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Direct Measurement of Local and Global Contributions in the Binding of Coformycin to Bovine Adenosine Deaminase

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A general method is outlined that determines quantitatively the extent to which tight ligand binding to an enzyme active site is facilitated by the adoption of a stabler macromolecular conformation in the complex. The method therefore rejects the general assumption that competitive inhibitor binding to enzyme active sites involves only local (active site) interactions. The procedure involves comparing the unfolding transition state free energies of the free and complexed enzyme from physiological conditions. For the interaction of the transition state analog coformycin with bovine adenosine deaminase we observed that the binding free energy by the physiological enzyme was ~92% due to the assumption of a stabler enzyme conformation in the complex. The significance of these findings in terms of general enzyme catalysis is discussed.

Keywords: Enzyme catalysis; Shifting specificity model; Adenosine deaminase; Coformycin; Transition state analog; Binding

INTRODUCTION

The primary purpose of this work is to explore the possibility that binding of the transition state analog coformycin to the bovine adenosine deaminase active site under physiological conditions may be facilitated by the adoption of a significantly stabler enzyme conformation. We also introduce a novel method for quantitating the degree to which ligand binding to enzyme active sites is facilitated by local (purely active site) versus global (the rest of the enzyme) interactions. The motivation for this work is the testing of a novel model for general enzyme catalysis which states that a primary source of the catalytic efficiency of enzymes is their ability to adopt, transiently, a conformational energy minimum at the reaction transition state.[1–3]

Adenosine deaminase (ADA) functions physiologically to convert adenosine or deoxyadenosine to inosine or deoxyinosine. Previous studies of the interactions of the enzyme with coformycin and deoxycoformycin, two potent transition state analogs, suggest that the ADA active site is not initially optimized for interaction with the transition state of the reaction but instead prefers interaction with the reaction ground state (substrate).[3,4] This seems at odds with the traditional view of general enzyme catalysis,[5] which states that enzyme-ground state interactions are inherently nonproductive[6,7] although sound reasons why an initial complementarity of the active site to the reaction ground state may actually facilitate the reaction have been offered.[1,2,8] In the Shifting Specificity model (SSM) for general enzyme catalysis,[1–3] the enzyme active site is initially complementary to the reaction ground state. Interaction of the ground state with the active site via the same noncovalent interactions that govern enzyme tertiary structure necessarily results in an enzyme global conformational change. It is proposed that nature has selected a conformational change that shifts the active site specificity from the ground state to the transition state, in the process transforming the substrate to the transition state. It is further proposed that catalysis is facilitated by the adoption of a stabler enzyme conformation at the reaction transition state. In other words, though the total energy at the transition state is, of course, maximal, the enzyme-localized energy achieves an energy minimum at this crucial point in catalysis. So, instead of a passive, structurally inert enzyme that will only catalyze a reaction for any collection of atoms that happens to wander into the active site

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and that can also assume the appropriate transition state structure—a picture the Haldane view leaves us with—the SSM gives us an enzyme that uses its full conformational potential to initiate the reaction, by initially favoring interaction with the ground state, and then facilitate its conversion.

Determining the binding affinities of ligands to enzyme active sites is conceptually trivial and can be done with great accuracy—one need only carefully determine the relative concentrations of the species in $K_b = [E - I]/[E][I]$ where K_b is the association binding constant, $[E - I]$ is the concentration of the complex, and $[E]$ and $[I]$ are the concentrations of free enzyme and inhibitor, respectively. Binding results from the net gain of the various favorable noncovalent interactions between the enzyme and inhibitor over the affinity each has for solvent. The energetic sum of these interactions may then be accurately calculated from $\Delta G^0 = -RT\ln(K_b)$. It is further generally assumed, particularly in the case of transition state analog binding to enzymes, that these interactions are purely local in nature; i.e., they involve only interactions at the active site. However, it is possible that favorable binding could result from the adoption of a stabler enzyme global conformation. A method for discerning between local and global contributions to binding is therefore necessary for a complete understanding of the interactions of ligands with enzymes.

We propose the following strategy for measuring the local and global contributions of the binding of ligands to enzyme active sites (Fig. 1). We define the folded form of the enzyme as that which predominates under physiological buffer conditions and are aware of the fact that the folded form of ADA is known to undergo a sharp, nondenaturational conformational transition at ~29°C.[9] Binding of the transition state analog coformycin to the enzyme active site stabilizes the enzyme regardless of whether a conformational change results. However, if ligand binding is purely local there is no alteration in enzyme conformational energy and the unfolding transition state free energy of the complex ($\Delta G^{\neq}_{dn\,(ADA/cof)}$) should be the same as the free enzyme ($\Delta G^{\neq}_{dn\,(ADA)}$) since unfolding is a property of the bulk enzyme conformation. If, on the other hand, the complex is not stabilized at all by active site interactions but only by a global conformational relaxation of the enzyme then one should expect $\Delta G^{\neq}_{dn\,(ADA/cof)}$ to be increased by an amount equivalent to the coformycin binding energy. These two scenarios represent extreme cases. If there is a partitioning of the energy between local and global interactions then its extent may be quantitated by defining a binding partioning parameter P_B^{global} as $P_B^{global} = -(\Delta G^{\neq}_{dn\,(ADA/cof)} - \Delta G^{\neq}_{dn\,(ADA)})/\Delta G_b$ where ΔG_b is the coformycin binding free energy and P_B^{global} gives the fraction of the total binding energy which may be attributed to interactions not involving the few residues that constitute the active site. If a temperature-dependent analysis of these unfolding rate constants is performed then one can also understand other basic thermodynamic aspects of the interaction of coformycin with ADA.

