Temperature Effects on the Catalytic Efficiency, Rate Enhancement, and Transition State Affinity of Cytidine Deaminase, and the Thermodynamic Consequences for Catalysis of Removing a Substrate "Anchor"†

Mark J. Snider,‡ Stefan Gaunitz,‡ Caroline Ridgway,‡ Steven A. Short,§ and Richard Wolfenden*,‡

Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599-7260, and Molecular Sciences, Venture 131, Glaxo-Wellcome, 5 Moore Drive, Research Triangle Park, North Carolina 27709

Received April 21, 2000

ABSTRACT: To obtain a clearer understanding of the forces involved in transition state stabilization by *Escherichia coli* cytidine deaminase, we investigated the thermodynamic changes that accompany substrate binding in the ground state and transition state for substrate hydrolysis. Viscosity studies indicate that the action of cytidine deaminase is not diffusion-limited. Thus, K_m appears to be a true dissociation constant, and k_{cat} describes the chemical reaction of the ES complex, not product release. Enzyme−substrate association is accompanied by a loss of entropy and a somewhat greater release of enthalpy. As the ES complex proceeds to the transition state ($ES^{‡}$), there is little further change in entropy, but heat is taken up that almost matches the heat that was released with ES formation. As a result, k_{cat}/K_m (describing the overall conversion of the free substrate to $ES^{‡}$) is almost invariant with changing temperature. The free energy barrier for the enzyme-catalyzed reaction (k_{cat}/K_m) is much lower than that for the spontaneous reaction (k_{non}) ($\Delta\Delta G^{‡} = -21.8$ kcal/mol at 25 °C). This difference, which also describes the virtual binding affinity of the enzyme for the activated substrate in the transition state ($S^{‡}$), is almost entirely enthalpic in origin ($\Delta\Delta H = -20.2$ kcal/mol), compatible with the formation of hydrogen bonds that stabilize the $ES^{‡}$ complex. Thus, the transition state affinity of cytidine deaminase increases rapidly with decreasing temperature. When a hydrogen bond between Glu-91 and the 3′-hydroxyl moiety of cytidine is disrupted by truncation of either group, k_{cat}/K_m and transition state affinity are each reduced by a factor of 10^4. This effect of mutation is entirely enthalpic in origin ($\Delta\Delta H \sim 7.9$ kcal/mol), somewhat offset by a favorable change in the entropy of transition state binding. This increase in entropy is attributed to a loss of constraints on the relative motions of the activated substrate within the $ES^{‡}$ complex. In an Appendix, some objections to the conventional scheme for transition state binding are discussed.

Many enzyme reactions roughly double in rate ($Q_{10} \sim 2$) with a 10 °C rise in temperature. A similar tendency, often attributed to spontaneous reactions in aqueous solution, would imply that enthalpies of activation are similar for enzymatic and nonenzymatic reactions. It was recently shown that this interpretation is misleading, and that Q_{10} values tend to be much larger than 2 for slow hydrolytic and single-substrate reactions proceeding spontaneously in the absence of enzymes. Thus, enzyme rate enhancements (k_{cat}/k_{non}) tend to increase with decreasing temperature for reactions of this type (*1*). To obtain a clearer view of the entropy and enthalpy changes that accompany progress along the reaction coordinate, it would be desirable to have information about the thermodynamic changes that accompany the formation of an enzyme−substrate complex in both the ground state and transition state.

Cytidine deaminase from *Escherichia coli* offers a favorable opportunity for such analysis, since interpretation of its kinetic constants appears to be relatively straightforward. Evidence presented below indicates that the enzyme−substrate complex is in rapid equilibrium with the free enzyme and substrate, and that k_{cat} describes the chemical transformation of the substrate at the active site. Both the uncatalyzed (*2*) and enzyme-catalyzed (*3*) reactions are insensitive to changing pH near neutrality, reducing the likelihood of complications that might arise from differing temperature effects on proton dissociation constants.

Cytidine deaminase catalyzes the hydrolytic displacement of ammonia from the 4-position of the substrate in a reaction that appears to proceed through a tetrahedral intermediate (Figure 1A). In an analogue of the partial reaction in which that intermediate is generated, the 4-H-substituted cytidine analogue zebularine undergoes covalent hydration after it enters the active site, to generate the inhibitor 3,4-dihydrouridine which is very tightly bound (Figure 1B) (*4*). The crystal structure of this inhibitory complex and the kinetic consequences of modifying either the ligand or the active site indicate the presence of several hydrogen bonds that help to stabilize the transition state for deamination, including interactions with substituent ribose (*5*). A succession of crystal structures indicates that during the catalytic process

† This work was supported by NIH Grant GM18325 and by NIH Training Grant GM-08570.

* To whom correspondence should be addressed. E-mail: water@med.unc.edu. Telephone: (919) 966-1203. Fax: (919) 966-2852.

‡ University of North Carolina.

§ Glaxo-Wellcome.

