Catalysis by Entropic Effects: The Action of Cytidine Deaminase on 5,6-Dihydrocytidine†

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ABSTRACT: In neutral solution, 5,6-dihydrocytidine undergoes spontaneous deamination ($k_{25} \sim 3.2 \times 10^{-5}$ s^{-1}) much more rapidly than does cytidine ($k_{25} \sim 3.0 \times 10^{-10}$ s^{-1}), with a more favorable enthalpy of activation ($\Delta\Delta H^{\ddagger} = -8.7$ kcal/mol) compensated by a less favorable entropy of activation ($T\Delta\Delta S^{\ddagger} = -1.8$ kcal/mol at 25 °C). *E. coli* cytidine deaminase enhances the rate of deamination of 5,6-dihydrocytidine ($k_{cat}/k_{non} = 4.4 \times 10^5$) by enhancing the entropy of activation ($\Delta\Delta H^{\ddagger} = 0$ kcal/mol; $T\Delta\Delta S^{\ddagger} = +7.6$ kcal/mol, at 25 °C). Binding of the competitive inhibitor 3,4,5,6-tetrahydrouridine (THU), a stable analogue of 5,6-dihydrocytidine in the transition state for its deamination, is accompanied by a release of enthalpy ($\Delta H = -7.1$ kcal/mol, $T\Delta\Delta S = +2.2$ kcal/mol) that approaches the estimated enthalpy of binding of the actual substrate in the transition state for deamination of 5,6-dihydrocytidine ($\Delta H = -8.1$ kcal/mol, $T\Delta\Delta S = +6.0$ kcal/mol). Thus, the shortcomings of THU in capturing all of the binding affinity expected of an ideal transition-state analogue reflect a less favorable entropy of association. That difference may arise from the analogue's inability to displace a water molecule from the "leaving group site" at which ammonia is generated in the normal reaction. The effect on binding of removing the 4-OH group from the transition-state analogue THU, to form 3,4,5,6-tetrahydrozebularine (THZ) ($\Delta\Delta H = -2.1$ kcal/mol, $T\Delta\Delta S = -4.4$ kcal/mol), is mainly entropic, consistent with the inability of THZ to displace water from the "attacking group site". These results are consistent with earlier indications [Snider, M. J., and Wolfenden, R. (2001) *Biochemistry 40,* 11364] that site-bound water plays a prominent role in substrate activation and inhibitor binding by cytidine deaminase.

The ability of a catalyst to lower the free energy of activation that limits the rate of a reaction depends on its ability to bind the altered substrate in the transition state more tightly than it binds the substrate in the ground state (*1*). Considering the general similarity in structure between these different forms of the substrate, and the large rate enhancements produced by most enzymes, their powers of binding discrimination are remarkable (*2*).

In an early discussion of possible sources of enzyme rate enhancement. Westheimer suggested that an enzyme might serve in part as an "entropy trap", gathering several substrates at the active site in positions conducive to reaction (*3*). Their subsequent activation might not be expected to require as much loss of entropy as would the same reaction proceeding spontaneously in dilute solution. In a later discussion of the potential advantages of intramolecularity (*4*), Page and Jencks estimated that a near-total loss of 3° of translational and 3° of rotational freedom might lead to rate accelerations [$k_{intra}(s^{-1})/k_{bimol}(M^{-1}\ s^{-1})$] as large as $\sim 10^8$ M, but cautioned that solvent effects might obscure their expression as an increase in the entropy of activation. Indeed, for many enzyme reactions, catalysis has been found to arise mainly from a reduction in the enthalpy of activation (*5*). Here, we describe an exceptional case in which catalysis is achieved by entropic effects alone.

The properties of *E. coli* cytidine deaminase (CDA)[1] render it attractive for comparing the activation parameters of a catalyzed with those of an uncatalyzed reaction. Experiments involving viscosity variation (*6*), as well as kinetic isotope effects on k_{cat}/K_m (*7*), indicate that K_m represents the dissociation constant of the ES complex, that k_{cat} describes the chemical transformation of the substrate rather than product release, and that the spontaneous and enzyme-catalyzed reactions proceed by mechanisms in which the making and breaking of bonds to the attacking and leaving groups have proceeded to a similar extent. Both the enzymatic (*8*) and the uncatalyzed (*9*) reactions are insensitive to changing pH near neutrality, reducing the likelihood of complications that might otherwise arise from differing heats of ionization. Moreover, crystal structures have been solved for cytidine deaminase complexes with a substrate analogue (*10*), a transition-state analogue (*11*), and product uridine (*12*).

