INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®
RICE UNIVERSITY

Structural Consequences of Base Modifications and Metal Ion Interactions on the Anticodon Stem-Loop from tRNA^Phe (Escherichia coli)

by

Javier Cabello-Villegas

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

APPROVED, THESIS COMMITTEE:

[Signatures of committee members]

Edward P. Nikonowicz, Associate Professor of Biochemistry and Cell Biology

Scott F. Singleton, Assistant Professor of Chemistry

Seiichi Matsuda, Associate Professor of Chemistry

Ronald Parry, Professor of Chemistry

HOUSTON, TEXAS

SEPTEMBER, 2001
September, 2001

ABSTRACT

Structural Consequences of Base Modifications and Metal Ion Interactions on the Anticodon Stem-Loop from tRNA^phe^ (Escherichia coli)

by

Javier Cabello-Villegas

The structural effects of naturally modified bases of RNA are mostly uncharacterized. The structural effects of two base modifications and of cations in the anticodon stem loop of tRNA^phe^ (Escherichia coli) were studied on a 17 mer RNA (ACSL^phe^) by NMR spectroscopy. The anticodon region in fully modified tRNAs is proposed to be in a U-turn conformation. Solution NMR studies of unmodified tRNA anticodon stem-loops suggest that base modifications and divalent cations have a minor impact on the structure of the anticodon loop. The unmodified ACSL^phe^ contains two extra base pairs, 32-38 and 33-37, precluding a U-turn conformation. ACSL^phe^ contains a tri-nucleotide loop composed of the anticodon residues. The attachment of a dimethylallyl group at the amino nitrogen of A1 (i^6A1) has been proposed to increase the stacking ability of the anticodon residues and residue 37. In the i^6A1-containing molecule (i6A37-ACSL^phe^), base pair 33-37 is broken, and base pair 32-38 is destabilized. Several loop resonances enter a regime of intermediate exchange. The presence of Mg^{2+} has been proposed to stabilize the U-turn in other studies. Mg^{2+} opens the loop region of ACSL^phe^ and makes it multi-conformational. Mg^{2+} causes a peak pattern that resembles that of i6A37-ACSL^phe^ and stabilizes the open conformation of i6A37-ACSL^phe^. However, i6A37-ACSL^phe^ in the presence of Mg^{2+} does not form a stable U-turn. Pseudouridine at position 32 (Ψ_32) is another naturally occurring modification on tRNA^phe^. Ψ_32 on ACSL^phe^ (Ψ32-ACSL^phe^) increases the stability of the stem and is base paired in anti conformation with A1. The structure of Ψ32-ACSL^phe^ is essentially the same as that of ACSL^phe^, two ion probes for Mg^{2+} binding sites, Mn^{2+} and Co(NH_3)_6^{3+}, were tested on ACSL^phe^ and i6A37-ACSL^phe^. Both ions bind to the 5' side of the stem regions and weakly to the loop regions. Co(NH_3)_6^{3+} induces a U-turn conformation in i6A37-ACSL^phe^ and ACSL^phe^ but in the latter case a second conformation coexists. It is concluded that the dimethylallyl modification and cations act synergistically in the stabilization of the U-turn. It is proposed that the greater charge density of Co(NH_3)_6^{3+}, relative to Mg^{2+}, is responsible for the formation of a stable U-turn.
Acknowledgements

The author thanks the following people and institutions:

Dr. Edward P. Nikonowicz, who provided extensive academic support and advice in the NMR and RNA structure fields.

Dr. Scott F. Singleton, Dr. Seiichi Matsuda, Dr. George N. Phillips, and Dr. Ronald Parry, who gave favorable observations and guidance as members of my thesis committee.

Dr. Malcolm M. Winkler, who provided clones for production of the MiaA enzyme and the ACSL$^{Phe}$ RNA, as well as protocols for purification of MiaA.

Dr. James Ofengand, who provided a clone for expression of the RluA enzyme.

Dr. Eric DeJong, Dr. Kumar Kalurachchi, Dr. Sean Moran and Jeff S. Smith, who shared their knowledge of computational and NMR spectroscopic techniques and help in the analysis of RNA structures. Timothy Olson, who participated in the assignment of ACSL$^{Phe}$ and generation of an initial constraint list. Michelle Callabretta, who participated in the purification of MiaA and RluA.

Dr. Margaret Michnicka, who provided advice in laboratory techniques and produced reagents required for RNA synthesis.

Dr. Jeff. Nichols, who provided instruction on the use of ultracentrifigation and CD equipment.

Dr. Yousif Shamoo, who allowed the use of his spectrophotometer.

Erin O’Neil, who proofread this dissertation.

Dr. Olga Cabello, who granted support on living logistics.

The Welch Foundation supplied a PhD student fellowship since 1997.

CONACYT lent money for personal health insurance.
Table of Contents

Title page i
Abstract ii
Acknowledgements iii
Table of Contents iv
List of Abbreviations viii
List of Illustrations x
List of Tables xiv

1. INTRODUCTION

1.1 The U-turn conformation of RNA 1
   i. Conformations of RNA loops 1
   ii. tRNA crystallographic studies 1
   iii. Function of the anticodon stem loop 2
   iv. Ubiquity of U-turns 4
   v. Extended anticodon hypothesis 5

1.2 Nucleotide modifications in RNA 5
   i. Relevance of modifications for RNA structure; function 6
   ii. Which RNAs are modified and frequent positions 6
   iii. Types of modifications
        A. Hydrophobic, hydrophilic, charged 7
        B. Base pair breaking modifications 7
        C. Helix stabilizing modifications 8
   iv. Pseudouridine
        A. Occurrence of pseudouridine 8
        B. Function 9

1.3 Cations and RNA conformation 11
   i. Physical properties of Mg 11
   ii. Mg\(^{2+}\) binding to nucleic acids 11
   iii. Mg\(^{2+}\) and nucleotide modifications 13
   iv. Co\((NH_2)_2\) as an analog for Mg\(^{2+}\) 14

2. STRUCTURES OF ANTICODON STEM-LOOPS 15
2.1 Structures of unmodified anticodon stem-loops
  i. Stacking of anticodon residues and base pairing
  ii. NMR studies of anticodon stem-loops

2.2 Structures of modified anticodon stem-loops
  i. Crystal structures
  iii. Solution structures

2.3 Modifications of nucleotides within the anticodon stem loop
  i. Modified nucleotide at position 37
     A. Functions
     B. The dimethylallyl modification
        a. MiaA enzyme
        b. MiaA mutant phenotypes
        c. Studies of the effect of \( \text{i}^{\text{A}} \text{A} \) - and its derivatives in translation
  ii. Modified nucleotide at position 32

2.4 E coli ACSL® as a model to study effects of modifications and cations on RNA

2.5 Use of NMR to study structure and dynamics of an anticodon stem-loop

3. OBJECTIVES
  3.1 General
  3.2 Specific
  3.3 Hypotheses

4. MATERIALS AND METHODS
  4.1 Materials
  4.2 Biochemical methods
     i. Isotopically enriched oligonucleotides
     ii. Recombinant protein production
     iii. Modification of RNAs
          A. Dimethylallyl modification
          B. Pseudouridylation
  4.3 Structural methods
     i. NMR spectroscopy
     ii. Structural constraints
     iii. Structure calculations
5. RESULTS

5.1 Distinguishing RNA duplex and hairpin forms

Summary

5.2 Unmodified ACSL structure

i. Spectral assignments
ii. Relevant constraints
iii. Structure calculations
iv. Loop structure
v. Stem structure
Summary

5.3 Dimethylallyl-modified ACSL

i. Spectral effects and assignments
ii. Relevant constraints and comparison with the unmodified structure
iii. Conformation of the loop with the dimethylallyl group
Summary

5.4 T₁, measurements

i. Information obtained from relaxation rates
ii. Relaxation rate measurements
Summary

5.5 Mg²⁺ before and after dimethylallyl modification

i. Spectral assignments
ii. Spectral similarities caused by the dimethylallyl modification and Mg²⁺
iii. Peak widths
iv. T₂, measurements
v. Relevant constraints for the dimethylallyl modified and unmodified ACSL
Summary

5.6 Pseudouridylylated ACSL

i. Spectral assignments
ii. Relevant constraints
iii. Loop structure and comparison with the unmodified molecule
Summary

5.7 Double modified ACSL

Summary

5.8 Mn²⁺, Co(NH₃)₆³⁺ and induction of a U-turn

i. Mn²⁺ and Co(NH₃)₆³⁺
ii. Effects of Co(NH₃)₆³⁺ and assignments
iii. Spectral and structural effects on the
5.9 ACSL-MiaA complex

i. MiaA and complex solubility 199
ii. Imino $^1\text{H}/^1\text{N}$ spectra 200
iii. Amide $^1\text{H}/^1\text{N}$ spectra 200
iv. Ultracentrifugation 203
Summary 206

6 DISCUSSION

i. Structure of ACSL$^{\text{Phe}}$ 208
ii. Effect of pseudouridine 32 213
iii. Effect of the dimethylallyl group 214
iv. Effect of Mg$^{2+}$ and synergy with the dimethylallyl modification 217
v. Effect of Co(NH$_3$)$_4$$^{2+}$ on ACSL$^{\text{Phe}}$ and i6A37-ACSL$^{\text{Phe}}$ 221
vi. Interaction of ACSL$^{\text{Phe}}$ with MiaA 225

7 CONCLUSIONS 228

8 APPENDICES 230

I. Chemical shifts of A37-ACSL 230
II. Structural constraints for A37-ACSL 231
III. Chemical shifts of i6A37-ACSL 235
IV. Identified NOES for i6A37-ACSL 236
V. Chemical shifts of i6A37-ACSL Co(NH$_3$)$_4$$^{2+}$ 237
VI. Structural constraints of i6A37-ACSL Co(NH$_3$)$_4$$^{2+}$ 238
VII. Chemical shifts of A37$\Psi$32-ACSL 239
VIII. Structural constraints of A37$\Psi$32-ACSL 240
IX. Expected or inconsistent NOES for calculated $\Psi$32-ACSL$^{\text{Phe}}$ loop regions 243

9 BIBLIOGRAPHY 244
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>1 dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>2 dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>3 dimensional</td>
</tr>
<tr>
<td>'J</td>
<td>three-bond coupling constant</td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>A'</td>
<td>adenine protonated at N1</td>
</tr>
<tr>
<td>ACSL</td>
<td>anticodon stem loop</td>
</tr>
<tr>
<td>ACSL\textsuperscript{Fhe}</td>
<td>anticodon stem loop from <em>E. coli</em> tRNA\textsuperscript{Fhe}</td>
</tr>
<tr>
<td>A-site</td>
<td>aminoacyl tRNA ribosomal binding site</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CH</td>
<td>methyne</td>
</tr>
<tr>
<td>CH\textsubscript{2}</td>
<td>methylene</td>
</tr>
<tr>
<td>CH\textsubscript{3}</td>
<td>methyl</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation spectroscopy</td>
</tr>
<tr>
<td>cttHSQC</td>
<td>constant time heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>D</td>
<td>deuterium</td>
</tr>
<tr>
<td>D\textsubscript{2}O</td>
<td>deuterium oxide</td>
</tr>
<tr>
<td>DIPSI</td>
<td>Decoupling in the presence of scalar interactions</td>
</tr>
<tr>
<td>DMAPP</td>
<td>dimethylallyl diphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DQF-COSY</td>
<td>double quantum filtered correlation spectroscopy</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>Elongation factor Tu</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>GDPNP</td>
<td>5\textsuperscript{'}-Guanlylimidodiphosphate</td>
</tr>
<tr>
<td>GMP</td>
<td>guanosine monophosphate</td>
</tr>
<tr>
<td>GNRA</td>
<td>G-any nucleotide-purine-A sequence</td>
</tr>
<tr>
<td>GpN</td>
<td>G-phosphate-any nucleotide</td>
</tr>
<tr>
<td>HetCor</td>
<td>heteronuclear correlation spectroscopy</td>
</tr>
<tr>
<td>His\textsubscript{6}-</td>
<td>histidine tagged</td>
</tr>
<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus type 1</td>
</tr>
<tr>
<td>HMOC</td>
<td>heteronuclear multiple quantum coherence spectroscopy</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence spectroscopy</td>
</tr>
<tr>
<td>i'A</td>
<td>N\textsuperscript{'}-(dimethylallyl)adenosine</td>
</tr>
<tr>
<td>i6A37Ψ32-ACSL\textsuperscript{Fhe}</td>
<td>i'A\textsubscript{37}, and Ψ\textsubscript{32} containing ACSL\textsuperscript{Fhe}</td>
</tr>
<tr>
<td>i6A37-ACSL\textsuperscript{Fhe}</td>
<td>i'A\textsubscript{37} containing ACSL\textsuperscript{Fhe}</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthiogalactoside</td>
</tr>
<tr>
<td>KDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>K\textsubscript{m}</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td><em>M. mycoides</em></td>
<td><em>Mycoplasma mycoides</em></td>
</tr>
<tr>
<td>m'G</td>
<td>1-(methyl)guanosine</td>
</tr>
<tr>
<td>m'C</td>
<td>5-(methyl)cytidine</td>
</tr>
<tr>
<td>MiaA</td>
<td>dimethylallyl diphosphate:tRNA transferase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>ms</td>
<td>methylthio group</td>
</tr>
<tr>
<td>ms'i'A</td>
<td>2-(methylthio)-N\textsuperscript{'}-(dimethylallyl)adenosine</td>
</tr>
<tr>
<td>ms'io\textsuperscript{6}A</td>
<td>cis-2-(methylthio)-N\textsuperscript{'}-(4-hydroxydimethylallyl)adenosine</td>
</tr>
<tr>
<td>ms't\textsuperscript{6}A</td>
<td>2-(methylthio)-N\textsuperscript{'}-(threonylcarbamoyl)adenosine</td>
</tr>
<tr>
<td>NH</td>
<td>imino</td>
</tr>
</tbody>
</table>
NH₂
NMR
NOE
NOESY
NTP
OD
ppm
P-site
Ψ
Ψ32-ACSL₅⁺
RLuA
RMSD
RNA
tRNA
S. typhimurium
snRNA
spp
T
t¹A
tDNA
TMP
TOCSY
tRNA
TROSY
TSP
U
X
Y

amino
nuclear magnetic resonance spectroscopy
nuclear Overhauser enhancement effect
nuclear Overhauser enhancement spectroscopy
nucleotide triphosphate
optical density
parts per million
peptidyl tRNA binding site
β-pseudouridine
Ψ₃² containing ACSL₅⁺
ribosomal large subunit pseudoU formation enzyme
root mean square deviation
ribonucleic acid
ribosomal ribonucleic acid
Salmonella typhimurium
small nuclear RNA
species
thymine
N6-threonylcarnamoyladenosine
DNA analog of tRNA
trimethyl phosphate
total correlation spectroscopy
transfer ribonucleic acid
transverse relaxation-optimized spectroscopy
(3-trimethylsilyl)-propionate
uracil
any aminoacid
wybutusine
List of Illustrations

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>10</td>
</tr>
<tr>
<td>2.1</td>
<td>24</td>
</tr>
<tr>
<td>2.2</td>
<td>32</td>
</tr>
<tr>
<td>2.3</td>
<td>33</td>
</tr>
<tr>
<td>4.1</td>
<td>47</td>
</tr>
<tr>
<td>5.1.1</td>
<td>51</td>
</tr>
<tr>
<td>5.1.2</td>
<td>53</td>
</tr>
<tr>
<td>5.1.3</td>
<td>54</td>
</tr>
<tr>
<td>5.1.4</td>
<td>55</td>
</tr>
<tr>
<td>5.1.5</td>
<td>59</td>
</tr>
<tr>
<td>5.1.6</td>
<td>60</td>
</tr>
<tr>
<td>5.2.1</td>
<td>63</td>
</tr>
<tr>
<td>5.2.2</td>
<td>65</td>
</tr>
<tr>
<td>5.2.3</td>
<td>67</td>
</tr>
<tr>
<td>5.2.4</td>
<td>68</td>
</tr>
<tr>
<td>5.2.5</td>
<td>70</td>
</tr>
<tr>
<td>5.2.6</td>
<td>71</td>
</tr>
<tr>
<td>5.2.7</td>
<td>73</td>
</tr>
<tr>
<td>5.2.8</td>
<td>75</td>
</tr>
<tr>
<td>5.2.9</td>
<td>77/78</td>
</tr>
</tbody>
</table>

1.1 Schematic representation showing structural features of an anticodon loop containing a canonical U-turn.

1.2 Structure of pseudouridine and possible conformations for base pairing.

2.1 Scheme showing the sequential modification of tRNA A₁-ro to m̈s^2i^7A₁-ro in E. coli and further modifications in other organisms.

2.2 Sequence of the fully modified anticodon stem loop from E. coli tRNA^{Ph} and the partially modified sequences studied.

2.3 Scheme showing the modification of U₁ to Ψ₁ by the enzyme RluA in some tRNAs.

4.1 Backbone torsion angles of nucleic acids.

5.1.1 2D 15N/1H HMQC spectrum of the imino region of ACSL^{Ph}.

5.1.2 Schematic representation of the chemical shift method for detection of duplex form of oligonucleotides.

5.1.3 RNAs sequences used to test duplex formation.

5.1.4 2D 15N/1H HMQC spectra of the imino regions of RNA I and a mixture of RNA I and RNA I', in presence and absence of NaCl.

5.1.5 2D 15N/1H HMQC spectra of the imino regions of ACSL^{Ph} and a mixture of ACSL^{Ph} and ACSL^{Ph} U₁-ro probe.

5.1.6 2D 15N/1H HMQC spectrum of the imino region of a mixture of ACSL^{Ph} and ACSL^{Ph}·U42 in presence of MgCl₂.

5.2.1 Schemes illustrating the strategy for the determination of molecular structures using NMR, NOE connectivities present on an RNA double helix, and the Newman projection of torsion angles.

5.2.2 Aromatic and anomeric regions from a 2D 13C/1H HMQC of ACSL^{Ph} in low salt buffer.

5.2.3 2D NOESY of ACSL^{Ph} showing the H6/H8 inter residue NOEs and the H6/H8 to anomeric NOE sequential walk.

5.2.4 2D NOESY of ACSL^{Ph} showing the H6/H8 to H2' sequential connectivities.

5.2.5 1^3P one dimensional spectra of ACSL^{Ph}.

5.2.6 2D NOESY of the imino region of ACSL^{Ph}.

5.2.7 HNN-COSY and 15N/1H HSQC spectra of ACSL^{Ph} in D₂O.

5.2.8 15N/1H HSQC for the amino region of ACSL^{Ph}.

5.2.9 Schematic representation of the inter-residue NOEs identified on ACSL^{Ph}.
Histograms of H1′-H2′ J couplings and chemical shifts of C3′ and C4′, from ACSL^Phe.

Histograms of C2′-P J couplings.

Superposition of ACSL^Phe convergent structures, and a representation of the minimized average structure.

2D \textsuperscript{13}N/\textsuperscript{1}H HMQC of the imino region of i6A37-ACSL^Phe.

Imino proton 1D spectra of ACSL^Phe and i6A37-ACSL^Phe.

2D \textsuperscript{13}N/\textsuperscript{1}H HSQC of the amino region of i6A37-ACSL^Phe.

H6/H8 to anomic NOE sequential walks of ACSL^Phe and i6A37-ACSL^Phe.

Aromatic and anomic regions from a 2D \textsuperscript{13}C/\textsuperscript{1}H HMQC of i6A37-ACSL^Phe.

Inter-residue NOE's identified in i6A37-ACSL^Phe residues G\textsubscript{12} to C\textsubscript{12}, in low salt buffer.

\textsuperscript{31}P one-dimensional spectra of i6A37-ACSL^Phe at different temperatures.

Melting curves at UV 260nm wavelength of ACSL^Phe and i6A37-ACSL^Phe.

Model of ACSL^Phe with an attached dimethylallyl group, in accordance with NOE information from i6A37-ACSL^Phe.

Histograms of \textsuperscript{13}C T\textsubscript{1\textsuperscript{n}} measurements for ACSL^Phe and i6A37-ACSL^Phe.

2D \textsuperscript{13}N/\textsuperscript{1}H HMQC of the imino resonance region of ACSL^Phe in the presence of 5 mM MgCl\textsubscript{2}.

\textsuperscript{15}N/\textsuperscript{1}H 2D HSQC of the amino resonance region of ACSL^Phe in the presence of 5 mM MgCl\textsubscript{2}.

H6/H8 to anomic crosspeaks of a 2D-NOESY acquired on ACSL^Phe in the presence of 7 mM MgCl\textsubscript{2}.

Aromatic and anomic regions from a 2D \textsuperscript{13}C/\textsuperscript{1}H HMQC of ACSL^Phe in presence of 10 mM MgCl\textsubscript{2}.

Histograms of Mg\textsuperscript{2\textsuperscript{+}} dependent chemical shift changes of ACSL^Phe and i6A37-ACSL^Phe.

H6/H8 to anomic spectral region of a 2D-NOESY acquired on i6A37-ACSL^Phe in the presence of 7 mM MgCl\textsubscript{2}.

Aromatic and anomic regions from a 2D \textsuperscript{13}C/\textsuperscript{1}H HMQC spectrum of i6A37-ACSL^Phe titrated to 10 mM MgCl\textsubscript{2}.

\textsuperscript{13}P 1D spectra of ACSL^Phe and i6A37-ACSL^Phe, in presence of 7 mM Mg\textsuperscript{2\textsuperscript{+}} at different temperatures.

C5/H5 region of 2D \textsuperscript{13}C/\textsuperscript{1}H HMQC spectra of ACSL^Phe and i6A37-ACSL^Phe in the absence and presence of Mg\textsuperscript{2\textsuperscript{+}}.

C6/H6 region of 2D \textsuperscript{13}C/\textsuperscript{1}H HMQC spectra of ACSL^Phe and i6A37-ACSL^Phe in the absence and presence of Mg\textsuperscript{2\textsuperscript{+}}.

\textsuperscript{1}H 1D spectra of imino protons of ACSL^Phe, i6A37-ACSL^Phe, and ACSL^Phe titrated with 10 mM MgCl\textsubscript{2}.
5.5.12 Melting curves at 260nm wavelength of i6A37-ACSL<sub>e</sub> in the absence and presence of Co(NH<sub>3</sub>)<sub>4</sub> and Mg<sup>2+</sup>.

5.6.1 Aromatic and anomeric regions from a 2D <sup>13</sup>C/<sup>1</sup>H HMOC spectrum of Ψ<sub>32-ACSL</sub><sup>phe</sup>.

5.6.2 H6/H8 to ribose proton crosspeaks of a 2D-NOESY acquired on Ψ<sub>32-ACSL</sub><sup>phe</sup>.

5.6.3 <sup>15</sup>N/<sup>1</sup>H 2D HMOC and <sup>1</sup>H 1D spectra, optimized for the imino resonance region, acquired on Ψ<sub>32-ACSL</sub><sup>phe</sup>.

5.6.4 2D NOESY in 90% H<sub>2</sub>O of the imino region of Ψ<sub>32-ACSL</sub><sup>phe</sup>.

5.6.5 Melting curves at 260nm wavelength of ACSL<sub>e</sub>, i6A37-ACSL<sub>e</sub>, and Ψ<sub>32-ACSL</sub><sup>phe</sup>.

5.6.6 HNN-COSY and <sup>15</sup>N/<sup>1</sup>H HSQC spectra of Ψ<sub>32-ACSL</sub><sup>phe</sup> in D<sub>2</sub>O.

5.6.7 Stereo views of minimized average structures of Ψ<sub>32-ACSL</sub><sup>phe</sup>.

5.7.1 C6-8/H6-8 and C2/H2 regions of 2D <sup>13</sup>C/<sup>1</sup>H HMOC spectra of i6A37Ψ<sub>32-ACSL</sub><sup>phe</sup> at 17, 25 and 30 °C.

5.7.2 C5/H5 and C1'/H1' regions of 2D <sup>13</sup>C/<sup>1</sup>H HMOC spectra of i6A37Ψ<sub>32-ACSL</sub><sup>phe</sup> at 17, 25 and 30 °C.

5.7.3 2D <sup>15</sup>N/<sup>1</sup>H HMOC spectrum of the imino resonance region of i6A37Ψ<sub>32-ACSL</sub><sup>phe</sup>.

5.7.4 Imino proton 1D spectra of i6A37Ψ<sub>32-ACSL</sub><sup>phe</sup> and i6A37-ACSL<sub>e</sub>.

5.7.5 Melting curves at 260nm wavelength of i6A37-ACSL<sub>e</sub>, Ψ<sub>32-ACSL</sub><sup>phe</sup>, and i6A37Ψ<sub>32-ACSL</sub><sup>phe</sup>.

5.8.1 C6-8/H6-8 regions of 2D <sup>13</sup>C/<sup>1</sup>H HMOC spectra of ACSL<sub>e</sub>, ACSL<sub>phe</sub> + MnCl<sub>2</sub>, i6A37-ACSL<sub>e</sub> and i6A37-ACSL<sub>phe</sub> + MnCl<sub>2</sub>.

5.8.2 H2/N1, H2/N1, H3/N7, and H8/N9 correlations of 2D <sup>15</sup>N/<sup>1</sup>H HSQC spectra of ACSL<sub>e</sub> in the absence and presence of MnCl<sub>2</sub>.

5.8.3 Schematic representation of ACSL<sub>e</sub> and i6A37-ACSL<sub>phe</sub> resonances broadened by Mn<sup>2+</sup>.

5.8.4 2D <sup>1</sup>P/<sup>1</sup>H Hetero-TOCSY-NOESY spectrum of i6A37-ACSL<sub>phe</sub> in the presence of Co(NH<sub>3</sub>)<sub>4</sub>.

5.8.5 Aromatic and anomeric regions from a 2D 13C/1H HMOC spectrum acquired on i6A37-ACSL<sub>phe</sub> in presence of Co(NH<sub>3</sub>)<sub>4</sub>.

5.8.6 H6/H8 to H6/H8 and H6/H8 to anomeric crosspeaks of a 2D-NOESY acquired on i6A37-ACSL<sub>phe</sub> in the presence of Co(NH<sub>3</sub>)<sub>4</sub>.

5.8.7 <sup>1</sup>P 1D spectrum of i6A37-ACSL<sub>phe</sub> in the presence of Co(NH<sub>3</sub>)<sub>4</sub>.

5.8.8 H8/N7 correlations of <sup>15</sup>N/<sup>1</sup>H HSQC spectra of i6A37-ACSL<sub>phe</sub> in the absence and presence of Co(NH<sub>3</sub>)<sub>4</sub>.

5.8.9 2D <sup>15</sup>N/<sup>1</sup>H HMOC spectrum of the imino resonance region of i6A37-ACSL<sub>phe</sub> in the presence of Co(NH<sub>3</sub>)<sub>4</sub>.

5.8.10 <sup>1</sup>H 1D spectra of i6A37-ACSL<sub>phe</sub> in the presence of Co(NH<sub>3</sub>)<sub>4</sub>.
5.8.11 2D NOESY spectrum of i6A37-ACSL<sup>Phe</sup> in 90% H<sub>2</sub>O in the presence of Co(NH<sub>3</sub>)<sub>4</sub><sup>2+</sup>. 185

5.8.12 Loop region from the lowest energy ACSL<sup>Phe</sup> structure calculated with constraints from i6a37-ACSL<sup>Phe</sup> in presence of Co(NH<sub>3</sub>)<sub>4</sub><sup>2+</sup>. 188-9

5.8.13 Aromatic and anomeric regions from a 2D <sup>13</sup>C/H HMQC acquired on ACSL<sup>Phe</sup> in the presence of Co(NH<sub>3</sub>)<sub>4</sub><sup>2+</sup>. 190

5.8.14 H6/C6/H2/C2 regions of 2D <sup>13</sup>C HMQC spectra of i6A37-ACSL<sup>Phe</sup>, i6A37-ACSL<sup>Phe</sup> + Mg<sup>2+</sup>, i6A37-ACSL<sup>Phe</sup> + Co(NH<sub>3</sub>)<sub>4</sub><sup>2+</sup>, and ACSL<sup>Phe</sup> + Co(NH<sub>3</sub>)<sub>4</sub><sup>2+</sup>. 193

5.8.15 2D <sup>15</sup>N-H HSQC multiple bond spectrum of i6A37-ACSL<sup>Phe</sup> + Mg<sup>2+</sup>. 198

5.9.1 2D <sup>15</sup>N/H HMQC spectrum of the imino resonance region of <sup>15</sup>N enriched ACSL<sup>Phe</sup> in complex with unlabeled MiaA. 201

5.9.2 2D <sup>15</sup>N/H HSQC spectrum of 0.5 mM <sup>15</sup>N enriched MiaA. 202

5.9.3 CD spectrum of MiaA. 204

5.9.4 Absorbance profile of a sedimentation equilibrium of MiaA. 205
### List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.1</td>
<td>Summary of experimental constraints and structure calculation statistics for ACSL$^{\text{Fe}}$.</td>
<td>85</td>
</tr>
<tr>
<td>5.5.1</td>
<td>$^1\text{H}$ chemical shifts of ACSL$^{\text{Fe}}$ and i6A37-ACSL$^{\text{Fe}}$ in the presence and absence of 5mM Mg$^{2+}$.</td>
<td>123</td>
</tr>
<tr>
<td>5.5.2</td>
<td>$^{13}\text{C}$ T$_2^*$ measurements for C6/C8, C2 and C1' of i6A37 in the presence of 10mM Mg.</td>
<td>135</td>
</tr>
<tr>
<td>5.6.1</td>
<td>Detectable H1'-H2' couplings in $\Psi$32-ACSL.</td>
<td>150</td>
</tr>
<tr>
<td>5.6.2</td>
<td>Summary of experimental constraints and structure calculation statistics for $\Psi$32-ACSL$^{\text{Fe}}$.</td>
<td>156</td>
</tr>
<tr>
<td>5.8.1</td>
<td>Resonances of ACSL$^{\text{Fe}}$ and i6A37-ACSL$^{\text{Fe}}$ broadened by Mn$^{2+}$.</td>
<td>171</td>
</tr>
<tr>
<td>5.8.2</td>
<td>$^{13}\text{C}$ T$_2^*$ measurements for C6/C8, C2, and C1' of i6A37 in presence the of 5mM Co(NH$_3$)$_2$$^{+}$.</td>
<td>197</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

1.1 The U-turn conformation of RNA

i. Conformations of RNA loops

RNA molecules can form hairpins and fold as independent units. These hairpins consist of base paired stems, unpaired bases in the loop and on occasion bulged residues in the stem (Tinoco and Bustamante 2000). RNA molecules are proposed to fold sequentially with secondary structures, such as hairpins, forming first and tertiary structure, or interactions between the secondary structures, later (Tinoco and Bustamante 2000). In spite of the multiple possible sequence combinations for terminal loops, relatively few families of stable conformations have been described (Moore 1999), and several consensus sequences occur in vivo in higher proportion than would be expected in random sequence. The identification of few families of structures might be a reflection of a relative scarcity of RNA structural information, but it also could be that there are RNA motifs that are abundant because they have a physiologically relevant role. A good example for a physiologically relevant conformation is the anticodon stem-loop from tRNAs. Different sequences are required to transfer the different aminoacids and the crystal structures of several tRNAs are very similar.

ii. tRNA crystallographic studies
In crystal structures, the conformations of the anticodon stem-loop regions of tRNAs were determined to consist of seven residue loops and five base pair stems. The conformation is called a U-turn (Quigley and Rich 1976), in part because a uridine residue at position 33 is highly conserved. A good example of this fold, because of the number of studies, is yeast tRNA<sup>phen</sup> (Goddard 1977, Shi and Moore 2000, Jovine et al. 2000). In the U-turn fold (Figure 1.1) the bases 32 and 33 are stacked with the rest of the 3′ side of the stem, there is a sharp turn between residues 33 and 34, and the bases of the anticodon residues (34 to 36) are stacked in the 5′ direction. Hydrogen bonds between U<sub>1</sub>2′ and A<sub>1</sub>7N7, as well as U<sub>1</sub>3N3 and A<sub>1</sub>7P-OL stabilize this structure (Goddard 1977, Moore 1999). The residue at position 32 is not Watson-Crick base paired and has been proposed to be involved in a bifurcated base pair with the residue at position 38, closing the loop (Aufinger and Westhof 1999).

iii. Function of the anticodon stem-loop

One of the functions of a tRNA is the recognition of the mRNA codon through the anticodon. In tRNAs the U-turn fold seems to be responsible for an increased tendency of the anticodon to base pair. tRNAs with complementary anticodons form stable complexes, with binding constants 6 orders of magnitude larger than those of the corresponding trinucleotides (Eisinger 1971). Selection of aptamers that bind to tRNA<sup>phen</sup> preferentially yields sequences that bind to the anticodon loop
Figure 1.1
Schematic representation showing structural features of an anticodon loop containing a canonical U-turn. The loop has a sharp turn between residues 33 and 34, which involves the α and ζ torsion angles. The anticodon residues are stacked in 3' direction. Residue 33 is hydrogen bonded to the phosphate 36 and stacks on the phosphate 35. Redrawn from Saenger (1984).
(Scarabino et al. 1999). The codon-anticodon pairing has been proposed to be analogous to intermolecular anticodon-anticodon base pairing present through packing in a tRNA$^{3\text{tp}}$ crystal structure, forming a continuous helical structure (Dirheimer et al. 1995, Moras et al. 1986). The anticodon stem-loop from E. coli tRNA$^{3\text{tp}}$ bound to its cognate codon in the A site has a U-turn conformation in crystal structures of the 30S ribosomal subunit from E. coli. The crystal structures have in the P site the 3' end of the 16S rRNA forming a U-turn like conformation and interacting with the mRNA. In this case the difference from a U-turn is that the 3' end of the 16S rRNA contains a base pair involving residues that correspond to nucleotides 32 and 38 of tRNA (Ogle et al. 2001, Carter et al. 2000). Carter et al. (2001) propose that the 3' end of the 16S rRNA in the P site is analogous to tRNA being bound there. In the A and P sites the residues in the position that corresponds to residue 37, 3' of the anticodon, are not base paired.

iv. Ubiquity of U-turns

The U-turn motif is known to occur in other RNA molecules besides tRNAs. NMR studies have shown that similar conformations are found in the GNRA type tetraloops (Jucker and Pardi, 1995), HIV-1 A-rich loop (Pugliisi and Pugliisi 1998), the hammerhead ribozyme (Pley et al. 1994), and an hexanucleotide loop found in 23S ribosomal RNA (Fountain et al. 1996, Huang et al. 1996). Interestingly in these cases the loop is smaller than 7 nucleotides and no modifications are present. The unmodified anticodon stem-loops from initiator and elongator tRNA$^{3\text{tp}}$ form a U-turn when free in solution, while the anticodon stem-loop of tRNA$^{lys}$...
(Human), the other NMR structure of an anticodon stem-loop available at high resolution, does not (see below) (Schweigeth and Moore 1997).

v. Extended anticodon hypothesis

Besides the anticodon sequence, the flanking sequences are important for forming functional tRNAs. Regularities were noted between certain bases at the third codon positions and the sequences 3' to them (Yarus 1982). The regularities were proposed to optimize the translation process, a proposal known as the extended anticodon hypothesis. The conservation of these residues might be required during the translation process in the ribosome. Alternatively, the modifications in the nucleotides of the anticodon loop could depend on the conserved residues, and the modified nucleotides would be the ones directly involved in increasing the efficiency of translation. The second possibility is at least partially true, as some modifying enzymes and tRNA synthetases show sequence dependent recognition of the anticodon stem-loop (Grcsjean et al. 1996, Arnes and Cavarelli 1996) and the modifications increase the efficiency of translation. The second possibility is also true for the system studied for this dissertation, the anticodon stem-loop from E. coli modified by the enzymes MiaA and RluA.

1.2 Nucleotide modifications in RNA

Over 100 modified nucleotides (DNA and RNA) are known (Agris 1996). Up to the present, 96 nucleotide modifications have been described in the RNA Modification Database (Rozenski et al 1999).
i Relevance of modifications for RNA structure and function

The naturally occurring nucleotide modifications are a strategy of the cell to regulate and modify the chemistry and structure of RNA. Their importance is suggested by the fact that, in E. coli for example, the amount of genetic material devoted to code for the tRNA modifying enzymes is at least four times the size of that which codes for all the tRNAs (Björk 1995-1). The presence of some modifications has been confirmed as advantageous for the cell in several cases, through mutational analyses of the genes that code for the modifying enzymes (Björk 1995-1, Hagervall et al. 1990, Janner et al. 1980, Connolly and Winkler 1990, Li et al. 1997, Winkler 1998). The study of the structural effects of modifications on RNA has lagged behind their identification. Multiple modifications occur in the anticodon regions of tRNAs but their interactions remain unexplored. Their individual effects can be very different from the collective effect. For instance, the undermodified anticodon stem-loop from yeast tRNA\textsuperscript{Phe}, containing only \textit{O}^\textit{m}-methylguanosine 34, binds to poly-U programmed 30S ribosomal subunits with an affinity two orders of magnitude lower than when it is fully modified (Koval'chuke et al. 1991). However, the corresponding affinities of the unmodified and the fully modified anticodon stem-loops are similar to that of the native tRNA (Uhlenbeck et al. 1982, Dao et al. 1994, Ashraf et al. 1999-1).

ii Which RNAs are modified and frequent positions
A survey of the 93 known nucleotide modifications up to 1994 found 28 in rRNA, 12 in mRNA, 11 in snRNA, and 79 in tRNAs (Limbach et al. 1994). 50 of the 79 modifications known in tRNAs occur in the anticodon region and positions 34 and 37 have the largest variety of modifications (Agris 1996, Björk 1995-2). Position 34 is the first anticodon position (the wobble residue) and position 37 is the residue adjacent to the anticodon in the 3' direction.

iii Types of modifications

A. Hydrophobic, hydrophilic, charged

The nucleotide modifications have such diverse physicochemical effects as an increase in hydrophobicity or hydrophilicity, charge etc. Modifications have been categorized by types such as those that increase polarity (31), those that increase hydrophobicity (33), and those that introduce charge (24) (Agris 1996). Hydrophobic modifications can enhance base stacking, as is the case for wybutosine in yeast tRNA<sup>thw</sup> (Nishimura 1972) while hydrophilic modifications could in principle provide groups for hydrogen bonding.

B. Base pair breaking modifications

Although different in their electric properties, base pair breaking modifications can affect the structure of nucleic acids functionally by blocking hydrogen bonding groups or creating steric hindrance. This effect has important consequences for RNA structure, since the stability of interactions such as base paring would otherwise limit available conformations. Recent examples of studies about hydrogen bonding prevention deal with modifications as simple as methylations.
Methylation can prevent the formation of a GNRA tetraloop on a ribosomal hairpin (Rife and Moore 1998) and break base pairing, thus preventing misfolding of human mitochondrial tRNA\textsuperscript{\textsc{lys}} (Helm et al. 1999) and opening the loop of a DNA analog for the anticodon domain of tRNA\textsuperscript{\textsc{phe}} (yeast) (Basti et al. 1996). Hyper-modifications, like the negatively charged N\textsubscript{6}-threonylcarbamoyladenosine (t\textsuperscript{\textsc{a}}\textsubscript{7}A\textsubscript{3}'), also have a role in breaking base pairing (Agris 1996, Stuart et al. 2000).

C. Helix stabilizing modifications

Methylation at position C5 of pyrimidines increase hydrophobicity and their ability to stack with other bases. There is an increase in the melting temperature when T is substituted for U and 5-methylcytidine (m\textsuperscript{5}C) is substituted for C (Agris 1996 review). 2-thio uridine also stabilizes RNA double helices, in this case by favoring a ribose C3' endo conformation (Smith et al. 1992, Kumar and Davis 1997). Pseudouridine (Ψ) also increases the stability of base paired regions as presented below.

iv Pseudouridine

A. Occurrence of pseudouridine

Pseudouridine is the most common modified nucleotide and is discussed here in detail because in this work the effect of such modification is studied in the context of an anticodon stem-loop (see below). Pseudouridine is a regioisomer of uridine where the C5 of uracil is bound to the ribose C1', leaving two imino groups in the base (Figure 1.2). The conformation of the monomer about that bond shows a slight preference for syn (Davis 1998, Davis et al. 1998). The glycosidic
conformation is preferentially anti in oligonucleotides and double helices, consequently pseudouridine base pairs with adenosine through its N3 imino group and the C2 carbonyl (Hruska et al 1970, Roy et al 1984, Griffey et al. 1985).

Pseudouridine occurs in the three phylogenetic domains -archaea, bacteria and eukarya- and organelles. It can be found in small nucleolar and nuclear RNAs, rRNA and tRNA (Charette and Gray 2000). The proportion of pseudouridine in ribosomal RNA is larger in eukarya than in bacteria and archaea. There are around 90 pseudouridines in mammalian rRNA while in E. coli there are 10 (Ofengand and Fournier 1998). The relative positions of pseudouridine in the rRNA are not universally conserved (Ofengand and Fournier 1998). tRNAs in contrast have specific positions with conserved pseudouridines. There is an almost universally conserved pseudouridine, Ψ15, in the ΨWC loop (Spinzi 1999) and several pseudouridines are highly conserved in different species of tRNAs at positions 13, 38 and 39.

IV. Function

Pseudouridine can stabilize base stacking when present within a double helix or at helix-loop junctions, or slightly destabilize it, when present in single stranded loop regions (Davis 1995, Meroueh et al. 2000). When present within a double helix, pseudouridine is proposed to restrict the mobility of the backbone through an H2O bridge between Ψ NH1 and the phosphates of Ψ and its 5' residue, as seen in the crystal structure of tRNA15 (Arnez and Steitz, 1994). This proposal is consistent with the slow exchange rate of the NH1 proton (Hall and McLaughlin 1992, Davis 1995), which is not involved in a base pair. Pseudouridine favors a ribose C3'-endo conformation when present in an oligonucleotide chain, thus limiting it's base moiety conformation to
Figure 1.2
Left) Structure of pseudouridine, Right top) pseudouridine in anti conformation base paired to adenosine, Right bottom) pseudouridine in syn conformation base paired with adenosine.
anti and enhancing local stacking (Davis 1998).

1.3 Cations and RNA conformation

i. Physical properties of Mg$^{2+}$

Unlike other biologically relevant cations such as Na$^+$, K$^+$, and Ca$^{2+}$, which coordinate mostly with oxygen atoms, Mg$^{2+}$ can coordinate strongly with N-ligands and phosphate oxygens. Mg$^{2+}$ has a strong tendency to have 6 coordinations with a near octahedral configuration. Mg$^{2+}$ has a 72 pm ionic radius and thus has a low radius to charge ratio relative to the aforementioned metals (Kaim and Schwederski 1994). It prefers coordinating with small ligands, and therefore tends to retain water molecules when interacting with biological ligands (Cowan 1995). Mg$^{2+}$ occurs in millimolar levels within the cells, 23 mM in E. coli and 8 mM in human muscle for example (Bloomfield et al 2000). The concentration of intracellular free Mg$^{2+}$ in solution is typically ~1mM (Cowan 1995).

ii. Mg$^{2+}$ binding to nucleic acids

Nucleic acids are polyanions with one negative charge per phosphate. These groups interact with metal ions through salt linkages. One of these cations is Mg$^{2+}$. Mg$^{2+}$ retains the water molecules of the inner hydration sphere upon binding to single stranded polynucleotides. Inner sphere coordination can occur as a slower process in particular cases, after formation of the outer sphere coordination (Porschke 1995).
When bound to ligands other than water, Mg$^{2+}$ prefers oxygen polyanions, particularly polyphosphates (Kain and Schwederski 1994, Cowan 1995). Mg$^{2+}$ and other divalent metals stabilize nucleic acid double helices and do this more effectively than monovalent cations. For example, the stabilization obtained by a given Na$^+$ concentration is achieved with a concentration of Mg$^{2+}$ two orders of magnitude lower. This is probably due to electrostatics (counterion condensation theory) and region specific binding on the double strand for Mg$^{2+}$ (Bloomfield et al 2000, Misra and Draper 2000, Robinson et al. 2000). In the counterion condensation theory a higher valence of the counterion is going to reduce the charge more effectively (Bloomfield et al. 2000). Region specific binding for Mg$^{2+}$ has been described in A-form helices by crystallographic results. Hydrated Mg$^{2+}$ forms hydrogen bonds to O6/N7 sites of G's in GpN steps in the deep major groove and also bridges phosphates across the narrow major groove, allowing the sugar phosphate backbones in RNA or A-DNA to approach each other with less electrostatic repulsion (Robinson et al. 2000). Site-specific binding of Mg$^{2+}$ has been proposed for tRNAs at several positions, based on crystallographic evidence (Holbrook et al. 1977, Hingerty et al. 1979, Shi and Moore 2000, Jovine et al 2000). Inner sphere coordination of Mg$^{2+}$ to the phosphate of wobutusine 37 of yeast tRNA$^\text{Thr}$, is supported by some crystal structures and by Mg$^{2+}$ binding dynamics (Holbrook et al. 1977, Shi and Moore 2000, Bujalowski 1996). This binding is proposed to be stabilized by interactions between unstacked conformations of base π electron systems and the cation (McFail-Isom et al 1998). A Mg$^{2+}$ induced increase of wobutusine 37 fluorescence in yeast tRNA$^\text{Thr}$ (Labuda and Porschke 1982, Bujalowski et al. 1986, Striker et al. 1989) has been attributed to specific Mg$^{2+}$ binding (Bujalowski et al. 1986). The increase in fluorescence is probably due to the stacking of residue 37 (Striker et
al. 1989). Some crystal structures do not support the presence of the Mg^{2+} binding site with coordination to the phosphate at residue 37 (Hingerty et al. 1978, Jovine et al. 2000).

