Transition-State Stabilization by Adenosine Deaminase: 1,6-Addition of Water to Purine Ribonucleoside, the Enzyme's Affinity for 6-Hydroxy-1,6-dihydropurine Ribonucleoside, and the Effective Concentration of Substrate Water at the Active Site†

Walda Jones, Linda C. Kurz, and Richard Wolfenden*

Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514, and Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

Received May 18, 1988; Revised Manuscript Received August 5, 1988

ABSTRACT: Positions of equilibria of highly unfavorable addition reactions, whose products are present at concentrations below the limits of detection, can be determined from equilibria of combination of anionic nucleophiles with quaternized enamines. Applied to the newly prepared 1-methylpurinium ribonucleoside cation, this method yields approximate equilibrium constants of 2×10^{-9} M^{-1} for addition of water and 4×10^{-5} M^{-1} for addition of *N*-acetylcysteine to neutral purine ribonucleoside, in dilute aqueous solution. Positions of ^{13}C magnetic resonances and UV absorption maxima of the above complexes and comparison with those of adenosine deaminase complexes strongly suggest that purine ribonucleoside is bound by adenosine deaminase as the 1,6 covalent hydrate, not as a covalently bonded complex formed by addition of a thiol group at the active site. The favorable position of equilibrium of the hydration reaction on the enzyme, together with its extremely unfavorable position in free solution, indicates that the effective activity of substrate water at the active site is in the neighborhood of 10^{10} M. The K_i value of the active diastereomer of 6-hydroxy-1,6-dihydropurine ribonucleoside is estimated as 1.6×10^{-13} M, more than 8 orders of magnitude lower than the apparent dissociation constants of enzyme complexes with the substrate adenosine or the product inosine. The enzyme's remarkable affinity for this hydrated species, which is vanishingly rare in free solution, seems understandable in terms of the hydrate's close resemblance to a hydrated intermediate approaching the transition state in direct water attack on adenosine.

Unsubstituted purine ribonucleoside is a competitive inhibitor of adenosine deaminase, with an affinity for the enzyme somewhat higher than the apparent affinities of substrates or products (Wolfenden et al., 1969). This preferential binding might be attributed to the modest space-filling requirements of the inhibitor, which are a little less than those of substrates or products. Newly determined resonances from [6-^{13}C]purine ribonucleoside indicate, however, that formation of the inhibitory complex is accompanied by addition of a nucleophile at C-6, probably a water molecule or a sulfur nucleophile at the enzyme's active site,[1] as indicated in Figure 1 (Kurz & Frieden, 1987). These alternative mechanisms of binding would result in complexes resembling intermediates in direct water attack on adenosine or in double displacement by a thiol nucleophile at the enzyme's active site (Figure 2). In order to decide between these alternatives, we have examined the stabilities and spectroscopic properties of model complexes. The results suggest that an oxygen adduct is formed at the active site and that this adduct is stabilized in the active site by attractive forces of remarkable magnitude.

Additions of neutral nucleophiles such as water and thiols to 9-substituted purines are so unfavorable that they have never been observed directly in aqueous solution. Figure 3 shows that, in principle, these reactions could be characterized if it were possible to examine the tendencies of hydroxide and thiolate anions (X^-) to combine with the conjugate acid of purine ribonucleoside, protonated at N-1. However, at pH values where the conjugate acid of purine ribonucleoside (pK_a = 2.05; Brown & Weliky, 1953) is present at significant concentrations, neither hydroxide nor thiolate anions exist at concentrations sufficient to produce detectable quantities of adduct. Equilibria of this kind could be determined in a virtual sense, if purine ribonucleoside were first modified by quaternization at the nitrogen atom at which proton addition would normally occur and its equilibria of reaction with anionic nucleophiles were determined.[2]

Figure 3 shows that the equilibrium constant for addition of a neutral nucleophile HX to a $\rangle C{=}N-$ bond must be equivalent to the product of K_1, the equilibrium constant for dissociation of HX to H^+ and X^-, K_2, the equilibrium constant for addition of H^+ to $\rangle C{=}N$, and K_3, the equilibrium constant for addition of X^- to $\rangle C{=}NH^+-$. K_1 and K_2 are directly related to acid dissociation constants and can be measured directly in many cases. K_3, the equilibrium constant for addition of the nucleophilic anion X^- to $\rangle C{=}NH^+-$, cannot normally be determined directly, because the concentration of $\rangle C{=}NH^+-$ becomes vanishingly small as the pH is elevated to the point where nucleophilic anions such as OH^- become sufficiently abundant for the addition reaction to proceed to a measurable extent. This difficulty can be overcome by making the reasonable assumption that $\rangle C{=}NH^+-$

†Supported by Research Grants GM18325 (W.J. and R.W.) and GM33851 (L.C.K.) from the National Institutes of Health and PCM-7823016 from the National Science Foundation (W.J. and R.W.).

[1] Earlier investigations have uncovered little evidence of nucleophilic amino acid residues other than cysteine that might participate directly in the catalytic process (see Discussion).

