Adenosine deaminase was overexpressed in a baculovirus system. The pure recombinant and native enzymes were identical in size, Zn$^{2+}$ content, and activity. Five amino acids, in proximity to the active site, were replaced by mutagenesis. The altered enzymes were purified to homogeneity. The altered enzymes were expressed at high levels along the entire murine gastrointestinal tract, in thymic T cells and in decidual cells of the developing mammalian tissues. From the Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

Adenosine deaminase (EC 3.5.4.4), an important enzyme of the purine salvage pathway, catalyzes the irreversible hydrolytic deamination of adenosine or deoxyadenosine to inosine or deoxyinosine. Adenosine deaminase is expressed at very high levels along the entire murine gastrointestinal tract, in thymic T cells and in decidual cells of the developing maternal-fetal interface (Lee, 1973; Knudsen et al., 1988 and 1989; Witte et al., 1991). In humans, the upper gastrointestinal tract is devoid of this enzyme activity, but high levels are expressed in the lower part of the tract.

The wide spectrum of adenosine deaminase activity in mammalian tissues portended an important role for purine metabolism in nutrition and reproduction. However, the entire purine salvage pathway, and adenosine deaminase in particular, became the focus of intense interest with the observation that hereditary deficiency of the enzyme in humans is invariably associated with a form of severe combined immunodeficiency (Giblett et al., 1972). These patients have no obvious gastrointestinal tract abnormalities, but they do exhibit a dramatic lymphopenia that seems to be a direct consequence of the absence of adenosine deaminase (Coleman et al., 1978; Donofrio et al., 1978).

Potent inhibitors of adenosine deaminase are lympholytic in humans, and this property has been exploited in the treatment of certain leukemias, the hallmark of which is accumulation of differentiation-arrested lymphocytes (Coleman, 1983). Ground and transition state analog inhibitors have also proven useful in studies of the reaction mechanism of adenosine deaminase. With a rate enhancement of about 10$^5$, this enzyme is among the most efficient that have been described (Frick et al., 1987). A hydrate tetrahedral intermediate has been postulated from a large number of chemical studies (Evans and Wolfenden, 1973; Wolfenden et al., 1969; Kurz and Frieden, 1983). The most convincing evidence for this intermediate reaction product is from $^{13}$C NMR studies of adenosine deaminase bound to purine riboside (1,6-dihydropurine riboside), in which a change of hybridization from sp$^3$ to sp$^2$ is detected (Kurz and Frieden, 1987). Subsequent UV and NMR studies confirmed that this inhibitor is bound as an oxygen adduct, presumably hydrated at the 1,6 position (Jones et al., 1989). This covalent hydrate with C6 in the adenosine deaminase-purine riboside complex has been confirmed recently by the determination of its structure by x-ray crystallography (Wilson et al., 1991). Unexpectedly, the crystal structure also revealed that adenosine deaminase is a metalloenzyme that complexes 1 mol of Zn$^{2+}$ per mol of protein.

Solution of the crystal structure of a mammalian adenosine deaminase provided knowledge of the amino acids in the active site. However, at pH 4.2, where crystals were generated for x-ray analysis, adenosine deaminase is almost completely inactive, and at this pH the substrate analogue, purine riboside is only weakly bound (Wolfenden and Kati, 1991). Construction of mutations in active site residues coupled with determination of functional consequences of each mutation under conditions of optimal enzyme activity, will permit detailed characterization of the reaction pathway and description of enzyme intermediates.

In this study, guided in selection of targets by the crystal structure, we have altered key amino acid residues within the active site of human adenosine deaminase, an enzyme that is highly homologous to its murine counterpart. The recombinant enzymes were expressed in a baculovirus system and purified to homogeneity on a monoclonal antibody affinity column. Kinetic characteristics, stabilities, and metal binding capacities of the altered enzymes were assessed and correlated with mechanistic models.

**EXPERIMENTAL PROCEDURES**

Materials—Oligodeoxynucleotide primers used in constructing mutations and sequencing were synthesized at the University of Ken-
tucky Macromolecular Structure Facility on an Applied Biosystems 380B DNA synthesizer. Restriction endonucleases were from United States Biochemicals and New England Biolabs. Sequence data from disulfide sequencing kits were obtained from United States Biochemicals. [α-35S]dATP and [35S]adenosine were from Du Pont-New England Nuclear Products. The polyclonal antibody used in these experiments was raised in rabbits against homogeneous human adenosine deaminase. The anti-adenosine deaminase monoclonal antibody (N1D1) used in the study was also generated by our laboratory and used in ascites fluid (Philips et al., 1987). All other reagents were of the highest commercial grade available.