The presence of specific binding sites in tRNA is not supported by some spectroscopic results (Guéron and Leroy 1982). Theoretical calculations suggest a more diffuse ion clustering instead, as those sites coincide with regions of high negative electrostatic potential (Misra and Draper 2000).

iii. Mg^{2+} and nucleotide modifications

The interaction of nucleotide modifications with Mg^{2+} has been studied using mainly tRNA as a model system. Comparison between unmodified transcripts and mature tRNAs has been one of the main sources of information. These data suggest that there are complementary effects between the modifications and Mg^{2+}. The unmodified tRNA^{35A} has weaker Mg^{2+} binding than the fully modified molecule (Yue et al. 1994), and it has been proposed that Mg^{2+} can compensate for the structural destabilization originating from the absence of modifications on tRNA^{35A} (E. coli) (Hall et al. 1989, Serebrov et al. 1998). A dissection of the effect of specific modifications and Mg^{2+} has been done only in undermodified yeast tRNA^{35A}. In the anticodon stem-loop sequence of yeast tRNA^{35A}, the isolated 5-methylcytidine at position 40 (mC40) leads to cooperative binding of Mg^{2+}. mC40, in combination with Mg^{2+} leads to the formation of base pairs 32-37 and 33-36 (Chen et al. 1993, Guenther et al. 1992).
iv. Co(NH₃)₄⁺⁺ as an analog for Mg²⁺

To study the Mg²⁺ outer sphere complexation through NMR, Co(NH₃)₄⁺⁺, an analog of the hexahydrated ion has been utilized. Co(NH₃)₄⁺⁺ has been proposed to work as a probe for hexahydrated Mg²⁺ (Cowan 1993), as it has octahedral geometry and a similar size (Rüdisser and Tinoco 1999). Co(NH₃)₄⁺⁺ has been shown in some cases to be able to substitute effectively for Mg²⁺ in enzymatic reactions (Bassi et al 1995, Hampel and Cowan 1997, Murray et al 1998), allows formation of four way DNA junctions more effectively than Mg²⁺ (Ducket et al 1990) and has a similar number of binding sites in 5S ribosomal RNA (Cowan 1993). The amino protons of this compound exchange relatively slowly, hence they are useful for detection of a binding site through NMR experiments that detect NOEs to other nearby protons (Robinson and Wang 1996, Kieft and Tinoco 1997). NMR and X-ray crystallographic studies have found Co(NH₃)₄⁺⁺ binds to several specific and general Mg²⁺ binding sites in nucleic acids in an analogous manner as Mg²⁺ (Gao et al. 1995, Gonzalez and Tinoco, 1999, Robinson et al. 2000, Rüdisser and Tinoco 2000).
2. STRUCTURES OF ANTICODON STEM-LOOPS

2.1 Structures of unmodified anticodon stem-loops

i. Stacking of anticodon residues and base pairing

Intermolecular base pairing can disrupt or stabilize a U-turn. The anticodons and their 5’ residues can base pair with complementary oligonucleotides, thereby forming a 5’ stacked anticodon instead of the 3’ stacked U-turn (Geerdes et al 1980, Freier and Tinoco Jr. 1975, Pulikowska et al 1988). There seems to be a tendency of native anticodon stem-loops to form a 5’ stacked base paired conformation, as suggested by higher binding constants for oligomers that bind to additional bases on the 5’ side of the anticodon than for those on its 3’ side (Freier and Tinoco 1975, Uhlenbeck 1972, Eisinger and Spahr 1973, Labuda et al. 1985). By in vitro selection, it has been shown that aptamers that bind to unmodified tRNA$^{Met}$ (yeast) have sequences complementary in the majority of cases to the anticodon loop, from residues 32 to 38 (Scarabino et al. 2000). Hence modifications in this region could also be important to regulate intermolecular interactions, preventing extra base pairs other than the ones involving the anticodon residues (Grosjean et al. 1998).

ii. NMR studies of anticodon stem-loops

NMR studies of unmodified models of the anticodon stem-loops of initiator and elongator tRNA$^{Met}$ have concluded that they take on a U-turn
conformation (Schweisguth and Moore 1997). Both tRNAs in yeast and in
E. coli contain the modified nucleotide tA at position 37. Residues 32
and 38 are C and A respectively. The same two residues occur in tRNA^{16S}.
A model RNA for the unmodified tRNA^{16S} anticodon stem-loop does not take
on a U-turn conformation, as was determined using NMR spectroscopy
(Durant and Davis 1999). The conditions in which this unmodified
molecule was studied included low pH, which is proposed to allow the
formation of a C_{12}A_{12}^{-} base pair. The molecule also is proposed to
contain a U_{11}^{-}A_{12} base pair on the basis of structure calculations, but
no direct evidence is provided. The unmodified anticodon stem-loop from
yeast tRNA^{16S} is proposed to have a 7 residue loop in the presence of
Mg^{2+}, based on 1D imino proton spectra (Chen et al 1993, Dao et al
1994). For the unmodified E. coli tRNA^{16S} in solution, no high-resolution
structural information has been reported. The full-length unmodified
transcript is functional in the transfer process with a decrease in the
accuracy of translation, compared to the modified form (Harrington et al
1993). The unmodified anticodon stem-loop from this molecule binds to
the 30S ribosomal subunit P site and is proposed to have a U turn
conformation when bound, based on phosphorothioate and deoxy-
ribonucleotide interference assays (Von Ahsen et al. 1997, Schnitzer and
Von Ahsen 1997). Crystal structures show that this unmodified anticodon
stem-loop makes a U-turn in the ribosomal A-site (Cgle et al. 2001).
This conformation is likely stabilized by the multiple contacts of the
anticodon stem-loop with the ribosome and maybe by the presence of
organic polycations and Mg^{2+}. 
2.2 Structures of modified anticodon stem-loops

i. Crystal structures

The crystal structures of tRNA<sup>phe</sup>, tRNA<sup>asp</sup>, tRNA<sup>t<sub>5</sub>v</sup> and tRNA<sup>t<sub>3</sub>v</sup> contain a classical U-turn in the anticodon stem-loop (Shi and Moore 2000, Moras et al. 1986, Bénas et al. 2000, Nissen et al. 1999). tRNA<sup>phe</sup> (yeast) contains the hypermodified base wybutine at residue 37. tRNA<sup>asp</sup> and tRNA<sup>t<sub>5</sub>v</sup> (human) contain the t<sup>5</sup>A<sub>12</sub> residue while tRNA<sup>t<sub>3</sub>v</sup> contains the 2-(methylthio)-N<sup>6</sup>-(dimethylallyl)adenosine residue (ms<sup>2</sup>i<sup>5</sup>A<sub>12</sub>). The dimethylallyl group of ms<sup>2</sup>i<sup>5</sup>A<sub>12</sub> in tRNA<sup>t<sub>3</sub>v</sup> could not be observed (Nissen et al. 1999). These crystallized tRNAs have packing interactions involving the anticodons. The tendency of anticodon bases to be non-isolated within the loop and base paired to complementary molecules, as indicated by solution studies (Eisinger 1971, Scarabino et al. 1999), is also supported by their crystal packing interactions. The tRNAs that form anticodon-anticodon interactions include the tRNA<sup>t<sub>3</sub>v</sub> EF-Tu complex, that crystallized forming intermolecular G<sub>18</sub>-C<sub>15</sub> base pairs (Nissen et al 1999); yeast tRNA<sup>asp</sup>, where the G and C of its GUC anticodon are base paired (Moras et al 1986) and methyoryl-tRNA<sup>t<sub>5</sub>v</sub>, where nucleotides C<sub>14</sub>, A<sub>15</sub>, U<sub>16</sub> and A<sub>17</sub> interact between two anticodons forming two C-A and two A-U base pairs (Schmitt et al 1993). In the tRNA<sup>t<sub>3</sub>v</sub> crystal, the anticodon of each tRNA base pairs with the CCA end of a symmetry related molecule (Bénas et al. 2000).

The flexibility of the anticodon stem-loop, deduced from binding of complementary oligonucleotides and NMR, seems to be maintained even in crystallized molecules. The anticodon loop is among the most flexible regions, based on the high temperature factors and low occupancies of
its residues. Formation of a minihelix of base paired anticodons is expected to reduce their temperature factors (Westhof et al. 1988). The temperature factors of the anticodon loop from yeast tRNA^{Phe} are higher than those of tRNA^{ACF}. Anticodon-anticodon base pairing occurs in the two crystal forms of tRNA^{ACF} (Westhof et al. 1988). In the two crystal forms of yeast tRNA^{Phe}, the anticodons have crystal packing interactions to the -CCA end and van der Waals and stacking interactions for residue 34. Not all the anticodon loop residues are equally dynamic or ordered. Residue 37 in E. coli initiator tRNA (Schmitt 1998) and the dimethylallyl group in tRNA^{51} (Nissen et al 1999) are more disordered than the rest of the loop even when the anticodon residues are involved in intermolecular base pairs.

ii. Solution structures

Sundaram et al. (2000) obtained for the E. coli tRNA^{phe} anticodon stem-loop an NMR model that contains a U-turn. The structure of a 15 nucleotide RNA molecule comprising the yeast tRNA^{Phe} fully modified anticodon stem-loop was studied by one dimensional NMR methods. It was concluded that the NOEs were consistent with the molecule containing a U-turn as in the crystal structure. However, due to the limitations of the solution NMR techniques used, relatively few structural constraints were collected and used for the modeling (Clore et al 1984). NMR data indicate that the anticodon stem of full length, fully modified E. coli tRNA^{Phe} extends to the Ψ_{i+1}-A_{i+1} base pair in the presence of Mg^{2+} (Hyde and Ryde 1985-1). This tRNA has two downfield shifted 31P resonances that are proposed to originate from the anticodon loop, and to indicate a backbone conformation similar to yeast tRNA^{Phe} (Hyde and Reid 1985-2).
The anticodon loops of E. coli and yeast tRNA\textsuperscript{thr} have similar \textsuperscript{31}P chemical shift changes in response to cations. Mg\textsuperscript{2+} and spermidine have the opposite effect as increased temperature on the chemical shift of one of the two downfield shifted \textsuperscript{31}P resonances identified in the anticodon loop of E. coli tRNA\textsuperscript{thr}. A similar effect on the \textsuperscript{31}P resonances in the anticodon stem-loop has been seen in tRNA\textsuperscript{phe} (yeast) (Gorenstein and Goldfield 1982).

2.3 Modification of nucleotides within the anticodon stem-loop

i. Modified nucleotide at position 37

Position 37 almost always contains a purine, and in 72.5% of the tRNAs sequenced up to 1998, a modified nucleotide (Auffinger and Westhof 1998-2, Sprinzl et al. 1998, Sprinzl et al. 1999). To illustrate all of the variety of natural nucleotides at position 37, the reader is referred to comprehensive compilations by Auffinger and Westhof (1998-2), and Björk (1998).

Modifications at position 37 can be simple methylations, for example 1-methylguanosine (m\textsuperscript{1}G) or hypermodifications like wibutusine (Y). The modifications may block functional groups that play a role in base pair formation. Residue 37 is modified in more than 95% of the tRNA molecules where it could be involved in a base pair and there could be another intra-loop base pair involving an anticodon residue (Dao et al 1994). Hydrophobic modifications are commonly present at residue 37 when an A-U interaction occurs in the first codon position (Cedergren et al 1981). Thus most tRNAs with an A at position 36 have a hydrophobic
modification at position 37. Hydrophilic modifications of position 37 are present in most tRNAs with a U at position 36 (Björk 1998). t"A and its hypermodification 2-methylthio-N6-threonylcarbamoyladenosine (ms"t"A-) are examples.

A. Functions

A-U base pairs have relatively low stacking energy (Borer et al. 1974). Since most modifications at position 37 follow A or U residues at position 36, a proposed role is the stabilization of anticodon stacking in the 3' direction (Cedergren et al. 1981). Native modifications at position 37 are important for keeping the translation reading frame, translational fidelity and ribosomal frameshifting. For example, undermodified tRNA^Pro containing m^1G._1 instead of Y._1 causes enhancement of -1 frameshift (Carlson et al. 2001). Given their prevalence in cases where base pairing could occur, one role of modifications of residue 37 is proposed to be to prevent it from base pairing, thus keeping an open conformation for the anticodon loop (Dao et al. 1994, Basti et al. 1996, Stuart et al. 2000). The unmodified anticodon stem-loop from yeast tRNA^Pro binds specifically to 30S ribosomes less tightly than the undermodified molecule containing m^1G._1. Tighter binding is attributed to opening of the loop by the methyl group (Dao et al. 1994).

Two model systems have been used to compare the effects of absence and presence of the modifications on the structure of anticodon regions. Those are tRNA^{Lys} from human and E. coli and a DNA analog of the anticodon stem-loop from tRNA^Pro (yeast). In both cases the absence of a modification at position 37 allowed its base to pair, either with base 33, for tRNA^{Lys}, or with base 32, for tDNA^Pro. Crystal structures of yeast tRNA^Pro and tRNA^{Lys} also show the hypermodified residues 37 not base paired (Shi and Moore 2000, Jovine et al 2000, Sénas et al. 2001).
The modified residue t'A₁₋ on the tRNA<sup>⁵'</sup> anticodon stem-loop is able to open the closed loop but by itself does not make this RNA take on a U-turn conformation (Stuart et al. 2000). Instead, the anticodon residues still have a significant C2' endo conformation, not present in the U-turn, and they are not stacked in the 3' direction with residue 37. Residue 34 is oriented to the inside of the helix instead of being at the tip of the loop (Stuart et al. 2000). This structure contrasts with the fully modified anticodon regions of tRNA<sup>⁵'</sup> from E. coli and tRNA<sup>⁵'</sup> from human, which contain a canonical U-turn conformation in solution and in crystal form respectively (Sundaram et al. 2000, Bénas et al. 2000). The other modifications, pseudouridine 39 and 5-methylaminomethyl-2-thioridine 34 or 5-methoxycarbonylmethyl-2-thioridine 34, work together to promote the U-turn conformation by stabilizing the A-form helix and the stacking of residue 34 on 35 (Sundaram et al. 2000, Bénas et al. 2000).

The DNA analog of yeast tRNA<sup>⁵'</sup> with modifications m'<sup>⁵</sup>C₃₋, instead of wybutosine, and m'<sup>⁵</sup>C₅₋, has a U-turn like conformation. The presence of this conformation was determined by theoretical calculation using NMR data (Basti et al. 1996). In the presence of Mg<sup>2+</sup> it is proposed to make a U₃₋-A₄₋ base pair, however it binds specifically to ribosomes. The G₃₋-containing molecule has base pairs C₃₋-G₄₋ and T₃₋-A₄₋ (Basti et al. 1996). Hence the simple methylation is proposed to open this loop. No U₃₋ and G₃₋-containing structure was determined, but interestingly such RNA binds to ribosomes even better than the m'<sup>⁵</sup>G₃₋-containing molecule (Dao et al. 1994). The interpretation of the DNA results is not straightforward with regard to RNA.

B. The dimethylallyl modification
One of the hydrophobic modifications at position 37 is the attachment of a dimethylallyl group to the exocyclic amino nitrogen of A$_1$ to yield $N'-(\text{dimethylallyl})$adenosine (Figure 2.1). In earlier literature the modified nucleotide was called $N'-(\Delta^1\text{-isopentenyl})$adenosine) and was abbreviated i'\text{A}_1$. This abbreviation is still used, to be consistent with the earlier literature, and is used in the present dissertation. i'\text{A} was first purified from yeast and calf tRNAs (Hall 1966). i'\text{A} or its derivatives have been identified at position 37 of most anticodon loops that recognize codons starting with U in bacteria and eukarya (Hall 1970, Persson et al 1994, Björk 1995-2). In eukarya, except plants, i'\text{A}_1$ is the final modified base. In some bacteria the base is further modified to ms'\text{i'A}_1$, and in most other organisms to cis-2-(methylthio)$-N'-(4\text{-hydroxydimethylallyl})$adenosine (ms'\text{i'o'A}_1$) (Persson et al. 1994).

a. MiaA enzyme

The dimethylallyl modification is carried out by the enzyme dimethylallyl diphosphate:tRNA dimethylallyltransferase. This enzyme catalyzes the transfer of the dimethylallyl group from dimethylallyl diphosphate (DMAPP) to the N6 of A$_1$ (Figure 2.1). In E. coli and Salmonella spp., the enzyme is named MiaA and is a 34 KDa protein (Connolly and Winkler, 1991). The structure of the protein is not known. It contains several conserved regions, two of which are speculated to bind the RNA and the DMAPP (Moore and Poulter 1997). One of the regions is analogous to an aspartic acid rich motif DDXXD which is present in other prenyltransferases. This region is hypothesized to bind DMAPP (Moore and Poulter 1997) and two mutations on it increase the $K_m$ of either DMAPP or RNA exclusively. The other region contains a conserved P loop motif, which is characteristic of ATP/GTP binding sequences. In
MiaA this motif is suggested to bind tRNA (Moore and Poulter 1997), but also to bind DMAPP, because the enzyme is competitively inhibited for DMAPP by ATP/ADP (Leung et al 1997) and mutations on the motif decrease catalysis and DMAPP binding (Soderberg and Poulter 2001). Residues in other regions of the protein have been implicated in tRNA binding and in catalysis (Soderberg and Poulter 2001).

MiaA can recognize features in the anticodon stem-loop alone, although is less active than with the full length tRNA. It requires adenosine residues at positions 36 and 38 for binding. Nucleotide 37 can be adenosine or guanosine (Leung et al 1997). MiaA also requires for binding a sequence pattern in the stem of the anticodon stem-loop (Mctorin et al 1997). Base pair 29-41 should be G-C or A-U, base pairs 29-41 or 30-40 should be G-C, base pair 30-40 should be G-C, C-G, or A-U, but not G-U, and base pair 31-39 should be A-U or G-C (Soderberg and Poulter 2000).

b. MiaA mutant phenotypes

MiaA mutants, lacking ms'i'1A1, exhibit a reduction of up to 50% in their growth rate as well as a reduced rate of polypeptide synthesis (Persson et al. 1994). The absence of i'1A1 in fungi slightly affects their growth rate (Janner et al. 1960). The physiological effects of the dimethylallyl group have been studied less than the collective effects with the subsequent modifications in bacteria. However the availability of mutants for the subsequent modifications allowed attributing a reduction of tetracycline resistance to the lack of the dimethylallyl group itself (Taylor et al 1998).

Some phenotypes in bacteria have been attributed to the methylthio group, such as the increased rate of GC to TA transversions (Zhao et al. 2001), and to the hydroxylation of ms'-io'1A1, such as the regulation of
Figure 2.1
Scheme showing the sequential modification of tRNA A$_{37}$ to 2-(methylthio)-N6-(dimethylallyl)adenosine in E. coli.
growth on citric acid cycle intermediates (Persson et al. 1998).

c. Studies of the effect of i\(^\ddagger\)A\(_{1}\)- and its derivatives upon translation

Common systems used to study the translational effects in vivo of MiaA mutations are transcriptional attenuation models. In these models the presence of A\(_{1}\)- instead of ms\(^\ddagger\)i\(^\ddagger\)A\(_{1}\)-, de-represses transcription, thus indicating that there is retardation of translation of the leader mRNA (reviewed in Björk 1995-1). Suppressor tRNAs are affected negatively in MiaA mutants in bacteria (Hargeval et al. 1990, Boudaloun et al 1986, Ericson and Björk 1986, Petrullo et al. 1983) and in fungi (Janner et al. 1980). Part of the physiological effect is due to the absence of the dimethylallyl group. The use of suppressor tRNAs has shown that when i\(^\ddagger\)A\(_{1}\)- is present, there is an increase in the efficiency of translation and a decrease of differences in suppression depending upon the identity of the residues 3' next to the codon (Ericsson and Björk 1991). The later effect is called codon context sensitivity. The dimethylallyl group reduces the codon context sensitivity, which is further reduced on a lesser degree with the subsequent modifications (Haguervall et al. 1990, Persson et al 1994). In vitro translation systems indicate that the i\(^\ddagger\)A\(_{1}\)- and ms\(^\ddagger\)i\(^\ddagger\)A\(_{1}\)- containing tRNAs have increased protein synthesis rate in comparison to the unmodified tRNA, and indicate that the major contributor is the dimethylallyl group (Gefter and Russel 1969). In contrast, thermodynamic and kinetic parameters support the idea that the ms\(^\ddagger\) group is responsible for most of the stabilization given by ms\(^\ddagger\)i\(^\ddagger\)A\(_{1}\)- on base paired complementary anticodons (Houssier and Grossjean 1985).

ms\(^\ddagger\)i\(^\ddagger\)A\(_{1}\)- does not seem to affect the codon-anticodon interaction while in the ternary complex, but enhances proofreading (Diaz and
Ehrenberg 1991). ms\textsuperscript{2}i\textsuperscript{A}_{77} prevents misreading of the CUU codon by E. coli tRNA\textsuperscript{Phe} (Wilson and Roe 1989), although misreading of the CGU codon by tRNA\textsuperscript{G \textsubscript{75}} is unaffected by the absence of ms\textsuperscript{2}i\textsuperscript{A}_{77} (Boudaloun et al. 1983). ms\textsuperscript{2}i\textsuperscript{A}_{77} also affects the translational reading frame. In contrast to the undermodification of Y in yeast tRNA\textsuperscript{Phe}, the absence of ms\textsuperscript{2}io\textsuperscript{A}_{37} in S. typhimurium tRNA\textsuperscript{Phe}_{sRNA} increases -1 frameshifting (Qian and Björk 1997, Schwartz and Curran 1997).

A higher tendency of the modified adenine to stack, is proposed as one of the causes for the effects of the dimethylallyl group in translation (Persson et al 1994, Ericson and Björk 1991). In a model, ms\textsuperscript{2}i\textsuperscript{A}_{77} stacks on the first residue of the codon and on residue 36 of the anticodon. A continuous stack would form in a similar fashion for the anticodon and codon bases, until base 34, which would stack on the residue 3' next to the codon (Ericson and Björk 1991). The differential stacking ability of different nucleotides is invoked as an explanation for the codon context sensitivity (Ericson and Björk 1991).

ii. Modified nucleotide at position 32

Residue 32 is always a pyrimidine, and is modified around 25% of the time (Auffinger and Westhof 1998-2). The residue at position 32 is frequently modified to nucleotides that stabilize a C3' endo conformation, such as 2'-O-methylation and 2-thiolation (Yokcyama and Nishimura 1995) or pseudouridine (Davis 1998).

Pseudouridine 32 occurs in the anticodon of E. coli tRNA\textsuperscript{Phe} and other 46 of the known tRNA sequences (Spinzi et al. 1999). Few structural studies have been done on pseudouridine 32. On the crystal structure of tRNA\textsuperscript{G \textsubscript{75}} in complex with EF-Tu and GDPNP, \Psi_{12} has a syn
conformation (Nissen et al. 1999). This pseudouridine 32 is involved in a bifurcated base pair with A38 (Auffinger and Westhof 1999). Residue 32 appears to be linked to the backbone through a near water molecule hydrogen bonding to its N3. The crystal structure of yeast tRNA$^{Ψ\text{G}}$ shows $Ψ_{11}$ with an anti conformation, forming a bifurcated base pair involving its O4, which is hydrogen bonded to the C11 N4 (Westhof et al. 1985). Similar bifurcated base pairs have been seen in other crystal structures (Auffinger and Westhof 1998-1, Auffinger and Westhof 1999). This hydrogen-bonding pattern stabilizes the U-turn and allows a more open loop conformation than a regular base pair.

2.4 E.coli ACSL$^{Ψ\text{G}}$ as a model to study effects of modifications and cations on RNA

The clustering of modifications in the anticodon region calls up the question of their cumulative effect. Addressing this question requires studying the molecules before and after each modification. The anticodon stem-loop from tRNA$^{Ψ\text{G}}$ (E. coli) contains three modified nucleotide residues, 2-(methylthio)-N'-(dimethylallyl)adenosine at position 37 (ms$^2$I'A$_{37}$), $Ψ_{11}$ and $Ψ_{11}$ (Figure 2.2). Only the individual structural role of residue 39 has been studied previously. It is known to be base paired in a regular Watson-Crick fashion to A$_{11}$ and to stabilize the stem. The anticodon stem-loop is a favorable system to study the effects of those modifications. A 17 residue oligonucleotide with the sequence of the unmodified anticodon stem-loop from E. coli (ACSL$^{Ψ\text{G}}$) can be modified enzymatically in vitro by the enzymes MiaA, that attaches the dimethylallyl group to A$_{11}$ (Connolly and Winkler 1991)
and we found also by RluA, that isomerizes U₃₃ into Ψ₃₃ (Figure 2.3) (Wlezinski et al. 1995). These modification capabilities allowed the synthesis of large amounts of the RNA containing either of the modifications or both (Figure 2.2), and the structural study reported in this dissertation. These reactions can be performed on isotopically enriched RNA oligonucleotides, allowing their study by heteronuclear NMR techniques. Due to its size, ACSL-Phe is an amenable system to study the individual and collective effects of nucleotide modifications through NMR spectroscopy. The structural NMR study of the individual and collective effects of modifications in isotopically enriched RNA, yields much more information than that of unlabeled material and a higher degree of reliability in the results.

Mg²⁺ and other cations are known to be important for the biological function of several RNAs and also seem to stabilize the U-turn (Gorenstein and Goldfield 1982, Sundaram et al. 2000). Given that Mg²⁺ has been proposed to interact with modifications to stabilize the U-turn conformation, the structural effects of Mg²⁺ in ACSL-Phe before and after the dimethylallyl modification were studied in the present work. Mn²⁺ and Co(NH₃)₆⁺⁺ were used as probes for cation binding sites, and the latter ion, as a probe for the effects of polycations in the ACSL-Phe and its dimethylallyl derivative.

2.5 Use of NMR to study structure and dynamics of an anticodon stem-loop
The present study has all the advantages that an NMR study has to offer. No crystals are needed, and the sample is intact after an experiment. The molecule is studied in solution hence crystal packing is not a possible source of artifacts. The study can be performed with samples in different solvent conditions. High concentrations of ions are required to crystallize RNA in order to promote ordered precipitation of the molecule. The need of high salt concentrations prevents the study of the effects of cations that do not bind to specific sites. For NMR samples, the temperature and salt conditions are close to physiological, allowing the study of physiologically relevant conformations of the RNA and their stability. NMR can provide insights into the dynamics of the bases, indicating precisely which bases are relatively more mobile. In some optimal cases NMR can be used to estimate the populations of conformers and the rate of their interconversion directly. Recently NMR has become a powerful tool to prove the presence of some hydrogen bonds directly, by taking advantage of their partial covalent character to transfer magnetization across them. Before these advances, hydrogen bonds were typically inferred from the proximity of donor and acceptor groups in crystal and NMR structures, and on the basis of characteristic NMR chemical shifts.

In the case of the study of base modifications, NMR allows one to examine their effects without the concern that intermolecular interactions in a crystal will affect the structure or dynamics. This is a relevant consideration, since most tRNA crystal structures have anticodons involved in intermolecular interactions given by the crystal packing. This calls up the question of what possible conformations of the RNA exist in the absence of such interactions or modifications. The molecular weight of the anticodon hairpin allows its study by NMR. The base modifications mentioned above have protons that are known to
possess characteristic chemical shifts and should be easily detectable by NMR. Detection of the dimethylallyl group on tRNA\(^{23}\) has not been possible using crystallography, presumably due to disorder in the crystal (Nissen et al. 1999). NMR should be able to identify available conformations of this group even if it is dynamic. In the tRNA\(^{23}\) crystal structure, pseudouridine 32 has unexpectedly a syn conformation and is not making any direct interaction in spite of being present in a U-turn. NMR should be useful technique to figuring out if this is the case in solution or if an imino group is hydrogen bonded. If pseudouridine 32 is involved in a regular base pair, as has been proposed for residues 32-38 of unmodified tRNA\(^{23}\) (Durant and Davis 1999), the use of NMR to detect an intervening hydrogen bond will provide for the first time a proof of a base pairing mode of pseudouridine (either syn or anti). It will also be a practical system to make a detailed dissection of the additive effect of base modifications. The expected characteristics of the U-turn, including non-sequential NOEs, NOEs indicating base stacking in an anticodon, and chemical shift changes for the phosphorus in the turning phosphate, would be evidence of such conformation. Optimally a large number of structural constraints can be obtained and allow the determination of a structure with high accuracy.

This study will enlighten the question of how RNA structure can be finely regulated by targeted chemical modifications. In particular, the additive effects of the anticodon stem-loop modifications and their individual roles are studied. The study makes a dissection of the factors involved in the formation of a U-turn in an anticodon stem-loop: solvent conditions, and modifications in solution. For utilitarian purposes, the study may contribute to understand and possibly design strategies to interfere with the retroviral translational frameshifting, which requires undermodification at residue 37 of tRNA\(^{Flm}\) (Carlson et al.
2001), or for engineering of molecules that can have a desired sequence yet can form a U-turn for binding other nucleic acids, or for designing sequences where this conformation is undesirable. Further, this study indicates under what buffer conditions a U-turn is promoted, which should be useful in cases where maximal binding of oligonucleotides with complementary loop is desired, given the increased tendency for base pairing present in tRNA's anticodons.
Figure 2.2  
Sequence of the fully modified anticodon stem loop from *E. coli* tRNA^{Phe} and the partially modified sequences studied for this dissertation.
Figure 2.3
Top) Scheme showing the modification of U₃₂ to Ψ₃₂ by the enzyme RluA. Bottom) Sequences known to be modified by RluA (Wrzesinski et al. 1995, Raychaudhuri et al. 1999). The U that is pseudouridylated by RluA is indicated by an arrow.
3. OBJECTIVES

3.1 General

Identify effects of base modifications and cations on RNA structure

3.2 Specific

Determine:
1. The structure of the unmodified anticodon stem-loop from tRNA\(^\text{Phe}\) (E. coli) in solution by NMR
2. The structural effect of the dimethylallyl modification at residue 37
3. The structural effect of the pseudouridine modification at residue 32
4. The combined effect of pseudouridine 32 and dimethylallyladencosine 37
5. The effects of Mg\(^{2+}\) and Co(NH\(_3\))\(_4^{3+}\) on the unmodified and dimethylallyl modified molecules

3.3 Hypotheses

tRNAs are molecules that interact with the ribosome. They have conserved sequence features and are expected to take on a similar
conformation that allows them to interact with the translation machinery. Differences in the tRNA structure are necessary in order for them to be recognized specifically by the aminoacyl-tRNA-synthetases. tRNAs also have different anticodon sequences. A way of achieving the result of different sequences taking on a similar structure would be through the modification of individual residues. Hence, modifications may be a way of fine-tuning the structure of RNA molecules.

The many modifications that occur in the anticodon stem-loops presumably stabilize the U-turn and the interaction with the translation machinery. Modifications might be required for a loop of seven nucleotides closed by a base pair to form a U-turn, while still accommodating the sequence variability required in the anticodon. If complementarity exists between residues corresponding to an anticodon stem-loop 32-38 and 33-37, the loop could alternatively form two extra base pairs and a trinucleotide loop, as proposed for tRNA\textsuperscript{\textsc{\textcopyright}} (human) at low pH.

Due to its position at the exocyclic amino nitrogen of A\textsubscript{\textcircled{1}} and its bulkiness, the dimethylallyl modification might prevent pairing of this base and the formation of a closed loop. Additionally, it might increase the stacking ability of A\textsubscript{\textcircled{1}} and promote a U-turn by stabilizing the 3' stacked conformation.

If pseudouridine 32 favors the C3' endo conformation, it would stabilize the A-form conformation around residue 32 and thus its stacking with residue 31. If a U-turn were the preferred conformation, \(\Psi\textsubscript{11}\) in tRNA\textsuperscript{\textsc{\textcopyright}} (E. coli) would be involved in a bifurcated base pair involving its C4 and the NH\textsubscript{6} of A\textsubscript{\textcircled{1}}.

The combination of \(\Psi\textsubscript{11}\) and \(1\textsuperscript{\textcircled{1}}\text{A}\textsubscript{\textcircled{1}}\) could stabilize a U-turn more than each individually. \(\Psi\textsubscript{11}\) would stiffen the 5' side of the loop and form a bifurcated base pair closing the stem, counteracting a possible
destabilization on that side of the loop by an opening effect from i''A\textsubscript{1}-. i''A\textsubscript{1}- would counteract a stabilization of an extended helix by $\Psi_{1\Sigma}$.

Mg\textsuperscript{2+} could stabilize the U-turn, by binding to the anticodon loop in one of the sites defined by X-ray crystallography (Hingerty et al 1978, Shi and Moore 2000, Jovine et al 2000). It may also contribute to the stacking of residue 37 and the stabilization of the backbone conformation, effects proposed for yeast tRNA\textsuperscript{Phe} (Striker et al. 1989, Gorenstein and Goldfield 1982). Co(NH\textsubscript{3})\textsubscript{6}\textsuperscript{3+} would be an adequate probe for an hexahydrated Mg\textsuperscript{2+} binding site.
4. MATERIALS AND METHODS

4.1 Materials

All enzymes were purchased from Sigma Chemical (St. Louis, MO.), except T7 RNA polymerase, His6-MiaA tRNA prenyl transferase, and His6-RluA, which were prepared as described (Uavanloo et al. 1984, Leung et al. 1997). Deoxyribonuclease I type II, pyruvate kinase, adenylate kinase, and nucleotide monophosphate kinase were obtained as powders, dissolved in 15% glycerol, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.4, and stored at -20°C. Guanylate kinase and nuclease P1 were obtained as solutions and stored at -20°C. Unlabeled 5' nucleoside triphosphates (5'-NTPs) were purchased from Sigma. Phosphoenolpyruvate (potassium salt) was purchased from Bachem, and 99% [2-15N]-ammonium sulfate and 99% [6-14C]-methanol were purchased from Cambridge Isotope Labs. 7H-dimethylallyl diphosphate was purchased from Sigma and American Radiolabeled Chemicals. MgCl₂ was purchased from Aldrich with 99.995% purity.

4.2 Biochemical methods.

i. Isotopically enriched oligonucleotides

The oligonucleotide sequences studied are shown on figure 2.2. They correspond to the anticodon stem-loop from E. coli tRNA^{23S}, residues 27-34, with different degrees of modification. Initially the unmodified
sequence was synthesized by in vitro transcription using T7 polymerase and \(^{15}N\) or \(^{13}C\) isotopically enriched ribonucleotide triphosphates. The 5′-NTPs were prepared from E. coli ribosomes as described (Nikonowicz et al. 1992). These molecules were modified enzymatically as described below.

The in vitro transcription reactions included synthetic oligonucleotides or linearized plasmids as templates (Batey et al. 1992, Nikonowicz et al. 1992). The plasmid sequence used as a template for ACSL\(^{59}\) was the Sma I-digested pTX539 (Leung et al. 1997). ACSL\(^{59}\) sequences were synthesized separately using 3 mM \(^{15}C\), 3 mM \(^{15}N\) labeled, or 5 mM unlabeled 5′-NTPs in 7-15 ml reactions. Typically the reaction conditions were as follows: 40 mM Tris pH 8.0, 25 mM MgCl\(_2\), 1 mM spermidine, 0.01% Triton, 5 mM DTT, 3 mM NTPs, 1 μM oligonucleotide template or 0.2 mg/ml plasmid template, 30 μg/ml T7 polymerase. 10 mM GMP was sometimes used to increase efficiency.

Sequences for two different CUUG tetraloop containing RNA hairpins, RNA-I (5′ GGCGCUUGCGCC 3′) and RNA-I\(^{5′}\) (5′ GGCGCUUGCGUC 3′), were synthesized using a synthetic template.

Purification of the RNA molecules was achieved through electrophoretic separation of the transcription products on 20% (w/v) preparative polyacrylamide gels. The bands corresponding to the products of interest were visualized by UV shadowing, excised from the gel and electroeluted (Schleicher & Schuell), and then ethanol precipitated. Ethanol precipitated pellets were dissolved in 1.0 M NaCl, 20 mM potassium phosphate, pH 6.8, and 2.0 mM EDTA, and dialyzed extensively against 10 mM NaCl, 10 mM potassium phosphate, pH 6.8, and 0.05 mM EDTA, using Centricon-3 concentrators (Amicon Inc.). The sample volumes were adjusted with the latter buffer to a volume of 200 μl and dried by lyophilization.
The dialysis conditions for RNA-I and RNA-T" were similar except that the second buffer used was 2.5 mM sodium phosphate pH 6.8, 0.1 mM EDTA to favor hairpin formation or 100 mM NaCl, 2.5 mM sodium phosphate pH 6.8, 0.1 mM EDTA, to favor duplex formation. For these samples the final volume was 500 μl.

For experiments used to observe the non-exchangeable protons, the lyophilized pellets were resuspended in 99.9% \textsuperscript{2}H\textsubscript{2}O and dried twice, and finally resuspended in 200 μl of 99.96% \textsuperscript{2}H\textsubscript{2}O. For experiments used to observe exchangeable protons, the pellets were resuspended in 200 μl of 90% \textsuperscript{2}H\textsubscript{2}O / 10% \textsuperscript{2}H\textsubscript{2}O. The samples contained ~50-130 A\textsubscript{260} OD units in 200 μl (~1.7-4.3 mM) for the ACSL\textsuperscript{Fm}, 75 to 100 OD units for the i\textsuperscript{A},\textsuperscript{J} containing ACSL\textsuperscript{Fm} (i6A37-ACSL\textsuperscript{Fm}) or Ψ\textsubscript{J} containing ACSL\textsuperscript{Fm} (Ψ32-ACSL\textsuperscript{Fm}) (2.5-3.4 mM), and 30 and 60 OD units of the \textsuperscript{13}C and \textsuperscript{15}N labeled i\textsuperscript{A},\textsuperscript{J}-Ψ\textsubscript{J} containing ACSL\textsuperscript{Fm} (i6A37Ψ32-ACSL\textsuperscript{Fm}) respectively. Immediately before collecting spectra the samples were annealed by warming to 65-80°C for 1 min and chilling on ice.

Addition of cations was done by titration of concentrated solutions of MgCl\textsubscript{2} (0.5M) or Co(\textsubscript{2}NH\textsubscript{3})\textsubscript{6}\textsuperscript{3+} (0.1M) into the NMR samples. To obtain the Mg\textsuperscript{2+} "saturated" form, the MgCl\textsubscript{2} was added to the samples to a concentration of 20mM and they were then dialyzed extensively with 5-10 mM MgCl\textsubscript{2} containing buffer. MnCl\textsubscript{2} was titrated in to final concentrations of 5 to 50μM.

ii. Recombinant protein purification

The enzymes, MiaA (dimethylallyl diphosphate:tRNA transferase) and RluA were overproduced from E. coli strains TX3371 and BL21(DE3)/p1uA" ///pET15b-rluA" (Leung et al 1999, Wrzesinsky et al 1996). Both are
expressed from pET plasmid vectors as fusions with a His-tag, which allows their purification in a single step by affinity to a Ni\(^2\) column (Leung et al. 1999).

The MiaA protein was also produced with \(^{15}\)N enrichment, with the objective of studying it spectroscopically by NMR. Enrichment was achieved through growth of the overproducing strain on minimal media containing 99\% \(^{15}\)N-ammonium sulfate as the sole nitrogen source in media as described for production of \(^{15}\)N labeled NTPs (Nikonowicz et al. 1992). The cells were initially grown overnight on -20 ml of LB medium with 100ug/ml ampicillin, centrifuged and washed once in minimal media. They were resuspended and grown in labeled minimal media with 60 \(\mu\)g/ml carbenicillin and incubated until they reached 0.5 OD\(_{600}\) (-20 hours). They were induced for 4-5 hours with 5 mM IPTG, harvested and kept frozen at -20°C until protein purification.

iii. Modifications of RNA

A. Dimethylallyl modification

RNA was enzymatically modified using MiaA. The modified molecules were 100 OD\(_{260}\) units of \(^{15}\)N labeled ACSL and 75 OD\(_{260}\) units of \(^{13}\)C labeled ACSL\(^{\text{DMAPP}}\). The reaction was done according to Leung et al. (1999) except that 2-mercapto ethanol was substituted for DTT. The DMAPP:RNA ratio was \(\approx 2.5\). The reaction conditions were 50mM Tris-HCl pH 7.6, 10mM MgCl\(_2\), 50 \(\mu\)M ACSL, 0.1 Mg/ml BSA, 180\(\mu\)M DMAPP, 5mM 2-mercapto ethanol, 26 \(\mu\)g/ml MiaA. The reactions were incubated overnight at 30°C. The RNA was purified by extraction with phenol:chloroform:isoamyl alcohol and chloroform (Sambrook et al. 1989) followed by ethanol precipitation. The reactions were monitored by the shift in electrophoretic migration.
of the modified molecule on 20% acrylamide-7M urea gels. The modified RNA migrates with a band shift of 1 additional nucleotide.

S. Pseudouridylation

The reaction conditions were scaled up from the reported conditions from Mrzesinsky et al. (1996). To ensure completion of the reaction an excess of the RluA enzyme was used, with a molar ratio of 1:48 enzyme to RNA. The reaction conditions were 50mM HEPES pH 7.5, 100 mM NH₄Cl, 0.03mg/ml RluA, 0.347 mg/ml ACSL₃⁺ or Δ6A37-ACSL₃⁺. The incubation was done overnight at 37°C. The RNA was purified from the protein by heating the reaction at ~80-95°C, which causes precipitation of RluA, followed by centrifugation. The supernatant was dialyzed with NMR buffer. Completion was estimated using NMR, either through COSY or 2D ³¹C-¹H HMQC, by the disappearance of the H5 resonance of U₅ (Figure 2.3).

4.3 Structural methods

i NMR spectroscopy

NMR spectra were acquired on a Bruker AMX-500 spectrometer equipped with a ¹H-(¹³C, ¹⁵N) triple resonance probe, except for the ³¹P-decoupled ¹³C-¹H constant time (ct) HSQC experiment, for which a ¹H-(¹³C, ³¹P) triple resonance probe was used. GARP broadband decoupling was used for ¹³C and ¹⁵N with γB₂ = 3125 Hz and γB₁ = 1570 Hz respectively. Quadrature detection was achieved using the States-TPPI method. Acquisition was delayed by a half-dwell in all indirectly detected
dimensions. Spectra of 90% $^2$H$_2$O samples were collected at 12°C
suppressing the solvent peak with spin lock pulses or binomial $1 \tilde{I}$ or
$1 \tilde{I}^3 \tilde{I}$ read pulses with maximum excitation at 12.5 ppm (Hore 1983).
For spectra of 100% $^2$H$_2$O samples the residual HDO was suppressed using
presaturation or spin lock pulses. Typically the data were apodized
using 1 Hz line broadening and 65 degree shifted sinebell functions and
the data points in the indirectly detected dimensions were extended 25%
by linear prediction (Evans 1995). $^1$H spectra were referenced to the
internal HDO resonance (4.75 ppm at 25°C). The $^{13}$C, $^{15}$N, and $^{31}$P spectra
were referenced to external standards of TSP, NH$_4$OH, and TMP,
respectively, defined as 0.30 ppm. Spectra were processed and analyzed
with Felix 98.0 (Molecular Simulations, Inc.).

The strategy used to assign the resonances consists in finding
spin systems belonging to each type of nucleotide residue and deducing
their position in the sequence through NOE connectivities. Most nuclei
within the residues have typical frequencies that allow their
identification, particularly the H1' and base resonances which are well
resolved. These were used as starting points for the identification of
the spin systems involving non-exchangeable protons. The H1' and base
resonances $^1$H/$^{13}$C chemical shifts were correlated using 2D $^{13}$C-$^1$H HMQC and
HSQC spectra. The remaining ribose resonances were correlated to the
H1'/$^{13}$C1' resonances using 2D HCCH-COSY and 3D HCCH-TOCSY (24 ms DIPSI-3
spin lock) experiments optimized for polarization transfer through the
ribose carbons (Pardi and Mikolowicz 1992). The H8/C8 and H2.C2
resonances of the adenines were correlated using a 2D 13C-1H HCCH-TOCSY
(52 ms DIPSI-3 spin lock) for polarization transfer through the adenine
bases (Legault et al 1995). Intraresidue correlations between the base
and the ribose resonances were obtained for the H1' and H6/H8 to N1/N9
through 2D $^{15}$N-$^1$H HSQC spectra optimized for two- and three-bond
couplings. The experiments were acquired as reported (Sklenar et al. 1994) except that no gradients were used, and the $^{14}$N-$^1$H anti-phase magnetization after t₁ was refocused to obtain in-phase cross peaks in ω₁ and ω₂, and to allow use of broad band $^{14}$N decoupling at t₂.

The assignment of exchangeable resonances also relies initially on the characteristic chemical shifts of the $^1$H/$^{14}$N pairs of each base. These shifts can further indicate whether the imino or amino groups are involved in base pairing (Varani et al. 1996). Sequence specific assignment is made using NOESY crosspeaks among these protons. The 2D $^{14}$N-$^1$H chemical shift correlations for amino protons were identified through $^{14}$N-$^1$H HSQC spectra, with acquisition time 40 ms. The imino proton correlations were identified using $^{14}$N-$^1$H HMQC spectra with $^{14}$N carrier at 150 ppm and acquisition times of 36-50 ms. For unambiguous identification of A-U base pairs, $^3$J$_{HN}$ HNN-COSY spectra, optimized for A H2 to U N3 correlations, were collected as described (Henning and Williamson 2000) with samples in $^2$H₂O.

