J. Enzyme Inhibition, 2001, Vol. 16, pp. 217–232
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Binding Thermodynamics of the Transition State Analogue Coformycin and of the Ground State Analogue 1-Deazaadenosine to Bovine Adenosine Deaminase

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(Received 21 June 2000)

Binding of the transition state analogue coformycin and the ground state analogue 1-deaazadenosine to bovine adenosine deaminase have been thermodynamically characterized. The heat capacity changes for coformycin and 1-deazaadenosine binding are -4.7 ± 0.8 kJ/mole-K and -1.2 ± 0.1 kJ/mole-K, respectively. Since the predominant source of heat capacity change in enzyme interactions are changes in the extent of exposure of nonpolar amino acid side chains to the aqueous environment and the hydrophobic effect is the predominant factor in native structure stabilization, we propose that the binding of either class of ligand is associated with a stabilizing enzyme conformational change with coformycin producing the far greater effect. Analysis of the *T* dependence of the second order rate constant for formation of the enzyme/coformycin complex further reveals that the conformational change is not rate limiting. We propose that the enzyme may facilitate catalysis via the formation of a stabilizing conformation at the reaction transition state.

Keywords: Adenosine deaminase, Coformycin, 1-deazaadenosine, Conformational change, Enzyme catalysis, Competitive inhibitors

INTRODUCTION

The conventional (Haldane) view of enzyme catalysis maintains that enzymes have evolved active sites which are complementary to the transition states of the reactions they catalyze.[1] Although the induced fit modification[2,3] recognizes that interaction of the substrate with the active site must result in a local perturbation of the enzyme, it is generally considered that enzyme tertiary structure plays only a passive role in the enzyme catalytic event – namely, one of maintaining the active site in a sole, transition state complementarity. A corollary to this model states that it is catalytically advantageous to bind the substrate ground state weakly[4,5] since interaction with the transition state is necessary and the enzyme cannot display multiple complementarities. The Haldane view therefore leaves

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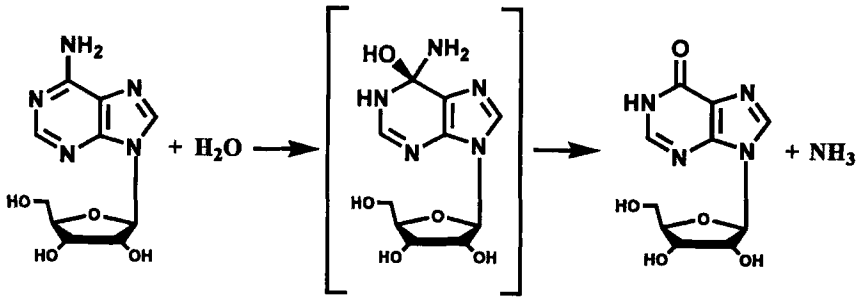
us with a picture of enzymes as highly efficient yet strangely static and passive catalysts: any ligand which can wander into the active site and which is also capable of undergoing the transformation dictated by the active site environment is a substrate. This conventional view of enzyme catalysis has been criticized and arguments suggesting that strong ground state binding may, in fact, facilitate catalysis have been offered.[6–8]

Our purpose here is to present further evidence that the enzyme adenosine deaminase (ADA) does not exist initially in a conformation complementary to the transition state analogue (TSA) coformycin[9,10] but is induced into one only after interaction of the inhibitor with the active site, to quantitate the extent of the global conformational change that results from TSA binding, and to discuss the significance of these findings as they relate to general enzyme catalysis. If the TSA is an adequate representation of the true transition state of the reaction catalyzed by the enzyme then the results reported here further challenge the notion that enzymes have evolved exclusively transition state-complementary active sites. To place the binding thermodynamics of the TSA into perspective, we also report parallel studies of the binding of the ground state analogue (GSA) 1-deazaadenosine to the enzyme.

We choose this particular system for the reasons that it is well studied and ADA is a relatively small (~ 40 kDaltons), single subunit enzyme. We consider this last aspect important for this seminal investigation since it is anticipated that the extent of enzyme global rearrangement that may result from ligand binding is a function of the relative size of the enzyme to ligand.[8] ADA catalyzes the conversion of the physiological substrates adenosine and deoxyadenosine to inosine and deoxyinosine via the formation of an sp^3-hybridized-like transition state at the C-6 position (Figure 1). It is thought that coformycin is a strong competitive inhibitor (K_I of the order of 10^{-11} M over the T range of the present study) due to the similarity of the geometry about its C-8 to that of the proposed transition state.[11,12] 1-Deazaadenosine is considered to be a ground state analogue due to its lack of an N-1 in the purine ring which facilitates attack of an hydroxide ion to the C-6 position.[13]

From a straightforward thermodynamic analysis of the T dependence of the binding constant (K_b) describing the reversible association for either the TSA or the GSA with ADA, values of ΔH, ΔS, and ΔG° are determined. It is not possible to use isothermal titration calorimetry to obtain this information due to the high affinity of the TSA for the enzyme. ΔH for the ADA/TSA interaction is much more T dependent than for the ADA/GSA interaction indicating that the former process is associated with a significantly greater heat capacity change (ΔC_p). Since the dominating factor in heat capacity changes involving enzyme interactions are changes in the exposure of nonpolar amino acid side chain surface area to the aqueous environment, the ΔC_ps can be expressed in terms of the reduction in amino acid side chain nonpolar surface area, ΔA_{np}, to provide a quantitative measure of the extent of enzyme conformational change.[14,15] As it is accepted that side chain nonpolar interactions are the major factor in stabilizing enzyme native structure we therefore associate a significantly negative ΔC_p with the formation of a much stabler enzyme conformation in the complex. In addition to understanding the overall thermodynamic changes in the interactions, we also determined the transition state thermodynamics for the slow binding TSA from measurements of the T dependence of the second order association rate constant and the application of transition state theory.

