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# The role of divalent cations in structure and function of murine adenosine deaminase

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**Abstract**

For murine adenosine deaminase, we have determined that a single zinc or cobalt cofactor bound in a high affinity site is required for catalytic function while metal ions bound at an additional site(s) inhibit the enzyme. A catalytically inactive apoenzyme of murine adenosine deaminase was produced by dialysis in the presence of specific zinc chelators in an acidic buffer. This represents the first production of the apoenzyme and demonstrates a rigorous method for removing the occult cofactor. Restoration to the holoenzyme is achieved with stoichiometric amounts of either $Zn^{2+}$ or $Co^{2+}$ yielding at least 95% of initial activity. Far UV CD and fluorescence spectra are the same for both the apo- and holoenzyme, providing evidence that removal of the cofactor does not alter secondary or tertiary structure. The substrate binding site remains functional as determined by similar quenching measured by tryptophan fluorescence of apo- or holoenzyme upon mixing with the transition state analog, deoxycoformycin. Excess levels of adenosine or $N^6$-methyladenosine incubated with the apoenzyme prior to the addition of metal prevent restoration, suggesting that the cofactor adds through the substrate binding cleft. The cations $Ca^{2+}$, $Cd^{2+}$, $Cr^{2+}$, $Cu^{+}$, $Cu^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Pb^{2+}$, or $Mg^{2+}$ did not restore adenosine deaminase activity to the apoenzyme. $Mn^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ were found to be competitive inhibitors of the holoenzyme with respect to substrate and $Cd^{2+}$ and $Co^{2+}$ were noncompetitive inhibitors. Weak inhibition ($K_i \geq 1000\ \mu M$) was noted for $Ca^{2+}$, $Fe^{2+}$, and $Fe^{3+}$.

**Keywords:** adenosine deaminase; apoenzyme; divalent cation; kinetic studies; metal inhibition; metalloenzyme; metal substitution; structural role of metal

Adenosine deaminase (ADA) (adenosine aminohydrolase, EC 3.5.4.4) catalyzes the deamination of adenosine (or 2′-deoxyadenosine) yielding inosine (or 2′-deoxyinosine) and ammonia. The physiological relevance of the reaction is well documented in association with congenital Severe Combined Immunodeficiency Syndrome that occurs when this enzymatic activity is absent. The immunodeficiency has been alleviated in humans by the administration of the active enzyme directly to the blood stream (Kredich & Hershfield, 1989) or by replacement of adenosine deaminase through gene therapy (Hershfield & Mitchell, 1995). This crucial role demonstrates the importance of understanding factors and cofactors that affect the stability and reactivity of the enzyme.

Prior to the crystallographic analysis, adenosine deaminase had been thought not to require a $Zn^{2+}$ cofactor (Frick, 1986). Although a pharmacological correlation between ADA activity and dietary levels of zinc suggested some role for the ion (Luo et al., 1989), no kinetic evidence for the zinc cofactor was reported. Due to the catalytic and active site similarities with AMP deaminase, which had been shown to require zinc or cobalt ions for activity (Stankiewicz et al., 1979), these results were puzzling. Furthermore, there were inconsistent effects of various metal ions inhibiting, activating, or not affecting adenosine deaminase isolated from several different organisms that lead to confusion about the exact role of the cations in the deaminase reaction (Rokosu, 1983).

The presence of a zinc cofactor and a catalytic role were identified in the first X-ray crystallographic analysis of adenosine deaminase (Wilson et al., 1991). The zinc ion is located deep within the substrate binding cleft and coordinated in a tetrahedral geometry to $His^{15}$, $His^{17}$, $His^{214}$, and $Asp^{295}$. A water molecule, which shares the ligand coordination site with $Asp^{295}$, is polarized by the metal giving rise to a hydroxylate ion that replaces the ammonia at the $C^6$ position of adenosine through a stereospecific addition-elimination mechanism (Wilson & Quiocho, 1993). Mutation studies of amino acids in the proposed active site near the zinc binding

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site in the adenosine deaminase confirmed the essential role of these residues in catalysis (Bhaumik et al., 1993; Mohamedali et al., 1996; Sideraki et al., 1996).

