Adenosine Deaminase and Adenylate Deaminase: Comparative Kinetic Studies with Transition State and Ground State Analogue Inhibitors†

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ABSTRACT: A number of transition state analogues and other inhibitors have been investigated by stopped-flow kinetic techniques with respect to inhibition of rabbit muscle adenylate deaminase and calf intestinal adenosine deaminase. The inhibitors are classified into three groups: (1) those which inhibit rapidly as exemplified by purine riboside or purine ribotide and which can be termed ground state analogue inhibitors, (2) those which inhibit rapidly but the initial enzyme–inhibitor complex undergoes a slow conformational change with further inhibition as exemplified by the inhibition of adenosine deaminase by 9-(*erythro*-2-hydroxy-3-nonyl)adenine (EHNA), and (3) those which appear to inhibit slowly as exemplified by the transition-state analogue coformycin. For this latter class, the rate constants for the on reaction have been determined as well as the overall inhibition constants. What appears to be characteristic of inhibition by different transition-state analogues is not the tightness of binding (which ranges from 5×10^{-6} to 10^{-12} M) but the apparent slow rate constant (k_{on}) with which inhibitor binds to the enzyme. It is concluded that the slow on rates reflect an extremely weak initial binding of the inhibitor followed by a conformational change. What appears to be characteristic of ground state analogue inhibitors is not the weakness of binding (which ranges from 2×10^{-7} to 1.2×10^{-5} M) but the apparent instantaneous inhibition. Thus, the initial structure of the active site of the enzyme appears to be appropriate for binding the ground state of the substrate and not the transition state, and considerable readjustment of the site seems required to bind the transition state effectively. By use of stopped-flow techniques, some estimates of the dissociation constant for the initial binding step for the transition-state analogues can be made. In the course of the work, two new transition state analogue inhibitors are reported for adenylate deaminase: coformycin 5′-phosphate and 1,6-dihydro-6-hydroxymethylpurine ribotide. The chemical synthesis of the latter compound and an improved synthesis of purine ribotide are reported.

There are now a number of compounds known which are potent inhibitors of adenosine deaminase (EC 3.5.4.4) and adenylate deaminase (EC 3.5.4.6). Since the reactions (and presumably the chemical mechanism) catalyzed by these enzymes are similar, it is not surprising that an inhibitor of one of the enzymes, with slight modification, may serve as an inhibitor of the other. Thus, coformycin and 2′-deoxycoformycin are potent inhibitors of adenosine deaminase (Sawa et al., 1967; Agarwal et al., 1977; Cha, 1976; Cha et al., 1975) while the 2′-deoxycoformycin 5′-phosphate and (as shown in the present paper) coformycin 5′-phosphate are potent inhibitors of the adenylate deaminase (Frieden et al., 1979). It has been proposed that these compounds are potent inhibitors because they approximate the structure of the tetrahedral intermediate (or the transition state leading to it) which results from direct water attack on the substrate (Cha et al., 1975; Wolfenden, 1972). The same explanation may also be extended to the methanol adduct of purine riboside, 1,6-dihydro-6-hydroxymethylpurine riboside (DHMPR),[1] although this compound binds to adenosine deaminase considerably less tightly than coformycin (Evans & Wolfenden, 1970). Other inhibitors of adenosine deaminase include 9-(*erythro*-2-hydroxy-3-nonyl)adenine (EHNA) (Schaeffer & Schwender, 1974) and the purine riboside itself. Probably neither of the latter two inhibitors are transition state analogue inhibitors although ENHA shows reasonably tight binding. In this paper we investigate the mechanism of inhibition of these and other compounds for either (or both) adenosine or adenylate deaminase. Structures of some of these compounds are shown in Chart I. These investigations have been performed primarily by stopped-flow kinetic techniques and demonstrate another experimental approach to the investigation of the mechanism of inhibition by these tight binding compounds. As will be shown, there are considerable differences in the kinetic characteristics of the inhibition process dependent on the type of inhibitor. However, when comparisons are possible, both the adenosine deaminase and adenylate deaminase behave in a similar manner to particular inhibitors.

Materials and Methods

Inhibitors. Coformycin and 2′-deoxycoformycin were obtained from Dr. John Douros of the Developmental Therapeutic Program, Chemotherapy, National Cancer Institute. Coformycin 5′-phosphate and 2′-deoxycoformycin 5′-phosphate were synthesized enzymatically from the corresponding nucleosides by using a preparation of a specific 5′-nucleoside phosphotransferase from *Serratia marcescens* as described previously (Frieden et al., 1979). We are indebted to Dr. Richard Miller, Wellcome Research Laboratories, for the preparation of the nucleotides. The 1,6-dihydro-6-hydroxymethylpurine riboside (DHMPR)[1] was prepared by the methods of Evans & Wolfenden (1970). IsoAMP, 3-β-D-ribofuranosyladenine 5′-phosphate, was synthesized as reported by Leonard & Laursen (1965). 9-(*erythro*-2-Hydroxy-3-nonyl)adenine (EHNA) was a product of the Burroughs Wellcome Co. This material is a 50:50 mixture of enantiomers, only one of which is the inhibitory material (see below).