10.1021/bi000914y CCC: $19.00
Published on Web 07/19/2000

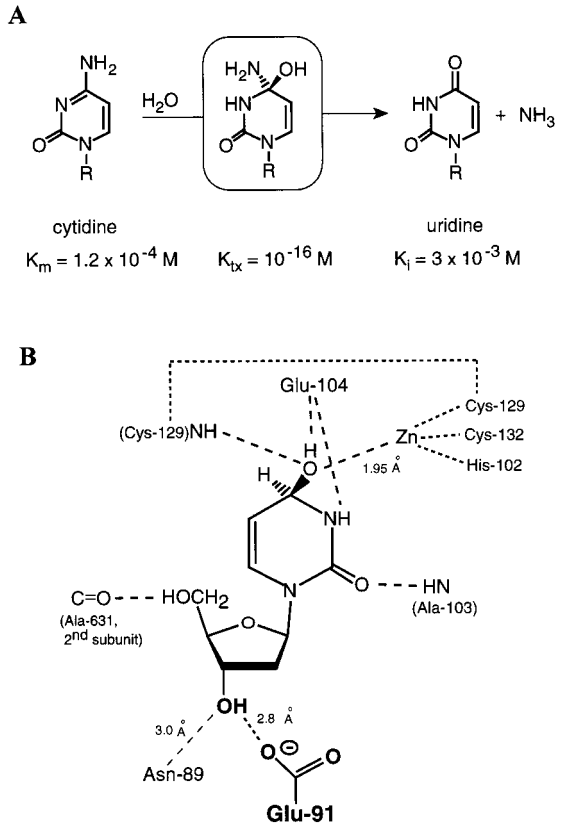


FIGURE 1: (A) Reaction catalyzed by cytidine deaminase. The proposed mechanism of catalysis involves direct zinc hydroxide attack on cytidine to generate a tetrahedral intermediate (boxed). (B) Interactions between cytidine deaminase and transition state analogue 3,4-dihydrouridine (*6*). The 2′-hydroxyl group, which makes no significant contribution to binding or catalysis (*7*), has been omitted for clarity.

the ribose moiety serves as an "anchor", remaining fixed in position within the active site while the pyrimidine ring migrates away from the leaving group ammonia and toward the attacking zinc-bound hydroxyl group (*6*).

Here, we examine the enthalpy and entropy changes associated with ground state and transition state stabilization by cytidine deaminase, as expressed in the relationship

$$K_{tx} = k_{non}/(k_{cat}/K_m) \quad (1)$$

where K_{tx} is the virtual dissociation constant of the enzyme−substrate complex in the transition state. We also examine the contributions made to transition state stabilization by enzyme interactions with the 3′-hydroxyl group of substituent ribose.

We began by testing the effect of varying viscosity, in the presence of the disaccharide trehalose, to determine the extent to which k_{cat}/K_m might be limited to productive enzyme−substrate association, and whether product release was rate-limiting for k_{cat}. Next we sought to determine whether the transition state affinity of cytidine deaminase is mainly enthalpic or entropic in origin, and the extent to which its properties may be foreshadowed by the properties of the enzyme−substrate complex in the ground state. Thus, we examined the effect of temperature on the values of k_{cat} and K_m for the enzyme reaction and on the rate constant of the uncatalyzed reaction (k_{non}). Finally, in view of the above-mentioned role of substituent ribose as an anchor relative to which the substrate's pyrimidine ring moves during the catalytic process, we examined the thermodynamic basis of the large contribution made to transition state stabilization by the interaction between Glu-91 and the 3′-hydroxyl group of cytidine. Truncation of either of these complementary groups on the enzyme or substrate had been shown to reduce the value of k_{cat}/K_m approximately 10^4-fold (*8*). To establish the enthalpic and entropic components of this contribution, we determined the effects of temperature on k_{cat}/K_m for the wild-type enzyme acting on 3′-deoxycytidine, and for the action on cytidine of an enzyme variant in which Glu-91 had been replaced with alanine.

EXPERIMENTAL PROCEDURES

Enzyme Preparation. Wild-type and E91A mutant cytidine deaminases were purified from cell extracts of *E. coli* SS6130 as described previously (*8*, *9*). This strain is unable to express the endogenous *cdd* gene and completely derepresses the expression of the plasmid-borne *cdd* gene, allowing cytidine deaminase to be expressed from only the plasmid-borne gene. Enzyme concentrations were determined from the absorbance at 280 nm using an extinction coefficient of 3.9×10^4 M^{-1} cm^{-1} (*10*).

Enzyme Assays. Nucleosides were purchased from Sigma Chemical Co. Rates of deamination were monitored using a Hewlett-Packard diode array spectrophotometer by following the change in UV absorbance of the cytosine nucleoside as it was converted to the corresponding uracil nucleoside (*11*). Enzyme assays were conducted in potassium phosphate buffer (5×10^{-2} M, pH 7.5). The temperature inside the water-jacketed cuvette was measured with a thermister probe. Reactions were initiated by addition of enzyme, and initial rates were obtained from the changes in absorbance monitored at several wavelengths depending on the concentration of substrate used, maintaining a total absorbance of less than 1.5 to minimize stray light error. The change in molar extinction coefficient was found to increase linearly with increasing temperature from 25 to 50 °C [$\Delta\epsilon_{282}$ increases from 3660 (25 °C) to 3720 (50 °C) M^{-1} cm^{-1} and $\Delta\epsilon_{298}$ increases from 120 (25 °C) to 180 (50 °C) M^{-1} cm^{-1}].

Temperature Dependence of Enzyme Reactions. The effect of temperature on the catalytic efficiency (k_{cat}/K_m) of the wild-type enzyme was determined using low cytidine concentrations ($\leq 0.1K_m$), with a thermostated cuvette with a 10 cm light path. The temperature dependence of k_{cat} was determined at a high concentration of cytidine ($\geq 10K_m$), using a thermostated cuvette with a 1 cm light path. Under the conditions of these experiments, irreversible loss of activity was observed only at temperatures of >57 °C for the wild-type enzyme, and >45 °C for the mutant enzyme E91A. Similar procedures were used for the wild-type enzyme acting on 3′-deoxycytidine, and for the mutant enzyme E91A acting on cytidine.

Uncatalyzed Deamination of Cytidine. The spontaneous rate of deamination of cytidine (1×10^{-3} M) was determined at temperatures between 80 and 157 °C in potassium phosphate buffer (0.1 M, pH 6.8), conducting reactions in triplicate in sealed quartz tubes. After various time intervals, the tubes were opened and the extent of reaction was determined spectrophotometrically. Under all conditions, deamination was found to proceed to completion following

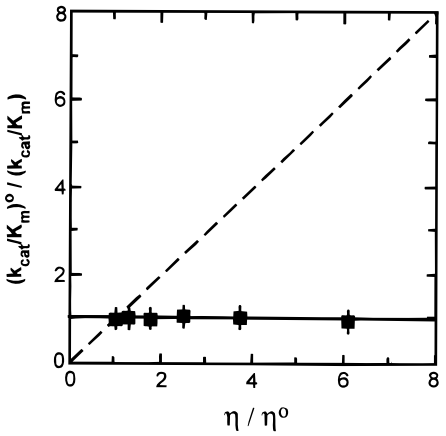


FIGURE 2: Plot of the reciprocal value of relative k_{cat}/K_m as a function of the relative viscosity in trehalose-containing buffers. The dashed line (slope of 1) shows the expected result of a diffusion-controlled reaction. Experimental points represent the average of four runs, with the standard deviation of the mean represented by the vertical bars.

