Site-Directed Mutagenesis of Active Site Glutamate-217 in Mouse Adenosine Deaminase†

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ABSTRACT: Mouse adenosine deaminase (ADA) contains an active site glutamate residue at position-217 that is highly conserved in other adenosine and AMP deaminases. Previous research has suggested that proton donation to N-1 of the adenosine ring occurs prior to catalysis and supports the mechanism as proceeding via formation of a tetrahedral intermediate at C-6 of adenosine. The proposed catalytic mechanism of ADA based on the recent elucidations of the crystal structure of this enzyme with transition- and ground-state analogs hypothesized that Glu^{217} was involved in this proton donation step [Wilson, D. K., Rudolph, F. B., & Quiocho, F. A. (1991) *Science 252*, 1278−1284; Wilson, D. K., & Quiocho, F. A. (1993) *Biochemistry 32*, 1689−1693]. Site-directed mutagenesis of the equivalent glutamate in human ADA resulted in a dramatic loss of enzyme activity [Bhaumik, D., Medin, J., Gathy, K., & Coleman, M. (1993) *J. Biol. Chem. 268*, 5464−5470]. To further study the importance of this residue, site-directed mutagenesis was used to create mouse ADA mutants. Glu^{217} was mutated to Asp, Gly, Gln, and Ser, and all mutants were successfully expressed and purified. Circular dichroism and zinc analysis showed no significant changes in secondary structure or zinc content, respectively, compared to the native protein. The mutants showed only a slight variation in K_m but dramatically reduced k_{cat}, less than 0.2% of wild-type activity. UV difference and ^{13}C NMR spectra conclusively demonstrated the failure of any of these mutants to hydrate purine riboside, a reaction carried out by the wild-type enzyme that results in formation of an enzyme−inhibitor complex. Surprisingly, K_i values for binding of the inhibitor to the mutants and to wild-type protein are similar, irrespective of whether the inhibitor is hydrated upon binding. These data confirm the importance of Glu^{217} in catalysis as suggested by the crystal structure of mouse ADA.

Adenosine deaminase (ADA, EC 3.5.4.4)[1] is a monomeric zinc metalloenzyme which catalyzes the irreversible deamination of adenosine and 2′-deoxyadenosine to their respective inosine derivatives. ADA is found in virtually all vertebrate tissues and plays a critical role in controlling the effects of these substrates in a variety of systems and includes immunological (Kredich & Hershfield, 1989), neurological (Senba et al., 1987), and vascular (Smits et al., 1987) roles. The phenotype of ADA deficiency in humans has focused attention on the essential role of the enzyme in immune function (Kredich & Hershfield, 1989; Parkman et al., 1975). ADA plays a central role in the development of the lymphoid system as genetic absence of the enzyme leads to severe combined immunodeficiency disease (SCID). In humans ADA levels are highest in the thymus, presumably reflecting the critical role of the enzyme in T-cell development (Chinsky et al., 1989; Kizaki et al., 1973). Comparable levels are present in the duodenum (Aronow et al., 1989). However, SCID patients have no obvious gastrointestinal tract abnormalities (Bhaumik et al., 1993). In addition to its role in SCID, ADA abnormalities have also been reported in other diseases of the immune system, including certain leukemias (Murray et al., 1985), acquired immune deficiency syndrome, anemia, and various lymphomas (Murray et al., 1985; Wilson et al., 1991; Renouf et al., 1989; Kanno et al., 1988). Lower than normal ADA levels have been noted in cancerous human laryngeal tissues (Durak et al., 1993). In mice, elevated levels of ADA exist in the alimentary tract (Chinsky et al., 1990) and in decidual cells of the developing fetal−maternal interface (Knudsen et al., 1989); these levels are not matched by other enzymes of the purine metabolic pathway (Mohamedali et al., 1993), suggesting unique roles for ADA relating to the growth rate of cells, embryo implantation (Knudsen et al., 1989, 1991), and other yet undetermined functions.

In addition to its deaminating activity, ADA will catalyze the hydrolysis of several 6-substituted purine derivatives including a number of clinically important antimetabolites (Zielke & Suelter, 1971). At least 80 compounds are known to bind to ADA, some as substrates but most as inhibitors

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[1] Abbreviations: ADA, adenosine deaminase; SCID, severe combined immunodeficiency disease; PMSF, phenylmethylsulfonyl fluoride; IPTG, isopropyl β-D-thiogalactopyranoside; PEG, polyethylene glycol; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; ssDNA, single-stranded DNA; CD, circular dichroism; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; HDPR, 6-hydroxyl-1,6-dihydropurine riboside; PR, purine riboside.

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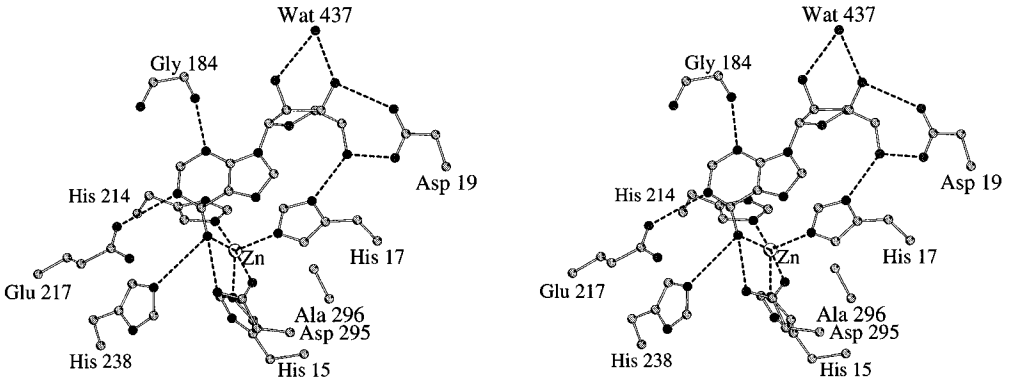

FIGURE 1: Stereoscopic view of the active site of mouse adenosine deaminase bound with 6-hydroxyl-1,6-dihydropurine riboside (HDPR). Dashed lines indicate non-covalent interactions between neighboring atoms. Atoms are coded as follows: carbon, lighter circles; nitrogen, black circles; oxygen, gray circles. Figure courtesy of Dave Wilson (Baylor College of Medicine).

that show different types of inhibition (Wilson & Quiocho, 1993); some of these compounds have been instrumental in postulating a reaction mechanism for ADA. Phosphorylated nucleosides are not substrates or inhibitors (Zielke & Suelter, 1971). Understanding the catalytic mechanism of ADA is of special interest because analogs of adenosine with chemotherapeutic potential (Agarwal, 1982) as well as drugs directed against other enzymes are degraded by ADA. These drugs could be modified to have a longer pharmacological half-life if it were understood how ADA interacts with these compounds.