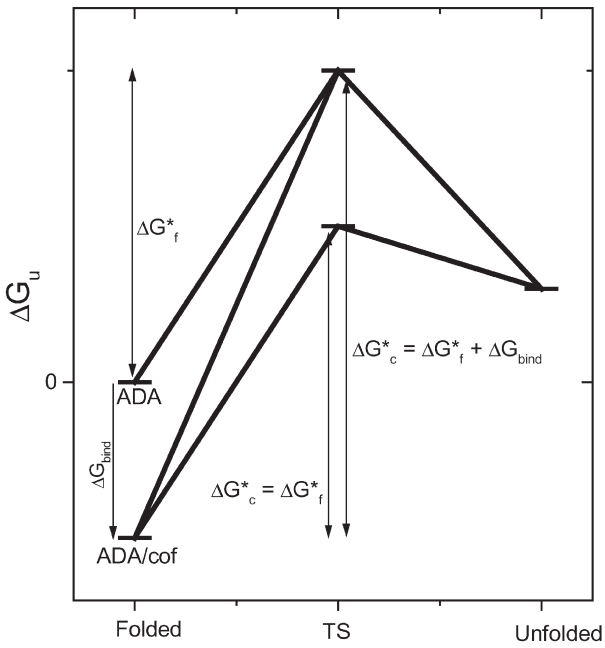

FIGURE 1 Rationale for determination of P_B^{global}, the fraction of coformycin binding energy which results from a stabilizing, global enzyme conformational change. Shown are the free energies of unfolding (ΔG_u) for free ADA and the ADA/coformycin complex. Coformycin binding (ΔG_{bind}) stabilizes the folded form of the enzyme regardless of whether a stabilizing enzyme conformational change results. One limiting case of coformycin binding energy partitioning occurs when the activation free energy for unfolding of the free enzyme (ΔG^*_f) equals that of the complex (ΔG^*_c). In this case, the interaction energy must be purely local (confined to the active site) and $P_B^{global} = 0$. The other limiting case occurs when the unfolding activation free energy of the complex equals the sum of the unfolding activation energy of the free enzyme and the coformycin binding energy. In this case, binding must result exclusively from a stabilizing global enzyme conformational change and $P_B^{global} = 1$. If binding is partitioned between local and global interactions—the most likely scenario—then $\Delta G^*_f < \Delta G^*_c < \Delta G^*_f + \Delta G_{blind}$ and, for a generalized case, $P_B^{global} = -(\Delta G^*_c - \Delta G^*_f)/\Delta G_{blind}$ and can take a value between 0 and 1.

We report our evaluation of the unfolding transition state thermodynamics for free and coformycin-complexed ADA from an analysis of the temperature dependence of the pseudofirst order rate constants for guanidine hydrochloride (GuHCl)-induced denaturation of both species from 5 to 50°C. Pseudofirst order rate constants for denaturation under physiological conditions (no GuHCl) are obtained from linear extrapolation procedures and the transition state thermodynamics ($\Delta G^{\neq}$, $\Delta H^{\neq}$, $\Delta S^{\neq}$, $\Delta C^{\neq}$) are calculated by standard methods. With knowledge of the binding thermodynamics of coformycin to ADA[3] and of the equilibrium unfolding thermodynamics of ADA,[9] both from

physiological conditions, we are able to at least partially understand the unfolding transition state thermodynamics as well as calculate P_B^{global}.

We find that the temperature dependence of the unfolding rate constants for either free ADA or its complex with coformycin offers further support for a sharp conformational transition of the enzyme at ~29°C.[9] The coformycin binding energy at 38.3°C, the bovine normal body temperature, appears to be ~92% global, in contrast to 4°C, where the crystals for X-ray structure analysis were grown, where binding appears to be only ~52% global. We also find that the unfolding transition state for either species (bound or free) at 38.3°C appears to occur early during unfolding while the unfolding transition state at 4°C for either species appears to occur late. These results are interpreted in terms of the specific interaction of ADA with adenosine and in terms of enzyme catalysis in general.

MATERIALS AND METHODS

ADA from calf spleen as an ammonium sulfate suspension and 99 + % guanidine hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO). Coformycin was a gift from Parke-Davis (Ann Arbor, MI).

Unfolding reactions were initiated by combining either free ADA or its complex with coformycin with a GuHCl solution in a 1:10 volume ratio (enzyme–denaturant) using an Applied Photophysics stopped flow reaction analyzer (model SX-17 MV) equipped with a Neslab RTE-111 circulating water bath. Temperatures were measured at the moment of mixing from the water bath which bathed the reaction syringes and are true to within 0.1°C. An appropriate concentration of ADA was prepared in 100 mM phosphate buffer, pH = 6.3 (the pH optimum of the enzyme) to yield a 3 μM solution after mixing. For measurements of the unfolding rates of the ADA/coformycin complex a previous study of the temperature dependence of K_b for the ADA/coformycin interaction revealed that making the final solution 4.5 μM in coformycin complexed essentially all of the enzyme at any temperature of interest.[3] GuHCl concentrations were determined gravimetricly at 25°C.[10] Free ADA was denatured in 3.00, 4.00, 5.00, and 6.00 M GuHCl (concentrations after mixing). The ADA/coformycin complex was denatured in 7.05, 6.82, 6.59, 6.36 M GuHCl (concentrations after mixing). This high and rather narrow range of GuHCl concentrations for unfolding of the complex was necessary to obtain kinetics on a reasonably fast time scale (unfolding at least two-thirds complete within 1000 s) given the solubility limit of the denaturant. GuHCl was chosen as the denaturant over urea because the complex would not denature, even in very concentrated urea, in the time frame of the stopped flow experiment. Unfolding rates for either enzyme species were determined from 5.0 to 50.0°C at 5°C intervals. Denaturation was monitored by the change in enzyme intrinsic fluorescence with excitation at 280 nm and emission at 320 nm. Pseudofirst order rate constants were determined by fits to single exponentials of the fluorescence decays. Unfolding appeared to be a two-state process in that fits of the fluorescence decays to two or more exponentials were no better than fits to one exponential. A denaturation at a certain temperature and [GuHCl] was repeated at least 3 times and error bars in the figures represent the uncertainties of the measurements in terms of single standard deviations. Values for the pseudo-first order rate constants (k_u^0) at zero molar GuHCl were determined from linear extrapolations of the ln(k_u) with [GuHCl]. R^2 values for these linear fits ranged from 0.999 to 0.946.

