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Adenosine Deaminase: Viscosity Studies and the Mechanism of Binding of Substrate and of Ground- and Transition-State Analogue Inhibitors†

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ABSTRACT: We have studied the effects of viscosogenic agents, sucrose and ficoll, on (1) the hydrolysis of adenosine and of 6-methoxypurine riboside catalyzed by adenosine deaminase and (2) the rates of association and dissociation of ground-state and transition-state analogue inhibitors. For adenosine, V_{max}/K_m is found to be inversely proportional to the relative viscosity with sucrose, an agent affecting the microscopic viscosity, while no effect is found with ficoll, an agent affecting the macroscopic viscosity. Viscosogenic agents have no effect on the kinetic constants for 6-methoxypurine riboside. Thus, the bimolecular rate constant, $V_{max}/K_m = 11.2 \pm 0.8\ \mu M^{-1}\ s^{-1}$, for the reaction with adenosine is found to be at the encounter-controlled limit while that for the reaction with the poor substrate 6-methoxypurine riboside, $0.040 \pm 0.004\ \mu M^{-1}\ s^{-1}$, is limited by some other process. Viscosity-dependent processes do not make a significant (<10%) contribution to V_{max}. The dissociation constants for inhibitors are unaffected by viscosity. The ground-state analogue inhibitor purine riboside appears to bind at a rate comparable to that of adenosine. However, the slower rates of association ($0.16–2.5\ \mu M^{-1}\ s^{-1}$) and dissociation ($5 \times 10^{-6}$ to $12\ s^{-1}$) of transition-state analogue inhibitors are affected by the viscosity of the medium to approximately the same extent as the encounter-controlled rates of association and dissociation of adenosine. A two-step mechanism, a weak prior-equilibrium binding step followed by a viscosity-dependent enzyme conformation change, seems to adequately account for both the low absolute magnitude and the viscosity dependence of the apparent second-order rate constants for transition-state analogue binding.

Adenosine deaminase (EC 3.5.4.4) catalyzes the hydrolysis of (deoxy)adenosine to (deoxy)inosine. An addition–elimination mechanism (Figure 1) has been proposed which proceeds via the addition of water to the 6-position of the purine ring, forming a tetrahedral intermediate (Wolfenden, 1968). Some of the evidence in support of this mechanism is based on the transition-state analogue inhibitor concept (Pauling, 1946; Wolfenden, 1972) in which molecules with structural features of the proposed transition state show unusually high affinity for the enzyme (Figure 2). Thus, nucleoside analogues (Wolfenden et al., 1977; Frieden et al., 1980; Frick et al., 1986) such as (deoxy)coformycin and 1,6-dihydro-6-(hydroxymethyl)purine riboside (DHMPR) with a tetrahedral carbon at the 6-position of the heterocyclic ring (as in the proposed tetrahedral intermediate or its adjacent transition states) have been shown to be unusually effective inhibitors (K_i for deoxycoformycin $< 10^{-12}$ M).

We have studied (Frieden et al., 1980) the kinetic behavior of the interaction of transition-state and ground-state analogue inhibitors with adenosine deaminase. The transition-state analogue systems exhibit unusual kinetics in that they typically bind 1–3 orders of magnitude more slowly than substrates or their analogues (Frieden et al., 1980; this work). Similar slow binding has been observed in several other enzyme–transi-

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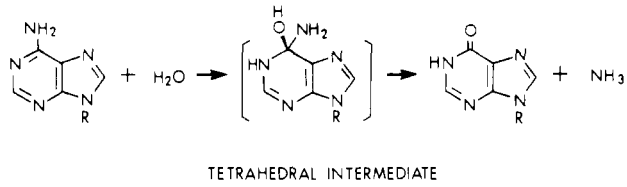

FIGURE 1: Addition–elimination mechanism for the hydrolysis of adenosine catalyzed by adenosine deaminase.

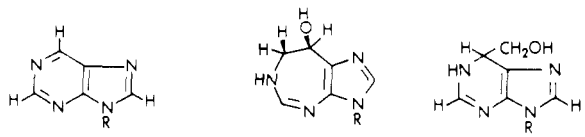

FIGURE 2: Structures of ground-state and transition-state analogue inhibitors of adenosine deaminase. R = (deoxy)ribose.

tion-state analogue inhibitor systems, but the reasons for this behavior have remained obscure [see Wolfenden (1976) and references cited therein].