The reaction catalyzed by ADA appears to be encounter limited (Kurz et al., 1992) with a k_{cat} of 375 s^{-1} and a k_{cat}/K_m of 1.4×10^7 M^{-1} s^{-1}. The rate enhancement of approximately 2×10^{12} (Frick et al., 1987) puts this enzyme among the more efficient that have been described (Radzicka & Wolfenden, 1995). Support for the enzyme mechanism proceeding via formation of a tetrahedral intermediate has come from several studies (Evans & Wolfenden, 1973; Wolfenden et al., 1969; Kurz & Frieden, 1983). ^{13}C NMR studies with purine riboside showed that hybridization of C-6 changes from sp^2 to sp^3 in the binary complex (Kurz & Frieden, 1987) with the nucleophile at C-6 being either a water molecule or a thiol at the enzyme's active site. Subsequent NMR and UV absorption maxima (Jones et al., 1989) gave evidence that the substrate was bound as a 1,6 covalent hydrate. The elucidation of the crystal structure of mouse ADA bound to a nearly ideal transition-state analog, 6-hydroxyl-1,6-dihydropurine riboside (HDPR) (Wilson et al., 1991), confirmed the presence of the covalent hydrate at C-6 of the substrate and revealed the existence of a zinc cofactor bound in the active site (Figure 1). More recently, the crystal structure of mouse ADA bound to 1-deazaadenosine, a ground-state analog, indicated the presence of a zinc-activated water molecule in the active site (Wilson & Quiocho, 1993).

The proposed mechanism of catalysis by this enzyme (Wilson et al., 1991; Wilson & Quiocho, 1993) calls for Glu^{217}, ideally located coplanar with the purine ring, to donate a proton to N-1 of adenosine, thereby reducing the N-1−C-6 double-bond character. The C-6 is thus susceptible to nucleophilic attack by the zinc-bound hydroxide (whose proton has been abstracted by His^{238}) and transformation from sp^2 to sp^3 in the tetrahedral adduct. Asp^{295} remains coordinated to the zinc and is thought to hydrogen bond to the activated water or hydroxide and optimize the geometry for the hydroxide addition to the C-6 of adenosine. Thus, the tetrahedral intermediate is formed. Formation rather than decay of the tetrahedral intermediate is believed to be the most difficult (possibly rate-determining) step (Kurz & Frieden, 1987). The proton source for the leaving $-NH_2$ group is far from certain since this leaving group would be pushed into an essentially hydrophobic environment; the proton may come from the incipient hydroxyl group, shuttled by the Asp^{295} (Wilson & Quiocho, 1994). The precise chirality of the chemical reaction is dictated by the favorable geometry together with the location of the zinc and Asp^{295} in the B-face side of the purine ring as hydroxide attack occurs on this side only.

The goal of this study was to elucidate the role of Glu^{217} in mouse ADA. In the X-ray structure of the enzyme complex with a tetrahedral intermediate analog, the carboxylic acid side chain of Glu^{217} is found to be hydrogen bonded to N-1 of the analog (Wilson et al., 1991). Presumably the carboxylic side chain of Glu^{217} is the proton donor required in formation of the actual tetrahedral intermediate. Additionally, it has been proposed that proton donation may precede formation of the tetrahedral intermediate, facilitating the attack of the hydroxide nucleophile on C-6. The existence of a pre-protonated intermediate at N-1 has remained attractive on theoretical grounds (Orozco et al., 1990a) and has been proposed by several researchers (Kurz et al., 1992; Weiss et al., 1987). It is now clear that if such an intermediate exists, it must be low in concentration and transient in nature in the case of the wild-type enzyme (Kurz et al., 1992). It is reasonable to examine mutants in which the putative proton donor residue has been changed in order to determine if a pre-protonated intermediate can be detected. Site-directed mutagenesis of Glu^{217} to alanine in human ADA resulted in a 10 000-fold decrease in substrate turnover, indicating that this residue plays a vital role in catalysis (Bhaumik et al., 1993). However, no further characterization was done in that study. We also chose site-directed mutagenesis to understand the role of this active site residue. This method was used to change Glu^{217} to Asp, Gly, Gln, and Ser. The mutants were purified using a novel purification procedure and characterized in detail.

MATERIALS AND METHODS

Materials. Phenylmethylsulfonyl fluoride (PMSF), xanthine oxidase, purine nucleoside phosphorylase, adenosine, ammonium sulfate, ampicillin, isopropyl β-D-thiogalactopy-

ranoside (IPTG), polyethylene glycol (PEG), and purine riboside were purchased from Sigma. 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) was obtained from Calbiochem, imidazole from Research Organics, bactotryptone from Difco, yeast extract from Becton Dickinson, and dithiothreitol from Boehringer Mannheim. The DEAE-cellulose column matrix was DE-52, obtained from Whatman. Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from GIBCO. Sequenase DNA sequencing kits were purchased from United States Biochemicals, and mutagenesis kits were purchased from Bio-Rad. [α-^{35}S]dATP was from DuPont. Oligonucleotide primers used in constructing mutations and sequencing were synthesized on a Biosearch 8600 DNA synthesizer. Restriction endonucleases were from Promega.

Bacterial Strains and Vectors. The *Escherichia coli* strains used for plasmid propagation were CJ236, BW313, BB4, and SØ3834 (Chang et al., 1991). The phagemid expression vector pRC4 (Chang, 1992) was used for site-directed mutagenesis of the mouse adenosine deaminase cDNA. All site-specific mutant forms of adenosine deaminase were developed with this construct. Both SØ3834 (an ADA-deficient *E. coli* strain) and pRC4 were generously provided by the laboratory of Dr. Rod Kellems, Baylor College of Medicine, Houston, TX. pRC4 contains a replication origin from the single-stranded phage f1 so that single-stranded phagemid DNA (ssDNA) can be isolated and used as a template for oligonucleotide primers that contain the mutation of choice. Superinfection with IR1 helper phage yields ssDNA.