We find that the ADA/TSA interaction is described by a $\Delta C_p = -4.7 \pm 0.8$ kJ/mole-K. The heat capacity change upon achieving the transition state for binding of the TSA, $\Delta C_p^{\neq}$, is apparently zero, based upon the linearity of the Arrhenius plot of the second order association rate constant. We therefore conclude that binding



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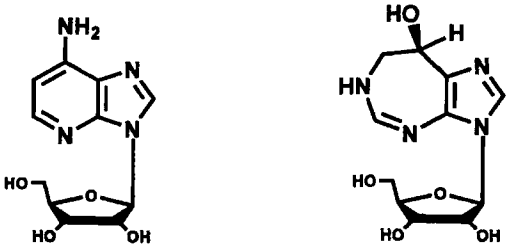

1- Deazaadenosine **Coformycin**

GSA ***TSA***

FIGURE 1 Reaction scheme for the deamination of adenosine by adenosine deaminase. 1-Deazaadenosine (a ground state analogue) and coformycin (a transition state analogue) each competitively inhibit the reaction.

of the TSA results in a significantly stabler enzyme conformation and that this conformational change is not a barrier to the binding reaction. Conversely, the ADA/GSA interaction is described by a $\Delta C_p = -1.2 \pm 0.1$ kJ/mole-K. As GSA binding apparently results in much less of a conformational alteration than TSA binding we conclude that the enzyme active site is initially optimized more for interaction with the ground state of the substrate rather than with the transition state. These results therefore challenge the conventional view of enzyme catalysis. Instead, the data support a model for general enzyme catalysis which requires an initial ground state complementarity and the facilitation of catalysis by the assumption of a stabler enzyme conformation at the reaction transition state.[7,8]

MATERIALS AND METHODS

Reagents

Adenosine and ADA from calf spleen as an ammonium sulfate suspension were from Sigma Chemical Company (St. Louis, MO). Coformycin was a gift from Parke-Davis (Ann Arbor, MI). 1-Deazaadenosine was a gift from Dr. Linda Kurz of Washington University. All reactions were performed in 10 mM phosphate buffer at pH = 6.3, the pH optimum of the enzyme.

Instrumentation

Measurements of the steady-state turnover of adenosine substrate by either TSA- or GSA-inhibited ADA (in determining the overall thermodynamic changes) and of the binding of coformycin to ADA (in determining the transition state thermodynamics) were performed on an Applied Photophysics stopped flow reaction analyzer (model SX-17MV) equipped with a Neslab RTE-111 circulating water bath. Temperatures were measured at the moment of mixing from the water reservoir which bathed the sample syringes and reaction cell and are accurate to within 0.1°C. Reaction rates were determined with the instrument software. Subsequent mathematical manipulations were performed with Microcal Origin (v. 4.10).

Temperature Dependence of K_b for TSA and GSA Binding to ADA

Determinations of the association constant K_b ($=1/K_I$) were made using the relation of Henderson developed for the study of steady state kinetics with tightly bound inhibitors,[17]

$$I_t/(1 - v_i/v_0) = E_t + K_I(1 + S/K_M)v_0/v_i, \quad (1)$$

where I_t is the total inhibitor concentration (13.5 nM for coformycin, 3.0 μM for 1-deazaadenosine), E_t is the total enzyme concentration (4.5 nM), v_i is the initial reaction velocity in the presence of inhibitor, v_0 is the initial reaction velocity in the absence of inhibitor, K_M is the Michaelis constant, K_I is the enzyme/ligand dissociation constant, and S is the substrate concentration (50 μM) (all concentrations are after mixing). K_M values used were from a previous T-dependent Michaelis-Menten analysis of the ADA/adenosine interaction.[18] Reaction progress was followed by monitoring the decrease in A_{260} as adenosine is converted to inosine. For assays with coformycin, enzyme and inhibitor were pre-equilibrated and combined with the substrate solution which contained an inhibitor concentration equal to that in the enzyme/inhibitor solution. To offset the acid instability of coformycin, a stock solution was prepared at pH = 10 and stored frozen. The extent of decomposition of the drug at pH = 6.3 on the time scale of our measurements is negligible (personal communication from Parke-Davis). For assays involving 1-deazaadenosine, enzyme was combined with the substrate/inhibitor mixture.

Temperature Dependence of the 2nd Order Rate Constant for TSA Binding to ADA

The reaction of 7.0 nM ADA with 9.0 nM coformycin was followed by monitoring the intrinsic enzyme fluorescence emission decrease at 340 nm with excitation at 280 nm. A previous study found this combination of excitation and emission wavelengths suitable for studying the binding of deoxycoformycin to ADA[19] and survey scans of ADA and coformycin-bound ADA (data not shown) reveal that this wavelength combination is also ideal for the present study. Since the binding of the TSA is very slow and the overall binding affinity is very large, the contribution of complex dissociation to the observed binding reaction is negligible. Second order rate constants are then calculated by standard methods assuming the equilibrium ADA + coformycin $\rightleftharpoons$ ADA · coformycin and knowledge of the T dependence of K_b. Rate constants were determined from five sets of averages of at least three kinetic runs at temperatures of 5.5, 10.0, 15.0, 20.0, 22.0, 24.0, 25.0, 26.0, 28.0, 30.0, 35.0, and 40.0 °C.

RESULTS

Given the truly reversible nature of the binding of the competitive inhibitors to ADA, it is possible to thoroughly describe the binding thermodynamics from an analysis of the T dependence of K_b, the association binding constant, for each

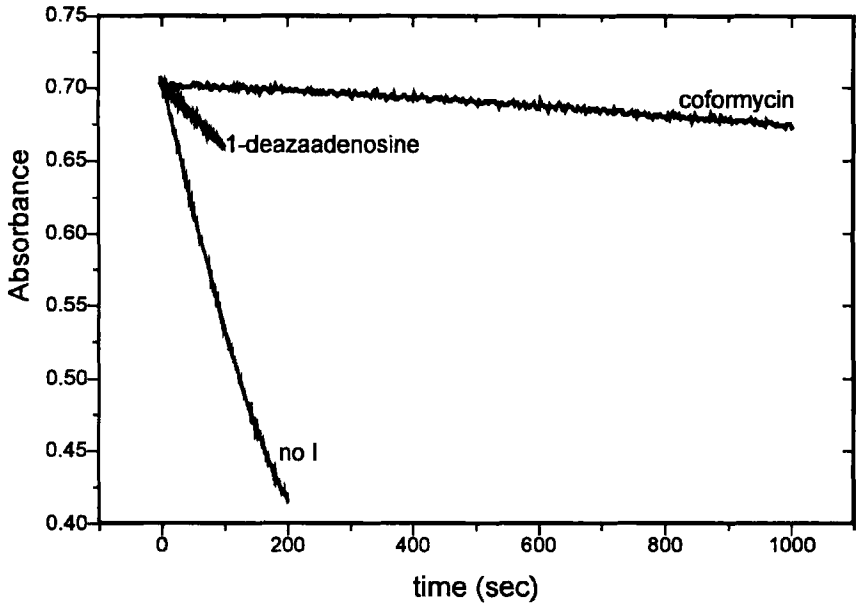

FIGURE 2 Representative kinetic traces of the ADA-catalyzed deamination of adenosine for the uninhibited, the 1-deazaadenosine-inhibited and the coformycin-inhibited enzyme at 25.0 °C. Conditions are as in "Materials and Methods".