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[1] Abbreviations: CDA, cytidine deaminase; DHU, 5,6-dihydrocytidine; DHC, 3,4-dihydrouridine; DHU, 3,4-dihydrozebularine; THU, 3,4,5,6-tetrahydrouridine; THZ, 3,4,5,6-tetrahydrozebularine; ITC, isothermal titration calorimetry.

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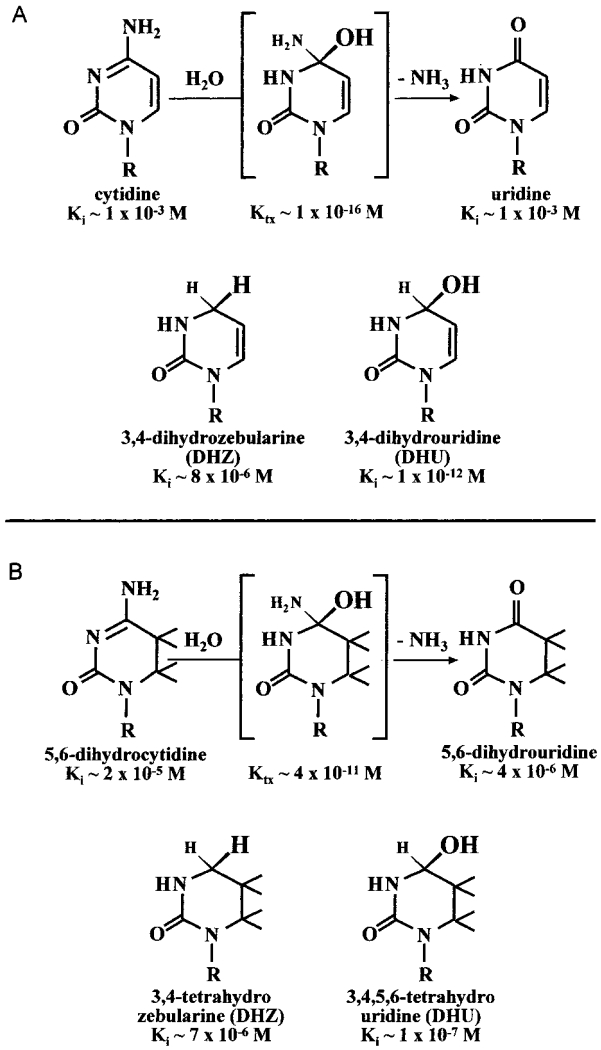

FIGURE 1: Reaction scheme for the deamination of (A) cytidine and (B) 5,6-dihydrocytidine and the structures of the corresponding transition-state analogue inhibitors.

Kinetic measurements have shown that the binding of cytidine is accompanied by a favorable change in enthalpy ($1/K_s$, $\Delta H = -13$ kcal/mol, $T\Delta S = -7.6$ kcal/mol at 25 °C), and that additional heat is released in binding the altered substrate in the transition state[2] ($1/K_{tx}$, $\Delta H = -20$ kcal/mol, $T\Delta S = +1.6$ kcal/mol at 25 °C) (*13*). Structural studies indicate that in the ground-state ES complex (but not in the transition-state complex) (*9, 10*), one excess water molecule is present in the attacking group site, split between Zn and Glu-104. The relatively favorable entropy of binding of the altered substrate in the transition state can be attributed to displacement of bound water from the active site into bulk solvent. In recent work, we used isothermal titration calorimetry and van't Hoff analysis to determine the thermodynamic changes associated with the equilibrium binding of inhibitors (Figure 1) by cytidine deaminase (*13*). The results were consistent with a scenario in which the binding of 3,4-dihydrouridine (DHU), a potential transition-state analogue, is accompanied by a favorable change in enthalpy ($\Delta H = -21$ kcal/mol), that is nearly equivalent to that of the altered substrate in the transition state ($\Delta H = -20$ kcal/mol). However, $T\Delta S$ for binding of 3,4-dihydrouridine is much less favorable ($T\Delta S = -5$ kcal/mol) than $T\Delta S$ for binding of the transition state itself ($T\Delta S = +1.6$ kcal/mol). In the transition-state analogue complex, but not in the actual transition state, one excess water molecule appears to be bound at the "leaving group site". In most respects, bonding appears to be similar in these two complexes, but the fact that water is trapped in the complex of the slightly smaller transition-state analogue was considered to explain its less favorable entropy of binding (*13*).

