Regulation of Tryptophan Synthase by Temperature, Monovalent Cations, and an Allosteric Ligand. Evidence from Arrhenius Plots, Absorption Spectra, and Primary Kinetic Isotope Effects

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ABSTRACT: To investigate the linkage between enzyme conformation and catalysis, we have determined the effects of temperature on catalytic properties of the tryptophan synthase $\alpha_2\beta_2$ complex and β_2 subunit in the absence or presence of different monovalent cations (Cs^+, Na^+, and GuH^+) and of an allosteric ligand, α-glycerol 3-phosphate. Arrhenius plots of the activity data between 5 and 50 °C are nonlinear in the presence of certain ligands but not others. The conditions that yield nonlinear Arrhenius plots also yield temperature-dependent changes in the equilibrium distribution of enzyme−substrate intermediates and in primary kinetic isotope effects. The results provide evidence that the nonlinear Arrhenius plots are caused by a temperature-dependent conformational change that precedes the rate-limiting step in catalysis. Thermodynamic analysis of the data associated with the conformational change shows that the activation energies are much higher at low temperatures than at high temperatures. We correlate the results with a model in which the enzyme is converted by increased temperature under certain conditions from a low-activity "open" conformation to a high-activity "closed" conformation. The allosteric ligand and different monovalent cations, including GuH^+, which also acts as a chaotropic agent, affect the equilibrium between the open and closed forms. The large positive entropy changes in the conformational conversion suggest that the closed conformation results from tightened hydrophobic interactions that exclude water from the active site of the β subunit.

Conformational changes play integral roles in both enzyme catalysis and allosteric regulation. Investigations of the effects of temperature on enzyme-catalyzed reactions are important tools for elucidating the mechanism of catalysis and the linkage between catalysis and conformational changes.

The tryptophan synthase $\alpha_2\beta_2$ complex (EC 4.1.2.20) has proved to be an important system for gaining insights into the relationships between protein structure, catalysis, and regulation (for reviews, see refs *1*−*4*). The three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* (*5*) revealed that the active sites of the α and β[1] subunits are ∼25 Å apart and are connected by a hydrophobic intramolecular tunnel. The tunnel is believed to facilitate the diffusion of indole from the active site of the α subunit, where it is produced by cleavage of indole-3-glycerol phosphate, to the active site of the β subunit, where indole reacts with L-serine to produce L-tryptophan (for a recent review of substrate channeling, see ref *6*). The reaction of L-serine with indole is a pyridoxal 5′-phosphate (PLP)[2]-dependent β-replacement reaction (reaction 1), which is also catalyzed by the isolated β_2 subunit.

$$\text{L-serine} + \text{indole} \rightarrow \text{L-tryptophan} + H_2O \qquad (1)$$

The $\alpha_2\beta_2$ complex and the β_2 subunit also catalyze a β-elimination reaction with L-serine (reaction 2). However, the $\alpha_2\beta_2$ complex has very low activity in reaction 2.

$$\text{L-serine} \rightarrow \text{pyruvate} + NH_3 \qquad (2)$$

Reactions at the β site proceed through a series of PLP intermediates that have characteristic spectroscopic properties (Scheme 1) (*7*−*10*). The reaction of the $\alpha_2\beta_2$ complex with L-serine yields the PLP aldimine of L-serine (E-Ser), which eliminates the α-proton to form a quinonoid intermediate, $E\text{-}Q_1$, followed by elimination of the hydroxyl group to yield the aldimine of aminoacrylate (E-AA) (stage I in reactions 1 and 2). In the absence of indole, an equilibrium mixture of the E-Ser (λ_{max} = 424 nm) and E-AA (λ_{max} = 350 nm) intermediates accumulates; E-AA is hydrolyzed to pyruvate and NH_3 by the β-elimination reaction (reaction 2). In the presence of indole, E-AA reacts with indole to form a quinonoid intermediate ($E\text{-}Q_2$), which is then protonated to form the aldimine of the product L-tryptophan (E-Trp)

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[1] The term β_2 subunit is used for the isolated enzyme in solution; β subunit is used for the enzyme in the $\alpha_2\beta_2$ complex, for a specific residue in the β subunit or for the dissociation of the α and β subunits.

[2] Abbreviations: GuHCl, guanidine hydrochloride; PLP, pyridoxal 5′-phosphate; GP, DL-α-glycerol 3-phosphate; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; E-Ser, external aldimine of L-serine; E-AA, external aldimine of aminoacrylate; E-Trp, external aldimine of L-tryptophan; $E\text{-}Q_1$ and $E\text{-}Q_2$, quinonoid intermediates; EIE*, apparent equilibrium isotope effect.

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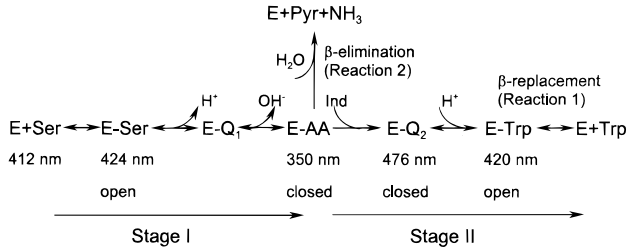
Scheme 1



(stage II in reaction 1). β-Chloro-L-alanine can substitute for L-serine in β-replacement and β-elimination reactions analogous to reactions 1 and 2.

Association of the α and β subunits increases activity in the β-replacement reaction with L-serine and indole (reaction 1) about 20-fold. We have proposed that association with the α subunit converts the β_2 subunit from a low activity, open conformation to a high activity, closed conformation (Table 1) (*11*). Association with the α subunit alters both the reaction specificity and the substrate specificity of the β_2 subunit (Table 1). The β_2 subunit (open) has approximately equal activities in β-replacement and β-elimination reactions whereas the $\alpha_2\beta_2$ complex (closed) has much higher activity in the β-replacement reaction than in the β-elimination reaction. Furthermore, β-chloro-L-alanine is a better substrate than L-serine for the β_2 subunit, whereas L-serine is a better substrate for the $\alpha_2\beta_2$ complex. Several $\alpha_2\beta_2$ complexes having single amino acid replacements in the β subunit have substrate and reaction specificities similar to those of the wild-type β_2 subunit, suggesting that the mutations stabilize the open form of the $\alpha_2\beta_2$ complexes (Table 1) (*11*). Solvents (*12*, *13*) and GuHCl or urea (*14*) also stabilize an alternative conformation (open) of the $\alpha_2\beta_2$ complex that has properties similar to those of the β_2 subunit.

The reaction of the β_2 subunit and $\alpha_2\beta_2$ complex with L-serine in the absence of a cosubstrate (i.e., indole) yields E-Ser and E-AA intermediates (Scheme 1). E-Ser is the predominant intermediate that accumulates under steady-state conditions with the β_2 subunit and mutant $\alpha_2\beta_2$ complexes (open), whereas E-AA is the predominant intermediate with the wild-type $\alpha_2\beta_2$ complex (closed) (Table 1 and Scheme 1). The equilibrium distribution of the E-Ser and E-AA intermediates is altered by a number of factors including pH, temperature and α subunit ligands (*15*), mutations (*11*, *16*−*18*), GuHCl or urea (*14*), solvents (*12*, *13*), and different cations (*16*, *19*). Most of the conditions that favor the low activity, open conformation (i.e., Na^+, K^+, mutations, solvents, GuHCl and urea) also result in accumulation of the E-Ser intermediate (Table 1).

Other solution studies of the tryptophan synthase $\alpha_2\beta_2$ complex provide evidence for ligand-mediated interactions between the α and β subunits that promote conformational transitions between putative open and closed structures (*4*, *20*−*25*). Because ligands can bind to either the α subunit or to the β subunit or to both subunits, several conformational states of the $\alpha_2\beta_2$ complex can be postulated (*20*). Investigations using the fluorophore 8-anilino-1-naphthalenesulfonate provide evidence for at least three conformational states of tryptophan synthase: a fully open state in the absence of ligands and partially or fully closed states associated with different intermediates along the reaction pathway in the presence of different ligands (*22*). Because the terms open and closed probably represent several different conformational states, our use of these terms herein is oversimplified but convenient. Indeed, crystallographic studies of wild-type and mutant forms of the $\alpha_2\beta_2$ complex have identified several different conformations in the presence of different monovalent cations and in the presence of ligands that bind to the α and β subunits (*5*, *26*−*30*).