Since diffusion coefficients vary inversely with the viscosity of the medium (Caldin, 1964), a direct measure of the extent to which an enzymatic rate process is diffusion limited can be obtained from a study of the dependence of the rate upon the viscosity of the medium. This method has been applied successfully to estimate the fractional diffusion-limited component of several enzymatic reactions involving enzyme–ligand encounter (Brouwer & Kirsch, 1982) as well as of other processes including conformation changes (Beece et al., 1981) and protein folding (Tsong, 1982). The present studies were undertaken to explore the extent to which the apparent association (and dissociation) rate constants for substrates, ground-state analogue inhibitors, and transition-state analogue inhibitors reflect a diffusion-limited process.

Materials and Methods

Materials. Adenosine deaminase (calf intestine) was obtained from Boehringer Mannheim Co. Coformycin was obtained from Dr. John Douros of the Developmental Therapeutic Program, Chemotherapy, National Cancer Institute. *N*-Benzylcoformycin was the generous gift of Dr. David C. Baker of the University of Alabama. Sucrose (99%+, Gold Label) was the product of Aldrich Chemical Co. Purine riboside, 6-methoxypurine riboside, and ficoll (M_r 400 000) were obtained from Sigma Chemical Co.

DHMPR was prepared by the methods of Wolfenden et al. (1977) and stored as described by Kurz and Frieden (1983). This material is now known to be an equimolar mixture of two diastereomers which differ in their absolute configuration at the tetrahedral 6-carbon (Buffel et al., 1985). The two diastereomers were separated by high-performance liquid chromatography (HPLC) on a Waters radial compression RCM-100 column (C-18, 10 μm) as follows: A 20-mg sample was prepared; methanol was removed under vacuum to yield a yellow oil which was dissolved in 1 mL of water. After being filtered, 50–100 μL was injected, and a linear gradient from 1% to 10% v/v methanol in water was run at the rate of 3 mL/min for 20 min. Two peaks eluted at 5.5 and 9 min monitoring the absorbance at 330 nm. The two fractions were separately pooled and lyophilized.

Before fractionation by HPLC, the ^{13}C NMR spectrum of DHMPR shows double resonances of approximately equal area for most carbons. After fractionation, the spectra of the two fractions isolated from the DHMPR preparation each show a single resonance for each carbon. This was expected on the basis of the work with triacetylated DHMPR reported by Buffel et al. (1985) which showed that the photoaddition of methanol to purine riboside is random, producing equal amounts of two diastereomers. The 6*S* diastereomer has been reported by Shimazaki et al. (1983) to have the greater affinity for the enzyme, and on this basis (together with our kinetic experiments), we have identified the peak eluting from the column at 5.5 min as the 6*R* isomer and that at 9 min as the 6*S* isomer. In contrast to the mixture of diastereomers which is an oil, the purified diastereomers are white solids. Molar extinction coefficients were obtained: 4.34×10^3 M^{-1} cm^{-1} and λ_{max} = 296 nm for the 6*R* isomer and 4.59×10^3 M^{-1} cm^{-1} and λ_{max} = 296.5 nm for the 6*S* isomer.

Buffers. Buffer solutions of 0.05 M potassium phosphate, I = 0.15 (KCl), and 10^{-4} M ethylenediaminetetraacetic acid (EDTA), pH 7.00, were prepared by mixing a solution containing 0.05 M K_2HPO_4 and 10^{-4} M EDTA with one containing 0.05 M KH_2PO_4, 0.1 M KCl, and 10^{-4} M EDTA until the desired pH was reached. In order to prepare sucrose- or ficoll-containing buffers, the desired weight of viscosogenic agent was added to the volumetric flask before the solution was diluted to volume; molar concentrations of viscosogenic agents are reported. The solutions were filtered through a 0.22-μm nylon membrane to remove suspended material.

The viscosities of buffer solutions were measured with an Ostwald viscometer at 20 °C and are reported relative to the buffer containing no viscosogenic agent. Densities of the solutions were measured at 20 °C.

