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Adenosine Deaminase: Solvent Isotope and pH Effects on the Binding of Transition-State and Ground-State Analogue Inhibitors†

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ABSTRACT: We have studied the pH(D) effects on the deamination of adenosine catalyzed by adenosine deaminase as well as on the binding of the inhibitors purine riboside, a ground-state analogue, and 1,6-dihydro-6-(hydroxymethyl)purine riboside (DHMPR), a transition-state analogue. The observed pK_a value of 4.9 for the free enzyme in H_2O buffers is found to be increased by 0.6 pK unit in the enzyme–substrate complex and decreased by 0.5–1.0 pK unit in the enzyme–inhibitor complexes. In D_2O buffers, the pK_as of the free enzyme and its complexes are all found to be increased by ~0.6 pK unit relative to their position in H_2O. A small inverse solvent isotope effect is observed on V_{max} while none is observed on K_m. Substantial solvent isotope effects [$K_i(H_2O)/K_i(D_2O)$ = 1.2–1.5] are found for the dissociation of both ground-state and transition-state analogue inhibitors from the enzyme–inhibitor complex. Fluorescence titrations of the enzyme with DHMPR in H_2O and D_2O confirm the equilibrium solvent isotope effect obtained from kinetic experiments. For the transition-state analogue, a small inverse kinetic effect, similar in magnitude to that on V_{max}, is found on the association rate constant, k_{on}, while a normal effect is observed on the dissociation rate constant, k_{off}. The intrinsic protein fluorescence is quenched 70% by the transition-state analogue and only 6% by the ground-state analogue supporting the idea that a greater structural reorganization of the enzyme is required to bind the transition state effectively in comparison to the ground state. In contrast, a large UV difference spectrum is observed upon formation of the complex with purine riboside, suggesting that the binding isotope effect may be interpretable in terms of structural changes in the ligand rather than in the enzyme. Three possible structures for the complexed inhibitors are discussed which could account for the observed solvent isotope effect. The data are most consistent with protonation of the purine ring at N-1 by an active-site sulfhydryl. However, hydration of the purine ring at C-6 or formation of a covalent sulfhydryl adduct at C-2 or C-6 cannot be excluded in view of the large pK_a shifts required to accomplish purine protonation with a sulfhydryl group.

For catalysis to occur, an enzyme must have a greater affinity for its substrates in the transition state than in the ground state (Pauling, 1946). Thus, an understanding of enzyme mechanisms requires an understanding of those interactions between the enzyme and its substrate which lead to transition-state stabilization. While the factors responsible for transition-state stabilization can be investigated through a study of the response of the kinetic constants of the catalytic reaction to perturbations, the development of stable analogues of the substrate portion of the activated complex (Wolfenden, 1972) has made possible a more direct approach. In general, transition-state analogues bind more tightly to the enzyme than do ground-state analogues (or substrates), but an apparent slow rate of binding also distinguishes transition-state from ground-state analogues in many cases (Wolfenden, 1976; Frieden et al., 1980).

These points are well illustrated by the binding of adenosine deaminase (EC 3.5.4.4) to the ground-state analogue purine riboside (PR) and the transition-state analogue 1,6-dihydro-6-(hydroxymethyl)purine riboside (DHMPR) (Wolfenden et al., 1977). It has been proposed that DHMPR approximates the structure of the tetrahedral intermediate (or the transition state leading to it) which results from water attack on the substrate adenosine (Figure 1). In a previous study of adenosine deaminase and AMP deaminase, we concluded (Frieden et al., 1980) that the initial structure of the active site of the enzyme appears to be appropriate for binding the ground state of the substrate rather than the transition state and that considerable readjustment of the enzyme structure seems to be required to bind the transition state effectively.

The importance of proton transfer processes to catalysis and the importance of hydrogen bonding to protein structure (and to protein conformation changes) make solvent isotope effects an ideal probe since such effects will be sensitive to bonding changes involving exchangeable hydrogens. In principle, isotope effects can be interpreted in terms of changes in bond force constants which in turn can allow a detailed molecular description of the processes accompanying catalysis.

Accordingly, we report here a study of the effects of pH(D) on the kinetic and equilibrium properties of the binding of these inhibitors to adenosine deaminase. Data for the effects on the

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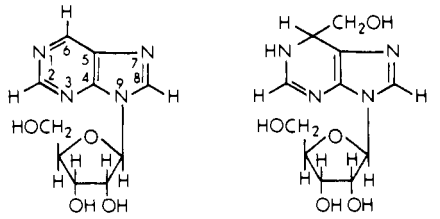

PURINE RIBOSIDE DHMPR

FIGURE 1: Structures of purine riboside, a ground-state analogue inhibitor, and DHMPR, a transition-state analogue inhibitor.

catalytic reaction itself are included since they are required for interpretation of the binding isotope effects.

It has become increasingly clear that binding processes themselves are fundamental contributors to enzyme catalysis (Jencks, 1975). There has been considerable speculation that binding processes might show substantial isotope effects ("substantial" is any effect that is unambiguously not 1.0), but data are scarce for enzyme systems probably because the difficulties discussed below have discouraged investigation. Apparently, there are no data in the literature for ligand binding solvent isotope effects of the magnitude we report here. However, effects of similar size for binding of various inhibitors to chymotrypsin have recently been observed (D. Julin and J. F. Kirsch, unpublished experiments).