[2] Bunting (1966) appears to have been the first to recognize this possibility, whose application to the hydration of purine ribonucleoside was described recently (Jones & Wolfenden, 1986).

0006-2960/89/0428-1242$01.50/0

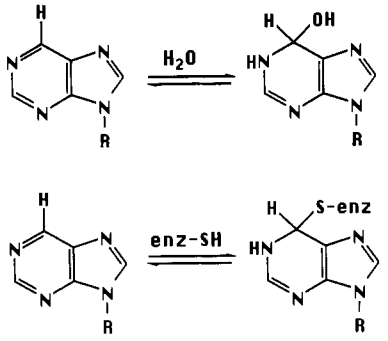


FIGURE 1: Alternative structures of adducts formed by purine ribonucleoside at the active site of adenosine deaminase.

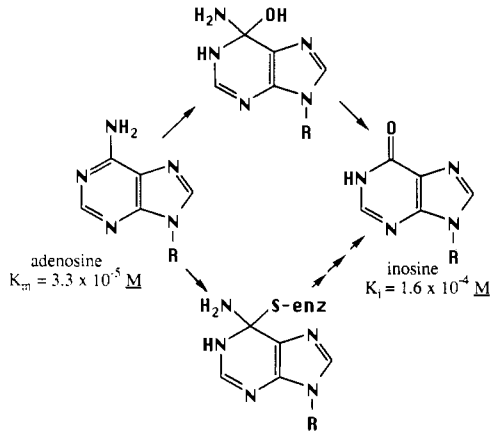
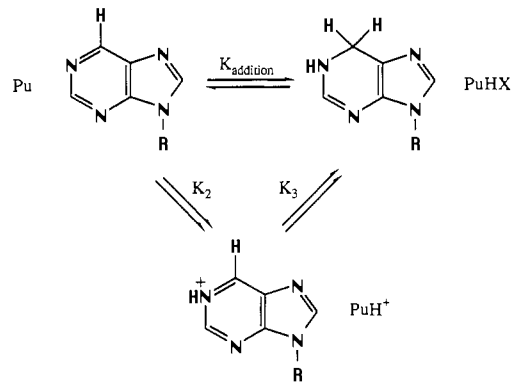


FIGURE 2: Alternative mechanisms of action of adenosine deaminase involving direct water attack (above) and double displacement by an active site thiol (below).



$$K_{addition} = [PuHX]/[Pu][HX] = K_1 \cdot K_2 \cdot K_3$$

where

$$K_1 = [H^+][X^-]/[HX] \qquad K_2 = [PuH^+]/[Pu][H^+]$$

$$K_3 = [PuHX]/[PuH^+][X^-]$$

FIGURE 3: Equilibrium of addition of a nucleophile HX, analyzed in terms of successive equilibria of addition of a proton and a nucleophilic anion.

and $)C{=}NCH_3^+{-}$ are similar in their affinities for X^-, as suggested in Figure 4. That assumption is supported by an earlier study showing that the 3-methylquinazolinium cation closely resembled the conjugate acid of quinazoline (protonated at N-3) in its affinity for the hydroxide ion (Albert et al., 1961). Thus, it should be possible to evaluate the susceptibility of purine ribonucleoside to 1,6-addition of nucleophiles by measuring equilibria of addition of anionic nucleophiles to the cation of 1-methylpurine ribonucleoside, since purine ribonucleoside has been shown to be protonated at N-1 (Coburn et al., 1965).

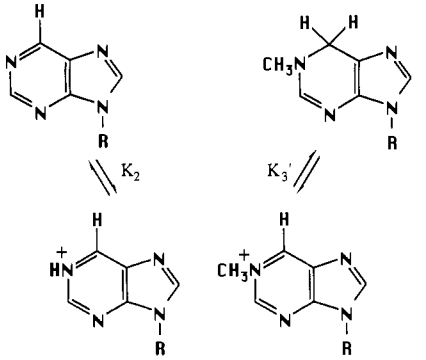


FIGURE 4: Equilibrium of addition of a nucleophile HX, analyzed in terms of equilibria of addition of a proton to purine ribonucleoside (K_2), and of a nucleophilic anion to 1-methylpurine ribonucleoside (K_3'). Equilibrium constants are analogous to those in Figure 3.

We have prepared the iodide salt of 1-methylpurinium ribonucleoside and used it to evaluate the tendency of purine ribonucleoside to combine with water and with other nucleophiles and to determine the spectroscopic properties of typical adducts for comparison with those of the enzyme complex with purine ribonucleoside.

Materials and Methods

^1H and ^{13}C NMR spectra were obtained with a Varian XL-400 NMR spectrometer, using a 5-mm multinuclear probe. Positive ^1H and ^{13}C chemical shifts, referred to an internal dioxane standard, were used to denote a resonance at lower shielding with respect to an external capillary containing Me_4Si. NOE spectra were obtained from a 512 × 512 data matrix, with 16 scans per t_1 value, and a delay of 1 s between scans. The rf carrier frequency was positioned at 6.6 ppm, and the mixing time was 1 s. The NMR spectrum of the pseudobase of 1-methyl[6-^{13}C]purine ribonucleoside required special precautions because of its limited lifetime. Its half-life was extended from 150 s at 20 °C to 20 min at 5 °C, where it proved possible to obtain a strong C-6 signal (s/n = 20) from 200 scans of a 0.01 M solution of ^{13}C-enriched material, accumulated over a period of 120 s after neutralization with KOH, before the pseudobase had undergone significant decomposition.

