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Type II dihydrofolate reductases: Probing the natural diversity of enzyme active sites

De Brito, Rui Manuel Pontes Meireles Ferreira, Ph.D.

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TYPE II DIHYDROFOLATE REDUCTASES:
PROBING THE NATURAL DIVERSITY OF ENZYME ACTIVE SITES

by

RUI MANUEL PONTES MEIRELES FERREIRA DE BRITO

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IN PARTIAL FULFILLMENT OF THE
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DOCTOR OF PHILOSOPHY

APPROVED, THESIS COMMITTEE

Frederick B. Rudolph
Professor
Biochemistry and Cell Biology

John S. Olson
Professor
Biochemistry and Cell Biology

George N. Bennett
Professor
Biochemistry and Cell Biology

Jacqueline V. Shanks
Assistant Professor
Chemical Engineering

Kathleen S. Matthews
Wiess Professor and Chair
Biochemistry and Cell Biology

Paul R. Rosevear
Associate Professor
Biochemistry and Molecular Biology
U. of Texas, Medical School, Houston

Houston, Texas
April, 1992
ABSTRACT

Type II Dihydrofolate Reductases:
Probing the Natural Diversity of Enzyme Active Sites

by

Rui Manuel Meireles Brito

Type II dihydrofolate reductases (DHFRs) encoded by the R67 and R388 plasmids are sequence and structurally different from known chromosomal DHFRs, and are responsible for conferring trimethoprim resistance to the host bacterial strain. An overproduced derivative of R388 DHFR, RBG200 DHFR, was purified to apparent homogeneity. The pH versus activity profile of RBG200 DHFR was found to be similar to that previously reported for R388 DHFR, but different from that of chromosomal DHFRs. Gel filtration and equilibrium ultracentrifugation experiments suggested that at pH 6, where the enzyme is most active, RBG200 DHFR is in equilibrium between tetramer and other protein species. Gel filtration studies suggested that the loss of enzyme activity between pH 6 and pH 5 is not due to tetramer dissociation. Gel filtration experiments with R67 DHFR produced similar results. RBG200 DHFR was found to catalyze the transfer of the pro-R hydrogen of NADPH to dihydrofolate, making it a type-A dehydrogenase, along with the chromosomal DHFRs. Addition of NADP⁺ to RBG200 DHFR results in the formation of an initial binary complex which slowly interconverts to a second binary complex, with an apparent first order rate constant of $1.0 \times 10^{-4} \text{ s}^{-1}$, at
25 °C. The binding of NADP+ to RBG200 DHFR in the second binary complex was found to be weak, $K_D = 1.9 \pm 0.4$ mM. The stoichiometry for coenzyme binding was found to be approximately one coenzyme per tetramer of RBG200 DHFR. Transferred NOEs were used to estimate internuclear distances, and it was found that NADP+ binds to RBG200 DHFR in the initial and final RBG200 DHFR•NADP+ binary complexes, as well as in the ternary complex with folate, with a *syn* conformation about the nicotinamide-ribose glycosidic bond and an *anti* conformation about the adenine-ribose glycosidic bond. From the stereochemistry of hydride transfer and the conformation of the enzyme-bound cofactor, a model was proposed for the orientation of coenzyme and substrate at the active site of RBG200 DHFR, which differs from that observed at the active site of chromosomal DHFRs, and may be responsible for the resistance of type II DHFRs to several antifolates.
This thesis is dedicated with gratitude to:

my teachers and professors that over the years kept stimulating my interest in science;

my parents, Manuela and Joaquim Brito, for their unconditional lifelong support;

my wife, Karla Dantas, for her patience, commitment and love.
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Portions of Chapters II, III and IV have been published elsewhere:

"Characterization and Stereochemistry of Cofactor Oxidation by a Type II Dihydrofolate Reductase"

"Conformation of NADP⁺ Bound to a Type II Dihydrofolate Reductase"
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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>1D</td>
<td>one-dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>AMN</td>
<td>adenine mononucleotide</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>DQF-COSY</td>
<td>double-quantum filtered two-dimensional correlated spectroscopy</td>
</tr>
<tr>
<td>DSS</td>
<td>sodium 2,2-dimethyl-2-silapentane-5-sulfonate</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>H₂F</td>
<td>7,8-dihydrofolate</td>
</tr>
<tr>
<td>H₄F</td>
<td>5,6,7,8-tetrahydrofolate</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IMP</td>
<td>inosine monophosphate</td>
</tr>
<tr>
<td>Kₐ</td>
<td>equilibrium association constant</td>
</tr>
<tr>
<td>Kₜd</td>
<td>equilibrium dissociation constant</td>
</tr>
<tr>
<td>MTX</td>
<td>methotrexate</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced form of nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>oxidized form of NADP</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced form of NADP</td>
</tr>
<tr>
<td>NMN</td>
<td>nicotinamide mononucleotide</td>
</tr>
</tbody>
</table>
NMR  nuclear magnetic resonance
NOE  nuclear Overhauser effect
NOESY two-dimensional nuclear Overhauser enhancement spectroscopy
PAGE polyacrylamide gel electrophoresis
SDS  sodium dodecyl sulphate
TMP  trimethoprim
TNOE transferred nuclear Overhauser effect
TOCSY two-dimensional total correlation spectroscopy
Tris tris(hydroxymethyl)aminomethane
Chapter I. INTRODUCTION.

1. Dihydrofolate Reductase and the Metabolism of Folate and One-Carbon Units.

The enzyme dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: NADP\(^+\) oxido-reductase, EC 1.5.1.3) exists in almost all living organisms, with the known exceptions being the archaebacteria and some protozoa (Kraut and Matthews, 1987). Dihydrofolate reductase catalyzes a central reaction in the metabolism of one-carbon units, the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. Tetrahydrofolate works as a shuttle of one-carbon units in metabolic processes as diverse as the biosynthesis of deoxythymidylate, the biosynthesis of purine nucleotides and the biosynthesis of methionine and glycine, as well as in several catabolic pathways (Figure I.1) (Kisliuk, 1984). These one-carbon units are carried in three different oxidation states attached to the N-5, N-10 or both positions of tetrahydrofolate (Figure I.2). The major entry route of one-carbon units into the intracellular pool is from \(\text{sérine} \) through the reaction catalyzed by serine hydroxymethyltransferase to form 5,10-methylenetetrahydrofolate (Figure I.1). The methylene group can then be transferred to deoxyuridylate to form deoxythymidylate; it can be reduced to 5-methyltetrahydrofolate for use in methionine biosynthesis; and it can be oxidized to 10-formyltetrahydrofolate for use in \textit{de novo} purine biosynthesis. The synthesis of deoxythymidylate from deoxyuridylate leads to the oxidation of tetrahydrofolate to dihydrofolate. DHFR is the enzyme responsible for reducing dihydrofolate
Figure I.1: Scheme of metabolic pathways involving dihydrofolate and tetrahydrofolate (from Kisliuk, 1984). H₂PteGlu and H₄PteGlu are 7,8-dihydrofolate and 5,6,7,8-tetrahydrofolate, respectively. Numbers 1 through 17 represent the following enzymes:

1. Dihydrofolate reductase (EC 1.5.1.3)
2. Serine hydroxymethyltransferase (EC 2.1.2.1)
3. Glycine synthase (EC 2.1.2.10)
4. Thymidylate synthase (EC 2.1.1.45)
5. Methylene tetrahydrofolate reductase (EC 1.1.99.15)
6. 5-Methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.13)
7. Methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5)
8. Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9)
9. 10-Formyltetrahydrofolate synthase (EC 6.3.4.3)
10. Phosphoribosylglycinamidase formyltransferase (EC 2.1.2.2)
11. Phosphoribosylaminomimidazolecarboxamide formyltransferase (EC 2.1.2.3)
12. Methenyltetrahydrofolate synthase (EC 6.3.3.2)
13. Glutamate formiminotransferase (EC 2.1.2.5)
14. Formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4)
15. 10-Formyltetrahydrofolate dehydrogenase (EC 1.5.1.6)
16. Dimethylglycine dehydrogenase (EC 1.5.99.2)
17. Sarcosine dehydrogenase (EC 1.5.99.1)
Figure I.2: Structural formulas of dihydrofolate, methotrexate, aminopterin and trimethoprim.
back to tetrahydrofolate, which then re-enters the one-carbon metabolism cycle. Inhibition of DHFR leads to the accumulation of dihydrofolate at the expense of the one-carbon adducts of tetrahydrofolate which consequently leads to inhibition of purine and thymidylate biosynthesis, decrease of nucleic acid biosynthesis and cell stasis. This scheme makes DHFR a prime target for chemotherapy against actively dividing cell populations, such as bacteria, protozoa, fungi or cancer cells. It should be noted that in cells not actively synthesizing thymidylate and DNA, because little tetrahydrofolate is oxidized to dihydrofolate in the thymidylate synthetase-catalyzed reaction, inhibition of DHFR should not result in cell death (Schweitzer et al., 1990).

The biosynthesis of the pterin ring of folate has been studied in several bacteria, spinach, Chinese hamsters, Drosophila melanogaster, chicken liver and rat brain (Brown, 1985). It was found that guanosine triphosphate (GTP) is the precursor in the biosynthesis of pterin. The reaction is catalyzed by GTP cyclohydrolase I and the products are dihydronopterin triphosphate and formate. The conversion of GTP to dihydronopterin triphosphate involves two successive hydrolytic steps, a rearrangement of the ribose moiety and ring closure. All these steps are catalyzed by the same enzyme and no free intermediates have been detected (Brown, 1985). Dihydronopterin triphosphate is at the branching point of several pathways leading to the synthesis of dihydrofolate, dihydrobiopterin, tetrahydrobiopterin, sepiapterin, drosopterin, and butterfly wing pigments, among others (Kisliuk, 1984). Tetrahydrobiopterin is a cofactor for the phenylalanine, tyrosine and tryptophan hydroxylases, which in animals are part of the pathways for the biosynthesis of amine hormones such as serotonin, adrenaline,
noradrenaline and dopamine (Curtius et al., 1985). Sepiapterin and
drosopterin are the yellow and red pigments, respectively, partially
responsible for the eye color in *Drosophila* (Brown, 1985). On the
dihydrofolate pathway, dihydronopteron triphosphate is converted to 6-
hydroxymethyl-dihydropterin, the precursor of dihydrofolate. In *E. coli*,
dihydronopteron triphosphate loses a pyrophosphate group in a reaction
catalyzed by dihydronopteron triphosphate pyrophosphohydrolase and the
third phosphate group is removed by a nonspecific phosphatase to form
dihydronopteron. Dihydronopteron is then sequentially converted to 6-
hydroxymethyl-dihydropterin, 6-hydroxymethyl-dihydropterin
pyrophosphate, dihydronopteroate and finally dihydrofolate, by the enzymes
dihydronopteron aldotase, 6-hydroxymethyl-dihydropterin
pyrophosphokinase, dihydronopteroate synthase and dihydrofolate synthase,
respectively (Kisliuk, 1984). A similar set of reactions have been reported for
pea seedlings and spinach extracts (Shiota, 1984). Animals can carry out the
synthesis of dihydrobiopterin and tetrahydrobiopterin from GTP, but are
unable to synthesize dihydrofolate, apparently due to the lack of the enzymes
that convert dihydronopteron triphosphate to dihydrofolate (Kisliuk, 1984).

Most eukaryotic cells and some bacteria are unable to synthesize folate
de novo and require an external source and a transport mechanism for folate
(Huennekens, et al., 1987). Fresh fruits and vegetables are good sources of
folates (Herbert, 1990). A substantial portion of folates in foods are present in
the polyglutamyl form — derivatives with several glutamate units linked via
γ-carboxyl peptide bonds. In mammalian intestine, the polyglutamyl folates
undergo hydrolysis to the monoglutamyl forms before they are absorbed
(Mason, 1990). This reaction is catalyzed by γ-glutamyl hydrolase in the intestinal brush border (Halsted, 1990). The monoglutamyl folates are then transported by a saturable carrier into the cytoplasm of the enterocytes where they are largely converted to reduced, methylated and formylated forms (Mason, 1990). Little is known about how the folates are transferred from the enterocytes to the bloodstream, but it appears that another saturable carrier-mediated transport system is involved (Mason, 1990). In the plasma, folates are transported mostly as monoglutamate derivatives (Shane, 1990).

After reaching the different tissues and being internalized, folates are converted to poly-γ-glutamyl derivatives of various glutamyl chain lengths, which ensures intracellular retention and enhances affinity for most enzymes of the folate metabolism (Huennekens et al., 1987). The polyglutamate chain length varies from tissue to tissue and also varies with dietary and growth conditions. In some organisms there is a broad distribution of polyglutamate chain lengths, while in others a single length predominates. The number of glutamic acid units in cellular folates most commonly vary between one and nine (Kisliuk, 1984). The enzyme folylpolyglutamate synthetase catalyzes the ATP-dependent addition of glutamic acid units to folate, and at least in mammals this enzyme can account for the synthesis of all the polyglutamyl forms of folate (Shane, 1990). Another enzyme, γ-glutamylhydrolase, catalyzes the hydrolysis of the γ-peptide bond of the polyglutamate chain of folates. In most mammalian tissues, glutamylhydrolase is located in the lysosomes (Shane, 1989). The role of the lysosomal glutamylhydrolase in folate homeostasis has not been established (Shane, 1989).
2. Pharmacological Interest of Dihydrofolate Reductase.

The key role played by DHFR and other enzymes of the one-carbon metabolism in nucleotide biosynthesis and hence in DNA replication and cell proliferation, makes these enzymes good potential targets for therapeutic antimicrobial and antineoplastic agents. Several clinically important antifolate drugs such as the antineoplastic agent methotrexate, the antibacterial agent trimethoprim and the antimalarial agent pyrimethamine have been used extensively.

A wide interest in the synthesis of antifolates was generated in the late 1940s following the characterization of the folic acid structure and the recognition that sulfanilamide was a competitive antagonist of \(p\)-aminobenzoic acid (Stokstad et al., 1946; Angier et al., 1946). Compounds such as aminopterin (Seeger et al., 1947) and methotrexate (Seeger et al., 1949; Cosulich and Smith, 1948) (Figure I.2), which were strong antagonists of folic acid in the growth of \textit{Streptococcus faecalis} (Hitchings and Baccanari, 1984) were produced. It was also recognized that derivatives of 2,4-diaminopyrimidines were antagonists of folic acid in the growth of \textit{Streptococcus faecalis}, \textit{Lactobacillus casei}, \textit{Escherichia coli}, \textit{Staphylococcus aureus} and \textit{Lactobacillus arabinosus} and that different species of bacteria responded differently to some of these compounds (Daniel and Norris, 1947; Daniel et al., 1947; Mallette et al., 1947; Hitchings et al., 1948, 1950). Falco and collaborators (Falco et al., 1949) discovered antimalarial activity among the 2,4-diaminopyrimidines, and soon after pyrimethamine was found to be the most active and useful antimalarial 2,4-diaminopyrimidine (Hitchings and Baccanari, 1984). The continued search for better antibacterial 2,4-
diaminopyrimidines led to the synthesis of trimethoprim in 1962 (Figure I.2) (Roth et al., 1962). Work with partially purified dihydrofolate reductases from several different species and determination of the 50% inhibitory concentration (IC$_{50}$) for several antifolates, showed that the trimethoprim IC$_{50}$ for human DHFR is approximately 30,000 times higher than that for the bacterial enzyme which opened the door for the use of trimethoprim as a therapeutically valuable antibacterial agent (Burchall and Hitchings, 1965). Analogously, pyrimethamine which is used as antimalarial agent, is 2000- to 3000-fold more active against the dihydrofolate reductase of *Plasmodium berghei*, one of the malaria parasites, than to the rat liver DHFR (Hitchings and Baccanari, 1984).

Testing of antifolates as therapeutic agents was done from the beginning in combination with sulfonamides. Daniel and collaborators (Daniel et al, 1947; Daniel and Norris, 1947) found synergism in the antibacterial action of 2,4-diaminopyrimidines and sulfonamides, and Greenberg and Richeson (1950) found similar synergism against *Plasmodium gallinaceum* in chicken malaria. The sulfonamides inhibit dihydrofolate biosynthesis at the level of dihydropteroate synthase, a reaction not present in animals. The action of the sulfonamides depends both on the presence of this biosynthetic pathway as well as on the absence of an uptake mechanism for folate in the pathogenic microorganism (Hitchings and Baccanari, 1984). The synergism observed in the combined action of sulfonamides and 2,4-diaminopyrimidines results from the cumulative action of both drugs in the reduction of the pathogen tetrahydrofolate pool (Hitchings and Baccanari, 1984).
The use since 1970 of a combination of trimethoprim and sulfa-
methoxazole under the commercial names of Bactrim (Roche) and
Septra or Septrim (Burroughs Wellcome) has been met with remark-
sable success in the prophylaxis and treatment of many bacterial infec-
tions due to its broad spectrum of action and excellent penetration into body tissues and
fluids (Green and Demos, 1984).

In the treatment of malaria, pyrimethamine is used in sequential
combination with quinine sulfate and sulfadiazine. Malaria is caused by four
species of protozoa — *Plasmodium falciparum, Plasmodium vivax,
Plasmodium ovale* and *Plasmodium malariae* — transmitted from the
infected saliva of the female anopheline mosquito. *P. falciparum* is the most
dangerous of the four parasites for humans because it may cause extensive
parasitemia, progressing to massive hemolysis and fatal cerebral and renal
abnormalities (Hughes, 1984). Plasmodial DHFR, the target for
pyrimethamine, exists as a bifunctional enzyme associated with thymidylate
synthetase activity (Blakley, 1984).

In 1948 Farber and collaborators (Farber et al., 1948) reported that
aminopterin (Figure I.2) produced temporary remissions in children with
leukemia. This was "the first demonstration that an antimetabolite could be
an antineoplastic agent" (Johns and Bertino, 1982). Shortly after,
methotrexate was synthesized (Cosulich and Smith, 1948; Seeger et al., 1949),
and its use in the treatment of childhood leukemia was reported (Burchenal
et al., 1949; Goldin et al., 1949). Since then, many other classic and
nonclassical antifolates\(^1\) have been synthesized and tested, but methotrexate, along with 5-fluorouracil which targets thymidylate synthase, is still one of the most commonly used antimetabolites in the management of cancer (Brown and Kaye, 1990). Methotrexate has been widely used in the cure of choriocarcinoma and acute lymphocytic leukemia of childhood, and it is of value alone or in combination with other drugs in the management of non-Hodgkins lymphomas, testicular cancer, medulloblastoma and carcinomas of the neck and head, breast, lung, cervix, ovary and bladder (Maxwell and Eckhardt, 1990). The therapeutic doses of methotrexate administered to cancer patients vary considerably with the type of neoplastic disease. In advanced cancer disease or in the case of tumors with low sensitivity to methotrexate, very high lethal doses of methotrexate may be used. In this case, the patient is given subsequently a rescue agent, commonly 5-formyl-tetrahydrofolate (leucovorin), in order to overcome the cytotoxic effects on the normal tissues. One of the challenges in cancer chemotherapy is to find agents with selectivity stringent enough to discriminate between normal and target cells. Cytostatic agents like methotrexate have marked inhibitory effects not only in cancer cells but also in normal rapidly replicating cells like those from bone marrow, gastrointestinal epithelium, germinal epithelium, hair follicles and lymphoid organs (Maxwell and Eckhardt, 1990). Contrary to

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\(^1\) The terms classic and nonclassic antifolates are not precisely defined, but their use is widespread in the literature. Classical antifolates are generally considered those with chemical structures very close to folate, like methotrexate and aminopterin, while nonclassical folates are those with chemical structures significantly different from folate, like trimethoprim and pyrimethamine (see Figure I.2) (Hitchings and Baccanari, 1984).
many other antineoplastic agents, there is no evidence that methotrexate has any mutagenic or carcinogenic effects (Bertino, 1989).

Methotrexate has also been used in the treatment of psoriasis, a chronic disease characterized by dry, scaling papules and plaques occurring in the scalp, elbows, knees, back and buttocks, and caused by an excessive epidermal cell proliferation (Maxwell and Eckhardt, 1990). More recently, low doses of methotrexate (around 10 mg/week) have been used in less classical situations such as the management of severe rheumatoid arthritis (Chassagne, 1990), severe ophthalmic (Hemady, 1991) or dermatologic conditions (Callen, 1990), and experimentally in the non-surgical treatment of ectopic pregnancies (Pansky et al., 1991).

The therapeutic efficiency of pharmacological agents acting on one-carbon metabolism is a result not only of their direct inhibition of specific target enzymes, but also a consequence of the metabolic imbalance created in other steps of the one-carbon metabolism. For example, in MCF-7 human breast tumor cells treated with methotrexate, the pentaglutamate form of dihydrofolate accumulates and inhibits the 5-amino-4-imidazole-carboxamide ribonucleotide transformylase, which catalyzes the formation of IMP, with concomitant inhibition of the de novo biosynthesis of purines (Allegra et al., 1987). Thus, in these cells methotrexate not only inhibits the synthesis of deoxythymidylate but also the synthesis of purine nucleotides. And in fact, today's view of the mechanism of action of methotrexate in animal cells encompasses several components: i) inhibition of DHFR by methotrexate; ii) inhibition of DHFR, thymidylate synthase and aminoimidazole carboxamide ribonucleotide (AICAR) transformylase by methotrexate polyglutamates; iii) inhibition of thymidylate synthase and AICAR transformylase by
dihydrofolate polyglutamates, which accumulate due to DHFR inhibition, and iv) inhibition of thymidylate synthase and glycinamide ribonucleotide (GAR) transformylase by 10-formyl-dihydrofolate polyglutamate (Knight et al., 1989).

One frequent and frustrating problem in cancer chemotherapy is the occurrence of cellular drug resistance. This resistance appears to show two main clinical forms: intrinsic drug resistance, which relates to those cancers that respond infrequently to initial treatment with cytotoxic drugs; and acquired drug resistance, which is confined to those cancers that do respond to initial treatment with cytotoxic drugs, but later regrow even in the presence of the cytotoxic drugs (Brown and Kaye, 1990). Resistance to methotrexate has been studied in vitro in mammalian tumor cells, and several mechanisms of resistance have been identified. Low levels of polyglutamylation of methotrexate have been associated with intrinsic resistance and resistance to high pulse doses of the drug; increased levels of DHFR, due to gene amplification or an impaired transport mechanism have been associated with resistance after exposure of the tumor cells to low continuous doses of methotrexate; and, even though relatively uncommon, mutations in the DHFR gene resulting in an enzyme with decreased affinity for the drug have also been observed (Bertino, 1989). Alternatively, cells in tissue culture may show multi-drug resistance. This phenomenon of broad resistance appears to be associated with overexpression of a plasma membrane-associated glycoprotein, P-glycoprotein, which acts as an energy-dependent drug efflux pump and leads to a lower intracellular concentration of the drug (Brown and Kaye, 1990).
Bacterial resistance to antibiotics is also a major medical problem. Bacteria have developed resistance to each new antibiotic used to date (Dever and Dermody, 1991). The use of antibiotics kills thousands or millions of nonpathogenic bacteria for each pathogenic bacteria killed. By killing the susceptible organisms an antibiotic selects for overgrowth rare bacterial strains that carry genes conferring some level of resistance to the antibiotic. Overgrowth then increases the chance for proliferation and maintenance of the gene in the bacterial population and for transmission to other bacterial strains and species (O'Brien, 1987).

Acquired resistance to antibiotics is the result of three major mechanisms: i) enzymatic degradation of the drugs; ii) alteration of the bacterial proteins that are the targets for the antibiotic; iii) alterations in the transport properties or membrane permeability to the antibiotic (Dever and Dermody, 1991). The principal cause of trimethoprim resistance is the production of altered plasmid-encoded DHFRs, some of them residing in transposons, which have a markedly reduced affinity for trimethoprim (Neu, 1989). Additionally, trimethoprim resistance may result from overproduction of the normal chromosomal DHFR, production of a chromosomal DHFR with decreased sensitivity for trimethoprim, decreased cell wall permeability to trimethoprim, or even the existence of alternative metabolic pathways (Huovinen, 1987).

Plasmid-mediated trimethoprim resistance is wide-spread around the world and is a significant medical problem specially in developing countries, most probably due to the less restricted use of trimethoprim in these countries. In industrialized countries trimethoprim resistance tends to be a
problem in hospitals and especially in geriatric units where long-term treatment of urinary tract infections is often associated with trimethoprim resistance. Combinations of trimethoprim with other antimicrobial agents, like sulphonamide, does not prevent the development of trimethoprim resistance. The best method to prevent the spread of resistance is the controlled use of trimethoprim and other antimicrobial agents (Huovinen, 1987).

Resistance to pyrimethamine has been identified in plasmodia, the malaria causing parasites. In the human parasite Plasmodium falciparum, resistance has been shown to be due to production of a mutated DHFR or increased levels of normal DHFR. In cell culture, resistance to pyrimethamine due to altered permeability of the cells has also been identified (Chen et al., 1987).


Dihydrofolate reductase catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate:

\[
\text{NADPH} + \text{H}^+ + 7,8\text{-dihydrofolate} \rightleftharpoons \text{NADP}^+ + 5,6,7,8\text{-tetrahydrofolate} \quad (I.1)
\]
DHFR has been the subject of extensive studies due to its critical role in cellular metabolism and its importance as a pharmacological target. Chromosomal DHFRs from different sources have been shown to have varied biochemical properties. For instances, the enzyme isolated from *E. coli, L. casei, S. faecium* (isoenzyme 2), chicken liver, rabbit brain and human placenta, not only catalyzes the reduction of dihydrofolate but also, at a much slower rate, the reduction of folate to tetrahydrofolate (Blakley, 1984):

\[
2\text{NADPH} + 2\text{H}^+ + \text{folate} \rightleftharpoons 2\text{NADP}^+ + 5,6,7,8\text{-tetrahydrofolate}
\]

However, folate is not a substrate for the plasmid-encoded type II DHFRs (Amyes and Smith 1976; 1978), *S. faecium* isoenzyme 1, and DHFRs from soybean and pea seedlings (Blakley, 1984).

For all known DHFRs, NADPH is the preferred coenzyme. However, some DHFRs can also use NADH, albeit with a less favorable \( K_M \) and with a \( V_{\text{max}} \) in most cases 10 to 30% that for the reaction with NADPH (Blakley, 1984). The stereochemistry for hydride transfer conforms to a type-A dehydrogenase. The hydride transfer takes place from the A-side (4-pro-R hydrogen) of the reduced nicotinamide ring to the same face of the pteridine ring in both the reduction of folate (the *re* face at C6) and dihydrofolate (the *si* face at C7) (Charlton et al., 1979, 1985).

The shape of the activity *versus* pH profile for DHFR is dependent on the buffer system, the ionic strength, the presence of additives such as urea and organic mercurials, and the source of enzyme. Therefore widely variable pH optimum values and activity *versus* pH profiles have been published (see review by Blakley, 1984). In a recent publication the activity *versus* pH
profiles for DHFRs from various sources, under identical experimental
conditions, were reported (Beard et al., 1989). In general the pH profiles show
a small variation between pH 6 and 8 and a decrease in activity above pH 8.
Exceptions to this behavior are the DHFRs from two strains of *L. casei*, one
resistant to methotrexate and another resistant to dichloromethotrexate.
Below pH 6 the profiles vary considerably depending on the enzyme source:
for *E. coli* DHFR the activity decreases (in fact decreases below pH 7), but for
chicken liver, bovine liver, L1210 murine leukemia cells, and recombinant
human DHFR the activity increases between pH 6 and pH 5 (Beard et al.,
1989).

Vertebrate and chromosome-encoded bacterial DHFRs are monomeric
proteins with molecular weights varying between 18 and 22 kDa (Blakley,
1984). Plasmid-encoded bacterial DHFRs show a wider distribution of
molecular weights and quaternary structures, and are discussed in more detail
in the next section. DHFR from T4 bacteriophage is a dimer with a molecular
weight of about 44.5 kDa (Purohit et al., 1981). DHFRs isolated from soybean
seedlings (Reddy and Rao, 1976) and carrot cells (Albani et al., 1985) were
shown to be trimers with a total molecular weight of 140 and 183 kDa,
respectively. DHFR activity in several protozoa is associated with a
bifunctional enzyme which also has thymidylate synthetase activity (Blakley,
1984).

Blakley (1984) reviewed and tabulated Michaelis constants for a series
of DHFRs from bacterial, protozoal, plant, avian and mammalian sources.
Most \( K_M \) values for dihydrofolate vary between 0.05 and 10 \( \mu M \) and for
NADPH between 0.5 and 450 \( \mu M \). However, no general comparisons between
DHFRs from different sources can be safely made because most of the reported values are apparent Michaelis constants (Blakley, 1984).

The equilibrium constant for the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate (equation I.1) has been determined in the presence of DHFRs from various sources. The equilibrium is highly shifted towards the formation of tetrahydrofolate. The various reported values of the overall equilibrium constant for the reaction (equation I.1), at pH 8, agree reasonably well with each other and they are: $5.6 \times 10^{11}$ M$^{-1}$ (Mathews and Huennekens, 1965), $8.4 \times 10^{10}$ M$^{-1}$ (Nixon and Blakley, 1968) and $1.3 \times 10^{11}$ M$^{-1}$ (Fierke et al., 1987) in the presence of DHFR from bacterial sources; and $1.4 \times 10^{11}$ M$^{-1}$ in the presence of recombinant human DHFR (Appleman et al., 1990a).