The kinetic complexity of enzyme reactions and the multiplicity of exchangeable protons which could contribute to any observed isotope effects make the interpretation of solvent isotope effects in enzyme systems a difficult undertaking. There are several potential sources of a solvent isotope effect on an equilibrium constant for the binding of a ligand to a protein. First, there are transfer effects such as those which arise when a substance containing exchangeable hydrogens is partitioned from water (H_2O or D_2O) into another solvent (the other "solvent" in the present case is the active site). Second, there are possible specific or nonspecific enzyme effects. Nonspecific effects include possible changes in the (ground state) structure of the free enzyme itself in the isotopic solvents. Specific effects might include an isotope effect on an enzyme conformation change (which results in a redistribution of hydrogen bonds) which accompanies ligand binding. An actual proton transfer could occur between residues remote from the active site as a consequence of the conformation change, and this could generate a solvent isotope effect. Third, a change in the structure of the ligand itself which results in a change of bond force constants involving exchangeable hydrogens (such as protonation of the purine nucleus by an active-site acid) could be responsible. These three classes of contributors to a solvent isotope effect on binding have been considered in some detail in the present work. We believe the data presented here allow an interpretation in terms of structural changes in the ligand upon binding to the enzyme, and the consequences for the chemical mechanism of adenosine deaminase are discussed.

Materials and Methods

Materials. Adenosine deaminase (calf intestine) was obtained from Sigma Chemical Co. and purified (when required by the experiment) as described earlier (Frieden et al., 1980). Purine riboside was obtained from Sigma. DHMPR was prepared according to Wolfenden et al. (1977). The solutions of DHMPR in anhydrous methanol were stored in serum-capped vials in liquid nitrogen. After the solutions were thawed, samples were withdrawn by syringe with care to keep the stock solution under nitrogen. D_2O (biograde) was obtained from Bio-Rad.

Temperature Measurements. Thermometers and thermistors were calibrated relative to a National Bureau of Standards certified platinum resistance thermometer. All experiments were performed at 20.0 ± 0.1 °C.

pH(D) Measurements. Measurements to ±0.001 pH(D) unit were made in a thermostated cell. Data for D_2O solutions were corrected to give the pD by adding 0.404 to the pH meter reading (Glasoe & Long, 1960).

UV Difference Spectra. Identical volumes of ligand and protein solutions (or ligand and acid; exact conditions given in figure legends) were pipetted into each side of stoppered split-compartment cells (0.438-cm path length, each side) and placed in the sample and reference turrets of a Cary 118 spectrophotometer with a thermostated cell compartment. After temperature equilibration, the base line was adjusted and recorded. The solutions in the sample cell were mixed, and the difference spectrum was recorded. Finally, the solutions in the reference cell were mixed, and the spectrum was recorded again to check the base line.

pK_a of DHMPR—Spectrophotometric Titration. Aliquots of DHMPR stock solutions were added to thermostated UV cells containing buffers of the desired pH, and the optical density was recorded at the maximum of the acid − neutral difference spectrum (310 nm). An attempt was made to include buffers in which DHMPR was either completely protonated or unprotonated. However, the acid instability of DHMPR made impractical direct determination of the extinction coefficient of the protonated form. Best values of K_a, A_{acid}, and A_{base} were obtained by a nonlinear least-squares fit of the observed A and a_{H^+} values to eq 1, where A is the

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

observed absorbance, a_{H^+} is the hydrogen ion activity (from the measured pH), A_{acid} is the absorbance of a solution containing only protonated ligand, A_{base} is the absorbance of a solution containing only unprotonated ligand, and K_a is the acid dissociation constant.

pK_a of Purine Riboside in H_2O and D_2O. The pK_a of purine riboside was determined spectrophotometrically. Aliquots of a purine riboside stock solution were pipetted into UV cuvettes (final concentration 8.08×10^{-4} M). The acid extinction coefficient at 280 nm was measured in a cell containing 1 M HCl in H_2O or D_2O. The basic extinction coefficient was measured in a cell containing a pH(D) 7.00, 50 mM potassium phosphate buffer with sufficient K_2SO_4 added to bring the ionic strength to 0.15. The absorbance at pH(D) 2.00 [small pH(D) corrections of ~0.02 were applied for consumption of acid by purine riboside] was measured in a cell containing 0.0131 M HCl and 0.14 M KCl, I = 0.15. The acid dissociation constant, K_a, was calculated from eq 1 where a_{H^+}, the hydrogen ion activity, was calculated by using activity coefficients from the Davies equation (Davies, 1961). A_{acid} is the absorbance of the solution in 1 M HCl. A_{base} is the absorbance of the solution at pH(D) 7.00. A is the absorbance of the solution at the hydrogen ion activity, a_{H^+}.

DHMPR Dissociation Constant—Fluorescence Titrations. DHMPR quenches the intrinsic protein fluorescence of adenosine deaminase. Fluorescence measurements were made with a Spex Fluorolog spectrofluorometer by using an excitation wavelength of 290 nm (band-pass of 5 nm) and observing the emission at 350 nm (band-pass of 40 nm). Aliquots of DHMPR were added to enzyme solutions [50 mM potas-

sium phosphate/sulfate, pH(D) 7.00, $I = 0.15$] in a constantly stirred, thermostated, 1.0 × 1.0 cm cell. Corrections were made for the presence of a fluorescent contaminant in DHMPR preparations by DHMPR titration of a tryptophan solution of approximately the same fluorescence as that of the protein. This correction was about 20% by the end of the titration. The value of ΔF_{max}, the total fluorescence quenching at saturation of binding sites, was determined by linear extrapolation of the double-reciprocal plot of $1/[DHMPR]_{total}$ vs. $1/\Delta F$ where ΔF is the observed fluorescence change. The concentration of free DHMPR was calculated from eq 2

$$[DHMPR]_{free} = [DHMPR]_{total} - (\Delta F/\Delta F_{max})[E] \quad (2)$$

where [E] is the enzyme concentration. The dissociation constant was determined from a plot of $Y/[DHMPR]_{free}$ vs. Y (Scatchard plot) where Y is the fraction of DHMPR bound to the enzyme.