Monovalent cations activate the β_2 subunit (*16*, *31*) and the $\alpha_2\beta_2$ complex (*16*, *19*, *24*, *25*, *32*). The order of activation of the $\alpha_2\beta_2$ complex by monovalent cations at 25 °C is $NH_4^+ > Cs^+ > Rb^+ > Li^+ > K^+ > Na^+$ with NH_4^+ increasing the reaction rate about 5-fold (*19*). GuHCl at low concentrations (0.02−0.08 M) is also a cation activator of the $\alpha_2\beta_2$ complex (*14*). Molecular modeling (*14*) shows that GuH^+ could bind to the β subunit site that binds Na^+, K^+, or Cs^+ (*26*). At higher concentrations, GuHCl serves a dual role as a cation activator and as a chaotropic agent that modulates the active-site conformation. GuHCl at ~0.2 M stabilizes the open conformation that preferentially accumulates E-Ser at 25 °C.

In the present work, we have investigated effects of temperature in the absence or presence of different monovalent cations and of an allosteric ligand, GP, on the activities of the tryptophan synthase $\alpha_2\beta_2$ complex and β_2 subunit, on the equilibrium distribution of enzyme−substrate intermediates, and on primary kinetic isotope effects. We have selected Na^+ and Cs^+ as monovalent cations for our studies because they have quite different effects on activity and on the equilibrium distribution of E-Ser and E-AA intermediates formed by the $\alpha_2\beta_2$ complex or β_2 subunit, as described above (see Table 1). We have included GuHCl in some of our experiments because this chaotropic agent at 0.2 M

Table 1: Conformational States of the β_2 Subunit and $\alpha_2\beta_2$ Complex

	open	closed
activity in reaction 1 (Ser + Ind → Trp)	low	high
reaction specificity	β-replacement = β-elimination	β-replacement ≫ β-elimination
substrate specificity	β-chloro-L-alanine > L-serine	L-serine > β-chloro-L-alanine
primary intermediate	E-Ser	E-AA
stabilizing factors	lower temperature	higher temperature
	higher pH	lower pH
		α subunit
		α subunit ligands
	Na^+, K^+	Cs^+, NH_4^+
	mutations	
	solvents	
	GuHCl, urea	

stabilizes the open conformation of the $\alpha_2\beta_2$ complex at 25 °C (*14*) (Table 1). Under the same conditions, 0.2 M NaCl stabilizes the closed conformation (*14*). The results provide evidence that the $\alpha_2\beta_2$ complex and β_2 subunit are converted by increased temperature under certain conditions from a low-activity open conformation to a high-activity closed conformation (Table 1).

MATERIALS AND METHODS

Chemicals and Buffers. L-Serine, DL-serine, DL-α-glycerophosphate disodium salt (GP), pyridoxal 5′-phosphate (PLP), and Bis-Tris propane were from Sigma. GuHCl was from Gibco-BRL. α-^{2}H-DL-Serine was prepared as reported previously (*33*). Sodium-free GP was prepared by repeated passage of the solution of the disodium salt over an ion-exchange column (DW-50 in the H^+-form, Sigma) (*32*); the pH of the final eluate was adjusted to 7.8 with Bis-Tris propane. All experiments were carried out in 50 mM Bis-Tris propane buffer containing 0.5 mM dithiothreitol. The pH of this buffer was adjusted to 7.8 by addition of HCl at 25 °C. We found that the pH of Bis-Tris propane buffer varies with temperature with a dpH/dT value of −0.018 unit/°C. If 50 mM Bis-Tris propane buffer was adjusted to pH 7.8 at 25 °C, the pH of the buffer changes to 8.1 at 5 °C and to 7.3 at 50 °C. Our results reported here carried out in 50 mM Bis-Tris propane buffer adjusted to 7.8 at pH 25 °C, with no compensation for pH variation with temperature. A control showing the similarities of results in buffers with and without pH adjustment is shown in the inset in Figure 1C. The similarity of the results is consistent with our finding that the kinetic properties of tryptophan synthase are relatively pH independent between pH 7.3 and 8.1 (*34*).

Bacterial Strain, Plasmids, and Enzymes. Plasmids pEBA-10 and pEBA-4A8 (*35*) were used to express wild-type tryptophan synthase $\alpha_2\beta_2$ complex and the α subunit from *S. typhimurium*, respectively, in *Escherichia coli* CB 149 (*36*), which lacks the *trp* operon. The $\alpha_2\beta_2$ complex (*37*) and the α subunit (*35*, *38*) were purified to homogeneity as described previously. The β_2 subunit was purified by heat treatment of the $\alpha_2\beta_2$ complex to denature the α subunit (*39*). The enzymes were dialyzed against monovalent cation free Bis-Tris propane buffer, pH 7.8, before use. Protein concentrations were determined from the specific absorbance at 278 nm using $A^{1\%}_{1cm} = 6.0$ for the $\alpha_2\beta_2$ complex, $A^{1\%}_{1cm} = 6.5$ for the β_2 subunit, and $A^{1\%}_{1cm} = 4.4$ for the α subunit (*39*).

Enzyme Assays and Spectroscopic Methods. One unit of activity in any reaction is the formation of 0.1 μmol of product in 20 min at the indicated temperature. Activity in reaction 1 was measured by a direct spectrophotometric assay (*39*). Activity in reaction 2 was measured by spectrophotometric assay coupled with lactate dehydrogenase (*16*) with modified components (50 mM L-serine, 0.2 mM NADH, and excess lactate dehydrogenase, which was dialyzed against 50 mM Bis-Tris propane buffer, pH 7.8 to remove ammonium sulfate). α-^{1}H-DL-Serine and α-^{2}H-DL-serine were used in the place of L-serine for investigations of isotope effects on the reaction kinetics and on the equilibrium distribution of enzyme−substrate intermediates. Control experiments showed identical results with α-^{1}H-DL-serine and α-^{1}H−L-serine. The reaction mixtures were equilibrated for at least 5 min at the desired temperature before addition of the enzymes. The concentration of L-serine (50 mM) used for assays was saturating because the same initial rates were obtained with 25, 50, and 100 mM L-serine for reactions 1 and 2 at 5 and 50 °C.

Absorption spectra and assays were measured using a Hewlett-Packard 8452 diode array spectrophotometer thermostated by a Peltier junction temperature-controlled cuvette holder, which was calibrated with a thermometer. Spectra were recorded immediately after mixing 1 vol of enzyme with 20 vol of buffer, which had been preequilibrated at the desired temperature in the cuvette with the indicated components for at least 5 min.

RESULTS

Effects of Temperature on the Activities of the $\alpha_2\beta_2$ Complex in the Absence or Presence of Monovalent Cations and an Allosteric Ligand. Figure 1A shows the effects of temperature on the initial rates of the $\alpha_2\beta_2$ complex (in the presence of the excess α subunit) in the β-replacement reaction with indole and L-serine (reaction 1) in the absence of salt or in the presence of 0.1 M NaCl, 0.1 M CsCl, or 0.2 M GuHCl. GuHCl was used at 0.2 M because E-Ser is the predominant form in 0.2 M GuHCl at 25 °C (*14*). At low temperatures (5−35 °C), the initial rates in the presence of CsCl are much higher than the rates in the presence of NaCl, GuHCl, or no salt. However, at temperatures above 35 °C, the rates in the presence of NaCl become similar to those in the presence of CsCl. The assay solution becomes turbid above 52 °C due to the precipitation of the α subunit. In the absence of excess α subunit, the $\alpha_2\beta_2$ complex dissociates significantly and shows decreased activity at high temperatures (Figure 1 legend). The data were plotted as logarithm of activity (ln activity) versus the reciprocal of the absolute temperature (K) ($1/T$) and fitted to the Arrhenius equation, eq 3, as described in the legend to Figure 1.

$$\ln \text{activity} = \ln Z - \frac{E_a}{RT} \tag{3}$$

where E_a is the energy of activation, R is the gas constant, and Z is the preexponential factor.

The results can be readily transformed into a rate constant, k_{cat}.[3] According to Eyring's transition-state theory, the temperature dependence of a rate constant is given by eq 4

$$k_{cat} = \frac{k_B T}{h}\exp\left(\frac{-\Delta G^{\neq}}{RT}\right) = \frac{k_B T}{h}\exp\left(\frac{-\Delta H^{\neq}}{RT}\right)\exp\left(\frac{\Delta S^{\neq}}{R}\right) \tag{4}$$

where k_B is the Boltzmann's constant, h is Planck's constant, R is the gas constant, and $\Delta G^{\neq}$, $\Delta H^{\neq}$, and $\Delta S^{\neq}$ are the free energy, enthalpy, and entropy of activation of the rate-limiting step in the reaction, respectively. From eqs 3 and 4, it is easy to show that

$$\Delta S^{\neq} = R\left(\ln Z - \ln\frac{k_B}{h}\right) \text{ and } \Delta H^{\neq} = E_a - RT \tag{5}$$

Thus, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ can be determined from the Arrhenius plots.