The main sources of information for NMR structure calculations are the distance constraints between pairs of protons. These are obtained from NOESY spectra, which give crosspeaks between resonances that transfer magnetization through space. The intensity of the peaks is approximately inversely proportional to the sixth power of the distance between pairs of protons (Varani et al. 1996). The proportion is not exact because only applies to atoms with the same correlation time. Internal motions, spin diffusion, base line distortions, and differential excitation of frequencies in some experiments, can change the intensity of an NOE crosspeak (Varani et al 1996, Wijmenga and van Buuren 1998). The distance constraints for the non-exchangeable resonances of the unmodified molecule were derived from 2D $^1$H-$^1$H NOESY spectra collected at 25-28°C for the unmodified molecule (120, 180, 360,
480 and 520 ms mixing times). Similar spectra were collected for the pseudouridylationd ACSL\textsuperscript{Fpr}. 2D \textsuperscript{1}H-\textsuperscript{1}H NOESY spectra of the dimethylallyl modified ACSL\textsuperscript{Fpr} were collected at 25\textdegree C (400, 500 ms mixing times) and at 12, 18 and 31\textdegree C (500ms mixing time). Heteronuclear spectra were also collected. These were: \textsuperscript{13}C-edited 2D HMOC-NOESY (460 ms mixing time), \textsuperscript{13}C-edited 3D NOESY-HMOC spectra (180 and 300 ms mixing time), \textsuperscript{13}C-edited 3D HMOC-NOESY (380 ms mixing time) and \textsuperscript{13}C-edited 3D NOESY-ctHSQC spectra (80 and 180 ms mixing time) optimized for the ribose or base resonances. Distance constraints for the exchangeable protons were derived from \textsuperscript{1}H-\textsuperscript{1}H NOESY spectra with a 180 ms mixing time in 90\% \textsuperscript{1}H\textsubscript{2}O.

Three-bond coupling constants for \textsuperscript{1}H-\textsuperscript{1}H, \textsuperscript{31}P-\textsuperscript{1}H, and \textsuperscript{13}C-\textsuperscript{31}P, were measured from the following experiments in order to estimate torsion angles. A \textsuperscript{31}P-decoupled DQF-COSY (for homonuclear H-H couplings) and a 2D HetCor (for \textsuperscript{1}H-\textsuperscript{31}P couplings) were collected in \textsuperscript{1}H\textsubscript{2}O using unlabeled RNA samples. A pair of 2D \textsuperscript{13}C-\textsuperscript{1}H ctHSQC experiments with and without \textsuperscript{31}P decoupling in \textit{t}\textsubscript{1} was used for measurement of three-bond \textsuperscript{31}P-\textsuperscript{13}C coupling constants (Legault et al. 1995).

\textsuperscript{13}C \textit{T}_{\text{1}} relaxtion times were measured using a series of 2D \textsuperscript{13}C-\textsuperscript{1}H ctHSQC based experiments (Yamazaki et al. 1994) separately optimized for the C2, C6/C8 and C1\textquotesingle regions. The \textsuperscript{13}C carrier positions were at 152.65 ppm for C2 and 138.4 for C6/C8. For C1\textquotesingle, three sets of experiments with the \textsuperscript{13}C carrier positions at 90.2 ppm, 90.2 ppm and 87.9 ppm were collected for ACSL\textsuperscript{Fpr} and one with the carrier at 87.2 ppm for the i6A37-ACSL\textsuperscript{Fpr}. A 2.3 kHz \textsuperscript{13}C spin lock field with delays of 4, 8, 12, 16, 24, 60 and 20 ms was used for the C6/C8 and C2 measurements, and 4,8,12,16,24,44,60,80 and 120 for the C1\textquotesingle measurements. The error for the measured intensities was estimated by collecting the 12 and 24 ms experiments twice, except for the C1\textquotesingle measurements of the ACSL\textsuperscript{Fpr}, where three complete sets of experiments were collected.
ii. Structural constraints

Semiquantitative distance constraints were estimated from crosspeak intensities of the 2D and 3D $^{13}$C edited NOESY spectra for ACSL™. Crosspeaks from non-exchangeable spectra were classified by intensity and assigned corresponding upper bound constraints as very strong, 3 A; strong, 4 A; medium, 5 A; weak, 6 A; and very weak, 7 A, using the pyrimidine H5-H6 (-2.46 A) and ribose H1'-H3' (-3.1 A) crosspeaks as references. Lower bound limits were set to 0 A except in the few cases where a very short distance was considered unlikely in the calculated structures. The very strong category was not used for peaks resolved only on 3D spectra or exchangeable $^1$H spectra. Only crosspeaks not seen at 180 ms or less were assigned to the very weak category. No intra-residue ribose-ribose distances were constrained. Exchangeable proton distance constraints were also estimated from NOESY spectra as medium, weak or very weak, with the exception of those of A H2-U NH3 and G NH1-C NH4, which were classified as medium (4 A). These NOE crosspeaks were indicative of A-U and G-C Watson-Crick base pairs respectively. These base pairs were also identified by the characteristic chemical shift of U and G imino $^1$H and $^2$N. Additionally A-U base pairs were confirmed through the $^1$JHN, HNN-COSY. The distance constraints for hydrogen bonds in base pairs were 2.9±0.3 A between donor and acceptor heavy atoms and 2.0±0.2 A between hydrogen and acceptor atoms, with the exception of the loop closing base pair, which was given a 1A larger upper constraint.

Ribose pucker was determined by measuring the $^1$JHN, HC from $^{31}$P-decoupled 2D DQF-COSY spectra. Couplings >7Hz were considered indicative
of C2' endo conformation, while those <5Hz of C3' endo conformation (Varani et al. 1996). Other evidence for sugar puckering includes the $^3J_{H_3, \cdot H_5}$ couplings (<5Hz for C2' endo), the C3' chemical shifts (~76-80ppm for C2' endo, 70-72ppm for C3' endo), and the C4' chemical shifts (~85-86ppm for C2' endo, 82-84ppm for C3' endo) (Santos et al. 1989). Residues with intermediate $^3J_{H_3, \cdot H_5}$ coupling and chemical shifts were left unconstrained.

$\gamma$ torsion angles (Figure 4.1) were estimated from a DQF-COSY and a 75ms 3D NOESY-HMQC. The $\gamma$ torsion angle can be constrained to a gauche$^+$ conformation if $^1J_{H_4, \cdot H_5}$ and $^1J_{H_4, \cdot H_5'}$ are <5Hz, which was considered the case if H4'-'H5' and H4'-H5'' were clearly absent from the DQF-COSY spectrum (Varani et al. 1996). The $\gamma$ torsion angle was considered trans and gauche$^-$ for residues with $J_{H_4, \cdot H_5}$ and $J_{H_4, \cdot H_5'}$ >5Hz and unequal H4'-H5' and H4'-H5'' crosspeak intensities in the 3D NOESY-HMQC. If $^1J_{H_4, \cdot H_5}$ and $^1J_{H_4, \cdot H_5'}$ were weakly detectable or not detectable and H4'-H5' and H4'-H5'' were of different intensities, $\gamma$ was not constrained because conformational averaging might exist.

$\beta$ torsion angles (Figure 4.1) were estimated from $^1J_{P, \cdot H_5}$ and $^1J_{P, \cdot H_5'}$ from 2D $^{13}$P-'H HetCor spectra (Varani et al. 1996). If both sets of crosspeaks were absent, couplings would be <5Hz and $\beta$ was constrained to trans. If both sets of crosspeaks were present, $\beta$ was not constrained because the $H_5'$ and $H_5''$ were not stereospecifically assigned.

$\epsilon$ torsion angles (Figure 4.1) were estimated from $^1J_{P, \cdot H_3}$ and $^1J_{P, \cdot H_3'}$ measured from 2D $^{13}$P-'H HetCor and 2D $^{13}$C-'H ct-HSQC (Legault et al. 1994) respectively. $^1J_{P, \cdot}$ was calculated from the fractional difference between the intensity of each H2'/C2' crosspeak in a $^{13}$C-'H ct-HSQC, and the intensity of its corresponding crosspeak in a $^{13}$P decoupled $^{13}$C-'H ct-HSQC ($\Delta S$), using the equation $^1J_{P, \cdot} = [\cos^{-1}(1-(\Delta S \pm \Delta S))]/(\pi \cdot ct)$. The error, $\Delta S$, is estimated as the largest value of ±2 standard deviations.
**Figure 4.1**
Top: Schematic representation for the backbone torsion angles of nucleic acids. Bottom: Backbone torsion angle values for A-form helix RNA and B-form helix DNA.
either from the ∆S values for H1'/Cl' crosspeaks (which are not coupled to 31P) or from three ∆S from different sections of each H2'/C2' crosspeak. If 1J P, H1 > 5Hz and 1J P, Cl < 5Hz, the angle was constrained trans (210±40°), and if 1J P, H1 > 5Hz and 1J P, Cl > 5Hz, it was constrained to gauche^- (260±40°). For residues whose error allowed a 1J P, Cl upper estimate >5Hz and a lower estimate <5 Hz, the ⅃ constraint included both trans and gauche^- conformations (235±65°) (Varani et al. 1996).

α and ζ torsion angles (Figure 4.1) were estimated from 31P chemical shifts (Gorenstein 1984). A trans conformation was loosely excluded for both angles if the involved phosphorus resonance did not show a large (=1-2 ppm) downfield shift from the main cluster, with the exception of residues 33-37, which were left unconstrained.

The terminal 5' monophosphate also resonates downfield from the main cluster. Calf intestinal alkaline phosphatase was used to remove the terminal phosphate in order to unambiguously identify it. The digestion was performed at 0.5 U/nmol (3.5 U/OD) of AC5L, according to standard protocols (Sampbrook et al. 1989).

iii. Structure calculations.

Model structures were calculated using X-PLOR 3.8 (Brünger, 1992) on Silicon Graphics O2 computers. Initial structures (60) were generated by randomizing the backbone torsion angles. These structures were subjected to three rounds of restrained molecular dynamics: global fold, refinement, and final minimization. The global fold was done including torsion angle constraints only for the sugar pucker. It consisted of 1000 cycles of unconstrained minimization, restrained molecular dynamics for 10 ps at 1000 K without applying a Van der Waals energy term, 9 ps
with gradual introduction of Van der Waals forces, and 14 ps lowering 25 K every 0.5 ps, and finally, a 1000 step minimization at 300 K. The refinement consisted of a constrained minimization without torsion angle constraints, constrained molecular dynamics for 2 ps at 1200 K with a 10 kcal/0.5 ps increase in torsion angle constraint energy, 4.5 ps lowering 25 K every 0.125 ps, a restrained minimization, 5 ps of restrained molecular dynamics and a 1000 step minimization at 300 K. The final minimization consisted of 1000 cycles of conjugate gradient restrained minimization. The final structures were analyzed using Insight 98 (Molecular Simulations, inc.).

For X-PLOR calculations of 32-ACSL\textsuperscript{phe}, the structure of pseudouridine was defined in the topology and parameter files editing uridine as a template. The experimental bond lengths and bond angles from the C1'-C5 bond and the base of the pseudouridine crystal structure (Hempel et al. 1997) were entered. Other variables for the simulation were analogous to those of uridine.
5. RESULTS

5.1 Distinguishing RNA duplex and hairpin forms

Nucleotide sequences that can form a hairpin by Watson-Crick complementarity, can also form a duplex. When the study of the ACST\textsuperscript{ph} started, it became apparent that extra base pairs other than the ones expected in a U-turn were manifested in the spectra. Namely two extra A-U base pairs were identified, since three $^1\text{H}/^1\text{N}$ imino crosspeaks were present in HMQC spectra with chemical shifts characteristic of Watson-Crick base paired uridines (Figure 5.1.1). This raised the possibility that these extra resonances were due to the formation of a duplex form of the molecule. The spectra commonly used for structural studies can not be used to distinguish between duplex and hairpin forms of a molecule, because the peak patterns for the bulges in duplexes and the loops in hairpins, are not typical for either. Both duplex and hairpins forms of a sequence would have the same residues in non-helical conformation flanked by a helical region. There are methods available for detection of duplex formation of oligonucleotides through NMR. They are based on the detection of diagnostic NOEs in mixtures of labeled and unlabeled molecules, relying either on $^1\text{N}$ filters or crosspeak splitting patterns (Aboul-ela et al 1994, Sich et al 1996). These methods are relatively insensitive and depend on adequate resolution to yield clear results.
Figure 5.1.1
Imino region of a "N-H HMOC of ACSL" in 10% D.O at 12°C.
We devised a technique that allowed us to detect the formation of duplexes through the use of a $^{15}$N-$^1$H 2D HMOC spectrum, which is much more sensitive than NOE spectra. The method is illustrated in Figure 5.1.2. It relies on the formation of a G-U wobble base pair between the $^{15}$N labeled native sequence and an unlabeled probe sequence. This sequence has a C to U substitution in the stem, and forms a wobble base pair. The C and U imino resonances in the wobble pair have characteristic chemical shifts of 10-12ppm for the G and 11-12ppm for the U, that are resolved from the standard base paired iminos of G at 12-13.5ppm and U at 13-15ppm. The appearance of the new G resonance in the HMOC spectra indicates the formation of the heterodimer, thus indicating that the molecules can form duplexes in the tested conditions. The G-U wobble pair also forms in the probe molecule hairpin, but since it is not $^{15}$N labeled, its resonances will not appear in the 2D $^{15}$N-$^1$H HMOC. The expected ratios for the $^{15}$N-$^1$H, $^{15}$N-$^2$H and $^{15}$N-$^3$H dimers are 1:1:2 if the C to U substitution does not greatly affect the stability of the dimer.

The technique was tested with a molecule known to form a hairpin or a duplex depending on the salt concentration. RNA-I (Figure 5.1.3) forms a hairpin in the absence of NaCl, and a duplex in 0.1 M NaCl (Abou-elia et al. 1994, Jucker and Pardi 1995). The sequence RNA-I and the probe RNA-I" (Figure 5.1.3) were synthesized using $^{15}$N labeled and unlabeled nucleotide triphosphates, respectively (Jucker and Pardi 1995). Imino $^1$H-$^{15}$N HMOC spectra were collected for 0.4 mM RNA-I in the absence of NaCl. The spectrum is shown in Figure 5.1.4A. Three G imino $^1$H/$^{15}$N resonances are present in the region of Watson-Crick base paired G's. Assignment through NOESY spectra indicates that G8 and G10
Figure 5.1.2
Schematic representation of the chemical shift method for detection of duplex form of oligonucleotides. Labeled strands are represented by solid lines, a striped line represents the unlabeled probe. Left) Expected G imino spectra $^1$$N$-$^1H$ HMQC for base paired residues for either hairpin or duplex forms of molecule. Right) Expected G imino spectra for the duplex between a labeled strand and an unlabeled C$\rightarrow$U substituted probe. The imino crosspeak of the shaded G is represented by a solid circle.
Figure 5.1.3
Sequences of RNAs used to test duplex formation. A) RNA I hairpin form. B) Duplex between RNA I and U₁, substituted RNA I. C) ACSLᵖʰᵉ and the U₁₄₂ probe.
Figure 5.1.4
2D $^1$N-$^1$H HMBC spectra in 90% H$_2$O optimized for the imino regions of: A) 0.4 mM RNA 1 in absence of NaCl. B) 0.4 mM each of RNA 1 and RNA 1'' in absence of NaCl. C) 0.4 mM each of RNA 1 and RNA 1'' in presence of 0.1 M NaCl. D) 0.078 mM each of RNA 1 and RNA 1'' in presence of 0.1 M NaCl.
E) 1D imino $^1$H spectrum of 0.4 mM each of RNA 1 and RNA 1'' in the absence of NaCl. Resonances clearly belonging RNA 1'' are marked by arrows. The temperature during acquisition was 12 °C.
crosspeaks are overlapped. Addition of an equimolar amount of RNA-\textsuperscript{15}N does not lead to the appearance of the G imino in a G-U wobble pair, a result consistent with the presence of the hairpin form in absence of NaCl (Figure 5.1.4B). This is confirmed by 1 dimensional proton spectra, that shows the presence of the G and U imino resonances in wobble pairs originating from the unlabeled RNA-\textsuperscript{15}N hairpin (Figure 5.1.4E). Addition of NaCl to a concentration of 0.1 M leads to the appearance in the HMQC of a crosspeak at a $^1$H chemical shift of 11.5 ppm (Figure 5.1.4C). This is the expected G NH crosspeak for a G-U base pair, indicating that a heterodimer has been formed. A labeled G$_i$ is base paired with the unlabeled U$_{\text{15}N}$ of the probe. In the spectrum, the G$_i$ and G$_i$ crosspeaks are doubled corresponding to RNA-\textsuperscript{15}N in homo- or hetero-dimer forms. The new crosspeak for G$_i$ is due to its proximity to the G$_i$-U$_{\text{15}N}$ wobble pair in heterodimers. The intensities of the G$_i$ resonance pair were used to estimate the relative population of homodimer to heterodimer as $\sim$1:2:1. The larger population of homodimers is presumably due to the higher stability of the G-C pair compared to a G-U pair.

The experiment is highly sensitive since the new crosspeak is detected in a five-fold diluted sample with 0.1 M NaCl. (0.078mM RNA-I) (Figure 5.1.4D). If the chemical shifts of the monomer and dimer forms are resolved, our chemical shift method can be used estimate the relative populations of monomer and dimer forms of the molecule by quantifying crosspeak intensities of each form. In the diluted sample a mixture of the two forms of the molecule was evidenced by the presence of crosspeaks corresponding to each. The relative population was estimated by comparing the sum of the two G$_i$ imino crosspeaks of the dimer forms to the G$_i$ crosspeak of the monomer. The two intensities were almost equal, indicating that RNA-I was almost equally distributed between duplex and hairpin forms. Since the duplex is half heterodimer,
the concentration of the detected G₁-U₁₁ G imino crosspeak is around 1/4 of the labeled RNA, ~20 μM. This illustrates the sensitivity of the experiment and its ability to identify and quantify the formation of duplex forms in duplex-hairpin mixtures. The concentration of the heteroduplex (20μM) would be undetectable with the other NMR methods (Aboul-Ela et al 1994, Sich et al 1996).

The RNA probe can contain a substitution other than C to U in order to make the diagnostic pair, if it forms a heteroduplex. In the case of a sequence that contains a G-U pair in a stem, the probe can have a U to C substitution and be 15N labeled. The original sequence would be unlabeled. Duplex formation is detected by the presence of a G in a G-U wobble pair in a way analogous to the technique described above. If the C containing probe is unlabeled and the U containing molecule is 15N labeled, the result would be the presence of a new G imino crosspeak in a G-C base pair, and a concomitant reduction of the G imino crosspeaks of the G-U wobble pair. These two crosspeaks would be 1/2 of the expected intensity of the U imino of the G-U pair. Another variation of the method would be the use of a probe where an A involved in an A-U pair in a stem, is substituted for a G. Again a G or U wobble resonance, depending on which molecule is 15N labeled, would indicate heteroduplex formation. Similarly to the G imino, the imino group from a U in a G-U wobble pair also has characteristic upfield 1H and 15N upfield shifts. Hence, this chemical shift would also be a useful probe in the same way described for the G imino resonances.

The new technique was used to confirm that the ACSL₉⁺ forms a hairpin. C₁₁ was substituted with a U in an unlabeled probe (Figure 5.1.3) and mixed with 15N labeled ACSL₉⁺ in equimolar amounts to a total concentration of ~2mM (1mM each). The HMQC spectrum was essentially identical to the spectrum of the ACSL₉⁺ alone; no G imino in a wobble
base pair was observed, indicating that the molecule is a hairpin in the conditions tested (Figure 5.1.5). The result suggests that the three downfield U iminos correspond to base pairs A$_{13}$-U$_{13}$, U$_{13}$-A$_{14}$, and U$_{12}$-A$_{14}$, the latter two are not present in a classical U-turn (Kim et al. 1974, Robertus et al. 1974, Hingerty et al. 1978).

Formation of a duplex in the presence of Mg$^{2+}$ was also tested. After dialysis with buffer containing 5 mM Mg$^{2+}$, a broadening of resonances occurred, most notably in NOESY spectra. This could be due to non-specific aggregation of the molecules due simply to the presence of the divalent metal, or to the formation of dimers. Both cases would increase the correlation time of the molecule leading to line broadening. Dialysis of the mixture of AC5 for the probe with MgCl$_2$ containing buffer followed by collection of HMQC spectra showed an extremely small crosspeak which could be due to formation of the G-U wobble pair (Figure 5.1.6). This hypothesis was confirmed by annealing of the molecule, (heating in a water bath at 95°C for 5 minutes followed by cooling over 1 hour to room temperature). The intensity of the new peak increased relative to G$_{13}$ paired with C$_{14}$, suggesting that it is G$_{14}$ paired with U$_{14}$ of the probe. Quantification indicates that only ~1/16 of the molecules are in duplex form in conditions used for NOESY spectra. Hence the general broadening seen with Mg$^{2+}$ is due to non-specific aggregation. The peaks of the NOESY experiments arise from the monomer form, because the dimers are in low proportion, and have a longer correlation time, which would act together with the non-specific Mg$^{2+}$ broadening and the low sensitivity of the NOESY to hinder their detection.

In conclusion the chemical shift method is able to detect formation of the duplex form of oligonucleotides with high sensitivity unambiguously. Additional advantages over the existing NOE based methods
Figure 5.1.5
2D $^{15}$N-$^1$H HMQC in 90% H$_2$O optimized for the imino regions of: A) 1 mM each of ACSL$^{13}$" and ACSL$^{13}$"-U$_6$ probe. B) 1mM ACSL$^{13}$". The temperature during acquisition was 12°C.
Figure 5.1.6
Imino region from a 13N-1H HMBC spectrum of a 1mM mixture of ACSL* and ACSL*- U, dialyzed with 5mM MgCl2. The crosspeak indicated by an arrow is from a G in wobble pair with U, from duplex. Its intensity indicates the duplex in presence of Mg2+ is very scarce. The temperature during acquisition was 12 °C.
used to detect duplex formation include shorter acquisition time and reduced spectral overlap. The new method is also potentially useful as an accurate quantification tool of duplex and hairpin content.

Summary

A method that detects the formation of duplex forms of oligonucleotides by NMR was devised. It consists of the use of an unlabeled RNA probe with a substitution of a U for a C in a stem region that base pairs with G in the original sequence either as hairpin or duplex. When mixed with a $^{15}$N labeled RNA, a G-U wobble base pair would form between the unlabeled probe U-containing and the labeled RNA if a duplex formed. The G imino crosspeak involved in the wobble base pair is identified by its characteristic chemical shift using a 2D $^{15}$N HMQC spectrum, indicating formation of the duplex. The effectiveness and sensitivity of the method was confirmed by applying it to a molecule known to form a duplex or a hairpin depending on the salt concentration. The formation of the duplex form was detected at a concentration as low as 20 μM. The method was used as a tool for quantification of duplex and hairpin content in this mixture. The method was applied to ACSL$^{\text{fix}}$; a probe with a C$^1$ to U$^1$ substitution was used with negative result. Hence ACSL$^{\text{fix}}$ is predominantly a hairpin in the NMR conditions.
5.2 Unmodified ACSL structure

As mentioned on chapter 2, two NMR structural studies on the effects of modifications have been performed on anticodon stem-loops. Only one unmodified structure has been determined at atomic resolution, but several base pairing assumptions were not proven. In order to determine the structure of an unmodified ACSL molecule and the cumulative effects of modifications, a structure of the unmodified ACSL was determined initially. The ACSL sequence was synthesized by in vitro transcription with $^{15}$N or $^{13}$C and studied through heteronuclear NMR.

i. Spectral assignments

The determination of RNA structure through NMR was accomplished in the following way (Figure 5.2.1A). The strategy consists in finding the resonance frequency of each $^1$H and of the directly attached heteronucleus if possible. $^{15}$N and $^{13}$C nuclei are NMR active and therefore are used as heteronuclei for this purpose. The process of determining the resonance corresponding to each nucleus is referred to as assignment. The assignment of the $^{13}$C and $^{15}$N resonances is useful because these nuclei can be used in 2D and 3D experiments, giving an extra dimension to resolve protons that have similar or the same chemical shifts but that differ in the frequencies of the directly attached heteronucleus. Additionally they provide a means to identify chemical groups and interactions such as hydrogen bonding using their
Figure 5.2.1
A) Strategy for the determination of molecular structures using NMR. B) Intraresidue and sequential H1'-H6/H8 and H2'-H6/H8 NOE connectivities present on an RNA double helix. C) Newman projection of torsion angles and a Karplus equation, that relates the torsion angle to a coupling constant, in this particular example for the two depicted hydrogen atoms. In the equation, A, B, and C are coefficients that depend on the substituent electronegativity.
characteristic chemical shifts. They also provide a way to transfer magnetization along several bonds, allowing one to identify spin systems. A spin system in a polynucleotide is defined as a subset of its resonances that belong to a particular residue and are assigned by through bond correlations among them. By knowing the spin systems and the type of residue they belong to (G, A, U or C), their order in the sequence can be deduced using inter-residue transfer of magnetization (Figure 5.2.1B) and the knowledge of the sequence of the molecule. Once the assignment of these nuclei is complete, the distances between the protons are estimated through the NOE. The distances serve as constraints to model the structure of the molecule. The torsion angles constraints are also defined. Torsion angles and three bond coupling constants between nuclei are related through the Karplus equations (Figure 5.2.1C). The angles are estimated from measurements of these coupling constants in different spectra.

The assignment of the resonances in ACSL\textsuperscript{Ac} (Figure 2.2) started with the identification of the H1\textsuperscript{t} and H6/H8 resonances belonging to the same residue. First, \textsuperscript{13}C correlation experiments allow identification of the \textsuperscript{1}H/\textsuperscript{13}C pairs for those groups (Figure 5.2.2). Then these protons are correlated to N1 (pyrimidines) or N9 (purines) using a multiple bond 2D HSQC on the \textsuperscript{15}N labeled molecule. The \textsuperscript{1}N1 resonances additionally are useful for identifying the purines U and C by their characteristic chemical shift ranges of 142-146 and 150-156 ppm respectively (Wijmenga et al 1998). The \textsuperscript{15}N9 resonances occur in the range of 166-172 ppm. Assignment of the base resonances was also facilitated by the easy identification of pyrimidine H5-H6 crosspeaks in NOESY and COSY spectra, and the C5 characteristic chemical shifts of U and C. The H5-H6 NOE crosspeaks are usually more intense than those of the H1\textsuperscript{t} to H6/H8 and H2 resonances, that occur in the same region of 2D NOESY spectra. The
Figure 5.2.2
Aromatic and anomeric regions from a 2D "C-1H HMQC of "C enriched ACSL"-" in low salt buffer. The temperature during acquisition was 25°C.
H5–H6 COSY crosspeaks are usually resolved. The \(^1^3\)C resonances of U and C are typically 102–107 ppm and 94–99 ppm, respectively, and in \(^1^3\)C/\(^1^H\) correlation spectra yield C5/H5 crosspeaks resolved from the Cl'/H1' crosspeaks because the \(^1^3\)Cl' resonances occur between 87–94 ppm.

With the above information, a sequential assignment based on NOEs between base and H1' regions was accomplished using 2D NOESY and 3D HMQC-NOESY spectra (Figure 5.2.3). At 25 °C (the temperature for most non-exchangeable \(^1^H\) observed spectra) U11 and A11, H1'/Cl' pairs are degenerate, but they are resolvable at 28 °C. The sequential assignment is contiguous throughout residues G2- to U11 and G11 to C41. There are very weak NOEs for U11/H1' to G4/H6 and A11/H8.

The assignment of the rest of the ribose \(^1^H/\(^1^3\)C pairs was done using a 2D \(^1^3\)C HCCH-COSY, which allows identification of H1'-H2' crosspeaks, and a 3D HCCH-TOCSY experiment, which allows through bond correlation of the H1' to the rest of the ribose \(^1^H/\(^1^3\)C pairs (2', 3', 4' and 5') (Appendix I). These experiments allowed the assignment of all the ribose \(^1^H/\(^1^3\)C correlations. A sequential assignment was also done for the H2' to H6/H8 region, with the help of a 3D NOESY-HMQC experiment, confirming the base \(^1^H\) assignments (Figure 5.2.4).

The assignment of the H2 resonances of adenines was particularly important because they are clustered in the loop region and their NOE crosspeaks can indicate their orientation and if they are base paired and stacked. The assignment of A11, A11 and A11 relied on a 2D HCCH-TOCSY, that correlates the H2/C2 pairs and H8/C8 pairs. There was not enough resolution to assign the H2 and H8 resonances of A11 and A11. Use of a 3D HCCH-TOCSY, that gave crosspeaks from the H2/C2 to C8, allowed unambiguous assignment of A11 and A11. Their \(^1^3\)C-\(^1^H\) HMQC correlations are shown in Figure 5.2.2.
Figure 5.2.3
2D NOESY (400 ms mixing time) of ACSL* showing the H6/H8 inter residue NOEs (left) and the H6/H8 to anomeric NOE sequential walk (right). In the sequential walk, the intra residue NOEs are labeled with the corresponding residue. The temperature during acquisition was 25 °C.
Figure 5.2.4
2D NOESY (400 ms mixing time) of ACSL showing the H6/H8 to H2' sequential walk connectivities. The intra residue NOEs are labeled with the corresponding residue. The temperature during acquisition was 25 °C.
The $^{31}$P resonances were assigned using a 2D $^1$H-$^{31}$P TOCSY-NOESY experiment. This experiment combines a through bond correlation of the n+1 $^{31}$P to the H3', through bond correlations of the H3' to the H2' and H4', and a $^1$H/$^1$H NOESY that correlates through space the H3', H2' and H4' to nearby protons. As a result the H1' and H6/H8 resonances, which are usually resolved from the rest of the $^1$H, are correlated to the n+1 $^{31}$P. The $^{31}$P resonances of ACSL$^{fr}$, with the exception of that belonging to the 5' terminal monophosphate, are clustered between -3.35 and -4.63 ppm (Appendix I). The 5' terminal $^{31}$P resonates at -1.3 ppm. This assignment was confirmed by the disappearance of the -1.3ppm peak after enzymatic removal of the terminal phosphate using calf intestinal alkaline phosphatase (Figure 5.2.5). The $^{31}$P assignments were confirmed by H3'-P, H4'-P, H5'-P and H5''-P crosspeaks in a HetCor spectrum. $^{31}$P 1d spectra were also collected at 12°C and 5°C in an attempt to stabilize backbone conformations that may be unstable at higher temperatures. No appreciable chemical shift changes were observed.

The exchangeable resonances NH and NH$_2$ were initially identified by base type using $^1$N-$^1$H through bond correlation HMBC and HSQC spectra respectively (Figure 5.1.1). The NH and NH$_2$ were assigned using NOESY spectra in 10% $^2$H$_2$O and 90% $^2$H$_2$O (Figure 5.2.6). The base paired G imino protons usually resonate between 13.5-12 ppm and the base paired U imino protons, between 14-15.3 ppm. When unpaired, the G and U imino protons resonate between 10.9-9.9 and 12-10 ppm respectively. The G and U imino protons are easily distinguished in the HMBC because the $^1$N's they are correlated to normally have very different chemical shifts, typically 144-149 for G and 156-162 ppm for U. Three G and three U imino crosspeaks with characteristic base paired chemical shifts are identified (Figure 5.1.1). The G imino to C amino NOE crosspeaks served to confirm the base pairs, while the sequential imino to imino walk
Figure 5.2.5
$^31P$ one dimensional spectrum of ACSL$^{	ext{I}}$. Top) containing terminal monophosphate. Bottom) dephosphorylated. The temperature during acquisition was 25°C.
Figure 5.2.6
2D NOESY in 90% H₂O of the imino region of ACSL™. Identified imino sequential connectivities are linked by a line. The diagonal peaks are folded in the second dimension (vertical). Folded peaks are above the dotted line. Imino assignments are indicated above the diagonal peaks. NOE crosspeaks of U₃ and U₃₂ to the H₂ of their base pairing partners are indicated. The temperature during acquisition was 12 °C.
allowed their sequential assignment. The three G imino resonances were assigned to G_{1s}, G_{1}, and G_{12}, while their C amino partners were assigned to C_{1s}, C_{1}, and C_{12}, respectively. The G imino corresponding to the 5' terminal G, is normally undetected. This is due to solvent exchange and the absence of labeling in reactions primed with GMP. Only two U NH resonances gave observable crosspeaks in the NOESY spectra. They were assigned to U_{1s} and U_{1}, through crosspeaks to A H2 and, in the case of U_{1}, to G_{1},NH. The third U imino observed in the HMQC, putatively U_{1s}, was not detectable in the NOESY spectra and the possibility existed that it was due to an alternative conformation of one of the other Us. Additionally, if it were U_{1s}, the base pairing partner was not identified in the NOESY. The three U imino resonances were assigned through their U 1\textsuperscript{5}N3-A H2 crosspeaks in a 1\textsuperscript{5}J_{HN} HNN-COSY spectrum (Figure 5.2.7), which allowed direct identification of all three A-U base pairs.

In the case where an A and a U are involved in a Watson-Crick base pair, the 1\textsuperscript{5}J_{HN} HNN-COSY experiment can be used to obtain a U 1\textsuperscript{5}N3 to A H2 correlation by means of a multi step transfer of magnetization that passes through the hydrogen bond between A 1\textsuperscript{5}N1 and U 1\textsuperscript{5}N3 (Henning and Williamson, 2000). Given the partial covalent character of a hydrogen bond, a scalar coupling exists between N1 and N3 of an 1\textsuperscript{5}N labeled A-U base pair, and magnetization can be transferred between these two nuclei. Magnetization is initially transferred in the adenine from the H2 to the N1. Next it is transferred to the uracil N3 across the hydrogen bond, back to the H2 through the reverse pathway and then detected. The 1\textsuperscript{5}J_{HN} HNN-COSY detects non-exchangeable resonances and thus can detect hydrogen bonds even when the involved hydrogen exchanges with the solvent. The identification of hydrogen bonds proves the presence of A-U base pairs. In ACSL\textsuperscript{AK}, the crosspeaks corresponding to A_{1s} H2-U_{1s} N3, U_{1s} N3-A_{1s} H2 and U_{1s} N3-A_{1s} H2 were identified. The latter crosspeak confirms
Figure 5.2.7
Left) HNN-COSY spectrum of ACSL, optimized for A H2 to U N1 correlations. Right) 1H-15N HSQC of ACSL, showing intra-residue U H5 to N1 correlations to indicate the positions of the U N3's. The HNN-COSY shows A H2-U N1 crosspeaks for residues 31-39, 38-32 and 37-31. The temperature during acquisition was 25°C.
the presence of the U$_{11}$-A$_{11}$ base pair. This is not as stable as the other two A-U base pairs, since the U$_{11}$-N3-A$_{11}$-H2 crosspeak is much weaker than the other N3-H2 crosspeaks.

A very weak crosspeak in the HMQC for the imino resonances, at (10.55 $^1$H, 143.3 $^{15}$N) ppm, was assigned to G$_{14}$NH because its chemical shifts are in the range of unpaired G resonances and no other G residue was expected to be unpaired. The HSQC spectrum optimized for the amino region accounts for the three pairs of C amino resonances assigned through the NOESY. It also contains four purine NH$_2$ correlations, two of them corresponding to unpaired adenines and two to unpaired guanines (Figure 5.2.8). They have been only tentatively assigned because the NOESY spectra did not give clear crosspeaks for them. The upfield most G NH$_2$ likely belongs to G$_{14}$ as a close $^1$H resonance had NOE crospeaks to the A$_{11}$ and A$_{11}$ H8 and H2 resonances. The second G NH$_2$ at (6.1, 70.27 ppm) is weak and may be caused by an alternate conformation of G$_{14}$. Long range correlation crosspeaks corresponding to the A amino N6/H2 were visible in the HNN-COSY spectrum and helped to assign tentatively the resonances of the two A amino H6/N6 crosspeaks identified in the HSQC spectrum. The base paired crosspeaks with N6 resonances at 80.68 ppm belong to A$_{11}$ or A$_{11}$. The single intense crosspeak at (6.44, 75.56) belongs to either A$_{11}$ or A$_{11}$. Accurate assignment was not possible due to low spectral resolution and expected small shift changes due to temperature and isotopic effect (the HNN-COSY was collected in 100% D$_2$O at 28 $^\circ$C and the HSQC in 90% H$_2$O at 12 $^\circ$C).
Figure 5.2.8
"$^{15}$N-"H HSQC for the amino region of "N labeled ACSL". The temperature during acquisition was 12 °C.
ii. Relevant constraints

The inter-residue NOEs for ACSL™ are shown in Figure 5.2.9. The base pair NOEs and the sequential connectivities in the base to anomeric region and base to H2' for the upper part of the ACSL™ "stem" clearly confirm a double stranded RNA helix with C1' endo sugar pucker. These connectivities were continuous from residues 27 to 33 and 34 to 43. A base paired helix is confirmed in the 90% H2O NOESY by the G imino to C amino crosspeaks as well as sequential imino to imino crosspeaks as described above. In a regular A-form helix, which has residues with C3' endo ribose puckers, very short distances on the order of 2 Å would occur between the H2' and the n+1 H6/8 giving very intense sequential NOE crosspeaks (Figure 5.2.1). The intranucleotide H2'-base crosspeaks would be weaker. ACSL™ showed strong H2' to base sequential internucleotide crosspeaks for residues 27-33 and 38-43. Other evidence further supports the presence of a C3' endo ribose sugar pucker for residues 27-32 and 38-43. The 13C chemical shifts for the C3' and C4' resonances are within the predicted range for a C3' endo conformation (Appendix I, Figure 5.2.10) and the H1'-H2' three bond coupling is < 5Hz (Figure 5.2.10).

Residues 32-33 are the region of most interest for the present study, the main point of investigation being whether they would take a U-turn or some other conformation before and after base modifications. Initially the absence of the sequential H1' to base NOE connectivity between residues U11 and G11 suggested that there was a sharp turn between them as would be expected for a U-turn. This interresidue distance is on the order of 6Å in a classical U-turn and several other NMR studies have seen the absence of such connectivity in U-turn containing molecules (Clore et al 1984, Schweisguth and Moore 1997,
Figure 5.2.9
A) Inter-residue H1' and base to base NOEs identified on ACSL."
Figure 5.2.9 B) Inter-residue NOEs involving the rest of the ribose protons.
Figure 5.2.10
Top) H1'-H2' coupling constants of loop nucleotides from a DQF-COSY for ACSL"'. Bottom) $^{13}$C chemical shifts for C3' and C4' resonances of ACSL"'. H1'-H2' couplings $>$7Hz, C3' chemical shift $>$76 ppm and C4' chemical shift $>$85 ppm are evidence for a C2' endo sugar pucker. The temperature for the measurements was 25 °C.
Sundaram et al. 2000). Additionally the non-sequential (U₁₁', A₃₇, 8) NOE was detected, which would be consistent with a U-turn conformation since studies done on U-turn containing RNAs also show this NOE. The unmodified ACSL does not contain a U-turn motif. The evidence of base pairing, even without structure calculations, indicates that the ACSL is essentially an extended hairpin closed by a weak U₁₁'-A₃₇ base pair and a trinucleotide loop. Other evidence indicates that the loop region residues 11, 14, 37 and 38 have certain A-form helix character. Residues 33 and 37 have intermediate characteristics of C2' and C3' endo conformation (Figure 5.2.10). Additionally, in a regular A-form helix, the base paired adenosine residue’s H2 is close to the H1' resonances of both its n-1 residue and the n-1 of its base pairing partner, allowing the observation of NOEs between those nuclei. Such crosspeaks are observable for all three A-U base pairs, although the (A₁,-H2, G₃₇,H1') NOE crosspeak is very weak. However this crosspeak would be expected to be completely absent in a U-turn, as the expected distance between those protons, based on yeast tRNA⁺⁺⁺ crystal structures, is on the order of 15 Å.

The conformation of the ribose-backbone in the trinucleotide loop is not consistent with a 3' stacked conformation. In a U-turn the ribose pucker is predominantly C3' endo for the anticodon residues, which is diagnostic of their stacking. The NMR evidence indicates that the anticodon residues (34-36) and residue 37, have a significant C2' endo conformation. When residues have a C2' endo conformation the H2'-H8 intranucleotide NOE crosspeaks will be strong and the internucleotide, weak. The residues G₃₇ to A₃₇ show very strong intranucleotide H2'-H8 crosspeaks and weak internucleotide connectivities (Figure 5.2.4). The H2'-H1' coupling would also indicate a C2' endo conformation if it were >7Hz. Measurements in a DQF-COSY for these couplings are shown in Figure
5.2.10. The largest couplings are for G_{14}, A_{15} and A_{16}. The third evidence for the ribose pucker was the $^{13}$C1′ and $^{13}$C4′ chemical shifts. Residues G_{14} to A_{31} show downfield shifts for these resonances (Figure 5.2.10, Appendix I), and again the largest deviation from the C3′ endo character is for residues G_{14} and A_{16}.

A residue's $\phi$ torsion angle (C4′-C3′-O3′-P) should be trans for its base to stack on the contiguous 3′ base (Yokoyama and Nishimura 1995). The $\phi$ torsion angle was estimated from the C2′-P coupling constant, according to Legault et al. (1995). The three anticodon residues are in a mixed conformation between trans and gauche′. A_{14} is significantly gauche′ (Figure 5.2.11). Hence the anticodon residues do not have a stable stacking in the 3′ direction.

Bases U_{11} and G_{14} are close at least part of the time. Even though there is almost no base-anomeric sequential connectivity between them, their H6 and H8 resonances, respectively, have a weak NOE crosspeak. The sequential connectivity (A_{15}H8, A_{16}H8) is absent, consistent with a disruption of base stacking between those residues. Important NOEs suggested that the A_{14} base points to its 3′ side and is 5′ stacked. These include the ones from A_{14}H2 to U_{11}H2′, G_{14}H4′, G_{16}H5′, A_{15}H1′, A_{16}H4′, A_{15}H1′, A_{16}H5′ and A_{17}H2 (Figure 5.2.9, Appendix II). Thus it is apparent that a turn occurs between G_{14} and A_{16}.

The absence of a 2-3ppm downfield shifted $^{31}$P resonance for ACSL_{Pig}, besides the 5′ terminal monophosphate, is relevant since a U-turn would be expected to contain a trans $\alpha$ torsion angle for residue 34, which would be associated with a downfield shift for the 33-P-34 resonance. The $^{31}$P resonance tends to shift downfield when one of the $\alpha$ or $\xi$ torsion angles is trans and the other gauche. Such a downfield shift has been seen for U-turn containing molecules (Gorenstein et al. 1981, Huang et al. 1996, Fountain et al. 1996, Schweisguth and Moore 1997, Puglisi...
**Figure 5.2.11**

C2'-P coupling constants measured from 31P modulation of peak intensities in non-decoupled and decoupled 13C HSQC collected from 13C labeled ACSL1 (Legault et al. 1995). Couplings of more than 5 Hz are indicative of gauche-gauche. Upper and lower estimates corresponding to ±1 standard deviation of the estimated peak intensities are plotted. The temperature during acquisition was 25 °C.
and Puglisi 1998, Sundaram et al 2000). The downfield shift is clearly not present in the unmodified ACSLPho, supporting the presence of a different conformation and the absence of such a type of sharp turn.

iii. Structure calculations

The approach to obtain model structures with the NMR data, relies on collecting as many interproton distance constraints as possible in a semiquantitative way. Distance constraints and torsion angles are defined and used in the simulated annealing protocol as described in the methods section.

A list of all the NOE estimated distance constraints and torsion angle constraints for ACSLPho is given in Appendix II. A total of 294 NOE defined distance constraints were used in the calculations, an average of 17.3 per residue. As can be seen, many constraints were present in the loop region allowing a good insight into its structure. Seventy torsion angle constraints were used. Since no downfield shifted phosphorus resonance was observed, $\alpha$ and $\zeta$ torsion angles were constrained to exclude the trans conformation except from $U_{12}^1$-(p)-$U_{11}$ to $A_{11}^1$-(p)-$A_{10}$ to allow the possibility of conformational averaging. The $\beta$-torsion angles were constrained to the trans conformation only for residues $G_{10}$ to $A_{11}$ as only the corresponding H5'-P crosspeaks were clearly absent in the HetCor spectrum. The $\gamma$ torsion angles for $C_{4i}$, $C_{4i}$ and $C_{4i}$ were constrained to gauche+ at their H4'-H5' and H4'-H5'' correlations were clearly absent in the DQF-COSY. The $G_{11}$ and $A_{11}$ $\gamma$ torsion angles were constrained to include the trans and gauche-conformations since they show H4'-H5' (or H5'') crosspeaks in the DQF-COSY and have slight intensity differences between their H4'-H5' and
H4'-H5' NOE crosspeaks. The $\tau$ torsion angle was constrained to gauche- for residue 35, trans-gauche for residues 34 and 36, and trans for the remaining residues. The sugar pucker was left unconstrained for 33, 36 and 37, constrained to C2' endo for residues 34 and 35, and constrained to C3' endo for the rest.

The structures were classified by families according to the conformation of the loop residues, particularly the ones in the anticodon. Two main families were defined by the orientation of residue G$_{11}$. The one with the least number of NOE and overall energy violations was considered the convergent structure. In this family G$_{11}$ is partially stacked with U$_{11}$, while in the other family it is oriented oppositely, protruding from the minor groove side of the helix. The convergence rate of the structures remained between 8 and 11 out of 60 starting structures. The convergent structures had the lowest number of distance constraint violations, with an average of 8 between 0.1 and 0.3 Å. No converged structures violated NOE constraints by more than 0.3Å. All torsion angle constraints were obeyed within 2°, and most structures violated not more than one constraint by 1°. The most common violations occurred in the loop region, as the residues seemed prone to clashing.

A superposition of the loop regions of 8 convergent structures is shown in Figure 5.2.12 and the statistics for the calculations in Table 5.2.1. There is good overall agreement between the distance constraints and the calculated distances; the average RMSD of the calculated distance to the defined distance constraints in the average structure is 0.03 Å.
### NOE distance constraints
- Intraresidue: 101
- Interresidue: 193
- Mean number per residue: 17

### NOE constraints by category
- Strong (0-3 Å): 7
- Medium (0-4 Å): 24
- Weak (0-5 Å): 69
- Very weak (0-6 Å): 108
- Extremely weak (0-7 Å): 78
- Super weak (0-8 Å): 8

### Base pair constraints
- Total: 36

### Torsion angle constraints
- Ribose ring: 24
- Backbone: 46
- Mean number per res: 4.1

### Convergent structures
- 8/60 (cutoff 1st 15)

### Violations
- Average distance constraints > 0.3 Å: 0
- RMSDs for distance constraints (Å): 0.0307 (average)
- Average dihedral constraints > 0.5 deg: 5.6
  - > 1.0 deg: 1.2
- RMSDs for dihedral constraints (deg): 0.28

### RMSDs from ideal geometry
- Bonds (Å): 0.0057
- Angles (deg): 1.369

### RMSDs from minimized mean structure
- Residues 32-18: 0.525
- Residues 27-31, 39-43: 1.572

---

**Table 5.2.1**

Summary of experimental constraints and structure calculation statistics for ACSL^−−^.
Figure 5.2.12
A) Superposition of the residues 12-38 from ACSL™ converged structures. The structures are the eight with the lowest violation energies, from 60 initial structures. They were calculated using experimental constraints with the program XPLOR 3.1.