Stopped-Flow Experiments. All stopped-flow experiments were performed with a Durrum stopped-flow instrument by using a thermostated cell with a path length of 2 cm. All experiments were performed at an nominal wavelength of 267 nm and a slit width of 0.3 mm. The spectral slit provided by the Durrum monochromator is sufficiently wide that the apparent difference extinction coefficients were found to depend somewhat upon the composition of the solution. For example, the apparent difference extinction coefficient for adenosine and inosine, $\epsilon_{Ado} - \epsilon_{Ino}$, differs by about 15% for identical buffers in H_2O and D_2O. Therefore, at the conclusion of each stopped-flow run, the substrate solution was removed from the drive syringe and its concentration determined from its abosrbance at 259 nm measured in a Cary 118 spectrophotometer. No difference between H_2O and D_2O was found for the extinction coefficient of adenosine at a spectral slit width of 1.93 nm in the Cary 118 spectrophotometer.

Kinetic constants were determined as discussed elsewhere (Bates & Frieden, 1973) by using computer simulation of full time course data. In our experience, full time course experiments yield accurate (±5%) kinetic constants (V_{max}, K_m) much more conveniently than do conventional initial velocity experiments. The mechanisms used for simulation were those previously described (Frieden et al., 1980) with the addition of prior equilibrium protonation of adenosine, pK_a of 3.54 at 20 °C (Suchorukow et al., 1964), and of DHMPR, pK_a of 4.83 at 20 °C (vide infra), when required at low pH. The mechanisms are shown in Scheme I.

Scheme I

Ground-State Analogues

$$E + A \underset{}{\overset{K_m}{\rightleftharpoons}} EA \xrightarrow{V_{max}} EP \overset{K_p}{\rightleftharpoons} E + P$$

$$E + I \overset{K_i}{\rightleftharpoons} EI$$

Transition-State Analogues

$$E + A \overset{K_m}{\rightleftharpoons} EA \xrightarrow{V_{max}} EP \overset{K_p}{\rightleftharpoons} E + P$$

$$E + I \overset{K_i}{\rightleftharpoons} EI \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI'$$

or alternatively if K_i is large

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI'$$

It was assumed that the protonated form of substrate or inhibitor did not bind to the enzyme. The validity of this assumption is discussed under Results and Discussion.

Table I: pH(D) Dependence of Kinetic Constants

kinetic constant	pK_a (low)	pK_a (high)
V_{max}	6.20 ± 0.08(D_2O)	
	5.64 ± 0.02(H_2O)	
V_{max}/K_m	5.68 ± 0.07(D_2O)	
	4.97 ± 0.05(H_2O)	
$(V_{max}/K_m)K_i$(PR)	4.05 ± 0.11	
$(V_{max}/K_m)K_i$(DHMPR)	4.39 ± 0.10	
k_{off}(DHMPR)	3.76 ± 0.18	9.39 ± 0.08
k_{on}(DHMPR)	4.72 ± 0.05	9.39 ± 0.07

Intrinsic kinetic constants [V_{max}, V_{max}/K_m, $(V_{max}/K_m)K_i$, k_{on} for DHMPR, k_{off} for DHMPR] were obtained from fits of the apparent kinetic constants as a function of pH to two- or three-parameter equations (eq 3 and 4) by using a nonlinear

$$Y = \frac{Y_{max}}{1 + a_{H^+}/K_{a1}} \quad (3)$$

$$Y = \frac{Y_{max}}{1 + a_{H^+}/K_{a1} + K_{a2}/a_{H^+}} \quad (4)$$

least-squares procedure (Busing & Levy, 1962) where Y is the apparent kinetic constant at hydrogen ion activity a_{H^+}, Y_{max} is the value of the intrinsic kinetic constant, K_{a1} is the first acid dissociation constant of free enzyme or complex, and K_{a2} is the second acid dissociation constant of free enzyme or complex. It is to be noted that a least-squares procedure assumes random error as opposed to (more likely) systematic ones. In addition, in any multiparameter fit, the errors are frequently correlated in such a way that a small "true" error in one parameter is reflected in the calculation of a smaller standard deviation for a second than seems warranted from inspection of the data (as illustrated by the low pK_a in the fit for k_{off} for DHMPR). While the standard deviations calculated do give some idea of the quality of the data and their consistency with the proposed models, the true error is probably larger.

As indicated in the legends to the figures, a series of overlapping buffers at constant ionic strength ($I = 0.15$) was used for these experiments. Thus, from pH 4.5 to 5.5, we used 50 mM potassium acetate buffers, from pH 5.5 to 8.5, potassium phosphate buffers, and from pH 8.5 to 9.0, potassium pyrophosphate buffers. K_2SO_4 or KCl was added to make up the ionic strength as required. In all cases, different buffers at the same pH were found to give the same values of the kinetic constants. At the highest pH used, pH 9.00, the enzyme was found to lose about 5% activity in 30 min, the time required for each stopped-flow run. At low pH values, the enzyme was found to be extremely stable.

Results and Discussion

pH(D) Dependences of V_{max}/K_m and V_{max}. The pH(D) profiles for V_{max}/K_m and V_{max} are shown in panels A and B, respectively, of Figure 2. For an enzyme utilizing a single substrate, the pH(D) dependences of V_{max}/K_m and V_{max} measure the pK_a values of ionizable groups important in enzymatic activity for the free enzyme and enzyme–substrate complex(es), respectively (Frieden & Alberty, 1955). The solid lines in the figure were calculated from the least-squares parameters from a fit of the data to eq 3 in which it is assumed that there is only one ionizable group. The pK_a values obtained from the least-squares fit are given in Table I.