interaction. Values of K_b are easily obtained from a measure of the ability of each inhibitor to inhibit the conversion of adenosine to inosine by the enzyme (Equation (1)) with a knowledge of the T dependence of K_M, the Michaelis constant describing the ADA/adenosine interaction. Figure 2 shows sample kinetic traces for the GSA- and TSA-inhibited enzymes. The trace which results from the combination of substrate and 1-deazaadenosine with the enzyme is typical of a weak, rapidly binding inhibitor such as a GSA – the onset of inhibition is immediate yet all the substrate is consumed after a relatively short amount of time (data not shown). When a mixture of the substrate and the TSA is combined with the enzyme, on the other hand, there is initially no inhibition of the reaction though the ligand has a much greater affinity for the active site than the GSA.[9,10] Soon, however, given an inhibitor concentration approximately equal to that of the enzyme, the reaction is all but shut down and complete substrate depletion occurs only after a very long time. This is typical of enzyme/TSA interactions. Attempts to obtain inhibited rates for use in Equation (1) from a procedure that combines an enzyme that has not achieved binding equilibrium with its TSA is therefore unsuitable for determining binding constants for high-affinity ligands. We find this difficulty is surmounted by pre-equilibrating the enzyme with coformycin and then initiating the reaction by combining this solution with a substrate solution containing an equal concentration of coformycin. Since in the process of mixing the ADA concentration halves while the coformycin concentration remains constant it is possible that K_b values are underestimated since at equilibrium more enzyme will be complexed. However, the agreement of our data with a previous study tends to validate the method. Frieden and coworkers determined K_b for the ADA/coformycin interaction at pH = 7.0 and 20 °C as $4.5 \times 10^9\,M^{-1}$.[9] This compares favorably with our value of $9.3 \times 10^{10}\,M^{-1}$ determined at the same temperature and pH = 6.3. Kurz and coworkers report an association constant for the ADA/1-deazaadenosine interaction at pH = 7.5 and 20 °C of $K_b = 5.56\,\mu M^{-1}$,[13] in good agreement

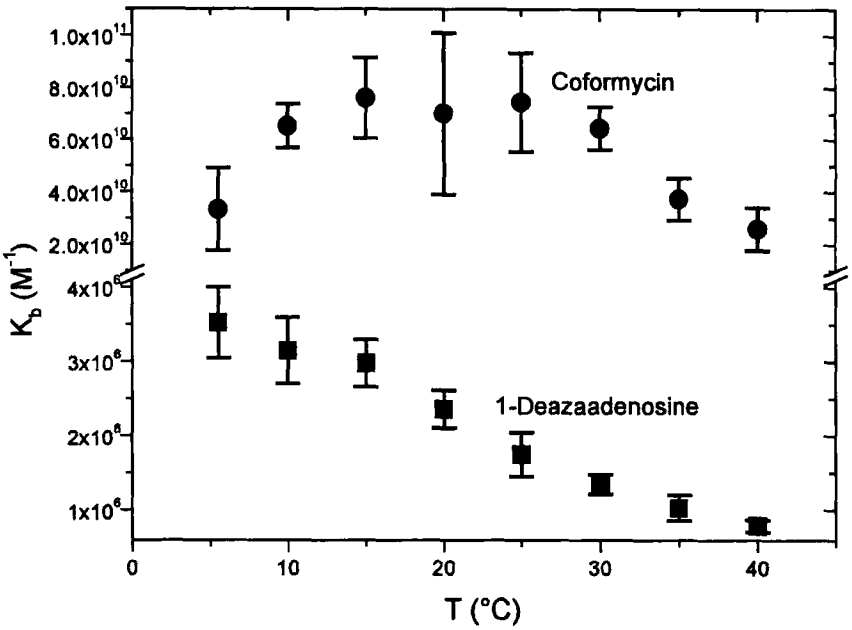

FIGURE 3 T dependence of the association constants for coformycin and 1-deazaadenosine binding to ADA. Results are the averages of at least five separate determinations, each the average of at least three kinetic scans such as shown in Figure 2. Error bars represent the uncertainties in terms of single standard deviations.

with our determination of $2.35 \pm 0.25\,\mu M^{-1}$ at pH = 6.3 and the same temperature. Figure 3 shows the T dependence of the association constants for TSA and GSA binding to ADA. The binding affinity of the TSA maximizes at a T well below the physiological T. The significance of this has been discussed.[10]

Next, the overall thermodynamic changes were determined in the ADA/TSA interaction from a consideration of the T dependence of $\ln(K_b)$ (Figure 4). The van't Hoff plot in Figure 4 shows a clear nonlinear trend indicative of a T-dependent ΔH and, therefore, a nonzero ΔC_p. Apparent values for ΔH, ΔS, $\Delta G°$, and ΔC_p are determined as follows. The data is first recast using the Gibbs-Helmholtz relation in the form,

$$\left[\partial(\Delta G°/T)/\partial(1/T)\right]_p = \Delta H \qquad (2)$$

with

$$\Delta G° = -RT\ln(K_b) \qquad (3)$$

(Figure 4). Rather than assuming an explicit dependence of the data on ΔH and ΔC_p we simply observe that the data are well modeled by a quadratic equation and that polynomials of higher order do not significantly improve the fit. Calculation of the derivative of this fit is then very straightforward and gives the linear dependence of ΔH on T (Figure 5). Using the standard assumption that the heat capacity change is constant over the T range of interest here, ΔC_p then follows from

$$\Delta C_p = \Delta(\Delta H)/\Delta T \qquad (4)$$

This analysis gives $\Delta C_p = -4.7 \pm 0.8\,kJ/mole\text{-}K$. $T\Delta S$ is then calculated from

$$T\Delta S = (\Delta H - \Delta G°). \qquad (5)$$

Figure 5 illustrates the compensatory interaction between enthalpy and entropy in the binding process. This observed enthalpy/entropy compensation is typical in binding processes