The iodide salt of 1-methylpurinium ribonucleoside was prepared by treating purine ribonucleoside (1 g, Sigma Chemical Co.) with 1 equiv of iodomethane in dry DMSO (3 mL) for 7 days at room temperature. The major product, purified by chromatography on a cellulose column (2 × 28 cm) with a gradient of increasing ethanol in chloroform, was eluted at an ethanol concentration of 100%. Crystallization from methanol yielded material (0.69 g; 44% yield) with the following C–H–N analysis: C, 33.3%; H, 3.9%; N, 14.0% ($C_{11}H_{15}N_4O_4I$ requires C, 33.5%; H, 3.8%; N, 14.2%). NMR analysis showed carbon and proton resonances with integrated intensities consistent with methylation of a single ring nitrogen. ^{13}C NMR (ppm, D_2O): 45.2 (CH_3N – 1); 60.9, 70.0, 74.3, 85.5, 89.2 (ribose); 133.1 (C-5); 143.9 (C-8); 149.0 (C-6); 151.2 (C-2); 152.9 (C-4). ^{1}H NMR (ppm, D_2O): 3.9 (m, 2 H, ribose); 4.2–4.9 (m, 3 H, ribose); 4.5 (s, 3 H, CH_3), 6.35 (d, 1 H, ribose); 9.15 (s, 1 H, H-8); 9.4 (s, 1 H, H-2), 9.62 (s, 1 H, H-6). The resonances of the aromatic protons were assigned as by comparison with Chenon et al. (1975). As expected for alkylation at N-1, we observed strong dipole–dipole interaction between the methyl protons and the protons at C-6 and C-2 but not between the methyl protons and the proton at C-8.

Table I: Addition of Nucleophiles to the 1-Methylpurinium Nucleoside Cation

nucleophile	pK_a of NuH^+	^{13}C shift (ppm)	λ_{max} (nm)
none[a]	none	144.7	269 (log ϵ = 3.8)
OH^-	15.7	81.3	283 (log ϵ = 3.9)
N-acetylcysteine-S^-	9.9	68.0	302 (log ϵ = 3.9)
CH_3NH_2	10.6	71.4	296 (log ϵ = 3.8)
purine ribonucleoside cation[b]		140	262 (log ϵ = 3.8)
purine ribonucleoside uncharged[b]		148	262 (log ϵ = 3.85)
purine ribonucleoside with adenosine deaminase[b]		75	280 (log ϵ = 3.67)

[a] 1-Methylpurinium ribonucleoside cation alone. [b] Kurz and Frieden (1987).

The iodide salt of [6-^{13}C]-1-methylpurinium ribonucleoside was prepared by the same procedure, from [6-^{13}C]purine ribonucleoside that had been synthesized as described previously (Kurz & Frieden, 1987). In D_2O, the methylated product showed a single major ^{13}C resonance at 144.7 ppm, at somewhat higher field than the major resonance of the original [6-^{13}C]purine ribonucleoside at 148 ppm but at lower field than that of the cation of [6-^{13}C]purine ribonucleoside at 140 ppm (Table I).

Results

Spectrophotometric titration of the iodide salt of 1-methylpurinium ribonucleoside (λ_{max} 270 nm, log ϵ = 3.8 in 0.1 N HCl) showed formation of an unstable conjugate base at high pH values (λ_{max} 284 nm, log ϵ = 3.9 in 0.1 N KOH). In 0.01 M KOH, the conjugate base was found to undergo first-order decomposition (presumably due to ring opening), with an increase of ultraviolet absorption at 320 nm. In 0.01 M KOH, decomposition occurred with a half-time of 2.3 min at 20 °C and of 20 min at 5 °C. In a series of 0.01 M potassium phosphate and potassium carbonate buffers, the rate of decomposition fell off rapidly with decreasing pH, with half-times of 29 min at pH 9.35 and 20 °C, 82 min at pH 8.5 and 20 °C, and 300 min at pH 7.9. The pK_a value of the conjugate acid, determined in these same buffers by scanning spectra with a Hewlett-Packard diode array spectrophotometer at 10-s intervals and extrapolating the results back to the time of mixing, was 9.0 ± 0.1.

