Substrate Connectivity Effects in the Transition State for Cytidine Deaminase†

Dean Carlow and Richard Wolfenden*

Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

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ABSTRACT: The binding properties of substrates and competitive inhibitors of *Escherichia coli* cytidine deaminase are compared with those of the fragments obtained by cutting these ligands at several positions including the glycosidic bond. In contrast with the normal substrate cytidine ($k_{cat}/K_m = 2.6 \times 10^6$ M^{-1} s^{-1}), cytosine is found to serve as an extremely slow substrate ($k_{cat}/K_m = 1.8 \times 10^{-3}$ M^{-1} s^{-1}), despite the ability of cytosine to enter any active site that can accommodate the normal substrate cytidine. Spontaneous nonenzymatic deamination proceeds at similar rates for cytosine and cytidine at pH 7 and 25 °C, indicating that substituent ribose exerts little effect on the intrinsic reactivity of cytidine in solution. Dividing k_{non} by k_{cat}/K_m, the maximal K_d value of the enzyme's complex with the altered substrate in the transition state is estimated as 6.1×10^{-8} M for cytosine, very much higher than the value (1.2×10^{-16} M) estimated for cytidine. The K_d value of ribofuranose, the missing substituent, is roughly 1.8×10^{-2} M, as indicated by the K_i values of D-ribose and 1-methyl-D-ribofuranoside as competitive inhibitors. Thus, the free energy of binding of the altered substrate in the transition state is 9.5 kcal/mol more favorable for the whole molecule cytidine than for the sum of those of its parts, cytosine plus ribofuranose. As a separate molecule, however, ribose shows no detectable effect on the enzyme's activity on cytosine. Connectivity effects of similar magnitude are indicated by the equilibrium binding affinities of inhibitors. Thus, the K_i value of the transition state analogue inhibitor zebularine hydrate (1.2×10^{-12} M) is very much lower than the combined affinities of *N*-ribofuranosylurea (1.6×10^{-4} M) and allyl alcohol (0.14 M), indicating that the glycoside bond, by its presence, exerts a connectivity effect of 9.9 kcal/mol on the observed free energy of binding.

As an enzyme reaction progresses toward the transition state in substrate transformation, attractive interactions between the enzyme and substrate increase in strength by a factor that matches or surpasses the large factor by which the enzyme enhances the rate of reaction. Later, that attraction subsides as products are formed and released. The transition state for deamination of cytidine (Figure 1) appears to be bound by the enzyme with a dissociation constant of approximately 4×10^{-16} M (*1*). The inhibitor zebularine hydrate (3,4-dihydrouridine) (I, Figure 1), formed by covalent hydration of zebularine is bound by *Escherichia coli* cytidine deaminase with a K_i value of 1×10^{-12} M, roughly 8 orders of magnitude lower than the K_m value of cytidine or the K_i value of product uridine (*2*). Thus, zebularine hydrate captures a considerable fraction of the total free energy of binding expected of an ideal transition state analogue inhibitor for the deamination of cytidine. Cytidine deaminase has been shown to envelop its substrate almost completely in the transition state, with numerous strong interactions between the enzyme and the bound transition state analogue, shown in Figure 2 (*3*). This paper describes an effort to explore the relative importance of substrate binding determinants in the transition state for cytidine deamination, and how their forces of attraction by the enzyme combine to supply the overall affinity that is needed to explain the level of catalysis that is observed.