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[1] Abbreviations used: DHMPR, 1,6-dihydro-6-hydroxymethylpurine riboside; DHMPR 5′-phosphate, 1,6-dihydro-6-hydroxymethylpurine ribotide; EHNA, 9-(*erythro*-2-hydroxy-3-nonyl)adenine; isoAMP, 3-β-D-ribofuranosyladenine 5′-phosphate; coformycin, (*R*)-3-β-D-*erythro*-pentofuranosyl-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol; NaDodSO$_4$, sodium dodecyl sulfate; AMP, adenosine 5′-monophosphate.

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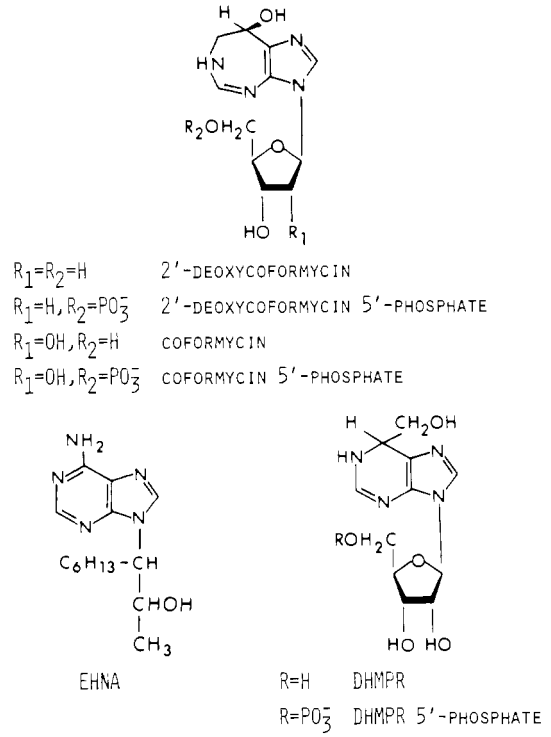
Chart I



Purine riboside and 6-mercaptopurine 5′-nucleoside monophosphate were obtained from Sigma Chemical Co. Purine ribotide was synthesized as follows. 6-Mercaptopurine 5′-nucleoside monophosphate was dissolved in H_2O and reduced with Raney nickel in boiling H_2O in a procedure similar to that described by Fox et al. (1958) for their preparation of 2-amino-9-β-D-ribofuranosylpurine. The crude material on thin-layer chromatography (TLC) (see below) had faint additional spots corresponding to 6-mercaptopurine 5′-nucleoside monophosphate (R_f = 0.33) and IMP (R_f = 0.52). The material was applied to a DE52 column (bicarbonate form) which was washed with H_2O. The purine ribotide was then eluted from the column with 50 mM NH_4HCO_3. The fractions containing purine ribotide were combined and lyophilized exhaustively to remove excess NH_4HCO_3. The yield was 80% and the material was pure as judged by the presence of a single spot on TLC (R_f = 0.62 on PEI–cellulose eluted with 0.5 M LiCl). For TLC, the materials were converted to the tetrabutylammonium salts and dissolved in methanol.

The 1,6-dihydro-6-hydroxymethylpurine ribotide (DHMPR 5′-phosphate) was synthesized in a manner similar to that described by Evans & Wolfenden (1970) for the corresponding riboside. The tetrabutylammonium salt of purine ribotide was dissolved in anhydrous methanol at a concentration of 6 mM. The slightly turbid solution was centrifuged and the clear supernatant was transferred to the quartz reaction tube for photolysis. In order to obtain good results, it was necessary to purge the methanolic solution with N_2 (dry, O_2 free) gas for at least 1 h prior to photolysis. Photolysis, with two GE germicidal lamps, was continued until no further change occurred in the A_{293}/A_{263} ratio (~100 min) and TLC on PEI–cellulose (1 M HCOOH) indicated all purine ribotide had been consumed. DHMPR 5′-phosphate is unstable in 1 M HCOOH, and degradation products appear as bright spots (under 254-nm illumination) which move with the solvent front.

Enzymes. Adenylate deaminase was prepared from fresh rabbit muscle according to procedure previously described (Ashby & Frieden, 1977). The enzyme concentration was determined from the absorbance at 280 mm by using $A_{280}^{1\%}$ = 9.13 (Zielke & Suelter, 1971). NaDodSO$_4$–polyacrylamide gels of the enzyme from fresh rabbit muscle yield a single band of molecular weight 80000, while enzyme prepared from frozen muscle frequently yields two closely spaced bands of slightly lower molecular weight (B. Barshop and C. Frieden, unpublished experiments). We consider the molecular weight of the native enzyme to be 320000.

Adenosine deaminase (calf intestine) was obtained from Sigma Chemical Co. This material contains several protein impurities and was purified by using a purine riboside affinity column prepared as described by Schrader et al. (1976) as modified by Buel (1979). The impurities wash through the column, while the enzyme itself is retarded. In a typical purification, adenosine deaminase, dialyzed against 20 mM phosphate, pH 7, at a concentration of 6 mg/mL was applied to the column of dimensions 0.7-cm diameter by 13-cm height and washed with the same buffer to remove the impurities. A gradient of 0–1 M KCl was applied and the enzyme eluted. Since the column only retards the elution of the enzyme, it can be washed off with buffer alone but only over a rather large volume. Purified enzyme (an ~1.4-fold increase in specific activity) has the same kinetic parameters as the impure enzyme and the impurities do not appear to bind any of the inhibitors. NaDodSO$_4$ electrophoresis of the purified enzyme showed a single band, while several other bands were found in the peak containing no enzymatic activity.