Formation of the conjugate base at pH 9 (Table I) was accompanied by a shift of maximum absorbancy (262 nm) to longer wavelength (280 nm). Somewhat larger red shifts have been observed in the 1,6-saturated derivatives of purine generated by electrochemical reduction (292 nm; Smith & Elving, 1962) and of purine ribonucleoside generated by photochemical alkylation (291 nm; Evans & Wolfenden, 1970). To determine whether addition of hydroxide ion had occurred at the 6-position of 1-methylpurine ribonucleoside, the ^{13}C chemical shift of [6-^{13}C]-1-methylpurine ribonucleoside was determined in base. Because of the limited lifetime of the pseudobase (see above), spectra were accumulated rapidly over a period of 3 min at 5 °C, where the half-time for decomposition of the pseudobase in 0.01 and 0.1 M KOH was approximately 20 min. In 0.1 M KOH containing 0.01 M [6-^{13}C]-1-methylpurine ribonucleoside (99 atom % excess), a single resonance was observed at 81.3 ppm (as compared with 144.7 ppm in the cation), confirming that there had been a change at C-6 from sp^2 to sp^3 geometry as expected for pseudobase formation. These results indicated that the pK_a value of 9.0 actually corresponded to combination of the 1-methylpurinium cation with hydroxide ion. Scheme I shows that this pK_a value is equivalent to an equilibrium constant of 1.0×10^{-5} M for dissociation of the hydroxide ion from the pseudobase (Table I).

Similar procedures were used to characterize complexes formed between 1-methylpurinium ribonucleoside and other nucleophiles. Buffer solutions were prepared containing equimolar concentrations of the conjugate acid and base of the nucleophile, by addition of 0.5 equiv of KOH to the conjugate acid. The resulting adducts proved to be very much more stable to decomposition than the pseudobase described above, with half-lives in excess of 20 min at 20 °C. The apparent dissociation constant was then determined by measuring the absorbancy of the purine derivative at the λ_{max} of the product, at each of a series of buffer concentrations ranging from 0.001 to 0.1 M. The apparent dissociation constant, referred to the fraction of nucleophile actually present as the anion and the fraction of 1-methylpurine ribonucleoside actually present as the reactive cation, was 3.8×10^{-4} M for the *N*-acetylcysteamine anion. ^{13}C chemical shifts of these relatively stable adducts, determined by dissolving the iodide salt of [6-^{13}C]-1-methylpurine ribonucleoside in a concentration of nucleophile (0.1 M) sufficient to produce at least 99% addition, are shown in Table I.

Discussion

Equilibria of Addition of Water and N-Acetylcysteamine to Purine Ribonucleoside. The conjugate acid of purine ribonucleoside is protonated at N-1 (Coburn et al., 1965), with a pK_a value of 2.05 (Brown & Weliky, 1953), corresponding to a value of 1.12×10^2 M^{-1} for the association constant K_2. On the basis of the actual molarity of water, K_1, the dissociation constant of water, is 1.82×10^{-16} M. The present observations, based on combination of the hydroxide ion with the 1-methylpurinium ribonucleoside cation, yield a K_3 value of 1.0×10^5 M^{-1} for the association constant describing pseudobase formation from the purine ribonucleoside cation and the hydroxide ion. On the basis of these values of K_1, K_2, and K_3, Scheme I yields an equilibrium constant of 2.0×10^{-9} M^{-1} for hydration of unsubstituted purine ribonucleoside, on the basis of the actual molarity of water. On the basis the alternative convention that treats water activity as unity in dilute aqueous solution, the equilibrium constant for hydration is 1.1×10^{-7}.

The observed equilibrium constant for addition of anionic *N*-acetylcysteamine to the 1-methylpurinium ribonucleoside cation, 3.8×10^{-4} M, yields an equilibrium constant of 3.7×10^{-5} M^{-1} for addition of the neutral thiol to unsubstituted purine ribonucleoside. Although thiol addition is considerably more favorable than hydration, these results explain why neither hydration nor thiol addition to purine ribonucleoside proceeds to an extent that is detectable by presently available spectroscopic methods in dilute aqueous solution.

Probable Nature of the Active Site Nucleophile That Combines with Purine Ribonucleoside in the Active Site. That purine ribonucleoside should be bound by adenosine deaminase entirely as the hydrate, *or* as the 1,6-adduct formed by an active site cysteine, would appear understandable if the enzyme's active site were designed to produce extreme stabilization of intermediates in deamination, formed as a result of 1,6-addition of substrate water *or* of an active site nucleophile to the substrate adenosine. A decision between these alternatives could provide an important indication of the mechanism that is followed during enzymatic deamination of adenosine.

Binding of purine ribonucleoside as a *sulfur adduct* would be consistent with several indications of the presence in