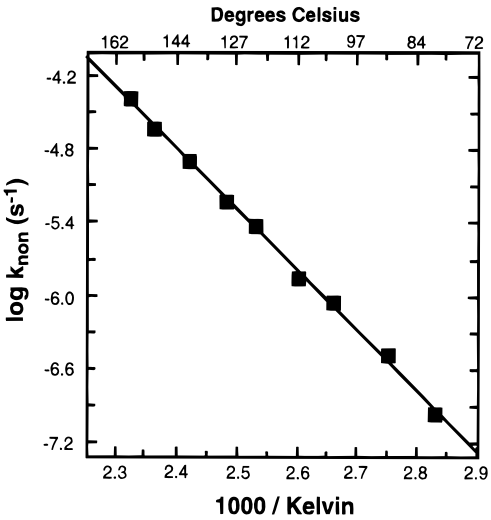


FIGURE 3: Arrhenius plot for the uncatalyzed deamination of cytidine. Each point represents the average of three determinations as described in the text.

satisfactory first-order kinetics, in agreement with earlier observations at <100 °C (*2*).

Viscosity Effects. To test the effects of viscosity on k_{cat}/K_m, solutions containing varying concentrations of D-(+)-trehalose dihydrate (Sigma) were prepared in potassium phosphate buffer (0.05 M, pH 7.5) containing 10^{-5} M cytidine. A substrate concentration of 2×10^{-3} M was used to determine the effect of viscosity on k_{cat}. Viscosities of buffered solutions were measured using a Cannon-Fenske kinematic viscometer at 25 °C, and are reported relative to buffer containing no trehalose. In both series, rates were determined in quadruplicate.

RESULTS

Effects of Viscosity Variation on the Steady State Parameters of Cytidine Deaminase. Figure 2 shows that increasing concentrations of trehalose had no effect on k_{cat}/K_m, even at relative viscosities as high as 6.1. If k_{cat}/K_m were limited by diffusion of cytidine into the enzyme's active site, the value of this apparent bimolecular rate constant would be expected to decrease linearly with increasing solution viscosity, as represented by the dashed line (Figure 2). Similar experiments performed on adenosine deaminase, for which k_{cat}/K_m is known to be diffusion-limited (*12*), yielded a slope of approximately unity. In separate experiments, increasing viscosity (trehalose) had no effect on k_{cat} for cytidine deaminase (data not shown), indicating that k_{cat} is not limited by product release.

Spontaneous Deamination of Cytidine. In agreement with earlier observations at two temperatures of <100 °C (*2*), cytidine was found to undergo conversion to uridine by a simple first-order process at temperatures between 80 and 157 °C. Under the conditions of these experiments, glycoside cleavage and opening of the pyrimidine ring occur at slower rates than deamination (C. Ridgway and R. Wolfenden, manuscript in preparation). The results presented here yielded a satisfactory Arrhenius plot (Figure 3), indicating that $\Delta H^{\ddagger} = 22.1 \pm 0.6$ kcal/mol. The entropy term ($T\Delta S^{\ddagger} = -8.3$ kcal/mol at 25 °C) is consistent with bimolecular attack by water. The rate constant of the uncatalyzed reaction, extrapolated to 25 °C, is 3×10^{-10} s^{-1}.

Temperature Dependence of k_{cat} and k_{cat}/K_m. Table 1 summarizes the thermodynamic activation parameters calculated from the observed effects of temperature on the enzymatic deamination of cytidine. Values of k_{cat} for wild-type cytidine deaminase, acting on cytidine, were found to yield a linear Arrhenius plot from 7 to 35 °C (Figure 4), with a slope corresponding to a $\Delta H^{\ddagger}$ of 14.9 ± 0.1 kcal/mol. It proved to be 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 value of k_{cat}/K_m for wild-type cytidine deaminase, acting on cytidine, yielded a linear Arrhenius plot from 20 to 51 °C (Figure 5), with a very small slope corresponding to a $\Delta H^{\ddagger}$ of 1.9 ± 0.1 kcal/mol. A similar analysis of the mutant enzyme E91A acting on cytidine showed a much greater sensitivity of k_{cat}/K_m to temperature than that of the wild-type enzyme acting on cytidine (Figure 5), with a $\Delta H^{\ddagger}$ of 9.9 ± 0.4 kcal/mol. Similarly, the wild-type enzyme acting on the slow substrate 3′-deoxycytidine showed that $\Delta H^{\ddagger} = 9.6 \pm 0.2$ kcal/mol.

DISCUSSION

Physical Significance of k_{cat}/K_m and k_{cat} for Cytidine Deaminase. Our primary goal in this work was to establish the entropy and enthalpy changes associated with transition state binding by cytidine deaminase, as expressed by the dissociation constant K_{tx} in eq 1. In the simplest analysis, k_{cat} is assumed to represent the rate constant for chemical transformation of the substrate at the enzyme's active site, and K_m is assumed to describe the dissociation constant of the enzyme−substrate complex. Transition state affinity would be underestimated if physical binding of the substrate were to limit the value of k_{cat}/K_m, or if product release were to limit the value of k_{cat} (*13*). The value of k_{cat}/K_m for cytidine deaminase (3×10^6 M^{-1} s^{-1}) is lower than values that have generally been observed for diffusion-controlled processes. However, steric obstacles, or a requirement for a rare form of the enzyme or the substrate for productive encounter, might cause k_{cat}/K_m to be lower than the theoretical limit for a diffusion process, yet still represent the activation barrier