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out by the method of Kunkel et al. (1987). pRC4 was transformed into *E. coli* strains CJ236 or BW313 (dut$^-$ ung$^-$). Single-stranded uracil containing pRC4 was isolated and annealed to synthetic phosphorylated mutagenic oligomers. Sequenase was used to synthesize the second strand of DNA. After ligation the reaction product was transformed into BB4 cells (dut$^+$ ung$^+$). The presence of the desired mutation was confirmed by dideoxy sequencing (Sanger et al., 1977) of the entire ADA cDNA in both directions.

Expression of Recombinant Wild-Type and Mutant Murine Adenosine Deaminase in E. coli SØ3834 Cells. The wild-type adenosine deaminase and mutants E217D, E217G, E217Q, and E217S were overexpressed in SØ3834 cells by IPTG induction. A 4.5 mL overnight culture was added to 6 L of Superbroth medium (32 g of bactotryptone/L, 20 g of yeast extract/L, 5 g of sodium chloride/L) also containing 1 mM zinc and 60 μg of ampicillin/mL and grown for 20 h. IPTG was added 10 h after seeding. The cells (25−35 grams) were harvested and frozen at −20 °C.

Purification of Recombinant Proteins. All steps were carried out at 4 °C except HPLC. Wild-type and mutant proteins were purified using the following protocol: Cells were suspended in 80 mL of DEAE buffer (50 mM imidazole, pH 6.8, 5% glycerol, 10 μM zinc, 1 mM dithiothreitol). Protease inhibitors PMSF (1 mM) and AEBSF (0.5 mM) were also present. Cell lysis was performed using a French pressure cell (SLM Instruments). The homogenate was centrifuged at 100 000*g* for 1 h (L5−50 ultracentrifuge, Beckman 55.2 rotor), and the supernatant was loaded onto a DEAE-cellulose column (5 × 20 cm) and eluted with a gradient of DEAE buffer containing 0.4 M sodium chloride. Fractions were collected (10 mL/tube) at a flow rate of 60 mL/h. Fractions containing ADA were determined by spectrophotometric assays and dot blots (see next section). Fifteen tubes of the eluent with the most ADA activity were pooled and brought to 60% ammonium sulfate saturation. The supernatant obtained following centrifugation was subjected to a second cycle of ammonium sulfate fractionation (80%). The pellet obtained following centrifugation was loaded onto a hydrophobic interaction HPLC column (polypropyl A, 5 μM, Custom LC, Inc.) by suspending it in 70% HIC buffer containing 2 M ammonium sulfate and eluted with HIC buffer (20 mM potassium phosphate, pH 7.0, 10 μM zinc, 5% glycerol, 1 mM dithiothreitol). ADA was concentrated overnight by dialysis against 20% PEG (M_r = 15 000−20 000) in AX300 buffer (20 mM imidazole, pH 7.0, 10 μM zinc, and 1 mM dithiothreitol). The precipitate was suspended in 75% AX300 buffer, loaded on a silica-based polyethyleneimine HPLC column (Synchropak AX300 250 × 10 mm, Synchrom, Inc.), and eluted with AX300 buffer containing 0.5 M sodium chloride. The purified protein was equilibrated with Tris-Zn buffer (10 mM Tris-HCl, pH 7.0, 10 μM zinc, 50% glycerol), gassed with argon, and stored at −20 °C.

SDS−Polyacrylamide Gel Electrophoresis, Dot Blots, Western Blots, and Zymograms. SDS−polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) on a minigel apparatus (from Bio-Rad). Proteins were visualized by staining with Coomassie Brilliant Blue.

DEAE fractions containing ADA mutants E217G and E217Q were identified by dot blots as their activity was too low to be measured spectrophotometrically. Dot blots and Western blots (both performed on PVDF transfer membranes) were analyzed with goat anti-mouse adenosine deaminase polyclonal antibody (1:7500 dilution). The secondary antibody was rabbit anti-goat IgG alkaline phosphatase conjugate (Sigma Immunochemicals). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard and reagents from Bio-Rad.

Dot blots were prepared by placing 1 μL of every other collected fraction on an activated PVDF transfer membrane. Excess protein binding sites were blocked for 1 h with 1% low fat milk in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Both primary and secondary antibodies were incubated for 30 min followed by three 5 min washes of the membrane with TBST buffer. The staining solution was 33 μL of NBT and 17 μL of BCIP in 10 mL of AP buffer (1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$).

Zymogram analysis was performed according to the method of Yeung et al. (1983). This method was used to identify adenosine deaminase activity after electrophoretic separation of fractions under non-denaturing conditions on an Authentifilm thin agarose gel (Innovative Chemistry) in a chilled electrophoresis chamber (150 V, 45 min).

Analysis of Secondary Structure of the Wild-Type and Mutant Proteins. Circular dichroism (CD) spectroscopy, a technique sensitive to structural perturbations in proteins, was used to compare the secondary structure of wild-type to mutant ADA proteins. Proteins were dialyzed against 20 mM HEPES buffer, pH 7.0, and their concentrations were adjusted to approximately 1 mg/mL. CD was measured with an AVIV spectrometer model 62DS at the laboratory of Dr.

James Lee, UTMB-Galveston, TX. Cuvettes of 1 cm path length were used for the near-UV CD region (320−240 nm) and 0.1 cm for the far-UV region (260−200 nm). For spectra, three scans were obtained for each sample and averaged. Each spectrum was recorded in 0.5 nm wavelength increments. Each spectrum was normalized for protein concentration and the observed ellipticity, θ, was background-corrected against the spectrum obtained for the dialysis buffer.

Analysis of Zinc Content. Flame atomic absorption spectrometry was used for zinc analysis. The absorbance peak was measured at 213.9 nm on an atomic absorption spectrophotometer (Perkin-Elmer model 2380) at the laboratory of Dr. David Giedroc, Texas A&M University, College Station, TX. Protein samples were extensively dialyzed in metal-free dialysis tubing (Auld, 1988) against 20 mM HEPES buffer, pH 7.0, that had been passed through a Chelex-100 column. All protein samples were between 2 and 6 μM concentration. Two determinations were made for each sample and averaged. No zinc was detected in the undialyzed duffer. The concentration of residual zinc in the dialyzed buffer ($\sim$1 μM) was subtracted from that in the unknown protein samples.

As a control, specific activities of two of the samples were measured prior to and following overnight dialysis. The dialysis procedure was the same as for the samples subjected to zinc analysis. The samples were assayed for activity as outlined in the next section. Protein concentration and specific activities were evaluated before and after dialysis and compared to protein/zinc ratios.

