Transition-State Stabilization by Adenosine Deaminase: Structural Studies of Its Inhibitory Complex with Deoxycoformycin†

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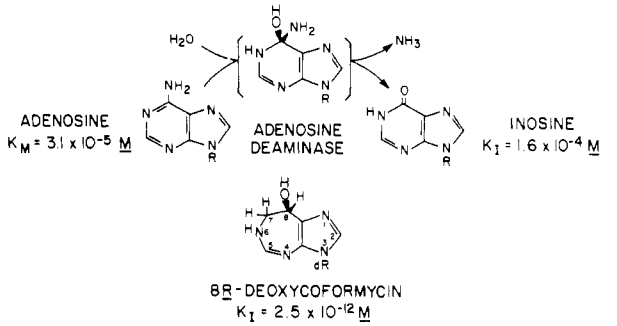
Received August 16, 1985

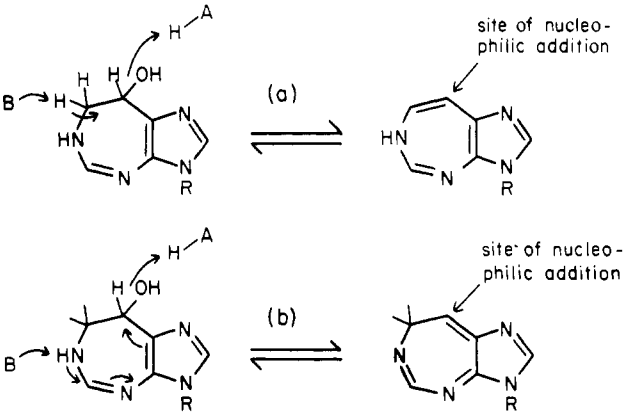
ABSTRACT: Experiments with radioactive deoxycoformycin indicate that the inhibitor is released from calf intestinal adenosine deaminase after the enzyme–inhibitor complex is disrupted by denaturation. Experiments with ^2H_2O and $H_2{}^{18}O$ indicate that the enzyme does not catalyze elimination–addition reactions that could have led to reversible covalent derivatization of the enzyme. Ultraviolet difference spectra and the influence of pH on inhibitor binding indicate that deoxycoformycin is bound intact as the neutral species, at a binding site that is less polar than solvent water. The enzyme–inhibitor complex appears to be held together by hydrogen bonds of extraordinary stability (ca. 10 kcal/mol). These results suggest that deamination proceeds by direct water attack, the enzyme acting as a general-base catalyst.

Adenosine deaminase catalyzes the hydrolytic cleavage of many leaving groups from the 6-position of purine ribonucleosides, producing one of the largest rate enhancements that appears to have been observed for any enzyme. It would be of interest to establish whether these reactions proceed by direct water attack or by a double-displacement mechanism. Several synthetic nucleosides, designed to resemble tetrahedral intermediates in adenosine hydrolysis by direct water attack, have been found to serve as strong antagonists of adenosine deaminase (Evans & Wolfenden, 1970). Still more effective were the natural products coformycin (Ohno et al., 1974; Nakamura et al., 1974) and its 2′-deoxy analogue[1] (Woo et al., 1974) (Scheme I), which were bound by the enzyme 6–8 orders of magnitude more tightly than substrates (Cha et al., 1975). Their special affinity depended critically on their stereochemistry at the hydroxyl-bearing C-8 position; thus the synthetic 8*S* analogue of deoxycoformycin was bound even more weakly than the substrate itself (Schramm & Baker, 1985). Nevertheless, significant doubt remains concerning the mechanism of binding of these compounds by the enzyme. Thus, the onset of inhibition by deoxycoformycin was slower than expected for a process governed by the frequency of encounter between enzyme and inhibitor in solution (Agarwal et al., 1977; Frieden et al., 1979), raising the possibility that chemical bonds might be broken and formed during assembly of the enzyme–inhibitor complex. Several studies have suggested that a thiol in or near the active site of the enzyme could play a role in catalysis (Wolfenden et al., 1967; Ronca et al., 1969), and thiols were found to be very effective nucleophiles in displacing leaving groups from the 6-position of purine nucleosides (Walsh & Wolfenden, 1967), in possible models for a double-displacement reaction. If the inhibitor deoxycoformycin were bound covalently, as for example by either of the mechanisms suggested in Scheme II, then the enzyme–inhibitor complex could resemble a tetrahedral intermediate in double displacement. A requirement for enzyme assistance in the reversible elimination of water from deoxycoformycin could help to explain its extreme stereospecificity with respect to the hydroxyl group at C-8 of deoxycoformycin and to account for the slow onset of inhibition. If, on the other hand, binding were noncovalent as implied by Scheme I, then the unusual stability and stereospecificity of the enzyme–in-