The binding kinetics of NADPH, H$_2$F, NADP$^+$ and H$_4$F to E. coli and L. casei DHFR, at saturating ligand concentrations, have been studied by quenching of the protein fluorescence, and are described by a sum of two exponentials: a rapid, ligand-concentration-dependent phase followed by a slower ligand-concentration-independent phase (Cayley et al., 1981; Fierke et al., 1987; Andrews et al., 1989). In contrast, in the formation of ternary complexes only a single ligand-concentration-dependent exponential is observed (Cayley et al., 1981; Fierke et al., 1987; Andrews et al., 1989). This behavior was interpreted as the result of a binding mechanism where substrate binds rapidly to the active form of the enzyme which is in a slow interconverting equilibrium with an inactive form of the enzyme (Cayley et al., 1981). The equilibrium between these conformers is pH dependent (Cayley et al., 1981). Alternatively, it has been shown that at higher concentrations of ligands, the slow phase for H$_2$F (Penner and Frieden, 1987)
and NADPH (Appleman et al., 1990b) binding also displays ligand-concentration-dependence, which implies that both \( \text{H}_2\text{F} \) and NADPH can bind to the inactive form of the enzyme. It has also been found that the amount of protein in each one of the two forms is different in the presence of NADPH or \( \text{H}_2\text{F} \) (Penner and Frieden, 1987). Thus, it is not clear if the conformers detected for NADPH, NADP\(^+\), \( \text{H}_2\text{F} \), \( \text{H}_4\text{F} \) as well as inhibitor binding correspond to the same two enzyme forms (Appleman et al., 1990b). Several mutations have been shown to alter the equilibrium interconversion between the two fluorescence-detectable enzyme conformers. While the mutants H45Q and L54G do not perturb the equilibrium, the mutants D27N, T113V, F31V and F31Y change the distribution of the two conformers (Chen et al., 1985; Mayer et al., 1986; Chen et al., 1987; Fierke and Benkovic, 1989; Appleman et al., 1990b). Among these mutants, the mutant D27N has the largest effect on the equilibrium, yet even this represents a free energy change of only 0.9 kcal (Appleman et al., 1990b). The dissociation constants (at pH 7.2 and 20 °C) determined from the rate constants of the fast phase binding are 0.4 \( \mu \text{M} \) for \( \text{H}_2\text{F} \) and 0.17 \( \mu \text{M} \) for NADPH in the binary complexes with DHFR (Table I.1) (Penner and Frieden, 1987). In the ternary complexes these dissociation constants are 0.21 \( \mu \text{M} \) for \( \text{H}_2\text{F} \) and 0.12 \( \mu \text{M} \) for NADPH (Penner and Frieden, 1987). The dissociation constants for the binary complexes obtained by kinetic analysis are somewhat higher than those determined by equilibrium titration experiments (0.23 \( \mu \text{M} \) for \( \text{H}_2\text{F} \) and 0.03 \( \mu \text{M} \) for NADPH; Table I.2) which is a consequence of the presence of multiple enzyme conformers (Penner and Frieden, 1987). Dissociation constants for \( \text{H}_4\text{F} \) and NADP\(^+\) are given in Table I.1 as determined by Penner and Frieden (1987). It should be noted that while the dissociation constants for \( \text{H}_2\text{F} \) and \( \text{H}_4\text{F} \) are of
TABLE I.1: Dissociation constants, Michaelis constants and turnover number for *E. coli* DHFR, at 20 °C and pH 7.2 (from Penner and Frieden, 1987).

<table>
<thead>
<tr>
<th>Constant</th>
<th>Process</th>
<th>Kinetic Value</th>
<th>Thermodynamic Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (s$^{-1}$)</td>
<td></td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>$K_M$ (μM)</td>
<td>$H_2F$</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>$K_M$ (μM)</td>
<td>NADPH</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td>$K_d$ (μM)</td>
<td>$E + \text{NADPH}$</td>
<td>0.17</td>
<td>0.03</td>
</tr>
<tr>
<td>$K_d$ (μM)</td>
<td>$E + H_2F$</td>
<td>0.4</td>
<td>0.23</td>
</tr>
<tr>
<td>$K_d$ (μM)</td>
<td>$E + \text{NADP}^+$</td>
<td>2.3</td>
<td>1.20</td>
</tr>
<tr>
<td>$K_d$ (μM)</td>
<td>$E + H_4F$</td>
<td>0.29</td>
<td>0.2</td>
</tr>
<tr>
<td>$K_d$ (μM)</td>
<td>$E\cdot H_2F + \text{NADPH}$</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>$K_d$ (μM)</td>
<td>$E\cdot \text{NADPH} + H_2F$</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>$K_d$ (μM)</td>
<td>$E\cdot H_4F + \text{NADP}^+$</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>$K_d$ (μM)</td>
<td>$E\cdot \text{NADP}^+ + H_4F$</td>
<td>0.33</td>
<td></td>
</tr>
</tbody>
</table>
**TABLE I.2:** Comparison of dissociation constants in the binary complexes of *E. coli* DHFR at 25 °C and pH 6.0 in MTEN buffer (*) (Fierke et al., 1987), at 20 °C and pH 7.2 in BTDE buffer (*) (Penner and Frieden, 1987), and of recombinant human DHFR at 20 °C and pH 7.65 in MATS buffer (*) (Appleman et al., 1990).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Kinetic Value</th>
<th>Thermodynamic Value</th>
<th>Ligand</th>
<th>Kinetic Value</th>
<th>Thermodynamic Value</th>
<th>Ligand</th>
<th>Kinetic Value</th>
<th>Thermodynamic Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>koff/kon (μM)</td>
<td>Kd (μM)</td>
<td></td>
<td>koff/kon (μM)</td>
<td>Kd (μM)</td>
<td></td>
<td>koff/kon (μM)</td>
<td>Kd (μM)</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.18</td>
<td>0.33</td>
<td></td>
<td>0.17</td>
<td>0.03</td>
<td></td>
<td>0.045</td>
<td>0.05</td>
</tr>
<tr>
<td>H₂F</td>
<td>0.5</td>
<td>0.22</td>
<td></td>
<td>0.4</td>
<td>0.23</td>
<td></td>
<td>0.053</td>
<td>0.12</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>22</td>
<td>24</td>
<td></td>
<td>2.3</td>
<td>1.20</td>
<td></td>
<td>1.9</td>
<td>2.3</td>
</tr>
<tr>
<td>H₄F</td>
<td>0.06</td>
<td>0.1</td>
<td></td>
<td>0.29</td>
<td>0.2</td>
<td></td>
<td>0.044</td>
<td>0.05</td>
</tr>
</tbody>
</table>

(*) BTDE buffer — 50 mM bis-tris, 1 mM dithioerythritol, and 40 μM EDTA
MTEN buffer — 50 mM 2-(N-morpholino)ethanesulfonic acid, 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM ethanolamine, and 100 mM sodium chloride
MATS buffer — 25 mM 4-morpholineethanesulfonic acid (MES), 25 mM acetate, 50 mM tris(hydroxymethyl) aminomethane (Tris), 100 mM sodium chloride, and 0.02% sodium azide.
the same order of magnitude, there is an approximately 40-fold increase in the $K_d$ for NADP$^+$ in comparison to the $K_d$ for NADPH.

Full time course studies of the kinetic activity of *E. coli* DHFR show that there is an increase in enzymatic activity with time (Penner and Frieden, 1985). When the reaction is started by simultaneous addition of $H_2F$ and NADPH to the enzyme, the reaction starts slowly and increases with time before the substrate is depleted. This process has a half-time of about 9 s (Penner and Frieden, 1985). If DHFR is preincubated with either $H_2F$ or NADPH the initial slow reaction rate is abolished and the initial velocity is 2 to 2.3-fold faster than that observed for the non-preincubated enzyme. This process, known as hysteresis, is characteristic of enzymes which, during the enzyme assay, slowly interconvert from one kinetic form to another. One of these conformers (E1) binds ligands more rapidly and tightly than does the other conformer (E2), and as the reaction proceeds there is slow interconversion between E2 and E1, rapid binding of more ligands to E1 with consequent increase in the catalytic activity until the steady-state rate is reached. Thus, it appears that the hysteretic behavior is a direct consequence of the multiple conformers with different catalytic activity observed in the formation of the binary complexes and mentioned in the previous paragraph (Appleman et al., 1990b). The DHFR from *L. casei* and the isoenzyme 2 from *S. faecium* exhibit the same type of hysteresis as the *E. coli* enzyme (Appleman et al., 1989). Baccanari and Joyner (1981) reported another type of hysteretic behavior for isoenzyme I of DHFR from *E. coli* RT500, a trimethoprim resistant strain of *E. coli*. In this case, hysteresis was observed only with $H_2F$-started reactions after preincubation of the enzyme with
NADPH for approximately 1 min. The half-time for the process was 3.5 min (Baccanari and Joyner, 1981).

Kinetic studies with vertebrate DHFRs from several sources — recombinant human DHFR (rhDHFR) and DHFR from chicken, bovine liver and L1210 mouse leukemia cells (mlDHFR) — did not show hysteretic behavior with a half-time of the order of 9 s, as is observed for E. coli DHFR (Appleman et al., 1989). However, both rhDHFR and mlDHFR showed a fast hysteretic behavior. Typically for enzyme-catalyzed reactions, product formation starts at a rapid rate and, after the first turnover, quickly decreases to the constant steady-state rate. For rhDHFR and mlDHFR, after the first very rapid "burst" of product formation there is an intermediate phase during which the reaction velocity decreases exponentially to the final steady-state rate. This intermediate phase has an half-time of 110 ms, and it was interpreted as a very short-lived period of hysteretic behavior (Appleman et al., 1989). The authors suggested that this behavior could result from the existence of two conformers of the enzyme, one of which has a higher turnover number than the other, and with the equilibrium shifting in favor of the less active conformer during the course of the reaction (Appleman et al., 1989). However, the same authors subsequently reported the kinetic scheme for the recombinant human DHFR, which we will discuss later, and concluded that the exponential decrease of the reaction velocity after the initial burst is a consequence of the extended period of time required to reach steady-state among all enzyme complexes involved in the kinetic scheme (Appleman et al., 1990a). Thus, there is no need to invoke the existence of two enzyme conformers to justify the apparent exponential decrease of the reaction rate in the milisecond time scale (Appleman et al., 1990a). The same
authors reported that a hrDHFR mutant where Phe-31 had been replaced by Leu didn't show this fast apparent hysteretic behavior (Appleman et al., 1989).

The steady-state kinetic mechanism for the reduction of 7,8-dihydrofolate by *E. coli* DHFR has been studied and shown to conform to a random mechanism (Stone and Morrison, 1982). The profile for the pH dependence of $V_{\text{max}}$ in steady-state kinetic experiments produced a pKa of 8.4 for the DHFR catalyzed reaction (Stone and Morrison, 1984). These same studies suggested that unprotonated dihydrofolate reacts, at neutral pH, with a protonated form of the enzyme. It was further suggested that the protonated side-chain of Asp-27, the only ionizing group in the active site, was responsible for the observed pH dependence (Stone and Morrison, 1984).

A kinetic scheme for *E. coli* DHFR that predicts steady-state kinetic parameters and full time course kinetics under a variety of substrate concentrations and pHs has been proposed (Fierke et al., 1987; Penner and Frieden, 1987). This scheme was derived from measurement of the various ligand association and dissociation rate constants and pre-steady-state transients, using stopped-flow fluorescence and absorbance spectroscopy (Fierke et al., 1987; Penner and Frieden, 1987). It has also been shown that *L. casei* DHFR follows an identical kinetic scheme, and none of the rate constants vary by more than 40-fold despite there being less than 30% amino acid homology between the two enzymes (Andrews et al., 1989). The proposed kinetic scheme is shown in Figure I.3. The heavy arrows (Figure I.3A) indicate the kinetic pathway for steady-state turnover at saturating substrate conditions. At low pH [i.e. below the pKa of 8.4 determined from the steady-state experiments (Stone and Morrison, 1984)], product dissociation is the rate-limiting step for steady-state turnover, and it follows a pathway in
Figure I.3: Kinetic scheme for *E. coli* DHFR at 25 °C in MTEN buffer (from Fierke et al., 1987). (A) pH-independent kinetic scheme; (B) pH-dependent kinetic scheme. The composition of the MTEN buffer is 50 mM 2-(N-morpholino)ethanesulfonic acid, 25 mM tris(hydroxymethyl)aminomethane, 25 mM ethanolamine, and 100 mM sodium chloride.
which tetrahydrofolate dissociation occurs after NADPH replaces NADP⁺ in the ternary complex. At high pH, the rate-limiting step is the hydride transfer. While the rate constant for tetrahydrofolate dissociation is pH independent, the rate constant for hydride transfer is pH dependent with a pKa of 6.5 and a maximum value of 950 s⁻¹. The pH-dependent switch of the rate-limiting step accounts for the apparent pKa of 8.4 observed in the steady-state experiments, even though a single pKa of 6.5 is observed for the ternary complex DHFR•NADPH•H₂F in the pre-steady-state experiments (Fierke et al., 1987). This pKa of 6.5 is due to the ionization of Asp-27 at the active site of DHFR. The pH-dependent portion of the kinetic scheme is shown in Figure I.3B. A similar pKa of 6.5 is estimated for the free enzyme and for the binary complexes with coenzyme or dihydrofolate since there is little pH dependence on substrate binding (Fierke et al., 1987), which agrees with the pKa of 6.3 for the free enzyme as determined from the binding of 2,4-diamino-6,7-dimethylpteridine (Stone and Morrison, 1983). From the the kinetic pathway for steady-state turnover at saturating substrate conditions (heavy arrows in Figure I.3) and from computer simulation of the full time course of enzyme complexes, it is clear that no appreciable concentration of apoenzyme or enzyme•H₂F complex exists during the normal catalytic cycle (Penner and Frieden, 1987). Thus, the potential multiple enzyme conformers of apoenzyme and binary complexes, to which we made reference in previous paragraphs, may be of little significance to the full time course of the reaction (Penner and Frieden, 1987). The Michaelis constants for NADPH and H₂F, calculated from simulations using the proposed kinetic mechanism, and the turnover number for the enzyme are: \( K_M(\text{NADPH}) = 1.05 \mu M, \ K_M(\text{H}_2\text{F}) = 0.27 \mu M, \ V_{\text{max}} = 14.3 \text{ s}^{-1} \) (Penner and Frieden, 1987). The hydride transfer reaction
is essentially irreversible with a equilibrium constant for hydride transfer of $K_{eq} = 1700$ (Frieken et al., 1987). Penner and Frieden (1987) also reported that simulation of the double-reciprocal plot for initial velocity versus NADPH concentration is nonlinear at concentrations of NADPH lower than 0.5 μM, which appears to be a consequence of the relative magnitude of the dissociation rate constants for $H_4F$ from the DHFR•$H_4F$ and DHFR•NADPH•$H_4F$ complexes.

Recently, the kinetic scheme for recombinant human DHFR (rhDHFR) was reported (Appleman et al., 1990a). The scheme is more complex than the one reported for *E. coli* and *L. casei* DHFR described above because, under saturating substrate concentrations, it involves branched pathways (Figure I.4). The major branch point in the catalytic cycle occurs at DHFR•NADP$^+$•$H_4F$. While the bacterial kinetic scheme defines the dissociation of NADP$^+$ from this ternary complex as the preferential route, the kinetic scheme for recombinant human DHFR allows the dissociation of NADP$^+$ or the dissociation of $H_4F$ from the ternary complex as plausible routes (compare Figures I.3A and I.4). For rhDHFR the rates of dissociation of the two products from the ternary complex are similar ($k_{off}$ (NADP$^+$) = 84 s$^{-1}$ and $k_{off}$ ($H_4F$) = 46 s$^{-1}$), and after conversion of the enzyme-bound substrates to products, 65% of the enzyme is converted to DHFR•$H_4F$ and the remaining 35% becomes DHFR•NADP$^+$. Thus, for rhDHFR both pathways of product release and subsequent substrate binding contribute significantly to transient and steady-state kinetics (Appleman et al., 1990a). These two alternate pathways are shown in Figure I.4 in bold arrows: the bold solid arrows representing the pathway equivalent to the major catalytic route in the bacterial kinetic scheme; the bold dashed arrows representing the alternate
Figure I.4: Kinetic scheme for human recombinant DHFR at 20 °C in MATS buffer, pH 7.65 (from Appleman et al., 1990). The composition of the MATS buffer is 25 mM 2-(N-morpholino)ethanesulfonic acid, 25 mM acetic acid, 50 mM tris(hydroxymethyl)aminomethane, 100 mM sodium chloride, 0.02% sodium azide.
pathway. If NADP\(^+\) dissociates first from the DHFR•NADP\(^+\)•H\(_2\)F complex (bold solid arrows), since release of H\(_2\)F is much faster when NADPH is bound to the enzyme, the flux through this branch of the kinetic scheme is greatest at high NADPH concentrations and H\(_2\)F concentrations sufficiently high that H\(_2\)F binding to the E•NADPH complex does not become rate limiting. Alternatively, if H\(_4\)F dissociates first from the DHFR•NADP\(^+\)•H\(_4\)F complex, the binary complex DHFR•NADP\(^+\) can follow two routes depending on the concentration of H\(_2\)F. At high H\(_2\)F concentration, H\(_2\)F binds to DHFR•NADP\(^+\) to form the ternary complex, but subsequent release of NADP\(^+\) is slow and limits cycling through this pathway. At low concentrations of H\(_2\)F, NADP\(^+\) dissociates from the DHFR•NADP\(^+\) complex and flux through this pathway is potentially faster than through the DHFR•NADP\(^+\)•H\(_2\)F complex. At very low concentrations of H\(_2\)F, binding of H\(_2\)F is rate limiting in the formation of the DHFR•NADPH•H\(_2\)F complex. At physiological concentrations of substrates and products\(^2\) the steady-state rate is limited primarily by the rate of H\(_2\)F binding to the DHFR•NADPH complex (Appleman et al., 1990a). The rate constants for all the steps are shown in Figure I.4 and can be compared with those for *E. coli* DHFR in Figure I.3A.

The rate for hydride transfer has been determined under the same conditions for DHFRs of various origins, and it varies considerably from a low 7 s\(^{-1}\) for DHFR from a methotrexate-resistant strain of *L. casei*, to 3000 s\(^{-1}\)

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\(^2\) In eukaryotic cells of mammalian origin the concentrations of substrates and products are assumed to be: NADPH 2000 \(\mu\)M, H\(_2\)F 0.1 \(\mu\)M, NADP\(^+\) 20 \(\mu\)M, and H\(_4\)F 1 \(\mu\)M (Greenbaum et al., 1971; Allegra et al., 1986; Appleman et al., 1990a).
for recombinant human DHFR (Beard et al., 1989). It is not clear what structural factors determine such different rates of catalysis. It has been suggested that very small changes in the distance between C-4 of the nicotinamide ring of NADPH and C-6 of dihydrofolate in the ternary substrate complex, as well as small differences in the orientation of the nicotinamide or pteridine rings could justify such differences in the rates of hydride transfer (Benkovic et al., 1988). In a series of mutants designed to test the role of several hydrophobic residues that line the active site of DHFR, Benkovic and collaborators (1988) showed that mutants like L54G even more than 10 Å away from the site of hydride transfer, perturb the rate of this chemical step. Even more interesting is the correlation in this series of mutants between the decrease of the rate of hydride transfer and the increase in the dissociation constant for dihydrofolate. This correlation is consistent with the hypothesis that in DHFR the active-site residues create a specific surface that position the hydride donor and acceptor at distances and orientations optimal for catalysis to occur (Benkovic et al., 1988).

A constant point of attention in the literature discussing the mechanism of action of DHFR has been the protonation state of the substrate, and what is the role of the enzyme in stabilizing this protonation state. Huennekens and Scrimgeour (1964) proposed that protonation of N8 of folate and N5 of 7,8-dihydrofolate would allow the reduction of folate to 7,8-

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3 Note that this value of 3000 s⁻¹ is not equal to the value reported by the same group (Appleman et al., 1990a) in the kinetic scheme for recombinant human DHFR under the same experimental conditions and shown in Figure I.4. This later value of 1360 s⁻¹ apparently refers to a combination of the hydride transfer step and a isomerization step of the ternary substrate complex.
dihydrofolate and the reduction of 7,8-dihydrofolate to tetrahydrofolate, respectively. Protonation at N8 or N5 leads to a transient displacement of electrons toward these nitrogen atoms resulting in a partial positive charge at C7 and C6 respectively, which in turn facilitates the nucleophilic addition of a hydride ion to carbons C7 and C6, i.e. the reduction of the -C=Н- bonds (Huennekens and Scrimgeour, 1964; Scrimgeour, 1975). Ab initio quantum-mechanical calculations confirmed that protonation at N8 and N5 is a plausible mechanism to enhance carbonium ion character at C7 and C6, respectively (Gready, 1985). The pKa of N5 in free dihydrofolate is 3.84 (Poe, 1977)(4). Thus, the enzyme at physiological pH has to provide an environment that increases the effective pKa of N5. There are no ionizable groups in the active site of the enzyme in a position to transfer a proton directly to N5 of folate (Filman et al., 1982; Bystroff et al., 1990). As we mentioned above, studies of the effects of pH on the magnitude of various kinetic parameters showed that unprotonated dihydrofolate reacts with a protonated form of the enzyme, and it was suggested that it is the protonated carboxyl of Asp-27, the only ionizing residue in the pteridine binding site of DHFR, which is somehow responsible for the protonation of the N5 nitrogen of dihydrofolate (Stone and Morrison, 1984). Mutation of Asp-27 to Asn and Ser decreased DHFR activity (kcat) at pH 7.0 by 300 and 68 fold respectively, and increased the Michaelis constant (Km) for dihydrofolate by 37 and 117 fold, respectively (Howell et al., 1986). The pH profiles of the kinetic parameters for the two mutant enzymes were very different from those of the

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4 Recently a new value for the pKa of N5 of dihydrofolate equal to 2.59 has been reported by Maharaj et al. (1990). This new pKa will give different values for some kinetic constants which were calculated based on Poe's pKa.
wild type enzyme. In both cases \( k_{\text{cat}} \) increased rapidly with decreasing pH. This observation shows that disabling the catalytic-site proton relay in DHFR by mutation of Asp-27 substantially decreases the enzyme's activity at pH 7, but the mutant enzymes can function at full catalytic efficiency by utilizing preprotonated substrate from solution at sufficiently low pH (Howell et al., 1986). Based on these observations and the crystal structure of the ternary complex of \textit{E. coli} DHFR with NADP\(^+\) and folate, which is discussed in the next section, a mechanism for protonation of N5 of 7,8-dihydrofolate has been proposed (Bystroff et al., 1990). In a first step, the carboxylic acid side-chain of Asp-27 promotes enolization of the pyrimidine ring by relaying a proton from N3 to O4 via a fixed crystallographic water molecule. Next it was postulated that a temporarily bound water molecule (not seen in the crystal structure) hydrogen bonds simultaneously to both O4 and N5. This water molecule protonates N5 and the substrate returns to the keto form. Finally, the movement of the hydrophobic side-chain of Met-20 towards N5 displaces the temporarily bound water molecule and favors the resonance form with enhanced carbonium ion character at C6 (\( \text{--HN}^+\text{C}-- \) \( \text{<-->} \) \( \text{--HN}\text{C}^+-- \)) which is also facilitated by the presence of the carbonyl oxygen of Ile-94 near C6 (Bystroff et al., 1990). Still this mechanism does not account for protonation of N8 and reduction of folate to 7,8-dihydrofolate, and we should point out that a mechanism involving protonation at O4 instead of N8 and N5 can not be ruled out (Gready, 1985). It was shown by \textit{ab initio} quantum-mechanical calculations that, even though less effectively than protonation at N8 and N5, protonation at O4 could facilitate reduction of both double bonds -N8=C7- and -N5=C6- (Gready, 1985). It is also interesting to note that NMR studies of binary complexes of bovine DHFR with folate, dihydrofolate,
dihydropteroylpentaglutamate and dihydrobiopterin labeled with $^{15}\text{N}$ at N5 and $^{13}\text{C}$ at C6 indicate that there is no protonation at N5 in these binary complexes. However these studies are consistent with deprotonation or at least partial loss of the proton at N3, as expected from the interaction of this nitrogen with the carboxyl side-chain of Glu-30 (Asp-27 in E. coli) (Selinsky et al., 1990).

3.2. Protein Structure and Ligand Binding.

The primary sequence of vertebrate DHFRs is highly conserved. In contrast, chromosome-encoded bacterial DHFRs have a smaller degree of homology. For example between E. coli and L. casei DHFR there is less than 30% homology (Blakley, 1984; Kraut and Matthews, 1987). Although vertebrate DHFRs are 25 to 30 residues longer than chromosome-encoded bacterial DHFRs and they show little sequence identity, comparison of the crystal structures of E. coli, L. casei, chicken and human DHFR shows a very similar overall backbone folding for all these proteins (Kraut and Matthews, 1987; Bolin et al., 1982; Volz et al., 1982; Oefner et al., 1988). The central feature of these structures is a twisted eight-stranded $\beta$-sheet consisting of seven parallel strands and a single antiparallel strand leading to the carboxyl terminus. The eight strands are designated $\beta A$ to $\beta H$, $\beta A$ being nearest the amino terminus and the $\beta H$ nearest the carboxyl terminus. Four $\alpha$-helices pack against the central $\beta$-sheet, and are designated $\alpha B$, $\alpha C$, $\alpha E$ and $\alpha F$, the letter designating the $\beta$-strand that follows the $\alpha$-helix in the primary sequence. The remaining residues lie within the loops connecting $\beta$-strands
and α-helices (Kraut and Matthews, 1987; Bolin et al., 1982; Volz et al., 1982; Oefner et al., 1988). Comparison of the crystal structures of chicken and bacterial DHFRs show that the extra amino acids in chicken DHFR are inserted mostly in loop regions (Volz et al., 1982). Chicken DHFR has an extra α-helix, αE', and the lengths of α-helices B, E and F are extended in comparison with the corresponding helices in bacterial DHFRs. The most unusual insertion in chicken DHFR occurs in the long β-strand βG causing a major disruption of the antiparallel interchain hydrogen bonding between βG and βH. However, this insertion does not significantly alter the overall tertiary structure of the enzyme (Volz et al., 1982; Kraut and Matthews, 1987). The relative position of secondary structural elements between chicken and bacterial DHFRs differ by 1 to 3 Å in the neighborhood of the substrate binding site, and most of the extra residues in the vertebrate enzyme are located more than 12 Å from the substrate binding site (Kraut and Matthews, 1987).

Recently three new crystal structures of E. coli DHFR in different liganded states have been reported (Bystroff et al., 1990; Bystroff and Kraut, 1991) and compared to the previously published crystal structure of E. coli DHFR with bound methotrexate (Bolin et al., 1982; Filman et al., 1982). This allowed the detailed study of the conformational changes induced by the binding of NADP⁺, folate and methotrexate to E. coli DHFR. The apo form of the enzyme shows two disordered backbone regions: residues 16 to 20 and residues 95 and 96 (Bystroff and Kraut, 1991). Residues 16 to 20, which belong to the Met-20 loop, are also disordered in the crystal structure of the holoenzyme but could be modeled as a type I β-turn in the ternary complex of the holoenzyme with folate (Bystroff et al., 1990). Glycines 95 and 96 form a
cis peptide bond in the crystal structures of the binary complexes of *E. coli* DHFR with methotrexate (Bolin et al., 1982), *E. coli* DHFR with NADP+ (Bystroff et al., 1990), and the ternary complex of *L. casei* DHFR with methotrexate and NADPH (Bolin et al., 1982). This Gly-Gly dipeptide is also observed in vertebrate DHFRs (Volz et al., 1982). In the apo form of *E. coli* DHFR, the backbone atoms of Gly-95 and Gly-96 produced electron density maps which were interpreted as an equilibrium between cis and trans conformations of the peptide bond (Bystroff and Kraut, 1991). This conformational equilibrium could account for the slow interconversion of the unliganded enzyme between an active form and an inactive form that does not bind NADPH or dihydrofolate (Bystroff and Kraut, 1991).

The structure of *E. coli* DHFR can be viewed as composed of two domains, and some of the conformational changes observed upon ligand binding can be described as a hinge motion of one domain relative to the other (Bystroff and Kraut, 1991). Residues 1 to 37 and 89 to 159 constitute the "major domain" and residues 38 to 88 constitute the smaller "adenosine binding domain". The major domain contains β-strands G, H, F, A and E and α-helices B and F. Most of the major domain exhibits little variance between the different liganded forms of the enzyme. The adenosine binding domain contains β-strands B, C and D and α-helices C and E. The coenzyme and the substrate or the inhibitor bind in the crevices between the two domains, except for the adenosine moiety which binds exclusively to the adenosine binding domain. There are two short backbone-chain connections between the adenosine binding domain and the major domain: αB-βB and αE-βE. The hinge motion between domains observed from comparison of the different liganded forms of the enzyme is restricted to a rotation of one domain about
an axis approximately halfway between β-strands B and E. The range of rotation is about 6°, with the extreme open conformation occurring in the apoenzyme and the extreme closed conformation in the methotrexate binary complex. No *E. coli* DHFR•NADP•methotrexate ternary complex has yet been analyzed by X-ray crystallography. The holoenzyme and the DHFR•NADP•folate complex showed an intermediate range of domain separation. The primary effect of the domain rotation from the apoenzyme to each one of the liganded complexes is to close the hydrophobic cleft between helices B and C where the p-aminobenzoylglutamate moiety of the substrate binds. Additionally, the domain rotation shrinks the pyrophosphate binding site, which lies between the N-termini of helices C and F, and opens slightly the hydrophobic adenine binding pocket. The pyrophosphate group of the coenzyme and the p-aminobenzoylglutamate moiety of the substrate appear to play the most important roles in inducing the domain rotation (Bystroff and Kraut, 1991).

Comparison between the different liganded forms of *E. coli* DHFR allows identification of a second mobile region of the molecule (Bystroff and Kraut, 1991). It comprises a shift of helix B and reordering of three loops: the Met-20 loop (residues 9 to 23) connecting βA and αB, the loop connecting βF and βG (residues 117 to 131) and the loop connecting βG and βH (residues 142 to 149). This loop region serves as a lid closing over the bound ligands in the ternary complex but remaining mobile in the apoenzyme and in the holoenzyme. The Met-20 loop folds over the binding sites for the NMN and pteridine moieties, and the loops βF-βG and βG-βH respond to the more compact folding of the Met-20 loop by collapsing toward it (Bystroff and Kraut, 1991).
NADP⁺ binds to *E. coli* DHFR (Bystroff et al., 1990) in a mode similar to that observed in *L. casei* DHFR (Filman et al., 1982). The coenzyme binds in an extended conformation, with a distance of about 17 Å between the nicotinamide and the adenine rings (Filman et al., 1982). NADP⁺ occupies a long cleft on the enzyme surface that stretches across five strands of β-sheet (Filman et al., 1982). The nicotinamide and the adenine rings bind at opposite sides of the β-sheet, with the pyrophosphate binding site at the crossing point (Kraut and Matthews, 1987). Qualitatively, the coenzyme binding mode conforms with the geometry generally observed for dehydrogenases. However, the relative position of the α-helices and β-strands forming the coenzyme binding domain in DHFR varies considerably when compared with that in the general coenzyme binding domain of the dehydrogenases (Kraut and Matthews, 1987).