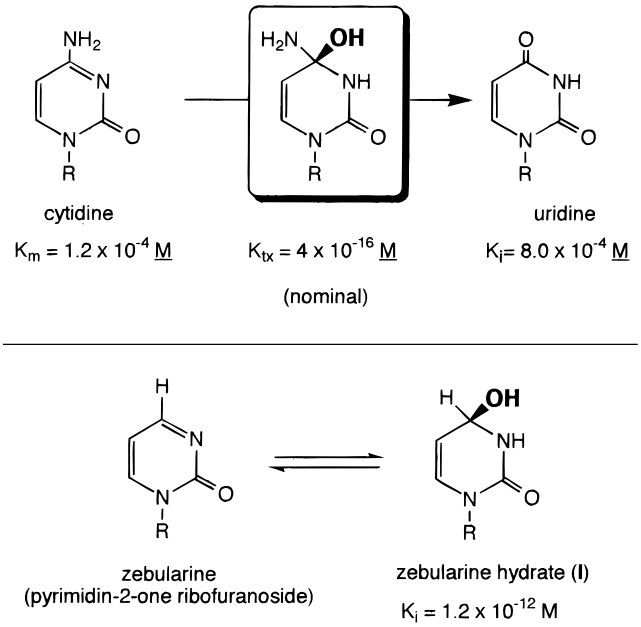

FIGURE 1: The reaction catalyzed by cytidine deaminase, showing the nominal binding affinity of the altered substrate in the transition state, and of zebularine hydrate, a rare species formed from the competitive inhibitor zebularine.

It was once believed that, in the absence of electronic effects, the free energy of binding of a substrate or inhibitor might tend to be equivalent to the sum of the free energies of binding of its constituent parts. It is now recognized, however, that marked departures from additivity are to be

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* Author to whom correspondence should be addressed.

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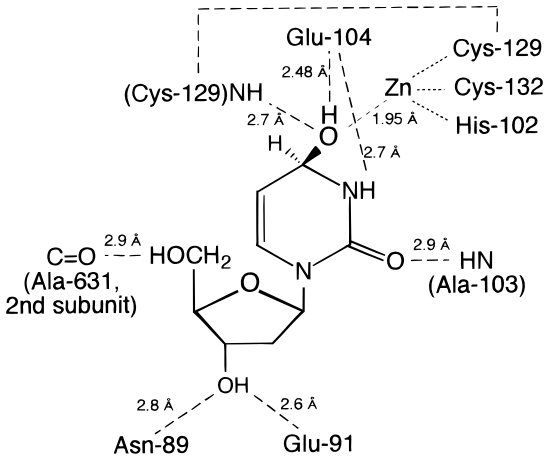

FIGURE 2: Enzyme interactions with the tetrahedral intermediate in deamination of cytidine, inferred from the crystal structure of the inhibitory complex formed between cytidine deaminase and the transition state analogue 5-fluoro-3,4-dihydrouridine (*3*).

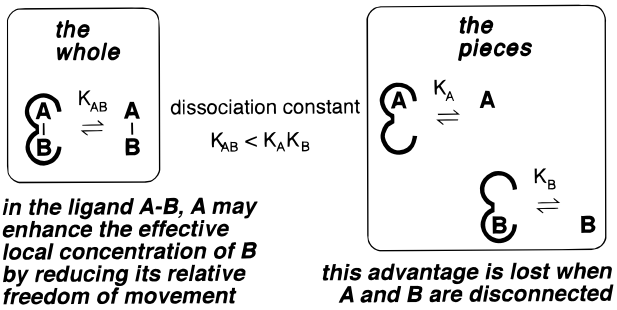

FIGURE 3: Affinity of a ligand, A-B, compared with the affinities of its pieces, A and B.

expected when enzyme binding of one part of a ligand limits the freedom of movement of another part in such a way as to assist or interfere with its binding (Figure 3). Connectivity effects can in principle be large is some cases, but are likely to be limited by any freedom of motion of the whole ligand in solution and by any residual freedom of motion that remains in its parts after they are bound (*4*). At the time of an early review, two examples were known (biotin/avidin and heavy meromyosin) in which discrepancies as large as $\sim 10^4$ M had been observed in K_d values (*5*).

Effects of translational and rotational constraints would be expected to be especially pronounced in those cases in which the different parts of a ligand A-B, in its unbound form, are already rigidly connected, so that the ligand has little internal freedom to lose when it is bound by the enzyme. Nucleosides, for example, have relatively narrowly defined structures in solution, with little rotational freedom except at the glycosidic bond. Even at that position, purines and pyrimidines show strong rotational preferences. Moreover, the crystal structures of cytidine deaminase ligands reveal that the glycosidic torsional angles of the enzyme-bound ligands are similar to the torsional angles observed in pyrimidine nucleosides in free solution (*3*, *6*). Accordingly, cytidine deaminase might be expected to afford a relatively uncomplicated setting for examining the effects of ligand connectivity on binding affinity.