Measurement of Kinetic Parameters (K_m, V_{max}, k_{cat}, and K_i). Adenosine deaminase activity was measured in a Cary 118 spectrophotometer by monitoring the increase in absorbance at 235 nm resulting from the formation of inosine from adenosine. Potassium phosphate (50 mM, pH 7.2) was used as the reaction buffer. One unit of ADA activity was defined as the amount of enzyme that produced 1 μmol of inosine/min. For each protein, kinetic measurements were done at a series of seven concentrations of adenosine ranging from 0.5K_m to 5K_m. The concentration of enzyme ranged from 0.14 nM for the wild-type to 0.2 μM for the least active mutant protein. K_m and V_{max} were determined using the Lineweaver−Burk method with a fourth-power weighting function on an enzyme kinetics program (EnzymeKinetics, Trinity Software). The k_{cat} was calculated from the equation $V_{max} = k_{cat}[E_0]$.

In order to further probe to what extent the structure of the active site pocket had been conserved upon mutation, the inhibition constant for purine riboside was determined. Purine riboside (300 μM) was prepared as stock. Kinetic measurements were done as described above using a concentration of purine riboside that inhibited maximal activity of the enzyme by 10%−20%. A second set using twice this inhibitor concentration was also recorded. Kinetic parameters were determined as outlined above.

UV Difference Spectroscopy. UV difference spectroscopy was performed according to the method of Kurz et al. (1983) in the laboratory of Dr. John Olson, Rice University, Houston, TX. Samples were placed in the sample and reference turrets of a UV-2101PC scanning spectrophotometer (Shimadzu Scientific Instruments) with a thermostated cell compartment. After temperature equilibration, the base line was adjusted and recorded. The solutions in the sample cell were mixed, and the difference spectrum was recorded. Finally, the solutions in the reference cell were mixed, and the spectrum was recorded again to check the base line. The interaction of protein with ligand can be obtained directly from the second spectrum.

Solvent Difference Spectroscopy. The usefulness of solvent difference spectroscopy has been demonstrated in understanding the interaction of a ligand with ADA in the event the ligand is susceptible to unexpected environmental factors within the active site (Kurz et al., 1992). In order to have a more complete picture of the interaction of purine riboside in the active site of ADA, we measured the effect of various solvents on the spectrum of purine riboside. The effect of the solvent on the spectrum of purine riboside was measured by difference spectra of this ligand with solvents of differing polarity. The solvents used were acetonitrile, ethanol, isopropyl alcohol, *tert*-butyl alcohol, and dimethyl sulfoxide. The spectra were obtained as outlined above.

NMR Spectroscopy. ^{13}C spectra were obtained at 150.7 MHz using a Varian Unity 600 spectrometer equipped with a 5 mm multinuclear probe. Proton-decoupled spectra were obtained using Waltz decoupling. The temperature of the sample was 10 °C. The sample buffer was 50 mM potassium phosphate, pH 7.5, and included 25% D_2O (for internal lock) and 0.15 M acetonitrile (as internal chemical shift standard). The cyano resonance of the standard was assigned the value of 118.9 ppm. Actual enzyme concentrations were measured by active site titration with deoxycoformycin as previously described (Kurz et al., 1985), since the use of phosphate buffer for these experiments resulted in some loss of active enzyme as a consequence of zinc loss (see below).

Dependence of k_{cat} and K_m on pH. Investigation of dependence of catalytic activity on pH can provide useful information about the ionization groups involved in catalysis. Such studies with mutants proteins may indicate the pH range over which the mutated residue contributes most efficiently to catalysis. To this end, values for K_m and k_{cat} for the deamination of adenosine by the wild-type and Glu^{217} mutant adenosine deaminases were determined at a series of different pH values from pH 4 to pH 10. pH buffers were 50 mM sodium acetate (pH 4 and 5.5), 50 mM potassium phosphate (pH 7), and 50 mM glycine (pH 8.5 and 10). All buffers were maintained at the same ionic strength ($I = 0.1$) with potassium sulfate. Assays were performed according to the method outlined above, with adenosine as the substrate.

RESULTS AND DISCUSSION

In order to determine the catalytic importance of the size and hydrophobicity of Glu^{217} in the active site of ADA, we mutated this residue to Asp, Gly, Gln, and Ser. Hirschhorn et al. (1992) have described the onset of ADA$^-$ SCID in a patient who carried a large deletion of the ADA gene on one allele and a Glu^{217} missense mutation (to lysine) on the other. The identical residue in human ADA was recently mutated to alanine and shown to be important for catalysis without further characterization (Bhaumik et al., 1993). Proton addition to N-1 is necessary to form the tetrahedral intermediate, and the X-ray structure showing the proximity of Glu^{217} to the N-1 of the bound tetrahedral analog implicates this amino acid in that role. Asp was chosen as it has a similar pK_a to Glu but is shorter by one carbon atom, thus potentially reducing the efficiency of hydrogen donation

Table 1: Purification of Recombinant Mouse Adenosine Deaminase from *E. coli* SØ3834 Using an Original Purification Method[a]

step	vol. (mL)	enzyme activity (units/mL)	protein (mg/mL)	total units (μmol/min)	specific activity (μmol/min/mg)	purification factor	% yield
crude homogenate	96	1954	105	187 600	18.6		100
DEAE-cellulose	168	809	5.3	135 900	151.5	8.1	72
ammonium sulfate fractionation	12	11 931	43.8	143 172	272	14.6	76
polypropyl A (HPLC)	24.8	4114	14.2	103 673	290	15.6	55
PEG dialysate	6.6	13 543	44.3	89 384	306	16.5	48
polyethyleneimine (HPLC)	7.8	9257	26.3	72 204	352	18.9	38

[a] The method did not use affinity chromatography because mutations were created within the active site. Instead, we incorporated the use of two HPLC methods. We observed extremely high expression of the recombinant protein (up to 5% of total soluble protein) with no formation of inclusion bodies. Similar yields were obtained for mutant ADAs. Protein concentrations were measured by the method of Bradford (1976). One unit of adenosine deaminase is defined as the amount of enzyme that produced 1 μmol of inosine per minute. The protocol for purification is explained in detail in the text.

to N-1 of the substrate. In contrast, Gln is the same size as Glu and can form hydrogen bonds but, unlike Asp, is unable to act as a proton donor. Ser is even smaller, and its γ-OH has a much higher pK_a than Glu. Gly was chosen to completely eliminate any possible interaction with N-1. The purification protocol used was devised to purposely exclude substrate affinity methods and to achieve a high degree of purity by use of HPLC. The four amino acid substitutions at position-217 were characterized for substrate and inhibitor binding, secondary structure conformation, metal binding, enzyme−inhibitor complex formation, and active site pK_a. The results were assessed and correlated with mechanistic models.

