Contribution of a Single Hydroxyl Group to Transition-State Discrimination by Adenosine Deaminase: Evidence for an "Entropy Trap" Mechanism†

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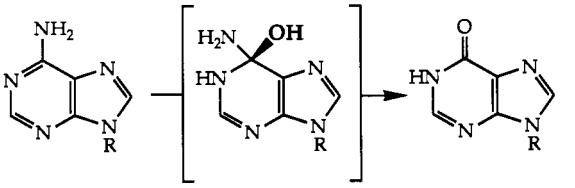
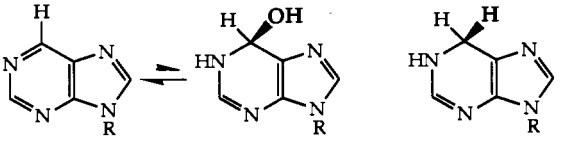
ABSTRACT: Adenosine deaminase was found to bind 6-hydroxy-1,6-dihydropurine ribonucleoside (II), formed by reversible addition of water to purine ribonucleoside (I) in a reaction analogous to formation of a tetrahedral intermediate in substrate deamination, with an apparent K_i value of 3×10^{-13} M at 20 °C. 1,6-Dihydropurine ribonucleoside (IV), synthesized by photolysis of purine ribonucleoside in the presence of $NaBH_4$, exhibited a K_i value of 5.4×10^{-6} M. After correction for differences between the relative free energies of solvation of II and IV, the 6-hydroxyl group of II was estimated to contribute more than 16 kcal to the free energy of binding, approaching the enthalpy of formation of a single hydrogen bond to a charged group in the vapor phase. The relatively weak binding of IV and of substrate water suggests that entropic effects, arising from the cooperative action of binding determinants contained within these separate molecules, contribute more than 10 kcal/mol to the free energy of binding of II in which these binding determinants are contained within a single molecule. In free solution, the entropy of reversible hydration of I was evaluated by measuring the temperature dependence of equilibria of protonation of I and of pseudobase formation from 1-methylpurinium ribonucleoside as −35 eu, comparable with the entropy of *activation* for the uncatalyzed hydrolysis of adenosine. In the active site of adenosine deaminase, this thermodynamic obstacle is evidently climbed spontaneously as a result of attractive interactions between the active site and the critical hydroxyl group at the 6-position. These findings accord with a mechanism in which binding interactions within a closely fitting active site supply the energy that is needed to overcome the unfavorable entropy change that would otherwise accompany the chemical combination of purine or adenosine with water.

The function of a catalyst is believed to depend on its ability to discriminate between the substrate in the ground state and the altered substrate in the transition state, binding the latter species more tightly and diminishing the difference in free energy that limits the rate of reaction (Polanyi, 1921). Enzymes have been observed to enhance reaction rates by as many as 17 orders of magnitude (Guthrie, 1977), and a simple algebraic analysis suggests that, during the central events in catalysis, increases in binding affinity are likely to match or even exceed this observed rate enhancement (Wolfenden, 1969a). This power of binding discrimination remains mysterious, because few bonds are typically formed or broken as a substrate passes from the ground state to the transition state. The present paper describes an attempt to analyze some of the forces responsible for transition-state discrimination by adenosine deaminase, using nonhydrolyzable analogues of a hypothetical intermediate that may approach the transition state in the enzyme-catalyzed reaction.

Adenosine deaminase catalyzes the hydrolytic removal of various leaving groups from 6-substituted purine ribonucleosides, as shown for adenosine in Scheme I. When a hydrogen atom takes the place of these leaving groups, hydrolysis cannot occur, and purine ribonucleoside (I, Scheme I) serves as a conventional competitive inhibitor (Wolfenden et al., 1969). A major upfield shift in the NMR resonance from C-6 indicates, however, that purine ribonucleoside is actually bound *not* in the form that is common in free solution (I, Scheme I) but in some other form that is sp^3 hybridized at C-6 (Kurz & Frieden, 1987). NMR and UV spectra of model adducts, recently prepared by addition of anionic nucleophiles to purine ribonucleoside quaternized at the 1-position, strongly suggest that the enzyme-bound inhibitor is present as a *covalent hydrate*, 6-hydroxy-1,6-dihydropurine ribonucleoside (II, Scheme I) (Jones et al., 1989). The covalent hydrate constitutes only 1 part in 10^7 of the total purine ribonucleoside that is present in free solution (Jones & Wolfenden, 1986), but since the inhibitor appears to be bound almost entirely as species II, enzyme binding evidently alters the position of *equilibrium* of the hydration reaction by a factor of at least 10^8.

Scheme I: Reaction Catalyzed by Adenosine Deaminase, Proceeding through a Tetrahedral Intermediate (III), and Competitive Inhibitors Formed by Covalent Hydration (II) and Photochemical Reduction (IV) of Purine Ribonucleoside (I)





The active diastereomer of II appears to be bound by adenosine deaminase with an affinity surpassing that of the natural products coformycin and 2′-deoxycoformycin and a K_i value (3×10^{-13} M) approximately 8 orders of magnitude

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lower than the K_m value for the substrate adenosine, the K_i value for the product inosine, and the estimated K_d value for unaltered purine ribonucleoside (Jones et al., 1989). The unusual affinity of the enzyme for II is presumably related to the enzyme's mechanism of action. After 1,6 addition of water, hydrated purine ribonucleoside II strongly resembles an sp^3-hybridized intermediate (III, Scheme I) that was postulated earlier to approach the chemical transition state for hydrolytic deamination of adenosine by a mechanism involving addition of water followed by elimination of the variable leaving group (Wolfenden, 1969b). Indeed, the only obvious difference between II and III is in the substitution of hydrogen for the leaving group, ammonia. As the enzyme shows remarkably little specificity with respect to the size or shape of the leaving group (Chassy & Suhadolnik, 1967), this substitution is expected to be well tolerated. In agreement with the likely structural analogy between II and III, a recent evaluation of the rate enhancement produced by adenosine deaminase suggests that II exhibits much of the binding affinity that might be expected of an ideal transition-state analogue.[1]

In view of this structural analogy, and its probable bearing on the mechanism of action of adenosine deaminase, it would be desirable to know what forces are responsible for the enzyme's exceptional affinity for II. The more than 10^8-fold tighter binding of compound II than of compound I is of particular interest, because the space-filling requirements of compound I are modest in comparison with those of compound II. In addition, compound I should be less difficult to extract from solvent water, a necessary part of the binding process. Structural features that distinguish II from I, and could aid the enzyme in making this distinction, include (a) the proton at N-1, which could form a hydrogen bond with the enzyme, (b) saturation of the 1,6 double bond, which results in modest distortion of the ring system (Shimazaki et al., 1983) and could alter noncovalent interactions with the enzyme, and (c) the hydroxyl group at C-6, which could form hydrogen bonds with the enzyme.