Uncertainties in the various thermodynamic parameters reported are reflections of the uncertainties in the values of the pseudofirst order rate constants, for calculations of the activation energies for unfolding ($\Delta G_u^{\neq}$), or reflect uncertainties in modeling of the data, as in fitting the data in Fig. 5 to a quadratic equation. Data sets were fitted using Microcal Origin software and using uncertainty weighting.

We calculated the unfolding thermodynamics at 38.3°C, since this is the normal physiological temperature of the organism from which the enzyme is isolated and the millions of years of evolution at this temperature has presumably driven its function, and at 4°C, since this is where the X-ray structural analyses were performed.[11–14] No measurements were actually made at either of these two temperatures; instead, calculations were made from extrapolations of trend lines of the rate data (see Fig. 5).

RESULTS

Figure 2 shows representative traces of the decrease in intrinsic fluorescence intensity accompanying unfolding of ADA and ADA/cof at various temperatures and [GuHCl]s to emphasize that the rates of denaturation are highly dependent upon these two variables. The decrease in fluorescence upon denaturation is presumably due to the quenching of the exposed aromatic residues upon exposure to the aqueous environment.

From the data at each [GuHCl] an Arrhenius plot may be calculated. Arrhenius plot progressions for the denaturation of ADA and of ADA/cof are shown in Fig. 3. The plots at all [GuHCl]s are concave downwards and the effect becomes more pronounced as the solution conditions approach

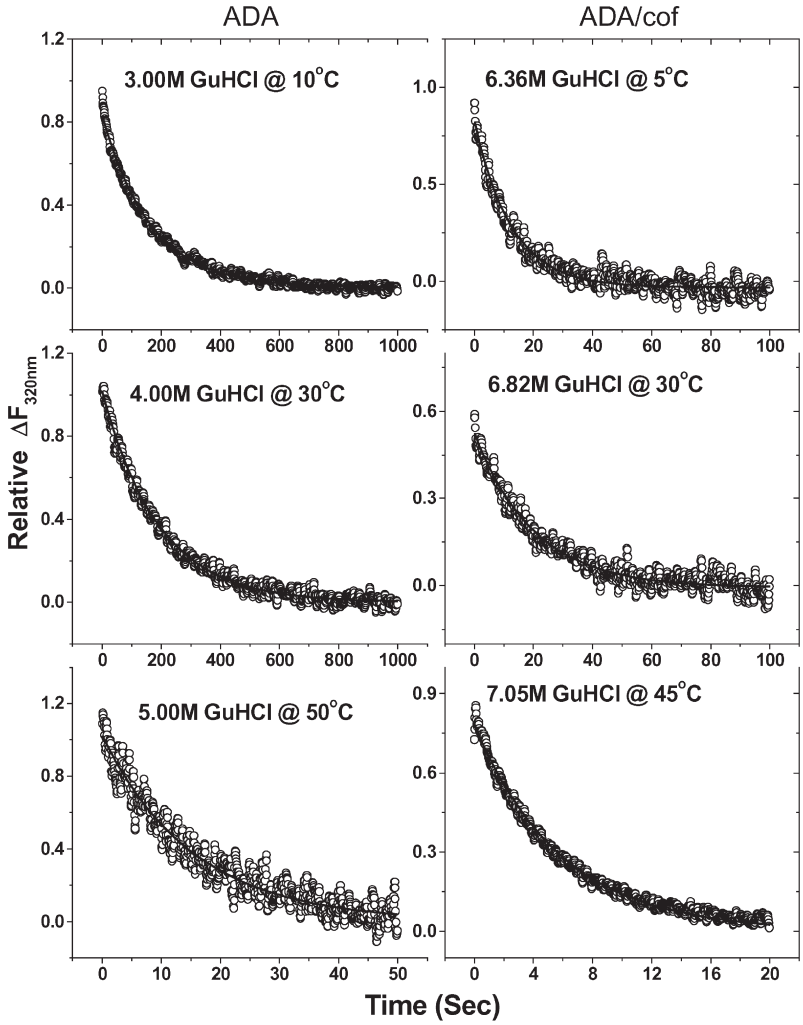

FIGURE 2 Representative kinetic traces of the GuHCl-induced denaturation of ADA as functions of T and [GuHCl]. Data are the fluorescence emission at 320 nm with excitation at 280 nm. Traces are single kinetic runs. Solid curves are single exponential fits to the data.

the physiological situation; i.e., with decreasing [GuHCl]. The downward curvature trend in the plots is attributed to the great increase in heat capacity of either system as it unfolds with the primary source for this increase being the net exposure of nonpolar amino acid side chain surface area.

As we are interested in the unfolding thermodynamics from physiological conditions, knowledge is required of the temperature dependence of these unfolding rate constants in the absence of denaturant. Figure 4 shows representative plots of $\ln(k_u)$ vs. [GuHCl]. We observe that all trends are linear—the worst R^2 observed for the linear fits is 0.946. Physiological values for the pseudofirst order rate constants for unfolding are then obtained from the y-intercepts.