Mutagenesis, Expression, and Purification of ADA. The phagemid expression vector pRC4 was mutated by the appropriate oligomers, and the recombinant plasmids were identified by restriction enzyme analysis and confirmed by sequencing. The plasmids were then transformed into SØ3834, an ADA-deficient *E. coli* strain, and ADA protein expression was verified using a Western blot. All transformants produced roughly equivalent amounts of ADA. For large-scale protein purification the plasmid was induced by IPTG. Consistently high amounts of all mutant and wild-type proteins were expressed by this method (around 5% of soluble protein), and the proteins were verified by enzyme assays, SDS−PAGE gels, and immunoblots. Electrophoretic mobility of wild-type and mutant ADAs were identical as judged by zymogram analysis (data not shown). For E217G and E217Q, dot blots were used in the initial stages of purification as the mutants were not detectable by spectrophotometric means until separation by the hydrophobic column. All proteins purified are stable although some degradation product is observed with Western blots run at the end of the purification procedure.

Both wild-type and mutant ADAs were purified by identical protocols. The procedure including ion exchange and hydrophobic columns was favored over affinity methods due to the unpredictability of binding affinity of each of the active site mutants. Consequently, methods relying on the binding property of the active site were not used. The retardation of all proteins throughout the purification process was similar. Table 1 shows the typical degree of purification obtained at each step. For wild-type, purification was typically about 20-fold and the yield was 30%−40%. Zinc and dithiothreitol were maintained throughout the purification process; we observed varying degrees of specific activity, which was dependent on the presence of zinc, and retardation on the ion exchange HPLC column, which was dependent on the oxidation state of the enzyme (unpublished). The specific activity values obtained for pure wild-type ADA were consistently higher than values presented in the literature using other methods and comparable to those that used affinity columns. Thus, this method of purification of ADA is an excellent substitute for other methods and is the method recommended for purification of ADA subjected to alterations in the active site.

Analysis of Secondary Structure of ADA by Circular Dichroism. The crystal structure of mouse ADA determined the protein to be composed of an eight-stranded α/β motif, with five additional α helices, and the active site located at the β barrel COOH terminal end (Wilson et al., 1991). Integrity of the secondary structure of the purified proteins was examined by CD spectroscopy (data not shown). Proteins were dialyzed in 20 mM HEPES buffer, pH 7.0, and their concentrations were adjusted to approximately 1 mg/mL. All proteins have the double minimum at 222 and 210 nm, characteristic for α helical content (Adler et al., 1973). E217Q exhibits a less intense minimum at 210 nm than the other proteins. The decrease in ellipticity at these wavelengths indicates a decrease in the number of amino acids in an α helical conformation. It should be noted, however, that data obtained for this mutant do not suggest that this change in α helical content in any way disturbed the active site of the enzyme.

Analysis of Zinc Content. The purified recombinant proteins were analyzed for zinc content with the use of a flame atomic absorption spectrometer. Proteins (stored in Tris-Zn buffer) were dialyzed extensively in 20 mM HEPES buffer, pH 7.0, that had been run through a Chelex-100 column. All samples are stored in 10 mM Tris-Zn buffer, pH 7.0, with 10 μM zinc because of loss of activity of the enzyme when stored in other buffers, such as potassium phosphate, or in the absence of zinc (unpublished data). The dialysis buffer was changed three times over an 18 h period before samples were adjusted to 2−6 μM concentration and analyzed for zinc content. Dialysis buffers were also subjected to zinc analysis. The initial buffer showed higher than expected amounts of zinc, whereas the final buffer had negligible amounts. Table 2 shows the results. Even though the ratios of zinc to enzyme are not 1:1 as expected, the ratios obtained for the mutants are very close to those obtained for wild-type ADA. To check if the discrepancy in the protein:zinc ratio was due to the dialysis procedure itself, specific activities of wild-type mADA and the E217D mutant were tested before and after dialysis (Table 3). The result clearly suggests that the observed protein to zinc ratios are due to loss of zinc from the enzyme during dialysis. Thus, the proteins are purified with an equimolar concentration of

Table 2: Zinc Analysis of Mouse ADA and Glu^{217} Mutants[a]

protein	Zn^{2+} (mol/mol of protein)
wild-type ADA	0.7 ± 0.2
E217D	0.6 ± 0.1
E217G	0.6 ± 0.1
E217Q	0.5 ± 0.1
E217S	0.6 ± 0.1

[a] Samples were dialyzed overnight in three sets of metal-free 20 mM HEPES buffer, pH 7.0. Protein concentration was adjusted to 2−6 μM before zinc analysis using flame atomic absorption spectroscopy. Two sets of data were recorded for each protein. The levels of zinc are below the 1:1 ratio expected with respect to protein. However, the difference in the ratio is similar for both wild-type and mutant proteins.

Table 3: Ratio of Specific Activity of Mouse ADA and E217D before and after Overnight Dialysis[a]

protein	specific activity before dialysis	specific activity after dialysis	ratio (before:after)
wild-type ADA	280	190	1:0.7
E217D	0.42	0.24	1:0.6

[a] Similar conditions to those in Table 2 were used. The data is an average of two samples. The proteins lost 30%−40% of their activity during dialysis, similar to the difference in protein:zinc ratios observed. The unexpectedly low stoichiometric levels of zinc to protein are thus due to zinc lost from the enzyme during dialysis. The ratio is not a reflection of the actual zinc content upon purification of the proteins.

zinc. On the basis of the similarity of protein to zinc ratios of wild-type and mutant ADAs, the mutation of Glu^{217} to Asp, Gly, Gln, and Ser apparently has little or no effect on the affinity of zinc in the active site.

Kinetic Characterization of Wild-Type and E217 Mutant ADAs. Kinetic parameters for the purified enzymes were determined using adenosine as a substrate (Table 4) at pH 7.2. Activity of E217S was too low for kinetic analysis. K_m values obtained for the other E217 mutants are similar to the wild-type. However, k_{cat} is reduced dramatically in all cases, ranging from 700 times (E217D) to 3200 times (E217G) less than wild-type activity. Thus, Glu^{217} does not seem to interfere with substrate binding but plays a key role in catalysis. The K_m of E217Q is slightly higher than that for wild-type mADA and may reflect the effect of the amino group, which is modestly larger than the original hydroxyl group, on binding of the substrate. The very low catalytic efficiency of E217G confirms the importance of Glu^{217}. The rate of catalysis may reflect the spurious protonation of N-1 by a local water molecule. The similarity of the rate of this mutant with that of E217Q underscores the importance of proton donation to N-1, since glutamine cannot donate its hydrogen even though it can hydrogen bond with the substrate. X-ray structures of all four mutants (in progress) will be most useful.