In earlier work, we investigated the effects of replacing active site residues that are involved in transition state stabilization by cytidine deaminase (Figure 2) (*7*−*10*). The present paper describes a complementary set of experiments in which the ligand rather than the active site was modified. The results indicate major departures from additivity, when the apparent free energy of binding of zebularine hydrate is compared with the affinities of fragments obtained when this transition state analogue is divided into fragments at several different positions. We observe similar departures from additivity when the affinity of the altered substrate in the transition state for cytidine deamination, determined kinetically, is compared with the combined affinities of (a) the transition state in enzymatic deamination of cytosine (from which the ribofuranosyl substituent has been removed) and of (b) ribose itself. These effects of connectivity appear to have significant practical implications for the design of inhibitors of enzymes, particularly enzymes involved in nucleoside metabolism. We also describe the effects of cutting ligands at other positions, and compare the effect of saturating the 5═6 double bond of substrate cytidine with its effect on the enzyme's affinity for the transition state analogue zebularine hydrate.

MATERIALS AND METHODS

Cytidine deaminase was prepared from an overproducing strain of *E. coli* as described by Yang et al. (*11*). Zebularine and 3,4-dihydrozebularine (II, Figure 4) were gifts from Dr. Victor Marquez (National Cancer Institute, NIH, Bethesda, MD). 3,4,5,6-Tetrahydrouridine (V, Figure 6) and 3,4,5,6-tetrahydrozebularine (VI, Figure 6) were prepared as described by Hanze (*12*), and *N*-ribofuranosylurea was prepared by the method of Ukita et al. (*13*). Allyl alcohol, D-ribose, ethylene glycol, and methanol were purchased from Aldrich Chemical Co. Cytosine, cytidine, and methyl-β-D-ribofuranoside were purchased from Sigma Chemical Co. The alternative substrates 1-[[2-hydroxy-1-(hydroxymethyl)-ethoxy]methyl]cytosine (III, Figure 5) and 1-[(2-hydroxy-ethoxy)methyl]cytosine (IV, Figure 5) were gifts from Dr. Steven Short of Wellcome Research Laboratory.

The activity of cytidine deaminase on various substrates was monitored by following the change in UV absorbance when cytosine and its derivatives were converted to the corresponding uracil derivatives, at the following wavelengths of maximum change in UV absorbance in Tris-HCl buffer (0.05 M, pH 7.0) at 25 °C: cytidine, 282 nm ($\Delta\epsilon = -3600$); cytosine, 259 nm ($\Delta\epsilon = +4340$); 1-[(2-hydroxy-ethoxy)methyl]cytosine, 260 nm ($\Delta\epsilon = +2197$); and 1-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]cytosine, 282 nm ($\Delta\epsilon = -2307$). In experiments with nucleoside concentrations greater than 1×10^{-4} M, aliquots of the reaction mixture were withdrawn at timed intervals and diluted into a 1-mL cuvette of 1-cm light path, and the absorbance was recorded. Concentrations of enzyme used ranged from 2×10^{-9} M (in subunits) when cytidine was the substrate to 5×10^{-5} M when cytosine was the substrate. Inhibition constants were determined from double reciprocal plots of enzyme activity as a function of changing substrate concentrations, in the presence and absence of inhibitors. With the exception of methanol and ethylene glycol, all inhibitors showed simple competitive inhibition. Inhibition by methanol and ethylene glycol was reversible, and the values reported for these inhibitors are the concentrations required to produce half-maximal activity when cytidine was present at a concentration of 1×10^{-4} M.

Table 1: Substrates and Inhibitors of *E. coli* Cytidine Deaminase, pH 6.8, 25 °C

substrates	K_m	k_{cat} (s^{-1})	k_{cat}/K_m	ref
cytidine	1.2×10^{-4} M	299 [$k_{non} = 3.2 \times 10^{-10}$ s^{-1}][a]	2.6×10^6 M^{-1} s^{-1}	(*10*)
cytosine	(*b*)	(*b*) [$k_{non} = 1.1 \times 10^{-10}$ s^{-1}][c]	1.8×10^{-3} M^{-1} s^{-1}	this work
1-[(2-hydroxyethoxy)methyl]cytosine (**III**, Figure 5)	(*b*)	(*b*)	48 M^{-1} s^{-1}	this work
1-[(2-hydroxy-1-hydroxymethyl)ethoxymethyl]cytosine (**IV**, Figure 5)	(*b*)	(*b*)	550 M^{-1} s^{-1}	this work