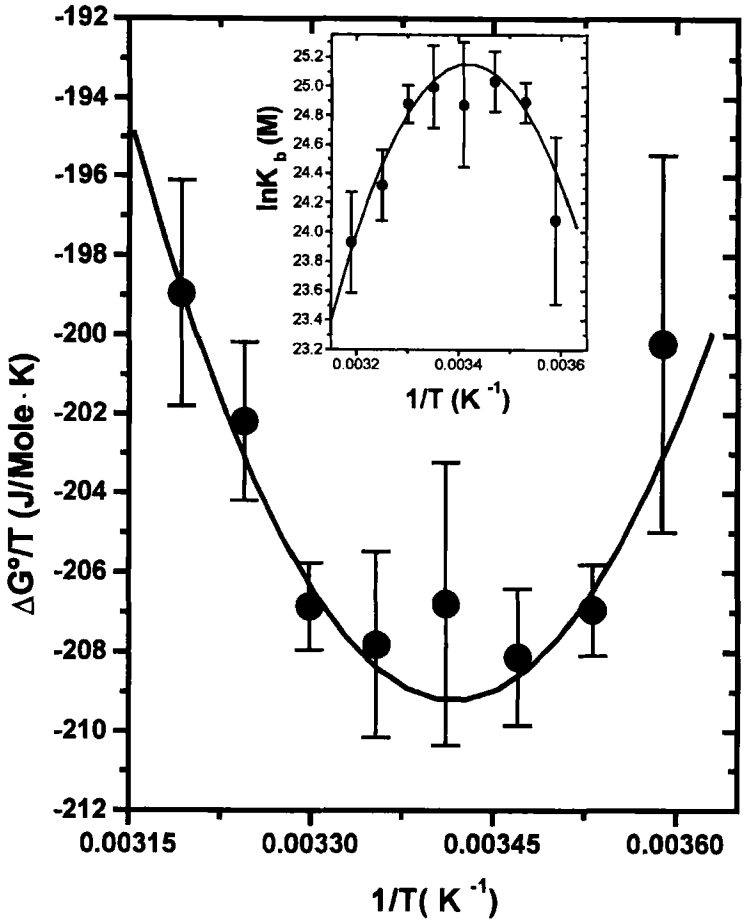

FIGURE 4 Plot of $\Delta G^\circ/T$ vs. $1/T$ calculated from the T dependence of K_b for coformycin binding to ADA. Error bars represent the uncertainties expressed in single standard deviations. Also shown is a quadratic fit to the data. Inset: a van't Hoff plot of the data.

involving enzymes[20–25] and reflects the large changes in heat capacity typically observed.

Change in the extent of exposure of nonpolar amino acid side chain groups to the aqueous environment is the overwhelming contributor to heat capacity changes in processes involving globular proteins.[15,26] The mechanism for altering the degree of exposure is enzyme conformational change with folding and unfolding constituting extreme cases. A decrease in heat capacity occurs when previously exposed groups lose some or all of their bound, immobile water molecules and become situated more in the enzyme interior, in better contact with other nonpolar groups; an increase corresponds to a net solvent exposure of previously partially or fully buried nonpolar groups. If this mechanism for heat capacity change is correct then there should exist a relationship between ΔC_p and change in water-accessible nonpolar surface area. Record and co-workers have discovered and quantitated this interrelationship as

$$\Delta C_p = -(0.25 \pm 0.03)\Delta A_{np}, \qquad (6)$$

where ΔC_p is expressed in cal/mole-K and ΔA_{np} is the reduction in water-accessible nonpolar surface area, expressed in A^2.[14,15] The

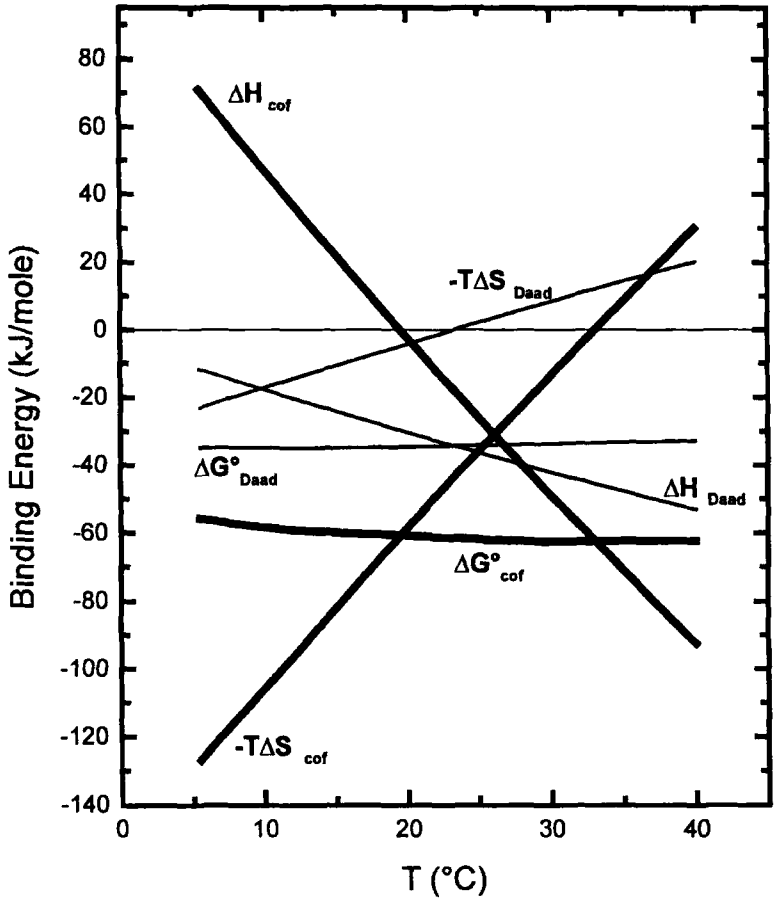

FIGURE 5 Temperature dependence of the energy breakdown for ADA/TSA binding (thicker lines) and ADA/GSA binding (thinner lines).

decrease in water accessible nonpolar side chain surface area that results from coformycin binding to ADA is apparently $(4.5 \pm 0.8) \times 10^3 A^2$.