[3] The turnover number, k_{cat}, is equal to a factor f times the specific activity for the $\alpha_2\beta_2$ complex ($f = 0.00594$) and for the β_2 subunit ($f = 0.00395$).

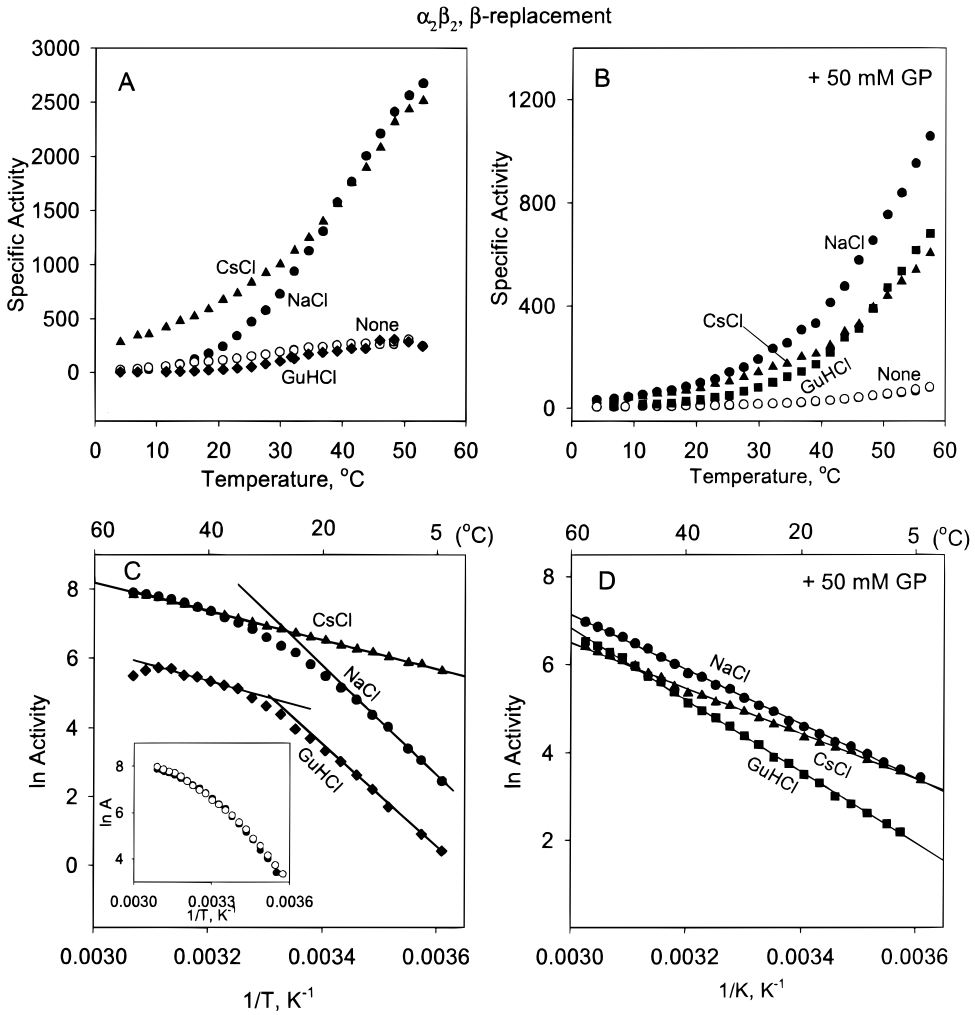


FIGURE 1: Effects of temperature and of monovalent cations on the activity of the $\alpha_2\beta_2$ complex in the β-replacement reaction. (A and B) The initial rates of the $\alpha_2\beta_2$ complex in the β-replacement reaction (reaction 1) were plotted as specific activity (units/mg) versus temperature (°C) in the absence of GP (A) or in the presence of 50 mM GP (B) and in the absence of salt (None) or in the presence of 0.1 M NaCl, 0.1 M CsCl, or 0.2 M GuHCl as indicated. (C and D) The data from panels A and B, respectively, were plotted as the logarithm of activity (ln Activity) versus the reciprocal of the absolute temperature (K). The data were fitted to the Arrhenius equation (eq 3), ln Activity = ln $Z - E_a/RT$. In cases where the data was nonlinear, the linear portions at low or high temperatures were fitted to the Arrhenius equation separately. The insert in the panel C compares the activities in reaction 1 in the presence of NaCl measured in buffer at constant pH 7.8 (○) with activities measured in buffer with no compensation for pH variation with temperature (●) (see Materials and Methods). The concentration of the $\alpha_2\beta_2$ complex varied from 160 μg/mL (~2 μM $\alpha\beta$ pair) for the conditions giving lowest activity (e.g., 0.2 M GuHCl at low temperature) to 5 μg/mL (0.07 μM $\alpha\beta$ pair) for the conditions giving highest activity (e.g., 0.1 M NaCl at high temperature). Activities in the panel A were measured in the presence of excess α subunit (0.5 μM). In the absence of excess α subunit, activity decreases at high temperatures due to subunit dissociation and inactivation. Addition of excess α subunit had no effect on the activities measured in panel B in the presence of GP, an α subunit ligand which inhibits subunit dissociation.

The Arrhenius plot (Figure 1C) of the data in the presence of CsCl is linear between 5 and 52 °C. In contrast, the Arrhenius plot of the data in the presence of NaCl or GuHCl is highly nonlinear in this temperature range. The low-temperature portion shows a much higher slope than the high-temperature portion. The high-temperature portions of the plots coincide in the presence of NaCl or CsCl. Activation energies (E_a) calculated from those plots are given in Table 2. The results show that E_a for the higher temperature portion in the presence of NaCl or GuHCl (34 kJ/mol) is the same as E_a in CsCl, whereas E_a for the lower temperature portion is much higher in the presence of NaCl (E_a = 128 kJ/mol) or GuHCl (E_a = 124 kJ/mol). The K_m for L-serine in the β-replacement reaction in the presence of NaCl (~0.4 mM) is essentially temperature-independent (data not shown).[4]

The α subunit ligand, GP, alters the temperature dependence of the activities of the $\alpha_2\beta_2$ complex in the presence of NaCl and CsCl (Figure 1B). The corresponding Arrhenius plots are linear up to 57 °C and yield E_a = 50 kJ/mol in NaCl, E_a = 43 kJ/mol in CsCl, and E_a = 67 kJ/mol in GuHCl (Figure 1D, Table 2). The addition of excess α subunit shows no effect on the initial rates at high temperatures, indicating that GP protects the enzyme from the heat-induced dissociation which is observed in the absence of GP (Figure 1 legend). We have reported previously that ligands that promote association of the α and β subunits markedly stabilize the more temperature labile α subunit in the $\alpha_2\beta_2$ complex from irreversible thermal denaturation and that the combination of GP with L-serine raised the inactivation temperature of the α subunit in the $\alpha_2\beta_2$ complex from 54

[4] K_m values were obtained from double-reciprocal plots of the initial velocity of the $\alpha_2\beta_2$ complex in reaction 1 versus L-serine concentration (0.1−4 mM) at 15, 20, 25, 30, 35, and 40 °C.

Table 2: Effects of Monovalent Cations on the Apparent Activation Energies and the Activation Entropies of Reactions of Tryptophan Synthase[a]

enzyme	cation	GP	reaction	Arrhenius plot	activity at 37 °C	E_a (kJ/mol)			$\Delta S^{\neq}$ (J/mol K)			$\Delta H_0^{\neq}$ (kJ/mol)	$\Delta C_p^{\neq}$ (kJ/mol/K)
						low T	high T	Δ	low T	high T	Δ		
$\alpha_2\beta_2$	Na^+	−	1	nonlinear	1310	128	34	94	187	−127	314	87.0	−2.76
$\alpha_2\beta_2$	Cs^+	−	1	linear	1400	34	34	0	−127	−127	0	32.3	−0.04
$\alpha_2\beta_2$	GuH^+	−	1	nonlinear	190	124	35	91	161	−151	312	89.1	−3.17
$\alpha_2\beta_2$	Na^+	+	1	linear	310	50	50	0	−85	−85	0	48.6	0.14
$\alpha_2\beta_2$	Cs^+	+	1	linear	200	43	43	0	−101	−101	0	39.8	0.17
$\alpha_2\beta_2$	GuH^+	+	1	linear	145	67	67	0	−38	−38	0	66.0	−0.04
β_2	Na^+	−	1	linear	45	122	122	0	130	130	0	81.7	−0.59
β_2	Cs^+	−	1	nonlinear	780	125	32	93	188	−140	328	85.4	−2.30
β_2	Na^+	−	2	linear	30	142	142	0	186	186	0	134.6	−1.00
β_2	Cs^+	−	2	nonlinear	310	148	38	110	219	−130	349	94.9	−4.14

[a] Conditions for activity measurements and data analysis by Arrhenius plots are given in Figures 1 and 2 in the text. Activity is specific activity (units/mg) at 37 °C. Values of $\Delta H_0^{\neq}$ and $\Delta C_p^{\neq}$ were obtained by simultaneous least-squares fit of the activity data to eq 6.

to 66 °C (*40*). These previous results explain why inactivation is observed in the absence of excess α subunit at high temperatures in the absence of GP but not in the presence of GP (Figure 1 legend).