inhibitors	K_i	ref
uridine	8×10^{-4} M	(*10*)
zebularine hydrate (**I**, Figures 1, 4, 6)	1.2×10^{-12} M	(*10*)
N-(ribofuranosyl)urea	1.6×10^{-4} M	this work
allyl alcohol	0.14 M	this work
1-methyl-D-ribofuranoside	2.4×10^{-2} M	this work
ethylene glycol[d]	~0.6 M	this work
2-propanol[d]	~2.4 M	this work
3,4-dihydrozebularine (**II**, Figures 4, 6)	3×10^{-5} M	(*2*)
3,4,5,6-tetrahydrouridine (**V**, Figure 6)	1×10^{-7} M	(*2*)
3,4,5,6-tetrahydrozebularine (**VI**, Figure 6)	4×10^{-5} M	this work
D-ribose	1.2×10^{-2} M	this work

[a] The rate constant for spontaneous deamination of cytidine is taken from ref 1. [b] K_m values of these substrates are in excess of 0.01 M, above the range where K_m and k_{cat} values could be measured accurately; for these substrates, values of k_{cat}/K_m were obtained from the slopes of double reciprocal plots. [c] Ridgway, C., and Wolfenden, R., unpublished results. [d] Inhibition by ethylene glycol and 2-propanol was reversible, and achieved half-maximal values at these concentrations.

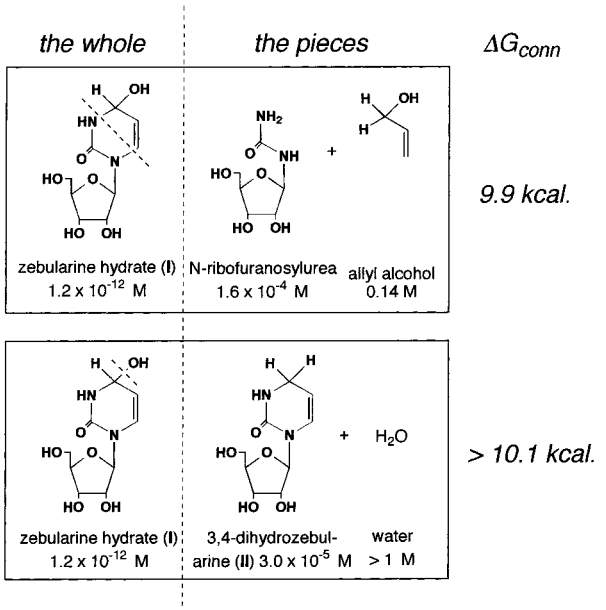

FIGURE 4: Effects on K_i of cutting the inhibitor zebularine hydrate into two pieces, represented by allyl alcohol and *N*-ribofuranosylurea, or 3,4-dihydrozebularine and water.

RESULTS

The results obtained in these experiments are summarized in Table 1.

Figure 4 shows the observed effects of "cutting" zebularine hydrate, either into allyl alcohol and *N*-(1-ribofuranosyl)-urea, or into 3,4-dihydrozebularine and water, on the affinities of these compounds as competitive inhibitors. The active site's affinity for water has not been clearly established, but appears to be weak ($K_d > 1$ M), as indicated by experiments in which an attempt was made to reduce solvent water content by admixture with other water-miscible solvents, in experiments similar to those described earlier for adenosine deaminase (*14*).

Figure 5 shows the effects on k_{cat}/K_m of cutting substrate cytidine at the glycosidic bond, and at two positions within the ribofuranosyl substituent. The value shown for ribose is an average of the K_i values observed for D-ribose (2.4×10^{-2} M) and β-1-methyl-D-ribofuranoside (1.2×10^{-2} M). D-Ribose, purely competitive in its effect on the deamination