When the 4-OH group is removed from the transition-state analogue DHU, the heat released upon binding becomes much less favorable ($\Delta\Delta H \sim -11$ kcal/mol), furnishing an indication of the value of enzyme interactions with the 4-OH group (*11*). The entropy of binding, however, is hardly affected ($T\Delta S = -3$ kcal/mol). This behavior seems consistent with the presence of one water molecule, this time trapped in the "attacking group site" (*13*).

Cytidine deaminase acts not only on cytidine, but also on a nucleoside of very different reactivity, 5,6-dihydrocytidine (*12*). This unnatural substrate furnishes an additional opportunity to explore the thermodynamic basis of catalysis by this enzyme. 5,6-Dihydrocytidine is deaminated almost as efficiently ($k_{cat}/K_m = 7.3 \times 10^5$ M^{-1} s^{-1}) as the natural substrate cytidine ($k_{cat}/K_m = 3.0 \times 10^6$ M^{-1} s^{-1}). However, its rate of spontaneous deamination ($k_{non} = 3.2 \times 10^{-5}$ s^{-1}) is much higher than that of cytidine ($k_{non} = 3.0 \times 10^{-10}$ s^{-1}) (*12*), the natural substrate. Because of that inherent difference in reactivity, the enzyme's affinity for activated 5,6-dihydrocytidine in the transition state is $\sim 10^5$-fold less than its affinity for activated cytidine in the transition state for hydrolytic deamination.[2] In the present work, we determined the enthalpy and entropy of activation for the uncatalyzed deamination of 5,6-dihydrocytidine, for comparison with those of the enzyme-catalyzed reaction (Figure 1).

In addition, we determined the thermodynamic changes associated with the equilibrium binding of the competitive inhibitor 3,4,5,6-tetrahydrouridine (THU). This compound, one of the earliest potential transition-state analogues to be identified for any enzyme (*15*), bears a closer resemblance to the probable transition state for deamination of the unnatural substrate 5,6-dihydrocytidine than to the transition state for deamination of cytidine itself. In addition to examining the behavior of this transition-state analogue, we determined the thermodynamic changes that accompany the binding of 3,4,5,6-tetrahydrozebularine (THZ), in which a hydrogen atom replaces the 4-OH group of THU (Figure 1).

MATERIALS AND METHODS

Spontaneous Deamination of 5,6-Dihydrocytidine. 5,6-Dihydrocytidine (DHC) was synthesized and characterized by the method of Evans et al. (*14*). Spontaneous deamination of DHC, in 0.1 M phosphate buffer (pH 7.3), was followed to completion by monitoring the decrease in absorbance at 247 nm ($\Delta\epsilon = -6445$ M^{-1} cm^{-1}) at temperatures between 25 and 76 °C. Nonlinear regression analysis of these kinetic data was used to obtain first-order rate constants at each temperature.

Enzymatic Deamination of 5,6-Dihydrocytidine. Enzymatic deamination of 5,6-dihydrocytidine with wild-type CDA was

[2] From the relationship that $K_{tx} = k_{non}/(k_{cat}/K_m)$, where K_{tx} is the virtual dissociation constant of the enzyme−substrate complex in the transition state.

monitored spectrophotometrically at 247 or 265 nm ($\Delta\epsilon_{265} = -800\ M^{-1}\ cm^{-1}$) in 0.1 M phosphate buffer (pH 7.3). The temperature dependence of k_{cat} was determined from 15 to 35 °C using saturating substrate concentrations ($>10 \times K_m$) in a thermostated cuvette of 0.2 cm path length. It proved impractical to extend these measurements above 35 °C because K_m increased to such an extent that very high concentrations of substrate were required to approach saturation, introducing stray light error that prevented continuous monitoring of activity. The temperature dependence of k_{cat}/K_m was measured from 20 to 50 °C under subsaturating substrate concentrations ($<0.1 \times K_m$) in a thermostated cuvette of 10 cm path length.

Temperature Dependence of Inhibition Constants. 3,4,5,6-Tetrahydrozebularine (THZ) and 3,4,5,6-tetrahydrouridine (THU) were synthesized and characterized by UV and ^1H NMR by the method of Hanze (*16*). The temperature dependences of the K_i values of THZ and 5,6-dihydrouridine (both competitive inhibitors) were determined over a temperature range from 20 to 45 °C, using a continuous spectrophotometric assay to measure CDA activity with cytidine at concentrations $<0.1 \times K_m$, to permit analysis of the effects of temperature on K_i without the complicating effects from the temperature dependence of K_m. 5,6-Dihydrouridine was synthesized enzymatically by deamination of 5,6-dihydrocytidine with cytidine deaminase.