To obtain an indication of the relative importance of these various features of II, it seemed desirable to test the enzyme's affinity for 1,6-dihydropurine ribonucleoside (IV, Scheme I). If ring distortion or protonation at N-1 contributed substantially to the stability of the enzyme's complex with II, then the enzyme would also be expected to interact strongly with IV. Differences between the binding affinities of II and IV, on the other hand, should be largely attributable to the presence of the 6-hydroxyl group of II. This paper describes the synthesis and inhibitory properties of IV, together with an analysis of thermodynamic changes associated with the nonenzymatic hydration of I and with the binding of II by adenosine deaminase. The results, some of which have been described in a preliminary communication (Kati & Wolfenden, 1989), suggest that the presence of the 6-hydroxyl group in II makes a very large contribution to its observed binding affinity.

Materials and Methods

Calf intestinal adenosine deaminase (type III) and purine ribonucleoside were obtained from Sigma Chemical Co. Sodium borohydride, sodium borodeuteride, silica gel (Merck, grade 60), phenyltrimethylammonium tribromide, and adenosine were obtained from the Aldrich Chemical Co. UV spectra were obtained with a Hewlett-Packard 8452A diode array spectrophotometer, and ^1H NMR spectra were obtained with a Varian XL-400 NMR spectrometer. A positive chemical shift was used to denote a resonance at lower shielding with respect to $Si(CH_3)_4$.

To prepare 1,6-dihydropurine ribonucleoside (IV), an aqueous solution of ethanolamine (0.05 M, 50 mL) containing purine ribonucleoside (200 mg, 0.8 mmol) and sodium borohydride (2.4 mmol) was transferred under a nitrogen atmosphere to a cylindrical quartz reaction vessel (5 × 40 cm), mounted on a Buchi rotavapor device and thoroughly flushed with nitrogen. The quartz reaction vessel was then placed in a horizontal position and rotated at a speed of about 200 rpm. This resulted in the spreading of a thin film of the optically dense solution, facilitating complete exposure of the reactants to the UV light provided by four 15-W germicidal lamps. The apparatus was continuously purged with a stream of nitrogen during irradiation for 2 h, and any remaining borohydride was then destroyed by adding a 10-fold excess of acetone followed by stirring for 2 h without irradiation. The mixture of products was evaporated nearly to dryness, diluted with water (5 mL) and analyzed by reverse-phase HPLC on a Whatman Partisil M9 10/50 ODS-2 column, using a linear gradient from 0 to 15% methanol in water over 60 min at a flow rate of 4 mL/min. The properties of the major product, emerging at a methanol concentration of 12%, are described under Results.

Deuterated IV was prepared by a similar procedure, except for the use of sodium borodeuteride in place of sodium borohydride. To determine the site of deuteration, the purified product (0.16 mmol) was reoxidized with 0.20 mmol of phenyltrimethylammonium tribromide in methanol over a period of 10 min (Shimazaki et al., 1983) and purified by silica gel chromatography (2 × 24 cm, methanol). Recrystallization from methanol yielded 16 mg (8% overall) of a mixture that was found to contain purine and 6-deuteriopurine ribonucleoside, in approximately equal proportions as judged by proton NMR.

The pK_a value of the conjugate acid of IV was determined by measuring its UV absorbance at 252 or 300 nm as a function of changing pH, in a series of buffers (Ellis & Morrison, 1982) in the pH range 5.5–7.5 containing MES (0.05 M) and Tris (0.05 M) adjusted with HCl or KOH to the appropriate pH, together with 0.10 M NaCl. These experiments yielded an apparent pK_a value of 6.5 at 25 °C.

Equilibrium constants for hydration of purine ribonucleoside were estimated as described by Jones et al. (1989), from equilibrium constants for the dissociation of water, for protonation of purine ribonucleoside and for pseudobase formation by hydroxide ion addition to the 1-methylpurinium ribonucleoside cation (see Results), using the equations shown in Scheme II. Values of K_1, the ionization constant of water, were obtained from the International Critical Tables and divided by 55.5 to allow expression in terms of the actual molarity of water. Values of K_2 were calculated from the dissociation constant of the conjugate acid of purine ribonucleoside, determined spectrophotometrically at 262 nm in HCl solutions adjusted with KCl to an ionic strength of 0.1. Values of K_3 were determined as the formation constant (expressed in terms of the concentration of OH^-) of the pseudobase of 1-methyllpurinium ribonucleoside iodide, determined spectrophotometrically at 286 nm in a series of 0.1 M Tris-HCl/0.1 M ethanolamine solutions over the pH range 7.1–10.6. The limited lifetime of the pseudobase (Jones et al., 1989) required extrapolation of the absorbance readings to

[1] From the rate enhancement produced by calf intestinal adenosine deaminase, the maximal K_i value of an ideal transition-state analogue of this enzyme, acting on adenosine, has been estimated as approximately 10^{-16} M (Frick et al., 1987).

the time of mixing. Best values of K_a, A_{acid}, and A_{base} were obtained by nonlinear regression analysis (Duggleby, 1984) of titration data from

$$A = (A_{acid} + A_{base}K_a/a^{H^+})/(1 + K_a/a^{H^+}) \quad (1)$$

where A is the observed absorbance, a^{H^+} is the hydrogen ion activity, A_{acid} is the absorbance of a solution containing positively charged ligand, A_{base} is the absorbance of a solution containing neutral ligand, and K_a is the acid dissociation constant. Values for a^{H^+} in Tris/ethanolamine buffers were corrected for temperature by a factor of 0.030 pH unit/deg (Ellis & Morrison, 1982).

Scheme II

water addition $>C{=}N- + HOH \overset{K_{addition}}{=} >C(OH)NH-$

first step $HOH \overset{K_1}{=} H^+ + OH^-$

second step $>C{=}N- + H^+ \overset{K_2}{=} >C{=}NH^+$

third step $>C{=}NH^+ + OH^- \overset{K_3}{=} >C(OH)NH-$

$$K_{addition} = [>C(OH)NH-]/[HOH][>C{=}N-] = K_1K_2K_3$$

Initial rates of enzymatic deamination of adenosine were measured over the temperature range 20–35 °C with cuvettes of 1.0-cm light path in potassium phosphate buffer (0.05 M, pH 7.5) containing variable concentrations of adenosine and inhibitor in a total volume of 3 mL. Reactions were initiated by addition of calf intestinal adenosine deaminase (0.063 unit) to thermally equilibrated cuvettes, and reaction rates were measured by following the decrease in absorbance at 265 nm with time. To obtain information about inhibitors, rates of reaction were measured at a minimum of four substrate concentrations, at each of four inhibitor concentrations. Slopes of double reciprocal plots were then replotted as a function of changing inhibitor concentration, and K_i values were obtained by linear regression analysis (Segel, 1975).