Stopped-Flow Experiments. All stopped-flow experiments were performed with a Durrum stopped-flow instrument using a 2-cm cell thermostated at 20 °C. The experiments were performed at a wavelength of 267 nm and a slit width of 0.3 mm. The concentrations of substrate stock solutions (prepared in the buffer for the experiment) were determined after appropriate dilution from their absorbance at 259 nm by using a Cary 118 spectrophotometer. Similarly, concentrations of solutions of inhibitors were determined from their absorbance at their wavelength maximum. Full time course data were collected continuously and stored in digital mode for later recall and analysis.

Kinetic Constants. Kinetic constants were determined from full time course, stopped-flow data by fitting progress curves (using a nonlinear least-squares regression analysis) to the appropriate kinetic scheme using the programs KINSIM and FITSIM on a Digital Electronics Corp. microVAX II. KINSIM allows simulation of kinetic mechanisms by numerical integration (Barshop et al., 1983). FITSIM (C. Zimmerle and C. Frieden, unpublished results) was used with the Marquardt algorithm (Marquardt, 1963) for the nonlinear least-squares analysis.

Full time course data for adenosine hydrolysis in aqueous and viscous buffers were initially fitted to the prior-equilibrium mechanism I:

$$E + A \underset{}{\overset{K_m}{\rightleftharpoons}} EA \overset{V_{max}}{\Rightarrow} E + P \qquad (I)$$

In a concentration range (as in our experiments) which avoids the substrate binding transient and in the absence of significant product inhibition (Kurz & Frieden, 1983), the correct kinetic constants, K_m and V_{max}, for a one-substrate reaction are obtained from fits to mechanism I even though those kinetic constants may be composed of several rate constants for elementary steps. In the absence of a perturbant (viscosity change

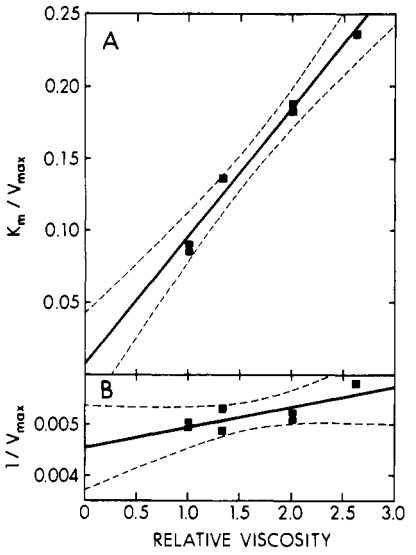

FIGURE 3: Dependence of the kinetic constants of adenosine deaminase catalyzed hydrolysis of adenosine upon relative viscosity. (Panel A) K_m/V_{max}. (Panel B) $1/V_{max}$. Solid lines are the least-squares lines of best fit. Dashed lines are the 95% confidence curves. Stopped-flow experiments were performed at 20 °C in 50 mM potassium phosphate, I = 0.15(KCl), pH 7.00, and kinetic parameters were determined by fitting full time course data as described under Materials and Methods.

in the present case), mechanism I cannot be distinguished from the more realistic mechanism II:[1]

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \overset{k_2}{\Rightarrow} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (II)$$

For full time course data in the presence of inhibitors, additional steps (as indicated where appropriate) were included in simulations using either mechanism I or mechanism II. The kinetic constants so obtained are accurate to within ±5%. A more detailed description of the experimental protocol for these experiments can be found elsewhere (Frieden et al., 1980; Kurz & Frieden, 1983).

Initial velocity data for 6-methoxypurine riboside were collected at 270 nm on a Cary 118 spectrophotometer using a $\Delta\epsilon$ of 3.90×10^3 M^{-1} cm^{-1} for the hydrolysis to inosine (Baer et al., 1968). Kinetic constants were determined by fitting initial velocity data by nonlinear regression to eq 1:

$$v_i/[E] = V_{max}[S]/(K_m + [S]) \qquad (1)$$

RESULTS

Adenosine Hydrolysis. Ficoll buffers (η_{rel} = 2.28) were found to have no effect on the kinetic constants.

