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MECHANISTIC AND STRUCTURAL STUDIES OF MOUSE ADENOSINE DEAMINASE

by

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

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ABSTRACT

Mechanistic and Structural Studies of Mouse Adenosine Deaminase

by

Vera Sideraki

Adenosine deaminase (ADA) catalyzes the irreversible deamination of (2'-deoxy)adenosine to (2'-deoxy)inosine. It is an indispensable enzyme, with a role in purine catabolism and in the development of a competent immune system. This work focuses on the study of the catalytic mechanism employed by the murine enzyme through the use of site-directed mutagenesis.

A glutamate mutation at the conserved active site Asp 295 shows that this residue is necessary for the proper orientation and placement of the catalytic hydroxylate. An alanine and an asparagine mutant of Asp 296 show that this residue functions mainly by anchoring the substrate in the active site via hydrogen bonding and thus reducing the aromaticity of the purine ring.

Alanine, glutamate and arginine mutations at the proposed base for the reaction, His 238, clearly show that it does not abstract the proton from the zinc-bound water, but rather promotes the formation of the hydroxylate through charge stabilization. Replacements of the conserved Cys 262 by alanine and serine clearly demonstrate that it is not directly involved in the reaction mechanism.

Structural studies with the ADA apoenzyme reveal that chelation of zinc does not result in structural rearrangements of either the active site or the secondary and tertiary structures of the enzyme. Loss of zinc is accompanied by loss of activity, which can be restored upon stoichiometric re-addition of zinc or cobalt. A transition-state analog such as deoxycoformycin can bind the
apoenzyme by inducing the same type of conformational change as it does when it binds the holoenzyme.

Mutants such as D296A and D296N denature more slowly compared to the wild-type, probably due to the better packing of an Ala or Asn side chain compared to the native Asp in the part of the enzyme surrounding residue 296. By contrast, mutants such as D295E, H238A, and H238E destabilize the holoenzyme, and mutants H238R, C262A, and C262S destabilize the holoenzyme and may also impede the in vivo folding pathway. Removal of the metal cofactor from wild-type or mutant ADA generally increases the enzyme's rate of denaturation.
ACKNOWLEDGMENTS

I would like to thank Fred Rudolph for his invaluable support and advice over the years, and for being the nicest boss I have ever had; Kathy Matthews for her encouragement and help; Khalid Mohamedali for starting me up in the lab and for his persistent good nature, silliness (sil) enhancer, and friendship; Bruce Cooper, for his unconditional help and advice, and the Bennett and Rudolph crews for creating a fun environment to work in. Many thanks to everyone in the Department for being so good to their graduate students (by all accounts a rare thing in Universities). And to the Olson and Phillips labs (specially Tromo and Mark) for letting and helping me mess around, occasionally including me in artistic endeavors, and for their general dementia.

I never thought I'd say it, but Houston has been a great city to live in during my graduate career, principally because of the many and dear friends I have made here. A huge thanks to Zeus and Co., Valentini, Nick, Litha, Hariklia, Alex, Billy Gibbons, JB, Maggie, Fredrik, Peeter, Sven, Anders B., Svante, Lisa, Annakarin, the New Swedes, the Old Swedes (specially Martini and Masse), the Frogs, the Greeks, the Norwegians, Valborgs Parties, the Chemical Brothers, McClendon parties, Yello, Rich's, Easter Egg Hunts, Big Bend, ZZTop, El diablo, Dr. Bocell, Drs. Mabuse & Dre, the Firebird, Texas Art Supply, the PCH and SF, Half-Price Books, Jane Williams, and specially Laurent.

My deepest, deepest love and thanks to my parents, Aspa and George, for all they have done for me. This thesis is for them.
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<td>Adenosine deaminase</td>
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<td>HEPES</td>
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CHAPTER I.
PHYSIOLOGICAL ROLES AND IMPORTANCE OF ADENOSINE DEAMINASE

1. Adenosine Deaminase and the Purine Catabolism Pathways
Adenosine deaminase catalyzes the irreversible deamination of 2'-deoxyadenosine and adenosine into 2'-deoxyinosine and inosine. It is a key enzyme involved in purine catabolism, and is present in virtually all mammalian cells. As part of the purine catabolism pathways, the enzyme deaminates adenosine into inosine, which is in turn converted to hypoxanthine by purine nucleoside phosphorylase (Figure I.1). Guanosine can be converted to guanine by purine nucleoside phosphorylase; guanine is then deaminated to xanthine by guanine deaminase. Xanthine oxidase converts hypoxanthine to xanthine and xanthine to uric acid. The ADA substrate, adenosine, derives from AMP, by the action of a nucleotidase. AMP can also be converted to IMP by an enzyme related to ADA, AMP deaminase. IMP can be acted upon by nucleotidase and give rise to inosine, which feeds into the rest of the pathway. In addition to inosine, xanthosine can also be converted to xanthine (not shown in the pathway) and this to uric acid. Thus, the final product of purine breakdown is uric acid, which is excreted in the urine of humans and primates.

2. Modulation of Adenosine Levels is Crucial to the Cell
Adenosine deaminase is exceedingly important in regulating the levels of adenosine in the cell. Adenosine pools are also depleted by the action of adenosine kinase, which phosphorylates adenosine to AMP. Purines are vital molecules for all cells, since they ultimately give rise to DNA and RNA
FIGURE 1.1 Purine catabolism in animals. All purine nucleotides are degraded to uric acid. PNP refers to purine nucleoside phosphorylase. Adapted from Voet and Voet (1990).
building blocks. Adenosine is produced intracellularly by two distinct metabolic pathways. One of these involves the conversion of AMP to adenosine; the other, the catabolism of S-adenosylhomocysteine (Stiles, 1992). In addition to its role in DNA synthesis and replication, adenosine is an important cellular messenger. Under conditions of cellular stress, such as hypoxia, there is a dramatic rise in the levels of adenosine within the cell. Adenosine is then released and bound by specific adenosine receptors. This event activates a second messenger system that will attempt to bring the cell back into a normal state. In addition, these receptors can regulate a wide variety of physiological functions such as cardiac rate and contractility, sedation, release of neurotransmitters, platelet function, lipolysis, and white blood cell function (Stiles, 1992). Based on its multiple physiological functions, regulation of intracellular adenosine levels is crucial. Upsetting the balance of intracellular adenosine pools can become life-threatening; such is the case when adenosine deaminase is genetically absent, as described below.

3. ADA Deficiency Leads to SCID
Deletions, splicing defects and mostly point mutations in the coding portion of the human ADA gene account for the majority of cases of ADA deficiency (Kredich and Hershfield, 1989). It is thought that the primary effect of these point mutations is to enhance enzyme degradation of ADA by destabilizing the mature protein or interfering with protein folding (Hershfield and Mitchell, 1995). Dearth of ADA results in elevated levels of adenosine and deoxyadenosine in the plasma, elevated levels of dATP in erythrocytes, and diminished S-adenosylhomocysteine hydrolase activity owing to inhibition by deoxyadenosine (Figure I.2). Ultimately, ADA deficiency leads to immune system dysfunction. Patients with ADA deficiency are lymphopenic and lack
**Figure I.2** The transmethylation pathway, showing relationships between methionine, homocysteine, and adenosine metabolism. Because the S-adenosylhomocysteine hydrolase reaction is reversible, a build-up in adenosine levels as a result of ADA deficiency results in higher levels of S-adenosylhomocysteine. This compound is a potent inhibitor of methyl transfer reactions (adapted from Hershfield and Mitchell, 1995).
T-cell and B-cell mediated immunity, which results in a condition known as severe combined immunodeficiency disease (SCID). Patients with this disorder suffer from recurrent infections involving pathogens and opportunistic organisms; untreated, ADA-deficient children die before the age of two from overwhelming infection.

The detailed mechanisms by which lack of ADA results in impaired immune system function are thought to be the following (also shown in Figure I.3):

i. High levels of dATP in immature thymocytes inhibit ribonucleotide reductase, blocking DNA replication and cell division.

ii. Increased levels of deoxyadenosine result in the accumulation of dATP, which leads to depletion of cellular ATP; in addition, dATP leads to increased DNA strand breaks.

iii. Elevated levels of deoxyadenosine inhibit S-adenosylhomocysteine hydrolase activity, leading to inhibition of vital transmethylation reactions.

Treatments for SCID have involved bone marrow transplantation, administration of the bovine enzyme covalently modified with polyethylene glycol (PEG) to improve its circulating time, and gene therapy. The latter treatment involves the administration to patients of mature T-cells in which the ADA cDNA has been introduced through a retroviral vector. Since ADA patients lack mature T cells, this treatment has been applied in combination with PEG-ADA; thus, its effects alone cannot be adequately assessed. Two patients receiving this combined treatment have been shown to have increased lymphocyte count and lymphocyte ADA activity (Hershfield and Mitchell, 1995).
Figure I.3 Effects of adenosine and deoxyadenosine that are potential causes of immune dysfunction in ADA deficiency. Adapted from Hershfield and Mitchell, 1995.
4. Tissue Distribution of ADA and Involvement in Other Diseases

In mice ADA levels are highest in the developing fetal-maternal interface (Knudsen et al., 1989) and the alimentary tract; however, in these same tissues, the other purine metabolism enzymes are not similarly abundant, suggesting that ADA may have unique roles in embryo implantation and other processes (Mohamedali et al., 1993; Knudsen et al., 1991). In humans, ADA levels are highest in the thymus and the duodenum (Kizaki et al., 1973; Aronow et al., 1989). The enzyme's high levels in the thymus are in keeping with its essential role in T-cell development; however, SCID patients do not have gastrointestinal tract abnormalities, as would be expected if ADA were defective or absent from these tissues.

The levels of ADA are changed in a number of immunological diseases such as acquired immune deficiency syndrome, anemia, various leukemias and lymphomas (Murray et al., 1985; Kanno et al., 1988; Renouf et al., 1989). Thus, adenosine deaminase appears to be an integral component and key indicator of the proper function and competence of the immune system. Impaired immune function and thymic atrophy are phenotypes involved in zinc deficiency (Cunningham-Rundles, 1988); this suggests that ADA, being a zinc enzyme and a component of the immune system, may play a part in this disease.

5. CD26 is the ADA-Binding Protein

For a long time, ADA purified from humans ran on SDS-PAGE gels in two forms: a smaller form of 40,000 (pure ADA) and a larger form of ~ 200,000 (Zielke and Suelter, 1971). This larger ADA isozyme proved to be a complex between human ADA and the ADA-binding protein (Dinjens et al., 1989). The ADA binding protein (a 110 kDa glycoprotein) was shown to be identical
to CD26, a T-cell surface marker; its role was postulated to be that of regulating ADA levels, which would in turn control levels of adenosine, a transducer between B and T-cells (Kameoka et al., 1993). Two different groups then revealed that CD26 had DPP IV activity in its extracellular domain (Morrison et al., 1993; Tanaka et al., 1993). DPP IV is a serine protease, which has been implicated in the cleavage and inactivation of growth hormone releasing factor (Morrison et al., 1993), and T-cell immune response and B-cell differentiation (Yaron and Naider, 1993). Association of ADA with CD26 did not require the DPP IV activity for binding, and both ADA and CD26 retained their enzymatic activities after their association (Kameoka, et al., 1993; De Meester et al., 1994). ADA was proposed to be involved in the stimulation of T-cells through protein binding (Martin et al., 1995). The exciting report that CD26 is required for entry and infection of T-cells by the human immunodeficiency virus (Callebaut et al., 1993) has been supported by other groups (Oravecz, 1995), but has also been refuted (Morimoto et al., 1994; Lazaro et al., 1994) and still remains controversial.
CHAPTER II.
CATALYTIC MECHANISM OF ADENOSINE DEAMINASE

1. Previous Work on the Elucidation of the Mechanism

1.1. Early Work on the Enzyme
Homogeneous preparations of ADA were obtained from calf and chicken as early as 1967. From these it was assessed that the enzyme is predominantly acidic, with an isoelectric point of 4.85-5.0 and a molecular weight of 31,000-35,000. It was concluded that no cofactors were necessary for catalysis, since addition of EDTA or extensive dialysis of the enzyme did not cause significant reduction in activity (Zielke and Sueltzer, 1971). An early kinetic study of ADA enzymes from various species yielded a $K_m$ value for adenosine in the range of 10-60 μM. The mechanism was thought to be an ordered uni-bi with ammonia the first product to be released from the enzyme (Orsi et al., 1972). The pH dependence of the $K_m$ value for the reaction yielded inflection points at pH 5.7-6.3 and pH 9.5-10.2. The lower $pK_a$ was attributed to a His residue (Orsi et al., 1972). The higher inflection point was attributed to the ionization of a sulphydryl residue, since the enzyme appeared to be inactivated after the addition of sulphydryl-specific reagents such as p-mercuribenzoate and phenylmercuriacetate (Ronca et al., 1967). The pH dependence for the rate-limiting step (log $V_{max}$ vs pH plot) appeared to be governed only by a single group with a $pK_a$ of 4.8, speculated to be a His in the basic form (Orsi et al., 1972). Examination of a variety of adenosine and alkylated adenine analogs led a group of investigators to propose an active site containing a purine binding region, an apolar region and a hydrophilic region (Schaeffer et al., 1968), and another group to assert that the 1' and 5' positions of the ribose
were important for substrate binding and enzymatic activity (Bloch et al., 1967).

1.2. Information About the Mechanism Prior to the Crystal Structure
With the knowledge of the substrates and the products of the reaction catalyzed by ADA (Figure II.1), investigators attempted to gain insight into the mechanism by a variety of experiments. The first notable observation was the apparent promiscuity of the enzyme: a number of 6-substituted purine derivatives were bound and hydrolyzed by the enzyme, albeit less efficiently than the natural substrates (Zielke and Suelter, 1971). This tolerance by ADA of such C-6 substituents as chloro-, iodo-, bromo-, and methoxy- as well as the observed back-incorporation of $^{18}$O into inosine led Wolfenden (1969) to suggest that the reaction was essentially a nucleophilic aromatic substitution, involving enzyme or enzyme-bound water as the attacking group in the formation of a tetrahedral intermediate at position 6 of the purine (C in Figure II.1). Support for a rate-limiting step which involved nucleophilic attack was gained by the observation that the $V_{\text{max}}$ for the reaction was increased by the presence of electron-withdrawing groups in the ring system (Wolfenden et al., 1969). The notion that ADA employed direct water attack on the substrate was reinforced by (a). the discovery or synthesis of tight-binding inhibitors such as deoxycoformycin and the methanol adduct of purine ribonucleoside (D and E in Figure II.1, respectively) in the 70's (discussed in Wolfenden et al., 1977) and (b). the finding that ADA catalyzes the stereospecific addition of water to pteridine (Evans and Wolfenden, 1973). However, as late as 1983, direct water attack was not a certainty. To explain the origin of solvent isotope effects on the binding of transition-state and ground-state analogs, Kurz and Frieden (1983) proposed that part of the reacti...
Figure II.1 Substrates, products, reaction intermediates and analogs of the ADA reaction. (A) Substrates adenosine and deoxyadenosine. (B) Products inosine and deoxyinosine. (C) Proposed tetrahedral intermediate for the reaction mechanism. (D) The tight-binding inhibitor deoxycoformycin is an analog of the tetrahedral intermediate. (E) The methanol adduct of purine ribonucleoside is also a transition state analog and potent ADA inhibitor.
involved protonation of the substrate on N-1 by an enzyme acid. Nevertheless, other chemical events such as the hydration on C-6 or formation of a covalent adduct between the substrate and an enzyme sulfhydryl could not be excluded as alternative explanations. Frick and Wolfenden (1986) were not able to isolate a stable covalent intermediate between the enzyme and the tight inhibitor deoxycoformycin. A $^{13}$C NMR study of the ground-state analog purine riboside complexed to ADA provided evidence that the inhibitor was bound with a change in its C-6 hybridization state from sp$^{2}$ to sp$^{3}$ with formation of a new bond to oxygen or sulfur (Kurz and Frieden, 1987). From these studies, an addition-elimination mechanism with direct water attack to form a single tetrahedral intermediate was proposed. Weiss et al. (1987) suggested that an enzyme sulfhydryl protonates N-1 of the substrate prior to water attack on C-6 based on $^{15}$N and solvent deuterium isotope effects.

Meanwhile, work with a variety of transition-state analogs for the reaction drew attention to the extreme preference of ADA for the 6R isomers and the unusually high contribution of an axial C-6 hydroxyl to inhibitor binding (9.8 kcal/mol). One or a few hydrogen bonds between an enzymic charged group and the hydroxyl as well as the entropic advantage of having the hydroxyl covalently bound to the inhibitor were postulated as an explanation. A possible candidate for this charged residue on the enzyme was an active site histidine (Kati and Wolfenden, 1989). The investigators were careful to point out that positions N-1, C-2, N-3, and the C-6 OH of the purine ring as well as ribose are all important determinants of binding affinity that act synergistically and must all be present for strong binding (Kati et al., 1992). The fact that 3-deazaadenosine bound ADA very poorly but was a substrate whereas 1-deazaadenosine could bind very well but was not turned over by
the enzyme led Kurz et al. (1992) to infer that the N-3 interaction was essential for binding but not catalysis whereas the N-1 interaction was required for catalysis but not binding. In that same work, the UV solvent difference spectra of adenosine analogs lacking N-1 showed them to be bound by the enzyme in an unprotonated and unhydrated state. Furthermore, analysis of progress curves of the association between ADA and a variety of inhibitors revealed only one kinetic phase; this observation led the investigators to propose that the reaction catalyzed by ADA was encounter-limited, at least for formation of the tetrahedral intermediate from substrate analogs, and possibly also for the natural substrate, adenosine. By contrast, the changes in the optical spectrum of a 6-methylamino, 2-amino-purine analog and the transient changes in the spectrum of adenosine bound to ADA were attributed to a detectable protonated intermediate formed between substrate or substrate analogs and the enzyme (Porter and Spector, 1993).

2. Crystal Structure of Mouse Adenosine Deaminase

2.1. Structure of ADA Complexed to HDPR

The determination of the atomic structure of the murine enzyme at 2.4 Å resolution by Wilson et al. (1991) clarified many key aspects of the mechanism employed by ADA. The substrate analog used to crystallize the enzyme was purine riboside (PR), an adenosine analog. However, it was found that the hydrated form of this compound, HDPR, was actually bound in the active site. Both compounds, along with the presumed transition state for the reaction, are shown in Figure II.2. It can readily be seen that HDPR mimicks the transition-state and thus is a very tight-binding inhibitor of ADA (Jones et al., 1989). This finding corroborated earlier results regarding the sp² to sp³ change
Figure II.2 Purine riboside (PR) is a ground-state analog of the ADA reaction. Its hydrated form (HDPR) was bound in the active site of the crystallized enzyme. HDPR is an analog of the tetrahedral intermediate of the reaction but lacks the C-6 amino leaving group.
in the hybridization state of C-6 of PR upon binding to ADA (Kurz and Frieden, 1987). A finding that directly refuted earlier results with EDTA and other metal chelators (Zielke and Suelter, 1971) was that of a zinc cofactor, buried in the deepest part of the active site cavity. The zinc exhibited penta-coordinate geometry, with the three Ne2 atoms of His 15, His 17, and His 214 being tetrahedral, and the O-6 of HDPR and O82 of Asp 295 sharing the remaining site.

ADA was found to have a parallel α/β barrel motif with eight central β strands and eight peripheral α helices, with five additional helices. The active site is an oblong cavity, located at the COOH-terminal of the β barrel and lined by the connecting loops and carboxy-terminal segments of the β strands. Deepest in the cavity is found the zinc atom; next is the purine ring of HDPR, and last, closest to the active site opening, is the ribose unit (Figure II.3). The narrow opening of the active site is capped by two helices, one of which contains several hydrophobic residues. Thus, as predicted much earlier, "a purine binding region, an apolar region and a hydrophilic region" make up the substrate binding site (Schaeffer et al., 1968).

HDPR is in the anti conformation and is clearly the 6R diastereomer. It is held in place by nine hydrogen bonds with the enzyme (Figure II.4). The ribose unit is hydrogen-bonded via its 5'-OH and its 3'-OH to Asp 19 and His 17 of the enzyme. Clearly, the 5'-OH position is important in substrate binding, as predicted by early work on the enzyme (Bloch et al., 1967). By contrast, the 2'-OH is not, since it interacts only with an ordered water molecule and points towards the opening of the cavity. The purine portion of the substrate is connected to the enzyme via a number of hydrogen bonds through its N-1, N-3, N-7 and 6-OH. Glu 217 is coplanar with HDPR and in good hydrogen bond distance (2.8 Å) to N-1. It is thought to be protonated because of its proximity
Figure II.3 Backbone trace of murine ADA perpendicular to the β-barrel. The deep active site cleft is occupied by the bound HDPR (ball and stick model) and the zinc (white sphere) located below the purine ring. Two peptide segments (residues 58-67 and 183-188) partially cover the cleft.
Figure II.4 Schematic drawing of active site of ADA complexed with the transition-state analog HDPR. Distances between refined non-hydrogen atoms are in Angstroms. Non-covalent interactions are represented by dashed lines. Dotted lines represent coordinating interactions with the zinc. The zinc is coordinated to Oδ2 of Asp 295 and to the C-6 OH of HDPR. The metal is also coordinated to the Ne2 atoms of His 15, His 17 and His 214 (not shown).
to three apolar residues and thus acts as a hydrogen bond donor. Asp 296, also coplanar with the ring, donates a hydrogen bond to N-7. It is thought to be in a neutral, non-ionized state because of its close proximity to 3 Phe residues. Gly 184 donates a hydrogen bond to N-3 via its NH group. The 6-OH of HDPR is coordinated to the zinc and donates a hydrogen-bond to the Oδ1 of Asp 295. The 6-OH also accepts a hydrogen bond from His 238. The Oδ2 of Asp 295 shares the fourth zinc ligand site with the hydroxyl group.

How did this structural information correlate with earlier predictions on the binding interactions between ADA and its substrates? Work with a variety of substrate and transition-state analogs correctly postulated the indispensability of the ribose and positions N-1, C-2, N-3, the C-6 OH of the purine ring to the binding of substrates (Kati et al., 1992; Kurz et al., 1992). On the other hand, the extreme stereoselectivity of the enzyme for the 6R isomers of transition-state analogs like HDPR or coformycin had been attributed to the participation of the 6-OH to hydrogen bonds of unusual strength, perhaps to a charged enzyme residue like histidine (Kati and Wolfenden, 1989). From the crystal structure, it became apparent that this was only partially true and that the stabilization of 6R isomers within the active site was mainly due to the interaction of the 6-OH with the zinc cofactor. Other factors explaining ADA's stereoselectivity were the anchoring of the hydroxyl by Asp 295 and His 238 and the nonpolar nature of that part of the active site which the hydroxyl on a 6S isomer would face. In accord with the enzyme's ability to catalyze the deamination of both adenosine and 2'-deoxyadenosine, the 2'-OH position is clearly not essential for binding.
2.2. Proposed Mechanism

On the basis of the crystallographic information as well as the earlier work on ADA, a stereospecific, addition-elimination mechanism was proposed (Figure II.5). In this mechanism, the interaction between the 6-OH of HDPR and the zinc was taken as evidence for a similar interaction between the catalytic water and the zinc, right before nucleophilic addition to C-6 of the substrate. Thus, the zinc, with its high electrophilicity, serves to polarize the electron density of the water molecule, so that a nearby base—in this model Asp 295—can abstract a proton and create a hydroxyl group. This hydroxyl is held in the correct orientation for attack on C-6 via its interaction with His 238 and Asp 295. As the OH\(^-\) attacks the purine ring, the hybridization of C-6 changes from sp\(^2\) to sp\(^3\) as previously observed by Kurz and Frieden (1987); the double bond character between N-1 and C-6 is lost, and N-1 can become protonated by Glu 217. The result is formation of a hydrated, tetrahedral intermediate in the active site, with a hydroxyl- and an amino- substituent on C-6. HDPR, formed after enzymic addition of water, is a mimick of this intermediate, with the exception of a hydrogen replacing the amino group.

Because of the unfavorable placing of the leaving group in a predominantly apolar environment, Wilson et al. suggested that the tetrahedral intermediate may be extremely short-lived. In order for the intermediate to collapse to the two products of the reaction, ammonia and inosine, protonation of the leaving group must occur. However, the structure did not present any residue as a good candidate for the acid. The closest possible proton sources were Asp 295 and His 238.

What aspects of this mechanism agreed with earlier work? Firstly, the notion that ADA employed direct water attack rather than covalent catalysis was correct (Wolfenden et al., 1969; Kurz and Frieden, 1987). Additionally, the
Figure II.5 Proposed mechanism for the ADA reaction. Asp 295 abstracts the proton from the zinc-bound water, creating the attacking hydroxylate. The tetrahedral intermediate formed collapses into ammonia and the enol form of inosine. The source of the ammonia proton is unclear (Asp 295 is shown as the donor here).
occurrence of protonation on N-1 during the reaction had correctly been identified earlier (Kurz and Frieden, 1983). The observation by Wolfenden et al. (1969) that electron-withdrawing substituents on the purine ring enhanced the catalytic rate of the enzyme agreed with the involvement of all the purine nitrogen lone pairs in electron-withdrawing hydrogen bonds. However, no evidence from the crystal structure was compatible with the assignment of a Cys to one of the primary catalytic residues (Ronca et al., 1967; Weiss et al., 1987). Only two out of five Cys residues were relatively close to the active site (Cys 262 and Cys 153). Nevertheless, they were either too far (Cys 153) or prevented from access to the substrate by other amino acids (Cys 262) to play a direct role in the catalytic events.

2.3. Modifications to the Mechanism: Structure of 1-DAA with ADA

Following the solution of the structure of ADA complexed to a transition-state analog, Wilson and Quirocho (1993) were able to solve the structure of the enzyme bound to the ground-state, or "pre-transition-state" analog 1-deazaadenosine (1-DAA). This tight-binding ADA inhibitor (Kᵢ = 0.18 μM) lacks the N-1 group and is therefore not protonated or turned over by the enzyme (Kurz et al., 1992). This structure revealed some important new aspects in the mechanism (Figure II.6). Firstly, coordinated to the zinc at a distance shorter than the other four ligands (1.91 Å) was a hydroxide ion, in lieu of the 6-OH of HDPR found in the previous structure (distance from zinc: 2.29 Å). It was evident that the enzyme base had been able to deprotonate the zinc-polarized water to produce a hydroxide, which was now "suspended", poised for attack. Also, the purine ring of 1-DAA had now moved slightly away from Glu 217 since the N-1/Glu 217 bonding interaction was absent. Having both oxygens involved in metal coordination and hydrogen-bonding
Figure II.6 Active site of ADA complexed with the ground-state analog 1-deazaadenosine. Distances between refined non-hydrogen atoms are in Angstroms, non-covalent interactions are represented by dashed lines. Dotted lines represent coordinating interactions with the zinc. The metal is also coordinated to the Ne2 atoms of His 15, His 17 and His 214 (not shown). An activated hydroxyl group is coordinated to the zinc. Addition of the hydroxylate cannot occur at C-6 due to the lack of a nitrogen on position 1 of this inhibitor.
interactions, Asp 295 was unlikely to be a good base. Instead, the investigators suggested that the nearby His 238, which had now moved closer to the hydroxide, fulfilled that role (Figure II.7). The function of Asp 295 was confined to that of locking the hydrogen of the attacking OH in a hydrogen bond, so that a lone pair on the hydroxyl was properly oriented for attack on C-6. Superposition of the HDPR and 1-DAA structures revealed that the distance between the hydroxide and C-6 of HDPR is approximately 2.5 Å, ideal for such an attack. The requirement for a His residue in its basic form in the rate-determining step agreed with early studies of the pH dependence for the ADA reaction (Orsi et al., 1972). The origin of the proton required for elimination of the leaving group, however, was still uncertain despite this newer data. Modeling of the axial amino group placed it somewhat far from the most probable candidates, Asp 295 and His 238 (3.2 and 4.5 Å, respectively). An intriguing possibility was that this structure represented a "snapshot" of the true pre-transition-state for the ADA reaction, with the free enzyme, after binding and "activating" the catalytic water, able to now bind and catalyze the substrate.