This is an enormous decrease and is far greater than can be accounted for by a simple docking of the inhibitor in the active site. This decrease in the heat capacity therefore suggests a major conformational rearrangement in the enzyme in which partially or fully exposed nonpolar side chains become situated more in the enzyme interior. As it is generally accepted that the hydrophobic effect is the dominant stabilizing force in native enzyme structure, these results are therefore consistent with the assumption of a much stabler enzyme conformation in coformycin-bound ADA relative to free ADA. Such correlations in enzyme/ligand interactions have been previously noted. For example, it was concluded that the binding of hirudin to thrombin resulted in what amounted to a folding transition based upon the large, negative heat capacity change for binding (−1.7 kcal/mole-K)[27] and it was found that binding of a TSA to glutamine synthetase produced more enzyme conformational change than binding of other ligand types.[28] TSA binding has also been observed to increase the temperature of enzyme thermal denaturation for glutamine synthetase[29] and β-lactamase[30] and we find that the temperature of irreversible thermal denaturation of ADA

increases from 77.0 ± 0.2 °C to 92.3 ± 0.4 °C when complexed with coformycin.[31] It has been previously noted that either coformycin or the similar transition state analogue deoxycoformycin greatly decreases the accessibility of ADA tryptophan side chains to acrylamide quenching.[19] We have also observed that complexation of ADA with coformycin decreases the number of binding sites but increases the average affinity of the hydrophobic probe 1-anilino-8-napthalenesulfonate for the enzyme, consistent with the formation of a tighter conformation in the complex.[32] All these observations lead us to conclude that coformycin binding to ADA results in an enzyme conformational change that more efficiently sequesters nonpolar amino acid side chain residues into the enzyme interior, resulting in a significantly stabler conformation.

Next, the thermodynamic changes associated with formation of the transition state for coformycin binding to ADA were examined from an analysis of the T dependence of the second order rate constant describing the association. Figure 6 shows representative traces of coformycin binding at several temperatures from 5.5 to 40.0 °C as measured by the rate of quenching of intrinsic enzyme fluorescence emission upon TSA binding. The observed process was clearly first order with respect to either reactant by the method of initial rates as determined at 15.0, 25.0, and 35.0 °C (data not shown). We find that the data at all temperatures are well modeled by single exponentials. This is a bit surprising as we anticipated the association would be a three-state process. However, scans at much shorter times than the ones presented here failed to reveal an extra step. The simplest interpretation is that binding appears to be an overall second order, two-state process. The simplest mechanism consistent with this data is therefore: ADA + coformycin $\rightleftharpoons$ ADA · coformycin.

Figure 7 shows the Arrhenius plot of the second order binding rate constant k_{on}. A lot of data was collected around 30 °C because it was suspected that the overall trend may not be necessarily linear as we have previously noted an inflection here in the steady state hydrolysis of adenosine substrate by the enzyme.[10] However, there was no evidence for any discontinuity and the data are well modeled by a linear fit.

Assuming a single-step binding mechanism, the transition state thermodynamic parameters are straightforwardly determined from standard transition state theory. The enthalpy change associated with obtaining the binding transition state, $\Delta H^{\neq}$, is calculated from

$$\Delta H^{\neq} = E_a - RT, \tag{7}$$

where E_a is the apparent Arrhenius activation energy, calculated from the slope of the Arrhenius plot (slope $= -E_a/R$) and R is the gas law constant. The Gibbs free energy change upon assuming the binding transition state, $\Delta G^{\neq}$, is calculated from

$$\Delta G^{\neq} = -RT \ln \left[k_{on} h / k_B T\right], \tag{8}$$

where h is Planck's constant and k_B is Boltzmann's constant. The entropy change upon forming the binding transition state, $\Delta S^{\neq}$, then follows from

$$\Delta S^{\neq} = (\Delta H^{\neq} - \Delta G^{\neq})/T. \tag{9}$$

We are concerned mostly with the overall binding heat capacity change (ΔC_p) but it is also of interest to determine the change in heat capacity associated with achieving the transition state ($\Delta C_p^{\neq}$). $\Delta C_p^{\neq}$ is negligible as revealed by the linearity of the Arrhenius plot (Figure 7); i.e., recasting the data in a plot of $\Delta G^{\neq}/T$ vs. $1/T$ is linear indicating a constant $\Delta H^{\neq}$ and therefore a zero $\Delta C_p^{\neq}$ over the temperature range of 5.5 to 40.0 °C. The simplest interpretation is that reduction in amino acid side chain nonpolar surface area in achieving the binding transition state, $\Delta A_{np}^{\neq}$, is therefore also zero although a conformational change with equally offsetting positive and negative contributions to $\Delta C_p^{\neq}$ cannot be ruled out by the present data.