Isothermal Titration Calorimetry. ITC experiments were performed using a Microcal MSC calorimeter (Northampton, MA). Solutions of CDA were dialyzed in 0.1 M potassium phosphate buffers (pH 7.3), and the dialysate was used to dissolve the inhibitor, THU. Enzyme solutions (30 μM) were titrated with a stirring speed of 400 rpm with ~20, 5−10 μL injections of THU (1.2 mM). The heat evolved after each inhibitor injection was obtained from the integrated intensity of the calorimetric signal. The heat of binding of the inhibitor was obtained from the difference between the heat of interaction and the corresponding heat of dilution. Integration of the calorimetric signal and nonlinear least-squares analysis of the data using a single binding site model were accomplished using Origin (version 5.0, MicroCal, Inc.) (*17*, *18*). The values reported represent the average of three titrations at 25 °C.

RESULTS

Thermodynamics of Uncatalyzed Deamination of 5,6-Dihydrocytidine. In agreement with earlier observations at room temperature (*14*, *19*), 5,6-dihydrocytidine was found to undergo conversion to 5,6-dihydrouridine by a simple first-order process at temperatures between 25 and 76 °C. The results yielded a satisfactory Arrhenius plot (Figure 2A), indicating that $\Delta H^{\ddagger} = 13.4\ (\pm 0.4)$ kcal/mol. From the relationship that $-RT \ln(k_{obs}h/k_bT) = \Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, the entropy of activation was estimated to be −10.1 (±0.8) kcal/mol at 25 °C, consistent with bimolecular attack by water. The rate constant at 25 °C for spontaneous deamination of this cytidine analogue, $3.2 \times 10^{-5}\ s^{-1}$, is much higher than that of cytidine itself, $3.0 \times 10^{-10}\ s^{-1}$ (extrapolated from values obtained at elevated temperatures) (*13*).

Thermodynamics of Enzymatic Deamination of 5,6-Dihydrocytidine. The temperature dependence of k_{cat} was found to yield a satisfactory Arrhenius plot (Figure 2B) with a slope corresponding to $\Delta H^{\ddagger} = 13.3\ (\pm 0.7)$ kcal/mol, and an