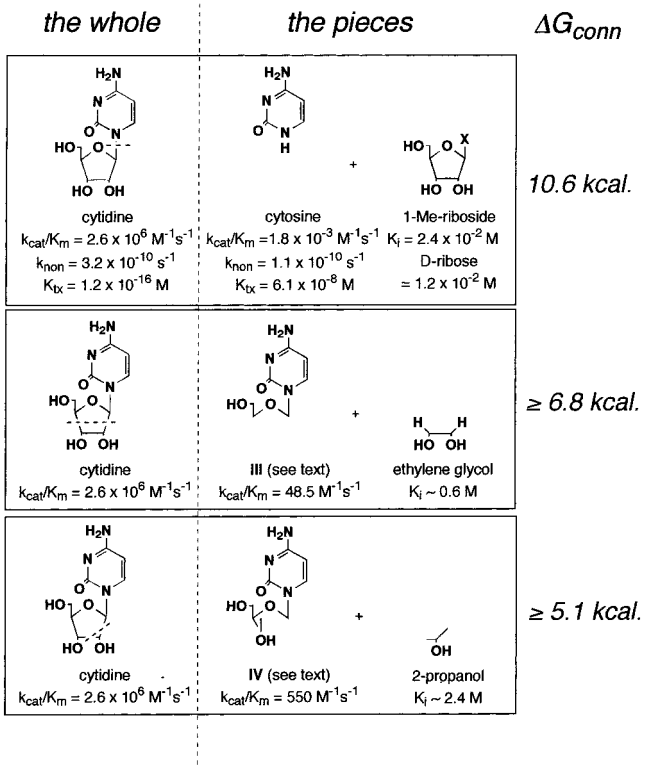

FIGURE 5: Effects of cutting the substrate cytidine into two pieces, represented by cytosine and ribose, on enzyme binding affinity for the transition state in enzymatic deamination. The value shown for ribose is an average of the K_i values observed for D-ribose (2.4×10^{-2} M) and β-1-methyl-D-ribofuranoside (1.2×10^{-2} M). In the comparison of cytidine with cytosine, K_{tx} is calculated to include the small difference between the rates of spontaneous deamination of cytosine and cytidine (see text). Other substrates, shown in the lower panels, are assumed to be equivalent to cytidine in their rates of spontaneous deamination.

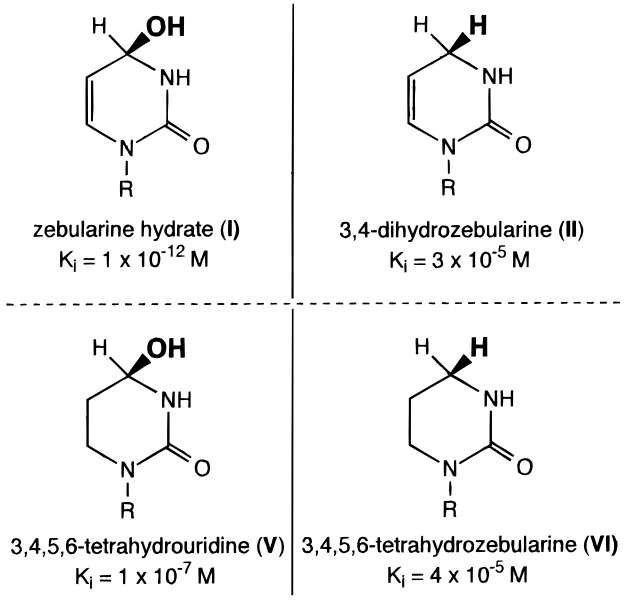

FIGURE 6: K_i values observed for derivatives of zebularine hydrate. Replacement of the 4-OH group by hydrogen gives 3,4-dihydrozebularine, which is accompanied by a 3×10^8-fold decrease in binding affinity (*2*). Saturation of the 5,6 C═C of gives 3,4,5,6-tetrahydrouridine, resulting in a 10^5-fold decrease in binding affinity (*22*). When both modifications are present together in the same molecule (3,4,5,6-tetrahydrozebularine), further reductions in binding affinity are much smaller.

of cytidine, was shown not to enhance the rate of enzymatic hydrolysis of cytosine, when present at a concentration equivalent to its K_i value. An Arrhenius plot of the rate of the nonenzymatic deamination of cytosine, measured in 0.1 M potassium phosphate buffer, pH 6.8 (Wolfenden, R., and Ridgway, C., unpublished) yielded a rate constant of 1.1×10^{-10} s^{-1} for the spontaneous reaction at 25 °C, slightly lower than the value (3.2×10^{-10} s^{-1}) obtained earlier for cytidine under the same conditions (*1*).