**Isolation of Mutant Viral Strains**—Plasmid carrying the human adenosine deaminase cDNA was injected into Sf-9 insect cells (Medin et al., 1990). Briefly, 10 μl of each viral stock was injected into the larvae at the fourth instar and purified as in our previous study (Medin et al., 1990). Purification of Recombinant Proteins—Wild-type adenosine deaminase was purified from frozen larvae by adenosine-affinity chromatography (Medin et al., 1990). The isolation of the proteins on the monoclonal antibody column was essentially the same as previously described (Philips et al., 1987) with modifications in the initial extraction procedure. Frozen larvae (3–10 g) were suspended in three volumes of cold extraction buffer containing several protease inhibitors (50 mM potassium phosphate, pH 6.8, 10 mM 6-aminocaproic acid, 5 mM benzamidine, 2 mM EDTA, 20 μM leupeptin, 5 μM aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and homogenized on ice with a Tekmar tissue homogenizer. The homogenate was centrifuged at 30,000 × g for 30 min. The pellet was re-extracted with half the original volume of buffer, homogenized, and centrifuged as before. Supernatants from both extractions were mixed (crude extract) and brought to 30% ammonium sulfate saturation. Further subjected to another cycle of fractionation with ammonium sulfate precipitation. The precipitate, resuspended in a minimum volume (~8 ml) of phosphate-buffered saline, pH 7.4, was applied to an adenosine-Sepharose column (110 × 1.5 cm) (Schrader and Stacy, 1977), and the fractions containing the major adenosine deaminase activity were pooled and concentrated by ultrafiltration.

**Site-directed Mutagenesis**—To facilitate site-directed mutagenesis of selected regions of the enzyme, the full length adenosine deaminase cDNA was cloned into the vector M13 mp18. The cDNA was released from the vector pBR8 (Medin et al., 1990) with the restriction enzymes NotI and HintI. Phosphorylated linkers that convert the HintI site to a NcoI site were ligated to the HintI-end of the cDNA. The phosphorylated cDNA was isolated after subcloning into the NcoI site of pAcC4 by digestion with NcoI. Phosphorylated NcoI to EcoRI linkers were ligated to the adenosine deaminase cDNA, and this cDNA was digested with EcoRI and ligated into the dephosphorylated M13 mp18 at the EcoRI site. This construct (M13 adenosine deaminase) was used in the development of all site-specific mutant forms of adenosine deaminase. Site-directed mutagenesis was carried out using the method of Kunkel et al. (1987). M13 adenosine deaminase was transfected into E. coli strain CJ36 (dut-, ung-). Single-stranded uracil containing M13 adenosine deaminase was isolated and annealed to synthetic phosphorylated mutagenic oligomers (Table 1). The second strand of DNA was synthesized using Sequenase version II and dNTPs. After ligation the reaction product containing the newly synthesized second strand was transformed into (dut-, ung-) E. coli strain DH5α. The presence of the desired mutation was confirmed by DNA sequencing using dideoxynucleotides.

**Expression of Recombinant Wild-type and Mutant Human Adenosine Deaminase**—The wild-type adenosine deaminase cDNA (including 5' and 3' flanking sequences) was inserted into the pAcC4 vector by EcoRI digestion. The mutant cDNA constructs were released from M13 mp18 by digestion with NcoI and subcloned into pAcC4 at the NcoI site. In order to introduce base changes into the amino acid sequence of human adenosine deaminase, the newly synthesized second strand was transformed into E. coli strain DH5α and grown overnight on a rotating platform.