Table 1: Thermodynamic Activation Parameters for the Uncatalyzed and Enzyme-Catalyzed Deamination of Cytidine and the Thermodynamic Parameters of Transition State Affinity ($1/K_{tx}$)[a]

uncatalyzed cytidine deamination		$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$T\Delta S^{\ddagger}$ (kcal/mol)
k_{non}	2.7×10^{-10} s^{-1}	30.4	22.1	−8.3
WT CDA and cytidine		ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
k_{cat}/K_m	2.8×10^6 M^{-1} s^{-1}	8.6	1.9	−6.7
$1/K_{tx}$	10^{16} M^{-1}	−21.8	−20.2	1.6
mutant E91A and cytidine		ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
k_{cat}/K_m	150 M^{-1} s^{-1}	14.4	9.9	−4.5
$1/K_{tx}$	5.6×10^{11} M^{-1}	−16.0	−12.2	3.8
relative to wild-type CDA and cytidine		$\Delta\Delta G = 5.8$	$\Delta\Delta H = 8.0$	$\Delta(T\Delta S) = 2.2$
WT CDA and 3′-deoxycytidine		ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
k_{cat}/K_m	660 M^{-1} s^{-1}	13.6	9.6	−4.0
$1/K_{tx}$	2.4×10^{12} M^{-1}	−16.8	−12.5	4.3
relative to wild-type CDA and cytidine		$\Delta\Delta G = 5.0$	$\Delta\Delta H = 7.7$	$\Delta(T\Delta S) = 2.7$

[a] Values are reported at 25 °C. Both the uncatalyzed and enzyme-catalyzed reactions are assumed to be similar in their dependence on water activity, which is taken as unity according to the usual convention.

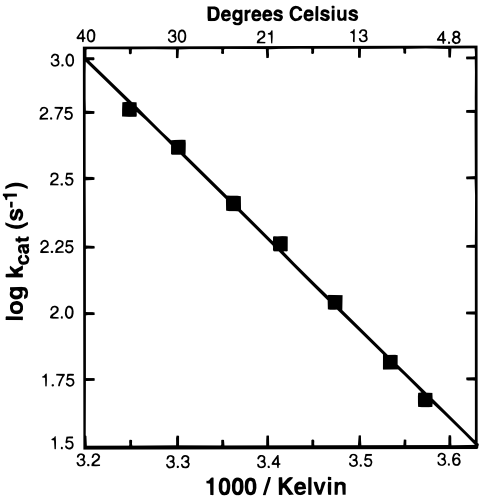


FIGURE 4: Arrhenius plot of k_{cat} (s^{-1}) for wild-type cytidine deaminase acting on cytidine. Each point represents the average of at least four runs; the deviation from the mean was found to be less than the size of the point representing the mean on this plot.

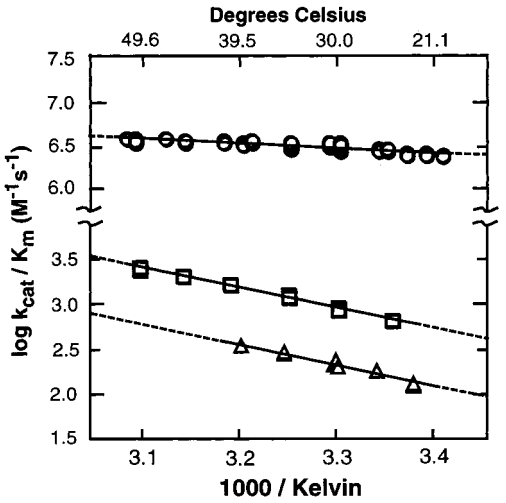


FIGURE 5: Arrhenius plots of k_{cat}/K_m for wild-type cytidine deaminase acting on cytidine (○, 56 data points), wild-type cytidine deaminase acting on 3′-deoxycytidine (□, 18 data points), and mutant cytidine deaminase E91A acting on cytidine (△, 18 data points).

for substrate binding. Indeed, several enzymes with lower values of k_{cat}/K_m have been shown to be at least partially limited by the rate of productive enzyme−substrate association (*14*, *15*). We therefore tested the sensitivity of cytidine deaminase's apparent bimolecular rate constant to viscosity variation. Earlier, Kurz et al. had shown that k_{cat}/K_m for adenosine deaminase, which resembles cytidine deaminase in some respects, is limited by the rate of encounter between enzyme and substrate in solution (*12*). In their experiments, k_{cat}/K_m was found to vary in inverse proportion to the solution's relative viscosity in the presence of sucrose. Using trehalose, a disaccharide devoid of reducing groups, we confirmed the viscosity effects reported earlier for calf intestinal adenosine deaminase. In contrast to adenosine deaminase, we found that for cytidine deaminase acting on cytidine, k_{cat}/K_m is unaffected by elevating the relative solution viscosity to a value as high as 6.1 (Figure 2). This implies that the apparent bimolecular rate constant, k_{cat}/K_m, does not describe the formation of the enzyme−substrate complex as in the case of adenosine deaminase, but that substrate cytidine is in rapid equilibrium between free and enzyme-bound forms.[1] The fact that k_{cat} is unaffected by increasing viscosity suggests that this rate constant is not limited by product release. Thus, neither k_{cat} nor k_{cat}/K_m is limited by diffusive processes, consistent with the view that K_{tx} represents the virtual dissociation constant of the ES complex in the transition state (eq 1).

Changes in Entropy and Enthalpy That Accompany Substrate Binding and Catalysis. Figure 6 shows that substrate binding ($1/K_m$) is accompanied by a loss of entropy ($T\Delta S = -7.6$ kcal/mol), such as might be expected to accompany the organization of two molecules in forming a single complex. Once the enzyme−substrate complex has formed, little further change in entropy occurs (0.9 kcal/mol) as the reaction progresses from ES to ES‡. Substrate binding is associated with a substantial release of enthalpy (−13 kcal/

[1] The possibility cannot be ruled out that the complete viscosity independence of the wild-type enzyme acting on cytidine might represent some form of compensation, such as activation combined with retardation by the viscogen (*16*). In fact, we observe a slight activation by trehalose (∼1.5-fold at a relative viscosity of 6.1) for wild-type cytidine deaminase acting on the slow substrate 3′-deoxycytidine. This observation does not alter the conclusion that enzyme−substrate association is of little importance in determining k_{cat}/K_m.