Results

1,6-Dihydropurine Ribonucleoside. 1,6-Dihydropurine ribonucleoside does not appear to have been described before, and efforts to prepare it by conventional methods of reduction of purine ribonucleoside were unavailing.[2] In view of the susceptibility of purine and its ribonucleoside to photochemical alkylation reactions (Connolly & Linschitz, 1968; Evans & Wolfenden, 1970), we decided to investigate the products of photolysis of purine ribonucleoside in water in the presence of sodium borohydride. In the presence of ultraviolet light, but not in its absence, borohydride reduction of I was found to proceed smoothly with generation of a major product absorbing maximally at 299 nm.

The major product of photochemical reduction (see Materials and Methods) was obtained by evaporation of the material emerging from the HPLC column in 12% methanol as a tan solid (40 mg, 25% yield). UV spectra (in water) λ_{max} 299 nm (log ϵ = 3.55); ^{1}H NMR (ppm, in Me_2SO-d_6) 7.58 (d, 1 H, D_2O exchanges, NH), 7.45 (s, 1 H, H-8), 6.89 (d, becomes s with D_2O exchange, 1 H, H-2), 5.95 (br s, D_2O exchanges, ribose OH), 5.47 (d, 1 H, H-1′), 5.29 (d, D_2O exchanges, ribose OH), 5.04 (d, D_2O exchanges, ribose OH), 4.61 (s, 2 H, H6), 4.41 (q, 1 H, H-2′), 4.00 (q, 1 H, H-3′), 3.88 (q, 1 H, H-4′), 3.50 (m, 2 H, H-5′). Fast atom bombardment with positive argon ions gave a mass/charge ratio of 255 ($M + H^+$). The conjugate acid of this compound exhibited a pK_a value (6.5) and ^{1}H NMR and UV absorption spectra similar to those observed for 6-alkylated 1,6-dihydropurine derivatives (Connolly & Linschitz, 1968; Evans & Wolfenden, 1970), suggesting that reduction had occurred at the 1,6-position to yield 1,6-dihydropurine ribonucleoside.

When purine ribonucleoside was treated with $NaBD_4$ in place of $NaBH_4$ and then reoxidized, the ^{1}H NMR spectrum of the product was identical with that of purine ribonucleoside except for a decrease in the intensity of the proton resonance at the 6-position (Chenon et al., 1975). Thus, purine ribonucleoside and [6-^{2}H]purine ribonucleoside were produced in roughly equivalent proportions, indicating that the 1,6 C=N bond had been reduced and reoxidized with little stereospecificity. From the earlier observation that the pK_a value associated with N-1 of the conjugate acid of purine is 2.39 in the ground state, but 5.1 in the lowest excited triplet state (Aaron & Winefordner, 1973), it seems probable that electron density may be shifted toward the nitrogen in the excited triplet state, rendering C-6 more electrophilic and susceptible to attack by borohydride. Flavins are known to be reduced by a number of amino acids and nitrogen-containing buffers in the presence of light (Frisell et al., 1958; Heelis et al., 1987), suggesting that photochemical reduction of heteroaromatic molecules may be a general phenomenon.

Equilibria and Temperature Dependence of Binding of Nucleosides. Equilibrium constants for the dissociation of water (K_1), for protonation of purine ribonucleoside (K_2), and for pseudobase formation from 1-methylpurinium ribonucleoside (K_3), which were used to calculate the equilibrium constant for purine ribonucleoside hydration according to

Table I: Equilibrium Constants Used for Calculating K_{add}

temp (°C)	K_1 ($\times 10^{16}$ M)	K_2 (M^{-1})	K_3 ($\times 10^{-5}$ M)	K_{add} ($\times 10^9$ M^{-1})[a]
11.1	0.575	170	3.16	3.09
17.0	0.977	132	2.24	2.89
23.0	1.55	112	2.19	3.80
30.1	2.69	100	1.07	2.88
38.1	4.68	95.5	0.832	3.72
43.1	6.46	102	0.562	3.70
48.1	8.71	93.3	0.490	3.98

[a] $K_1K_2K_3$ as described in Scheme II.

Table II: Enzyme/Nucleoside Dissociation Constants as a Function of Temperature

temp (°C)	1,6-dihydropurine ribonucleoside (IV) (μM)	6-hydroxy-1,6-dihydropurine ribonucleoside (II) (pM)	adenosine (μM)	(8*R*)-2′-deoxycoformycin (V) (pM)
20.0	5.39	0.303	21.5	
22.5	7.30	0.432	23.5	2.5[a]
25.0	7.77	0.567	26.5	
27.5	9.12	0.644	28.5	
30.0	9.57	0.962	36	
32.5	10.3	1.13	36	
35.0	11.7	1.40	39.5	

[a] At room temperature (Agarwal et al., 1977).

[2] Trace quantities of the aglycon of IV have been generated in small quantities by controlled-potential reduction of unsubstituted purine at a massive mercury electrode (Smith & Elving, 1962). This was not attempted with the nucleoside because of the small yield and known susceptibility of deaminases to inhibition by mercurials (Wolfenden et al., 1967). Purines containing an electron-withdrawing group at the 8-position (e.g., 8-azapurine and 8-trifluoromethylpurine) can be reduced to the corresponding 1,6-dihydropurine by catalytic hydrogenation (Albert, 1981; Albert & Pendergast, 1972).

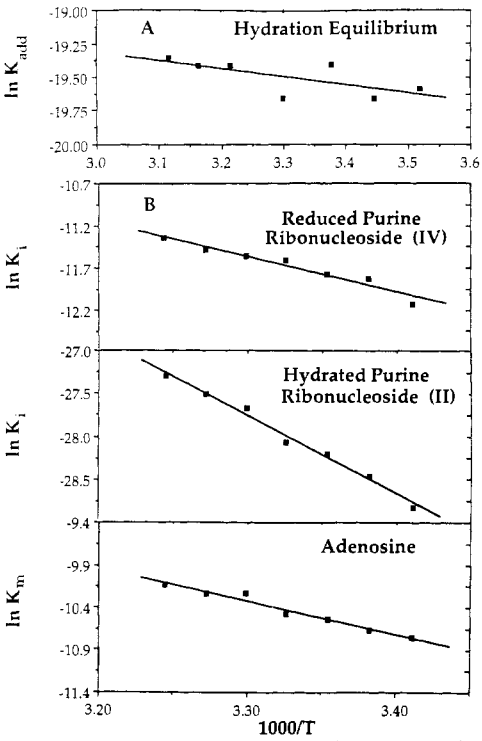


FIGURE 1: van't Hoff plots illustrating the dependence of the equilibrium constants as a function of changing temperature. (A) Equilibrium constant for 1,6 hydration of purine ribonucleoside. Values for K_{add} were obtained from Table I. (B) Apparent equilibrium constants for the dissociation of nucleosides from adenosine deaminase: (top panel) measured K_i values for compound IV; (middle panel) calculated K_i values for the active isomer of compound II (see text); (bottom panel) apparent K_m values for adenosine.