Four aspects of these data should be noted. First, the pK_a values for the free enzyme and for the enzyme–substrate complex is lower in H_2O relative to D_2O by 0.68 and 0.56,

Table II: Solvent Isotope Effects

	ratios[a] of values of kinetic constants								
pH(D)	V_{max}	V_{max}/K_m	K_m	K_i (PR)	$(V_{max}/K_m)K_i$(PR)	k_{off} (DHMPR)	k_{on} (DHMPR)	K_i (DHMPR)	$(V_{max}/K_m)K_i$ (DHMPR)
7.00	0.92	0.92	1.00	1.48	1.36	1.12	0.94	1.19	1.10
7.51	0.92	0.90	1.03	1.50	1.35	1.23	0.88	1.40	1.25
7.99	0.88	0.85	1.03	1.53	1.30	1.43	1.01	1.42	1.21

[a] $V_{max}(H_2O)/V_{max}(D_2O)$.

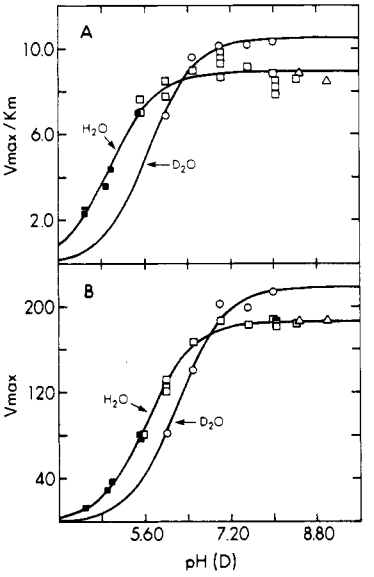

FIGURE 2: pH(D) dependence of (A) V_{max}/K_m (s^{-1} μM^{-1}) and (B) V_{max} (s^{-1}). For pH 4.5–5.5, 50 mM potassium acetate/chloride buffers, I = 0.15 (■); for pH(D) 5.5–8.5, 50 mM potassium phosphate/sulfate buffers, I = 0.15, (□) H_2O, (○) D_2O; for pH 8.5–9.0, 13 mM potassium pyrophosphate/chloride buffers, I = 0.15 (△). Solid lines in the figures were calculated from the least-squares parameters in Table I.

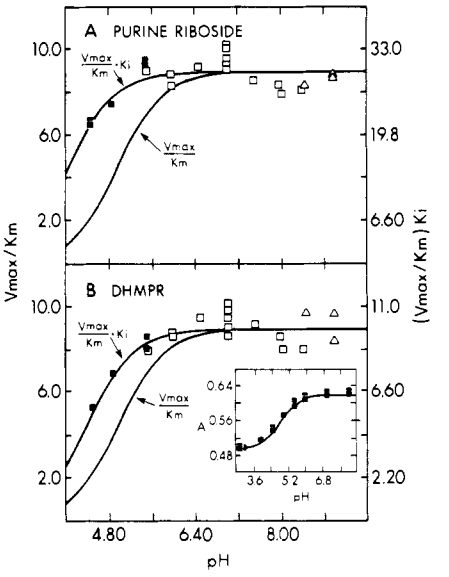

FIGURE 3: pH dependence of V_{max}/K_m (s^{-1} μM^{-1}) and $(V_{max}/K_m)K_i$ (s^{-1}) for (A) purine riboside and (B) DHMPR. Symbols are defined as in Figure 2. Solid lines were calculated from the least-squares parameters in Table I. (Inset) Spectrophotometric titration of DHMPR at 310 nm. Solid line calculated from the least-squares parameters given in the text.

respectively. These observed differences [$\Delta pK_a = pK_a(H_2O) - pK_a(D_2O)$] for the acid group are compatible with histidine or carboxyl ionizations (Laughton & Robertson, 1969), but the actual pK_a value, 4.97, is probably more consistent with a carboxyl residue. Second, in both solvents, the pK_a for the enzyme–substrate complex is higher than that for the free enzyme by 0.52 (D_2O) and 0.67 (H_2O) pK units, indicating that the binding of the substrate shifts the pK_a of this group. The data obtained in H_2O are in agreement (vide infra) with previously reported values (Baer et al., 1968).

Third, an inverse kinetic solvent isotope effect of 0.9 is found in the intrinsic (pH independent) V_{max} and V_{max}/K_m values (see Table II). We have confidence that this small effect is real because over the course of several independent experiments using different enzyme and solution preparations we have never observed any overlap in the values of the kinetic constants obtained in the two solvents for the plateau region of the pH(D) profiles (see Figure 2). In addition, a small inverse kinetic solvent isotope effect on V_{max} has been reported for the adenosine deaminase catalyzed hydration of pteridine (Evans & Wolfenden, 1973). The pH(D) data illustrate why determination of the complete pH(D)–rate profiles is required to understand solvent isotope effects in enzyme systems. Thus, at pH values ≤6.5, an apparent normal isotope effect would be observed as reported by Wolfenden (1969). Fourth, it is interesting that there is apparently no solvent isotope effect on substrate binding as K_m is the same for both solvents, ±5%, in the plateau region of the pH(D)–rate profiles. Thus, the solvent isotope effects obtained in this region for V_{max} and V_{max}/K_m are equal and most likely reflect the intrinsic values. The identity of the isotope effects for V_{max} and V_{max}/K_m supports the validity of the prior equilibrium mechanism since the only steady-state mechanism in which this would be found is one in which the rate constant for substrate dissociation from the ES complex equaled that for product release from the EP complex. However, since the observed isotope effects are so small and consequently uncertain, some caution must remain in accepting a prior equilibrium mechanism. Studies of the solvent isotope effects on the binding of inhibitors (discussed below) were confined to this plateau region. It is of interest that product inhibition (not shown) is apparent in D_2O buffers (at pD 6.5 and 6.0) but not in H_2O buffers, making it difficult to expand the pD range to lower pD values by using full time course simulation. The apparent tighter binding of the product in D_2O is not surprising in light of the tighter binding of purine riboside and DHMPR (vide infra) but contrasts with the lack of any effect on the substrate.

