Site-Bound Water and the Shortcomings of a Less than Perfect Transition State Analogue†

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Received June 8, 2001

ABSTRACT: Kinetic measurements have shown that substantial enthalpy changes accompany substrate binding by cytidine deaminase, increasing markedly as the reaction proceeds from the ground state ($1/K_m$, $\Delta H = -13$ kcal/mol) to the transition state ($1/K_{tx}$, $\Delta H = -20$ kcal/mol) [Snider, M. J., et al. (2000) *Biochemistry 39*, 9746−9753]. In the present work, we determined the thermodynamic changes associated with the equilibrium binding of inhibitors by cytidine deaminase by isothermal titration calorimetry and van't Hoff analysis of the temperature dependence of their inhibition constants. The results indicate that the binding of the transition state analogue 3,4-dihydrouridine $\Delta H = -21$ kcal/mol), like that of the transition state itself ($\Delta H = -20$ kcal/mol), is associated with a large favorable change in enthalpy. The significantly smaller enthalpy change that accompanies the binding of 3,4-dihydrozebularine ($\Delta H = -10$ kcal/mol), an analogue of 3,4-dihydrouridine in which a hydrogen atom replaces this inhibitor's 4-OH group, is consistent with the view that polar interactions with the substrate at the site of its chemical transformation play a critical role in reducing the enthalpy of activation for substrate hydrolysis. The entropic shortcomings of 3,4-dihydrouridine, in capturing all of the free energy involved in binding the actual transition state, may arise from its inability to displace a water molecule that occupies the binding site normally occupied by product ammonia.

The ability of a catalyst to enhance the rate of a reaction depends on its ability to discriminate between the substrate in the ground state and its activated form in the transition state, binding the latter species more tightly and diminishing the difference in free energy that limits the rate of reaction (*1*). Enzymes have been observed to enhance reaction rates by factors as large as 10^{19}-fold (*2*), and a simple algebraic analysis suggests that, during the central events in catalysis, increases in binding affinity are likely to match or surpass this observed rate enhancement (*3*). That power of binding discrimination seems especially remarkable in that few bonds are typically formed or broken as a substrate passes from the ground state to the transition state. However, the interactions involved exhibit very high levels of synergism, as implied by the great losses in binding affinity that are produced upon disrupting a single interaction (*4*) and by experiments that involve "cutting" the enzyme or the substrate in two and comparing the binding or transition state affinities of the "pieces" with the "whole" (*5*−*7*). To obtain further understanding of the forces responsible for transition state binding by *Escherichia coli* cytidine deaminase, it seemed desirable to determine the enthalpy and entropy changes that accompany the binding of inhibitors that resemble the substrate in the ground state and transition state for hydrolytic deamination.

Cytidine deaminase, a zinc metalloenzyme, catalyzes the hydrolytic displacement of ammonia from the 4-position of cytidine in a reaction that appears to proceed through a tetrahedral intermediate (Figure 1). In an analogue of the partial reaction by which that intermediate is generated, the 4-H-substituted cytidine analogue pyrimidin-2-one ribonucleoside (zebularine) undergoes covalent hydration after it enters the active site to generate the inhibitor 3,4-dihydrouridine (zebularine hydrate). The dissociation constant of the 3,4-dihydrouridine enzyme complex, estimated at 1.3×10^{-12} M, is roughly 8 orders of magnitude lower than the K_s value of cytidine, whereas the enzyme enhances the rate of reaction (k_{cat}/k_{non}) by nearly 12 orders in magnitude (*8*). Accordingly, this complex captures a considerable fraction of the total free energy of binding expected for the binding of an ideal transition state analogue inhibitor.

This enzyme reaction appears particularly suitable for thermodynamic analysis for several reasons. Extensive information is available concerning the crystal structures of enzyme complexes with transition state analogues (*9*, *10*), with a substrate analogue (*11*), and with the product uridine (*12*). Site-directed mutagenesis has been used to identify and evaluate polar interactions that are important for transition state stabilization (*13*, *14*), indicating an essential role for Glu-104 (*15*). The rates of both the enzyme-catalyzed (*15*) and the uncatalyzed (*8*) deamination of cytidine are pH-invariant in the neutral range, reducing the likelihood of complications that might otherwise arise from differing temperature effects on proton dissociation constants. Moreover, viscosity variation studies with trehalose (*16*) and ^{15}N kinetic isotope effects experiments (*17*) suggest that K_m represents the dissociation constant of the ES complex and

† This work was supported by NIH Grant GM18325.

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10.1021/bi011189+ CCC: $20.00
Published on Web 08/30/2001