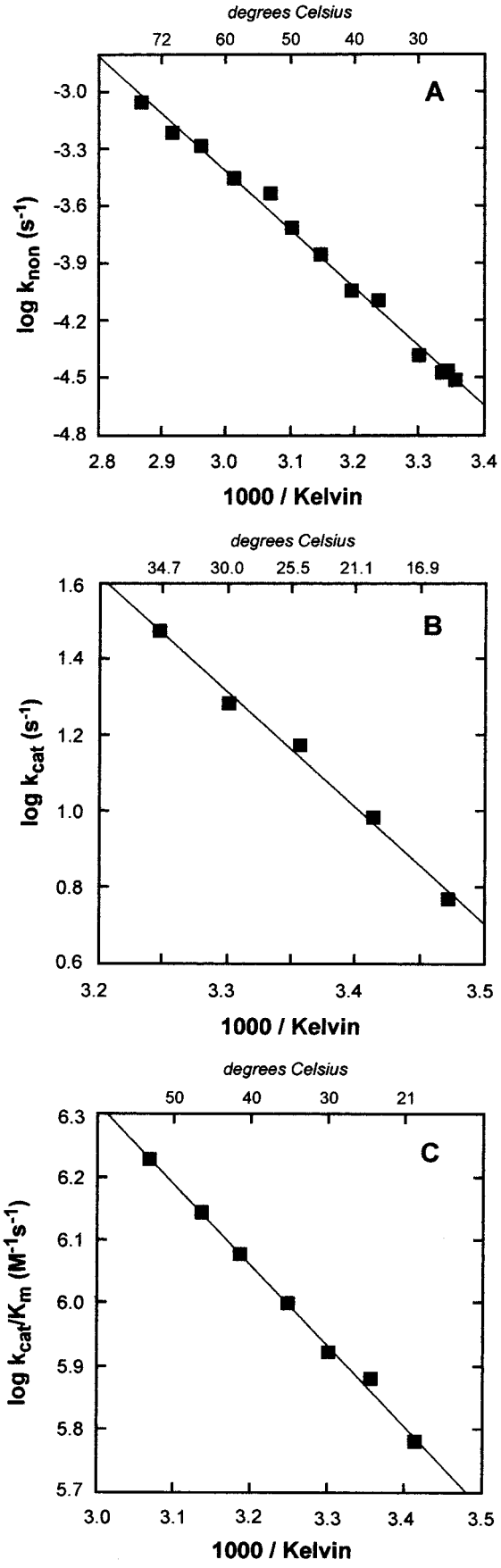

FIGURE 2: Arrhenius plots for (A) uncatalyzed (k_{non}, s^{-1}) deamination of 5,6-dihydrocytidine; (B) k_{cat} (s^{-1}) and (C) k_{cat}/K_m ($M^{-1}\ s^{-1}$) for enzymatic deamination of 5,6-dihydrocytidine in potassium phosphate buffer (0.10 M, pH 7.3).

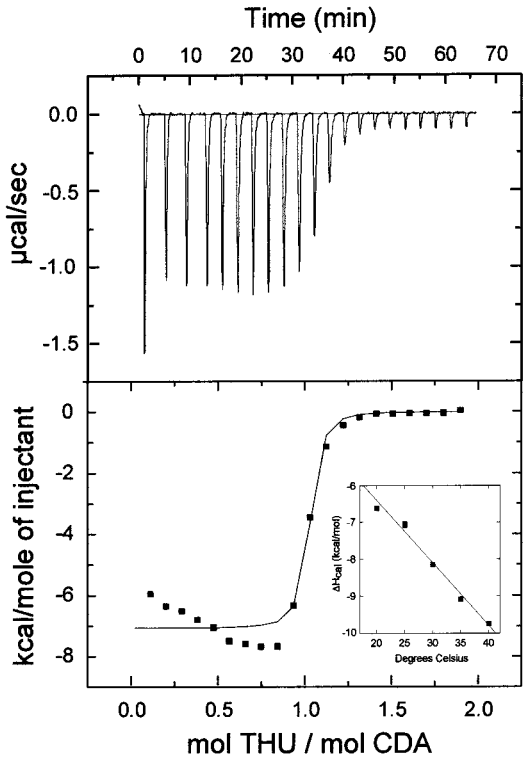

FIGURE 3: Isothermal calorimetric titration of cytidine deaminase with 3,4,5,6-tetrahydrouridine (THU) at 25 °C in phosphate buffer (0.10 M, pH 7.3). The upper panel shows the heat effects associated with each injection after baseline correction. The lower panel shows the integrated heats (■) and the best-fitted curve to a 1:1 binding model. Inset: Calorimetric enthalpy change for THU binding by CDA as a function of temperature.

activation entropy of −2.5 (±1) kcal/mol at 25 °C. The temperature dependence of the catalytic efficiency (k_{cat}/K_m) of cytidine deaminase for deamination of DHC also yielded a linear Arrhenius plot from 20 to 53 °C (Figure 2C), indicating that $\Delta H^{\ddagger} = 5.2$ (±0.2) kcal/mol and $T\Delta S^{\ddagger} = -4.2$ (±0.4) kcal/mol at 25 °C.

Thermodynamics of Inhibitor Binding. 4,5,6-Tetrahydrouridine (THU) is bound with sufficiently high affinity by CDA ($K_i = 1.3 \times 10^{-7}$ M) to allow the thermodynamics of its binding to be measured directly by isothermal titration calorimetry. The binding of THU was found to be exothermic [$\Delta H_{cal} = -7.1$ (±0.03) kcal/mol] and was associated with a favorable change of entropy [$T\Delta S = +2.2$ (±0.3) kcal/mol at 25 °C] (Figure 3).[3] The value of ΔH_{cal} for THU binding was found to increase as a linear function of increasing temperature (measured every 5 °C over a temperature range of 20−40 °C), with a slope that corresponded to a heat capacity change of −170 (±10) cal mol^{-1} deg^{-1} (Figure 3, inset). Because of the relatively weak binding affinity of 3,4,5,6-tetrahydrozebularine (K_i of THZ $= 1 \times 10^{-5}$ M), the thermodynamic changes associated with its binding were estimated from the temperature dependence of its inhibition constant using a van't Hoff plot. Values of K_i of THZ were found to increase with increasing temperature, from 20 to 45 °C (Figure 4), indicating that $\Delta H_{assoc} = -9.2$ (±0.5) kcal/mol and $T\Delta S_{assoc} = -2.2$ (±1) kcal/mol at 25 °C. The binding affinity of 5,6-dihydrouridine, the product of 5,6-dihydrocytidine deamination, was also found to decrease with increasing temperature, indicating that $\Delta H_{assoc} = -9.2$ (±1.7) kcal/mol and $T\Delta S_{assoc} = -1.8$ (±1) kcal/mol at 25 °C.