Stopped-Flow Experiments. All stopped-flow experiments were performed at 20 °C with a Durrum stopped-flow apparatus using a 2-cm cell. Kinetic data were obtained as described previously (Ashby & Frieden, 1978), and kinetic constants were determined as discussed elsewhere (Bates & Frieden, 1973) by using computer simulation of the mechanisms in the text. Some figures shown are hard-copy outputs of digitized data plotted on a Versatec printer–plotter. Fits of experimental data and computer simulation were made by visual inspection when the two plots are overlaid. The stopped-flow experiments were performed at either 267 or 280 nm (depending on the concentration of substrate) by using an extinction coefficient difference between substrate and product of −3.25 mM^{-1} cm^{-1} and +0.237 mM^{-1} cm^{-1}, respectively.

Results

Experiments with Adenylate Deaminase: Time Dependence of Inhibition. We have shown previously that inhibition of adenylate deaminase by 2′-deoxycoformycin 5′-phosphate is a time-dependent process. This time dependence was observed by incubating the enzyme with inhibitor and, at given time intervals, measuring, after a 100-fold dilution, the residual activity. This procedure is satisfactory for an inhibitor which is so tightly bound to the enzyme that it does not measurably dissociate from the enzyme during the time of the assay. As indicated by the previous results (Frieden et al., 1979) (and as shown below), this requirement is met when studying the 2′-deoxycoformycin 5′-phosphate as well as by coformycin, 2′-deoxycoformycin, and coformycin 5′-phosphate. In the previous study (Frieden et al., 1979), we obtained a value of 9×10^4 s^{-1} mol^{-1} for the second-order rate constant, k_{on}, using 2′-deoxycoformycin 5′-phosphate and assuming that k_{off} = 0.

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI \qquad (I)$$

A different, perhaps more useful, procedure is to measure the time course of inhibition by stopped-flow methods during the deamination of the substrate as catalyzed by the enzyme. This procedure is useful not only because one can obtain the mechanism of the reaction and of the inhibition, but also

Table I: Rate and Overall Inhibition Constants for Adenylate Deaminase[a]

inhibitor	K_I (M)	k_{on} (s^{-1} mol^{-1})	k_{off} (s^{-1}) from K_I	k_{off} (s^{-1}) from off rates
2'-deoxycoformycin	3.6×10^{-7}	1.3×10^{4}	4.6×10^{-3}	4×10^{-3}
coformycin	2×10^{-8}	9×10^{3}	1.8×10^{-4}	1×10^{-4}
2'-deoxycoformycin 5'-phosphate	1×10^{-9}	9×10^{4}	9×10^{-5}	2×10^{-5}
coformycin 5'-phosphate	5.5×10^{-11} [b]	1×10^{5}	5×10^{-6} [b]	
purine ribotide	6.5×10^{-6}			
isoAMP	12×10^{-6}			
DHMPR 5'-phosphate[c]	2.5×10^{-6}	4×10^{4}	0.1[d]	

[a] With the assumption of the process shown in mechanism I, values for the rate constants were calculated as described in the text at 20 °C in 0.15 M KCl and 10 mM imidazole buffer, pH 6.5. Computer simulation of mechanism II assumed 180 μM and 800 s^{-1} for the Michaelis constant and maximum velocity of the uninhibited enzyme and a molecular weight of 80 000 for the subunit. [b] Value estimated by assuming that the ratio of constants for 2'-deoxycoformycin to 2'-deoxycoformycin 5'-phosphate is the same as for coformycin to coformycin 5'-phosphate. [c] It is assumed that this material is a 50:50 mixture of enantiomers, only one of which is inhibitory. [d] k_{on} and k_{off} can be uniquely determined from the full time course in this case.

because if the rate constant, k_{on}, is much larger than 10^5 s^{-1} mol^{-1}, it would otherwise be difficult to follow the time course of inhibition by the usual methods.

In a typical stopped-flow experiment, AMP and inhibitor in one syringe are mixed with the enzyme in the other syringe to start the reaction. The dead time of the instrument is ~4 ms. The concentration of enzyme and inhibitor may be adjusted so that inhibition occurs during substrate depletion. Typical experiments are shown in Figure 1 with coformycin (50 μM) and 2'-deoxycoformycin 5'-phosphate (10 μM) compared to the reaction in the absence of inhibitor. It may be seen that under these conditions, the enzymatic reaction is totally inhibited before the substrate is completely depleted. In addition, close examination of the data shows that neither of these inhibitors change the initial portion of the substrate-depletion curve. Thus, at the levels of inhibitor used, there is no decrease in the initial velocity of the reaction, i.e., there is no "instantaneous" inhibition. This result is observed with several other inhibitors as well, and we shall return to it under Discussion.