B) Ribbon backbone representation of the minimized average structure calculated from the 8 convergent structures of ACSL™ (corresponding to residues 27 to 41). Residue 37 is colored green.
iv. Loop structure

The loop region of the convergent structure is a trinucleotide loop composed of the anticodon residues closed by the U\textsubscript{15}-A\textsubscript{11} base pair. The bases of residues G\textsubscript{14} and A\textsubscript{15} are nearly coplanar and are oriented with their base pairing groups towards the major groove, as defined by the residues above. G\textsubscript{14} stacks on U\textsubscript{15} while A\textsubscript{15} appears mostly unrestricted. A\textsubscript{14} is positioned between the planes defined by the U\textsubscript{15} and G\textsubscript{14} bases. A\textsubscript{14} stacks partially with A\textsubscript{11} and tends to bulge. Its C8-N1 vector is antiparallel to that of A\textsubscript{11}. This position is consistent with the A\textsubscript{14}H2 NOEs mentioned above. The position of A\textsubscript{14} distorts base pairs 32-38 and 33-37 making them non planar.

The turn of the loop backbone is gradual instead of sharp, and all three anticodon residues have a C2' endo conformation. A C2' endo conformation allows for a more extended backbone, increasing the distance between the phosphorus atoms (Sanger, 1984). The ribose puckers in the model are consistent with the C2' endo character observed spectroscopically. Only the G\textsubscript{14} and A\textsubscript{15} ribose puckers were constrained to C2' endo. In the average structure, A\textsubscript{14} and A\textsubscript{11}, which were left unconstrained, also have C2' endo conformation. A\textsubscript{14} and A\textsubscript{11} had mostly C2' endo character in the spectra. U\textsubscript{14} has a C3' endo pucker consistent with its mostly C3' endo spectral character (Figure 5.2.10). The distances between the P atoms from U\textsubscript{15}-U\textsubscript{14}, G\textsubscript{14}-A\textsubscript{14}, A\textsubscript{14}-A\textsubscript{11} and A\textsubscript{11}-A\textsubscript{15} are particularly long, >7Å. The convergent structures are very similar as the average RMSD of residues 32-38 to the mean convergent structure is 0.525 Å.

In order to confirm that the constraints are not compatible with a U-turn conformation, a simulation was done enforcing the torsion angle
constraints $\alpha$, $\beta$, $\gamma$ and $\xi$ found between residues 33 and 34 in a yeast tRNA$^{\text{Phe}}$ crystal structure (Shi and Moore 2000), and the ACSL$^{\text{Phe}}$ experimental constraints, except the 33-37 base pair constraint. The lowest energy structures had the same overall conformation of the anticodon residues as the convergent structures, and violated the U-turn torsion angle constraints.

v. Stem structure

The stem is an A-form helix, composed of base pairs G$_2$-C$_4$, to U$_1$-A$_1$. The helix is distorted and starts opening at the junction with the loop, that is, base pairs 32-38 and 33-37. The RMSD of the stem residues 27-32 and 38-43 with an ideal A-form helix is 3.75 Å. The ideal helix was constructed using the biopolymer module of the Insight 98 software (Molecular Simulations Inc.). The main difference between the ideal and the calculated stem is that the two terminal base pairs are tilted towards the major groove in the calculated average structure. This is a reflection of the lack of restraints on an RNA terminus.

The charge density in the ACSL$^{\text{Phe}}$ should be comparatively lower than that of a U-turn, at least in the case of yeast tRNA$^{\text{Phe}}$. Although a charge density was not calculated, it is clear that in a U-turn it would be higher, due to its compactness. For example, each phosphate group in the ACSL$^{\text{Phe}}$ has three or less non-neighboring phosphate groups at less than 15 Å, while in yeast tRNA$^{\text{Phe}}$ the same phosphates have 4 or more. The unmodified ACSL$^{\text{Phe}}$ might be affected by multivalent cations, as these have been proposed to allow approach of the phosphate backbones (Robinson and Wang 1996). The results dealing with this subject are reported in sections 5.5 and 5.8.
Since the U₁₁-A₁₇ base pair is weak, the distance constraint upper limits for the two hydrogen bonds of this base pair were increased by 1 Å, to allow greater flexibility. Bases U₁₁ and A₁₇ have a propeller twist of 29.8°. The distance between U₁₁-N4 and A₁₇-N6 is 3.58Å and between U₁₁-H3 and A₁₇-N1 is 3.17Å. The long distances are a result of the tilting of the U₁₁ base caused by the NOE constraints of its H1' to G₁₄ and A₁₁. The tilting of U₁₁ might not be present in the molecule since no particular interaction seems to promote it, except possibly stacking of U₁₁ with G₁₄. Structure calculations without the U₁₁-A₁₇ base pair showed that the rest of the constraints still position these bases with the potential for pairing, as they are facing each other with a distance of <5Å between the U₁₁-H3 and A₁₇-N1. When regular hydrogen bonding distance constraints were used, the U₁₁-A₁₇ base pair favored having the G₁₄ base bulging out on the minor groove side, and increased the NOE violations.

Thus the unmodified ACSL₇₅ is an extended double helix, and has base pairs U₁₁-A₁₇ and U₁₆-A₁₆. The former is relatively weak and is a transition region between the loop and stem regions. The loop is composed of the anticodon residues 34-36 and has a gradual turn in which G₁₄ partially stacks with U₁₁ and A₁₆ with A₁₇. A₁₅ is almost coplanar with G₁₄. G₁₄ and A₁₆ have an opposite orientation in their base pairing groups from those of A₁₅ (Figure 5.2.12). The structure does not contain a U-turn and is not as compact because the turn in the loop is gradual instead of sharp.
Summary

The structure ACSL\textsuperscript{Ph} was determined using NMR. All non-exchangeable and most exchangeable \textsuperscript{1}H resonances were assigned together with their corresponding bound heteronucleus. \textsuperscript{31}P resonances were also assigned. Distance and torsion angle constraints were defined based on several homo- and hetero-nuclear NMR experiments. Additionally base pairs involving residues 32-38 and 33-37 were confirmed experimentally by the use of an HNN-COSY experiment. The structure is clearly not a U-turn. The spectra indicate that ACSL\textsuperscript{Ph} consists of an A-form helix that extends to base 33-37, and a trinucleotide loop consisting of residues 34-36. The anticodon residues have significant C2' endo conformation, which is not consistent with a U-turn motif. The experimental constraints indicate that A\textsubscript{16} is oriented towards its 3' flanking residues. The structures calculated for ACSL\textsuperscript{Ph} indicate that G\textsubscript{14} and A\textsubscript{17} are almost coplanar and are oriented towards the major groove side of the helix, while A\textsubscript{15} has its base pairing groups oriented towards the minor groove. The bases of G\textsubscript{14}, A\textsubscript{17}, and A\textsubscript{16} do not form a 3'-stack as seen in the U-turn.
5.3 Dimethylallyl-modified ACSL

The dimethylallyl modification occurs in most tRNAs that have an adenosine residue at position 36. Because of its hydrophobic nature this modification has been proposed to stabilize the stacking of the anticodon with the codon during the decoding process. Given that the modification occurs at the NG of adenine, it might destabilize A-U base pairs. The unmodified ACSL\textsuperscript{U-turn} structure is an extended helix that includes two base pairs that should not be present in a U-turn. One question to be addressed is the role of this modification and whether the dimethylallyl modification alone can break the trinucleotide loop structure and induce formation of a U-turn.

The availability of the MiaA enzyme to do this reaction in vitro on the ACSL\textsuperscript{U-turn} gives an ideal system to study a modified RNA through heteronuclear NMR. To our knowledge this is the first study of the effect of an isolated modification on fully isotopically $^{13}$C or $^{15}$N enriched RNA molecules. Without the advantage of isotopic enrichment the assignment and structural analysis would have been too complicated, because, as described below, the modified molecule had marked resonance broadening. Furthermore, as described in next chapter, a dynamic analysis was made possible by the $^{13}$C enrichment.

i. Spectral effects and assignments

The dimethylallyl modification had a destabilizing effect in the loop base pairs. Comparison of the imino region shows broadening of the U\textsubscript{12} and U\textsubscript{11} resonances, while neither the U\textsubscript{13} nor G\textsubscript{14} resonances are
detected (Figure 5.3.1). The destabilization reaches into the stem region up to $G_{13}$ as evidenced in the 1D imino $^1H$ spectrum (Figure 5.3.2). The C amino resonances are mostly unaffected by the modification, with the exception of $C_{15}$, which has some broadening (Figure 5.3.3). Neither the $A_{13}$ H6/N6 crosspeaks seen in the ACSL$^{ph}$ nor any other amino resonance crosspeaks from a base paired A are visible in the $^{15}N-^1H$ HSQC (Figure 5.3.3). In the same spectrum, the $^1H_{A_{13}-N^6}$ secondary amine resonance is observed as a new crosspeak at 6.71 $^1H$, 89.47 $^{15}N$ ppm (Figure 5.3.3). The $G_{13}N2/H2$ resonances shift upfield 1.7 and 0.13 ppm respectively. Two unpaired A NH$_2$ crosspeaks are seen. They probably correspond to $A_{11}$ and $A_{14}$ as deduced by comparison with the unmodified molecule, although they could not be unambiguously assigned. There are also two much weaker A amino crosspeaks in the same region, that could not be assigned.

The dimethylallyl group protons were identified in NOESY spectra and their resonances assigned based on the reported chemical shifts for the free dimethylallyl group (Davisson et al. 1986). The secondary amine resonance had crosspeaks to all the dimethylallyl group protons in a 500 ms H$_2$O NOESY. These proton resonances could not be assigned through a COSY spectrum because they have large linewidths (between 25 and 40 Hz). The assignments are included in Appendix III. The assignment of the methyl groups was done through their NOE crosspeaks to the methylene (CH$_2$) and methyne (CH) protons respectively.

A comparison of the base to anomic walks on ACSL$^{ph}$ and i6a37-ACSL$^{ph}$ shows that most of the loop region has dynamics of intermediate exchange (Figure 5.3.4). The assignments for the dimethylallyl modified molecule were obtained using similar techniques as were used for ACSL$^{ph}$. The assignments are reported in Appendix III. In the stem region the molecule is essentially unaffected until residue $G_{13}$ and after $C_{15}$. The resonance assignment was complicated by the broadening of the base and
Figure 5.3.1
In the region $^1H-^15N$ HMQC collected from $^14N$ labeled 16A37-A GSL. Crosspeaks marked by asterisk are due to a contaminant.
The temperature during acquisition was 12 °C.
Figure 5.3.2
Imino proton 1D spectra of ACSL\textsuperscript{\textprime \prime} and i6A37-ACSL\textsuperscript{\textprime \prime}. The temperature during acquisition was 12 °C.
Figure 5.3.3
$^{15}N$-$^1$H HSQC for the amino region of $^{15}N$ labeled i6A37-AcsL$^{25}$. The temperature during acquisition was 12 °C. "Assignments based on chemical shift conservation with AcsL$^{25}$."
H1' resonances, as evidenced in the 13C HMQC (Figure 5.3.5). The ct-HSQC spectrum optimized for the H1'/Cl' region does not show resonances U1 to A11. For most of these residues, long-range correlations of the base and anomic resonances to N1/N9 in the 'H/13N HSQC were also missing, complicating the assignment. Assignment of the H1' resonances in the loop residues was difficult because of their broadness. HCCH-COSY and 2D NOESY spectra, in combination with the long range HSQC, were sufficient to assign all but the G14, A15, and A16 H1' resonances, based on the conservation of crosspeaks to H2' and base protons, respectively.

The U13H6 and H5 resonances were identified by their mutual crosspeaks in a 3D NOESY-HMQC and a DQF-COSY. The U13 H1' was assigned by the H1'-N1 crosspeak in the long range HSQC with the characteristic N1 resonance of uridines and by its crosspeak with U13H6 in NOESY spectra.

G14 H8 was assigned by analogy to ACSLPhβ, based on the similar chemical shifts of its intranucleotide NOE crosspeaks to H2' and H3', the similar H8/C8 crosspeak in the 2D HMQC, with the absence of a correlation to the H2/C2 in the 2D HCCH-TOCSY, and a very weak NOE crosspeak with U13H1' in a 2D-NOESY. G14H1' was tentatively assigned by the analogy of a P/H1' crosspeak to the ACSLPhβ (G14H1', A11P) crosspeak in hetero-TOCSY-NOESY spectra. These 31P resonances are the downfield most in ACSLPhβ and i6A37-ACSLPhβ.

A11H8 was assigned by analogy to its chemical shift in ACSLPhβ and conserved NOE crosspeaks. The relative position of the chemical shift with respect to the rest of base 'H resonances in i6A37-ACSLPhβ is analogous to that in ACSLPhβ. A11H8 has NOE crosspeaks to three very upfield shifted resonances that correspond to A11 H5', H5'', and H4' in ACSLPhβ. The same proton also shows a weak NOE crosspeak to U13H1' in 2D and 3D 13C edited NOESY spectra.
Figure 5.3.4
Left) 2D NOESY of ACSL1 showing the H6/H8 to anomeric NOE sequential walk. Right) Analogous spectrum of i6A17-ACS1. In the sequential walks of both spectra, the intra-residue NOEs are labeled with the corresponding residue. The shown spectra were collected with a 400 ms mixing time in 100% D,0 at 25°C. Dotted line indicates tentative connectivities. The temperature during acquisition was 25°C.
Figure 5.3.5
Aromatic and anomeric regions from a 2D "C-1H HMQC of "C enriched 16A37-ACSL". The temperature during acquisition was 25. °C. Crosspeaks from residual unmodified ACSL."
A$_{1}$ H8 was assigned through the chemical shift analogy of the H8-H1' NOE crosspeak to that of the unmodified molecule. It has conserved crosspeaks to A$_{13}$ H4' and H5'. The A$_{13}$ C8 chemical shift is conserved.

A$_{1}$-H8 is still the most downfield base resonance and retains a strong intranucleotide H8 to H2' crosspeak. It also has a very broad crosspeak in the H1' region, presumably to the A$_{1}$-H1' resonance.

Of the 5 adenine H2 protons in the molecule, four are observed in the $^{13}$C-$^{1}$H HMOC spectrum (Figure 5.3.4). Using an HCCH-TOCSY spectrum they were assigned to residues 31, 35, 36 and 38. A$_{1}$-H2 was not observed in this spectrum or an HSQC optimized for H2 to N3 and N1 correlations. It is most likely broadened by exchange, since it is unlikely that it would have its H2, N1, C2 and N3 resonances degenerate with others.

ii. Relevant constraints and comparison with the unmodified structure

Relatively few NOEs have been identified for i6A37-ACSL$^{fr}$ due to the broadness of the loop resonances. A highly precise structure would not be expected from these constraints unless unreasonably restrictive distance constraints were used. The qualitative NOE derived information is described below. It is important because it yields an insight into the dynamic structure of the modified molecule. The only distinguishable inter-residue base to base NOE crosspeaks involve U$_{n}$H6 to U$_{n}$H6 and U$_{n}$H5 to U$_{n}$H5 at 12°C but not 15°C or above. The relative scarcity of NOE crosspeaks suggests that the loop residues are not stably stacked above 12°C. The internucleotide constraints are shown in Figure 5.3.6 and tabulated in Appendix IV. The i6A37-ACSL$^{fr}$ shows some similarity to ACSL$^{fr}$ since its U$_{n}$H1' resonance has NOE crosspeaks to G$_{n}$H8 and A$_{1}$H8.
Crosspeaks $A_{11}H1'$ to $A_{11}H8$ and to $A_{7}H8$ are extremely weak due to their broadness. $A_{11}H2$ shows a weak crosspeak to $A_{11}H1'$, suggesting it is still stacked with that residue. $A_{7}H8$, although broad, has strong $H1'$ and $H2'$ intraresidue crosspeaks, suggesting that its ribose still has some $C2'$ endo character. $A_{11}H1'$ has a sequential connectivity to $A_{7}H8$. $A_{7}H2$ still shows crosspeaks to $U_{11}H1'$ and $U_{11}H1''$, indicating that this region still has some double helical character and that there is, at least part of the time, a short distance between the "opposite strands" of the loop. This short distance might not be preserved because the intermediate exchange for the loop resonances indicates that their nuclei do not have stable conformation on the NMR time scale.

The most notable effect of the dimethylallyl modification on the linewidths involves the $U_{11}H5$ and $H6$ resonances (Figures 5.2.2 and 5.3.5). The $U_{11}H5$ and $H6$ linewidths increase from 21.6 Hz and 18.9 Hz in the ACSL$^Phe$ to 80.8 Hz and 29 Hz in i6A37-ACSL$^Phe$, respectively. The particularly intense broadening effect on these resonances and on the resonances from i'A$_{1}$ supports the proposal that the modification destabilizes the U$_{11}$-A$_{1}$ base pair. The localization of the effect of the dimethylallyl modification to the loop region is illustrated by the other H5/C5 resonances in the $^{13}C$ HMOC spectra (Figure 5.3.5). The U$_{11}$ and U$_{1}$, H5/C5 crosspeaks are also broadened. This broadening in the loop contrasts with the H5/C5 crosspeaks of the C residues in the stem, which are mostly unaffected.

The dimethylallyl group protons have several NOE crosspeaks to U$_{11}$, U$_{12}$, G$_{14}$ and A$_{11}$, positioning the group in the vicinity of those residues (Figure 5.3.6). The CH$_2$ protons have two strong NOE crosspeaks, one to U$_{11}H4'$ and the other probably to U$_{12}H2'$, as identified in a 3D HSQC-NOESY. The CH$_3$ shows NOE crosspeaks to U$_{11}H1'$ and to U$_{12}$ H6 and H5 at all temperatures tested. It shows weak NOE crosspeaks to U$_{11}H5$ at 18°C
Figure 5.3.6
Inter-residue NOEs identified in i6A37-ACSL 5*, residues G1 to C30, in low salt buffer.
and 12°C and to U₁₅H6 at 12°C. The CH₂ protons show weak NOE crosspeaks to the H5 and H6 resonances of U₁₅ and U₁₆ and to the A₁₄H2 resonance.

Most ³¹P resonances were assigned using a ³¹P/¹H hetero-TOCSY NOESY spectrum, with the exception of G₁₄, A₁₅ and A₁₆. ³¹P 1D spectra show that all the resonances, with the exception of the 5' terminal monophosphate, are clustered within -3.4 and -4.6 ppm as in the unmodified molecule. 1D spectra were collected at different temperatures as shown in Figure 5.3.7. No new downfield shifted phosphorus was observed with the exception of an extremely broad peak at 5°C at -2.6 ppm, a slight downfield shift. Thus the trans conformation is not likely adopted for any α or ζ torsion angles in a stable form.

i6A37-ACSL₇⁷ has a larger decrease in UV hypochromicity at low temperatures than ACSL₇⁷ (Figure 5.3.8). This suggests the dimethylallyl group facilitates local unstacking of some bases with increasing temperature.

iii. Conformation of the loop with the dimethylallyl group

The evidence indicates that the dimethylallyl group indeed destabilizes the unmodified trinucleotide loop structure. It breaks base pairs 33-37 and most likely also 32-38, while it also leads to destabilization of the 31-39 base pair. The dimethylallyl group is clearly reaching toward the residues opposite to A₇, to what could be called the opposite strand, and interacting predominantly with U₂₄ and U₂₅. The ACSL₇⁷ structure must change upon attachment of the dimethylallyl group. Manual modeling of this group on the unmodified ACSL₇⁷ shows the NOEs detected for this group are not compatible with its available conformations. This is particularly clear for the CH₂.
Figure 5.3.7
$^{31}$P one-dimensional spectra of i6A37-ACSL" at the indicated temperatures. The 5' terminal monophosphate is the only downfield shifted resonance.
Figure 5.3.8
Melting curves at UV 260nm wavelength of ACSL and 16A37-ACSL. Each curve is an average of three independent measurements. For each measurement, the minimum absorbance was set to 0 and the data normalized to the maximum absorbance defined as one.
to $A_{14}H2$ NOE crosspeaks. These atoms are always too distant to give an NOE. Additionally, any attempt to bring the CH$_2$ groups near the opposite strand caused a large amount of clashes between the dimethylallyl group and the RNA. One way of resolving the structure with the NOE information was achieved by simply shifting the $i'A_{1}$ base horizontally, with respect to the rest of the helix, towards the minor groove (Figure 5.3.9). This movement breaks the base pair involving residues 33 and 37.

Based on this approach, the orientation of the dimethylallyl group is proposed to be one of close to maximum extension towards the opposite strand. The torsion angles of the dimethylallyl group in the above model are -44° for N1-C6-N6-CH$_2$, -160° for C6-N6-CH$_2$-CH, and -137° for N6-CH$_2$-CH-C. These torsion angles are only tentative. The conformation of the dimethylallyl group differs from that calculated by Tewari (1988) for the free modified base, where the corresponding angles are 0°, 0° and 120°.

**Summary**

In i6A37-ACSL$^{28}$, the chemical shifts for the stem resonances up to G$_{14}$ are essentially identical to those of ACSL$^{28}$. Assignment of the base and H1' resonances in the loop region employed multiple strategies that included the analogy with resonances of the unmodified molecule and the use of heteronuclear spectra. The dimethylallyl group in i6A37-ACSL$^{28}$ does not allow the formation of the base pair 33-37 and destabilizes the base pair 32-38, as indicated by resonance broadening, and HNN-COSY and NOE data. The non-exchangeable resonances from A$_{1}$ to U$_{1}$, as well as the imino protons from G$_{1}$, on to the loop, are broad, particularly those of U$_{1}$ and $i'A_{1}$. The dimethylallyl group is oriented
Figure 5.3.9
Model of ACSL\textsuperscript{11c} with an attached dimethylallyl group, in accordance with NOE information from i6A37-ACSL\textsuperscript{11c}. A\textsubscript{11} was manually flipped horizontally towards the minor groove, the structure was subjected to a short constrained molecular dynamics and refined. Then the dimethylallyl group was positioned so as to be consistent with the observed NOEs for its protons. Residue 37 is colored green, except the dimethylallyl group, which is colored yellow.
towards the opposite strand and positioned between the planes defined by the bases of U₁₂ and U₃₇. Residues from U₃₇ to A₁₄ have a few conserved NOEs that indicate that the overall loop structure of i6A37-ACSL₃₇ is similar to the unmodified molecule. No inter-residue ³¹P resonance with a downfield shift is detected which would be indicative of a U-turn. Hence, a U-turn is not induced by the breaking of the 33-37 base pair, the strong destabilization of the 32-38 base pair, and the increased hydrophobicity conferred by the dimethylallyl group on ACSL₃₇. Instead, an open and dynamic structure is induced.
5.4 $T_1\rho$ measurements

1. Information obtained from relaxation rates

$T_1\rho$ relaxation rates are informative of exchange processes that occur on the 0.01-1 msec time scale, such as conformational transitions (Lane and Lefèvre 1994). Comparison of the $^{13}C\ T_1\rho$'s between different parts of a molecule can indicate regions with different dynamic behavior. When single resonances are observed, they either are not exchanging or are in fast exchange, and when they broaden, they enter intermediate exchange. The line width in intermediate exchange is directly proportional to the sum of the lifetimes, in the simplest case, of two conformations. This is the process that is being monitored by the transverse relaxation measurements, as the line width is inversely proportional to the transverse relaxation time $T_2$. In solution NMR, $T_1$ is equivalent to $T_1\rho$. A pair of exchange conformations of an atom with long lifetimes will have a shorter $T_1\rho$ and larger line width (Lane and Lefèvre 1994). A relatively long $T_1\rho$ indicates that a C-H or N-H vector reorients faster, i.e. the line width is inversely proportional to the rate constant (or directly proportional to the lifetime of a state). Such simple analysis has been used to confirm the dynamic nature of particular RNA residues (Zimmermann et al. 1998, Smith and Nikonowicz 1998). We used the strategy of measuring $^{13}C\ T_1\rho$’s as a quick way of evaluating the dynamics of the ACSL $^{35}S$ and i6A37-ACSL $^{35}S$. The observed resonances were C6/C8, the adenine C2 and the ribose C1’.
ii. Relaxation rate measurements

$T_\text{1,9}$ relaxation measurements are depicted in Figure 5.4.1 for both molecules. The relaxation rates are clearly very similar between the ACSL$_\text{Fp}$ and i6A37-ACSL$_\text{Fp}$s. Hence the dimethylallyl modification, although it destabilizes the loop structure of the unmodified molecule, does not alter the fast dynamics. The uncertainty of the measurements is estimated as $\pm 5\%$ by collecting duplicate experiments for two time points on each relaxation series. The base C8 resonances of G$_\text{14}$ and A$_\text{15}$ have notably higher $T_\text{1,9}$ than their stem congeners, indicating they have increased mobility, although their amplitudes are unknown. There is also a reduction of $T_\text{1,9}$ for A$_\text{14}$- and A$_\text{15}$ C8, caused by the dimethylallyl modification, suggesting a slight stabilization of those bases.

The adenine C2 $T_\text{1,9}$'s were unaffected by the presence of the dimethylallyl modification. A$_\text{14}$ had slightly higher $T_\text{1,9}$, consistent again with this base as being the least restricted. A$_\text{15}$- C2 was not measured, because it had not been assigned and the H2 resonance is presumed broadened beyond detection. The identification of the proton resonance is necessary for the measurements, because the $^{13}$C intensity variations are identified and measured from $^{13}$C/$^1$H crosspeak intensities at increasing $^{13}$C spin lock times, in a series of $^{13}$C/$^1$H HSQC experiments.

The C1' $T_\text{1,9}$ of residues 34 to 37 in ACSL$_\text{Fp}$, are significantly lower than all the rest in the stem. This is consistent with a more stable ribose backbone in this region. The C1' linewidths measured directly in a ct-HSQC were not broader than those from the stem residues, indicating that the short $T_\text{1,9}$'s are not caused by broadening due to a slow conformational exchange process present only in the loop. This sort of process prevented the measurement of the C1' $T_\text{1,9}$ for residues 34-37 on i6A37-ACSL$_\text{Fp}$, because they were broadened in the $^{13}$C and $^1$H dimensions to
Figure 5.4.1
Histograms of $^{13}$C T1p measurements for AC5L$^{+}$ (black bars) and 16A37-AC5L$^{-}$ (gray bars): Top) C6/C8, Middle) C1' and Bottom) Adenine C2. The estimated error is 5%. The bars that are labeled with two assignments correspond to resonances that were nearly degenerate, the first assignment on each correspond to the resonance nearest to the measured intensity. The temperature during spectra acquisition was 28 °C.
the point of being undetectable with the ct-HSQC experiment used. The
ACSL™ C1' values were means of three independent sets of relaxation
series, which allowed determination of standard deviations for each
measurement. Each experiment had a different position of the 13C
carrier, within a small range, in order to discard the possibility that
it was responsible for the increased relaxation. This possibility was
considered, since the C1' resonances of residues 34-37 are upfield from
the rest. These C1' T2 measurements have small dispersions, hence the
small T2's for the loop residues are not attributable to the carrier
position. The T1 (longitudinal relaxation) measurements in the loop are
very similar to the rest of the stem, ~0.3 sec. Hence the fast
relaxation for the C1' resonances of residues 34 to 37 attributed to
transverse relaxation is not due to a side effect of T1.

The shorter C1' T2's for the loop residues G14 to A17 in ACSL™
suggests their riboses are in a more stable conformation than the
riboses in the stem. The structure of the unmodified molecule gives an
insight into the observed T2, in the loop region. Bases G14 and A17 do not
have extensive hydrogen bonding and should be less restricted in their
motion, consistent with their C8 and C2 T2's, which are larger than
those of the stem residues. The C1'-H1' vectors of the loop residues 34
to 37 conversely reorient more slowly than the ones in the rest of the
molecule. This is consistent with them being more stretched and maybe
more tense, as they all have a significant C2' endo conformation, which
is associated with increased inter-phosphate distances.
Summary

The T1' measurements of the C6 resonances indicate that the bases of residues G1' and A1' are more dynamic than the stem purines in both AC53FPane and i6A37-AC53FPane. The pyrimidine C6 T1' are all similar. The C1' T1' s from residue 34 to 37 are significantly shorter than those in the rest of AC53FPane, suggesting that the ribose moieties of those residues are more static, perhaps more tense. It was not possible to measure C1' T1' s of i6A37-AC53FPane due to their broadness. The C2 T1' between AC53FPane and i6A37-AC53FPane was essentially identical, with the exception of i5A1', which could not be measured because its H2 was not identified. These results indicate that the fast dynamics of AC53FPane are not altered by the dimethylallyl modification, and agree with the determined structure of AC53FPane.
5.5 Mg$^{2+}$ before and after dimethylallyl modification

i. Spectral assignments

Given that the crystal structures of some tRNAs contain Mg$^{2+}$ ions bound to the anticodon stem-loop, their effect on ACȘL$^{\text{Phe}}$ and i6A37-ACȘL$^{\text{Phe}}$ was investigated, with the hypothesis that they help in the formation of a U-turn.

Mg$^{2+}$ has a general broadening effect on both ACȘL$^{\text{Phe}}$ and i6A37-ACȘL$^{\text{Phe}}$. The effect is strongest on the imino resonances in the loop region for both molecules. There are similarities between the ACȘL$^{\text{Phe}}$ saturated with 20mM Mg$^{2+}$ and dialyzed to a Mg$^{2+}$ concentration of 5 mM, and the Mg$^{2+}$ free molecule. The U$_{11}$ imino resonance still can be identified in a 2D $^1$H-$^1$H HMQC with a chemical shift characteristic of base paired uridines (Figure 5.5.1). Only minor chemical shift changes are seen for the G imino resonances. Direct addition of Mg$^{2+}$ to above 10 mM final concentrations gives different U imino peaks and U$_{11}$H3 becomes undetectable. This suggests that upon saturation by dialysis, the loop is in a similar conformation to the Mg$^{2+}$ free molecule and with partial saturation an alternate more open conformation is present and increases the exchange rate with H$_2$O. The increase in broadening of the U imino peaks in the loop region seems to be due to a more open loop, because the general broadening by Mg$^{2+}$ is not expected to cause such a strong effect and should not prevent detection of the loop resonances.

Comparison of the 1D spectra shows that the broadening is only ~2 Hz for the G imino resonances.

The $^1$H-$^1$H HSQC optimized for the amino region of ACȘL$^{\text{Phe}}$ does not differ markedly when Mg$^{2+}$ is added by titration or dialysis. In these
Figure 5.5.1
"N-H 2D HMOC optimized for the imino resonance region, acquired on ACSL" in 5 mM MgCl₂ containing buffer. The molecule was dialyzed in buffer containing 5 mM MgCl₂. The temperature during acquisition was 12 °C.
spectra (Figure 5.5.2) the $\text{A}_{11}\text{NH}_2$ crosspeaks are not detected, and only one of the two putative G amino crosspeaks seen without Mg$^{2+}$, at (6.1, 70.27) ppm, is observable. The G amino is fairly intense and presumably belongs to G$_{11}$. In the Mg$^{2+}$ free form the crosspeak at that position is very weak. This suggests that the very weak $^{15}\text{N}/^1\text{H}$ pair with that frequency increases in population and is more stable. An intense A amino crosspeak at (6.2, 76.79) ppm and a very broad crosspeak at (6.46, 77.32) ppm are observed. These peaks might correspond to the intense A amino crosspeak seen in the absence of Mg$^{2+}$ at (6.43, 75.57) ppm. Therefore it appears that the addition of Mg$^{2+}$ causes a shift of the intense A amino resonance, resolves a weak A amino resonance and shifts or increases the intensity of a G amino crosspeak. The spectrum in the $^{13}\text{N}$ C amino region shows that the C’s are still base paired, as resonance pairs corresponding to the frequencies of amino protons, both hydrogen bonded and not, are conserved for residues 40-43.

The non-exchangeable resonances of ACSL$_{\text{Ph\text{e}}}^\text{Ph\text{e}}$ were assigned through a combination of $^{13}\text{C}/^1\text{H}$ HMOC spectra of a Mg$^{2+}$ titration of the i6a37-ACSL$_{\text{Ph\text{e}}}^\text{Ph\text{e}}$, and 2D NOESY spectra. Also long range $^{15}\text{N}/^1\text{H}$ HSQC, HCCH-COSY and HCCH-TOCSY spectra were utilized to help in the assignment of the H2-H6 and H2' resonances. In the presence of Mg$^{2+}$, the 2D NOESY (Figure 5.5.3) and $^{13}\text{C}/^1\text{H}$ HMOC (Figure 5.5.4) spectra show that the upper stem region, residues 26-31 and 40-43, has small chemical shift changes for the base and H1' region. The loop region, including residues 32 to 39 has more pronounced effects. The largest chemical shift changes cluster in this region, which is multiconformational. The intensity of the peaks suggests that one conformation is more populated. The C5/H5 resonances of U$_{11}$, U$_{12}$ and U$_1$, are doubled, with weak crosspeaks corresponding to the Mg$^{2+}$ free form and stronger new crosspeaks, which are analogous to those seen in i6a37-ACSL$_{\text{Ph\text{e}}}^\text{Ph\text{e}}$ (See below). Also, there is an extra C8/H8 purine
Figure 5.5.2
$^1$H-$^1$N2D HSQC optimized for the amino resonance region acquired on ACSL$^{\text{TM}}$ in 5 mM MgCl$_2$ containing buffer. The molecule was dialyzed in buffer containing 5mM MgCl$_2$. The temperature during acquisition was 12 °C.
Figure 5.5.3
H6/H3 to anomeric spectral region of a 2D-NOESY acquired on ACSL™ in presence of 7 mM MgCl2. The mixing time was 400 ms. The sequential assignment is traced and the intranucleotide crosspeaks are labeled with the residue assignment. Tentative connectivities are traced with small dotted lines. A stippled line marks the A1,H2 resonance. The temperature during acquisition was 25 °C.
Figure 5.5.4
Aromatic and anomeric regions from a 2D 13C-1H HMOC of 13C enriched ACSL"" titrated to 10 mM MgCl₂. The temperature during acquisition was 25°C.
crosspeak than expected. The A₁₆ H₈ and A₁₇ H₈ resonances were not identified but probably correspond to two of the three unassigned C8/H8 crosspeaks in the 2D ¹³C-¹H HMQC, which have only small chemical shift differences. There are also multiple resonances in the C2/H2 region of the spectrum, with three resolved and one intense overlapped crosspeak, and weak crosspeaks corresponding to the Mg²⁺ free form of residues 35, 36, 37 and probably 31. In contrast to the uridines, the intense C2/H2 resonances did not have a clear correspondence to the Mg²⁺ free 16A37-ACSLₚₚ. The A₁₅ and A₁₇ H₂ were assigned by NOESY H1' crosspeaks. The C2/H2 crosspeaks from A₁₅ and A₁₆ were assigned by analogy to 16a37-ACSLₚₚ titrated with Mg²⁺. The A₁₆ H2 resonance shifted downfield at low concentrations of Mg²⁺ and could not be assigned unambiguously at concentrations >7mM.

The chemical shift changes may indicate a region of preferential Mg²⁺ binding or a region where structure is particularly affected by Mg²⁺. The largest chemical shift changes on ACSLₚₚ after addition of Mg²⁺ were: U₁₃ H₅ +0.21ppm, U₁₃ H₅ +0.41ppm, U₁₅ C6/H6 +1.33/0.2ppm, G₁₄ H₈ -0.13ppm, A₁₅ H1' +0.35, A₁₁ H₈ +0.19ppm, A₁₂ H₂ +0.20ppm, U₁₃ H₁' -0.17ppm and C₁₂ H₆ +0.11ppm. The chemical shift changes for the assigned resonances are depicted in Figure 5.5.5 and Table 5.5.1. Thus the largest Mg²⁺ dependent chemical shift effects were found in the 5' terminal residue, the loop residues 31-35 and in the stem residues 38-41. The C1' resonances from residues 34-37 notably shift downfield towards the rest of the C1' resonances, suggesting that their riboses do not have a C2' endo pucker anymore (Figures 5.2.2 and 5.5.4). A₁₅ and A₁₆ C1' resonances have not been assigned with certainty. Twenty-one ²Cl'/H1' crosspeaks are visible, indicating that at least 4 resonances have duplicate peaks.

16A37-ACSLₚₚ in the presence of Mg²⁺ has almost complete absence of the U imino resonances both in 1D and 2D ¹⁵N-¹H HMQC spectra. The effect
Figure 5.5.5

Histograms of Mg\(^{2+}\) dependent chemical shift changes of ACSL\(^{15N}\) and 16A37-ACSL\(^{15N}\). The positions marked by asterisk correspond to resonances not assigned with or without Mg\(^{2+}\), or both. The temperature the data was obtained at was 25 °C.
<table>
<thead>
<tr>
<th>G1a</th>
<th>G1b</th>
<th>G2a</th>
<th>G2b</th>
<th>G3a</th>
<th>G3b</th>
<th>A3a</th>
<th>A3b</th>
<th>U3a</th>
<th>U3b</th>
<th>C3a</th>
<th>C3b</th>
<th>A4a</th>
<th>A4b</th>
<th>A4c</th>
<th>A4d</th>
<th>C4a</th>
<th>C4b</th>
<th>C4c</th>
<th>C4d</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.73</td>
<td>5.78</td>
<td>5.76</td>
<td>5.74</td>
<td>5.74</td>
<td>5.77</td>
<td>5.97</td>
<td>5.97</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td></td>
</tr>
<tr>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td>7.43</td>
<td></td>
</tr>
<tr>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td>8.08</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A5a</th>
<th>A5b</th>
<th>A5c</th>
<th>A5d</th>
<th>C5a</th>
<th>C5b</th>
<th>C5c</th>
<th>C5d</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.62</td>
<td>5.62</td>
<td>5.62</td>
<td>5.62</td>
<td>5.62</td>
<td>5.62</td>
<td>5.62</td>
<td>5.62</td>
</tr>
</tbody>
</table>

**Table 5.5.1**

"H chemical shifts of ACSL'" and i6A17-ACSL'" in the presence and absence of 5mM Mg" by dialysis.
of Mg\textsuperscript{2+} titration on the imino G resonances of the stem is essentially identical to that seen for ACSL\textsuperscript{Phe}. No amino HSQC was collected for the 16A37-ACSL\textsuperscript{Phe} in the presence of Mg\textsuperscript{2+}.

Titration experiments combined with 2D NOESY sequential assignments showed similarities in the chemical shifts between ACSL\textsuperscript{Phe} and 16A37-ACSL\textsuperscript{Phe} non exchangeable resonances C\textsubscript{4}H\textsubscript{6}, U\textsubscript{13}H\textsubscript{1'}, G\textsubscript{3}H\textsubscript{8}, A\textsubscript{13}H\textsubscript{8}, A\textsubscript{3}H\textsubscript{2}, and A\textsubscript{13}H\textsubscript{2} (Figure 5.5.5 and Table 5.5.1). The 2D NOESY and \textsuperscript{13}C-\textsuperscript{1}H HMOC base and H\textsubscript{1'} regions of the Mg\textsuperscript{2+} saturated 16A37-ACSL\textsuperscript{Phe} are shown in Figures 5.5.6 and 5.5.7, where the similarities of the above mentioned resonances with Figures 5.5.3 and 5.5.4 can be seen. Notably the C\textsubscript{1'} resonances that were very broad in the absence of Mg\textsuperscript{2+} also shift downfield to the C\textsubscript{1'} endo sugar pucker region.

The \textsuperscript{31}P resonances of ACSL\textsuperscript{Phe} and 16A37-ACSL\textsuperscript{Phe} were not assigned due to the increased linewidth caused by Mg\textsuperscript{2+}. Titration with Mg\textsuperscript{2+} and dialysis, give similar \textsuperscript{31}P 1D spectra. With titration to 7mM Mg\textsuperscript{2+}, both molecules have the \textsuperscript{31}P resonances still clustered between -3 and -5 ppm, with the exception of the 5' terminal monophosphate resonance at --1ppm. Spectra collected at 5, 12, 22 and 28°C have the same resonance clustering. Only a weak and broad peak appears at -3 ppm, a little downfield from the rest, at 12°C in both molecules (Figure 5.5.8). It is not possible to assign it but if it were the U\textsubscript{13}-P-G\textsubscript{1}, giving a U-turn, this would be only a minor conformation. A small hump close to the terminal resonance peak is unlikely to be a new resonance because it is also visible in the Mg\textsuperscript{2+} free form but not in the dephosphorylated sample. Thus it might be just an alternate conformation of the 3' terminal phosphate.
Figure 5.5.6
H6/H8 to anomic spectral region of a 2D-NOESY acquired on i6A37-ACSL™ in presence of 7 mM MgCl2. The mixing time was 500 ms. The sequential assignment is traced and the intranucleotide crosspeaks are labeled with the residue assignment. Tentative connectivities are traced with small dotted lines. A stippled line marks the A3, H2 resonance. The temperature during acquisition was 25 °C.
Figure 5.5.7
Aromatic and anomeric regions from a 2D $^{13}$C-$^1$H HMOC spectrum of $^{13}$C enriched 16A37-ACSL$^{13}$C titrated to 10 mM MgCl$_2$. The temperature during acquisition was 25 °C.
ii. Spectral similarities caused by the dimethylallyl modification and \( \text{Mg}^{2+} \)

In the presence of \( \text{Mg}^{2+} \) the new chemical shifts of ACCL\( ^{\text{Pro}} \) for the H5/C5 resonances of U1, U2, and U3 (Figure 5.5.9) and the H6 of U1 (Figure 5.5.10) resemble those seen in i6A37-ACSL\( ^{\text{Pro}} \) in the absence of \( \text{Mg}^{2+} \), suggesting that \( \text{Mg}^{2+} \) has the same loop opening effect as the dimethylallyl group. Both \( \text{Mg}^{2+} \) and the dimethylallyl modification increase the exchange rate of the U imino \( ^1\text{H} \) resonances (Figure 5.5.11). Another similarity is the absence of the A1-H2 resonance in the presence of \( \text{Mg}^{2+} \) and after the dimethylallyl modification, although crosspeaks in the spectrum with \( \text{Mg}^{2+} \) might be overlapped. Also, the intense G amino \( ^{13}\text{N}/^1\text{H} \) crosspeak seen with \( \text{Mg}^{2+} \) is seen in the dimethylallyl modified molecule at (6.13, 70.33) ppm, while in the \( \text{Mg}^{2+} \) free ACCL\( ^{\text{Pro}} \) the corresponding peak is very weak and a different G amino crosspeak is seen, suggesting that the G amino has an alternate conformation enhanced by the dimethylallyl modification and \( \text{Mg}^{2+} \).

The dimethylallyl modification also causes a downfield shift of the C1' resonances of the loop residues 34-37, indicating that there is not a stable C2' endo conformation in the loop but a mixture of C2' and C3' endo caused by both modification and \( \text{Mg}^{2+} \).

iii. Peak widths

The most notable effect of \( \text{Mg}^{2+} \) on i6A37-ACSL\( ^{\text{Pro}} \) is the sharpening of resonances in the loop region, particularly those most broadened by the dimethylallyl group. The linewidth of the most intense and broad U1,H5 peak decreases from ~81 Hz in the \( \text{Mg}^{2+} \) free form to 30 Hz with \( \text{Mg}^{2+} \).
"P 1D spectra at the indicated temperatures, in presence of 7mM Mg²⁺, of Left: ACSL⁻ and Right: i6A37-ACSL⁻. Both samples contain 5' a terminal monophosphate. The temperature at which each row of spectra was collected is indicated on the left of the figure.
Figure 5.5.9

C5-H5 region of 2D 13C-1H HMBC spectra of ACSL1- and 16A37-ACSL in the absence and presence of 7mM Mg2+ as indicated. The spectra were acquired at 25 °C.
Figure 5.5.10
C6-H6 region of 2D 1H-13C HMOC spectra of ACCL and i6A37-ACCL in the absence and presence of 7mM Mg2+ as indicated. The spectra were acquired at 25°C.
Figure 5.5.11
'H 1D spectra of imino protons of Top)ACSL''', Middle)i6A37-ACSL'' and Bottom)ACSL'' titrated with MgCl₂ to 10 mM. The temperature during acquisition was 12°C.
(Figure 5.5.9). The two $U_{ij}$ H6 resonances in the Mg$^{2+}$ free form, with linewidths of 35 and 30 Hz, become a single peak of 31 Hz linewidth with Mg$^{2+}$ (Figure 5.5.10). All the dimethylallyl group proton resonances also sharpen significantly. The proton resonances of the two dimethylallyl CH$_{3}$ groups are overlapped and have an apparent width of 100 Hz without Mg$^{2+}$, while in the presence of Mg$^{2+}$ they are better resolved and have a width of ~30 Hz together. The melting curves of ~1.8 µM i6A37-ACSL in the absence and presence of 100 µM Mg$^{2+}$ are very similar (Figure 5.5.12).