Scheme I: Direct-Displacement Mechanism for Hydrolytic Deamination of Adenosine (Hydrated Intermediate in Brackets) and Structure of Competitive Inhibitor Deoxycoformycin



Scheme II: Alternatives for Water Elimination from Deoxycoformycin To Generate a Species Unsaturated (a) at the 7–8 Position or (b) at the 8–9 Position[a]



[a] In either case, reaction of the unsaturated species with a nucleophilic residue at the enzyme's active site would lead to reversible inactivation.

†Supported by Grant GM-18325 from the National Institutes of Health.

[1] Synonyms for deoxycoformycin are pentostatin and (8*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol.

0006-2960/86/0425-1616$01.50/0

hibitor complex would require other explanations.

Forces involved in stabilizing an enzyme–substrate complex in the transition state should in principle resemble those involved in binding an ideal transition-state analogue; thus, the presence or absence of covalent bonds between adenosine deaminase and deoxycoformycin could be helpful in establishing whether this enzyme acts by catalyzing direct water attack on the substrate or by a double-displacement mechanism. The present study was undertaken in an attempt to test these alternatives. Radiolabeled inhibitor was used to determine the influence of pH on inhibitor binding and to detect enzyme–inhibitor complexes that might be stable to denaturation. Next, the mechanisms in Scheme II were tested with ^2H_2O and $H_2{}^{18}O$. According to these mechanisms, water should first be eliminated from deoxycoformycin to form an unsaturated species[2] that could act as a Michael acceptor. Its reaction with a nucleophile at the active site would result in reversible covalent binding, by a mechanism similar to those involved in the inhibition of papain by aldehydes (Lewis & Wolfenden, 1977) or the inhibition of β-galactosidase by D-galactal (Wentworth & Wolfenden, 1974). Reversal of these reactions would be expected to result in incorporation of solvent deuterium according to Scheme IIa and of solvent oxygen according to either mechanism in Scheme II. In these experiments, inhibitor was isolated after several turnovers of the enzyme–inhibitor complex, and its isotopic enrichment was determined by mass spectrometry. The results of these experiments, in conjunction with further spectroscopic observations, suggest that the enzyme–inhibitor complex may be stabilized by hydrogen bonds of unusual strength.

Materials and Methods

Calf intestinal adenosine deaminase (type VIII) was obtained from Sigma Chemical Co. $H_2{}^{18}O$ (95 atom % excess) was obtained from Monsanto Corp. [8-^{3}H]Deoxycoformycin (207 mCi/mmole) was prepared as described by Putt et al. (1981), and nonradioactive deoxycoformycin was prepared by the procedure of Chan et al. (1982). Diaion HP-20 resin was obtained from Mitsubishi, Inc.

Solvent Isotope Incorporation into Deoxycoformycin. Adenosine deaminase (3.5 mg, 100 nmol) was dialyzed against NH_4HCO_3 (10 mM), lyophilized, and then incubated with deoxycoformycin (300 nmol) in $H_2{}^{18}O$ (0.5 mL) containing NH_4HCO_3 (10 mM, pH 7.8) and sodium azide (1 mM) for 16 days at 25 °C in the dark. Control experiments with deoxycoformycin omitted showed no significant loss of enzyme activity as a result of this treatment. After incubation, samples were pertrimethylsilylated with bis(trimethylsilyl)trifluoroacetamide (20 mg) in anhydrous pyridine (20 mg) under nitrogen at room temperature for 15 h and then analyzed on a Hewlett-Packard Model 5985A mass spectrometry system integrated with a Hewlett-Packard Model 5840A gas chromatography unit. Chromatography was performed with a fused silica capillary column [CP Sil 5 CB (WCOT), Chrompack, Inc., 10 m × 0.32 mm] with an oven-temperature programming from 100 to 250 °C at a rate of 20 °C/min. Samples incubated with ^2H_2O were treated similarly.