The common structural feature among dehydrogenases is a central parallel β-sheet composed of six β-strands and four helices, two on each side of the β-sheet (Rossmann et al., 1975). This structural motif forms the coenzyme binding domain and is termed the "dinucleotide fold" or "Rossmann fold". The amino end of this structural motif is at the middle of the β-sheet. Starting from the middle of the β-sheet, the polypeptide chain forms three β-strands interconnected by two α-helices — βA-αB-βB-αC-βC —, and then passes back to the center of the sheet to form another sequence of three β-strands and two α-helices — βD-αE-βE-αF-βF. In the molecular structure, βA and βD are adjacent strands and helices αB and αC are in the opposite side of the sheet from helices αE and αF. Each one of the β-α-β-α sequences form a mononucleotide binding unit, and the two units are related to each other by an approximate two-fold axis of symmetry running parallel
to the strands, between βA and βD (Rossmann et al., 1975). The dinucleotide binds with the nicotinamide and the adenine rings situated on opposite sides of the β-sheet and with the crossover occurring at the pyrophosphate-binding site. This binding geometry leaves the pyrophosphate group of the coenzyme close to the amino end of the nearby α-helices, leading to stabilization of the negatively charged oxygens by the α-helix dipoles (Kraut and Matthews, 1987).

In *E. coli* DHFR, the pyrophosphate of the coenzyme contributes most of the energetically favorable binding interactions with the enzyme. Ten hydrogen-bonds, one ionic interaction and two helix dipoles stabilize the bound pyrophosphate (Bystroff et al., 1990). The 2'-phosphate of the ADP moiety makes also several hydrogen bonds. Especially important is Arg-44, a conserved basic side chain among DHFRs and NADPH-utilizing dehydrogenases, which forms an ion pair and a hydrogen bond with the 2'-phosphate (Bystroff et al., 1990). NADH-utilizing dehydrogenases have an aspartate at this position (Eklund and Brändén, 1987). Feeney and collaborators using 31P-NMR demonstrated that the 2'-phosphate of NADPH and NADP⁺ binds to *L. casei* DHFR in the dianionic form (Feeney et al., 1975). Thus the positively charged guanidinium group of Arg-44 in *E. coli* (Arg-43 in *L. casei*) must play a major role in the stabilization of the double charged phosphate group (Kraut and Matthews, 1987). The interactions with the adenine ring are less specific and mostly hydrophobic in nature (Kraut and Matthews, 1987).

The crystal structure of the binary complex of *E. coli* DHFR with NADP⁺ (Bystroff et al., 1990) shows the absence of continuous electron density corresponding to the NMN moiety of NADP⁺. This particular feature of the electron density maps was interpreted as suggestive of a
conformational transition between two positions of the NMN moiety involving a rotation of about 120° between the bridging pyrophosphate oxygen and the nicotinamide phosphorous, which would allow the NMN moiety to rotate out of its binding pocket toward the solvent (Bystroff et al., 1990). Interestingly, ¹H, ¹³C and ³¹P NMR studies with *L. casei* DHFR showed that while only one conformation was identified in the binary complex with NADP⁺, two conformations of the NMN moiety could be identified for bound NADP⁺ in the ternary complex with trimethoprim (Gronenborn et al., 1981; Birdsall et al., 1984). While in conformation I, the nicotinamide ring interacts with the enzyme in a fashion very similar to that in the binary complex DHFR•NADP⁺ and in the ternary complex DHFR•NADP⁺•methotrexate; in conformation II the nicotinamide moiety of the bound NADP⁺ swings away from the enzyme into the solvent (Birdsall et al., 1984).

The nicotinamide binding pocket does not contain negatively charged nor aromatic side chains to specifically interact with the positive charge of the aromatic oxidized nicotinamide, which could justify why the enzyme binds NADPH about 100 times tighter than NADP⁺ (Bystroff et al., 1990). In the ternary complex of *E. coli* DHFR with NADP and folate (Bystroff et al., 1990), and the ternary complex of *L. casei* DHFR with NADP and methotrexate (Filman et al., 1982), the nicotinamide binding pocket has three highly conserved residues which position a main chain or a side chain oxygen atom around the perimeter and nearly coplanar with the pyridine ring, at close distance from the ring carbons C2, C4 and C6. These partial negative charges around the pyridine ring could stabilize resonance forms with partial positive
charges on the C2, C4 and C6 atoms of the ring in the enzymic transition state (Filman et al., 1982).

The carboxamide group of the nicotinamide is bound to the enzyme with its oxygen cis to C4, while in solution the lowest energy conformation for the carboxamide has the oxygen trans to C4 of the pyridine ring (Bystroff et al., 1990). The nicotinamide group of bound NADPH is not measurably distorted from planarity (Filman et al., 1982). The planarity of both the oxidized and reduced nicotinamide group has also been observed in the crystal structures of N1-substitute nicotinamides (Karle, 1961), in the crystal structures of the coenzyme bound to several dehydrogenases (Eklund and Brändén, 1987) and in ab initio calculations with pyridine and dihydropyridine (Raber and Rodriguez, 1985).

The conformation of bound NADP+ in E. coli DHFR (Bystroff et al., 1990) and in L. casei DHFR (Filman et al., 1982) is similar. The adenosine ribose and the nicotinamide ribose conformations are C3'-endo (3E) and C2'-endo (2E), respectively. These are the most common ribose conformations in enzyme-bound NAD(P) molecules (Eklund and Brändén, 1987). The adenine and nicotinamide glycosidic torsional angles are both anti. The torsion angles \( \phi \) and \( \psi(5) \), which define the geometry of the pyrophosphate bridge, are such that the pyrophosphate oxygens are arranged in an eclipsed conformation with each one of the ribose 5'-oxygen nearly trans to one another (Filman et al., 1982). The torsion angle \( \theta(5) \) about the C5'-O5' bond of the AMN and

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5 The torsion angles \( \phi, \psi \) and \( \theta \) for the NMN and AMN moieties of NADP+ are defined by the following atoms: 
- \( \phi \rightarrow \text{C4'-C5'-O5'-P} \)
- \( \psi \rightarrow \text{C5'-O5'-P-O3P} \)
- \( \theta \rightarrow \text{C4'-C5'-O5'-P} \)
NMN portions of the coenzyme have values of $\theta_{AMN} = -163^\circ$ and $\theta_{NMN} = 129^\circ$. The value for $\theta_{AMN}$ is within the minimum energy value of $180 \pm 22^\circ$, but the value for $\theta_{NMN}$ deviates significantly from this value (Filman et al., 1982). This difference in the values of the torsion angles $\theta$ was also observed for binary and ternary complexes of *L. casei* DHFR in solution, using estimates of the three-bond $^{31}\text{P}$$\text{H}$ coupling constants in $^{31}\text{P}$-NMR spectra (Feeney et al., 1975; Hyde et al., 1980).

Folate binds to DHFR in a deep hydrophobic cleft bisecting the DHFR molecule and containing a single polar residue, Asp-27 (*E. coli* numbering) (Bystroff et al., 1990). This residue is also conserved in *L. casei* DHFR (Asp-26) and in vertebrate DHFRs is replaced by glutamate, Glu-30 (Kraut and Matthews, 1987). Both folate and methotrexate bind to DHFR in a somewhat bent conformation with the pteridine ring nearly perpendicular to the $p$-aminobenzoyl group (Kraut and Matthews, 1987; Bystroff et al., 1990). In the ternary complex of *E. coli* DHFR with folate, the angle between the planes of the pteridine ring and the $p$-aminobenzoyl ring of folate is $75^\circ$ (Bystroff et al., 1990). In contrast, in solution (Lam and Kotowycz, 1972) and in the crystal structure (Mastropaolo et al., 1980) folic acid by itself assumes an extended conformation, with an angle between the planes of the pteridine ring and the $p$-aminobenzoyl ring close to $180^\circ$. The C4-O4 bond length in the crystal structure of free folate demonstrates that the keto-enol equilibrium at N3-C4-O4 strongly favors the keto form of folate (Mastropaolo et al., 1980).

In the crystal structures of bacterial DHFRs the pyrimidine portion of folate is deeply buried in the hydrophobic pocket where it interacts with Asp-27. The carboxyl side-chain of Asp-27 makes two hydrogen bonds with N3 and the amino group at C2 of the pteridine. The $p$-aminobenzoylglutamate
moiety leads away from the interior of the protein to the surface, where its α-carboxylate interacts with the guanidinium side-chain of an invariant arginine residue, Arg-57 in both *E. coli* and *L. casei* (Kraut and Matthews, 1987; Bystroff et al., 1990). When compared with the 2,4-diaminopteridine ring of methotrexate (Bolin et al., 1982), the 2-amino-4-oxopteridine ring of folate is rotated roughly 180° about an axis approximately collinear with a line through N5 and the amino nitrogen at C2 (Figure I.2; Figure I.5). The same type of folate orientation has also been observed in the binary complex of human DHFR with folate (Oefner et al., 1988). Close comparison of the crystal structures of the ternary complex of *E. coli* DHFR with coenzyme and folate (Bystroff et al., 1990) and the binary complex of *E. coli* DHFR with methotrexate (Bolin et al., 1982) also shows that the pteridine ring of folate is twisted about 7° counterclockwise about a perpendicular axis through N3 and is rotated approximately 15° out of the plane of the methotrexate pteridine about an axis approximately collinear with the C4-C4a bond. With this folate orientation Asp-27 hydrogen bonds N3 and the amino group at C2 of folate, rather than N1 and the amino group at C2 as it happens in methotrexate. The rotation of the pteridine ring changes the position of C6 and consequently the position of the *p*-aminobenzoylglutamate moiety (Bystroff et al., 1990). In the crystal structure of the ternary complex of *E. coli* DHFR with NADP⁺ and folate, there is no evidence that the pteridine ring of folate binds in a manner similar to that of methotrexate. A similar conclusion was obtained in solution for the binary complex of *E. coli* DHFR with folate, where only a single conformation of the complex was detected by NMR (Falzone et al., 1990). In this study, observed NOEs between the C7H of folate and the γ-methyl protons of Ile-5 and the CβH of Ile-94 were consistent with the
Figure I.5: Comparison between folate and methotrexate binding in chromosomal DHFRs. The coordinates for folate and methotrexate were obtained from the crystal structures of the ternary complexes *E. coli DHFR* • *NADP*⁺ • folate (Bystroff et al., 1990) and *L. casei DHFR* • *NADPH* • methotrexate (Filman et al., 1982), respectively.
orientation of the pteridine ring of folate observed in the crystal structure. However, NMR studies of the ternary complex of *L. casei* DHFR with NADP$^+$ and folate show an equilibrium between three slowly interconverting conformations of the complex whose proportions are pH dependent (Birdsall et al., 1982; Birdsall et al., 1989). In conformations I and IIa the pteridine ring of folate seams to bind in a manner similar to that of the methotrexate pteridine. In conformation IIb the NMR observations are consistent with an orientation of the pteridine ring of folate rotated by about 180° from the orientation of the methotrexate pteridine (Birdsall et al., 1989). This result would imply that while folate can bind to *L. casei* DHFR in a "productive" mode (conformation IIb) and in a "non-productive" mode (conformations I and IIa), methotrexate can bind essentially only in one mode corresponding to the "non-productive" one (Birdsall et al., 1989). Therefore, despite the similarities of the global fold and architecture of the binding site between *E. coli* and *L. casei* DHFR, it is apparent that at least in solution there are differences in the number and type of ligand conformational states that each one of these enzymes is able to stabilize. NMR studies of binary complexes of bovine DHFR with folate, dihydrofolate, dihydropteroylpentaglutamate and dihydrobiopterin revealed the presence of only one conformation for each one of these binary complexes in solution (Selinsky et al., 1990).

Based on the crystal structure of the ternary complex of *E. coli* DHFR with NADP$^+$ and folate, hypothetical models for the ternary complex with NADPH and dihydrofolate and for the transition-state ternary complex have been proposed (Bystroff et al., 1990). To model dihydrofolate from folate, hydrogen atoms were attached to C7 and N8 of folate. A hydrogen bond was modeled between N8 and the carbonyl oxygen of Ile-5, which led to a
clockwise 7° in-plane rotation of the pteridine ring. This rotation positioned
the C6 of dihydrofolate close to the ideal geometrical relationship with the
nicotinamide C4 for hydride transfer. This rotation also allowed the distorted
hydrogen bonds between the pteridine ring and Asp-27, observed in the
ternary complex of DHFR with NADP+ and folate, to approach their normal
geometry. However this rotation of the rigidly planar pteridine ring would
put C7 of dihydrofolate too close to the carbonyl oxygen of Ile-94. Since this
carbonyl group is below the plane of the pteridine ring, it was proposed that
C7 would tend to pucker upward, out of the plane of the ring. Such an
enforced upward puckering at C7 accompanied by a downward puckering at
C6, would bring C6 of dihydrofolate closer to C4 of NADPH (Bystroff et al.,
1990). Additionally, the nicotinamide ring was modeled in the transition-
state complex based on previous theoretical studies. In the ground state the
dihydronicotinamide ring is planar (Filman et al., 1982; Eklund and Brändén,
1987). However, theoretical studies on hydride transfer from 1,4-
dihydronicotinamide suggest that in the transition state C4 of the
dihydronicotinamide is puckered slightly out of the plane of the ring in the
direction of hydride transfer (Donkersloot and Buck, 1981a,b). In the model of
the transition-state complex for DHFR, puckering of C4 out of the plane of the
nicotinamide ring leads to partial relief of an unfavorably close contact
between C4 and the hydroxyl group of Tyr-100, observed in the ternary
complex of DHFR with NADP+ and folate. The combined puckering of C4 of
dihydronicotinamide upward and of C6 of dihydrofolate downward brings
the two carbon atoms within 2.6 Å which is the theoretical carbon-carbon
distance for the hydride-transfer transition-state (Wu and Houk, 1987; Bystroff
et al., 1990). Thus, this model for the transition-state complex proposed by
Bystroff et al. (1990) is able to accommodate the optimal theoretical distances for hydride transfer as well as to relieve a series of strained contacts between ligands and protein observed in the crystal structure of the ternary complex of \textit{E. coli} DHFR with NADP\textsuperscript{+} and folate.

4. Antifolate Insensitive Dihydrofolate Reductases.

4.1. General Overview.

Only three years after the introduction of the antibiotic trimethoprim in clinical use, strains of Enterobacteriaceae (\textit{Klebsiella aerogenes} and \textit{Escherichia coli}) carrying R-plasmids which conferred high trimethoprim and sulphonamide resistance to their host were isolated from urine of patients in a London hospital (Fleming et al., 1972). Contrary to all previous plasmid-encoded resistance genes which manifested their resistance either by modifying the drug or by preventing the accumulation of the drug within the cell, this new plasmid-encoded resistance to trimethoprim was mediated by the production of a new DHFR insensitive to the action of the antibiotic (Amyes and Smith, 1974; Sköld and Widh, 1974; Amyes, 1989). Since then, seven major types (I-VII) of plasmid-encoded DHFRs present in gram-negative bacteria and one type (S1) present in gram-positive bacteria have been identified (Amyes, 1989). Some of these major groups have more than one characteristic enzyme. In those cases the enzymes do not differ significantly in their inhibition properties and are usually related by similar amino acid sequences. Bacterial strains producing any one of the enzyme
types I, II, V, VI, VII and S1 exhibit a characteristic high level of resistance to trimethoprim (MIC\(^6\) > 1000 mg/l). In contrast, bacterial strains producing the types III and IV DHFR are only moderately resistant to trimethoprim (MIC = 64 - 256 mg/l) (Thomson et al., 1990a). All the plasmid-encoded DHFRs from gram-negative bacteria that confer high level of trimethoprim resistance (types I, II, V, VI and VII) are also resistant to the inhibitory action of methotrexate. This occurrence is quite remarkable, taking into account that the affinity of these plasmid-encoded DHFRs for dihydrofolate is not much smaller than the affinity of the chromosomal DHFR, even though methotrexate and dihydrofolate show a close structural similarity (Table I.3 and Figure I.2) (Amyes and Towner, 1990). The most common of the plasmid-encoded DHFRs are those belonging to types I and II; in particular, the type Ia is widely spread around the world due to its presence in the promiscuous transposon Tn7 (Amyes et al., 1989). The biochemical properties of the plasmid-encoded DHFRs known to date are summarized in Table I.3.

There are two type I DHFRs. The type Ia was first identified in the plasmid R483 (Sköld and Widh, 1974) and is also associated with transposon Tn7. The transposon Tn7 also encodes resistance to streptomycin and spectinomycin. Studies of the occurrence of Tn7 in enterobacteria have shown a general increase in the percentage of enterobacteria harboring the transposon. This increase has been accompanied by the movement of Tn7 into the the bacterial chromosome. Thus, even bacteria that lose their R-plasmid will remain resistant to the antibiotics as a result of the presence of Tn7 in the bacterial chromosome (Amyes and Towner, 1990). The type Ib was

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\(^6\) MIC, the minimum inhibitory concentration, is the minimum concentration of drug that is able to inhibit bacterial growth.

<table>
<thead>
<tr>
<th>Type</th>
<th>Source</th>
<th>Molecular Weight (kDa)</th>
<th>Number of Subunits</th>
<th>TD50 (min)</th>
<th>MIC TMP (mg/l)</th>
<th>ID50 TMP (μM)</th>
<th>ID50 MTX (μM)</th>
<th>Km H2F (μM)</th>
<th>Kj TMP (μM)</th>
<th>Kj MTX (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>R483 (Tn7)</td>
<td>35.0</td>
<td>2</td>
<td>0.5</td>
<td>&gt;1000</td>
<td>57</td>
<td>4.4</td>
<td>5.6</td>
<td>7.4</td>
<td>0.58</td>
</tr>
<tr>
<td>Ib</td>
<td>pUK163 (Tn4132)</td>
<td>24.5</td>
<td>—</td>
<td>1.2</td>
<td>&gt;1000</td>
<td>32</td>
<td>2.8</td>
<td>11</td>
<td>41</td>
<td>—</td>
</tr>
<tr>
<td>Ila</td>
<td>R67</td>
<td>35.0</td>
<td>4</td>
<td>&gt;12</td>
<td>&gt;1000</td>
<td>70000</td>
<td>1100</td>
<td>4.6</td>
<td>6100</td>
<td>90</td>
</tr>
<tr>
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<td>R388</td>
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<td>4</td>
<td>&gt;12</td>
<td>&gt;1000</td>
<td>80000</td>
<td>750</td>
<td>8.3</td>
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<td>—</td>
</tr>
<tr>
<td>Iic</td>
<td>R751 (Tn402)</td>
<td>34.0</td>
<td>4</td>
<td>&gt;12</td>
<td>&gt;1000</td>
<td>20000</td>
<td>1000</td>
<td>4.2</td>
<td>400</td>
<td>—</td>
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<td>0.4</td>
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<tr>
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<td>pBH600</td>
<td>17.0</td>
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<td>&gt;12</td>
<td>128</td>
<td>2.0</td>
<td>0.02</td>
<td>9.5</td>
<td>0.4</td>
<td>—</td>
</tr>
<tr>
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<td>22.0</td>
<td>—</td>
<td>8</td>
<td>256</td>
<td>3.0</td>
<td>0.007</td>
<td>3.12</td>
<td>0.52</td>
<td>—</td>
</tr>
<tr>
<td>IV</td>
<td>pUK1123</td>
<td>46.7</td>
<td>1+1</td>
<td>&gt;12</td>
<td>160</td>
<td>0.2</td>
<td>0.02</td>
<td>0.02</td>
<td>0.03</td>
<td>—</td>
</tr>
<tr>
<td>V</td>
<td>pLMO20</td>
<td>17.5 (1)</td>
<td>—</td>
<td>3.0</td>
<td>&gt;1000</td>
<td>23</td>
<td>3.5</td>
<td>15.5</td>
<td>3.2</td>
<td>—</td>
</tr>
<tr>
<td>VI</td>
<td>pUK672</td>
<td>10.0</td>
<td>—</td>
<td>0.4</td>
<td>&gt;1000</td>
<td>200</td>
<td>7.25</td>
<td>31</td>
<td>75</td>
<td>—</td>
</tr>
<tr>
<td>VII</td>
<td>pUN835</td>
<td>11.5</td>
<td>—</td>
<td>1.5</td>
<td>&gt;1000</td>
<td>30</td>
<td>3.0</td>
<td>20</td>
<td>7.0</td>
<td>—</td>
</tr>
<tr>
<td>S1</td>
<td>pSK1 (Tn4003)</td>
<td>19.7</td>
<td>1</td>
<td>&gt;12</td>
<td>&gt;1000</td>
<td>50</td>
<td>0.002</td>
<td>10.8</td>
<td>11.6</td>
<td>—</td>
</tr>
</tbody>
</table>

E. coli (2) 18.0 1 >12 0.1 0.02 0.01 1.2 0.006 2.1 x 10^-5

TD50 — Incubation time at 45 °C that produces 50% inhibition of the enzyme.
ID50 — Dose of drug that produces 50% inhibition of the enzyme.
(1) Molecular Weight calculated from gene sequence. Gel filtration on Sephadex G-50 gives an apparent MW of 5000 (Towner et al., 1990).
(2) This refers to the chromosome-encoded DHFR. The values shown are those reported by Amyes (1989).
identified in the plasmid pUK163 and resides in the transposon Tn4132 (Young and Amyes, 1985). The two enzymes differ significantly in their molecular weight: the type Ia enzyme is a homodimer with a total molecular weight of approximately 35 kDa; the type Ib enzyme has a molecular weight of approximately 24.5 kDa, and its subunit composition has not yet been determined (Amyes and Towner, 1990). The two enzymes were classified in the same group due to their similar biochemical properties (Table I.3) (Huovinen, 1987). Type I DHFRs confer a high degree of resistance to trimethoprim and methotrexate, are heat labile, and are produced within the bacterial cell to a level ten times higher than the chromosomal enzyme (Huovinen, 1987; Amyes, 1989). Type I DHFRs have a pH profile with a peak of activity at pH 6 and 95% activity over a range of one pH unit (Young and Amyes, 1985).

Type III DHFRs are only moderately inhibited by trimethoprim. Sequence analysis of the type IIIa enzyme has shown it to share 51% homology with the E. coli chromosomal DHFR, suggesting that the type IIIa enzyme may have originated from the chromosomal enzyme (Amyes and Towner, 1990). The trimethoprim resistance provided by type IIIa DHFR is a consequence of combining a slightly higher $K_i$ for trimethoprim and a lower $K_M$ for $H_2F$ when compared to the chromosomal DHFR (Table I.3) (Joyner et al., 1984).

The type IV DHFR differs from all the other plasmid-encoded DHFRs because it is an inducible enzyme, which is rare for a plasmid-encoded resistance enzyme in gram-negative bacteria, it has a large molecular weight (46.7 kDa), and it confers only partial resistance to trimethoprim (Young and Amyes, 1986; Thomson et al., 1990c). The mechanism of resistance provided
by type IV DHFR is more similar to the mechanism characteristic of some strains of *E. coli* which overproduce the chromosome-encoded DHFR rather than the more common by-pass mechanism mediated by trimethoprim R-plasmids (Thomson et al., 1990c). Sequence comparison of the first fifty amino acids of the type IV enzyme shows no sequence homology with other plasmid-encoded DHFRs but shows significant homology with chromosome-encoded DHFR (29 out of the first 50 amino-acids are homologous) (Thomson et al., 1990c). Another characteristic that distinguishes the type IV DHFR when compared with other plasmid-encoded DHFRs, is that the native type IV enzyme is a complex of two proteins: the functional DHFR, with a molecular weight of approximately 33.0 kDa, and a DNA binding protein (NS1) which could be involved in the induction mechanism for expression of the enzyme (Thomson et al., 1990c).

Inhibition data and the gene sequence for type V DHFR revealed that this DHFR is closely related to type Ia DHFR (Sundström et al., 1988; Table I.3). Both enzymes are comprised by 157 amino acid residues, and the sequence homology between them is 75% (Sundström et al., 1988). However at the nucleotide level the homology is just 68% which could have contributed to a lack of hybridization between the type V gene and DNA probes for the type I enzyme (Sundström et al., 1987). It is also interesting to note that the amino acid sequence of type V DHFR shows 37% homology with *E. coli* K-12 chromosomal DHFR (Sundström et al., 1988). The type V DHFR molecular weight deduced from the nucleotide sequence is 17.531 kDa (Sundström et al., 1988). However, when run on a Sephadex G-50 column the apparent molecular weight of type V DHFR is 5.0 kDa, which is probably due to
interaction between the Sephadex matrix and protein hydrophobic residues (Towner et al., 1990).

The type VI DHFR with a molecular weight of 10.0 kDa is the smallest plasmid-encoded DHFR known to date (Table I.3) (Wylie et al., 1988). Note however that this is an apparent molecular weight determined on a Sephadex G-50 column in presence of 10 mg/l of bovine serum albumin. In the absence of this additional protein no trimethoprim-resistant DHFR activity could be found off the column (Wylie et al., 1988). Thus, a true molecular weight remains to be determined. Type VI DHFR confers a high level of resistance to trimethoprim and methotrexate, which is just surpassed by the type II enzymes (Table I.3) (Wylie et al., 1988).

Type VII DHFR is relatively insensitive to inhibition by trimethoprim and confers high level resistance to the antibiotic (Table I.3). The enzyme shares with the types I and V DHFRs similar inhibition profiles for trimethoprim and methotrexate, as well as the heat lability of these enzymes. However the type VII DHFR is much smaller than the type I and V DHFRs (Table I.3). Additionally, genetic probes made from the type I and V genes did not hybridize with the gene for type VII demonstrating the lack of DNA sequence homology between the genes coding for these enzymes (Amyes et al., 1989).

All the seven types of plasmid-encoded DHFRs we have discussed until now were isolated from gram-negative bacteria. But, contrary to what happens in gram-negative bacteria, the incidence of trimethoprim resistance in gram-positive bacteria is very low (Amyes and Towner, 1990). To date only one plasmid-encoded DHFR (type S1) from gram-positive bacteria (Staphylococcus aureus, S. epidermidis and S. hominis) has been isolated and
characterized (Young et al., 1987; Burdeska et al., 1990). The enzyme confers a high degree of trimethoprim resistance to its host. However, and contrary to what is observed for all the plasmid-encoded gram-negative DHFRs that are highly resistant to trimethoprim, type S1 DHFR does not confer methotrexate resistance (Table I.3) (Amyes and Towner, 1990).

From the discussion above it is clear that the number of different plasmid-encoded DHFR enzymes is large, and it is increasing. It is also possible to conclude that some of the enzymes are directly related in evolutionary terms, while other enzymes seem to be complete new DHFR innovations.

4.2. Type II Dihydrofolate Reductases: work by other authors.

Among all the plasmid-encoded DHFRs known to date, the type II DHFRs are the most insensitive to trimethoprim, and they confer to their host high resistance to this antibiotic (Table I.3). The type II enzymes have 50% inhibitory concentrations (ID50) for trimethoprim more than one million times higher than those for the *E. coli* chromosomal DHFR and more than one hundred times higher than any other plasmid-encoded DHFR (Table I.3). Another distinctive characteristic of the type II DHFRs is their high insensitivity to methotrexate. They have 50% inhibitory concentrations for methotrexate one hundred thousand times higher than the *E. coli* chromosomal DHFR and one thousand times higher than any other plasmid-encoded DHFR (Table I.3). Unlike some of the other plasmid-encoded DHFRs, type II DHFRs are heat stable, as defined by maintenance of catalytic
activity after incubation at 45 °C for more than twelve minutes (Table I.3) (Huovinen, 1987). It has even been reported that R388 DHFR maintains full catalytic activity after being kept at 100 °C for 20 minutes (Zolg et al., 1978).

There are three subgroups of type II DHFRs: types IIa, IIb and IIc encoded by the plasmids R67 (Pattishall et al., 1977), R388 (Amyes and Smith, 1974) and R751 (Shapiro and Sporn, 1977; Flensburg and Steen, 1986), respectively. All these enzymes have 78 amino acid residues per subunit and high amino acid sequence homology. Between both the R67 and R388 enzymes and the R67 and R751 enzymes there is 78% amino acid sequence identity, and between the R388 and R751 enzymes there is 86% amino acid sequence identity (Stone and Smith, 1979; Swift et al., 1981; Zolg and Hänggi, 1981; Flensburg and Steen, 1986). Most of the amino acid sequence differences among the type II DHFRs are concentrated within the first 21 amino acid residues of the N-terminus. The R388 and R751 DHFRs share 100% sequence identity between residues 22 and 78 (GenBank sequence database search (7)). The R388 and the R67 enzymes differ in 17 amino acid residues but only four residues fall within the region between amino acids 22 and 76, and two of these four substitutions are conservative (Swift et al., 1981). The amino acid sequence differences among type II DHFRs produce a different isoelectric point (pI) for the R751 enzyme. While both the R67 and R388 enzymes have a pI of 5.5, the R751 enzyme has a pI of 7.2 (Broad and Smith, 1982). The pI for chromosomal E. coli K12 DHFR is 4.4 (Broad and Smith, 1982). No sequence homology has been observed between the type II DHFRs and any other of the plasmid-encoded DHFRs or any known chromosomal DHFR. It has been suggested that the type II DHFRs may have evolved from an oxidoreductase which has lost its earlier substrate specificity and has acquired the capacity to
reduce dihydrofolate (Smith et al., 1979). However no such related enzyme has yet been found. As late as November 1991, I searched the "GenBank" sequence database (September 1991 release), and there were no known DNA or amino acid sequences that had any extensive homology to the type II DHFRs (7).