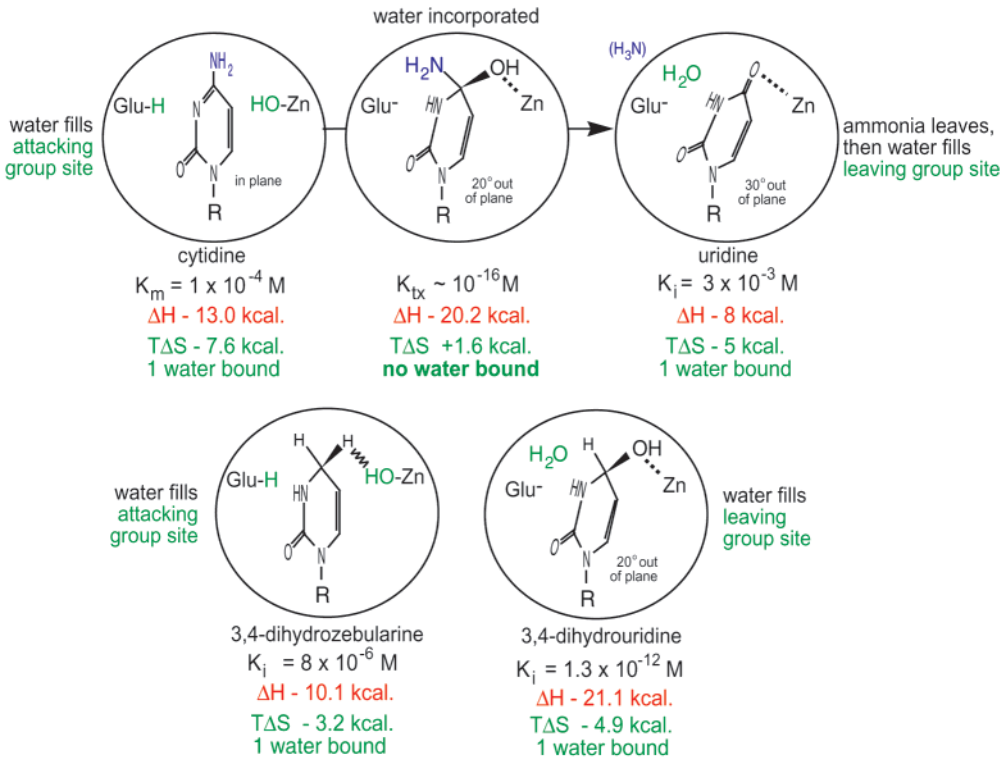

FIGURE 1: Top: Thermodynamic changes (*16*) that accompany the binding of cytidine in the ground state, cytidine in the transition state for deamination, and uridine in the enzyme−product complex, illustrating the presence or absence of site-bound water molecules as inferred from X-ray diffraction data (*11*, *12*). In this figure, equilibrium constants are shown for *dissociation*, whereas thermodynamic changes are shown for *association*. Bottom: Thermodynamic changes that accompany the equilibrium binding of inhibitors, illustrating the trapping of split substrate water and steric strain (zigzag line) in the 3,4-dihydrozebularine complex (*10*), and a water molecule trapped in the ammonia binding site of the 3,4-dihydrouridine complex (*10*). The significant H-bonds between the carboxylate group of Glu-104 and the ligands have been omitted for illustration clarity. R = ribose, held in fixed position in each complex as an anchor.

k_{cat} describes the chemical transformation of the ES complex to the EP complex.

If the enthalpy and entropy changes that accompany progress along the reaction coordinate are compared for the enzymatic and the uncatalyzed deamination of cytidine (Figure 1), the enzyme can be seen to produce favorable changes in both the enthalpy and entropy of activation (*16*). The favorable enthalpy change is compatible with the development of polar interactions in the transition state complex, involving Glu-104 and zinc, that were not present in the ground state ES complex (*10*, *11*). The favorable entropy of transition state binding was tentatively ascribed to the role of substrate water in this reaction.

Here, we analyze the thermodynamic changes associated with the equilibrium binding of the product uridine and transition state analogue inhibitors by cytidine deaminase for comparison with the results obtained by kinetic measurements of the enzyme's affinity for cytidine in the ground state and transition state.

EXPERIMENTAL PROCEDURES

Enzyme Preparation. Wild-type cytidine deaminase was purified from cell extracts of *E. coli* SS6130 as described previously (*13*). 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. Concentrations of enzyme stocks (found to be greater than 95% homogeneous as analyzed by SDS−PAGE) were determined from the absorbance at 280 nm using an extinction coefficient of 3.9 × 10^4 $M^{-1}cm^{-1}$ (*18*).

Thermodynamics of Zebularine Hydrate Formation in Solution. The constant for zebularine hydration (to form 3,4-dihydrouridine), in free solution, can be estimated by measuring the equilibrium constants for the ionization of water (K_w), for protonation of 1-methylpyrimidin-2-one (K_1), and for pseudobase formation by hydroxide ion addition to the 1,3-dimethyl-2-oxopyrimidinium cation (K'_2) using the equation shown in Figure 2 (*19*). The temperature dependence of each equilibrium constant was measured to establish the enthalpy and entropy changes associated with the hydration of zebularine in free solution. Values for the ionization constant of water were obtained over a temperature range of 15−65 °C from Mesmer and Herting (*20*). 1-Methylpyrimidin-2-one and 1,3-dimethyl-2-oxopyrimidinium sulfate were prepared by the method of Tee and Endo (*21*). The temperature dependence of K_1 was determined from the dissociation constant of the conjugate acid of 1-methylpyrimidin-2-one, measured spectrophotometrically at 312 nm in dilute HCl or 0.05 M phosphate and malonate buffers over a pH range of 1−3.9 at temperatures between 14 and 63 °C. The temperature dependence of K'_2, for 1,3-dimethyl-2-oxopyrimidinium pseudobase formation, was determined by two methods: (1) spectrophotometrically at 315 nm in 0.05 M bicine, phosphate, Tris-HCl, and MES buffers over a pH range of 5.0−8.8 and temperature range of 10−65 °C and (2) by titrating a 0.05 M solution of 1,3-dimethyl-2-oxopyrimidinium sulfate to its pK_a at 25 °C and measuring

the pH of the solution using a pH meter equipped with a thermocouple over a temperature range of 15−70 °C.