Denaturation of the Enzyme–Inhibitor Complex. Adenosine deaminase (200 μg, 0.57 nmol) was incubated with a 3-fold excess (1.7 mmol) of labeled deoxycoformycin in potassium phosphate buffer (0.1 mL, 0.1 M, pH 7.0) for 16 h at 25 °C. Sodium dodecyl sulfate was added to a concentration of 1%, and the mixture was placed in a boiling water bath. Aliquots (40 μL) were removed after 2 and 6 min for analysis on a column of Sephadex G-25 (1 × 9 cm) equilibrated with tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (50 mM, pH 8.0, containing 0.1 M NaCl). Radioactivity that was eluted with the void volume was used to determine the amount of inhibitor that remained bound by the enzyme after this treatment.

Influence of pH on the K_i of Deoxycoformycin. Rates of release of radioactive deoxycoformycin from adenosine deaminase were determined by incubating radioactive enzyme–inhibitor complex (isolated as described above with Sephadex G-25) in various buffers containing unlabeled deoxycoformycin (1 mM) at 25 °C; buffers employed were sodium acetate (pH 4.5–6.0; 0.1 M), potassium phosphate (pH 6.0–7.0; 0.1 M), Tris-HCl (pH 7.0–8.5, 0.1 M), and glycine–NaOH (pH 8.5–10.0; 0.1 M). Aliquots (40 μL) were removed at intervals and analyzed by Sephadex G-25 chromatography as described above. Rates of association of deoxycoformycin with adenosine deaminase were determined in the same buffers, by observing the rate of onset of inhibition, essentially as described by Agarwal et al. (1977).

Difference Spectra. To obtain difference spectra for binding of deoxycoformycin by enzyme, affinity-purified (Schrader et al., 1976) adenosine deaminase (12.4 μM) was incubated with deoxycoformycin (12.4 μM) in potassium phosphate buffer (0.05 M, pH 7.0, containing 0.1 M NaCl and 0.015 M 2-mercaptoethanol) for 10 min at 25 °C. The reference cell in the spectrophotometer contained the enzyme and inhibitor unmixed, in the separate compartments of a 1-cm tandem cuvette. Other difference spectra were obtained by comparing spectra obtained in 2-propanol with those obtained in water and by comparing spectra obtained in sodium buffer (0.1 M, pH 4.0) with those obtained in Tris-HCl buffer (0.1 M, pH 8.0).

Results

Adenosine deaminase did not catalyze incorporation of either deuterium or ^{18}O from solvent water into deoxycoformycin. Mass spectrometry of the persilylated inhibitor yielded two useful clusters of peaks, one at m/z 556 corresponding to the molecular ion and one at m/z 296 corresponding to the silylated aglycon. A third cluster, at m/z 206, corresponded to the aglycon from which the trimethylsilyloxy group at C-8 had been eliminated and was useful only in testing for deuterium incorporation at C-7 or C-8. Normalized intensities of the peaks that were observed, and the effects of incubation with $H_2{}^{18}O$ and ^2H_2O with and without adenosine deaminase, are shown in Table I. Since the half-time for dissociation of the enzyme–inhibitor complex is approximately 24 h (Agarwal et al., 1977; this present study), the average molecule of deoxycoformycin, present in 3-fold excess over the enzyme, was bound and released approximately 2.7 times during the 16-day incubation period. If incorporation of ^{18}O had occurred, then the peaks at m/z 558 and 298 would have been expected to be more intense in samples incubated with the enzyme, but this was not observed. We estimate that a 10% change in peak intensity should have been observable, which would have corresponded to isotope exchange on 4% of the occasions on which enzyme and inhibitor would be expected to have combined. Table I also presents the results of enzyme–inhibitor incubations in ^2H_2O, where the results were similar. Since the proton at C-2 of deoxycoformycin is exchangeable,[3] the peaks are positioned at m/z values differing

[2] It is suggested that such a species would be antiaromatic and therefore of limited stability. However, such an elimination is observed in the mass spectrum of deoxycoformycin derivatives, leading to the unsaturated species as a principal ion (Smal, 1985).