[3] The ITC data for titration with THU can be fit by a two-site binding model, with endothermic binding at the higher affinity site and exothermic binding at the lower affinity site. However, the estimated K_d values of these sites differ by a factor of less than 1.5 so that any distinction between them does not appear to be statistically significant.

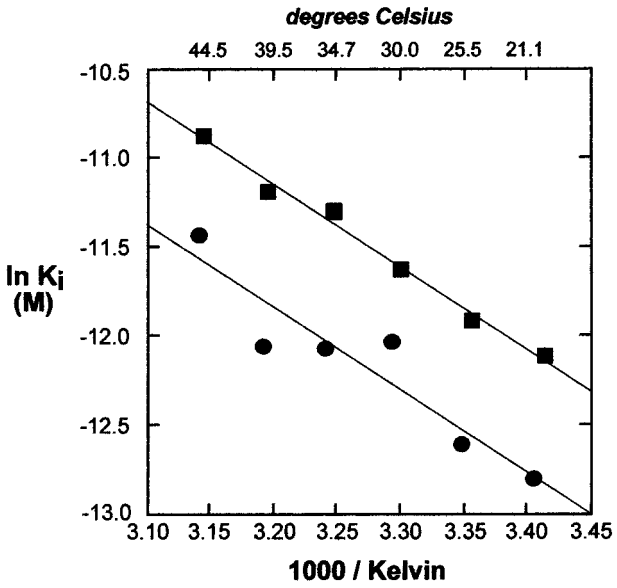

FIGURE 4: van't Hoff plots of the dissociation constants of the cytidine deaminase−3,4,5,6-tetrahydrozebularine (■) and −5,6-dihydrouridine (●) complexes in potassium phosphate buffer (0.10 M, pH 7.3).

DISCUSSION

Figure 5 shows the enthalpy and entropy changes that accompany progress along the reaction coordinate for the deamination of 5,6-dihydrocytidine (DHC). Earlier, we showed that CDA enhances the rate of deamination of cytidine by lowering the enthalpy of activation ($\Delta\Delta H^{\ddagger} = -7.2$ kcal/mol) and by raising the entropy of activation ($T\Delta\Delta S^{\ddagger} = +9.2$ kcal/mol) (*13*). The present results show that catalysis of deamination of DHC arises *entirely* from an increase in the entropy of activation ($T\Delta\Delta S^{\ddagger} = +7.6$ kcal/mol at 25 °C).

In attempting to understand this behavior, let us first consider the uncatalyzed reaction. During the hydrolytic deamination of cytidine, addition of water across the N3═C4 double bond disrupts the aromatic stability of the pyrimidine ring. When the conjugated C5═C6 double bond of cytidine is reduced (in DHC), the enthalpy of its covalent hydration would be expected to become less positive, as is observed, increasing its rate of spontaneous deamination.

The enthalpy changes that accompany the binding of DHC by cytidine deaminase in the ground state and in the transition state for deamination are found to be equivalent. Accordingly, this enzyme's greater affinity for the altered substrate in the transition state arises entirely from a more favorable entropy of binding (Figure 5). Earlier, the spontaneous deamination of cytidine was found to immobilize at least two water molecules (one presumably substrate water) in the transition state for hydrolytic deamination, as indicated by experiments in mixed solvents (*7*). It seems reasonable to conjecture that the enzyme reaction may incur an entropic advantage from the positioning of Glu-104 in such a way as to obviate the involvement of a second water molecule (*13*).