Figure 6 shows the effects of (a) reduction of the 5═6 double bond and (b) removal of the 4-OH group by hydrogen, and of both these changes in combination, on the binding affinity of zebularine hydrate as a competitive inhibitor. In Figures 4 and 5, the effect of connectivity (equivalent to an "effective molarity", ref *16*) was obtained by comparing the binding affinity of the whole substrate or inhibitor with the sum of the affinities of its pieces, and is shown here as a free energy of connection (ΔG_{conn}).

DISCUSSION

Synergistic Effects of Binding Determinants. In model systems, and in theory, the potential advantage of limiting the rotational and translational freedom of reactants becomes evident when the first-order rate constant of a typical intramolecular reaction is compared with the second-order rate constant of an intermolecular reaction that is similar in other respects. In some model systems, the "effective concentration" of the second reactant, that would be required to cause the two-molecule reaction to proceed as rapidly as the one-molecule reaction, has been shown to be very large indeed (10^5-fold in an early example, ref *15*; for a review, see ref *16*). Similarly, in reactions that involve more than a single substrate, it should be possible in principle for an enzyme to produce a major rate enhancement, simply by binding two substrates at the active site in a configuration that is appropriate for reaction (*17*).

In reactions catalyzed by lyases and isomerases, no second substrate is involved, and an enzyme can only enhance the reaction rate by stabilizing chemically altered intermediates in substrate transformation, relative to the substrate in the ground-state enzyme−substrate complex. Hydrolytic reactions, in which the effective concentration of water, the second substrate, is presumably already high, might be expected to exhibit the same tendency. Even in one-substrate and hydrolytic reactions, however, k_{cat} may be greatly affected by an interaction that seems distant from the site of bond making and breaking. Here, we show that the ribofuranosyl substituent of cytidine greatly enhances its reactivity with cytidine deaminase, but has little effect on the rate of the spontaneous reaction. In such a case, the binding effect of a distant substrate substituent is manifested in the transition state, so that the substrate's observed binding affinity in the transition state includes the contribution of that distant functionality in addition to the region of the substrate where bonds are being made and broken. These combined influences on transition state binding can be synergistic, if restrictions on the relative rotational and translational mobility of binding determinants are important in optimizing structural complementarity to the active site.

Effects of Cutting Zebularine Hydrate and Cytidine. In earlier experiments, comparing the catalytic proficiencies of cytidine deaminase with those of fragments obtained by mutagenesis, the active site binding determinants of the enzyme were shown to act synergistically (*10*). In the present experiments, the binding properties of substrates and competitive inhibitors of *E. coli* cytidine deaminase were compared with those of their fragments, obtained by cutting the ligand at various positions including the glycosidic bond. In experiments involving the kinetic properties of substrates (Figure 5), the aglycone cytosine was found to serve as an extremely slow substrate ($k_{cat}/K_m = 1.8 \times 10^{-3}$ M) compared with the normal substrate cytidine, for which $k_{cat}/K_m = 2.6 \times 10^6$ M^{-1} s^{-1}. The spontaneous, nonenzymatic deamination of cytosine proceeds at a rate ($k_{non} = 1.1 \times 10^{-10}$ s^{-1}, pH 7, 25 °C) similar to that of cytidine ($k_{non} = 3.2 \times 10^{-10}$ s^{-1}), indicating that the ribofuranosyl substituent exerts only a small effect on the intrinsic reactivity of cytidine in solution. Dividing k_{non} by k_{cat}/K_m, the maximal K_d value of the enzyme's complex with the altered substrate in the transition state is estimated as 6.1×10^{-8} M for cytosine, as compared with 1.2×10^{-16} M for cytidine. The K_d value of ribose, the "missing piece", is roughly 1.8×10^{-2} M, as indicated by averaging the K_i values observed for D-ribose (2.4×10^{-2} M) and 1-methyl-D-ribofuranoside (1.2×10^{-2} M). As a separate molecule, ribose has no significant effect on the enzyme's activity on cytosine. As a substituent, however, the ribofuranosyl group enhances the effective concentration of the altered substrate in the transition state by a factor of approximately 10^7 M. Thus, the free energy of binding of the altered substrate in the transition state appears to be ~9.5 kcal/mol more favorable for the whole molecule cytidine than for its constituent fragments, cytosine and D-ribofuranose.