2.4. Structure of Inosine with ADA
Inosine is a competitive inhibitor of ADA with a Kᵢ of 1 mM (Shih and Wolfenden, 1996). When this compound was used in the crystallization mixture, the resulting structure revealed that it was bound as a hydrated adduct, with two oxygen substituents on C-6 (which now had an sp³ hybridization). It appears that ADA catalyzes the addition of a hydroxide on C-6 of inosine, creating a gem-diol, in which the old oxygen anion (O6A) is in the same position with the leaving amino group from adenosine (Figure II.8). In this unexpected adduct, the proton on the new hydroxyl group (O6B) is
Figure II.7 Modified ADA mechanism from the 1-DAA structure. His 238 abstracts the proton from the zinc-bound water forming the attacking hydroxylate. Glu 217 donates a proton to N-1 and the tetrahedral intermediate forms. The tetrahedral intermediate collapses into ammonia and the enolate form of inosine.
Figure II.8 Schematic diagram of the inosine \textit{gem}-diolate and its non-covalent interactions with enzymic residues. The numbers represent distances in Angstroms. O6B corresponds to the C-6 OH of HDPR; O6A to the leaving amino group in adenosine or 1-DAA. The angles of the hydrogen bonds between the O6A and the fully extended side chain of Glu 217 or Asp 295 are 95° (adapted from Wilson and Quiocho, 1994).
thought to be transferred to the original oxygen anion \((O6A^-)\). Proton shuttling could be mediated by Asp 295, which is located in between the two oxygen groups. Displacing the proton from O6B to O6A removes negative charge from an apolar environment and allows for favorable interactions between the anionic O6B and the zinc or the positively charged His 238. That the O6B oxyanion is coordinated to the zinc is evident from the distance between the two atoms \([1.96 \, \AA], a shorter distance than that observed between the 6-OH of HDPR and zinc (2.29 \, \AA), but slightly longer than the zinc-hydroxylate distance in the 1-DAA structure (1.91 \, \AA)]. In addition, the transfer of a proton from the incoming group to the leaving group via Asp 295 may be relevant to the actual mechanism by which the amino leaving group on adenosine becomes protonated prior to departure. This new finding helps shed light to the mysterious origin of the ammonia proton.

2.5. Summary of the ADA Mechanism

The salient features of the mechanism emerging from the three crystal structures of ADA as well as earlier work are the following:

-The enzyme employs direct water attack on the substrate by utilizing a zinc-bound water as the source of the hydroxyl. The residues proposed to act as the base are either Asp 295 or His 238.

-The substrate must be protonated by the enzyme on N-1 for hydration to occur. The enzyme acid postulated to donate a proton is Glu 217.

-The incipient hydroxyl is correctly oriented for attack by its coordination to the zinc and its hydrogen bonding interactions with Asp 295 and His 238.

-The resulting tetrahedral intermediate may have a very short lifetime due to unfavorable interactions between the leaving group and the surrounding apolar regions.
- The ammonia proton may originate from Asp 295 or His 238 or may be shuttled from the attacking hydroxyl to the leaving amino group via Asp 295.
- There is evidence for binding and activation of the catalytic water prior to substrate binding. Thus the mechanism is probably an ordered uni-bi, in which substrate binds the ADA active site with an already activated water poised for attack, and ammonia is the first and inosine the second product to leave.

3. Similarity to Other Enzymatic Mechanisms
ADA is an enzyme which utilizes metal ion catalysis through its zinc cofactor. A number of zinc enzymes have been described in the literature, which share some common mechanistic features with ADA. These include carbonic anhydrase, carboxypeptidase, and thermolysin. In addition, the enzyme cytidine deaminase, which catalyzes a similar reaction, has recently been described mechanistically in great detail. Together with ADA, they are an example of convergent evolution, because of their similarities in transition-state stabilization despite differences in zinc ligands and tertiary structure. Another enzyme, urease, catalyzes the hydrolysis of urea and shares a great deal of structural similarity in its catalytic domain with ADA, thus providing an example of divergent evolution.

3.1. Zinc Proteases: Thermolysin and Carboxypeptidase
Thermolysin is a zinc endopeptidase, with an approximately tetrahedral zinc having 2 His, one Glu, and a water as ligands (Matthews, 1988). A carbonyl oxygen of the incoming peptide substrate displaces the water (which is still bound to the zinc) towards Glu 143. The zinc-water interaction as well as hydrogen bonds between both water protons and Glu 143 serve to orient a
lone pair towards the carbonyl carbon and enhance its nucleophilicity. General-base catalysis occurs by Glu 143 and the OH\(^-\) adds to the carbonyl carbon to form a penta-coordinate intermediate. The proton is immediately transferred from Glu 143 to the nitrogen of the peptide amido group. A second transfer of a proton accepted by Glu 143 from the hydrated peptide and donated to the leaving nitrogen mediates collapse to products. A nearby His 231 must be in its protonated state in order to stabilize the hydrated peptide.

Carboxypeptidase is a zinc exopeptidase, with a zinc coordinated to two His residues, a Glu and the catalytic water. If the active sites of thermolysin and carboxypeptidase are superimposed, one can detect a number of common elements. Thus the catalytic glutamate in thermolysin, Glu 143, superimposes with Glu 270 in carboxypeptidase. Glu 270 is thought to create the hydroxyl attacking group from the zinc-polarized water for attack on the carbonyl carbon of the substrate (Christianson and Lipscomb, 1989). Similarly to thermolysin, this glutamate residue also donates two more protons to the substrate before product formation. In place of His 231 in thermolysin, there is Arg 127 in carboxypeptidase. This residue serves to stabilize negative charge in the tetrahedral intermediate in both enzymes. The metal, in both systems, starts with four ligands, becomes penta-coordinated as reaction proceeds, and is finally tetracoordinated at the end of the reaction.

The zinc proteases share a number of common features with the ADA mechanism: they involve a zinc cofactor in the polarization of the catalytic water; employ direct water attack on the substrate to generate a tetrahedral intermediate, and the zinc cofactor oscillates between tetra- and penta-coordination during the course of the reaction. If the ADA mechanism employs Asp 295 as the base creating the hydroxylate, and Asp 295 again as the acid protonating the leaving group, then there is an equivalence between the
catalytic glutamate of the zinc proteases and Asp 295 of ADA.

3.2. Carbonic Anhydrase

Carbonic anhydrase catalyzes the hydration of carbon dioxide and dehydration of bicarbonate. The three human isozymes are all found to contain 1 mol of zinc per subunit. The zinc is approximately tetrahedral, coordinated to 3 His ligands and the catalytic water. The initial step in the reaction involves the direct nucleophilic attack of zinc-bound hydroxide on carbon dioxide (Silverman and Lindskog, 1988). The hydroxide donates a hydrogen bond to the hydroxyl of Thr 199, which in turn donates a hydrogen bond to Glu 105. The Zn-bound water is rendered remarkably acidic (pKa=6.8) through the hydrogen-bonding network, as a Thr 199 to Ala mutation shows (Liang et al., 1993). The interaction between Thr 199 and the zinc-bound water may also be important in properly orienting the hydroxyl group for attack on CO₂ (Liang et al., 1993). Bicarbonate is formed following addition of the hydroxylate to CO₂. The bicarbonate appears to be coordinated to the zinc in a bidentate fashion, resulting in penta-coordinate zinc; it is later replaced from the metal coordination sphere by a water (Xue et al., 1993). In a separate step from the conversion of CO₂ to HCO₃⁻, a proton is transferred from this zinc-bound water via a network of hydrogen bonds to His 64 and from there to the buffer, a process which regenerates the zinc hydroxide (Silverman and Lindskog, 1988; Tu et al., 1989).

Obvious similarities between the ADA reaction and this mechanism include the direct nucleophilic attack of a hydroxide on the substrate, a hydroxide originating from a zinc-bound polarized water. The water is rendered more acidic and is oriented for attack through its donation of a hydrogen-bond to Thr 199, similarly to the interaction seen between the OH and Asp 295 and/or
His 238 in ADA. His 64 (through a lattice of hydrogen-bonds) abstracts the zinc-water proton, as His 238 may be doing in ADA. The zinc cofactor itself is coordinated to 3 His residues and the catalytic water in both enzymes and oscillates between tetra- and penta-coordinated states as reaction proceeds.

3.3. Cytidine Deaminase: An Example of Convergent Evolution

Cytidine deaminase (CDA) catalyzes the hydrolytic deamination of cytidine to uridine. The active site contains a zinc atom coordinated to four ligands (the catalytic water, 2 Cys and one His residue). A nearby Glu 104 is postulated to abstract a proton from the zinc-bound water to form the attacking hydroxyl group. The same Glu residue is thought to protonate N-3 (the equivalent of N-1 on adenosine) and shuttle the proton from the C-6 OH to the amino leaving group (Betts et al., 1994). Despite the similarity of the two reactions and the similar functions of the zinc atoms in their active sites, the mechanisms which are employed by CDA and ADA appear to be different. The zinc atom in CDA is tetra-coordinated versus the initially penta-coordinated metal in ADA. The leaving amino group appears to bind via well-defined hydrogen bonds in a specific pocket of the CDA active site and the pyrimidine ring of the substrate moves away from it as cytidine approaches the transition-state (Xiang et al., 1996). By contrast, the amino leaving group in ADA faces a very apolar region of the enzyme, and little is known on the mode of its departure (Wilson et al., 1991). The most important difference between the two enzymes is that Glu 104 of CDA is able to catalyze all proton-abstracting and proton-donating functions, including the donation of the proton to the leaving amino group. Asp 295 or His 238 and Glu 217 are instead employed by ADA to perform these roles, and leaving group protonation is an aspect of the mechanism still not clearly defined. Thus,
CDA appears to require fewer primary catalytic residues than does ADA. This is reflected in the absence of conservation when the two enzyme sequences are compared. The only common stretch is the sequence TVHAGE (residues 212-217 in ADA) and TVHAE (residues 100-104 in CDA; Yang et al., 1992). The conserved His in the above sequences is a zinc ligand in both enzymes. In addition, the conserved Glu is the primary catalytic residue in CDA and performs the important function of donating the proton to N-1 in ADA. None of the other primary catalytic or zinc-coordinating ADA residues (His 238, Asp 295 etc.) are obviously conserved in the CDA sequence. The differences in zinc ligands and overall tertiary structure of CDA and ADA and the similarities in transition-state stabilization appear to be a product of convergent evolution (Betts et al., 1994).

3.4. Urease: An Example of Divergent Evolution

Urease catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. The crystal structure of unbound urease from Klebsiella aerogenes was recently determined (Jabri et al., 1995). It is a nickel-dependent trimeric metalloenzyme. One of the subunits has a (α/β)₈ domain; in it lies the active site which contains two nickel atoms. Ni-1 is coordinated by three ligands: two His and a carbamylated Lys. Ni-2 is coordinated by five ligands: 2 His, an Asp, the carbamylated Lys, and the catalytic water. Modeling of urea in the active site suggests that the mechanism proceeds as follows: urea is bound through its oxygen by Ni-1 and oriented and polarized for attack by nearby His 219. The catalytic water is bound to the other nickel, Ni-2. A base, possibly Asp 360, abstracts a proton from the water and the resulting hydroxide attacks urea to form a tetrahedral intermediate. The intermediate decomposes into the products of the reaction, ammonia and carbon dioxide, with aid from a
general acid, whose identity is unknown.

Despite the complete lack of primary sequence homology, there is a high degree of structural homology between urease and ADA, not only in their $\alpha/\beta$ barrels, but also in their active site architecture. If the two active sites are superimposed, it is clear that Ni-2 and its water ligand are structurally equivalent to zinc and the water in ADA. The other 2 His and one Asp ligands of Ni-2 are also equivalent to His 14, His 17 and Asp 295 in ADA. The last ADA zinc ligand, His 214, corresponds to the Ni-1 His 246 ligand in urease. Another His ligand of the Ni-1 atom of urease (His 272) is equivalent to the catalytic His 238 of ADA. The reactions catalyzed by the two enzymes are very similar, and if the above urease mechanism proves to be correct, there are important mechanistic similarities as well. For example, the metal-polarized water is oriented for attack by His 219 in urease as it may be by His 238 in ADA. If Asp 295 is the base creating the hydroxylate in ADA, it plays a role very similar to that of Asp 360 in urease. This lack of primary sequence homology but high degree of structural similarity and the common mechanistic features despite the difference in metal cofactors between the two enzymes are examples of active site divergence.

4. Sequence Homology Among Deaminases

A comparison of ADA sequences from mouse, human, and *E. coli* demonstrates their extensive homology (Figure II.9). The *E. coli* sequence is 32% identical and 47% similar to the human ADA sequence. (Chang et al., 1991). All the important catalytic and structural active site residues have been conserved: His 15, 17 and 214 (the zinc ligands), Asp 19, 295, and 296 (substrate and zinc binding), Gly 184 (substrate binding), Glu 217 and His 238 (catalysis).
Figure II.9 Amino acid sequence comparison of E. coli (a), mouse (b), and human (c) adenosine deaminases. Identical amino acids are shown in the bottom line (d), with (+) used to designate a conservative and (-) a non-conservative amino acid substitution. Blocks of conserved amino acids are boxed. The vertical arrow is on His 238, a residue conserved among the three sequences. Adapted from Chang et al., 1991.
In addition, certain of these residues have been conserved in the sequences of AMP deaminase and cytidine and dCMP deaminase, enzymes mediating related reactions. The mechanism of cytidine deaminase was described in Section 3.3. AMP deaminase is a tetrameric, zinc-containing enzyme catalyzing the hydrolysis of monophosphorylated adenosine (Zielke and Suelter, 1971; Merkler and Schramm, 1993). Even though the crystal structure of the enzyme has not been solved, AMP deaminase is believed to employ a related mechanism to that of ADA, from the similarity in reaction and substrates as well as the fact it is inhibited by the potent ADA transition-state analog deoxycoformycin and its derivatives (Frieden et al, 1980).

The sequence alignment in Figure II.10 shows the remarkable retention of one block of amino acids, (T)VHA(G)(E), among all four deaminases. In ADA and CDA deaminases, this block contains important catalytic and structural residues, such as the metal-bound His (His 214 in ADA and His 102 in CDA), and a catalytic glutamate essential to both reactions (Glu 217 in ADA and 104 in CDA). The functions of these conserved residues in AMP and dCMP deaminases are not yet known, but they can be expected to be of importance to catalysis and/or metal coordination. A second well-conserved block of amino acids found in ADA and AMP deaminases is S(L)(N)TDDP, which contains the two important aspartates, 295 and 296, in mouse ADA. Since E. coli CDA has a tetra-coordinate zinc, there is no residue with a role equivalent to that of Asp 295. The same goes for Asp 296, which forms a hydrogen bond with N-7 of the substrate: cytidine lacks the imidazole moiety of adenosine, and therefore does not require such a binding interaction. Therefore, these residues have not been conserved in CDA and dCMP deaminases.

In addition to the two amino acid blocks, a cysteine residue is also conserved among ADA and AMP deaminases. The degree to which this residue is
Adenosine Deaminase

E. coli  HRHLGD-----DLAGDE-----TVHAGE-----H------C------SINTDDP
Mouse  \textsuperscript{15}HVHLGD--\textsuperscript{181}DLAGDE--\textsuperscript{212}TVHAGE--\textsuperscript{238}H--\textsuperscript{262}C--\textsuperscript{291}SLNTDDP
Human  HVHLGD-----DLAGDE-----TVHAGE-----H------C------SINTDDP

AMP Deaminase

Rat  \textsuperscript{362}VHAG---------\textsuperscript{573}C------SLSTDDP
Human M form  \textsuperscript{362}VHAG---------\textsuperscript{573}C------SLSTDDP
Yeast  \textsuperscript{362}MHAH---------\textsuperscript{573}C------SLSTDDP

Cytidine Deaminase

E. coli  \textsuperscript{100}TVHAE----\textsuperscript{126}Y--\textsuperscript{128}PC--\textsuperscript{132}C

dCMP Deaminase

S. cerevisiae  \textsuperscript{}TVHAE-------Y-----PC------C
Bacteriophage T2  \textsuperscript{}TVHAE-------Y-----PC------C
Bacteriophage T4  \textsuperscript{}TVHAE-------Y-----PC------C

Figure II.10 Sequence homology of ADA, CDA and AMP and dCMP deaminases. For mouse ADA and E. coli CDA those residues proven to be important in catalysis, substrate binding and metal coordination are shown in bold. The ADA/AMP deaminase sequence alignment was adapted from Chang et al. (1991). The CDA and dCMP sequence information was adapted from Yang et al. (1992).
conserved is remarkable if one looks at its distances from the first and second conserved amino acid block (TVHAGE and SLNTDDP). The first distance is 44 amino acids in all ADA sequences, and 206-207 amino acids in AMP deaminases. The second distance is 31 amino acids in ADA and 74 in AMP deaminases. From these observations, an essential role was expected for this cysteine in the enzymic mechanism (Chang et al., 1991). Surprisingly, data from the mouse ADA crystal structure showed this cysteine (Cys 262) as being close to the active site but clearly not involved in the catalytic process. In CDA and dCMP deaminase, the two cysteine residues that are completely conserved are ligands to the zinc metal cofactor (at least in the E. coli CDA active site). It is tempting to speculate that one of them is a cysteine conserved among all deaminases which found use as a zinc ligand in CDA.

His 238 is conserved in all ADA sequences, where it is located 22 amino acids away from Ala in the TVHAGE sequence and 24 amino acids away from the conserved cysteine. It lies in between two conserved glycine residues. In rat AMP deaminase, there is a histidine next to a glycine (His 594 and Gly 595 in rat AMP deaminase) between these conserved elements, which may correspond to His 238. In E. coli CDA, there is a similar histidine and glycine at positions 182 and 183. Interestingly, in CDA and dCMP deaminases, there is a tyrosine residue (Y 126 in E. coli CDA) which is 23 residues away from Ala in TVHAE, the same distance from TVHAGE at which we find His 238 in adenosine deaminases. This tyrosine is in the active site of CDA and plays important roles in aromatic stacking with the substrate and facilitating exit of the leaving group (Betts et al., 1994).
CHAPTER III.

SITE-DIRECTED MUTAGENESIS OF HIS 238 IN MURINE ADENOSINE DEAMINASE

1. Objectives
As discussed in the previous chapter, a number of proposals for the role of His 238 in the ADA mechanism were put forth on the basis of the three ADA crystal structures. The HDPR structure suggests that His 238 functions in orienting the hydroxylate and stabilizing its negative charge. It may also donate a proton to the leaving group, thus acting as a general acid. The 1-DAA structure proposes His 238 to be the base, forming a hydroxylate anion from the zinc-bound water. Moreover, based on this structure, His 238 orients the hydroxylate for attack, together with Asp 295, and may donate the proton to the leaving group. Thus, it is proposed to act both as a general base and a general acid. From the inosine structure, His 238 acts only as a general base, and the proton for the leaving group is shuttled from the attacking hydroxylate via Asp 295. We employed the powerful technique of site-directed mutagenesis in order to examine, and hopefully dissect, the above suggested functions of this conserved residue, His 238, in the ADA mechanism.

2. Choice of Mutations
The residues by which His 238 was replaced were chosen according to the following criteria: their relative size, based on the area occupied by each side chain; their acidity or basicity; their ability to participate in hydrogen bonds; and their β-sheet forming propensity, since His 238 is located within the β-barrel. However, this last criterion was the least strict, since His 238 is found
in a loop connecting two β-sheets of the barrel. A search of the literature revealed that catalytic histidines were most commonly replaced by Ala, Gln, and Arg/Lys residues. Our choices were alanine, glutamate, glutamine and arginine.

Alanine is a nonpolar, non-ionizable residue with an area of 118 Å² versus 202 Å² of the histidyl side chain (Moody and Wilkinson, 1990). Its methyl side chain is not able to donate or receive hydrogen bonds. According to the Chou and Fasman rules, it is an indifferent β-sheet former as compared to histidine, which is a former of this type of secondary structure (Voet & Voet, 1990).

Glutamine is an uncharged, polar residue with an area of 193 Å². Together with glutamate, an acidic, charged amino acid residue (pKₐ = 4.07; area = 186 Å²) they are the closest, in terms of size, to histidine. While glutamine is a β-sheet former, glutamate is not frequently found in sheets but rather prefers α-helices. Both residues can easily participate in hydrogen bonds.

Arginine is a basic, charged residue with a pKₐ of 12.5 for its side chain. It is the most drastic departure from histidine in terms of relative area (256 Å²). It can participate in hydrogen bonds and is an indifferent β-sheet former. The arginine and glutamate mutations were modeled in murine ADA and theoretical energy minimizations were made using the Quanta Program (MSI). Mutant enzymes with a stable conformation (i.e. a global energy minimum) were the result of these calculations.

Assuming that the above mutations result in enzymes expressed and folded correctly, various predictions can be made in terms of their catalytic behavior. If the arginine mutation at 238 can be accommodated without major active site disruption, the residue will be positively charged at physiological pH, where ADA is the most active. If the primary function of His is that of orienting the hydroxylate for attack and stabilizing its negative charge, the Arg mutant will
have some activity. However, the arginine side chain cannot donate a proton to the leaving group. Thus, if His 238 only acts as a general acid, Arg mutants will be inactive, with the substrate stuck in the hydrated tetrahedral intermediate form. If His 238 acts as a base, or both an acid and a base, Arg will not be able to mimick it catalytically, and the Arg 238 mutant will be inactive. Glutamate causes a drastic ionic disruption of the active site, since it introduces a negative charge (Glu 238 will be anionic at pH 7.0) where a basic, positively charged residue used to be. Therefore, if His 238 primarily stabilizes negative charge, the glutamate at this position will have a destabilizing effect. This situation may affect the ionization state of neighboring amino acids such as Asp 295 and Glu 217; this substitution may also reduce the acidity of the zinc-water proton. If His 238 is the source of the leaving group proton, Glu 238 will be an inactive enzyme. A similarly inactive Glu 238 enzyme is expected if His 238 is the base. In all cases, then, a Glu at position 238 is expected to produce an inactive enzyme.

Glutamine may be able to orient the hydroxylate correctly for attack, but will not stabilize negative charge, nor be able to abstract or donate protons. Thus, if His 238 does nothing more than align things properly in the active site, this mutant will be active; in all other cases, this mutation will result in an inactive deaminase.

Finally the Ala 238 mutation introduces a small apolar acid at that location. Alanine cannot orient the hydroxylate, stabilize charge, hydrogen-bond or donate/abstract a proton. This substitution would lead to an inactive mutant ADA. However, a water molecule may fit in the large space now left vacant by the alanine. The water could hydrogen-bond with and orient the hydroxylate, but cannot act as the base or protonate the leaving group. As is the case with the glutamine and arginine mutants, an Ala 238/water mutant will have
activity only if the role of His 238 is confined to orienting the OH⁻ via hydrogen bonds.

3. Methods

3.1 Bacterial Strains and Vectors
The mouse adenosine deaminase cDNA was inserted in the pRC4 phagemid expression vector (Figure III.1) under control of an IPTG-inducible tac promoter (Chang et al., 1992). The f1 locus on this vector allows for superinfection with IR1/M13 helper phage and isolation of single-stranded (ss) DNA. The amp resistance gene allows for colony selection on ampicillin-containing agar plates. *E. coli* strains CJ236 and BW313 (genotype: dut⁻ ung⁻) were used for the production of uridine-rich ss DNA. A third *E. coli* strain, 71-18, was used for mutant selection and plasmid propagation. Mutant plasmids were expressed in the ADA⁻ strain SΦ3834 (Chang et al., 1991). This strain contains a deletion of the adenosine deaminase (*add*) gene and two neighbouring genes. Both the pRC4 vector and the SΦ3834 strain were developed in Dr. Rodney Kellems' laboratory at Baylor College of Medicine, Houston.

3.2 Site-Directed Mutagenesis
The Kunkel method for producing site-specific mutants was used (Kunkel et al., 1987). Uridine-rich ss DNA was isolated from pRC4-transformed CJ236/BW313 cells (*dut⁻ ung⁻* genotype). First, mutagenic primers were designed for annealing onto the template ss DNA based on the following sequence information for mouse ADA:
Glu Arg Val Gly His\textsuperscript{238} Gly Tyr His Thr
5' GAG AGG GTG GGA CAT GGT TAT CAC ACC 3'
3' CTC TCC CAC CCT GTA CCA ATA GTG TGG 5'
The following four mutagenic primers were used:

Ala 238:
5' GGT GTG ATA ACC CGC TCC CAC CCT CTC 3'

Arg 238:
5' GGT GTG ATA ACC ACG TCC CAC CCT CTC 3'

Gln 238:
5' GGT GTG ATA ACC CTG TCC CAC CCT CTC 3'

Glu 238:
5' GGT GTG ATA ACC TTC TCC CAC CCT CTC 3'
The above primers were either 17-mers if the mutant codon contained one base different from the wild-type His codon (as was the case for Gln and Arg) or 25-mers if the codon had 2 or 3 bases different (Glu and Ala). The second strand was synthesized using the Muta-Gene M13 \textit{in vitro} Mutagenesis Kit (Bio-Rad). The synthesized double stranded DNA products were transformed into a wild-type strain (71-18) and selected on LB-ampicillin plates. Ss DNA from individual colonies was isolated and sequenced in the area of the mutation (Sequenase kit, United States Biochemicals). Once mutant colonies were identified (only 15-25\% of all colonies), the entire cDNA was sequenced to ensure that no other alterations were present.

