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A Pre-Transition-State Mimic of an Enzyme: X-ray Structure of Adenosine Deaminase with Bound 1-Deazaadenosine and Zinc-Activated Water†

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ABSTRACT: The refined 2.4-Å structure of adenosine deaminase, recently discovered to be a zinc metalloenzyme [Wilson, D. K., Rudolph, F. B., & Quiocho, F. A. (1991) *Science 252*, 1278–1284], complexed with the ground-state analog 1-deazaadenosine shows the mode of binding of the analog and, unexpectedly, a zinc-activated water (hydroxide). This structure of a pre-transition-state mimic, combined with that previously determined for the complex with 6(*R*)-hydroxy-1,6-dihydropurine ribonucleoside, a nearly ideal transition-state analog, sheds new understanding of the precise stereospecificity and hydrolytic catalysis of an important and well-characterized member of a large group of zinc metalloenzymes. As both of these excellent mimics were generated in the active site, they demonstrate a powerful means of dissecting the course of an enzymatic reaction by direct crystallographic analysis.

Three-dimensional structures of complexes of enzymes with catalytically relevant ligands have provided detailed understanding of enzyme mechanisms. While several X-ray structures of complexes with transition-state analogs have been analyzed, contributing key results, rarely have there been structures determined of complexes that closely mimic the pre-transition state. Here we report the refined X-ray structure of a mimic of the pre-transition state of adenosine deaminase.

Adenosine deaminase (ADA),[1] present in virtually all mammalian cells and a key enzyme in purine metabolism, catalyzes the irreversible hydrolysis of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively. ADA plays key roles in a variety of biological processes [briefly reviewed in Wilson et al. (1991)] and is particularly crucial in the development of the lymphoid system (Kredich & Hersfield, 1989). There are at least 80 compounds that bind to ADA; some are substrates, but many more are inhibitors that display different types of inhibitions (Zielke & Suelter, 1971; Schaeffer, 1971; Agarwal, 1982; Centelles et al., 1988). This makes the enzyme an excellent model for exploring the active site and catalytic mechanism. The function of ADA is also related to the fact that adenosine and several analogs have biological (e.g., neurotransmitters, modulators) (McIlwin, 1983; Schabe, 1981; Centelles et al., 1988) as well as a variety of pharmacological activities (e.g., antibiotics, anticancer, analgesics, coronary vasodilators) (Glazer, 1980; Agarwal, 1982; Dawick et al., 1988; Centelles et al., 1988).

The recent structure determination of the murine ADA (Wilson et al., 1991) led to four major discoveries concerning catalytic activity: (i) a Zn^{2+} cofactor is bound in the active site located in a deep pocket at the C-terminus of the β barrel; (ii) although ADA was crystallized in the presence of an excess of purine ribonucleoside, the ligand bound in the site is 6(*R*)-hydroxy-1,6-dihydropurine ribonucleoside (HDPR); (iii) the location of the zinc and key catalytic residues confer the precise stereospecificity of the site and the hydrolytic reaction; and (iv) the active site with bound HDPR is buried and made inaccessible to the bulk solvent by a hinged motion of one or two peptide loops that serve as lids to the active site pocket. The Zn^{2+} is pentacoordinated to the side chains of three His residues, one Asp residue, and the 6(*R*)-hydroxy of HDPR.

† Crystallographic coordinates have been deposited in the Brookhaven Protein Data Bank.

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[1] Abbreviations: ADA, adenosine deaminase, DAA, 1-deazaadenosine; HDPR, 6(*R*)-hydroxy-1,6-dihydropurine ribonucleoside.

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HDPR is the product of the enzyme-catalyzed hydration of purine ribonucleoside at the active site, representing the first half of the chemical reaction. Sequestering the HDPR in the active site pocket contributes to the stability of the transition-state analog. In addition to the zinc, the residues involved in ligand recognition, catalysis, and stereospecificity have been identified. The original structure determined at pH 4.2 (Wilson et al., 1991) is virtually identical to that subsequently determined at pH 6, the pH at which the enzyme activity begins to exhibit maximum activity (Sharff et al., 1992). Maximum activity extends to about pH 8.5.