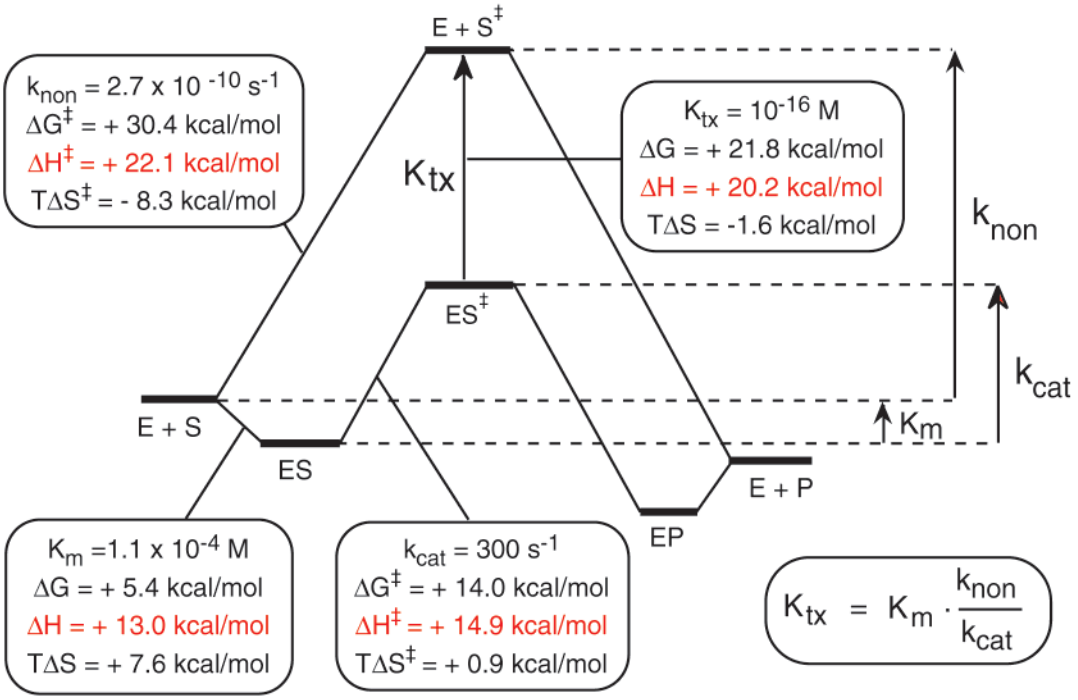


FIGURE 6: Thermodynamic changes that occur for the uncatalyzed and enzyme-catalyzed deamination of cytidine.

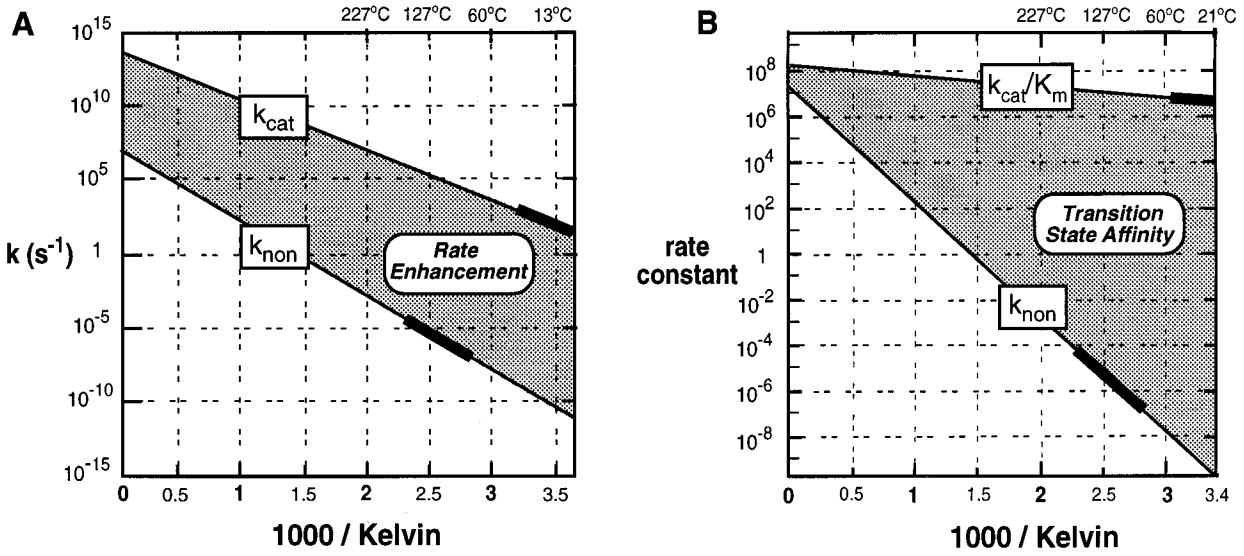


FIGURE 7: (A) Effect of temperature on the rate enhancement (k_{cat}/k_{non}) produced by cytidine deaminase. (B) Effect of temperature on transition state affinity [$(k_{cat}/K_m)/k_{non}$] (M^{-1}) for cytidine deaminase. Bold lines show the actual ranges of temperature over which the enzyme-catalyzed and uncatalyzed reaction rate constants were determined.

mol), which seems to be compatible with the formation of hydrogen bonds that hold the substrate within the active site. Interestingly, this release of enthalpy almost matches the enthalpy of activation associated with the next step of the reaction (14.9 kcal/mol). The overall result of this near equivalence is that k_{cat}/K_m is almost temperature-independent ($\Delta H^{\ddagger} = 1.9$ kcal/mol). This very small heat of activation, arising from the two-step nature of k_{cat}/K_m (*17*), is lower than that expected for a diffusion-limited process which is typically in the neighborhood of 4 kcal/mol (*18*). A similar insensitivity of k_{cat}/K_m to changing temperature has also been reported for several enzymes, including cytochrome *c* peroxidase (*19*), carbonic anhydrase II (*20*), lactate dehydrogenase (*21*), horseradish peroxidase (*22*), and ribonuclease A (*23*). Thus, at the subsaturating substrate concentrations at which many enzymes operate in vivo (*24*), the action of enzymes of this type is almost temperature-independent.