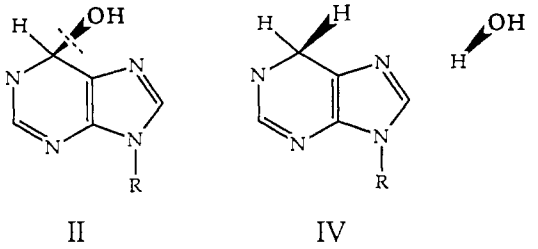
Scheme II, are listed in Table I. Figure 1A shows a van't Hoff plot of the temperature dependence of equilibrium constants for purine ribonucleoside hydration calculated from these results. Linear regression analysis yielded $\Delta H = 1.2 \pm 0.6$ kcal/mol and $\Delta S = -34.9 \pm 2.0$ cal/(mol deg).

Steady-state kinetic experiments at pH 7.5 showed that both II and IV served as competitive inhibitors with respect to adenosine. The observed temperature dependence of K_i values for II and IV and of K_m values for adenosine are shown in Table II and as van't Hoff plots in Figure 1B. The top panel shows the temperature dependence of K_i values observed for IV, yielding $\Delta H = 8.3 \pm 0.90$ kcal/mol and $\Delta S = 4.6 \pm 2.9$ cal/(mol deg). The middle panel shows the temperature dependence of corrected K_i values for the active isomer of II, calculated from

$$K_i = K_d K_{eq}/2 \quad (2)$$

where K_i = corrected dissociation constant of the EI complex containing the active isomer of II, K_d = apparent K_i value of I, and K_{eq} = equilibrium constant for conversion of I to II in free solution (i.e., 55.5 K_{add} from Table II). A numerical correction factor of 2 was included in eq 2 on the assumption that only one isomer of II is bound by the active site, as expected from the known stereoselectivity of the enzyme as a catalyst for pteridine hydration (Evans & Wolfenden, 1972), and with respect to binding of the diastereomers of 2′-deoxycoformycin (Schramm & Baker, 1985; see Discussion). These results yielded values of $\Delta H = +18.1 \pm 0.9$ kcal/mol and $\Delta S = +4.6 \pm 2.9$ cal/(mol deg) for the binding of the active isomer of II by adenosine deaminase. The bottom panel shows the temperature dependence of the K_m value of adenosine, yielding $\Delta H = +7.7 \pm 0.65$ kcal/mol and $\Delta S = +4.9 \pm 2.2$ cal/(mol deg).

Chart I: 6-Hydroxy-1,6-dihydropurine Ribonucleoside (II) and Its Parts, As Represented by 1,6-Dihydropurine Ribonucleoside (II) and Water, Obtained by Cleaving the C–O Bond at the 6-Position[a]



II IV

[a] Note that these parts can occupy, individually, any space occupied by II.

In summary, comparison of the observed K_i values for II and IV at 25 °C (Table II) indicates that the presence of the 6-hydroxyl substituent of II contributes a factor of approximately 1.4×10^7, or −9.8 kcal/mol, to its binding affinity. Despite major differences in apparent binding affinity between adenosine, 1,6-dihydropurine ribonucleoside, and 6-hydroxy-1,6-dihydropurine ribonucleoside, all three nucleosides appear to be bound by adenosine deaminase with an overall *loss* of entropy of −4.6 to −4.9 cal/(mol deg) (Table II).[3]

Discussion

Mechanism of Binding of 6-Hydroxy-1,6-dihydropurine Ribonucleoside (II). Combination of the enzyme with a species as rare as the hydrate would be expected to result in slow onset of inhibition, but no delay is observed in the onset of inhibition by I (Kurz & Frieden, 1987; Jones et al., 1989). This indicates that inhibition does not depend on such infrequent encounters but rather that 6-hydroxy-1,6-dihydropurine ribonucleoside (II) is usually generated by attack of water on I within the active site of adenosine deaminase. The equilibrium process by which II is formed from I is probably analogous to the quasi-equilibrium process by which a near-tetrahedral intermediate III, approaching the transition state in structure, is formed from adenosine in the normal catalytic process (Scheme I). That analogy is supported by the fact that II captures a substantial fraction of the minimal binding affinity expected of an ideal transition-state analogue for this reaction [K_d = approximately 10^{-16} M (Frick et al., 1987)] and that II differs from III only in replacement of the variable leaving group by a hydrogen atom. This latter substitution is expected to be well tolerated, since the enzyme catalyzes the departure of widely varying leaving groups at similar rates (Chassy & Suhadolnik, 1967; Wolfenden, 1967, 1969b).

We therefore sought to analyze the remarkable affinity of II in terms of its constituent parts: reduced purine ribonucleoside and its 6-hydroxyl group. In addition, it seemed desirable to determine whether the highly unfavorable free energy of 1,6 hydration of purine ribonucleoside (and, by extension, adenosine) is primarily enthalpic or entropic in origin. Evidently the enzyme is designed to overcome this obstacle, which also arises during the deamination of adenosine, since the position of equilibrium is at least 10^8-fold more

[3] The observed entropy loss of about −5 eu for the binding of all three ligands should not be overinterpreted as the true entropy loss that the nucleoside experiences when it is bound by adenosine deaminase. In dilute aqueous solution the true ΔH and ΔS values are perturbed by the release of constrained water molecules during ligand binding that give a positive conribution to the observed ΔS value and by changes in the conformation of the protein that may alter solvent structure (Jencks, 1981). However, for a series of related ligands these perturbing factors are expected to be similar in magnitude. Thus, the true value for ΔS is probably about the same for the binding of II, IV, and adenosine.

favorable in the enzyme's active site than in free solution.

Binding Affinity of II: Comparison with IV and Water. In view of the likelihood that formation of II at the active site resembles a partial reaction in catalysis, it would be desirable to understand its high affinity for the enzyme in structural and thermodynamic terms. This question can be approached by breaking II into two parts, IV and a water molecule, as shown in Chart I. When a ligand such as II is broken into two parts, its observed free energy of binding is not expected to be equivalent to the sum of the individual free energies of binding for the component parts (Page & Jencks, 1971; Jencks, 1981). This situation is more appropriately described by

$$\Delta G_{II} = \Delta G_{IV} + \Delta G_{H_2O} - \Delta G_S \quad (3)$$

in which ΔG_{II}, ΔG_{IV}, and ΔG_{H_2O} are the observed free energies of binding of II, IV, and a water molecule, respectively. It should be noted that IV and a water molecule must, individually, be able to bind to the enzyme in the space that would accommodate II. In eq 3, the term ΔG_S includes differences in entropy between II and its constituent parts and arises from any change in the probability of binding these two parts (IV and a water molecule) when they are joined by a covalent bond to form II.