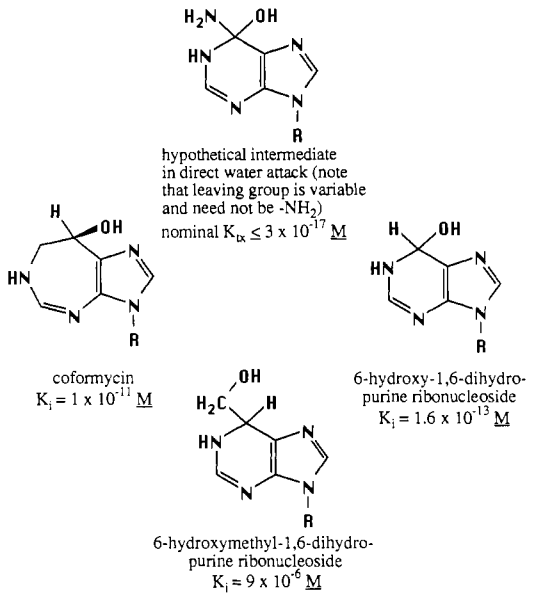


FIGURE 5: Structural relationship between a hypothetical intermediate approaching the transition state for direct water attack on adenosine and adenosine deaminase inhibitors of unusual potency. The nominal K_{tx}, describing the formal dissociation constant of the hypothetical intermediate from the enzyme, is taken from Frick et al. (1987). The K_m value of the substrate adenosine is 10^{-5} M, and the K_i value of the product inosine is 1.6×10^{-4} M.

adenosine deaminase of an essential sulfhydryl group that could participate in substrate binding or catalysis (Wolfenden et al., 1967; Ronca et al., 1967; Orsi et al., 1972; Weiss et al., 1987).[1] Binding of purine ribonucleoside as a sulfur adduct would also be consistent with the effectiveness of sulfur nucleophiles in aromatic displacements on 6-chloropurine ribonucleoside (Walsh & Wolfenden, 1967). However, it has recently been found that in displacements on less activated species such as adenosine itself the reactivity of thiols does not exceed that of water itself (Frick et al., 1987).

Other evidence favors the alternative view that adenosine hydrolysis proceeds by *direct water attack*, involving a 1,6-hydrated species, rather than by a double-displacement mechanism. First, the enzyme is strongly inhibited by apparent analogues of 1,6-hydrated intermediates (Figure 5). In one diastereomer of 6-(hydroxymethyl)-1,6-dihydropurine ribonucleoside, the hydroxymethyl group can be made to adopt a position similar to that postulated for the attacking hydroxyl group in deamination (Evans & Wolfenden, 1970). The stereochemical orientation of this hydroxyl group has been found (Shimazaki et al., 1983) to be identical with the orientation that would permit superimposition of the oxygen atom of its hydroxyl group on the oxygen atom of the hydroxyl group of the active isomer of the more powderful inhibitors coformycin and 2′-deoxycoformycin. Deoxycoformycin appears to be bound intact, without covalent addition (Frick et al., 1986). If these compounds are transition-state analogues, then one is led to infer that the enzyme-catalyzed reaction proceeds by direct water attack on adenosine. In addition, adenosine deaminase catalyzes the hydrolytic deamination of 4-aminopteridine and serves as an efficient catalyst for hydration of unsubstituted pteridine at the corresponding position in that ring system (Evans & Wolfenden, 1972, 1973). It seems natural to suppose that hydration and deamination of these pteridine derivatives proceed by a similar mechanism and to infer that direct water attack is involved in both cases.

The present results (Table I) offer strong support for this conclusion. Thus, the ^{13}C resonance corresponding to C-6 of purine ribonucleoside bound by adenosine deaminase (Kurz & Frieden, 1987) was situated 65 ppm upfield from that of the purine ribonucleoside *cation*.[3] We observed a similar shift (63.4 ppm upfield) when the resonance from C-6 of the model hydroxide ion adduct corresponding to the 1,6-hydrate of purine ribonucleoside was compared with that from the 1-methyl-1,6-dihydropurine ribonucleoside cation. In contrast, the upfield shift observed for the model thiolate adduct was much larger, 76.7 ppm (Table I). In the 13.3-ppm range defined by the shifts observed for oxygen and sulfur adducts,[4] the shift observed for the enzyme–inhibitor complex fell 88% toward the extreme expected for an oxygen adduct.

Changes in UV absorption suggest a similar conclusion. Thus, enzyme binding of purine ribonucleoside resulted in an 18-nm red shift in the apparent wavelength of maximum absorption (Kurz & Frieden, 1983), compared with the position observed for the cation[3] of purine ribonucleoside (Table I). A 15-nm red shift was observed for the oxygen adduct, relative to the λ_{max} observed for the 1-methylpurinium ribonucleoside cation. In contrast, a 33-nm red shift was observed for the model sulfur adduct. In the 18-nm range defined by the shifts observed for oxygen and sulfur adducts, the shift observed for the enzyme–inhibitor complex fell 83% toward the extreme expected for an oxygen adduct.