Temperature Dependence of Inhibition Constants. Zebularine and 3,4-dihydrozebularine were gifts from Dr. Victor Marquez (National Cancer Institute, NIH, Bethesda, MD). The temperature dependence of each inhibitor's dissociation constant (K_i) was determined over a temperature range from 20 to 45 °C using a continuous spectrophotometric assay to measure cytidine deaminase activity with cytidine concentrations $<0.1K_m$. Subsaturating substrate concentrations are required to monitor the temperature dependence of inhibitor binding so as to limit the effect of temperature on K_m. It can be shown that under conditions where $[S] \ll K_m$ the fractional decrease in activity due to the presence of a competitive inhibitor (I) is simply

$$\frac{v_I}{v_0} = \frac{K_I}{K_I + [I]}$$

indicating that, under these conditions, the temperature dependence of K_i can be isolated from that of K_m. Inhibition constants are reported as the average of five assays.

Isothermal Titration Calorimetry. ITC experiments were performed using a Microcal MSC calorimeter (Northampton, MA). Enzyme solutions were dialyzed in 0.1 M potassium phosphate buffers (pH 7.3), and the dialysate was used to dissolve zebularine. Enzyme and zebularine solutions were degassed before use. Enzyme solutions (30 μM) were titrated with a stirring speed of 400 rpm with ~20 5−10 μL injections of zebularine (1.22 mM). The heat evolved after each inhibitor injection was obtained from the integral of the calorimetric signal. The heat due to the binding reaction between the inhibitor and the enzyme 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 fitting of the data to a single binding site model were accomplished using Origin (v5.0, MicroCal, Inc.) (*22*, *23*). Thermodynamic data reported herein are the average of three independent titrations at 25 °C.

RESULTS

Temperature Dependence of Zebularine Hydrate Formation ($K_{hydration}$). Earlier work (*19*, *24*) established that the equilibria of addition of water to derivatives of pyrimidin-2-one were unfavorable. In the present study, the thermodynamic changes associated with the hydration of zebularine free in solution were estimated by measuring the temperature dependence for the protonation of 1-methylpyrimidin-2-one (K_1) and for 1,3-dimethyl-2-oxopyrimidinium pseudobase formation (K'_2) and the ionization constant of water, as shown in Figure 2. The value of K_2 is not directly accessible using the N-protonated species, because it is present only in vanishingly small amounts at pH values where the concentration of hydroxide ion becomes significant. Its value can, however, be estimated indirectly by measuring the affinity of the parent compound, quaternized by methylation, for hydroxide ion, i.e., pseudobase formation (K'_2). This method involves the assumption that a quaternized ring $=NCH_3^+-$ and ring $=NH^+-$ groups are approximately equivalent in their electronic effects. That assumption is supported by the similar hydroxide ion affinity of the conjugate acid of the 3-methylquinazolinium cation ($pK_a = 7.77$) and the 3-protonated conjugate acid ($pK_a = 7.64$) of quinazoline itself (*25*).

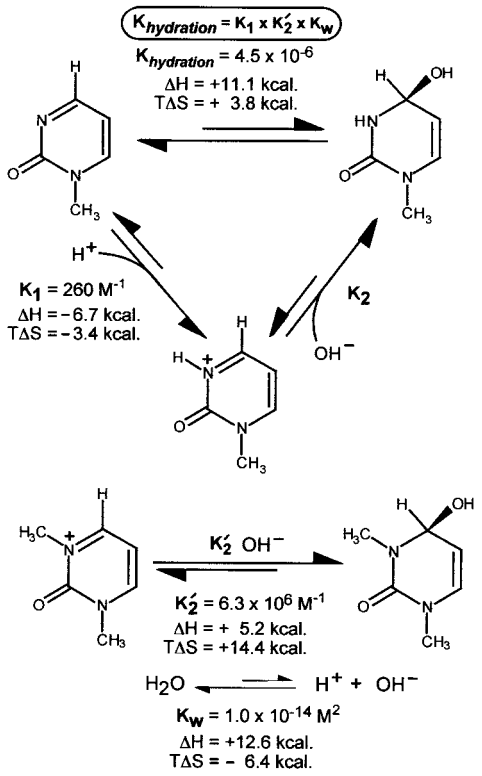

FIGURE 2: Thermodynamic changes associated with the equilibrium constant for 1-methylpyrimidin-2-one hydration, analyzed in terms of the thermodynamic changes that accompany the successive equilibrium constants for the ionization of water to form a proton and nucleophilic hydroxide anion (K_w), addition of a proton to the C=N bond (K_1), and addition of hydroxide to the protonated C=N bond (K_2). This last equilibrium constant (K_2) can only be modeled by addition of hydroxide anion to the quaternary amine formed by *N*-methylation (K'_2).