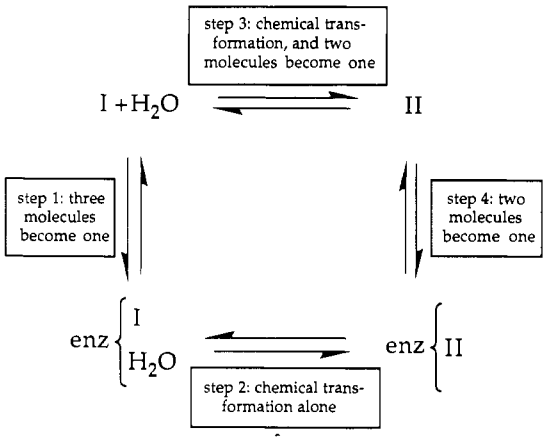
The present results indicate that II is more tightly bound than IV by a factor of 1.7×10^7, so that the difference in free energy of binding between II and IV amounts of −9.8 kcal/mol. The free energy of binding of substrate water is not known for this or any other hydrolytic enzyme.[4] Because water is abundantly available, it seems reasonable to guess that the "binding affinity" of water, ΔG_{H_2O} may lie in the range between +1.0 and +3.8 kcal/mol, corresponding to a K_m value lying between 5 and 550 M. Substitution of these values into eq 4 yields estimated values of ΔG_S lying in the range between +10.8 and +13.6 kcal/mol.

The contribution of the reduced 1,6 double bond, including ring distortion and the presence of a proton at N-1, could be determined if it were possible to compare the binding affinity of IV with that of *intact* purine ribonucleoside (I), as shown in eq 4. In this equation, ΔG_I represents the overall free

$$\Delta G_{IV} = \Delta G_I + \Delta G_R \quad (4)$$

energy of binding of I, ΔG_R represents the combined contribution of the reduced 1,6 double bond and the N-1 proton, and ΔG_R is assumed to be similar in magnitude for IV and II.[5] The present results show that $K_i = 7.6 \times 10^{-6}$ M for IV, corresponding to $\Delta G_{IV} = -7.0$ kcal/mol. The binding affinity of I is not directly accessible, since purine ribonucleoside does not appear to be bound unhydrated to a significant extent. However, the apparent K_i value of purine ribonucleoside is approximately 10^{-6} M, and since the bound nucleoside appears to be more than 90% hydrated (Kurz & Frieden, 1987), the true K_d value of species I appears to be larger than 10^{-5} M. K_m values for a variety of 6-substituted ribonucleosides lie between 7×10^{-4} M and 5×10^{-5} M (Chassy & Suhadolnik, 1967), so that it seems reasonable to suppose that the true K_d value of species I falls in the same range and that ΔG_I lies between −4.1 and −5.9 kcal/mol. Thus, ΔG_R may contribute −1.1 to −2.9 kcal/mol toward the binding affinity of II.[6]

Scheme III: Possible Mechanism for Hydration of I at the Active Site[a]



[a] I and reactant water are bound first, with much loss of entropy, followed by their chemical combination in a second step. Much more entropy is lost when the chemical reaction occurs in solution than when it occurs at the active site.

In summary, the reduced 1,6 double bond appears to contribute relatively little (ca. −2 kcal) to the free energy of binding of II, whereas the greater part (ca. −12 kcal) of its exceptional affinity arises from differences in entropy between II and its constituent parts, reflecting the increased probability of binding these two parts (IV and a water molecule) when they are joined by a covalent bond to form II. This large entropic contribution suggests that the 6-hydroxyl group of II might be bound by the enzyme with no further loss of entropy, allowing most of the intrinsic binding energy of the 6-hydroxyl group to be manifested as observed binding affinity (Jencks, 1981). This interpretation is supported by experimental data which indicate that both II and IV are bound by the enzyme with roughly the same entropy loss.

Entropy Changes during Generation of II from I and

[4] Because ground-state stabilization tends to be counterproductive for catalysis, substrate water would *not* be expected to be bound tightly. Although not measurable directly, K_m values for substrate water in excess of 10 M have been estimated for glycosidases, on the basis of studies in mixed solvents (Nelson & Schubert, 1928) and on extrapolation from apparent dissociation constants observed for glycosyl acceptors other than water (Dale et al., 1985). From a physiological standpoint, K_m values of this magnitude appear reasonable for hydrolytic enzymes in general, if [as suggested by Cleland (1967)] evolutionary pressures have tended to result in enzymes that exhibit K_m values that equal or exceed the usual physiological concentration of the substrate. If, as seems probable, charged groups are located at the active site of adenosine deaminase (see text), then "bound" water molecules are likely to be present in positions appropriate for reaction. It will be impossible in principle to identify any of these rigorously as that water molecule which serves as the actual substrate (as opposed to water molecules attacking directly from solution), because pathways from ground states to transition states are operationally unobservable (Hammett, 1970). In practice, however, the structures of enzyme–ligand complexes may be found to reveal the presence of a single water molecule, with the active site "closed off" in such a way as to suggest no reasonable alternative to attack by that water molecule in the ES complex [see discussion of thermolysin by Monzingo and Matthews (1984)].

[5] Hydroxyl substituents are somewhat electron withdrawing, and the resulting electronic effects could influence the relative ability of N-1 to act as a proton donor in hydrogen bonding to groups in the active site when II and IV are compared. II would be expected to be the stronger donor, but these effects are not expected to be large. For example, glycolic acid is a stronger acid than acetic acid, and ethylene glycol is a stronger acid than ethanol; but the differences in pK_a values amount to less than 1 unit (Jencks & Regenstein, 1968). In addition, free energies of solvation suggest that the strengths of hydrogen bonds tend to vary as an insensitive function of the acidities of the proton donor, as suggested from values compiled for phenols by Hine and Mookerjee (1975) and by Brønsted β values in the neighborhood of 0.2 for quinuclidines (Jencks et al., 1986). Therefore, it seems probable that differences in hydrogen bond strengths involving the 1-NH group may contribute only a few tenths of a kilocalorie, at most, to adenosine deaminase's preference for II.

[6] Weiss et al. (1987) have suggested that an enzyme thiol group may interact with the N-1 of adenosine during the catalytic process, so that an enzyme thiolate group could be responsible for this modest attraction.

Reactant Water. The rate of onset of inhibition indicates that II is formed after I combines with the active site of adenosine deaminase (Kurz & Frieden, 1987). Its subsequent hydration involves reaction with a water molecule that may come either from a position in which it may be weakly bound at the active site or directly from solution. From a thermodynamic standpoint, this mechanistic distinction is not significant, since the structures of the starting materials (purine ribonucleoside, water, and enzyme) and product (enzyme-bound II) are the same in either case. For the sake of argument, we will assume that complex formation occurs by the first of these alternative mechanisms.[4]

According to this mechanism, hydration at the active site can be considered to involve two steps, as shown in Scheme III. In step 1, compound I and reactant water would be bound by the active site in a position and orientation appropriate for reaction. In this first step, each substrate would be bound with an unfavorable loss of translational and rotational entropy, needed to fix each molecule at the active site; the free energy needed to fix these molecules at the binding site would be supplied (or "paid for") by part of the binding energy itself. In step 2, occurring within the active site, compound I and the reactant water molecule would combine to form II. With the reactants fixed in place, and no need for a further major loss of entropy, this second step could then proceed with a free energy change determined mainly by differences between the inherent free energies of bonds present in the reactants and product.