Effects of Temperature on the Activities of the β_2 Subunit in the Absence or Presence of Monovalent Cations. Dissociation of the β subunit from the α subunit alters its activity and reaction specificity (*11*) (see the introductory portion of this paper). For example, the $\alpha_2\beta_2$ complex has much lower activity in the β-elimination reaction (reaction 2) than in the β-replacement reaction (reaction 1), whereas the β_2 subunit has similar activities in the two reactions (Table 1). These changes in activity are attributed to conformational changes that occur upon the disassembly of the complex. Panels A and B of Figure 2 show the effects of temperature on the initial rates of the β_2 subunit in reactions 1 and 2, respectively. Although NaCl and CsCl both stimulate the activity in the β-replacement reaction, CsCl gives much greater stimulation. The maximum activity was obtained at 43 °C in the presence of CsCl, but at 50 °C in the presence of NaCl. As shown in Figure 2B, NaCl inhibits the β-elimination activity of the β_2 subunit, whereas CsCl strongly stimulates this activity. The maximum activity was obtained at 47 °C in the presence of CsCl and at 52 °C in the presence of NaCl. At higher temperatures, the β_2 subunit is converted to an inactive form (*41*).

The Arrhenius plots of the data for reactions 1 and 2 are shown in Figure 2, panels C and D, respectively. The plots for both reactions are linear in the presence of NaCl below 45 °C as shown previously (*41*), but are nonlinear in the presence of CsCl. The activation energies and activation entropies calculated from these plots are listed in Table 2. The results show that the E_as for the low-temperature portions of both reactions in the presence of CsCl (125 and 148 kJ/mol) are similar to the corresponding E_as in the presence of NaCl (122 and 142 kJ/mol). The high-temperature portions in the presence of CsCl yield much lower E_as (45 and 30 kJ/mol).

The activation energies in the presence of NaCl (122 and 142 kJ/mol in reactions 1 and 2, respectively) are close to those reported previously (122 and 130 kJ/mol) (*41*). The β_2 subunit undergoes a temperature-dependent reversible conformational transition with $T_i = \sim 52$ °C (*41*, *42*). Irreversible inactivation of the β_2 subunit occurs at ~80 °C (*40*).

Alternative Analysis of Activity Data. Curved Arrhenius plots may also be observed for simple mechanisms in which one step is rate-limiting under all circumstances. Differences in specific heat between reactants and the transition state will result in temperature-dependent enthalpies and entropies of activation (*43*). Such differences in specific heat can result from changes in hydration, loss of intramolecular vibrations, or large changes in pK_as of titratable groups in the reactants. All of these could be involved in an enzymatic reaction, which involves a postulated conformational change in the protein. To test the validity of this alternative cause of the nonlinear Arrhenius plots, the activity data shown in Figures 1, panels A and B, and 2, panels A and B, were fitted to a modified form of the Eyring equation (eq 6):

$$\frac{k_{cat}}{T} = \frac{k_{cat}^0}{T_0}\exp\left\{\frac{\Delta H_0^{\neq}}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)+\frac{\Delta C_p^{\neq}}{R}\left[1-\frac{T}{T_0}-\ln\left(\frac{T}{T_0}\right)\right]\right\} \quad (6)$$

where k_{cat}^o is the rate constant, $\Delta H_o^{\neq}$ is the temperature-dependent enthalpy of activation, and $\Delta C_p^{\neq}$ is the temperature-independent change in specific heat at an arbitrary reference temperature ($T_0 = 25$ °C $= 298.15$ K). The results of these fits are shown in Table 2. Conditions that yielded linear Arrhenius plots also yielded linear Eyring plots with derived values of $\Delta C_p^{\neq}$ less than 1 kJ/mol/K. Conditions that yielded nonlinear Arrhenius plots also yielded nonlinear Eyring plots with larger values of $\Delta C_p^{\neq}$. The derived changes in specific heat are within the range expected for processes involving proteins (*44*).

Effects of Temperature on the Absorption Spectra of the $\alpha_2\beta_2$ Complex in the Presence of L-Serine and Other Ligands. The reaction of the $\alpha_2\beta_2$ complex with L-serine in the presence of 0.1 M CsCl yields a complex spectrum with a major peak centered at 350 nm (dotted curve in Figure 3A), which is ascribed to E-AA (Scheme 1). The absorbance at 424 nm in the presence of CsCl and L-serine is essentially independent of temperature between 4 and 50 °C (Figure 3C). The spectra obtained from the reaction of the $\alpha_2\beta_2$ complex with L-serine in the presence of NaCl (Figure 3A) and GuHCl (Figure 3B) are strongly temperature dependent. Decreasing the temperature results in decreased absorbance at 350 nm (E-AA) and increased absorbance at 424 nm (E-Ser) (Scheme 1). The spectra in Figure 3, panels A and B,