By use of a computer simulation procedure described elsewhere (Bates & Frieden, 1973), data such as shown in Figure 1 can be fit if mechanism II is assumed where A is the

$$\begin{gathered} E + A \rightleftharpoons EA \rightleftharpoons EP \rightleftharpoons E + P \\ E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI \end{gathered} \qquad (II)$$

substrate, P the product, and I the inhibitor. In accordance with previous kinetic studies utilizing the full time course of the reaction (Ashby & Frieden, 1978), we assume that the enzymatic reaction is essentially irreversible and that the product does not inhibit. The mechanism also assumes that the inhibitor is competitive with the substrate since no allowance is made for an EAI complex. By use of values of 180 μM and 800 s^{-1} for the Michaelis constant and maximum velocity (determined by computer simulation in the absence of inhibitor) and with the assumption, for a first approximation, that inhibitor binding is essentially irreversible, the data of Figure 1, as well as more extensive data as a function of inhibitor concentration, can be fit by assuming specific values for k_{on} of mechanism II. Table I lists the values obtained for the various inhibitors of adenylate deaminase. These values range from 9×10^3 to 1×10^5 s^{-1} mol^{-1} for the different transition state analogue inhibitors and are, as discussed later, considerably lower than would be expected for a diffusion-controlled process (Eigen & Hammes, 1963). This would suggest that mechanism II needs to be expanded to include an initial binding step followed by a slower conformational change. This mechanism will be discussed later.

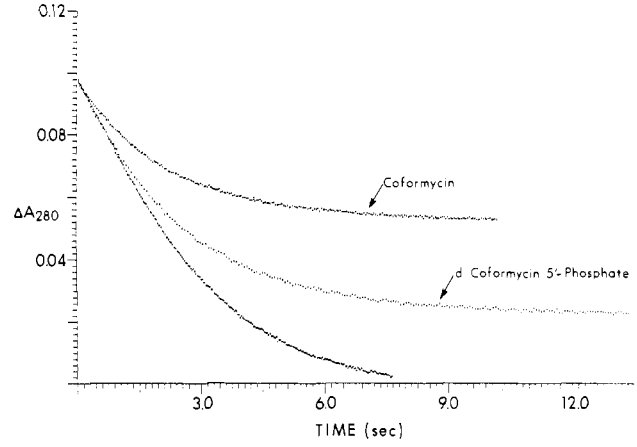


FIGURE 1: Stopped-flow kinetic measurement of disappearance of AMP catalyzed by adenylate deaminase in the absence of inhibitor (lower line) and in the presence of 10 μM 2'-deoxycoformycin 5'-phosphate or 50 μM coformycin. Experiments were performed at 20 °C in 0.01 M imidazole buffer, pH 6.5, in the presence of 0.15 M KCl. AMP (final concentration after mixing = 200 μM) and inhibitor were in one syringe of the stopped-flow apparatus, and enzyme (final concentration of 0.125 μM based on a subunit molecular weight of 80 000) was in the other syringe. The stopped-flow cell was 2 cm in length.

Determination of Inhibition Constants for Adenylate Deaminase. Inhibition constants were determined by two-stopped-flow methods, one involving the initial velocity of the reaction and the other involving the full time course of the reaction. In both methods, the enzyme was preincubated with inhibitor.

For simple cases of competitive inhibition, Henderson (1972) has derived a steady-state equation which may be written as

$$\frac{I_t}{(1 - v_i/v_0)} = E_t + K_I(1 + S/K_m)v_0/v_i \qquad (1)$$

where I_t and E_t are the total inhibitor and enzyme concentrations, v_i is the velocity in the presence of inhibitor, v_0 is that in the absence of inhibitor, K_I and K_m are the inhibition and Michaelis constants, and S is the substrate concentration. Enzyme and varying concentrations of inhibitor were preincubated in one syringe of the stopped-flow apparatus, and the time course was followed after mixing with substrate. From these curves, the initial velocity, as well as the full time course of the reaction, can be obtained. The results, using initial velocities, were plotted according to eq 1 from which the inhibition constant and total enzyme concentration may be obtained. Since the off rate of the inhibitor is quite slow, the inhibitor and enzyme concentrations used in the calculation of the inhibitor constant are those in the preincubation mixture

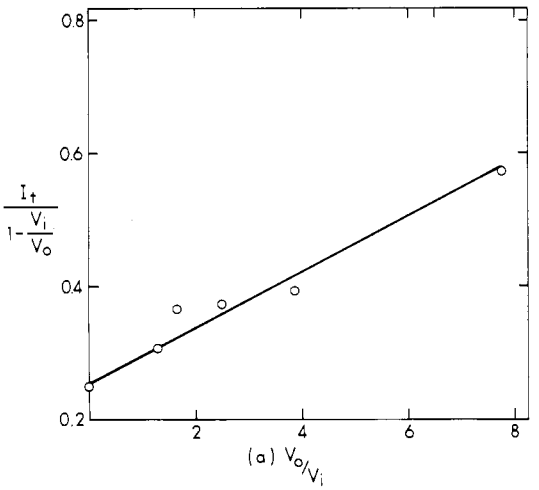


FIGURE 2: Plot to determine the inhibition constant for coformycin with adenylate deaminase. Initial velocities determined with stopped flow where enzyme and inhibitor were preincubated for 20 min before addition of the substrate. The experiment was performed at 20 °C in 0.01 M imidazole buffer, pH 6.5, in the presence of 0.15 M KCl. For a competitive inhibitor $a = 1 + K_m/S$ (see eq 1) which under the conditions of this experiment has a value of 2 ($K_m = S = 180$ μM). The intercept on the ordinate is the total enzyme concentration under conditions prior to mixing with the substrate.