### Table 1

| Name | Synthetic primers | Codon change | Amino acid change |
|------|-------------------|--------------|------------------|
| H17A | GAGCGGCTGGGAECGGCTACCACACC | CAC - GCC | His - Ala |
| H214N | CACCGTACTGTCCTCGCCGGGGAGGTGGGC | CAC - GCC | His - Ala |
| H217A | CACCGTACTGCTCCGCCGCGGGATGGTGCC | CAC - CTG | His - Leu |
| H218N | CACCGTACTGCTCCGCCGCGGGATGGTGCC | CAC - AAC | His - Aan |
| C822A | CATCCATTCCGAGATGCGCCGCCCTGTCGCA | CAC - GCC | His - Ala |

### Mutational Analysis of Adenosine Deaminase

The adenosine deaminase cDNA was isolated after subcloning into the plasmid vector M13 mp18. The plasmid M13 mp18 was used for site-directed mutagenesis of the adenosine deaminase cDNA. The baculovirus transfer vector pAcC4, a generous gift from Cetus, was used in homologous recombination experiments to construct specific baculovirus variants.

**Viruses, Cells, and Larvae**—Autographa californica nuclear polyhedrosis virus (ACMNPV strain Li, Invitrogen Corp.) and Spodoptera frugiperda (SF-9) insect cells (Invitrogen) were cultured in Grace's insect cell medium (Invitrogen). The plasmid vector M13 mp18 was used for site-directed mutagenesis of the adenosine deaminase cDNA. The baculovirus transfer vector pAcC4, a generous gift from Cetus, was used in homologous recombination experiments to construct specific baculovirus variants.
Macromolecular Structure Facility.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—**
SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) in a mini-gel apparatus (Bio-Rad). To visualize proteins the gels were either stained with Coomassie Brilliant Blue or with silver staining procedures (Wray et al., 1981). Adenosine deaminase in the crude extracts was determined by Western blot analysis. Electrotransfer of proteins onto nitrocellulose sheets was performed according to Burnette (1981). Blots of the protein were analyzed with anti-human adenosine deaminase monoclonal antibody (1:250, dilution) or anti-human adenosine deaminase polyclonal antibody (1:500, dilution). The second antibody used in the procedure was goat anti-rabbit or anti-mouse IgG alkaline phosphatase conjugate (Bio-Rad). The immunoreactive proteins were detected with an alkaline phosphatase conjugate substrate kit from Bio-Rad.

**Enzyme Assay and Kinetic Studies—**
Adenosine deaminase activity was detected by using the radiochemical assay described before (Coleman and Hutton, 1975) with [3H]adenosine as the substrate. One unit of adenosine deaminase activity is defined as the amount of enzyme required to produce 1 μm of inosine/min at 37 °C. For each protein, kinetic measurements were done at a series of six concentrations of adenosine so as to bracket the expected Km value. The concentration of enzyme ranged from 0.75 to 1 μM for the wild type to 1 μM for the least active mutant proteins. Kinetic constants were determined by an enzyme kinetics program (Trinity software, Campton, NH). The nonlinear regression analyses from this program are reported in this paper.

**Thermolysin Digestion of Wild Type and Mutant Adenosine Deaminase—**
The structural integrity of each mutant protein was studied by performing a thermolysin digestion at a variety of temperatures. The method used by Polesky et al. (1990) was followed with slight modifications. The proteins were stable to thermolysin at an enzyme:protein ratio of 1:50. Therefore an enzyme:protein ratio of 1:10 was used. Digestions were carried out for 45 min and were terminated by the addition of protein gel sample buffer. The digestion products were electrophoresed on a 12.5% polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue.

**Zinc Content of Wild-type and Mutant Adenosine Deaminase—**
Two methods, flame atomic absorption spectrometry and graphite furnace atomic absorption spectrometry, were used for zinc analysis. Absorbance peaks by both methods were measured at 213.9 nm. All values shown are averages of duplicate determinations. The detection limits for zinc by the above two methods were 120 and 1.5 nM, respectively.

For zinc analysis the protein samples were extensively dialyzed against 5 mM HEPES buffer (passed through a Chelex-100 column) in metal-free dialysis tubing prepared as suggested by Auld (1988). The concentration of residual zinc in the buffer was subtracted from those in the unknown protein samples.