Titration of 1-methylpyrimidin-2-one in 0.05 M buffers at 25 °C resulted in a K_1 of 255 (±25) M^{-1}, in good agreement with an earlier determination of 263 M^{-1} at 25 °C and ionic strength 0.10 (*19*). The temperature dependence of K_1 yielded a linear van't Hoff plot from 14 to 63 °C (Figure 3A) which corresponded to an $\Delta H = -6.7$ (±0.4) kcal/mol. The affinity of the 1,3-dimethyl-2-oxopyrimidinium cation for hydroxide ion at 25 °C was obtained by spectrophotometric titration of its hydrogen sulfate salt under similar conditions. Its apparent formation constant (K'_2) of 7.1 (±0.2) $\times 10^6\ M^{-1}$ is in excellent agreement with earlier determinations of the chloride salt at 25 °C and ionic strength 0.10 (*19*, *21*). A van't Hoff plot over a temperature range of 10−65 °C for K'_2 (Figure 3C) yielded a ΔH value of +5.2 (±0.4) kcal/mol. Using a different method, by monitoring the temperature dependence (from 15 to 70 °C) of the pH of a 0.05 M solution of 1,3-dimethyl-2-oxopyrimidinium titrated to its pK_a at 25 °C, the enthalpy change for pseudobase formation was found to be +5.4 (±0.05) kcal/mol (Figure 3D), in good agreement with the ΔH determined by spectrophotometric titration at various temperatures. The temperature dependence of K_w, taken from 15 to 65 °C from ref *20* (Figure 3B), yields a satisfactory van't Hoff plot indicating $\Delta H = +12.6$ (±1.1) kcal/mol.

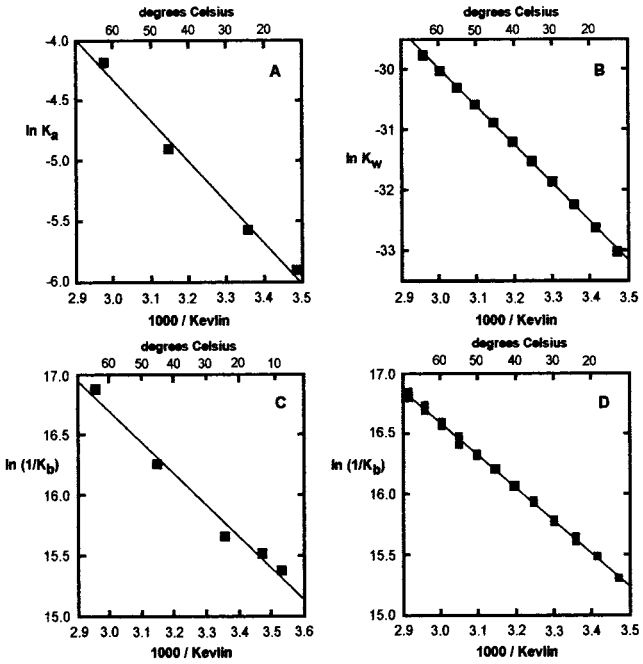

FIGURE 3: van't Hoff plots of the equilibrium constants for (A) proton dissociation from 1-methylpyrimidin-2-one ($1/K_1$), (B) the ionization of water (K_w), values taken from ref *19*, (C) addition of hydroxide ion to 1,3-dimethyl-2-oxopyrimidinium cation (K'_2) determined by spectrophotometric analysis, and (D) addition of hydroxide ion to 1,3-dimethyl-2-oxopyrimidinium cation, obtained from the temperature dependence of its pK_a.

The equilibrium constant for zebularine hydrate (3,4-dihydrouridine) formation in free solution at pH 7.0 and 25 °C, obtained from the product of K_1 (255 M^{-1}) × K'_2 (7.1 × 10^6 M^{-1}) × K_w (1 × 10^{-14} M^{-2}) is 1.8 (±0.4) × 10^{-5}, expressed in terms of unit water activity. Taking into account the statistical factor associated with the stereospecific hydration of zebularine,[1] the formation constant for active zebularine hydrate in free solution is estimated to be 4.5 (±0.4) × 10^{-6}. The summation of the enthalpy changes associated with each equilibrium constant (K_1, K'_2, and K_w) results in an approximate ΔH of +11.2 (±1.9) kcal/mol for zebularine hydrate formation in free solution with a corresponding entropy change of +3.8 (±2.3) kcal/mol at 25 °C, using the relationship $-RT \ln K_{eq} = \Delta G = \Delta H - T\Delta S$.

Isothermal Titration Calorimetric Study of Zebularine Binding by Cytidine Deaminase. Zebularine binding was found to be exothermic, yielding a ΔH_{cal} of −10.0 (±0.1) kcal/mol, after subtracting the heat for dilution of the inhibitor measured after saturation had been achieved (Figure 4). The K_d measured at 25 °C by a nonlinear fit of the titration data using a single binding site model matched the K_i value for this complex. From the relationship $\Delta G = \Delta H - T\Delta S$, an entropy change of −1.1 (±0.6) kcal/mol at 25 °C was estimated for zebularine binding. The heat capacity change, ΔC_p, can be experimentally obtained from the temperature dependence of the binding enthalpy according to the equation $\Delta C_p = (\delta\Delta H/\delta T)_p$. The temperature dependence of the ΔH_{cal} for zebularine binding was measured every 5 °C over a temperature range of 20−40 °C (Figure 4, inset). The

[1] A correction factor of 1/4 is applied for forming the one diastereomer of zebularine hydrate (3,4-dihydrouridine) formed in solution that would have high affinity for the active site (for a discussion, see ref *18*). This probability factor affects only the entropy term.