Surmising that 1-deazadenosine (DAA) does indeed represent a nearly ideal "ground-state" analog (Ikehara & Fukui, 1974; Kurz et al., 1992), having all the attributes for molecular recognition of a substrate but incapable of being protonated by an acidic residue (Wilson et al., 1991) (a requirement in the catalytic reaction) because of the conversion of N1 of adenosine to C1, we embarked on the determination of the structure of its complex with ADA. The K_i value of DAA of 0.18 μM is about 100 times smaller than the K_m of adenosine (Kurz et al., 1992).

EXPERIMENTAL PROCEDURES

Crystals of the ADA–DAA complex were obtained by a procedure similar to that used for the ADA–HDPR crystallization (Wilson et al., 1988, 1991). They were grown by suspending a droplet containing 10 mg/mL ADA, 50 μM DAA, 3% poly(ethylene glycol) 6000, and 50 mM citrate, pH 4.2, over a well of 10.5% poly(ethylene glycol) 6000 in the same buffer. The recombinant enzyme was purified from an *Escherichia coli* strain that contained a plasmid with the cloned gene for murine ADA (Wilson et al., 1988).

Diffraction data were collected from a single ADA crystal at 4 °C on an ADSC two-area detector system mounted on a Rigaku RU-200 rotating anode X-ray generator (Cu Kα radiation) equipped with a graphite crystal monochromator and operated at 40 kV and 110 mA. The ADA–DAA complex crystal belongs to space group $C2$ with unit cell dimensions of a = 102.39 Å, b = 94.28 Å, c = 73.92 Å, and β = 127.05°. The asymmetric unit contains one ADA molecule of 40 000 Da. Merging of the 63 922 observations in the shell from 12 to 2.4 Å produced 20 925 unique intensities with an R-merge of 0.067.

The XPLOR suite of programs (Brünger, 1990) was used in the refinement of the ADA structure. The program CHAIN (Sack, 1988) was used in the density fitting, molecular modeling, and analysis of the structure.

RESULTS AND DISCUSSION

As the crystal of the ADA–DAA complex was isomorphous with that of the ADA–HDPR complex (Wilson et al., 1991), the structure determination and sbsequent refinement were straightforward. Initial ($|F_o| - |F_c|$) and ($2|F_o| - |F_c|$) maps, calculated with structure factors from the refined structure of the ADA–HDPR with the contribution of the HDPR and bound water molecules omitted (Wilson et al., 1991), were examined in order to identify any ligand in the active site and bound water molecules. These maps showed a large, well-resolved electron density in the enclosed active site to which a DAA model could be easily fitted in an orientation very similar to that observed for HDPR. They also revealed a new, well-defined spherical density very close to the Zn^{2+}, which indicates a zinc-bound water or hydroxide. After modeling the DAA and ordered water molecules as oxygen atoms (with the exception of the one bound to the zinc) to these maps, the structure was extensively refined using XPLOR to an R-factor of 0.187 and was used to calculate a new ($|F_o| - |F_c|, \alpha_c$) map. The zinc-coordinated water or hydroxide was then fitted accurately as an oxygen atom to the centroid of the difference peak, and the distance to the refined zinc position was measured to be 1.95 Å (in order to obtain a distance between the zinc and oxygen of the hydroxide that was unbiased by the Zn density during refinement and on which to set the distance restraint in the succeeding refinement). After the completion of the last round of refinement, the distance is 1.91 Å.

A value of 0.181 for the crystallographic R-factor was achieved for the refinement of the 2922 coordinates (2792 protein non-hydrogen atoms, one Zn^{2+}, 19 DAA atoms, and 110 water molecules modeled as oxygen atoms) against 20 925 reflections (with $F \geq 1\sigma_F$ and 97.6% of complete data) in the shell from 12 to 2.4 Å. The rms deviations from ideal geometry are 0.01 Å for bond distances and 1.71° for bond angles. Although the mean of the coordinate errors estimated by a Luzzati plot is about 0.2 Å, the finding that the bound DAA and adjacent residues have well-defined density and an averaged B-factor of about 10 Å^2 which is 3 times lower than for the entire ADA molecule indicates the error in the active site is significantly lower.