The pH-rate profiles for type II DHFRs are very different from that of chromosome-encoded DHFR. Amyes and Smith (1976) compared the pH-rate profiles for R388 DHFR and for the chromosomal DHFR from the host E. coli 114. While the chromosomal enzyme has a much flatter pH-rate dependence, with an activity maximum at pH 6.5-7.0 and 95% activity over a range of two pH units, the R388 enzyme show a much sharper activity peak at pH 6.0 and 95% activity over a narrow range of 0.4 pH units (Amyes and Smith, 1976). The pH profile for R67 DHFR is qualitatively similar to the pH profile for R388 DHFR with an activity maximum at pH 6.5 (Pattishall et al., 1977).

The molecular weight of type II DHFRs determined by gel filtration chromatography and gel electrophoresis in native conditions was reported to be 35 to 37 kDa (Amyes and Smith, 1974; Pattishall et al., 1977; Smith et al., 1979; Zolg and Hänggi, 1981). However a single protein band of molecular weight 8500 was observed on sodium dodecyl sulfate gel electrophoresis (Smith et al., 1979; Zolg and Hänggi, 1981), and amino acid and gene sequencing of the R67 and R388 enzymes produced a polypeptide chain of 78 amino acid residues with a theoretical molecular weight around 8500 (Smith et al., 1979; Stone and Smith, 1979; Zolg and Hänggi, 1981). These results are consistent with a native tetrameric structure for the type II DHFRs (Smith et

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7 This search was done on a μVAX running the "Sequence Analysis Software Package" by Devereux, Haeberli and Smithies (1984) Nucleic Acids Res. 12, 387-395.
al., 1979; Zolg and Hänggi, 1981). However, several other interconverting protein bands in native polyacrylamide gels of R67 DHFR with apparent molecular weights of approximately 27 kDa, 36 kDa, 45 kDa and higher values ranging up to 81 kDa have been observed (Smith et al., 1979). To date, no other known DHFRs have been shown to be tetramers (Table I.3). Recently, in an abstract it was reported that tetrameric R67 DHFR dissociates to dimers at low enzyme concentrations or low pH, with a \( pK_a \) of approximately 6.0 for the pH-dependent dissociation transition (Nichols et al., 1991).

Even though the affinity of the type II DHFRs for the antifolates trimethoprim and methotrexate is remarkably low, the Michaelis constant (\( K_M \)) for dihydrofolate is similar to that of the chromosomal enzyme. Amyes and Smith (1976) reported for R388 DHFR values of \( K_M \) for dihydrofolate and \( K_I \) for trimethoprim equal to 8.3 \( \mu \)M and 0.15 mM, respectively, and for the chromosomal DHFR of the host *E. coli* strain a \( K_M \) for dihydrofolate equal to 20 \( \mu \)M and a \( K_I \) for trimethoprim equal to 6.0 nM. These authors also reported that no enzymatic activity was observed when folate was used as substrate (Amyes and Smith, 1976). However, folate does bind to the R67 enzyme with a \( K_I \) similar to that for chromosomal enzyme (Pattishall et al., 1977). In an abstract Smith and Burchall (1980) reported that the R67 DHFR tetramer contains four folate binding sites but only one NADPH site. The catalytic efficiency (\( k_{cat}/K_M \)) is approximately 100-fold smaller in R67 DHFR than in the *E. coli* chromosomal enzyme (Reece et al., 1991).

The Michaelis constant for NADPH is similar in type II DHFRs (3.9 \( \mu \)M) and in *E. coli* chromosomal DHFR (2.7 \( \mu \)M) (Smith and Burchall, 1983). However, while the \( \alpha \)-epimers of the pyridine nucleotides are almost totally inactive as reductants in dehydrogenase reactions, R67 DHFR can utilize both
α-NADPH and α-NADH in addition to the normally biological active β-NADPH and β-NADH (Smith and Burchall, 1983). The Michaelis constant (K_M) for α-NADPH (16.6 μM) was approximately 4-fold greater than that for β-NADPH (3.9 μM), and the maximal velocity of the α-NADPH catalyzed reaction was 70% of that observed with β-NADPH. For α- and β-NADH the K_M values were higher than those for the NADPH epimers (K_M (β-NADH) = 82 μM and K_M (α-NADH) = 490 μM). The oxidized forms of both α- and β-epimers of NADP were competitive inhibitors of R67 DHFR (Smith and Burchall, 1983). However, it had been previously reported that no enzymatic activity was observed with R388 DHFR when β-NADH was used instead of β-NADPH (Amyes and Smith, 1976). Chromosomal DHFRs from several bacterial, protozoan and mammalian sources do not utilize α-NADPH or α-NADH for catalysis (Smith and Burchall, 1983).

The nucleotide sequence for R388 DHFR contains two termination codons in phase. In some suppressor positive strains, it is possible the first termination codon to be translated, which would extend the 78 amino acid sequence by 13 extra residues at the C-terminus (Zoln and Hänggi, 1981). In agreement with this possibility, it has been reported that in E. coli minicells the R388 monomer has a molecular weight of 10500, somewhat larger than the normal R388 and R67 monomers with molecular weight 8500 (Fling and Elwell, 1980). If these differences in the molecular weight for the R388 monomer are real and reflect the proposed extension at the C-terminus, it can be concluded that this extension does not inactivate the R388 enzyme because the cells were able to grow in presence of trimethoprim.

Several derivatives of R67 and R388 DHFR with C-terminus peptide fusions have been engineered, cloned and expressed in E. coli (Brisson and
Hohn, 1984; Vermersch et al., 1986; Vermersch, 1988). Extension of the 3' end of the R67 DHFR gene to encode an additional 20 amino acid residues at the C-terminus did not produce any apparent loss of trimethoprim-resistant DHFR activity (Brisson and Hohn, 1984). Analogously, C-terminus extensions of R388 DHFR varying from 7 to 21 amino acids did not alter severely the activity of the enzyme as judged by the ability of the host strains to grow in presence of trimethoprim (Gayle et al., 1986; Vermersch et al., 1986; Vermersch, 1988).

Deletions at the N-terminus of R388 and R67 DHFR have also been studied (Matthews et al., 1986; Vermersch and Bennett, 1987; Vermersch and Bennett, 1988; Reece et al., 1991). Digestion of R67 DHFR with chymotrypsin in presence of NADPH cuts the protein at Phe-16, but full enzymatic activity is retained (Matthews et al., 1986; Reece et al., 1991). A derivative of R388 DHFR with amino acid residues 2 to 7 deleted has been cloned, and the protein was able to confer trimethoprim resistance to the host cells (Vermersch and Bennett, 1987). Additionally, a R388 DHFR derivative with the first 10 amino acid residues deleted and residues 11 to 20 changed has been cloned and overexpressed in E. coli (Vermersch and Bennett, 1988). However, attempts to express cloned derivatives of R388 DHFR (Vermersch and Bennett, 1988) and R67 DHFR (Reece et al., 1991) shortened by 18 and 15 amino acids respectively did not succeed, probably due to instability of these derivatives in the bacterial cell. Based on guanidinium hydrochloride unfolding experiments at pH 5, it has been reported that the truncated version of R67 DHFR is 2.6 kcal/mol less stable than the normal protein (Reece et al., 1991).
The crystal structure of a dimeric form of R67 DHFR has been solved to 2.8 Å resolution and an R factor of 0.28 (Matthews et al., 1986). Each subunit is folded into a β-barrel conformation approximating a pure six stranded antiparallel β-barrel where the fifth stranded has been replaced by three consecutive residues in 3_10 conformation. The outer surfaces of the three longest β-strands in each subunit form a third six stranded β-barrel at the subunit interface. An 8 Å cleft runs the full length of the intersubunit β-barrel (Matthews et al., 1986). The architecture of this protein formed by β-barrels, and the absence of α-helices immediately suggests that the coenzyme binding motif in R67 DHFR is distinct from the common nucleotide binding domain observed for other pyridine dinucleotide dependent enzymes (Matthews et al., 1986). The crystal structure of the dimeric form of the protein was solved in the absence of coenzyme, substrate or antifolates, but based on molecular modeling studies the authors proposed that NADPH would bind in the deep intersubunit cleft with an anti conformation about both the nicotinamide and the adenine glycosidic torsion angles (Matthews et al., 1986).

Very recently Dr. David Matthews communicated to us the coordinates for the crystal structure of a tetrameric form of R67 DHFR. The protein was crystallized in presence of coenzyme, but no electron density has been found that could correspond to the coenzyme. This model was constructed by molecular replacement methods using the monomer of the crystal structure of the dimeric form of R67 DHFR. In this structure the tetramer is essentially assembled from two dimers that resemble the dimer in the previous crystal structure. A peculiar characteristic of this model for the tetrameric crystal
structure is the presence of a sizable pore (approximately 13 x 8 Å) at the center of the molecule.

5. Objectives.

The broad goal of the research reported here is to elucidate the functional and structural properties of type II dihydrofolate reductases, and compare these properties with those of chromosomal DHFRs, knowing that type II and chromosomal DHFRs do not share sequence or structural homology. Additionally, it is our intention to investigate the mechanisms that confer to type II dihydrofolate reductases low sensitivity to drugs of pharmacological interest such as trimethoprim and methotrexate. The search for new antimicrobial and antineoplastic agents, which can circumvent acquired resistances, are able to produce more efficient therapeutic responses, and demonstrate lower toxicity, is a constant of today's pharmaceutical research. Thus, it is essential to understand the basic molecular mechanisms by which resistances to drugs are acquired, and in the particular case of type II DHFRs, why are these enzymes so insensitive to common antifolates.

The study of the unique biochemical and structural properties of type II DHFRs and comparison with the well studied chromosomal DHFRs, will also allow, from a more basic point of view, identification of structural and mechanistic determinants essential to the catalytic reduction of dihydrofolate to tetrahydrofolate. This achievement, in the long run, could teach valuable lessons useful to the efforts being made in the fields of protein engineering and artificial enzymes.
Additionally, with the recently reported crystal structure of a tetrameric form of a type II DHFR, it seems plausible to use the enzyme as a scaffold on which to attach different functional groups as a means of constructing semi-artificial enzymes with a variety of catalytical properties. Finally, the absence in type II DHFRs of a tertiary fold identifiable as the widely conserved dinucleotide binding domain, could lead to the identification and description of a new motif for dinucleotide binding.

Toward these broad goals we will report in this work the results of a series of biochemical and biophysical studies on a derivative of R388 DHFR, RBG200 DHFR, having the sequence Thr-Thr-Ser-Arg-Thr-Leu at the carboxy terminus in addition to the 78 amino acids of the R388 DHFR (Vermersch et al., 1986). RBG200 DHFR was produced as result of earlier work on the use of R388 DHFR as a carrier for protein segments of different origins (Vermersch, 1988). The advantage of using RBG200 DHFR for the studies reported here is that this derivative is highly overproduced, stable and soluble.
Chapter II. METHODS.

1. Bacterial Strains and Growth

Frozen stocks of *Escherichia coli* C600\(^1\) bearing the plasmid pRBG200 were kindly supplied by Dr. George Bennett. Plasmid pRBG200 codes for a derivative of R388 DHFR, having the sequence Thr-Thr-Ser-Arg-Thr-Leu at the carboxy terminus in addition to the 78 amino acids of the R388 DHFR (Vermersch et al., 1986; Vermersch, 1988). pRBG200 is a high copy number plasmid, which leads to expression of large amounts of RBG200 DHFR in bacterial cells grown to saturation (Vermersch, 1988). The original clinical isolates coding for R388 DHFR and R67 DHFR are expressed at modest levels, similar to the expression levels of *E. coli* chromosomal DHFR (Amyes and Smith, 1974; Pattishall et al., 1977).

*Escherichia coli* C600 cells bearing the plasmid pRBG200 were stored frozen in vials containing 2 ml of a 1:1 mixture of glycerol and a freshly saturated bacterial culture, and kept at -70 °C. Bacteria were recovered from these frozen stocks by inoculating tubes containing 5 ml of LB liquid medium (Maniatis et al., 1982) with splinters scrapped off the frozen stock, and incubated at 37 °C for 12 to 15 hours. Single bacterial colonies were then selected by streaking agar plates with small inoculums of bacteria from the 5 ml cultures. The agar plates contained M9 minimal medium (Maniatis et al., 1982) supplemented with 0.2% casein amino acids and 50 mg/l trimethoprim.

\(^1\) The genotype of this *E. coli* strain is: lacY1 galK leuB6 supE44 thr-1 tonA21
and were incubated at 37 °C. Single colonies were then picked and used to inoculate several tubes with 5 ml of liquid medium (M9 minimal medium supplemented with 0.2% casein amino acids and 50 mg/l trimethoprim). These 5 ml cultures were grown at 37 °C to saturation and the total bacterial protein analysed in the following way. An aliquot (1 ml) of each culture was removed and the cells lysed by sonication. The resulting suspension was centrifuged in a bench top centrifuge for 10 minutes to remove cell debris. Then, 20 µl of a 10 % aqueous solution of Polymin P (polyethyleneimine; Sigma) were added, the mixture incubated at 4 °C for 5 min, and centrifuged in a bench top centrifuge for 10 minutes. Finally, the supernatant was analysed by SDS-PAGE. The 5 ml culture showing the best overproduction of RBG200 DHFR was used to inoculate (1%) a 100 ml bacterial culture, which was then used as starting culture for several 1 l batches.

For RBG200 DHFR production, 1 l liquid cultures of E. coli C600 bearing the plasmid pRBG200 (1% inoculation) were grown to saturation (12 to 15 hours) in M9 minimal medium supplemented with 0.2% casein amino acids and 50 mg/l trimethoprim, at 37 °C. The cells were then harvested by centrifugation at 4 °C and 4000 x g for 20 min, mixed with an equal weight of 50 mM Tris buffer (Tris(hydroxymethyl)aminomethane), pH 7.5, containing 10% sucrose, added dropwise to liquid nitrogen in a Dewar flask, and the frozen pellets stored at -70 °C.

Most of the RBG200 DHFR used in the studies reported here was purified from a large (190 l) growth in a 250 l industrial fermentator (New Brunswick Scientific Co., Edison, New Jersey) which yielded 514 g of cells.
In order to incorporate deuterium labeled aromatic amino acids into RBG200 DHFR, an *E. coli* strain auxotrophic for aromatic amino acids, strain AB2849 (Pittard and Wallace, 1966), was transformed with the plasmid pRBG200. This *E. coli* strain is an auxotroph for tryptophan, phenylalanine and tyrosine due to a mutation in the gene *aroC* that codes for chorismate synthetase in the synthetic pathway of chorismate, the last intermediate in the common pathway leading to tyrosine, phenylalanine, tryptophan, ubiquinone, vitamin K, and folic acid (Pittard and Wallace, 1966). *E. coli* AB2849 was obtained from the "*E. Coli* Genetic Stock Center", Department of Biology, Yale University, New Haven, Connecticut.

pRBG200 plasmid DNA was isolated from liquid cultures of *E. coli* C600 carrying the plasmid pRBG200 following a general procedure for small scale plasmid DNA isolation (Davis et al., 1986). This plasmid DNA was then used to transform the aromatic auxotrophic strain *E. coli* AB2849 using the procedure developed by Hanahan (1983). The transformed bacteria were then grown in presence of TMP to select those colonies that carried the plasmid pRBG200. The transformants were unable to grow in the absence of aromatic amino acids which confirmed their auxotrophy.

*E. coli* AB2849 cells carrying the plasmid pRBG200 were grown in M9 minimal medium supplemented with 50 mg/l trimethoprim, 100 mg/l aromatic amino acids (tryptophan, tyrosine and phenylalanine), and 1 µM each of the following benzoic acid derivatives: 4-aminobenzoic acid, 4-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid and 2,4-dihydroxybenzoic acid. Three 6 l batches of cells were grown in presence of three different combinations of deuterated amino acids, each batch having two deuterated aromatic amino acids and one non-labeled aromatic amino acid.
The perdeuterated aromatic amino acids were synthesized by Dr. George Krudy in Dr. Paul Rosevear's laboratory using variations of published synthetic methods (Krudy, G. A., unpublished results; Griffiths et al., 1976; Matthews et al., 1977). NMR analysis of the deuterated aromatic amino acids revealed 95% or higher deuterium incorporation at the aromatic rings of tyrosine, phenylalanine and tryptophan (Krudy, G. A., unpublished results).

To find out if some of the peculiar biochemical properties of RBG200 DHFR were a consequence of the short C-terminal tail that this type II DHFR has in comparison to R388 and R67 DHFR, we purified R67 DHFR. We obtained from Dr. George Bennett a lyophilized powder of E. coli cells bearing the plasmid pFE364 which codes for R67 DHFR, and which was initially obtained from the Wellcome Research Laboratories, Research Triangle Park, North Carolina (Fling and Elwell, 1980). These cells were grown and their R67 DHFR production monitored using methods and conditions similar to those described above for RBG200 DHFR.

2. Protein Purification

When we initially started the work with RBG200 DHFR, we followed a purification procedure based on selective precipitation of cellular proteins at high pH (0.1 M NaHO) and low pH (pH ~ 3) (Vermersch et al., 1986; Vermersch, 1988). Even though this method worked as a general screening procedure in the genetic studies (Vermersch et al., 1986; Vermersch, 1988), it became clear during the progress of initial biochemical and biophysical
studies that we could not obtain reproducible results. The specific activity of the purified protein was low and varied from preparation to preparation between 0.5 and 1 unit/mg. Additionally, the aromatic region of the $^1$H-NMR spectrum presented a large hump underneath an envelope of sharper resonances, and the magnitude of the hump varied between protein preparations. These and other observations suggested the presence of a significant and variable amount of denatured protein in the RBG200 DHFR preparations and showed the absolute need to develop a more gentle purification procedure for RBG200 DHFR which we describe next.

*Escherichia coli* C600 cells bearing the plasmid pRBG200 were grown to stationary phase, harvested by centrifugation, mixed with an equal weight of 50 mM Tris buffer containing 10% sucrose at pH 7.5, poured into liquid nitrogen, and stored at -70 °C. RBG200 DHFR was then purified using a procedure consisting of four basic steps: cell lysis, nucleic acid precipitation with streptomycin sulfate, ammonium sulfate precipitation, and ion-exchange chromatography in DEAE-Sepharose. All steps are carried out at 4 °C. The following purification procedure is based on 20 g of cell paste (1:1 mixture of cells and Tris/sucrose buffer) as starting material.

**Buffers for protein purification:** Cell lysis buffer contained 50 mM Tris, pH 8.0, 5% glycerol, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM 1,4-dithiothreitol (DTT), 0.24 M NaCl, 1.4 mM β-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). TGED buffer is 10 mM Tris, pH 8.0, 5% glycerol, 0.1 mM EDTA, 0.2 M NaCl, 0.1 mM PMSF, and 1 mM DTT. TME buffer contained 10 mM Tris, pH 8.5, 10 mM β-mercaptoethanol, and 1 mM EDTA. PMSF was added to the buffers immediately prior to use.
(I) **Cell Lysis:** Frozen cell paste (20 g) is stirred at room temperature in 55 ml lysis buffer until homogeneous. Lysozyme (20 mg) is added and the suspension stirred for 25 min. Freshly made 4% sodium deoxycholate is added to a final concentration of 0.1%; the solution is stirred an additional 2 min and incubated for an additional 20 min. To this solution, 55 ml TGED buffer and 100 ml of 50 mM Tris/HCL, pH 7.5 containing 10 mM β-mercaptoethanol are added with stirring, and the chromosomal DNA sheared in a blender at medium speed for 30 s. The cell lysate is then centrifuged at 10,000 g for 45 min. The supernatant is fraction I.

(II) **Streptomycin Sulfate Precipitation:** A 10% solution of streptomycin sulfate is added dropwise to fraction I with constant stirring to a final concentration of 1%. After complete addition of the streptomycin sulfate, the suspension is stirred for an additional 15 min and then centrifuged at 10,000 g for 15 min. The nucleic acid precipitate is discarded and the supernatant, fraction II, is considered the crude extract.

(III) **Ammonium Sulfate Precipitation:** Solid ammonium sulfate is slowly added to fraction II with constant stirring over a 30 min period to 35% saturation. The suspension is stirred for 30 min and centrifuged at 10,000 g for 45 min. The precipitate is discarded and solid ammonium sulfate is slowly added over a 30 min period to 50% saturation. After complete addition of the ammonium sulfate, the suspension is stirred for an additional 30 min. The ammonium sulfate precipitate is collected by centrifugation at 10,000 g for 45 min. The supernatant is discarded. The precipitate is dissolved in 20 ml TME buffer and dialyzed twice against 3 liters of TME buffer to give fraction III.
(IV) **Ion-Exchange Chromatography:** Fraction III is centrifuged at 10,000 g for 15 min and the supernatant loaded onto a 2.5 x 45 cm DEAE-Sepharose Fast Flow (from Pharmacia) column equilibrated in TME buffer. After loading the supernatant onto the column, the column is washed with 1 l of TME buffer. RBG200 DHFR is eluted with a 1.6 l linear gradient from 0 to 0.5 M NaCl in TME buffer. The purified RBG200 DHFR was dialyzed against 50 mM ammonium carbonate, pH 6.5, lyophilized, and stored at -20 °C with no measurable loss of activity over a period of several months.

RBG200 DHFR labeled with deuterated aromatic amino acids was isolated from an auxotrophic strain of *E. coli* transformed with the plasmid pRBG200 (see section II.1.1.). The purification procedure for deuterated RBG200 DHFR includes an extra step of gel filtration because the level of protein overproduction achieved with the auxotrophic strain is smaller than with the C600 strain. In this case the RBG200 DHFR fraction eluted from the DEAE-Sepharose column is concentrated by ammonium sulphate precipitation at 80% saturation. The precipitate is dissolved in the minimum volume of 50 mM potassium phosphate, pH 6.0, and loaded onto a 2.5 x 45 cm Sephacryl S-100 (Pharmacia) column equilibrated in 50 mM potassium phosphate, pH 6.0. The column is eluted with the same buffer and the fractions containing DHFR are pooled.

We have also purified R67 DHFR using the same purification procedure developed for RBG200 DHFR.
3. Determination of Protein Concentration and the Extinction Coefficient

Several methods were used to determine the extinction coefficient and protein concentration of solutions of RBG200 DHFR. The extinction coefficient at 280 nm ($\varepsilon_{280}$) was determined by three different methods: i) aromatic amino acid composition (Cantor and Schimmel, 1980); ii) absorbance ratio at 205 nm and 280 nm (Scopes, 1974; 1982); iii) absorbance at 280 nm in presence of 6 M guanidinium hydrochloride (Elweel and Schellman, 1977; Edelhoch, 1967). The protein concentration of RBG200 DHFR solutions was also determined using the Biuret (Gornall et al., 1949) or Bradford (1976) methods, using a solution of bovine serum albumin as standard.

All the spectrophotometric measurements were made in a Hewlett-Packard spectrophotometer model 8450A, using a measuring integration time of 15 s. For the extinction coefficient determinations, quartz cuvettes were washed with concentrated nitric acid before they were used.

3.1. $\varepsilon_{280}$ Based on Aromatic Amino Acid Composition. The strong absorption band at 280 nm observed in proteins, in the absence of cofactors, is due to the effects of the aromatic amino acids tryptophan, tyrosine and phenylalanine, as well as histidine and disulfide bonds (cystine). However the most significant contribution is made by tryptophan and tyrosine. The extinction coefficient at 280 nm for each of these amino acids is 5700 M$^{-1}$cm$^{-1}$ for tryptophan and 1300 M$^{-1}$cm$^{-1}$ for tyrosine. Thus, assuming that the extinction coefficients of these amino acids do not change drastically when
they are present in a protein, the protein extinction coefficient at 280 nm can be estimated from the following equation:

$$\varepsilon_{280} \text{ (mg}^{-1} \cdot \text{ml} \cdot \text{cm}^{-1}) = (5700 \times n_{\text{Trp}} + 1300 \times n_{\text{Tyr}}) / \text{MW}$$

where $n_{\text{Trp}}$ and $n_{\text{Tyr}}$ are the number of tryptophan and tyrosine residues per protein molecule and MW is the molecular weight of the protein molecule (Cantor and Schimmel, 1980).

RBG200 DHFR has two tryptophan and three tyrosine residues per subunit and a subunit molecular weight determined from its amino acid sequence equal to 8855. Thus, for RBG200 DHFR $\varepsilon_{280}$ can be estimated to be $1.73 \text{ mg}^{-1} \cdot \text{ml} \cdot \text{cm}^{-1}$.

3.2. $\varepsilon_{280}$ Based on the Absorbance Ratio at 205 nm and 280 nm. The peptide bond in proteins absorbs in the far-UV region, with a maximum at 192 nm (Scopes, 1982). However, due to oxygen and solvent interferences it is not possible to measure absorbances at that low wavelength in most routine spectrophotometers, and measurements are usually performed on the side of the absorbance band (Scopes, 1982). Most proteins have extinction coefficients at 205 nm for 1 mg/ml solutions between 30 and 35 (Goldfarb et al., 1951). The variation in the absorbance at 205 nm observed for different proteins is the result of the contribution of aromatic side-chains, in particular tryptophan and tyrosine, as well as the smaller contribution of other amino acid residues and different percentages of secondary structure. It has been shown that using the following semi-empirical expression:

$$\varepsilon_{205} \text{ (mg}^{-1} \cdot \text{ml} \cdot \text{cm}^{-1}) = 27.0 + 120 \times (A_{280} / A_{205})$$

the extinction coefficient at 205 nm can be determined for several proteins with an accuracy of $\pm 2\%$ (Scopes, 1974). However this method does not take into account contributions of secondary structure or other amino acid side-
chains other than tryptophan or tyrosine for the absorbance at 205 nm. The measurement of $A_{280}$ is done in a solution approximately 10 times more concentrated than the solution used for measurement of $A_{205}$.

Knowing the extinction coefficient at 205 nm, the protein concentration of a RBG200 DHFR solution and the extinction coefficient at 280 nm can be determined by the Beer-Lambert law:

$$A = \varepsilon \cdot c \cdot l$$

where $A$ is the absorbance, $\varepsilon$ is the extinction coefficient, $c$ is the protein concentration and $l$ is the path length of light through the solution.

3.3. $\varepsilon_{280}$ Based on the Absorbance in Presence of 6M Guanidinium Hydrochloride. In presence of 6 M guanidinium hydrochloride most proteins are unfolded and highly solvated, and their aromatic absorption spectrum is closely approximated by the sum of the spectra of suitable derivatives of their constitutive aromatic amino acids (Elwell and Schellman, 1977). Due to the influence of the ionizable amino and carboxyl groups in the absorption spectra of free amino acids, amino acid derivatives with both ionizable groups blocked produce spectra closer to those of the aromatic residues in a polypeptide chain (Edelhoch, 1967). It has been shown that N-acetyl-L-tryptophanamide and glycyl-L-tyrosyl-glycine are the most suitable model compounds to mimic the aromatic absorption spectra of tryptophan and tyrosine residues in proteins (Edelhoch, 1967). The extinction coefficient at 280 nm for these model compounds in 6 M guanidinium hydrochloride, 20 mM phosphate, pH 6.5 is 5690 M$^{-1}$•cm$^{-1}$ for N-acetyl-L-tryptophanamide and 1280 M$^{-1}$•cm$^{-1}$ for glycyl-L-tyrosyl-glycine (Edelhoch, 1967). Knowing the aromatic amino acid composition of a protein, the protein concentration can be determined comparing its absorption spectrum in 6 M guanidinium
hydrochloride with that calculated for its amino acids. Then the protein extinction coefficient in native conditions is determined from the absorption spectrum of the protein solution of known concentration (Elwell and Schellman, 1977).

3.4. Determination of Protein Concentration by the Methods of Biuret and Bradford. The Biuret reaction involves the formation of a copper complex with the peptide bonds of the protein. The complex has a purple coloration and can be quantified by spectrophotometry at 540 nm (Gornall et al., 1949). The Biuret method shows little variation in color yield from protein to protein because color formation is not dependent on the presence of particular amino acid residues. In this work we followed a general procedure for the Biuret method described in "Experimental Biochemistry" (Stenesh, 1984).

The Bradford method (Bradford, 1976) involves the binding of a dye (Coomassie Brilliant Blue G-250) to the protein under assay. The binding of the dye to the protein causes a shift in the absorption maximum of the dye from 465 nm to 595 nm. In this method the increase in absorption at 595 nm in the presence of protein is monitored. The Bradford method shows considerable variation in color yield from protein to protein because different proteins have significantly different dye-binding capacities (Dawson et al., 1986). However the method has the advantage of being very simple, rapid, reproducible and sensitive. Thus, we used the Bradford method only for relative estimations of protein concentration and specially to monitor protein concentration during protein purification procedures. Routinely we used the dye solution and the experimental protocol from Bio-Rad Laboratories (Technical Bulletin 1051 (1977)).
Protein concentrations determined by the Biuret or Bradford methods were obtained by interpolation in calibration curves prepared with solutions of bovine serum albumin (BSA) which were standardised by spectrophotometry at 280 nm using an extinction coefficient for BSA of $\varepsilon_{280} = 0.66 \text{ mg}^{-1} \cdot \text{ml} \cdot \text{cm}^{-1}$ (Fasman, 1976).

4. Enzyme Assay

Dihydrofolate reductase activity was assayed as described by Smith and Burchall (1983), except the buffer used was 50 mM potassium phosphate, 1 mM EDTA and 1 mM DTT at pH 5.9. The assay is based on the absorbance change at 340 nm due to NADPH oxidation and dihydrofolate reduction. The assay is performed at 30 °C in a spectrophotometer with temperature control capability. The assay mixture contains 70 $\mu$M NADPH, 45 $\mu$M dihydrofolate, 12 mM $\beta$-mercaptoethanol, and sufficient amount of enzyme to produce a linear record for at least 30 s. Routinely the reaction is started by addition of enzyme to the reaction mixture. One enzyme unit is the quantity of enzyme required to convert 1 $\mu$mol of NADPH and dihydrofolate to NADP$^+$ and tetrahydrofolate per min calculated based on an absorption coefficient of 12,300 M$^{-1} \cdot \text{cm}^{-1}$ at 340 nm (Smith and Burchall, 1983).