3.3 Protein Expression and Purification

Wild-type and mutant plasmids were expressed in the ADA\textsuperscript{-} strain \textit{Sφ3834}. Overnight cultures were inoculated into 6 liters of superbroth medium (32 g/l
Figure III.1 Representation of the phagemid expression vector pRC4 constructed by Yeung et al., 1985. This vector was used in the purification of single-stranded template DNA for site-directed mutagenesis. The mADA cDNA has been inserted into a Nco I and an Eco RI site. The designated unique Nco I and Cla I sites were digested during mutagenesis to ensure integrity of the vector. Digestion at these sites yields a 3 kb and a 2.2 kb fragment, which contains the mADA cDNA. The p^tac promoter allows strong induction by IPTG. Amp^r = gene conferring ampicillin resistance; Ori = origin of replication; F_1 = replication origin for the single stranded phage f1.
bactotryptone, 20 g/l yeast extract, 5 g/l sodium chloride) containing 1 mM zinc sulfate and 60 μg/ml ampicillin. Ten hours into the growth, 4 mg/l IPTG (Sigma) was added, and growth was continued for 20 hours. The cells were harvested and frozen at -20°C. Cell pellets from the entire 6 liter culture were resuspended in 80 mls of DEAE buffer (50 mM imidazole, pH 6.8, 10 μM zinc sulfate, 5% glycerol, 1 mM dithiothreitol) containing 1 mM AEBSF protease inhibitor (Sigma). A French Pressure Cell (SLM Instruments) was used for cell lysis, and the resulting homogenate was centrifuged at 100,000 X g for one hour in a Beckman L5-50 ultracentrifuge using a 55.2 rotor. The supernatant was loaded onto a DEAE-cellulose column (5 X 20 cm, Whatman) and eluted using a 0-0.5 M NaCl salt gradient in DEAE buffer. Fractions were collected at a flow rate of 1 ml/min. The ADA proteins eluted between 10 and 17 hours into the gradient. ADA-containing fractions were determined by spectrophotometric assays or dot blots (as described below), pooled, and brought to 60% saturation with ammonium sulfate. After centrifugation, the supernatant was saturated to 80% with ammonium sulfate and centrifuged. The pellet was dissolved in 70% HIC buffer (20 mM potassium sulfate, pH 7.0, 10 μM zinc sulfate, 5% glycerol, and 1 mM dithiothreitol) containing 2 M ammonium sulfate and loaded onto a hydrophobic interaction HPLC column (Polypropyl A, 5 μM, Custom LC, Inc.). Proteins were eluted with HIC buffer at a flow rate of 1 ml/min. The eluate was monitored at 280 nm and the ADA peak typically eluted at 25-30 min. The ADA-containing fractions were concentrated by dialysis overnight against 20% PEG, mol. weight 15-20,000 (Sigma) in AX300 buffer (20 mM imidazole, pH 6.8, 10 μM zinc sulfate, 5% glycerol, 1 mM dithiothreitol). The dialysate was washed with 75% AX300 buffer, loaded onto a silica based polyethyleneimine HPLC column (SynChropak AX300 10 X 250 mm, Synchrom, Inc) and eluted with AX300
buffer containing 500 mM sodium chloride. The ADA peak eluted at 25-30 min at a flow rate of 1 ml/min. The purified protein was concentrated in Tris-Zn buffer (10 mM Tris-HCl, pH 7.0, 10 μM zinc sulfate, 50% glycerol, 1 mM dithiothreitol) and stored in air-tight vials under argon at -20°C. All purification steps except HPLC were performed at 4°C.

3.4 SDS-Polyacrylamide Gel Electrophoresis, Western Blots and Dot Blots

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) on a mini-gel apparatus (Bio-Rad). Proteins were stained with Coomassie Brilliant Blue. The Rainbow molecular weight markers (Amersham Corp.) were used as standards. For western blots, proteins separated by SDS-PAGE were blotted onto Immobilon-P transfer membranes (Millipore). The primary antibody was goat anti-mADA polyclonal antibody, and the secondary was alkaline-phosphatase conjugated rabbit anti-goat IgG (Sigma Immunochemicals). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard and the Bio-Rad Protein Assay Reagent. Dot blots were performed by pipeting 1 μL of DEAE fractions onto an activated PVDF membrane. The membrane was pre-hybridized in 1% low fat dried milk for 1 hour in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The membrane was then incubated with the primary and secondary antibody (as used in Western blots) for 30 min.

3.5 Inclusion Body Assays

Inclusion bodies were prepared from SΦ3834 by a modification of the protocol in Sambrook et al. (1989). A 5 ml overnight culture of SΦ3834 harboring ADA was inoculated into 500 ml of superbroth medium containing 1 mM zinc
sulphate and 60 mg/ml ampicillin and expression of pRC4 was induced with IPTG at 10 hours into the growth. The cells were allowed to grow for 22 hours and were lysed by French Press. The cell lysate was centrifuged at 100,000 X g for 45 minutes in a Beckman L5-50 ultracentrifuge using a 55.2 rotor. The pellet, containing cell debris and inclusion bodies, was resuspended in 15 ml DEAE buffer (Section 3.3) and the supernatant, containing soluble proteins, was saved. The resuspended pellet was centrifuged again for 45 minutes in the ultracentrifuge. The supernatant was discarded and the pellet was resuspended in 100 μl of 0.5% Triton X-100 buffer (50 mM Tris, 100 mM NaCl, 10 mM EDTA, 0.5% Triton X-100). After resuspension was complete, an additional 900 μl of buffer were added and the solution was left to stand at room temperature for 10 minutes. The solution was centrifuged for 20 minutes at 12,000 X g in a microfuge, and the supernatant was removed and saved. The pellet, containing purified inclusion body proteins, was resuspended in 100 μl of H₂O. The cytosolic soluble proteins and those present in the purified inclusion bodies were run on SDS-PAGE and blotted with anti-ADA antibodies.

3.6 Enzyme Assays and Kinetic Analyses
Enzymatic activities were assayed on a Cary 118 spectrophotometer by measuring the rate of ADA-dependent increase of inosine absorption at 235 nm at 30°C, using an extinction coefficient of 3.5 mM⁻¹cm⁻¹. For H238A, the rate of ADA-dependent decrease of adenosine absorption was measured at 265 nm at 30°C, using an extinction coefficient of 8.4 mM⁻¹cm⁻¹. One unit of ADA activity is defined as the amount of enzyme that produces 1 μmol of inosine per minute. Cuvettes of 1 cm pathlength were used. Assays were carried out in 50 mM potassium phosphate buffer, pH 7.2. Activities were measured over
at least six different concentrations of adenosine and the assays were repeated at least three times. Adenosine concentrations ranged from 0.5-5 X K_m.

The concentration of enzyme in the assay mixture ranged from 0.1 nM for the wild-type to 0.2 μM for H238R and 0.3 μM for H238A and H238E. Kinetic parameters were determined by Lineweaver-Burk plots with a fourth power weighting function using the Enzyme Kinetics (Trinity Software) program. K_i values for two ADA inhibitors, N6-methyl adenosine (N6MA) and purine riboside (PR) were determined for the enzymes. Assays were performed as above, with thorough mixing of the substrate and inhibitor in the reaction mixture prior to addition of the enzyme. Two to four different inhibitor concentrations bracketing the K_i value were used in the assays, and each velocity vs substrate curve was determined at least three times. Inhibition plots and K_i values were obtained as described above. The kinetic parameters K_m and k_cat refer to the following mechanism for a one-substrate reaction:

\[
E + A \xrightleftharpoons{\text{k}_1}{\text{k}_-1} EA \xrightarrow{\text{k}_2} E + P
\]

where A is adenosine and E is ADA, and K_m = (k_1 + k_2)/k_1. Kurz et al. (1992) calculated the individual rate constants for the hydrolysis of adenosine by calf intestine ADA. From their data, K_m was not equal to the dissociation constant (k_1 was predicted from their data to be close to zero, k_1 was 11 μM\(^{-1}\) s\(^{-1}\) and k_2 was 246 s\(^{-1}\)). By contrast, Porter and Spector (1993) calculated K_m (24 μM) to be close to the dissociation constant (16 μM) with a k_1 value close to 500 s\(^{-1}\), k_1 equal to 31 μM\(^{-1}\) s\(^{-1}\) and k_2 equal to 244 s\(^{-1}\). In the present study, we assume that the value for the K_m is not equal to K_d for the wild-type; with our less active mutants, k_2 is extremely low, and K_m approaches K_d.
3.7 pH Dependence of the Reaction Catalyzed by ADA

pH profiles for the reaction catalyzed by the mutant and wild-type enzymes were determined over the range of pH 4-10 using the following buffers: 50 mM sodium acetate (pH 4, 5.5), 50 mM potassium phosphate (pH 7.0), and 50 mM glycine (pH 8.5, 10). The ionic strength of each buffer was adjusted to 0.1 with potassium sulfate. Reaction rates were measured by performing the enzyme assays in triplicate in each buffer at five different substrate concentrations.

3.8 Flame Atomic Absorption Spectroscopy

Flame atomic absorption spectroscopy (FAAS) was performed on a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer at the laboratory of Dr. David Giedroc, Texas A&M University, College Station, TX. The absorption of protein samples at 213.9 nm (λ characteristic for zinc) was measured using seven zinc nitrate solutions (from 0.5-8 μM concentration) as standards. All protein samples were extensively dialyzed in metal-free dialysis tubing (Auld, 1988) against metal-free 20 mM Hepes buffer, pH 7.0, prepared by passage through a Chelex-100 (Sigma) column. The dialysis buffer was changed three times during the experiment. Protein concentrations were between 2-6 μM. Measurements for each sample were taken twice, background-subtracted, and averaged.

3.9 Circular Dichroism Spectroscopy

CD spectra were measured in the far UV (200-260 nm) and the near UV (250-340 nm) region on an Aviv 6100 Spectrometer. Measurements were made using a 0.02 cm path-length cuvette (Starna Cells, Inc). Each scan was recorded
in 1 nm increments at 25°C, repeated three times and averaged. The proteins were in 20 mM Hepes buffer, pH 7.0, and their concentration was adjusted to 1 mg/ml (25 μM) prior to spectral acquisition. Protein concentration assays were done to determine the exact amount of enzyme in each sample. The spectrum of the buffer was subtracted from all protein spectra, and the observed ellipticities, θ, were normalized for protein concentration.

The observed signal was converted to ellipticity from the formula

$$[\theta] = 10[\theta]_{\text{obs}}/cl$$

where $[\theta]$ is the ellipticity measured in degrees, $c$ is the protein concentration in mol/L, and $l$ is the optical path of the cell in dm. The final ellipticity is reported in deg dm$^{-1}$ mM$^{-1}$.

3.10 Fluorescence Spectroscopy

Fluorescence spectroscopy was performed on a SLM 8100 spectrofluorimeter. The excitation wavelength was 290 nm, and the emission was scanned from 300-400 nm every 1 nm. Measurements were made using a 1 cm path-length fluorescence cuvette (Starna Cells, Inc). Samples of 25 μM ADA were in 20 mM Hepes buffer, pH 7.0. All spectra were normalized for protein concentration.

3.11 Crystallography

Crystals for two of our mutants, H238A and H238E, were successfully grown in the presence of purine ribonucleoside as described before (Wilson et al., 1991). These were then harvested into a solution containing 10.5% polyethylene glycol 6000, 50 mM sodium citrate, pH 4.2 and mounted in thin walled capillaries for data collection. Intensities were collected on an ADSC
multiwire area detector mounted on a Rigaku RU-200 generator operated at 40 kV and 110 mA. Since the mutant crystals were much more sensitive to radiation decay, a special protocol was developed by which they were frozen in liquid nitrogen prior to data collection. Protocols and refinement methods are described in detail by Wilson (1996).

3.12 NMR Spectroscopy

$^{13}$C NMR spectroscopy was performed in the laboratory of Dr. L.C. Kurz on purine riboside complexes of wild-type ADA and the H238A mutant in 50 mM potassium phosphate buffer, pH 7.5 in 99.996% D$_2$O and 0.15 M acetonitrile. Conventional proton-decoupled $^{13}$C spectra were obtained at 150.7 MHz using a Varian Unity 600 spectrometer. Protocols are described in more detail in Sideraki et al. (1996).

4. Results

4.1 Site-Directed Mutagenesis

Three out of the four mutants (H238A, H238E and H238R) were constructed by site-directed mutagenesis. After annealing of the mutagenic primers to single-stranded, uridine-rich template DNA, the mutagenic strand was synthesized in vitro, and the resulting double-stranded plasmids were transformed into wild-type and dut-ung$^-$ E. coli. The same template DNA batch was used for the synthesis of all 4 mutants. Double-stranded DNA from ampicillin-resistant colonies growing on wild-type E. coli was isolated and restriction digests were performed to confirm integrity of the plasmids. The presence of the desired mutation was identified by dideoxy sequencing (Sanger et al., 1977) close to the area of residue 238. Despite the reported
frequency of mutant colonies at ~50% (Kunkel et al., 1987), we found this ratio to be closer to 15% in our experiments. In the case of H238Q, screening of more than 30 colonies produced only wild-type ADA. Two new mutagenic oligonucleotides were synthesized in the course of the next two years, and approximately 100 colonies were sequenced, with no success. Once a mutation at residue 238 was identified, sequencing was performed on the entire ADA cDNA in order to ascertain its integrity. All three of our mutant cDNA's were found to be identical to the wild-type, which was sequenced in its entirety as well. Our sequence of wild-type murine ADA completely agreed with the published results, which were thus independently confirmed (Yeung et al., 1985).

4.2 Expression and Purification of Mutants

The mutant plasmids H238A, H238E and H238R were transformed into the ADA-deficient strain SΦ3834. Transformation efficiencies for this strain were low, and typically fewer than 10 mutant colonies appeared per plate. Their small size often required 2 overnight incubations at 37 °C. Colonies were picked and grown in liquid cultures containing ampicillin; these overnight liquid cultures were used to inoculate our 6-liter batch. The growth medium was supplemented with extra zinc to avoid low protein production from the bacteria due to limiting amounts of the cofactor. We found that ADA activity was highest if the inoculae were grown in the presence of 100 μM to 1 mM zinc. Zinc and 5% glycerol were also added in all buffers throughout purification to ensure stability of the proteins. At first, we avoided the use of dithiothreitol because of its metal-chelating properties. However, this led to the appearance of a second ADA peak in HPLC, which had ADA activity and similar mobility to native ADA on SDS-PAGE but was more unstable and had
slightly different mobility on native gels (data not shown). When we combined the two peaks, dialyzed them in the presence of DTT and ran them again on HPLC, one, presumably the reduced, form of ADA appeared, with native ADA properties. From then on, we faithfully included 1 mM DTT in all of our purification and storage buffers.

The purification protocol which had been developed in our laboratory (Mohamedali, 1994; Mohamedali et al., 1996) resulted in good yields of pure products with both mutant and wild-type ADA enzymes. An affinity chromatography step had been intentionally excluded from this protocol, because of the unknown affinities of our active site mutants for a substrate or inhibitor bound to the column. As hoped for, the three His 238 mutants behaved very similarly to wild-type throughout the various stages of purification by eluting at wild-type times from the DEAE, HIC and AX300 columns. Table III.1 shows the purification profile for H238R. One problem was the detection of mutants with low activity: for H238A, dot blots were used to identify the mutant-containing fractions during the DEAE step; for the extremely inactive H238E mutant, dot blots were used during DEAE and SDS-PAGE was performed on the HIC fractions in order to identify ADA-containing fractions. The purity of the enzymes following purification was 85-95% (Figure III.2). A small amount of degradation product is seen in all cases below the main ADA band (Figure III.2 A and B); this product, which may arise during electrophoresis, did not increase over time after prolonged storage of the enzymes (data not shown). ADA was stored in air-tight vials under argon in Tris-zinc-50% glycerol-DTT buffer at -20 °C. Under these conditions, its activity remained stable for many months.

The yields for the mutant enzymes following purification were lower than those obtained with wild-type ADA. For example, H238R was obtained at an
<table>
<thead>
<tr>
<th>step</th>
<th>total activity (units)</th>
<th>total protein (mg)</th>
<th>specific activity (units/mg)</th>
<th>Purific. factor</th>
<th>yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude extract</td>
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<td>100</td>
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<tr>
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<td>25</td>
<td>0.188</td>
<td>92</td>
<td>25</td>
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</tbody>
</table>

Table III.1 Purification table for H238R. H238A and H238E yielded similar results. The total activity of the HIC-HPLC fractions is probably an underestimate, due to the inhibitory effect of high salt. Protein concentrations were assessed by the method of Bradford (1976). One unit of activity is the amount of adenosine deaminase that produces 1 μmol of inosine per minute.
Figure III.2 SDS-PAGE and Western Blot of purified H238 mutants and wild-type ADA. Panel A: SDS-PAGE of adenosine deaminases. Lane 1: wild-type; Lane 2: H238R; Lane 3: H238E; Lane 4: H238A; Lane 5: Rainbow molecular standards. The 39.9 kilodalton ADA band is designated by the arrow. Some amount of impurity is seen below the main band; most of it reacted with the anti-ADA antibody (Panel B) and represents degradation product of ADA. Approximately 12 µg of protein were loaded in each lane. Panel B: An identical protein gel to the one shown in Panel A was blotted with anti-ADA antibody. The main band is ADA, and the lower bands and the smear probably represent degradation products.
<table>
<thead>
<tr>
<th>enzyme</th>
<th>Average Yield (mg of protein)</th>
<th>Inclusion Body (% of total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt mADA</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>H238A</td>
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<td>70</td>
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<tr>
<td>H238R</td>
<td>20</td>
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</tbody>
</table>

Table III.2 Yields and inclusion body content of mutant and wild-type ADA proteins. Average yields were calculated from three or more protein preparations of each sample. The amounts of mutant and wild-type ADA present in bacterial inclusion bodies were estimated from Western blots of total inclusion body solubilized protein; per cent values were assigned after comparing the intensity of the bands to blots of cytoplasmic proteins. Total protein refers to total amount of ADA found in inclusion bodies and cytoplasm.
average of 20 mg per purification, H238E at 50 mg and H238A at 70 mg compared to the average 100 mg yield of the wild-type enzyme. These yields correlate well with the estimated amounts of these enzymes found in bacterial inclusion bodies (Table III.2). The reasons behind the elevated shunting of His 238 mutants into inclusion bodies are not clear, but they may be related to the decreased in vitro stability of the mutants versus the wild-type (see Chapter VI). Despite their lower yields, the similarity between mutants and wild-type during purification and their recognition by the ADA-specific antibody hints at the absence of gross alterations in the mutant enzymes' tertiary structures.

4.3 Evaluation of Zinc Content in Mutant and Wild-Type ADA
In order to ascertain that the ability of our mutants to bind zinc was not affected, the zinc content of mutant and wild-type ADA was measured by FAAS after extensive dialysis (18 hours) of the proteins in zinc-free buffer. The amount of zinc in the final buffer was measured and subtracted from that of the samples. With the exception of H238E, the ratios of zinc to enzyme for the mutants are comparable to those for the wild-type mADA. All ratios, including that of the wild-type, were 30-40% lower than the expected 1:1 ratio (Table III.3). To test for possible denaturation of the proteins and concomitant loss of zinc during the long dialysis step, enzyme activities were measured before and after dialysis. We saw a 30% drop in activity as a result of the dialysis step, which correlates to the 30% drop in the metal/protein ratios, suggesting that denaturation was the cause of the observed zinc loss. As the protein denatures, it unfolds and loses zinc (or vice versa), which can easily diffuse through the pores of the dialysis bag into the external buffer. These results confirm that our mutations do not impair ADA's ability to bind zinc.
<table>
<thead>
<tr>
<th>enzyme</th>
<th>Zn$^{2+}$ (mol/mol protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt mADA</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>H238A</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>H238E</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>H238R</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

Table III.3 Analysis of zinc content in mutant and wild-type ADA by FAAS. Protein samples (2-6 μM) were extensively dialyzed for 18 hours in metal-free dialysis tubing against three changes of metal-free 20 mM Hepes buffer, pH 7.0. Some amount of protein denaturation and zinc loss occurred during dialysis, resulting in ratios of zinc to protein lower than one.
This conclusion is supported by the H238A and H238E crystal structures (described below), where a zinc atom is clearly present in both active sites.

4.4 Evaluation of Secondary Structure in Mutant and Wild-Type ADA by Far UV Circular Dichroism Spectroscopy

To examine whether the mutations at position 238 had disrupted the enzyme's secondary structure, circular dichroism spectroscopy was performed on His 238 mutants and compared to wild-type spectra. Wild-type murine ADA has a parallel α/β barrel motif with eight central β-strands and eight peripheral α-helices, and five additional helices (Wilson et al., 1991). The CD spectrum of wild-type mADA exhibits the minimum at 222 nm characteristic of α-helical content and a minimum at 218 nm reflecting β-sheet structure (Adler et al., 1973). The CD spectra of all three ADA mutants are very similar to the wild-type (Figure III.3). This result confirms that the mutated sites did not cause alterations in ADA's overall secondary structure.

4.5 Evaluation of Tertiary Structure of ADA by Near UV Circular Dichroism Spectroscopy

Murine ADA contains thirteen phenylalanine, fourteen tyrosine and four tryptophan residues. The environment around the aromatic residues can be probed by the use of near UV circular dichroism spectroscopy; the environment around the tryptophans can additionally be examined by the use of fluorescence spectroscopy. Figure III.4 shows the near UV CD spectra for mutant and wild-type ADA. The wild-type ADA spectrum in this region is very complex, undoubtedly due to the contributions of the multiple Phe, Tyr and Trp residues. Aromatic CD bands are poorly understood in terms of sign, magnitude or wavelength, despite recent advances in the literature.
Figure III.3 Far UV circular dichroism spectra of His 238 mutants and wild-type ADA. Samples were at 1 mg/ml in 20 mM Hepes buffer, pH 7.0. Each spectrum shown represents the average of three scans. Little difference is seen in the secondary structure between mutants and wild-type. Minimum at 222 nm arises from α-helical structure; minimum at 218 nm from β-sheet.
Figure III.4 Near UV circular dichroism spectra of His 238 mutants and wild-type ADA. Samples were at 1 mg/ml in 20 mM Hapes buffer, pH 7.0. Each spectrum shown represents the average of three scans. No major differences are noticeable in the spectra of H238A, H238E and wild-type. The H238R spectrum, however, is altered in the 250-270 nm region, where contributions from all aromatic amino acids and histidines can be found.
(Vuilleumier et al., 1993; Freskard et al. 1994). Nevertheless, the spectra of H238A, H238E and wild-type ADA are composed of the same peaks and troughs at the same wavelength values. Despite the difference in intensities, which are small and may arise from errors in the relative protein concentrations, we can conclude that the environment around the various aromatic residues has not been severely perturbed as a consequence of the H238E and the H238A mutation. The H238R mutation, however, has resulted in an altered near UV CD spectrum, especially in the region 250-270 nm. In this region, all three aromatic amino acids as well as histidines can contribute to the ellipticity (Vuilleumier et al., 1993; Freskard et al. 1994). This observation was our first indication that H238R may have a slightly altered tertiary structure, probably localized around certain aromatic residues.

4.6 Evaluation of Tryptophan Environment in Wild-Type and Mutant ADA by Fluorescence Spectroscopy

Tryptophan emission fluorescence spectroscopy can be used to probe protein areas surrounding Trp residues. Depending on the species, ADA contains 3 or 4 such residues, and fluorescence studies have been used in the past to examine their solvent exposure, shed light into the types of conformational changes that occur upon binding of the enzyme to its substrates, and measure rates of association between the enzyme and its substrates or inhibitors (Kurz et al., 1985 & 1992; Philips et al., 1987 & 1989; Porter and Spector, 1992). The four tryptophans of murine ADA are mostly buried within the protein, with Trp 264 being the most exposed (with 31% of its surface accessible to solvent) and Trp 117, Trp 161, and Trp 272 the least exposed (11%, 7%, and 16% of their surface respectively is accessible to solvent). We used fluorescence spectroscopy to examine whether mutations at residue 238 had any effect on
Figure III.5 Tryptophan emission fluorescence spectroscopy of wild-type murine ADA and His 238 mutants. The excitation wavelength was 290 nm and emission was scanned from 300-400 nm. Protein concentrations were 25 μM in 20 mM Hepes buffer, pH 7.0. The above spectra were normalized for protein concentration differences. H238A, H238E and wild-type ADA have similar emission spectra. The H238R tryptophan emission is significantly quenched (by 25%) due to changes in the environment surrounding tryptophan residues as a result of the Arg mutation.
the fluorescence emission of the four Trp residues. The fluorescence spectra of His 238 mutants along with that of the wild-type enzyme are shown in Figure III.5. Wild-type murine ADA emission has a maximum at 328 nm, similar to the maximum of human (335 nm) and calf (340 nm) enzymes (Kurz et al., 1985; Philips et al., 1989). The spectrum of H238A is superimposable with that of the wild-type protein, with a maximum at 331 nm. The spectrum of H238E is slightly red-shifted, with a maximum at 332 nm. H238R is the only mutant which exhibits a drastically altered spectrum, with emission quenched by 25% and a maximum at 333 nm. In addition, a shoulder at 352 nm has become prominent in the spectrum. It appears that substitution of His 238 by the bulkier arginine has resulted in one or more tryptophans becoming more exposed to solvent. An alteration of the local environment surrounding one or more tryptophans supports and partly explains the changes seen in the near UV CD spectrum of this mutant (Figure III.4).

4.7 Effects of His 238 Substitutions on Substrate and Inhibitor Binding and Catalysis

The steady-state parameters for His 238 mutants and wild-type ADA are shown in Table III.5. Murine ADA is a remarkably efficient enzyme, with a \( k_{\text{cat}}/K_m \) value in the order of \( 1 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \). This value compares well to the calf (Kurz et al., 1992) and human ADA (Bhaumik et al., 1993) \( k_{\text{cat}}/K_m \) values. The \( K_m \) value for adenosine is approximately 20 \( \mu M \); the inhibition constants for two adenosine analogs, both competitive inhibitors of the reaction, are slightly lower, around 10 \( \mu M \). The rate enhancement ADA affords compared to the non-enzymatic deamination of adenosine is \( 2 \times 10^{12} \) (Frick et al., 1987). This means that the half-life for the non-enzymatic
<table>
<thead>
<tr>
<th>sample</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (μM$^{-1}$s$^{-1}$)</th>
<th>$K_i$-PR (μM)</th>
<th>$K_i$-N$^6$MA (μM)</th>
</tr>
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<tbody>
<tr>
<td>wild-type</td>
<td>21 ± 2</td>
<td>240 ± 20</td>
<td>11</td>
<td>9 ± 2</td>
<td>12 ± 1</td>
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<tr>
<td>H238A</td>
<td>1.0 ± 0.2</td>
<td>0.020 ± 0.001</td>
<td>0.020</td>
<td>50 ± 20</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>H238E</td>
<td>29 ± 6</td>
<td>0.0010 ± 0.0001</td>
<td>0.00004</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>H238R</td>
<td>24 ± 2</td>
<td>0.17 ± 0.01</td>
<td>0.007</td>
<td>19 ± 4</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

**Table III.4** Steady-state kinetic and inhibition parameters of His 238 mutants and wild-type murine ADA. Assays were performed at 30°C in 50 mM phosphate buffer, pH 7.2 with adenosine as the substrate. Each value is the average of at least three separate determinations. Inhibition constants for purine riboside and N$^6$-methyladenosine, two ground-state inhibitors, were determined for all enzymes except H238E, due to this mutant's extremely low enzymatic activity.
reaction would be approximately 120 years (Radzicka and Wolfenden, 1995). Changing the His 238 residue to Ala, Glu, or Arg dramatically reduces the efficiency of ADA as an enzyme. The most inactive of the three mutants is H238E, with a $k_{cat}/K_m$ value $4 \times 10^{-6}$ lower than the wild-type. The most active of the three is H238A, with a $k_{cat}/K_m$ value of $2 \times 10^{-3}$ of the wild-type; H238R is in between the other two mutants, with a $k_{cat}/K_m$ value of $6 \times 10^{-4}$ that of the wild-type. Neither the glutamate nor the arginine mutation affect the affinity of the enzyme for its substrate adenosine. This result for H238R suggests that despite tertiary structure differences, the mutant has an intact active site, able to bind substrate with wild-type affinity. While H238E is too inactive to permit measurement of the inhibition constants, H238R is competitively inhibited by both PR and N$^6$MA, with a slightly increased $K_i$ value of 19 $\mu$M for PR and a value of 10 $\mu$M for N$^6$MA.