Temperature Dependence of Transition State Binding by Cytidine Deaminase. Because of the insensitivity of k_{cat}/K_m to temperature (in contrast to the temperature sensitivity of the uncatalyzed reaction), the transition state affinity can be seen (Figure 7B) to increase steeply with decreasing temperature. This effect is much more pronounced than the increase in rate enhancement with decreasing temperature (Figure 7A), a simple result of the effect of temperature on K_m. If the enthalpy of activation associated with the spontaneous deamination of cytidine (22.1 kcal/mol) is subtracted from that of k_{cat}/K_m ($\Delta H^{\ddagger} = 1.9$ kcal/mol), the enthalpy change associated with the binding of the activated substrate in the transition state ($1/K_{tx}$) can be estimated to

be −20.1 kcal/mol (Table 1). The entropy change associated with transition state binding is much smaller, and slightly positive ($T\Delta S$ = 1.6 kcal/mol at 25 °C).[2] Thus, the total forces involved in transition state binding are mostly enthalpic in origin, consistent with the numerous polar interactions, mostly hydrogen bonds, that have been shown to be present in the enzyme complex with the transition state analogue inhibitor zebularine hydrate (Figure 1B) (*5*).

Catalytic Role of an Enzyme−Ribose Anchor. Earlier, a hydrogen bonding interaction between the 3′-hydroxyl group of cytidine and the carboxylate of Glu-91 at the active site was shown to contribute ∼5.5 kcal/mol to the transition state affinity of cytidine deaminase. This analysis was carried out by mutating Glu-91 to alanine, or by truncating the substrate cytidine to 3′-deoxycytidine. The rough agreement between the results produced by these differing truncations suggests that the contribution made to transition state binding by the 3′-hydroxyl group's hydrogen bond to Asn-89 (Figure 1B) is relatively minor, consistent with the fact that this hydrogen bond is longer than the 3′-hydroxyl group's hydrogen bond to Glu-91, and that it does not involve a charged partner (*8*).

In the work presented here, activation parameters were determined for the action of wild-type cytidine deaminase on 3′-deoxycytidine, and for the mutant enzyme E91A acting on cytidine, for comparison with those of the wild-type enzyme acting on its natural substrate, cytidine. Each of these activity-lowering mutations caused k_{cat}/K_m to become much more sensitive to increasing temperature (Figure 5). The slopes of the resulting Arrhenius plots of k_{cat}/K_m show that the enthalpy of activation rises from 1.9 kcal/mol for the wild-type enzyme acting on cytidine to 9.9 kcal/mol for the mutant enzyme E91A acting on cytidine, and to 9.6 kcal/mol for the wild-type enzyme acting on 3′-deoxycytidine (Table 1). This increase in activation enthalpy is consistent with the loss of a polar interaction involved in stabilizing the activated substrate in the transition state. Comparable effects on the *equilibrium* binding affinity ($\Delta\Delta H \sim 8$ kcal/mol) have been observed when amino acid residues (Tyr-43 and Asn-23) of streptavidin are mutated to alanine, disrupting hydrogen bonds to the ureido oxygen atom of biotin (*25*). In the case of cytidine deaminase, although the enthalpy of activation becomes less favorable by ∼7.9 kcal/mol, the entropy of activation becomes somewhat *more* favorable [$\Delta(T\Delta S) \sim 2.5$ kcal/mol at 25 °C] with either deletion, consistent with the possibility that the substrate's range of motion within the active site increases in such a way as to interfere with catalysis.[3] As mentioned in the introductory section, the ribofuranosyl group appears to serve as a kind of anchor during the catalytic process.[4] With deletion of the interaction between the carboxylate of Glu-91 and the substrate 3′-hydroxyl group, the anchor appears to have lost its grip.

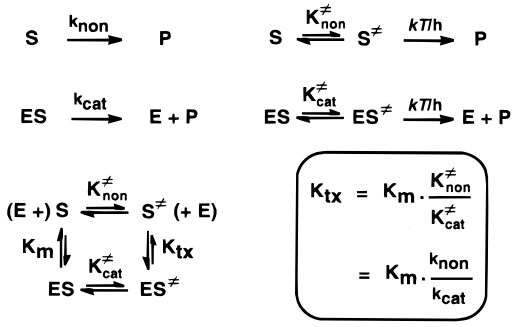
CONCLUSIONS

Slow reactions, proceeding spontaneously in the absence of enzymes, tend to be highly sensitive to temperature. In contrast, values of k_{cat}/K_m for enzyme-catalyzed reactions, typified by cytidine deaminase, tend to be relatively insensitive to changing temperature because of compensating effects on substrate binding and catalysis. As a result, transition state affinity increases steeply with decreasing temperature, and is largely enthalpy-based. That tendency is compatible with the common involvement of hydrogen bonds in transition state stabilization, as revealed by the structures of transition state analogue complexes. Deletion of a single such interaction in cytidine deaminase is found to result in a major increase in the enthalpy of transition state binding, accompanied by a small increase in entropy that is attributed to a loosening of constraints on the bound ligand.