To exclude the possibility that the chemical shift effects attributed to the dimethylallyl group were due to residual Mg$^{2+}$ from the dimethylallyl modification reaction or any other previous conditions, i6A37-ACSL was dialyzed extensively with a 1M NaCl and 10 mM EDTA containing buffer followed by dialysis with a 5 mM EDTA containing buffer. Spectra in the later buffer were essentially the same as in the Mg$^{2+}$ free and EDTA free buffer, hence there is considered to be no residual Mg$^{2+}$ in the molecule and the chemical shifts similar to the Mg$^{2+}$ form of the unmodified molecule are indeed caused by the dimethylallyl group.

iv $T_{1s}$ measurements

$T_{1s}$ relaxation rates were measured for i6A37-ACSL in the presence of Mg$^{2+}$. As expected, the $^{13}$C $T_{1s}$ values are smaller than in the absence of this ion (Table 5.5.2), indicating a general broadening of the $^{13}$C resonances. In the presence of Mg$^{2+}$, the C8 resonances of the loop
Figure 5.5.12
Melting curves at UV 260nm wavelength of i6A37-ACSL"-" in the absence and presence of Co(NH₃)₆³⁺ or Mg²⁺. The concentrations for the Co(NH₃)₆³⁺ sample were 5.8 μM i6A37-ACSL"-", 29 μM Co(NH₃)₆³⁺. The concentrations for the Mg²⁺ sample were 1.8 μM i6A37-ACSL"-" and 100 μM MgCl₂. The percent change of the absorbance on each sample is plotted.
residues \( G_{14} \) and \( A_{15} \) have significantly longer \( T_{2p} \)'s than the rest of the purines. Likewise the \( C6 \) of \( U_{12} \) still relaxes faster than the \( C6 \) of \( A_{12} \), although in this case the goodness of fit to the exponential decay curve is around 95% for both (typical values for resolved intense resonances are >98%). These effects are similar to what was seen in the Mg\(^{2+}\) free form. However, the adenine \( C2 \) resonances for residues \( A_{13} \) and \( A_{14} \) have shorter \( T_{2p} \)'s than their stem congeners \( A_{11} \) and \( A_{12} \). In the absence of Mg\(^{2+}\), \( A_{15} \) and \( A_{16} \) \( C2 \)'s relax more slowly than the stem \( C2 \) nuclei, thus suggesting that Mg\(^{2+}\) restricts their dynamics (Table 5.5.2). \( T_{2p} \)'s for the \( Cl' \) resonances in the loop region, residues \( U_{12} \) to \( A_{15} \), were not measured due to assignment uncertainties and extreme broadness. \( U_{12} \) and \( U_{13} \) are only tentatively measured, because their assignment is uncertain (Table 5.5.2). The stem \( Cl' \) resonances have \( T_{2p} \)'s within 46 to 52 ms, indicating similar dynamic characteristics.

v. Relevant constraints for the dimethylallyl modified and unmodified ACSL

Since Mg\(^{2+}\) has a general broadening effect, a highly precise structure determination is prevented due to spectral overlap. However in the base to \( H1' \) region some crosspeaks are fairly well resolved, allowing their identification and giving insight into the molecules' structures.

The Mg\(^{2+}\) saturated ACSL\(^{pm}\) in the 2D NOESY still has double stranded helix characteristics up to residues \( U_{12} \) and \( A_{13} \). The \( H2 \) to \( H1' \) crosspeaks (\( A_{12} H2, U_{13} H1' \)) (\( A_{13} H2, U_{14} H1' \)) as well as the \( U_{12} \) imino resonance indicate that the 32-38 base pair is at least partially present. The presence of the \( (U_{14} H1', G_{14} H8) \) NOE crosspeak suggests the
<table>
<thead>
<tr>
<th>Residue</th>
<th>$T_\sigma$</th>
<th>Residue</th>
<th>$T_\sigma$</th>
<th>Residue</th>
<th>$T_\sigma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G27??</td>
<td>53.4</td>
<td>G27??</td>
<td>38.2</td>
<td>A31</td>
<td>38.1</td>
</tr>
<tr>
<td>G28</td>
<td>38.7</td>
<td>G29</td>
<td>43.6</td>
<td>A35</td>
<td>36.5</td>
</tr>
<tr>
<td>G29</td>
<td>38.5</td>
<td>G30</td>
<td>50.5</td>
<td>A36</td>
<td>36.3</td>
</tr>
<tr>
<td>G30</td>
<td>39.3</td>
<td>A31</td>
<td>39.3</td>
<td>A37</td>
<td>n.d.</td>
</tr>
<tr>
<td>A31</td>
<td>39.3</td>
<td>U32</td>
<td>31.9*</td>
<td>A38</td>
<td>37.6</td>
</tr>
<tr>
<td>U32</td>
<td>31.9*</td>
<td>U33</td>
<td>36.9*</td>
<td>U337</td>
<td>44.8</td>
</tr>
<tr>
<td>U33</td>
<td>36.9*</td>
<td>G34??</td>
<td>43.3</td>
<td>G14</td>
<td>n.d.</td>
</tr>
<tr>
<td>A35</td>
<td>41.2</td>
<td>A35</td>
<td>n.d.</td>
<td>A36</td>
<td>n.d.</td>
</tr>
<tr>
<td>A36</td>
<td>33.1</td>
<td>A36</td>
<td>n.d.</td>
<td>16A37</td>
<td>n.d.</td>
</tr>
<tr>
<td>16A37</td>
<td>n.d.</td>
<td>G14</td>
<td>n.d.</td>
<td>A38??</td>
<td>10.9</td>
</tr>
<tr>
<td>A38??</td>
<td>10.9</td>
<td>A38</td>
<td>n.d.</td>
<td>U39</td>
<td>n.d.</td>
</tr>
<tr>
<td>U39</td>
<td>31.2*</td>
<td>C43</td>
<td>34.9*</td>
<td>C40</td>
<td>47.9</td>
</tr>
<tr>
<td>C40</td>
<td>34.9*</td>
<td>C41</td>
<td>34.9*</td>
<td>C41</td>
<td>50.2</td>
</tr>
<tr>
<td>C41</td>
<td>34.9*</td>
<td>C42</td>
<td>34.2</td>
<td>C42</td>
<td>47.5</td>
</tr>
<tr>
<td>C42</td>
<td>34.2</td>
<td>C43</td>
<td>31.4</td>
<td>C43</td>
<td>31.4</td>
</tr>
</tbody>
</table>

**Table 5.5.2**

$^1$C $T_\sigma$ measurements, in milliseconds, for C6, C8, C2, and C1' of 16A37 in the presence of MgCl$_2$ titrated to 10mM. n.d.=nh data. Resonances marked by an asterisk are an average of the values for the two pyrimidines C6 split crosspeaks.
loop still does not contain a U-turn. The NOE crosspeaks (U₃,H1',,A₄,H8) and (U₃,H2',,A₄,H8) become more intense. These intensity changes suggest a more U-turn like conformation in the presence of Mg²⁺.

The Mg²⁺ saturated i6A37-ACSLₚₚs notably no longer has the NOE crosspeak (A₄,H2, U₃,H1'), which is present in the absence of Mg²⁺ and in ACSLₚₚw both with and without Mg²⁺. In contrast, the NOE crosspeak (A₄,H2, U₃,H1') becomes more intense than in the absence of Mg²⁺ and more intense than that of ACSLₚₚw with or without Mg²⁺. Mg²⁺ also causes the appearance of the new crosspeaks (A₄,H2, dimethylallyl CH) and (U₃,H1',, dimethylallyl CH₃). All other dimethylallyl proton NOEs identified in the absence of Mg²⁺, are present and sharpened. The above evidence suggests that the synergy of Mg²⁺ and the dimethylallyl group goes beyond the spectrum, both facilitating the opening the loop and making the base of A₄ more tightly stacked between the U₃ ribose and i'A₅. As in the unmodified molecule, the (U₃,H1',, A₄,H6) crosspeak is more intense in non Mg²⁺ saturating conditions but in the saturated form it would be overlapped with the (A₄,H1',,H8) crosspeak. Therefore it is very likely that the molecule again has more U-turn like characteristics than in the previous cases but still not a stable U-turn, since the (U₃,H1',,G₄,H8) crosspeak is still present and there is no downfield shifted "P resonance as would be expected for a sharp turn.

The presence of duplicate resonances with Mg²⁺ for U₃,H5 and H6, and G₄,NH₂, and the analogy of a resonance of each duplicate with those of the Mg²⁺ free form, suggests that ACSLₚₚw has two conformations in slow exchange on the NMR time scale, one of which corresponds to the Mg²⁺ free form and the other to the Mg²⁺ bound form. The similarity of the "Mg²⁺ form" resonances of those nuclei with the corresponding ones of i6A37-ACSLₚₚs suggests that Mg²⁺ promotes the same conformation as the dimethylallyl modification. The disappearance of the multiple
conformations of i6A37-ACSL\textsuperscript{Ph} upon addition of Mg\textsuperscript{2+} and the sharpening of very broad loop resonances, particularly of residues 33 and 37, supports the hypothesis that there is a synergistic effect for Mg\textsuperscript{2+} and the dimethylallyl modification. Both would stabilize the same conformation, alternate to that of the Mg\textsuperscript{2+} free ACSL. The broadening of U imino resonances in the loop by Mg\textsuperscript{2+}, and the chemical shift and NOE similarities between the Mg\textsuperscript{2+} forms of ACSL\textsuperscript{Ph} and i6A37-ACSL\textsuperscript{Ph}, indicate that this metal opens the loop. The absence of cross-strand NOEs for A\textsubscript{14}, and the sharpening of the dimethylallyl group protons and their NOEs, indicate that the new structure, although opened, is neither unstructured nor yet a U-turn.

Summary

The Mg\textsuperscript{2+} forms of ACSL\textsuperscript{Ph} show broadening of the imino resonances of U\textsubscript{11} and U\textsubscript{21} in a similar fashion as was seen for i6A37-ACSL\textsuperscript{Ph} in the absence of Mg\textsuperscript{2+}. In ACSL\textsuperscript{Ph}, Mg\textsuperscript{2+} leads to doubling of the U C5/H5 resonances and multiple H2/C2 and H1'/C1' resonances, with one predominating species apparently. Differences between the chemical shifts of ACSL\textsuperscript{Ph} in the presence and absence of Mg\textsuperscript{2+} occur on the regions of residues 33-35 and 38-41. The C1' chemical shifts from residues 34 to 37 of ACSL\textsuperscript{Ph} shift downfield. ACSL\textsuperscript{Ph} has NOEs in the anticodon region that are similar to those in the absence of Mg\textsuperscript{2+}. i6A37-ACSL\textsuperscript{Ph} in the presence of Mg\textsuperscript{2+} has similar shift changes as ACSL\textsuperscript{Ph} and its C1' resonances shift downfield and sharpen. The \textsuperscript{31}P spectra of ACSL\textsuperscript{Ph} and i6A37-ACSL\textsuperscript{Ph} do not show evidence of a new stable downfield shifted resonance. The chemical shifts of ACSL\textsuperscript{Ph} in the presence of Mg\textsuperscript{2+} for the U's H5/C5, U\textsubscript{14}H6/C6, and presumably the G\textsubscript{14} H2/N2 resonances resemble
those of 16A37-ACSL\textsuperscript{ph} in the absence of Mg\textsuperscript{2+}. Mg\textsuperscript{2+} induces sharpening of
the non-exchangeable broad loop resonances in 16A37-ACSL\textsuperscript{ph}, particularly
those of U\textsubscript{i,1} and all the dimethylallyl group protons. The U imino
resonances in 16A37-ACSL\textsuperscript{ph} are almost completely absent in the presence
of Mg\textsuperscript{2+}. The T\textsubscript{i,1}'s of 16A37-ACSL\textsuperscript{ph} C6 and C8 have a similar pattern in
the presence of Mg\textsuperscript{2+} as in its absence. The A\textsubscript{i,1} and A\textsubscript{i,2} T\textsubscript{i,1}'s are shorter
than those of A\textsubscript{i,1} and A\textsubscript{i,2}, suggesting that they become less dynamic in the
presence of Mg\textsuperscript{2+}. NOE evidence indicates that with Mg\textsuperscript{2+}, the U\textsubscript{i,1}'-A\textsubscript{i,1} base
pair is still present in ACSL\textsuperscript{ph} while in 16A37-ACSL\textsuperscript{ph} it is broken and
A\textsubscript{i,2} stacks more tightly with i'A\textsubscript{i,1} and U\textsubscript{i,1}. There is still a sequential
base to anomeric U\textsubscript{i,1} to G\textsubscript{i} NOE crosspeak. The evidence indicates that
Mg\textsuperscript{2+} stabilizes an open conformation of the loop, and based on the
chemical shift evidence, the conformation promoted by the dimethylallyl
modification is the same as the one promoted by Mg\textsuperscript{2+} in ACSL\textsuperscript{ph}, and they
act synergistically to stabilize this open conformation. There is no
evidence however to indicate formation of a U-turn.
5.6 Pseudouridylated ACSL

Pseudouridine is the most common modified nucleotide. In the case of the anticodon stem loop of E. coli tRNA^{Phe}, two pseudouridines are present at positions 32 and 39. The interest in pseudouridine 32 is that it would be at a loop-stem junction, if the fully modified ACSL^{Phe} has a U-turn conformation. The structural and functional effects of pseudouridines in such regions is mostly unknown. The hypothesis then was that the presence of pseudouridine would help form the U-turn conformation, by making a bifurcated base pair involving O2 of \( \psi_{32} \) and N6 of \( \Lambda_{32} \). This proposal is based on analogy to the proposed bifurcated base pairs seen in crystallographic and theoretical studies of tRNAs (Auffinger and Westhof 1999). The data of the singly modified molecule clearly disproved the hypothesis and proved that in this case a regular base pair involving \( \psi_{32} \) and \( \Lambda_{32} \) formed. The use of the ACSL^{Phe} as our model system allowed the enzymatic modification of \( U_{32} \) to \( \psi_{32} \) in a labeled RNA using the pseudouridine synthetase RluA. This capability made it possible to assign pseudouridine \( ^{13}C \) shifts for the first time and to study the effects of the modification through heteronuclear NMR techniques.

i. Spectral assignments

The ACSL^{Phe} was modified at position 32 to a pseudouridine as described in chapter 4. Eighty and 100 OD_{260} of \( ^{13}C \) and \( ^{15}N \) labeled ACSL^{Phe}, respectively, were pseudouridylated. Completion of the reaction was confirmed through the disappearance of the H5-H6 crosspeak.
corresponding to residue 32 in the unmodified molecule, in a COSY spectrum. The $U_{12} H5/C5$ crosspeak of ACSL$^{\text{Fhe}}$ was absent in the $^{13}$C-$^1$H HMQC spectrum of the $^{13}$C labeled $\Psi_{32}$-ACSL$^{\text{Fhe}}$ (Figure 5.6.1), also confirming the completion of the reaction.

The assignment strategy was the same as that used for ACSL$^{\text{Fhe}}$. The spectra of ACSL$^{\text{Fhe}}$ and $\Psi_{32}$-ACSL$^{\text{Fhe}}$ are very similar (Figure 5.6.2). Some differences are that $\Psi_{12} H6$, shifts upfield with respect to $U_{12} H6$, to 6.92 ppm, and $\Psi_{12} H1'$ shifts upfield with respect to $U_{12} H1'$, to 4.75 ppm (Appendix VII, Figure 5.6.1). Additionally a small chemical shift change for $A_{12} H1'$ makes it degenerate with $G_{12} H1'$. Identification of the $\Psi_{12}$ resonances is as follows. The $\Psi_{12} H6$ and $H1'$ can not be correlated in a long range $^{1}$N HSQC because C5 is involved in the glycosidic bond instead of N1. The $\Psi_{12} H6$ was identified by its sequential crosspeak with $A_{12} H1'$ in 2D NOESY spectra and its $H6/C6$ crosspeak in a non refocused $^{13}$C-$^1$H HMQC. The crosspeak clearly contains a pyrimidine splitting pattern in the $^{13}$C dimension caused by coupling to C5, while the rest of the pyrimidines are essentially unchanged (Figure 5.6.1). The $\Psi_{12} H1'$ was identified through its intense sequential crosspeak to $A_{12} H2$, which is expected for a base paired region (Figure 5.6.2). The assignment of the rest of the non exchangeable $^1$H resonances was achieved using a 2D HCCH-COSY, a 3D HCCH-TOCSY optimized for the ribose protons and a 2D HCCH-TOCSY optimized for H2/C2-H8/C3 correlations (Appendix VII). A sequential assignment was also traced for the H2'-H6/H8 region of a 2D NOESY spectrum, confirming these assignments. The $\Psi_{12} C1'$ resonance is upfield shifted, it resonates in the region of the C4' resonances, at 82.78 ppm, consistent with it being bonded to another carbon instead to a more electronegative nitrogen. To our knowledge this is the first assignment of the $^{13}$C resonances of a pseudouridine.

Assignment of the $^{31}$P resonances was achieved using a $^{31}$P/$^1$H Hetero-
Figure 5.6.1
Aromatic and anomic regions from a 2D $^{13}$C-$^1$H HMOC spectrum of $^{13}$C enriched $\Psi 32$-ACSL$^\text{32}$. The temperature during acquisition was 25 $^\circ$C.
Figure 5.6.2

H6/H8 to ribose proton spectral region of a 2D-HOESY acquired on Ψ32-ACSL™ at 25°C. The mixing time was 400 ms. The H6/H8 to H1' sequential assignment is traced by a solid line and that for the base to H2' is traced by a dotted line. The intranucleotide crosspeaks are labeled with the residue assignment.
TOCSY NOESY. All of these resonances are clustered within -3.65 and
-4.65 ppm and have only small differences from those of ACSLα* (Appendix
VII).

The assignment of the exchangeable resonances was analogous to
that for ACSLα*. A 2D 15N-1H HMOC optimized for the imino region shows
the appearance of a strong imino crosspeak at (10.31, 128.59 ppm) in the
region reported for the Ψ11 N1 imino at around 10 ppm for 1H and 130 ppm
for 15N (Roy et al 1984, Griffey et al 1985). A very strong NOE
crosspeak between the imino resonance at 10.31 ppm and the Ψ12 H6
confirms its identity as Ψ12 N1, which is further supported by an intense
crosspeak from the Ψ12 H6 to the 15N resonance at 127.57 ppm in the long
range HSQC. This crosspeak suggests a modest coupling between the two
nuclei, as would be expected for a two-bond coupling to N1 as opposed to
a four-bond coupling to N3. The imino 1D and 15N-1H HMOC spectra also
show a typical U imino resonance at 13.86 and two extremely weak
resonances at 13.21 and 13.05 ppm (Appendix VII, Figure 5.6.3). In this
order, they were assigned to U11, U13, and U1, N3/H3 by a 2D NOESY in 90% H2O and an HNN-COSY in 100% 2H2O. The H2 to H3 NOE crosspeaks and H2 to
N3 through bond correlation clearly confirm that U11 is base paired with
Ψ11 and Ψ12 is base paired with Ψ1, through its N3 imino group (Figures
5.6.4 and 5.6.3). An Ψ12 H2 to U11 N3 crosspeak is also weakly visible in
the HNN-COSY. There are no appreciable chemical shift differences caused
by the pseudouridine modification on the amino regions of ACSLα* and
Ψ32-ACSLα*. The amino intensities were not analyzed because they are
more difficult to evaluate in 2D H2O spectra, and because of resonance
overlap in 1D spectra. Only minor shifts are discernible for the C12
amino resonances. The same NOE crosspeaks of the proposed G amino of
ACSLα*, to the Ψ11 and Ψ13 H2 and H8 resonances, are present in Ψ32-
ACSLα*. The chemical shifts of the proposed G amino in ACSLα* are 6.28
Figure 5.6.3
Top) 1H-1H 2D HMQC acquired on $\Psi^{32-\text{ACSL}^{\text{in}}}^{\text{in}}$ optimized for the imino resonance region. Bottom) Imino proton I D of $\Psi^{32-\text{ACSL}^{\text{in}}}^{\text{in}}$ (upper spectrum) and of $\text{ACSL}^{\text{in}}$ (lower spectrum). The temperature during acquisition was 12°C.
Figure 5.6.1
2D NOESY in 90% H₂O-10% D₂O of the imino region of Ψ32-AcSL⁻. The G imino assignments are indicated above the diagonal peaks and the U imino assignments at the top of the spectrum. NOE crosspeaks of U, and Ψ, H3 to the H2 of their base pairing partners are indicated. Also indicated are the crosspeaks to Ψ, H1. The temperature during acquisition was 15°C.
The chemical shifts of the proposed G amino in $\Psi_{32}$-ACSL$^{\text{Ph}}$ are 6.26 $^1H$, 71.65 $^{15}N$ ppm. These amins are presumably belong to a G because their $^{15}N$ chemical shifts are between 70 and 75 ppm, which is typical of guanosine amino nitrogens. These amins might belong to G$_{14}$, because G$_{14}$ is in the loop and would be expected to be closer to A$_{10}$ and A$_{14}$ than the stem G's.

### ii Relevant constraints

Overall the $\Psi_{32}$-ACSL$^{\text{Ph}}$ has the same structural features as the ACSL$^{\text{Ph}}$. Similar to the ACSL$^{\text{Ph}}$, $\Psi_{32}$-ACSL$^{\text{Ph}}$ H$_2$O NOESY spectra show the same sequential imino to imino connectivities between G$_{2}$ and G$_{1}$, and the same G imino to C amino NOE pattern, confirming base pairs G$_{25}$-C$_{24}$, G$_{26}$-C$_{25}$, G$_{1}$-C$_{43}$ (Figure 5.6.4). The base to anomic and base to H2' region connectivities for residues 27 to 33 and 34 to 43 had very similar intensities to those of the unmodified molecule. The evidence indicates that after the pseudouridylation, the stem residues continue to have a C3' endo character. There are strong sequential H2' to base connectivities between residues 27-33 and 38-43; the C3' and C4' chemical shifts are consistent with this conformation (Appendix VII) and the H1'-H2' couplings are <5Hz.

As noted above, $\Psi_{12}$ base pairs with A$_{1}$ through its N3 imino group. Notably its H1 imino is remarkably more stable, and yields strong NOESY crosspeaks. The NOE crosspeaks indicate that the conformation of $\Psi_{12}$ and its surroundings is similar to that of U$_{12}$ as they are analogous to the H5 crosspeaks seen in U$_{12}$ of ACSL$^{\text{Ph}}$ (Appendix VIII). Examples of protons that give analogous NOE crosspeaks with U$_{12}$H5 of ACSL$^{\text{Ph}}$ and $\Psi_{12}$H1 of $\Psi_{32}$-ACSL$^{\text{Ph}}$ are A$_{1}$H8, A$_{1}$H2', A$_{1}$H3', U$_{12}$H5' or $\Psi_{1}$H5' respectively, and
U_{i1}, H5. Further support for a similar position of \( \Psi_{12} \) comes from the NOE crosspeaks \( A_{i1}, H2 \) to \( \Psi_{11}, H1' \) and sequential \( A_{i1}, H8 \) to \( \Psi_{12}, H6 \) and H1, indicating that \( \Psi_{12} \) is part of an A-form type helix. The same is true for its base pairing partner \( A_{i1} \). The imino resonances of the stem guanines proximal to \( \Psi_{12} \) are more intense than those farther away (Figure 5.6.3). This pattern contrasts with that seen in ACSL\(^{Ph} \), where the NH resonance of \( G_{i1} \), is more intense than that of \( G_{i2} \) (Figure 5.1.1). The \( U_{i1} \) and \( \Psi_{12}, H3 \) resonances are broader than the corresponding peaks in ACSL\(^{Ph} \), indicating that these iminos have a higher exchange rate in \( \Psi_{32}-ACSL\(^{Ph} \). The increase of the intensity for the \( G_{i1} \) imino proton resonance with respect to that of \( G_{i2} \) and the similar intensities of the \( G_{i2} \) and \( G_{i1} \) resonances in ACSL\(^{Ph} \), indicate that there is less solvent exchange. The lower solvent exchange for the imino resonance of \( G_{i1} \) and the higher one for the imino resonance of \( U_{i1} \), compared to ACSL\(^{Ph} \), suggests that \( \Psi_{12} \) stabilizes the \( G_{i1}-C4 \) base pair, but destabilizes base pair \( U_{i1}-A_{i1} \). The melting curve at 260 nm wavelength of \( \Psi_{32}-ACSL\(^{Ph} \), indicates base stacking in this molecule is more temperature stable than in ACSL\(^{Ph} \) (Figure 5.6.5).

The HNN-COSY spectrum indicated the presence of the \( U_{i1}-A_{i1} \) base pair, albeit with a weaker crosspeak than that in ACSL\(^{Ph} \); it is barely visible (Figure 5.6.6). This weakness is not likely due to the lower concentration of the \( \Psi_{32}-ACSL\(^{Ph} \), as the intensity ratio of the crosspeak \( (U_{i1}, N3, A_{i1}, H2) \) to other crosspeaks in the HNN-COSY, is lower than the corresponding ratios in the spectrum of ACSL\(^{Ph} \). The presence of this base pair is further supported by the conserved \( A_{i1}, H2 \) crosspeaks to \( G_{i2} \) and \( A_{i1}, H1' \) and the very weak \( U_{i1} \) imino 2'N-1'H crosspeak in the 2D HMQC, which has a characteristic chemical shift for base paired uridines. Still, \( U_{i1} \) and \( A_{i1} \) show intermediate characteristics of C2'-C3' endo, as their coupling constants are detectable in a COSY (Table 5.6.1).
Figure 5.6.5
Melting curves at UV 260nm wavelength of ACSL™, i6A37-ACSL™, and Ψ32-ACSL™. Each curve is an average of three independent measurements. For each measurement, the minimum absorbance was set to 0 and the data normalized to the maximum absorbance defined as one.
Figure 5.6.6

Left) HNN COSY spectrum acquired on Ψ32-ACSL. Right) Long range "N-H HSQC of Ψ32-ACSL" showing intra-residue U H5 to N3 and Ψ H6 to N1 correlations, to indicate the positions of the U N3s and Ψ N1. The HNN-COSY shows cross-strand H2-N3 crosspeaks for residues 31-39, 38-32 and absence of a (Ψ, N1, A, H2) crosspeak. The temperature during acquisition was 25 °C.
<table>
<thead>
<tr>
<th>Residue</th>
<th>$J_{\chi_i-\chi}$ (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U33</td>
<td>3.7</td>
</tr>
<tr>
<td>G34</td>
<td>6.2</td>
</tr>
<tr>
<td>A35</td>
<td>6.9</td>
</tr>
<tr>
<td>A36</td>
<td>5</td>
</tr>
<tr>
<td>A37</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 5.6.1**
Detectable H1'-H2' couplings in Ψ32-ACS1. Couplings were measured from a DQF-COSY.

and their C3' and C4' chemical shifts deviate from those expected for an A-form helix but are still below the C2' endo limit. Additionally the H2'-H8 intraresidue A₃ crosspeak is very intense. The above spectral data is consistent with this region as a transition between the helix and the loop region. The base pair distance constraint utilized for the simulations was increased by 1 Å as it was for ACSL²⁹⁻. Likewise the ribose pucker was left unconstrained.

The loop region shows again a very weak sequential NOE base-H1' connectivity between U₁, H₁' and G₄, H₈. Also present is an extremely weak U₁, H₁' to A₃, H₈ crosspeak seen only at long mixing times (360 ms or more). A sequential NOE crosspeak between U₁, H₆ and G₄, H₈ supports the existence of partial stacking of the U₁ and G₄ bases. The anticodon residues continue to have a C2' endo character after the modification. Strong intraresidue and weak sequential H2' to H₈ NOE crosspeaks are present between residues G₄, A₃, and A₄. Their C4' and C3' resonances are close to those expected for a C2' endo conformation (Appendix VII) and their H1'-H2' couplings are close to or above the 7 Hz boundary typical of such conformation (Table 5.6.1). Residues G₄ and A₃ were constrained to C2' endo while A₄ was left unconstrained.

A₃, H₂ crosspeaks to U₁, H₂', A₄', H₄ and H₅' indicate that Watson-Crick base pair functional groups are oriented to the interior of the
molecule, pointed toward the residues flanking its 5' side. The A16H2 crosspeak to G14 and/or A15 H1', although these are degenerate, lends further support to this orientation. The conserved NOE crosspeaks A16H2 to A17 H2 and H1' suggest that A17 is partially stacked with A17'. An overlapped crosspeak between A16H2 and A15H1' suggests that A16 has slid towards A15.

Based on the 31P chemical shifts, the α and ζ torsion angles were constrained to exclude the trans conformation as in ACSL\textsuperscript{Phe} for the stem residues and left unconstrained for the loop residues. The β torsion angles for G34 and A35 were constrained to trans as the corresponding H5'-P crosspeaks were clearly absent in the HetCor spectrum. The γ torsion angle can be constrained to gauche if there is an absence of the H4'-H5' and H4'-H5''' crosspeaks in the COSY spectrum. As in the unmodified molecule, only residues C45, C46 and C47 were constrained to this conformation because the presence or absence of other H4'-H5'/5''' crosspeaks cannot be confirmed due to spectral overlap. In the loop region G34 and A35, again show, particularly for the latter, intense H4'-H5' and H4'-H5''' crosspeaks. Since the H4' to H5' and H5''' crosspeaks would be expected to have spectral overlap as was the case for the majority in the unmodified molecule, no attempt was made to distinguish whether or not unequal intensities of H4'-H5' to H4'-H5''' NOEs were present in 3D 13C edited NOESY experiments.

Important constraints from A15 and A16 H2s were not detected, were overlapped, or were extremely weak, especially those involving A15.

Important missing constraints are (A16H1', A16H2) and (A15H1', A15H2). Among the new constraints, not seen in the ACSL\textsuperscript{Phe}, are (A12H2, Ψ12H6) (A12H2, A12H), (U13H1', U13H5). Most of the constraints of the ACSL\textsuperscript{Phe} that are not present in the Ψ32-ACSL\textsuperscript{Phe} belong to inter-ribose or exchangeable resonances. The inter-ribose constraints did not have a large
conformational effect in the structure determination of ACSL\textsuperscript{PH*}, hence no
effort was put into collecting and analyzing the required 3D spectra. In
the exchangeable NOESY spectra some of the weakest resonances were
undetectable. This seems to be in part due to a lower concentration of
the molecule because some of these resonances occurred in the stem
region, but may also be due to the broader lines of the \(\Psi\)_\text{II} and \(U\)_\text{II} \(H^3\)
resonances.

iii. Loop structure and comparison with the unmodified molecule

To calculate \(\Psi32\)-ACSL\textsuperscript{PH*} structures the same computational
strategy that was used for ACSL\textsuperscript{PH*} was employed. The topology and
parameter files for the standard nucleic acid residues were modified as
described in the methods section to include pseudouridine. A total 36
base pair constraints, 232 NOE distance constraints and 54 torsion angle
constraints were defined. The lower number of constraints than those
used for ACSL\textsuperscript{PH*} is attributed in part to some degeneracy, particularly
of resonances \(G^2\_H1\) with \(A^1\_H1\)\', \(A^1\_H2\) with \(G^2\_H8\), and to a lower
concentration of the \(\Psi32\)-ACSL\textsuperscript{PH*}. 2.6 mM compared to 3.5mM of the ACSL\textsuperscript{PH*}.
Constraints for the \(\epsilon\) torsion angles were not derived due to a lack of
required instrumentation.

Using the distance constraints derived only from NOEs of clearly
resolved resonances, the calculations produced lowest energy structures
analogous to the second most populated structure of the simulations that
included the full set of constraints (Figure 5.6.7). The family of
structures with the second lowest energy in the calculations using the
partial set of constraints, has the same conformation as the convergent
ACSL\textsuperscript{PH*}, with \(G^1\) flipped towards the major groove side of the helix. With
inclusion of all the constraints, the obtained \( \Psi^{32} \)-ACSL\textsuperscript{Fhe} lowest energy structures are the same as the ACSL\textsuperscript{Fhe} convergent structure (Figure 5.6.7). The statistics for these convergent structures are given in Table 5.6.2. The RMSD of the \( \Psi^{32} \)-ACSL\textsuperscript{Fhe} and ACSL\textsuperscript{Fhe} minimized average convergent structures is 1.13 Å. The energy difference between the two structures of \( \Psi^{32} \)-ACSL\textsuperscript{Fhe} is small, but predicted NOEs for both agree better with the structure that includes the constraints analogous to those used in ACSL\textsuperscript{Fhe}. For example, \( G_{11}H8 \) is farther apart from \( U_{11}H1' \) in the structure with the extra H2 constraints. The other structure has a distance of <4 Å for this proton pair, which does not agree with the very weak intensity of the corresponding crosspeak seen in NOESY spectra. The disagreements are not sufficient to qualify the structure that is not analogous to ACSL\textsuperscript{Fhe} as inaccurate, because \( T_1 \), local spin density and rapid dynamics could affect the NOE intensity and are not known. NOEs for non-exchangeable protons in the loop region, that should be observed or that do not agree with the intensities of those observed in the spectra are listed in Appendix IX.

It is concluded that the pseudouridylation at residue 32 has some spectral effects that indicate it increases the stability of the end of the run of G-C base pairs in the stem and is base paired to \( A_{14} \) in anti conformation. The spectral effects are specifically: \( \Psi_{12} \) sharpens and increases the relative intensity of \( G_{12} \) imino proton compared to that of \( G_{12} \), has similar H1 NOE crosspeaks as those of \( U_{11}H5 \), has \( \Psi_{11}:N3 \) to \( A_{14}:H2 \) correlation in an HMN-COSY. The stability of the \( \Psi_{12} \) H1 imino proton is remarkable, but is not unexpected based on other reports, and suggests it is protected from exchange. The loop is prone to solvent exchange as evidenced by broadening of the U H3 resonances. The modification leaves
Figure 5.6.7 (Next page)
Stereo views of minimized average structures of Ψ32-ACSL™: A) Calculated from the eight lowest energy structures obtained with uncertain H2 constraints in addition to those used for B. These H2 constraints were included by analogy to ACSL™, they were either too weak or ambiguous because of degeneracy. B) Calculated from the seven lowest energy structures obtained with constraints from only clearly present and resolved NOE crosspeaks. The base in Ψ1 is color-coded by atom type (red O, blue N). A, is colored purple.
### NOE distance constraints
- **intraresidue**: 38
- **interresidue**: 144
- **mean number per residue**: 13.6

### NOE constraints by category
- **strong**: $0-3$ A, 7
- **medium**: $4-6$ A, 24
- **weak**: $6-9$ A, 67
- **very weak**: $9-12$ A, 65
- **extremely weak**: $>12$ A, 47
- **super weak**: $>15$ A, 1

### Base pair constraints
**total**: 4

### Torsion angle constraints
- **ribose ring**: 24
- **backbone**: 28
- **mean number per res**: 3.1

### Convergent structures
15/60 (cutoff last 20)

### Violations
- **average distance constraints**: $>0.3$ A
  - RMSDs for distance constraints (Å): 0.0273 (average: $-0.00626$)
  - RMSDs for distance constraints deg: 1.7
- **average dihedral constraints**: $>1.5$ deg
  - RMSDs for dihedral constraints deg: 1.24 ($-1.156$)
- **RMSDs from ideal geometry**
  - dihedral A: 1.0169 ($-1.0012$)
  - angles deg: 1.165 ($-1.125$
- **RMSDs from minimized mean structure**
  - residues 1-38: 1.631 ($-1.156$)
  - residues 1-51, 39-43: 1.664 ($-1.122$

---

**Table 5.6.2**

Summary of experimental constraints and structure calculation statistics for $\Psi_{32}$-ACSL$^\text{32}$. 
the ACSL\textsuperscript{Phe} structure essentially unchanged, allowing formation of the
weak base pair U\textsubscript{11}-A\textsubscript{1}, and keeping a trinucleotide loop formed by the
anticodon residues.

Summary

Pseudouridine 32 formation was achieved in ACSL\textsuperscript{Phe} by simple
addition of the RiuA enzyme. The chemical shifts of \(\Psi_{32}\)-ACSL\textsuperscript{Phe}
resonances are very similar to those of ACSL\textsuperscript{Phe}. All the \(^1H\), \(^1C\), \(^15N\) and
\(^31P\) assignments of \(\Psi_{12}\) were obtained using NOE, 2D and 3D heteronuclear
experiments. The \(\Psi_{12}\)-H1 peak is intense, indicating that it is protected
from solvent exchange. The stem G imino resonances are sharper the
closer they are in sequence to \(\Psi_{12}\). A slight broadening occurs in the
loop U iminos relative to ACSL\textsuperscript{Phe}. \(\Psi_{32}\)-ACSL\textsuperscript{Phe} has similar NOEs to those
of ACSL\textsuperscript{Phe}. The molecule contains a stem that includes base pairs U\textsubscript{11}-A\textsubscript{1},
and U\textsubscript{11}-A\textsubscript{11}, the latter base pair is relatively weaker than in ACSL\textsuperscript{Phe}.
The conformation of \(\Psi_{12}\) is similar to that of U\textsubscript{11} as the H1 NOE
crosspeaks resemble those of the H5 from U\textsubscript{11}, it is stacked between U\textsubscript{11}
and A\textsubscript{11}, and is base paired with A\textsubscript{11} in an anti conformation. \(\Psi_{32}\)-ACSL\textsuperscript{Phe}
has a higher melting temperature than ACSL\textsuperscript{Phe}. The NOEs and the coupling
constants that could be measured (\(\beta\), \(\gamma\)) for the loop region are
analogous to those of ACSL\textsuperscript{Phe} with the exception that G\textsubscript{14} and A\textsubscript{14} H1'
resonances are degenerate and the crosspeaks to the H2 protons of A\textsubscript{14}
and A\textsubscript{14} are extremely weak. The calculated structure is essentially
identical to that of ACSL\textsuperscript{Phe} when all the constraints, including those
degenerate but deduced by analogy to ACSL\textsuperscript{Phe}, are used. The evidence
shows \(\Psi_{12}\) in the context of an unmodified ACSL preferentially base pairs
with an A\textsubscript{1}, in a Watson-Crick fashion and stabilizes the extended stem.
5.7 Double modified ACSL

i. Dynamic loop

The study of i6A37Ψ32-ACSL^Phe is in its early stages. The results indicate however that pseudouridine at position 32 has a notable effect in stabilizing the loop of the molecule, in comparison with i6A37-ACSL^Phe. The double modified molecule, i6A37Ψ32-ACSL^Phe, was obtained by enzymatic pseudouridylation of i6A37-ACSL^Phe using the RluA enzyme. Thirty and 60 OD units of ^1^C and ^1^N labeled material respectively were obtained. The ^1^C 2D ^1^C-^1^H HMQC spectra show completion of the reaction based upon the characteristic upfield shift of both the H6 and C6 resonances of Ψ^;^; and the absence of the U^;^;H5/C5 crosspeak (Figures 5.7.1 and 5.7.2). The ^1^N labeled molecule also seemed to reach completion. Four Ψ^;^;H1/N1 crosspeaks appear, one of them belonging to Ψ^32-ACSL^Phe. The crosspeak corresponding to U^;^;H3/N3 and probably U^;^;H3/N3 is gone, and two new U imino crosspeaks appear in the base paired region of the spectrum (Figure 5.7.3). These new crosspeaks are tentatively assigned to U^;^; and Ψ^;^;N3/H3.

In the 1D imino proton spectrum the G^;^; and particularly G^;^; imino peaks are more intense and sharper than in i6A37-ACSL^Phe (Figure 5.7.4). Therefore Ψ^;^; counteracts the increased exchange of these G imino protons caused by the dimethylallyl group. The new peak at 13.59ppm is tentatively assigned to the Ψ^;^; imino resonance as it is only visible with pseudouridylation and is still in the region of base paired uridines. This resonance is broad in the ^1^N and ^1^H dimensions in the 2D ^1^N-^1^H HMQC. The melting curve of i6A37Ψ32-ACSL^Phe at 260 nm UV is very similar to that of i6A37-ACSL^Phe (Figure 5.7.5).
Figure 5.7.1
C6-8/H6-8 and C2:H2 regions of 2D "C-H HMQC spectra of i6A37Ψ32-ACSL" at 17, 25 and 30 °C. The asterisks mark the resonances corresponding to the single modified Ψ32-ACSL present in a small amount on this sample.
Figure 5.7.2
C5/H5 and C1'/H1' regions of 2D 13C-1H HMQC spectra of i6A37Ψ32-ACSL"" at 17, 25 and 30 °C. The asterisks mark the resonances corresponding to the single modified Ψ32-ACSL"" present in a small amount on this sample. Two U33H5/C5 crosspeaks that become more intense at 17 or 30 °C are indicated by >17 and >30 respectively.
Figure 5.7.3

$^1$H-$^1$H 2D HMOC spectrum optimized for the imino resonance region acquired on 16A37$\psi\psi$-ACSL<sup>+</sup>. The temperature during acquisition was 12 °C. $\psi_{\psi}$ N1/H1 imino group shows two crosspeaks. * Crosspeaks due to a contaminant.

Figure 5.7.4

Imino proton 1D spectrum of 16A37$\psi\psi$-ACSL<sup>+</sup> (upper spectrum) and of 16A37-ACSL<sup>+</sup> (lower spectrum). The temperature during acquisition was 12 °C.
Figure 5.7.5
Melting curves at UV 260nm wavelength of i6A37-ACSL Phe, Ψ32-ACSL Phe, and i6A37Ψ32-ACSL Phe. Each curve is an average of three independent measurements. For each measurement, the minimum absorbance was set to 0 and the data normalized to the maximum absorbance defined as one.
The multiple $\Psi_{12}$ NL/H1 crosspeaks indicate that some conformational heterogeneity exists. The $^{13}$C-$^1$H HMQC spectra also indicate conformational heterogeneity for i6A37Ψ32-ACSL$^{\phi \psi}$. At 25 °C the molecule shows characteristics of intermediate exchange for the C6/H6 pairs of $\Psi_{12}$, U$_{11}$ and U$_{13}$, and some purine C8/H8 yet to be assigned. Multiple resonances are also seen for U$_{11}$ C5/H5 and a broadened U$_{13}$ C5/H5 (Figure 5.7.2).

At 17 °C and 30 °C the C6/H6 crosspeaks of $\Psi_{12}$, U$_{11}$, and U$_{13}$ sharpen (Figure 5.7.1). Two of the three U$_{13}$ C5/H5 crosspeaks change their intensities in such a way that one is more intense at 17 °C and weaker at 30 °C and the other one is more intense at 30 °C and weaker at 17 °C. The third U$_{11}$ C5/H5 crosspeak is weak at 17 °C, 25 °C and 30 °C.

The U$_{13}$ C5/H5 crosspeak at 17 °C is almost unique. At 17 °C, new duplicate crosspeaks are distinguishable for U$_{13}$C6/H6, $\Psi_{12}$C6/H6 and U$_{13}$C5/H5. These new crosspeaks are identified based on their proximity to the most intense crosspeak of U$_{13}$, $\Psi_{12}$, or U$_{13}$.

After the pseudouridylation, the loop C1'/H1' upfield crosspeaks in a 2D HMQC spectrum are still very broad, mainly in the $^{13}$C dimension (Figure 5.7.2). Pseudouridylation causes the C1' of i$^5$A$_{1}$ and A$_{21}$ to shift downfield about 0.87ppm and 1.17ppm respectively, at 25 °C (Figure 5.7.2). The downfield shifts are counteracted by high temperature. At 30 °C these resonances resemble those of i6A37-ACSL$^{\phi \psi}$ more than they do at 25°C. An increase in temperature presumably counteracts the stabilization of C3' endo conformation induced by pseudouridine and promotes a regime of intermediate exchange similar to that conferred by the dimethylallyl modification on ACSL$^{\phi \psi}$. At 17 °C, A$_{35}$ and another residue with an upfield shifted C1' in i6A37-ACSL have a more downfield shift than at 25 °C. The C1'/H1' crosspeaks upfield from the main C1' cluster sharpen. One of these $^{13}$C resonances shifts upfield instead and
is still yet to be assigned. An analogous upfield shifted resonance is also present in i6A37-ACSL\textsuperscript{Phe} at 12 °C, and hence seems to be associated with a conformation that is stabilized at low temperature. Given the multiple conformations present at 25°C, the molecule will be studied at 17°C, to enrich for the conformation stabilized at lower temperature. The conformation at 30°C is considered the melted form, and resembles \textit{i6A37-ACSL\textsuperscript{Phe}}. This consideration is based on the similarity of the chemical shifts and peak broadness of the Cl'/H1' crosspeaks with upfield Cl' resonances, to the Cl'/H1' crosspeaks from i6A37-ACSL\textsuperscript{Phe}. 

\textbf{Summary}

Double modified ACSL\textsuperscript{Phe} molecules containing \textit{i}^6A'\textsubscript{i} and $\Psi$\textsubscript{i} were synthesized by pseudouridylation of the \textsuperscript{13}C and \textsuperscript{15}N labeled i6A37-ACSL\textsuperscript{Phe} molecules. $\Psi$\textsubscript{i} decreased the broadness of the stem G imino residues compared with i6A37-ACSL\textsuperscript{Phe}. The $\Psi$\textsubscript{i} H1'/N1 and the U H6/C6 and H5/C5 resonances show multiple conformations. The U\textsubscript{i}, H5/C5 resonances show one crosspeak that increases in intensity by reducing the temperature from 25 to 17 °C, while another pair of crosspeaks, that are analogous to those of i6A37-ACSL\textsuperscript{Phe}, become more intense by increasing the temperature to 30°C. At 25°C the C6/H6 crosspeaks of $\Psi$\textsubscript{i}, U\textsubscript{i}, and U\textsubscript{i} are in intermediate exchange and they sharpen up both at 17 °C and 30 °C. Pseudouridylation causes a slight downfield shift of the Cl' resonances of \textit{i}^6A'\textsubscript{i} and A'\textsubscript{i}, which is enhanced at 17 °C and counteracted at 30 °C. Hence it appears that the dimethylallyl modification still destabilizes the ACSL\textsuperscript{Phe} loop structure, while $\Psi$\textsubscript{i} in part counteracts this effect, promoting a new structure.
5.8 Mn²⁺, Co(NH₃)₆³⁺ and induction of a U-turn

i. Mn²⁺ and Co(NH₃)₆³⁺

To probe for a binding site for divalent cations in both molecules we used two strategies. One was the use of Mn²⁺. It is a paramagnetic ion and broadens resonances that are within a distance of 10 Å. The effect is proportional to 1/ᵣ⁴. Typically the metal ion bound to RNA has a short residence lifetime and the interaction is in the fast exchange regime (off rates ≤ 1000 s⁻¹). A substoichiometric concentration, in the order of micromolar, can yield appreciable effects because a single ion can affect multiple sites (Allain and Varani 1995, Butcher et al. 2000, Gonzalez and Tinoco 2001).