In solution, the free energy change that accompanies the nonenzymatic hydration of I (step 3, Scheme III) includes contributions from the free energies of the bonds that are formed and broken and also from any loss of entropy that accompanies the addition reaction, in which two molecules combine to form a single product. The position of equilibrium is expected to be correspondingly less favorable in solution than at the active site by a factor that includes any difference in entropy between product II and the substrates, I and water. Consequently, as the cycle shown in Scheme III suggests, II is expected to be *bound* more tightly than the sum of I plus water (step 4 minus step 1) by a factor that includes this same entropy term [see discussion by Jencks (1975)].

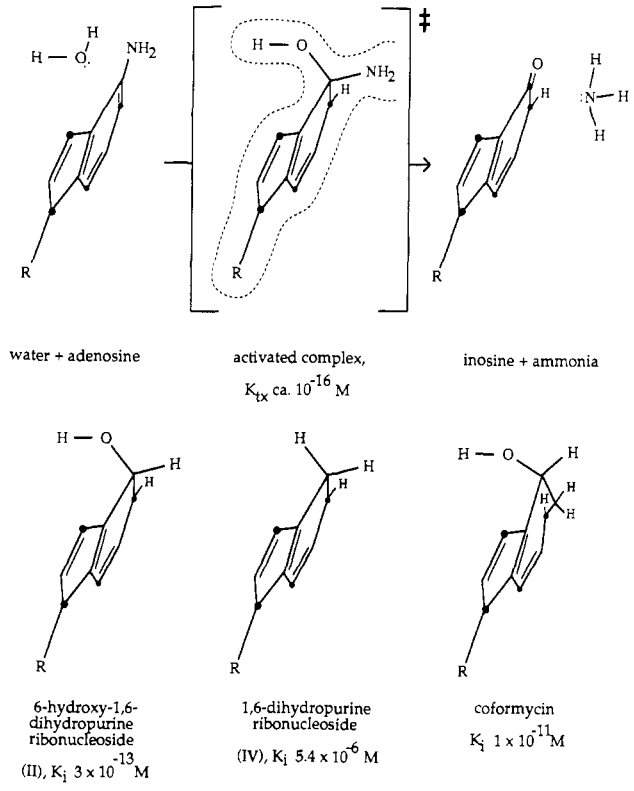
Because this entropic term might constitute a major thermodynamic difference between steps 2 and 3, we sought to determine the actual amount of entropy that is lost when purine ribonucleoside is hydrated in free solution (step 3). van't Hoff plots of equilibria of successive uptake of a proton and a hydroxide ion by I, in conjunction with the temperature dependence of the dissociation of water, show that ΔH for step 3 is small (+1.2 kcal/mol) whereas ΔS is highly unfavorable, approximately −35 cal/(mol deg) in dilute solution.[7] If that loss in entropy ($T\Delta S$ = −10.5 kcal/mol at 25 °C) could be completely avoided by "fixing" the reactants in the active site in a position appropriate for reaction in step 2, then entropic effects alone could account for most of the factor of at least 10^8 by which the equilibrium of hydration is enhanced at the enzyme's active site (Jones et al., 1989).

Combined Entropy Results. The combined observations suggest, first, that II is bound very much more tightly than its constituent parts, as represented by IV and a water molecule, and, second, that II is bound very much more tightly than I plus a water molecule. In both cases an entropic term contributes at least 10.5 kcal/mol in favorable free energy of binding. This coincidence seems unlikely to be fortuitous. If decomposition of II to form I and a water molecule resulted in the loss of a major entropic contribution to the binding of II, then breaking of the C-6–OH bond in a slightly different way to form IV and a water molecule might be expected to result in a similar disappearance of this entropic advantage. The fact that these values are similar suggests that the loss of rotational and translational entropy when I is bound by the enzyme resembles in magnitude the loss of rotational and translational entropy when IV is bound. This may not be surprising, since I and IV differ by only two molecular weight units, and entropy differences exhibit only a small dependence on changing molecular size (Page & Jencks, 1971). The present results indicate that II, IV, and adenosine are bound by adenosine deaminase with a loss of about −4.6 eu, and it seems probable that the entropy loss that accompanies binding of I may be similar in magnitude.

Whether or not one chooses to assume that the reacting water molecule is bound before it attacks I, it seems clear that a great deal of entropy is lost when I is hydrated to form II and that a large part of the catalytic activity of adenosine deaminase arises from its ability to act as an "entropy trap", using binding energy to overcome the unfavorable entropy needed for hydration of the purine ring system.

Mechanism of Inhibition by 2'-Deoxycoformycin. Scheme IV shows the structural relationship between compounds believed to be involved in the catalytic action of adenosine deaminase and several inhibitors. These inhibitors include II and IV and coformycin, a natural product with a K_i value of

Scheme IV: Structural Relationship between Adenosine Deaminase Ligands, Showing the Estimated Dissociation Constants of Their Enzyme Complexes[a]



[a] Ring nitrogen atoms are indicated by the symbol ●. The affinity of tetrahedral intermediate III, which may approach the transition state in deamination, was estimated from the approximate rate enhancement observed for adenosine deaminase (Frick et al., 1986). The stereochemical arrangement of the 6-hydroxyl group is assumed to be the same in all cases as in the active isomer of coformycin (Nakamura et al., 1974).

[7] Entropies of comparable magnitude have been reported for the covalent hydration of chloral (Bell & McDougall, 1960; Gruen & McTigue, 1963) and isobutyraldehyde (Gruen & McTigue, 1963).

approximately 10^{-11} M. The 2′-deoxy derivative of coformycin is somewhat more tightly bound that coformycin itself (Agarwal et al., 1977). These structures are shown with the 6-hydroxyl stereochemistry that has been established for the active isomer of coformycin (Nakamura et al., 1974), from the perspective used to present the crystal structure of a related inhibitor (Shimazaki et al., 1983).