Binding coformycin to ADA greatly decreases the rate at which the enzyme unfolds under physiological conditions. The values of the pseudo-first order rate constants for unfolding of ADA and the coformycin complex at 38.3°C are 6.48 $(\pm 0.57) \times 10^{-6}\,s^{-1}$ and 1.50 $(\pm 0.13) \times 10^{-15}\,s^{-1}$, respectively. These correspond to unfolding half lifes of 1.07×10^5 s or 29.7 h for ADA and 4.62×10^{14} s or 14.7 million years for the complex! The Arrhenius plots for the unfolding of either species from physiological conditions may be seen in Fig. 5. The data as presented in Fig. 5 may be used to calculate the unfolding transition state thermodynamics.

Before calculating these values, we first observe that the trends of the physiological Arrhenius plots show a sharp discontinuity at ~29°C. A previous study revealed that the ADA stability curve (a plot of the Gibbs free energy of unfolding, ΔG_u, versus temperature) also displays a sharp discontinuity at this temperature which we attribute to an abrupt conformational change in the enzyme.[9] Accordingly, we model the high- and low-temperature data separately.

The final results of this analysis are shown in Fig. 6. The figure contains information determined from previous studies on the binding of coformycin to ADA[3] and on the calculation of the ADA stability curve.[9] Due to the shape of the stability curves

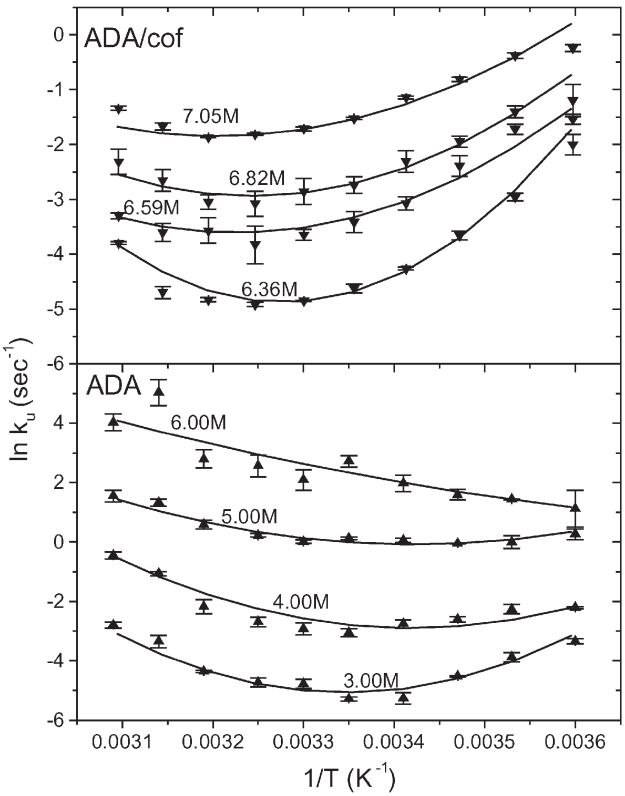

FIGURE 3 Arrhenius plot progressions for the unfolding of coformycin-bound ADA and of free ADA as functions of [GuHCl]. Trend lines are quadratic equation fits to the data.

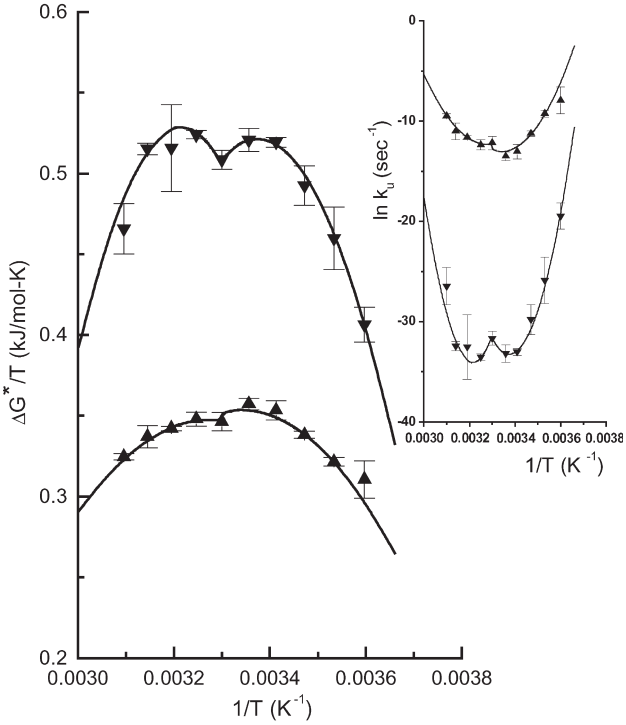

FIGURE 5 Gibbs–Helmholtz plots for the physiological pseudo-first order rate constants for unfolding of free ADA (▲) and coformycin-complexed ADA (▼). Inset is the corresponding Arrhenius plots. Trend lines are quadratic equation fits to the data.