**RESULTS**

The goal of this study was to produce recombinant human adenosine deaminase proteins in which amino acid residues that have been implicated in or are close to the enzyme active site (Wilson et al., 1991) have been mutationally altered. 5 amino acid residues were targeted for substitution, His238, Glu217, His214, His17, and Cys262 (Fig. 1). Each of these was converted to Ala except the His17 residue which was changed to Asn, Leu, and Ala. The first 4 residues in this group are predicted to be involved in ligand binding or catalysis. The Cys262 is close to the active site pocket, but is blocked by a wall of residues (His238, Ser265, and Asp295).

We have previously demonstrated that wild-type human adenosine deaminase is produced in very high amounts in T. ni larvae and can be readily purified from larvae infected with recombinant baculovirus containing the adenosine deaminase open reading frame under the control of the polyhedrin promoter (Medin et al., 1990). Therefore, we selected the baculovirus system for production of the seven mutant proteins.

**Expression and Immunodetection of Recombinant Proteins—**
Each adenosine deaminase mutant was generated by subcloning the cDNA into M13 and using the mutagenesis technique pioneered by Kunkel et al. (1987). Recombinant plasmids were isolated, identified by restriction analysis, confirmed by sequence analysis, and subcloned into the transfer vector.

Each of the transfer vector DNAs containing the adenosine deaminase open reading frame was then used in homologous recombination with wild-type baculovirus DNA to generate recombinant baculovirus for each mutation. The relative levels of wild-type and mutant adenosine deaminases produced in infected larvae were assessed by immunoblot analyses of crude extracts. No immunoreactive protein was detected in crude extract of insect larvae infected with nonrecombinant virus (Fig. 2, lane 9).

**Immunoblot analysis of recombinant human wild-type and mutant adenosine deaminases produced in insect larvae and cells.** Equivalent amounts of crude extract protein (30 μg) or 200 ng of the purified wild-type adenosine deaminase were applied to 12% polyacrylamide gels and subjected to SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose as described under "Experimental Procedures" and probed with rabbit anti-human adenosine deaminase polyclonal antibody. Lane 1, wild-type (larvae); lane 2, H238A (larvae); lane 3, E217A (larvae); lane 4, C262A (larvae); lane 5, H214N (larvae); lane 6, H214A (larvae); lane 7, H214L (larvae); lane 8, H17A (larvae); lane 9, uninfected larval protein; lane 10, purified recombinant wild-type adenosine deaminase; lane 11, blank; lane 12 H17A (cells); lane 13, H214L (cells).

**FIG. 1. Proposed active site amino acid residues in adenosine deaminases.** Residues depicted are based on the solution of the crystal structure of murine adenosine deaminase complexed to purine riboside (Wilson et al., 1991). These amino acids are identical in human adenosine deaminase. The residues targeted for mutagenesis in this study are boxed in bold lettering.

**FIG. 2. Immunoblot analysis of recombinant human wild-type and mutant adenosine deaminases produced in insect larvae and cells.** Equivalent amounts of crude extract protein (30 μg) or 200 ng of the purified wild-type adenosine deaminase were applied to 12% polyacrylamide gels and subjected to SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose as described under "Experimental Procedures" and probed with rabbit anti-human adenosine deaminase polyclonal antibody. Lane 1, wild-type (larvae); lane 2, H238A (larvae); lane 3, E217A (larvae); lane 4, C262A (larvae); lane 5, H214N (larvae); lane 6, H214A (larvae); lane 7, H214L (larvae); lane 8, H17A (larvae); lane 9, uninfected larval protein; lane 10, purified recombinant wild-type adenosine deaminase; lane 11, blank; lane 12 H17A (cells); lane 13, H214L (cells).
that are predicted to interact with the substrate, H238A and E217A, either at position 1 of the heterocyclic ring (lane 3) or with the incoming hydroxyl residue (lane 2), were produced in quantities equivalent to the wild-type recombinant protein (lane 1).

Proteins containing mutations in the 2 residues that are thought to be coordinated to Zn^{2+} (H17A and H214A, H214N and H214L), were present at lower levels (lanes 5–8). In fact, immunodetection of antigen in the crude extracts containing H214L and H17A (lanes 7 and 8) was difficult, indicating that these proteins accumulated to much lower levels than wild-type adenosine deaminase in infected larvae. Therefore, expression of these two mutants in infected Sf9 and T. ni (High-5) insect cell cultures was compared. Accumulation of both mutant proteins in these cell lines was equally improved (Fig. 2, lanes 12 and 13), though it was still much lower than for wild-type or any of the other mutant constructs (data not shown). The reason for the low accumulation of these mutant proteins has not been resolved.