Sucrose buffers were found to profoundly affect K_m. For the steady-state mechanism II, the ratio of the apparent kinetic constants, $K_m/V_{max} = (k_{-1} + k_2)/k_1k_2$, is predicted (Brouwer & Kirsch, 1982) to depend upon the relative viscosity of the solution according to eq 2:

$$K_m/V_{max} = \eta_{rel}/k_1 + k_{-1}/k_1k_2 \qquad (2)$$

A plot of K_m/V_{max} vs. η_{rel} and the linear least-squares line together with the 95% confidence level curves are shown in panel A of Figure 3. The slope yielded a value for k_1 of 11.2 ± 0.8 μM^{-1} s^{-1}. The value of the intercept, 0.007 ± 0.01, indicates that the partition ratio, k_{-1}/k_2, is very small [adenosine is a very "sticky" (Cleland, 1977) substrate]. Nonlinear regression fits of the data to mechanism II with the value of k_1 fixed at 11.2 μM^{-1} s^{-1} yield values of k_{-1} close to zero within experimental error.

If the rate of product dissociation is kinetically significant, $1/V_{max}$ [$=(k_2 + k_3)/k_2k_3$] is predicted to depend upon the relative viscosity of the solution according to eq 3:

$$1/V_{max} = \eta_{rel}/k_3 + 1/k_2 \qquad (3)$$

A plot of $1/V_{max}$ vs. η_{rel} and the linear least-squares line together with the 95% confidence level curves are shown in panel B of Figure 3. The slope yielded a value of 2500 ± 1000 s^{-1} for k_3 and the intercept a value of 219 ± 12 for k_2. However, the significance level of the fit is very low (R^2 = 0.598), the slope being mainly determined by the data at the highest viscosity.

6-Methoxypurine Riboside Hydrolysis. The kinetic constants for the poor substrate 6-methoxypurine riboside (V_{max} relative to adenosine = 0.0019) were found to be independent of the presence of the viscosogenic agents ficoll and sucrose and are given in Table I.

Ground-State Analogue Purine Riboside. The effect of viscosity upon the kinetics of the reaction in the presence of the ground-state analogue purine riboside was measured. The kinetic constants in the absence of the inhibitor as determined above were used, and values for K_3 (or k_3 and k_{-3}) were determined by using KINSIM. The mechanism used for purine riboside binding was either the rapid-equilibrium one shown in mechanism III or a steady-state mechanism (IV) with an

$$EI \overset{K_3}{\rightleftharpoons} E + I \qquad (III)$$

$$E + I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EI \qquad (IV)$$

assumed association rate constant equal to that for adenosine. Identical inhibitor dissociation constants, K_3 (or k_{-3}/k_3), were obtained by using either mechanism and were found to be independent of the viscosity of the solution as shown in Table II.

Table I: Kinetic Constants for Adenosine Deaminase Catalyzed Hydrolysis of 6-Methoxypurine Riboside[a]

buffer	K_m (μM)	V_{max} (s^{-1})	η_{rel}
aqueous	8.3 ± 0.8	0.33 ± 0.01	1.00
0.88 M sucrose	8.7 ± 1.7	0.39 ± 0.02	2.62
0.13 mM ficoll	9.2 ± 1.4	0.35 ± 0.02	2.48

[a] Initial velocity experiments were performed at 20 °C in 50 mM potassium phosphate, I = 0.15(KCl), pH 7.00, and kinetic parameters were determined by fitting the data by nonlinear regression to eq 1.

Table II: Lack of Effect of Viscosity on Dissociation Constants for Inhibitors[a]

inhibitor	buffer	η_{rel}	K_i (μM)
purine riboside	aqueous	1.00	2.9 ± 0.2
	0.35 M sucrose	1.33	3.2 ± 0.2
	0.67 M sucrose	2.01	2.8 ± 0.2
	0.82 M sucrose	2.62	2.7 ± 0.2
	0.13 mM ficoll	2.28	2.9 ± 0.2
DHMPR (mixture)	aqueous	1.00	0.81 ± 0.02
	0.67 M sucrose	2.09	0.82 ± 0.02
	0.13 mM ficoll	2.38	0.81 ± 0.02
DHMPR (*S* isomer)	aqueous	1.00	0.29 ± 0.01
	0.67 M sucrose	2.09	0.27 ± 0.01
	0.13 mM ficoll	2.38	0.29 ± 0.01

[a] Stopped-flow experiments were performed at 20 °C in 50 mM potassium phosphate, I = 0.15(KCl), pH 7.00, and kinetic parameters were determined by fitting full time-course data as described under Materials and Methods.