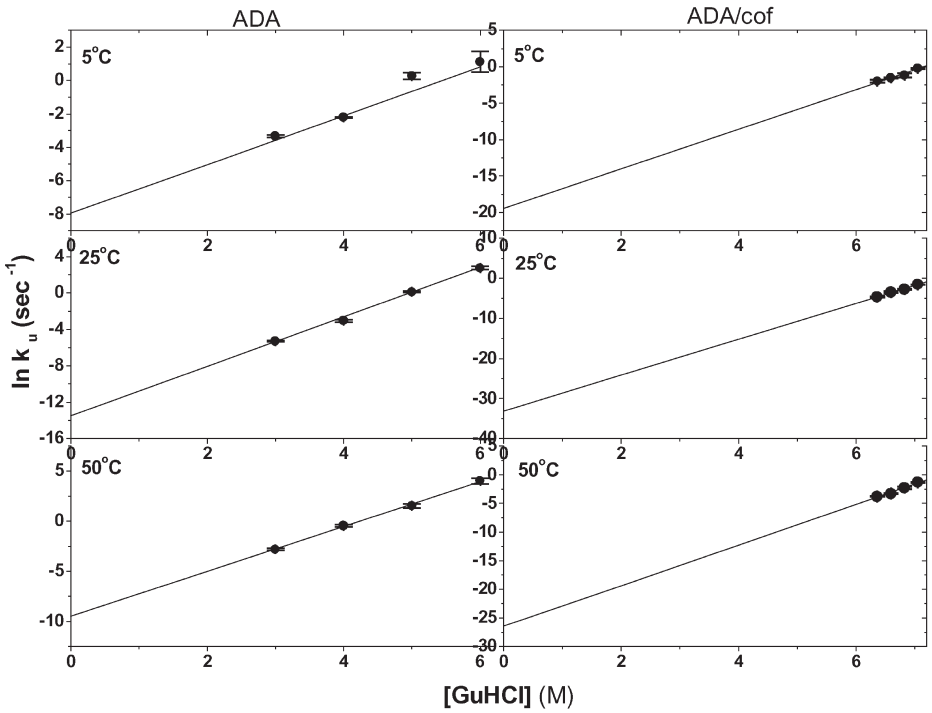

FIGURE 4 Representative linear extrapolations of ln(k_u) vs. [GuHCl]. These plots were made to obtain ln(k_u^0), the natural logarithm of the pseudo-first order rate constant for unfolding from physiological conditions, from the *y*-intercept of the plots. The worst R^2 value for any fit was 0.946.

for either the high- or low-temperature species, there is essentially no contribution from the low-temperature form of the enzyme for calculations at 38.3°C nor from the high-temperature form of the enzyme for calculations at 4°C. We assume that the affinity of coformycin for the unfolded enzyme is negligible so that either species unfolds to the same final state.

Analysis was begun at the physiological temperature of 38.3°C ($1/T = 0.003211\,K^{-1}$), the normal bovine body temperature. At this temperature the Gibbs free energy for coformycin binding ΔG_b is -62 ± 1 kJ/mole and the free energy of ADA unfolding ΔG_u is 23 ± 3 kJ/mole. Not surprisingly, the transition state analog has a great affinity for the enzyme active site and the enzyme native form is only marginally stable under physiological conditions. The free energy change for achieving the transition state is calculated from basic transition state theory using $\Delta G_u^{\neq} = -RT\ln(k_u^0 h/k_B T)$ where $\Delta G_u^{\neq}$ is the unfolding activation energy, R is the gas law constant, k_u^0 is the pseudo-first order rate constant for unfolding of either the free enzyme or the complex from physiological conditions, h is Planck's constant, and k_B is Boltzmann's constant. The activation free energy for unfolding of ADA $\Delta G_{u(ADA)}^{\neq}$ is 107 ± 1 kJ/mole and that for the unfolding of the ADA/coformycin complex $\Delta G_{u(ADA/cof)}^{\neq}$ is 165 ± 1 kJ/mole. Due to the high reproducibility of our rate constant determinations, the unfolding transition state free energy changes are precisely known.

To further understand the interaction between ADA and coformycin we calculated the enthalpy, entropy, and heat capacity unfolding transition state changes and, with prior knowledge of the complete equilibrium thermodynamics of coformycin binding and of ADA denaturation, sought to understand how unfolding of the free enzyme differs from that of the complex. At 38.3°C the equilibrium binding enthalpy $\Delta H_b = -85 \pm 9$ kJ/mole and the equilibrium unfolding enthalpy of ADA $\Delta H_u = -149 \pm 37$ kJ/mole. Our procedure for calculating the unfolding transition state enthalpy changes for ADA ($\Delta H_{u(ADA)}^{\neq}$) and for the complex ($\Delta H_{u(ADA/cof)}^{\neq}$) was to first recast the physiological rate constant data in the form of $\Delta G_u^{\neq}/T$ versus $1/T$ and use the general Gibbs–Helmholtz relation $\Delta H^{\neq} = [\partial(\Delta G^{\neq}/T)/\,\partial(1/T)]_P$ to calculate the transition state enthalpy changes (Fig. 5). We observed that the trend for all four temperature regions (the high- and low-temperature regions for either the free enzyme or the complex) are well modeled by quadratic equations. Calculation of the derivative was then trivial and the trend of $\Delta H^{\neq}$ with temperature was then easily calculated. At 38.3°C, this analysis gave $\Delta H_{u(ADA)}^{\neq} = 95 \pm 8$ kJ/mole and $\Delta H_{u(ADA/cof)}^{\neq} = 13 \pm 14$ kJ/mole.

The very large and negative ΔH_u is indicative of a major hydrophobic effect in the unfolding process. As conventional wisdom suggests that it is the nonpolar amino acid side chain contacts that stabilize the folded state, this is an expected result which is confirmed by the equilibrium entropy and heat capacity changes in unfolding.[9] The positive transition state enthalpy changes for either the free enzyme or the complex then suggest that these nonpolar effects are not manifest to any great extent in the unfolding transition state. Instead, the positive values suggest transition states that are formed early in the unfolding process and that are characterized by conventional deoptimization of all stabilizing interactions—not just the nonpolar ones. Previous observations of positive $\Delta H_u^{\neq}$s have led to the same conclusion.[15–17] The much smaller $\Delta H_{u(ADA/cof)}^{\neq}$ suggests, but does not prove, that net exposure of previously buried nonpolar amino acid side chains to the solvent may contribute more to the unfolding transition state of the complex though it must be concluded that the unfolding transition state for the complex also resembles more the folded form than the unfolded form.