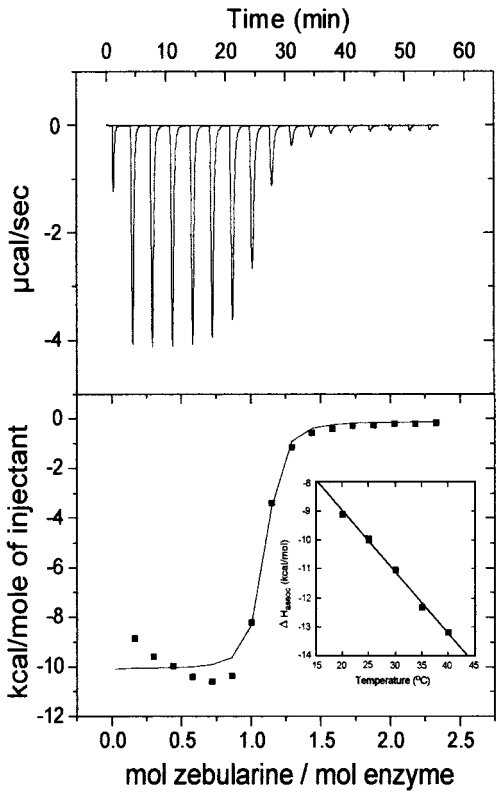

FIGURE 4: Isothermal calorimetric titration of cytidine deaminase with zebularine at 25 °C in phosphate buffer (0.1 M, pH 7.3). The upper panel shows the heat effects associated with each injection after baseline correction. The lower panel shows the integrated heats (■) and the best fitted curve to a one-site binding model. Inset: The calorimetric enthalpy change for zebularine binding as a function of temperature.

enthalpy change was found to increase linearly with increasing temperature over this range, yielding a ΔC_p of −212 (±7) cal mol^{-1} K^{-1}. Negative heat capacity changes for protein−ligand interactions may arise from the burial of solvent-accessible surfaces, changes in conformational entropy, and changes in intramolecular vibrations (*26*). In the case of zebularine binding by cytidine deaminase, it seems reasonable to attribute the observed negative value of ΔC_p to the sequestration of water molecules in the active site (*27*), since this possibility is supported by structural evidence (*10*−*12*).

Temperature Dependence of Inhibition Constants. Because of the relatively weak binding affinities for 3,4-dihydrozebularine and the product uridine by cytidine deaminase, the thermodynamic parameters associated with the binding of each of these ligands were estimated from the temperature dependence of their inhibition constants **(**Figure 5) using van't Hoff plots. The enthalpy changes associated with the binding of 3,4-dihydrozebularine and uridine were −10.1 (±0.6) and −8.3 (±0.9) kcal/mol, respectively. The corresponding entropy changes at 25 °C associated with the binding of 3,4-dihydrozebularine and uridine were estimated to be −3.2 (±1.2) and −4.9 (±1.6) kcal/mol, respectively. The temperature dependence of zebularine's dissociation constant also yielded a linear van't Hoff plot (Figure 5) with a slope corresponding to a ΔH value of −12.9 (±0.8) kcal/mol, slightly larger than the value obtained from calorimetric measurements (ΔH_{cal} = −10.0 kcal/mol). This difference

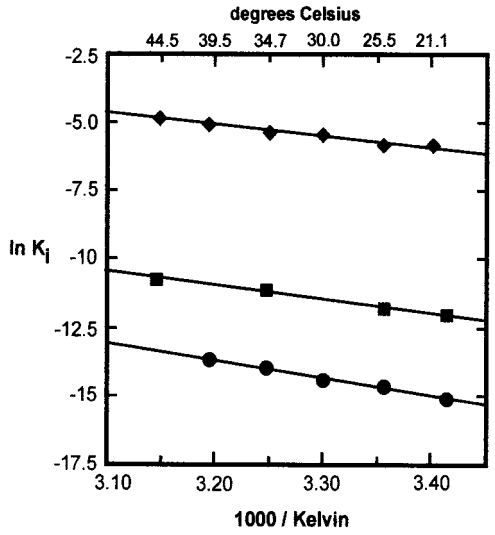

FIGURE 5: van't Hoff plots of the dissociation constants of the cytidine deaminase−uridine complex (▲) and the 3,4-dihydrozebularine complex (■) and the observed dissociation constant of the cytidine deaminase−zebularine complex (●).

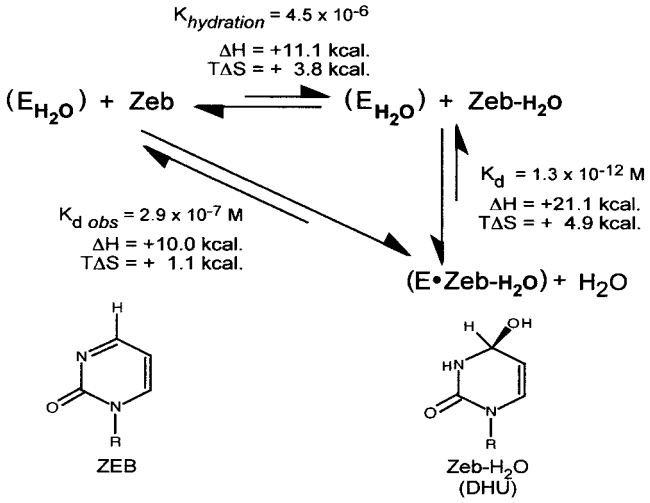

FIGURE 6: Equilibrium thermodynamic cycle for estimating the equilibrium constant and the associated enthalpy and entropy changes for dissociation of the enzyme−3,4-dihydrouridine (DHU) complex.

seems explainable in terms of the heat capacity change noted above.