5. pH-rate Profile

The enzyme activity of RBG200 DHFR was determined at pH values varying between 4.2 and 7.9 using the standard assay buffer and coenzyme and
substrate concentrations described in the previous section. The pH of the reaction mixture was measured prior to and immediately after the reaction was monitored, using a microelectrode and found to vary less than ±0.05 units. For each pH value at least three separate assays were performed.

6. Native Molecular Weight Determination by Gel Filtration

Apparent native molecular weights of RBG200 DHFR and R67 DHFR were determined by gel filtration using high performance liquid chromatography (HPLC). These experiments were performed on gel filtration Superose 12 HR and Superdex 75 HR HPLC columns (Pharmacia), measuring 10 X 300 mm, at room temperature (22 °C) in 50 mM potassium phosphate buffer, and several pH values and potassium chloride concentrations. A wide range of protein concentrations, between 19 mg/ml and 1.6 μg/ml, was used. Sample volumes of 100 μl, approximately 0.4% of the column bed volume, and a flow rate of 0.5 ml/min were used to maximize separation resolution. DHFR sample concentrations were determined using an extinction coefficient of $\varepsilon_{280} = 1.8 \text{ cm}^{-1} \cdot \text{mg}^{-1} \cdot \text{ml}$ (see section III.1.2.). Protein elution off the column was followed by absorbance at 280, 295 or 220 nm, depending on the protein concentration of the sample under analysis. Apparent molecular weights were determined by interpolation of the retention times for RBG200 DHFR in calibration curves expressing the logarithm of the molecular weight as a function of the retention time. These calibration curves were prepared with the following molecular weight standards: bovine serum albumin (66.0
kDa), carbonic anhydrase (29.0 kDa), cytochrome c (12.4 kDa) and aprotinin (6.5 kDa).

Two columns, Superose 12 HR and Superdex 75 HR, were used to test if some of the results obtained were due to protein-matrix interactions. While Superose is a cross-linked agarose based matrix, Superdex is produced by the covalent bonding of dextran to highly cross-linked agarose beads with the separation properties of this matrix being mostly determined by the dextran component\(^2\). Some hydrophobic interactions between proteins and column matrix have been reported for Superose but no hydrophobic interactions have yet been found with Superdex\(^2\).

The protein concentrations reported in this section are concentrations of the solution applied to the gel filtration column. The approximately 10 fold dilution that the protein samples experience through the column was not taken into account. Thus, the protein concentrations reported in this section can only be used as an initial qualitative approach to the study of the apparent molecular weight of RBG200 and R67 DHFR as a function of pH, ionic strength and protein concentration.

7. Laser Desorption Mass Spectrometry

In order to further characterize the quaternary structure of RBG200 DHFR we made use of matrix-assisted UV-laser desorption mass spectrometry (LD-MS). This technique allows the determination of a wide range of

\(^2\) This description of the matrices was obtained from the Pharmacia Instruction Sheets for the Superose 12 HR and Superdex 75 HR columns.
molecular weights of proteins (Beavis and Chait, 1989; Karas, 1990). However to study multimeric proteins there is the need to use cross-linking agents to avoid dissociation of the proteins during the experiment or sample preparation (Farmer and Caprioli, 1992). We used glutaraldehyde which is a cross-linking agent that reacts with the free amino groups in the protein. In solution, glutaraldehyde polymerizes and forms cross-linking arms of various lengths that can fit the distances between intersubunit amino groups, holding the associated subunits together (Farmer and Caprioli, 1992).

The spectra were acquired in a Finnigan MAT LaserMAT laser desorption time-of-flight mass spectrometer equipped with a nitrogen laser emitting at 337 nm. The spectrometer was calibrated with several proteins of known molecular weight used as external standards. A 10 mg/ml solution of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid; Aldrich Chemical Co., Milwaukee, Wisconsin) in 70% acetonitrile, 30% water, 0.1% trifluoroacetic acid was used as matrix.

RBG200 DHFR samples were prepared in 100 mM Bis-Tris (2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol) buffer at pH 6.0 and 100 mM sodium acetate buffer at pH 5.0. Protein concentrations varied between 11 mg/ml and 3.5 μg/ml. Protein samples to be cross-linked were incubated with glutaraldehyde at a final concentration of 0.8%. The reaction mixture was incubated for 15 to 60 min and then analyzed. For LD-MS analysis, 0.5 μl of the sinapinic acid solution was placed on a stainless steel target (Finnigan MAT) immediately followed by 0.5 μl of the protein solution, and the mixture dried under a lamp. Mass spectra were obtained by summing 5 to 10 runs.
8. Analytical Ultracentrifugation

Analytical ultracentrifugation was performed using a Beckman Model E analytical ultracentrifuge equipped with a scanning absorption optical system. The scanner output was digitized and transferred to a personal computer. The experiments were run at 20 °C and 28000 rpm. The loading subunit concentration of RBG200 DHFR was 12 μM. The results were plotted and analyzed as absorbance values at 280 nm as a function of the radial position in the ultracentrifuge cell. The concentration distribution, given in absorbance units, as a function of the radial position was then fitted by a nonlinear least-squares fitting procedure to several models representing different types of reversible associating systems.

The theory for the concentration distribution of a solute at sedimentation equilibrium in a centrifugal field is well established. The sedimentation equilibrium is attained when the transport of solute due to sedimentation ($J_s$) is in equilibrium with the reverse transport due to diffusion ($J_d$). The flow of solute due to sedimentation is given by:

$$J_s = S \omega^2 r C_r$$

where $S$ is the sedimentation coefficient, $\omega$ the angular velocity, $r$ the radial distance, and $C_r$ the solute concentration at any point $r$. The flow due to diffusion is given by Fick's first law:

$$J_d = -D \left( \frac{d C_r}{d r} \right)$$

where $D$ is the diffusion coefficient. When the sedimentation equilibrium is reached we have:

$$J_s + J_d = S \omega^2 r C_r - D \left( \frac{d C_r}{d r} \right) = 0$$
and given Svedberg's and Einstein's law defining $S$ and $D$, respectively, we have:

$$\frac{M(1-\nu)p\omega^2 r C_r}{N f} \cdot \frac{RT}{N f} \cdot \frac{d C_r}{d r} = 0$$

Rearranging:

$$\frac{d C_r}{C_r} = \frac{M(1-\nu)p\omega^2}{RT} rd r$$

And integrating to get the equation in terms of concentration we have:

$$\ln \frac{C_r}{C_b} = \frac{M(1-\nu)p\omega^2}{RT} \frac{r^2 - r_b^2}{2}$$

where $M$ is the molecular mass of the solute, $\nu$ is the partial specific volume of the solute, $p$ is the solution density, $\omega$ is the rotor angular velocity, $R$ is the gas constant, $T$ is the absolute temperature, $r$ is the radial position at any point, $r_b$ is the radial position at the bottom of the cell, $C_r$ is the solute concentration at any point $r$, and $C_b$ is the solute concentration at the bottom of the cell.

If instead of a single species in solution there is a reversible associating system such as a monomer-dimer (M-D) equilibrium with an association constant $K_{a1}$:

$$2M \leftrightarrow D \quad K_{a1} = [D] / [M]^2$$

then the observed concentration distribution of the species as a function of radial position at ultracentrifugal and chemical equilibrium is described by (Becerra et al., 1991):

$$C_r = C_{b,M} \exp(A M_M \delta r^2) + \exp(\ln K_{a1}) C_{b,M}^2 \exp(2 A M_M \delta r^2) + \varepsilon \quad (II.1)$$
where \( A = (1 - \nu \rho) \sigma^2 / 2RT \), \( M_M \) is the molecular mass of the monomer, \( \delta r^2 = (r^2 - r_b^2) \), and \( \varepsilon \) is the base-line error.

For an equilibrium involving monomer (M), dimer (D) and tetramer (T):

\[
2M \rightleftharpoons D \quad K_{a1} = [D] / [M]^2 \\
2D \rightleftharpoons T \quad K_{a2} = [T] / [D]^2
\]

the observed concentration distribution of the species as a function of radial position at ultracentrifugal and chemical equilibrium is described by:

\[
C_r = C_{b,M} \exp(A M_M \delta r^2) + \exp(\ln K_{a1}) C_{b,M}^2 \exp(2 A M_M \delta r^2) + \\
K_{a1}^2 K_{a2} C_{b,M}^4 \exp(4 A M_M \delta r^2) + \varepsilon \quad (II.2)
\]

The concentration \( C_r \) can be expressed in gram per liter, mole per liter, or UV absorbance. The values for \( \nu \) and \( \rho \) were assumed to be 0.74 and 1.0, respectively. Most proteins which do not contain large amounts of carbohydrate or lipid, have partial specific volumes (\( \nu \)) close to 0.74. The terms \( C_{b,M}, K_{a1}, K_{a2} \) and \( \varepsilon \) were the fitting parameters which were determined in the nonlinear least-squares fitting procedure.

9. Spectrofluorometry

Spectrofluorometric measurements with RBG200 DHFR were carried out on a Perkin-Elmer LS-5 spectrofluorimeter. The excitation and emission maxima were determined to be 280 nm and 342 nm respectively, for a solution of RBG200 DHFR at a subunit concentration of 6.5 \( \mu \)M.
DHFR solutions were prepared in 50 mM potassium phosphate, 50 mM potassium chloride, pH 6.0. The protein concentration was estimated by spectrophotometry at 280 nm using an extinction coefficient of 1.8 mg\(^{-1}\) \(\cdot\)ml\(\cdot\)cm\(^{-1}\).

10. Circular Dichroism

Circular dichroism spectra of RBG200 DHFR were measured in an Aviv 62DS spectrometer under constant nitrogen flush. Spectra were measured between 190 and 280 nm every 0.5 nm, using a band width of 1.5 nm and an averaging time of 1 s. Protein solutions were prepared in 50 mM potassium phosphate, 50 mM potassium chloride, pH 6.0 and filtered through 0.45 μm Millipore filters before use. The protein concentration was estimated by spectrophotometry at 280 nm using an extinction coefficient of 1.8 mg\(^{-1}\) \(\cdot\)ml\(\cdot\)cm\(^{-1}\).

11. Determination of the Stereochemistry of NADPH Oxidation

The strategy to determine the stereochemistry for hydride transfer catalyzed by RBG200 DHFR consisted of three steps: i) synthesis of [4-\(^2\)H]NADP\(^+\); ii) stereospecific reduction of [4-\(^2\)H]NADP\(^+\) to [4S-\(^2\)H, 4R-\(^1\)H]NADPH using a type-A dehydrogenase; iii) incubation of [4S-\(^2\)H, 4R-\(^1\)H]NADPH with dihydrofolate and RBG200 DHFR, and NMR analysis of the
oxidized coenzyme in order to determine which hydride (pro-R or pro-S) is transferred by RBG200 DHFR.

[4-2H]NADP+ was prepared by alkaline cyanide treatment of NADP+ in 2H2O according to the procedure of San Pietro (1955). 1H NMR spectroscopy was used to confirm the position of the isotopic label and estimate the extent of deuteration (>95%). [4S-2H, 4R-1H]NADPH was prepared using isocitrate dehydrogenase. The reaction mixture contained 50 mM Tris-HCl buffer at pH 7.2, 10 mM isocitric acid, 5 mM [4-2H]NADP+, 50 μM MnSO4 and 2 units of isocitrate dehydrogenase in a final volume of 1.5 ml. The mixture was incubated for 2 hours at 37° C, and the extent of reaction monitored by the increase in absorption at 340 nm. The reaction mixture was diluted to 50 ml with 10 mM triethylammonium bicarbonate buffer, pH 7.5 and loaded onto a 1.5 x 90 cm Pharmacia Fast Flow DEAE-Sepharose column equilibrated in 10 mM triethylammonium bicarbonate buffer, pH 7.5. The labeled NADPH was eluted with a 500 ml linear gradient from 10 to 500 mM triethylammonium bicarbonate, pH 7.5, at a flow rate of 1.5 ml/min. Fractions having A260/A340 ratios of approximately 2.5 were pooled and the isotopically labeled NADPH precipitated with a 10-fold excess of cold acetone. The chemical shift and coupling constant of the C4 methylene proton of the isotopically labeled NADPH confirmed the stereochemistry at C4 to be [4S-2H, 4R-1H] (Arnold et al., 1976; Seyama et al., 1977; Arnold and You, 1978). The [4S-2H, 4R-1H]NADPH (70 μM) was incubated with RBG200 DHFR (0.11 mg) in a reaction mixture which contained 45 mM potassium phosphate buffer, pH 5.9, 120 μM dihydrofolate, and 12 mM 2-mercaptoethanol for 1 hr at 22° C. The reaction mixture was diluted with 50 ml of 10 mM triethylammonium bicarbonate buffer, pH 7.5 and loaded onto a 1.5 x 30 cm Pharmacia Fast Flow DEAE-
Sepharose column. NADP⁺ was eluted with a 500 ml linear gradient from 10 to 500 mM triethylammonium bicarbonate, pH 7.5. Fractions containing the purified NADP⁺ were pooled and the NADP⁺ collected by precipitation with a 10-fold excess of cold acetone. The precipitated NADP⁺ was dissolved in ²H₂O for analysis by ¹H NMR. Proton NMR spectra were obtained at 22° C and 500 MHz. Spectra were obtained by collecting 128 transients using 16K data points, a spectral width of 10000 Hz, a 90° pulse and a 5 s recycle time. DSS was used as an internal standard and the pH of the samples adjusted to 7.0.

12. Synthesis and Purification of [³H]NADP⁺

[⁴⁻³H]NADP⁺ was prepared by alkaline cyanide treatment of NADP⁺ in ³H₂O according to the general procedure of San Pietro (1955). Potassium cyanide (65 mg) was dissolved in 1 ml of ³H₂O (100 mCi/g) to which 20.6 mg of solid NADP⁺ was added. The pH was adjusted to 11.3 with 6 M NaOH and reaction incubated for 90 min at room temperature. After incubation, 30 mg KH₂PO₄ was added to acidify the reaction mixture and the excess cyanide removed by bubbling nitrogen through the reaction mixture. The [⁴⁻³H]NADP⁺ was precipitated with 10 ml of cold acetone and collected by centrifugation. The crude [⁴⁻³H]NADP⁺ was washed twice by re-dissolving the crude material in 0.5 ml H₂O, precipitating with 5 ml acetone, and collecting the precipitate by centrifugation. The precipitate was dried overnight under vacuum, using a oil pump and an acetone-dry ice trap to avoid contamination by residual ³H₂O.
The crude [4-³H]NADP⁺ was then purified by ion exchange chromatography. The [4-³H]NADP⁺ precipitate was dissolved in 1.5 ml of 10 mM ammonium carbonate, pH 8.0, and loaded onto a 1.5 x 40 cm Fast Flow DEAE-Sepharose (Pharmacia) column equilibrated in 10 mM ammonium carbonate, pH 8.0. The column was washed with 210 ml of 10 mM ammonium carbonate, pH 8.0, and eluted with a 500 ml linear gradient from 10 mM to 600 mM ammonium carbonate, pH 8.0. Approximately 2.5 ml fractions were collected and 30 μl aliquots were removed and added to 5 ml of scintillation counting fluid ("Liquiscint" from National Diagnostics, Manville, New Jersey) in scintillation vials. Radioactivity was then counted on a liquid scintillation counter (LKB Wallac, model 1209 Rackbeta). Three peaks of radioactivity were found, but only one of the peaks was found to consist of NADP⁺, as judged by thin layer chromatography using commercial NADP⁺ as reference. Thin layer chromatography was performed on pre-coated silica gel plates (E. Merck, Darmstadt, Germany) developed in isopropanol:H₂O:ammonia (8:3:1). The isolated [4-³H]NADP⁺ was shown to be pure by thin layer chromatography. The concentration of the isolated [4-³H]NADP⁺ was measured by absorption at 259 nm, using an extinction coefficient of ε = 18000 M⁻¹cm⁻¹ (Rizzo et al., 1987). The specific activity of [4-³H]NADP⁺ was found to be 202 cpm/nmol.

13. Equilibrium Dialysis

13.1. Binding assays with [4-³H]NADP⁺. The RBG200 DHFR number of binding sites and the dissociation constant (K_d) for NADP⁺ were estimated by
equilibrium dialysis in ~1 ml micro-dialysis cells, using [4-3H]NADP+. The two halves of the cells were assembled placing 6000 to 8000 Mr cutoff dialysis membrane (Spectra/Por, Spectrum Medical Industries, Houston) between the two cell halves to form the dialysis chamber. On each micro-dialysis cell, 450 µl of approximately 1.5 mM RBG200 DHFR (concentration calculated as a tetramer) was placed on one side of the dialysis chamber, and 450 µl of [4-3H]NADP+ was placed on the other side. Five different concentrations of [4-3H]NADP+ were used. [4-3H]NADP+ with a specific activity of 202 cpm/nmol was diluted with cold NADP+ to produce [4-3H]NADP+ with a specific activity of 14.2 cpm/nmol. DHFR and [4-3H]NADP+ solutions were prepared in 50 mM sodium citrate, pH 6.0. The dialysis was carried out at room temperature (~ 22 °C) by continuously rotating the microcells for 55 h. This long dialysis time was used to allow for complete interconversion between conformations I and II of the the RBG200 DHFR•NADP+ complex (see section III.2.1). Aliquots of the dialyzed solutions were added to scintillation fluid and the radioactivity counted. Protein concentrations were determined using the Biuret method (Gornall et al., 1949). Binding data were analyzed by curve fitting to the Scatchard equation of the form

\[
[L]_b / [L]_f = -\frac{(1/K_d)}{[L]_b} + \frac{[P]_t}{K_d}
\]

where [L]_b and [L]_f are the concentrations of bound and free ligand respectively, and [P]_t is the total concentration of binding sites. Thus, on a graph of [L]_b/[L]_f versus [L]_b, the slope of the line that best fits the experimental data is given by -(1/K_d) and the abscissa intercept is equal to [P]_t. The number of ligand binding sites can then be determined by dividing the abscissa intercept by the total concentration of protein (Boeynaems and Dumont, 1980).
13.2. Binding assays with NADPH. The RBG200 DHFR number of binding sites for NADPH was estimated by equilibrium dialysis. Caps of 1.5 ml microcentrifuge tubes, capable of holding a volume of approximately 200 µl, were cut with the top ring of the tube from microcentrifuge tubes and used as microdialysis chambers. A solution of RBG200 DHFR (200 µl) was placed in each microcentrifuge tube cap which then was covered with dialysis membrane (6000 to 8000 Mr cutoff) (Spectra/Por, Spectrum Medical Industries, Houston). The dialysis membrane was held in place by closing the top ring of the microcentrifuge tube over the membrane. The caps were then placed inside 50 ml conic polypropylene tubes containing 2 ml of NADPH at several different concentrations. The tubes were then capped, wrapped in aluminium foil to decrease NADPH degradation and maintained at 4 °C with continuous agitation for 44 hours. The RBG200 DHFR and NADPH solutions were prepared in 50 mM potassium phosphate, pH 7.0. This pH was used instead of pH 6.0, where the enzyme is maximally active, to decrease the degradation of NADPH which is faster at acidic pH. The NADPH equilibrium concentrations were determined after dialysis by spectrophotometry using an extinction coefficient of ε\textsubscript{334} = 6.178 x 10\textsuperscript{3} M\textsuperscript{-1}cm\textsuperscript{-1} (Ziegenhorn et al., 1976). The protein concentration was determined by spectrophotometry at 280 nm before dialysis. To evaluate possible dilution of the protein during dialysis, no NADPH was added to one of the dialysis tubes and the protein concentration was measured at the end of the experiment. No significant dilution of the protein was observed (< 5%). The influence of RBG200 DHFR on the NADPH absorbance at 334 nm was also studied in order to evaluate the possible changes in the extinction coefficient of NADPH in the presence of
RBG200 DHFR which would introduce errors in the evaluation of the NADPH concentrations. At the concentrations of NADPH and DHFR used, no significant changes were observed in the spectral properties of NADPH between 320 and 420 nm. A small contribution (~ 0.02) for the absorbance at 334 nm was a direct consequence of the presence of high protein concentrations, and because the amount of protein was maintained constant in all the dialysis cells, all the absorbance values at 334 nm in presence of DHFR were corrected by 0.02.

14. Nuclear Magnetic Resonance Methods

14.1. Sample Preparation. Purified RBG200 DHFR for NMR studies was dialyzed twice against at least a 300 fold volume of 50 mM ammonium carbonate, pH 6.5. The ammonium carbonate solution was prepared and its pH adjusted by bubbling CO₂ in a 50 mM solution of ammonium hydroxide, to minimize the amount of contaminant metals. The protein concentration of the dialysate was measured, and the dialysate was divided and lyophilized in batches of 10 to 15 mg of protein. A batch of lyophilized RBG200 DHFR was then dissolved in 480 to 500 μl of 50 mM potassium phosphate buffer, pH* 6.0(3), in 2H₂O (Cambridge Isotope Laboratories) and the solution placed in a 5 mm NMR tube. Samples which were used in the analysis of the aromatic region of one-dimensional ¹H-NMR spectra, were incubated for 6 to 12 h at 30

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³ pH* denotes a pH meter reading uncorrected for the isotope effect on the equilibrium and on the glass electrode.
0°C in order to exchange with 2H the vast number of slow exchanging proton amides of RBG200 DHFR.

All buffers used in the NMR samples were treated with Chelex 100 (Bio-Rad Laboratories) before use to remove trace metal contaminants. No detectable loss in the activity of RBG200 DHFR was observed at the end of the NMR experiments.

14.2. NMR Data Collection. All the 1H-NMR spectra reported in this work were obtained at 500 MHz in a GN500 spectrometer. One-dimensional NMR data were processed on the spectrometer, on an adjacent Nicolet 1280 data station, or on IBM-compatible personal computers running the program FELIX. Two-dimensional NMR data were transferred to a DEC MicroVAX II computer and processed with the program FTNMR, or were transferred to a Silicon Graphics Personal Iris workstation and processed with the program FELIX. The programs FTNMR and FELIX were obtained from Hare Research, Inc.

14.3. Aromatic Region of the 1H-NMR Spectrum of RBG200 DHFR. The aromatic region of the 1H-NMR spectrum of RBG200 DHFR in the absence and presence of NADP+ and/or folate was analysed using double-quantum filtered two-dimensional correlated spectroscopy (DQF-COSY), two-dimensional total correlation spectroscopy (TOCSY) and two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) of RBG200 DHFR samples with selectively deuterated aromatic residues as well as unlabeled protein.
14.4. Dissociation Constant (K_d) for NADP⁺. Titration of RBG200 DHFR with oxidized coenzyme while monitoring the line width of the nicotinamide HN2 resonance (see Figure III.10 for proton naming convention) was used to determine the K_d for NADP⁺ binding to conformation II of the RBG200 DHFR • NADP⁺ complex (see section III.2.1). Titrations were performed by addition of known aliquots of NADP⁺ over the range 0.5 to 25 mM, to a solution of 0.4 mM RBG200 DHFR (tetramer) in 50 mM potassium phosphate, pH 5.9, in ²H₂O. After each addition of coenzyme, the sample was incubated at 25 °C until equilibrium between conformations I and II of the binary complex was reached. Proton NMR spectra were collected at 25° C and 500 MHz. Spectra were recorded with 64 transients, each with 16K data points, double precision, a spectral width of 7042 Hz, a 90° pulse and a 2.5 s recycle time. The FID was zero filled once but no window function was applied for resolution enhancement or line broadening. Line widths of the HN2 resonance were measured at half peak height, and alternatively were measured using the peak fitting routine of the NMR processing software. The line widths of the HN2 resonance were plotted as a function of the total NADP⁺ concentration and the data fitted with the nonlinear least squares program MINSQ (MicroMath Scientific Software, Utah) using equations II.5 and II.7 below.

If a nucleus exchanges between two chemical environments, the NMR parameters for this nucleus will be affected differently, depending on the exchange rate. If the rate of exchange is much larger than the difference between the values of the NMR parameter in each chemical environment, the observed NMR parameter is given by a weighted average and the nucleus is said to be in the fast exchange regime. For a ligand in fast exchange between
free and bound states, the observed line width ($LW_{obsd}$) of a ligand resonance is given by

$$LW_{obs} = f_{L}(LW_{L}) + f_{PL}(LW_{PL})$$  \hspace{1cm} (II.4)

where $f_{L}$ and $f_{PL}$ are the molar fractions and $LW_{L}$ and $LW_{PL}$ are the line widths of free and enzyme bound ligands, respectively (Feeney et al., 1979; Campbell and Dwek, 1984). Rearrangement of equation II.4 permits $LW_{obsd}$ to be expressed as a function of $f_{PL}$, $LW_{L}$, and $LW_{PL}$ (eq II.5).

$$LW_{obsd} = LW_{L} + (LW_{PL} - LW_{L})*f_{PL}$$  \hspace{1cm} (II.5)

The dissociation constant, $K_{D}$, which quantitates the affinity of the ligand (L) for the protein (P) is given by

$$K_{D} = ([P_{T} - PL] - ([L_{T}] - [PL]) / [PL]$$  \hspace{1cm} (II.6)

where $[L_{T}]$ and $[P_{T}]$ are the total concentrations of ligand and non-interacting homogeneous binding sites, respectively. When equation II.6 is solved for $[PL]$ with $f_{PL}$ defined as $[PL]/[L_{T}]$, a quadratic expression for $f_{PL}$ is obtained (eq II.7).

$$f_{PL} = \{( [L_{T}] + [P_{T}] + K_{D} ) - \sqrt{([L_{T}] + [P_{T}] + K_{D})^2 - 4[L_{T}]*[P_{T}] / [L_{T}]^2} / 2[L_{T}]$$  \hspace{1cm} (II.7)

If equations II.5 and II.7 are combined, $K_{D}$ can be determined from nonlinear least-squares analysis of plots of $LW_{obsd}$ as a function of $[L_{T}]$.

14.5. Assignment of NADP$^+$ resonances. The $^1$H-NMR assignment of the resonances of free and bound NADP$^+$ was made by comparison with previously published assignments (Oppenheimer, 1982) and confirmed by double quantum filtered two dimensional correlated spectroscopy (DQF-COSY) and two dimensional nuclear Overhauser enhancement spectroscopy (NOESY). The NOESY experiments are described in detail in the next paragraph. DQF-COSY spectra (Piantini et al., 1982; Rance et al., 1983) were
collected at 15 °C as 512 t₁ × 2048 t₂ complex data points with a sweep width of 6250 Hz in both dimensions and 16 transients per t₁ point. Residual H₂O was suppressed by continuous irradiation during the 1.5 s relaxation delay. The DQF-COSY spectra were processed with a 45° shifted skew bell squared function in t₂ and by a 90° shifted sinebell squared function in t₁, and zero-filled in t₁ to obtain a final matrix of 1024 × 1024 real data points. Samples of free NADP⁺ and NADP⁺ in the presence of RBG200 DHFR were prepared in 50 mM potassium phosphate, pH 6.0, in H₂O. Chemical shifts are reported relative to external DSS at 0 ppm, in the same experimental conditions.

14.6. Conformation of NADP⁺ in the binary complex RBG200 DHFR•NADP⁺. The theory for investigating the structure of enzyme-bound ligands using transferred nuclear Overhauser effects (TNOEs) has been developed (Clore and Gronenborn, 1982; 1983) and the methodology used to determine the conformation of a number of enzyme-bound flexible ligands (Rosevear and Mildvan, 1989; Levy et al., 1983; Ehrlich and Colman, 1985). The conformation of NADP⁺ bound to RBG200 DHFR was investigated by measuring the time dependence of the transferred NOE in two dimensional phase-sensitive NOESY spectra.

Phase sensitive NOESY spectra were obtained at 500 MHz and 15°C with 50, 75, 100, 125, and 150 ms mixing times using the method of States et al. (1982). These spectra were collected in arbitrary order during a single 4 day period without removing the sample from the spectrometer or changing the spectrometer settings (Banks et al., 1989). A 180° composite pulse was used during the mixing period to eliminate the contribution of J cross-peaks to the NOE cross-peaks (Macura et al., 1982). The 2D spectra were acquired with 4096
points in $t_2$, 256 points in $t_1$, and a spectral width of 6250 Hz in both dimensions. For each $t_1$ value, 32 transients were collected with a relaxation delay of 2 s. The time domain in $t_1$ was expanded to 1024 points by zero filling giving 6 Hz resolution in $t_1$ and 3 Hz resolution in $t_2$. The sample used to collect the NOESY spectra contained 0.5 mM RBG200 DHFR, 9 mM NADP+, 50 mM potassium phosphate buffer, pH* 5.95, in $^2$H$_2$O. Following data collection, the 2D data sets were transferred to a MicroVax II for processing using the FTNMR program (Hare Research Inc.). The NOESY data sets were processed using a 45° phase-shifted sine bell in $t_2$, and a Kaiser window in $t_1$ to avoid truncation effects (Hamming 1983). The first $t_1$ value was multiplied by 0.5 to attenuate $t_1$ ridges in the spectra (Otting et al., 1986). The use of these window functions was shown not to alter the relative magnitude of the NOE cross-peaks by comparing intensity ratios of isolated NOE cross-peaks from the same data sets processed with different window functions. Intensities of the cross peaks were estimated by measuring volume integrals using FTNMR (Banks et al., 1989; Nerdal et. al., 1989). FTNMR sums all points within the operator specified square footprint.

The time dependence of the NOE buildup curves were used to separate primary NOEs from secondary or spin diffusion effects (Rosevear and Mildvan, 1989). Secondary or spin diffusion effects arising from the indirect transfer of magnetization exhibit a lag in their time dependent NOE build-up. NOEs resulting from such effects cannot be used directly to determine internuclear distances. Primary NOEs, as judged from the time dependence of the NOE build-up, were used to estimate internuclear distances. The initial slopes of the time dependent NOE build-up were determined from the linear coefficient of a second order polynomial fit to the experimental NOE
intensities (Hyberts and Wagner, 1989; Baleja et al., 1990). All experimental NOE intensities were utilized in the calculation up to and including the maximum cross-peak intensity for any internuclear pair (Hyberts and Wagner, 1989). Distances were estimated using equation II.8, where $R_{\text{ref}}$ and $R_{ij}$ are the initial slopes, determined from the time dependence of the NOE, of the known ($R_{\text{ref}}$) and unknown ($R_{ij}$) interproton distances, respectively.

$$\frac{R_{\text{ref}}}{R_{ij}} = \frac{r_{\text{ref}}^6}{r_{ij}^6} \quad \text{(II.8)}$$

The fixed nicotinamide HN4 to HN5 distance, 2.48 Å, and the fixed ribose H1' to ribose H2' distance, 2.9 ± 0.2 Å (Rosevear and Mildvan, 1989), were used as reference distances to estimate unknown distances, $r_{ij}$, in the NMN and AMN portions of NADP⁺, respectively. The use of equation II.8 assumes isotropic rotation of the molecule as a whole and makes the assumption that all internuclear vectors in either the NMN or AMN portion of NADP⁺ have the same correlation time (Rosevear and Mildvan, 1989).