The recombinant adenosine deaminase produced in this system was not totally soluble. The pellet fractions obtained from the initial clarification of the crude extracts from wild-type and mutant proteins contained large quantities of immunoreactive antigen (data not shown). Attempts to solubilize recombinant adenosine deaminase from the pellets (using nonionic detergents, urea, or high salt) yielded only an additional 10–15% of the antigen (data not shown). The two mutants (H214L and H17A) that were present in the extracts in low quantities exhibited correspondingly smaller amounts of antigen in the pellet fractions, suggesting that solubility of these proteins was about the same as that of the wild-type adenosine deaminase.

**Purification of Wild-type and Mutant Adenosine Deaminase**—The wild-type adenosine deaminase was purified using a two-column procedure (including an adenosine-Sepharose column) that we have previously described (Medin et al., 1990). In the present studies the procedure was scaled to about 30 g of larvae from which 25–30 mg of recombinant protein was purified, corresponding to a yield of 25%. The purified enzyme is shown in Fig. 3 (lane 1). This purification procedure was next applied to mutants C262A, E217A, H214A, H214L, and H214A. The retardation of wild-type and all mutant proteins by the adenosine-Sepharose matrix was similar. Thus binding of substrate was not dramatically altered in any of the mutant proteins.

Unexpectedly, a low level of enzyme activity was detected in all mutant preparations except C262A, which exhibited high levels of adenosine deaminase activity. Therefore, we re-examined the uninfected larvae and larvae infected with baculovirus (that did not contain any adenosine deaminase coding sequences) for a deaminase-like activity. Both sets of larvae were subjected to the purification procedure. Indeed, a deaminating activity (1–5 units/mg) was detected in the eluant from the adenosine-Sepharose column from uninfected larval extract. However when infected larval extract was subjected to the purification protocol, the deaminating activity dropped to 0.1–0.5 units per mg. Undoubtedly, viral replication repressed host protein synthesis (O’Reilly et al., 1992). This remaining deaminase-like activity was insignificant when compared to wild-type adenosine deaminase (400 units/mg), but might have interfered with detection of authentic activity in some of the mutant enzymes. In order to eliminate the insect deaminating activity from the mutant protein preparations, an alternate procedure was introduced for purification of all mutant recombinant proteins.

Monoclonal antibody (N1D1) directed against human adenosine deaminase (Philips et al., 1987) was used to construct an immunoaffinity column. This antibody, when tested in a Western blot against deaminating activity purified from uninfected larvae, showed no detectable antigen (data not shown). In addition, no adenosine deaminase antigen or activity was recovered from uninfected larval extracts when subjected to a purification procedure using the immunoaffinity column. As a further precaution against cross-contamination of the mutant proteins, individual monoclonal antibody affinity columns were constructed for each mutant and used only in the purification of that protein.

The results of the purification of the seven mutants are shown in Fig. 3. Each of these proteins has been purified 10 to 25 times. The proteins, detected by silver staining, were produced in excellent yield and purity, except for two constructs. H214L and H17A consistently gave low yields when purified from either infected larvae or cells. These two mutants have been purified 15 times from different cell preparations with similar results. The two preparations shown in the figure were from recombinant virus-infected cells and exhibited significant degradation of adenosine deaminase, as confirmed by Western blotting (data not shown). These data suggested that the very low antigen yields for these two mutants may reflect an altered protein stability.

**Thermolabilities of Wild-type and Mutant Proteins**—We explored thermolabilities of the proteins by comparing the patterns of products obtained from thermolysin digestion generated over a range of temperatures of all seven mutant and wild-type adenosine deaminases. The procedure was designed to detect differences in thermolability of wild-type and mutationally altered proteins. The rationale for this approach is based on differential sensitivities of denatured and native proteins and differential thermolabilities of wild-type and mutationally altered proteins. Each of the purified proteins was subjected to digestion by thermolysin at varying temperatures. The results of this analysis for two of the mutants is shown in Fig. 4. Across the entire temperature spectrum, the wild-type protein and the two mutants, H238A and C262A, exhibited similar digestion patterns. Other mutants, H214A, H214N, and E217A (not shown in this figure) showed identical digestion patterns. The two low yield mutants, H17A and H214L, were also subjected to thermolysin digestion. However, the limited quantities of these proteins made the analysis difficult to interpret. The overall digestion patterns and temperature dependence appeared similar, but with small amounts of these two proteins available, subtle changes in the
purified by using an immunoaffinity column. When these proteins were purified by conventional chromatography the following values were obtained from five different purified adenosine deaminase preparations.