The second strategy is the use of Co(NH₃)₆³⁺, which is an analog for hexahydrated Mg²⁺ and has amino protons which can be detected through NMR. NOE crosspeaks between the RNA protons and these amino protons localize the binding site. There are differences however between hexahydrated Mg²⁺ and Co(NH₃)₆³⁺. The higher positive charge of Co(NH₃)₆³⁺ leads to a stronger electrostatic effect when interacting with the RNA and more effectively neutralizes the backbone negative charge. Co(NH₃)₆³⁺ has been found to have around 10 fold lower Kᵦ’s than Mg²⁺, for metal binding sites in nucleic acids (Gonzalez and Tinoco 1999, Gonzalez and Tinoco 2001). Co(NH₃)₆³⁺ has also been proposed as an inorganic analog for organic polyamines such as spermine and spermidine.

MnCl₂ was titrated into ACSL²⁺ and i6A37-ACSL²⁺ to concentrations of up to 50 micromolar as described in Materials and Methods. The non-exchangeable resonances of ¹³C bound protons as well as ¹⁵N and ³¹P resonances were monitored. Both molecules showed a strong broadening
effect in the first four G residues' H6/C8 and \(^{31}\)P resonances (Figures 5.8.1 and 5.8.3). In the presence of 5 μM MnCl\(_2\), the ACSSL\(\text{Fm}^{\text{es}}\) N7 and N9 resonances of the first four residues are absent and A\(_{11}\)N7 is broadened, while its N9 is unaffected (Figure 5.8.2). i6A37-ACSSL\(\text{Fm}^{\text{es}}\) was not tested for these two types of nuclei. There is a gradually weaker effect for residues 31 to 34 (Figure 5.8.3 and Table 5.8.1). Both molecules also showed some broadening in residues 38 of i6A37-ACSSL\(\text{Fm}^{\text{es}}\) and 38 and 39 of ACSSL\(\text{Fm}^{\text{es}}\). A very limited effect was seen in the U\(_{13}\) and U\(_{14}\) resonances of both molecules. No effect was seen on the A H2 resonances.

The broadening of the stem G's is consistent with the known preference of Mn\(^{2+}\) for sequences of consecutive G residues (Allain and Varani 1995). The N7 resonance is particularly indicative of the interaction of the cation along the major groove. The differential broadening of N9 and N7 of A\(_{11}\) suggests that the interaction with Mn\(^{2+}\) occurs along the major groove, but is tight enough at G\(_{11}\) to G\(_{13}\) to broaden the N9 resonances. The broadening on residues 38 and 39 suggests that there might be a weaker binding site in the loop region. The explanation that this is due to a "spill over" effect from the Mn\(^{2+}\) bound to the stem G's can not be discarded but long range effect of a tightly bound Mn\(^{2+}\) seems unlikely because some resonances closer to the stem broadened nuclei are not broadened while others farther apart are. For example, the A\(_{11}\)H2 in the convergent structures is 2A closer to J\(_{3}\)N7 than A\(_{11}\)H8 and is not broadened, while the H8 is. The binding mode indicated by the broadening results resembles the crystallographic results on RNA-DNA hybrids from Robinson et al. (2000). In that case it was shown that Mg\(^{2+}\) can bind to the deep major groove of A-form helices, hydrogen bonding to the N7 and O6 of guanines. This might be a general binding mode for hexahydrated divalent ions.
Figure 5.8.1
C6-8/H6-8 regions of 2D "C-"H HMQC spectra of A) ACSL<sup>−</sup>−, B) ACSL<sup>−</sup>− + 25μM MnCl<sub>2</sub>, C) 16A37-ACSL<sup>−</sup>- and D) 16A37-ACSL<sup>−</sup>- + 25μM MnCl<sub>2</sub>. The temperature during acquisition was 28°C.
Figure 5.8.2
Spectral region containing crosspeaks H2/N3, H2/N1, H8/N7, and H8.N9 of 2D 15N–H HSQC spectra of ACSL in the absence of Mn2+ and B) in the presence of 5 μM MnCl2. The temperature during acquisition was 25 °C. The resonances of N3, N7 and N9 are folded once in the spectra.
Figure 5.8.3 (Next page)
Schematic representation of resonances broadened by Mn" on Left) ACSL" and Right) 15A37=ACSL". The identified resonances that were broadened are indicated by depicting their atoms as spheres. The intensity of the effect of the Mn" is qualitatively classified by the color of the spheres.
<table>
<thead>
<tr>
<th>ACSL&quot;&quot;&quot;, Residue</th>
<th>H6-8/C6-8</th>
<th>H5/C5</th>
<th>H1' C1</th>
<th>H2/C2</th>
<th>H3'/P</th>
<th>3' P 1D</th>
<th>N7</th>
<th>N9</th>
</tr>
</thead>
<tbody>
<tr>
<td>G27</td>
<td>&lt;</td>
<td>n.d.</td>
<td>x</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>x</td>
<td>&lt;</td>
<td>x</td>
<td>&lt;?</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G29</td>
<td>x</td>
<td>n.d.</td>
<td>x</td>
<td>&lt;?</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G30</td>
<td>x</td>
<td>n.d.</td>
<td>x</td>
<td>&lt;?</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A31</td>
<td>&lt;</td>
<td>=</td>
<td>&lt;</td>
<td>n.d.</td>
<td>&lt;</td>
<td>=</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U32</td>
<td>&lt;</td>
<td>&lt;</td>
<td>n.d.</td>
<td>&lt;</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U33</td>
<td>&lt;</td>
<td>&lt;</td>
<td>n.d.</td>
<td>&lt;</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G34</td>
<td>&lt;</td>
<td>=</td>
<td>=</td>
<td>&lt;</td>
<td>=</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>A35</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>A36</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>A37</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>A38</td>
<td>=</td>
<td>n.d.</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>U39</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>C40</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>C41</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>C42</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>C43</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>i6A37-ACSL&quot;&quot;&quot;, Residue</th>
<th>H6-8/C6-8</th>
<th>H5/C5</th>
<th>H1' C1</th>
<th>H2/C2</th>
<th>ribose</th>
</tr>
</thead>
<tbody>
<tr>
<td>G27</td>
<td>n.d.</td>
<td>n.d.</td>
<td>H5''</td>
<td>&lt;</td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>x</td>
<td>x</td>
<td>H2'&lt;, H3'</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>G29</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G30</td>
<td>x</td>
<td>x</td>
<td>H5'&amp;' x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>A31</td>
<td>x</td>
<td>&lt;</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>U32</td>
<td>&lt;</td>
<td>x</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>U33</td>
<td>&lt;</td>
<td>&lt;</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>C34</td>
<td>x?</td>
<td>n.d.</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>A35</td>
<td>&lt;</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>A36</td>
<td>x?</td>
<td>n.d.</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>i6A37</td>
<td>x</td>
<td>a</td>
<td>n.d.</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>A38</td>
<td>&lt;</td>
<td>n.d.</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>U39</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>C40</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>C41</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>C42</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>C43</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td>=</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.8.1**

Resonances of ACSL""" and i6A37-ACSL"""" broadened by Mn"""". The concentration of Mn"""" was 15μM for ACSL"""" and 55μM for i6A37-ACSL"""". A higher concentration was required to obtain similar effects on i6A37-ACSL"""" as the ones in ACSL"""" due to the presence of residual EDTA in the former sample. N.D. = no data, in most cases due to spectral overlap. = no broadening. = slight broadening. < = broadening. x = not detected.
ii. Effects of Co(NH$_3$)$_4^{2-}$ and assignments

The binding of this [Mg(H$_2$O)$_4$]$^{2+}$ analog to the RNA can be detected by the NOE crosspeaks of its amino protons to the RNA protons. Since the amino protons are exchangeable, their spectra have to be collected in H$_2$O. Initially, to facilitate the assignments and to provide better solvent suppression to study its structural effects, the spectra were collected in D$_2$O. Cu(NH$_3$)$_4^{2-}$ was titrated in the same solvent, to $^{15}$N and $^{13}$C labeled ACSL$^{\text{Ph}}$ and i6A37-ACSL$^{\text{Ph}}$ until 3.5 fold the molar concentration of the RNA. At this concentration the chemical shifts do not change further hence the molecule binding sites are considered saturated. There are analogous chemical shift effects for both molecules, but the changes are more pronounced in i6a37-ACSL$^{\text{Ph}}$. ACSL$^{\text{Ph}}$ has intermediate chemical shifts for resonances A$_{14}$ and A$_{1}$-H8/C8. The differences with i6A37-ACSL$^{\text{Ph}}$ are 0.4 ppm in $^{13}$C for A$_{14}$, and 0.21 ppm and 1.04 ppm, in $^1$H and $^{13}$C respectively, for residue 37.

One of most notable effects of the Co(NH$_3$)$_4^{2-}$ is the sharpening of all the resonances broadened by the dimethylallyl modification. For example, the H5 of U$_{11}$ changes its width at half height from 81 to 26 Hz. However, as is the case with Mg$^{2+}$, there is a general broadening of peaks, most evident in NOESY spectra, due most likely to the aggregation of the RNA caused by the polyvalent cation. Due to this reduced resolution, the assignment was accomplished by following the displacement of the chemical shifts in both ACSL$^{\text{Ph}}$ and i6A37-ACSL$^{\text{Ph}}$ as a function of Co(NH$_3$)$_4^{2-}$ concentration. The displacement was best observed with i6A37-ACSL$^{\text{Ph}}$. The assignments for this molecule are described, and are similar to those of the unmodified molecule with Co(NH$_3$)$_4^{2-}$. The displacements were observed in $^{13}$C-1H HMQC and NOESY spectra and used to assign all the H1' and the base H6/8 resonances. These were used to
supplement the standard sequential assignment strategy of base-to-anomeric NOE connectivities and multiple bond $^1\text{H}-^1\text{H}$ HSQC spectra. Adenine C2/H2 resonances were correlated to the intraresidue C8/H8 through an HCCH-TOCSY spectrum and their assignments are consistent with the H2-H1' crosspeaks seen in 2D NOESY spectra (see below). The available assignments are shown in Appendix V. H2' chemical shifts were assigned by correlation to the H1' resonances in an HCCH-COSY spectrum. H3', H4', H5' and H5'' were not assigned due to the broadness of the resonances which led to very weak crosspeaks in a 3D HCCH-TOCSY.

$^{31}\text{P}$ resonances were partially assigned (Appendix V) because they could not be correlated through bond using $^{31}\text{P}/^1\text{H}$ 2D HETCOR spectra, but only through very weak NOE crosspeaks detected in a $^{31}\text{P}/^1\text{H}$ hetero TOCSY-NOESY (Figure 5.3.4).

The exchangeable proton assignments relied on the same strategy described for the unmodified molecule. Briefly, $^1\text{H}-^1\text{H}$ HSQC and HMQC spectra in H$_2$O were used to identify the amino and imino resonances respectively by their characteristic chemical shift ranges. Additionally the $^{1}\text{A}_{1g}$ N6 secondary amine was identified in the HSQC and in NOESY spectra in 90% H$_2$O by its $^1\text{H}$ crosspeaks to the other dimethylallyl group protons. The Co(NH$_3$)$_4$$^1\text{H}$ proton resonance was identified by its strong intensity and the chemical shift coincident with that reported in the literature (Kieft and Tinoco 1997). A 2'-OH was identified in NOESY and 1D $^1\text{H}$ spectra through being an exchangeable resonance not coupled to either $^1\text{C}$ or $^1\text{N}$. Its assignment as the U$_{1\text{h}}$2'-OH is described below.

iii. Spectral and structural effects on i6A37-ACSL$^{\text{Phe}}$

The Co(NH$_3$)$_4$$^1\text{H}$ protons have intense NOE crosspeaks with the 5'
Figure 5.8.4

3D "P-1H Hetero-TOCSY NOESY spectrum of 2 mM i6Al7-ACSL" in the presence of 8 mM Co(NH3)63+. The identified crosspeaks are labeled with the proton assignment. 1H crosspeaks between 5.1 and 6.1 ppm involve 1H1' resonances and between 7.2 and 8 ppm involve the H6/H8 protons. The temperature during acquisition was 25 °C.
region of the stem and weak crosspeaks with the 3' region. Due to
spectral overlap and a t1 streaking at the Co(NH4)$_4$$^{13}$ proton resonance,
only NOEs to the H2/H6/H8 base region were identified. The crosspeaks,
identified at 25°C in a 360ms NOESY in H2O, were to G12, G13, A12, U12 and
U13. Weaker crosspeaks were visible for G2,H8, A1,H2, and A1,H8. The imino
groups from G12 to G13 have intense Co(NH4)$_4$$^{13}$' crosspeaks. The strongest of
the upfield shifted U imino protons, most likely U13, has an intense
Co(NH4)$_4$$^{13}$' NOE crosspeak at 5°C. The C amino protons also have diffuse
NOE crosspeaks to Co(NH4)$_4$$^{13}$'. All these crosspeaks suggest binding of the
Co(NH4)$_4$$^{13}$' along the major groove, close to the 5' side of the stem, in a
similar fashion to Mn$^{2+}$. This arrangement is consistent with the Mn$^{2+}$
titration data which led to broadening of many of the same resonances.

Dramatic changes are seen for the i6A37-ACSL$^{2+}$ by addition of
Co(NH4)$_4$$^{13}$'. There are large chemical shift changes for the A14 and i5A1- H8
resonances (>0.7 ppm) (Figure 5.8.5, Appendix III, Appendix V) and their
H8/C8 crosspeaks are weakened. The A1- H2/C2 correlation is clearly
visible and as sharp as the other H2 resonances (Figure 5.8.5). All the
U H5 and H6 resonances as well as the dimethylallyl group protons are
sharpened by Co(NH4)$_4$$^{13}$'. At the highest concentration of Co(NH4)$_4$$^{13}$', i6A37-
ACSL$^{2+}$ exhibits characteristics consistent with a U-turn conformation in
the loop (Figure 5.8.6A and B). In the NOESY spectra, there is no
sequential (U13,H1',G14,H8) crosspeak. The U13,H1' has an intense crosspeak
with A14,H8 and a weak crosspeak with A14,H8, consistent with a sharp turn
between U13 and G14. No (U13,H6,G14,H8) NOE is detected. The bases 5' of U13
have helix type stacking and C3' endo character. There are intense
crosspeaks between each of the A H2 resonances and the H1' of their
respective n-1 residues. The sequential H6/H8 NOE connectivities are
continuous on the 3' side of the loop, starting from G14 (Figure
5.8.6B). The (A14,H2, A13,H2) NOE indicates that they stack with the same
**Figure 5.8.5**

Aromatic and anomeric regions from a 2D $^1$C-$^1$H HMQC spectrum acquired on 1.4mM $^{13}$C enriched i6A37-ACSL$^{+}$ titrated to a final concentration of 5mM Co(NH)$_4$Cl$_2$.$^2$ The temperature during acquisition was 25°C.
orientation. The intraresidue G$_{11}$, A$_{16}$ and A$_{17}$ H8 to H2$'$ NOE crosspeaks are very weak. The H8-H2$'$ intraresidue NOE crosspeak of A$_{15}$ has a medium intensity but is clearly weaker than in the absence of Co(NH$_3$)$_4$$^{2+}$. The intensities of the sequential H2$'$-H8 connectivities for residues 34 to 39 increase with Co(NH$_3$)$_4$$^{2+}$. The C1$'$ resonances of the loop residues 34 to 37 are neither shifted upfield nor broadened as they were in the absence of Co(NH$_3$)$_4$$^{2+}$ (Figure 5.8.5). There are no H1$'$-H2$'$ crosspeaks in the DQF-COSY spectrum, although the increased line width could also contribute to their absence. There are no other H2 to H1$'$ NOE crosspeaks for residues 35 to 38. The cross strand NOEs between the H2 of adenines and the H1$'$ of the residues immediately 3$'$ of their base pairing partners are absent. Thus, the loop adenosine residues do not appear to base pair. The A$_{15}$H2 is the only A residue that has such a crosspeak (to C$_{6}$H1$'$) confirming it is base paired to U$_{15}$, and that C$_{6}$ stacks above U$_{15}$. The dimethylallyl protons have similar NOE crosspeaks to those seen in the presence and absence of Mg$^{2+}$, but they are much sharper.

Again there are cross-strand NOEs from the CH$_2$ protons to U$_{15}$H2$'$, and U$_{15}$H1$'$, H6 and H4$'$ and/or H5$'$. The latter is very intense. Other intense NOE crosspeaks are those of A$_{15}$H2 to both of the CH$_2$ protons.

The $^{31}$P resonances also suggest a change in the backbone conformation in the loop region. Most resonances are clustered within -3.6 and -4.9 ppm, indicating no unusual conformation for most of the backbone. The G$_{15}$P-A$_{15}$ $^{31}$P resonance shifts downfield from -3.5 ppm to -2.76 ppm, and there are two other downfield shifted resonances at -0.95 and -1.59. The former corresponds to the terminal monophosphate; the latter may correspond to the U-turn turning phosphate, although that resonance and five others in the main cluster remain unassigned (Figure 5.8.7).
One notable shifted resonance is the A₇₇ N7. In the absence of Co(NH₃)₆³⁺ it is extremely broad, spanning from 228.43 and 231.06 ppm, and all the identifiable N7 resonances occur between 232 and 226 ppm (Figure 5.8.3). In the presence of Co(NH₃)₆³⁺ A₇₇N7 resonates at 221.9 ppm, and is identified in the long range HSQC by its crosspeak with the H8. All the other identified purine N7 resonances have typical chemical shifts downfield from 228 ppm. The upfield shift suggests that A₇₇N7 is involved in a hydrogen bond (James et al. 1981).

Exchangeable proton information is as follows. In the ¹⁵N-¹H HMOC, two U imino resonances of i6A37-ACSL₉₂° appear unusually upfield, at (11.70 ¹H, 155.76 ¹⁵N) ppm and (11.52 ¹H, 156.40 ¹⁵N) ppm, a region typical of non-base paired or oxygen H-bonded U iminos (Figure 5.8.9). Two extremely weak U imino crosspeaks are discernible in the base paired region of the same spectrum. This is evidence that Co(NH₃)₆³⁺ causes additional destabilization of the A-U base pairs to that already caused by the dimethylallyl group. Therefore two of the U iminos, not base paired anymore, are either hydrogen bonded or protected/sequestered from the solvent as they do not disappear through solvent exchange. Note that one of the resonances is intense, and thus must be protected from exchange. These resonances belong to U₁₀ and U₁₁, and were identified through their ¹⁵N chemical shifts based on the U N3/H5 crosspeaks in the 2D long range ¹⁵N-¹H HSQC. The most intense crosspeak belongs to U₁₁ because at 25°C, it is more upfield than U₁₂. The most intense of the two resonances has NOE crosspeaks to A₁₄H8, A₁₄H1', and A₁₁H2 or A₁₁H3, suggesting that it is closer to the end of the loop, consistent with its assignment as the U₁₁ imino. Given the presence of the other two small crosspeaks, indicative of paired Us, two conformations might be present. The N3 of U₁₁ is downfield from the other two U N3s, in agreement with the base paired imino N/H crosspeaks seen at low temperature. A 2D NOESY
Figure 5.8.7

$^31$P one dimensional spectrum of 2.3 mM i6A37-ACSL in the presence of 8 mM Co(NH$_2$)$_2$. Each identified phosphorus resonance is assigned with the number of the residue immediately 3'. The temperature during acquisition was 25 °C.
Figure 5.8.8
Top) "N-H HSQC spectrum of 16A37-ACSL". the H8/N7 spectral region is shown.
Bottom) "N-H HSQC of 2.3 mM 16A37-ACSL - 8 mM Co(NH)₂"., the spectral region
containing H8, N7 and H2/N1-N3 crosspeaks is shown. A₂₇N₁ is broadened in the
absence of Co(NH)₂ and resonates upfield from the regular N7 resonances in
presence of Co(NH)₂. The temperature during the acquisition of both spectra
was 25°C.
Figure 5.8.9

'N-H 2D HMBC optimized for the imino resonance region, acquired on 2.3 mM i6A37-ACSL' in the presence of 8mM Co(NH3)$_3$Cl. Resonances marked with asterisks are due to a small amount of a contaminant RNA. The temperature during acquisition was 15°C.
spectrum indicates that \( A_{11} \) is base paired. It has a NOE crosspeak to the imino resonance at 13.36 ppm and hence is most likely \( U_{3j} \)H3.

\( U_{3j} \) 2'-OH proton was identified at 8.6 ppm as follows. It is an exchangeable resonance, it is not visible with i6A37-ACSL\textsuperscript{RNA} + Co(NH\textsubscript{2})\textsubscript{4}\textsuperscript{2+} dissolved in 100% D\textsubscript{2}O. It does not yield crosspeaks in \(^{15}\text{N}\) or \(^{13}\text{C}\) 2D spectra and is not sharpened by decoupling of those nuclei in 1D \(^{1}\text{H}\) spectra of the corresponding labeled molecules. Therefore it is not coupled to either heteronucleus (Figure 5.8.10). The only exchangeable resonance with such characteristics in RNA would be a 2'-OH. It has weak NOE crosspeaks with \( U_{3j} \)H6 and \( A_{11} \)H3 and strong NOEs with \( U_{3j} \)H1' and either \( U_{3j} \)H2' or \( G_{3j} \)H2' (Figure 5.8.11). Therefore it is concluded that it belonged to \( U_{3j} \). Observation of 2'-OH resonances in RNA is very unusual. It is only possible when the 2'-OH is highly sequestered or hydrogen bonded. Our resonance has an unusual chemical shift. The 2'-OH protons reported in the literature resonate at around 6.1-6.9 ppm in RNA and in simple carbohydrates (Leroy et al. 1985, Pope and Halbeek 1994, Allain and Varani 1995, Conte et al. 1996, Gygi et al. 1998, Hsu et al. 2000). The chemical shift difference suggests that this 2'-OH is in a very different environment, possibly hydrogen bonded.

All the above evidence supports the idea that i6A37-ACSL\textsuperscript{RNA} is forming a U-turn when Co(NH\textsubscript{2})\textsubscript{4}\textsuperscript{2+} is present. In crystal structures of tRNA\textsuperscript{RNA} from yeast, all the anticodon residues are stacked in a 3' direction and their riboses have C3' endo conformations. Such a structure would cause strong sequential H2 to H1' and H2' to H8 NOE crosspeaks, and the absence of upfield shifted C1' resonances and of H1'-H2' COSY crosspeaks. The crystal structures also have hydrogen bonds between the 2'-OH of \( U_{3j} \) and the \( A_{11} \) N7 and between \( U_{3j} \)N3 H and a phosphoryl oxygen immediately 3' to \( A_{11} \). The \( U_{3j} \) 2'-OH-A15 N7 hydrogen bond would both stabilize the \( U_{3j} \) 2'-OH, and cause large chemical shift.
Figure 5.8.10
\(^1H\) one-dimensional spectra of 2.3 mM i6A37-ACSL in the presence of 8mM Co(NH\(_3\))\(_6^{3+}\). Spectra were collected at 5°C. "\(^{13}C\)" or "\(^{15}N\)" indicate the isotopic enrichment of the samples. "D\(_2\)O" or "H\(_2\)O" indicate whether the sample was dissolved in 100% D\(_2\)O or 10% D\(_2\)O-90% H\(_2\)O respectively. It is indicated whether or not the experiment had decoupling for the enriched heteronucleus in the sample. The proton resonating at 8.6 ppm is exchangeable and is apparently not coupled to either \(^{13}C\) or \(^{15}N\), hence is identified as an OH2.
Figure 5.8.11
Imino region of a 2D NOESY in 90% D.O of 2.1 mM 16A17-ACSL in the presence of 8mM Co(NH$_3$)$_6$$^{3+}$. The spectrum was collected at 5°C. Resonances giving crosspeaks to the exchangeable resonance at 8.6ppm are linked by a line to their crosspeaks in the base to anomeric region.
changes for those two chemical groups, from those reported in the
literature. The hydrogen bonding of the U_{11} imino could be the cause of
the strong upfield shifted U_{11} N/H imino crosspeak in the HMQC spectrum.
The NOE crosspeaks identified for the strongest of the upfield shifted
iminos are consistent with its assignment as U_{11}H3. In a U-turn, such as
that of yeast tRNA^{\text{yea}}, U_{11}H3 would be <5Å from A_{14}H8, while U_{11}
would be >8 Å away. The distance to A_{14}H1' would be 6.8 Å for U_{11}H3 and 10.17 Å for
U_{11}H3. A similar 'H chemical shift, at 11.29 ppm, was attributed by
"default" to U_{11}H3 in the fully modified yeast tRNA^{\text{yea}} ACSL pentadecamer,
since it did not yield NOE crosspeaks that would reveal its identity
(Clore et al 1984).

Fifty NOE constraints were collected for residues 32-38 of i6A37-
ACSL^{\text{yea}} in the presence of Co(NH$_3$)$_4^{2+}$ (Appendix VI). The loop constraints
also included the hydrogen bond between U_{11} 2'-OH and A_{14} N7. No backbone
torsion angles or ribose puckers were constrained for residues 32-38.
The loop distance constraints were used together with the constraints
collected for the stem of ACSL^{\text{yea}} in the absence of Co(NH$_3$)$_4^{2+}$ or Mg$^{2+}$ for
structure calculations with ACSL^{\text{yea}}. The dimethylallyl group was not
modeled in the calculations for practical reasons. The structures had a
very low convergence rate, but the three with the lowest energies had a
conformation similar to a U-turn. These structures had the expected 3' stacked pattern for the anticodon residues, except G_{14}, which took
different positions but was at the tip of the loop in all of the
structures. There is a sharp turn between U_{11} and G_{14}, and the α torsion
angle for G_{14} is trans for the three lowest energy structures. The
lowest energy structure is shown in Figure 5.8.12.
iv. Spectral and structural effects on the unmodified molecule

With Co(NH$_3$)$_5^{3+}$, ACSL$^{\text{Phe}}$ has the same NOE pattern as i6a37-ACSL$^{\text{Phe}}$ in the base to anomic region of the 2D NOESY spectra. The ACSL$^{\text{Phe}}$ has the same intense H2 to H1$'$ n+1 crosspeaks, from A$_{13}$ to A$_{12}$, and only A$_{14}$H2 has a crosspeak to the H1$'$ n+1 of its base-pairing partner. The chemical shift changes brought about by Co(NH$_3$)$_5^{3+}$ follow the same pattern as those in the i6a37-ACSL$^{\text{Phe}}$ molecule, with a few differences around the modification site. The A$_{13}$N7 resonance also has a large upfield shift, to 220.3 ppm. Other similarities include the A$_{15}$C8/H8 and the U$_{11}$C6/H6 crosspeaks. The latter shifts downfield in both dimensions, although it appears to have two conformations (figure 5.8.13). The A$_{14}$C2/H2 crosspeak, that in the absence of Co(NH$_3$)$_5^{3+}$ was the upfield most base resonance in both dimensions, shifts downfield and clusters with the rest of the crosspeaks in the 2D $^1$H- $^1$H HMOC, to a final position very close to that of i$^3$A$_{14}$ C2/H2 with Co(NH$_3$)$_5^{3+}$. With the exception of A$_{14}$, the rest of the intense C2/H2 crosspeaks have the same chemical shift changes as i6a37-ACSL$^{\text{Phe}}$ with Co(NH$_3$)$_5^{3+}$. The A$_{13}$H8/C8 crosspeak is slightly more upfield in both dimensions than it is in i6A37-ACSL$^{\text{Phe}}$. Chemical shift changes similar to the dimethylallyl modified molecule are seen for the H5 resonances of U$_{12}$, U$_{14}$, and U$_{15}$. There is also a downfield shift of the C1$'$ resonances for residues 34-37, that indicates the loop residues do not have a stable C2$'$ endc character anymore. Notably the U$_{14}$ and A$_{15}$ C1$'$/H1$'$ resonances are essentially identical to the corresponding resonances in i6A37-ACSL$^{\text{Phe}}$.

The imino resonances of U$_{12}$ and U$_{14}$ re-distribute as they do in i6A37-ACSL$^{\text{Phe}}$. They move upfield to the region of non-base paired uridines, but are broader than the i6A37-ACSL$^{\text{Phe}}$ resonances. The two
Figure 5.8.12
Stereo view of residues 31-39 of the ACSP, the lowest energy structure, obtained using constraints derived from residues 32-38 of 16aY7 ACSP, in presence of Co(NH)₃⁺, and from the stem of ACSP, in absence of Co(NH)₃⁺ or Mg⁺.
Figure 5.8.13
Aromatic and anomeric regions from a 2D 13C-1H HMBC acquired on 1.4mM 13C-enriched ACSL® titrated to a concentration of 5mM Co(NH)2. Unidentified extra crosspeaks are marked with a "?". The temperature during acquisition was 25 °C.
crosspeaks in the base paired region are more intense than those of
i6A37-ACSL\textsuperscript{Fm}. The largest differences with i6A37-ACSL\textsuperscript{Fm} are seen for the A\textsubscript{14} and
A\textsubscript{27} C8/H8 crosspeaks. These crosspeaks still have the largest chemical
shift changes, but migrate less at the same RNA and Co(NH\textsubscript{4})\textsuperscript{1+}
concentrations than i6a37-ACSL\textsuperscript{Fm} (Figure 5.8.13). Their chemical shifts are
(7.18 H, 139.64 C\textsuperscript{14}) and (7.48 H, 138.57 C\textsuperscript{14}) respectively. This
compares with the values for i6A37-ACSL\textsuperscript{Fm} of (7.14 H, 139.21 C\textsuperscript{14}) and
(7.27 H, 137.53 C\textsuperscript{14}) for A\textsubscript{14} and i\textsuperscript{14}A\textsubscript{14} (Appendix V). There are a few
differences in the C1'/H1' region for residues A\textsubscript{17}, A\textsubscript{16} and U\textsubscript{12} (figure
5.8.13).

The U H5 resonances, as in the presence of Co(NH\textsubscript{4})\textsuperscript{1+}, have very
weak alternate C5/H5 crosspeaks, suggesting that two alternative
conformations exist in slow exchange. This phenomenon was not seen in
the i6A37-ACSL\textsuperscript{Fm} molecule. In the long range HSQC, it is evident that
four additional H2/N3-N1 and H8/N9-N7 crosspeak pairs are present. These
crosspeaks are weaker than those that are analogous to the single
crosspeaks in the long range HSQC of i6A37-ACSL\textsuperscript{Fm} in the presence of
Co(NH\textsubscript{4})\textsuperscript{1+}. Some of the multiple base peaks are resolved in the "C HMQC.

The highest ratio tested of Co(NH\textsubscript{4})\textsuperscript{1+} to ACRL\textsuperscript{Fm} RNA was four times
the highest used for i6A37-ACSL\textsuperscript{Fm}. This ratio did not lead to chemical
shift changes as large as those seen in the modified molecule,
particularly for A\textsubscript{27}C8/H8. Also, there were still crosspeaks in
intermediate exchange. Thus Co(NH\textsubscript{4})\textsuperscript{1+} by itself does not substitute for
the dimethylallyl modification.

The chemical shift changes together with the NOE information
indicate that the unmodified molecule tends to resemble a U-turn
conformation. The dimethylallyl group either stabilizes the U-turn
together with Co(NH\textsubscript{4})\textsuperscript{1+} or destabilizes a different conformation. This
seems to involve base pairing based on the U imino crosspeaks. The 
Co(NH₃)₆⁺⁺ induction of a stable U-turn in i6A37-ACSL₆₊, larger chemical 
shift changes as compared with ACSL₆₊, and resonance sharpening, 
indicate that the dimethylallyl group and Co(NH₃)₆⁺⁺ act synergistically. 
A synergistic effect was also proposed for Mg²⁺ (See chapter 5.5), and 
some parallels were noted between the effects of the two ions.

v. Comparison with Mg²⁺ effects

Similar spectral effects are caused by Co(NH₃)₆⁺⁺ and Mg²⁺ 
suggesting that they affect the ACSL structures in a similar manner. For 
i6A37-ACSL₆₊, both ions reduce the line widths of the dimethylallyl 
group protons and the resonances most broadened by this group: U₁, H5 and 
H6, and U₂, H5. They cause similar chemical shift changes for U₁, H6/C6 in 
ACSL₆₊ (Figure 5.5.4), and those perturbations are analogous to the ones 
caused by the dimethylallyl modification alone (5.3.5). They also yield 
similar chemical shift changes in resonances U₂, H1', C₆₄, H6 and G₁₄ and A₁₁, 
H8 and H1'. Both ions open the loop: the base pairs 32-38 and 33-37 are 
derstabilized and there is no (A₁, H2, U₁, H1') NOE crosspeak, while the 
(A₁, H2, U₁, H1') crosspeak is very intense in both cases. The ions induce 
the formation of intense (A₁, H2, i', A₁, CH₆) NOE crosspeaks, likely by 
stabilizing stacking between the dimethylallyl group and A₁₁. There is 
a weak downfield shifted ³¹P peak in the presence of Mg²⁺ that might 
correspond to the downfield shifted ³¹P resonance seen with Co(NH₃)₆⁺⁺ 
(Figure 5.5.8). However this peak could not be assigned. The H6/8-C6/8 
and H2/C2 crosspeaks from ³¹C-¹H HMQC spectra of i6A37-ACSL₆₊ in low salt 
buffer, in the presence of Mg²⁺, in the presence of Co(NH₃)₆⁺⁺, and of 
ACSL₆₊ in the presence of Co(NH₃)₆⁺⁺, are shown in Figure 5.8.14.
Figure 5.9.14
H6/C6/8 and H2/C2 regions of 2D $^1$C HMQC spectra of A16A37-ACSL$^{15b}$, B)16A17-ACSL$^{15b}$ in presence of 7 mM Mg$^{2+}$, C)16A37-
ACSL$^{15b}$ in presence of 5 mM Co(NH)$_3$Cl,$^{15a}$, D) ACSL$^{15b}$ in presence of 5 mM Co(NH)$_3$Cl.$^{15a}$
Mg\(^{2+}\) does not appear to cause AC\(\text{SL}^{\text{Fse}}\) or i6A37-AC\(\text{SL}^{\text{Fse}}\) to adopt a U-turn. The Mg\(^{2+}\) titrated i6A37-AC\(\text{SL}^{\text{Fse}}\) did not have an upfield resonance shift for A\(_{14}\)N7 or H2/N3 correlations in the long range ¹⁵N-H HSQC spectrum (Figure 5.8.15). The A\(_{14}\)N7 probably had a crosspeak with A\(_{14}\)H8 at (8.0, 226.63) ppm. Although this peak could also be A\(_{14}\)H2/N3. In any case the A\(_{14}\)N7 resonance is broadened in the presence of Mg\(^{2+}\) as it is in its absence. In the same spectrum in the presence of Mg\(^{2+}\) no H2/N1 correlations are seen. This is most likely caused by an intermediate exchange and not non-specific aggregation, since the spectrum also showed several intense H8/N9, H8/N7 and H6/N1 correlations. The U\(_{14}\) 2′-OH, was not identified in the presence of Mg\(^{2+}\) for either of the two molecules at 12°C. If present, the U\(_{14}\) 2′-OH resonance would not be at 8.6 ppm but upfield. The melting curve of i6A37-AC\(\text{SL}^{\text{Fse}}\) in the presence Co(NH\(_3\))\(_{4}\)^{3+} in a 1:5 ratio, shows that Co(NH\(_3\))\(_{4}\)^{3+} increased the thermal stability of base stacking (Figure5.5.12). Mg\(^{2+}\) did not have such effect.

It is interesting to note that Clore et al. (1985) used a very low temperature and high salt concentration for their yeast t\(\text{R}N\)A\(\text{F}^{\text{He}}\) ACSL structure (0.5 M KCl, 0-5 °C). These conditions together with the modifications presumably would stabilize this turn, because Mg\(^{2+}\) and low temperature give effects consistent with such stabilization (Sundaram et al. 2000, Labuda and Porscche 1982, Gorenstein and Goldfield 1982), and the effects of Mg\(^{2+}\) can sometimes be mimicked by high concentrations of monovalent ions. Here the conditions were 8mM Co(NH\(_3\))\(_{4}\)^{3+} and 12-25 °C. This shows the remarkable stabilization of the U-turn conformation achieved with a polycation. This conformation is presumably promoted by the lower charged Mg\(^{2+}\), which stabilizes the open loop conformation caused by the dimethylallyl group.
Figure 5.8.15
2D 'N-'H HSQC spectrum of 2.3 mM i6A17-ACSL in presence of 5 mM MgCl₂. The spectral region containing the H8, N7, H8/N9, H6/N1 and H2/N1-N3 crosspeaks is shown. N7 resonances are folded twice. The upfield most 'N resonance detected is 226 ppm. The temperature during the acquisition was 25 °C.
vi. T_{1p} measurements

As with Mg^{2+}, there is general broadening of the $^{13}$C resonances caused by the presence of Co(NH$_3$)$_{5}$' (Table 5.8.2). Likewise the C8’s of G$_{14}$ and A$_{14}$ still relax more slowly than those of the other purines, indicating that on the nanosecond time scale they are still the most dynamic purines. However, in contrast with Mg^{2+}, U$_{13}$C6 and U$_{13}$C6 have similar values. The C1' resonances in the loop region do not relax quickly compared to the stem region, as they do in the unmodified Co(NH$_3$)$_{5}$' free molecule. Hence they are not less mobile than the corresponding nuclei in the stem.

Summary

Mn^{2+} and Co(NH$_3$)$_{5}$' were used as probes for Mg^{2+} binding sites. The N7, N9, H8, and $^{31}$P resonances were observed for Mn^{2+} broadening. Mn^{2+} broadened resonances on the 5' side of the stem of both ACSL$^{Ph}$ and i6A37-ACSL$^{Ph}$, particularly from G$_{13}$ to G$_{14}$. A lesser effect was observed for resonances in residues U$_{13}$, U$_{14}$, and A$_{14}$. The above information indicates that Mn^{2+} interacts primarily with the G residues along the major groove. Co(NH$_3$)$_{5}$' was titrated into ACSL$^{Ph}$ and i6A37-ACSL$^{Ph}$ as a means to follow the displacements of the chemical shifts for their assignments. Assignments were also obtained using heteronuclear spectra for the base and H1' resonances. Co(NH$_3$)$_{5}$' sharpens all the resonances that are broadened by the dimethylallyl modification and this group's protons. Co(NH$_3$)$_{5}$' has NGE crosspeaks to the 5' side of the stem (H6/H8 of G$_{13}$ to U$_{13}$), the C amino groups and weak crosspeaks with A$_{13}$H2 and A$_{13}$H8, A$_{13}$ and i$''$A$_{13}$ H8/C6 resonances show large chemical shift changes.
<table>
<thead>
<tr>
<th>Residue</th>
<th>C6/C8</th>
<th>Cl'</th>
<th>C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2</td>
<td>39.9</td>
<td>46.4</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>39.6</td>
<td>45.7</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>38.2</td>
<td>46.2</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>39.2</td>
<td>50.0</td>
<td>40.2</td>
</tr>
<tr>
<td>U6</td>
<td>*33.5</td>
<td>46.1</td>
<td></td>
</tr>
<tr>
<td>C7</td>
<td>*34.0</td>
<td>46.6</td>
<td></td>
</tr>
<tr>
<td>G8</td>
<td>42.4</td>
<td>45.8</td>
<td></td>
</tr>
<tr>
<td>A9</td>
<td>42.1</td>
<td>51.1</td>
<td>35.9</td>
</tr>
<tr>
<td>A10</td>
<td>37.2</td>
<td>47.5</td>
<td>32.1</td>
</tr>
<tr>
<td>A11</td>
<td>36.3</td>
<td>49.7</td>
<td>39.3</td>
</tr>
<tr>
<td>A12</td>
<td>32.3</td>
<td>42.6</td>
<td>38.3</td>
</tr>
<tr>
<td>U13</td>
<td>*32.2</td>
<td>48.5</td>
<td></td>
</tr>
<tr>
<td>C14</td>
<td>*35.8</td>
<td>47.3</td>
<td></td>
</tr>
<tr>
<td>T15</td>
<td>*35.4</td>
<td>49.9</td>
<td></td>
</tr>
<tr>
<td>C16</td>
<td>*33.2</td>
<td>46.6</td>
<td></td>
</tr>
<tr>
<td>C17</td>
<td>*32.6</td>
<td>42.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.8.2

$^{13}$C T$_1$ measurements, in milliseconds, for C6/C8, C2, and Cl' of i6A37 in the presence of Co(NH$_3$)$_6$ titrated to 5mM. Resonances marked by asterisk are an average of the values for the two pyrimidines C6 split crosspeaks.
upon addition of Co(NH$_3$)$_4^{3+}$. Co(NH$_3$)$_4^{3+}$ induces a U-turn conformation in the loop region; NOE crosspeaks indicate that bases A$_{15}$, A$_{14}$, and i$^6$A$_{12}$ are stacked in a 3' direction and U$_{13}$ is not stacked with G$_{14}$, but is close to A$_{15}$. There are no cross-strand H2-H1' NOE crosspeaks. A$_{15}$N7 resonates at an unusual 221.9 ppm, while U$_{13}$ 2'-OH is identified at 8.6 ppm, supporting the idea that these two groups are hydrogen bonded. The U$_{13}$NH 'H/$^1$N crosspeak is intense and upfield shifted indicating that it is protected from exchange, consistent with it being hydrogen bonded and not base paired. An unidentified $^3$P resonance appears at $\sim$1.5 ppm, and might belong to U$_{13}$-P-G$_{14}$. A calculation that included the loop NOE distance constraints and the hydrogen bond between U$_{13}$2'-OH and A$_{15}$N7 yields lowest energy structures with clear resemblance to a U-turn.

ACSL$^{Phe}$ has similar characteristics to those of i6A37-ACSL$^{Phe}$ but has extra resonances indicating that it is muticonformational. Co(NH$_3$)$_4^{3+}$ has some similar effects as those caused by Mg$^{2+}$ on ACSL$^{Phe}$ and i6A37-ACSL$^{Phe}$ including chemical shift changes for U$_{13}$ and U$_{12}$ resonances, sharpening of the resonances broadened by the dimethylallyl modification, opening of the loop, and common NOE crosspeaks. However the evidence of a U-turn in the presence of Co(NH$_3$)$_4^{3+}$ is not seen in the presence of Mg$^{2+}$. The T$_{19}$'s of the C1' resonances in the anticodon are similar to those in the stem, indicating they are not as relatively mobile as in ACSL$^{Phe}$. Thus the induction of a U-turn by Co(NH$_3$)$_4^{3+}$ is synergistic with the opening of the loop by the dimethylallyl modification. A possible basis for the U-turn induction by Co(NH$_3$)$_4^{3+}$ and not by Mg$^{2+}$ is the charge density difference of these ions.
5.9 ACSL-MiaA complex

i. MiaA and complex solubility

It is of interest to use the ACSL<sup>His<sub>6</sub></sup> system as a model for protein-RNA interaction. The MiaA enzyme is 34 KDa. Thus the size of the complex of MiaA with ACSL<sup>His<sub>6</sub></sup> is within the current limits for NMR structure determination. The complex can be studied in two parts, the RNA and the protein, with either of them isotopically labeled and the other one unlabeled. This simplifies the spectra. A significant effort was put into obtaining the preliminary results described below. The results support the feasibility of such a project but point to the need for optimization of experimental conditions, specifically to prevent protein aggregation and precipitation.

The His-tagged MiaA protein was produced from the clone pTX439 on BL21(DE3) E. coli cells, and purified using a Ni affinity column, as described in the methods chapter. From large scale (3L) cultures in rich media, up to 300 mg of protein have been produced with a high degree of purity. However the protein is not very soluble at high concentrations (>5mg/ml) after the removal of imidazole and reduction of the concentration of NaCl, both of which are present in the elution buffer for the Ni column. After several solubility tests it was concluded that polyanion containing buffers stabilize the MiaA protein. Phosphate (~50 mM, 100mM NaCl, pH6.8) and citrate buffers worked particularly well to keep MiaA in solution. Interestingly the presence of the RNA was also able to promote solubility, most probably because it acts as a co-solvent through the presence of its polyphosphate. The protein also requires reducing agents, such as 2-mercapto-ethanol or DTT, indicating
its tendency to oxidize. The highest concentration of MiaA achieved was 20 mg/ml (0.57 mM).

ii. ACSL<sup>15N</sup> imino <sup>1</sup>H/<sup>15</sup>N spectra in the complex

A 1:0.75 molar complex of unlabeled MiaA and <sup>15</sup>N labeled ACSL<sup>15N</sup> was made. The MiaA concentration was 0.57 mM. The imino <sup>1</sup>N-<sup>1</sup>H HMQC spectra show the disappearance of the downfield U<sub>1</sub> and U<sub>12</sub> crosspeaks, while an intense U imino crosspeak at 13.13 ppm appears (Figure 5.9.1). Weak crosspeaks corresponding to the G iminos of the free RNA are still visible, indicating that not all ACSL<sup>15N</sup> is bound to MiaA. There is a slightly downfield G N1, close to the G<sub>18</sub> of the free RNA, suggesting that it belongs to that residue. The most notable change is the appearance of a crosspeak at 9.23 ppm <sup>1</sup>H, 151.5 ppm <sup>15</sup>N. These are very unusual chemical shifts and preclude assignment as G or U iminos (Varani et al. 1996, Wijmenga and Van Buuren 1998). The crosspeak likely belongs to a U given that there are three relatively intense G imino crosspeaks, which should correspond to G<sub>20</sub>-G<sub>32</sub> involved in base pairs.

iii. MiaA amide <sup>1</sup>H/<sup>15</sup>N spectra

The MiaA protein was overproduced with <sup>15</sup>N labeling by expression in bacteria grown in minimal medium containing <sup>15</sup>N enriched (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as the sole nitrogen source. Around 80 crosspeaks in the amide region are resolved (Figure 5.9.2). Three hundred and thirty-six amide protons should be present in the protein, thus an increase in resolution is required. This reflects the α-helical nature of the protein, as
Figure 5.9.1
{'H-'H 2D HMQC spectrum optimized for the imino resonance region acquired on ^14N enriched ACSL(0.43mM) in complex with unlabeled MiaA (0.57mM). Resonances marked with asterisk correspond to the free ACSL. No assignments have been done yet. The crosspeak at 9.23, 150 ppm has unusual chemical shifts and cannot be assigned to a U or G imino based on comparison with free RNA chemical shifts. The temperature during acquisition was 12 °C.
Figure 5.9.2
$^1$H-$^1$N 2D HMQC spectrum acquired on $^1$N enriched MiaA (0.5 mM) at 22 °C in phosphate buffer. The intense crosspeaks at -108ppm $^1$N are presumed to belong to a mobile region of the protein. ~80 out of 336 crosspeaks are resolved in the spectrum.
indicated by its CD spectrum (Figure 5.9.3). A few crosspeaks are more intense than the rest. They were confirmed not to belong to the His-tag by its removal with thrombin. These crosspeaks likely belong to a dynamic region of the protein as the rest of the resonances have higher intensity with the increase in temperature from 17 °C to 25 °C. The generally reduced peak intensity at low temperatures suggested that the protein was aggregating leading to broadening of most peaks except in the most mobile regions. Acquisition of the spectra at 22 °C allowed a good compromise between protein stability and peak intensity.