Although these results indicate a zinc ion is tightly bound and directly involved in catalysis, the possible structural role for the cation has not been established and the effects of other metal ions has not been understood for the deaminase. In addition, quantitation of the zinc content of wild-type and mutant forms of human adenosine deaminase yielded ratios of 1:1 to 2:1 of zinc ion to enzyme (Bhaumik et al., 1993) and allows for speculation that more than one zinc ion may be involved. To further investigate the role of the metal ion, we synthesized the apo- form of murine adenosine deaminase to study the correlation between zinc content and activity and to determine the structural role of the cofactor. A survey of other cations was undertaken to determine which could functionally substitute for the zinc ion. In this report, we provide kinetic evidence of a catalytic role for the single zinc ion bound in the enzyme and show that the cation is not required for the proper structure of the substrate binding site in adenosine deaminase. We also report that secondary metal binding site(s) are available that allow some metal ions to inhibit the holoenzyme.

## Results

### *Reduction of deaminase activity by zinc chelators*

The decrease in the catalytic activity of adenosine deaminase by zinc chelators is time- and pH-dependent. A half-life of approximately 36 hours was observed for zinc removal by dialysis against solutions of 10 mM dipicolinate or 1,10 phenanthroline in 10 mM MES at pH 5.5 in the presence of 1 mM dithiothreitol (Table 1). A control solution of adenosine deaminase dialyzed against 4,7 phenanthroline retained >95% of the original activity during the dialysis. Zinc could be removed from the deaminase by these chelators only at acidic pH. At neutral pH, no loss of zinc or reduction in activity was measured after a 5-day dialysis of adenosine deaminase against the above chelators. Incubations below pH 4.5 resulted in significant (>50%) loss of activity in the control solutions. No loss of zinc or activity was measured when the enzyme was dialyzed against 15% (w/v) Chelex 100 or 10 mM EDTA at pH 5.0 to 8.0 in 5-day incubations. This demonstrates the stability that had prevented earlier detection of the bound cofactor (Zielke and Suelter, 1971). The presence of up to 1 mM of any of the chelators in the assay solution had no effect on the reaction rate over the course of an assay. Cyanide (0.5 M) in Tris pH 9.0 irreversibly inactivates the enzyme in less than 3 min and was not investigated further.

Zinc quantitation of partial apo-adenosine deaminase preparations (i.e., shorter dialysis times) by flame atomic absorption confirmed that zinc was present at a level corresponding to a 1:1 molar ratio of zinc to active enzyme (Table 2). By using scrupulous technique and extending dialysis times, the level of enzyme activity can be reduced to <3% of control with a corresponding decrease in zinc levels.

**Table 1.** *Effectiveness of selected metal chelators for inactivating adenosine deaminase*

| Chelator | Half-life of Inactivation[a] | % Activity[b] (time dialyzed) | % Activity Restored by Added Zinc[c] |
|---|---|---|---|
| Cyanide pH 9.0 | <10 min | 0% | 0% |
| Dipicolinic acid pH 5.5 | 1.5 days | ≤5% (5 days) | >97% |
| 1,10 phenanthroline[d] pH 5.5 | 1.5 days | ≤5% (5 days) | >97% |
| 1,10 phenanthroline pH 7.0 | N.D.[e] | 100% (5 days) | N.D. |
| EDTA pH 5.5 | N.D. | 100% (5 days) | N.D. |

[a]Half-life estimated from the percent of recovered activity after the indicated times of exposure to the chelator.

[b]Percent activity calculated from control solution treated in the same manner but without chelator. Less than 7% loss of initial activity occurred during 5 days of dialysis of the control.

[c]Zinc chloride was incubated at a 1:1 molar ratio with enzyme for 5 min before the substrate was added.

[d]Incubations with 4,7 phenanthroline (a non-chelating analog of 1,10 phenanthroline) produced no change in activity versus controls.

[e]N.D. Not determined; no loss of activity versus controls was measured in the enzyme solutions treated for up to 7 days.

### *Restoration of metalloenzyme*

Titration of adenosine deaminase with zinc shows that maximum deaminase activity is achieved at a 1:1 molar ratio of zinc or cobalt to adenosine deaminase and no cooperativity is evident (Fig. 1). Restoration by the metal ions is immediate (<1 min). These studies clearly define the stoichiometry but the binding of these ions in adenosine deaminase is sufficiently tight to preclude the determination of a $K_d$ from a binding plot. This experiment does establish that the $K_d$ is significantly lower than $10^{-9}$ M.

Restoration was independent of the counter ions in the metal solution. Metal salts of chloride, acetate, or sulfate worked equally well in reconstituting the enzyme activity.