APPENDIX ON THE VALIDITY OF THE TRANSITION STATE BINDING RELATIONSHIP

The transition state binding relationship (Scheme 1) implies that during enzyme reactions the substrate becomes bound by the enzyme much more tightly in the transition state than in the Michaelis complex. In a recent discussion of the probable importance of near-attack conformations in the ES complex (*28*), the authors take issue with this relationship, objecting that it is incompatible with the tendency of K_{tx} to vary with the rate of the spontaneous reaction (k_{non}), rather than with the rate of the enzyme reaction (k_{cat}, or k_{cat}/K_m), and that it fails to take the solvent environment into consideration. It seems appropriate to consider these objections because of their bearing on the findings presented here and their interpretation.

Scheme 1



Many enzyme reactions proceed at roughly similar rates, as indicated by k_{cat}/K_m (range of $\sim 10^4$-fold), whereas the rates of the corresponding uncatalyzed reactions (k_{non}) show much wider variation (range of $>10^{14}$-fold) (*29*). Bruice and Benkovic (see also ref *30*) state "That the narrow range of log k_{cat} values does not correlate with the much larger range

[2] It appears puzzling at first glance that K_{tx} is associated with a small negative entropy change, considering that this term describes the dissociation of one molecule to yield two. However, this dissociation also involves fixation of a water molecule at the enzyme's active site.

[3] These entropic effects, also observed in the streptavidin−biotin complex (*25*), might also arise, at least in part, from changes in solvation by water. However, the most likely scenario, in which water fills the gap vacated by the group that has been removed from the enzyme or the substrate, would not help to explain the observation of a positive change in entropy. Had water been "trapped" in that way, isolating it from bulk solvent, a negative change in entropy might have been expected (for examples, see ref *26*).

[4] For a discussion of the anchor principle, see ref *27*.

[5] Although the idea behind Scheme 1 is sometimes attributed to Pauling, M. Polanyi appears to have been the first to state explicitly that catalysis requires enhanced affinity for the altered substrate in the transition state (*36*; see also ref *37*). For an excellent account of these issues, see ref *38*.

of log K_{tx} is contrary to Pauling's proposal that the tighter the enzyme binds the transition state the greater the rate of catalysis." Such a proposal, omitting consideration of K_m or k_{non}, would not be expected to be valid when different enzymes are compared. In our view, the observation that variation in K_{tx} arises largely from variations in k_{non}, rather than from variations in k_{cat}/K_m, is compatible with Scheme 1 and would also be expected *a priori*. The fact that most enzyme reactions proceed at roughly comparable rates seems understandable when one considers that most enzymes must operate with second-order rate constants (k_{cat}/K_m) that are not far from the diffusion limit if they are to be useful at the limited concentrations at which enzymes are present within the cell. In contrast, rate constants of uncatalyzed reactions (k_{non}) are subject to no such constraints. Thus, while k_{non} might appear statistically to be the variable that determines K_{tx}, that is simply the result of the difference between the numerical ranges of the rate constants for the enzymatic and nonenzymatic reactions, and should not obscure the fact that some enzymes have vastly more difficult tasks to perform than do others, and can be considered to be more *proficient* (i.e., exhibit high transition state affinities). From a mechanistic standpoint, the relative invariance of k_{cat}/K_m can be considered information-free.

One-substrate and hydrolytic enzymes show a consistent tendency to reduce the heats of activation of the reactions that they catalyze, but show no consistent effects on entropies of activation. As a result, rate enhancements increase sharply with decreasing temperature (*1*). Bruice and Benkovic suggest that this reduction in enthalpy of activation is compatible with the importance of near-attack conformations (or propinquity effects) in the ES complex rather than in the $ES^{\ddagger}$ complex. We concur with the view that near-attack conformations may play important roles in certain reactions, but doubt that enthalpy reduction need be given that interpretation as a general rule. First, activation barriers appear to be chiefly enthalpic for very slow one-substrate and hydrolytic reactions in the absence of enzymes. Thus, there is relatively little benefit to be gained by raising the entropy of activation, and the tendency of enzymes to reduce the enthalpies of activation of these reactions seems inevitable, regardless of the detailed mechanism by which they accomplish this in any particular case. Second, the largely enthalpic origin of transition state affinity, as exemplified by the behavior of cytidine deaminase in the present experiments, is consistent with the development of numerous polar interactions (H-bonds and electrostatic forces) that help to stabilize the enzyme-bound transition state (Figure 6). Interactions of this kind have been observed in the crystal structures of transition state analogue complexes formed by cytidine deaminase (*5*, *6*), triose phosphate isomerase (*31*), OMP decarboxylase (*32*), and numerous other enzymes. Intermolecular forces of this type differ from hydrophobic interactions in that they tend to grow stronger with decreasing temperature. Working together, several such bonds are presumably responsible for the high transition state affinity observed in these reactions, as well as its remarkable sensitivity to temperature (*1*).

Bruice and Benkovic (see also ref *30*) object that the transition state binding principle fails to take into consideration the solvent environment. This objection neglects the fact that all relevant experiments are performed in aqueous solution. Scheme 1 is entirely general and valid so long as water is in equilibrium with the transition state. Thus, solvent water retards some nonenzymatic reactions in which the number of charges is reduced or charge is dispersed in the transition state, solvating the ground state more effectively than the transition state. Nonpolar transition state analogues have been successfully designed to exploit this special characteristic of enzymes that catalyze reactions of this type (see, for example, refs *33* and *34*). In the majority of enzyme reactions, such as that catalyzed by cytidine deaminase, the altered substrate in the transition state appears to be more polar than the reactants. Its superior affinity for the active site arises from interactions with polar groups in the active site that are so well-organized that they are able to compete successfully with water, in much the same manner as EDTA is able to compete with water in binding divalent cations. The interesting possibility remains that solvent *relaxation* effects (as distinct from equilibrium solvation effects, which we have just considered) could limit the rate of some nonenzymatic reactions involving major changes in electrostatic charge or charge dispersal. If an enzyme were to catalyze such a reaction by removing "solvent friction", then the enzyme's transition state binding affinity might be overestimated, on the basis of simple comparisons of rate constants. However, solvent water relaxes very rapidly, and solvent friction has been observed only in fast reactions such as photolysis (*35*). Most biological reactions, like that catalyzed by cytidine deaminase, proceed very slowly in the absence of enzymes. In our view, it would be surprising if solvent friction were responsible for the extreme sluggishness of these reactions, or reduce their rates to more than a modest extent.