Comparisons of this kind are clearly approximate, in that it is not easy to rule out, or even to test, the possibility that structural changes might have caused the position of the

transition state for the enzyme reaction to shift along the reaction coordinate. Thus, it is of special interest that a connectivity effect of very similar magnitude is indicated by experiments in which the equilibrium binding affinity of the transition state analogue inhibitor zebularine hydrate is compared with the affinities of its fragments, obtained by cutting this inhibitor at various positions (Figure 4). Thus, the K_i value of the transition state analogue inhibitor zebularine hydrate (1.2×10^{-12} M) is very much lower than those of its pieces, *N*-ribofuranosylurea (1.6×10^{-4} M) and allyl alcohol (0.14 M), corresponding to a connectivity effect of 9.9 kcal/mol on the observed free energy of binding.

In the present experiments on cytidine deaminase, as in earlier experiments on adenosine deaminase (*18*), free energies (ΔG_{conn}) associated with these connectivity effects are found to approach the maximal values that have been estimated for such effects from model studies, ~11 kcal/mol (*4*). These comparisons show the magnitude of the potential advantage that can be gained by a substrate from the fact that its parts are properly connected to fit the active site, to produce a high affinity that is of special importance in the transition state. These comparisons also furnish a striking illustration of how an enzyme reaction rate can be affected by a binding determinant that is distant (at least in electronic terms) from the site of its chemical modification. Effects of this magnitude seem unlikely to be encountered except in those cases in which the parts of the substrate or inhibitor are joined in such a way that within the ligand molecule, little internal freedom is lost when binding occurs. It is therefore of special interest that nucleosides strongly favor the high-anti configuration in solution, and that in the crystal structures of complexes of cytidine deaminase (*3*, *6*) and adenosine deaminase (*19*) with transition state analogue inhibitors the configurations of the glycosidic bonds of the bound inhibitors are roughly the same as their configurations in free solution.

Can a more specific role be assigned to ribose, in the action of cytidine deaminase? Recently, the structures of a series of cytidine deaminase complexes with structures representing several frames in a motion picture along the reaction coordinate: ES, EP, and ES‡, the enzyme's complex with the altered substrate in the transition state (*20*). Unexpectedly, these structures show that substrate's ribofuranosyl group remains anchored in the protein structure by H-bonds involving the 3′- and 5′-OH groups, while a change in the glycosidic torsion angle allows the pyrimidine ring to describe a trajectory in which it leaves the leaving amino group of cytidine behind, near Glu-104. At the same time, the pyrimidine ring moves toward the attacking group (a zinc-bound water molecule) to form the product uridine. During that movement, the carboxylate group of Glu-104 rotates in such a way that its original H-bond to the substrate's O-4 is broken, and a new H-bond to the leaving ammonia is formed. Concomitant changes in the length of the $S_{\gamma 132}$ bond allow the zinc atom's electrophilicity to adjust to fluctuations in the negative charge on the activated water molecule, buffering its interactions with other zinc ligands and with the carboxylate group of Glu-104. The estimated cost in free energy of glycoside distortion in the EP complex with uridine is modest (~1 kcal/mol, in accord with the existence of several compounds in the Cambridge Small Molecule Data Base in which comparable distortion has been reported), but may help to facilitate product release (*20*).

Apparent Interactions between the 4-OH Group and the 5═6 Double Bond of Zebularine Hydrate. Interaction effects of a different kind are indicated by experiments showing that the 4-OH group and the 5═6 double bond of zebularine hydrate (I) are strongly interdependent in their effects on its free energy of binding. Earlier, the contribution of the 4-OH group of I was tested by comparing the binding affinities of I ($K_i = 1 \times 10^{-12}$ M) with that of II (3,4-dihydrozebularine, $K_i = 3 \times 10^{-5}$ M, ref *2*) which differs from I in that the 4-OH group has been replaced by a hydrogen atom. Thus, by its presence, the 4-OH group of zebularine hydrate (I) contributes 10.1 kcal/mol to binding, the largest contribution of a single OH group to protein−ligand binding that appears to have been reported (*21*). X-ray structures (*6*) reveal that the 4-OH group of zebularine hydrate interacts with the active site in several ways, including a short H-bond to the carboxylate side chain of Glu-104, an H-bond to the amide proton of Cys-129, and a coordinate bond to the active site zinc atom (Figure 2). The structure of the enzyme complex with II differs from the enzyme complex with I only in that a trapped water molecule now occupies the position vacated by the 4-OH group of 3,4-dihydrouridine. Since water is observed to fill this gap, it seems reasonable to infer that the resulting complex must be more stable than any (hypothetical) complex from which that water molecule is absent; otherwise, a complex of the latter kind would have been observed. If the latter (hypothetical) complex is even less stable than the complex observed, then the contribution of the 4-OH group to the binding affinity would have been estimated only as a lower limit, by comparing the binding affinities of I and II.