In terms of substrate and inhibitor affinities, H238A yielded the most surprising results. The $K_m$ value for adenosine was increased by about 20-fold relative to wild-type, and this result was echoed in the 10-fold lower inhibition constant for N$^6$MA (1 $\mu$M vs 12 $\mu$M for the wild-type). Nevertheless, PR was not as an effective inhibitor of this enzyme as of the wild-type, with a 5-fold increased $K_i$ value. Both inhibitors were competitive with respect to adenosine. The kinetic results with the H238E and the H238R mutations suggest that altering residue 238 causes a profound decrease in the catalytic activity of ADA, as expected from the postulated role of His 238 in the mechanism. Results with H238A, however, also suggest that His 238 may interact with the substrate by destabilizing it in the ground state.
Figure III.6 pH profiles for H238A, H238R and wild-type ADA. (A)-(C): log $k_{cat}$ versus pH. (A): wild-type; (B): H238A; (C): H238R. (D)-(F): log $k_{cat}/K_m$ versus pH. (D): wild-type; (E): H238A; (F): H238R.
4.8 Dependence of Steady-State Kinetic Parameters on pH

In order to examine whether mutations of an active site residue like His 238 produced alterations in the pH dependence of catalysis, steady-state kinetic parameters for H238A, H238R and wild-type ADA were determined in buffers of various pH values. For the two mutants and wild-type ADA, control experiments showed that the decrease in catalytic activity at the extremes of the pH range was not caused by irreversible enzyme denaturation (data not shown). In wild-type ADA, values of $k_{\text{cat}}$ and $k_{\text{cat}}/K_{\text{m}}$ are maximal at pH 7 and decrease on either side of this pH value in a bell-shaped curve with an acidic pKa at approximately 5.5 and a basic one at 8.5 (Figure III.6). Similar behavior is exhibited by the H238R mutant, although its $k_{\text{cat}}/K_{\text{m}}$ and, more markedly, its $k_{\text{cat}}$ value decrease at acidic pH more rapidly than do the wild-type kinetic parameters. The catalytic process in the H238A mutant exhibits a very different pH dependence. The mutant $k_{\text{cat}}$ value is now maximal at pH 5.5 rather than pH 7.0. Its $k_{\text{cat}}/K_{\text{m}}$ value is, within experimental error, also maximal at pH 5.5 but decreases sharply at basic pH values above 8.5. These results demonstrate that replacement of His 238 by alanine considerably affects the pH dependence of the ADA-catalyzed reaction.

4.9 Crystallography on H238A and H238E

The crystal structures of two His 238 mutants, H238A and H238E, were solved to 2.4 Å resolution and compared to the previously solved structure of the wild-type enzyme complexed to HDPR, a transition-state analog (Wilson, 1991). The crystals were grown in the presence of PR, a substrate analog and inhibitor of the enzyme. As Table III.5 shows, PR was observed to be bound in the active site of H238E, but not in the wild-type or H238A active sites. An
<table>
<thead>
<tr>
<th>Sample</th>
<th>Ligand</th>
<th>$R_{\text{cryst}}$</th>
<th>Resolution Range (Å)</th>
<th>r.m.s.d. bond distance (Å)</th>
<th>r.m.s.d. angle (deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>HDPR</td>
<td>0.195</td>
<td>10 -&gt; 2.4</td>
<td>0.003</td>
<td>1.366</td>
</tr>
<tr>
<td>H238A</td>
<td>HDPR</td>
<td>0.203</td>
<td>10 -&gt; 2.4</td>
<td>0.017</td>
<td>1.98</td>
</tr>
<tr>
<td>H238E</td>
<td>PR</td>
<td>0.203</td>
<td>10 -&gt; 2.4</td>
<td>0.014</td>
<td>1.78</td>
</tr>
</tbody>
</table>

Table III.5 Crystallographic data for H238A and H238E and wild-type ADA. Crystals were grown in the presence of PR, which was the ligand bound in the H238E structure. The hydrated analog of PR, HDPR, was bound to H238A and wild-type ADA. Definitions: r.m.s.d. is root mean square deviation from ideal bond lengths and ideal angles. $R_{\text{cryst}}$ is the crystallographic R factor calculated from $\Sigma |F_o - F_c| / \Sigma |F_o|$ where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively. Adapted from Wilson, 1996.
active adenosine deaminase enzyme, over the long period of crystallization, is capable of carrying out the deprotonation of the zinc-bound water and addition of the hydroxylate on C-6 of the substrate. PR is able to participate in this half of the reaction, but lacks a leaving group for the reaction to be completed. Consequently a hydrated, tetrahedral version of PR (HDPR) is bound in the active site, and this adduct is observed in H238A and wild-type but not the H238E structures.

The overall structures of the mutants showed few deviations between alpha-carbons or differences in zinc coordination when overlapped with the wild-type structure. A zinc atom is bound in each of the three proteins, confirming the lack of an effect by His 238 mutations on ADA's affinity for zinc. An important feature revealed by the H238A structure is a water molecule occupying the space vacated by the imidazole ring of the histidine. As shown by the schematic of the H238A active site in Figure III.7, the alanine substitution leaves enough room for a water molecule to fit. The distance between the oxygen of this water and the oxygen of the C6-OH of HDPR is 3.43 Å, somewhat long for a hydrogen-bonding interaction. The remaining components of the active site are situated similarly to the wild-type structure.

The main feature in the H238E structure is the presence of the non-hydrated inhibitor, PR, in the active site. Refinement of the metal coordination sphere shows that the zinc-coordinated water is not clearly defined in this structure, suggesting that it occupies this site only partly, perhaps due to unfavorable electrostatic interactions with the newly-introduced Glu at position 238. Nevertheless, a water molecule is shown as a ligand to the metal in the H238E active site (Figure III.8).

In summary, these crystallographic results reveal that the two His 238 mutants sustained differences only in the active site but not in the overall
Figure III.7 Schematic of the H238A active site bound to HDPR. The zinc cofactor is in green, nitrogen atoms are in blue, oxygen atoms in red, and carbon atoms in white. Non-covalent interactions between atoms are represented by the yellow dotted lines. The engineered Ala at position 238 is labeled in red. The new water molecule is shown in red, and is at a distance of 3.4 Å from the C6-OH of the HDPR analog.
Figure III.8 Schematic of the H238E active site bound to PR. The zinc cofactor is in green, nitrogen atoms are in blue, oxygen atoms in red, and carbon atoms in white. Non-covalent interactions between atoms are represented by the yellow dotted lines. The engineered Glu at position 238 is labeled in red. Even though the zinc-water is not clearly defined in the structure, it is shown here as coordinated to the zinc cofactor.
tertiary structure, in agreement with our results from circular dichroism and fluorescence spectroscopy. A zinc atom is present in both mutants, as FAA spectroscopy already indicated. The H238E mutant has PR bound in its active site and a metal-coordinated water that is possibly less well-coordinated than in wild-type. The finding of PR in this structure agrees with the extremely low catalytic efficiency of this mutant (4 X 10^-6 lower than the wild-type). The H238A structure reveals a water in addition to the Ala at position 238, and the inhibitor bound is HDPR. This result suggests that H238A may be able to catalyze the deprotonation of the metal-bound water to create the hydroxylate, and thus eliminates His 238 from being the base in the reaction, despite this mutant's higher substrate affinity and lower kcat/Km value compared to wild-type ADA.

4.10 13C NMR Spectroscopy on H238A

In view of the finding of a hydrated inhibitor in the H238A active site by X-ray crystallography, we wanted to assess whether this mutant was able to carry out the first half of the reaction in a much shorter time-scale than that required for crystallization. To this end, 13C NMR studies were performed on purine riboside complexes of H238A and wild-type ADA. Figure III.9 shows the spectra of 6-13C PR free in solution (A), bound to wild-type ADA (B) and bound to H238A (C). In its complex with the wild-type enzyme, the 6-13C carbon of hydrated purine riboside (HDPR) resonates at 74.7 ppm and its attached proton at 7.03 ppm (not shown). In its complex with H238A, the 6-13C carbon of HDPR resonates at 73.7 ppm and its attached proton at 6.84 ppm. These results show that within the limits of experimental detection, the only form of purine riboside bound in the active site of H238A is the hydrated form. The values of both the carbon and proton chemical shifts of the bound
Figure III.9 $^{13}$C NMR spectra of purine riboside and its complexes with wild-type ADA and H238A. (A) 1 mM purine riboside, 500 transients; (B) 0.9 mM complex of purine riboside with wild-type ADA, 1400 transients; (C) 0.7 mM complex of purine riboside with H238A, 37000 transients. In both (B) and (C), the sharp resonance at 71 ppm is due to glycerol remaining from the storage solution of the enzyme.
hydrate are perturbed only minimally in the mutant compared to those values for the wild-type. These results provide additional evidence that, despite the absence of His 238, H238A is able to catalyze the formation and addition of a hydroxylate to C-6 of the substrate.

5. Discussion

5.1 Effects of His 238 Mutations on ADA Structure

Neither an alanine nor a glutamate at position 238 drastically altered the structure of ADA as judged by the elution characteristics of these mutants, their recognition by the anti-ADA antibody, their far and near UV circular dichroism spectra, fluorescence emission, crystal structures, and their substrate and inhibitor binding properties. The arginine mutation, however, resulted in some unforeseen structural differences, as the near UV circular dichroism and the tryptophan emission fluorescence spectra show.

From the similarity between the wild-type and the H238R far UV CD spectrum, we know that the secondary structural elements, the β-sheets and α-helices of this mutant, have not been perturbed. From the fact that adenosine binds H238R with almost wild-type affinity and is able to turn over once every 5 seconds, and from the similar to wild-type Kᵢ values for two competitive inhibitors, we know that the active site must be largely intact. Yet the tertiary structure of this mutant, especially in the vicinity of some of its aromatic residues, must have sustained some change. It is noteworthy here that we have not been able to grow crystals of this mutant ADA, despite repeated efforts and using the same conditions at which many other mutants crystallized.

The fluorescence spectrum of H238R (Figure III.5) has a maximum at 333 nm
vs 328 of the wild-type, and is quenched by approximately 25% relative to the wild-type. This spectrum does not resemble the emission from guanidinium-chloride unfolded ADA, which has a maximum at 349 nm and is quenched by 60-70%, ruling out global unfolding of the mutant as the cause of the spectral changes. Instead, it resembles the spectrum of calf ADA complexed to adenosine or adenosine analogs (Porter & Spector, 1992). Binding of these substrates to ADA causes the enzyme's tryptophan fluorescence emission to become quenched by 25-27% and the maximum to be slightly red-shifted.

What is the nature of this change? It has long been thought that the binding of substrates and ground- or transition-state analogs to ADA brings about a global conformational change (Frieden et al., 1980; Kurz et al., 1985 & 1987; Philips et al., 1987 & 1989). Fluorescence quenching experiments attribute this change to long-range resonance energy transfer from tryptophan residues to the ligand upon binding (Kurz et al., 1985). Similar experiments by Philips et al. (1987) supported a conformational change which quenches the emission of tryptophans remote from the active site. Time-resolved fluorescence spectroscopy was used by Philips et al. (1989) to evaluate the tryptophan environments in human ADA (4 Trp residues). They separated those into three lifetime components: component 1 has a maximum at 330 nm and contributes 10% of the intensity; component 2 has a maximum at 330 nm and contributes 55% of the intensity; component 3 has a maximum at 340 nm and contributes 35% of the intensity. Component 2 is quenched by both purine riboside and deoxycoformycin binding and may be near the active site, in a relatively buried environment. In mouse ADA, there are 4 Trp residues, but only two are within 11-13 Å from the active site: W 264 and W 272. Of these, W 272 is 16% solvent-exposed while W 264 is more accessible, with 31% of its surface area in contact with the solvent. Assuming similarity between Trp
environments in human and mouse ADA, component 2 could correspond to Trp 272 in murine ADA, which is 12.6 Å away from the substrate and relatively buried within the protein. Thus, it is possible that substrate binding in murine ADA could quench the emission of Trp 272.

This hypothesis is very intriguing in view of the following finding: molecular modeling of an arginine at position 238 of ADA reveals that its side chain can rotate to within 6 Å of Trp 272. This rotation also brings it in close contact with Tyr 240. If the bulky arginine cannot be accommodated in the ADA active site and must rotate away, it could re-position Tyr 240, whose near UV CD signal would change. In addition, such a rotation could re-orient Trp 272, altering its near UV CD signal and quenching its fluorescence emission. Consequently, as a result of the Arg 238 rotation, the changes in the vicinity of Trp 272 may parallel those which occur upon ligand binding. Thus the mutant fluorescence spectrum is as quenched as the wild-type emission is upon ligand binding.

5.2 Effects of His 238 Mutations on Substrate and Inhibitor Binding.

Replacement of His 238 by an acidic glutamate or a bulky arginine did not affect the enzyme's affinity for adenosine. These results are consistent with structural information on the absence of bonding interactions between residue 238 and the substrate. Because of the low but measurable catalytic activity of H238R, inhibition studies could be carried out. Little or no difference was seen in the Ki values for PR and N\(^6\)MA, two competitive inhibitors of the enzyme. The absence of an effect on the Km or Ki values of the H238R mutant suggests that the arginine substitution has not greatly disrupted the active site of the enzyme.

A 20-fold increase in the affinity of H238A for adenosine was observed with
this mutant. This result was surprising, since no contact between His 238 and the substrate was predicted from the crystal structures (Wilson et al., 1991; Wilson and Quiocho, 1993 and 1994). Calculation of the distance between Ne2 of the histidine and N-6 of the substrate from the 1-deazaadenosine structure shows it to be 3.8 Å, too far for a hydrogen bond interaction. However, Kurz et al. (1992) have pointed out that there must be an interaction between the 6-NH₂ substituent and ADA since 1-deazaadenosine (N-1 missing) is bound 100-fold more tightly by the enzyme than 1-deazapurine riboside (both N-1 and N-6 missing). The increased affinity for substrate by H238A was corroborated by the 10-fold lower Kᵢ value of N⁶-methyladenosine, an adenosine analog and weak substrate of ADA [(Kᵢ of 5 μM, kcat of 0.3 s⁻¹ (Porter and Spector, 1993)], as well as a competitive inhibitor of the enzyme. A three-fold reduction in substrate affinity and 1000-fold drop in kcat/Kᵢ was observed for a similar H238A mutant in human ADA (Bhaumik et al., 1995). These combined results suggest that His 238 does not only function in catalysis by lowering the activation barrier to the transition state through hydroxylate charge-stabilization (see next section), but also by raising the energy of the enzyme-substrate complex.

Interestingly, an alanine mutation of Glu 104 (the primary catalytic residue) in cytidine deaminase results in a 10⁸ reduction in kcat but also a 30-fold reduction in Kₘ (Carlow et al., 1995). No binding interactions between Glu 104 and cytidine were predicted from the crystal structure (Betts et al., 1994). In fact, there seems to be a wealth of residues implicated in catalysis but not in substrate binding which, when mutated to alanine, exhibit higher affinities for substrate. Some histidines which belong to this group are His 372 in alkaline phosphatase (10-fold lower kcat and 30-fold lower Kₘ; Xu et al., 1994) and His 64 in carbonic anhydrase (40-fold lower Kₘ, 70-fold lower kcat; Liang
et al., 1993), both zinc-utilizing enzymes.

There are no good explanations offered for the observed improvement in substrate binding when a catalytic histidine is replaced by alanine. One possible mechanism by which such an effect takes place in ADA is steric hindrance. Removing histidine from the ADA active site and replacing it by alanine and a water molecule may free up space, making substrate binding more favorable. This theory would explain the lower $K_m$ value for adenosine, with its C-6 amino group, and $K_i$ value for N$^6$MA, with its C-6 methylamino group. However, an inhibitor like PR, with only a hydrogen on C-6, might be bound too loosely in the more spacious mutant active site, thus becoming a poorer inhibitor of adenosine (hence its 5-fold higher $K_i$ in H238A versus the wild-type).

A second theory is that His 238 may somehow constrain the substrate, thus destabilizing it; removal of this residue would result in better binding. Weiss et al. (1987) and Jones et al. (1989) have both suggested that the substrate in the reaction must be distorted in order for N-1 to become protonated and aromaticity of the purine ring to be destroyed. The various wild-type and mutant ADA structures do not show any obvious distortion in substrate/inhibitor purine rings. It is interesting, however, to note that, of all the residues in ADA structures, His 238 and Asp 295 are the only ones in high-energy, disallowed conformations (Wilson, 1996). This could mean that they are strained in their effort to maintain the substrate in an unfavorable conformation. Alternatively, this may be a consequence of the specific electrostatic and hydrogen-bonding interactions between His 238 and Asp 295, and Asp 295 and the zinc. Nevertheless, if His 238 constrains the substrate, and its absence results in tighter binding, it is difficult to rationalize the $K_m$ values observed with H238E and H238R, which are not different from those
of wild-type ADA.

5.3 Effects of His 238 Mutations on the Catalytic Efficiency of ADA

As discussed in the beginning of the chapter, our objectives in mutating His 238 were to decipher which role this histidine plays in the reaction. It has been proposed to act as a general base, creating the hydroxylate from the zinc-bound water; a general acid, protonating the leaving group; and as base and acid, performing both functions. In addition, it has been postulated to stabilize the developing negative charge on the hydroxylate and orient it for attack on C-6 of adenosine. Our results with His 238 mutants give us considerable insight into the mechanism of the reaction.

All three mutations of histidine 238 reduced, but did not abolish, the catalytic activity. This result, combined with the finding of the hydrated form of PR in the H238A active site by both crystallography and NMR spectroscopy, confirms that histidine 238 is not the base which creates the attacking OH group. Clearly, however, His 238 is important to the reaction, as the low \( \text{kcat}/\text{Km} \) values of the mutants show. H238E exhibits the lowest activity (\( \text{kcat}/\text{Km} \) only \( 4 \times 10^6 \) of the wild-type), suggesting that the negative charge on the glutamate is greatly detrimental to catalysis. By comparison, an uncharged (Ala-water) or positively charged (Arg) residue at position 238 is more easily tolerated. In addition, the H238E structure shows that this mutant is unable to catalyze the hydration of PR to form HDPR. It is possible that having an acidic residue at position 238 may result in such destabilization of the hydroxylate that the zinc-water is a metal ligand only part of the time (Section 4.9). Thus, even though His 238 is not the base, its presence promotes and ensures deprotonation of the zinc-water. These results support an electrostatic role for His 238 in stabilizing the developing negative charge on
the hydroxylate.

One would think that Arg 238 with its positive charge would successfully balance the hydroxylate charge. Spectroscopic evidence however suggests that its side chain may have moved away from the position His 238 normally occupies. Thus, any contribution of the guanidinium positive charge to hydroxylate stabilization is lost. In addition, the high pKₐ of the Arg side chain precludes protonation of the amino group of the tetrahedral intermediate. This mutant has therefore only 0.06% of the wild-type catalytic efficiency.

The importance of a positive charge at position 238 is underscored by pH studies of the kinetic behavior of H238A. In this mutant, which is normally 500-fold less active than wild-type ADA, both the kₐ and kₐ/Kₘ values increase at pH 5.5 relative to pH 7.0, opposite from wild-type behavior. As the crystal structure shows, a water is found next to the engineered alanine at this position. At acidic pH values, a hydronium ion can replace this water in the H238A active site. The positive charge on the hydronium ion can stabilize the incipient hydroxylate negative charge. In addition, a proton from the hydronium could be transferred to the leaving group, allowing elimination to occur and completion of the reaction. The second hypothesis is consistent with the greater enhancement of the H238A kₐ rather than kₐ/Kₘ value at pH 5.5, which shows that at this pH, it is the collapse of the ES complex to the products of the reaction that is facilitated, rather than the association of substrate with the enzyme. The altered pH profiles for the reaction catalyzed by H238A also suggest that the rate-determining step has now changed. This is not surprising, since, in the absence of His 238, formation of the hydroxylate and/or collapse of the tetrahedral intermediate into products may be the most difficult steps.
How does His 238 stabilize the hydroxylate negative charge? One explanation may be found in a His->Ala mutant with another zinc-containing enzyme, alkaline phosphatase. The mutated His 372 hydrogen-bonds with one of the carboxyl oxygens of Asp 327, which is a bidentate ligand to Zn₁. In alkaline phosphatase, Zn₁ is thought to activate the water which is the nucleophile attacking the phosphoserine intermediate. A H372A mutant alkaline phosphatase had a 10-fold lower hydrolysis activity and 30-fold higher substrate affinity compared to wild-type (Xu et al., 1994). In the wild-type enzyme, His 372 is thought to neutralize the negative charge of the Asp 327, which in turn becomes a worse electron donor to the zinc. That makes the metal more electrophilic, and the bound water a better nucleophile. In the H372A mutant the aspartate charge is not neutralized and the water is a worse nucleophile (i.e. its pKₐ is higher); thus, reaction slows down. We may be witnessing a very similar effect with our H238A mutant. Instead of the His 238 directly stabilizing the hydroxylate, it could be doing so indirectly by stabilizing the Asp 295 charge. In the H238A mutant, this electrostatic effect is lost (unless the water found next to Ala 238 becomes charged, as in acidic pH values) and the water is much less polarized, because it is bound to the zinc less tightly. If this is true, then we would expect the Zn-water bond to be longer and, by inference, the zinc-Asp 295 bond to be shorter (stronger) in the H238A structure than it is in the wild-type. This is in fact true. Wilson (1996) measured the distances between zinc and all its ligands in various ADA structures. The zinc-Asp 295 distance in H238A is 2.1 versus the wild-type 2.4 Å. In H238E, where there is an added negative charge in the immediate vicinity of Asp 295 and uncertainty as to the presence of the water ligand, this distance drops to 2 Å. None of the other zinc-histidine distances have been affected.
Since HDPR cannot eliminate its leaving group (a hydrogen), the present data does not allow us to distinguish between a role of the histidine in charge stabilization of the hydroxylate versus protonation of the leaving group. Nevertheless, whether His 238 acts as a general acid or merely has an electrostatic effect (or performs both functions), this study clearly eliminates His 238 from being the base required to bring about the first part of the ADA-mediated reaction. More experiments which would help pinpoint this residue's precise function are discussed below.

5.4 Implications for the Mechanism and Future Work
If His 238 is not the base, then which residue creates a hydroxylate out of the zinc-coordinated water in the ADA reaction? There are two alternative possibilities. One is that Asp 295 may act as the base. It is certainly located close to the catalytic water (both its oxygen atoms are 2.5-2.6 Å away from the activated hydroxylate in the 1-deazaadenosine wild-type structure). In this mechanism, a carboxylate residue creates the nucleophile in the reaction, similarly to what occurs in other enzyme systems described in Chapter II, such as carboxypeptidase and thermolysin, as well as the homologous enzyme cytidine deaminase. However, the fact that Asp 295 has one of its oxygens coordinated to the zinc, while the other oxygen accepts a hydrogen bond from Ser 265 makes the likelihood of this residue acting as a base remote. In wild-type ADA, furthermore, His 238 "neutralizes" part of the aspartate's negative charge, further reducing its basicity. As will be seen in the next chapter, a D295E mutation reduces significantly, but does not eradicate, the enzyme's catalytic efficiency. These observations argue against Asp 295 being the base.

The second possibility involves Glu 217, the residue shown to donate a proton to N-1 of the substrate. If this residue is the base, the ADA mechanism
will be yet another version of the reaction pathways in cytidine deaminase and the zinc proteases, where glutamate residues create the nucleophile. Glu 217 is homologous to Glu 104, the residue thought to deprotonate the water, protonate N-3 of the cytidine and shuttle the proton to the leaving group in cytidine deaminase (Betts et al., 1994). One of the oxygens on Glu 217 interacts with an ordered water molecule and is 2.8 Å away from N-1 of the substrate. The residue is roughly coplanar with the purine ring, and is in van der Waals distance from Leu 58, Thr 269 and Val 218, which suggests a higher $pK_a$ than normal. Thus, it is ideally situated to donate the proton on N-1 during the reaction. But does it abstract this proton from the water? The Glu 217 distance from the activated hydroxylate in the 1-deazaadenosine structure is greater than its distance from the C-6 OH in the HDPR structure; both are greater than 3 Å, which seems a little far for proton abstraction. In cytidine deaminase, where Glu 104 is the base, one of its oxygens is 2.4 Å away from the -OH group. However, Glu 217 mutants are very inactive (Mohamedali, 1994), which makes the possibility of this residue acting as the base in the ADA-catalyzed reaction more attractive. In addition, information from the crystal structures may mislead one away from what can happen in solution: a rotation of the Glu 217 side chain can bring it into closer proximity to the catalytic water (Wilson, 1996). It is encouraging that in crystals of two of the Glu 217 mutants, E217S and E217Q, purine riboside, and not its hydrated analog HDPR, is bound in the active sites (Wilson, unpublished results). This information renders Glu 217 the most likely candidate for the general base in the ADA reaction to date.

In order to examine whether His 238 provides the proton to the leaving group, a number of experiments can be done using our H238A mutant. Stopped-flow spectrophotometry can be used to detect the formation of a
tetrahedral intermediate in the H238A active site, as has been used by others to study enzyme-inhibitor complex formation (Kurz et al., 1992). If His 238 protonates the leaving group, and the Ala-water at position 238 cannot readily do the same, then the lifetime of the tetrahedral intermediate would be increased in this mutant relative to wild-type, and its formation and decay could be observed spectroscopically. In addition, if proton transfer from the enzyme to the leaving group differs in the H238A and H238R mutants, the solvent isotope effect is also expected to be different. These two kinds of experiments should help determine whether His 238 is involved in leaving group protonation or not.
CHAPTER IV.

EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF ASP 295 AND ASP 296 MUTANTS IN MURINE ADENOSINE DEAMINASE

1. Introduction

As discussed in Chapter II, both aspartates at positions 295 and 296 of murine ADA have been conserved in all adenosine deaminase and AMP deaminase sequences. Based on this data alone, their potential importance in the reaction was recognized early on (Chang et al., 1991). The elucidation of the crystal structure of the murine enzyme verified these predictions (Wilson et al., 1991). In the complex of wild-type ADA with HDPR, the Oδ2 of Asp 295 is the fourth ligand to the zinc cofactor, while the Oδ1 accepts a hydrogen-bond from the 6-OH of HDPR and the γ-OH of Ser 265 (Figure IV.1). In the mechanism proposed based on this structure, Asp 295 acts as the base, abstracting the proton from the zinc-bound water to create the attacking hydroxylate. However, data from a subsequent structure of the wild-type enzyme complexed to the ground-state analog 1-deazaadenosine revealed that another neighboring residue, His 238, was more likely to be the base in the reaction (Wilson & Quiocio, 1993). Accordingly, the function of Asp 295 was modified to be that of locking the hydrogen of the attacking OH in a hydrogen bond, so that a lone pair on the oxygen was properly oriented for attack on C-6. Yet a third structure of wild-type ADA complexed to the product of the reaction inosine, revealed that the Asp 295 was located in between the two C-6 substituents of the tetrahedral intermediate, and suggested the possibility that Asp might function as a proton shuttle between them (Wilson & Quiocio, 1994). In this scenario, a proton from the hydroxylate passes on to Asp 295 and from there to the departing amino group, which becomes ammonia, the
Figure IV.1 Schematic drawing of active site of ADA complexed with the transition-state analog HDPR. Distances between refined non-hydrogen atoms are in Angstroms. Non-covalent interactions are represented by dashed lines. Dotted lines represent coordinating interactions with the zinc. The zinc is coordinated to O82 of Asp 295 and to the C-6 OH of HDPR. The metal is also coordinated to the Ne2 atoms of His 15, His 17 and His 214 (not shown).
second reaction product.

Thus, in addition to coordinating the zinc, three separate chemical functions were assigned to this residue at various times: that of a general base, creating the hydroxylate; a positional role, with Asp 295 mainly anchoring the hydroxylate in the proper orientation for attack; and that of a proton shuttle, with Asp 295 picking up and immediately donating a proton.

By contrast, there was only one proposal for the function of the other aspartate, Asp 296, in the reaction mechanism. As this residue was perfectly coplanar with the purine ring and in excellent distance (2.8 Å) to N-7 of the substrate, it was proposed to participate in a hydrogen bond with that nitrogen. The fact that Asp 296 makes van der Waals contacts with three Phe residues suggested that its pKₐ may be displaced upward from the usual value of 4.0, necessitating that this residue be the donor of the hydrogen bond.

Thus, the function assigned to this residue was in substrate binding only, and not in the chemical steps occurring during catalysis. Nevertheless, this hydrogen bond between N-7 and Asp 296 is only one of many such bonds between the enzyme and the nitrogens of the purine ring. All these interactions serve to reduce the aromatic character of the ring and enhance catalysis by promoting a nucleophilic attack on C-6. Thus, in addition to anchoring the substrate, Asp 296 plays a role in destabilizing it in the ground-state and lowering the energy barrier to the transition state.

2. Construction of Mutant ADA Enzymes and Preliminary Work

Chang et al. (1992) initiated site-directed mutagenesis studies on the two aspartates. In order to probe the catalytic role of Asp 295, this residue was modified to a glutamate and an asparagine. The substrate binding role of Asp 296 was probed by changing it to an alanine, an asparagine and a glutamate
residue. The five mutants were synthesized and purified, and preliminary kinetic analyses were performed (Chang et al., 1992). The D295N mutant was not purified and seemed to be very unstable during the purification process. Little experimental work was performed on the other four mutants, and the kinetic characterization was incomplete, with steady-state kinetic parameters determined using adenosine at levels which were barely saturating.

In collaboration with Dr. Kellems' laboratory, where these mutants were created, we re-purified D295E, D296A, and D296N, and characterized them in great detail both kinetically and structurally. Important insights into the roles played by the two conserved aspartates in the ADA reaction mechanism were the outcome of these experiments.

3. Methods

3.1 Bacterial Strains, Vectors and Site-Directed Mutagenesis

The mouse adenosine deaminase cDNA was inserted in the pRC4 phagemid expression vector under control of an IPTG-inducible tac promoter (Chang et al., 1992). The f1 locus on this vector allows for superinfection with IR1/M13 helper phage and isolation of single-stranded (ss) DNA. The amp resistance gene allows for colony selection on ampicillin-containing agar plates. Site-directed mutants were constructed using this vector by the Kunkel mutagenesis protocol (Kunkel et al., 1987) as described in Chang (1992). Mutants were screened by sequencing in the area of the mutation. Mutant plasmids were expressed in the ADA− strain SF3834 (Chang et al., 1991). This strain contains a deletion of the adenosine deaminase (add) gene and two neighbouring genes. A wild-type Escherichia coli strain, 71-18, was used for the production of plasmid DNA used in restriction digests, transformation,
and sequencing.

3.2 Restriction Digests and Sequencing of Mutant ADA Plasmids

As shown in Figure III.1 of Chapter III, the mADA cDNA has been inserted into a Nco I and an Eco RI site within the pRC4 expression vector. In order to ensure integrity of the vector, plasmid DNA was prepared from the five mutant plasmids (D295N, D295E, D296A, D296N, and D296E) in the SΦ3834 strain. The plasmid DNA was then digested using the Nco I and Cla I restriction enzymes (Promega). Digestion of pRC4 at these unique sites yields a 3 kb and a 2.2 kb fragment, the latter containing the mADA cDNA. For sequencing, mutant plasmid DNA was transformed into the wild-type 71-18 strain. Single-stranded DNA was prepared as described in the Kunkel mutagenesis method (Kunkel et al., 1987), without the addition of uridine. Single-stranded sequencing was then performed using the Sequenase Kit (United States Biochemicals) first in the vicinity of the mutation and then throughout the entire cDNA.

3.3 Protein Expression and Purification

Three mutant plasmids, D295E, D296A, and D296N, were expressed in the ADA-deficient strain SΦ3834 and used for protein purification. The detailed protocol was described in Section 3.3 of Chapter III. The ADA proteins eluted between 12-17 hours from the DEAE chromatography step; activity assays were used to identify Asp 296 mutants but dot blots were used for D295E. The ADA peak typically eluted at 30 min from HIC-HPLC and 25-30 min from AX300-HPLC. The purified proteins were stored as described before.

3.4 SDS-Polyacrylamide Gel Electrophoresis, Western Blots, Dot Blots and
Inclusion Body Assays
The detailed protocols were described in Sections 3.4 and 3.5 of Chapter III.

3.5 Enzyme Assays and Kinetic Analyses
Enzymatic activities were assayed on a Cary 118 spectrophotometer by measuring the rate of ADA-dependent increase of inosine absorption at 235 nm at 30°C, using an extinction coefficient of 3.5 mM⁻¹cm⁻¹. The assays were performed as described in previous chapters. Cuvettes of 1 cm (for the wild-type and D295E enzymes) or 0.2 cm pathlength (for D296N) were used. For D295E, D296N, and wild-type ADA, adenosine concentrations ranged from 0.5-5 X Kₘ. For D296A, an alternative assay was used (see below). The concentration of enzyme in the assay mixture ranged from 0.1 nM for the wild-type to 0.3 μM for the least active mutant. Kᵢ values for two ADA inhibitors, N⁶-methyl adenosine (N⁶MA) and purine riboside (PR) were determined for the enzymes, as previously described.

For the determination of the Kₘ, k_cat, and Kᵢ values of D296A, an ammonia detection assay was used. The amount of ammonia produced from the ADA-dependent adenosine deamination was measured on a Cary 118 spectrophotometer at 400 nm at 30°C. Reaction mixtures contained 0.625 to 5 mM adenosine, 0.8 μM D296A and either 1.5 and 2.5 mM N⁶MA or 5 and 7.5 mM PR in 50 mM potassium phosphate buffer, pH 7.4. Reactions were incubated in 30°C for 8 minutes (control experiments determined linearity of the reaction between 2 and 12 minutes) and then stopped by the addition of 30 μl of Ammonia Color Reagent (Sigma). Their absorbance at 400 nm was recorded and converted to amount of ammonia produced using a standard curve for absorbance at 400 nm by a range of ammonium sulfate concentrations from 12.5-200 μM. Kinetic parameters were calculated from
rates plotted against substrate levels using the Enzyme Kinetics program (Trinity Software).

3.6 pH Dependence Measurements and pH Stability Assays
The profile of the ADA-catalyzed reaction versus pH was determined by assays performed in buffers of pH 4.0-10.0 as described in Section 3.7, Chapter III, for the three mutants and wild-type ADA.

pH stability assays were performed by incubating the enzymes in buffers of pH 4.0-10.0 used in the kinetic pH profiles for 4 minutes (duration of a typical kinetic assay). The samples were then diluted in 50 mM potassium phosphate buffer, pH 7.2, and their activity was measured.

3.7 Flame Atomic Absorption Spectroscopy, Circular Dichroism Spectroscopy and Fluorescence Spectroscopy
All samples were prepared and spectroscopic assays were performed as described previously (Sections 3.8-3.10, Chapter III).

3.8 Crystallography
Crystals of the mutant ADA's were grown in the presence of purine ribonucleoside as described before (Wilson et al., 1991). These were then harvested into a solution containing 10.5% polyethylene glycol 6000, 50 mM sodium citrate, pH 4.2 and mounted in thin walled capillaries for data collection. Intensities were collected on an ADSC multiwire area detector mounted on a Rigaku RU-200 generator operated at 40 kV and 110 mA. To minimize radiation damage during data collection, crystals were cooled to 4°C. Crystals belonged to spacegroup C2 and since they were isomorphous
with those of the wild-type enzyme complexed with HDPR, refinement commenced using this structure as a starting point. The refinement procedure is explained in more detail in Sideraki et al. (1996).

4. Results

4.1 Restriction Digests and Sequencing of Mutant ADA Plasmids
Digests of plasmid DNA from mutants D295E, D296A, and D296N exhibited the same restriction bands as did wild-type pRC4. However, D295N and D296E produced bands of different sizes (data not shown). Re-transformation of these plasmids into wild-type 71-18 E. coli and re-digestion gave indications that in these samples, the pRC4 plasmid had been rearranged or was contaminated by other plasmids. Thus, only the first three mutant plasmids were sequenced in their entirety. Sequencing confirmed the presence of the mutations at residue 295 or residue 296; in addition, the rest of the mADA cDNA was sequenced, and determined to be identical to wild-type ADA for all three mutants.

4.2 Protein Expression and Purification
After completion of sequencing, the three mutant plasmids (D295E, D296A, and D296N) were expressed in the ADA− strain SΦ3834. Purification after induction by IPTG yielded 20 mg of pure protein for the D295E mutant and 160-220 mg for the Asp 296 mutants from the 6 liter cultures. The wild-type ADA yield from the same culture volume was 100 mg on average. Comparison of these yields with the amounts of ADA present in inclusion bodies reveals that for wild-type and Asp 296 mutants, most of the protein is present in the cytoplasm (Table IV.1). This result correlates well with their
high yields after purification. However, little of the D295E mutant is present in inclusion bodies, yet its average yield never exceeds 20 mg. We noticed that about 80% of the D295E enzymatic activity was lost during the DEAE column step (Table IV.2, Panel A). Our efforts to retrieve D295E from different fractions of the DEAE column were fruitless, and gave evidence that this sample was unstable, breaking down during this step. This observation correlates well with the tremendous instability of another Asp 295 mutant, D295N, observed during the DEAE purification step by Chang (1992). The remaining D295E, the Asp 296 mutants, and wild-type ADA eluted at similar times during hydrophobic and AX300 ion-exchange column chromatography, suggesting that the tertiary structure of the mutant proteins had not been affected by each mutation. Purified ADA proteins were 85-95% homogeneous, as shown in Lanes 5, 6 and 7 of a mutant D296N denaturing protein gel throughout the various stages of purification (Figure IV.2). The purified proteins appear as single bands with a molecular weight of about 40,000 daltons; a few faint bands of lower molecular weight can also be observed by SDS-PAGE in some cases (Figure IV.2). These bands correspond to degradation products of ADA in Western blots, which may be arising during purification or electrophoresis (data not shown). We do not see an increase in the amount of the degradation product in SDS-PAGE of mutant proteins at later time points following purification and storage, which suggests stability of pure samples under our storage conditions.

4.3 Evaluation of Secondary and Tertiary Structure of Mutant ADA by Circular Dichroism Spectroscopy and Fluorescence Spectroscopy

Mutation of a single active site residue can conceivably have long-range effects, disrupting secondary structure elements such as β-sheets and α-helices
<table>
<thead>
<tr>
<th>enzyme</th>
<th>Average Yield (mg of protein)</th>
<th>Inclusion Body (% of total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt mADA</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>D295E</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>D296A</td>
<td>220</td>
<td>10</td>
</tr>
<tr>
<td>D296N</td>
<td>160</td>
<td>10</td>
</tr>
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**Table IV.1** Yields and inclusion body content of mutant and wild-type ADA proteins. Average yields were calculated from three or more protein preparations of each sample. The amounts of mutant and wild-type ADA present in bacterial inclusion bodies were estimated from Western blots of total inclusion body solubilized protein; per cent values were assigned after comparing the intensity of the bands to blots of cytoplasmic proteins. Total protein refers to total amount of ADA found in inclusion bodies and cytoplasm.
### A.

<table>
<thead>
<tr>
<th>step</th>
<th>total activity (units)</th>
<th>total protein (mg)</th>
<th>specific activity (units/mg)</th>
<th>Purific. factor</th>
<th>yield (%)</th>
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<td>AX300 HPLC</td>
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<td>65</td>
<td>11</td>
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### B.

<table>
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<th>total protein (mg)</th>
<th>specific activity (units/mg)</th>
<th>Purific. factor</th>
<th>yield (%)</th>
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<tr>
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<td>0.303</td>
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<td>51</td>
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**Table IV.2** Purification tables for D295E (A), and D296A (B). Unlike D296A and D296N, most of the D295E was lost due to instability during the DEAE step. The total activity of the HIC-HPLC fractions is probably an underestimate, due to the inhibitory effect of high salt. Protein concentrations were assessed by the method of Bradford (1976). One unit of activity is the amount of adenosine deaminase that produces 1 μmol of inosine per minute.
Figure IV.2 SDS-PAGE of D296N mutant during purification. Approximately 20 µg of protein were loaded in each lane. Lane 1: DEAE pool; Lane 2: 60-80% ammonium sulfate precipitation; Lane 3: HIC-HPLC pool; Lane 4: Blank; Lanes 5-7: Pools from three separate AX300-HPLC columns; Lane 8: Molecular weight standards (sizes are indicated in kDa). ADA is marked by the arrow.
or altering smaller or bigger portions of the protein’s tertiary structure. As reported in Chapter III, H238R is an example of this phenomenon. The far UV CD spectra of the Asp 295 and the two Asp 296 mutants are completely superimposable with each other and with the spectrum of wild-type ADA (Figure IV.3). This result demonstrates that none of the mutations has altered ADA’s secondary structure.

Near UV CD spectra demonstrate little change in the environment around the protein’s many aromatic residues, as a result of each mutation (Figure IV.4). The absence of drastic changes in tertiary structure is also evident in the tryptophan fluorescence emission spectra of the mutants (Figure IV.5). Excitation of tryptophan fluorescence is possible in ADA by virtue of its four tryptophan residues; if mutations at a certain site have altered the environment around a Trp residue, the intrinsic fluorescence emission spectrum of that mutant will differ from the wild-type spectrum. As we saw, an Arg at position 238 of ADA has just such an effect, resulting in decreased intensity of the spectrum, probably by exposing one or more tryptophans to solvent. However, mutations at Asp 295 and Asp 296 do not have a similar effect. The mutant fluorescence emission spectra are very similar to the wild-type, with maxima ranging from 328 to 331 nm.

4.4 Zinc Content of ADA

One of the mutants, D295E, alters a zinc-coordinating residue from aspartic to a longer glutamic acid. This may perturb the metal-coordination sphere, resulting in less tight zinc binding by the mutant enzyme. In order to measure the amount of zinc present in the purified D295E, and D296A and D296N enzymes, FAAS was performed after extensive dialysis (18 hours) of the proteins in zinc-free buffer. The amount of zinc in the final buffer was
Figure IV.3 Far UV circular dichroism spectra of Asp 295 and Asp 296 mutants and wild-type ADA. Samples were at 1 mg/ml in 20 mM Hepes buffer, pH 7.0. Each spectrum shown represents the average of three scans. Little difference is seen in the secondary structure between mutants and wild-type. Minima at 208 and 222 nm arise from $\alpha$-helical structure; minimum at 218 nm from $\beta$-sheet.
Figure IV.4 Near UV circular dichroism spectra of Asp 295 and Asp 296 mutants and wild-type ADA. Samples were at 1 mg/ml in 20 mM Hepes buffer, pH 7.0. Each spectrum shown represents the average of three scans. No major differences are noticeable between mutant and wild-type ADA; minor differences are seen in the 280-290 nm region of the spectrum. The tertiary structure of ADA has not been significantly perturbed as a result of either of the three mutations.
Figure IV.5 Tryptophan emission fluorescence spectroscopy of wild-type murine ADA and Asp 295 and Asp 296 mutants. The excitation wavelength was 290 nm and emission was scanned from 300-400 nm. Protein concentrations were approximately 5 µM in 20 mM Hepes buffer, pH 7.0. Small intensity differences arise from errors in protein concentration. The maxima of each spectrum are: wild-type, 328 nm; D295E, 330 nm; D296A, 331 nm; D296N, 328 nm. The ADA tryptophan environment has not been altered as a result of mutations on either Asp 295 or Asp 296.
measured and subtracted from that of the samples. As explained for the histidine mutants, all zinc-to-protein ratios were below the expected value of one, due to protein denaturation which occurred during the long dialysis step. Nevertheless, the ratios of zinc to enzyme for the mutants are comparable to those for the wild-type mADA (Table IV.3). This result suggests that the zinc content of ADA mutants was unaffected by Asp 295 or Asp 296 mutations. Results from the crystal structures of two of the mutants, D295E and D296A, later confirmed this observation (see below).

4.5 Kinetic Properties of Mutants

To examine the effect of our mutations on the steady-state kinetic parameters of the enzyme, $k_{\text{cat}}$, $k_{\text{cat}}/K_m$ and $K_m$ values for adenosine for mutant and wild-type ADA's were obtained (Table IV.4). All mutants have greatly impaired catalytic efficiencies ($k_{\text{cat}}/K_m$) compared to the wild-type enzyme. The most active of the mutants, D296N, has a $k_{\text{cat}}/K_m$ of 1%, while D295E and D296A retain 0.04% and 0.001% of the wild-type catalytic efficiency, respectively. Differences among Asp 295 and Asp 296 mutants are seen in their ability to bind substrate. While a glutamate in place of Asp 295 has no effect on the $K_m$ value for adenosine, mutations at residue 296 cause dramatic decreases in substrate affinity. Thus, the $K_m$ value for adenosine for D296N is 10-fold higher than wild-type, and for the D296A mutant 70-fold higher. The reduced efficiency in substrate binding observed with these two mutants is reflected in the altered inhibition constants for the two adenosine analogs, PR and N$^6$MA. For D296N, the $K_i$ value for N$^6$MA is comparable to this mutant's affinity for substrate (approximately 200 μM). However, purine riboside is a much poorer competitive inhibitor of this mutant enzyme, with a $K_i$ value 8-fold higher than that of N$^6$MA and 160-fold higher than the
<table>
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<th>enzyme</th>
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<tr>
<td>wt mADA</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>D295E</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>D296A</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>D296N</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

*Table IV.3* Analysis of zinc content in mutant and wild-type ADA by FAAAS. Protein samples (2-6 μM) were extensively dialyzed for 18 hours in metal-free dialysis tubing against three changes of metal-free 20 mM Hepes buffer, pH 7.0. Some amount of protein denaturation and zinc loss occurred during dialysis, resulting in ratios of zinc to protein lower than one.
<table>
<thead>
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<th>$k_{cat}/K_m$</th>
<th>$K_i$ PR</th>
<th>$K_i$ N\textsuperscript{6}MA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>($\mu$M)</td>
<td>($s^{-1}$)</td>
<td>($\mu$M\textsuperscript{-1}s\textsuperscript{-1})</td>
<td>($\mu$M)</td>
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<tr>
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<td>240 $\pm$ 20</td>
<td>11</td>
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<td>0.004</td>
<td>9 $\pm$ 2</td>
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<td>0.0001</td>
<td>6000 $\pm$ 2000</td>
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<tr>
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<td>17 $\pm$ 1</td>
<td>0.11</td>
<td>1440 $\pm$ 40</td>
<td>170 $\pm$ 20</td>
</tr>
</tbody>
</table>

Table IV.4 Steady-state kinetic and inhibition parameters of Asp 295 and Asp 296 mutants and wild-type murine ADA. Assays were performed at 30°C in 50 mM phosphate buffer, pH 7.2 with adenosine as the substrate, except for D296A, where an ammonia detection assay was used as described in the text. Each value is the average of at least three separate determinations.
wild-type inhibition constant.

Judging from the D296A mutant's sharply reduced substrate affinities, we expected poorer inhibition. N⁶-methyl adenosine can compete with adenosine for binding with a Kᵢ value comparable to the D296N Kᵢ value (Kᵢ is 2.1 mM; Kᵢ is 1.4 mM). However, just as occured with the asparagine mutant, purine riboside is a poorer inhibitor of D296A, with an estimated Kᵢ value of 6 mM. This inhibition constant is about 4-fold higher than the mutant's Kᵢ value for adenosine, and approximately 600-fold higher than PR's Kᵢ value with wild-type ADA.

These findings clearly support the minimal role of Asp 295 in substrate binding and stabilization, and verify predictions that Asp 296 is essential in anchoring the substrate to the active site.

4.6 pH Profiles of Wild-Type and Mutant Enzymes

The pH dependence for the reaction catalyzed by ADA is a bell-shaped curve, with an acidic pKᵢ of about 5.5 and a basic pKᵢ of 8.5. If either of the two aspartates were responsible for one of the pKᵢ values, our mutants might be expected to have different pH profiles compared to the wild-type enzyme. To examine this possibility, we measured the steady-state kinetic parameters of mutants at different pH values. The profiles for the k₅ and k₅/Kᵢ pH dependence are shown in Figure IV.6. Essentially wild-type pH dependence is seen with both profiles of D295E and D296A. The k₅ and k₅/Kᵢ profiles of D296N have somewhat higher values at pH 5.5 when compared to wild-type, and the free enzyme D296N pH profile has a slightly lower basic pKᵢ of about 8 (Figure IV.6, Panel B). Control experiments where all four enzymes were incubated in buffers of pH 4.0-10.0 for the duration of a typical assay (4-5 minutes) and then assayed at pH 7.2 showed that the activity drop at the
Figure IV.6 pH profiles of D295E, D296A, D296N, and wild-type ADA. Panel (A): log $k_{cat}$ versus pH; Panel (B): log $k_{cat}/K_m$ versus pH.
extremes of the pH range is not caused from denaturation of the enzymes (data not shown). All three mutants and the wild-type enzyme retained 80-100% of their activity after incubation at pH 4.0-10.0. Overall, these data suggest that our mutations at residues 295 and 296 do not significantly alter the pH dependence of the ADA reaction under steady-state conditions.

4.7 Crystallography

The crystal structures of D295E and D296A were solved using purine riboside as the inhibitor. When this substrate analog is used to grow ADA crystals, the catalytic water attacks C-6 to form 6(R)-hydroxy-1,6-dihydropurine ribonucleoside (HDPR). The reaction then stalls at this point due to the lack of a leaving group. The resulting complex, which would dehydrate very quickly in solution (Jones et al., 1989), is stable within the confines of the active site for the weeks in which it takes to grow the crystals and collect the data.

The crystals of D295E showed that while the overall structure was not perturbed, the inhibitor that was bound to the protein was PR and not HDPR (Figure IV.7). An activated water molecule, poised to attack as in the case of the wild-type structure complexed with 1-deazaadenosine (Wilson et al., 1993) was not observed. Instead, the extra methylene in the glutamate side chain has caused its carboxylate group to displace the catalytic water which is liganded to the zinc in wild-type ADA. The glutamate still chelates the zinc but makes contacts with the purine ring which is displaced by approximately 0.50 Å relative to the wild-type structure. The displaced purine ring no longer engages in hydrogen bonding to Oe1 of Glu-217 via N-1. Without this interaction, the side chain of Glu-217 adopts a different conformation.

The crystal structure of D296A (Figure IV.8) is generally similar to that of the wild-type enzyme. When superimposed on each other, the two active sites
Figure IV.7 Schematic of the D295E active site bound to PR. The zinc cofactor is in green, nitrogen atoms are in blue, oxygen atoms in red, and carbon atoms in white. Non-covalent interactions between atoms are represented by the yellow dotted lines. The engineered Glu at position 295 is labeled in red. The longer glutamate chain has displaced the catalytic water from the zinc coordination sphere. The purine ring of the inhibitor has been displaced by approximately 0.50 Å relative to the wild-type structure.
Figure IV.8 Schematic of the D296A active site bound to HDPR. The zinc cofactor is in green, nitrogen atoms are in blue, oxygen atoms in red, and carbon atoms in white. Non-covalent interactions between atoms are represented by the yellow dotted lines. The engineered Ala at position 296 is labeled in red. The plane of the purine ring is shifted by approximately 0.40 Å relative to the wild-type structure. This perturbation may be a direct consequence of the lack of the hydrogen bond to N-7 due to the mutation.
show no substantial differences in the positions of their amino acids or the zinc cofactor. The plane of the purine ring in D296A is, however, shifted slightly by approximately 0.40 Å, which may be a direct consequence of the lack of the hydrogen bond to N-7 due to the mutation. Examination of the electron density in the active site shows that the purine riboside, which was included in the crystallization, is bound as an adduct at the 6 position. These results agree with the increased $K_m$ and $K_i$ values observed with D296A in our kinetic studies and confirm the proposed role of this residue in substrate binding.

5. Discussion

5.1 Effects of the Asp 295 -> Glu Mutation on Structure and Function of ADA

Asp 295 was predicted to be an essential residue for the structure and function of ADA from sequence homology and crystallography data. It chelates the zinc cofactor, and assists in the proper orientation of the catalytic water via hydrogen-bonding. Our data reveals that from a structural point of view position 295 appears to be very sensitive to replacements. A glutamate mutation resulted in an unstable protein, the majority of which was degraded in the early stages of purification. A protein with glutamine engineered at this position by Chang (1992) completely disintegrated and was unretrievable after the first purification stages. The portion of the D295E mutant which we were able to purify appeared identical to wild-type ADA in secondary and tertiary structure and zinc content as judged by circular dichroism, FAA and fluorescence spectroscopy and crystallography. However, denaturation studies show that this mutant is much more easily denatured in the presence of guanidinium chloride relative to the wild-type enzyme (Chapter VI).
Therefore, replacement of this critical aspartate in ADA compromises the enzyme in terms of its compactness and stability, without major secondary or tertiary structure rearrangements.