Inhibitor Binding. In order to further probe the extent of conservation of the structure of the active site pocket upon mutation and also as a means of further probing the role of Glu^{217}, the inhibition constants for purine riboside with wild-type and Glu^{217} mutants was determined. The data are presented in Table 4. The inhibition constant (K_i) obtained for wild-type mouse ADA is similar to that obtained for ADAs of other species (Kurz et al., 1992). Purine riboside was found to inhibit competitively with respect to substrate with K_i's of the mutants similar to that of wild-type ADA. The rate of hydration of purine riboside in the active site of wild-type ADA has been shown to be very fast and is faster than the binding of the inhibitor to the enzyme (Kurz et al., 1992); unhydrated purine riboside enters the active site, and those molecules that become subsequently hydrated remain bound to the wild-type enzyme (Kurz & Frieden, 1987). Since only wild-type ADA hydrates purine riboside (see below), forming an analog of a reactive intermediate when bound in the active site, the identity of K_i's of purine riboside for the wild-type and mutant ADAs is unexpected and requires a remarkable cancellation of opposing forces.[2]

Difference Spectra. Difference spectra for the binding of purine riboside to wild-type and mutant adenosine deaminases were compared to the acid−neutral difference spectra for this inhibitor (Figure 2). The changes in the UV spectrum for purine riboside upon formation of the wild-type enzyme−inhibitor complex result from formation of the tetrahedral intermediate analog, the purine riboside hydrate, in the enzyme active site (Jones et al., 1989). Our data clearly show that this does not occur in any of the Glu^{217} mutant complexes. In addition, none of the complexes has difference spectra similar to that which results from simple protonation at N-1. Instead, a peak is observed at 260 nm which increases in intensity inversely with the polarity of the amino acid substituted at position-217. To understand the cause of the peak we obtained solvent difference spectra of purine riboside in different solvents. The spectra are characterized by peaks at approximately 270 and 255 nm, with the latter becoming more prominent in progressively apolar solvents (data not shown). Because the spectrum of E217G at 260 nm resembles that of purine riboside in dimethyl sulfoxide, the difference spectra results obtained for the mutants can most easily be explained by environmental differences in their active sites affecting the absorption spectrum of the bound inhibitor with no covalent bond changes taking place. The stability of purine riboside binding in the active sites should not be the cause since inhibition constants were found to be similar for all the proteins.

^{13}C NMR Spectra of Purine Riboside Complexes with Wild-Type ADA and E217D. The ^{13}C NMR spectra of purine riboside and its complexes with wild-type ADA and E217D are shown in Figure 3. The resonance at 118.9 ppm is the CN resonance of acetonitrile which is included in all samples as a chemical shift standard. The C-6 resonance of the free ligand is found at 148 ppm (spectrum A). In the complex with the wild-type enzyme (spectrum B), the bound ligand peak is found at 74.7 ppm with a line width of 54 Hz and is characteristic of the tetrahedral intermediate analog, the hydrate of purine riboside, which is formed in the active site (Wilson et al., 1991; Kurz & Frieden, 1987). This sample contains 2 equiv of inhibitor. The position of the bound resonance of the wild-type enzyme complex is unaffected by the presence of excess ligand. The small increase in line width of the free ligand resonance in the presence of the enzyme complex has been used to calculate the purine riboside dissociation rate constant (Kurz & Frieden, 1987).

The E217D sample in Spectrum C contains 1.2 equiv of purine riboside. The bound inhibitor resonates at 153 ppm with a line width of 100 Hz. The unbound ligand is found at its expected position with a line width of 25 Hz. The

[2] Similar cancellation of opposing forces has been proposed to occur with the mechanistically similar enzyme, cytidine deaminase (R. V. Wolfenden, personal communication).

Table 4: Compilation of Kinetic Data for Mouse ADA and the Glu^{217} Mutants[a]

protein	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	relative K_m/k_{cat} (wt/mutant)	$\Delta\Delta G^{\ddagger}_T$ ($kcal^{-1}$ mol^{-1})	K_i with purine riboside (μM)
wild-type ADA	19 ± 4	250 ± 30	13 ± 1	1	N/A	9 ± 2
E217D	19 ± 3	0.357 ± 0.006	0.019 ± 0.003	680	-3.9	10 ± 4
E217G	20 ± 5	0.075 ± 0.004	0.004 ± 0.001	3250	-4.8	7 ± 1
E217Q	31 ± 1	0.085 ± 0.006	0.0027 ± 0.0001	4800	-5.0	10 ± 5

[a] Activity of E217S was too low for measurement. All assays were performed in 50 mM potassium phosphate buffer, pH 7.2, 25 °C. The large change in k_{cat} along with negligible change in K_m reflects the role that Glu^{217} plays in catalysis rather than substrate binding. $\Delta\Delta G^{\ddagger}_T$ represents the change in the transitional-state binding energy, assuming that all structural perturbations are confined to the site of the mutation. The inhibition constant with purine riboside is similar for all proteins, further underscoring the minimal role this residue plays in substrate binding.