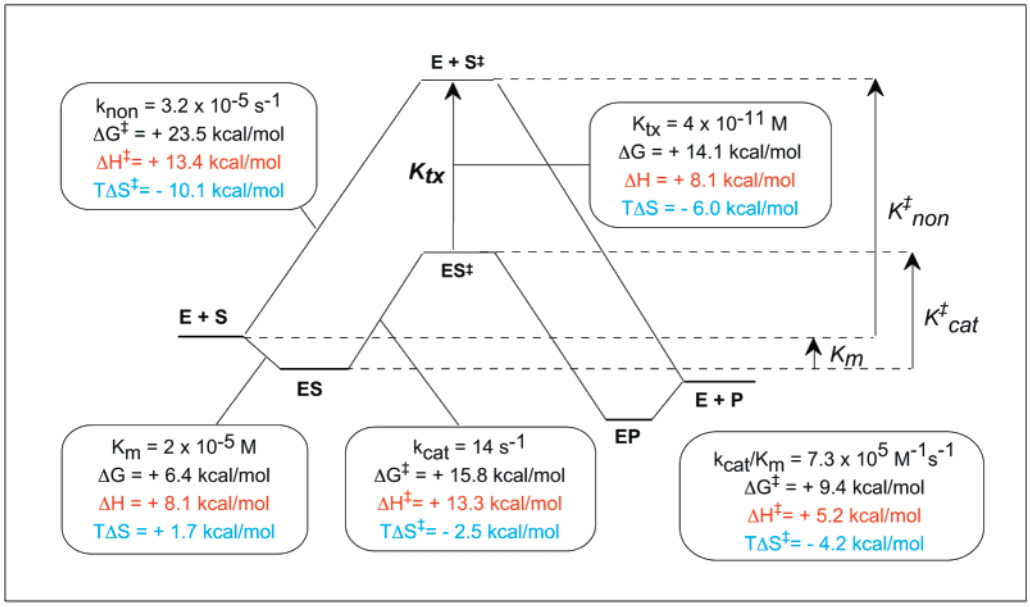

FIGURE 5: Enthalpy and entropy changes that accompany progress along the reaction coordinate for uncatalyzed and enzymatic deamination of 5,6-dihydrocytidine in potassium phosphate buffer (0.10 M, pH 7.3) at 25 °C.

This interesting behavior, also observed for substrate cytidine (*6*), may be understandable by considering the role of substrate water in closer detail. Before cytidine is bound in the ES complex, the elements of substrate water are divided between Zn and Glu-104, and are thus localized (*7*). In contrast, the covalently hydrated substrate in the transition state already incorporates a water molecule. Thus, the virtual process by which that *already*-hydrated species is bound from free solution (in the equilibrium described by the dissociation constant K_{tx}) involves displacement of substrate water from the active site into bulk solvent. That difference in behavior would be expected to lead to a more favorable entropy of binding of the substrate in the transition state than in the ground state.

The more positive entropy of binding of the unnatural substrate DHC, compared with that of cytidine, is of special interest. In the transition state, the active site appears to enclose the natural substrate in such a way as to sequester cytidine completely from bulk solvent, as inferred from the structure of the enzyme's complex with the transition-state analogue 3,4-dihydrouridine (*11*). In that complex, the phenyl group of Phe165 is situated perpendicular to the pyrimidine ring, forming interactions with the 5═6 double bond that seem likely to constrain the motions of the pyrimidine ring. Reduction of that double bond, in the alternate substrate 5,6-dihydrocytidine, would be expected to relax those constraints, accounting for the more favorable entropy of binding for DHC than cytidine in the transition state. Binding of DHC in the transition state is accompanied by a less favorable change of enthalpy, as would be expected if the alteration of the substrate interfered with these interactions.

In evaluating potential inhibitors as transition-state analogues, it is of interest to compare their equilibrium binding properties with those of the activated substrate in the transition state, as inferred from kinetic measurements. The affinity of CDA for THU ($K_i = 1.3 \times 10^{-7}$ M) is much weaker than its affinity for 3,4-dihydrouridine (DHU) ($K_i = 1.2 \times 10^{-12}$ M). However, when these dissociation constants are compared with those estimated for the actual transition

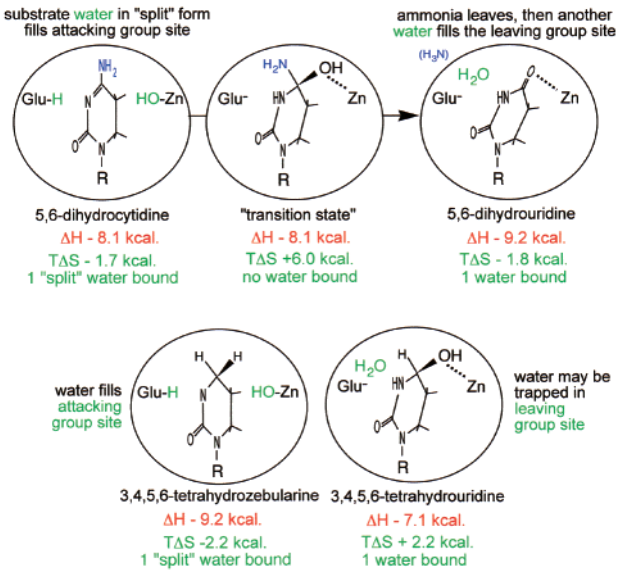

FIGURE 6: Comparison of the thermodynamic changes (at 25 °C) that accompanying the binding of 5,6-dihydrocytidine (DHC) in the ground state and transition state, with those of the binding of the competitive inhibitors 3,4,5,6-tetrahydrouridine (THU) and 3,4,5,6-tetrahydrozebularine (THZ).