Baer et al. (1968) have reported data in H_2O buffers indicating a second pK_a at high pH for the free enzyme. The data presented here suggest that if this group can be titrated in the free enzyme, its pK_a must be greater than 9.5. Instability of the enzyme at high pH precludes experiments above pH 9.0. As discussed later, a second ionizable group is found in the enzyme–DHMPR complex. In agreement with our results, Baer et al. (1968) found a single pK_a for the enzyme–substrate complex.

pH(D) Dependences of $(V_{max}/K_m)K_i$ for DHMPR and Purine Riboside. For the single substrate case in the presence of a competitive inhibitor, a plot of the quantity $(V_{max}/K_m)K_i$

vs. pH should yield the pK_as of the enzyme–inhibitor complex. The data for the enzyme–inhibitor complex with the ground-state inhibitor purine riboside and with the transition-state analogue inhibitor DHMPR are shown in panels A and B, respectively, of Figure 3. The V_{max}/K_m profile (from Figure 2A) is given for comparison. The pK_a values obtained from these data are summarized in Table I.

Data for DHMPR are complicated by the fact that the ligand is protonated in the low-pH region. A pK_a of 4.83 ± 0.03 was found for DHMPR at 20.0 ± 0.1 °C. The spectrophotometric titration of DHMPR is shown in the inset to Figure 3B. In determining the pK_a value of the enzyme–inhibitor complex from $(V_{max}/K_m)K_i$, it was assumed that the protonated form of DHMPR did not bind. Certainly, the binding of the protonated form is looser than that of the unprotonated form since if the assumption is made that both forms bind equally well, the plot shows a marked upward curvature at low pH. For example, at pH 4.5, assuming both forms to bind equally well results in a value for $(V_{max}/K_m)K_i$ over 4 times greater than that given in the figure. However, we cannot be certain that the protonated form does not bind at all.

It is clear for purine riboside (and probably also for DHMPR despite the complication of ligand protonation) that the low pK_a found for the free enzyme (which was increased in the enzyme–substrate complex) has been decreased substantially (by 0.9 and 0.5 pK_a units for purine riboside and DHMPR, respectively; Table I) in the enzyme–inhibitor complexes. The observation that the binding of competitive inhibitors shifts the low pK_a of the enzyme should in principle make possible a test of the prior equilibrium mechanism since it should be possible to fit K_i data by using the pK_as found for V_{max}/K_m (ostensibly the free enzyme) and $(V_{max}/K_m)K_i$ (the enzyme–inhibitor complex) pH profiles. Unfortunatly, the quality of the data in the low-pH region is not sufficiently good at this time to make this test.

pH(D) Dependences of On and Off Rate Constants for DHMPR. As reported previously (Frieden et al., 1980), the inhibitory behavior of this transition-state analogue can be described by using single off and on rate constants, the ratio of the two being an overall dissociation constant. As discussed elsewhere (Frieden et al., 1980), a more accurate representation would be a weak initial binding followed by an isomerization to yield a final tight complex. However, the initial binding appears to be so weak that the representation as a single-step on and off rate is adequate.

The pH dependences of the on and off rate constants, k_{on} and k_{off}, are shown in panels A and B, respectively, of Figure 4. The curves are bell shaped, reflecting two ionizable groups on the enzyme which are related to DHMPR binding. The pK_a values obtained from the least-squares fit to eq 4 (two ionizations) are given in Table I, and the solid lines in the figure are calculated from the least-squares parameters. A mechanism involving a single ionizable group is clearly inadequate to describe the data.

For a simple mechanism, the titration curve for k_{on} should superimpose with that of the free enzyme if the inhibitor associates with a single form of the enzyme (the active one), assuming a pH-independent rate constant. Furthermore, for this simple mechanism, k_{off} should titrate with the enzyme–inhibitor complex. Examination of Table I shows that neither k_{on} nor k_{off} titrates as expected for the free enzyme or the enzyme–DHMPR complex, respectively. Instead, a pK_a value at high pH is observed (pK_a = 9.4) where no ionizable group has been previously observed in either the free enzyme or the

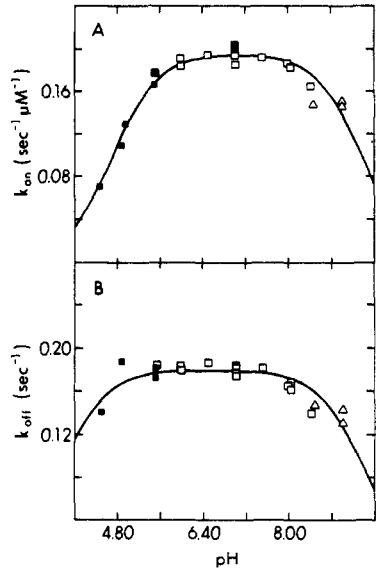

FIGURE 4: pH dependence of (A) k_{on} (s^{-1} μM^{-1}) and (B) k_{off} (s^{-1}) for DHMPR. Symbols are defined as in Figure 2. Solid lines were calculated from the least-squares parameters in Table I.

complex. The k_{on} shows a low pK_a value (4.7) which is slightly lower than the pK_a for the free enzyme (4.95) while k_{off} shows a pK_a value (3.7) lower than that for the enzyme–DHMPR complex (4.4). These results suggest that a more complicated mechanism, involving one or more intermediates in the formation of the enzyme–inhibitor complex, is required; this is in agreement with the mechanism we have previously proposed (Frieden et al., 1980) in which DHMPR first binds weakly to one or more forms of E followed by an isomerization to give the final form(s) of EI. Unfortunately, this mechanism has a sufficient number of variable parameters that no unique fit of the data would be found given the extent of the data.

It is interesting to note that a small inverse solvent isotope effect is observed for k_{on} similar in magnitude to that on V_{max} while a larger normal effect is observed for k_{off}. The small inverse effect on k_{on} might have been predicted if we assume that the same processes involved in reaching the transition state for *binding* a transition-state analogue are also involved in reaching the actual catalytic transition state.