Table I: Mass Spectroscopy of Deoxycoformycin Treated with Labeled Water in the Presence and Absence of Adenosine Deaminase

	normalized intensities for sample				
m/z	A[a]	B[b]	C[c]	D[d]	E[e]
		Molecular Ion			
556	11		100	100	100
557	100	100	54	51	53
558	54	f	25	28	27
559	29	f		2	3
		Silylated Aglycon			
295					
296	18	21	100	100	100
297	100	100	24	28	24
298	28	28	11	14	12
299	11			2	2
		Aglycon			
206	13	20			100
207	100	100			62
208	53	56			15
209	13				5
210	5				

[a] A, deoxycoformycin in D_2O. [b] B, deoxycoformycin in D_2O with adenosine deaminase. [c] C, deoxycoformycin in $H_2{}^{18}O$. [d] D, deoxycoformycin in $H_2{}^{18}O$ with adenosine deaminase. [e] E, deoxycoformycin in H_2O (control). [f] No peaks observed.

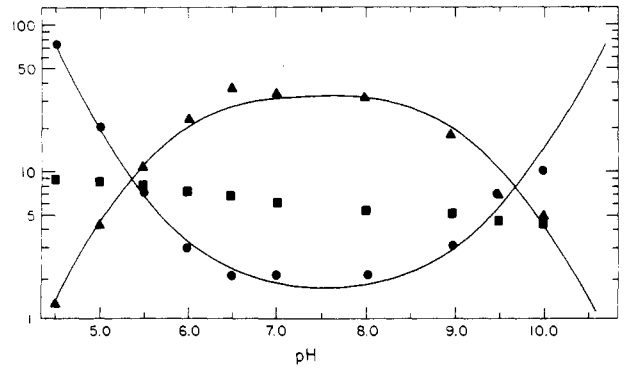


FIGURE 1: Effects of pH on formation and stability of the EI complex. k_{on}, k_{off}, and K_i of the EI complex were determined as described in the text. (▲) k_{on} ($\times 10^{-6}$ s^{-1} M^{-1}); (■) k_{off} ($\times 10^{6}$ s^{-1}); (●) K_i ($\times 10^{12}$ M).

from those observed in experiments with ^{18}O. Again, however, there were no significant differences between samples of deoxycoformycin incubated in the presence and absence of the enzyme.

Efforts to isolate a stable covalent adduct of deoxycoformycin and adenosine deaminase, by heating the complex in sodium dodecyl sulfate at neutral pH, were unsuccessful. Sephadex G-25 chromatography showed no significant amounts of radioactive inhibitor remaining associated with the enzyme after these treatments.

The influence of pH on the rates of deoxycoformycin association with, and dissociation from, adenosine deaminase is shown in Figure 1. Values obtained at pH 7.5 were in satisfactory agreement with those reported by Agarwal et al. (1977). K_i values increased below pH 6.0 and above pH 9.1. Values of k_{on} decreased below pH 5.8 and above pH 9.2, but k_{off} remained almost unaffected by changes in pH.

[3] A proton magnetic resonance experiment, in which deoxycoformycin (7 mM) was equilibrated for 6 weeks at room temperature in ^2H_2O, with a crystal of sodium azide added, demonstrated essentially complete exchange of the proton at C-2. This phenomenon is well documented for imidazole-containing nucleosides [Boerth et al. (1985) and references cited therein].