The refined structure of the complex shows the atomic interaction between ADA and DAA as well as a hydroxide bound to the zinc at a distance of 1.91 Å (Figures 1 and 2). The distance is shorter than the distances between the zinc and the other four coordinating atoms [Figure 2B; see also Wilson et al. (1991)]. The location of the zinc-bound hydroxide in ADA is clearly different from the zinc coordination position of the 6(R)-hydroxy of HDPR, which is 2.29 Å away from the zinc (Figure 2C) (Wilson et al., 1991). The distance of 1.91 Å between the zinc and hydroxide falls close to the low end of the range of 1.891–2.096 Å for 9 Zn^{2+}–hydroxide distances and shorter than the mean distance of 2.085(0.055) Å for 155 Zn^{2+}–waters observed in accurate small-molecule crystal structures surveyed in the Cambridge Structural Data Base (V4.10) (Allen et al., 1979).

The formation and stability of the hydroxide anion are further favored by the interactions with Asp^{295} and the now protonated His^{238} as well as a sequestered site (Figures 1 and 2B). Moreover, as discussed below, these interactions contribute to the stereospecific hydroxide addition in the catalytic mechanism. Although the crystals of ADA in the presence of 1-deazaadenosine reported herein or purine ribonucleoside (Wilson et al., 1991) were obtained at pH 4.2 where the enzyme in solution is only 20% active (Sharff et al., 1992), the long period of cocrystallization and data collection is more than ample time to generate the two complexes observed in the crystal structures (Figures 1 and 2). This includes the activation of the zinc-bound water that is required for the stereospecific hydroxide addition to the C6 of the purine ribonucleoside to produce HDPR (Wilson et al., 1991) as well as that observed in the ADA–DAA complex. While the pK_a of a zinc-bound water in an environment such as the one in the active site of ADA (Figure 1) is not known, nevertheless, it is expected to be quite low due to the combined enhancing influence of the zinc, Asp^{295}, and His^{238} as well as the hydrophobic effect. Even with the availability of the structure of the ADA–HDPR complex, it is very difficult to unambiguously assigned the several different pK_a values (in both the acidic and basic range) deduced from the effects of pH on activity to specific ionizing groups in the active site (Sharff et al., 1992). A zinc-coordinated water in model compounds

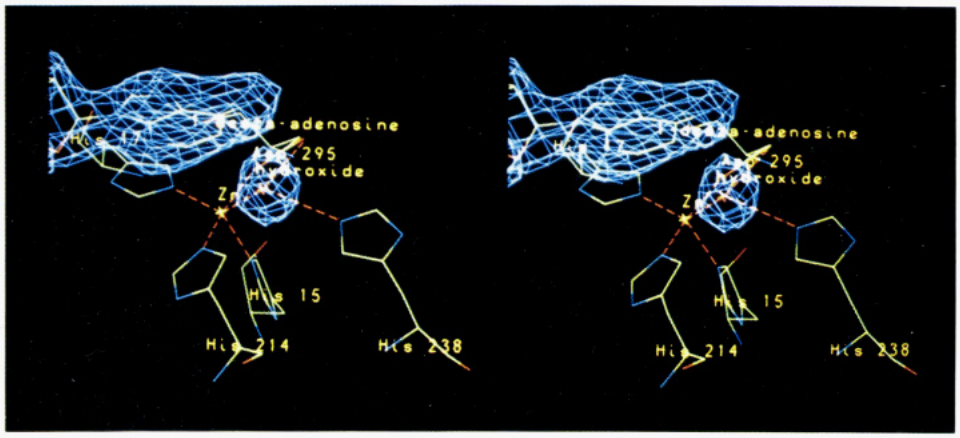
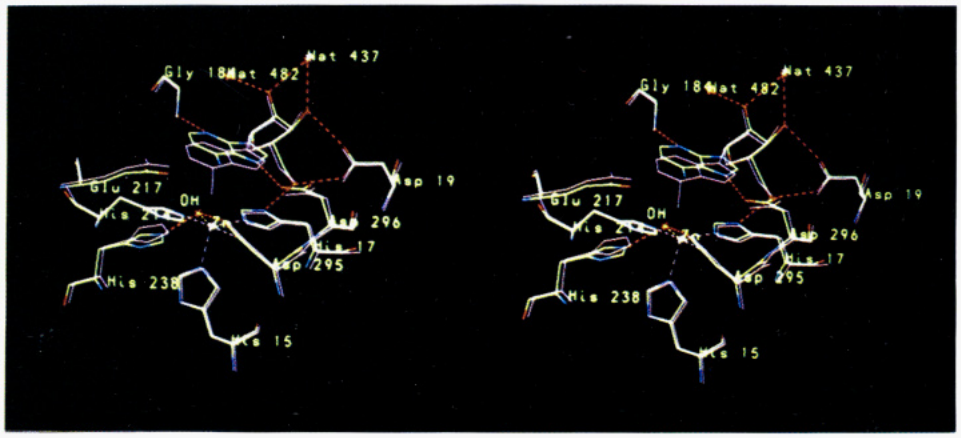