In summary, we see no conflict between high enzyme−substrate affinity in the transition state, which can be regarded as a universal characteristic of catalytic systems,[5] and near-attack conformations that may lie on the path to the transition state in certain reactions. Scheme 1 follows directly from the theory of absolute reaction rates, and is so general that it makes no assumptions concerning (nor can it be invalidated by) details such as the structure of the ES complex in the ground state.[6] Scheme 1 does require that the substrate be bound weakly in the ground state to produce a large value of k_{cat}, but is silent concerning events that transpire along the subsequent path to the transition state. A detailed description of the dynamic events that accompany enzyme catalysis will continue to engage the best efforts of theoreticians and experimentalists.

[6] Indeed, the minimal affinity of the enzyme for the chemically activated substrate in the transition state can even be derived without explicit reference to formation of a ground state ES complex if the second-order rate constant (k_{cat}/K_m) is known for the enzyme reaction (*39*).

REFERENCES

1. Wolfenden, R., Snider, M., Ridgway, C., and Miller, B. (1999) *J. Am. Chem. Soc. 121*, 7419.
2. Frick, L., MacNeela, J. P., and Wolfenden, R. (1987) *Bioorg. Chem. 15*, 100.
3. Carlow, D. C., Smith, A. A., Yang, C. C., Short, S. A., and Wolfenden, R. (1995) *Biochemistry 34*, 4220.
4. Frick, L., Yang, C., Marquez, V. E., and Wolfenden, R. (1989) *Biochemistry 28*, 9423.

5. Betts, L., Xiang, S., Short, S. A., Wolfenden, R., and Carter, C. W. (1994) *J. Mol. Biol. 235*, 635.
6. Xiang, S., Short, S. A., Wolfenden, R., and Carter, C. W. (1997) *Biochemistry 36*, 4768.
7. Hasono, H., and Kuno, S. (1973) *J. Biochem. 74*, 797.
8. Carlow, D. C., Short, S. A., and Wolfenden, R. (1998) *Biochemistry 37*, 1199.
9. Smith, A. A., Carlow, D. C., Wolfenden, R., and Short, S. A. (1994) *Biochemistry 33*, 6468.
10. Yang, C., Carlow, D., Wolfenden, R., and Short, S. A. (1992) *Biochemistry 31*, 4168.
11. Cohen, R. M., and Wolfenden, R. (1971) *J. Biol. Chem. 246*, 7561.
12. Kurz, L. C., Weitkamp, E., and Frieden, C. (1987) *Biochemistry 26*, 3027.
13. Wolfenden, R. (1972) *Acc. Chem. Res. 5*, 10.
14. Feng, L., Li, Y., and Kirsch, J. F. (1998) *J. Phys. Org. Chem. 11*, 536.
15. Mattei, P., Kast, P., and Hilvert, D. (1999) *Eur. J. Biochem. 261*, 25.
16. Bazelyansky, M., Robey, E., and Kirsch, J. F. (1986) *Biochemistry 25*, 125.
17. Laidler, K. J., and Peterman, B. F. (1979) *Methods Enzymol. 202*, 234.
18. Job, D., Jones, P., and Dunford, H. B. (1993) *J. Phys. Chem. 97*, 9259.
19. Loo, S., and Erman, J. E. (1977) *Biochim. Biophys. Acta 481*, 279.
20. Ghannam, A. F., Tsen, W., and Rowlett, R. S. (1986) *J. Biol. Chem. 261*, 1164.
21. Borgmann, U., Moon, T. W., and Laidler, K. J. (1974) *Biochemistry 13*, 5152.
22. Hewson, W. D., and Dunford, H. B. (1975) *Can. J. Chem. 53*, 1928.
23. Eftink, M. R., and Biltonen, R. L. (1983) *Biochemistry 22*, 5140.
24. Cleland, W. W. (1967) *Annu. Rev. Biochem. 36*, 77.
25. Klumb, L. A., Chu, V., and Stayton, P. S. (1998) *Biochemistry 37*, 7657.
26. Holdgate, G. A., Tunnicliffe, A., Ward, W. H. J., Weston, S. A., Rosenbrock, G., Barth, P. T., Taylor, I. W. F., Pauptit, R. A., and Timms, D. (1997) *Biochemistry 36*, 9663.
27. Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 296−305.
28. Bruice, T. C., and Benkovic, S. J. (2000) *Biochemistry 39*, 6267.
29. Radzicka, A., and Wolfenden, R. (1995) *Science 267*, 90.
30. Cannon, W. R., Singleton, S. F., and Benkovic, S. J. (1996) *Nat. Struct. Biol. 3*, 821.
31. Lolis, E., and Petsko, G. A. (1990) *Biochemistry 29*, 6619.
32. Miller, B. G., Hassell, A. M., Wolfenden, R., Milburn, M. V., and Short, S. A. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 2011.
33. Gutowski, J. A., and Lienhard, G. E. (1976) *J. Biol. Chem. 251*, 2863.
34. Tang, K.-C., Pegg, A. E., and Coward, J. K. (1980) *Biochem. Biophys. Res. Commun. 96*, 1371.
35. Maroncelli, M., MacInnis, J., and Fleming, G. R. (1989) *Science 243*, 1674.
36. Polyani, M. (1921) *Z. Elektrochem. 27*, 143.
37. Schwab, G.-M. (1937) *Catalysis*, pp 241−243, MacMillan, London.
38. Schowen, R. L. (1978) in *Transition States of Biochemical Processes* (Grandour, R. D., and Schowen, R. L., Eds.) pp 77−114, Plenum Press, New York.
39. Wolfenden, R. (1974) *Mol. Cell Biochem. 3*, 207.

BI000914Y