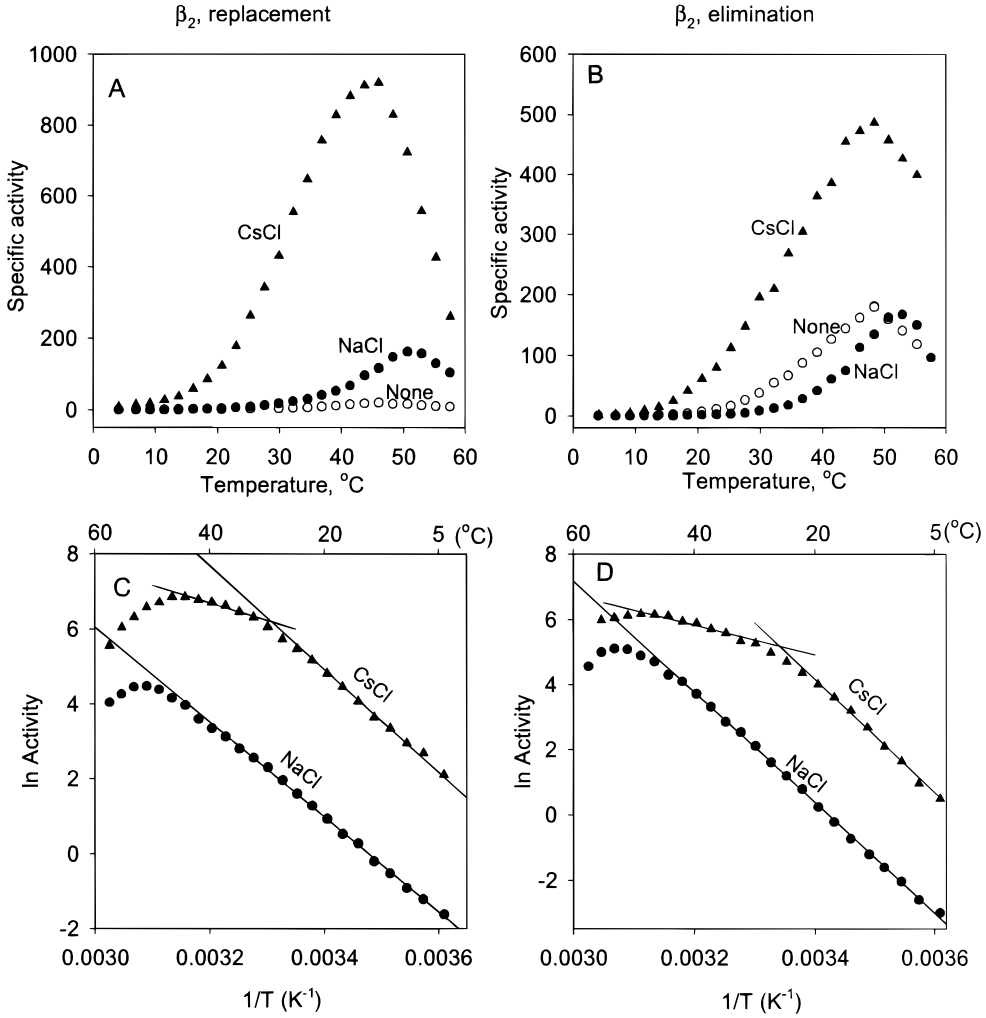


FIGURE 2: Effects of temperature and of monovalent cations on the activities of the β_2 subunit. (A and B) The initial rates of the β2 subunit in reaction 1 (A) and reaction 2 (B) were measured in the absence of salt (None) or in the presence of 0.1 M NaCl or 0.1 M CsCl, as indicated, and were plotted as specific activity (units/mg) versus temperature (°C). (C and D) The data from panels A and B, respectively, were plotted as the logarithm of activity (ln Activity) versus the reciprocal of the absolute temperature (K). The data in the presence of NaCl below 45 °C were fitted to the Arrhenius equation (eq 3). The data in the presence of CsCl at low (5−25 °C) or moderate (30−45 °C) temperatures were fitted separately to the Arrhenius equation. The concentration of the β_2 subunit varied from 320 μg/mL (7 μM β) at low temperature to 6 μg/mL (0.13 μM β) at high temperature.

show approximate isosbestic points. The results indicate that decreasing temperature shifts the equilibrium distribution of the two species from E-AA to E-Ser, but do not rule out the presence of other minor species. The temperature that produces a half-maximal change in the presence of GuHCl (25 °C) is higher than that in the presence of NaCl (8 °C) (Table 3). This result suggests that a higher temperature is needed to shift the equilibrium from the open form to the closed form because GuHCl stabilizes the open form of the $\alpha_2\beta_2$ complex (*14*) (Table 1).

The equilibrium constant, K_{eq}, for the E-Ser to E-AA conversion can be calculated from the temperature dependence of the absorbance using eq 7:

$$K_{eq}(T) = \frac{[\text{E} - \text{AA}]}{[\text{E} - \text{Ser}]} = \frac{A_T - A_{\text{E-ser}}}{A_{\text{E-AA}} - A_T} \qquad (7)$$

where A_T is the absorbance of the solution at absolute temperature T and $A_{\text{E-Ser}}$ and $A_{\text{E-AA}}$ are the absorbance of E-AA obtained at higher temperature and of E-Ser obtained at low temperature, respectively.

Thermodynamic parameters for the E-Ser to E-AA conversion can be calculated by eq 8:

$$\Delta G(T)_{eq} = -RT \ln K_{eq}(T) = \Delta H_{eq} - T\Delta S_{eq} = \Delta H_{eq}\left(1 - \frac{T}{T_m}\right) \qquad (8)$$

where $\Delta G(T)_{eq}$ and ΔS_{eq} are the changes in free energy and entropy of the reaction, respectively, and T_m is the midpoint, where $K_{eq}(T) = 1$, assuming no change in enthalpy with temperature.

Analysis of the absorbance data for the reaction of the $\alpha_2\beta_2$ complex with α-^{1}H-serine in the presence of NaCl (Figure 3C) or GuHCl (Figure 3D) yields the thermodynamic parameters and T_m values in Table 3. van't Hoff plots of the data are linear (not shown). Results with α-^{2}H-serine are discussed below. The absorption spectra observed upon the reaction of the $\alpha_2\beta_2$ complex with L-serine in the presence of 50 mM GP and NaCl, GuHCl, or CsCl exhibit a peak at 350 nm and are essentially temperature independent at 4−50 °C (Figure 3, panels C and D and legend). These results show

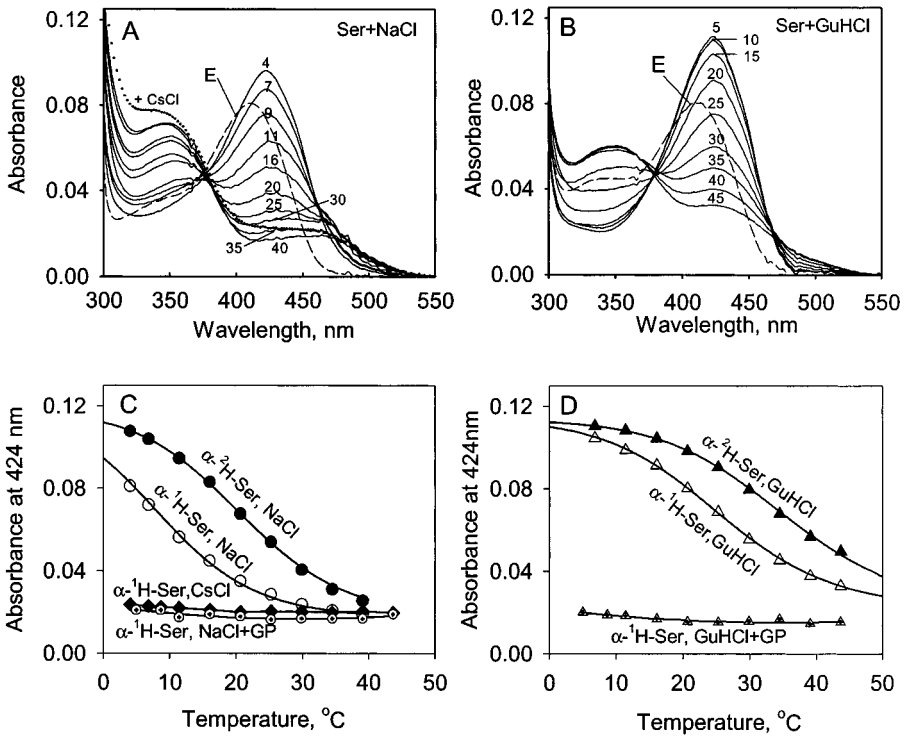


FIGURE 3: Effects of temperature and of monovalent cations on the intermediates formed by the $\alpha_2\beta_2$ complex in the reaction with L-serine. (A and B) The absorption spectra of the $\alpha_2\beta_2$ complex (7 μM $\alpha\beta$ pair) were measured in the presence of 50 mM α-^{1}H-DL-serine (α-^{1}H-serine) and 0.1 M NaCl or 0.1 M CsCl (A) or 0.2 M GuHCl (B). Spectra were recorded immediately after addition of the enzyme (0.025 mL) to Bis-Tris propane buffer containing 50 mM α-^{1}H-DL-serine (α-^{1}H-serine) and indicated salts (0.975 mL), which had been preincubated at the indicated temperature (°C) for at least 5 min. (C and D) Plots of absorbance at 424 nm versus temperature (°C) for the reaction of the $\alpha_2\beta_2$ complex in the presence of 0.1 M NaCl (C) or 0.2 M GuHCl (D) and either α-^{1}H-DL-serine (α-^{1}H-serine) (data from A and B) or α-^{2}H-DL-serine (α-^{2}H-serine) (data from analogous spectra not shown). Control experiments showed that identical results were obtained with α-^{1}H-DL-serine and with α-^{1}H-L-serine. Results are also shown from analogous experiments with α-^{1}H-L-serine in the presence of 0.1 M CsCl or 0.1 M NaCl + 50 mM GP (C) and with α-^{1}H-L-serine in the presence of 0.2 M GuHCl + 50 mM GP (D). The data were fit to eqs 7, 8, and 9 using the PC-MLAB program (Civilized Software, Bethesda MD) to obtain values of ΔH_{eq}, ΔS_{eq}, T_m, and the equilibrium isotope effect (EIE*) given in Table 3.

Table 3: Thermodynamic Changes for the Equilibrium between E-Ser and E-AA[a]

enzyme	cation	ΔH_{eq} (kJ/mol)	ΔS_{eq} (J/mol K)	$K_{eq}(^1H)$ at 20 °C	$K_{eq}(^2H)$ at 20 °C	T_m (°C)	figure	EIE*
$\alpha_2\beta_2$	Na^+	92 ± 5	+327	4.90	0.83	8.4 ± 0.4	3	5.1 ± 0.4
$\alpha_2\beta_2$	GuH^+	86 ± 3	+288	0.50	0.18	25.3 ± 0.4	3	2.9 ± 0.2
β_2	Cs^+	64 ± 12	+211	0.45	0.13	29.8 ± 2.3	4	3.5 ± 0.8

[a] Spectroscopic measurements were made as described in the indicated Figures. Values of ΔH_{eq}, ΔS_{eq}, T_m, and EIE* were obtained by combined fits of the absorbance changes at 424 nm with α-^{1}H-serine and with α-^{2}H-serine to eqs 7, 8, and 9 using the PC-MLAB program (Civilized Software, Bethesda, MD). The ΔH_{eq}, ΔS_{eq}, and T_m values shown are for α-^{1}H-serine. Representative values of equilibrium constants, $K_{eq}(^1H)$ and $K_{eq}(^2H)$, at 20 °C were calculated from eqs 8 and 9 using the derived parameters.

that GP stabilizes E-AA in the presence of NaCl, CsCl, or GuHCl.