We turn now to the calculations of the entropy changes for the processes at 38.3°C. For all calculations we use $\Delta S = (\Delta H - \Delta G)/T$. Our earlier studies revealed the equilibrium entropy change for coformycin binding ΔS_b to be -74 ± 29 J/mole–K and the equilibrium entropy change for enzyme unfolding ΔS_u to be -553 ± 150 J/mole–K. $\Delta S_{u(ADA)}^{\neq} = -41 \pm 27$ J/mole–K and $\Delta S_{u(ADA/cof)}^{\neq} = -486 \pm 49$ J/ mole–K. Uncertainties in these values reflect the uncertainties in the determinations of $\Delta H_u^{\neq}$ as obtained from the graphical procedure outlined above. The large and negative ΔS_u further suggests that nonpolar amino acid side chain contacts contribute significantly to the stability of the folded form as the decrease in entropy here suggests a net increase in the number of bound water molecules to the enzyme. The relatively small $\Delta S_{u(ADA)}^{\neq}$ again suggests that the unfolding transition state of the free enzyme more closely resembles the folded form than the unfolded form. The large and negative $\Delta S_{u(ADA/cof)}^{\neq}$ is more difficult to interpret. Within experimental error, its value is equal to ΔS_u and therefore suggests the unfolding transition state for the complex more closely resembles the final, unfolded state. This lies in contrast to our interpretation of the enthalpy data. However, an examination of the heat capacity changes suggests that there are a greater number of bound water molecules in the transition state of the complex than in the final state (see below). This would then tend to make $\Delta S_{u(ADA/cof)}^{\neq}$ substantially negative.

Finally, we turn to a discussion of the heat capacity changes at the physiological temperature. Our previous studies found the heat capacity change for coformycin binding, ΔC_b, to be -4.7 ± 0.8 kJ/mole–K and the overall heat capacity change of ADA unfolding, ΔC_u, to be 23 ± 2 kJ/mole–K.

We suggested earlier that the negative ΔC_b alone is evidence for the production of a stabilizing enzyme conformational change upon coformycin binding.[3] The sign and magnitude of ΔC_u is typical for an enzyme of the size of ADA.[18,19] We calculate the transition state heat capacity changes by the usual method of $\Delta C = \Delta(\Delta H)/\Delta T$ and obtain $\Delta C^{\neq}_{u(ADA)} = 15 \pm 1$ kJ/mole–K and $\Delta C^{\neq}_{u(ADA/cof)} = 60 \pm 1$ kJ/mole–K. The value of $\Delta C^{\neq}_{u(ADA)}$ suggests that the unfolding transition state for the free enzyme is approximately halfway between the folded and unfolded forms. This is in contrast to the enthalpy and entropy results which suggest a transition state more closely resembling the folded form. We have no good explanation for this discrepancy other than to remind the reader that there are several contributors to heat capacity effects such as the hydration of polar groups and vibrational entropy effects and that a straightforward interpretation of these data may not be possible. In contrast, the large, positive value of $\Delta C^{\neq}_{u(ADA/cof)}$ suggests a greater number of bound water molecules in the transition state than in the final state. The most straightforward explanation of this is that the semi-folded nature of the transition state permits the formation of an extensive bridgework of water molecules from one nonpolar surface to another with this extensive structure becoming lost in the final, random coil state. This tends to be supported by the very negative value of $\Delta S^{\neq}_{u(ADA/cof)}$. Further experiments are necessary to verify this assumption.

Next we perform an analogous analysis of the unfolding thermodynamics at 4°C ($1/T = 0.003608\ K^{-1}$). This is an important temperature to consider as this is where the crystals of ADA and of its complex with bound catalytically relevant ligands were formed.[11–14] Though the resulting structures as determined by X-ray crystallographic analysis are generally considered to be the physiological structures we have demonstrated by a study of the temperature dependence of the denaturation free energy that this cannot be the case for ADA as we observe an abrupt conformational transition at ~29°C.[9] Nevertheless, it is still of historical interest to consider the nature of the interaction of coformycin with ADA at this temperature.

Starting again with an evaluation of the denaturation free energy changes of the two species we recall that the binding free energy, ΔG_b, of coformycin to ADA at 4°C is -56 ± 1 kJ/mole and the equilibrium free energy for unfolding, ΔG_u, is 18 ± 3 kJ/mole. Our analysis gives $\Delta G^{\neq}_{u(ADA)} = 81 \pm 2$ kJ/mole and $\Delta G^{\neq}_{u(ADA/cof)} = 110 \pm 1$ kJ/mole. Coformycin binding is somewhat less favorable at this temperature and the folded form of the low-temperature enzyme conformer is only marginally stable. The transition state free energy changes are more similar here than at 38.3°C suggesting that ligand binding has less influence on the enzyme global structure in the low-temperature regime. This aspect will be discussed in greater detail below.

As for enthalpy considerations, binding of coformycin at this temperature is characterized by a ΔH_b of 83 ± 9 kJ/mole and a ΔH_u of -813 ± 94 kJ/mole. At 4°C we calculate $\Delta H^{\neq}_{u(ADA)}$ to be -461 ± 114 kJ/mole and $\Delta H^{\neq}_{u(ADA/cof)}$ to be -1076 ± 47 kJ/mole. We have previously discussed how the large, negative ΔH_u is characteristic of folded enzyme stabilization by nonpolar interactions. The more negative ΔH_u observed at 4°C and the signs and magnitudes of the other equilibrium thermodynamic parameters at this temperature suggest a significantly more compact structure of the enzyme at low temperature versus high temperature.[9] Unlike the situation for the 38.3°C data, the large and negative transition state values observed for either the free enzyme or the complex suggest that nonpolar amino acid side chain interactions with water are a major contributor to the transition state. We therefore conclude that the unfolding transition state for either species much more closely resembles the final unfolded state than the initial folded state. Once again, the fact that $\Delta H^{\neq}_{u(ADA/cof)}$ is more negative than ΔH_u suggests an extended bridgework of water molecules caught by the enzyme matrix though this must be achieved from a more extended structure of the polypeptide chain. This is supported by the entropy data below.