The crystal structure shows that the glutamate at 295, longer than aspartate by one methylene group, displaces the catalytic water from the zinc coordination sphere, where it is thought to share a ligand site with Asp 295. With the exception of this change and a slight shift of the purine ring of the substrate by 0.5 Å, we observe no other obvious reason why D295E would be less stable than wild-type ADA, especially since the glutamate can still be a ligand to the zinc just as aspartate was.

This very displacement of the catalytic water, however, is the key to the mutant's diminished catalytic efficiency (its $k_{\text{cat}}/K_m$ value is 0.04% that of the wild-type). Without a catalytic water, no reaction can take place. From the non-zero $k_{\text{cat}}/K_m$ value, it is evident that reaction does occur a small percentage of the time in solution, where it must still be possible, albeit rare, for a water molecule to bind the active site and fit as a ligand to the zinc.

Fitting the water in the metal coordination sphere may necessitate the displacement of Glu 295 or the weakening of its interaction with the metal cofactor. Either Glu 295 itself or, more likely, another residue in the active site such as Glu 217 can then function as a general base, activating the water to a hydroxylate. These possibilities are discussed in more detail below.

The reaction catalyzed by D295E varies with pH exactly as the wild-type reaction does, with an acidic $pK_a$ of 5.5 and a basic $pK_a$ of 8.5. This observation is not surprising, since, in wild-type ADA, Asp 295 has its Oδ2 coordinated to the zinc, and its Oδ1 participating in hydrogen bonds with the catalytic water and the OH of Ser 265. In addition, His 238, with its positive charge, is within 3.7 Å of Asp 295. Therefore, the $pK_a$ of the aspartyl side chain is expected to be
lower than 4.0, and will not be reflected in the pH profile, which ranges from pH values 4 to 10. Since the engineered glutamate is also a zinc ligand, the same forces that depress the aspartate's pK_a value will operate on Glu 295, and no change will be seen in the pH dependence of the D295E reaction. Significantly, "no change" implies that none of the pK_a values of the catalytic ADA residues (Glu 217, His 238) have been displaced from their wild-type values as a result of the D295E mutation. Thus, whenever a water molecule can squeeze into the coordination sphere of the zinc, catalysis occurs in exactly the same manner as it does in wild-type ADA.

The kinetic and inhibition data suggest that the D295E mutation does not impede substrate or inhibitor binding, even if the crystal structure shows that the longer glutamate displaces the purine ring by 0.5 Å. In solution, the enzyme must be flexible enough to allow substrate to bind as tightly as it does in wild-type ADA. These observations are in support of the minimal interaction between Asp 295 and substrate predicted from the crystal structure, and have important implications for the catalytic mechanism of ADA, as will be discussed below.

5.2 Effects of the Asp 296 -> Ala and the Asp 296->Asn Mutations on Structure and Function of ADA

Mutating the other conserved aspartate in adenosine deaminase, Asp 296, into an uncharged asparagine or an apolar alanine did not affect the secondary or tertiary structure of the molecule or its zinc content, as the CD, FAA and fluorescence spectra show. For D296A, these results are confirmed by the crystal structure, where no rearrangements of active site or other residues are discernible.

The predicted role of this residue is in substrate binding, via a hydrogen bond
donated by Asp 296 to N-7 of the purine. This interaction is expected to also reduce the aromaticity of the purine ring, facilitating the nucleophilic attack on C-6. In agreement with these predictions, the substrate and inhibitor affinities of D296A, and to a lesser extent D296N, were dramatically reduced, and their catalytic efficiencies were diminished. The substitution of an alanine for an aspartate in a very hydrophobic patch of the active site (3 Phe residues are nearby) may be favorable structurally, but it completely abolishes the hydrogen bond with N-7. The absence of electron density between Ala 296 and substrate is clear from the crystal structure of the mutant and demonstrates that a water molecule has not taken the place of the missing aspartyl side chain. Because of the loss of this hydrogen bond, D296A binds adenosine 70-fold less effectively than wild-type ADA does. Using the difference in the $K_m$ values between mutant and native enzyme, the loss in binding energy as a result of the mutation is calculated to be 2.5 kcal/mol. The binding energy assigned to hydrogen bonds in vacuum has been calculated to be in the order of 3-9 kcal/mol (Fersht, 1985). Values for hydrogen bonds measured in solution range between 0.5-1.5 kcal/mol (Moody and Wilkinson, 1990). A hydrogen bond maintained in a highly hydrophobic part of the active site may easily be worth more than 1.5 kcal/mol. From our results, the N-7/Asp 296 bond appears to contribute 2.5 kcal/mol to the binding energy. The major contribution of this specific hydrogen bond to the enzyme's ability to bind adenosine is not surprising in view of the fact that 7-deazaadenosine is neither a substrate nor a tightly bound inhibitor (Wilson et al., 1991).

When Asp 296 is replaced by asparagine, the ability to bind substrate is not as impaired. The $K_m$ value is 10-fold higher than the wild-type value, which gives a loss in binding energy of 1.4 kcal/mol. Thus, the amide of Asn 296 may be able to engage in a weak hydrogen bond with N-7, binding substrate
more tightly than D296A, but certainly not as tightly as the wild-type enzyme does. The crystal structure of this mutant would be helpful in elucidating the nature of the interaction between Asn 296 and adenosine. The detrimental effects of both the asparagine and the alanine mutations of Asp 296 on substrate binding are matched by the higher $K_i$ values for the substrate analog $N^6$-methyladenosine. Purine riboside (PR) is yet another analog of adenosine with a hydrogen instead of a C-6 amino group. Surprisingly, with both mutants, purine riboside is a much worse inhibitor of the enzyme than $N^6$MA. In wild-type ADA, PR is an equally effective inhibitor of adenosine as is $N^6$MA, so why does it differ in Asp 296 mutants? An explanation may be connected to the lack of a substituent on C-6 of PR. Kurz et al. (1992) have proposed that a C-6 substituent on a substrate or inhibitor makes favorable binding contacts with ADA. Our results show that these contacts become especially important to substrate and inhibitor binding when other anchoring interactions, such as the strong hydrogen bond between Asp 296 and N-7, are lost. Interestingly, PR was also a worse inhibitor of another mutant ADA, H238A, than $N^6$MA (Chapter III). The purine riboside ring, with no C-6 substituent, and an absent or weak hydrogen bond between its N-7 and Ala 296 or Asn 296, can make fewer binding contacts with the enzyme than $N^6$MA and consequently becomes a much weaker inhibitor of adenosine.

The two Asp 296 mutations not only decreased substrate affinities, but also reduced the turnover rate of the enzyme. The $k_{cat}$ value was diminished by the Ala mutation much more than by Asn at 296 (the D296A $k_{cat}$ is 0.07%; the D296N $k_{cat}$ is 7% of the wild-type value). Due to its poor substrate binding, D296A was the least efficient of the three mutant enzymes, with a $k_{cat}/K_m$ value of 0.001% of the wild-type. Nevertheless, this amount of activity was
enough for the enzyme to catalyze the hydration of purine riboside into HDPR over the long crystallization time (approximately two weeks). The reduced turnover rates of the two Asp 296 mutants suggest that residue 296 affects not only the ground state of the reaction, but also its transition state. The engagement of every lone pair of the nitrogens of the purine in hydrogen bonds has been postulated to reduce the ring's aromaticity and assist nucleophilic attack on C-6 (Wilson, 1991). This phenomenon lowers the energy barrier to the transition state; consequently, a mutation that impairs the enzyme's ability to abstract electron density from the purine ring, such as D296N and, to a much greater extent, D296A, will make it more difficult for the ADA reaction to reach its transition state. This conclusion is supported by our steady-state kinetic results.

From the crystal structure, Asp 296 is embedded in a highly hydrophobic environment, in close proximity to three Phe residues. Therefore, it is thought to be protonated and able to donate a hydrogen bond to N-7 of the substrate. Its pKa is thus expected to be much higher than 4.0, and probably higher than 7.0; deprotonation of this residue would result in less tight substrate binding and decrease in the catalytic activity. Ionization constants from pH 6.0 to above 8.0 have been reported for protonated carboxyl groups in enzymes (Tipton and Dixon, 1983; Fersht, 1985). If either the acidic pKa of 5.5 or the basic pKa of 8.5 in the wild-type enzyme represented the deprotonation of this aspartate, we would expect to see a grossly altered pH profile with our Asp 296 mutants. However, our pH profiles do not support this theory (Figure IV.6). The D296A free enzyme and enzyme-substrate complex pH profile is identical to the wild-type in both the basic and acidic limbs. In the case of the D296N mutant, both of its profiles show elevated kcat and kcat/Km values at pH 5.5 relative to the wild-type, perhaps because at those
pH values N-7 of the substrate can be protonated by the solvent. Protonation at N-7 of imidazole rings is favorable due to resonance stabilization within the ring. Such protonation would have the same effect that the N-7/D296 hydrogen bond has: it would reduce the aromaticity of the substrate's purine ring, and enhance its catalysis by the D296N mutant. It appears then that even if neither of the pKa values reflects solely the ionization of Asp 296, they can be affected by mutations at this position, as is the case with D296N. Our pH stability experiments rule out the possibility that the activity decrease at the acidic or basic ends of the pH range is simply caused by the denaturation of either wild-type or mutant ADA.

Our combined results with D296A and D296N underscore the importance of Asp 296 in substrate binding. In addition, our results suggest that Asp 296 is instrumental in lowering the activation barrier between ground state and transition state by reducing the aromaticity of the purine ring.

5.3 Implications for the Mechanism and Future Work

Work with histidine 238 mutations (Chapter III) suggested that this residue is not the base creating the attacking nucleophile, the hydroxylate. This left two alternative possibilities: Asp 295 and Glu 217, as the candidates for the base. Data presented in this chapter with a glutamate mutation at position 295 can be interpreted in two ways, since the catalytic water was absent from the structure of the mutant enzyme.

1. On the rare occasions when a water molecule fits into the active site and the zinc coordination sphere, Glu 295 may serve the same function as Asp 295, performing all of the duties aspartate has. In this case, we cannot draw any conclusion as to what role residue 295 plays in the reaction, because the glutamate could pick up the water proton, orient the hydroxylate or shuttle
the leaving group proton just as aspartate does.

2. Alternatively, again on the rare occasion that a water fits the D295E active site and becomes properly oriented for deprotonation (by Glu 295?), other residues abstract the proton creating the hydroxylate, and protonate the leaving group. The only other residue in the vicinity that can act as the base is Glu 217; the candidate for donating the leaving group proton can still be His 238. Indeed, work with site-directed mutants of Glu 217 enhances the probability that this residue, in addition to protonating N-1 of the reaction, is also the base (Mohamedali, 1994).

We favor Glu 217 as the base, on the basis of the following observations: First, Asp 295 has one of its oxygens coordinated to the zinc, while the other oxygen accepts a hydrogen bond from Ser 265, and probably has a $pK_a$ value lower than 4.0. Thus it is unlikely to deprotonate the zinc-bound water (probable $pK_a$ of 7.0). Secondly, D295E retains a modicum of catalytic activity, and the pH profile for the D295E-catalyzed reaction is identical to the wild-type profile, suggesting that the same residues that have the acid/base functions in native ADA can occasionally confer activity to D295E. Thirdly, when Chang (1992) attempted to purify the D295N mutant, residual ADA activity in the cell extract was observed. If this is true, Asp 295 is clearly not essential to the reaction for abstracting the zinc-water proton.

From our results to date, the ADA mechanism is likely as follows:

- The catalytic water binds ADA, coordinates the zinc and is oriented for attack by Asp 295.
- Glu 217 deprotonates the water and His 238 stabilizes the OH$^-$ negative charge.
- Substrate binds, and the aromaticity of the purine ring is reduced by a number of hydrogen bonds with the enzyme, such as the N-7/Asp 296 bond.
- The hydroxylate attacks C-6 and the proton from Glu 217 is transferred to N-1 of the substrate.

- The proton from the C-6 OH is transferred to NH2 by the action of His 238 or Asp 295.

Clearly, more work is needed to establish this mechanistic scenario. If the key D295N mutant can be purified and is a relatively stable ADA with an intact zinc cofactor, its catalytic properties should be determined. Since the side chains of asparagine and aspartic acid do not differ in length, a water molecule should bind this mutant as it does wild-type ADA. If this mutant is proven to retain catalytic activity, this result will clearly eliminate Asp 295 from being the base.

In the event of D295N being impossible to purify in a stable form, cysteine could be an alternative mutation at this position. Sulfhydryl groups frequently coordinate zinc atoms (Vallee and Auld, 1990). If a stable D295C mutant can be expressed, the basic function of residue 295 will not be carried out by a cysteine. Thus, should the mutant have activity, the role of Asp 295 as a base will be disproved.

Since all four Glu 217 mutants had little or no catalytic activity (Mohamedali, 1992) an E217H mutant, where some activity could be retained, can be synthesized. At physiological pH, histidines are ideal residues for acid/base dual roles. Assuming that the imidazole ring can be fit at position 217, and that its pKa is not displaced from the usual value, it will be interesting to examine if this mutant can carry out N-1 protonation and proton abstraction (in which case there will be catalysis) or just proton abstraction without N-1 protonation (no catalysis).
CHAPTER V.

EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF CYS 262 MUTANTS IN MURINE ADENOSINE DEAMINASE

1. Introduction

Early experiments with sulfhydryl-specific reagents had suggested that a sulfhydryl residue, e.g. a cysteine, was required for the reaction catalyzed by ADA (Ronca et al., 1967). Sulfhydryl-specific modifiers, such as mercurials, were excluded by substrate from binding in the active site. (Wolfenden et al., 1967). Protonation of the substrate by an active site cysteine was proposed, and the high pK_{a} value (8.5) of the V_{max}/K_{m} profile was attributed to this cysteine residue (Maguire and Sim, 1971; Orsi et al., 1972). One of the explanations for the solvent isotope effect on the binding of ground-state and transition-state analogs was the formation of a covalent adduct between the substrate and an enzyme sulfhydryl (Kurz and Frieden, 1983). Weiss et al. (1987) suggested that an enzyme sulfhydryl protonates N-1 of the substrate prior to water attack on C-6 based on ^{15}N and solvent deuterium isotope effects.

A comparison of primary sequences between adenosine deaminases from E. coli, mouse and human and AMP deaminases from yeast, rat and human revealed a great deal of homology between the two types of deaminases (Figure V.1). Two blocks of amino acids, TVHAGE and SL(I)NTDDP were conserved, and the distances between them had also been conserved at 74 amino acids in adenosine and 280 in AMP deaminases. A single conserved cysteine was found in between these two blocks, again at distances conserved in the two enzyme groups. This observation led Chang et al. (1991) to propose Cys 262 as the catalytic sulfhydryl of the reaction, which protonates N-1 of the
Adenosine Deaminase

E. coli 194TVHAGE-------(44)-------C244-------(31)-----SINTDDP279-----
Mouse 212TVHAGE-------(44)-------C262-------(31)-----SLNTDDP297-----
Human 212TVHAGE-------(44)-------C262-------(31)-----SLNTDDP297-----

AMP Deaminase

Rat 362VHAG-------(207)-------C573-------(74)-----SLSTDDP651-----
Human (M) 362VHAG-------(207)-------C573-------(74)-----SLSTDDP651-----
Yeast 421VHAG-------(206)-------C631-------(74)-----SLSTDDP709-----

Figure V.1 Sequence alignment of adenosine and AMP deaminases. The number of amino acids between conserved residues is indicated in parentheses. Adapted from Chang et al. (1991).
substrate. Information on Cys 262 came from the crystal structure of murine adenosine deaminase complexed to the transition-state analog, HDPR (Wilson et al., 1991). The γ-SH of this residue was 7.9 Å away from N-1 and 7.3 Å away from C-6 of the substrate, distances too great for the thiol to play a direct catalytic role. In addition, a wall of residues such as His 238, Ser 265 and Asp 295 prevent Cys 262 from accessing the active site. Of the other five cysteines of mADA, only Cys 153 was close to the active site, but too far (~9 Å) from N-1 and C-6 of the substrate to have a direct impact on catalysis.

Chang (1992) created two site-directed mutants at this residue, C262A and C262S. These mutants were expressed in an ADA-deficient strain and characterized in a preliminary fashion. In order to examine their kinetic and structural parameters in more detail, these two site-directed mutants were re-sequenced, purified using the ADA-specific purification protocol developed in our laboratory, and characterized by a variety of methods. The results suggest that Cys 262 is not required for the proper function of adenosine deaminase.

2. Methods

Methods pertaining to sequencing, expression and purification, measurement of the steady-state kinetic and inhibition parameters, zinc content analysis, circular dichroism and fluorescence spectroscopy, and crystallography of wild-type and mutant ADA have been described in detail in Chapters III and IV, Sections III.3 and IV.3, respectively.

2.1 Overexpression of pGroESL in SΦ3834 strain harboring mutant ADA plasmids

pGroESL was a generous gift from Dr. H.F. Gilbert at Baylor College of
The plasmid contains the *E. coli groE* operon, under the control of both p\text{lac} and the heat shock promoter. This yields very high levels of GroEL expression, often leading to 30\% of total cell protein. pRC4, harboring wild-type or mutant ADA cDNA, was co-transformed with pGroESL into the ADA-deficient bacterial strain, *S.\Phi3834*. Colonies harboring both plasmids were selected on chloramphenicol (30 \mu g/ml)-ampicillin plates (60 \mu g/ml); colonies grew only after a two-day incubation at 37 °C. The presence of both plasmids was confirmed by digesting plasmid DNA with *Cla* I/Nco I (for pRC4) and with *Eco* RI and *Hind* III (for pGroESL). Single colonies were inoculated in 5-ml cultures in superbroth medium (32 g/l bactotryptone, 20 g/l yeast extract, 5 g/l sodium chloride) containing 1 mM zinc sulfate and 60 \mu g/ml ampicillin, 30 \mu g/ml chloramphenicol, and grown overnight. In order to confirm that neither of the two plasmids had been dropped from the overnight cultures, plasmids were prepared and digested as described above. From each overnight culture, 0.5 ml were used to inoculate 500 ml of superbroth medium containing zinc sulfate, ampicillin and chloramphenicol as above. At 10 hours into the growth, 4 mg/l IPTG (Sigma) was added, and growth was continued for 22 hours. Cells were lysed and inclusion bodies were prepared as described in Section 3.5 of Chapter III. These experiments were performed for wild-type, C262A, and C262S ADA. The ADA inclusion body content was compared for each sample from two types of cell extracts: extracts from colonies harboring only pRC4 and colonies harboring both pRC4 and pGroESL.
3. Results

3.1 Sequencing, Expression, and Purification of C262A and C262S
Plasmid DNA from colonies of the two Cys 262 mutants was digested with Nco I and Cla I and integrity of the phagemid expression vector, pRC4, was verified. Single-stranded DNA was prepared and sequenced, and the presence of an Ala and a Ser mutation at residue 262 was established. Sequencing of the whole mutant ADA cDNA confirmed the absence of other mutations.

The two mutants were purified to homogeneity using our purification protocol. It was immediately obvious from the early stages of purification that they both retained ADA activity; their elution characteristics matched those of wild-type ADA, suggesting that these mutants did not sustain drastic tertiary structure changes. For both of them however, purification yields were very low, with an average of 7 mg per 6 liters of cells for C262A and 10 mg for C262S per purification. Purification of proteins present in inclusion bodies revealed that 90% of these mutants had been shunted by the cell into these vesicles (Table V.1). By contrast, about 10% of wild-type ADA is present in inclusion bodies, and the typical yield is 100 mg of pure protein per purification.

In order to address the question of whether cells experienced difficulty in folding these particular mutants, the pGroESL plasmid, containing both the groES and groEL genes under control of the p1ac promoter, was co-transformed with pRC4 into our ADA-deficient strain. The groES and groEL genes encode chaperonins, which assist folding of misfolded proteins in bacteria (Ang et al., 1991). Overexpression of these chaperonins did not alter the relative amounts of C262A or C262S protein shunted into inclusion bodies (data not shown).
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<td>90</td>
</tr>
<tr>
<td>C262S</td>
<td>10</td>
<td>90</td>
</tr>
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**Table V.1** Yields and inclusion body content of mutant and wild-type ADA proteins. Average yields were calculated from three or more protein preparations of each sample. The amounts of mutant and wild-type ADA present in bacterial inclusion bodies were estimated from Western blots of total inclusion body solubilized protein; per cent values were assigned after comparing the intensity of the bands to blots of cytoplasmic proteins. Total protein refers to total amount of ADA found in inclusion bodies and cytoplasm.
3.2 Evaluation of Zinc Content in C262A, C262S and Wild-type ADA

The mol/mol ratio of zinc to protein determined by FAA spectroscopy was 0.5 ± 0.1 for both Cys 262 mutants and 0.7 ± 0.2 for wild-type ADA (Table V.2). All these ratios were lower than 1.0, due to denaturation of ADA occurring during the experiment. Within the limits of the experiment, both Cys mutants had essentially wild-type zinc content. This finding was later confirmed by X-ray crystallography for the C262S mutant.

3.3 Evaluation of Secondary and Tertiary Structure in Mutant and Wild-Type ADA by Circular Dichroism and Fluorescence Spectroscopy

Neither an alanine at position 262 nor a serine perturbed the secondary structure of ADA, as the far UV CD spectra show (Figure V.2). The tertiary structure of the mutants was almost identical to wild-type, based on results from near UV CD spectroscopy (Figure V.3) and tryptophan emission fluorescence spectroscopy (Figure V.4).

3.4 Steady-State Kinetic and Inhibition Parameters of Mutants

Cysteine 262 is clearly not required for catalysis or substrate binding, as the kinetic results demonstrate (Table V.3). The substrate or inhibitor affinities of the two mutant ADA enzymes were not significantly altered as a result of either mutation. Both mutants were somewhat less efficient deaminases; the alanine mutation decreased the $k_{cat}/K_m$ value to 64% of the wild-type value, while the serine mutation decreased it to about 55%. Despite the decrease in catalytic efficiency, both mutants are able to bring about the deamination of adenosine without a requirement for Cys 262.
<table>
<thead>
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<th>enzyme</th>
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<tr>
<td>wt mADA</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>C262A</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>C262S</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

**Table V.2** Analysis of zinc content in mutant and wild-type ADA by FAAS. Protein samples (2-6 μM) were extensively dialyzed for 18 hours in metal-free dialysis tubing against three changes of metal-free 20 mM Hepes buffer, pH 7.0. Some amount of protein denaturation and zinc loss occurred during dialysis, resulting in ratios of zinc to protein lower than one.
Figure V.2 Far UV circular dichroism spectra of Cys 262 mutants and wild-type ADA. Samples were at 1 mg/ml in 20 mM Hepes buffer, pH 7.0. Each spectrum shown represents the average of three scans. Little difference is seen in the secondary structure between mutants and wild-type. Minima at 208 and 222 nm arise from $\alpha$-helical structure; minimum at 218 nm from $\beta$-sheet.
Figure V.3 Near UV circular dichroism spectra of Cys 262 mutants and wild-type ADA. Samples were at 1 mg/ml in 20 mM Hepes buffer, pH 7.0. Each spectrum shown represents the average of three scans. No major differences are noticeable between the spectra of C262A, C262S and wild-type.
Figure V.4 Tryptophan emission fluorescence spectroscopy of wild-type murine ADA and Cys 262 mutants. The excitation wavelength was 290 nm and emission was scanned from 300-400 nm. Protein concentrations were 5 µM in 20 mM Hepes buffer, pH 7.0. The above spectra were normalized for protein concentration differences. C262A, C262S and wild-type ADA have similar emission spectra.
<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (μM$^{-1}$s$^{-1}$)</th>
<th>$K_i$ PR (μM)</th>
<th>$K_i$ N$^6$MA (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>21 ± 2</td>
<td>240 ± 20</td>
<td>11</td>
<td>9 ± 2</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>C262A</td>
<td>31 ± 4</td>
<td>220 ± 40</td>
<td>7</td>
<td>14 ± 2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>C262S</td>
<td>29 ± 3</td>
<td>163 ± 7</td>
<td>6</td>
<td>15 ± 2</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

**Table V.3** Steady-state kinetic and inhibition parameters of C262A and C262S mutants and wild-type murine ADA. Assays were performed at 30°C in 50 mM phosphate buffer, pH 7.2 with adenosine as the substrate. Each value is the average of at least three separate determinations.
3.5 pH Profiles of Wild-Type and Mutant Enzymes

C262A retained 90-100% of its catalytic activity for the duration of the assays in pH values of 5.5-10.0; however, a 35% loss of activity was seen when this enzyme was incubated at pH 4.0 for 4 minutes. This result suggested that at very acidic pH values, this enzyme is unstable and denatures easily. Both C262A and C262S showed lower catalytic activities at acidic pH values than the wild-type; this difference is evident in the log $k_{cat}$ pH profile in Figure V.5 (Panel A). Thus, the acidic pKa of 5.5 appears to be affected by mutations on Cys 262. This result is somewhat complicated by the fact that, at least for C262A, the $k_{cat}$ value at 4.0 is a combination of loss of enzymatic activity due to pH effects as well as denaturation of the enzyme. Arguing against a significant change in the pKa values of the ADA reaction are the pH profiles of log $k_{cat}/K_m$ for both mutants, which show great similarity with the corresponding wild-type profile (Figure V.5, Panel B).

3.6 Crystallography of C262S

Crystals of this mutant were grown in the presence of the ground-state analog, PR. As expected from the mutant's high catalytic activity, the hydrated form of PR, i.e. HDPR, was found in the active site of the solved structure. The serine side chain adopted a conformation almost identical to that of the cysteine side chain (Figure V.6). To explain the slight decrease in this mutant's activity, Wilson (1996) looked at the contacts between the original sulfur atom of the cysteine and other amino acids in the active site. The $\text{S}_{\gamma}$ of Cys 262 makes two contacts under 4 Å with His 15 (a zinc ligand) and one with Asp 295 (zinc ligand and catalytic residue). However, the $\text{O}_{\gamma}$ of the Ser 262 does not interact with His 15 but makes two contacts with Asp 295. This
Figure V.5 pH profiles of C262A, C262S, and wild-type ADA. Panel (A): log $k_{cat}$ vs pH; Panel (B): log $k_{cat}/K_m$ vs pH.
**Figure V.6** Superposition of the C262S and the wild-type murine ADA active sites. Wild-type residues are shown in light blue. C262S residues are in color. The engineered serine side chain occupies the same position as the wild-type cysteine. HDPR is bound in both active sites.
arrangement may result in less optimal positioning of the attacking hydroxylate and therefore a slight loss in activity.