The use of TROSY methodology (Pervushin et al 1997) on a 750 MHz spectrometer, allowed a significant improvement in resolution. The TROSY type experiments observe one the components of the 1H/1H multiplet, that has a slow relaxation rate due to interference effects at high fields. The resolution was further increased by the use of 15N and 2H enriched protein. The absence of protons bound to the alpha carbons eliminates an extra path for relaxation of the magnetization, allowing longer T2 and sharper lines. Around 160 peaks are clearly resolved in a TROSY acquired on a 0.33mM complex of 15N-2H enriched MiaA and ACSL™.

iv Ultracentrifugation

To test whether MiaA was aggregating, analytical ultracentrifugation was performed at close to NMR concentrations (0.12 mM). Monomeric molecules are expected to give a sedimentation curve with a linear dependence of ln of the concentration on the square of the radius (McRorie and Voelker 1993). The absorbance-radius points, fitted to the model for a single ideal solute, had residuals that are typical of aggregates (McRorie and Voelker 1993) (Figure 5.9.4). Residuals
**Figure 5.9.3**
CD spectrum of MiaA. The plot is an average of three measurements of 0.1 mg/ml MiaA.
Figure 5.9.4
Absorbance profile and residuals of an ultracentrifugation of MiaA in phosphate buffer at 0.15mM initial concentration. The data were collected after 20 hours of ultracentrifugation at 15000 rpm and a temperature of 17 °C. The residuals pattern is typical of non-ideality due to aggregation.
are defined as the difference between each experimental data point and the corresponding calculated points. Hence it was concluded that the protein was aggregating during the NMR experiments and that these experiments should be collected at higher temperatures or in conditions optimized to prevent aggregation. Formation of MiaA multimers has been reported (Leung et al. 1998). Alternatively, given the requirement for reducing agents to prevent precipitation, the aggregation could be related to its oxidation. Storage and processing of the protein in an O₂-free atmosphere might help to prevent its aggregation.

Summary

The MiaA protein was found to be soluble in buffers containing polyanions, such as phosphate and citrate. RNA can function as a cosolvent. ACSL²⁻ in complex with MiaA keeps the stem G-C base pairs and shows in 2D ²⁰N-¹H HMBC spectra the presence of a single base paired U imino crosspeak and the appearance of a ¹H-¹⁵N crosspeak with unusual chemical shifts of (9.23, 151.5) ppm. The MiaA protein has a predominantly α-helical conformation. It has been soluble up to concentrations of 0.33mM. NMR and ultracentrifugation indicate that MiaA tends to aggregate while in solution at 17 °C. At 25 °C or in the absence of a reducing agent, MiaA tends to precipitate. ²⁰N-¹H HSQC spectra of MiaA contains ~80 resolved peaks. ²⁰N-¹H TROSY spectra of the complex between deuterated MiaA and unlabeled ACSL²⁻ contains ~160 resolved peaks. Hence a remarkable increase in spectral resolution is achieved by deuteration and TROSY methodology. Three hundred sixty amide protons should be present. The lack of resolution is a reflection of the
α-helix predominance and maybe of the need for solvent optimization to prevent aggregation.
6. DISCUSSION

i. Structure of ACSL^{Pho}

The base pair constraints indicate that ACSL^{Pho} is an extended double helix and tri-nucleotide residue turn. In the absence of divalent or trivalent cations it is not a U-turn. It contains base pairs 31-39, 32-38 and 33-37. The trinucleotide loop comprises the three anticodon residues. In the convergent structures, G_{14} and A_{11} are at the end of the loop, are oriented toward the major groove side of the helix, and are nearly coplanar. G_{14} partially stacks beneath U_{13}. The planar positions of G_{14} and A_{11} at the tip of the loop do not seem to be stabilized by particular interactions. This is consistent with their long C8 T_{1p} values that indicate a higher mobility than the stem bases on the picosecond time scale. A_{14} partially stacks on A_{12}, slides towards the minor groove and is oriented with its base pairing groups toward U_{13} and G_{14}. Residues 33 to 37 have significant C2' endo ribose pucker, which is a reflection of the deviation from A-form helix in the loop. The high C1' T_{1p} relaxation rates for these loop residues indicates they have a rigid structure. Hence the ribose backbone is less dynamic than the stem probably due to it being stretched in the turn.

The chemical shifts for C3' and C4' resonances together with H1'-H2', J_{HH} couplings indicate that the riboses at positions 34-36 have a C2' endo ribose pucker. U_{13} and A_{12} seem to be in an intermediate conformation. The epsilon torsion angles are not trans for the anticodon residues. Consequently they are not stacked in a 3' direction, as they would be in a classical U-turn. An alpha gauche-zeta trans conformation is expected for a classical U-turn (Shi and Moore 2000) and a large 18
downfield shift is associated with it (Gorenstein and Goldfield 1991, Schweiguth and Moore 1997, Huang et al. 1996, Fountain et al. 1996, Puglisi and Puglisi 1998, Sundaram et al. 2000). The absence of a downfield shifted resonance in ACSL^{3+} agrees with the calculated structure even though no alpha or zeta torsion angles were constrained in the loop region. Absence of the downfield shifted \(^{31}P\) resonances has been seen for the unmodified tRNA^{\text{V_{1}}}, that does not make a U-turn either (Durant and Davis 1999), and for unmodified yeast tRNA^{\text{V_{2}}} (Ashraf et al. 1999-2).

Caution needs to be taken when interpreting isolated NOEs as evidence for a U-turn. In particular, the ACSL^{3+} has in the base to anomic region an NOE pattern similar to the ones of yeast tRNA^{\text{V_{1}}}, tRNA^{\text{V_{2}}} and tRNA^{\text{V_{3}}} (Clore et al. 1984, Schweiguth and Moore 1997, Sundaram et al. 2000). A loss of the connectivity between U_{i};H_{i}' to the next H_{i+1} or H_{i}, and a non sequential U_{i};H_{i}' to residue 35 H_{i} or H_{i}, are two evidences expected for U-turns. ACSL^{3+} has a very weak sequential H_{i}' to H_{i} connectivity between U_{i}; and G_{i}' and a U_{i};H_{i}'-A_{i};H_{i} non-sequential connectivity, but its conformation is distinctively different from a U-turn. This NOE pattern is also present in the 16A37-ACSL^{3+} and \(\Psi32\)-ACSL^{3+}, but many other evidences show that the structures resemble the unmodified molecule, with an extended stem and a gradual turn along the anticodon residues.

An extended stem has previously been proposed to exist in the unmodified tRNA^{\text{V_{1}}}; anticodon stem-loop (Durant and Davis 1999). At pH 5, it is an A-form helix stem, expected to have the A_{i};U_{i}, base pair, ending in the base pair C_{i};A_{i}', and having “intermediate dynamic characteristics” for residues U_{i} and A_{i}'. There has been evidence for the presence of the 32-48 base pair in the presence of Mg\(^{2+}\) in the fully modified tRNA (Hyde and Reid 1985-1). However in the E. coli purified
tRNA\textsuperscript{\textit{Ph}}. Davis and Poulter (1991) only detected base pair 31-39 in the presence of Ψ₁, but not base pairs 32-38 or 33-37 using 2D NMR experiments. In the absence of Ψ₁, none of the N3-H correlations of residues 39, 32 or 33 were detected. Their failure to detect these base pairs in 2D experiments may be due to destabilization by \textit{ms}i\textsuperscript{i}A₁- or Ψ₁.

If a U-turn is the preferred conformation, the role of Ψ₁ would be to stabilize its base pair, limiting the destabilization by \textit{ms}i\textsuperscript{i}A₁- or Ψ₁ to residues 32 to 38.

Solution studies show the presence of U-turn type structures in loops that contain less than 7 nucleotides (Jucker and Pardi 1995, Huang et al. 1996, Puglisi and Puglisi 1998, Fountain et al. 1996, Sundaram et al. 2000), suggesting that this conformation is more stable in loops smaller than anticodon loops. With the exception of initiator and elongator tRNA\textsuperscript{\textit{Ph}} anticodon stem-loops (Schweiguth and Moore, 1997), only fully modified anticodon stem-loops have been seen to take this conformation in solution (Clore et al. 1984, Sundaram et al 2000) while unmodified or undermodified molecules do not (Durant and Davis 1999, Stuart et al 2000). This fact points to the requirement for the nucleotide modifications in order for some larger loops to make a U-turn when free in solution. Probably the formation of extra base pairs in the seven residues and a trinucleotide loop would be favored to having a stretch of seven non-base paired residues.

Base pairing should be one of the major factors for the formation of a U-turn. The unmodified ACSL\textsuperscript{\textit{Ph}} from \textit{E. coli}, exactly the same sequence used in our work, makes a canonical U-turn in crystal structures of the 30S ribosomal subunit (Ogle et al. 2001). The turn in the A-site is stabilized by codon-anticodon base pairing and extensive hydrogen bonding of residues A₁₂, A₁₃, and their respective codon partners to the rRNA residues A1492, A1493 and G530 along the minor groove of the
mihelix. For the tRNA_F_{\text{Fm}} anticodon in solution, the formation of a kissing hairpin with itself through Watson-Crick base pairing is not possible. This could explain the distinctive conformation of ACSL_{\text{Fm}} instead of a U-turn in solution. Anticodons have a tendency to form intermolecular base pairs. The unmodified yeast tRNA_F_{\text{Fm}} binds mostly aptamers that bind to its anticodon loop (Scarabino et al. 1999). Solution studies show the tendency of modified anticodon regions to form base pairing between complementary anticodons (Eisinger 1971, Grosjean et al. 1998). In several crystal structures, tRNA anticodon residues are also base paired by crystal packing, and some anticodons that are at least partly complementary form anticodon-anticodon interactions (Moras et al. 1986, Schmitt et al. 1998, Nissen et al. 1999). Evidence for stabilization of the U-turn by base pairing comes from the correlation of comparatively low temperature factors for the anticodon loops when their anticodon residues are involved in intermolecular base pairs (Westhof et al. 1988). Solution NMR indicates that intermolecular base pairing alters the conformation of the anticodon loop. $^{15}$P spectra of a tRNA_F_{\text{Fm}}·tRNA_{\text{2A}} anticodon-anticodon dimer show that their anticodons undergo a conformational change when they base pair; $^{15}$P and several shifted resonances disappear while the main cluster of resonances grows (Gorenstein and Goldfield 1982), suggesting unfolding upon interaction. Given that E. coli tRNA_F_{\text{Fm}} binds E. coli tRNA_{\text{2A}5} with similar equilibrium binding constants as Yeast tRNA_F_{\text{Fm}}, it may have a similar base paired anticodon loop structure in solution (Eisinger and Gross 1975).

The possibility that ACSL_{\text{Fm}} would take a GNRA tetraloop type structure in solution was considered because of the presence of the sequence GAAA (Heus and Pardi 1991, Jucker et al. 1995). Our spectroscopic evidence disproves this possibility. If that type of structure were present, there would be intense NOE crosspeaks $\text{H8}_4$,
A_{15}H2') and (A_{14}H8, A_{15}H3'). In ACSL™, these crosspeaks are relatively weak. A GNRA type structure would have a change in the direction of the phosphate backbone similar to that found in the U-turn of anticodon loops (Jucker and Pardi 1995). This turn would cause very weak sequential crosspeaks of the G_{14}H8 to A_{15}H1' (>9Å) and H2' (>7Å), and a fairly intense non-sequential crosspeak to A_{14}H1' (Jucker et al. 1996). The NOESY spectra show those sequential crosspeaks with moderate to strong intensity, while no (G_{14}H8, A_{15}H1') is detected.

The structure of the unmodified molecule might be relevant for the function of anticodons which have unmodified bases U_{14}, U_{15}, A_{14}, and A_{15}. This is the case for E. coli tRNA^Gly and several other tRNAs in bacteria, mitochondria and archaea (Spinzi et al 1998). It has been shown in studies of E. coli tRNA^Gly and M. mycoides tRNA^Gly that with U_{14} the standard wobble rules are obeyed, while the C_{14} substitution as found in the mycoplasma ACSL allows recognition of all four Gly codons (Claesson et al. 1995, Lustig et al 1993, Lustig et al 1989). C_{14} also enhances -1 frameshifting at near cognate GGA codons in E. coli (O'Connor 1998). Thus, it is possible that base pair 32-38 decreases wobbling and -1 frameshifting in tRNA^Gly. It can be hypothesized that the destabilization of this base pair by the dimethylallyl modification at position 37 increases such wobbling and -1 frameshifting. Although ms^iA_{15} decreases first position errors, it increases the wobble effect (Persson et al. 1994). miaA mutants have higher +1 frameshifting, hence i^4A_{15} or its derivatives decrease -1 frameshifting (Qian and Björk 1997, Schwartz and Curran 1997). Maybe this effect can be considered an increased "-1 frameshifting". Substitution of the hypermodified residue Y_{14} in yeast tRNA^Gly to m^5G increases -1 frameshifting. A regular 32-38 base pair is not possible in this sequence (Carlson et al 2001),
suggesting that the bulkiness of the modification regulates ribosomal frameshifting.

ii. Effect of pseudouridine 32

The change of $U_{32}$ to $\Psi_{12}$ sharpens the $G_{32}$ imino resonance in the lower part of the stem in ACSL$^{ph}$ and i6A37-ACSL$^{ph}$. However it increases the linewidths of the $U_{32}$ and $\Psi_{12}$ imino resonances. It may be that stabilization of the stem also destabilizes the loop structure, as the relative intensity of the HNN-COSY $U_{32}$ N3-A1-H2 crosspeak compared to the other N3-H3 crosspeaks is lower than the relative intensity of the corresponding crosspeak in ACSL$^{ph}$. The $\Psi_{12}$-H3 resonance is very weak too, in spite of the fact that it is clearly involved in a base pair as indicated by the HNN-COSY spectrum. This base pair may be stable but simply more solvent exposed. Still, the structure has very similar characteristics to ACSL$^{ph}$, including the conserved sequential connectivities and NOEs, the C2' endo character for the loop residues, and the base pairs 32-38 and 33-37. $\Psi_{12}$ is base paired in anti conformation with A32. The two lower energy families of structures are very similar to ACSL$^{ph}$ in the vicinity of $\Psi_{12}$, but one of the families differs in the loop region. This difference is attributed to the lack of constraints due to spectral overlap in $\Psi32$-ACSL$^{ph}$, although $\Psi_{12}$ also causes lack of NOE constraints due to broadening, as seen for the imino resonances in the loop region.

Given the stabilization in the stem and the structural similarity to the unmodified molecule, it can be concluded that the stabilization comes from the new imino NH1. The slow exchange rate of $\Psi_{12}$ H1 is not the result of intramolecular hydrogen bonding directly, since no
acceptor is in its vicinity. This is not known for sure in the molecule but all the calculated structures lack a hydrogen bond acceptor close enough for bonding. Hence an H₂O mediated H-bond is plausible (Davis 1998), although we have no direct evidence. In the Ψ32-ACSLₜₜ structure, a water bridge to a Ψₗ₂ phosphoryl oxygen is possible, since the distance between the pro(S) oxygen and the H1 is <4.5Å.

The Watson-Crick base pair Ψₗ₂-A₁₄ contrasts with the conformations proposed in other U-turn studies. In the anticodon loop from the crystal structure of tRNA^Cys in a ternary complex with EF-Tu -GDPNP, Ψₗ₂ has a sym conformation and the modified anticodon loop only differs with tRNA^Phe at residue 35 (Nissen et al 1999). Residue 32 has been proposed to be involved in a bifurcated base pair to residue 38 based on a phylogenetic and molecular dynamics study (Aufinger and Westhof 1998, Aufinger and Westhof 1999). If such an interaction occurred in tRNA^Phe of E. coli, by analogy with those base pairs, it would involve hydrogen bonding between O₄ of Ψₗ₂ and N₆ of A₁₄. The A₁₄ amino protons are not assigned for Ψ32-ACSLₜₜ and are presumably broadened beyond detection in the ¹H-¹H HSQC. The A₁₄ N₆ is identified in the HNN-COSY by a crosspeak to A₁₄ H₂. It shows no unusual chemical shift.

iii. Effect of the dimethylallyl group

The spectroscopic data indicate that the loop region of i6A37-ACSLₜₜ is destabilized by the modification, which increases nucleotide flexibility. The modification destabilizes base pairs 31-39, 32-38 and 33-37. The Uₗ₂-Aₙ₄ base pair is barely detected while there is no evidence for a Uₗ₁-iₕA₁₄ base pair. The weakness of the anticodon sequential connectivities indicates that those residues are not in a
stable stack, suggesting the absence of a stable U-turn. There are conserved structural features from the unmodified molecule in the loop region. The A-form character of the extended stem is indicated by the NOE crosspeaks \((U_{12} H1', G_{12} H8)\) and \((U_{12} H1', A_{12} H2)\). At low temperature the \(U_{12}\) and \(U_{11}\) bases still stack. The anticodon residues maintain a C2’ ribose pucker, as indicated by the strong intraresidue H2’-to-H8 NOE crosspeaks and the still upfield shifted C1’ resonances. No unusual backbone torsion angles are hinted by the \(^{13}P\) chemical shifts. Instead the \(^{13}P\) resonances are still clustered and those from the loop become broadened.

The similar \(^{13}C\) \(T_1\), relaxation rates for \(G_{12}\), \(A_{12}\), and \(A_{14}\) C8 indicate that both ACCL\(^{Pm}\) and i6A37-ACCL\(^{Pm}\) have not only a similar structure in the stem but similar dynamics in the loop, on the picosecond time scale. However, they differ on the microsecond-to-millisecond time scale as can be seen in the HMQC spectra. The corresponding \(^1H\) resonances, particularly the \(G_{12}\) and \(A_{14}\) base and H1’, are in intermediate exchange.

The dimethylallyl group is pointing towards the opposite side of the loop from \(i' A_{12}\), between residues 32 and 33. Its protons have cross strand NOE crosspeaks, and the dimethylallyl group has strong effects on the base chemical shifts of \(U_{12}\) and on the linewidths of \(U_{12}\) H5 and H6, and \(U_{11}\) H5. Since these residues show inter base NOEs, it is clear that the dimethylallyl group does not break their stacking. A possible way of preventing base pairing is by preventing \(U_{12}\) from aligning with \(A_{12}\) or by disrupting the alignment of \(U_{12}\) and \(U_{11}\) to their corresponding base pairing partners by steric clashes. For example, the manually modeled dimethylallyl group in the unmodified structure bumps with the RNA at many positions, hence the structure must change to a more open conformation to accommodate this group. This modeling indicates that the NOE crosspeaks of the \(CH_2\) in the dimethylallyl group to \(A_{12} H2\) would not be present if \(i' A_{12}\) had the conformation as \(A_{12}\) in ACCL\(^{Pm}\). Horizontal
displacement of \( i^tA_{37} \) towards the minor groove is sufficient for the
dimethylallyl protons to comply with the observed NOEs. A molecular
dynamics approach would possibly yield a great array of conformations,
but the lack of constraints prevents the attainment of a highly precise
structure. This situation would be expected since the RNA itself does
not have "one" structure. Thus, the dimethylallyl group does not
completely disrupt the unmodified structure, but opens the loop and
makes it more dynamic. It is clear that the i6A37-ACSL\(^{\text{Phe}} \) does not form a
U-turn, therefore the prevention of base pairing in the loop region
together with the predicted increase in stacking ability by a
hydrophobic moiety are not sufficient changes to induce the U-turn
conformation.

A role for the other modifications and base pairing is suggested
by a tRNACYS crystal structure of the ternary complex with EF-tu and
GDPNP (Nissen et al. 1999). With the exception of residue 35 (C)
residues 29 to 41 are identical to those of tRNA\(^{\text{Phe}} \), including
 pseudouridines at positions 32 and 39 as well as ms\(^2i^tA_{37} \). This ACSL has
a classical U-turn conformation. However its anticodon forms a kissing
complex to another anticodon due to crystal packing. The results
presented in this dissertation as well as those from others (Durant and
support the claim that formation of a U-turn in an anticodon stem loop
requires the modifications, the presence of cations, or base pairing of
the anticodon. In the fully modified \( E. \ coli \) tRNA\(^{\text{Phe}} \), it can be
hypothesized that the role of \( \Psi_{1,1} \) is to strengthen the base pair with
\( A_{1,1} \). \( \Psi_{1,1} \) would have a role in stabilizing the next part of the stem
through interactions of the N1 imino to the backbone. Finally ms\(^2i^tA_{1,1}
would keep the 7 member loop open by preventing \( U_{1,1} \) and \( \Psi_{1,1} \) from forming
Watson-Crick base pairs with \( A_{1,1} \) and \( A_{1,8} \) respectively. \( \Psi_{1,1} \) and \( A_{1,8} \) would
form a bifurcated base pair instead, as proposed by Auffinger and Westhof (1999). The results for 56A37-ACSL\textsuperscript{phe} in the presence of Co(NH\textsubscript{4})\textsuperscript{+} do not support or disprove this postulate. The A\textsubscript{c}NH\textsubscript{2} resonance has not been assigned. The distance of U\textsubscript{c}O4 to A\textsubscript{c}NH\textsubscript{2} in the lowest energy structure modeled with the constraints of 56A37-ACSL\textsuperscript{phe} in the presence of Co(NH\textsubscript{4})\textsuperscript{+} is 4.8 Å. It is possible that the hydrogen bond can form in solution; no hindrance for approach of the strands with U\textsubscript{c} and A\textsubscript{c} is obvious in the calculated structure.

iv. Effect of Mg\textsuperscript{2+} and synergy with the dimethylallyl modification

Previous studies have seen that nucleotide modifications can affect affinity for Mg\textsuperscript{2+} and that this metal can promote a single RNA conformation. Our results further suggest that in ACSL\textsuperscript{phe}, the structure individually favored by Mg\textsuperscript{2+} is the same structure that is favored by the dimethylallyl modification. Although obtaining a highly precise structure is hindered by the line broadening caused by Mg\textsuperscript{2+}, it can be said that Mg\textsuperscript{2+} destabilizes base pairs 32-38 and 33-37 on ACSL\textsuperscript{phe} and stabilizes an open conformation present in 56A37-ACSL\textsuperscript{phe}. A similar effect happens in the full length modified E. coli tRNA\textsuperscript{phe}. Addition of Mg\textsuperscript{2+} shifts Ψ39 H3 which is labile indicating flexibility (Hyde and Reid 1985b). Base breaking through a combination of the modification and Mg\textsuperscript{2+} is not a general effect, but sequence dependent. In a poly(N\textsuperscript{6}-dimethylallyladenosine) oligonucleotide, base pairing with poly(uridylate) is absent without Mg\textsuperscript{2+} and present with it (Thedford and Straus 1974). The homopolynucleotide should not be an adequate model for the isolated modified base in the context of ACSL\textsuperscript{phe}. 
The similarities between the effects of Mg$^{2+}$ and the dimethylallyl modification on the chemical shifts of $U_{11}$, $U_{13}$, and $U_{15}$, $H5/C5$, and $U_{13}$, $H6/C6$, suggest that they cause similar structural effects. The sharpening of the $U_{11}$ resonances and the disappearance of duplicate resonances in i6A37-ACSL$^{2+}$ upon addition of Mg$^{2+}$ suggest that Mg$^{2+}$ and the dimethylallyl group have a synergistic effect in stabilizing the open structure, but each separately is not sufficient for this. The absence of the cross-strand H2-H1' NOE, and the presence of an intense sequential H2-H1' for $A_{14}$,H2, indicate that $A_{14}$ is more stably stacked with $U_{13}$. Given that the cross-strand H2-H1' crosspeak is absent in i6A37-ACSL$^{2+}$ with Mg$^{2+}$, and that there is a similarity of spectral effects with Co(NH$_3$)$_6$$^{2+}$, it appears that the dimethylallyl group with Mg$^{2+}$ makes the upper part of the loop resemble a U-turn (see below).

The Mg$^{2+}$ dependent doubling of resonances of ACSL$^{2+}$ and the stabilization of the i6A37-ACSL$^{2+}$ dimethylallyl group, positioned in the vicinity of $U_{11}$ and $U_{13}$, is similar to the effects seen for the yeast tRNA$^{2+}$ in solution. Hall et al. (1989) have proposed that, in the full length unmodified yeast tRNA$^{2+}$, base modifications and Mg$^{2+}$ stabilize particular conformations, as there is a Mg$^{2+}$ dependent transition, although not in the fully modified molecule. Transition was called a structural rearrangement of the unmodified RNA, evidenced by proton chemical shifts and NOEs in the presence of low concentrations of Mg$^{2+}$ compared to high concentrations of Mg$^{2+}$. The transition includes the formation of a GU base pair not present in the fully modified molecule. The anticodon loop region has been found to have multiple ion dependent conformations in solution (Striker et al. 1989). Mg$^{2+}$ affects this anticodon loop by increasing its rigidity (Turner et al. 1975) and enhancing a conformation associated with an increased stacking of $Y_{13}$. 
(Striker et al. 1989). Mg\(^{2+}\) however was not sufficient to yield large \(^{31}P\) changes in our work, whereas a conformational transition has been proposed where Mg\(^{2+}\) would have the opposite effect from temperature on the \(^{31}P\) chemical shifts of U1τ and Y1τ (Gorenstein and Goldfield 1982). Alternate conformations dependent upon the presence of Mg\(^{2+}\) in the anticodon stem have also been reported for tRNA\(^{16}\) (Yue et al 1994).

Given that a more stable structure results by combination of the dimethylallyl modification and Mg\(^{2+}\), it is conceivable that the modification could stabilize Mg\(^{2+}\) binding. Mg\(^{2+}\) binds more tightly to poly(N\(^{\prime}\)-dimethylallyladénylate) than to poly(adénylate) (Thedford and Straus 1974). In tRNA\(^{29}\) (E.coli), base modifications stabilize Mg\(^{2+}\) binding at the anticodon stem (Yue et al. 1994). A methylation of a cytosine in unmodified DNA and RNA oligonucleotide models for yeast tRNA\(^{29}\) ACSL also allows cooperative binding of Mg\(^{2+}\) but in contrast to modifications in the loop, induces base pairing (32-37, and 33-36) in the anticodon loop (Chen et al. 1993, Guenther et al. 1992). These studies underscore the importance of modifications on residue 37 for anticodon function. Methylation of G1τ in the m\(^5\)C modified DNA analog prevented pairing of residues 32-37 and 33-36, and induced a 3' stacked anticodon and ribosomal binding (Basti et al. 1996, Dao et al. 1994). The same modification on the corresponding RNA sequence increased its ability to compete with tRNA\(^{29}\) binding to ribosomes (Dao et al. 1994). The dimethylallyl group may have a similar function in E.coli tRNA\(^{29}\), as base pairing is broken and the base paired loop residues are less restricted.

tDNA ACSL from yeast also shows duplicate resonances in the presence of Mg for dT\(_{1\tau}\) (Basti et al. 1996). However the secondary structure of the unmodified tDNA was little affected by Mg\(^{2+}\), is still biologically inactive and with a methylation at position 1 of G\(_{1\tau}\) shows
broad methyl peaks in the presence of Mg$^{2+}$. The dimethylallyl modification on ACSL$^{\text{he}}$ also has the effects of base breaking and doubling of resonances. However the ACSL$^{\text{he}}$ resonances are broadened by the presence of Mg$^{2+}$ or by the dimethylallyl modification in the absence of Mg$^{2+}$. The synergy of Mg$^{2+}$ and the modification promotes one conformation. These differences are attributable to the inability of the tDNA sequence to form base pairs 32 and 38, to the higher steric restrictions in RNA than in DNA, and to the different interaction of Mg$^{2+}$ with DNA. This prevents extrapolation of the DNA results to RNA.

The relaxation analysis indicates that the rates of reorientation of the C-H vectors of ACSL$^{\text{he}}$ and i6A37-ACSL$^{\text{he}}$ on the nanosecond timescale are similar. The proton broadening in i6A37-ACSL$^{\text{he}}$ is due to a sampling of different electronic environments for the protons, which does not affect their fast dynamics. The Mg$^{2+}$ $T_{1p}$ relaxation pattern for C6 and C8 resonances in i6A37-ACSL$^{\text{he}}$ is similar to the one in its absence, hence the loop still has G$_{14}$ and A$_{15}$ as the most dynamic bases. The relaxation pattern suggests that the loop bases still have less dynamic restriction. However the relaxation pattern of the C2s suggests that the A$_{14}$ and A$_{15}$ base pairing groups are now more dynamic than their loop congeners. This further supports a role for Mg$^{2+}$ in stabilizing the loop region. An NMR study on the dynamics of yeast tRNA$^{\text{he}}$ methyl protons in presence of Mg$^{2+}$, concluded that the motions of the methyls in the anticodon loop are more restricted than those of other methyls in the same molecule (Schmidt et al. 1987).

The conclusion is that Mg$^{2+}$ and the dimethylallyl group destabilize the loop structure of the unmodified molecule. The structure of ACSL$^{\text{he}}$ would otherwise hinder the anticodon residues, particularly A$_{14}$, from being exposed for base pairing. Mg$^{2+}$ and the dimethylallyl
modification act synergistically in stabilizing, at least partially, an open conformation.

v. Effects of Co(NH$_3$)$_5$$^{1+}$ on ACSL$^{Phe}$ and i6A37-ACSL$^{Phe}$

The NMR evidence is compelling in support of a U-turn induction by an excess of Co(NH$_3$)$_5$$^{1+}$. In this conformation the anticodon residues stack having a C3'endo ribose pucker. The universal U$_{11}$ has hydrogen bonds involving its 2' OH and imino groups to A$_{15}$N7 and the phosphate 3' of this residue, respectively. Destacking of U$_{11}$ and G$_{14}$ causes the absence of sequential NOE connectivities between U$_{11}$ and G$_{14}$. The sharp turn in the phosphate backbone causes a downfield shift of the $^{31}$P turning phosphate and non-sequential connectivities between U$_{11}$ and A$_{15}$. The spectroscopic evidence is consistent with all the above characteristics. The strong sequential H$_2$-H1' crosspeaks for all of the A residues in the loop indicates that they have a helical character and are stacked. The anticodon residues' C3' endo character is supported by their C1' chemical shifts. The presence of the U$_{12}$OH2', its unusual chemical shift, and the upfield shift of A$_{15}$N7 are evidence that each is hydrogen bonded. The $^{15}$N chemical shifts of the U$_{12}$ and U$_{15}$ imino groups indicate that both are not base paired and suggest that the U$_{12}$ imino proton is protected from exchange presumably by being hydrogen bonded to the A$_{15}$-P-A$_{14}$ phosphate. There is a clear non-sequential (U$_{12}$H1', A$_{15}$H8) NOE crosspeak and complete absence of the (U$_{12}$H1', G$_{14}$H8) crosspeak. There is also a weak non sequential (U$_{12}$H1', A$_{15}$H8) NOE crosspeak. Finally there is a downfield shifted $^{31}$P resonance, which could not be assigned to U$_{12}$-P-G$_{14}$, but is suggestive of a large conformational change in the phosphate backbone.
The spectral effects of Mg$^{2+}$ and Co(NH$_3$)$_6^{3+}$ indicate that the latter has a more pronounced impact and induces a U-turn while the extent that Mg$^{2+}$ induces a U-turn not clear. The dimethylallyl group peaks become sharper with Co(NH$_3$)$_6^{3+}$ than with Mg$^{2+}$. However, both ions stabilize the dimethylallyl group in a similar way as both cause the appearance of an intense (A$_x$H2, i$^1$A$_x$, CH) while the crosspeaks seen in their absence remain present in the spectra. Both ions open the loop, but with Mg$^{2+}$, AC3L$_{RM}$ still shows the (U$_y$H1', G$_z$H8), (A$_y$H2, U$_y$H1'), while with Co(NH$_3$)$_6^{3+}$ it does not. Thus both ions destabilize the loop base pairs similarly. The lack of resonance doubling indicates that they act synergistically with the dimethylallyl modification. The stabilization of the U-turn with Co(NH$_3$)$_6^{3+}$ could be due to its higher charge.

Co(NH$_3$)$_6^{3+}$ and Mn$^{2+}$ binding to both molecules occurs primarily along the 5' side of the stem. This binding is evidenced by the Co(NH$_3$)$_6^{3+}$ NOE crosspeaks to the base and imino protons and the particularly strong broadening effect of the G base and $^1$P resonances in the stem caused by Mn$^{2+}$. Mn$^{2+}$ binds to three or more consecutive G-C base pairs, through G N7 and has been used as a probe for divalent metal ion binding sites in other RNAs (Allain and Varani 1995, Butcher et al. 2000). Co(NH$_3$)$_6^{3+}$ binds similarly to Mn$^{2+}$, at G-C triplets, through hydrogen bonds to sequential N7 and O6 atoms and is a specific probe of A-form geometry (Hingerty et al 1982, Kieft and Tinoco, 1997, Gao et al. 1995). A crystallographic study found that Mg(H$_2$O)$_6^{2+}$ binds to A-form nucleic acids in two modes. One mode is identical to the one described for Co(NH$_3$)$_6^{3+}$, i.e. through hydrogen bonds to the deep major groove. The other is by bridging the phosphates across the narrow major groove (Robinson et al. 2000). Co(NH$_3$)$_6^{3+}$ also can bridge phosphates across the major groove and from adjacent residues (Gao et al 1995).
The stabilization of the U-turn in the loop region could be due to these bridges as there are no runs of Gs in the anticodon loop. Co(NH₃)₅²⁺ binds that region, as shown by its proton NOEs to the U₁₂ and U₃₃ base resonances. The weak broadening of resonances in the loop by Mn²⁺ indicates that multi-valent cations interact with the loop region with higher affinity than they do non-specifically with RNA. ACSLₚₓₑ, in the absence of Mg²⁺ or Co(NH₃)₅²⁺, has larger distances between non-sequential phosphates than in a U-turn. The shielding of the same PO₃⁻ charges by these cations would help U-turn formation.

Conversion of the C₂' endo anticodon residues to the more compact A-form like C₃' endo found in a U-turn could explain the induction of the U-turn, by the neutralization of a high electrostatic potential. It is not obvious whether or not the C₃' endo ribose pucker is a consequence of the U-turn rather than a driving force for it.

Misra and Draper have shown that the anticodon region has a high electrostatic potential that correlates with the proposed binding sites of Mg²⁺ from X-ray crystallography data (Misra and Draper 2000). Several bound Mg²⁺ ions have been found by these studies, but not all consistently appear. The Mg²⁺ coordinated by inner sphere to Y₁₀-O1P in yeast tRNAₚₓₑ seemed to be specific for the orthorhombic crystal form (Quigley et al 1978, Shi and Moore 2000). However it was not observed by another group (Jovine et al 2000), which in an orthorhombic crystal form located two "weak" Mg²⁺ binding sites in the anticodon region, to the phosphates of residues 30 and 36 (Jovine et al 2000). The other "strong" binding site of Mg²⁺ defined by crystallography in the anticodon stem loop is at the junction between the D and anticodon arms. Mg²⁺ has in this site octahedral coordination and hydrogen bonds to 5MC₄₆, U₄₇, G₄₉, C₅₁, G₵₄ (Shi and Moore 2000, Jovine et al. 2000).
Our evidence agrees better with the presence of non-specific or weak counterion binding to the anticodon region, instead of strong single ion binding sites. Binding could be specific but weak, making an "outer sphere complex". Non-specific and outer sphere bindings are difficult to distinguish energetically and experimentally (Misra and Draper 1999). The binding of Mn\(^{2+}\) and Co(NH\(_3\))\(_4\)\(^{3+}\) seen in the loop region is weaker than that in the stem. These ions localize to the 5' side of the loop and don't match the proposed binding sites. Previous spectroscopic evidence, including \(^{31}P\) NMR broadening by Mn\(^{2+}\) binding to tRNA has also supported a non-specific or weak type of binding (Guéron and Leroy 1982). The amino groups of Co(NH\(_3\))\(_4\)\(^{3+}\) prevent the metal from forming inner sphere coordination. Our data show that such interaction is not necessary for the cation induction of a U-turn.

The U-turn might be further induced by the Co(NH\(_3\))\(_4\)\(^{3+}\) stabilization of the A-form character in the stem, avoiding the need for pseudouridine 39 that stabilizes this conformation (Davis and Poulter 1991, Durant and Davis 1999). The binding mode of Co(NH\(_3\))\(_4\)\(^{3+}\) to GG containing sequences confers on it the ability to convert B-form duplex DNA into an A-form duplex DNA. This effect is attributed to the neutralization of the charge of the phosphate backbones that approach each other more closely in A-form DNA than B-form DNA (Robinson and Wang 1996). Although they yield similar effects in some of the loop resonances of ACSL\(^{3+}\), Mg\(^{2+}\) and high concentrations of Na\(^+\) (data not shown) failed to stabilize a U-turn. This may correlate with DNA results in which Mg\(^{2+}\) and high concentrations of Na\(^+\) do not induce B- to A-form transition, while Co(NH\(_3\))\(_4\)\(^{3+}\) does (Xu et al. 1993). Spermine and neomycin also convert DNA from B- to A-form (Robinson and Wang 1996). Co(NH\(_3\))\(_4\)\(^{3+}\) binds in an analogous form as spermine to a site on crystallized yeast tRNA\(^{3+}\) (Hingerty et al. 1982). Co(NH\(_3\))\(_4\)\(^{3+}\) has also been utilized as an
inorganic analog of biological organic polyamines, like putrescine, spermine and spermidine for nucleic acid condensation, Holliday junction formation and B-DNA to Z-DNA transitions (Widom and Baldwin 1980, Duckett et al. 1990, Gessner et al. 1985). Our results show that the formation of U-turns is ion-condition dependent and suggest its presence in crystal structures could be induced by the presence of the polycations used to neutralize RNA electrostatic repulsion for crystallizations.

vi. Interaction of ACSL\(^\text{Phe}\) with MiaA

The structural features that MiaA recognizes in the unmodified ACSL should not be affected by pseudouridines 32 and 39. The local ACSL structure seems to determine the catalytic rate of the dimethylallyl attachment, since MiaA shows similar "substrate activity" for the unmodified tRNA\(^{\text{Phe}}\) (Leung et al 1997), the unmodified ACSL (Soderberg and Poulter 2000) and the undermodified tRNA at position 37 (Moore and Poulter 1997, Soderberg and Poulter 2000). The enzyme requires the full length tRNA for minimum \(K_e\). The \(K_e\) is similar for the unmodified and A37 undermodified tRNA (Moore and Poulter 1997, Winkler personal communication). This fact suggests that additional contacts are made but probably only facilitate stable substrate binding. The ultracentrifugation results support MiaA's tendency to aggregate in the NMR buffer. This may be related to the proposed catalytic action on the full length tRNA as a multimer, though it seems to act as a monomer on the anticodon stem-loop (Leung et al. 1997).

In the absence of DMAPP, MiaA causes alteration of the chemical shifts of most imino resonances, but particularly the loop residues.
Only one intense base paired U imino resonance is present in the HMQC spectra, most likely U_{13}. Additionally an upfield imino resonance at 9.23 ppm is present, suggesting that a non-base paired imino is protected from exchange by MiaA. Good candidates for this group are the iminos from U_{13}, U_{14} or G_{13}. Since three intense new G imino resonances in the region of base paired Gs can be seen, the stem appears to be intact. Thus it is clear that the enzyme is specifically altering the loop structure. This is consistent with the fact that the strongest requirements for binding occur in the loop. The enzyme is known to recognize a particular consensus, mainly A residues at positions 36 and 38, and a purine at position 37 (although a G residue cannot be modified). Based on experiments using cell extracts from E. coli, it was determined that MiaA prefers a G_{14}:C_{14} pair, a purine at position 29 and a pyrimidine at 41 (Motorin et al. 1997). However a purine at position 30 and a pyrimidine at 40 yield similar kinetic parameters when using the recombinant purified MiaA (Sodeberg and Poulter 2000). The reaction kinetics are not affected by the C_{14} substitution for U_{13}. Since the reactions are done in the presence of Mg^{2+}, the destabilization of base pairs 31-39 and 32-38 by Mg^{2+} may be a factor in the similar kinetics. MiaA residues K56, R167, R170, and K280 are proposed to be involved in RNA binding, based on a mutational study (Soderberg and Poulter 2001). These mutations should be useful guides for future studies.

Motorin et al. (1997) have proposed a model of anticodon interaction with MiaA based on the assumption of a U-turn conformation and the requirement for G_{14} and C_{14}. In it, the G_{14} exocyclic amino and the C_{14} oxygen 2 are candidates for binding through the minor groove. Our lowest energy structures suggest a model where Mia A recognizes the ACSL through the major groove. Although A_{14} tends to orient to the minor groove, A_{13} is exposed and stacks with A_{13}, though not perfectly. A_{14} NH_{2}
is also very exposed, suggesting that the A amino groups are important for recognition, which could explain the requirement of A₁₆ and A₁₈ for recognition. Likewise the exposition of A₁₇ would be in agreement that a purine at that position is sufficient for binding (Leung et al 1997). Possibly the presence of Mg⁡⁺⁺ and/or C₃₂ could help expose A₁₆ NH₂. The enzyme can recognize both the hairpin and duplex forms of the sequence we used (Sodeberg and Poulter, 2000). Since A₁₆ and A₁₈ are required for activity, it seems that the stacking of these residues but not base pairing would be essential for activity, and that the enzyme would recognize the RNA also as an extended A-form helix.
7. CONCLUSIONS

A. The unmodified ACSL is a tri-nucleotide loop hairpin

B. The dimethylallyl modification destabilizes the loop

C. Mg$^{2+}$ stabilizes the open conformation synergistically with the dimethylallyl modification

D. Pseudouridine 32 alone is Watson-Crick base paired, stabilizes the stem and has little effect on the loop

E. Co(NH$_3$)$_6^{3+}$ induces a U-turn, which is stabilized by the dimethylallyl modification

F. Cations bind preferentially to the stem region and presumably stabilize the A-form helix, thus indirectly stabilizing the U-turn.

G. The double modified ACSL has at least two conformations; pseudouridine 32 still stabilizes the stem while the dimethylallyl modification opens the loop

This work indicates that the formation of a U-turn depends on several factors. In the absence of modifications an extended loop is formed due to extra base pairs involving residues 32, 33, 37 and 38. The presence of an hydrophobic modification on residue 37 prevents base pairing but does not stabilize a 3' stacked conformation. The presence of a helix stabilizing modification on residue 32 alone stabilizes the
closed stem but might cause a slight opening in the loop. The presence of cations is necessary to stabilize the open conformation and the U-turn presumably by allowing a more compact helix. The higher the charge of the cation the more likely it seems to induce a U-turn. This conformation does not require direct coordination to the metal.