Effects of Temperature on the Absorption Spectra of the β_2 Subunit in the Presence of L-Serine and NaCl or CsCl. The reaction of the β_2 subunit with α-^{1}H-L-serine in 0.1 M NaCl yields an absorption spectrum with maximum absorbance at 424 nm, which is essentially temperature independent (Figure 4). Thus, E-Ser is the predominant intermediate in the presence of NaCl and temperature does not alter the equilibrium distribution of the E-Ser and E-AA intermediates. The reaction of the β_2 subunit with α-^{1}H-serine in 0.1 M CsCl also yields an absorption spectrum with a major peak at 424 nm at low temperatures. Increasing temperature results in decreased absorbance at 424 nm (Figure 4) and increased absorbance at 350 nm. Thus, temperature alters the equilibrium distribution of the E-Ser and E-AA intermediates in the presence of CsCl. Analysis of the temperature dependence of the absorbance at 424 nm in the presence of 0.1 M CsCl (Figure 4) yields ΔH_{eq} = 64 kJ/mol, ΔS_{eq} = 211 J/mol K, T_m = 30 °C (Table 3). (Results with α-^{2}H-serine are discussed below.)

Temperature Dependence of Isotope Effects. To investigate the effects of temperature on the rate-limiting steps, we measured the primary kinetic isotope effects for abstraction of the α-proton of L-serine as a function of temperature. Figure 5 shows the effects of isotopic substitution of the α proton of serine on the Arrhenius plots for the $\alpha_2\beta_2$ complex in the β-replacement reaction (reaction 1) in the presence of NaCl or CsCl. The Arrhenius plot for the reaction with indole and α-^{2}H-serine in the presence of CsCl is linear and parallel to that for the reaction with indole and α-^{1}H-serine. In contrast, the Arrhenius plot for the reaction with indole and α-^{2}H-serine is nonlinear in the presence of NaCl. This plot is closer to the plot for the reaction with indole and α-^{1}H-

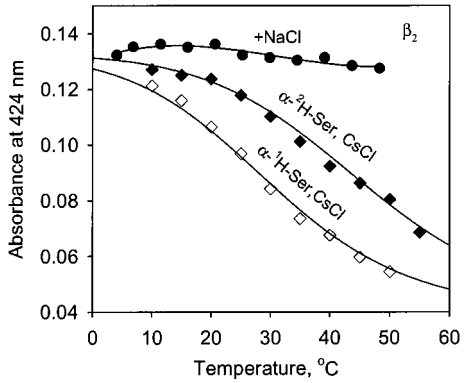


FIGURE 4: Effects of temperature on the intermediates formed by the β_2 subunit in the reaction with L-serine. The absorbance at 424 nm in the presence of 0.1 M NaCl and α-^{1}H-DL-serine (α-^{1}H-serine) and in the presence of 0.1 M CsCl and α-^{1}H-DL-serine (α-^{1}H-serine) or α-^{2}H-DL-serine (α-^{2}H-serine) was measured at the indicated temperatures as described in the legend of Figure 3 and was plotted versus temperature (°C). It is important to measure the absorbance immediately upon mixing because rapid changes in absorbance occur as a function of time in the presence of CsCl as the result of substrate turnover, especially at higher temperatures. The data were fit to eqs 7, 8, and 9 using the PC-MLAB program (Civilized Software, Bethesda MD) to obtain values of ΔH_{eq}, ΔS_{eq}, T_m, and the equilibrium isotope effect (EIE*) given in Table 3.

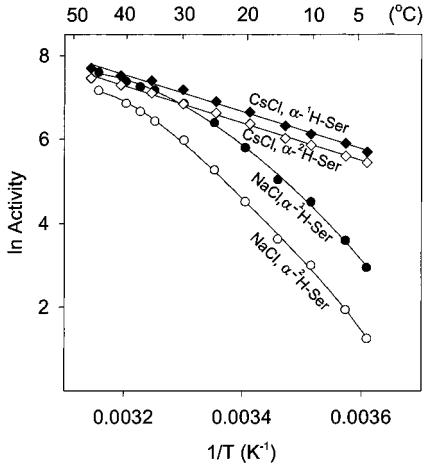


FIGURE 5: Effects of substituting the α-proton of L-serine with deuterium on the temperature dependence of the activity of the $\alpha_2\beta_2$ complex. The initial rates of the $\alpha_2\beta_2$ complex in the β-replacement reaction (reaction 1) were determined as described in Figure 1A in the presence of 0.1 M NaCl or 0.1 M CsCl and 0.5 μM α subunit with 50 mM α-^{1}H-DL-serine (α-^{1}H-serine) or α-^{2}H-DL-serine (α-^{2}H-serine). The data were plotted as the logarithm of specific activity (units/mg) (ln Activity) versus the reciprocal of the absolute temperature (K).

serine at higher temperatures than that at lower temperatures. As shown in Figure 6A, the primary kinetic isotope effect for the activity of the $\alpha_2\beta_2$ complex in the reaction with indole and L-serine in the presence of CsCl is small (~1.3) and essentially temperature independent. In contrast, the primary kinetic isotope effect in the presence of NaCl is temperature dependent and decreases from 5.5 at 5 °C to 1.5 at 45 °C. The primary kinetic isotope effect also decreases in the presence of 0.2 M GuHCl from 2.8 at 5 °C to 1.3 at 45 °C. The primary kinetic isotope effects are negligible (i.e., ~1) and are temperature independent in the presence of GP in combination with CsCl, NaCl, or GuHCl.

The primary kinetic isotope effects for the β_2 subunit in the β-replacement and β-elimination reactions are temper-

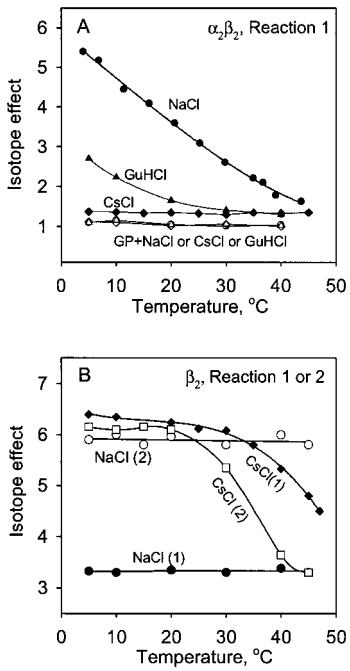


FIGURE 6: Temperature dependence of the isotope effects for the activities of the $\alpha_2\beta_2$ complex and the β_2 subunit and for the intermediates formed by the $\alpha_2\beta_2$ complex in the reaction with L-serine. The primary kinetic isotope effect is the ratio of the activity with α-^{1}H-DL-serine to the activity with α-^{2}H-DL-serine. Specific activities (units/mg) were determined for the $\alpha_2\beta_2$ complex in the β-replacement reaction (A) and for the β_2 subunit in the β-replacement reaction (reaction 1) or β-elimination reaction (reaction 2) (B) and plotted versus temperature.

ature dependent in the presence of CsCl but temperature independent in the presence of NaCl (Figure 6B). The primary kinetic isotope effect in the presence of NaCl is ~3.3 in the β-replacement reaction and is ~6.0 in the β-elimination reaction between 10 and 47 °C (Figure 6B). The primary kinetic isotope effect in the presence of CsCl decreases from ~6.3 at 10 °C to ~4.5 at 47 °C in the β-replacement reaction and from ~6 at 10 °C to ~ 3 at 47 °C in the β-elimination reaction (Figure 6B).