Restoration of catalytic activity requires that reducing conditions are maintained during the formation and storage of apoenzyme. Dithiothreitol or β-mercaptoethanol added to the dialysis buffers just before use provided effective protection of the apoenzyme. Although the absence or presence of reducing agents had no apparent effect on the rate of zinc removal or of activity loss during dialysis, only apoenzyme protected by thiol reagents could be restored to an active holoenzyme by the addition of the ions. The presence of thiol reagents at 0.1 to 5 times the enzyme concentration provided sufficient protection and had no effect on the restoration titrations.

**Table 2.** *Activity of adenosine deaminase correlated to the level of* $Zn^{2+}$

| Sample (time of dialysis) | % Activity | % $Zn^{2+}$ |
|---|---|---|
| Control (4 days) | 100[a] | 100[b] |
| Sample 1 (2 days) | 36 | 43 |
| Sample 2 (3 days) | 22 | 21 |
| Sample 3 (4 days) | 11 | 13 |

[a]Percent activity relative to control sample treated in a similar manner but without the chelator, dipicolinic acid.

[b]Percent zinc present relative to the control sample treated in a similar manner but without chelator. After the dialysis, the control sample yielded 103% of the pre-dialysis activity. Zinc concentrations were determined by flame atomic absorption and a linear standard curve generated from zinc standards of 0.5–6 μM. All sample values were interpolations.

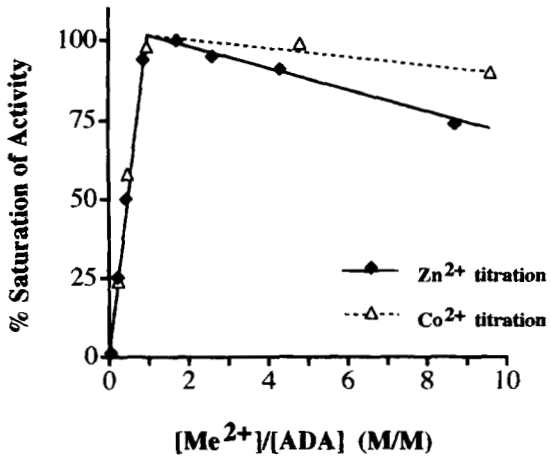


**Fig. 1.** Fractional saturation of activity versus the molar ratio of $Zn^{2+}$ or $Co^{2+}$ to adenosine deaminase. The apoenzyme (230 $\mu$M) was prepared using dipicolinic acid and assayed using $N^6$-methyladenosine as described in the materials and methods. The $K_m$ for this substrate is similar to that of adenosine but the compound is catalyzed nearly 500-fold slower than adenosine thus allowing activity measurements of up to 1 $\mu$M enzyme. Note that excess levels of either cation begin to inhibit the enzyme.

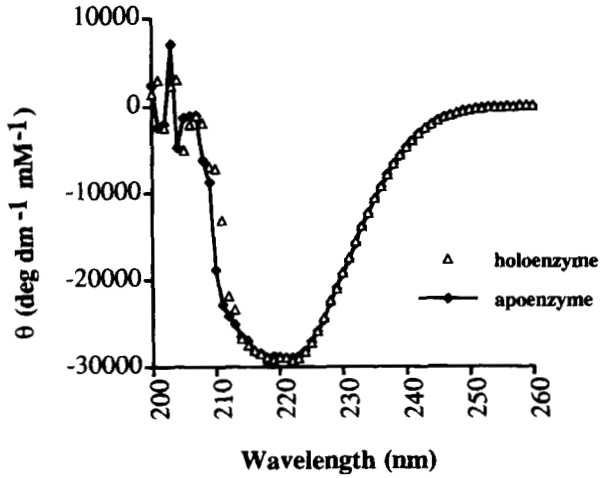


**Fig. 2.** Far UV circular dichroism spectroscopy of adenosine deaminase apoenzyme and holoenzyme. Spectra were normalized for protein concentration and are the average of three scans. Protein concentrations were 25 $\mu$M adenosine deaminase in metal-free 100 mM potassium phosphate buffer, pH 7.5. The well-defined secondary structures of adenosine deaminase give rise to the features at 218 nM ($\beta$-sheet) and 222 nm ($\alpha$-helix).