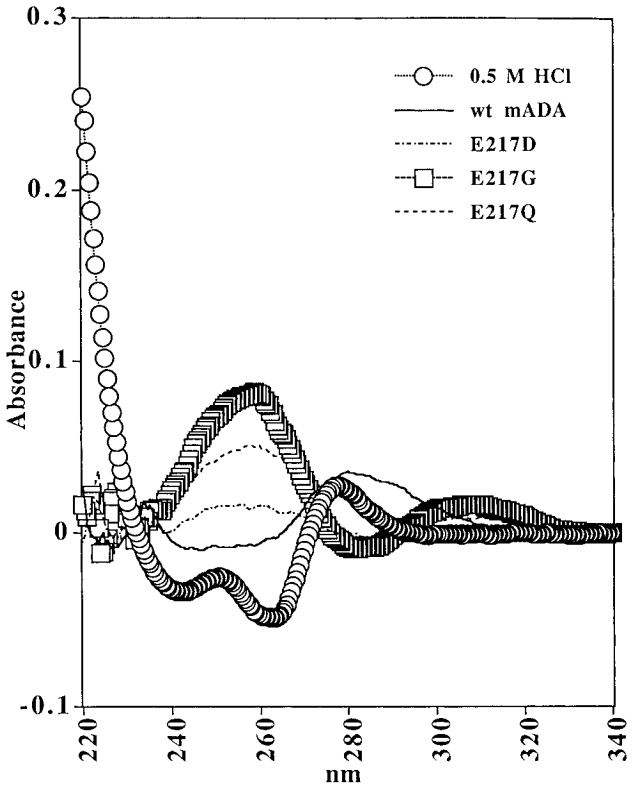

FIGURE 2: UV difference spectroscopy for purine riboside with wild-type mouse ADA, E217 mutants, and various solvents. (a) Experiments performed using 50 mM potassium phosphate/sulfate buffer (pH 7.0), $I = 0.15$, 20 °C. For protein spectra, final concentrations in sample cells 12.5 μM protein and 150 μM purine riboside (PR). For HCl-purine riboside spectra (pH 2.5), final concentration of purine riboside was 36 μM. Only wild-type ADA protonated N-1 of purine riboside.

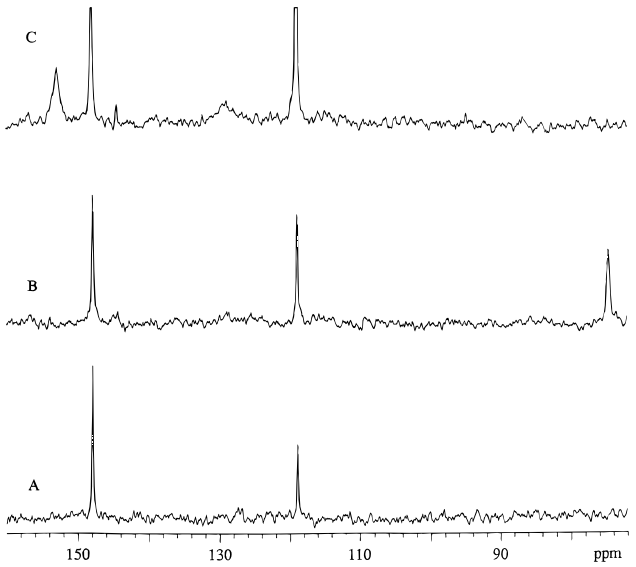

FIGURE 3: ^{13}C NMR spectra of purine riboside and its complexes with wild-type ADA and E217D. (A) 0.88 mM 6-^{13}C purine riboside, 491 transients; (B) 0.88 mM mADA containing 2 equiv of purine riboside, 1443 transients; (C) 0.44 mM E217D containing 1.2 equiv of purine riboside, 32 320 transients. Spectra were processed with a 30 Hz exponential filter.

line width of the methyl resonance of the acetonitrile standard is 1.2 Hz in this experiment. The line width of the free ligand resonance in the absence of enzyme has a comparable value. There is no resonance in the region of 75 ppm, indicating that no detectable hydrate is formed by the mutant. Increasing the amount of purine riboside to 2 equiv increases the bound line width to 200 Hz and decreased the free ligand resonance line width to 8 Hz (data not shown). No changes in chemical shift are observed.

The chemical shift of the ^{13}C-6 resonance of purine riboside bound to E217D is only 5 ppm *deshielded* from the free resonance in contrast to the 75 ppm *shielding* of the resonance which occurs upon hydration of the ligand in the active site of the wild-type enzyme. The mutant enzyme is seriously defective in its ability to form the tetrahedral intermediate analog. The NMR data are consistent with the low specific activity of this mutant which is 500−1000-fold less than wild-type. Protonation of purine riboside at N-1 shields the resonance by 8 ppm (Kurz & Frieden, 1987), again in the opposite direction of the changes we have observed in the E217D complex. A pre-protonated intermediate is not formed by this mutant.

The deshielding of the chemical shift of the bound resonance by 5 ppm is in the upper range of effects which may be simply interpreted as resulting from "environmental" factors. Solvent effects do not usually exceed 1−2 ppm. Variations in the chemical shifts of aromatic amino acid residues in proteins rarely are as large as 5 ppm. The combined proximity of several aromatic residues to the C-6 of the ligand may indeed be responsible for the observed effect. However, the chemical shift change may also reflect an active site environment induced decrease in electron density at C-6, an increase in positive charge there, and a consequential increased reactivity of C-6 toward the Zn−H_2O or Zn−OH^- nucleophile.

The line width changes observed in the free and bound resonances which accompany changes in the ligand concentration are consistent with the border between slow and intermediate exchange regimes. For a separation of bound and free resonances of 5 ppm (755 Hz), slow exchange behavior would be observed for an off-rate constant with a value less than about 76 s^{-1}. If the on-rate constant is similar to that observed for the bovine enzyme, ~ 10 μM^{-1} s^{-1} (Kurz et al., 1992), then an off-rate constant of 100 s^{-1} is predicted for a K_d of 10 μM. This is in the borderline region.

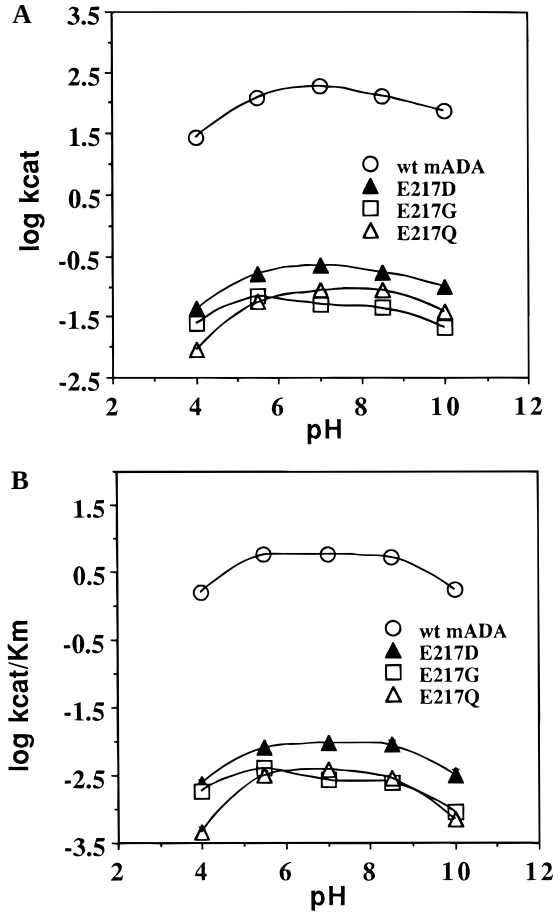

FIGURE 4: pH dependence of ADA and its E217 mutants. Activity of E217S was too low for measurement. Both plots shown are for the deamination of adenosine. Lines drawn through the points are illustrative of the shape of the profile. (A) Plot of log k_{cat} vs pH. (B) Plot of log k_{cat}/K_m vs pH.