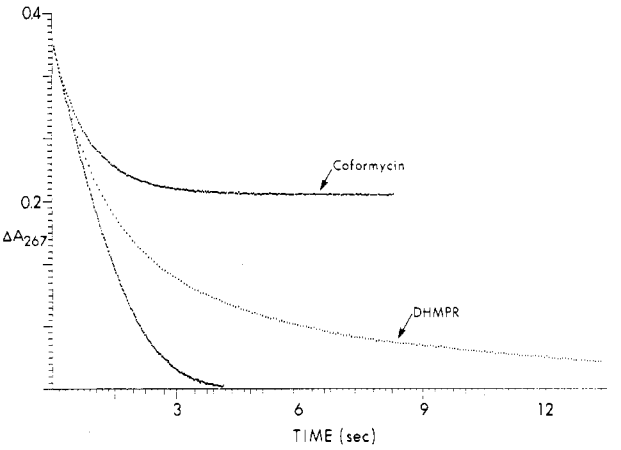


FIGURE 3: Stopped-flow kinetic measurement of disappearance of adenosine catalyzed by adenosine deaminase in the absence (lower line) and presence of 3 μM coformycin or 11 μM DHMPR. Experiments were performed at 20 °C in 0.02 M phosphate buffer, pH 7, in the presence of 0.1 mM EDTA. Adenosine (concentration after mixing = 50 μM) and inhibitor in one syringe of the stopped-flow apparatus were added to enzyme in the other syringe (final concentration = 0.18 μM). The stopped-flow cell was 2 cm in length.

in the drive syringe of the stopped-flow apparatus. Figure 2 shows results plotted with coformycin as the inhibitor. Values of the off rate constant could then be calculated from the inhibition constant and the on rate constant, as determined above (see Table I).

For the same experiment, the values for the inhibition constant can also be obtained by computer simulation of the full time course of the reaction with the assumption of mechanism II. In these cases, the time course was fit by determining the concentration of the free enzyme which gave the proper fit for the time course using the known values for the maximum velocity and Michaelis constant under these conditions. If the total enzyme concentration is known (from Figure 2, for example), the concentration of the enzyme–inhibitor complex and of the free inhibitor may be determined, and the inhibition constant is calculated from the relationship $K_I = E_f I_f / EI$. The agreement between the two methods was good for coformycin and deoxycoformycin as inhibitors. However, with the nucleotides, the inhibition constants are sufficiently low to require that a lower enzyme concentration be used. It has been shown elsewhere that the enzyme loses activity at low enzyme concentration (<2 μg/mL) (Ashby & Frieden, 1978). While this problem is alleviated somewhat by the observation that the inhibitors protect against this loss, the overall result is that the inhibition constant is somewhat less reliable for the 2′-deoxycoformycin 5′-phosphate and essentially impossible to obtain for the coformycin 5′-phosphate since the enzyme concentration required is so low that extensive inactivation occurs. The value listed in Table I for the coformycin 5′-phosphate is therefore calculated by assuming that the ratio of inhibition constants for deoxycoformycin to coformycin is the same for the 5′-phosphates.

The inhibition constants for these inhibitors may, in theory, be determined by a different procedure—that of directly obtaining the off rate of the enzyme–inhibitor complex. Thus a sufficient dilution of a complex of enzyme and inhibitor will result in some dissociation of the inhibitor and concomitant recovery of enzymatic activity. These experiments can be performed by diluting the enzyme–inhibitor complex into an assay mixture and following recovery of activity. In these experiments, the extent of activity recovered was kept small, and the off rates were calculated by the computer simulation program using mechanism II. As in the experiments described earlier, instability of the enzyme may give values which are too low for the off rate. Table I also lists off rate constants for coformycin, 2′-deoxycoformycin, and 2′-deoxycoformycin 5′-phosphate determined by this method. For the first two, the rate constants are in good agreement with the values calculated from the initial velocity experiments. However, for the 2′-deoxycoformycin 5′-phosphate the value is too low, indicating some enzyme instability. This problem would also make any value for coformycin 5′-phosphate too low since experimentally a very long time is required for even a small recovery of activity with coformycin 5′-phosphate.

The determination of the inhibition constant for more classical competitive inhibitors, purine ribotide and isoAMP, is considerably easier than that for the transition-state analogues. In these cases, inhibition occurs within the dead time of the stopped-flow apparatus (<4 ms) and is therefore considered as instantaneous. By use of mechanism II and the assumption that EA and EI are in rapid equilibrium with E, A, and I, the data are fit by using an inhibition constant of 6.5×10^{-6} M for the purine ribotide and 12×10^{-6} M for isoAMP. It has been shown that isoAMP is a competitive inhibitor of adenylate deaminase (Zielke & Suelter, 1971; Setlow & Lowenstein, 1968), although the value obtained here is considerably lower than those obtained by other workers. Of interest is the comparison of purine ribotide binding to adenylate deaminase with purine riboside binding to adenosine deaminase as described later.