14.7. Qualitative Evaluation of the NADP⁺ conformation in the ternary complex RBG200 DHFR•NADP⁺•Folate. The conformation of NADP⁺ in the ternary complex with RBG200 DHFR and folate was qualitatively evaluated by comparing the pattern and intensities of the NOE cross-peaks in phase-sensitive NOESY spectra of NADP⁺ in the presence of RBG200 DHFR samples saturated with folate, with the NOEs previously observed in the RBG200 DHFR•NADP⁺ binary complex. RBG200 DHFR samples in 50 mM potassium phosphate, pH 6.0, in $^2$H₂O, were initially titrated with folate. A stock solution of folate was prepared in water and its pH adjusted to 6.0. The concentration was measured by spectrophotometry at 350 nm using an extinction coefficient of 7000 M⁻¹cm⁻¹ (Dawson et al., 1986),
and the stock solution divided in 1 equivalent portions and lyophilized. These lyophilized portions of folate were then used in the titration. After four equivalents of folate were added to the RBG200 DHFR sample, the ternary complex was formed by addition of 8 mM NADP⁺.

NOESY spectra at several temperatures and mixing times were acquired with 2048 points in $t_2$, 450 points in $t_1$, 32 transients per $t_1$ value, and a spectral width of 6250 Hz in both dimensions. The NOESY spectra were transferred to a Silicon Graphics Personal Iris workstation and processed with a 90° phase-shifted sine bell squared function in both $t_2$ and $t_1$, using the program FELIX (Hare Research, Inc). The first $t_1$ value was multiplied by 0.5 to attenuate $t_1$ ridges in the spectra (Otting et al., 1986). The time domain in $t_1$ was zero filled to 1024 points to obtain a final square matrix of 1024×1024 real points.

15. Distance Geometry and Structure Determination

Internuclear distances, estimated from the time dependence of the NOE, were used in defining upper and lower bound distance constraints in distance geometry calculations to determine the conformations of the NMN and AMN moieties of NADP⁺ bound to RBG200 DHFR (Kuntz et al., 1979; Havel et al., 1983; Rosevear and Mildvan, 1989). Interproton pairs yielding an estimated distance, using equation II.8, of $<3.6$ Å were given an upper bound distance constraint of $<3.6$ Å. Pairs of protons giving internuclear distances $>3.6$ Å were given lower bound distance constraints of $>3.6$ Å and upper bound distance constraints of $<5.0$ Å. Upper and lower bound distance
constraints were used, in lieu of absolute distances, to minimize errors introduced into the distances from inaccuracies in measurement of initial NOE build-up rates and estimation of correlation times. Since no NOEs were observed between the NMN and AMN moieties of NADP+, we modeled the bound conformations of the NMN and AMN moieties of NADP+ separately. In the conformational search procedure, each atom of either the NMN or AMN moiety is represented by a point at a known distance from the other atoms. If the distance between any two nonbonded atoms is unknown, the lower bounds are set to the sum of the van der Waals radii, and the upper bounds set at 50 Å to permit the conformation of the molecule to vary freely. Bond lengths and bond angles, expressed as 1-2 and 1-3 distances, respectively, were allowed to vary by 0.01 Å and 0.1 Å. All dihedral angles were expressed as 1-4 distances and allowed to vary by 1.8 Å to permit a complete conformational search. The planarity of the nicotinamide and adenine rings was maintained using a subroutine that minimizes the weighted sum of the squares of the distances through the atoms of the ring (Rosevear et al., 1983). The correct chirality of the asymmetric atoms was maintained by addition of a penalty term based on quantitative deviations from the chiral volume (Rosevear et al., 1983). All internuclear distance constraints, in the form of upper and lower distance bounds, were subjected to a smoothing procedure based on triangle inequalities (Crippen, 1981). Random distances between the upper and lower bounds are chosen and an embedding procedure utilized to reduce the problem from distance space to three-dimensional space. The embedded structures are then refined using steepest descent and conjugate gradient minimization. Conjugate gradient minimization is continued until the distance violations in the embedded structures are reduced to the
associated input errors. Usually, less than 500 iterations were required to reach suitable low error structures. The fit of each computed structure to the input data is expressed quantitatively by a score that measures the fourth power of the total deviations of the computed and input distances, \( < 1 \ \text{Å}^4 \). This low error deviation was consistent with violations no greater than 1% in the bond lengths and bond angles of the computed structures, and observance of planarity of the aromatic rings, and the correct chirality of asymmetric carbons. To effectively search the conformational space consistent with the stereochemical and experimental restraints, 400 structures were calculated. Only the structures that satisfied the experimental constraints were kept. No attempt was made to further refine the calculated structures since enzymes do not necessarily bind low-energy substrate conformations (Mildvan, 1981; Fersht, 1987).

The glycosidic torsional angle \( (\chi) \) was evaluated for all the final computed structures of the NMN and AMN moieties of NADP\(^+\). For NMN, \( \chi \) is defined by atoms O4'-C1'-N1-C2, and for AMN \( \chi \) is defined by atoms O4'-C1'-N9-C4 (Saenger, 1984). The glycosidic torsion angle \( (\chi) \) defines the orientation of the base relative to the sugar moiety. Two main orientations about the glycosidic torsion bond, called \textit{syn} and \textit{anti}, can be adopted. In an \textit{anti} conformation, the bulk of the base points away from the ribose, and in a \textit{syn} conformation the base points toward the ribose (Figure III.10) (Saenger, 1984).
Chapter III. RESULTS.

1. RBG200 DHFR Purification and Biochemical Characterization.

1.1. Protein Purification

The purification procedure for RBG200 DHFR from *E. coli* C600 cells carrying the plasmid pRBG200 is summarized in Table III.1 and the SDS-polyacrylamide gel electrophoresis monitoring the purification from crude extracts is shown in Figure III.1. The overall purification from 10 g of cell paste (20 g of 1:1 mixture of cells and Tris/sucrose buffer) is 31 mg of apparently homogeneous enzyme, representing a 40% yield. RBG200 DHFR elutes from the DEAE-Sepharose column at approximately 0.15 M NaCl (Figure III.2, fractions 55-66). This behavior closely mimics that observed for R388 DHFR, where the protein is eluted from a DEAE-Cellulose column at 0.16 M NaCl (Broad and Smith, 1982). The purity of the enzyme was constant across the elution peak, as judged by SDS-polyacrylamide gel electrophoresis. An SDS-polyacrylamide gel of the pooled fraction from the DEAE-Sepharose column revealed one major band (>95%) corresponding approximately to the predicted molecular weight of 8855 dalton for RBG200 DHFR (Figure III.1). The specific activity of RBG200 DHFR, 2.8 units/mg, is significantly higher than the specific activity previously reported for the similar R388 enzyme, 1.5 units/mg (Zolg and Hänggi, 1981). The increased specific activity of RBG200 DHFR, a derivative of the R388 enzyme, most likely results from the more gentle purification procedure used to purify the overproduced protein. The
Table III.1: Purification of RBG200 DHFR.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (Units)</th>
<th>Sp. Activity (Units/mg)</th>
<th>X-fold Purification</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Lysate</td>
<td>671</td>
<td>217</td>
<td>0.3</td>
<td>--</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>378</td>
<td>206</td>
<td>0.5</td>
<td>1.7</td>
<td>95</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>31</td>
<td>86</td>
<td>2.8</td>
<td>9.3</td>
<td>40</td>
</tr>
</tbody>
</table>
Figure III.1: SDS-polyacrylamide gel electrophoresis monitoring the purification of RBG200 DHFR from *E. coli* C600 cells carrying the plasmid pRBG200. (Lanes 1 and 5) Molecular weight standards: bovine serum albumin (68 kDa), hen egg albumin (45 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa). (Lane 2) Supernatant from streptomycin sulfate precipitation. (Lane 3) Dialyzed fraction from the 35-50% ammonium sulfate cut. (Lane 4) RBG200 DHFR eluted from the DEAE-Sepharose column. The 20% polyacrylamide gel was stained with Coomassie Brilliant Blue.
Figure III.2: Ion exchange chromatography of RBG200 DHFR on DEAE-Sepharose. The dialyzed 35-50% ammonium sulfate fraction was applied to a 2.5 X 45 cm DEAE-Sepharose fast flow column equilibrated in TME buffer and washed in TME buffer. RBG200 DHFR was eluted with a 1.6 l gradient from 0 to 0.5 M NaCl in TME buffer. A flow rate of 40 ml/h was maintained throughout and 8 ml fractions were collected. RBG200 DHFR elutes at approximately 0.15 M NaCl with a specific activity of 2.8 units/mg.
Zolg and Hänggi (1981) purification procedure included a step where the enzyme is denatured by boiling in 6 M guanidinium hydrochloride and passed over a gel filtration column in 6 M urea. RBG200 DHFR purified using the procedure of Zolg and Hänggi (1981) was also found to have a lower specific activity, approximately 1.5 units/mg. Thus, it is possible that once the type II DHFR is partially unfolded, it does not re-fold completely to its native conformation. RBG200 DHFR purified by the acid and base precipitation procedure (Vermersch et al., 1986; Vermersch, 1988) also showed significantly lower specific activity, between 0.5 and 1 units/mg. Protein concentrations used to calculate these specific activities were estimated by the method of Bradford (1976).

With the overproduction of RBG200 DHFR and the simplified purification scheme, large amounts of RBG200 DHFR can be prepared for biochemical and biophysical studies aimed at understanding the unique properties of this protein. In addition, this type of purification scheme will help in the efficient incorporation of stable isotopes into RBG200 DHFR for use in detailed magnetic resonance studies. A major advantage of RBG200 DHFR is its greatly increased solubility (> 25 mg/ml) in 50 mM potassium phosphate, 50 mM KCl, pH 6.0, when compared to the solubility of R67 DHFR (< 8 mg/ml) in the same buffer.

1.2. Extinction Coefficient

The extinction coefficient at 280 nm (ε_{280}) for RBG200 DHFR estimated from the aromatic amino acid composition is equal to 1.73 mg^{-1}\cdot ml\cdot cm^{-1} (see section II.1.3.1.).
Using the ratio between the absorbance at 280 nm and 205 nm and the semi-empirical expression (see section II.1.3.2.):

\[ \varepsilon_{205} \text{ (mg}^{-1} \cdot \text{ml} \cdot \text{cm}^{-1}) = 27.0 + 120 \times (A_{280}/A_{205}) \]

the extinction coefficient at 205 nm for RBG200 DHFR was determined to be 34.1 mg\(^{-1}\)ml\(\cdot\)cm\(^{-1}\). This value was obtained as an average of 5 independent measurements of absorbance at 205 nm and 3 independent measurements of absorbance at 280 nm. From the extinction coefficient at 205 nm and the absorbance ratio at 280 nm and 205 nm, an extinction coefficient at 280 nm for RBG200 DHFR equal to 2.00 mg\(^{-1}\)ml\(\cdot\)cm\(^{-1}\) was determined.

If a simple average value for the extinction coefficient at 205 nm equal to 31.0 mg\(^{-1}\)ml\(\cdot\)cm\(^{-1}\), instead of 34.1 mg\(^{-1}\)ml\(\cdot\)cm\(^{-1}\), is assumed (Scopes, 1974), then taking the experimentally obtained ratio \(A_{280}/A_{205} = 0.05885\), we have for the extinction coefficient of RBG200 DHFR at 280 nm the value 1.82 mg\(^{-1}\)ml\(\cdot\)cm\(^{-1}\). Shown in Figure III.3 is the absorbance spectrum of RBG200 DHFR in 50 mM potassium phosphate, pH 6.0.

Based on the absorbance at 280 nm in presence of 6 M guanidinium hydrochloride and the aromatic residue composition of RBG200 DHFR (see section II.1.3.3.), we obtain \(\varepsilon_{280} = 1.87 \text{ mg}^{-1} \cdot \text{ml} \cdot \text{cm}^{-1}\). This value was obtained from 4 independent measurements in presence of 6 M guanidinium hydrochloride and 3 independent measurements in the absence of guanidinium hydrochloride. After subtraction of the appropriate blanks, no differences were observed between samples that were boiled 15 minutes in presence of 6 M guanidinium hydrochloride and samples that were not boiled.
Figure III.3: Absorbance spectrum of RBG200 DHFR in 50 mM potassium phosphate, pH 6.0.
Taking into account the methods used and the values presented above, we will use as extinction coefficient of RBG200 DHFR at 280 nm ($\varepsilon_{280}$) the value $1.8 \text{ mg}^{-1} \cdot \text{ml} \cdot \text{cm}^{-1}$.

1.3. pH-Rate Profile

The pH versus activity profile of RBG200 DHFR is shown in Figure III.4. RBG200 DHFR has a sharp pH optimum at approximately 5.9, similar to that observed for R388 DHFR (Amyes and Smith, 1976). At pH 7.5 the enzyme exhibits approximately 25% of its maximal activity. However, at pH 5.0, less than a pH unit from the pH of maximal activity, RBG200 DHFR exhibits only 5% of its maximal activity. The sharp decrease in activity over approximately 0.9 pH units may result from protonation of a functional group essential in binding, catalysis or in maintaining the proper quaternary or tertiary structure of the enzyme. This behavior is in contrast with the *E. coli* chromosomal DHFR which exhibits a broad pH optimum between pH 6 and 8 (Mathews and Sutherland, 1965; Amyes and Smith, 1976; Beard et al., 1989).

1.4. Native Molecular Weight

1.4.1. HPLC gel filtration. The native molecular weight of RBG200 DHFR was determined by HPLC gel filtration at pH 6.0 and pH 5.0, at two different ionic strengths and over a wide range of protein concentrations
Figure III.4: pH versus activity profile for RBG200 DHFR. The percentage of RBG200 DHFR activity is shown at different pH values. Potassium phosphate buffer, 50 mM, containing 1 mM EDTA and 1 mM DTT was used for the pH range 4.2 to 7.9. The pH was measured prior to, and immediately after, monitoring the absorbance change of reaction using a microelectrode and found to vary less than ±0.05 unit. Each data point is the average of three separate assays.
(Figure III.5; Table III.2). Figure III.5 shows a clear concentration dependence of the apparent molecular weight of RBG200 DHFR. A single peak with an apparent molecular weight between 27.7 kDa and 29.7 kDa is observed at pH 6.0 for protein subunit concentrations approximately between 2 μM and 40 μM (Figure III.5; Figure III.6; Table III.2). At pH 5.0, this range of apparent molecular weights is extended to subunit concentrations as high as 2.0 mM (17.4 mg/ml). From the subunit molecular weight calculated from the amino acid sequence of RBG200 DHFR (Vermesch et al., 1986) and equal to 8855 Da, a molecular weight between 27.7 and 29.7 kDa corresponds to 3.1 to 3.4 subunits.

Previous gel filtration studies with R388 and R67 DHFR predicted a tetrameric structure for the type II DHFRs (Amyes and Smith, 1974; Pattishall et al., 1977; Smith et al., 1979; Zolg and Hänggi, 1981). Additionally the crystal structure of a dimeric form of R67 DHFR has been reported (Matthews et al., 1986). Thus the apparent molecular weight corresponding to slightly more than a trimer, observed between subunit concentrations of 2 μM and 40 μM at pH 6 and above 2 μM at pH 5, could be the result of a pure tetramer slightly retarded by interactions between the protein and the column matrix, or could be the result of a fast equilibrium between a dimer and a tetrameric form of RBG200 DHFR. To try to address this question we made use of other techniques such as laser desorption mass spectroscopy and analytical ultracentrifugation which we will discuss in the next section. The laser desorption mass spectroscopy results were inconclusive because we were unable to identify, even at high protein concentrations, any large amount of RBG200 DHFR species larger than the dimer, which is inconsistent with the HPLC and the analytical ultracentrifugation data. In fact, in all conditions
Figure III.5: Apparent native molecular weight of RBG200 DHFR determined by HPLC gel filtration as a function of the protein subunit concentration, at different pH and ionic strengths. Protein solutions were prepared and the gel filtration column was equilibrated in 50 mM potassium phosphate, 150 mM potassium chloride, pH 6.0 (▲); 50 mM potassium phosphate, 50 mM potassium chloride, pH 6.0 (○); and 50 mM potassium phosphate, 150 mM potassium chloride, pH 5.0 (□).
Table III.2: Apparent Molecular Weight for RBG200 DHFR as a Function of Protein Concentration, pH and Salt Concentration.

<table>
<thead>
<tr>
<th>DHFR Subunit Concentration (µM)</th>
<th>Apparent Molecular Weight (kDa)</th>
<th>Apparent Subunit Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. 50 mM potassium phosphate, 150 mM potassium chloride, pH 6.0</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 e-1</td>
<td>26.5</td>
<td>3.0</td>
</tr>
<tr>
<td>4.4 e-1</td>
<td>26.9</td>
<td>3.0</td>
</tr>
<tr>
<td>1.8 e+0</td>
<td>27.7</td>
<td>3.1</td>
</tr>
<tr>
<td>3.5 e+0</td>
<td>27.8</td>
<td>3.1</td>
</tr>
<tr>
<td>1.8 e+1</td>
<td>28.6</td>
<td>3.2</td>
</tr>
<tr>
<td>4.4 e+1</td>
<td>29.7</td>
<td>3.4</td>
</tr>
<tr>
<td>8.9 e+1</td>
<td>31.0</td>
<td>3.5</td>
</tr>
<tr>
<td>1.8 e+2</td>
<td>32.6</td>
<td>3.7</td>
</tr>
<tr>
<td>3.0 e+2</td>
<td>34.6</td>
<td>3.9</td>
</tr>
<tr>
<td>4.4 e+2</td>
<td>36.1</td>
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</tr>
<tr>
<td>8.9 e+2</td>
<td>39.0</td>
<td>4.4</td>
</tr>
<tr>
<td>1.8 e+3</td>
<td>41.5</td>
<td>4.7</td>
</tr>
<tr>
<td><strong>B. 50 mM potassium phosphate, 150 mM potassium chloride, pH 5.0</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 e-1</td>
<td>24.2</td>
<td>2.7</td>
</tr>
<tr>
<td>4.9 e-1</td>
<td>26.7</td>
<td>3.0</td>
</tr>
<tr>
<td>2.0 e+0</td>
<td>27.7</td>
<td>3.1</td>
</tr>
<tr>
<td>4.9 e+1</td>
<td>28.3</td>
<td>3.2</td>
</tr>
<tr>
<td>4.9 e+2</td>
<td>28.3</td>
<td>3.2</td>
</tr>
<tr>
<td>2.0 e+3</td>
<td>28.2</td>
<td>3.2</td>
</tr>
<tr>
<td><strong>C. 50 mM potassium phosphate, 50 mM potassium chloride, pH 6.0</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0 e-1</td>
<td>26.7</td>
<td>3.0</td>
</tr>
<tr>
<td>1.1 e+0</td>
<td>27.4</td>
<td>3.1</td>
</tr>
<tr>
<td>2.1 e+0</td>
<td>28.6</td>
<td>3.2</td>
</tr>
<tr>
<td>4.2 e+0</td>
<td>28.9</td>
<td>3.3</td>
</tr>
<tr>
<td>2.1 e+1</td>
<td>29.6</td>
<td>3.3</td>
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<tr>
<td>1.1 e+2</td>
<td>30.7</td>
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<td>2.1 e+2</td>
<td>31.7</td>
<td>3.6</td>
</tr>
<tr>
<td>5.3 e+2</td>
<td>33.2</td>
<td>3.7</td>
</tr>
<tr>
<td>2.1 e+3</td>
<td>38.9</td>
<td>4.4</td>
</tr>
</tbody>
</table>
Figure III.6: HPLC gel filtration elution profiles of RBG200 DHFR as a function of protein concentration and pH. The experiments were performed in 50 mM potassium phosphate, 150 mM potassium chloride, pH 6.0 (A) or pH 5.0 (B). RBG200 DHFR subunit concentrations are as follows: 1.8 mM (A-i), 44 µM (A-ii), 1.8 µM (A-iii), 0.18 µM (A-iv), 2.0 mM (B-i), 49 µM (B-ii), 2.0 µM (B-iii), 0.20 µM (B-iv). Some retention times are indicated at the top of the peaks. Other experimental details are described in section II.6.
A

pH 6.0

(i) 18.34

(ii) 19.87

(iii) 20.19

(iv) 6.32

Retention Time (min)
B
pH 5.0

(i)

(ii)

(iii)

(iv)

Retention Time (min)
tested the monomer was the dominant species. This observation could be the result of unavailability of free amino groups sufficiently close to the subunit interfaces in order for the glutaraldehyde to cross-link the subunits (section II.7).

At protein subunit concentrations above 40 μM and pH 6.0, the apparent molecular weight increases significantly with increasing protein concentration. For example, at a subunit concentration of 1.8 mM, which was the highest protein concentration tested at pH 6.0 in 50 mM potassium phosphate and 150 mM potassium chloride, the apparent molecular weight is 41.5 kDa which corresponds to 4.7 subunits (Figure III.5; Table III.2). Thus, at high protein concentrations and pH 6.0 there are protein species in solution larger than the tetramer. However such aggregates are not observed at pH 5.0, even at subunit concentrations as high as 2.0 mM.

At protein subunit concentrations below 2 μM, the apparent molecular weight of RBG200 DHFR decreases as judged by the retention time of the main peak whose apparent molecular weight is shown in Figure III.5. The change in retention time of the main peak is accompanied by a decrease in the size of the this peak, and the appearance of a series of multiple broad peaks. At very low concentrations (≤ 0.2 μM subunit) no defined main peak is observed and there is a series of broad peaks some of which have retention times even longer than what would be expected for the RBG200 DHFR monomer (Figure III.6). This result indicates the presence, at low protein concentrations, of RBG200 DHFR species which interact with the column matrix or are in some other way retarded. It is possible that at low protein concentrations some partial unfolding of the species in solution could occur. To test this hypothesis we measured the fluorescence emission intensity at
Figure III.7: (A) Fluorescence emission spectrum of RBG200 DHFR at a subunit concentration of 6.5 μM. (B) Fluorescence emission intensity as a function of RBG200 DHFR subunit concentration. Emission was measured at 342 nm with excitation at 280 nm in 50 mM potassium phosphate, 50 mM potassium chloride, pH 6.0.
342 nm (emission maximum) using an excitation wavelength of 280 nm (Figure III.7). Unfolding of the polypeptide chain would lead to a change in the environment of the tryptophan and tyrosine residues which should lead to changes in the intensity of fluorescence emission. However, between subunit concentrations of 6.5 μM and 6.5 nM, the fluorescence emission intensity decreased linearly with the protein concentration (Figure III.7B), suggesting that the environment of tryptophan and tyrosine residues is not drastically affected between subunit concentrations of 6.5 μM and 6.5 nM. A more sensitive tool to study possible environment changes of the aromatic residues as a function of protein concentration, is fluorescence anisotropy which should be tried in the future. We also tried to investigate possible protein unfolding by circular dichroism but due to the large percentage of β-sheet present in type II DHFRs, as revealed by the crystal structure of R67 DHFR (Matthews et al., 1986), and consequently the low CD signal, it was not possible to obtain spectra below subunit concentrations of 6.5 μM.

The RBG200 DHFR enzyme assay is routinely carried out at subunit concentrations in the range of 1 to 2 μM. At these concentrations there is no significant difference in the apparent molecular weight of RBG200 DHFR at pH 6.0, where the enzyme is maximally active, and pH 5.0, where the enzyme loses 95% of the activity (Table III.2; Figure III.4). Thus, from these gel filtration experiments it appears that the loss of enzyme activity between pH 6.0 and pH 5.0 is not due to drastic alterations of the quaternary structure of the enzyme.

To test if RBG200 DHFR behaved differently than other type II DHFRs, we purified R67 DHFR and compared the apparent molecular weight of RBG200 DHFR and R67 DHFR at pH 5, 6 and 8 (Table III.3). Qualitatively both
Table III.3: Comparison of the Apparent Molecular Weight of RBG200 DHFR and R67 DHFR at Several pH Values.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition(^{(a)})</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 mM KPi</td>
<td>50 mM KPi</td>
<td>50 mM KPi</td>
</tr>
<tr>
<td></td>
<td>150 mM KCl</td>
<td>pH 5.0</td>
<td>pH 6.0</td>
</tr>
<tr>
<td>RBG200</td>
<td>27.2</td>
<td>30.7(^{(c)})</td>
<td>48.4</td>
</tr>
<tr>
<td>Apparent</td>
<td>DHFR</td>
<td>28.9(^{(d)})</td>
<td></td>
</tr>
<tr>
<td>Molecular</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight</td>
<td>R67</td>
<td>22.6</td>
<td>26.1</td>
</tr>
<tr>
<td>(kDa)</td>
<td>DHFR</td>
<td>23.4(^{(e)})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RBG200</td>
<td>3.1</td>
<td>3.5(^{(c)})</td>
</tr>
<tr>
<td>Apparent</td>
<td>DHFR</td>
<td>3.3(^{(d)})</td>
<td></td>
</tr>
<tr>
<td>Subunit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number(^{(b)})</td>
<td>R67</td>
<td>2.7</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>DHFR</td>
<td>2.8(^{(e)})</td>
<td></td>
</tr>
</tbody>
</table>

(a) KPi, potassium phosphate; KCl, potassium chloride.

(b) Subunit molecular weights for RBG200 DHFR and R67 DHFR are 8855 and 8444, respectively.

(c), (d) and (e) Values obtained at subunit concentrations of 110 μM, 4.2 μM and 4.6 μM, respectively. All other values were obtained at a subunit concentration of 80 μM.
proteins behave similarly in the conditions tested. Thus, the complex solution behavior of RBG200 DHFR is not a consequence of the presence of the six extra amino acid residues in its carboxy terminus, but apparently a characteristic of all type II DHFRs.

1.4.2. Analytical Ultracentrifugation. Preliminary analytical ultracentrifugation experiments for RBG200 DHFR were performed at only one loading protein concentration (12 μM subunit concentration) and in 50 mM potassium phosphate, 50 mM potassium chloride, pH 6.0. This protein concentration was chosen because it corresponds to the middle of the plateau in the HPLC versus protein concentration profile (Figure III.5). Figure III.8 shows the concentration distribution of protein, in absorbance units, as a function of radial position in the ultracentrifuge cell. The two large panels in Figure III.8 represent the fit of the experimental data to two different reversible associating models. The two small panels in Figure III.8 show the distribution of the residuals as a function of the radial position for each one of the fitting models. The models and equations to which the experimental data were fitted are described under Methods (section II.8). Panel A (Figure III.8) shows the data fit to an equilibrium between monomer, dimer and tetramer with association constants of $K_{a1} = 1.35 \times 10^4$ M$^{-1}$ and $K_{a2} = 5.56 \times 10^6$ M$^{-1}$. Panel B (Figure III.8) shows the data fit to an equilibrium between monomer and dimer with an association constant $K_{a1} = 7.14 \times 10^7$ M$^{-1}$. It should be noted that the ΔAbs scale for the residual distribution is doubled in panel B (Figure III.8) and consequently the apparent deviations of the predicted from the experimental values are much larger in panel B (Figure III.8). Thus, it appears that under the solution conditions used and at RBG200 DHFR
Figure III.8: Concentration distribution and distribution of the residuals as functions of radial position for RBG200 DHFR at ultracentrifugal equilibrium at 28000 rpm and 20 °C, in 50 mM potassium phosphate, 50 mM potassium chloride, pH 6.0. The loading protein subunit concentration was 12 µM. Panel A shows the fit for a monomer-dimer-tetramer association equilibrium. Panel B shows the fit for a monomer-dimer association equilibrium.
subunit concentrations of 12 μM, the experimental data best fits an equilibrium between monomer, dimer and tetramer. Attempts to fit the experimental data to models involving a dimer-tetramer equilibrium or a single trimer resulted in much poorer fits. It should be noted however that the most stringent criterion for establishing the validity of a reversible associating model is the simultaneous fit of the model to experimental data involving more than one loading protein concentration (Becerra et al., 1991; Roark, 1976). Thus, the confirmation of the model here proposed waits further analytical ultracentrifugation experiments at different protein concentrations.

In order to better characterize the complex solution behavior of RBG200 DHFR, additional analytical ultracentrifugation experiments must be performed at different pHs, specially pH 5.0, different ionic strengths, different protein concentrations, and in the presence of substrate and coenzyme in order to evaluate the influence of ligand binding on the association equilibrium.

1.5. Hydride Transfer Stereospecificity

[4S-2H, 4R-1H]NADPH was synthesized from [4-2H]NADP+ using isocitrate dehydrogenase and isocitric acid (see section II.11). It is well established that isocitrate dehydrogenase transfers a hydrogen from the C2 of isocitric acid to the A face (pro-R position) of NADPH (Englard and Colowick, 1957; Nakamoto and Vennesland, 1960; Freudenthal et al., 1973). Additionally, the stereochemistry and isotopic purity of the synthesized
NADPH was confirmed by $^1$H NMR spectroscopy (Arnold et al., 1976; Seyama et al., 1977; Arnold and You, 1978).