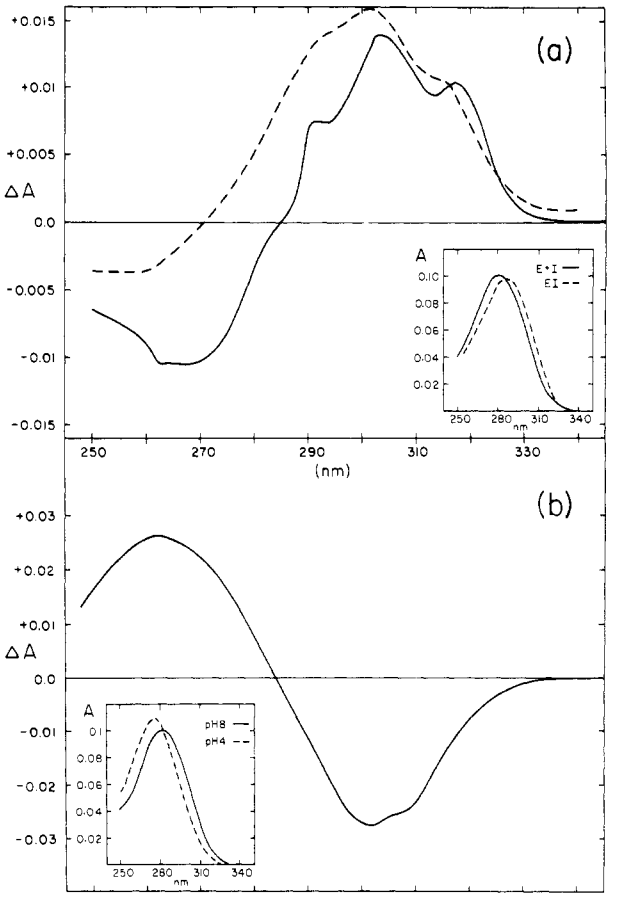


FIGURE 2: Difference spectra of deoxycoformycin, determined as described in the text. (a) Difference spectra for EI complex formation (solid line) and for transfer from water to 2-propanol (broken line). (b) Difference spectrum for protonation. (Insets) Actual spectra, compared with the appropriate reference buffers.

Figure 2 shows ultraviolet difference spectra observed for protonation of deoxycoformycin, for which a pK_a value of 5.8 was observed by spectrophotometric titration. Protonation resulted in a shift of maximal absorption to shorter wavelengths. In contrast, binding of deoxycoformycin by adenosine deaminase resulted in a shift to longer wavelengths. Figure 2 shows that similar shifts were associated with the transfer of deoxycoformycin from water to 2-propanol.

Discussion

Probable Structure of the Enzyme–Inhibitor Complex. If adenosine deaminase had catalyzed its own derivatization, as indicated in Scheme II, then radioactive deoxycoformycin might have been adhered to the enzyme after the complex had been denatured and its catalytic apparatus presumably destroyed. This was not observed. In similar experiments, Frieden et al. (1979) showed that the analogous inhibitor deoxycoformycin 5′-phosphate was released from adenylate deaminase when the complex was denatured by boiling. These results favor noncovalent binding, although a heat-labile covalent bond would have escaped detection. In experiments using milder methods, such as treatment with 6 M guanidine hydrochloride, we found that the deoxycoformycin complex with adenosine deaminase was not denatured, retaining its compact shape as assessed by chromatography on Bio-Gel P-60, whereas the native enzyme was denatured rapidly under the same conditions.

In experiments with isotopically labeled waters, adenosine deaminase did not catalyze solvent isotope exchanges into deoxycoformycin, as might have been expected if inhibition

had involved reversible covalent derivatization of the enzyme by either of the mechanisms shown in Scheme II. In these experiments, deoxycoformycin was incubated with enzyme for a period of time sufficient for several turnovers of the enzyme–inhibitor complex to have occurred, so that even a small fraction of oxygen or hydrogen exchange with the solvednt would have been detected. These latter experiments do not rule out the possibility that a molecule of water might have been eliminated and sequestered by enzyme from the solvent, returning without solvent exchange. Taken together, however, the present findings suggest that adenosine deaminase binds deoxycoformycin intact and that the inhibitor does not undergo elimination of water and addition of an enzyme nucleophile by either of the mechanisms indicated in Scheme II.

The UV spectrum of the enzyme–inhibitor complex suggests that the structure of the chromophoric portion of bound deoxycoformycin does not differ very much from its structure in free solution, and bound deoxycoformycin does not appear to be protonated. Binding of deoxycoformycin by adenosine deaminase results in a bathochromic shift qualitatively similar to the shift observed in transferring deoxycoformycin from water to 2-propanol (Figure 2). Protonation in solution, on the other hand, results in a hypsochromic shift (Figure 2). The direction of the shift appears compatible with binding in a nonpolar environment, with the interesting additional feature that the difference spectrum for inhibitor binding exhibits considerable fine structure compared with the relatively smooth difference spectrum observed for inhibitor transfer from water to 2-propanol. This apparent sharpening of vibronic structure also exceeds that observed in an earlier study of 1,6-dihydro-6-(hydroxymethyl)purine ribonucleoside (Wolfenden et al., 1977) and suggests that the chromophore may be bound in an environment where vibrational relaxation occurs less readily than in water. Binding of deoxycoformycin as a neutral species, as indicated by these spectrophotometric observations, accords with the lack of significant variation of K_i through the pH range in which deoxycoformycin is uncharged in free solution (Figure 3). Binding, if it had required a protonated form of the inhibitor, might have been expected to have become progressively weaker with elevation of the pH above the pK_a of deoxycoformycin's conjugate acid (i.e., above pH 5.8).