Agarwal & Parks (1977) reported that both coformycin and 2′-deoxycoformycin were noncompetitive inhibitors for adenylate deaminase, which is not consistent with the results presented here. Both inhibitors, as well as their 5′-phosphates, appear as competitive inhibitors. While direct comparisons of our inhibition constants with those reported by Agarwal and Parks may not be appropriate, the values shown in Table I are lower than both the inhibition constants reported for each inhibitor.

Experiments with Adenosine Deaminase: Time Dependence of Inhibition. As with adenylate deaminase, there is time-dependent inhibition of adenosine deaminase with some of the tight binding inhibitors. Figure 3 shows stopped-flow full time course experiments with adenosine deaminase in the presence

Table II: Rate and Overall Inhibition Constants for Adenosine Deaminase[a]

inhibitor	k_{on} (s^{-1} mol^{-1})	k_{off} (s^{-1})	K_I (M)
2′-deoxycoformycin	2×10^6	5×10^{-6} [c]	2.5×10^{-12} [b]
coformycin	0.9×10^6	2×10^{-4} [c]	2.2×10^{-10} [d]
DHMPR[e]	7.5×10^4	0.21	7×10^{-7}

inhibitor	K_D (M)	k_+ (s^{-1})	k_- (s^{-1})	K_I (M)
EHNA[e]	2×10^{-7}	0.6	0.005	1.7×10^{-9}
purine riboside	4×10^{-6}			4×10^{-6}

[a] Stopped-flow experiments were performed at 20 °C in 0.02 M phosphate buffer, pH 7.0. Computer simulation results were obtained by using 22 μM and 180 s^{-1} as the Michaelis constant and maximum velocity, respectively, of uninhibited enzyme and by assuming a molecular weight of 35 000 for the enzyme. [b] Value obtained by Agarwal et al. (1977) for the human erythrocyte adenosine deaminase. [c] Value calculated from the relationship $k_{off} = k_{on}K_I$. [d] Value obtained by Cha (1976). [e] Values corrected for the EHNA and DHMPR containing 50% isomer which is not an inhibitor.

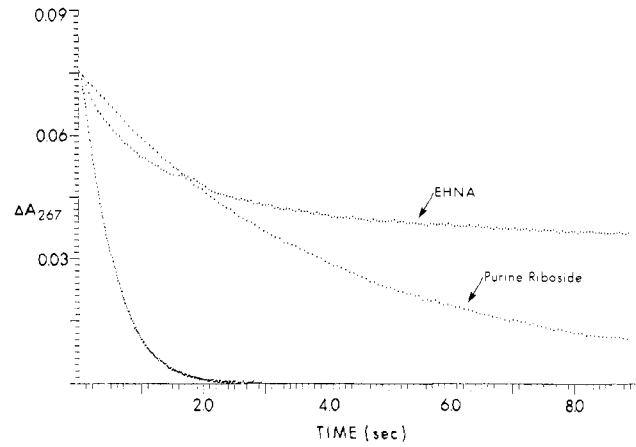


FIGURE 4: Stopped-flow kinetic measurement of disappearance of adenosine catalyzed by adenosine deaminase in the absence (lower line) or presence of 2 μM EHNA or 30 μM purine riboside. Conditions were the same as described in the legend for Figure 3 except that the adenosine concentration (final after mixing) was 10 μM. Note instantaneous inhibition compared to that shown in Figures 1 and 3.

of 3 μM coformycin and 11 μM DHMPR. As observed with the adenylate deaminase and coformycin (and its analogues), these inhibitors do not appear to affect the initial velocity of the reaction. Rather, the inhibition appears as a rapid decrease in velocity without any instantaneous component. With coformycin (as shown in Figure 2) and with 2′-deoxycoformycin (data not shown), the inhibition is essentially irreversible and substrate depletion rapidly stops. With DHMPR, the reaction goes to completion indicating that the inhibition is relatively reversible. The data for these three inhibitors has been analyzed by computer simulation, and the results are shown in Table II. For coformycin and 2′-deoxycoformycin, the overall inhibition constants were assumed to be those obtained by Agarwal et al. (1977) and Cha et al. (1975), for human erythrocyte adenosine deaminase, and the values of k_{off} were calculated from these inhibition constants and the k_{on} values. The values of k_{on} for these two inhibitors are in good agreement with those obtained by these authors for this enzyme. The computer simulation of the data with DHMPR gives a unique set of values for k_{on} and k_{off} and the rate of these values gives an overall inhibition constant of 1.7×10^{-6} M, which is somewhat lower than the value published previously (Evans & Wolfenden, 1970; Agarwal et al., 1977).

The nature of the inhibition observed in the presence of EHNA or purine riboside differs from that observed with 2′-deoxycoformycin, coformycin, or DHMPR. These data are shown in Figure 4. Examination of this figure shows that both EHNA and purine riboside inhibit the initial velocity of the reaction. The data for the purine riboside are fit by making the assumption that this compound is strictly a competitive inhibitor which is in rapid equilibrium with the enzyme. Thus, no time-dependent inhibition is observable with this inhibitor (K_D and K_I of Table II are the same). The data for EHNA, on the other hand, can be fit only with a mechanism which assumes a rapid equilibration of enzyme and inhibitor followed by a slow conformational change:

$$E + I \underset{}{\overset{K_D}{\rightleftharpoons}} EI \underset{k_-}{\overset{k_+}{\rightleftharpoons}} E'I \qquad \text{(III)}$$

Values of K_D and k_+ can be uniquely determined from stopped-flow experiments when EHNA and adenosine are added simultaneously to the enzyme. The overall inhibition constant can be determined in the stopped-flow experiments when EHNA and enzyme are preincubated prior to addition of substrate, and the data can be plotted according to eq 1. The values so obtained and the calculated off rate constant, k_-, are also given in Table II. As shown below, EHNA is a mixture of two enantiomers, only one of which binds to the adenosine deaminase. The nature of inhibition of purine riboside is identical with that observed for purine ribotide inhibition of adenylate deaminase (data not shown).