The synthesized [4S-2H, 4R-1H]NADPH was incubated with dihydrofolate and RGB200 DHFR and the enzymatically oxidized NADPH isolated by ion-exchange chromatography. If the Type II plasmid DHFR transferred the A-side (pro-R) hydrogen, the isolated NADP$^+$ would contain deuterium at the N4 position. However, if the enzyme transferred the B-side (pro-S) hydrogen, the isolated NADP$^+$ would contain a proton at the N4 position. The $^1$H NMR spectrum of the aromatic region of commercial NADP$^+$ and NADP$^+$ isolated from incubation of [4S-2H, 4R-1H]NADPH with RGB200 DHFR are compared in Figure III.9. The isolated NADP$^+$ is shown to contain deuterium at N4 (Figure III.9A; Figure III.10). Therefore, RGB200 DHFR specifically removes the pro-R hydrogen leaving behind the pro-S deuterium. Chromosomal DHFRs are known to transfer the pro-R hydrogen from NADPH to dihydrofolate (Charlton et al., 1979). Thus, the stereochemistry of hydrogen transfer from NADPH to dihydrofolate by two evolutionarily distinct reductases is identical. This was predicted to be a general rule by Colowick et al. (1966) and shown to be correct for evolutionarily divergent sources of L-lactate dehydrogenase and malate dehydrogenase, both A-stereospecific enzymes (Arnold et al., 1976).

NMR experiments using the chromosomal DHFR and [4-2H]NADPH have previously shown that hydride ion transfer is to the C6 si face of the pteridine ring in dihydrofolate producing the biologically active isomer of tetrahydrofolate (Pastore and Friedkin, 1962). Under the growth conditions utilized, the E. coli strain C600 will not grow in the presence of trimethoprim without the plasmid carrying the gene for RGB200 (Vermersch et al., 1986).
Figure III.9: Comparison of the aromatic region of the 500 MHz $^1$H-NMR spectrum of (A) NADP$^+$ isolated from the oxidation of $[4R^{-1}H, 4S^{-2}H]$NADPH by RBG200 DHFR and (B) commercial NADP$^+$. The nicotinamide protons are labeled N2, N6 and N4. The adenine H8 proton is labeled A8.
Figure III.10: Numbering system for proton identification in NADP$^+$ and NADPH (insert). Protons from the AMN and NMN moieties of NADP$^+$ are labeled with A and N, respectively. NADP$^+$ is drawn with the adenosine in the anti conformation and the nicotinamide in the syn conformation. The pro-R and pro-S hydrogens of NADPH are labeled H$_R$ and H$_S$, respectively.
Therefore, the only source of tetrahydrofolate in the presence of trimethoprim is that synthesized by the type II DHFR encoded by the plasmid RBG200. Since the tetrahydrofolate synthesized by RBG200 DHFR is necessary for cell growth and only the C6 si isomer of tetrahydrofolate is biologically active (Charlton et al., 1979; Fontecilla-Champs et al., 1979), RBG200 DHFR transfers the C4 pro-R hydrogen of NADPH to the C6 si face of the pteridine ring of dihydrofolate.

2. Coenzyme Binding

2.1. Evidence for Two Interconverting RBG200 DHFR•NADP⁺ Binary Complexes

Addition of NADP⁺ to RBG200 DHFR followed by ¹H-NMR spectroscopy revealed two sets of well resolved resonances associated with NADP⁺ protons. Figure III.11(B-D) shows the nicotinamide HN2 and HN6 proton resonances of 5 mM NADP⁺ at several different time intervals after addition to 0.5 mM RBG200 DHFR. The presence of enzyme caused extensive broadening and upfield chemical shifts of both HN2 and HN6 nicotinamide resonances (Figure III.11B). Chemical shift changes and line broadening for the adenine and ribose resonances of NADP⁺ were also observed immediately upon addition to RBG200 DHFR. A second set of nicotinamide proton resonances were also observed downfield of the exchange broadened resonances having chemical shifts closer to those of the free coenzyme (Figure III.11B and E). After incubating NADP⁺ with RBG200 DHFR at 25°C
Figure III.11: HN2 and HN6 proton resonances of NADP⁺ in the presence of RBG200 DHFR showing the time dependent interconversion from the initial RBG200 DHFR•NADP⁺ binary complex (Conformation I) to the final binary complex (Conformation II), at 25 °C. The sample contained 0.5 mM RBG200 DHFR and 5 mM NADP⁺ in 50 mM potassium phosphate buffer in 2H₂O at pH* 5.9. (A) Portion of the downfield aromatic ¹H-NMR spectrum of RBG200 DHFR in the absence of NADP⁺. (B-D) Nicotinamide HN2 and HN6 proton resonances of 5 mM NADP⁺ immediately after addition to 0.5 mM RBG200 DHFR (B); after 1 hour incubation with RBG200 DHFR showing two sets of HN2 and HN6 proton resonances of the coenzyme (C); and after 10.5 hours incubation with RBG200 DHFR showing the HN2 and HN6 proton resonances of the coenzyme in the final RBG200 DHFR•NADP⁺ binary complex (D). (E) Nicotinamide HN2 and HN6 proton resonances of free NADP⁺ in solution under identical conditions. NMR spectra were obtained at 500 MHz by accumulating 32 transients with 16K data points each, a spectral width of 7042 Hz and a recycle time of 3 sec. Spectra were processed with an exponential multiplication with a line broadening of 2 Hz.
for 1 hour two distinct sets of HN2 and HN6 proton resonances are more clearly observed (Figure III.11C). Continued incubation for 10.5 hours results in the observation of just a single set of HN2 and HN6 proton resonances (Figure III.11D) with chemical shifts closer to those of the free coenzyme (Figure III.11E). This behavior is consistent with the slow time dependent conformational interconversion between two distinct binary RBG200 DHFR•NADP⁺ complexes. In the initial conformation, conformation I, the nicotinamide portion of the coenzyme is in an environment which produces upfield chemical shifts of 0.021, and 0.049 ppm in the HN2 and HN6 protons, respectively. The chemical shift changes in the nicotinamide proton resonances indicate that the oxidized nicotinamide ring does interact with RBG200 DHFR in a highly specific manner. In contrast, the chemical shift changes on coenzyme binding to the final binary conformation, conformation II, are smaller, 0.011 ppm, for both HN2 and HN6 and downfield from the resonances of the free coenzyme. However, in both conformation I and conformation II NADP⁺ is in fast exchange between the free and bound states and consequently the observed chemical shifts are weighted averages between the chemical shifts for the free and bound species. The broadening of the HN2 and HN6 resonances in conformation I, compared with conformation II, implies that the rate of dissociation of NADP⁺ in conformation I is slower than in conformation II.

The rate of interconversion of conformation I to conformation II was determined by measuring the area of the HN2 resonance in both conformations I and II as a function of time (Figure III.12). Figure III.12A shows the decrease in area of HN2 resonance in conformation I with the corresponding increase in area of the HN2 resonance in conformation II. The
Figure III.12: Kinetics of interconversion at 25 °C of the initial RBG200 DHFR•NADP+ binary complex (Conformation I) to the final binary complex (Conformation II). (A) The area under the HN2 resonance in the $^1$H-NMR spectrum was evaluated by integration and used to monitor the interconversion. (△) Area of the HN2 resonance in conformation I. (■) Area of the HN2 resonance in conformation II. (□) Sum of the area of HN2 in both conformation I and II. (B) Plot of the natural logarithm of the concentration of conformation I as a function of time. Sample conditions are those given in the legend of Figure III.11.
total intensity of the HN2 resonance from both conformations I and II remained constant throughout the time course of the interconversion indicating that a two state model of the interconversion process is adequate (Figure III.12A). A linear dependence of the natural logarithm of the concentration of conformation I as a function of time is observed indicating that the conformation interconversion follows a first order rate process with an apparent first order rate constant of $1.0 \times 10^{-4} \text{s}^{-1}$ at $25^\circ \text{C}$ (Figure III.12B). A similar experiment was carried out at $5^\circ \text{C}$ and an apparent first order rate constant of $9.3 \times 10^{-6} \text{s}^{-1}$ for the interconversion was determined.

The binding of NADP$^+$ and formation of the initial binary complex (conformation I) can also be detected by monitoring the chemical shifts of the upfield shifted methyl resonances of RBG200 DHFR (Figure III.13). Comparison of the upfield shifted methyl regions of RBG200 DHFR in the absence (Figure III.13A) and immediately after addition of NADP$^+$ (Figure III.13B) reveals several chemical shift changes in the methyl resonances upon addition of coenzyme and formation of binary conformation I. After 10.5 hours, time sufficient for complete interconversion of conformation I to II, at $25^\circ \text{C}$, a different pattern of chemical shifts for the methyl resonances is observed for the complex in conformation II (Figure III.13C). This pattern is more similar to that found for enzyme alone. However, the line widths of the methyl resonances are broader than found for enzyme alone due to the exchange contribution from the binding of NADP$^+$. Although conformational changes in the protein observed upon interconversion of the two complexes cannot yet be described in detail, alterations in the upfield-shifted methyl protons are most simply explained by altered geometric relationships of the methyl groups to aromatic rings of either the protein or
Figure III.13: $^1$H-NMR spectra of the upfield shifted methyl resonances in RBG200 DHFR showing the effect of NADP$^+$ addition and the time dependent interconversion from conformation I to II. Shown is the upfield shifted methyl region of RBG200 DHFR in the absence of NADP$^+$ (A), 9 min after the addition of 5 mM NADP$^+$ (B), and 10.5 h after the addition of NADP$^+$ (C). The sample contained 0.5 mM RBG200 DHFR and 5 mM NADP$^+$ in 50 mM potassium phosphate buffer in $^2$H$_2$O at pH 5.9. NMR spectra were obtained using conditions described in Figure III.11.
coenzyme. Chemical shift changes observed in this region are consistent with the formation of an initial binary RBG200 DHFR•NADP⁺ complex, involving an initial conformational alteration in the protein, which slowly converts to a second binary complex in which the coenzyme or the protein exists in a different environment or conformation. The existence of two or more conformational states have been detected in ternary complexes of chromosomal *L. casei* DHFR•trimethoprim•NADP⁺, but not in binary complexes with NADP⁺. (Hyde et al., 1980; Gronenborn et al., 1981).

2.2. NADP⁺ Stoichiometry and K_d by Equilibrium Dialysis

The number of binding sites and the dissociation constant (K_d) for NADP⁺ were estimated by equilibrium dialysis using [4-³H]NADP⁺. The determination of the stoichiometry for NADP⁺ binding to RBG200 DHFR entails special problems due to the low affinity of RBG200 DHFR for NADP⁺ and consequently the need to use very high protein concentrations, of the order of 50 mg/ml. Measurement of protein concentration by the Biuret method (Gornall et al., 1949) at the end of the dialysis yielded concentrations of 53 ± 3 mg/ml. Figure III.14 shows a Scatchard plot for the binding of [4-³H]NADP⁺ to RBG200 DHFR. The data points are significantly scattered which most probably is a consequence of the error associated with pipetting very viscous solutions. The best linear fit to the data yields a K_d of 1.5 mM and a stoichiometry of 0.95 coenzyme per RBG200 DHFR tetramer.
Figure III.14: Scatchard plot of $^3$H-NADP\(^+\) binding to RBG200 DHFR. The data were obtained by equilibrium dialysis of variable concentrations of $^3$H-NADP\(^+\) against 1.5 mM RBG200 DHFR (tetramer) in 50 mM sodium citrate, pH 6.0.
2.3. NADP⁺ Dissociation Constant (Kd) by NMR

Binding of NADP⁺ in conformation II of the binary complex RBG200 DHFR•NADP⁺ was also monitored by measuring the concentration dependent broadening of the nicotinamide HN2 resonance in the presence of RBG200 DHFR (Figure III.15). The change in line width as a function of coenzyme concentration was used to calculate the dissociation constant, Kd, for NADP⁺ binding to RBG200 DHFR. Nonlinear least squares analysis as detailed under Methods (section II.14.4) yielded a Kd = 1.9 ± 0.4 mM (Figure III.15), in good agreement with the value obtained by equilibrium dialysis using [4-³H]NADP⁺.

2.4. NADPH Stoichiometry by Equilibrium Dialysis

The number of binding sites for NADPH was determined by equilibrium dialysis measuring the equilibrium concentration of NADPH by spectrophotometry at 334 nm (ε = 6.178 x 10³ M⁻¹cm⁻¹; Ziegenhorn et al., 1976). Figure III.16 shows a plot of the concentration of bound NADPH, calculated as the difference between the concentrations of free and total NADPH, versus the concentration of total NADPH. The binding curve consists of an initial portion (below 0.3 mM total NADPH) showing a linear dependence between concentration of bound NADPH, and a second portion (above 0.3 mM total NADPH) where the amount of bound NADPH does not increase with increasing concentrations of total NADPH. This particular shape for a binding curve is characteristic of stoichiometric binding. In this case, the
Figure III.15: Titration of RBG200 DHFR with NADP⁺ measuring the concentration dependence of the line width of the nicotinamide HN2 resonance. The sample contained 0.4 mM RBG200 DHFR in 50 mM potassium phosphate at pH 5.9 and 25 °C. The curve is a nonlinear least-squares computer fit to the data using eqs II.5 and II.7 with three variables. The best fit was obtained with a $K_d = 1.9 \pm 0.4$ mM, assuming one NADP⁺ binding site per RBG200 DHFR tetramer.
Figure III.16: NADPH binding to RBG200 DHFR. The data were obtained by equilibrium dialysis of NADPH at several different concentrations against 0.23 mM RBG200 DHFR (tetramer) in 50 mM potassium phosphate, pH 7.0.
number of binding sites can be directly estimated from a plot such as the one in Figure III.16 by taking the abscissa of the intersection point between the two straight lines and dividing it by the protein concentration. This yielded a value of 1.3 NADPH binding sites per tetramer of RBG200 DHFR.

Taken together the estimated stoichiometries for NADP\(^+\) and for NADPH indicate that RBG200 DHFR binds one coenzyme molecule per tetramer of protein. This value would limit the smallest active species of RBG200 DHFR to a tetramer. However, it is possible that the experimentally observed stoichiometry for NADPH, slightly higher than one, could represent coenzyme binding to protein species smaller than the tetramer. An answer to this question has to await the complete characterization of the protein association equilibrium in the conditions used in the equilibrium dialysis in order to establish how much of the RBG200 DHFR is present in solution in the monomeric and dimeric forms.

2.5. NADPH Binding Monitored by NMR

Binding of NADPH to RBG200 DHFR was also monitored by NMR. Figure III.17 shows the aromatic region of the \(^1\text{H-}\text{NMR}\) spectra of RBG200 DHFR in the absence and presence of NADPH, in 50 mM potassium phosphate, pH 7.5, in \(^2\text{H}_2\text{O}\). This pH was used to decrease NADPH degradation, which is faster at acidic pH. The aromatic region of the \(^1\text{H-}\text{NMR}\) spectrum of 0.6 mM RBG200 DHFR at 20 °C shows a complex envelope of relatively broad resonances (Figure III.17A). Addition of 1.6 mM NADPH to the enzyme leads to broadening of the protein resonances, in particular the
Figure III.17: NADPH binding to RBG200 DHFR monitored by NMR. Aromatic region of the $^1$H-NMR spectrum of 0.6 mM RBG200 DHFR in 50 mM potassium phosphate, pH 7.5, at 20 °C, in the absence of NADPH (A), immediately after addition of 1.6 mM of NADPH (B), and approximately 36 hours after addition of 1.6 mM NADPH. Also shown is the same region of the $^1$H-NMR spectrum of 2.5 mM NADPH in 50 mM potassium phosphate, pH 7.5, at 20 °C. The dashed lines indicate resonances that are clearly perturbed by NADPH addition and conformation interconversion.
most upfield resonances, between 6.0 and 6.5 ppm, and the most downfield resonance, between 7.5 and 8.0 ppm, indicating interaction of the coenzyme with the protein (Figure III.17A, B, C). Immediately after addition of NADPH to RBG200 DHFR the coenzyme HA8 and HN6 resonances show extensive line broadening (Figure III.17B) when compared to the line widths of the resonances of free NADPH (Figure III.17D). After incubating NADPH with RBG200 DHFR at 4 °C for approximately 36 hours, analysis of the 1H-NMR spectrum at 20 °C reveals that the HA8 and HN6 resonances of the coenzyme are sharper, and that the HA1' and HN6 resonances are shifted upfield (Figure III.17C). This behavior is consistent with a slow conformational change of the environment of the reduced coenzyme at pH 7.5, similar to what was observed for the oxidized coenzyme at pH 6.0 (section III.2.1), where RBG200 DHFR is most active.

2.6. Conformation of bound NADP⁺ in the RBG200 DHFR•NADP⁺ Binary Complex

The conformation of NADP⁺ bound to RBG200 DHFR was investigated by measuring the time dependence of the transferred NOE in two dimensional phase-sensitive NOESY spectra, as detailed under Methods (section II.14.6). Figure III.18 shows an expansion of the contour plot from a 100 ms NOESY of 9 mM NADP⁺ in the presence of 0.5 mM RBG200 DHFR at 15° C, demonstrating selective NOEs between the ribose protons and the nicotinamide and adenine base protons. Assignments of the NADP⁺ resonances in the presence of RBG200 DHFR were obtained from DQF-COSY
Figure III.18: Contour plot of the 500 MHz phase-sensitive NOESY spectrum of NADP⁺ in the presence of RBG200DHFR, in the binary conformation II, at 15 °C. The sample contained 0.5 mM RBG200 DHFR and 9 mM NADP⁺ in 50 mM potassium phosphate buffer in ²H₂O, pH* 5.9, and was incubated at 25 °C until interconversion of conformation I to conformation II was completed. The spectrum was recorded with a 100 ms mixing time. Chemical shifts are referenced to external DSS. The labels indicate the assignments of the NOE cross-peaks.
Figure III.19: DQF-COSY of 10 mM NADP⁺ in 50 mM potassium phosphate, pH* 6.0, at 15 °C. Shown are two expanded spectral regions (A and B) corresponding to the NADP⁺ ribose protons with the assignments indicated. Chemical shifts are from internal DSS.
spectra (Figure III.19) and are consistent with the known chemical shifts for NADP+ (Oppenheimer, 1982). Large negative internuclear NOEs were observed between the HN2/HN1’, HN2/HN2’, and HN6/HN1’ proton pairs in the NMN portion of NADP+ (Figure III.18). Smaller NOEs were also observed between the HN6/HN2’ and HN2/HN3’ proton pairs (Figure III.18). In the adenosine moiety, a large NOE was observed between the HA8/HA2’ proton pair, with smaller NOEs between the HA8/HA1’ and HA8/HA3’ proton pairs (Figure III.18). Under identical conditions, in the absence of RBG200 DHFR, negative NOEs were not observed up to 500 ms mixing times. Thus, the observed NOEs in the presence of RBG200 DHFR arise from cross-relaxation between protons of NADP+ in the enzyme-bound state and can be used to evaluate the bound conformation of the coenzyme.

The time dependence of the NOE was measured to separate primary NOEs from secondary or spin diffusion effects (Figure III.20). Secondary or spin diffusion effects are characterized by a longer lag in the development of the NOE build-up curve than observed for primary effects, resulting from the time required to transfer magnetization via intermediate spins (Rosevear and Mildvan, 1989). From the time dependence of the NOE build-up curve, primary NOEs were observed between the HN2/HN1’, HN2/HN2’, and HN2/HN3’ proton pairs (Figure III.20A). No significant NOE was observed between the HN2/HN4’ proton pair. Figure III.20B shows the time dependent NOE build-up curves for NOEs involving the nicotinamide HN6 proton. A large primary NOE was observed between HN6 and HN1’ (Figure III.20B). A small primary NOE was also observed between HN6 and HN2’. The NOEs between the HN6/HN3’ and HN6/HN4’ proton pairs were judged to be secondary due to the lag in the NOE build-up curve (Figure III.20B).
Figure III.20: Plots of the NOESY cross-peak intensities as a function of the mixing time at 15° C. (A) NOE build-up curves are shown for HN2/HN1' (■), HN2/HN2' (△), HN2/HN3' (□), and HN2/HN4' (▲) proton pairs. (B) NOE build-up curves are shown for HN6/HN1' (■), HN6/HN2' (▲), HN6/HN3' (□), and HN6/HN4' (△) proton pairs. (C) NOE build-up curves are shown for HA1'/HA2' (■), HA8/HA1' (□), HA8/HA2' (△), and HA8/HA3' (▲) proton pairs. The curves are second order polynomial fits to the experimental data. Experimental conditions are given in Figure III.18.
Primary NOEs were also observed between the HN4/HN5 and HN5/HN6 proton pairs as expected since the distance between these proton pairs is fixed at 2.48 Å (Clore and Gronenborn, 1983). This pattern of primary NOEs is only consistent with a syn conformation about the nicotinamide - ribose glycosidic bond for enzyme-bound NADP\(^+\) (Figures III.10, III.20A and B).

Primary NOEs, as judged from the time dependence of the NOE build-up curves, were also observed between the HA8/HA1', HA8/HA2', HA8/HA3' and the HA1'/HA2' proton pairs (Figure III.20C). The large primary NOE between the HA8/HA2' proton pair and the smaller primary NOEs between HA8/HA1' and HA8/HA3' proton pairs confine the adenine-ribose glycosidic torsional angle of the AMN moiety of the enzyme-bound NADP\(^+\) to an anti conformation.

The lack of internuclear NOEs between the adenine and nicotinamide bases of enzyme-bound NADP\(^+\), suggests that NADP\(^+\) is bound in an open conformation with the adenine and nicotinamide rings separated by at least 5.0 Å. Previously, a NOE between the HA2 proton and the HN2 or HN6 protons was used to demonstrate that NADPH exists in a folded conformation when bound to isocitrate dehydrogenase (Ehrlich and Colman, 1985).

Initial slopes of the primary NOEs were calculated from the linear coefficient of a second order polynomial fit to the experimental NOE intensities as described under Methods (Hyberts and Wagner, 1989; Baleja et al., 1990). Internuclear distances (Table III.4), within the NMN and AMN moieties of enzyme-bound NADP\(^+\), were estimated from the initial slopes using the fixed nicotinamide HN4 to HN5 distance, 2.48 Å, and the fixed ribose H1' to ribose H2' distance, 2.9 ± 0.2 Å, as reference distances in equation...
**Table III.4:** Estimated Distances and Upper and Lower Bound Distance Constraints Derived from the NOE build-up Rates for NADP⁺ Bound to RBG200 DHFR in the Binary Complex II.

<table>
<thead>
<tr>
<th>Spin pair (AB)</th>
<th>( r_{AB}(\text{Å})^a )</th>
<th>Lower bound (Å)</th>
<th>Upper bound (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN2/HN1'</td>
<td>3.0</td>
<td>vdw(^b)</td>
<td>&lt; 3.6</td>
</tr>
<tr>
<td>HN2/HN2'</td>
<td>2.7</td>
<td>vdw</td>
<td>&lt; 3.6</td>
</tr>
<tr>
<td>HN2/HN3'</td>
<td>4.7</td>
<td>&gt;3.6</td>
<td>&lt; 5.0</td>
</tr>
<tr>
<td>HN6/HN1'</td>
<td>2.5</td>
<td>vdw</td>
<td>&lt; 3.6</td>
</tr>
<tr>
<td>HN6/HN2'</td>
<td>3.9</td>
<td>&gt;3.6</td>
<td>&lt; 5.0</td>
</tr>
<tr>
<td>HN1'/HN4'</td>
<td>4.2</td>
<td>&gt;3.6</td>
<td>&lt; 5.0</td>
</tr>
<tr>
<td>HA8/HA1'</td>
<td>3.7</td>
<td>&gt;3.6</td>
<td>&lt; 5.0</td>
</tr>
<tr>
<td>HA8/HA2'</td>
<td>2.7</td>
<td>vdw</td>
<td>&lt; 3.6</td>
</tr>
<tr>
<td>HA8/HA3'</td>
<td>3.8</td>
<td>&gt;3.6</td>
<td>&lt; 5.0</td>
</tr>
</tbody>
</table>

\(^a\) Internuclear distances within the NMN portion of enzyme-bound NADP⁺ were estimated using the initial slope of the HN4/HN5 proton pair and known HN4/HN5 distance (2.48 Å). Internuclear distances within the AMN portion of enzyme-bound NADP⁺ were estimated using the initial slope of the HA1'/HA2' proton pair of the known HA1'/HA2' distance (2.9 ± 0.2 Å).

\(^b\) Lower limit distance set at the sum of the Van der Waals radii of the two atoms.
II.8 for the NMN and AMN portions of NADP\(^+\), respectively. Hand-built models of the NMN and AMN portions of NADP\(^+\) based on the values of the measured distances (Table III.4) were consistent with syn and anti conformations about the nicotinamide-ribose and adenine-ribose torsional angles of NMN and AMN, respectively.

In a more general and objective approach, the experimentally measured internuclear distances (Table III.4) were used to set upper and lower bound constraints in a distance geometry algorithm to determine the conformations of the NMN and AMN moieties of NADP\(^+\) bound to RGB200 DHFR. This approach permits the conformational space of the NMN and AMN moieties of NADP\(^+\), defined by the experimental distance constraints, to be evaluated. Proton pairs yielding estimated distances <3.6 Å were given upper bound constraints of 3.6 Å and lower bound constraints equal to the sum of their Van der Waals radii (Table III.4). Proton pairs yielding estimated distances greater than 3.6 Å, but less than 5 Å were given an upper bound constraint of 5.0 Å and a lower bound constraint of 3.6 Å (Table III.4). Using these constraints, 400 computer generated structures were obtained for the NMN and AMN portion of NADP\(^+\), respectively. All acceptable structures were found to have internuclear distances which did not deviate by more than 1% from the input bond lengths, bond angles, Van der Waals values, and experimental distance constraints. No attempt was made to further refine these structures. Using these criteria, all acceptable NMN structures were found to have syn nicotinamide-ribose glycosidic torsional angles (\(\chi = 94 \pm 26^\circ\); Figure III.21A). Use of the estimated internuclear distances, with a \(\pm 0.4\) Å error (Table III.4), as constraints in the distance geometry calculation did not significantly alter the average or the range of acceptable nicotinamide-
Figure III.21: Glycosidic torsion angle wheels for the NMN and AMN portions of NADP$^+$ bound to RBG200 DHFR in binary conformation II. (A) The conformational space, defined by the upper and lower bound constraints (Table III.4), available to the NMN portion of NADP$^+$. (B) The conformational space, defined by the upper and lower bound constraints (Table III.4), available to the AMN portion of NADP$^+$. 
ribose glycosidic torsional angles. These results demonstrate that qualitative interpretation of the observed NOEs in combination with distance geometry calculations is sufficient to evaluate the range of conformations available to the NMN moiety of NADP$^+$ bound to RBG200 DHFR. For AMN, all of the acceptable solutions were found to have anti adenine-ribose glycosidic torsional angles ($\chi = -92 \pm 32^\circ$; Figure III.21B). Thus, in conformation II of the binary RBG200 DHFR•NADP$^+$ complex, the oxidized coenzyme is bound with a syn conformation about the nicotinamide–ribose and an anti conformation about the adenine–ribose glycosidic bond (Figure III.21).

2.7. Qualitative Comparison of Enzyme–Bound NADP$^+$ in Conformations I and II of the RBG200 DHFR•NADP$^+$ Binary Complex

In order to qualitatively compare the enzyme-bound conformations of NADP$^+$ in both binary complexes I and II, NOESY spectra were collected at 5 °C. Under these conditions, at the end of the experiment approximately 25% of the initial binary complex, conformation I, had converted to conformation II (section III.2.1). Figure III.22 shows an expanded region of the 150 ms NOESY at 5°C of NADP$^+$ bound to RBG200 DHFR in conformations I and II. In conformation I, large NOE cross-peaks were observed between the HN6/HN1' and the HN2/HN2' proton pairs (Figure III.22A). Smaller NOE cross-peaks were observed between the HN2/HN1' and the HN2/HN3' proton pairs. This pattern of NOEs qualitatively confines the glycosidic torsional angle about the nicotinamide–ribose bond to a syn conformation for NADP$^+$ bound in conformation I (Figure III.10). In conformation I, a large
Figure III.22: Contour plots of the 500 MHz phase-sensitive NOESY spectra of NADP\(^+\) bound to RBG200 DHFR in the binary complexes I and II at 5 °C. (A) Expanded region of the 150 ms NOESY spectrum showing the NOE cross-peak intensities for enzyme-bound NADP\(^+\) in binary conformation I. (B) Expanded region of the 150 ms NOESY spectrum showing the NOE cross-peak intensities for enzyme-bound NADP\(^+\) in binary conformation II. Experimental conditions, except for the temperature, are similar to those given in Figure III.18.
NOE was also observed between the HA8/HA3' proton pair, and a smaller NOE observed between the HA8/HA2' proton pair (Figure III.22A). These NOEs qualitatively constrain the glycosidic torsional angle about the adenine-ribose bond to an anti conformation for NADP⁺ bound in conformation I.

After complete interconversion of conformation I to II, as determined by the chemical shifts of NADP⁺ (section III.2.1), a second NOESY spectrum was obtained under identical conditions. The pattern of NOE cross-peaks observed at 5° C for NADP⁺ bound to RBG200 DHFR in conformation II (Figure III.22B) was similar to that observed for conformation I (Figure III.22A). However, intensity ratios between identical NOEs were found to vary (Figure III.22). The NOE cross-peak intensity ratio between the HN6/HN1' and the HN2/HN1' proton pairs in conformation II was found to be closer to unity than observed in conformation I (Figure III.22). Additionally, a small NOE was observed between the HN6/HN2' proton pair in conformation II (Figure III.22B). These differences in the NOE cross-peak intensity ratios between conformation I and conformation II of the NMN moiety of NADP⁺ could be the result of a tighter syn conformation around the nicotinamide-ribose glycosidic torsion angle in conformation I, and a higher degree of mobility around the glycosidic torsion angle in conformation II.