Similarly, the significance of the 5═6 double bond of zebularine hydrate for binding can be assessed by comparing the binding affinity of zebularine hydrate ($K_i = 1 \times 10^{-12}$ M) with that of 3,4,5,6-tetrahydruridine ($K_i = 1 \times 10^{-7}$ M, ref *22*; V, Figure 6). That comparison suggests that the presence of the C═C bond in zebularine hydrate contributes roughly 5 orders of magnitude to the binding of this inhibitor. However, an inhibitor with both a reduced 5═6 double bond and its 4-OH group replaced by hydrogen (VI, Figure 6) shows only a modest further reduction in binding affinity, compared with compounds possessing one of these modifications. Replacement of the 4-OH group of 3,4,5,6-tetrahydrouridine by hydrogen results in only a 400-fold reduction in binding affinity, and saturation of the 5═6 double bond of 3,4-dihydrozebularine results in virtually no effect on binding. Thus, the 5═6 double bond and the crucial 4-OH group appear to be interdependent in their effects on binding affinity.

The significance of the 5═6 double bond for transition state stabilization can be explored in a different way by examining the effect of saturation on the rate of deamination of cytidine. *E. coli* cytidine deaminase has been shown to serve as an inefficient catalyst of the deamination of 5,6-dihydrocytidine (DHC) to 5,6-dihydrouridine (DHU), enhancing the rate of this reaction by a factor of only 1.4×10^5 (*23*), much less than the rate enhancement (4×10^{11}-fold) observed for cytidine (*1*). The 5═6 double bond appears to have little effect on the binding of ground-state structures, as the Michaelis constant observed for 5,6-DHC and the K_i value observed for 5,6-DHU are similar to those of the 5,6-unsaturated parent compounds (*23*). Thus, the

contribution of the 5═6 double bond, like that of the 4-OH group, is evidently manifested only in the transition state.

At present, we do not understand the role of the 5═6 double bond in further detail. Unsaturation at this position might be expected to enhance the acidity of the 4-OH group of zebularine hydrate, enhancing the strengths of H-bonds in which it serves as a donor (*24*). However, allyl alcohol exhibits a pK_a value (15.5) only 0.5 units lower than that of 1-propanol (*25*). Such a small difference in acidity would be expected to have only a slight effect on H-bonding capacity and seems unlikely, by itself, to be responsible for the large contribution of this group to binding affinity. It appears that the 5═6 double bond may help to maintain the structure of the bound inhibitor in the exact form that is needed for optimal organization of binding determinants to fit the binding site. However, more exact structural information will be needed to understand the means by which this is accomplished.

CONCLUSIONS

In earlier work on the effects of replacing active site residues that are involved in transition state stabilization by cytidine deaminase (Figure 2) (*7*−*10*), these residues were found to behave in a strongly synergistic manner. In another manifestation of the importance of connectivity, a mutant cytidine deaminase was also found to exhibit moderate levels of catalytic rescue (*8*), reminiscent of the pioneering experiments of Toney and Kirsch (*24*) on aspartate aminotransferase. These observations point to the presence of high relative concentrations, or effective molarities, of catalytic residues at the active site of the native enzyme. The present experiments indicate that the same is true of the binding properties of substrates and competitive inhibitors. The major losses of binding affinity that are found to result from apparently minor structural modifications of substrates and their analogues, in this and in an earlier study of adenosine deaminase (*18*), have encouraging implications for inhibitor design. Thus, under certain circumstances, one or two simple modifications can improve the effectiveness of a weak inhibitor to an extraordinary extent.

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