Stoichiometry of Inhibition for Adenosine Deaminase. In experiments to measure the stoichiometry of the inhibition, we observed, as did Wolfenden et al. (1977), that 2′-deoxycoformycin gave an apparent minimum molecular weight of 53 000 per binding site, although the molecular weight of the enzyme is known to be 34 000 (Murphy et al., 1969). When the enzyme was purified by using a purine riboside affinity column (see Materials and Methods), the stoichiometric titration gave 38 000 as the minimum molecular weight, indicating not only that the original enzyme was impure but also that the impurities do not bind 2′-deoxycoformycin. However, the stoichiometric titration of the adenosine deaminase with EHNA always gave values of the molecular weight which were twice those obtained with 2′-deoxycoformycin or coformycin. This result shows that the EHNA only contains 50% of material which binds tightly to adenosine deaminase. We interpret this to mean that EHNA which is the erythro compound is, in fact, a 50:50 mixture of two enantiomers and that one of the forms binds tightly while the other does not.

Discussion

By use of either the adenylate or adenosine deaminase, it is clear from the data presented here that there appear to be three classes of competitive inhibitors: (1) those which appear to inhibit instantaneously, (2) those which inhibit rapidly but the binding is followed by a slow conformational change, and (3) those which appear to inhibit slowly.

Purine riboside, purine ribotide, and isoAMP fall into the first class. Inhibition occurs within the dead time of the stopped-flow apparatus (<4 ms), and no further inhibition occurs as a function of time over the full time course of substrate depletion. This would be classical competitive inhibition. The inhibition constants for purine riboside, with adenosine deaminase, and purine ribotide, with adenylate deaminase, are remarkably similar, being 4×10^{-6} and 6.5×10^{-6} M, respectively. The value obtained for purine riboside is about twofold lower than that reported by Evans & Wolfenden (1970). Estimates of the on rate constants which would result in inhibition by these compounds within the dead time of the

stopped-flow apparatus yield values of 10^8 s^{-1} mol^{-1} or greater, similar to values calculated for diffusion control (Eigen & Hammes, 1963). These compounds, which are analogues of the substrate, could be termed ground state analogue inhibitors.

EHNA inhibition of adenosine deaminase falls into the second class listed above. The inhibition process may be clearly separated into two steps—initial binding followed by a further conformational change. The initial binding step is rapid and characterized by an inhibition constant of 2×10^{-7} M. The second step is characterized by rate constants of 0.6 and 0.005 s^{-1} in the forward and reverse direction to give an overall inhibition constant of 1.7×10^{-9} M. This value is slightly less than that of 6.5×10^{-9} obtained by Agarwal et al. (1977) for this enzyme. While EHNA has been classed as a tight binding inhibitor of adenosine deaminase, it is unlikely to bind to the enzyme in the same way as either the purine riboside or the coformycin analogues (discussed below). Thus, even though there is an amino group at the 6 position of the purine ring, the compound is not deaminated by the enzyme. Further, the inhibition constant for the initial binding step is considerably lower than that of purine riboside or the adenosine substrate itself ($K_m = 20 \times 10^{-6}$ M) and suggests that the nonyl side chain is important in this binding. It is likely that EHNA, although a tight binding inhibitor, is binding incorrectly at the active site to give classical competitive inhibition and that a subsequent rearrangement of either the inhibitor or the enzyme results in an enzyme–inhibitor species from which the inhibitor escapes only slowly.

DHMPR, coformycin, and its analogues fit a third category of inhibition in which inhibition is characterized by an apparent slow on rate constant for the formation of the enzyme–inhibitor complex. Such behavior has been observed for adenosine deaminase as well as for several other systems which involve transition-state inhibitors (Wolfenden, 1976). The observed (or apparent) on rate constant in these cases is considerably smaller than that expected from simple diffusion control (Eigen & Hammes, 1963), while this is not true for the inhibitors such as the purine riboside or ribotide discussed above, which exist in rapid equilibrium with the enzyme. We have shown previously (Frieden et al., 1979) that the slow inhibition of adenylate deaminase by 2′-deoxycoformycin 5′-phosphate is not a consequence of chemical modification. This is presumably also true of the other transition state analogue inhibitors used here.