*Restoration blocked by substrate*

Reactivation by $Zn^{2+}$ or $Co^{2+}$ can be blocked by the presence of substrate or substrate analog. No measurable activity was restored when the enzyme was pre-incubated for 1 min with 2 mM (100 × $K_m$) of adenosine or $N^6$-methyladenosine followed by the addition of a two-fold excess of zinc over enzyme concentration. The product, inosine, yielded only 66% inhibition of zinc uptake at 2 mM ($K_i$ 120 $\mu$M) (Agarwal & Parks, 1978) confirming that the active site of the apoenzyme retains the same specificity as the holoenzyme. Physical evidence for the presence of substrate in the active site of the apoenzyme and for the blocking of zinc uptake by this substrate is provided by the prevention of a fluorescence emission change associated with $Co^{2+}$ restoration to the unbound apoenzyme (see below).

*Structural analysis*

The far UV CD spectrum of adenosine deaminase exhibited a minimum at 222 nm characteristic of $\alpha$-helical content and a minimum at 218 nm reflecting $\beta$-sheet structure (Adler et al., 1973). Wild-type murine adenosine deaminase has a parallel $\alpha/\beta$ barrel motif with eight central $\beta$-strands and eight peripheral $\alpha$-helices and five additional helices (Wilson et al., 1991), giving strong characteristic features to the spectrum. Figure 2 shows that the far UV circular dichroism spectra for the apoenzyme and holoenzyme are essentially identical, suggesting that the secondary structural elements of holoadenosine deaminase are preserved in the apoenzyme.

Fluorescence emission spectroscopy was performed on the apo- and holodeaminase to probe changes in the environment surrounding the four tryptophan residues contained in the protein. The tryptophan emission fluorescence spectrum of both apo- and holoenzyme had a maximum at 328 nm and produced identical intensities across the spectra (Fig. 3). This maximum compares well to the emission maxima of the calf (340 nm) and human (335 nm) adenosine deaminase (Kurz et al., 1985; Philips et al., 1989). Furthermore, apoadenosine deaminase mixed with stoichiometric amounts of zinc had restored activity but showed no observable changes in tryptophan fluorescence (data not shown). However, reconstitution to the holoenzyme with cobalt resulted in a small perturbation reflected in a 6–15% quenching of fluorescence emission intensity (data not shown).

When equimolar amounts of deoxycoformycin were added to the holoenzyme, a 47% quench in fluorescence intensity was measured (Fig. 3). A similar decrease in intensity (46%) was observed when deoxycoformycin was added to the apoenzyme. When cobalt

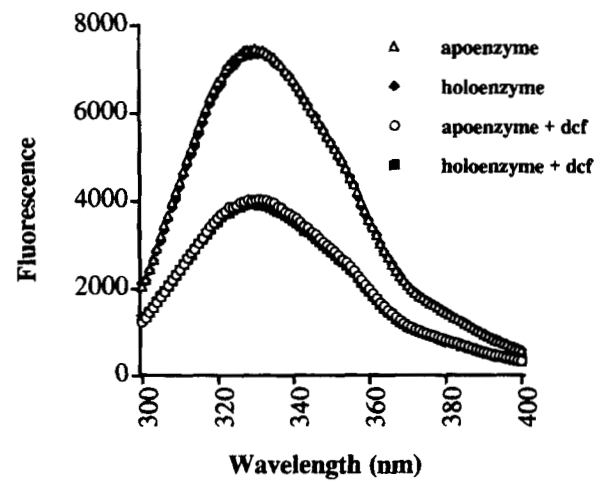


**Fig. 3.** Tryptophan emission fluorescence spectra of adenosine deaminase apoenzyme and holoenzyme in the presence and absence of deoxycoformycin. The excitation wavelength was 290 nm. Samples of 25 $\mu$M protein were in metal-free 100 mM potassium phosphate buffer, pH 7.5, with or without 25 $\mu$M deoxycoformycin. Tryptophan emission without inhibitor is identical in both the apoenzyme and the holoenzyme giving a $\lambda_{max}$ at 330 nm. Fluorescence intensity is quenched by 47% upon binding of the inhibitor to both forms of adenosine deaminase.

replaced zinc in the active site, fluorescence was quenched by 51% upon deoxycoformycin binding (data not shown). Addition of zinc or cobalt after binding of deoxycoformycin had no effect upon the fluorescence quenching (data not shown). The protection provided by the inhibitor against the small fluorescence change expected to occur with the binding of cobalt is consistent with the restoration studies presented above in which occupation of substrate site blocked access to the metal binding site.

*Effects with other metals*

Apoenzyme preparations were used to test several metal cations for their ability to serve as cofactors. Equimolar, 10-fold, and 100-fold excess amounts of $Ca^{2+}$, $Cd^{2+}$, $Cr^{2+}$, $Cu^{+}$, $Cu^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Pb^{2+}$, or $Mg^{2+}$ were added to the apoenzyme solution that was then tested for deaminase activity. None of these ions restored measurable deaminase activity to the apoenzyme.