| Wild type | Phosphate | 400 |
| H238A | Tris\(^\text{a}\) | 0.12 |
| H238A | Phosphate | 0.09 |
| E217A | Phosphate | 0.04 |
| E217A | Tris\(^\text{a}\) | 0.02 |
| H214N | Phosphate | 0.03 |
| H214N | Tris\(^\text{a}\) | 0.15 |
| H214A | Phosphate | 0.05 |
| H214A | Tris\(^\text{a}\) | 0.35 |

* The addition of Zn\(^{2+}\) during the purification did not alter activity.

\(^{\text{a}}\) Average enzyme activity obtained from five different purified adenosine deaminase preparations.

was C262A. This residue is highly conserved from humans to bacteria in all deaminases (Chang et al., 1991), and it lies in the crystal structure in the entrance to the enzyme active site (Wilson et al., 1991). When this protein was purified using the standard two-column procedure, specific activities in homogenous preparations ranged from 120 to 160 units/mg. In contrast, when this protein was purified by using the monoclonal antibody column procedure, specific activities of the purified preparations ranged from 24 to 137 units/mg. The most common activities in these preparations in 15 separately purified samples of C262A was 50–90 units/mg. The lower activity of the proteins purified by an immunoaffinity procedure probably reflected the conditions required (4–6 M urea) to elute the pure protein from the column. Alteration of this conserved residue may impede refolding of the protein during the procedure used for removal of urea and result in a lower enzyme activity than that observed in the native enzyme. In contrast, wild-type adenosine deaminase purified by both methods exhibits identical specific activities (Philips et al., 1987). None of the other mutant enzymes exhibited differential specific activities as a result of conventional purification or the immunoaffinity column procedure (see Footnote a in Table II).

The second mutant adenosine deaminase that appeared to exhibit activity was H17A. Enzymatic activity varied dramatically among the 15 preparations of this mutant purified on the immunoaffinity column. The lowest activity observed was 0.2 units/mg and the highest activity was 28 units/mg. This residue and H214 are postulated to be involved in coordinating the zinc atom. Therefore we examined the possibility that variations in Zn\(^{2+}\) content throughout the purification procedures were related to the retention of adenosine deaminase activity in these altered enzymes.

Our standard purification scheme utilized throughout potassium phosphate buffer, pH 6.8. Since phosphate is known to chelate Zn\(^{2+}\), we substituted for this buffer, Tris (which does not chelate Zn\(^{2+}\) (Sellin and Manneevvik, 1984)) and purified wild-type, H214A, H214N, H238A, and E217A in the presence or absence of exogenously added Zn\(^{2+}\). The results are shown in Table III. In the presence of Zn\(^{2+}\), either by exogenous addition throughout the purification or by use of a nonchelating buffer, enzyme activity was increased by 5- to 6-fold for the H214 mutants. We confirmed that this apparent stimulation of adenosine deaminase activity was not a general phenomenon since the specific activities of wild-type, H238A, H214N, H214A, H214N, or H214A variant.

The V\(_{\text{max}}\) and k\(_{\text{cat}}\) values were dramatically altered in the mutant proteins (Table II). The most active of the proteins

- **Kinetic Parameters of Wild-type and Mutant Derivatives**—Kinetic properties of the purified wild-type and mutant adenosine deaminases were determined using adenosine as substrate (Table II). The apparent K\(_{\text{m}}\) values for the mutants were similar to the wild-type enzyme. Thus, alterations in neither the side chains of the two-Zn\(^{2+}\) coordinating residues (His\(^1\) and His\(^10\)) nor in the N1 interacting residue (Glu\(^32\)) interfered with productive substrate binding. The only significant variation in K\(_{\text{m}}\) (3-fold) was observed for the H238A variant.

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and E217A enzymes were identical to those obtained by using phosphate buffer in the purification procedures (Table III).