In summary, the ^{13}C chemical shift of enzyme-bound purine ribonucleoside and the ultraviolet absorption maximum indicated by the enzyme–inhibitor UV difference spectrum are much closer to those of an oxygen than to those of a sulfur adduct. The present results cannot rule out the possibility that this oxygen nucleophile is provided by an amino acid side chain rather than by water. However, the lack of susceptibility of adenosine deaminase to derivatizing agents for any amino acid other than cysteine (Wolfenden et al., 1969; Zielke & Suelter, 1971), the enzyme's sensitivity to inhibition by analogues of hydrated intermediates that are bound noncovalently, and its activity as a catalyst for pteridine hydration (Evans & Wolfenden, 1972, 1973) argue against that possibility.[5]

These results strongly suggest that the enzyme stabilizes a form of the inhibitor, the 1,6-hydrate, that is extremely rare in free solution. This appears reasonable in view of the close resemblance between the hydrate and a covalently hydrated

Scheme I

$$K_{eq} = 1.1 \times 10^{-7}$$

Purine = Hydrate

$$K_{eq} = 3.2 \times 10^{-13}\ M \qquad \text{<- true } K_i \text{ value}$$

Hydrate + Enzyme = Enzyme-Hydrate

Overall reaction:

$$K_{eq} = 2.9 \times 10^{-6}\ M \qquad \text{<- apparent } K_i \text{ value}$$

Enzyme + Purine = Enzyme-Hydrate

[3] Before addition, the 1-methylpurinium ribonucleoside cation exists in a form analogous to the conjugate acid cation of unsubstituted purine ribonucleoside, so that it is necessary to use the cation as a starting point in this comparison.

[4] This difference between oxygen and sulfur adducts agrees closely with the observed difference between the positions of resonances from C-1 of acetaldehyde in *gem*-diols and in thiohemiacetals (Gamcskik et al., 1983).

[5] In a recent study exploiting the influence of isotopically labeled waters on ^{13}C chemical shifts, Schmidt et al. (1985) were able to show that an inhibitory ketone appears to be covalently hydrated at the active site of pepsin. The results were interpreted as indicating a catalytic mechanism involving attach on the peptide bond by water, rather than by an oxygen nucleophile contained in an amino acid side chain of pepsin.

intermediate in adenosine deaminase (Figure 5) and implies that water attacks adenosine directly during the normal catalytic process.

Comparison of 6-Hydroxy-1,6-dihydropurine Ribonucleoside with Other Inhibitors: Its True K_i Value and Mechanism of Binding. The observed K_i value of purine ribonucleoside is 2.9×10^{-6} M (Kurz & Frieden, 1987). Since the equilibrium constant for hydration is 1.1×10^{-7} (based on unit water activity; see above), the total concentration of the hydrate 6-hydroxy-1,6-dihydropurine ribonucleoside required to half-saturate the enzyme is 3.2×10^{-13} M, as indicated in Scheme I.[6] Earlier results have shown that only one diastereomer of the inhibitor 2′-deoxycoformycin is active (Schramm & Baker, 1986) and that adenosine deaminase catalyzes the reversible formation of only one enantiomer of pteridine 3,4-hydrate (Evans & Wolfenden, 1972, 1973). If this is assumed also to be true of 1,6-hydrated purine ribonucleoside, then the K_i value of the more active diastereomer [probably analogous in its hydroxyl group stereochemistry to (8*R*)-2′-deoxycoformycin and to the active isomer of 6-(hydroxymethyl)-1,6-dihydropurine ribonucleoside discussed above] would be 1.6×10^{-13} M.

The true K_i value estimated for the active isomer of the hydrate, 1.6×10^{-13} M, is somewhat more favorable than the values that have been estimated for coformycin, 1×10^{-11} M, and its deoxy analogue, approximately 2.5×10^{-12} M (Cha et al., 1975), whose structures are shown in Figure 5. This improved binding affinity seems understandable in view of the closer resemblance between 1,6-hydrated purine ribonucleoside and the proposed tetrahedral intermediate in adenosine deamination.

Evidently the affinity of the enzyme for 6-hydroxy-1,6-dihydropurine ribonucleoside surpasses its affinity for the substrate adenosine by more than 8 orders of magnitude. Comparison of the rates of deamination of adenosine in neutral aqueous solution, in the presence and absence of the enzyme, indicates a rate enhancement of approximately 10^{12}-fold by the enzyme (Frick et al., 1987). Accordingly, the enzyme's affinity for the altered substrate in the transition state for adenosine deamination should be bound by the enzyme at least 12 orders of magnitude more tightly than adenosine itself. The hydrate expresses the greater part of this binding enhancement, and its minor shortcomings may be explained by the absence of a 6-substituent corresponding to the departing ammonia substituent, for which the enzyme shows limited specificity (Figure 5).

Purine ribonucleoside is bound rapidly enough to preclude the possibility that hydration precedes its successful encounter with the enzyme (Kurz & Frieden, 1987). It seems natural to suppose that hydration may be catalyzed by the enzyme as in the case of pteridine. However, because the nonenzymatic hydration of purine ribonucleoside does not proceed to a measurable extent *in free solution*, the problem of demonstrating catalysis of this reaction is probably insurmountable (i.e., the rate of the nonenzymatic hydration reaction remains unknown and cannot be compared with the rate of hydration on the enzyme).