The pattern of NOE cross-peak intensities for bound NADP⁺ to RBG200 DHFR in conformation II at 5 °C was found to be identical to that observed at 15° C (Figure III.18). The pattern of interproton NOEs observed in the NMN portion of NADP⁺ bound to RBG200 DHFR in conformation II at 5° C (Figure III.22B) confines the nicotinamide–ribose glycosidic torsional angle to a syn conformation, as was previously observed at 15° C (Figure
III.18). Within the AMN moiety of NADP⁺ bound in conformation II, the large NOE cross-peak between the HA8/HA2' proton pair and the smaller NOE cross-peaks between the HA8/HA3' and the HA8/HA1' proton pairs qualitatively constrain the adenine–ribose glycosidic torsional angle to an anti conformation (Figure III.10). However, several interesting features are revealed when cross-peak intensities within the AMN moiety of NADP⁺ bound to RBG200 DHFR in conformations I and II are compared (Figure III.22). In conformation II, the ratio of interproton NOE cross-peaks in the AMN moiety was found to be HA8/HA2' >> HA8/HA3' ≈ HA8/HA1' (Figure III.22B). However, in conformation I the ratio of interproton NOE cross-peaks was found to be HA8/HA3' >> HA8/HA2' (Figure III.22A). Thus, conversion of binary conformation I to II appears to involve a change in the adenine–ribose glycosidic torsional angle within the range of anti conformations.

2.8. Conformation of NADP⁺ in the Ternary Complex RBG200 DHFR•NADP⁺•Folate

The conformation of NADP⁺ in the ternary complex RBG200 DHFR•NADP⁺•Folate was qualitatively evaluated by comparison of the pattern and intensity of the NOEs associated with NADP⁺ in the binary complex with RBG200 DHFR, and the ternary complex with RBG200 DHFR and folate. Figure III.23 shows an expanded region of the NOESY spectrum of NADP⁺ in the presence of RBG200 DHFR and saturating concentrations of folate. Four equivalents of folate to RBG200 DHFR (tetramer) were added. Addition of a fifth equivalent of folate led to folate precipitation. The pattern
Figure III.23: Contour plot of the 500 MHz phase-sensitive NOESY spectrum of NADP⁺ in the presence of RBG200 DHFR and folate, at 15° C. The sample contained 0.5 mM RBG200 DHFR, 2 mM folate and 8 mM NADP⁺ in 50 mM potassium phosphate buffer in ²H₂O, pH 6.0. The spectrum was recorded with a 250 ms mixing time. The labels indicate the assignments of the NOE cross-peaks.
of internuclear NOEs for bound NADP$^+$ in the ternary complex (Figure III.23) is extremely similar to the pattern of NOEs observed for conformation II of the binary complex (Figure III.18). Large NOE cross-peaks were observed between the HN6/HN1', the HN2/HN2' and the HN2/HN1' proton pairs (Figure III.23). Very small NOE cross-peaks were observed between the HN6/HN2' and the HN2/HN3' proton pairs (Figure III.23). This pattern of NOEs qualitatively confines the glycosidic torsional angle about the nicotinamide–ribose bond to a syn conformation for NADP$^+$ bound in the ternary complex (Figure III.10).

For the AMN moiety of NADP$^+$, a large NOE was observed for the HA8/HA2' proton pair, and smaller NOEs were observed for the HA8/HA1' and HA8/HA3' proton pairs (Figure III.23). This pattern of NOEs is very similar to the one observed for conformation II of the binary complex, and qualitatively constrains the glycosidic torsional angle about the adenine–ribose bond to an anti conformation for NADP$^+$ bound in the ternary complex.
Chapter IV. DISCUSSION

A rapid and gentle purification procedure for RBG200 DHFR, a cloned and overproduced derivative of R388 DHFR having the sequence Thr-Thr-Ser-Arg-Thr-Leu at the carboxy terminus, has been developed. This procedure yields milligram quantities of apparently homogeneous protein with a specific activity 1.9-fold greater than that previously reported for the R388 protein (Zolg and Hänggi, 1981). The Zolg and Hänggi procedure included a step in which the enzyme was denatured in 6 M guanidinium hydrochloride and chromatographed in 6 M urea. Thus, it is possible that once the type II DHFR is partially unfolded it does not refold completely to its native conformation. Additionally, RBG200 DHFR purified by a procedure based on precipitation of cellular proteins by pH variation (Vermersch et al., 1986; Vermersch, 1988) also showed significantly lower specific activity. Even though this method worked as a general screening procedure during genetic studies (Vermersch et al., 1986; Vermersch, 1988), it became clear during the progress of initial biochemical and biophysical studies that a significant and variable amount of denatured protein was present in these RBG200 DHFR preparations. This showed the absolute need to develop a more gentle purification procedure for the enzyme.

RBG200 DHFR was found to have a sharp pH optimum around 5.9, with 90% activity over less than 0.5 pH unit (Figure III.4). This behavior mimics that observed for R388 DHFR (Amyes and Smith, 1976). Deserving special attention is the sharp decrease in activity between pH 5.9 and pH 5.0 which is significantly different from what is found in most chromosomal
DHFRs. Chromosomal DHFRs have a broad pH optimum with 95% activity over a range of two pH units (Mathews and Sutherland, 1965; Amyes and Smith, 1976; Stone and Morrison, 1984; Beard et al., 1989).

Determination of the native molecular weight of RBG200 DHFR by gel filtration HPLC and analytical ultracentrifugation suggested that between subunit concentrations of approximately 2 μM and 40 μM, and at pH 6.0, the enzyme exists in solution as a mixture of monomer, dimer and tetramer. At pH 5.0, and subunit concentrations between 2 μM and 40 μM, HPLC studies showed that the apparent molecular weight of RBG200 DHFR does not significantly differ from that at pH 6.0. Additionally at both pH 5.0 and pH 6.0 and below subunit concentrations of 2μM, the apparent molecular weight of RBG200 DHFR decreases and a complex behavior is observed in the gel filtration HPLC profiles (Figure III.6). Taking into account that RBG200 DHFR is routinely assayed at subunit concentrations between 1 μM and 2 μM, it appears that the loss of enzyme activity between pH 5.9 and 5.0 is not caused by dissociation of the active enzyme. This conclusion contrasts with previous results alluded to in an abstract (Nichols et al., 1991) and a footnote of a paper (Reece et al., 1991) which stated that the active tetrameric R67 DHFR dissociates to inactive dimers at low enzyme concentrations or low pH with a pKₐ of approximately 6.0. We have purified R67 DHFR and compared the gel filtration behavior of RBG200 DHFR and R67 DHFR at pH 5, 6 and 8 at a subunit concentration of approximately 80 μM, and additionally at pH 6 and subunit concentration of approximately 4.5 μM (Table III.3). Both enzymes show a similar qualitative dependence of the apparent molecular weight on pH and protein concentration, even though it appears that there may be a slight shift on the position of the association equilibrium (Table III.3).
Previously reported gel filtration experiments of R388 and R67 DHFR at pH 7 suggested that the native molecular weight of these proteins was 35 to 37 kDa, consistent with a native tetrameric structure for type II DHFRs (Amyes and Smith, 1974; Pattishall et al., 1977; Smith et al., 1979; Zolg and Hänggi, 1981). This conclusion is consistent with our present results which show that the apparent number of subunits varies between 5.5 and 5.0 at pH 8.0, and 3.5 and 3.1 at pH 6.0 for RBG200 DHFR and R67 DHFR, respectively (Table III.3). Thus it is likely that at pH 7, even though the apparent molecular weight of type II DHFRs approaches the expected molecular weight for a pure tetramer, the observed molecular weight is the result of fast exchange between several multimeric species.

At protein subunit concentrations above 40 μM the increase in the apparent molecular weight at pH 6.0 is not accompanied by a similar increase at pH 5.0 (Figure III.5). The increase in the apparent molecular weight above what is expected for a tetramer indicates the presence of aggregated species larger than the tetramer at pH 6.0 but not at pH 5.0. This result is consistent with NMR observations where the aromatic region of TOCSY spectra of 2 mM RBG200 DHFR (subunit concentration) show broader line widths at pH 6.1 than at pH 4.8. However, these spectra do not show any significant differences in the chemical shifts of the aromatic resonances leading to the belief that species larger than the tetramer are loosely formed or that the aromatic residues are not involved in intersubunit interactions at this level. To clarify the questions involving the subunit association equilibria in type II DHFRs, more analytical ultracentrifugation studies are necessary at different protein concentrations, pH and ionic strength values, and in the
absence and presence of coenzyme and substrate in order to evaluate the influence of the ligands in the association equilibriums.

NMR studies monitoring the proton chemical shifts of NADP$^+$ upon addition of RBG200 DHFR have permitted the detection of two distinct binary RBG200 DHFR•NADP$^+$ complexes in solution (Figure III.11). Addition of NADP$^+$ to the enzyme results in the formation of an initial binary complex (conformation I) which slowly interconverts to a more stable binary complex (conformation II). At 25 °C, the apparent first order rate constant for the interconversion between conformation I and II was determined to be approximately $1.0 \times 10^{-4}$ s$^{-1}$. Conformations I and II are characterized by upfield and downfield chemical shift changes of the nicotinamide proton resonances from their positions in the free coenzyme, respectively. Changes in the $^1$H NMR chemical shifts of the upfield shifted methyl resonances of RBG200 DHFR were also observed upon NADP$^+$ binding and accompanying the interconversion between conformations I and II. The nature of these two distinct conformational states and their structural and functional role in catalysis remains to be determined.

Equilibrium dialysis experiments with $^3$H-NADP$^+$ and NADPH showed that approximately one coenzyme molecule binds per tetramer of RBG200 DHFR. This stoichiometry would limit the smallest active species of RBG200 DHFR to a tetramer. However, coenzyme binding to protein species smaller than the tetramer cannot be ruled out, particularly taking into account the presence of other protein species in solution as revealed by the gel filtration and analytical ultracentrifugation experiments. From the equilibrium dialysis studies, the binding of NADP$^+$ to RBG200 DHFR in
conformation II was found to be weak, \( K_d = 1.5 \pm 0.4 \text{ mM} \). The binding of NADP\(^+\) in conformation II was also monitored by NMR and yielded a \( K_d = 1.9 \pm 0.4 \text{ mM} \) (Figure III.15), in relative good agreement with the equilibrium dialysis experiments.

RBG200 DHFR was shown to stereospecifically transfer the \textit{pro-R} hydride of NADPH to dihydrofolate making it a member of the \textit{A} stereospecific class of dehydrogenases. Dehydrogenases catalyze the stereospecific transfer of hydrogen between their coenzyme, NADH or NADPH, and substrate. These enzymes can be grouped into two classes: \textit{A} stereospecific which transfer the C4 \textit{pro-R} hydrogen and \textit{B} stereospecific which transfer the C4 \textit{pro-S} hydrogen of the reduced nicotinamide ring (You, 1985). As a general rule, dehydrogenases transferring the \textit{pro-R} hydrogen have been shown to bind the coenzyme with an \textit{anti} conformation about the nicotinamide–ribose glycosidic bond. In contrast, enzymes transferring the \textit{pro-S} hydrogen have been shown to bind the coenzyme with a \textit{syn} conformation about the nicotinamide–ribose bond (Benner, 1982; Levy et al., 1983; You, 1985). As pointed out by Oppenheimer (1986), this makes the assumption that the substrate binds on top of the coenzyme. If the coenzyme binds on top of the substrate, the opposite correlation would apply. An example of this is glutathione reductase which is known to transfer the \textit{pro-S} hydrogen of NADPH, but to bind NADPH with an \textit{anti} conformation about the nicotinamide–ribose glycosidic bond (Pai and Schulz, 1983). Examination of the crystallographic structure of glutathione reductase shows that NADPH is stacked on top of the flavin part of the bound FAD (Pai and Schulz, 1983).
Chromosomal dihydrofolate reductases are known to transfer the pro-\(R\) hydrogen from NADPH to dihydrofolate and to bind NADPH with an anti conformation about both the nicotinamide–ribose and adenine–ribose glycosidic bonds (Charlton et al., 1979; You, 1982; Matthews et al., 1979). For example, the NMN moiety of NADPH bound to chromosomal *L. casei* DHFR has been shown by crystallography to have an anti glycosidic torsional angle, \(\chi = -141^\circ\) (Matthews et al., 1979). The arrangement of coenzyme and substrate initially proposed by Filman and collaborators (1982) at the active site of *L. casei* DHFR based on the stereochemistry of hydride transfer and the enzyme-bound conformation of NADPH and confirmed by X-ray crystallography of the ternary complex *E. coli* DHFR•NADP•Folate (Bystroff et al., 1990) is shown in Figure IV.1A. Dihydrofolate is located above the plane of the nicotinamide ring such that the pro-\(R\) hydrogen can be transferred to C6 of the pteridine ring of dihydrofolate (Figure IV.1A).

Type II DHFRs encoded by the R67 and R388 plasmids are structurally different from the chromosomal DHFRs and relatively insensitive to antifolate compounds such as trimethoprim and methotrexate (Pattishall et al., 1977). The crystal structure of a dimeric form of R67 DHFR in the absence of cofactor and substrate was found to be constituted by two identical subunits each folded into a \(\beta\)-barrel conformation which then form an intersubunit \(\beta\)–barrel. Based on modelling studies using the R67 DHFR dimer, the coenzyme was proposed to bind in a "gap" along one face of the intersubunit antiparallel \(\beta\)-barrel (Matthews et al., 1986). In contrast, the cofactor binding domain at the active site of chromosomal DHFRs structurally resembles the cofactor binding domains in other NAD\(^+\)–dependent dehydrogenases, commonly known as the Rossmann fold (Kraut and Matthews, 1986). Modelling studies,
Figure IV.1: Comparison of the proposed model for NADPH and H₂F bound to RBG200 DHFR with that proposed for L. casei DHFR and recently confirmed by X-ray crystallography for E. coli DHFR. (A) Spatial relationship between NADPH and the pteridine ring of H₂F in the active site of L. casei DHFR as modeled by Filman et al. (1982) and on the crystal structure of the E. coli DHFR•NADP•folate ternary complex (Bystroff et al., 1990). The NMN portion of NADPH is bound in an anti conformation and the nicotinamide ring is located in a plane below the plane of the pteridine ring of H₂F. (B) Proposed model of the spatial relation between NADPH and the pteridine ring of H₂F in the binding site of RBG200 DHFR. The NMN portion of NADPH is bound in a syn conformation and the nicotinamide ring is located in a plane above the plane of the pteridine ring, permitting the pro-R hydrogen of NADPH to be transferred to the pteridine.
assuming that type II DHFRs transferred the pro-R hydrogen of the reduced nicotinamide, also suggested that NADPH would bind to R67 DHFR with an anti conformation about both the nicotinamide–ribose and adenine–ribose glycosidic bonds (Matthews et al., 1986). Attempts to construct a model of NADP⁺ bound the the dimeric form of R67 DHFR with a syn conformation about the nicotinamide-ribose glycosidic bond were unsuccessful (Matthews et al., 1986).

We have studied by NMR the conformation of NADP⁺ when bound to RBG200 DHFR. The combination of internuclear distances, estimated from the initial slope of NOE build-up curves, Table III.4, and distance geometry calculations were used to define the conformational space available to the nicotinamide–ribose and adenine–ribose glycosidic torsional angles of NADP⁺ bound to RBG200 DHFR in conformation II. Unexpectedly, the average nicotinamide–ribose glycosidic torsional angle for enzyme-bound NADP⁺ was found to be syn, \( \chi = 94 \pm 26^\circ \), for all acceptable solutions (Figure IV.1A). The internuclear distances estimated for the NMN portion of enzyme–bound NADP⁺ were consistent with a unique conformation for the nicotinamide–ribose glycosidic torsional angle. However, the wide range of acceptable syn conformations suggests that the NMN portion of enzyme-bound NADP⁺ may have considerable mobility within this range of syn conformations. Multiple nicotinamide–ribose conformations, syn and anti, for NADP⁺ at the active site of RBG200 DHFR cannot unequivocally be ruled out by use of a single technique (Rosevear et al., 1983), although NOEs have previously been used to demonstrate multiple conformations for enzyme-bound nucleotides (Rosevear et al., 1987). In addition, the small initial build-up rate of the HN6/HN2' NOE rules out the presence of a significant
population of enzyme-bound NADP$^+$ having an *anti* conformation about the nicotinamide–ribose bond (Levy et al., 1983). Our data are consistent only with a *syn* conformation about the nicotinamide–ribose glycosidic bond in the binary complex. We have also shown that qualitatively the conformation of NADP$^+$ bound to RBG200 DHFR in the ternary complex Enzyme•NADP$^+$•Folate is very similar to that of conformation II of the binary complex Enzyme•NADP$^+$.

Based on the observed stereochemistry of hydride transfer and the enzyme-bound conformation of the NMN portion of NADP$^+$ both in the binary and ternary complexes, a hypothetical model for the arrangement of cofactor and dihydrofolate at the active site of RBG200 DHFR is shown in Figure IV.1B. In this model, the nicotinamide–ribose glycosidic torsional angle of NADPH is *syn* with the pro-R hydrogen below the plane of the nicotinamide ring. For this hydrogen to be transferred to the C6 *si*-face of the pteridine ring in dihydrofolate, the pteridine portion of H$_2$F must be below the plane of the nicotinamide ring, and rotated 180° with respect to H$_2$F binding in chromosomal DHFRs (Figure IV.1A and B). This arrangement of substrates in the active site of RBG200 DHFR may explain the weaker binding of 2,4-diamino heterocycles to Type II DHFRs, since the location of hydrogen bonding donors and acceptors at the pteridine binding site on type II DHFRs must be different from that found in chromosomal enzymes.

The enzyme-bound conformation of the AMN portion of NADP$^+$ in conformation II was also investigated using the estimated internuclear distances (Table III.4) and distance geometry calculations. Upper and lower bound constraints, obtained from the estimated internuclear distances, were sufficient to define the adenine–ribose glycosidic torsional angle, $\chi = -92 \pm 32^\circ$.
(Figure III.21B). An *anti* conformation about the adenine–ribose glycosidic bond has also been found for NADPH bound to chromosomal DHFRs (Matthews et al., 1979) and other NAD(P) binding proteins (Levy et al., 1983; Ehrlich and Colman, 1985).

The conformation of NADP⁺ bound to RBG200 DHFR in the initial binary complex, conformation I, was qualitatively evaluated at 5 °C, to decrease the interconversion rate between conformation I and II. The pattern of internuclear NOEs in conformation I, Figure III.22A, was only consistent with *syn* and *anti* conformations about the nicotinamide–ribose and adenine–ribose glycosidic bonds for the NMN and AMN portions of NADP⁺ bound to RBG200 DHFR. The pattern of internuclear NOEs at 5° C, after conversion to conformation II, was qualitatively similar to that obtained at 15° C (Figure III.18, Figure III.22B). Thus, the nicotinamide–ribose and the adenine–ribose glycosidic torsional angles of the NMN and AMN moieties of enzyme–bound NADP⁺ were found to be *syn* and *anti*, respectively, in both conformation I and II of the binary complex as well as in the ternary complex. However, from the magnitude of the NOE cross-peak intensities, some conformational differences were detected in conformation I of the enzyme-bound NADP⁺ when compared with conformation II of the binary complex and the ternary complex.

The lack of observed internuclear NOEs between the adenine and nicotinamide bases suggests that NADP⁺ is not bound to RBG200 DHFR in a stacked or folded conformation and that the distance between the two aromatic rings in the bound coenzyme must be at least 5 Å. Nuclear Overhauser effects between the two nucleotide rings have previously been used to demonstrate that NADPH exists in a stacked or folded conformation
when bound to isocitrate dehydrogenase (Ehrlich and Colman, 1985). Folded conformations of the coenzyme have also been observed for NADH bound to the allosteric effector site and the nucleoside inhibitor site of phosphorylase b (Stura et al., 1983).

In conclusion, the stereochemistry of hydride transfer and the enzyme-bound conformation of NADP$^+$ have permitted us to propose an unique arrangement of coenzyme and dihydrofolate at the active site of a type II DHFR (Figure IV.1B). This novel arrangement of substrates, compared to chromosomal DHFRs, may be responsible for the resistance of type II DHFRs to antifolate compounds. Future studies, leading to the identification of the amino acids involved in substrate binding and catalysis, will permit refinement of the proposed model for cofactor binding and better understanding of the resistance of type II DHFRs to the antifolate compounds. Among the research avenues to explore is the use of spin labeled ligands. In the NOESY spectra of binary or ternary complexes of RBG200 DHFR with NADP$^+$ and/or folate run to date, no NOEs were observed between the coenzyme or substrate and the aromatic region of the enzyme. Taking into account that NOEs are observed for short internuclear distances, 4 to 5 Å, and that paramagnetic effects extend to longer distances, 15 to 20 Å, the use of spin labeled ligands could lead to identification of aminoacid residues involved in ligand binding. In particular, the combined use of paramagnetic analogs of NADP$^+$ (Chang and Hammes, 1986) or folate, selective $^{13}$C-labeled RBG200 DHFR, and T$_1$-HMQC NMR experiments, could allow mapping of the aminoacid residues forming the enzyme active site.
BIBLIOGRAPHY


APPENDIX.

Abstracts of other published work by the author:

1. "The Low-Affinity Ca\(^{2+}\)-Binding Sites in Cardiac / Slow Skeletal Muscle Troponin C Perform Distinct Functions: Site I Alone Cannot Trigger Contraction"

ABSTRACT: Both troponin C (TnC) and calmodulin share a remarkably similar tertiary structure motif that may be common to other Ca\(^{2+}\)-binding proteins with activator activity. TnC plays a critical role in regulating muscle contraction and is particularly well-suited for structural analysis by site-direct mutation. Fast-twitch skeletal muscle TnC has two low-affinity Ca\(^{2+}\)-binding sites (site I and site II), while in cardiac and slow-twitch skeletal muscle TnC site I is inactive. Recently, using protein engineering, we directly demonstrated that binding of Ca\(^{2+}\) to the low-affinity site(s) initiates muscle contraction. In the present study, we use mutagenesis to determine whether either of the low-affinity sites in cardiac TnC can trigger contraction in slow-twitch skeletal muscle fibers. In one Ca\(^{2+}\)-binding mutant, Ca\(^{2+}\)-binding to the dormant low-affinity site I was restored (CBM+I). In a second mutant, site I was activated while site II was inactivated (CBM+I-IIA). Both proteins had the predicted Ca\(^{2+}\)-binding characteristics, and both were able to associate with troponin I and troponin T to form a troponin complex and integrate into permeabilized slow-twitch skeletal muscle fibers. A
comparison of NMR spectra shows the aromatic regions in the two proteins to be qualitatively similar without divalent cations but markedly different with Ca\(^{2+}\). Mutant CBM+I supported force generation in skinned slow skeletal muscle fibers but had Sr\(^{2+}\) and Ca\(^{2+}\) sensitivities similar to fast skeletal TnC. Mutant CBM+I-IIA was unable to restore Ca\(^{2+}\)-dependent contraction to TnC-depleted skinned slow muscle fibers. The data directly demonstrate that low-affinity sites I and II have distinct functions and that only site II in cardiac TnC can trigger muscle contraction in slow-twitch skeletal muscle fibers. This principle of distinct, modular activities for Ca\(^{2+}\)-binding sites in the same protein may apply to other members of the TnC/calmodulin family.

2. "Comparative NMR Studies on Cardiac Troponin C and a Mutant Incapable of Binding Calcium at Site II"  

ABSTRACT: One- and two-dimensional NMR techniques were used to study both the influence of mutations on the structure of recombinant normal cardiac troponin C (cTnC3) and the conformational changes induced by Ca\(^{2+}\) binding to site II, the site responsible for triggering muscle contraction. Spin systems of the 9 Phe and 3 Tyr residues were elucidated from DQF-COSY and NOESY spectra. Comparison of the pattern of NOE connectivities obtained from a NOESY spectrum of cTnC3 with a model of cTnC based on the crystal structure of skeletal TnC permitted sequence specific
assignment of all 3 Tyr residues, as well as Phe-101 and Phe-153. NOESY spectra and calcium titrations of cTnC3 monitoring the aromatic region of the $^1$H NMR spectrum permitted localization of 6 of the 9 Phe residues to either the N- or C-terminal domain of cTnC3. Analysis of the downfield shifted CaH resonances permitted sequence specific assignment of those residues involved in the $\beta$-strand structures which are part of the Ca$^{2+}$ binding loops in both the N- and C-terminal domains of cTnC3. The short $\beta$-strands in the N-terminal domain of cTnC3 were found to be present and in close proximity even in the absence of Ca$^{2+}$ bound at site II. Using these assignments, we have examined the effects of mutating Asp-65 to Ala, CBM-IIA, a functionally inactive mutant which is incapable of binding Ca$^{2+}$ at site II [Putkey, J. A., Sweeney, H. L., and Campbell, S. T. (1989) J. Biol. Chem. 264, 12370]. Comparison of the apo-, Mg$^{2+}$, and Ca$^{2+}$-bound forms of cTnC3 and CBM-IIA demonstrates that the inability of CBM-IIA to trigger muscle contraction is not due to global structural changes in the mutant protein but is a consequence of the inability of CBM-IIA to bind Ca$^{2+}$ at site II. The pattern of NOEs between aromatic residues in the C-terminal domain is nearly identical in cTnC3 and CBM-IIA. Similar inter-residue NOEs were also observed between Phe residues assigned to the N-terminal domain in the Ca$^{2+}$-saturated forms of both cTnC3 and CBM-IIA. However chemical shift changes were observed for the N-terminal Phe residues in CBM-IIA. This suggests that binding of Ca$^{2+}$ to site II alters the chemical environment of the residues in the N-terminal hydrophobic cluster without disrupting the spatial relationship between the Phe residues located in helices A and D.
3. "Conformational Changes in the Metal-Binding Sites of Cardiac Troponin C Induced by Calcium Binding"

ABSTRACT: Isotope labeling of recombinant normal cardiac troponin C (cTnC3) with 15N enriched amino acids and multidimensional NMR were used to assign the downfield shifted amide protons of Gly residues at position 6 in Ca2+ binding loops II, III, and IV, as well as tightly hydrogen bonded amides within the short antiparallel β-sheets between pairs of Ca2+ binding loops. The amide protons of Gly70, Gly110, and Gly146 were found to be shifted significantly downfield from the remaining amide proton resonances in Ca2+- saturated cTnC3. No downfield shifted Gly resonance was observed from the naturally inactive site I. Comparison of downfield shifted amide protons in the Ca2+ saturated forms of cTnC3 and CBM-IIA, a mutant having Asp65 replaced by Ala, demonstrated that Gly70 is hydrogen bonded to the carboxylate side-chain of Asp65. Thus, the hydrogen bond between Gly and Asp in positions 6 and 1, respectively, of the Ca2+ binding loop appears crucial for maintaining the integrity of the helix-loop-helix Ca2+ binding sites. In the apo-form of cTnC3, only Gly70 was found to be shifted significantly downfield with respect to the remaining amide proton resonances. Thus, even in the absence of Ca2+ at binding site II, the amide proton of Gly70 is strongly hydrogen bonded to the side-chain carboxylate of Asp65. The amide protons of Ile112 and Ile148 in the C-terminal domain and Ile36 in the N-terminal domain β-sheets exhibit chemical shifts consistent with hydrogen bond formation between the pair of Ca2+ binding loops in each domain of
Ca$^{2+}$-saturated cTnC3. In the absence of Ca$^{2+}$, no strong hydrogen bonds were detected between the β-strands in the N-terminal domain of cTnC3. Thus, Ca$^{2+}$ binding at site II results in a tightening of the Ca$^{2+}$ binding loop and formation of one strong hydrogen bond between β-strands in the N-terminal domain. These changes may initiate movement of helices in the N-terminal domain responsible for the interaction of TnC with troponin I.

4. "Calcium Plays Distinctive Structural Roles in the N- and C- Terminal Domains of Cardiac Troponin C"


ABSTRACT: One- and two-dimensional NMR techniques were used to compare the structural consequences of Ca$^{2+}$ binding to the low and the high affinity Ca$^{2+}$ sites in recombinant cardiac troponin C (cTnC3). Cardiac or slow skeletal troponin C (TnC) contains two high affinity Ca$^{2+}$/Mg$^{2+}$ binding sites, sites III and IV, located in the C-terminal domain and a single active Ca$^{2+}$ specific site in the N-terminal domain. In the absence of Ca$^{2+}$, the short β-sheet located between the high affinity Ca$^{2+}$/Mg$^{2+}$ binding sites in the C-terminal domain was found to be absent or loosely formed as judged by the chemical shifts and inter-residue NOEs within the binding loops. In contrast, the N-terminal domain β-sheet located between site II and the naturally inactive site I was present even in the absence of bound Ca$^{2+}$ in the N-terminal domain. Recently, cTnC3 was systematically altered by mutagenesis to generate calcium binding mutant proteins having either an inactive Ca$^{2+}$
binding site III (CBM-III), Asp105 to Ala, or an inactive Ca$^{2+}$ binding site IV (CBM-IV), Asp141 to Ala [Negele, J. C., Dotson, D., Liu, W., Sweeney, H. L., and Putkey, J. A. (1992) *J. Biol. Chem.* 267, 825]. Analysis of the downfield-shifted amide and CαH regions of the Ca$^{2+}$ binding mutant proteins have permitted sequence specific assignment of those resonances involved in the short b-sheets between Ca$^{2+}$ binding loops III and IV, as well as aromatic residues involved in the C-terminal hydrophobic cluster. Comparison of the downfield shifted amide protons in CBM-III and CBM-IV with those in cTnC3 have permitted the structural consequences of sequentially mutating each Ca$^{2+}$/Mg$^{2+}$ binding site in the C-terminal domain to be evaluated. Only a single active Ca$^{2+}$ binding site was found necessary for formation of the short β-sheet between Ca$^{2+}$ binding sites III and IV as judged by interstrand NOEs between the downfield shifted CαH resonances of Tyr111 and Asp149 and Asp113 and Arg147 located between the Ca$^{2+}$ binding loops in the wild type protein. The amide protons of Ile112 and Ile148, located in the center of the β-sheet in the wild type protein, were also shifted downfield and exhibited an inter-residue NOE consistent with hydrogen bond formation between the C-terminal domain Ca$^{2+}$ binding loops in Ca$^{2+}$ saturated CBM-III and CBM-IV. The absence of bound Ca$^{2+}$ at site III was found to produce greater instability in the C-terminal domain as judged from the mobility of aromatic hydrophobic cluster residues located between helices E and H within the C-terminal domain. Thus, our results demonstrate that Ca$^{2+}$ binding plays distinctive structural roles in the N- and C-terminal domains of cTnC. While progress has been made at discerning the structural consequences of Ca$^{2+}$ binding to each of these domains, the physico-chemical basis for this difference remains to be elucidated.