DISCUSSION

The ability of enzymes to enhance the rate of biological reactions is believed to depend on their ability to bind the activated substrate in the transition state very much more tightly than the substrate in the ground state in water (*28*−*30*). Exploring the thermodynamic basis of this difference in affinity for cytidine deaminase acting on cytidine, we showed earlier that the rate enhancement ($k_{cat}/k_{non} = 10^{12}$) was achieved by nearly equivalent changes in enthalpy ($\Delta\Delta H^{\ddagger} = -7.2$ kcal/mol) and entropy ($\Delta(T\Delta S)^{\ddagger} = +9.2$ kcal/mol) of activation at 25 °C (*16*). In the present work, we determined the thermodynamic basis of the equilibrium binding affinities of the inhibitors 3,4-dihydrouridine (a transition state analogue), 3,4-dihydrozebularine (in which a hydrogen atom replaces the 4-OH group of 3,4-dihydrouridine), and uridine [a substrate for the reverse reaction (*31*) and the product of cytidine deamination]. The results of this analysis can be considered in terms of structural information obtained by X-ray diffraction from single crystals.

Thermodynamic Changes That Accompany Ground State Binding. The binding of substrate cytidine in the ground state ($K_s = 1 \times 10^{-4}$ M) was shown to be driven by a favorable enthalpy change ($\Delta H = -13$ kcal/mol), partly compensated by an unfavorable change in the entropy of binding ($T\Delta S = -7.6$ kcal/mol at 25 °C) (*16*). Structural information regarding the 3-deazacytidine−enzyme complex (*12*), presumed to be analogous to the ES complex, suggests that the glycosidic bond lies within the plane of the pyrimidine ring and that N-3 lies close enough to accept an H-bond from the −COOH group of Glu-104. Moreover, a linear-scaling quantum mechanical semiempirical simulation predicts that the lowest energy structure of the ES complex includes an H-bond between these groups (*32*). This potential hydrogen bond may restrict the relative mobility of the pyrimidine ring in the ES complex as reflected in the unfavorable entropy term, to align it with the zinc-bound hydroxide nucleophile.

The product uridine is relatively weakly bound within the active site ($K_d \sim 3 \times 10^{-3}$ M). The crystal structure of the enzyme−uridine complex shows that the plane of the pyrimidine ring is bent with respect to the glycosidic bond approximately 30° toward zinc, bringing the 4-keto group of uridine within its coordination sphere, and that the substrate water molecule originally bound by zinc is displaced. Electron density ascribable to a water molecule occupies the proposed binding site for the product ammonia (Figure 1) (*12*).

The temperature dependence of the K_i value of product uridine shows that its enthalpy of binding ($\Delta H = -8$ kcal/mol) is considerably less favorable than that of substrate cytidine ($\Delta H = -13$ kcal/mol). The glycosidic bond angle strain induced by uridine binding [estimated to be around 1 kcal/mol by semiempirical quantum mechanical calculations (*12*)] is too small to account for this difference. However, the fact that the 4-keto group of uridine is coordinated to zinc requires that water be displaced from zinc in the enzyme−uridine complex. If, at the outset of reaction, water is "split", with its proton on Glu-104 and its hydroxide moiety attached to zinc (*32*), then the difference in enthalpies may simply reflect the difference between the stabilities of a water molecule in these two forms (intact water in the uridine complex and split water in the cytidine complex) (Figure 1).

Thermodynamic Changes That Accompany the Equilibrium Binding of a Analogue Inhibitor. Transition State Analogue Inhibitor. As enzymatic deamination progresses, the enthalpy of binding culminates in the transition state ($\Delta H = -20$ kcal/mol for $1/K_{tx}$). Evidence from changes in UV absorption spectra (*19*), ^{19}F NMR spectroscopy (*33*), and crystal structures of enzyme complexes with zebularine and 5-fluorozebularine (*9*, *10*) indicates that the binding of both inhibitors is accompanied by covalent hydration across the double bond from N-3 to C-4 to generate 3,4-dihydrouridine and 5-fluoro-3,4-dihydrouridine, respectively. These hydrated inhibitors are analogous in structure to a plausible tetrahedral intermediate in the hydrolytic deamination of cytidine (*34*). The binding of these reversible inhibitors offers an unusual opportunity to compare the equilibrium thermodynamics of