4. Discussion

4.1 Cysteine 262 Is Not Essential to Catalysis
Many literature reports have postulated an essential sulfhydryl in the reaction catalyzed by ADA; this cysteine has been proposed to protonate N-1 (Weiss et al., 1987), form a covalent intermediate with C-6 of the substrate (Kurz and frieden, 1983), and be the residue with pK_a of 8.5 in the pH profile of the enzyme (Maguire and Sim, 1971; Orsi et al., 1972). Moreover, the inactivation of the enzyme by organic mercurials was attributed to this thiol, and it was shown that substrate excluded these reagents from the active site (Wolfenden, 1967). From these results, and sequence alignments between adenosine deaminase and AMP deaminase sequences, Cys 262 appeared to be the ideal candidate for this essential thiol (Chang et al., 1991). The crystal structure of the murine enzyme, however, showed Cys 262 as being quite far from the substrate binding area of the active site, although it was close to a number of important residues, such as His 238 and Asp 295.

Our results with two Cys 262 mutants, an alanine and a serine, confirm the crystallographic predictions that this cysteine does not play a role in the reaction. Substrate or inhibitor binding is not affected, and the two mutants retain 55-65% of the wild-type activity. This result is in fairly good agreement with Chang (1992), who conducted the preliminary kinetic studies on these mutants. From his results, the mutants had 30-40% lower than wild-type k_{cat}/K_m values. Substrate binding was 2-fold lower in the two Cys 262 mutants; however, our results with adenosine and two adenosine analogs
clearly show that substrate binding is not affected. Similarly, kinetic studies with a similar C262A mutant in human ADA show that the $K_m$ value is not affected by this mutation; however, that mutant has a $k_{cat}/K_m$ value of only 15% of the wild-type (Bhaumik et al., 1993).

Mutation at Cys 262 in murine ADA may have subtle effects on catalysis, as the C262S structure demonstrates. The $\gamma$ of Cys 262 is proximal to His 15, a zinc-coordinating residue, and to Asp 295. This residue also coordinates the metal cofactor and has been proposed to position the hydroxylate group for attack on C-6. A change in the vicinity of Asp 295 may upset its orienting function. This situation appears to apply to the serine substitution of cysteine. Sulfur has a larger atomic radius than oxygen, and the C262S structure clearly shows that the oxygen makes different contacts with Asp 295 than the original sulfur did. The reduction in the catalytic activity of C262S must originate in such subtle steric effects. It is surprising then that the C262A mutant is not less active than the Ser. It would be helpful here to have a crystal structure of this mutant.

Since some mutations at Cys 262 can have slight detrimental effects on ADA activity, it seems logical that formation of adducts between the thiol of Cys 262 and organic mercurial reagents could result in inactivation, in agreement with early work on the enzyme. This speculation, however, seems to be only part of the answer. Chang (1992) reported that both C262A and C262S were inactivated after incubation with p-chloromercuribenzoate (PCMB), a sulfhydryl-specific reagent. This result suggests that another cysteine (perhaps in addition to Cys 262), may have been responsible for the inactivation. From the crystal structure, Cys 153 is the only other candidate: it lies far from N-1 and C-6 of HDPR, but close (~4 Å) to the C-5' of the ribose unit. Thus, substrate binding may have protected this cysteine from being chemically
modified, as reported (Wolfenden et al., 1967).

From our pH dependence studies, neither of the two Cys 262 mutations had any effect on the basic limb of the pH profiles, disproving the proposal that the basic pK_a of 8.5 belongs to a cysteine residue (Maguire and Sim, 1971; Orsi et al., 1972). By contrast, both mutations resulted in a decreased acidic pK_a in the log k_cat pH profile. The acidic pK_a of approximately 5.5 has been assigned to an imidazole group (Orsi et al., 1972); however, a H238R mutant exhibits a wild-type pH profile, suggesting that this pK_a does not represent ionization of His 238. We know that Asp 295 has a lower pK_a than 4.0 due to zinc coordination and hydrogen-bonding interactions (Chapter IV). A possible candidate for the acidic pK_a is Glu 217. If this residue functions as the base in the reaction, and also re-protonates N-1, it must certainly have a pK_a higher than 4.0. A higher pK_a was predicted from the crystal structure, since Glu 217 is in van der Waals distance from 3 Phe residues (Wilson, 1991). If ADA is maximally active at pH 7.0, and Glu 217 must function as both a general base/acid, then its pK_a may be somewhere in the range of 5.5-7.0. The zinc-bound water must be more acidic than the glutamate, with a pK_a as low as 5.0. Zinc-water pK_a values in the vicinity of 6.0 have been reported for carbonic anhydrase and carboxypeptidase (Vallee et al., 1983; Hakansson et al., 1992). Thus, the acidic pK_a may also reflect in part the zinc-water ionization constant.

Since Cys 262 contacts Asp 295, which in turn hydrogen-bonds the catalytic water and coordinates the zinc, it follows that mutations at this residue will perturb the zinc-water pK_a value. Information from the C262S crystal structure shows that the new serine makes altered contacts with Asp 295, which translates to differences in orientation and polarization of the zinc-water. The distance between zinc and the C-6 OH in this structure is shorter
than the corresponding distance in the wild-type HDPR complex (2.04 vs 2.29 Å), as is the zinc-Asp 295 bond length (1.9 vs 2.4 Å). These observations potentiate the argument that Cys 262 mutations result in subtle rearrangements within the metal coordination sphere which cause decreases in catalytic activity and altered pH dependence.

4.2 Why Has Cysteine 262 Been Conserved in Adenosine Deaminase Sequences?

An alanine and a serine mutation of Cys 262 produced ADA enzymes that were able to efficiently turn adenosine into inosine. Clearly, Cys 262 is not essential for catalytic activity. Both mutants were indistinguishable from wild-type murine ADA in secondary and tertiary structure and zinc content. Nevertheless, the average yields of these mutants were only 10% of wild-type after a typical purification. The finding of most of the mutant proteins within bacterial inclusion bodies suggested that mutations of Cys 262 may delay or alter the in vivo folding of the enzyme. An experiment where chaperonins were overexpressed in the bacterial strain carrying C262A or C262S failed to show changes in the distribution of these mutants between the cytoplasm and inclusion bodies. Nevertheless, there still remains a strong possibility that Cys 262 mutants are important in the folding process of ADA. When a human ADA C262A mutant was purified using a monoclonal antibody affinity column, lower yields were obtained after refolding this enzyme from urea compared to wild-type human ADA (Bhaumik et al., 1993). Other site-directed mutants purified in a similar manner, such as H238A, E217A, and H214A, appeared to refold from urea without problems.

Empirically, we observed decreased stabilities of the Cys 262 mutants during our kinetic assays compared to wild-type ADA or other mutant ADA
enzymes such as the Asp 296 mutants. C262A appeared to be especially sensitive, and this is reflected in its instability during incubation in acidic pH buffers. During a 4 minute incubation at pH 4.0, C262A lost 35% of its activity. These observations collectively hint that Cys 262 may be a residue important for the assembly and the stability of adenosine deaminase. Denaturation studies presented in the next chapter show that this hypothesis is true.

5. Summary of Information about the ADA Mechanism from Asp 295, Asp 296, His 238, and Cys 262 Site-Directed Mutants

A number of important propositions regarding the mechanism of adenosine deamination catalyzed by ADA have been verified or disproved by our site-directed mutagenesis work. These are summarized as follows:

1. H238A showed that His 238 is not the base which abstracts the zinc-water proton to create the attacking hydroxylate. Thus, out of the two residues proposed to perform this function, namely Asp 295 and His 238, His 238 has been eliminated. The function of His 238 has been limited to that of stabilizing the negative hydroxylate charge and/or donating a proton to the leaving group.

2. Results with D295E argue against Asp 295 being the base in the reaction. The role of Asp 295 seems likely to be that of chelating the metal and correctly positioning the hydroxylate. Results with Cys 262 mutants suggest that subtle alterations in Asp 295-catalytic water contacts may result in differences in ionization constants and decreases in catalytic activity.

3. Asp 296 has been verified as being important to the enzyme for binding the
substrate. In addition, the residue has been shown to assist in the formation of the transition-state by abstracting electron density from the substrate.

4. Cys 262 is clearly not essential for the catalytic activity of the enzyme.

The reaction base is likely to be Glu 217, a residue already proven to protonate N-1 of the substrate (Mohamedali, 1992). As discussed in Chapter IV, more site-directed mutations at this residue and at Asp 295 will unambiguously identify the base. Additional experiments with existing mutants such as H238A will prove/disprove the histidine's ability to protonate the leaving group.
CHAPTER VI.

STRUCTURAL STUDIES OF MUTANT AND WILD-TYPE ADENOSINE DEAMINASE

1. Objectives

Our investigation of the structural properties of ADA was instigated for two reasons. First, there are certain point mutations in human ADA cDNA's derived from patients suffering from ADA deficiency (e.g. Ala 329–> Val) which do not affect residues near or in the active site and are very conservative in nature (Wilson et al., 1991). It would be of interest to examine whether such mutations lead to ADA enzymes with altered stabilities and conformations. Secondly, during the study of our site-directed mutants, we observed that some of them were produced from bacteria at consistently lower amounts than wild-type ADA. A large percentage of poorer producers, such as C262A, C262S, H238R, was found in bacterial inclusion bodies (Table VI.1). Other mutant proteins, such as D296A and D296N, were always produced in far greater amounts than wild-type, and negligible amounts of them were found in inclusion bodies. We wanted to investigate the correlation between the ability of these mutant proteins to fold in vivo and the amount of protein that is produced in the cytoplasm. In order to study this question indirectly, we examined the in vitro unfolding properties of eight murine ADA mutants under denaturing conditions using fluorescence spectroscopy. The effect of each mutation on the observed denaturation rate of ADA was evaluated. In addition, insights were gained into the role played by the zinc cofactor in the structure of ADA.
<table>
<thead>
<tr>
<th>sample</th>
<th>average yield (mg of protein)</th>
<th>inclusion body content (% of total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>100</td>
<td>10%</td>
</tr>
<tr>
<td>D295E</td>
<td>20</td>
<td>10%</td>
</tr>
<tr>
<td>D296A</td>
<td>220</td>
<td>10%</td>
</tr>
<tr>
<td>D296N</td>
<td>160</td>
<td>10%</td>
</tr>
<tr>
<td>C262A</td>
<td>7</td>
<td>90%</td>
</tr>
<tr>
<td>C262S</td>
<td>10</td>
<td>90%</td>
</tr>
<tr>
<td>H238A</td>
<td>70</td>
<td>20%</td>
</tr>
<tr>
<td>H238E</td>
<td>50</td>
<td>70%</td>
</tr>
<tr>
<td>H238R</td>
<td>20</td>
<td>90%</td>
</tr>
</tbody>
</table>

Table VI.1 Yields and inclusion body content of mutant and wild-type ADA proteins. Average yields were calculated from three or more protein preparations of each sample. The amounts of mutant and wild-type ADA present in bacterial inclusion bodies were estimated from Western blots of total inclusion body solubilized protein; per cent values were assigned after comparing the intensity of the bands to blots of cytoplasmic proteins. Total protein refers to total amount of ADA found in inclusion bodies and cytoplasm.
2. Early Structural Work on the Enzyme

Prior to the determination of the crystal structure of murine adenosine deaminase, little was known about its structural properties. The first structural studies were initiated with the calf enzyme because transition-state analogs for the ADA reaction, such as coformycin and deoxycoformycin, were observed by stopped-flow kinetics to bind with a slow $k_{on}$. This slow binding was in contrast to the rapid inhibition of the enzyme by ground-state analogs such as purine riboside and was proposed to reflect a weak initial binding followed by a conformational change (Frieden et al., 1980). The proposed conformational change was reflected in the binding of transition-state analogs which quenched the intrinsic fluorescence spectrum of the enzyme by 40-70%. Ground-state analogs such as purine riboside caused only a minimal quench of 6%, in agreement with their fast $k_{on}$ values (Kurz and Frieden, 1983). The nature of the conformational change was investigated by measuring the solvent accessibility of the enzyme tryptophans in free calf ADA and ADA-inhibitor complexes via the effect of external quenchers on the protein's tryptophan fluorescence (Kurz et al., 1985). The results suggested that all three Trp residues were accessible to solvent in the free enzyme, but were more buried in ADA complexes with ground-state analogs and much more buried in complexes with transition-state analogs. It was proposed that the protein became more "compact" as the complexes were formed, and that all tryptophans had similar solvent accessibilities, with limited contact with water at the protein surface. The mechanism by which Trp fluorescence was quenched after ligand binding was proposed to be that of energy transfer from the donor (Trp) to the ligand. The distance between donor and acceptor was calculated to be 16 Å, suggesting that ligand binding resulted in conformational changes remote from the binding site. Studies on the
dependence of transition-state analog binding of ADA on viscosogenic agents reinforced the notion that a viscosity-dependent conformational change occurred upon ligand binding (Kurz et al., 1987). Fluorescence spectroscopy with the human enzyme revealed that its tryptophan fluorescence was also quenched by ground-state analogs (e.g. PR, causing a 10% decrease in intensity) and more so by transition-state analogs (e.g. deoxycoformycin, causing a 30% decrease). Quenching was caused in two ways: a tryptophan residue was postulated to be close to the active site whose emission was quenched upon ligand binding. Additionally, the emission of tryptophans remote from the active site was quenched due to a ligand-induced conformational change (Philips et al., 1987). So far, the results obtained with the human enzyme were in accord with those from calf ADA.

Adenosine itself was used in a later study with calf ADA to examine substrate binding effects on the protein fluorescence. A 27% quenching of the intrinsic fluorescence was observed and was attributed to a conformational change of the enzyme (Porter and Spector, 1993). Thus, prior to the determination of the crystal structure for murine ADA, studies on both the human and calf enzymes with the substrate itself, and with ground-state and transition-state analogs, had suggested a conformational change occurring upon ligand binding. This was the extent of information on the structure of ADA.

Some of the above proposals were evaluated when the murine crystal structure was determined. The enzyme folds in an $\alpha/\beta$ barrel motif, and has a tightly bound zinc cofactor in its active site, in addition to the bound ligand, HDPR (a transition-state analog). Of the four tryptophan residues in mouse ADA, W 161 is completely buried (7% of its surface is solvent-accessible), W 117 and 272 are relatively less buried (11% & 16% accessible, respectively) and W 264 is partly accessible (31%). Two of these tryptophans are relatively close
to the C-6 OH of HDPR; these are W 264 and W 272 with 10.8 Å and 12.6 Å distances, respectively. The other two Trp residues are further away, at 19-20 Å distances. This information agrees with data from the calf enzyme suggesting that in complexes of the enzyme with transition-state analogs all Trp residues are buried (Kurz et al., 1985).

Regarding the conformational change, only 0.5% of the surface of HDPR is accessible to solvent when the molecule is bound by ADA (Wilson et al., 1991). The authors suggested that a structural change had to occur for the ligand to dissociate from the active site pocket, and, by inference, for a preformed transition-state analog to bind the active site. Either a large loop of residues (Wilson, 1996) or two loops that form partial lids covering the active site (Wilson, 1991) could move to mediate such a conformational change.

One of the tryptophans in mouse ADA, Trp 117, is situated in the middle of the loop that has been speculated to move upon ligand binding. None of the four are within the two smaller partial lids capping the active site. If a conformational change occurs upon binding of transition-state analogs to the murine enzyme, and if this involves the loop containing Trp 117, then a quench of the emission could be observed. Additionally, binding of some ligands could quench the emission of the two tryptophans close to the active site (W 264 and W 272).

Despite this wealth of information from crystallography, little is yet known about the manner in which adenosine deaminase folds in vivo or in vitro, the role that the zinc cofactor plays in the structural assembly of the enzyme, and what factors promote or reduce stability in the structure. We used eight site-directed mutants and wild-type murine ADA to get a first glimpse of the answers to these questions.
3. Methods

3.1 Fluorescence Spectroscopy

Fluorescence spectroscopy was performed on a SLM 8100 spectrofluorimeter. The excitation wavelength was 290 nm and the emission was scanned from 300-400 nm every 1 nm. Measurements were made using a 1 cm path-length fluorescence cuvette (Starna Cells, Inc).

For the intrinsic fluorescence studies, samples of 25 μM ADA were in 20 mM Hepes buffer, pH 7.0. All spectra were normalized for protein concentration. Unfolding studies were performed at room temperature with enzymes diluted to 1 μM final concentration in 100 mM potassium phosphate buffer, pH 7.5 containing either 2 M or 3 M guanidinium hydrochloride [GdnHCl, prepared as described by Pace et al. (1990)]. The samples were mixed and scanned immediately. The amount of fluorescence intensity at 328 nm at time zero was assigned the value of 100%. The drop in fluorescence intensity at 328 nm was monitored at several time points and normalized by the fluorescence intensity at 328 nm at t=0. Spectra of each sample in buffer lacking GdnHCl were taken as a control before and after the unfolding experiment to check for possible denaturation of the sample in the absence of GdnHCl. Data were plotted as % fluorescence intensity at 328 nm over time. The linear portions of the plots were used to calculate their slopes; these were then compared to the slope of wild-type ADA fluorescence decrease with time. Relative rates of denaturation were assigned to samples from a comparison of these slopes.

As a control for unfolding, the far UV CD spectrum of each sample was obtained in the beginning and at the end of the experiments. In some cases, enzymatic activities were also assayed before and after denaturation of ADA.
3.2 Circular Dichroism Spectroscopy

CD spectra were measured in the far UV (215-260 nm) region on an Aviv 6100 Spectrometer. Measurements were made using a 0.02 cm path-length cuvette (Starna Cells, Inc). For spectra taken prior to and immediately after the unfolding studies, 1 μM samples were in 100 mM potassium phosphate buffer, pH 7.5 with or without 2 M or 3 M GdnHCl.

3.3 Zinc Chelation and Retitration

Apoenzymes were prepared as described in Wagner (1988). Samples were concentrated and exchanged against metal-free 20 mM HEPES, pH 7.0, 1 mM DTT using an Amicon Centriflo (CF25) device to 11 mg/ml (275 μM) ADA. Metal removal was initiated by placing the samples in metal-free dialysis bags (Spectrapor, 12-14 K molecular weight cut-off) and dialyzing them for 15 hours against 50 mM MES, 1 mM DTT, 10 mM dipicolinic acid (Sigma), pH 5.5 at 4°C. Control samples were dialyzed against 50 mM MES, 1 mM DTT, pH 5.5 buffer without chelator for the same amount of time. Activity assays were performed on the samples before, during and at the end of dialysis. To remove all traces of chelator, the samples were dialyzed against 3-4 washes of 50 mM HEPES, 1 mM DTT, pH 7.0, over 24 hours. The samples were stored over Chelex 100 resin (BioRad) in metal-free tubes at 4°C. All plasticware was rinsed thoroughly with 18 MW Milli-Q water (Auld, 1988) and buffers were stored over Chelex resin to eliminate adventitious metals.

Retitrations were performed by incubating the apo-enzymes for 4 minutes at room temperature with stoichiometric amounts of ZnSO₄ and measuring enzymatic activities as described in previous chapters. CD measurements and fluorescence spectroscopy were performed by diluting the samples in metal-free 100 mM potassium phosphate buffer, pH 7.5 with or
without 3 M GdnHCl. The cuvettes were rinsed with 20% HNO₃ followed by Milli-Q water to remove all traces of metals prior to spectral acquisition (Auld, 1988).

3.4 Correlation of Fluorescence, Circular Dichroism, and Enzyme Activities
Samples were exchanged into 100 mM potassium phosphate buffer, pH 7.5 and assayed for activity. Guanidinium hydrochloride was added to a final concentration of 1-6 M, and fluorescence emission and CD spectra were taken, followed by activity measurements. Samples were at 1 μM final concentration. This series of experiments was repeated at 2, 9.5, 26, and 75 hours. The fluorescence signal at 328 nm at 0 hours in the absence of denaturant was assigned the value of 100%, and all fluorescence intensity values were normalized using this value. The circular dichroism signal at 222 nm, time zero, was similarly used for the normalization of the CD values. Enzymatic activities, measured as described earlier, were normalized by the value of the ADA activity at time zero, in the absence of GdnHCl.

4. Results

4.1 Correlation of Fluorescence Emission, Helicity, and Enzymatic Activity with Wild-Type ADA in the Presence of Guanidinium Hydrochloride
The intrinsic fluorescence spectrum of wild-type murine ADA (Figure VI.1) has a maximum at 328 nm, due to the emission of one or more of the four tryptophans of the molecule. When ADA is denatured by guanidinium hydrochloride, the emission maximum shifts to 347 nm and fluorescence becomes quenched by 70%. The wavelength shift is consistent with the
Figure VI.1 Tryptophan emission fluorescence spectroscopy of native and denatured wild-type murine ADA. The excitation wavelength was 290 nm and emission was scanned from 300-400 nm. Protein concentrations were 1 μM ADA in 100 mM potassium phosphate buffer, pH 7.5 in the absence or the presence of 6 M guanidinium hydrochloride (GdnHCl).
Figure VI.2 Far UV circular dichroism spectroscopy of native and denatured wild-type murine ADA. Spectra were taken in the region 215-260 nm and normalized for protein concentration. Each spectrum shown represents the average of three scans. Protein concentrations were 1 μM ADA in 100 mM potassium phosphate buffer, pH 7.5 in the absence or the presence of 6 M guanidinium hydrochloride (GdnHCl).
emission maximum of tryptophan fluorescence in apolar (300 nm in hexane) and aqueous solvents (348 nm in water; Philips et al., 1987). This denaturation is accompanied by a decrease in the helicity of the protein, and the theta value at 222 nm decreases to ~20% of its value in the native protein (Figure VI.2). Figure VI.3 shows the correlation between fluorescence intensity of the wild-type tryptophan emission at 328 nm and the intensity of the CD signal at 222 nm, attributed to the \( \alpha \)-helix content of the protein (Adler et al., 1973). The correlation is shown for wild-type ADA in the absence and in the presence of varying amounts of GdnHCl, immediately after the denaturant was added to the enzyme. The fluorescence intensity at time zero for both the 0 M and the 1 M GdnHCl is 100%; the CD spectra of both samples are superimposable. The enzymatic activity, assayed within the next hour following sample preparation, shows that the 1 M sample has started to unfold and has already lost 13% of its catalytic activity. At time zero, both the fluorescence and CD signals of the 2 M sample have started to decrease, as reflected by the sample's 30% lower activity. For the 3 M sample, fluorescence was taken immediately after dilution, but the CD spectrum 30 minutes later. This is the cause of the sample's high fluorescence value but low CD signal and activity value. The 5 M sample unfolds extremely fast, with its fluorescence already at 30% immediately after mixing, and most of its helicity and activity lost within the next half hour.

Figure VI.4 shows similar data for the same samples 9.5 hours into the experiment. As the graph suggests, by this time the 3 M GdnHCl sample has almost entirely lost its secondary structure. Its quenched fluorescence and shifted \( \lambda \) max to 346 are characteristic of unfolding; the sample's activity is now zero. Also visible in the graph is the loss of secondary structure in the 1 M and 2 M GdnHCl samples. The 2 M sample has lost roughly half its helicity,
Figure VI.3 Top panel: correlation between fluorescence intensity at 328 nm, intensity of the $\theta_{222}$ signal and enzymatic activity of wild-type ADA in the presence or absence of GdnHCl at time zero. Samples were at 1 $\mu$M in KPi buffer containing 0-6 M GdnHCl. Bottom panel: Far UV CD spectra of 0-6 M GdnHCl/wild-type samples at time zero.
**Figure VI.4** Top panel: correlation between fluorescence intensity at 328 nm, intensity of the $\theta_{222}$ signal and enzymatic activity of wild-type ADA in the presence or absence of GdnHCl at 9.5 hours. Bottom panel: Far UV CD spectra of 0-6 M GdnHCl/wild-type samples at 9.5 hours.
Figure VI.5 Top panel: correlation between fluorescence intensity at 328 nm, intensity of the $\theta_{222}$ signal and enzymatic activity of wild-type ADA in the presence or absence of GdnHCl at 26 hours. Bottom panel: Far UV CD spectra of 0-6 M GdnHCl/wild-type samples at 26 hours.
fluorescence, and activity. The λ max for the fluorescence emission, however, has still not appreciably increased. The 1 M sample has lost 10% helicity and 20% fluorescence. Interestingly, the 0 M sample, while experiencing no secondary structure loss, has a somewhat quenched fluorescent emission (by 15%) and decreased activity (20%). This observation suggests that the emission of one or more tryptophan is quenched due to local unfolding; perhaps this residue is close to the active site of the protein. No change occurs in the 6 M sample’s parameters, signifying that denaturation at this level of GdnHCl has already reached equilibrium.

By 26 hours, the 3 M GdnHCl sample has also completely unfolded, but the 2 M sample is still equilibrating, with 50% of its α-helix intact, most of its fluorescence quenched, and most of its activity lost (Figure VI.5). The maximum of the emission is 336 nm. The 1 M sample is denaturing very slowly, a long way from equilibrium (the sample was still unfolding by 75 hours, data not shown). The 0 M sample shows no more quenching of fluorescence or loss of activity.

Overall these data suggest that guanidinium chloride causes the slow unfolding of ADA, as judged by the loss of secondary structure and enzymatic activity and that monitoring of the decrease in fluorescence intensity at 328 nm as well as the increase in λ max of the emission is an accurate indication of the degree to which the protein has unfolded.

4.2 Kinetics of Unfolding with Wild-Type and Mutant ADA
Fluorescence spectroscopy was used to measure the degree of unfolding in 2 M or 3 M GdnHCl samples of mutant and wild-type ADA immediately after dilution. The decrease in intensity at 328 nm, which is the wavelength maximum of the fluorescence emission for fully folded, wild-type ADA, was
**Figure VI.6** Panels A and B: Unfolding kinetics for wild-type, D296A, D296N and D295E ADA in 3 M GdnHCl. Panel C: Unfolding kinetics for wild-type, C262A and C262S in 2 M GdnHCl. Unfolding was monitored by the decrease in fluorescence intensity at 328 nm. The slope of the data was used to calculate relative denaturation rates.
Figure VI.7 Panels A and B: Unfolding kinetics for wild-type, H238A, H238E and H238R ADA in 2 M GdnHCl. Panel B is the data for the His 238 mutants plotted without the wild-type. Unfolding was monitored by the decrease in fluorescence intensity at 328 nm. The slope of the data was used to calculate relative denaturation rates.
plotted versus time. The plots were typically multiphasic; therefore, to estimate a slope, only their first, linear portion was used. The results are shown in Figures VI.6-7. Two mutants, D296A and D296N, unfolded 1.65 and 3.7 times more slowly than wild-type ADA, respectively (Figure VI.6, Panel A). C262A and C262S, on the other hand, unfolded 2.2 and 6.5 times more quickly (Panel C). D295E unfolded as quickly as C262S (Panel B). The His 238 mutants were exceedingly unstable, with H238E and H238R denaturing approximately 100 times faster than the wild-type, while H238A unfolded 27 times faster. The mutants are shown compared to wild-type ADA in Panel A and by themselves in Panel B of Figure VI.7, to better illustrate differences among them.

In order to ascertain that this rapid decrease in fluorescence intensity actually represented protein unfolding, CD spectra were taken at time zero and at the end of the experiments in the region of 215-260 nm. These showed that the amount of secondary structure in unfolding ADA samples had indeed decreased during denaturation in accord with the fluorescence data (data not shown).