[1] Inability to distinguish by simple kinetic experiments between a prior-equilibrium mechanism (I) and a steady-state mechanism (II) is not true in general. However, that is the case for adenosine deaminase full time course studies for feasible experimental conditions. In principle, single-turnover experiments should allow one to measure the substrate on-rate constant directly. However, no experimental conditions exist where such measurements could be made with available instrumentation.

Table III: Viscosity Dependence of the Rates of Association and Dissociation of Transition-State Analogue Inhibitors

inhibitor	buffer	k_3[a] (μM^{-1} s^{-1})	k_{-3}[a] (μM^{-1} s^{-1})	η_{rel}	$(k_3)_{rel}$[b]	$(k_{-3})_{rel}$[b]
coformycin	aqueous	1.18		1.00	1.00	
	0.67 M sucrose	0.57		2.09	2.07	
	0.13 mM ficoll	1.18		2.38	1.00	
DHMPR (mixture)	aqueous	0.21	0.17	1.00	1.00	1.00
	0.67 M sucrose	0.11	0.09	2.09	1.91	1.88
	0.13 mM ficoll	0.21	0.17	2.38	1.00	1.00
DHMPR (*S* isomer)	aqueous	0.51	0.15	1.00	1.00	1.00
	0.67 M sucrose	0.30	0.08	2.09	1.70	1.87
	0.13 mM ficoll	0.51	0.15	2.38	1.00	1.00

[a] Stopped-flow experiments were performed at 20 °C in 50 mM potassium phosphate, I = 0.15(KCl), pH 7.00, and kinetic parameters were determined by fitting full time course data as described under Materials and Methods. Uncertainties (standard deviations) are 4–7%. [b] Ratio of rate constant in aqueous buffer to that in viscosogenic buffer.

Transition-State Analogue Inhibitors. The progress curves in the presence of transition-state analogue inhibitors were simulated by using mechanism I or II plus mechanism IV for inhibitor binding. The off rate for coformycin ($K_i \sim 10^{-10}$ M; Cha, 1976; Frieden et al., 1980) was assumed to be zero in the simulation since essentially no dissociation occurs during the time of the experiment.

It was of some interest to obtain the kinetic constants for the purified DHMPR diastereomers. Full time course data for the 6*S* isomer were collected at a concentration of 0.2 μM adenosine deaminase, 54 μM adenosine, and 6.7 μM (6*S*)-DHMPR. For the 6*R* isomer, concentrations of 0.75 μM adenosine deaminase, 11 μM adenosine, and 133 μM (6*R*)-DHMPR were used. The 6*S* diastereomer was found to have k_3 = 0.51 μM^{-1} s^{-1} and k_{-3} = 0.15 s^{-1} for a K_i = 0.29 μM. Interestingly, the 6*R* diastereomer was also found to be a "slow" binding inhibitor with k_3 = 0.16 μM^{-1} s^{-1} and k_{-3} = 12 s^{-1} for a K_i = 75 μM.

The kinetic constants for *N*-benzylcoformycin were also obtained. Full time course data were collected at a concentration of 0.2 μM adenosine deaminase, 55 μM adenosine, and 102 μM *N*-benzylcoformycin. Using mechanism I or II plus mechanism IV, this inhibitor was found to have k_3 = 2.5 μM^{-1} s^{-1} and k_{-3} = 6.5 s^{-1} for a K_i of 2.6 μM. This material is a mixture of enantiomers, and it is not known if both bind to the enzyme. By analogy with the nucleoside deoxycoformycin (Frick et al., 1986), it was assumed that only one enantiomer of the *N*-benzylcoformycin was inhibitory.

The effects of viscosogenic agents on the rate of association and dissociation of the transition-state analogue inhibitors are given in Table III. As with adenosine, association and dissociation rates show a proportional decrease in the presence of the microscopic viscosogenic agent sucrose while the macroscopic viscosogenic agent ficoll has no effect.