FIGURE 1: Polar interaction between adenosine deaminase, the zinc cofactor, and 1-deazaadenosine (DAA). Stereoview of the difference electron density surface (blue) showing for clarity only a portion (the purine ring) of DAA and zinc-bound hydroxide superimposed with the refined structure (color by atom types). Note that the difference density map was calculated with coefficients ($|F_o| - |F_c|$) and α_c phases calculated from the final refined structure with the exclusion of the contributions of the entire DAA molecule and the hydroxide modeled as oxygen. The difference map is contoured at 3 σ positive density levels and a grid spacing of 0.6 Å. Red dashed lines represent the hydrogen bond between the hydroxide and Asp[295] and zinc coordinations. More complete atomic interactions between ADA and DAA are shown in Figure 2. They are also schematically portrayed in Figure 2B.



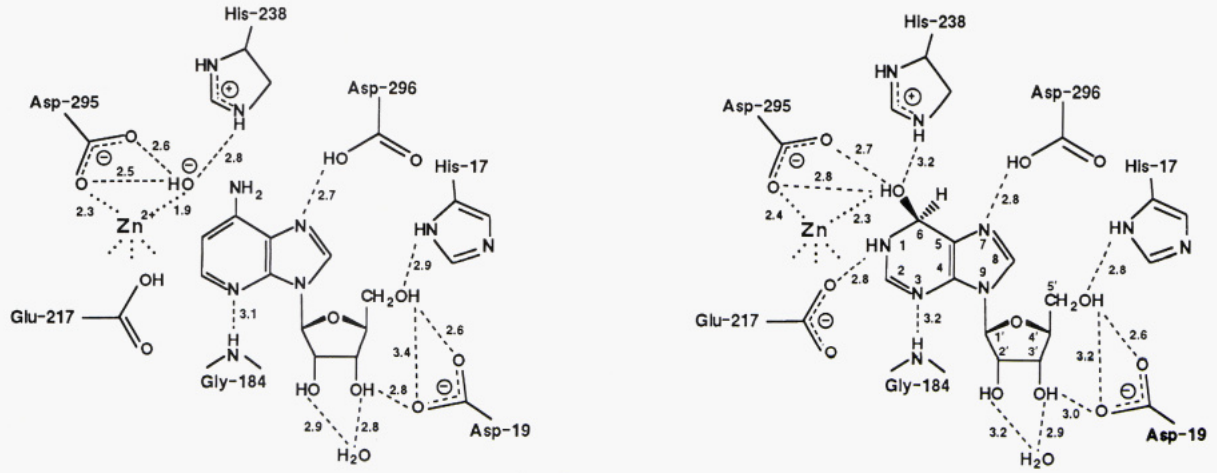


FIGURE 2: Complexes of adenosine deaminase with 2-deazaadenosine and 6(*R*)-hydroxy-1,6-dihydropurine ribonucleoside (HDPR). (A, top) Stereoview of the active site of the superimposed refined 2.4-Å structures of ADA–DAA (color by atom types) and ADA–HDPR (magenta) (Wilson et al., 1991). (B, bottom left) Schematic diagram of the ADA–DAA interaction. Numbers near dashed lines indicate distances (Å) between refined non-hydrogen atoms. Dotted lines represent coordinating interactions with the zinc. In addition to the hydroxide, the zinc is coordinated to the Nε2 atoms of His[15] (2.26-Å distance), His[17] (2.07 Å), and His[214] (2.04 Å) and the Oδ2 of Asp[295] (2.27 Å). (C, bottom right) Schematic diagram of the ADA–HDPR interaction as adapted from Wilson et al. (1991) using data from further refinement of the 2.4-Å structure.

can display a pK_a as low as 7 (Groves & Olson, 1985).

In both ADA–DAA and ADA–HDPR complexes, the coordinating and hydrogen-bonding interactions associated with Oδ1 and Oδ2 of the carboxylate of Asp[295] display the syn stereochemistry (Figures 1 and 2). It has been observed that the syn stereochemistry is preferred in both these types of interactions with carboxylate groups (Gandour, 1981; Carrell et al., 1988).

As shown in Figure 2, the binding of DAA to ADA is very similar to that observed for HDPR binding. With the