The apparent binding of deoxycoformycin as a neutral species is interesting in view of the possibility, suggested by Kurz and Frieden (1983), that the substrate analogue purine ribonucleoside and possibly 1,6-dihydro-6-(hydroxymethyl)-purine ribonucleoside may be protonated in their complexes with adenosine deaminase. Changes in the absorption spectrum of 1,6-dihydro-6-(hydroxymethyl)purine ribonucleoside, when it was bound by adenosine deaminase in their study, were different from those that accompanied protonation in solution. That was interpreted as indicating a different site of protonation under the two conditions, although no spectrum was available as a model that could be compared with that of the enzyme–inhibitor complex. In addition, K_i did not show the pH dependence that was to be expected if binding had required protonated inhibitor. This behavior was attributed to the possibility that a proton might be transferred to the inhibitor from an acidic group on the enzyme with a high pK_a value, possibly a thiol residue. Had a thiol group been responsible, a solvent deuterium isotope effect on K_i in the neighborhood of 2.5 might have been expected from the work of Creighton and Schamp (1980). The solvent effect that was actually observed for 1,6-dihydro-6-(hydroxymethyl)purine ribonucleoside binding to adenosine deaminase was 1.2, near enough to unity so that it would appear difficult to exclude the possibility that it results from minor effects of D_2O on enzyme conformation or on the strengths of certain hydrogen bonds that may be critically involved in ligand binding. Kurz and Frieden were careful to note that proton transfer was only one of several mechanisms that might account for their observations and clearly recognized the difficulties associated with proton transfer from a weak acid to a weak base.

Strong Hydrogen Bonds between Enzyme and Deoxycoformycin. If the unusual potency of deoxycoformycin as an inhibitor can be ascribed to noncovalent forces of attraction, then several tentative inferences about these interactions appear reasonable, even in the absence of definitive information concerning the three-dimensional structure of the active site of adenosine deaminase. The stereochemical arrangement at C-8 of deoxycoformycin is especially critical, as discussed in the introduction. The 10-kcal difference in binding affinity between the 8*R* and the 8*S* isomers might alternatively be considered to arise from steric exclusion of the 8*S* isomer (but not the 8*R* isomer) from proper binding at the active site, due to possibly unfavorable contacts between the protein and the hydroxyl group. However, if it is accepted that deoxycoformycin mimics a tetrahedral intermediate in substrate hydrolysis, then steric exclusion can also probably be rejected as an explanation of the very large difference in binding activity between the two isomers. The enzyme serves as an efficient catalyst for hydrolytic removal of several leaving groups of different sizes from the 6-position of purine nucleosides [for a review, see Zielke & Suelter (1971)]. During their hydrolysis, these leaving groups (some of which are considerably bulkier than a hydroxyl group) presumably occupy positions that would be similar to the position occupied by the hydroxyl group of the "wrong" isomer of deoxycoformycin. If serious steric constraints existed at the leaving group position, as implied by any mechanism that might tend to exclude the 8*S* isomer in this way, then substrates with larger leaving groups would presumably have been found to be unreactive. Since this does not appear to be the case, one is led to suppose that specific hydrogen bonds to the protein may account for the 10^7-fold higher affinity of the 8*R* as compared with the 8*S* isomer of deoxycoformycin.

When an enzyme binds a ligand, forces of attraction that arise between them presumably take the place, at least to some extent, of previously existing solvent interactions with the inhibitor and with the active site. Accordingly, the observed free energy of enzyme–ligand association is expected to reflect the difference between the free energies of the combined and solvent-separated molecules. In one recent study, effects of amino acid alterations in tyrosyl-tRNA synthetase, for example, in conjunction with crystal structures, were used to infer the existence of free-energy hydrogen bonds involving a charged group, in interactions between ligands and the enzyme (Fersht et al., 1985). It is not yet clear whether one or as many as three hydrogen bonds to the C-8 hydroxyl group are mainly responsible for the special stability of the complex formed between adenosine deaminase and deoxycoformycin, which surpasses enzyme complexes with substrates, substrate analogues, the aglycon of coformycin, or (8*S*)-deoxycoformycin by some 10 kcal/mol in negative free energy of formation. It should be remembered, however, that any attempt to analyze the individual energies of bonds that stabilize complexes between ligands and tightly fitting biological receptors, in simple additive terms, is likely to be misleading. Individual binding interactions can cooperate to enhance the apparent stability of single bonds (analyzed on the assumption of additivity of free energy) above any value that would be observed if they