Effective Concentration of Water at the Active Site and the Probable Equilibrium Constant for 1,6-Hydration of Adenosine. Adopting a convention suggested by Jencks (1975), the present equilibria can be used to estimate the "effective molarity" of substrate water that would have to be present in the active site to account for binding exclusively as a 1,6-adduct. NMR observations (Kurz & Frieden, 1987) suggest that purine ribonucleoside is at least 95% converted to the hydrate at the active site of adenosine deaminase. We have estimated the equilibrium constant for hydration of purine ribonucleoside in dilute solution as 2.0×10^{-9} M^{-1}, as described above. According to these estimates, the effective molarity of water at the active site is in the neighborhood of 10^{10} M, with respect to the equilibrium of hydration of purine ribonucleoside.

Carbon substitution strongly disfavors water addition to C=N groups (Albert, 1976). Accordingly, the equilibrium constant for 1,6-hydration of adenosine is presumably some orders of magnitude less favorable than the value presently estimated for 1,6-hydration of purine ribonucleoside, 10^{-9} M^{-1} on the basis of the molarity of water in dilute solution. In the uncatalyzed hydrolytic deamination of adenosine, which probably also proceeds by water addition at the 1,6-position, the equilibrium constant for activation is approximately $10^{-15}M^{-1}$ (Frick et al., 1987). Thus, the instability of the 1,6-hydration product of adenosine may approach that of the transition state for uncatalyzed deamination of adenosine.

Forces Involved in Stabilizing the Transition State. On the basis of present evidence, it seems reasonable to consider several kinds of forces that could be at work in stabilizing the inhibitory complex at the active site, since these might correspond to forces involved in the catalytic process itself.

First, the observation that purine ribonucleoside appears to be bound as the rare 1,6-hydrate and the fact that adenosine, inosine, and purine ribonucleoside itself are bound 8 orders of magnitude less tightly than the hydrate suggest the likelihood that hydrogen bonds of remarkable stability may be formed between the enzyme's active site and the 6-OH group of the inhibitor or hydrated reaction intermediate. These hydrogen bonds might represent a "snapshot" of the enzyme caught in the act of delivering a water nucleophile at C-6 of adenosine. In this catalytic process, the most likely role for the enzyme would be to serve as a general base.

Second, model building, as well as the observed crystal structure of 6-(hydroxymethyl)-1,6-dihydropurine ribonucleoside (Shimazaki, 1983), suggests that the purine ring system becomes somewhat distorted from its native, planar, structure when 1,6-hydration occurs. The enzyme might fit such a distorted ring precisely, accounting for its very high affinity for the hydrate. If that were the case, the enzyme might promote catalysis by providing an exact fit to the similarly distorted ring structure of a hydrate intermediate in adenosine deamination.

Third, on the basis of solvent isotope effects on catalysis by adenosine deaminase, Weiss et al. (1987) have suggested that

[6] A reviewer has noted that this treatment neglects possible catalysis of inhibitor hydration by the enzyme. The dissociation constant of an enzyme complex with a competitive inhibitor is a thermodynamic property, independent of the pathway by which that complex is formed. Therefore, if the apparent K_i value of an inhibitor is known and if it is bound as a rare species whose equilibrium of formation can be established in free solution (a major goal of the present work), then its true K_i value can be calculated (Scheme I). It has also been suggested that if the hydrate were the inhibitor, then inhibition should increase with increasing pH. That would be true if the inhibitor were the pseudobase formed by 1-methylpurinium ribonucleoside, since its formation is pH dependent (see Figure 3). However, in the present study, the 1-methylpurinium cation was used only as a model for the hydroxide addition reaction (see Figure 4), and unpublished experiments have shown that *neither 1-methylpurinium ribonucleoside nor the pseudobase formed by 1-methylpurinium ribonucleoside exhibits significant inhibitory activity.* It seems probable that the 1-methyl group provides a steric impediment to binding of the pseudobase and eliminates the possibility of forming a hydrogen bond between the enzyme and N-1 of the purine ring. The state of hydration of purine ribonucleoside, the actual inhibitor, does not change with changing pH, so that its inhibitory effect is not expected to be pH dependent.

an enzyme SH group may serve as a proton donor to N-1 of the substrate, assisting combination of a nucleophile with C-6. After transfer, such a proton might be expected to appear at N-1 of the hydrate, hydrogen bonded to the thiolate group from which it originated.

The second and third of these effects (interactions with N-1 and with "puckered" forms of the 1,6-hydrate) should also be possible in the case of 1,6-dihydropurine ribonucleoside, which possesses both these capabilities and has slightly reduced steric requirements as compared with 6-hydroxy-1,6-dihydropurine ribonucleoside and the reaction intermediate. That possibility is now being investigated.

Relevance to the Chemical Mechanism of Action of Adenosine Deaminase. Evidence suggesting that enzymatic deamination of adenosine proceeds by direct water attack, rather than by a double-displacement mechanism, may be summarized as follows.

(1) Adenosine deaminase catalyzes the stereospecific hydration of pteridine, in a process analogous to formation of a hydrated intermediate in hydrolysis of 4-aminopteridine and to formation of the inhibitory complex described here. Adenosine deaminase is also an efficient catalyst of 4-aminopteridine hydrolysis.