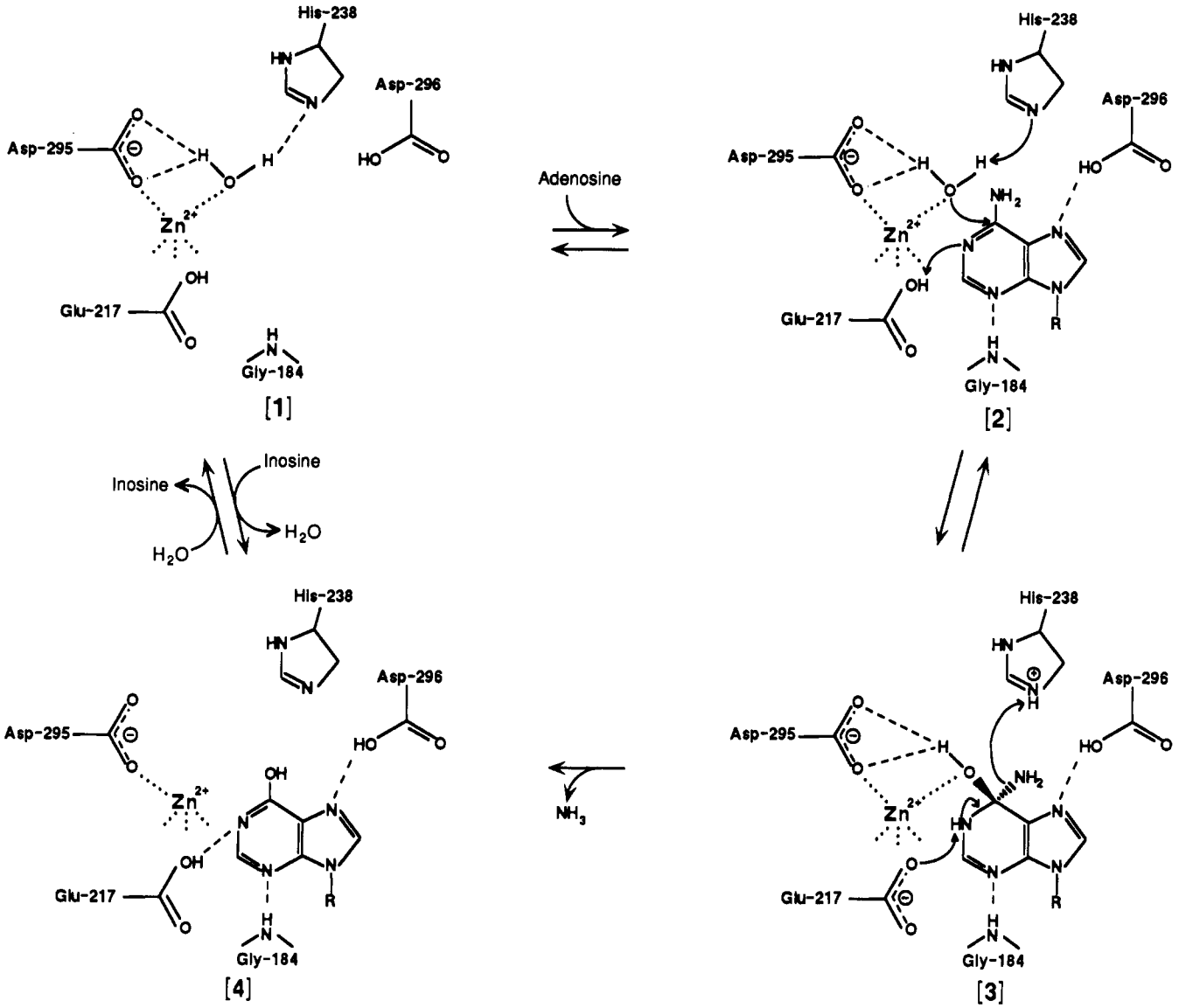


FIGURE 3: Mechanism of the reaction catalyzed by adenosine deaminase. See text for elaboration. It is also possible that a pre-transition state (in place of [2]) is formed by the binding of adenosine to ADA with an already zinc-bound hydroxide and a protonated His-238. In the bulk solvent inosine will exist almost entirely in the keto tautomer.

exception of the hydrogen bond between the N1 of HDPR and Glu^{217}, which is not possible with the C1 of DAA, all hydrogen bonds between ADA and HDPR are retained in the ADA–DAA complex. However, the superimposed structures show that the purine ring of DAA is slightly displaced from the position of the HDPR, away from Glu^{217}, and that His^{238} has moved closer to the hydroxide.

On the basis of the structure of the ADA–HDPR complex, a nearly ideal mimic of the transition state, we have determined the residues involved in substrate binding and proposed a mechanism of the enzyme-catalyzed stereospecific addition of hydroxide and elimination of ammonia (Wilson et al., 1991). Together with the structure of the ADA–DAA complex, several features of catalytic mechanism have been solidified and/or refined (Figure 3). Glu^{217} remains in an ideal alignment, because of its coplanarity with the purine ring (Figure 2), to donate a proton to N1 of adenosine, thereby reducing the N1 to C6 double bond character and making the C6 susceptible to nucleophilic attack and transformation from sp^2 to sp^3 in the tetrahedral adduct. There is very little doubt that a zinc-activated water or hydroxide is the attacking nucleophile. Our structure data do not support the proposed double displacement mechanism, analogous to that of the serine proteases, with an oxygen or sulfur atom of a side chain as the nucleophile (Orsi et al., 1972; Kurz & Frieden, 1987).