From the slopes of the pictured unfolding curves, a denaturation rate series was constructed for mutant and wild-type ADA enzymes. In this series, samples on the left are denatured more quickly, while samples on the right are denatured more slowly:

H238E>H238R>H238A>D295E, C262S>C262A>wild-type>D296A>D296N

Thus, as this series demonstrates, the Asp 296 mutants are denatured very slowly, even more so than the wild-type, while the His 238 mutants unfold very readily. These results raise interesting questions on the relation between the observed rates of unfolding and the ability of ADA mutants to fold properly in vivo, as will be discussed below.
4.3 Preparation of Wild-Type Apoenzyme and Reconstitution with Zinc

To probe the role of the metal cofactor in the structure and function of ADA, the apoenzyme was prepared. Wild-type ADA was dialyzed against MES buffer containing excess (10 mM) dipicolinic acid at pH 5.5. The sample lost 90% of its enzymatic activity after 1 day of incubation (Figure VI.8). When another chelator, EDTA, was used in the experiment, little activity was lost, in agreement with previous work on ADA-metal chelation (Cooper, unpublished results). Incubation of the enzyme with Chelex 100 resin also failed to remove the zinc (not shown). A control wild-type sample incubated under the same conditions but in the absence of chelator showed little activity loss.

To examine whether the activity loss during chelation could be restored by addition of zinc, stoichiometric amounts of ZnSO₄ were added to wild-type apoenzyme. The results (Figure VI.9, top panel) show that one equivalent of zinc fully restores enzymatic activity. Ratios much higher than 10:1 zinc/protein start inhibiting enzymatic activity, with 50% inhibition occurring at 1 mM zinc. Cobalt is also able to restore activity to the apoenzyme, but the maximal activity achieved is 90 versus 100% (Figure VI.9-bottom panel). Reactivation of the activity by zinc is immediate (< 1 minute). However, zinc was not able to restore enzymatic activity if Km levels of the substrate, adenosine, were pre-incubated with the apoenzyme for 5 minutes. This observation suggests that the bulkier substrate prevents zinc from accessing the correct part of the active site for binding, in accord with data from the crystal structure that show zinc to be bound in the deepest part of the active site cavity (Wilson et al., 1991).
Figure VI.8 Activity loss during dialysis of wild-type ADA against 50 mM MES, 1 mM DTT, 10 mM dipicolinic acid (Sigma), pH 5.5 at 4°C. The activity of a wild-type control sample, incubated against 50 mM MES, 1 mM DTT, pH 5.5 buffer for the same length of time was not reduced. Dialysis of wild-type ADA against 10 mM EDTA was not effective in chelating the zinc.
Figure VI.9 Reactivation of wild-type apoenzyme by zinc (top panel) or cobalt (bottom panel) addition. The metal was mixed with the apoenzyme, incubated for 4 minutes, and assayed for activity as described before. Zinc restores maximal activity at 1:1 mol/mol ratio; cobalt restores 90% of the activity. ADA activities following zinc restoration represent averages of three separate experiments.
4.4 Structural Properties of the Apoenzyme

The intrinsic fluorescence emission spectra of wild-type apoenzyme and holoenzyme are shown in Figure VI.10. The two spectra superimpose, with similar intensities and emission maxima. Addition of stoichiometric amounts of zinc to the apoenzyme does not affect the protein fluorescence, even though it restores the activity of these samples (data not shown). By contrast, addition of one equivalent of cobalt to the apoenzyme quenches the fluorescence intensity by 6-15%. This result suggests that cobalt perturbs Trp emission while binding, in contrast to zinc. The secondary structure of ADA is similarly unperturbed by the absence of the metal cofactor (Figure VI.11). These data suggest that zinc can be removed from and added back to the active site of ADA in the absence of substrate without major structural rearrangements.

Addition of equimolar deoxycoformycin to wild-type holoenzyme quenches its emission by 47%, in agreement with other reports (Kurz and Frieden, 1983; Philips et al., 1987). When this transition-state analog is added to the apoenzyme, a quench of similar magnitude (46%) is observed (Figure VI.12). This result suggests that the inhibitor binds ADA in a similar manner whether the cofactor is present or absent. When cobalt is present in the active site, deoxycoformycin quenches the fluorescence by 51%. The CD spectra of the free and bound enzymes are similar (data not shown). Once deoxycoformycin is bound in the apoenzyme active site, addition of zinc or cobalt does not alleviate the quenching. This agrees with the kinetic experiments where pre-incubation of the apoenzyme with substrate precluded reactivation by the metal. Overall, these results suggest that
Figure VI.10 Intrinsic fluorescence emission spectra of wild-type apo- and holoenzyme. The excitation wavelength was 290 nm and emission was scanned from 300-400 nm. Apo ADA samples were in metal-free 100 mM potassium phosphate buffer, pH 7.5.
Figure VI.11 Far UV circular dichroism spectroscopy of wild-type apo- and holoenzyme. Spectra were taken in the region 200-260 nm and normalized for protein concentration. Each spectrum shown represents the average of three scans. Protein concentrations were 1 μM ADA in metal-free 100 mM potassium phosphate buffer, pH 7.5.
Figure VI.12 Tryptophan emission fluorescence spectra of wild-type ADA apo- and holo-enzyme either free or complexed to an equimolar amount of the transition-state analog deoxycoformycin. Fluorescence intensity is quenched by 47% upon inhibitor binding in both the apoenzyme and the holoenzyme.
binding of the analog and the postulated concomitant conformational change
do not depend on the presence or identity of the metal cofactor.

4.5 Preparation of Three Mutant ADA Apoenzymes and Reconstitution with
Zinc

The Asp 296 mutants (D296A and D296N) unfolded more slowly than the
wild-type in our denaturation experiments. By contrast, D295E unfolded faster
than wild-type ADA. We used this group of mutants to examine if the metal
cofactor plays a role in the rate of denaturation of ADA. To this end, the
apoenzymes of D295E, D296A, and D296N were prepared using dipicolinic
acid, as described for the wild-type apoenzyme. All three enzymes lost most of
their activity within 1 day. Control samples of D296A and D296N incubated in
the absence of chelator for the same amount of time did not lose appreciable
amounts of activity; D295E, however, was more unstable losing 30-40% of its
activity after the lengthy dialysis. Neither EDTA nor Chelex were able to
remove zinc from the mutants.

Stoichiometric amounts of zinc added back to the mutant apoenzymes
restored their enzymatic activity (Figure VI.13 shows retitration data for
D296A and D296N). As seen with wild-type ADA, excess zinc inhibited the
activity. Reactivation was immediate, but an important difference was seen
between the Asp 296 mutants and the wild-type: for both apoenzymes, if
adenosine was pre-incubated with the apoenzyme prior to zinc addition,
incubation with K_m levels of adenosine did not prevent reactivation by zinc.

The structure of the three apoenzymes was probed by fluorescence and
circular dichroism spectroscopy, and compared to wild-type apoenzyme
prepared under the same conditions, as well as holoenzyme, incubated in
Figure VI.13 Reactivation of D296A (top panel) or D296N apoenzyme (bottom panel) by zinc addition. The metal was mixed with the apoenzyme, incubated for 4 minutes, and assayed for activity as described before. Zinc restores maximal activity at 1:1 mol/mol ratio.
Figure VI.14 Intrinsic fluorescence emission spectra of wild-type apo- and holoenzyme, and D295E, D296A and D296N apoenzymes. The excitation wavelength was 290 nm and emission was scanned from 300-400 nm. Apo ADA samples were in metal-free 100 mM potassium phosphate buffer, pH 7.5.
MES buffer, pH 5.5 for the same amount of time. The fluorimetry results are shown in Figure VI.14. The wild-type apo- and holo-enzyme spectra superimpose well, but the emission maximum of the apoenzyme is slightly red-shifted, to 332 nm. Both apo D296A and apo D296N have higher emission intensities than the wild-type. The emission spectrum of apo D295E is quenched by 25% and has a maximum of 335 nm, signifying that some denaturation has occurred in this sample.

The CD spectra show that apo D295E has indeed lost secondary structure (its \( \theta_{222} \) is 73% compared to the wild-type holoenzyme) whereas apo D296A and apo D296N are both somewhat more helical than the other samples (Figure VI.15). These results are in accord with the previously observed instability of D295E holoenzyme and the greater stability of the D296A and D296N holoenzymes.

4.6 Kinetics of Unfolding of Wild-Type and Mutant ADA Apoenzymes

The unfolding of all four apoenzymes at 3 M guanidinium chloride was followed by the decrease in fluorescence signal at 328 nm (Figure VI.16). Wild type apoADA loses 40% of its fluorescent signal at 328 nm within the time required to add it to the denaturing solution, mix it, and insert it in the instrument (20 sec). The control ADA holoenzyme unfolds more slowly and requires 15 minutes to lose 40% of its signal. The lengthy dialysis of the wild-type holoenzyme at pH 5.5 increases the sample's rate of denaturation, since the holoenzyme now unfolds 3 times faster than it did in the experiments described in Section 4.2.

Of the mutant apoenzymes, D295E is the most unstable, losing 60% of its fluorescent signal within 30 sec. Circular dichroism spectra taken in 1 hour after unfolding for wild-type holoADA and 15-30 minutes for apo wild type
Figure VI.15 Far UV circular dichroism spectroscopy of wild-type apo- and holoenzyme, and D295E, D296A and D296N apoenzymes. Spectra were taken in the region 200-260 nm and normalized for protein concentration. Each spectrum shown represents the average of three scans. Protein concentrations were 1 μM ADA in metal-free 100 mM potassium phosphate buffer, pH 7.5.
Figure VI.16 Unfolding kinetics for wild-type apo-and holo-enzyme, apoD295E, apoD296A and apoD296N in 3M GdnHCl. Unfolding was monitored by the decrease in fluorescence intensity at 328 nm.
and apo D295E ADA demonstrate the lack of any secondary structure (data not shown). The apoenzyme of D296N unfolds at the same rate as the wild type holoenzyme and therefore more slowly than the wild type apoenzyme. The most stable apoADA is D296A, which requires 45 minutes to lose 40% of its fluorescence signal (3-fold more time than the wild type holoenzyme). This reluctance to unfold is also demonstrated by its CD spectrum taken 1 hour after the initiation of unfolding, which shows that the mutant retains 60% of its \( \theta_{222} \). By comparison, apo D296N has virtually no secondary structure left within an hour. Based on this unfolding data, a series of rate of unfolding can be constructed for the apoenzymes:

\[
\text{apo D295E} \rightarrow \text{apo wild-type} \rightarrow \text{apo D296N} \rightarrow \text{apo D296A}
\]

In this series, the apoenzyme with the highest denaturation rate is D295E while the apoenzyme with the lowest rate of denaturation is D296A.

5. Discussion

5.1 Properties of the ADA Apoenzyme

The information that ADA was a zinc-utilizing enzyme came as a surprise, since early work had not identified a cofactor as part of the enzyme (Frick et al., 1987). However, this finding made sense from the point of view that enzymes homologous to ADA, such as AMP and cytidine deaminase, contain and utilize zinc (Merkler and Schramm, 1993; Yang et al., 1992). In addition, other enzymes which catalyze nucleophilic additions of hydroxylates such as the zinc proteases rely on zinc for activating water (reviewed in Chapter II). Zinc is the only metal which is present in active or structural sites of enzymes belonging to each one of the six enzyme classes (Vallee and Auld, 1990). In all cases where a zinc cofactor plays a catalytic role, it is coordinated to two
ligands closely spaced to each other which are thought to assist in forming a primary bidentate zinc complex. The third ligand is separated by a relatively long spacer (20-120 amino acids) from the first two ligands. It has been suggested that this long spacer arm could interact with the primary zinc bidentate complex, forming a more stable metal coordination sphere and positioning important catalytic and substrate-binding groups into the developing active site (Vallee and Auld, 1990).

The zinc cofactor in ADA is coordinated to His 15, His 17, and His 214, as well as to Asp 295 and the catalytic water, which share the remaining site. It is obvious that zinc coordination in ADA falls into the pattern of a short spacer separating the first two ligands (one amino acid in this case) and a longer spacer bridging them to the third ligand (198 amino acids). This zinc ligand arrangement is the same that carbonic anhydrase employs, where the first two His ligands are one amino acid apart, and the third histidine ligand is 122 amino acids away. This coincidence in the architecture of the zinc coordination sphere between ADA and carbonic anhydrase may be related to the similarity in their catalytic mechanisms. If the precise arrangement of zinc ligands is tailored to meet the requirements of specific active sites and reaction mechanisms as has been suggested (Vallee and Auld, 1990), this would imply that cytidine deaminase (CDA) catalyzes the hydrolytic deamination of cytosine by a different reaction pathway than adenosine deaminase employs. In CDA, the zinc ligands are two closely spaced cysteines (Cys 129 and 132) and a further histidine (His 102) in addition to the catalytic water.

By removing the zinc cofactor from ADA, we were able to examine the structural and functional properties of the apoenzyme. Removal of the metal was only possible using dipicolinic acid at acidic pH (pH 5.5). Neither EDTA or
Chelex 100 succeeded in chelating the metal from ADA. Chelation was relatively slow (15-24 hours), suggesting that the mechanism of metal removal is the depletion of free metal ion concentration by the chelator which promotes metal dissociation from the enzyme (Wagner, 1988). Zinc chelation resulted in complete loss of adenosine deaminase activity, which was restored following addition of stoichiometric amounts of the metal. Maximal activity was restored when one mol zinc was added to one mol apoADA. Cobalt was able to replace zinc in the active site, restoring 90% of the activity. A first indication that zinc loss did not cause major structural alteration was that zinc was able to re-associate with the apoenzyme rapidly (< 1 min) and immediately allow for substrate binding and reactivation. This finding was confirmed by the identity between the fluorescence and circular dichroism spectra of the apo- and holo-enzymes.

A very interesting finding was that preincubation of the substrate with the apoenzyme precluded zinc from binding to the active site and reactivating the enzyme. Crystallographic data showed zinc to be bound in the deepest part of the active site, with the purine ring of the substrate binding directly above it (Wilson et al., 1991). Thus, when substrate binds first, zinc access to its binding site is blocked. Zinc probably enters the active site only from the top, where the opening to the cleft is found.

That the substrate was able to bind apoenzyme supports the lack of alterations in the configuration of the active site. Deoxycoformycin, like other transition-state analogs of the reaction, quenches the enzyme's fluorescence when it binds ADA (Kurz and Frieden, 1983; this work). The effect on fluorescence has been postulated to be indicative of an overall conformational change in enzyme structure (Kurz and Frieden, 1983; Porter and Spector, 1993). Our results with fluorimetry suggest that not only is the apoADA active site able
to bind deoxycoformycin but also that its binding is accompanied by a quench in the emission similar in magnitude to that observed with the holoenzyme. Thus, if a conformational change is needed for accommodation of the analog, absence of the cofactor does not impede it. In other words, the cofactor appears to play no role in mediating the conformational change. This conclusion is also suggested from the observed quench when deoxycoformycin binds the ADA-cobalt enzyme.

In retitration experiments, cobalt could only restore 90 vs 100% of the ADA activity. A small quench in the fluorescence intensity of the ADA apoenzyme reconstituted with cobalt suggests that the mode of cobalt reactivation may differ from that of the zinc. More experiments with the cobalt derivative of ADA are in progress in our laboratory to investigate this possibility.

Chelation of the zinc from three ADA site-directed mutants, D295E, D296A, and D296N was only possible using dipicolinic acid and resulted in complete activity loss after a day of incubation, similarly to wild-type. Addition of one mol zinc per mol protein was required for activity to be restored. Zinc re-associated with mutant apoenzymes as fast as it did with the wild-type. Nevertheless, preincubation of D296A or D296N with adenosine did not preclude zinc from binding their active sites, in marked contrast with the wild-type apoenzyme results. This finding is not surprising when one considers the greatly decreased substrate affinities of these mutants (Km for adenosine is 200 μM for D296N, 1400 μM for D296A and 21 μM for wild-type). Substrate does not bind either apo D296A or apo D296N tightly enough to prevent zinc from entering and binding the active site.

D295E is a mutant which has been observed to have stability problems during purification (Chapter IV); therefore, it was not surprising that incubation of this mutant for many hours in buffer of acidic pH as required for the
chelation process resulted in its partial denaturation. Incubation of a control D295E sample in buffer lacking chelator caused its activity to drop by 30-40%. The apoenzyme itself had a fluorescence emission spectrum quenched from that of the wild-type apoenzyme by 25% with an red-shifted emission maximum of 335 nm; both these results suggest that denaturation has occurred to a certain degree. This was corroborated by the CD spectrum, which had a θ222 of 73% of the wild-type.

The fluorescence and CD spectra of both apo D296A and apo D296N compare well to the wild-type apoenzyme spectra, suggesting structural similarities among all three apoADA's. If anything, the two mutant apoenzymes are more helical and less quenched than wild-type apoADA, or even the wild-type holoenzyme, incubated under the same conditions. This result is a first indication that the enhanced resilience of the Asp 296 mutants compared to wild-type ADA is also present in their apoenzyme form.

5.2 Factors that Enhance or Decrease Rate of Denaturation of ADA

From the average yield of each of our eight site-directed mutants during purification, it was obvious that some samples were expressed in our bacterial expression system much better than others. Measurement of mutant ADA amounts present within inclusion bodies completely agreed with the observed yields, with the exception of one mutant, D295E. In this case, the low yield was explained by the observation that activity was lost during the first ion-exchange chromatography step of the purification. This suggested that the particular mutant was unstable *in vitro* if not *in vivo*.

The factors that determine shunting of bacterially expressed proteins into inclusion bodies are still being avidly investigated. Generally, a number of mechanisms are possible (Chrunyk et al., 1993): (i) an intermediate in the
folding process may aggregate; (ii). the poorly soluble native state may precipitate when it accumulates in high levels in the cell; (iii). the low stability of the native state may shift the equilibrium to a poorly soluble unfolded state which will then aggregate. Thus, any mutation in the protein sequence which alters the *in vivo* folding process, or changes the solubility or stability of the native state, could result in enhanced/decreased amounts of the protein to be found in inclusion bodies.

We studied the propensity of our mutants to unfold in the presence of guanidinium hydrochloride by monitoring the change in tryptophan fluorescence intensity. This experiment allowed us to calculate a series for mutant and wild-type ADA proteins based on their rates of denaturation, in which His 238 mutants were the fastest to unfold and Asp 296 mutants were the slowest. We can correlate the yields, inclusion body contents, and denaturation rates of these enzymes as shown in Table VI.2.

As is readily obvious, the correlation between how much recombinant protein is expressed and how unstable it is under denaturing conditions *in vitro* is poor for most of the samples. A qualitative agreement is seen only in the case of the Asp 296 mutants: both are expressed at higher levels than the wild-type and both exhibit the lowest rates of unfolding as well.

From these results, it is not obvious why 90% of a relatively stable mutant, such as C262A, should be shunted into inclusion bodies where only 20% of a much more unstable mutant (H238A) is found. Thus, a mutant's *in vitro* propensity to unfold cannot be the only criterion which determines inclusion body formation. Similar conclusions have been reached in other studies where inclusion body formation failed to correlate with the thermal or thermodynamic stability of mutant proteins (Chrunyk et al., 1993; Krebs and Fierke, 1993). The lack of such a correlation was interpreted as evidence that
<table>
<thead>
<tr>
<th>average yield (mg of protein)</th>
<th>inclusion body content (% of total protein)</th>
<th>rate of denaturation (lowest to highest)</th>
<th>denaturation rate ratio (mutant/wild-type)</th>
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<tr>
<td>160</td>
<td>10%</td>
<td>D296N</td>
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</tr>
<tr>
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<td>10%</td>
<td>D296A</td>
<td>0.61</td>
</tr>
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<td>100</td>
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<td>wild-type</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>90%</td>
<td>C262A</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>90%</td>
<td>C262S</td>
<td>6.7</td>
</tr>
<tr>
<td>20</td>
<td>10%</td>
<td>D295E</td>
<td>6.7</td>
</tr>
<tr>
<td>70</td>
<td>20%</td>
<td>H238A</td>
<td>27</td>
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<tr>
<td>50</td>
<td>70%</td>
<td>H238E</td>
<td>100</td>
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**Table VI.2** Yields, inclusion body content, and denaturation rates of mutant and wild-type ADA proteins. Average yields were calculated from three or more protein preparations of each sample. Denaturation rate ratios were calculated from slopes of plots of fluorescence intensity versus time as described in Methods.
the site-specific mutations resulted in the formation of an aggregation-prone folding intermediate (Chrunyk et al., 1993). We can speculate on how our specific mutations could be altering the folding process in ADA.

Unfolding the apoenzymes of the two Asp 296 mutants shows that apo D296A is twice as stable than apo D296N, as predicted from the higher yields of the alanine mutant. Little is known on the role of metal cofactors in the folding and assembly of proteins. One idea is that the metal does not get incorporated into the ribosome-bound polypeptide until after the protein is fully formed (Vallee et al, 1983). In this view, if a mutation enhances the productive folding of the apoenzyme, it will also enhance the productive folding of the holoenzyme, assuming sufficient levels of the cofactor are present in the cell. Both the asparagine, and, more so, the alanine substitution seem to belong to this class of mutations, because they cause greater amounts of the fully folded mutant ADA to be expressed compared to wild-type. In addition, the in vitro data suggests that an alanine or an asparagine at position 296 confers greater resistance to protein denaturation. Asp 296 is lodged in a β-sheet (β8) of the murine α/β barrel. According to the Chou and Fasman rules, both asparagine and alanine are indifferent β-sheet formers, but aspartates are β-sheet breakers. Therefore, simply based on secondary structure formation propensities, either of the substitutions on position 296 is preferable to the wild-type aspartate. Moreover, inspection of the three-dimensional structure around residue 296 reveals that three Phe residues are in van der Waals distance and that the environment as a whole is highly hydrophobic. It is tempting to speculate that the more apolar alanine substitution results in better packing of the local side chains than the original aspartate. The increased hydrophobicity of this region renders it more resistant to denaturation and may also facilitate its collapse in vivo, resulting in more
productive folding and higher yields of folded protein.

The different behavior of mutations at the highly conserved cysteine 262 is extremely interesting. Both are very poorly expressed from bacteria, yet they are not the least stable of our mutants. C262A is two times less stable than the wild-type, whereas the serine mutant is six times less stable. Residue 262 is located in a coil, between an $\alpha$-helix and a $\beta$-sheet; thus, secondary structure preferences do not come into play in this case. Other workers have observed that a C262A mutation obstructs the in vitro refolding capacity of urea-denatured human ADA (Bhaumik et al., 1993). Clearly, Cys 262 mutations appear to affect the folding pathway, possibly by causing an intermediate to aggregate. In an attempt to ameliorate the folding of these mutants within the cell, chaperonins were overexpressed, and inclusion body formation was evaluated. No improvement was seen in the amount of soluble C262A or C262S as a result of chaperonin overexpression. Thus, the role of Cys 262 in the folding of ADA is clearly important, but still remains to be elucidated.

The His 238 mutants were by far the most prone to denaturation in vitro. Once again, their yield from *E. coli* does not match the observed denaturation rate: H238A, which unfolds 27 times faster than wild-type ADA, is produced in high yields (70 mg on average). Moreover, H238E, which is unfolds as fast as H238R, yields 50 mg on average compared to H238R's 20 mg. The only mutant for which low expression could be correlated to high in vitro denaturation rate is H238R. We already know from spectroscopic studies that there are differences in the tertiary structure of this mutant localized around the tryptophan residues. Thus, it is possible that the His 238-> Arg mutation results in a native protein that is less stable and for which the equilibrium is shifted towards an unfolded species that aggregates within the cell.

The most stable His 238 mutant is H238A, which denatures 3-fold more
slowly than either H238E or H238R. Neither the glutamate at position 238 nor the alanine appear to alter the in vivo folding pathway drastically, but they both result in loss of stability in the mutant molecule as a whole. That glutamate does so is not surprising, since His 238 is close to Glu 217 and Glu 260, as well as to Asp 295. This close proximity of acidic residues could compromise the H238E structure, resulting in faster denaturation rates. Finally, the D295E mutant is not shunted in inclusion bodies, suggesting that the mutation does not alter ADA folding. However, its stability is compromised as a result of the glutamate substitution, and most of its activity becomes dissipated during purification. Not surprisingly, the purified protein unfolds 6 times faster than wild-type under denaturing conditions. In view of the coordination of this residue to the zinc cofactor, this mutant's decreased stability is not unexpected. In all four samples, removal of zinc from ADA resulted in faster rates of unfolding. This result implies that the metal cofactor is instrumental in keeping the structure of the molecule intact, through stabilization of its ligands. Mutations such as D296A and D296N not only render the apoenzyme more resistant to denaturation, but also stabilize the holoenzyme compared to wild-type ADA. However, apo D295E now unfolds as fast as wild-type apoADA does. In other words, apo D295E has the same denaturation propensity as apo wild-type, yet apo D295E + zinc denatures faster than holo wild-type. This result suggests that zinc may not be held as tightly in this mutant as it is in wild-type, causing the mutant to unfold at faster rates than the wild-type. More studies need to be done on the kinetics of zinc loss during metal chelation for the two enzymes. In fact, the kinetics of zinc loss should be determined for all mutant holoenzymes, in order to evaluate the correlation of kinetic stability to zinc binding.
Figure VI.17 Proposed Model of Effects of Site-Directed Mutants on Folding and Stability of ADA. Mutations are designated by a "x". Mutants have been grouped according to their proposed effects on ADA folding.
5.3 Conclusions on Effects of Mutations on Protein Folding and Stability

Figure VI.17 summarizes the effects of groups of mutants on the steps comprising the folding pathway. One possibility is not accounted for in this model: certain of the mutations could result in ADA proteins which are more susceptible to proteolytic degradation and become degraded in the cell. The schematic assumes that, as the protein is translated *in vivo*, it is largely unfolded (U) and then becomes folded into the apoenzyme structure F. This process is governed by the equilibrium constant $K_a$. Zinc binds the folded apoenzyme, to form native holoADA (N). The equilibrium constant for this process is $K_z$. The folded holoenzyme co-exists in equilibrium with its unfolded form and the free cofactor.

Mutations such as D296A and D296N accelerate the folding of the apoenzyme ($K_a$ decreased), do not affect the $K_z$, and shift the equilibrium of the third step towards the folded, native holoenzyme ($K_U$ increased). Thus, the native, folded enzyme concentration is increased within the cell.

Mutations such as C262A, C262S, or H238R, increase $K_a$ (thus inhibiting the folding of the apoenzyme), do not affect the association of metal, and decrease $K_U$, thus promoting the unfolding of the holoenzyme. This situation results in very little folded holoenzyme to be produced.

H238A and H238E may not affect the first two processes, but they destabilize the fully folded holoenzyme. D295E, acts similarly, but could also slow down the association of metal with folded apoenzyme ($K_z$ affected), resulting in low amounts of the holoenzyme to be produced. The holoenzyme could be destabilized in this case due to less optimal zinc binding. Preparation of the apoenzymes of these groups of site-directed mutants and examination of zinc binding and folding parameters would help evaluate this model.
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