Discussion

Steady-State Mechanism for Adenosine Deaminase. Adenosine deaminase is an efficient enzyme with a V_{max}/K_m of $\sim$10 μM^{-1} s^{-1} (Murphy et al., 1969). The upper limit of encounter-controlled enzyme ligand reactions has been estimated to be in the range 1–10^3 μM^{-1} s^{-1} (Hiromi, 1979). For neutral substrates, the chief factor reducing this value from the 10^3 μM^{-1} s^{-1} enzyme–ligand collision rate is the requirement that for an effective collision the ligand must hit the active site (a small fraction of the enzyme surface) in the proper orientation (Hiromi, 1979; Berg & von Hippel, 1985). In the absence of very detailed structural information, it is not possible to estimate the reduction resulting from this steric factor with any certainty. The viscosity variation method (Brouwer & Kirsch, 1982; Hardy & Kirsch, 1984) provides a means for determining the fractional diffusion-limited component for an enzyme-catalyzed reaction.

We have applied this method to the hydrolysis of adenosine catalyzed by adenosine deaminase. The data show that the value of k_1k_2 is very much greater than that of k_{-1} (mechanism II and eq 2) and that the bimolecular reaction rate (V_{max}/K_m) is entirely encounter-controlled since $V_{max}/K_m = k_1/\eta_{rel}$ within experimental error.

It is necessary to be sure that the effects we observe derive from the viscosity changes and not from a nonspecific "solvent effect". This conclusion is supported by two essential controls. The first of these is provided by the results with the poor substrate, 6-methoxypurine riboside. The value of V_{max}/K_m $\sim$ 0.04 μM^{-1} s^{-1} is too small to be encounter-limited. In accord with the principle of minimum hypothesis, we would then expect this poor substrate to follow a prior-equilibrium mechanism (I), and thus, we would expect no viscosity effect on any of the kinetic constants for this reaction as is observed (Table I). Thus, the K_m value observed for this poor substrate, $\sim$8 μM, should equal its K_d. The second control is provided by the absence of any viscosity effect on the K_i values for the ground-state and transition-state analogue inhibitors (Table II). For an inhibitor such as purine riboside which presumably binds with an on-rate constant comparable to that of adenosine, the concentration of [EI] in our experiments rapidly reaches steady state, and no effect of viscosity is expected as is observed (Table II). For the transition-state analogue inhibitors where the binding and dissociation transients are observable and viscosity dependent, the ratios of the rate constants should remain unaffected since equilibrium constants should not depend on the solution viscosity (Table II). Thus, we are observing viscosity effects on a dynamic process; diffusion and nonspecific solvent effects are not likely to be involved.

The absence of any effect (Table III) with the viscosogenic agent ficoll (a sucrose polymer of M_r $\sim$400 000) indicates that the reaction rate is primarily sensitive to the diffusion rate of the small ligand. Polymeric viscosogenic agents do not affect the diffusion of small hydrophilic species at concentrations which substantially affect the macroscopic viscosity (Stokes & Weeks, 1964; Hardy & Kirsch, 1984). However, the distinction between microscopic and macroscopic viscosity is only a qualitative one depending upon the relative sizes of the viscosogenic agent and the species under investigation.

Our experiments are not precise enough to unambiguously confirm a viscosity dependence for V_{max}. The observed viscosity dependence suggests that if k_3 is viscosity dependent, then its value is at least 10-fold greater than k_2. This viscosity-dependent rate constant may be that for inosine dissociation (mechanism II). The observed value for k_3, 2500 $\pm$ 1000 s^{-1}, combined with an assumed association rate constant equal to that for adenosine is approximately consistent with a K_i for inosine of $\sim$300–400 μM as is obtained under our

conditions (L. C. Kurz, unpublished results). It is clear that product off rates (or other viscosity-dependent processes) do not make a substantial contribution (<10%) to whatever processes are rate limiting for V_{max}. Of course, we do not exclude the possibility that V_{max} includes rate constants for viscosity-independent isomerizations etc. after product formation.

Mechanism of Binding of Transition-State Analogue Inhibitors. The usefulness of transition-state analogue inhibitors lies in the fact that they are analogues of the *substrate* portion of a state which cannot be observed directly, the activated complex, and the kinetic and equilibrium properties of their stable complexes with the enzyme may allow us to gain insight into the *enzyme* portion of the activated complex. While the rate constants for the association of transition-state analogue inhibitors with the enzyme are substantially smaller than that for adenosine, they are nearly as viscosity dependent (Table III), indicating a substantial diffusion-limited component. The nature of this diffusion-limited process is the subject of the following discussion.