As the carboxylate side chain of Asp^{295} remains coordinated to the zinc and accepts a hydrogen bond from the Ser^{265} γ-OH in both complexes with DAA and HDPR, it becomes a much weaker base. Therefore, instead of Asp^{295} acting as a catalytic base as originally proposed (Wilson et al., 1991), its function is achieved by the hydrogen bonding with the water and the hydroxide (see also Figures 1 and 2B). Tieing down the hydrogen of the hydroxide away from the C6 of the substrate optimizes the geometry for the hydroxide addition to the C6 of adenosine; a lone pair on the hydroxide oxygen atom is aligned for the addition. This favorable geometry, together with the location of the zinc and Asp^{295} on the B-face side of the purine ring (Figures 1 and 2), dictates the precise chirality of the chemical reaction. Hydroxide attack occurs only on the B-face side of the purine ring, resulting in a 6(*R*)-hydroxy adduct as clearly demonstrated in the ADA–HDPR structure.

Activation of the water to hydroxide is greatly facilitated by the coordination of the oxygen to the powerful zinc electrophile, the hydrogen bonding to Asp^{295}, and the action

of His[238] as a general base in abstracting the proton (Figure 3). Thus, it is possible that substrates bind to ADA with an already zinc-activated water (hydroxide) and a proton abstracted by His[238], creating a true pre-transition state which takes the place of [2] in the mechanism (Figure 3).