**Zn**\(^{2+}\) **Content of Wild-type and Mutant Adenosine Deaminases**—The purified recombinant proteins were analyzed for **Zn**\(^{2+}\) content using two methods: graphite furnace atomic absorption spectrometry and flame atomic absorption spectrometry (Table IV). The graphite furnace method is more sensitive, but since small quantities of protein are required (nanomolar range), the signal-to-noise ratio is smaller than with the atomic absorption method. Previous analyses, including the x-ray crystallography studies, revealed a single **Zn**\(^{2+}\) residue at the active site. In these experiments, wild-type enzyme, E217A, H238A, and C262A were demonstrated to contain a single **Zn**\(^{2+}\) when the atomic absorption spectrometry method was used. The graphite furnace method indicated 2 mol of **Zn**\(^{2+}\) per mol of protein for these enzyme preparations. We suspect that this higher ratio is simply due to the inherent problem in accurately measuring **Zn**\(^{2+}\) from small quantities of protein. Both types of H17A preparations (high and low activity) contained **Zn**\(^{2+}\), though the amounts of these proteins were so limited that accurate quantitation of molar ratios was not feasible. The His\(^{2+}\) mutants also contained **Zn**\(^{2+}\) (with the exception of H214L) even though these preparations exhibited no enzyme activity in the absence of added **Zn**\(^{2+}\), but were slightly activated (H214A and H214N) when **Zn**\(^{2+}\) was added throughout the enzyme purification.

**DISCUSSION**

Adenosine deaminase is an enzyme of enormous interest. It plays important roles in nutrition and reproduction, and it serves a protective role against accumulation of deoxyadenosine in cells of the immune system. The catalytic power of this enzyme is among the highest known and extensive chemical studies with inhibitors have generated testable hypotheses about the active site. As expected for a protein with a central role in metabolism, the primary amino acid sequence of adenosine deaminase is highly conserved across species (Chang et al., 1991). The crystal structure of murine adenosine deaminase, complexed to the inhibitor purine riboside at pH 4.2, has recently been elucidated (Wilson et al., 1991), and active site residues have been identified. This information, coupled with access to the coding sequences of adenosine deaminases from other species, has made the testing of amino acid side chains in the active site feasible.

Human adenosine deaminase is slightly longer than the murine enzyme (by 11 residues at the carboxyl end of the molecule) but shares greater than 80% identical side chains (Wiginton et al., 1984; Ingolia et al., 1985). Of the 59 amino acid differences between the two proteins, 26 are conservative substitutions. All of the residues implicated in binding substrate or catalyzing the hydrolytic reaction are identical in the human and mouse proteins (Chang et al., 1991). Therefore, we anticipated that residues identified in the mouse enzyme would serve identical functions in both enzymes. The major goal of this study was to test selected amino acid residues in the active site at a pH of optimal enzyme activity (6.8). Of particular interest were a Cys residue close to the active site, 3 His, and a Glu residue in the active site.

Chemical studies with adenosine deaminase from a number of sources have previously implicated a sulfhydryl group as a catalytically important residue, perhaps as the source for protonation of N1 of adenosine (Orsi et al., 1972; Wolfenden et al., 1967; Weiss et al., 1987). Furthermore, comparison of adenosine deaminase protein sequences from *Escherichia coli* to humans has demonstrated the presence of a conserved Cys (Chang et al., 1991) which is also positionally conserved in all known AMP deaminases. Thus, it seemed highly likely that this residue would be essential for enzyme catalysis or conformation.

In the mouse and human enzymes this conserved residue is at position 262. The interpretation of the crystal structure places this Cys out of the range of the active site and suggests that access by this sulfhydryl group to the active site is blocked by 3 residues involved in substrate binding or catalysis (Wilson et al., 1991). When this amino acid was converted to Ala as described in this study, there was only a modest effect on enzyme activity or kinetic parameters. The mutant protein was apparently correctly folded in vivo, since enzyme which retained 30–40% of wild-type activity was obtained following conventional purification. When the protein was exposed to reduced glutathione during purification on an ion exchange column, about 20% of native enzyme activity was normally recovered under urea removal conditions in which 100% of wild-type adenosine deaminase activity and other mutant enzyme activities are recovered. The thermolability studies of the C262A mutant showed that there was no evidence of a drastically altered proteolytic digestion pattern upon heating. Thus, while this Cys residue may play an important, but as yet poorly understood, structural role in the enzyme structure, it is clearly not essential for catalysis.