pH Dependence of Enzymes. Values of k_{cat} and K_m for the deamination of adenosine by all four mutants at pH values within the stability range of the enzyme were determined. Values were obtained at pH 4, 5.5, 7, 8.5, and 10. The buffers used were similar to those of Sharff et al. (1992) in measuring pH dependence on wild-type mADA with the exception that all buffers were of equal ionic strength (I = 0.1). The results are presented graphically in Figure 4a,b along with the comparable profiles with native enzyme. Within experimental error, the profiles of log k_{cat} or log k_{cat}/K_m vs pH for wild-type enzyme is similar to E217D, indicating that ionization groups in both enzymes have very similar pK_a values. The mutant data indicate that Glu^{217} is not the group with a pK_a of 8−9 indicated by the pH−rate profile of the wild-type enzyme and which is required to be protonated for activity. Relatively little difference is observed between the acidic limbs for the native and mutant enzymes. The exception is E217G, which shows a gradual decrease in activity from pH 5.5 to pH 8.5 where the dropoff in activity is more steep. Optimal activity at pH 5.5 can be explained in terms hydronium ions acting as hydrogen bond donors to N-1. Decrease in activity of E217G in the pH range 5.5−8.5, in contrast to wild-type enzyme, most likely follows the reduction in the numbers of hydronium ions that act as potential stabilizers of the transition-state intermediate, however inefficiently.

Conclusions. Theoretical calculations (Orozco et al., 1989, 1990a,b) indicate that protonation of N-1 occurs prior to tetrahedral intermediate formation and favor the protonation step as the rate-determining step in catalysis. One piece of evidence supporting the existence of a pre-protonated intermediate in the ADA reaction comes from the study of a fluorescent azido analog of *erythro*-9-(2-hydroxy-3-nonyl)-adenine (EHNA) (Caiolfa et al., 1990). This is far from conclusive for several reasons. It is known that EHNA does not bind like adenosine in the active site (or else it would be deaminated). The additional steric hindrance of the azido ring may displace the inhibitor even further from proper binding interactions. The fluorescence spectrum of the complex is suggestive but its interpretation in terms of protonation is as subject to question as is any other structural interpretation based on an indirect spectroscopic study. Indeed, the data reported here show that the pre-protonated intermediate is not present.

The crystal structure of ADA bound to HDPR shows one arm of Glu^{217} 2.8 Å away from N-1 of the substrate and the zinc just 2.3 Å from the C-6 hydroxyl group (Wilson et al., 1991). The structure with 1-deazaadenosine also shows Glu^{217} 2.8 Å away from the C-1 of the inhibitor even though no hydrogen bond is possible. A distance of 2.8 Å is too long to be a low-barrier, short, strong hydrogen bond which requires a distance of 2.55 Å or less. A low fractionation factor hydrogen bond has been proposed to exist in the ground state of the enzyme−substrate complex, which is converted to a normal hydrogen bond (fractionation factor approaching unity) in the tetrahedral intermediate (Cleland, 1992). On the basis of the structural evidence and the data presented here, it does not seem likely that Glu^{217} forms a low-barrier hydrogen bond with adenosine N-1. Additional isotope effect measurements with the mutants should give further insight into this question.

Glu^{217} is a residue important to transition-state stabilization as is indicated by the severely decreased k_{cat} values for all four mutants together with no effect on the K_m of the substrate. The decrease in activity is due to perturbations at the catalytic site as secondary structure and zinc content do not differ from native protein. Nevertheless, the fact that substitution of an Asp for Glu^{217} produces an enzyme that is unable to promote formation of any detectable tetrahedral analog is startling. The driving force for tetrahedral intermediate formation in the wild-type enzyme has been estimated to be enormously great. The hydration equilibrium is shifted by the enzyme by greater than 10^8 (Kati & Wolfenden, 1989). The stabilization has been attributed to a single interaction of the enzyme with the 6-hydroxyl of the intermediate analog.

The role of Glu^{217} in formation and stabilization of the tetrahedral-state intermediate is clearly illustrated by UV difference and NMR spectroscopies. Hydration of purine riboside is observed only with wild-type ADA and not with any other mutants. Solvent difference spectroscopy of purine riboside with solvents of differing polarity suggests that the mutant difference spectrum peaks may be due to environmental effects imposed upon the ligand while in the active site, with no covalent bond changes having taken place. The peak is not due to exposure of aromatic side chains as a result of the mutation since certain solvents, in particular dimethyl sulfoxide, were able to duplicate the effect with purine riboside. Dissimilar protonation and enzyme−inhibitor difference spectra for 1-deazaadenosine have also been attributed to environmental factors (Kurz et al., 1992). The

observed peak shift of 8−10 nm (from 250 to 260 nm) in the order isopropyl alcohol < dimethyl sulfoxide < [mutant ADA−ligand] (data not shown) is analogous to that observed for 1-deazaadenosine (Kurz et al., 1992).

Some estimate of the extent to which the transition-state stability has been compromised can be obtained from calculating the energetic contributions that Glu^{217} and its mutants contribute to enzyme catalysis (Table 4). The entire amount may be attributed to change in the enzyme−substrate transition-state energy as structural perturbations have been shown to be confined to the site of the mutation and K_m and K_i values are similar for wild-type and mutant enzymes. The minimal change that results from substitution of Glu^{217} is 3.9 kcal mol^{-1} (based on the E217D mutant). The actual contribution is probably in the 5−6 kcal mol^{-1} range, lower than the estimated entropic contributions by ADA for the 6-hydroxyl group of purine riboside hydrate (9.9 kcal mol^{-1}) and atoms 1−3 of the pyrimidine ring (10.2 kcal mol^{-1}) (Kati et al., 1992). Note that, even though the contribution of Glu^{217} is far lower than that of the 6-OH, the former represents an energy barrier that must be overcome before attack of the hydroxyl group on C-6.

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