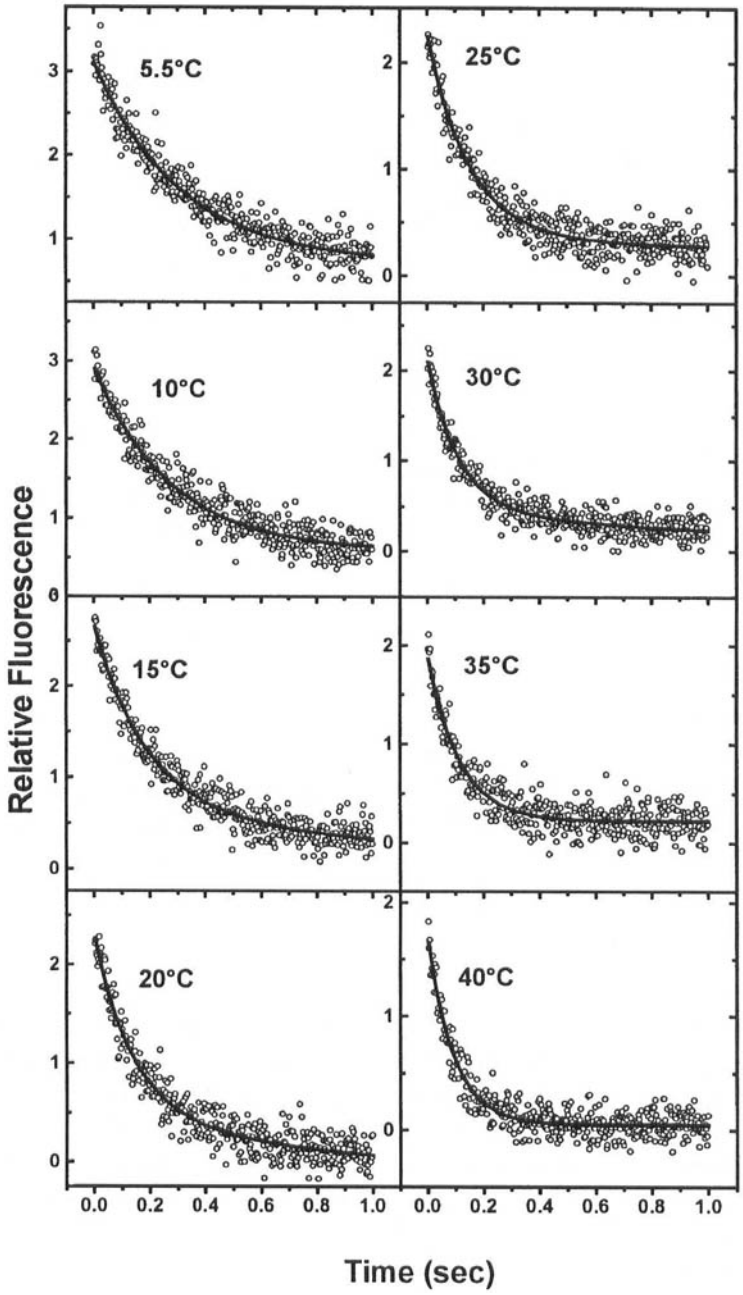

FIGURE 6 Representative kinetic traces of the binding of coformycin to ADA as determined by stopped flow fluorescence spectroscopy. Final concentrations after mixing: [ADA] = 7.0 nM, [coformycin] = 9.0 nM. Also shown are single exponential fits to the data. Excitation $\lambda = 280$ nm; emission $\lambda = 340$ nm. The kinetic traces are averages of at least three runs.

At 38.3 °C, the normal bovine body T, the overall coformycin binding thermodynamics are: $\Delta G° = -62 \pm 1$ kJ/mole, $\Delta H = -85 \pm 9$ kJ/mole, $-T\Delta S = 23 \pm 9$ kJ/mole (Figure 8). At the physiological T binding appears to be enthalpically driven, presumably by the favorable gain in TSA/active site interactions and, most importantly, by favorable global inter-

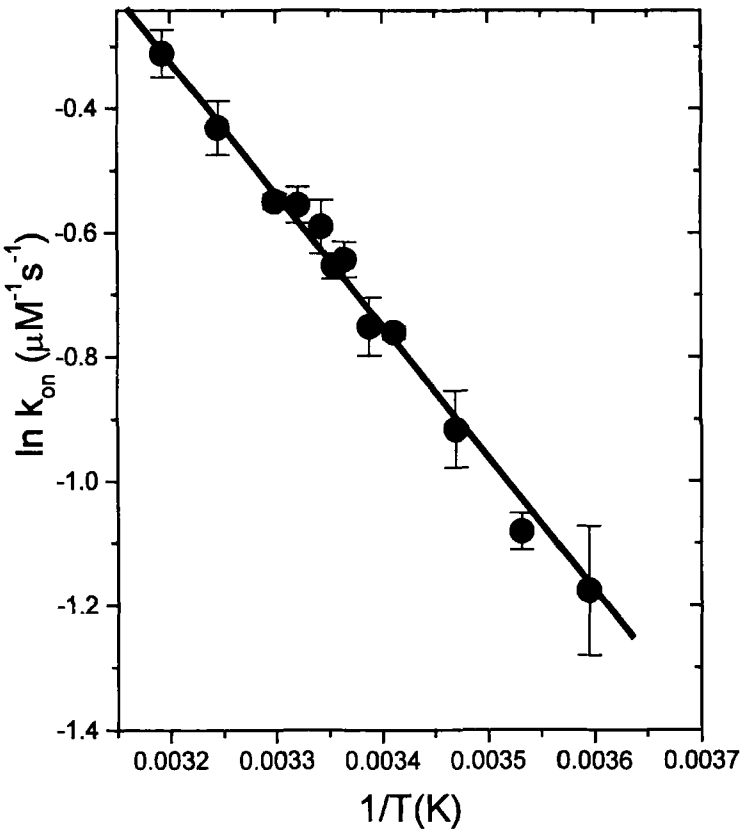

FIGURE 7 Arrhenius plot of the second order rate constant for coformycin binding to ADA. Each point represents the average of five data sets. Error bars are uncertainties expressed in terms of single standard deviations. Also shown is a linear fit to the data.

actions that result from the stabilizing enzyme conformational change. The entropy change does not favor binding. If there is in fact a stabilizing conformational change in the enzyme, then it appears that the loss in conformational entropy offsets significantly the entropy increase gained by the liberation of nonpolar surface area-complexed water molecules. At the physiological T, values for the transition state thermodynamics are: $\Delta G^{\neq} = 42 \pm 1$ kJ/mole, $\Delta H^{\neq} = 16 \pm 2$ kJ/mole, $-T\Delta S^{\neq} = 26 \pm 2$ kJ/mole. The transition state thermodynamics are therefore typical of a simple binding reaction with the positive $\Delta H^{\neq}$ reflecting the usual bond distortions that occur when two molecular force fields encounter one another and the negative $\Delta S^{\neq}$ reflecting the association of the two molecules. The zero $\Delta C_p^{\neq}$ suggests no significant reordering of the enzyme tertiary structure in the transition state. We therefore conclude that the transition state energy is composed primarily of local (active site) interactions between ADA and coformycin and that the subsequent conformational rearrangement is not a barrier to binding.