The entropy of coformycin binding, ΔS_b, at 4°C is 502 ± 29 J/mole–K and the equilibrium unfolding entropy difference, ΔS_u, is -3002 ± 332 J/mole–K. We calculate $\Delta S^{\neq}_{u(ADA)} = -1957 \pm 405$ J/mole–K and $\Delta S^{\neq}_{u(ADA/cof)} = -4279 \pm 170$ J/mole–K. Values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ which are both negative and large in magnitude point to a major nonpolar effect in the unfolding transition state.

Finally, we turn to a discussion of heat capacity effects at 4°C. At this temperature, ΔC_b is -4.7 ± 0.8 kJ/mole–K and the overall heat capacity change of ADA unfolding ΔC_u is 73 ± 5 kJ/mole–K. We calculate $\Delta C^{\neq}_{u(ADA)}$ to be 20 ± 1 kJ/mole–K and $\Delta C^{\neq}_{u(ADA/cof)} = 54 \pm 1$ kJ/mole–K. These data offer further support that extensive nonpolar amino acid side chain interactions with solvent water characterize the transition state.

DISCUSSION

In understanding the temperature dependence of the denaturation of ADA and coformycin-complexed ADA several points become clear. The thermodynamics of unfolding are very dependent upon both the bound state of the enzyme and on the

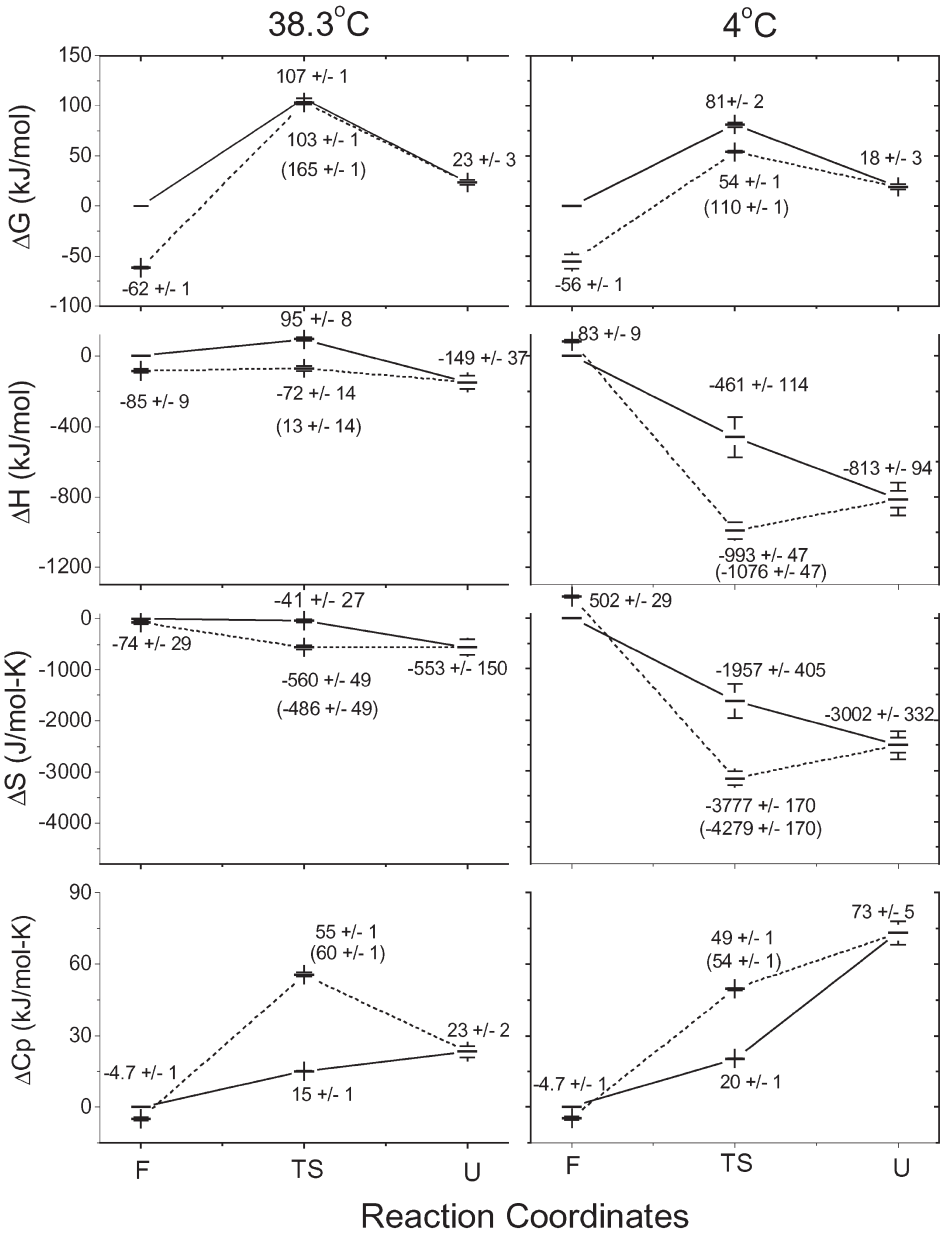

FIGURE 6 Thermodynamic changes in the unfolding of ADA (solid lines) and of coformycin-complexed ADA (dashed lines) from 100 mM phosphate buffer, pH = 6.3 from the folded state (F), through the transition state (TS), to the final, unfolded state (U). All plots are made relative to folded ADA = 0 for the particular thermodynamic value of interest. Numbers in parentheses are the activation parameters for the complex relative to the folded form of the complex set to zero. Corresponding numbers outside of parentheses are the activation parameters relative to free ADA set to zero.

particular folded form of the enzyme; i.e., whether unfolding occurs above or below ~29°C. Binding of coformycin has clear effects in either temperature regime with these effects being more pronounced in the physiological temperature regime. The thermodynamic analysis performed here confirms a parallel study which indicates that the low-temperature form of the enzyme is more compact structurally and stabilized to a greater extent by nonpolar interactions.[9] Finally, a qualitative comparison of the trends observed in Fig. 6 with Fig. 1 suggest that coformycin binding is much more local at low temperature than at the physiological temperature.