could be studied individually in isolation (Gurney, 1953; Westheimer, 1962; Page & Jencks, 1971). Thus, removal of *any* of the bonds that stabilize a very tightly sequestered transition state, or one of its stable analogues, held by the enzyme in a manner similar to that by which a polyvalent cation is held by a chelating agent, may be expected to lead to drastic losses in catalysis or inhibitor binding. In the case of adenosine deaminase, another illustration of this principle is the effect on catalysis of substituent ribose, which is considerably removed from the site of transformation of the substrate. Substituent ribose seems to exert little influence on the stability of the Michaelis complex, although the possibility of nonproductive binding of adenine (an alternate substrate) cannot be excluded. Yet, this group contributes approximately 7.5 kcal to the free energy of stabilization of the transition state for substrate transformation (Wolfenden et al., 1969) and a similar amount to the binding of potential transition-state analogues (Wolfenden et al., 1977; D. C. Baker et al., unpublished experiments). Parallel observations have been reported for proteolytic enzymes, where groups distant from the site of chemical transformation of a substrate acquire a special importance in the transition state that they did not have in the ground state (Thompson, 1974; Bartlett et al., 1983).

Implications for Catalysis. Forces involved in stabilizing an enzyme–substrate complex in the transition state are presumably the same as those involved in binding an ideal transition-state analogue. Thus, the presence or absence of covalent bonds between adenosine deaminase and deoxycoformycin could indicate whether this enzyme acts by catalyzing direct water attack or by double displacement. The present findings appear to remove any remaining doubt that deoxycoformycin is bound intact; similar conclusions were drawn by Frieden et al. (1979) from their analysis of the binding of deoxycoformycin 5′-phosphate by adenylate deaminase and by Ashley and Bartlett (1984) from their analysis of the interaction of cytidine deaminase with a phosphopyrimidine nucleoside inhibitor. These findings accord with the ability of adenosine deaminase to catalyze stereospecific addition of water to pteridine (Evans & Wolfenden, 1972, 1973) in indicating that adenosine deaminase mediates direct water attack on substrate adenosine. These findings also tend to rule out the making and breaking of covalent bonds as an explanation of the rather slow binding of transition-state analogues by these enzymes. Thus, the rate constant for the onset of inhibition of adenosine deaminase by deoxycoformycin is about 3×10^6 M^{-1} s^{-1} (Agarwal, 1982), somewhat slower than the second-order rate constant, approximately 1×10^7 M^{-1} s^{-2}, for substrate turnover. Such behavior, common but not universal among transition-state analogues that have been described to date,[4] is probably a consequence of the near-perfect fit, and large solid angle of contact, that is needed to account for the very tight binding of compounds of this kind (Wolfenden, 1976; Frieden et al., 1980). To provide for optimal affinity in the transition state, it may be necessary for enzyme-binding determinants to rearrange themselves in such a way as to maximize interactions with the substrate. In the course of the normal catalytic process, there is probably ample time during the approximately 1-ms lifetime of the ES complex for such adjustments to occur; certainly, the active site can be expected to have evolved in such a way as to minimize kinetic obstacles to the entrance of substrates and egress of products. On the other hand, encounter with a transition state (or its analogue) is an event for which the active site has not been prepared through natural selection, so that many nonproductive encounters may result[4] (Wolfenden, 1974). In the particular case of adenosine deaminase, there is evidence of a change in protein conformation when the inhibitor deoxycoformycin is bound (Kurz et al., 1985). In addition, it appears to us possible that the entrance to the active site may have some of the properties of a coil slot. Thus, "flat" coins like the planar purine rings of the substrates adenosine and inosine may be admitted without impediment, whereas "bent" coins like deoxycoformycin, with projections that depart from a planar arrangement,[5] may suffer many nonproductive encounters.

Registry No. Adenosine deaminase, 9026-93-1; deoxycoformycin, 53910-25-1.