Finally, we turn to the determination of the heat capacity change in the binding of the ground state analogue 1-deazaadenosine to ADA. Figure 9 shows a Gibbs-Helmholtz plot for the binding constant K_b describing the interaction between 1-deazaadenosine and ADA. As with the ADA/coformycin binding data, the T dependence of this data is well modeled by a quadratic fit, though the curvature here is much less pronounced. The nonlinear trend of the data

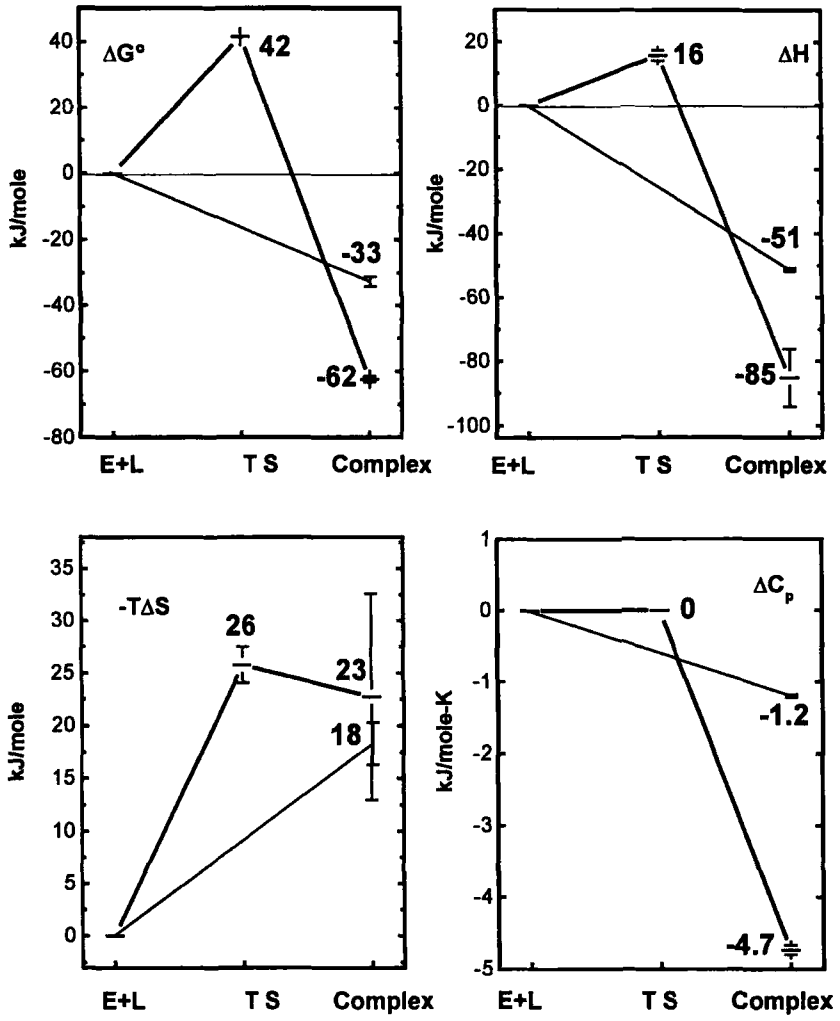

FIGURE 8 Binding thermodynamics for formation of the ADA/TSA (thicker line) and ADA/GSA (thinner line) complexes at 38.3 °C.

reveals a compensatory interaction for ΔH and ΔS (Figure 5) and a nonzero ΔC_p ($\Delta C_p = -1.2 \pm 0.1$ kJ/mole-K).

This ΔC_p corresponds to a ΔA_{np} of $(1.1 \pm 0.1) \times 10^3$ A^2. Thus, while interaction of the GSA with the enzyme apparently results in a stabilizing enzyme conformational change, the magnitude of this change is much less than for TSA binding. The thermodynamic parameters for GSA binding at 38.3 °C are: $\Delta G° = -33 \pm 1$ kJ/mole, $\Delta H = -51 \pm 1$ kJ/mole, $-T\Delta S = 18 \pm 2$ kJ/mole (Figure 8).

DISCUSSION

The Shifting Specificity model for enzyme catalysis[7,8] maintains that the free enzyme, at the physiological T, exists in a relatively high-energy state with an active site initially optimized for interaction with the ground state rather than the transition state. To a first approximation, then, it may be expected that binding of a GSA to an enzyme active site should be associated with a zero heat capacity change as this would indicate no conformational change resulting from bind-

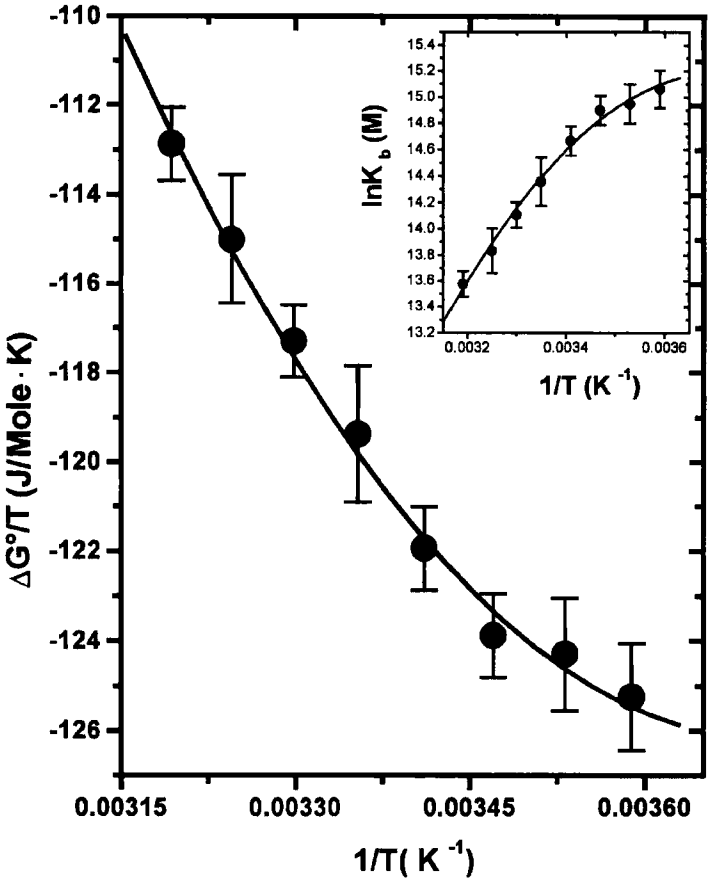

FIGURE 9 Plot of $\Delta G°/T$ vs. $1/T$ calculated from the T dependence of K_b for 1-deazaadenosine binding to ADA. Error bars represent the uncertainties, expressed in single standard deviations. Also shown is a quadratic fit to the data. Inset: a van't Hoff plot of the data.

ing. However, the model states the favorable enzyme/ground state interaction provides the activation energy for shifting the specificity of the active site, via a global conformation relaxation, to a transition state complementarity. As soon as the substrate binds, the enzyme undergoes the stabilizing conformational change which simultaneously transforms the substrate from the ground state to the transition state. We therefore attribute the nonzero heat capacity change observed in the ADA/GSA interaction to an arrested stabilizing enzyme conformational change resulting from the inability of the GSA to assume a transition state geometry. One possibility is that the enzyme conformation becomes trapped in some intermediate state between the free enzyme and the structure presumed to result from the binding of the true transition state. Another possibility is that the resulting geometry is completely catalytically irrelevant – an artefact resulting from the introduction of a ligand for which the enzyme has evolved no interaction. Conversely, binding of the TSA coformycin is associated with a substantially more negative heat capacity change indicating the formation of a much stabler enzyme conformation. Furthermore, this conformational change is apparently not rate limiting. It is highly unlikely that the observed differences in binding heat capacity of the two classes of ligands results primarily from local (only active site) interactions since the observed ΔC_ps are of an order of

magnitude greater than that typically observed for the transfer of small organic molecules from water to a nonpolar environment[33] and the ligands are structurally very similar.