We have obtained the *apparent* second-order rate constants for transition-state analogue inhibitor association assuming the simple bimolecular step shown in mechanism IV. This simple bimolecular mechanism cannot be an adequate description of transition-state analogue inhibitor binding since it cannot explain (1) why the rate constants are so low, 0.1 μM^{-1} s^{-1} (outside the range usually considered to be encounter-controlled and indeed much lower than those for adenosine, 10 μM^{-1} s^{-1}), and (2) why they differ for different analogues (0.16–2.5 μM^{-1} s^{-1}). The major factor reducing the value of encounter-controlled rate constants below the value usually assumed for enzyme–ligand collision is the small area (the active site) on the enzyme surface with which the ligand must collide in order to bind. This target area is unlikely to be different for adenosine and for transition-state analogues. Since all the ligands we have studied are uncharged and in fact very similar in structure, it is unlikely that long-range electrostatic forces affect the rate of diffusion of the ligands differently.

Thus, for transition-state analogues, a simple binding process (i.e., mechanism IV) must be oversimplified. There are two possible multistep mechanisms which account for the apparent second-order nature of the binding process and its viscosity dependence together with the low value for its rate constant.

The first[2] assumes (mechanism V) that the enzyme preexists in two forms, E and E′. The transition-state analogue inhibitor

$$E \underset{}{\overset{K_E}{\rightleftharpoons}} E' + I \underset{k_{-I}}{\overset{k_I}{\rightleftharpoons}} E'I \qquad (V)$$

can only bind to E′ (with an encounter-controlled rate). The absence of instantaneous inhibition and the apparent second-order nature of the process (Frieden et al., 1980) require that E′ be a rare form of the enzyme. For an inhibitor with K_i ($=K_E k_{-I}/k_I$) $\sim$ 1 μM (such as DHMPR) and assuming E′ represents 1% of the total enzyme, k_I = 20 μM^{-1} s^{-1} and k_{-I} = 0.2 s^{-1}; with only k_I and k_{-I} sensitive to viscosity, all the observations can be accommodated.

We have shown previously (Kurz et al., 1985) that substantial protein structural changes accompany transition-state analogue binding, and within the limited sensitivity of the methods employed, these structural changes were the same for all transition-state analogue inhibitors. The validity of mechanism V would imply that an enzyme form appropriate for binding the transition state preexists in equilibrium with that appropriate for binding the ground state.

In its simplest form, this mechanism would predict that all transition-state analogue inhibitors should associate with the enzyme with the same apparent second-order rate constant. However, these values vary $\sim$20-fold (Frieden et al., 1980; this work). If mechanism V is valid, we must postulate either a different rare enzyme form for each inhibitor or a different encounter-controlled association rate constant for each.[3] The differing effectiveness of short-range forces in prolonging the lifetime of the encounter complex with different analogues might be an adequate explanation for differences in the values of encounter-controlled rate constants (Berg & von Hippel, 1985). However, we have been unable to imagine a molecular model rationalizing the action of such forces.

The second possible mechanism (VI) assumes a very weak prior-equilibrium binding of inhibitor to the enzyme followed

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \underset{k_{-E}}{\overset{k_E}{\rightleftharpoons}} EI' \qquad (VI)$$

by a slow rate-determining conformation change. For an inhibitor with K_i ($=K_I k_{-E}/k_E$) = 1 μM and with K_I = 1 mM, values of k_E = 200 s^{-1} and k_{-E} = 0.2 s^{-1} accommodate the observations in aqueous solution. We have previously proposed this mechanism (Frieden et al., 1980) for the binding of transition-state analogue inhibitors, and there seems to be no difficulty in accounting for different association rate constants through differences in either the initial binding or the subsequent conformation change. It was also noted previously that the rate constant, k_E, of the enzyme conformation change could approximate the turnover number for adenosine.