Solvent Isotope Effects on the Dissociation Constants of Purine Riboside and DHMPR. A plateau region exists from pH 7.00 to 8.00 in all the pH profiles of kinetic parameters. Thus, solvent isotope effects in this region should represent isotope effects on the intrinsic constants. Solvent isotope effects on the dissociation constants of purine riboside and DHMPR are given in Table II.

Since most of these equilibrium constants are derived from kinetic measurements, the solvent isotope effect on the dissociation constant of DHMPR from the enzyme was determined independently and directly, utilizing the observation that DHMPR quenches the intrinsic protein fluorescence by 70%. Figure 5 shows a Scatchard plot of the fluorescence titration data in H_2O and D_2O. The plots are linear, consistent with simple hyperbolic binding, and indicate a site occupancy of 1.009 and 0.999 and dissociation constants of 0.75 ± 0.01 μM and 0.91 ± 0.01 μM in D_2O and H_2O, respectively. The solvent isotope effect on the dissociation constant $[K_i(H_2O)/K_i(D_2O)]$ is then 1.21. These values are in gratifyingly good agreement with those obtained from full time course simulation of kinetic data at pH(D) 7.00, where the dissociation constants obtained are 0.74 (D_2O) and 0.88 μM (H_2O) with a solvent isotope effect of 1.19. This agreement between

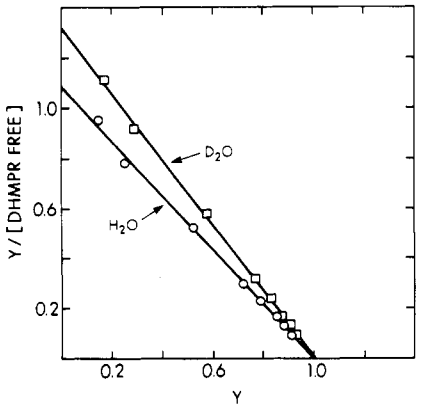

FIGURE 5: Scatchard plots for fluorescence titration of adenosine deaminase in 50 mM potassium phosphate/sulfate buffer, $I = 0.15$, in H_2O (○) and D_2O (□), pH(D) 7.00. Y is the fraction of DHMPR bound to the enzyme.

parameters obtained by kinetic methods and by static titration supports the validity of the mechanism selected.

The intrinsic protein fluorescence spectrum is found to have an excitation maximum at 280 nm with an emission maximum at 340 nm. After correction for fluorescent contaminants in DHMPR (which are assumed not to bind), no shifts are observed in the protein spectra. No low-wavelength shoulder from tyrosine fluorescence is visible in the emission spectrum [the enzyme, a single chain of molecular weight 35 000, contains 11 tyrosines and 3 tryptophans per mole (Phelan et al., 1970)] in either the presence of the absence of DHMPR even though the protein fluorescence is quenched by 70% in the enzyme–DHMPR complex. The large protein fluorescence quench is apparently another unique property of transition-state analogues since similar results are obtained for deoxycoformycin (data not shown). Further experiments to elucidate the nature of these observations in molecular terms are in progress.

It is not possible to confirm the purine riboside binding isotope effect by using fluorescence titration because the maximum protein fluorescence quench observed for the enzyme in the presence of saturating amounts of this inhibitor is only 6%.

Difference Spectra. Difference spectra for the binding of DHMPR and purine riboside to adenosine deaminase are compared to the acid – neutral difference spectra for the two inhibitors in panels A and B, respectively, of Figure 6. The DHMPR difference spectrum qualitatively resembles that previously reported (Wolfenden et al., 1977), but there are significant quantitative differences. The primary difference between the present and previously reported spectrum is in the location of the zero-absorbance line.

The changes in the UV spectrum of purine riboside generated upon formation of the enzyme–inhibitor complex are similar to those generated upon protonation of the purine ring [protonation is at N-1, Figure 1 (Izatt et al., 1971)] although the difference spectra cannot be superimposed. About the same degree of similarity is found between the acid – neutral difference spectrum of methotrexate and the methotrexate – dihydrofolate reductase difference spectrum (Erickson & Mathews, 1972). In this case, the evidence for protonation of the pteridine ring of methotrexate by dihydrofolate reductase has been recently strengthened by ^{13}C NMR studies of the enzyme–inhibitor complex (Cocco et al., 1981). The ^{13}C chemical shift of the 2-carbon of methotrexate in the complex was found to be close to that of methotrexate free in solution protonated on the adjacent nitrogen.

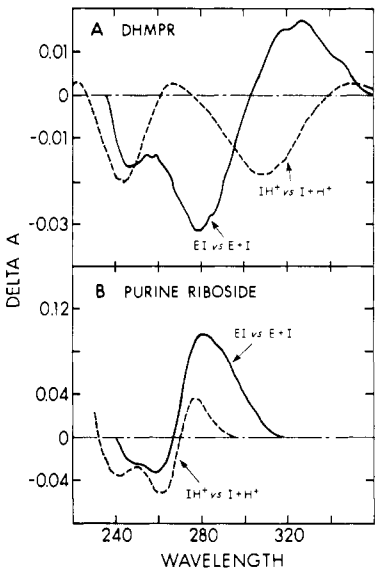

FIGURE 6: Difference spectra for (A) DHMPR. Solid line, pH 7.00, final concentrations in sample cell 23 μM adenosine deaminase and 75 μM DHMPR in 50 mM potassium phosphate/sulfate buffer, $I = 0.15$. Dashed line, pH 2.96, final concentrations in sample cell 37 μM DHMPR and 50 mM potassium phosphate buffer. (B) Purine riboside difference spectra. Solid line, pH 7.00, final concentrations in sample cell 23 μM adenosine deaminase and 150 μM purine riboside in 50 mM potassium phosphate/sulfate buffer, $I = 0.15$. Dashed line, 0.5 M HCl, final concentration in sample cell 36 μM purine riboside.