We also measured the deuterium isotope effects on the temperature dependence of absorbance on the equilibrium distribution of the E-Ser and E-AA intermediates under conditions that give nonlinear Arrhenius plots. The results show a pronounced deuterium isotope effect on temperature dependence of absorbance at 424 nm of the $\alpha_2\beta_2$ complex in the presence of serine and NaCl (Figure 3C) or GuHCl (Figure 3D) and for the β_2 subunit in the presence of L-serine and CsCl (Figure 4). Analysis of the three data sets yields values of apparent equilibrium isotope effects (EIE*) of 2.9−5.1 (Table 3) where EIE* is defined by eq 9:

$$\text{EIE*} = \frac{K_{eq}(^1\text{H})}{K_{eq}(^2\text{H})} = \frac{[\text{E} - \text{AA}]/[\text{E} - (^1\text{H}) - \text{Ser}]}{[\text{E} - \text{AA}]/[\text{E} - (^2\text{H}) - \text{Ser}]} \quad (9)$$

EIE* is an apparent equilibrium isotope effect because a true equilibrium is not measured (see Discussion).

DISCUSSION

The origin of nonlinearity in Arrhenius plots for enzyme catalyzed reactions has been the subject of much interest.

Table 4: Correlation between Arrhenius Plots and Temperature-Dependent Changes in Absorption Spectra and in Primary Kinetic Isotope Effects[a]

enzyme	cation	GP	reaction	Arrhenius plot	figure	spectral changes	figure	KIE[a] changes	figure
$\alpha_2\beta_2$	Na^+	−	1	nonlinear	1C	+	3AC	+	5,6A
$\alpha_2\beta_2$	Cs^+	−	1	linear	1C	−	3C	−	5,6A
$\alpha_2\beta_2$	GuH^+	−	1	nonlinear	1C	+	3BD	+	6A
$\alpha_2\beta_2$	Na^+	+	1	linear	1D	−	3C	−	6A
$\alpha_2\beta_2$	Cs^+	+	1	linear	1D	−	3C	−	6A
$\alpha_2\beta_2$	GuH^+	+	1	linear	1D	−	3D	−	6A
β_2	Na^+	−	1	linear	2B	−	4	−	6B
β_2	Cs^+	−	1	nonlinear	2B	+	4	+	6B
β_2	Na^+	−	2	linear	2D	−	4	−	6B
β_2	Cs^+	−	2	nonlinear	2D	+	4	+	6B

[a] KIE, kinetic isotope effects.

Nonlinear Arrhenius plots are attributed either to a change in the rate-limiting step, to a conformational change in the enzyme, or to a change in the specific heat of the reactant (*43*, *45*−*47*).

Evidence for conformational transitions associated with nonlinear Arrhenius plots has been reported for a number of enzymes including D-amino acid oxidase (*46*) and penicillopepsin (*47*). Similar results with pyruvate kinase have been related to the allosteric conversion of the T form of pyruvate kinase at low temperatures to an R form at higher temperatures (*48*). The presence of an allosteric effector, fructose-1, 6-bisphosphate, stabilized the R form of the enzyme, which had a lower activation energy than the T form and gave a linear plot. A decrease in the activation energy of the reaction catalyzed by D-glyceraldehyde 3-phosphate dehydrogenase from *Thermatoga maritima* has been related to a temperature-dependent structural reorganization of the enzyme that results in tightened hydrophobic interactions at high temperature (*49*). A nonlinear van't Hoff plot for L-glutamine binding to glutamine synthetase has been attributed to the temperature dependence of the enthalpy of binding [i.e., a change in the specific heat (ΔC_p)] (*50*).

In the present investigation, Arrhenius plots of the activity data for the tryptophan synthase $\alpha_2\beta_2$ complex and β_2 subunit are nonlinear in four cases and linear in six cases (see data in Table 2 and Figures 1 and 2 and summary in Table 4). A key finding is that there is an exact correlation between the conditions of monovalent cations and of an allosteric ligand (GP) that yield nonlinear Arrhenius plots and the conditions that yield temperature-dependent spectral changes and changes in primary kinetic isotope effects (Table 4). Furthermore, conditions that yield linear Arrhenius plots yield no changes in spectra or isotope effects (Table 4).

Another important observation is that the differences between the activation energies (E_a) at low and high temperatures derived from the nonlinear Arrhenius plots (91−110 kJ/mol in Table 2) are closely similar to the ΔH_{eq} values for the conversion of E-Ser to E-AA obtained under analogous conditions (64−92 kJ/mol in Table 3). Furthermore, the differences between $\Delta S^{\neq}$ values at low and high temperatures derived from the nonlinear Arrhenius plots (314−349 J/mol K in Table 2) are closely similar to the ΔS_{eq} values for equilibrium between E-Ser to E-AA under analogous conditions (211−327 J/mol K in Table 3). To explain this remarkable quantitative correlation between these two types of results, we will discuss the thermodynamic parameters for the cases that yield nonlinear Arrhenius plots in relation to the simple reaction mechanism shown in eq 10:

$$E + Ser \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E - Ser \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} E - AA \xrightarrow{k_{31}} E + \text{products} \quad (10)$$

The last step in the β-elimination reaction is hydrolysis of E-AA to pyruvate and ammonia. The last step in the β-replacement reaction is protonation of E-Q_2 (Scheme 1). Because the reaction of E-AA with indole to form E-Q_2 is very rapid (*51*, *52*), whereas the second step (E-Q_2 → E-Trp) is rate limiting (*53*), the effective rate constant for the last step is (k_{31})·[indole] for the conversion of E-Q_2 to E-Trp. The intermediate E-Q_1 between E-Ser and E-AA (Scheme 1) is not included in eq 10 because the conversion of E-Q to E-AA is very fast and does not affect the kinetic scheme. The steady-state solution for eq 10 is eq 11:

$$k_{cat} = \frac{k_{23}k_{31}}{k_{23} + k_{32} + k_{31}} \quad (11)$$

In terms of this mechanism, two different scenarios could account for the curved Arrhenius plots: (1) $k_{23} > k_{31}$ at low temperature, but $k_{32} \gg k_{23}$. Then, the prior equilibrium between E-Ser and E-AA is partially rate limiting at low temperature. (2) $k_{23} < k_{31}$ at low temperature. Then k_{23} will determine the rate and will show a large primary isotope effect.

We can define an apparent equilibrium constant between E-Ser and E-AA by eq 12:

$$K_{eq} = \frac{[E - AA]}{[E - Ser]} \quad (12)$$

It can be readily shown that under steady state, initial velocity conditions

$$\frac{[E - AA]}{[E - Ser]} = \frac{k_{23}}{k_{32} + k_{31}} \quad (13)$$

so,

$$k_{cat} = \frac{K_{eq}k_{31}}{K_{eq} + 1} \quad (14)$$

At low temperature, K_{eq} is small, the enzyme is mostly present as E-Ser ($K_{eq} \ll 1$), and k_{cat} is defined by eq 15:

$$k_{cat} \approx \frac{K_{eq}}{1} k_{31} = K_{eq} k_{31} \quad (15)$$

and

$$\log k_{cat} = \log K_{eq} + \log k_{31} \quad (16)$$

Then,

$$\Delta G^{\neq}_{cat} = \Delta G_{eq} + \Delta G^{\neq}_{31} \quad (17a)$$

$$\Delta H^{\neq}_{cat} = \Delta H_{eq} + \Delta H^{\neq}_{31} \quad (17b)$$

$$\Delta S^{\neq}_{cat} = \Delta S_{eq} + \Delta S^{\neq}_{31} \quad (17c)$$

Under scenario 1, where $k_{23} > k_{31}$ at low temperature, the interconversion of E-Ser and E-AA acts as a partially rate limiting, prior equilibrium. The release of the α-proton of L-serine occurs between E-Ser and E-AA, so the primary kinetic isotope effect is on k_{23}, which affects both K_{eq} and k_{cat}. Under scenario 2, where $k_{23} < k_{31}$ at low temperature, the rate of conversion of E-Ser to E-AA (k_{23}) becomes rate limiting. Under these circumstances, we may modify eq 11 to give eq 18, which is analogous to eq 15:

$$k_{cat} = \frac{k_{23} k_{31}}{k_{32} + k_{31}} = K_{eq} k_{31} \quad (18)$$

Steady-state measurements do not distinguish between these two scenarios, which can only be distinguished by rapid kinetic experiments as a function of temperature. Although values of k_{23} have been reported at 25 °C and above (*24*, *25*, *33*), no measurements have been made at lower temperatures. Therefore, future rapid kinetic experiments are needed to distinguish between these two interpretations of the results.