The apoenzyme preparations used for the reconstitution studies retained 15–30% activity of control. Fortuitously, this low activity identified inhibition by some of the metals. Weak inhibition by $Ca^{2+}$($K_i$ 1000 $\mu$M), $Fe^{2+}$ ($K_i$ 1000 $\mu$M), Fe3+ ($K_i$ >2000 $\mu$M) was noted and $Cu^{+}$ and $Mg^{2+}$ produced no effect on the enzyme up to 10 mM, which was the highest amount tested. Zinc contamination was likely responsible for a slight activation upon addition of 10 mM $Pb^{2+}$. Metals that showed significant inhibition at 100 $\mu$M in the initial experiments were used in inhibition studies to determine a $K_i$ and mode of inhibition with respect to substrate (Table 3). Figure 4 shows the results for the competitive inhibition by $Mn^{2+}$ with respect to adenosine. The inhibition by these added metals was immediately reversed by the addition of EDTA to the reaction solution. As was reported in the zinc restoration studies, the inhibition effects were independent of the counter ion used.

## Discussion

Zinc quantitation combined with kinetic analysis establish a direct correlation between the level of zinc present in adenosine deaminase and the activity of the enzyme (Fig. 1 and Table 2) and indicate that the fully active holoenzyme is formed by the combination of one molecule of the apoenzyme of adenosine deaminase with one molecule $Zn^{2+}$. The apoenzyme has no measurable catalytic activity to a limit of detection of 0.1% of the rate of the holoenzyme. The sharp inflection point in Figure 1 where $Zn^{2+}$ concentration is equal to the level of adenosine deaminase clearly establishes that one zinc molecule is contained in the active enzyme. These results support the vital catalytic role proposed for zinc in the deamination of adenosine (Wilson et al., 1991; Wilson & Quiocho, 1993).

**Table 3.** *Inhibition of adenosine deaminase by metal ions*

| Cation | Inhibition type with Respect to Substrate | $K_i$ ($\mu$M) ± S.D. (n) |
|---|---|---|
| $Zn^{2+}$ | Competitive | 7 ± 3 (4) |
| $Co^{2+}$ | Noncompetitive | 670 ± 68 (2) |
| $Cu^{2+}$ | Competitive | 119 ± 38 (2) |
| $Mn^{2+}$ | Competitive | 381 ± 16 (2) |
| $Cd^{2+}$ | Noncompetitive | 19 ± 3 (5) |

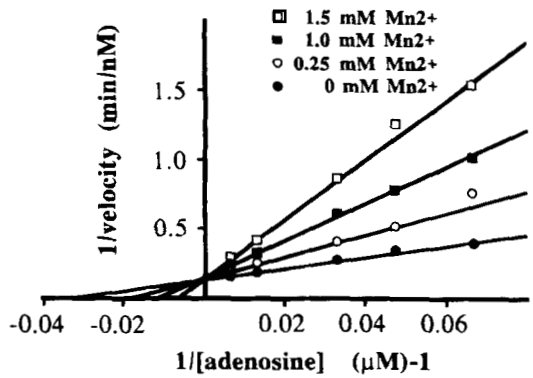


**Fig. 4.** Lineweaver-Burk plot of 1/velocity versus 1/[adenosine] in the presence of different levels of $Mn^{2+}$. The 1 mL assay solution contained 50 mM $KPO_4$, pH 7.4 and adenosine at the indicated concentrations. The $K_i$ of 380 $\mu$M for $Mn^{2+}$ was determined for competitive inhibition using Enzyme Kinetics (Trinity Software).

Adenosine deaminase tenaciously holds the single zinc ion using a high affinity motif which is common among Zn-binding proteins (Vallee & Auld, 1990). In all cases where a zinc cofactor plays a catalytic role in an enzyme, the metal ion is coordinated to two ligands closely spaced to each other ($His^{15}$ and $His^{17}$ in adenosine deaminase) which are thought to assist in forming a primary bidentate zinc complex. The third ligand ($His^{214}$ in adenosine deaminase) is separated typically by a relatively long spacer (20–120 amino acids) from the first two ligands. Although the spacer is longer than expected in adenosine deaminase, the crystal structure confirms that the $Zn^{2+}$ is coordinated to the three histidines and the fourth ligand site is shared between the carboxylate moiety from $Asp^{295}$ and a water molecule, which is activated to participate in the deamination reaction (Wilson et al., 1991).