In contrast, the changes in the UV spectrum of DHMPR do not resemble those which occur upon protonation of the heterocyclic ring. In the case of DHMPR, it is likely that a secondary amine is the site of protonation. However, it is not certain whether this will be the 1- or 3-nitrogen (Figure 1) since a simple isomerization will interconvert the more basic of these nitrogens. If free ligand solutions are mixtures of both isomers while enzyme binding (and subsequent protonation) occurs preferentially to only one isomer (as is likely), then we might expect little resemblance between the acid/neutral and enzyme bound/free ligand difference spectra. In addition, we know that DHMPR binding is accompanied by a large enzyme conformation change (vide supra), and the consequent perturbation of the protein spectrum may be obscuring that of the ligand.

Source of the Solvent Isotope Effect on Ligand Binding. We believe that the solvent isotope effect on the binding of inhibitors to adenosine deaminase may be explained in terms of structural changes in the ligand which occur upon binding to the enzyme. However, for systems as complex as these, it is necessary to consider a number of other possibilities. For convenience, the solvent isotope effect will be factored into three possible contributions: *transfer effects*, *specific and nonspecific enzyme effects*, and finally, effects on the *structure of the enzyme-bound ligand.*

The first possible contribution to the solvent isotope effect is that arising from transfer of a nucleoside from free solution to the active site, an environment of lower dielectric constant. Such transfer isotope effects are commonly though to be small (Schowen, 1978). Unfortunately, neither data concerning differential solubilities of nucleosides in D_2O and H_2O nor distribution coefficient isotope effects between aqueous and hydrocarbon solvents exist which could be used to estimate the magnitude of this contribution. However, H/D exchange studies with DNA and RNA indicate that all the exchangeable hydrogens in these nucleic acid polymers (regardless of whether in ordered or disordered regions) have fractionation fractions close to 1.0 (Lees & von Hippel, 1968; Printz & von Hippel,

1965; Englander & Englander, 1965). Therefore, we feel reasonably safe in neglecting this possible contribution to the observed isotope effect.

A second contribution might arise from whatever conformation changes may occur in the enzyme in the isotopic solvents themselves. It should be noted that incubating the enzyme in D_2O does not change the kinetic parameters measured in H_2O. However, there might be a solvent isotope effect on the conformation change which is known to occur upon the binding of inhibitors (a more extensive conformation change is indicated upon binding the transition-state analogue than the ground-state analogue, yet they yield about the same isotope effect). The magnitude of these possible contributions is difficult to assess, and it is certainly possible that they may account for the observed isotope effect. Using precise fluorescence titration methods, it may be barely feasible to use proton inventory techniques to distinguish whether more than one proton is involved in the observed solvent isotope effect. An unambiguous finding of more then one proton would center attention on a protein conformation change as being responsible for the observed effect.

The final contribution centers on the structural changes in the ligand itself which might occur upon binding to the enzyme. On the basis of the known chemistry of the purine ring and other similar heterocyclic systems, there are several possibilities. These include hydration of purine at C-6, protonation at N-1, and formation of a covalent sulfhydryl adduct at C-2 or C-6.

The enzyme has hydratase activity toward pteridine and hydrolase activity toward 4-aminopteridine (Evans & Wolfenden, 1973). Does an analogous situation exist with purine riboside and adenosine? Conversion of the 6 carbon of the purine ring from sp^2 to sp^3 as in the formation of DHMPR does induce UV spectrum changes somewhat similar to those which we have observed upon binding of purine riboside to the enzyme (Linschitz & Connolly, 1968). Equilibrium solvent isotope effects for the hydration of aldehydes (Laughton & Robertson, 1969) are usually around 0.8–0.9 (1.1–1.2 for dissociation). For reasons that are not entirely clear at this time, hydration of the seemingly analogous aromatic heterocycles is accompanied by a much smaller solvent isotope effect. Recent measurements of the equilibrium solvent isotope effect for formation of pteridine hydrate indicate a value very close to unity (K. R. Davis and R. Wolfenden, unpublished results). While this result does not rule out hydration as the source of the binding isotope effect for purine riboside, it makes it seem less likely. In addition, a separate mechanism would have to be formulated to account for the solvent isotope effect observed for the binding of DHMPR which already has a sp^3 6-carbon.

Protonation of the purine 1-nitrogen in the enzyme–inhibitor complex in a manner analogous to that of methotrexate in the dihydrofolate reductase–methotrexate complex is an attractive possibility. (We predict that methotrexate binding to dihydrofolate reductase should be accompanied by a comparable or larger solvent isotope effect.) Protonation would be advantagous to catalysis since decreasing the electron density in the ring would facilitate addition of nucleophilic water to the 6-position to form the tetrahedral intermediate. The events which would then accompany binding of an inhibitor to adenosine deaminase may be summarized according to eq 5:

$$\bar{E}H + I \rightleftharpoons [E\text{–}HI] \qquad (5)$$

The solvent isotope effect on the dissociation constant of inhibitor from the enzyme is then given by eq 6:

$$\frac{K_i(H_2O)}{K_i(D_2O)} = \frac{\phi_{[E\text{–}HI]}}{\phi_{EH}} \qquad (6)$$

where ϕ_{EH} is the fractionation factor of the free enzyme and $\phi_{[E\text{–}HI]}$ is the fractionation factor for protonated purine riboside in the enzyme–inhibitor complex. The brackets around the enzyme–inhibitor complex, [E–HI], are meant to emphasize the possibility that the structures of E and/or HI may not be the same as those in free solution. The environment (dielectric constant etc.) of protonated purine riboside in the active site may be significantly different than that in free solution. In general, the isotopic fractionation factor for a molecular site is defined as the ratio of its preference for deuterium over protium relative to a similar ratio for a site in solvent—the equilibrium constant for an isotope exchange reaction. A value <1 implies that protium prefers the molecular site over the solvent site relative to the preference of deuterium.