An important point to be made from examination of Table II is that, in some cases, the overall inhibition constant for two different inhibitors is similar, but the mechanism of inhibition is quite different. For example, with adenosine deaminase, the overall inhibition constants for purine riboside and DHMPR differ by less than sixfold, yet one is an instantaneous inhibitor while the other is a slow inhibitor. This difference is almost certainly related to the requirement for binding a transition-state analogue correctly. On the other hand, a high affinity is not a necessary criterion for a compound to be a transition-state analogue, since inhibition constants for inhibitors which fall into this class vary by a factor of $\sim 10^6$ (Tables I and II). Rather, they characteristically appear to have on rate constants which are considerably smaller than those calculated for diffusion-control processes. It could be postulated that the slow on rate may be a consequence of the presence of a very low concentration of a form of either the enzyme or the inhibitor that binds tightly or that it reflects a weak initial binding followed by a conformational change. Attempts to computer simulate the full time course of substrate depletion over a range of inhibitor concentration data assuming a rare species of enzyme or inhibitor have not been successful in our hands.

From the data, it is not possible to distinguish between a single-step mechanism (II) and one with a prior equilibrium (with poor affinity) followed by a slow conformational change (mechanism III). However, it seems likely that the slow on rate reflects an initial weak binding phenomenon. In this regard, it is of interest to observe in Tables I and II that there is not much difference, for a given enzyme, in the apparent on rate constant between coformycin and deoxycoformycin or (for adenylate deaminase) between coformycin 5′-phosphate and deoxycoformycin 5′-phosphate. Even for adenylate deaminase where there is an $\sim$10-fold difference between these on rate constants for the nucleosides compared to that for the nucleotides, the overall inhibition constants are, in these cases, strikingly different. These results imply that the off rate constants and therefore the final conformational form present after the initial binding may reflect the importance of both the ribose and the phosphate moiety of the inhibitors.

It is of interest to attempt to calculate the magnitude of the initial binding step. As described in the results, stopped-flow experiments could be performed by the simultaneous addition of substrate and inhibitor to the enzyme. With DHMPR and the coformycin-type compounds, no decrease in initial velocity was noted regardless of the concentration of inhibitor used (see Figures 1 and 3). Although technical considerations limit the concentrations of inhibitor that can be used, some details might be useful. With adenosine deaminase, levels of coformycin (overall $K_I = \sim 10^{-10}$ M) as high as 7×10^{-5} M give no inhibition of the initial portion of the reaction. With adenylate deaminase, 10^{-4} M coformycin (overall $K_I = 2 \times 10^{-8}$ M) and 10^{-5} M 2′-deoxycoformycin 5′-phosphate (overall $K_I = 10^{-9}$ M) did not influence the initial portion of the substrate-depletion curve. Assuming that we would be able to detect a 10–20% decrease in this portion of the curve, one can obtain *minimal* estimates for the value of the dissociation constant (K_D) for the initial step of mechanism III. These range from 10^{-4} to 10^{-3} M. Under conditions where there is no decrease in the initial velocity, the apparent on rate constant for the inhibitor k_{on} in mechanism II is equal to the ratio k_+/K_D of mechanism III. Thus, K_D can be any value greater than 10^{-3}–10^{-4} M with the appropriate value for the rate constant describing the conformational change. If we assume, for example, that the rate of the conformational change is the same as that of the catalytic turnover, it is possible to calculate the dissociation constants, and these range from 1.2×10^{-4} M (for deoxycoformycin binding to adenosine deaminase) to 9×10^{-2} M (for coformycin binding to adenylate deaminase). Thus, the ratio of the initial dissociation constant and the final dissociation constant (the overall inhibition constant) may be as large as 10^8. As expected from this calculation, since it is related to the on rates, there is not much difference in the initial binding between the ribo or deoxyribo inhibitors, and the marked differences in overall inhibition constants are related primarily to the rate constant describing the reverse process.

As stated above, the forward rate constant for transition state analogue binding shows a remarkable lack of sensitivity to variation in the overall inhibition constant (Tables I and II). For adenosine deaminase, the range of inhibition constants exceeds a factor of 10^6 while that for the forward rate constants changes by $\sim$10. For adenylate deaminase, a similar observation can be made. If Leffler's reasoning (Leffler & Grunwald, 1963) is applicable in this case, the transition state is reached very early along the reaction coordinate. Thus, those

factors responsible for the overall exceedingly high affinity of these analogues are only poorly expressed at the transition state for binding.

Another unusual feature of transition state analogue binding is the unexpectedly high activation barrier. The rate constants for binding range from 3 to 5 orders of magnitude below those expected for a diffusion-controlled process. On the other hand, ground-state analogues (such as purine riboside, purine ribotide, and isoAMP) bind rapidly and presumably at diffusion-controlled rates. Despite the few unique structural features of transition-state analogues, the overall structures of the two types of inhibitors in terms of possible sites of interaction with the enzyme are probably very similar. Thus, it seems that initially the structure of the active site is appropriate for binding the ground state and inappropriate for the transition state. In fact, some considerable readjustment is required to bind the transition state effectively. Recently, Schloss et al. (1980) reached a similar conclusion when studying the inhibition of aconitase by transition-state analogues.

Use of the stopped-flow apparatus allows determination of the apparent on rates for the inhibitors. In general, the values obtained here agree with those reported in the literature for which similar experiments were performed, i.e., coformycin and deoxycoformycin binding to adenosine deaminase. However, stopped-flow experiments also allow a determination of a minimum value of the initial binding process. The experiments reported here extend the study of these two deaminases to provide information on the type and mechanism of inhibition.

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