The consequences of this work include a better understanding of the interaction between RNA modifications and Mg$^{2+}$. It shows how RNA structure can be regulated and dramatically altered by disrupting a base pair and including a polyvalent ion. It shows that in order for the anticodon loop to form a U-turn, base modifications and cations are required. These two factors contribute individually to form the U-turn, but it does not form stably.
### 8 APPENDICES

#### I. Chemical shifts of A37-ACSL

<table>
<thead>
<tr>
<th></th>
<th>H6/H8</th>
<th>C6/C8</th>
<th>H5/H2</th>
<th>C5/C2</th>
<th>H1′</th>
<th>C1′</th>
<th>H2′</th>
<th>C2′</th>
<th>H3′</th>
<th>C3′</th>
<th>H4′</th>
<th>C4′</th>
</tr>
</thead>
<tbody>
<tr>
<td>G27</td>
<td>8.11</td>
<td>139.12</td>
<td>5.78</td>
<td>92.34</td>
<td>4.87?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>7.49</td>
<td>136.64</td>
<td>5.90</td>
<td>92.81</td>
<td>4.64</td>
<td>75.57</td>
<td>4.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G29</td>
<td>7.19</td>
<td>135.94</td>
<td>5.79</td>
<td>92.87</td>
<td>4.63</td>
<td>75.74</td>
<td>4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G30</td>
<td>7.11</td>
<td>136.21</td>
<td>5.73</td>
<td>93.15</td>
<td>4.63</td>
<td>75.98</td>
<td>4.46</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A31</td>
<td>7.67</td>
<td>139.45</td>
<td>7.77</td>
<td>153.93</td>
<td>5.96</td>
<td>93.33</td>
<td>4.49</td>
<td>75.57</td>
<td>4.53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U32</td>
<td>7.48</td>
<td>141.34</td>
<td>5.00</td>
<td>103.09</td>
<td>5.49</td>
<td>93.37</td>
<td>4.19</td>
<td>75.65</td>
<td>4.36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U33</td>
<td>7.54</td>
<td>141.56</td>
<td>5.27</td>
<td>104.01</td>
<td>5.75</td>
<td>92.95</td>
<td>4.31</td>
<td>76.12</td>
<td>4.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G34</td>
<td>7.95</td>
<td>139.41</td>
<td>5.49</td>
<td>88.27</td>
<td>4.71</td>
<td>74.95</td>
<td>4.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A35</td>
<td>8.1</td>
<td>141.96</td>
<td>8.04</td>
<td>155.24</td>
<td>5.46</td>
<td>88.39</td>
<td>4.35</td>
<td>77.31</td>
<td>4.55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A36</td>
<td>7.88</td>
<td>140.97</td>
<td>7.91</td>
<td>155.67</td>
<td>5.68</td>
<td>88.90</td>
<td>4.18</td>
<td>75.32</td>
<td>4.81</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A37</td>
<td>7.22</td>
<td>141.50</td>
<td>7.19</td>
<td>153.41</td>
<td>5.78</td>
<td>89.32</td>
<td>4.82</td>
<td>75.30</td>
<td>4.66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A38</td>
<td>7.82</td>
<td>139.93</td>
<td>7.82</td>
<td>154.06</td>
<td>5.76</td>
<td>92.87</td>
<td>4.41</td>
<td>75.30</td>
<td>4.39</td>
<td>41.74</td>
<td>0.08</td>
<td>4.59</td>
</tr>
<tr>
<td>U39</td>
<td>7.68</td>
<td>141.81</td>
<td>4.94</td>
<td>102.54</td>
<td>5.50</td>
<td>93.37</td>
<td>4.31</td>
<td>75.52</td>
<td>4.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C40</td>
<td>7.84</td>
<td>141.71</td>
<td>5.54</td>
<td>97.39</td>
<td>5.56</td>
<td>93.86</td>
<td>4.29</td>
<td>75.50</td>
<td>4.46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C41</td>
<td>7.78</td>
<td>-141.5</td>
<td>5.4512</td>
<td>97.41</td>
<td>5.43</td>
<td>94.05</td>
<td>4.35</td>
<td>75.36</td>
<td>4.46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C42</td>
<td>7.78</td>
<td>-141.5</td>
<td>5.4512</td>
<td>97.41</td>
<td>5.46</td>
<td>94.21</td>
<td>4.25</td>
<td>75.46</td>
<td>4.48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C43</td>
<td>7.66</td>
<td>141.81</td>
<td>5.46</td>
<td>97.92</td>
<td>5.71</td>
<td>92.87</td>
<td>3.98</td>
<td>77.43</td>
<td>4.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18</td>
<td>7.67</td>
<td>5.62</td>
<td>92.79</td>
<td>3.88</td>
<td>77.25</td>
<td>4.11</td>
<td>69.95</td>
<td>4.13</td>
<td>83.77</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>H5′</th>
<th>H5′′</th>
<th>C5′</th>
<th>H1′/H3′</th>
<th>N1/H3′</th>
<th>H2′/4′/6′</th>
<th>P</th>
<th>N2/4/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>G27</td>
<td>4.11?</td>
<td></td>
<td></td>
<td>12.53?</td>
<td>144.79?</td>
<td>-1.44  or -1.088</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>4.52</td>
<td>4.21</td>
<td>65.72</td>
<td>12.71</td>
<td>144.62</td>
<td>-3.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G29</td>
<td>4.49</td>
<td>4.08</td>
<td>65.48</td>
<td>12.54</td>
<td>144.4</td>
<td>-3.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G30</td>
<td>4.43</td>
<td>4.05</td>
<td>65.80</td>
<td>12.28</td>
<td>144.28</td>
<td>-3.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A31</td>
<td>4.53</td>
<td>4.09</td>
<td>65.21</td>
<td>6.51??/7.93?</td>
<td>80.64?/.8?</td>
<td>-4.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U32</td>
<td>4.51</td>
<td>4.05</td>
<td>64.87</td>
<td>13.59</td>
<td>158.32</td>
<td>-4.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U33</td>
<td>4.39</td>
<td>4.06</td>
<td>65.96</td>
<td>13.23</td>
<td>158.47</td>
<td>-4.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G34</td>
<td>4.17</td>
<td>4.03</td>
<td>67.04</td>
<td>10.55</td>
<td>143.3</td>
<td>6.28?</td>
<td>71.97?</td>
<td>-4.03</td>
</tr>
<tr>
<td>A35</td>
<td>3.79</td>
<td>3.51</td>
<td>67.72</td>
<td>6.43?</td>
<td>75.52?</td>
<td>-3.3?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A36</td>
<td>4.09</td>
<td>3.97</td>
<td>68.55</td>
<td>&quot;</td>
<td>&quot;</td>
<td>-3.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A37</td>
<td>4.35</td>
<td></td>
<td>68.94</td>
<td>&quot;</td>
<td>&quot;</td>
<td>-3.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A38</td>
<td>4.36</td>
<td></td>
<td>68.04</td>
<td>&quot;</td>
<td>&quot;</td>
<td>-4.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U39</td>
<td>4.52</td>
<td>4.05</td>
<td>64.39</td>
<td>14.08</td>
<td>159.53</td>
<td>-4.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C40</td>
<td>4.50</td>
<td>4.07</td>
<td>64.83</td>
<td>6.93 / 8.44</td>
<td>95.52</td>
<td>-4.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C41</td>
<td>4.54</td>
<td>4.06</td>
<td>64.54</td>
<td>6.89 / 8.46</td>
<td>95.27</td>
<td>-4.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C42</td>
<td>4.53</td>
<td>4.04</td>
<td>64.61</td>
<td>6.93 / 8.46</td>
<td>95.29?</td>
<td>-4.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C43</td>
<td>4.47</td>
<td>4.01</td>
<td>65.43</td>
<td>7.04 / 8.29</td>
<td>94.23</td>
<td>-4.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cin1</td>
<td>4.35</td>
<td>3.98</td>
<td>65.34</td>
<td>&quot;</td>
<td>&quot;</td>
<td>-4.41</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
II. Structural constraints for A37-ACSL

Base-pairing constraints

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G34</td>
<td>C34</td>
<td>A36</td>
</tr>
<tr>
<td>U33</td>
<td>A37</td>
<td></td>
</tr>
<tr>
<td>U32</td>
<td>A38</td>
<td></td>
</tr>
<tr>
<td>A31</td>
<td>C39</td>
<td></td>
</tr>
<tr>
<td>C30</td>
<td>C40</td>
<td></td>
</tr>
<tr>
<td>G29</td>
<td>C41</td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>C42</td>
<td></td>
</tr>
<tr>
<td>G27</td>
<td>C43</td>
<td></td>
</tr>
<tr>
<td>5'</td>
<td></td>
<td>3'</td>
</tr>
</tbody>
</table>

Base pair hydrogen bond distances from Varani.

G-C

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>G O6</td>
<td>C H42</td>
<td>2.80 ± 1.2</td>
</tr>
<tr>
<td>G O2</td>
<td>C N4</td>
<td>1.99 ± 1.3</td>
</tr>
<tr>
<td>G H1</td>
<td>C N3</td>
<td>2.50 ± 1.2</td>
</tr>
<tr>
<td>G NL</td>
<td>C N3</td>
<td>2.90 ± 1.3</td>
</tr>
<tr>
<td>G H22</td>
<td>C O2</td>
<td>2.10 ± 1.2</td>
</tr>
<tr>
<td>G N2</td>
<td>C O2</td>
<td>2.40 ± 1.3</td>
</tr>
</tbody>
</table>

A-T

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A H61</td>
<td>U H44</td>
<td>2.19 ± 1.1</td>
</tr>
<tr>
<td>A N6</td>
<td>U N4</td>
<td>2.90 ± 1.3</td>
</tr>
<tr>
<td>A N1</td>
<td>U N1</td>
<td>2.30 ± 1.3</td>
</tr>
<tr>
<td>A N1</td>
<td>U N1</td>
<td>2.30 ± 1.3</td>
</tr>
</tbody>
</table>

U33-A37

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A O4</td>
<td>U O61</td>
<td>2.70 ± 1.2</td>
</tr>
<tr>
<td>A O4</td>
<td>U O61</td>
<td>2.90 ± 1.3</td>
</tr>
<tr>
<td>A H3</td>
<td>U N1</td>
<td>2.10 ± 1.2</td>
</tr>
<tr>
<td>A N3</td>
<td>U N1</td>
<td>2.90 ± 1.3</td>
</tr>
</tbody>
</table>
Inter-proton distance constraints (A)
Numbering starts with 1, G1 corresponds to G27.
Alpha torsion angle constraints

$\alpha$ is constrained to exclude the trans conformation for:

$\beta_2$, $\beta_3$, $\beta_4$, $\beta_9$, $\beta_{12}$, $\beta_{19}$, $\beta_{40}$, $\beta_{41}$, $\beta_{42}$, $\beta_{43}$

$\beta_3'$, $\beta_3'$, $\beta_5'$: $0.5\pm120.0^\circ$

Beta torsion angle constraints

$\beta$ is constrained to exclude the trans conformation for:

$\gamma_2$, $\gamma_3$, $\gamma_4$, $\gamma_9$, $\gamma_{12}$, $\gamma_{19}$, $\gamma_{40}$, $\gamma_{41}$, $\gamma_{42}$

$\beta_3'$, $\beta_3'$, $\beta_5'$: $0.5\pm120.0^\circ$

Beta torsion angle constraints

trans: $\gamma_2$, $\gamma_3$, $\gamma_4$

$\beta_3'$, $\beta_5'$, $\gamma_1'$, $\gamma_2'$: $136\pm41^\circ$

Epsilon torsion angle constraints

trans: $\gamma_7$, $\gamma_8$, $\gamma_9$, $\gamma_{12}$, $\gamma_{13}$, $\gamma_{16}$, $\gamma_{33}$, $\gamma_{37}$, $\gamma_{38}$, $\gamma_{39}$, $\gamma_{42}$, $\gamma_{43}$

$\gamma_4'$, $\gamma_5'$, $\gamma_5'$, $\beta_2$: $215\pm47^\circ$

trans or gauche: $\gamma_34$ and $\gamma_36$

$\gamma_4'$, $\gamma_5'$, $\gamma_5'$, $\beta_2$: $215\pm45^\circ$

gauche: $\gamma_{36}$

$\gamma_4'$, $\gamma_5'$, $\gamma_5'$, $\beta_2$: $260\pm45^\circ$

Gamma torsion angle constraints

trans or gauche: $\gamma_34$ and $\gamma_36$

$\gamma_5'$, $\gamma_5'$, $\gamma_5'$, $\gamma_5'$: $120.0\pm120.0^\circ$

gauche: $\gamma_{34}$, $\gamma_{34}$ and $\gamma_{34}$

$\gamma_5'$, $\gamma_5'$, $\gamma_5'$, $\gamma_5'$: $11.0\pm11.0^\circ$

Torsion angle constraints about delta and nu 0

to apply C3 or C1 prime end sugar puckers

Angles determined from model built A-form/B-form ribose

$3^\prime$ ENDO sugar puckers for residues 27, 29, 33, 30, 31, 33, 38, 39, 40, 41, 42, 43

$3^\prime$ $\gamma_4'$, $\gamma_4'$, $\gamma_4'$, $\gamma_4'$: $1.4\pm1.0^\circ$

$3^\prime$ $\gamma_4'$, $\gamma_4'$, $\gamma_4'$, $\gamma_4'$: $124\pm2.5^\circ$

$2^\prime$ ENDO sugar puckers for residues 34 and 35

$2^\prime$ $\gamma_4'$, $\gamma_4'$, $\gamma_4'$: $156\pm2.5^\circ$

$2^\prime$ $\gamma_4'$, $\gamma_4'$, $\gamma_4'$: $156\pm2.5^\circ$

UNCONSTRAINED SUGAR PUCKER for residues 33, 34, 37
### III. Chemical shifts of i6A37-ACSL

<table>
<thead>
<tr>
<th></th>
<th>H6/H8</th>
<th>C6/C8</th>
<th>H5/H2</th>
<th>C5/C2</th>
<th>H1'</th>
<th>C1'</th>
<th>H2'</th>
<th>C2'</th>
<th>H3'</th>
<th>C3'</th>
<th>H4'</th>
<th>C4'</th>
</tr>
</thead>
<tbody>
<tr>
<td>G27</td>
<td>8.08</td>
<td>5.81</td>
<td>5.92</td>
<td>92.05</td>
<td>4.66</td>
<td>75.55</td>
<td>4.59</td>
<td>72.56</td>
<td>4.51</td>
<td>81.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>7.47</td>
<td>136.81</td>
<td>5.8</td>
<td>92.88</td>
<td>4.637</td>
<td>75.6</td>
<td>4.46</td>
<td>72.28</td>
<td>4.45</td>
<td>81.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G29</td>
<td>7.15</td>
<td>136.2</td>
<td>5.8</td>
<td>93.13</td>
<td>4.63</td>
<td>75.43</td>
<td>4.43</td>
<td>73.1</td>
<td>4.46</td>
<td>81.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G30</td>
<td>7.06</td>
<td>136.43</td>
<td>5.76</td>
<td>93.47</td>
<td>4.45</td>
<td>75.57</td>
<td>4.51</td>
<td>72.59</td>
<td>4.45</td>
<td>82.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A31</td>
<td>7.62</td>
<td>139.68</td>
<td>7.80</td>
<td>154.17</td>
<td>5.6</td>
<td>92.03</td>
<td>4.085</td>
<td>76.0</td>
<td>4.39</td>
<td>73.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U32</td>
<td>7.40</td>
<td>141.55</td>
<td>5.02</td>
<td>103.3</td>
<td>5.6</td>
<td>91.69</td>
<td>4.34</td>
<td>75.57</td>
<td>A38*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U33</td>
<td>7.655</td>
<td>143.2</td>
<td>~5.54</td>
<td>104.9</td>
<td>5.7</td>
<td>91.69</td>
<td>4.34</td>
<td>75.57</td>
<td>A38*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G34</td>
<td>7.87</td>
<td>140.04</td>
<td>5.53</td>
<td>88.36-23</td>
<td>4.65</td>
<td>75.03</td>
<td>A35*</td>
<td>4.62</td>
<td>A35*</td>
<td>76.8-78.1</td>
<td>4.33</td>
<td>84.17</td>
</tr>
<tr>
<td>A35</td>
<td>8.04</td>
<td>141.01</td>
<td>7.92</td>
<td>155</td>
<td>5.66</td>
<td>92.332</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A36</td>
<td>7.89</td>
<td>141.5</td>
<td>7.79</td>
<td>154.84</td>
<td>5.66</td>
<td>92.332</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i*A37</td>
<td>8.09</td>
<td>140.98</td>
<td>?</td>
<td>?</td>
<td>5.85</td>
<td>90.3-88.8</td>
<td>4.86</td>
<td>75.41</td>
<td>4.7</td>
<td>75.93</td>
<td>4.58</td>
<td>84.24</td>
</tr>
<tr>
<td>A38</td>
<td>7.88</td>
<td>140.31</td>
<td>?</td>
<td>7.80</td>
<td>5.82</td>
<td>91.69</td>
<td>4.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U39</td>
<td>7.63</td>
<td>142.07</td>
<td>5.01</td>
<td>~103</td>
<td>5.5</td>
<td>93.5</td>
<td>4.34</td>
<td>75.2</td>
<td>~4.4</td>
<td>72.2</td>
<td>4.37</td>
<td>81.95</td>
</tr>
<tr>
<td>C40</td>
<td>7.83</td>
<td>142.07</td>
<td>5.54</td>
<td>97.39</td>
<td>5.5</td>
<td>93.98</td>
<td>4.29</td>
<td>75.46</td>
<td>4.45</td>
<td>72.2</td>
<td>4.39</td>
<td>81.93</td>
</tr>
<tr>
<td>C41</td>
<td>7.77</td>
<td>141.6</td>
<td>~5.4</td>
<td>97.45</td>
<td>~5.45</td>
<td>94.15</td>
<td>4.35</td>
<td>75.22</td>
<td>~4.45</td>
<td>71.94</td>
<td>4.37</td>
<td>81.82</td>
</tr>
<tr>
<td>C42</td>
<td>?</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>94.35</td>
<td>4.24</td>
<td>75.44</td>
<td>4.45</td>
<td>71.83</td>
<td>~3.37</td>
<td>~81.95</td>
<td></td>
</tr>
<tr>
<td>C43</td>
<td>7.62</td>
<td>~141.8</td>
<td>5.42</td>
<td>97.9?</td>
<td>5.73</td>
<td>92.96</td>
<td>3.98</td>
<td>77.44</td>
<td>4.14</td>
<td>69.56</td>
<td>4.14</td>
<td>83.43</td>
</tr>
<tr>
<td>n1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>92.85</td>
<td>77.33</td>
<td>4.08</td>
<td>69.57</td>
<td>4.09</td>
<td>83.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H5'</td>
<td>4.01</td>
<td>3.89</td>
<td>62.3-48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H5'''</td>
<td>4.5</td>
<td>4.06</td>
<td>65.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1/H3</td>
<td>12.71</td>
<td>144.78</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1/H3</td>
<td>12.53</td>
<td>144.86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2/4/6</td>
<td>~0.99</td>
<td>-3.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U32</td>
<td>13.98</td>
<td>159.34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U33</td>
<td>4.14</td>
<td>4.0</td>
<td>66.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G34</td>
<td>4.11</td>
<td>4.02</td>
<td>67.15</td>
<td>A35*</td>
<td></td>
<td>6.14</td>
<td>70.37</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A35</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A36</td>
<td>6.42</td>
<td>68.08</td>
<td></td>
<td></td>
<td></td>
<td>6.71</td>
<td>89.45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A38</td>
<td>4.49</td>
<td>4.04</td>
<td>64.31-45</td>
<td>14.06</td>
<td>159.58</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C40</td>
<td>4.49</td>
<td>4.05</td>
<td>64.67</td>
<td>U32*</td>
<td><del>6.95</del>8.41</td>
<td>~95.51</td>
<td>~4.31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C41</td>
<td>4.53</td>
<td>4.03</td>
<td>64.76</td>
<td></td>
<td></td>
<td>6.849</td>
<td>81.41</td>
<td>95.19</td>
<td>~4.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C42</td>
<td>~4.52</td>
<td>~4.03</td>
<td>64.06-63.85</td>
<td>6.871</td>
<td>84.7</td>
<td>95.15</td>
<td>~4.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C43</td>
<td>4.45</td>
<td>4.0</td>
<td>64.95</td>
<td></td>
<td></td>
<td>6.951</td>
<td>82.22</td>
<td>94.1</td>
<td>~4.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n1</td>
<td>4.34</td>
<td>3.97</td>
<td>64.89-96</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

i*A37 dimethyallyl at 12 °C: CH 6.78, CH₃ 3.76 and 3.56, CH₂ 1.558 and 1.52 ppm
IV. Identified NOES for i6A37-ACSL
Only looked for the ones in the loop region.

<table>
<thead>
<tr>
<th></th>
<th>no Mg²⁺</th>
<th>Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁₂H₂</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>A₁₁H₂</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₂H₆</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₆</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₁'</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₅</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₂H₁'</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₄'</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₂H₂'</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₅</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>G₁₁H₁'</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₁'</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>A₁₁H₂</td>
<td>CH, dma</td>
<td>⊕</td>
</tr>
<tr>
<td>A₁₂H₂</td>
<td>U₁₂H₁'</td>
<td>⊕</td>
</tr>
<tr>
<td>A₁₁H₂</td>
<td>U₁₁H₁'</td>
<td>⊕</td>
</tr>
<tr>
<td>A₁₁H₂</td>
<td>A₁₁H₁'</td>
<td>⊕</td>
</tr>
<tr>
<td>A₁₁H₂</td>
<td>U₁₁H₁' &amp;or C₁₂H₂</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₂H₆</td>
<td>A₁₂H₈</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₆</td>
<td>C₁₂H₆</td>
<td>⊕</td>
</tr>
<tr>
<td>G₁₁H₈</td>
<td>A₁₂H₈</td>
<td>⊕</td>
</tr>
<tr>
<td>A₁₁H₈</td>
<td>U₁₁H₆</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₅</td>
<td>U₁₁H₆</td>
<td>⊕12°C</td>
</tr>
<tr>
<td>U₁₂H₆</td>
<td>U₁₂H₆</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₂H₅</td>
<td>A₁₂H₈</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₅</td>
<td>C₁₂H₅</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₅</td>
<td>U₁₁H₅</td>
<td>⊕</td>
</tr>
<tr>
<td>U₁₁H₁'</td>
<td>C₁₂H₅</td>
<td>⊕</td>
</tr>
<tr>
<td>G₁₁H₁'</td>
<td>A₁₁H₁'</td>
<td>⊕vvw</td>
</tr>
<tr>
<td>U₁₁H₅</td>
<td>A₁₁H₃</td>
<td>⊕</td>
</tr>
</tbody>
</table>

* Most NOES marked with ? are dubious due to spectral overlap
dma = dimethylallyl
### V. Chemical shifts of i6A37-ACSL Co(NH₂)₆³⁺

**Chemical shifts i6a37 CoN₅H₅M₅, 40 Ods i6a37.**

<table>
<thead>
<tr>
<th></th>
<th>H6/8</th>
<th>C6/8</th>
<th>H5/2</th>
<th>C5/2</th>
<th>H1'</th>
<th>C1'</th>
<th>H2'</th>
</tr>
</thead>
<tbody>
<tr>
<td>G27</td>
<td>7.54</td>
<td>136.69</td>
<td>5.79</td>
<td>92.73</td>
<td>5.37</td>
<td>92.73</td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>7.3</td>
<td>136.02</td>
<td>5.77</td>
<td>92.69</td>
<td>4.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G29</td>
<td>7.23</td>
<td>135.92</td>
<td>5.77</td>
<td>92.69</td>
<td>4.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G30</td>
<td>7.44</td>
<td>138.93</td>
<td>7.97</td>
<td>155.26</td>
<td>6.01</td>
<td>93.22</td>
<td>4.53</td>
</tr>
<tr>
<td>A31</td>
<td>7.63</td>
<td>140.12</td>
<td>5.16</td>
<td>92.57</td>
<td>4.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U32</td>
<td>7.63</td>
<td>142.51</td>
<td>5.16</td>
<td>92.57</td>
<td>4.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U33</td>
<td>7.632</td>
<td>142.51</td>
<td>5.52</td>
<td>103.74</td>
<td>5.92</td>
<td>94.67</td>
<td>4.73</td>
</tr>
<tr>
<td>G34</td>
<td>7.82</td>
<td>139.56</td>
<td>5.48</td>
<td>93.91</td>
<td>4.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A35</td>
<td>7.89</td>
<td>140.68</td>
<td>7.59</td>
<td>154.08</td>
<td>5.89</td>
<td>92.49</td>
<td>4.85</td>
</tr>
<tr>
<td>A36</td>
<td>7.14</td>
<td>139.21</td>
<td>7.80</td>
<td>154.07</td>
<td>5.38</td>
<td>93.04</td>
<td>4.71</td>
</tr>
<tr>
<td>i'A37</td>
<td>7.27</td>
<td>137.53</td>
<td>7.90</td>
<td>154.73</td>
<td>5.37</td>
<td>93.10</td>
<td>4.66</td>
</tr>
<tr>
<td>A38</td>
<td>7.77</td>
<td>138.51</td>
<td>7.99</td>
<td>154.90</td>
<td>5.34</td>
<td>92.49</td>
<td>4.45</td>
</tr>
<tr>
<td>U39</td>
<td>7.66</td>
<td>141.49</td>
<td>5.24</td>
<td>103.42</td>
<td>5.13</td>
<td>93.40</td>
<td>4.06</td>
</tr>
<tr>
<td>C40</td>
<td>8.03</td>
<td>142.54</td>
<td>5.59</td>
<td>97.07</td>
<td>5.64</td>
<td>93.62</td>
<td>4.18</td>
</tr>
<tr>
<td>C41</td>
<td>7.87</td>
<td>141.68</td>
<td>5.41</td>
<td>97.28</td>
<td>5.46</td>
<td>94.21</td>
<td>4.32</td>
</tr>
<tr>
<td>C42</td>
<td>7.78</td>
<td>141.45</td>
<td>5.44</td>
<td>97.50</td>
<td>5.45</td>
<td>94.21</td>
<td>4.28</td>
</tr>
<tr>
<td>C43</td>
<td>7.66</td>
<td>140.71</td>
<td>5.50</td>
<td>98.18</td>
<td>5.64</td>
<td>93.32</td>
<td>4.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>H(n)</th>
<th>N</th>
<th>H2(N)</th>
<th>Nami</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>G27</td>
<td>12.29</td>
<td>143.85</td>
<td>-0.94</td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>12.71</td>
<td>144.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G29</td>
<td>12.56</td>
<td>144.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G30</td>
<td>12.25</td>
<td>143.85</td>
<td>-3.86</td>
<td></td>
</tr>
<tr>
<td>A31</td>
<td>11.70</td>
<td>155.76</td>
<td>-4.21</td>
<td></td>
</tr>
<tr>
<td>U32</td>
<td>11.52</td>
<td>156.40</td>
<td>-4.44</td>
<td></td>
</tr>
<tr>
<td>U33</td>
<td>11.52</td>
<td>156.40</td>
<td>-4.73</td>
<td></td>
</tr>
<tr>
<td>G34</td>
<td>5.97</td>
<td>69.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A35</td>
<td>13.97</td>
<td>159.10</td>
<td>-2.86</td>
<td></td>
</tr>
<tr>
<td>A36</td>
<td>14.64</td>
<td>-4.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i'A37</td>
<td>14.64</td>
<td>90.67</td>
<td>-3.67 or A12</td>
<td></td>
</tr>
<tr>
<td>A38</td>
<td>14.64</td>
<td>158.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U39</td>
<td>13.97</td>
<td>159.10</td>
<td>-4.87</td>
<td></td>
</tr>
<tr>
<td>C40</td>
<td>14.64</td>
<td>-4.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C41</td>
<td>14.64</td>
<td>-4.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C42</td>
<td>14.64</td>
<td>-4.31 or C17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C43</td>
<td>14.64</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

237
VI. Structural constraints for 16A37-ACSL Co(NH)\textsubscript{4}\textsuperscript{2+}

The numbering for the NOE derived distance constraints is analogous to the one described for ACSL\textsuperscript{15} in Appendix II.

7: H1 9: H8
7: H3 10: H8
7: H3 9: H1
8: H6 8: H8
9: H3 9: H6
9: H3 10: H8
9: H2 11: H1
11: H6 12: H5
12: H5 13: H6
5: H1 6: H8
6: H5 5: H8
6: H5 6: H8
6: H5 7: H6
6: H5 5: H2
7: H1 7: H6
7: H1 8: H8
7: H1 9: H9
7: H1 10: H9
8: H1 9: H9
5: H1 6: H8
9: H1 5: H8
9: H1 10: H8
9: H1 9: H2
17: H1 11: H8
11: H1 11: H8
10: H1 9: H2
11: H1 12: H8
11: H1 13: H2
12: H2 12: H8
12: H2 13: H4
12: H2 14: H1
13: H4 12: H8
13: H4 12: H8
7: H2 5: H8
8: H2 6: H8
9: H2 11: H3
10: H2 10: H8
11: H2 11: H3
11: H2 12: H8
12: H2 12: H8
12: H2 13: H8
9: H3 9: H9
5: H3 6: H5
6: H5 6: H5
6: H5 7: H5
7: H5 7: H5

Hydrogen bonding constraints

U OH2' A\textsubscript{15}N7 2.0 ± 0.2
U O2' A\textsubscript{15}N7 2.9 ± 0.3

A31-U39
A H61 U O4 2.00 ± 0.2
A N6 U O4 2.90 ± 0.3
A N1 U H3 2.00 ± 0.2
A N1 U N3 2.90 ± 0.3

The rest of the constraints were taken from the ones used on ACSL\textsuperscript{15} stem region, residues 27-31 and 32-43.
### VII. Chemical shifts of \( ^{31}P \)-ACS

<table>
<thead>
<tr>
<th></th>
<th>H6/H8</th>
<th>C6/C8</th>
<th>H5/H2</th>
<th>C5/C2</th>
<th>H1'</th>
<th>C1'</th>
<th>H2'</th>
<th>C2'</th>
<th>H3'</th>
<th>C3'</th>
<th>H4'</th>
<th>C4'</th>
</tr>
</thead>
<tbody>
<tr>
<td>G27</td>
<td>7.99</td>
<td>5.74</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>7.47</td>
<td>136.74</td>
<td>5.90</td>
<td>92.86</td>
<td>4.64</td>
<td>75.65</td>
<td>4.56</td>
<td>72.81</td>
<td>4.509</td>
<td>82.274</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G29</td>
<td>7.17</td>
<td>135.98</td>
<td>5.79</td>
<td>92.87</td>
<td>4.62</td>
<td>75.69</td>
<td>4.477</td>
<td>72.82</td>
<td>4.45</td>
<td>81.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G30</td>
<td>7.10</td>
<td>136.17</td>
<td>5.75</td>
<td>93.12</td>
<td>4.617</td>
<td>75.53</td>
<td>4.42</td>
<td>73.14</td>
<td>4.66</td>
<td>-82.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A31</td>
<td>7.68</td>
<td>139.63</td>
<td>7.74</td>
<td>154.26</td>
<td>5.99</td>
<td>93.15</td>
<td>4.65</td>
<td>75.687</td>
<td>4.579</td>
<td>72.558</td>
<td>4.68</td>
<td>82.092</td>
</tr>
<tr>
<td>Psa32</td>
<td>6.92</td>
<td>140.05</td>
<td>5.46</td>
<td>82.70</td>
<td>4.12</td>
<td>75.16?</td>
<td>4.287</td>
<td>73.65?</td>
<td>4.135</td>
<td>79.917</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U33</td>
<td>7.51</td>
<td>141.49</td>
<td>5.25</td>
<td>103.83</td>
<td>5.70</td>
<td>93.29</td>
<td>4.31</td>
<td>76.23</td>
<td>4.365</td>
<td>73.25</td>
<td>4.306</td>
<td>82.856</td>
</tr>
<tr>
<td>C34</td>
<td>7.95</td>
<td>139.43</td>
<td>5.49</td>
<td>88.67</td>
<td>4.70</td>
<td>74.97</td>
<td>4.56</td>
<td>77.54</td>
<td>4.39</td>
<td>84.815</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A35</td>
<td>8.09</td>
<td>141.95</td>
<td>8.01</td>
<td>155.17</td>
<td>5.49</td>
<td>88.67</td>
<td>4.35</td>
<td>77.47</td>
<td>4.56</td>
<td>77.54</td>
<td>3.69</td>
<td>84.82</td>
</tr>
<tr>
<td>A36</td>
<td>7.88</td>
<td>141.16</td>
<td>7.91</td>
<td>155.06</td>
<td>5.72</td>
<td>89.13</td>
<td>4.74</td>
<td>75.57</td>
<td>4.806</td>
<td>77.00</td>
<td>4.44</td>
<td>84.78</td>
</tr>
<tr>
<td>A37</td>
<td>8.18</td>
<td>141.51</td>
<td>7.19</td>
<td>153.46</td>
<td>5.76</td>
<td>89.36</td>
<td>4.80</td>
<td>75.25</td>
<td>4.65</td>
<td>75.76</td>
<td>4.59</td>
<td>84.34</td>
</tr>
<tr>
<td>A38</td>
<td>7.77</td>
<td>139.90</td>
<td>7.86</td>
<td>154.09</td>
<td>5.75</td>
<td>-93</td>
<td>4.41</td>
<td>75.29</td>
<td>4.36</td>
<td>73.818</td>
<td>4.56</td>
<td>83.06</td>
</tr>
<tr>
<td>U39</td>
<td>7.61</td>
<td>141.64</td>
<td>4.92</td>
<td>102.6</td>
<td>5.46</td>
<td>93.49</td>
<td>4.29</td>
<td>75.33</td>
<td>4.407</td>
<td>72.104</td>
<td>4.386</td>
<td>82.11</td>
</tr>
<tr>
<td>C40</td>
<td>7.88</td>
<td>141.80</td>
<td>5.54</td>
<td>97.33</td>
<td>5.98</td>
<td>93.83</td>
<td>4.27</td>
<td>75.58</td>
<td>4.45</td>
<td>72.216</td>
<td>4.395</td>
<td>81.898</td>
</tr>
<tr>
<td>C41</td>
<td>7.79</td>
<td>-141.65</td>
<td>5.4512</td>
<td>97.44</td>
<td>5.43</td>
<td>94.09</td>
<td>4.44</td>
<td>75.37</td>
<td>4.447</td>
<td>72.064</td>
<td>4.37</td>
<td>81.99</td>
</tr>
<tr>
<td>C42</td>
<td>7.80</td>
<td>-141.65</td>
<td>5.4512</td>
<td>97.44</td>
<td>5.46</td>
<td>94.35</td>
<td>4.24</td>
<td>75.53</td>
<td>4.47</td>
<td>71.91</td>
<td>4.347</td>
<td>-82.07</td>
</tr>
<tr>
<td>C43</td>
<td>7.66</td>
<td>141.88</td>
<td>5.46</td>
<td>97.97</td>
<td>5.71</td>
<td>92.87</td>
<td>4.36</td>
<td>77.48</td>
<td>4.146</td>
<td>69.63</td>
<td>4.137</td>
<td>83.66</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>H5'</th>
<th>H5''</th>
<th>C5'</th>
<th>H1' / H3</th>
<th>H1' / H3</th>
<th>H2' / H3</th>
<th>N2' / H3</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>G27</td>
<td>4.49</td>
<td>4.19</td>
<td>65.77</td>
<td>12.7</td>
<td>144.8</td>
<td></td>
<td></td>
<td>-3.878</td>
</tr>
<tr>
<td>G28</td>
<td>4.48</td>
<td>4.06</td>
<td>65.41</td>
<td>12.51</td>
<td>144.76</td>
<td></td>
<td></td>
<td>-3.69</td>
</tr>
<tr>
<td>G30</td>
<td>4.407</td>
<td>4.029</td>
<td>66.05</td>
<td>12.27</td>
<td>144.3</td>
<td></td>
<td></td>
<td>-3.68</td>
</tr>
<tr>
<td>A31</td>
<td>4.53</td>
<td>4.09</td>
<td>64.86</td>
<td>7.71?</td>
<td>6.51?</td>
<td>80.13?</td>
<td></td>
<td>-4.007</td>
</tr>
<tr>
<td>Psa32</td>
<td>4.37</td>
<td>4.94</td>
<td>65.33</td>
<td>3) 13.21184.17</td>
<td>10.31128.59</td>
<td></td>
<td></td>
<td>-3.77</td>
</tr>
<tr>
<td>U33</td>
<td>4.40</td>
<td>4.02</td>
<td>65.02</td>
<td>13.05</td>
<td>157.98</td>
<td></td>
<td></td>
<td>-3.85</td>
</tr>
<tr>
<td>G34</td>
<td>4.15</td>
<td>4.01</td>
<td>66.93</td>
<td>10.49</td>
<td>144.16</td>
<td></td>
<td></td>
<td>-3.86</td>
</tr>
<tr>
<td>A35</td>
<td>4.77</td>
<td>4.51</td>
<td>67.8</td>
<td>6.42</td>
<td>75.71?</td>
<td></td>
<td></td>
<td>-3.311</td>
</tr>
<tr>
<td>A36</td>
<td>4.1</td>
<td>4.97</td>
<td>68.59</td>
<td>6.64</td>
<td>76.9?</td>
<td></td>
<td></td>
<td>-3.986</td>
</tr>
<tr>
<td>A37</td>
<td>4.35</td>
<td>4.18</td>
<td>68.84</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-3.397</td>
</tr>
<tr>
<td>A38</td>
<td>4.33</td>
<td>68.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-3.376</td>
</tr>
<tr>
<td>U39</td>
<td>4.506</td>
<td>4.048</td>
<td>64.26</td>
<td>13.86</td>
<td>159.41</td>
<td></td>
<td></td>
<td>-4.628</td>
</tr>
<tr>
<td>C40</td>
<td>4.49</td>
<td>4.046</td>
<td>64.62</td>
<td>6.91</td>
<td>8.39</td>
<td>95.65</td>
<td></td>
<td>-4.423</td>
</tr>
<tr>
<td>C41</td>
<td>4.52</td>
<td>4.03</td>
<td>64.42</td>
<td>6.85</td>
<td>8.42</td>
<td>95.269</td>
<td></td>
<td>-4.45</td>
</tr>
<tr>
<td>C42</td>
<td>4.53</td>
<td>-4.02</td>
<td>64.3</td>
<td>6.90</td>
<td>8.40</td>
<td>95.292</td>
<td></td>
<td>-4.423</td>
</tr>
</tbody>
</table>

* = dephosphorylated
VIII. Structural constraints for ψ32-ACSL
Base-pairing constraints

A35
G34 - A36
G33 - A37
Pse12 - A36
A31 - C33
G30 - C34
C29 - C34
C28 - C34
G27 - C33
5' - 3'

base pair hydrogen bond distances
G-C
G C6  C H42  2.13  1.3  1.3
G C6  C H43  2.40  1.3  1.3
G H1  C N3  2.10  1.5  1.5
G N1  C N3  2.10  1.5  1.5
G H22  C O2  2.10  1.8  1.8
G N2  C O2  2.10  1.8  1.8
A-C
A H61  U C4  2.10  1.2  1.2
A H6  U C4  2.90  1.3  1.3
A H1  U N3  2.10  1.2  1.2
A H1  U N3  2.10  1.2  1.2

C33-A37
A C4  U H61  2.13  1.3  1.3
A C4  U H6  2.40  1.3  1.3
A H3  U N3  2.10  1.2  1.2
A N3  U N3  2.30  1.3  1.3

inter proton distance constraints A
Numbering starts with 1, G1 corresponds to G2°.

A3
G1 - A3
Pse6 - A11
A5 - C13
G4 - C14
G3 - C15
G2 - C16
G1 - C17
5' - 3'

14:H42  13:H6  7  3:H1  16:H41  6  2:H1  3:H1  4
2:H1  17:H42  5  4:H1  15:H42  5  2:H1  17:H6  6
2:H1  17:H42  6  4:H1  15:H41  6  2:H1  3:H8  6
2:H1  17:H42  4  4:H1  14:H42  4  3:H1  4:H8  7
Alpha torsion angle constraints

alpha is constrained to exclude the trans conformation for:

329, 329', 333, A31, U32, A33, U39, G40, C41, C42, C43

33', P, CS', CE': 1.0 ± 120.0°

Beta torsion angle constraints

beta is constrained to exclude the trans conformation for:

327, 329, 331, A31, A33, U39, G40, C41, C42, C43

CS', CS', P, CS' 5.0° ± 120.0°

Beta torsion angle constraints

trans: 331, A31

P, CS', CS', C4', 180 ± 45°

Gamma torsion angle constraints

trans and gauche: 334 and A36

325', 335', C4', 120.0 ± 120.0°

gauche: 341, C41 and C42

325', 335', C4', 60.0 ± 120.0°

Torsion angle constraints about delta and nu 1
to apply ce or cs prime endo sugar puckers

Angles determined from model built A-form/B-form ribose

1'-ENDO sugar puckers for residues 27, 28, 30, 32, 33, 35, 41, 42, 43

CE' CE' CE' 1.4 ± 1.2°

C4' C4' C4' 2.2 ± 2.5°

1'-ENDO sugar puckers for residues 34 and 35

CE' CE' CE' 1.5 ± 1.5°

C4' C4' C4' 2.5 ± 1.5°

UNCONSTRAINED SUGAR PUCKER for residues 33, 34, 37
IX. Expected or inconsistent NOEs for Ψ32-ACSL\textsuperscript{per} structures' loop regions. The convergent structure has G\textsubscript{14} oriented towards the major groove. The alternate convergent structure has G\textsubscript{14} bulged on the minor groove side. The alternate convergent structure was calculated excluding some constraints from unresolved resonances or extremely weak. These constraints are marked by an asterisk in appendix VIII.

<table>
<thead>
<tr>
<th>NOE</th>
<th>distance in convergent structure (Å)</th>
<th>distance in alternate convergent structure (Å)</th>
<th>constraint (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U\textsubscript{13}H4', G\textsubscript{14}H8</td>
<td>&gt;5</td>
<td>2.7</td>
<td>7</td>
</tr>
<tr>
<td>U\textsubscript{13}H1', G\textsubscript{14}H8</td>
<td>&gt;5</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>U\textsubscript{13}H1', A\textsubscript{14}H2</td>
<td>&gt;5</td>
<td>4.6</td>
<td>-</td>
</tr>
<tr>
<td>U\textsubscript{13}H1', A\textsubscript{14}H2</td>
<td>2.8</td>
<td>4.8</td>
<td>-</td>
</tr>
<tr>
<td>U\textsubscript{13}H2', G\textsubscript{14}H8</td>
<td>3.1</td>
<td>4.6</td>
<td>5</td>
</tr>
<tr>
<td>U\textsubscript{13}H3', G\textsubscript{14}H8</td>
<td>2.7</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>G\textsubscript{14}H1', A\textsubscript{15}H8</td>
<td>2.5</td>
<td>4.9</td>
<td>5</td>
</tr>
<tr>
<td>G\textsubscript{14}H3', A\textsubscript{15}H8</td>
<td>&gt;5</td>
<td>2.7</td>
<td>-</td>
</tr>
<tr>
<td>G\textsubscript{14}H2', U\textsubscript{13}H1'</td>
<td>&gt;5</td>
<td>2.6</td>
<td>?</td>
</tr>
<tr>
<td>A\textsubscript{13}H8, A\textsubscript{14}H2</td>
<td>&gt;5</td>
<td>4.4</td>
<td>-</td>
</tr>
<tr>
<td>A\textsubscript{13}H8, A\textsubscript{14}H2</td>
<td>4.5</td>
<td>&gt;5</td>
<td>7</td>
</tr>
<tr>
<td>A\textsubscript{13}H2, A\textsubscript{14}H2</td>
<td>5.9</td>
<td>2.9</td>
<td>7</td>
</tr>
</tbody>
</table>
9 BIBLIOGRAPHY


base at position 37 in phenylalanine tRNA is responsible for its
shiftiness in retroviral ribosomal frameshifting. Virology 279(1), 130-
135.

Carter, A. P., Clemons, W. M., Brodersen, D. E., Morgan-Warren, R. J.,
structure of the 30S ribosomal subunit and its interactions with

Cedergren, R. J., Sankoff, D., LaRue, B. & Grosjean, H. (1981). The
evolving tRNA molecule. CRC Critical Reviews in Biochemistry 11(1), 35-
104.

how, and why. IUBMB Life 49(5), 341-351.

Chen, Y., Sierzputowska-Gracz, H., Guenther, R., Everet, K. & Agris, P.
F. (1992). 5-methylcytidine is required for cooperative binding of Mg$^{2+}$
and a conformational transition at the anticodon stem-loop of yeast
phenylalanyne tRNA. Biochemistry 32(38), 10249-10253.

Claesson, C., Lustig, F., Borén, T., Simonsson, C., Barciszewska, M. &
in position 32 of the anticodon loop. Journal of Molecular Biology
247(2), 191-196.

Clore, G. M., Gronenborn, A. M., Piper, E. A., McLaughlin, L. W.,
pentadecamer comprising the anticodon loop and stem of yeast tRNA$^{\text{Phe}}$. A
500 MHz 1H-n.m.r. study. Biochemical Journal 221(3), 737-51.

K-12 miaA and characterization of the mutator phenotype caused by miaA

the RNA duplex r(GCGAAAUUUGCG)$_2$ determined by NMR. Nucleic Acids
Research 24(19), 3693-3699.

Cowan, J. A. (1993). Metallobiochemistry of RNA. Co(NH$_3$)$_6$$^{3+}$ as a probe
for Mg$^{2+}$(aq) binding sites. Journal of Inorganic Biochemistry 49(3),
171-175.

Cowan, J. A. (1995). Introduction to the biological chemistry of
magnesium ion. In The Biological Chemistry of Magnesium (Cowan, J. A.,

Dao, V., Guenther, R., Malkiewicz, A., Nawrot, B., Sochacka, E.,


stem-loop of tRNA^Lys,3 by an A-C base-pair and by pseudouridine.

Eisinger, J. (1971). Visible gel electrophoresis and the determination of

complexes of tRNA^{Glu}_2 (Escherichia coli). Biochemistry 14(18), 4031-
4041.

pentanucleotides to the anticodon loop of transfer RNA. Journal of
Molecular Biology 73(1), 131-137.

nucleoside 2-methylthio-N^6-(4-hydroxyisopentenyl)adenosine distinguish
between bases 3' of the codon. Journal of Molecular Biology 218(3), 509-
516.

Press, New York.

Structural features of a six-nucleotide RNA hairpin loop found in
ribosomal RNA. Biochemistry 35(21), 6539-6548.

Freier, S. M. & Tinoco, I. J. (1975). The binding of complementary
oligoribonucleotides to yeast initiator transfer RNA. Biochemistry
14(15), 3310-3314.

Influence of counter-ions on the crystal structures of DNA decamers:
binding of [Co(NH_3)_6]^3+ and Ba^{2+} to A-DNA. Biophysical Journal 69(2),
559-568.

magnetic resonance studies of codon-anticodon interaction in tRNA^{Phe}. I.
Effect of binding complementantary tetra and pentanucleotides to the

DNA by cobalt hexaammine and magnesium cations. Biochemistry 24(2), 237-
240.

Goddard, J. P. (1977). The structures and functions of transfer RNA.
Progress in Biophysics and Molecular Biology 32(3), 233-308.


Kieft, J. S. & Tinoco, I. J. (1997). Solution structure of a metal-
binding site in the major groove of RNA complexed with cobalt (III) hexamine. Structure 5(5), 713-721.


A-DNA. Nucleic Acids Research 24(4), 676-682.


Stuart, J. W., Gdaniec, Z., Gunther, R., Marsza\l ek, M., Sochacka, E.,