(2) Adenosine deaminase is strongly inhibited by 6-(hydroxymethyl)-1,6-dihydropurine ribonucleoside and by 2′-deoxycoformycin, both of which bear an obvious resemblance to a 1,6-hydrated intermediate. If deamination were to proceed by double displacement, the reason for the potency of these inhibitors would not be apparent. These compounds resemble each other, and the proposed hydrated intermediate in substrate hydrolysis, and are bound 7–8 orders of magnitude more tightly than substrates or products. The rate enhancement produced by the enzyme amounts to approximately 12 orders of magnitude, so that 6-hydroxy-1,6-dihydropurine ribonucleoside expresses a substantial fraction of the free energy of binding that distinguishes the transition state from the ground state in the enzymatic reaction.

(3) Forces involved in the extraordinary stabilization of these complexes, and of the "tetrahedral" intermediate in deamination, almost certainly include hydrogen bonds of extraordinary stability between the active site and the inhibitor's 6-OH group. Obvious candidates for partnership in this interaction are active site residues whose normal function would be to serve as general base catalysts for water attack and as general acid catalysts for ammonia elimination.

Registry No. OH^-, 14280-30-9; CH_3NH_2, 74-89-5; H_2O, 7732-18-5; adenosine deaminase, 9026-93-1; 1-methylpurinium ribonucleoside iodide salt, 105018-75-5; purine ribonucleoside, 550-33-4; [6-^{13}C]-1-methylpurinium ribonucleoside, 118043-56-4; [6-^{13}C]purine ribonucleoside, 112043-66-0; 1-methylpurinium nucleoside, 118043-57-5; L-*N*-acetylcysteine, 616-91-1; L-*N*-acetylcysteine-S^-, 41079-67-8.

References

Albert, A. (1976) *Adv. Heterocycl. Chem. 20*, 117.

Albert, A., Armarego, W. L. F., & Spinner, E. (1961) *J. Chem. Soc. 161*, 5267.

Brown, G. B., & Weliky, V. S. (1983) *J. Biol. Chem. 204*, 1019.

Bunting, J. W. (1966) *Adv. Heterocycl. Chem. 25*, 1.

Cha, S., Agarwal, R. P., & Parks, R. E., Jr. (1975) *Biochem. Pharmacol. 24*, 2187.

Chenon, M. T., Pugmire, R. J., Grant, D. M., Panzika, R. P., & Townsend, L. B. (1975) *J. Am. Chem. Soc. 97*, 4627.

Coburn, W. C., Thorpe, M. C., Montgomery, J. C., & Hewson, K. (1965) *J. Org. Chem. 30*, 110.

Evans, B., & Wolfenden, R. (1970) *J. Am. Chem. Soc. 92*, 4751.

Evans, B., & Wolfenden, R. (1972) *J. Am. Chem. Soc. 94*, 5902.

Evans, B., & Wolfenden, R. (1973) *Biochemistry 12*, 392.

Frick, L., Wolfenden, R., Smal, E., & Baker, D. C. (1986) *Biochemistry 25*, 1616.

Frick, L., Mac Neela, J. P., & Wolfenden, R. (1987) *Bioorg. Chem. 15*, 100.

Gamcsik, M. P., Malthouse, J. P. G., Primrose, W. U., Mackenzie, N. E., Boyd, A. S. F., Russell, R. A., & Scott, A. I. (1983) *J. Am. Chem. Soc. 105*, 6324.

Jencks, W. P. (1975) *Adv. Enzymol. Rel. Areas Mol. Biol. 43*, 219.

Jones, W., & Wolfenden, R. (1986) *J. Am. Chem. Soc. 108*, 7444.

Kurz, L. C., & Frieden, C. (1983) *Biochemistry 22*, 382.

Kurz, L. C., & Frieden, C. (1987) *Biochemistry 26*, 8450.

Orsi, B. A., McFerran, N., Hill, A., & Bingham, A. (1972) *Biochemistry 11*, 3386.

Ronca, G., Bauer, C., & Rossi, C. A. (1967) *Eur. J. Biochem. 1*, 434.

Schmidt, P. G., Holladay, M. W., Salituro, F. G., & Rich, D. H. (1985) *Biochem. Biophys. Res. Commun. 129*, 597.

Schramm, V. L., & Bker, D. C. (1985) *Biochemistry 24*, 641.

Shimazaki, M. (1983) *Chem. Pharmacol. 31*, 3104.

Smith, D. L., & Elving, P. J. (1962) *J. Am. Chem. Soc. 84*, 1412.

Walsh, B. T., & Wolfenden, R. (1967) *J. Am. Chem. Soc. 89*, 6221.

Weiss, P. M., Cook, P. F., Hermes, J. D., & Cleland, W. W. (1987) *Biochemistry 26*, 7378.

Wolfenden, R., Sharpless, T. K., & Allen, R. (1967) *J. Biol. Chem. 242*, 977.

Wolfenden, R., Kaufman, J., & Macon, J. B. (1969) *Biochemistry 8*, 2412.

Zielke, C. L., & Suelter, C. H. (1971) *Enzymes (3rd Ed.) 4*, 47.