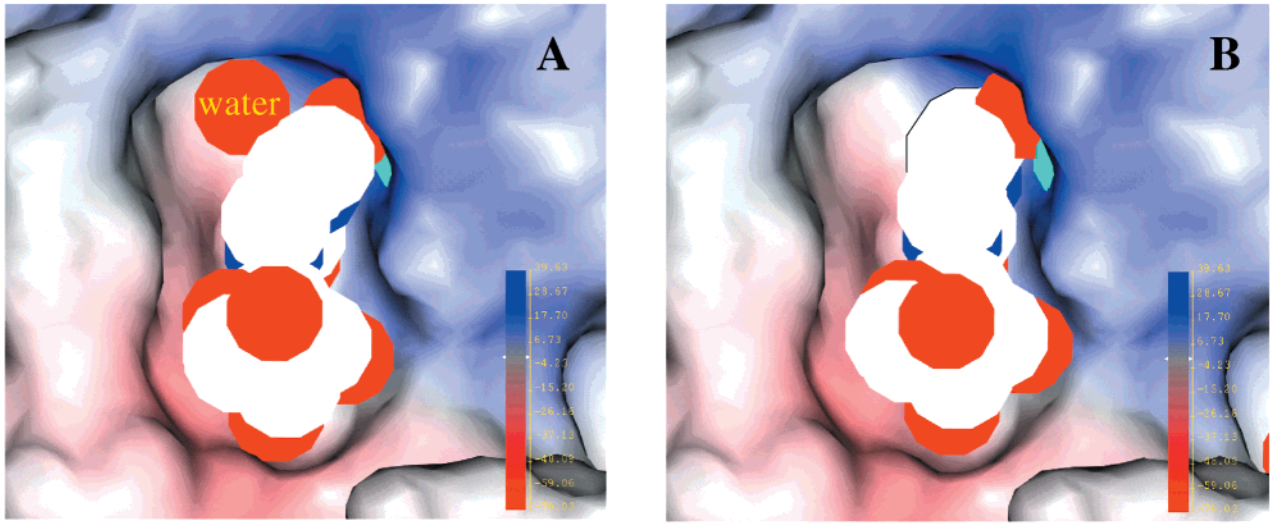

FIGURE 7: Molecular surface rendering of one active site subunit of cytidine deaminase (oxygen atoms in red) in complex with (A) uridine (*12*), showing a water molecule trapped in the ammonia binding site, and (B) 3,4-dihydrouridine. Scales in the lower right of each picture show that the electrostatic surfaces are equivalent. Surfaces were rendered with the Structural Properties and Calculation Kit (Center for Molecular Design, Texas A&M University, College Station, TX.

transition state analogue binding with the quasi-equilibrium thermodynamics of the binding of the activated substrate in the transition state.

The equilibrium for hydrating pyrimidin-2-one ribonucleosides in solution is known to be very unfavorable (*19*, *21*). To compare the thermodynamic changes that accompany the binding of 3,4-dihydrouridine with those established earlier for the actual transition state by kinetic methods, enthalpy and entropy changes were determined for the apparent equilibrium constant of zebularine binding and for the equilibrium constant of zebularine hydration to form 3,4-dihydrouridine in free solution (Figure 2). The present experiments show that the unfavorable position of the hydration equilibrium to form 3,4-dihydrouridine in solution is due to a large, unfavorable ΔH (+11 kcal/mol) which is partly compensated by a favorable entropy change ($T\Delta S \sim$ 4 kcal/mol at 25 °C). Isothermal titration calorimetry measurements indicate that the observed binding constant of zebularine by cytidine deaminase is associated with a ΔH of −10 kcal/mol at 25 °C (Figure 4). Thus, an enthalpy change of −21 kcal/mol accompanies the binding of 3,4-dihydrouridine from free solution ($\Delta H_{DHUdiss} = \Delta H_{ZEBdiss} + \Delta H_{ZEBhyd}$) (Figure 6). Comparing this value with the enthalpy change associated with the binding of the actual transition state ($\Delta H = -20$ kcal/mol), the binding of 3,4-dihydrouridine can be seen to capture all of the available enthalpy expected of an ideal transition state analogue inhibitor. Just as transition state affinity increases steeply with decreasing temperature (*16*), inhibition by 3,4-dihydrouridine becomes much more effective with decreasing temperature. The decisive influence of enthalpy on both catalysis and transition state analogue binding is consistent with other evidence that both of these functions are critically dependent on the electrostatic and H-bonds that form at the site of hydration, especially the relatively short H-bond (2.5 Å) between Glu-104 and the 4-OH group which is essential for catalysis (*15*) and zebularine hydration (*33*).

Cytidine deaminase binds 3,4-dihydrouridine approximately 10^7-fold more tightly than 3,4-dihydrozebularine, an analogue of 3,4-dihydrouridine in which a proton replaces the 4-OH group. Previously, difference Fourier map analysis of the 3,4-dihydrouridine and the 3,4-dihydrozebularine crystal structure complexes showed that the "substrate" water molecule used to hydrate zebularine at the active site was "trapped" in the complex with 3,4-dihydrozebularine (*10*) (Figure 1). Aside from interactions at the site of chemical transformation, no further change in enzyme conformation was observed. Most of the large difference in free energy of binding between these two inhibitors ($\Delta\Delta G = 9.3$ kcal/mol) arises from the difference ($\Delta\Delta H = 11$ kcal/mol) between the enthalpies of binding of 3,4-dihydrouridine ($\Delta H = -21$ kcal/mol) and 3,4-dihydrozebularine ($\Delta H = -10$ kcal/mol). This difference can be attributed to differences in H-bonding (involving interactions between the carboxylate group of Glu-104 and the 3-NH and 4-OH groups of 3,4-dihydrouridine), to the polar interaction between the 4-OH group of 3,4-dihydrouridine and zinc, and to an unfavorable steric interaction between the 4-H group and the substrate water molecule trapped in the 3,4-dihydrozebularine complex.[2]

Entropic Effects on Ligand Binding: A Role for Site-Bound Water. In an earlier survey of the temperature dependence of enzyme rate enhancements, we noted that single substrate and hydrolytic enzymes invariably lower the large enthalpies of activation of reactions that proceed slowly in their absence in water (*35*). Close inspection reveals that hydrolytic enzymes tend to increase the entropy of activation as well. That behavior appears understandable if one considers the role of substrate water in those reactions, in which the activated substrate in the transition state is often covalently hydrated. Since the activated substrate in the transition state *already* incorporates a substrate water molecule, the virtual