The zinc cofactor exchanges with the solvent extremely slowly from the high affinity site on the enzyme. Acidic conditions (pH <5.5), long dialysis times (1.5-day half-life), and large excess concentrations (>1000-fold over enzyme) of specific zinc chelators were required to create the apoenzyme. The requirement for extended dialysis usually indicates that the mechanism of zinc removal is through dissociation from the enzyme followed by scavenging by the chelators (Wagner, 1988). If this were the case, excess EDTA and Chelex 100 should also be effective in removing the cofactor but only dipicolinic acid or 1,10 phenanthroline at acidic pH slowly removed the zinc from adenosine deaminase. These chelators contain hydrophobic conjugated rings that could allow the molecules to access the zinc buried within the protein to effect the removal. In general, phenanthroline reportedly removes zinc by an $S_N2$ reaction through the rapid formation of a ternary complex with accessible zinc (Wagner, 1988). However, the presence of these chelators in the assay solution for adenosine deaminase caused no loss of activity during the time course of the assay, indicating that these molecules do not disrupt or block the nucleoside binding site or significantly affect the protein structure. We conclude that the bound metal cofactor is inaccessible to chelators, is held in a high affinity site, and is not exchanged with solvent to any appreciable extent at neutral pH.

Removing the metal cofactor does not change the gross structure of adenosine deaminase. The apoenzyme of adenosine deaminase retains the same spectroscopic features as the metalloenzyme as determined by the following: First, the apoenzyme is reconstituted

to the metalloenzyme by addition of zinc in a matter of seconds, suggesting that no major structural alterations accompany this event. The reconstituted deaminase binds substrate or substrate analogs readily and the reaction proceeds at the same rate as with the native enzyme. Second, based on the binding of substrate to the apoenzyme and on similar CD spectra of the apo- and holoenzyme, the presence or absence of the metal cofactor causes no measurable differences in the secondary structure of the enzyme. Third, the similarity in fluorescence spectra suggests that the tertiary structure of adenosine deaminase in the vicinity of its four tryptophans is not altered as a consequence of zinc removal because the emission intensity and wavelength maximum from the tryptophan residues in the apoenzyme are not altered by the removal of the cofactor.

The observation that substrate or substrate analogs can bind to the apoenzyme and that their presence can prevent the reconstitution of activity by added zinc suggests that the metal adds via the substrate binding cleft. The crystal structure of the deaminase places the $Zn^{2+}$ cofactor in the deepest part of the active site cleft and positions adenosine to block access to the zinc binding site with the purine moiety directly above the metal site and with the ribose ring closest to the exit of the active site (Wilson et al., 1991). A similar finding of substrate interfering with the uptake of metal ions has been reported for cytosine deaminase (Porter & Austin, 1993).

Previous findings with calf and human adenosine deaminase have suggested that binding of transition-state analogs to the enzyme (such as coformycin and deoxycoformycin) is accompanied by a conformational change (Kurz & Frieden, 1983; Philips et al., 1987). This change is manifested by a large (40–50%) decrease in the intensity of the tryptophan emission fluorescence spectrum of the enzyme. Binding of deoxycoformycin to wild-type (zinc) or $Co^{2+}$ forms of mouse adenosine deaminase quenches the fluorescence intensity by 47% and 51%, respectively (Fig. 3). Similar quenching is observed in the spectra of the apoenzyme-deoxycoformycin complex. These findings show that the inhibitor can bind in the absence of the metal cofactor and still effect the gross structural changes in the protein. The saturation of the signal at 1:1 ratios of deoxycoformycin to apoenzyme attests that the affinity is still quite high. Although some binding energy may be supplied by the cofactor in the metalloenzyme, the bulk of the binding energy for deoxycoformycin appears to occur through interactions independent of the presence of metal ion.

The preceding evidence demonstrates that the structure of the protein and the maintenance of the binding site is independent of the presence or absence of the metal cofactor. However, the removal of the cofactor increases the sensitivity of the enzyme to oxidation. This increase may reflect a shift in equilibrium between the fully folded form and a partially denatured state which exposes internal cysteines. As long as the denatured form represents less than 2% of the total protein, this shift would not be detected spectroscopically,

The presence of a thiol reagent in the apoenzyme solutions is sufficient to protect against the loss of recoverable activity but does not interfere with the reconstitution by added metals. Identical reconstitution curves are generated in the presence of thiol reagents at 0.1 to 5 times the concentration of enzyme suggesting that the metal binding site on the enzyme competes effectively for the ions possibly bound to the thiol reagents.