We now used the transition state free energies associated with denaturation of the free and bound enzyme forms to calculate the partitioning of binding energy between local and global contributions. Recalling that the binding partition which is global may be calculated from $P_B^{global} = -(\Delta G^{\neq}_{dn\,(ADA/cof)} - \Delta G^{\neq}_{dn\,(ADA)})/\Delta G_b$ we calculated $P_B^{global} = 0.92$ at 38.3°C and $P_B^{global} = 0.52$ at 4°C. While it is possible to model the docking of transition state analogues into rigid enzyme active sites to obtain similar binding

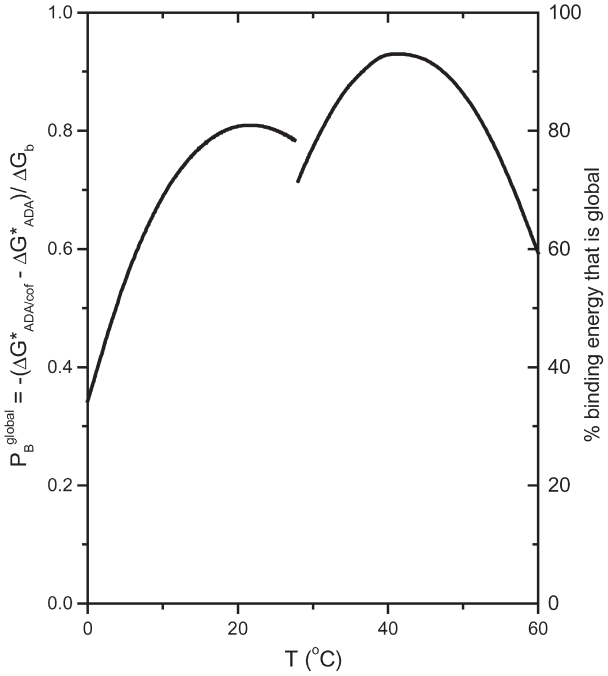

FIGURE 7 Temperature dependence of P_B^{global} for coformycin binding to ADA.

energies as observed here, we suggest that our data point to a stabilizing enzyme conformational change resulting from binding with the effect far greater at near the physiological temperature than at any other temperature (Fig. 7).

ADA is known to stabilize the transition state for deamination of adenosine by 70 kJ/mole.[20] If it is assumed that coformycin is an adequate representation of the true transition state of the reaction, then the present study suggests that ~92% of the 62 kJ/mole of binding energy of the reaction transition state, or ~57 kJ/mole, results from the adoption of a stabler enzyme conformation. This leaves only ~13 kJ/mole for transition state stabilization to arise from purely active site stabilizations (Fig. 8). The mechanism by which the enzyme assumes this stabler state is currently unknown. As the London force which governs the interactions between nonpolar surfaces is known to depend upon the sixth power of interatomic distance we speculate that it may involve only a slight optimization of nonpolar contacts within the enzyme interior. We are currently attempting to better understand the nature of this transition.

To conclude, we have presented a general method for discerning the local and global contributions in the interaction of a tight binding ligand to a protein receptor. For the specific case of the binding of coformycin to ADA, we observe that coformycin binding at the physiological temperature results overwhelmingly from the adoption of a much stabler enzyme conformation. We propose that the characteristics observed in the interaction of ADA with coformycin may generally extend to all enzyme/-transition state analog interactions and that the adoption of a stabler conformation by the enzyme at the transition state of reactions the enzymes have evolved to catalyze may be a general feature of enzyme catalysis.

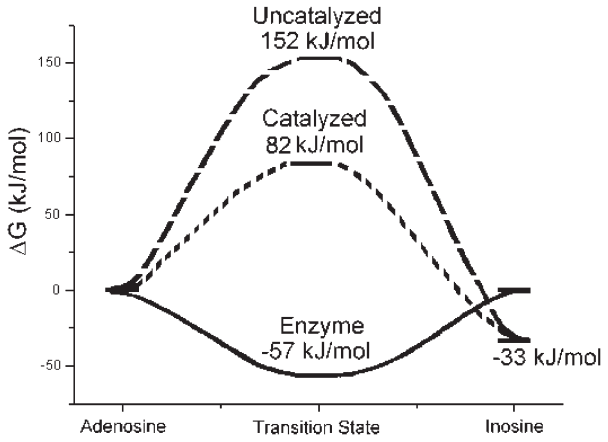

FIGURE 8 Free energy changes for the deamination of adenosine by ADA. Dashed line is the reaction profile for the uncatalyzed reaction. Dotted line is the reaction profile for the ADA-catalyzed reaction. The activation energy for the catalyzed reaction was calculated from $\Delta G_u^{\neq} = -RT\ln(k_{cat}h/k_BT)$ where k_{cat} for the deamination of adenosine by ADA at 38.3°C is 99 s^{-1}.[21] The noncatalyzed activation energy is set to 70 kJ/mole above the catalyzed activation energy.[20] The solid line suggests that the 70 kJ/mole reduction in the activation free energy for the ADA-catalyzed reaction is facilitated by a 57 kJ/mole reduction in the enzyme conformational energy at the reaction transition state.

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