The abstracted proton on Nε2 of His[238] makes sense as a donor to the N6 amino leaving group (Figure 3). However, while the location of the general base/acid His[238] (slightly on the B-face side of the purine ring) is very favorable for proton abstraction, it is less than ideal for proton transfer (Figures 1 and 2A). Model building of a 6(*S*)-amino on the C6 of the HDPR in the refined ADA–HDPR complex structure, in order to portray the complete transition-state intermediate (Figure 3, [4]), places the amino group (axially projecting out of the A-face side of the purine ring) in an environment that is highly nonpolar and lacking an ideally positioned proton donor group (Wilson et al., 1991). It also indicates that the Nε2 of His[238] is at a somewhat long distance (approximately 4.5 Å) from the N6 of the axial amino group.

An important feature revealed by the superpositioning of the ADA–DAA–hydroxide and ADA–HDPR structures (Figure 2) is the close proximity (about 2.5 Å) of the C6 of HDPR to the hydroxide at its position found in the ADA–DAA complex. This is an excellent distance of approach for hydroxide addition, assuming that the orientation of the purine ring of adenosine is identical to that of HDPR. Indeed, our previous results not only led to the unambiguous demonstration of the existence and structure of the bound HDPR, which appears to be extremely stable within the enclosed confine of the active site, but also indicated that the potent transition-state analog is the product of the stereospecific hydration of the purine ribonucleoside or of half of the chemical reaction (Wilson et al., 1991).

Kurz et al. (1992) have made a puzzling observation of a large solvent isotope effect on the K_i equilibrium constants of the binding of DAA and other ground-state analogs which presumably do not form a tetrahedral intermediate. We attribute this observation to an isotope effect on the activation of the zinc-bound water to hydroxide with the proton transfer to His[238] (Figure 2B) rather than, as suggested by Kurz et al. (1992), to a combined large transfer isotope effect on the free inhibitor and effect on the inhibitor bound in the active site. An alternative proposal is that the solvent isotope effect observed particularly on the binding affinity of the 1-deazanucleoside inhibitors might be due to a displacement of the zinc-bound water by the inhibitors (Kurz et al., 1992). This proposal is clearly not supported by our structural data (Figure 1).

Two features of the proposed catalytic mechanism of ADA (Figure 3) bear some resemblance to ones proposed for other well-studied zinc metalloenzymes (especially the carbonic anhydrase and the peptidases thermolysin and carboxypeptidase-A) that also involve the addition of a zinc-activated water to a carbon atom (Eriksson et al., 1988a,b; Matthews, 1988; Christianson & Lipscomb, 1989; Coleman, 1992). The important role of hydrogen-bonding interactions and geometry of the hydroxide coordinated to the zinc (Figures 1–3) applies to carbonic anhydrase as well. However, the more direct and simple interactions of the hydroxide with the general base/acid His[238] and Asp[295] residues in ADA (Figures 1 and 2) differ considerably from those observed in carbonic anhydrase. In carbonic anhydrase (Eriksson et al., 1988a,b; Merz, 1990; Coleman, 1992), the zinc-bound hydroxide [with slightly longer distance (2.1 Å) as reported here for ADA] is indirectly linked to a Thr-Glu hydrogen bond network which has a similar function as Asp[295] and to a His via a network of water molecules which mimics His[238]. In thermolysin and carboxypeptidase-A, it has been proposed that a general base (in this case a glutamate which is close to but not coordinated to the zinc) abstracts the proton of a presumed zinc-bound water which is then subsequently transferred to the leaving peptide bond amino group (Matthews, 1988; Christianson & Lipscomb, 1989; Coleman, 1992).

It is noteworthy that, to our knowledge, the refined structure of the adenosine deaminase reported here is the first to show a zinc-activated water in a complex with an almost perfect substrate analog. This structure, combined with that of the bound HDPR transition-state analog (Wilson et al., 1991), has enabled us to have a rare look at the precise stereospecificity and the mimics of two key intermediates of the catalytic reaction. The two structures also demonstrate a powerful and somewhat unique means for dissecting the anatomy of the catalytic reaction by using analogs that allow the different intermediates to be formed catalytically and trapped in the active site for direct crystallographic analysis.

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