The failure to detect a large conformational change in ADA upon binding the TSA by the application of traditional x-ray crystallographic techniques is inconsistent with the results presented here.[34,35] However, a major fault in the application of x-ray crystallography to structural studies of the interaction of enzymes with ligands is that the methodology assumes no major enzyme conformational changes upon introduction of the ligand to the active site. Crystals of enzymes with bound catalytically relevant ligands are typically formed from infusion of the ligands into preformed crystals. If binding of the ligand in solution results in a global conformational change it is very likely that it may not be realized in the crystalline state due to the crystal packing forces which favor the pre-existing conformation. This difficulty may also not be circumvented by forming ligand-complexed enzyme from solution by seeding with a pure enzyme crystal, as was done in forming the ADA/deoxycoformycin complex.[35] (Deoxycoformycin is a potent TSA similar in structure to coformycin – its structure differs only in the absence of an oxygen at the 2′-position of the ribose ring.) Another significant difficulty with the traditional application of x-ray diffraction in elucidating enzyme structure is the unfounded assumption that the structure which exists at crystal growth temperatures (typically 4 °C though occasionally as high as 25 °C) is the same which exists at the physiological T (~38 °C for mammals). It is known that several enzymes undergo sharp, reversible, T-dependent conformational transitions at temperatures between 4 °C and the physiological T: *Pseudomonas* urocanate hydrotase,[36] *Penicillium janthinellum* penicillopepsin,[37] porcine D-amino acid oxidase,[38] rabbit pyruvate kinase,[39] and yeast 3-phosphoglycerate kinase.[40] Other apparent T-induced conformational changes have also been observed.[41–43] Structures of these enzymes elucidated from traditional x-ray crystallographic techniques therefore cannot pertain to the physiological structure.

With these facts and ideas in mind, we would like to propose a mode of interaction of ADA with the two classes of inhibitors. Binding of the GSA to the enzyme produces a relatively small conformational change. As our results seem to confirm previous observations that the ADA active site appears to be initially complementary to the reaction ground state rather than the transition state,[9] we suggest the following mode of interaction between ADA and 1-deazaadenosine:

$$E_{GS} + GSA \rightleftharpoons E^{*}_{GS} \cdot GSA$$

Here, E_{GS} is the conformation of the free enzyme at the physiological T (presumed to be complementary to the reaction ground state), GSA is the ground state analogue, and $E^{*}_{GS} \cdot GSA$ is the bound complex. The asterisk emphasizes the uncertainty concerning the catalytic relevancy of the conformation. The binding of coformycin is similarly described by a scheme:

$$E_{GS} + TSA \rightleftharpoons E_{TS} \cdot TSA$$

where TSA is the transition state analogue and the resulting complex is one in which the enzyme-localized energy is substantially lower than the free enzyme energy and the active site has relinquished its specificity for the ground state in favor of the transition state (E_{TS}).

To extend this description to turnover of adenosine substrate, the scheme becomes:

$$E_{GS} + GS \rightleftharpoons E_{TS} \cdot TS \rightleftharpoons E_{GS} + P$$

Here, GS and TS refer to the true ground and transition states of the substrate and P is the reaction product. Thus, the enzyme exists initially in a relatively high energy conformation with an active site geometry complementary to the ground state. Evolution has selected for an interaction of the substrate with the active site which necessarily results in a global conforma-

tional change that transforms the active site into a transition state complementarity. Occurring simultaneously with this conformational change is the transformation of the ground state to the transition state. Furthermore, evolution has selected for the adoption of an enzyme-localized energy minimum at the reaction transition state to facilitate catalysis. We propose that this mode of action is generally extendable to all enzyme catalyzed reactions for which the substrate ground and transition states possess distinct spatial geometries.

The failure to detect a large conformational change upon binding a TSA to ADA by x-ray crystallographic techniques is inconsistent with the results presented here and in earlier studies on the interaction.[31–33] In the Shifting Specificity model for enzyme catalysis, the free enzyme, presumed to possess a ground state complementarity, should ideally be on the thermodynamic brink of assuming the lower energy E_{TS}. Under normal (physiological) conditions the activation energy for this isomerization is provided by the substrate binding energy. We propose that in the absence of this binding energy, E_{TS} may be artificially induced by lowering the enzyme a few degrees below its physiological T. This idea arises from the repeated observation of breaks in Arrhenius plots for rates or rate constants describing the interactions of enzymes with their physiological substrates where a sharp, global conformational change in the enzyme is implicated as the source of the discontinuities. We therefore propose that the crystal studies performed on ADA with its catalytically relevant ligands are examining the E_{TS} since the studies are performed at 4 °C.[34,35] It is therefore not surprising that binding of deoxycoformycin does not produce a drastic conformational change. One may then expect that binding a GSA should induce a conformational change to the higher energy E_{GS} but this may be impossible due to the stabilization of the lower energy conformation by the crystal packing forces. Indeed, an informal survey of the literature reveals that binding of GSAs to enzyme crystals are more likely to crack or disrupt them than the binding of TSAs. We therefore propose that the x-ray crystallographic studies on ADA and of most other enzymes done by traditional methods are examining E_{TS} and not the physiological enzyme, E_{GS}. This has perpetuated what we feel is the incorrect notion that enzymes exist in their stablest conformations at physiological conditions and possess exclusive transition state complementarities.

Acknowledgments

We thank Dr. Linda Kurz for the gift of 1-deazaadenosine and for helpful discussion and Parke-Davis for the gift of coformycin. This work was supported by Baylor University and the Robert A. Welch Foundation.

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