states in deamination of the corresponding substrates (compare K_{tx} for cytidine, $\sim 1 \times 10^{-16}$ M, with K_{tx} for DHC, $\sim 4 \times 10^{-11}$ M), THU can be seen to be comparable with DHU in its ability to capture a significant fraction of the free energy of binding expected for a true transition-state analogue (Figure 1). The present results show that the binding of THU is associated with a favorable enthalpy change ($\Delta H = -7.2$ kcal/mol) (Figure 6) that approaches the value associated with the binding of the actual transition state ($\Delta H = -8.1$ kcal/mol) for deamination of 5,6-dihydrocytidine. Earlier, we reported that the enthalpy of binding of DHU (*13*) ($\Delta H = -21$ kcal/mol) was comparable with that of the altered substrate in the transition state for enzymatic deamination of cytidine ($\Delta H = -20$ kcal/mol). These findings indicate that the shortcomings of DHU and THU, in capturing a larger fraction of the total free energy of binding a true transition-

state analogue, arise from an entropy of binding that is less favorable than expected for an ideal transition-state analogue for the corresponding reaction. As in the case of DHU (*13*), it seems reasonable to speculate that this entropic deficit arises from the inability of either inhibitor to displace a water molecule from the ammonia binding site, due to the smaller bulk of the hydrogen atom that replaces the ammonia leaving group.[4]

Replacement of the 4-OH group of THU by a hydrogen atom, in 3,4,5,6-tetrahydrozebularine (THZ), results in ~100-fold decrease in affinity, arising mainly from a change in the entropy of binding (Figure 6). Earlier crystallographic observations showed that substrate water remains bound to Zn when the substrate analogue DHZ is bound (*11*). It seems reasonable to suppose that a water molecule is "trapped" in the same way in the THZ complex, although that remains to be demonstrated by exact structural methods. Based on our earlier observations (*13*), we speculate that the 4-OH group of the transition-state analogue THU (like the 4-OH group of the altered substrate in the actual transition state) displaces water from Zn into bulk solvent, rendering the entropy change for binding more favorable.

The entropy increase observed here to favor the equilibrium binding of the substrate in its altered form in the transition state compared with its form in the ground state ($T\Delta\Delta S \sim +8$ kcal/mol at 25 °C) is larger than that which has been observed for release of a single water molecule from ice or crystalline salts to bulk solvent ($T\Delta S \sim +3$ kcal/mol at 25 °C) (*20*). Earlier, we showed (*13*) that the equilibrium for covalent hydration across the N3═C4 bond of the pyrimidine ring is accompanied by a favorable change of entropy ($T\Delta S \sim +4$ kcal/mol at 25 °C). If water addition to 5,6-dihydrocytidine is accompanied by a similar change of entropy, that could account for most of the entropy change observed.

In summary, enzyme catalysis of the deamination of 5,6-dihydrocytidine appears to arise from entropic effects alone. Moreover, the equilibrium binding properties of the competitive inhibitors THU and THZ accord with that view. As in deamination of the normal substrate cytidine, site-bound water molecules probably play a major role in determining the binding affinities on which catalysis depends.

[4] The question we seek to address is why replacement of an −OH group by a smaller hydrogen atom might render the entropy of inhibitor binding less favorable. We speculate that trapping of water is responsible, as in the similar behavior reported in our earlier observations on 3,4-dihydrouridine (*7*). A reviewer has suggested, as an alternative explanation, that there might be greater constraints on the motions of the H-containing nucleoside within the active site, or on groups of the active site itself. In the absence of the bound water molecule whose presence we are postulating for the H-containing nucleoside, it does not seem obvious how greater "freezing" might occur in the case of the H-containing nucleoside than in the case of the OH-containing nucleoside. If the presence of water is assumed, then, from an operational standpoint, these explanations appear to be identical.

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