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[4] It would be interesting, although very difficult, to distinguish cases where slow binding is due to the frequency of nonproductive encounters from cases where a loose complex is formed rapidly and isomerizes slowly to a stable complex. The problem of determining whether loose complexes fall on the pathway to products (or in this case, tight complexes) appears to have been first recognized by Henri in his pioneering work on substrate binding by enzymes [see discussion by Segal (1959)]. Viale (1970) has proposed an ingenious method of making such distinctions in the case of enzyme–substrate complexes, by observing the pre-steady-state rate of appearance of product. Given favorable rate constants, and an appropriate spectrophotometric method for observing each of the two complexes in question, it seems possible that Viale's method could be applied to enzyme–inhibitor complexes as well.

[5] Inspection of Pauling–Corey models, in conjunction with the crystal structure of coformycin (Nakamura et al., 1974), shows that the diazepine ring can be made roughly coextensive in space with the pyridine ring of adenosine but that substituents at the 8-position (a hydrogen atom and a hydroxyl group) are *not* arranged in a manner that is even approximately tetrahedral. Instead, one substituent approaches the plane of the ring, while the other projects at approximately right angles to the ring. Either substituent can be made to adopt this arrangement without appreciable steric hindrance, although the crystal structure of coformycin (Nakamura et al., 1974) shows the C-8 hydroxyl group in the latter configuration. This is interesting because the latter position corresponds approximately to the expected angle of approach of a water molecule attacking C-6 of adenosine and other evidence suggests that the transition state is reached very early during the enzyme reaction, before bonds in the substrates water and adenosine are stretched appreciably (Wolfenden, 1969).

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Kinetic Properties of Cyanase†

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Received August 22, 1985

ABSTRACT: Cyanase is an inducible enzyme in *Escherichia coli* that catalyzes the hydrolysis of cyanate. Bicarbonate is required for activity, perhaps as a substrate, and the initial product of the reaction is carbamate, which spontaneously breaks down to ammonia and bicarbonate [Anderson, P. M. (1980) *Biochemistry 19*, 2882]. The purpose of this study was to characterize the kinetic properties of cyanase. Initial velocity studies showed that both cyanate and bicarbonate act as competitive substrate inhibitors. A number of monovalent anions act as inhibitors. Azide and acetate appear to act as competitive inhibitors with respect to cyanate and bicarbonate, respectively. Chloride, bromide, nitrate, nitrite, and formate also inhibit, apparently as the result of binding at either substrate site. Malonate and several other dicarboxylic dianions at very low concentrations display "slow-binding", reversible inhibition which can be prevented by saturating concentrations of either substrate. The results are consistent with a rapid equilibrium random mechanism in which bicarbonate acts as a substrate, bicarbonate and cyanate bind at adjacent anion-binding sites, and both substrates can bind at the other substrate anion binding site to give a dead-end complex.

Cyanase is an inducible enzyme in *Escherichia coli* that catalyzes the hydrolysis of cyanate to give ammonia and bicarbonate. The enzyme has been highly purified in our laboratory, and several unusual and interesting aspects of the structural and kinetic properties have been identified (Anderson, 1980). The presence of bicarbonate is required for catalytic activity. The initial product of the reaction is carbamate, which spontaneously breaks down to bicarbonate and ammonia. The enzyme has a molecular weight of $\simeq$150000 and is composed of 8–10 identical subunits. Amino acid analysis and sequence studies have shown that the cyanase subunit (M_r 16750) is composed of 156 amino acid residues (one histidine, one cysteine, and no tryptophan residues) with no evidence for any of the common secondary structural features (Chin et al., 1983).

We have initiated studies aimed at elucidating the nature of the catalytic mechanism, the role of bicarbonate, and the biological role of this unusual enzyme. The purpose of this study was to characterize the kinetic properties of cyanase. A preliminary report has appeared (Anderson & Little, 1985).

Materials and Methods

Cyanase was isolated as previously described (Anderson, 1980). Cyanate was recrystallized from a water–ethanol mixture before use. Other reagents were obtained from Sigma Chemical Co.

Assay. Kinetic studies with this enzyme are complicated by several features of the reaction system. It is very difficult and impractical to prepare buffers totally free of low concentrations of bicarbonate. Cyanate is labile in aqueous solutions at pH 6–7 and lower due to hydrolysis of the protonated form of cyanate (Labbe, 1973), and highly purified cyanate

† Supported by National Institutes of Health Grant GM33842.

0006-2960/86/0425-1621$01.50/0