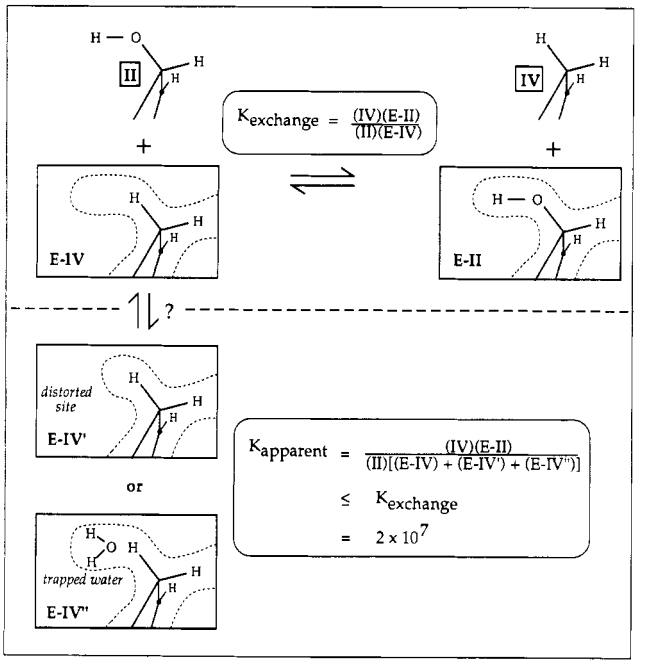
The naturally occurring 8*R* isomer of 2′-deoxycoformycin, an analogue of adducts II and III, inhibits adenosine deaminase with a K_i value of 2.5×10^{-12} M (Agarwal et al., 1977), whereas the 8*S* isomer exhibits a K_i value of only 3.3×10^{-5} M (Schramm & Baker, 1985). Steric hindrance by the 8*S*-hydroxyl group might account for this sharply reduced binding affinity. However, the 8*S* position corresponds to the position that would be occupied by the variable leaving group in substrate hydrolysis. The enzyme serves as an efficient catalyst for the hydrolytic removal of a variety of leaving groups (e.g., OCH_3, $NHCH_3$, $NHNH_2$) that have more pronounced space-filling requirements than a hydroxyl group (Chassy & Suhadolnik, 1967). Thus, there is no reason to expect steric hindrance from the hydroxyl group of the 8*S* isomer. For that reason, it seems likely that the preferential binding of the 8*R* isomer of 2′-deoxycoformycin is not due to steric hindrance of the 8*S* isomer but to attraction of the critical hydroxyl group of the 8*R* isomer by the active site. The force of this attraction (−9.6 kcal/mol) is comparable with the force of attraction of the −OH group of II inferred from the present results, and the factors responsible for its manifestation seem likely to be similar in both cases. Thus, it seems probable that in 2′-deoxycoformycin, and to an even greater degree in II, the critical hydroxyl group of the inhibitor is brought into nearly ideal contact with the active site.

Intrinsic vs Observed Binding Energy: Apparent Contribution of the 6-Hydroxyl Group of II. The observed H-bonding potential of the 6-hydroxyl group of II is expressed at a level surpassing those that have been reported for other oxy–deoxy comparisons of inhibitors (Kati & Wolfenden, 1989). In contrast with other examples in the literature, the 6-hydroxyl group of II has very limited freedom of movement with respect to the rest of the ligand. When the diazapine ring of deoxycoformycin is cleaved and otherwise modified, most of its distinctive binding affinity is lost (Montgomery et al., 1975). The resulting weakness of binding may arise in part from an increase in mobility of the critical hydroxyl group of the modified inhibitor.

These observations suggest that in 2′-deoxycoformycin, and to an even greater degree in II, a critical hydroxyl group of the inhibitor can be bound by the enzyme with little additional loss of entropy, so that most of its *intrinsic* binding energy can be manifested as observed binding energy (see below). It is of interest to inquire how strong such interactions might be in principle.

Intrinsic binding energy may be defined as the maximal amount of binding energy that would be possible between a particular substituent group and an enzyme in the absence of requirements that normally conflict with full expression of their affinity (Jencks, 1981). Ordinarily, some of that intrinsic binding energy would be spent (1) in overcoming any loss of *rotational and translational entropy* that the partners experience when they come together, (2) in bringing about any *changes in conformation* of the ligand or enzyme that may accompany binding, and (3) in *desolvating portions of the substituent and enzyme* that make contact. Any free energy that is "left over" after these processes have been paid for will be available as *observed* binding energy. One may attempt

Scheme V[a]



[a] $K_{exchange}$, the equilibrium constant for displacement of IV by II at the active site, gives an indication of the contribution of the −OH group to the binding affinity of II. Because compound II is very tightly bound, II probably fits the native active site snugly as shown in E–II. Any site that can accommodate II must in principle be able to accommodate the smaller compound IV, as shown in E–IV. It is possible that the active site may collapse slightly around the smaller IV, as shown in E–IV′, or that a water molecule may be trapped in the empty space, as shown in E–IV″. If that is the case, then the resulting structure must be favored thermodynamically compared with E–IV, and the equilibrium constant measured experimentally, $K_{apparent}$ (2×10^7), is expected to be less than the true value of $K_{exchange}$.

to estimate the contributions of the three unfavorable processes outlined above, and use these to correct the observed binding affinities of II and IV, in order to estimate the magnitude of the enzyme's intrinsic affinity for the 6-hydroxyl group of II.

Considering the first of these influences, the observed entropies of binding of II and IV are similar, suggesting that no major loss of entropy accompanies the binding of the 6-hydroxyl group. Movements of this hydroxyl group, with respect to the structure of the rest of the molecule, are presumably restricted to rotation around the C–O bond.

Second, the enzyme's very high affinity for II suggests that the active site of the native enzyme may be well shaped to accommodate II, with little or no need for an energetically costly change in conformation away from that of the native structure (Scheme V). Any site that can accommodate II should be able to accommodate IV because of its smaller size. Therefore, it seems *unlikely* that the weak binding of IV is due to some requirement for an energetically unfavorable change in the conformation of the active site.

Since IV is smaller than II, it is necessary to consider the possibility that a water molecule might be trapped in the E–IV complex or that the active site might collapse slightly around IV in such a way as to permit more intimate contact. Water trapping or a conformation change would not be expected to have occurred if it were not thermodynamically *favorable*. Therefore, the resulting E–IV complex would be expected to be more stable than the (hypothetical) E–IV complex depicted in Scheme V as most closely resembling the E–II complex. Conversely, displacement of a bound water molecule by the 6-hydroxyl group of II, in the exchange of II for IV at the active site, would then require expenditure of some of the

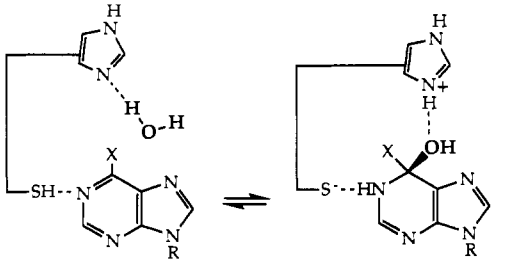
intrinsic binding energy of II. One is led to conclude that the observed difference in binding affinity between II and IV would have been greater than −9.8 kcal/mol if the suggested conformation change, or water trapping, had *not* occurred. If in fact water trapping or a conformation change occurs during formation of the E–IV complex (but not the E–II complex), then the intrinsic binding affinity of the 6-hydroxyl group will have been underestimated to the extent that these processes are energetically favorable.