[2] The enthalpy of binding of cytidine in the ground state ($\Delta H = -13$ kcal/mol) is more favorable than that of 3,4-dihydrozebularine ($\Delta H = -10$ kcal/mol), consistent with a role for enthalpic strain in the 3,4-dihydrozebularine complex due to the trapped "substrate" water molecule and the absence of the H-bond between N-3 and Glu-104. 3,4-Dihydrozebularine can be seen (Figure 1) to be bound more tightly than cytidine because of its more favorable entropy of binding, which is attributable to the presence of fewer constraints in the 3,4-dihydrozebularine complex.

equilibrium process by which that hydrated species is bound *from free solution* must involve displacement of the substrate water molecule from the active site into bulk solvent. That release would not occur with the binding of substrate in the ground state, and would be expected to render the entropy of binding of the activated substrate in the transition state more favorable. Numerous examples have been reported in which the displacement of site-bound water into bulk solvent appears to enhance the affinity of a covalently hydrated ligand (*36*). There are comparatively few reports that combine both structural and thermodynamic evidence in support of that hypothesis. These include descriptions of the stereoselectivity of a secondary alcohol dehydrogenase (*37*), the binding of azobenzene derivatives by streptavidin (*38*), and several additional examples (*27*).

Earlier, we showed that a significant contribution to catalysis by cytidine deaminase arises from an increase in the entropy of activation, to such an extent as to render transition state binding entropically favorable (*16*), rather than unfavorable as would ordinarily be expected for a process in which two species combine to form a single, tightly associated complex (Figure 1). In the enzyme's complex with the product uridine, a water molecule occupies the site at which the leaving group ammonia is normally generated by deamination (*12*). Formation of the ES complex requires that a solvent water molecule be bound in split form, with its proton on Glu-104 and its hydroxide attached to zinc. It is reasonable to assume that cytidine, like uridine in the reverse reaction (*39*), is not covalently hydrated in the ground state. In the transition state for hydrolytic deamination of cytidine, both the attacking and leaving group sites are occupied with no excess room for water (Figure 1). It seems reasonable to suppose that the more favorable entropy change that accompanies the binding of the activated substrate in the transition state ($T\Delta S_{25°C} = +1.6$ kcal/mol), relative to substrate cytidine in the ground state ($T\Delta S_{25°C} = -7.6$ kcal/mol) and product uridine in the ground state ($T\Delta S_{25°C} = -5$ kcal/mol), results from the release of an additional bound water molecule into bulk solvent.

3,4-Dihydrouridine can be regarded as a kind of bisubstrate analogue, in the sense that it contains the elements of both substrate cytidine and substrate water. Even though the binding of 3,4-dihydrouridine is accompanied by the same enthalpy change as is the binding of the actual transition state, there remains an ~7 kcal/mol difference in free energy. Taking into account the available structural information about the enzyme complexes of each inhibitor and of the product uridine, it seems reasonable to speculate that the entropy change that accompanies 3,4-dihydrouridine binding is due to partial occupancy by water of the binding site that is normally occupied by product ammonia in hydrolytic deamination. The 3,4-dihydrouridine complex does not show enough electron density to model a water molecule in that site (*10*). However, a pocket is visible in that complex that appears large enough to host a water molecule (Figure 7). Moreover, DOWSER, a program that calculates the free energy for introducing water molecules into buried cavities in proteins based on atomic force potentials (*40*), predicts that the energetics of burying a water in the pocket with 3,4-dihydrouridine bound is within the threshold limit. Thus, the hydrogen atom in 3,4-dihydrouridine that replaces the more bulky leaving group ($-NH_2$ group) of the altered substrate in the transition state appears to leave room for a trapped water molecule, resulting in a cost in entropy that seems to explain the shortcomings of 3,4-dihydrouridine as an ideal transition state analogue.

CONCLUSIONS

Thermodynamic changes that accompany the equilibrium binding of the transition state analogue 3,4-dihydrouridine support our earlier conclusion, from the quasi-equilibrium thermodynamics of binding of the actual transition state, that catalysis by cytidine deaminase is achieved largely by enthalpy reduction. That behavior is due to hydrogen-bonding interactions and electrostatic interactions that develop in the transition state. Interactions of this type are commonly involved in transition state stabilization, as revealed by the structures of transition state complexes with cytidine deaminase (*10*), adenosine deaminase (*41*), orotidine-5′-monophosphate decarboxylase (*42*), triosephosphate isomerase (*43*), and many other enzymes. Disrupting interactions of this type, by replacement of the 4-OH group of 3,4-dihydrouridine with a hydrogen atom, results in an unfavorable increase in the enthalpy of binding, similar to the effect on transition state binding of removing the substrate 3′-OH group (*16*). In addition, entropy changes play a significant role in determining relative binding affinities, depending on whether water molecules are trapped or displaced during ligand binding. These results suggest that the transition state analogue 3,4-dihydrouridine's shortcomings, in capturing all of the free energy of binding of the actual transition state, may arise from its inability to displace a water molecule that occupies the binding site normally occupied by product ammonia.

ACKNOWLEDGMENT

We thank V. Marquez for the inhibitors, S. Short for assistance with enzyme purification, and B. Temple for performing DOWSER calculations and preparing Figure 7.

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BI011189+