However, in order to account for the observed viscosity effect, we must postulate that the rate-controlling conformation change in mechanism VI is diffusion limited. Whether or not this is likely depends upon the exact nature of the conformation change. If relative displacements of parts of the enzyme structure are large compared to the size of the viscosogenic agent, so that the medium can be thought of as a continuum, we would expect a viscosity dependence although smaller relative displacements would not be expected to be systematically affected. The viscosity dependence of the rate of the later stages of the photoreaction cycle of bacteriorhodopsin has been proposed to result from a viscosity effect on the rate of protein conformational changes (Beece et al., 1981). A viscosity-dependent conformational relaxation of ribonuclease A has been detected in the thermal unfolding zone (Tsong, 1982). This latter case is particularly interesting since it was

[2] The possible existence of a rare form of the enzyme, E′, which is appropriate for binding transition-state analogues does not affect the accuracy of simulations of the full time course progress curves using mechanisms I or II. Our data require that such an enzyme form be present in very low concentrations in order to account for the lack of instantaneous inhibition with transition-state analogues. Furthermore, it is likely that such an enzyme form could not bind substrate.

[3] A kinetically equivalent mechanism would be one in which the inhibitor exists in a number of conformers. Only a small concentration of the appropriate conformer exists for each inhibitor in order to account for the lack of instantaneous inhibition. Further, the amount of appropriate conformer differs for each inhibitor. It is important to keep in mind that all of the compounds studied are nucleosides of very similar structures. A rare inhibitor form mechanism would have to explain why only transition-state analogues have this rare form requirement and not adenosine or ground-state analogue inhibitors. Initially, this would suggest that attention should be focused on conformers around the tetrahedral 6-carbon and sp^3 nitrogen. Thus, the amount of appropriate conformer in this region should not differ between coformycin and deoxycoformycin which have identical base structures. Yet, the association rate constants for these two compounds differ by a factor of 2 (Frieden et al., 1980). We feel that a rare inhibitor form mechanism is unlikely for the same reasons we disfavor the rare enzyme form mechanism.

possible to show that the hinge-bending motion associated with substrate binding was not viscosity dependent. Unless the hinge bending involves motion of a peptide chain longer than several peptide units (Hass et al., 1978), no viscosity effect is expected.

Overall, we favor mechanism VI with a viscosity-dependent conformation change of the protein. Conformation changes have been shown to accompany transition-state analogue inhibitor binding (Kurz et al., 1985). Furthermore, the nature of this conformation change seems to be independent of the structure of the transition-state analogue (or its affinity). Thus, it seems reasonable to account for the variation in the values of the apparent second-order rate constants for inhibitor binding as the result of differences in the prior-equilibrium binding constants, and for their viscosity dependence as resulting from that of the rate-determining conformation change. However, if this viscosity-dependent structural change is part of the catalytic cycle of the enzyme (which seems likely), then its rate must be substantially greater than the turnover number of the enzyme, $\sim 200\ s^{-1}$, or otherwise we would have detected a greater viscosity effect on V_{max}.

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Electronic Probes of the Mechanism of Substrate Oxidation by Buttermilk Xanthine Oxidase: Role of the Active-Site Nucleophile in Oxidation†

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ABSTRACT: Quinazolin-4(3*H*)-one derivatives substituted at the 6- and/or 7-position were studied as electronic probes of substrate oxidation by buttermilk xanthine oxidase. Since the enzyme active site possesses dimensional tolerance, the substituents exert an electronic effect rather than a steric effect on the catalytic parameters for oxidation. This feature permitted a Hammett plot to be made for quinazoline–oxygen substrate activity. The concave downward nature of this plot indicates that the rate-determining step for oxidation changes when electron-withdrawing substituents are placed on the substrate. This plot and kinetic isotope effects obtained with 2-deuterio derivatives of the substrates indicate the following: (i) oxidation involves nucleophile transfer to the C(2) center in concert with hydride transfer to the molybdenum center, and (ii) the formation of oxidized product is a three-step process, i.e., Michaelis complex formation, oxidation, and hydrolysis of the oxidized substrate–enzyme adduct. The role of the nucleophile in oxidation appears to be to increase the electron density in the substrate and thereby facilitate hydride transfer. The implication of this study is that similar electronic probes may be designed to study other purine-utilizing enzymes possessing a dimensionally tolerant active site.

Buttermilk xanthine oxidase is a complex molybdenum-containing enzyme that oxidizes a variety of substrates including hydroxypurines and quinazolines (Bray, 1975, pp 346–349; Krenitsky et al., 1972). The catalytic mechanism of oxidation is thought to involve hydride transfer (or its equivalent) from the substrate in concert with nucleophile transfer to the substrate (Stiefel, 1973; Edmondson et al., 1973;

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