A number of zinc metalloenzymes have been successfully converted to cobalt enzymes. Thus, it was no surprise that of the metals tested, only $Co^{2+}$ replaced $Zn^{2+}$ to fully restore catalytic activity to the deaminase. The metalloenzyme containing $Co^{2+}$ appears to mimic the $Zn^{2+}$ metalloenzyme in nearly all aspects measured. The only measured difference was that the fluorescence spectrum of the cobalt adenosine deaminase exhibited a 6–15% lower intensity than that of the native enzyme indicating a slightly different environment for the tryptophan(s). This perturbation likely results from the smaller atomic radius of $Co^{2+}$ but this change does not affect the reaction velocity or substrate binding parameters. The availability of the $Co^{2+}$ enzyme will facilitate spectroscopic investigation of the coordination of this metal within the active site.

A variety of effects of metal ions, including inhibition and activation, have been reported for adenosine deaminase but the findings have been inconsistent. After noticing inhibition effects in our titrations with zinc and other metals in the reconstitution studies, we performed kinetic studies to survey the effects of the cations. $Zn^{2+}$ proved to be the most potent inhibitor followed by $Cd^{2+}$, $Cu^{2+}$, $Mn^{2+}$ then $Co^{2+}$ (Table 3). The cations, $Zn^{2+}$, $Cu^{2+}$, and $Mn^{2+}$, were competitive with respect to substrate; $Cd^{2+}$ and $Co^{2+}$ were noncompetitive. Any inhibition by these added metals is immediately eliminated upon the addition of excess EDTA to the assay solution indicating that the inhibitory cations are associating at sites readily accessible to the solvent. Although the nature of the inhibitory interaction is not defined by these studies, competitive inhibition with respect to substrate suggests that the inhibitory cation(s) are located within the active site or the binding cleft thus interfering with the binding of the substrate. One possibility is the coordination of the cation to the activated water molecule formed in the active site by metal cofactor. A second possibility is coordination to the solvent exposed $Cys^{153}$ located in the active site approximately 4 Å from the nearest atom in the ribose moiety of the substrate. This thiol, along with neighboring charged residues such as Asp 181 (3.5 Å away from the cysteine sulfur) and His 17 (4.6 Å away), could conceivably form a metal binding site similar to that found in "zinc finger" structures. This coordination site would occupy a substantial portion of the substrate binding site, and could be responsible for the competitive inhibition observed with various metals with respect to substrate.

The noncompetitive inhibition by $Co^{2+}$ and $Cd^{2+}$ indicates that the substrate can bind, albeit not as well, in the presence of these metals.

In conclusion, this study shows that adenosine deaminase requires a single, bound zinc or cobalt ion for catalytic activity and that the cofactor is not required for the gross conformation of the enzyme. This metal cofactor is taken up by the apoenzyme through the substrate binding cleft and the presence of substrate or inhibitor prevents restoration of cofactor into the metal binding site. Some divalent cations, including zinc, may bind elsewhere within the active site and inhibit the holoenzyme by blocking the binding of the substrate.

## Materials and methods

### *Sample preparation*

Cloned mouse adenosine deaminase was purified from *Escherichia coli* culture AR/120 pots/adenosine deaminase NE5 according to Ingolia et al. (1985). Purification was accomplished using a 40–65% ammonium sulfate precipitation followed by anion exchange on DEAE and affinity chromatography on an

adenosine-sepharose column to yield >95% pure adenosine deaminase (SA > 300 μmol/min/mg). Protein concentrations were determined using $\epsilon_{280}$ = 1.14 mL/mg/cm (L.C. Kurz, personal communication) or a BioRad Protein Assay with bovine serum albumin as standard (Bradford, 1976). To maintain strict reducing conditions for the apoenzyme, all buffers and samples were maintained with 1 mM DTT or β-mercaptoethanol and purged with argon frequently during handling and use.