Third, the possible influences of differing free energies of desolvation on the relative binding affinities of II and IV remain to be considered. Compound II is so tightly bound as to suggest that solvent water is stripped almost completely from those regions in which its makes contact with the enzyme. The thermodynamic cost of removing solvent molecules from the active site itself is probably about the same for both ligands (with the possible exception noted in the preceding paragraph), but the costs of desolvating II and IV differ considerably. Free energies of solvation of organic molecules have been found to be a nearly additive function of their constituent groups (Butler, 1937; Hine & Mookerjee, 1975; Wolfenden, 1983), so that the free energies of desolvation of the critical regions of II and IV can be estimated with reasonable confidence by comparing the vapor pressures of secondary alcohols with those of alkanes, over their dilute aqueous solutions. Alcohols are found to exceed alkanes in hydrophilic character by a factor of approximately 10^5 (Butler, 1937), so that desolvation of IV is expected to occur more easily than that of II by a similar factor. As the observed affinities of solvated II and IV for the enzyme differ by a factor of 1.4×10^7, the desolvated ligands may be estimated to differ in affinity for the active site by a *minimum* factor of 1.4×10^{12}, equivalent to −16.5 kcal/mol in free energy.

It is of interest to consider what interactions with the enzyme could be responsible for the remarkable affinity that appears top be conferred on II by its 6-hydroxyl group, after appropriate correction has been made for the leveling influence of solvation. Mass spectrometric studies of cluster formation, in the vapor phase where competition with solvent water does not occur, have yielded enthalpies of formation for single hydrogen bonds between a single hydroxyl group and a positively or negatively charged group of −14 to −21 kcal/mol (Meot-Nir & Sieck, 1986; Meot-Nir, 1984), and molecular dynamics calculations yield similar results (Weiner et al., 1984). Much smaller values, in the neighborhood of −3 to −7 kcal/mol, appear to be typical of hydrogen bonds in which neither partner carries a net electrostatic charge (Weiner et al., 1984; Fersht et al., 1985). Formation of a single hydrogen bond to a charged active-site residue might therefore suffice to explain the negative free energy of binding of the hydroxyl group of II. Nevertheless, because these results suggest only a *lower limit* on the strength of this interaction (see above and legend to Scheme V), more extensive contacts may well be necessary. We are led to conclude that at least one charged active-site residue is likely to be involved in a hydrogen bond to the 6-hydroxyl group of II.

Probable Identity of a Charged Group at the Active Site: Hydration as an Analogue of a Partial Reaction in General Acid–Base Catalysis. Pending solution of the crystal structure of the enzyme–inhibitor complex (Wilson et al., 1988), one can only speculate about the active-site residues that may be responsible for the difference in affinity between II and IV. However, earlier observations on the kinetic properties of the enzyme suggest an arrangement that would be consistent with the simple mechanism for hydration of purine ribonucleoside and catalysis of adenosine deamination shown in Scheme VI.

Scheme VI: Possible Chemical Mechanism for Hydration of Purine Ribonucleoside (X = H) or Formation of a Tetrahedral Intermediate in Deamination of Adenosine (X = NH_2) at the Active Site of Adenosine Deaminase[a]



[a] A protonated histidine residue, generated by the action of a neutral histidine residue in abstracting a proton from attacking water, forms a hydrogen bond to the critical 6-OH group of the hydrate. In the inhibitory complex (X = H), the structure on the right remains static. In the catalytic process, one of the histidine protons may be transferred to leaving group X.

Two ionizable groups appear to participate in the enzyme reaction, whose rate remains approximately constant in the neutral pH range. For calf intestinal adenosine deaminase, K_m increases above apparent pK_a 8.7, and V_{max} decreases below apparent pK_a 4.8 (Orsi et al., 1972). The higher pK_a value, also observed in the enzyme from *Aspergillus oryzae*, is probably associated with an enzyme sulfhydryl group that must be protonated if formation of a productive ES complex is to occur. The susceptibility of the enzyme to competitive inhibition by mersalyl suggests that an active-site SH group is covered when the ES complex is formed (Wolfenden et al., 1967), and the pH dependence of irreversible inactivation by *p*-mercuribenzoate suggests that this group has a pK_a value of 8.7, identical with the inflection observed in V_{max} (Orsi et al., 1972). Solvent deuterium isotope effects on catalysis furnish the additional information that an SH group, not ionized at the outset of the reaction, donates its proton to the substrate, probably to N-1 of the substrate adenosine (Weiss et al., 1987). As this group remains buried during the course of the reaction, its ionization is reflected only in K_m.

A second ionizable group, responsible for the downward inflection in V_{max} that is observed below apparent pK_a 4.8, has been tentatively identified as a histidine residue from the effect of changing solvent polarity on its acid dissociation constant (Orsi et al., 1972). It seems reasonable to suppose that this group, which must be in basic form at the outset of the reaction, abstracts a proton from the attacking water molecule as the reaction proceeds from the ES complex to the transition state. According to Scheme VI, the overall electrical neutrality of the ES complex would be preserved through the reaction, as required by the constancy of k_{cat}/K_m through the neutral pH range.

A similar proton abstraction, occurring during the enzymatic hydration of purine ribonucleoside, would be compatible with the present results, indicating that a charged group on the enzyme interacts with the critical 6-hydroxyl group of II. If this scheme is correct, then the enzymatic hydration of I to form II represents an analogue of a partial reaction in general acid–base catalysis.

Conclusion

In a typical enzyme reaction, many of a substrate's binding determinants tend to be maintained in roughly the same configuration as the substrate passes from the ground state to the transition state. To serve as an efficient catalyst, an enzyme needs to manifest strong preferential binding of the

altered substrate in the transition state. Under these circumstances, natural selection might be expected to have explored the limits of protein chemistry in producing a highly discriminating binding site. The generation of II at the active site of adenosine deaminase appears to provide an experimentally accessible analogue of the process by which the enzyme favors the generation of a tetrahedral intermediate or intermediates in substrate hydrolysis. The 6-hydrogen atom of II occupies the one position, normally occupied by the leaving group, for which the enzyme exhibits little binding specificity in the transition state.

The entropy that is lost when I combines with water in aqueous solution, −35 eu, is similar in magnitude to the entropy that is lost when adenosine proceeds from the ground state to the transition state in nonenzymatic deamination in water, approximately −37 eu (Frick et al., 1987). At the active site of adenosine deaminase, these highly unfavorable processes may be rendered favorable by an exact match between binding determinants of the hydrate and the binding site.

Depending on one's point of view, one may perceive *either* that the 6-hydroxyl group of II appears to be very tightly bound compared with substrate water in an equivalent position *or* that the rest of II (apart from its hydroxyl group) appears to be tightly bound compared with IV. These two viewpoints are equivalent because the active site's much higher affinity for II can be considered to arise from the fact that these binding determinants are joined in a single compound. "Connectivity", or the change of entropy that arises from their conjunction, allows more of their intrinsic binding affinities to be manifested as observed binding affinity, because the entropy loss is not as great as that which their individual binding would entail. As a result, bonds with unusual chemical properties may not need to be invoked to explain the overall binding affinity that is observed for hydrated purine ribonucleoside or the large jump in affinity that occurs as adenosine passes to the transition state in its deamination.

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