If we assume that the fractionation factor for protonated purine riboside in the complex, $\phi_{[E\text{–}HI]}$, is approximated by the fractionation factor for protonated purine riboside free in solution, ϕ_{HI}, we can calculate ϕ_{EH}. We obtain pK_as for purine riboside in H_2O and D_2O of 2.044 ± 0.006 and 2.395 ± 0.012, respectively, for a ΔpK_a of 0.351 ± 0.013. This ΔpK_a is somewhat smaller than might have been expected but is not outside the range of values observed for other weak acids (Laughton & Robertson, 1969). Using a hydronium ion fractionation factor of 0.69, we calculate $\phi_{HI} = 0.74$. Then $\phi_{EH} = 0.49$, a value characteristic of sulfhydryls (Pohl, 1961). The higher pK_a of DHMPR would lead us to expect a larger ΔpK_a and consequently a smaller solvent isotope effect for its dissociation from the enzyme as is observed. Unfortunately, the acid instability prevents measurements of ΔpK_a for DHMPR.

Indeed, protonation of substrate by an active-site sulfhydryl has been postulated (Maguire & Sim, 1971; Orsi et al., 1972). One of the unusual properties of this sulfhydryl concerns its modification by mercurials (other modifying reagents are ineffective). A covalent adduct with the modifier has been unambiguously detected from its characteristic UV spectrum. The mercurial reagent was found to be competitive with substrate. Apparently, binding of substrate excludes the reagents from the active site (Wolfenden et al., 1967), a result consistent with the idea that the sulfhydryl is intimately involved in substrate binding. This sulfhydryl has also been postulated to be the high pK_a residue found in the V_{max}/K_m pH profile (Maguire & Sim, 1971). If this is the case, it is difficult to imagine how the enzyme succeeds in protonating a base with a pK_a of 2.0 with an acid of $pK_a = 9.5$. For the case of dihydrofolate reductase, an aspartate residue with a pK_a of 6.6 in the free enzyme is thought to be responsible for protonation of methotrexate, $pK_a = 5.7$ (Williams & Morrison, 1981; Cocco et al., 1981). However, protonation of the purine remains a viable possibility. Very large pK_a shifts have been observed in many enzyme systems. For example, the pK_a of bound methotrexate in the dihydrofolate reductase complex exceeds 10.0.

An interesting alternative to a sulfhydryl as the low fractionation factor acid responsible for purine protonation has been suggested by M. M. Kreevoy (personal communication). Any hydrogen-bonded structure in which the linear motion of the bridging hydrogen is governed by a double minimum potential function will have an abnormally low fractionation factor. Kreevoy & Liang (1980) have studied a number of biphenolate and bicarboxylate complexes with fractionation factors as low as 0.3 which illustrate this effect. An acidic protein residue which participates in such a hydrogen-bonded

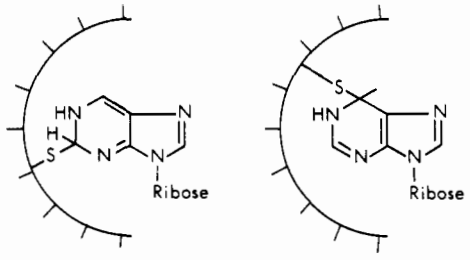

FIGURE 7: Structures of possible sulfhydryl adducts of purine riboside.

structure could have a pK_a more compatible with protonation of the purine base as well as a low fractionation factor.

An alternative to purine protonation has been suggested (R. Schowen, personal communication). The sulfhydryl may form a covalent adduct with the purine at either the 2- or the 6-carbon (Figure 7). Formation of an adduct at the 2-carbon would promote catalysis by destroying the aromaticity of the ring and thus activate the 6-position. Formation of an adduct at the 6-carbon would promote catalysis by means of conventional covalent catalysis. The possibility of a reaction involving enzyme addition at C-6 can probably be discounted because of the nature of the transition-state analogues as well as the ability of the enzyme to catalyze direct addition of water to pteridine. However, addition of the enzyme to C-2 remains an attractice alternative. A solvent isotope effect of 0.44 (2.3 for dissociation) has been reported for the formation of hemithioacetals (Lienhard & Jencks, 1966). While this is larger than we have observed, we might expect a smaller value for formation of the sulfhydryl adduct with these aromatic heterocycles on the basis of the observed diminution of the solvent isotope effect for hydration of pteridine when compared to aldehydes (K. R. Davis and R. Wolfenden, unpublished results).

Conclusions

We have studied the pH(D) effects on the deamination of adenosine catalyzed by adenosine deaminase and on the binding of a ground-state and a transition-state analogue inhibitor. The low pK_a of the free enzyme was found to be decreased in the enzyme–inhibitor complexes. A small inverse solvent isotope effect was observed on V_{max} while none was observed for K_m. Substantial solvent isotope effects were found for the binding of both ground-state and transition-state analogue inhibitors. For adenosine deaminase, we believe these binding isotope effects may be interpreted in terms of structural changes in the ligand rather than in the enzyme because of the large UV difference spectra observed upon formation of the complexes (particularly purine riboside). Three possible structures for the complexed inhibitors have been discussed. While we believe the evidence favors protonation of the purine ring, hydration or formation of a sulfhydryl adduct cannot be excluded in view of the large pK_a shifts which would be required to accomplish purine protonation. A more direct study of the structure of these complexes using NMR techniques is in progress.

Registry No. DHMPR, 63813-87-6; purine riboside, 550-33-4; adenosine deaminase, 9026-93-1.

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