*Cofactor removal*

Adenosine deaminase was concentrated to 1 mM adenosine deaminase (41 mg/mL) using an Amicon Centriflo (CF25) device and dialyzed against 3–5 changes of metal free 20 mM HEPES, pH 7.0, 1 mM DTT and stored at 4 °C. To remove the zinc, the enzyme solution was diluted 60-fold in 10 mM dipicolinate (Sigma) in 50 mM MES, 1 mM DTT, pH 5.5 and dialyzed against 3–5 changes of the same buffer over 3–5 days followed by 4 changes of 50 mM HEPES, 1 mM DTT, pH 7.0, over 8 h. All glassware was rinsed with 1 N $HNO_3$ and virgin plasticware was rinsed several times with 18 MΩ Milli-Q water before use (Holmquist, 1988). Dialysis tubing (12–14K MWCO, Spectrapor) was heated 3 times to 60–70 °C for 2–3 h with fresh changes of Milli-Q water (Auld, 1988). Other chelators used under similar conditions include 5 mM EDTA (Sigma), 10 mM 1,10 phenanthroline (Sigma), and Chelex 100 (BioRad) maintained at 15% (w/v) outside the dialysis tubing. Cyanide at 1 mM in 20 mM Tris, pH 9.0 was also used. A negative control of only buffer or 10 mM 4,7 phenanthroline (Aldrich) was included for each dialysis to check for retention of enzyme activity during the extensive dialysis.

All buffer solutions except the metal solutions were purged at 1.5 mL/min over a column of Chelex 100 (1.5 × 15 cm) (BioRad) then stored over a few beads of Chelex 100. The Chelex column was regenerated according to the manufacturer's instructions after each 500 mL of solution.

*Zinc quantitation*

Zinc concentrations were determined by flame atomic absorption spectroscopy at 213.9 nm on a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer at the laboratory of Dr. David Giedroc, Texas A&M University, College Station, Texas. A linear standard curve was generated from 0.5 μM to 6 μM zinc nitrate. Samples were diluted with the corresponding buffer if necessary to fall within the linear portion of the curve.

*Enzymatic activity assay*

Adenosine deaminase activity measurements were recorded on a Cary 118 at 0.1 AUFS at the indicated wavelengths. The assay solution contained 40 mM HEPES, pH 7.4 and 0.1 mM substrate. When adenosine was the substrate the reaction was monitored at 265 nm ($\epsilon_{265}$ = 8.5 × $10^3$ $M^{-1}cm^{-1}$) (Nygaard, 1978). In the presence of $N^6$-methyladenosine, the assay was measured using $\epsilon_{280}$ = 7.3 × $10^3$ $M^{-1}$ $cm^{-1}$ determined from a difference spectrum of 0.1 mM solutions of inosine and $N^6$-methyladenosine measured on a Cary 118. A $k_{cat\ (N^6\text{-methyladenosine})}$ of 0.35 $sec^{-1}$ was calculated for adenosine deaminase compared to a $k_{cat\ (adenosine)}$ of 180 $sec^{-1}$. The 0.19% relative rate of utilization of $N^6$-methyladenosine as substrate is consistent with the previous value of 0.16% (Chassy and Suhadolnik, 1967). The use of this substrate analog allows the use of 500-fold higher enzyme levels in the assay that reduces the affects of adventitious zinc.

*Metal replacement and inhibition*

For titrations with zinc and cobalt, 230 μM apoenzyme was diluted two-fold with 20–2000 μM metal ion solutions, incubated at room temperature for 3–10 min, then added as a 250-fold dilution into metal free assay solution containing N6-methyladenosine.

During the surveys of other metals, 10 μM apo-adenosine deaminase was incubated with 10 to 1000 μM of the indicated metal solution for 3–10 min at room temperature prior to dilution by 100-fold into the assay solution.

*Circular dichroism and fluorescence spectroscopy*

CD measurements and fluorescence spectroscopy were performed by diluting the samples in metal-free 100 mM potassium phosphate buffer, pH 7.5. The cuvettes were rinsed with 20% $HNO_3$ followed by several rinses with Milli-Q water prior to addition of each sample.

CD spectra were the average of three scans measured at 1 nm/sec from 200–260 nm on an Aviv 6100 Spectrometer using a 0.2 cm path-length cuvette (Starna Cells, Inc.) at 25 °C. Samples were 1 mg/mL (25 μM) adenosine deaminase. The spectrum of the buffer was subtracted from all protein spectra, and the observed ellipticities were normalized for protein concentration.

The observed signal was converted to ellipticity from the formula

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

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 $dm^{-1}$ $mM^{-1}$.

Fluorescence spectroscopy was performed on a SLM 8100 spectrofluorimeter using an excitation wavelength of 290 nm and the emission was scanned from 300–400 nm at 1 nm/sec. Samples of 25 μM adenosine deaminase were prepared in a 1 cm path-length fluorescence cuvette (Starna Cells, Inc.). All spectra were normalized for protein concentration.

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