The most surprising revelation about adenosine deaminase which occurred with the solution of the crystal structure was the role of **Zn**\(^{2+}\) in the enzyme active site (Wilson et al., 1991). This feature of the enzyme was unanticipated since standard studies with metal chelators failed to detect any enzyme sensitivity to such agents (Zielke and Suelter, 1971). The central role of **Zn**\(^{2+}\) in the functioning of adenosine deaminase helps to explain in part earlier observations of the effects on the immune system produced by **Zn**\(^{2+}\) deficiency in humans and animals. Zinc deficiency causes atrophy of lymphoid tissues and abnormalities in T and B cell function (Cossack and Prasad, 1991) reminiscent of the severe combined immunodeficiency observed in children who lack adenosine deaminase (Coleman, et al., 1978).

The human recombinant adenosine deaminase also contained **Zn**\(^{2+}\) as shown by analytical techniques. 2 residues were altered that have been implicated in **Zn**\(^{2+}\) binding, His\(^{2+}\) and His\(^{2+}\). The H17A was an unstable recombinant protein that exhibited an unaltered **K**\(_m\) for substrate binding. The His\(^{2+}\) mutants varied in stability. Substitution of His with Ala or Asn produced mutants that could be obtained in reasonable

| Protein          | GAAS\(^a\)  | FAAS\(^b\)  |
|-----------------|------------|------------|
| Wild type       | 1.9 ± 0.1  | 1.0 ± 0.1  |
| C262A           | 2.0 ± 0.2  | 1.2 ± 0.1  |
| H17A            | *          | ND\(^d\)   |
| H214A           | 1.4 ± 0.2  | 1.6 ± 0.06 |
| H214L           | <0.1 ND    |
| H214N           | 1.3 ± 0.2  | ND         |
| E217A           | 1.4 ± 0.3  | 1.2 ± 0.13 |
| H238A           | 1.9 ± 0.2  | 1.2 ± 0.06 |

\(^a\) GAAS, graphite furnace atomic absorption spectrometry. Standards containing zinc ranged from 15.4 to 92.4 nM. Measured values ranged from 18.5 to 65.2 nM.

\(^b\) FAAS, flame atomic absorption spectrometry. Standards containing zinc ranged from 0.385 to 6.16 μM. Measured values ranged from 1.16 to 3.08 μM.

\(^c\) H17A, **Zn**\(^{2+}\) was detected in this preparation (designated *), but the small quantity of protein did not permit accurate quantitation.

\(^d\) ND, not determined.
yield. The Leu substitution, however, produced a protein that was as unstable as the H17A. The concentration of Zn\[^{2+}\] available at all stages of the expression and purification of the recombinant proteins appeared to be important for recovery of enzyme activity. Use of a non-Zn\[^{2+}\]-chelating buffer and addition of Zn\[^{2+}\] throughout the purification usually resulted in recovery of a small percentage of wild-type enzyme activity (0.05–0.12%). However, the addition of Zn\[^{2+}\] during prolonged dialysis of purified enzyme preparations exhibiting less than 0.02% activity was not effective in restoring any additional activity. These mutants were not devoid of Zn\[^{2+}\] as shown by the atomic absorption analyses. If Zn\[^{2+}\] was not efficiently bound from the active site during purification by the use of phosphate buffer subsequent recovery of activity was never observed. These studies support the notion that His\(^{17}\) and His\(^{34}\) coordinate Zn\[^{2+}\].

Glu\(^{17}\) is proposed, on the basis of its proximity to the N1 of purine riboside in the crystal structure, to be involved in protonation of N1 of the adenosine purine ring (Wilson et al., 1991). The pK\(_{a}\) of the side chain can explain the Glu\(^{17}\) as a proton acceptor. In an alternate reaction, a fact that can be established only when we have a crystal structure (crystals were grown at pH 4.2), Asp\(^{Zg5}\) is observed. These studies support the notion that Hid\(^{7}\) and Kellems, R. E. (1991) Biochemistry 30, 2273–2280.

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