At high temperature, K_{eq} is large, the enzyme is mostly present as E-AA ($K_{eq} \gg 1$), and k_{cat} is defined by eq 19:

$$k_{cat} \approx \frac{K_{eq} k_{31}}{K_{eq}} = k_{31} \quad (19)$$

and,

$$\log k_{cat} = \log k_{31} \quad (20)$$

Then,

$$\Delta G^{\neq}_{cat} = \Delta G^{\neq}_{31} \quad (21a)$$

$$\Delta H^{\neq}_{cat} = \Delta H^{\neq}_{31} \quad (21b)$$

$$\Delta S^{\neq}_{cat} = S^{\neq}_{31} \quad (21c)$$

Thus, the prior equilibrium or k_{23} is no longer partially rate limiting at high temperature, and the primary kinetic isotope effect is removed. The differences between the activation free energy ($\Delta G^{\neq}$), activation enthalpy ($\Delta H^{\neq}$), and activation entropy ($\Delta S^{\neq}$) at low temperature and at high temperature, which are given by eq 17 minus eq 20, are equal to ΔG_{eq}, ΔH_{eq}, and ΔS_{eq}, respectively. Figure 7 illustrates the relationships between thermodynamic data for the β-replacement reaction of the $\alpha_2\beta_2$ complex in the presence of NaCl, where ΔH_{eq} (92 kJ/mol) ≅ $\Delta H^{\neq}_{L}$ (128 kJ/mol) − $\Delta H^{\neq}_{H}$ (34 kJ/mol) and ΔS_{eq} (327 J/mol/K) ≅ $\Delta S^{\neq}_{L}$ (187 J/mol/K) − $\Delta S^{\neq}_{H}$ (−127 J/mol/K). This analysis thus explains the quantitative relationships between the spectroscopic and activity data. The results provide evidence that the nonlinear Arrhenius plots result from a conformational change in the enzyme.

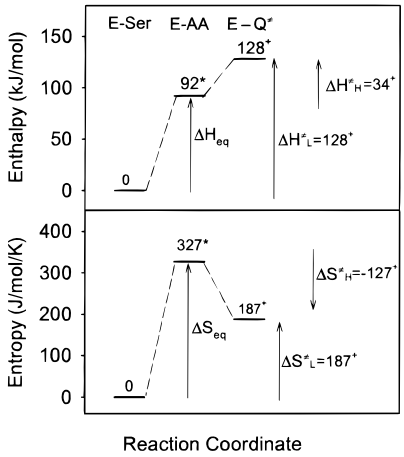


FIGURE 7: Diagram of enthalpy and entropy changes in the β-replacement reaction of the $\alpha_2\beta_2$ complex in the presence of NaCl. (*) Values of ΔH_{eq} and ΔS_{eq} were obtained by analysis of spectroscopic data (Table 3). (+) Values of $\Delta H^{\neq}_{H}$ and $\Delta S^{\neq}_{H}$ at high temperature and of $\Delta H^{\neq}_{L}$ and $\Delta S^{\neq}_{L}$ at low temperature were obtained from the corresponding values of E_a (∼Δ*H*) and $\Delta S^{\neq}$ in Table 2. The results show that $\Delta H_{eq} \cong \Delta H^{\neq}_{H} - \Delta H^{\neq}_{L}$ and that $\Delta S_{eq} \cong \Delta S^{\neq}_{H} - \Delta S^{\neq}_{L}$ (see Discussion). The results provide evidence that high-temperature induces a conformational change that removes the prior equilibrium between E-Ser and E-AA.

We have also determined the temperature dependence of the transition between E-Ser and E-AA by absorption spectroscopy with α-^{1}H-serine and α-^{2}H-serine (Figure 3) and have determined the apparent equilibrium isotope effects (EIE*) by eq 9 (see Table 3). The interconversion of E−^{2}H−Ser and E-AA measured is not a true equilibrium reaction because a true equilibrium reaction would be the reaction of E−^{2}H−Ser to produce ^{2}HOH in the forward reaction (k_{23}) and the reaction of E-AA with ^{2}HOH to form E−^{2}H−Ser in the reverse reaction (k_{32}). In the reaction measured, it is likely that E-AA reacts with HOH in the reverse reaction to yield E−^{1}H−Ser. Thus, the isotope effect observed is a steady-state kinetic isotope effect or apparent equilibrium isotope effect (EIE*) rather than a true equilibrium isotope effect.[5] The observed values of EIE* (2.9−5.1) are much higher than reported values of true equilibrium isotope effects, which range between 1 and 2 (*54*, *55*).

The low-temperature kinetic isotope effect of ∼6 indicates a 6-fold reduction in k_{23}, which will result in a 6-fold reduction in $K_{eq} = k_{23}/(k_{32} + k_{31})$ at all temperatures and an ∼10 °C increase in the T_m of the transition in the presence of α-^{2}H-serine, close to that observed for the $\alpha_2\beta_2$ complex in the presence of NaCl or GuHCl (Figure 3, panels C and D) or the β_2 subunit in the presence of CsCl (Figure 4). Within the temperature range of these experiments, changes in intrinsic chemical isotope effects are insignificant (*56*). Hence, changes in apparent isotope effects can be attributed

[5] We gratefully acknowledge a reviewer for suggesting this interpretation.

to changes in the mechanism of the enzymatic reaction with increasing temperature (*57*). The isotope effect results are not compatible with the alternative analysis of the activity data by eq 6 (Table 2) in which the same step, which is accompanied by a change in specific heat, is rate limiting under all conditions. Furthermore, the alternative analysis cannot explain why some Arrhenius plots, but not others, are linear. Finally, the alternative analysis is inconsistent with the results of calorimetric studies that have demonstrated that serine binding to the $\alpha_2\beta_2$ complex is not associated with a ΔC_p (*58*).

Conditions that stabilize the closed conformation of the $\alpha_2\beta_2$ complex (i.e., CsCl and the α subunit ligand GP, Table 1) also yield linear Arrhenius plots and no changes in spectra and kinetic isotope effects (Table 4). Interestingly, addition of GP in the presence of GuHCl or NaCl stabilizes E-AA even at low temperatures and yields linear Arrhenius plots with decreased activation energies. Thus, GP stabilizes a closed form of the $\alpha_2\beta_2$ complex in the presence of monovalent cations between 5 and 50 °C and overcomes destabilization of the closed form by GuHCl or NaCl. The observed reaction rates are lower in the presence of GP because GP increases the activation energy, E_a, from ∼34 kJ/mol to 43−67 kJ/mol (Table 2). The rate-limiting step in the presence of GP is the conversion of E-Q_2 to E-Trp, as shown by the steady-state accumulation of E-Q_2 in the reaction of L-serine with indole in the presence of GP (*59*). Our finding that GP stabilizes a closed form of the $\alpha_2\beta_2$ complex is analogous to the finding that fructose-1,6-bisphosphate, an allosteric effector, stabilizes the R form of pyruvate kinase, which has a lower activation energy than the T form (*48*).

The β_2 subunit in the presence of NaCl exhibits a linear Arrhenius plot with a high activation energy and forms E-Ser over the temperature range investigated. These results indicate that the equilibrium distribution of intermediates strongly favors E-Ser and cannot be shifted by increasing temperature. In contrast, the β_2 subunit in the presence of CsCl exhibits a nonlinear Arrhenius plot and a temperature-dependent decrease in E-Ser and in the kinetic isotope effect. These results indicate that increasing temperature and CsCl combine to induce a conformational change in the β_2 subunit. This conclusion is consistent with the previous finding that high concentrations of CsCl promote the closed conformation of the β_2 subunit at 25 °C (*16*).

The large values for ΔS_{eq} suggest that an increase in entropy drives the open to closed conformational transition (see the introductory portion of this paper, Table 1 and Scheme 1). Positive ΔS_{eq} values of this magnitude in enzyme catalysis or ligand binding are often thought to be associated with release of H_2O (*60*). Release of H_2O could switch the enzyme from a state in which the cofactor-binding site is open to solvent to a state in which the cofactor-binding site is desolvated and covered by another domain of the enzyme, as suggested for ribonucleoside triphosphate reductase (*60*). A decrease in the activation energy of the reaction catalyzed by D-glyceraldehyde 3-phosphate dehydrogenase from *T. maritima* has also been related to a temperature-dependent structural reorganization of the enzyme that results in tightened hydrophobic interactions at high temperature (*49*).

Our results suggest that under certain conditions increasing temperature shifts the conformation of the tryptophan synthase $\alpha_2\beta_2$ complex and β_2 subunit from the open, less active conformation toward the closed, active conformation by tightened hydrophobic interactions and by loss of water. This proposal is consistent with a number of earlier investigations (Table 1). Our previous studies of the effects of solvents (*12*, *13*) and of GuHCl or urea (*14*) on the equilibrium distribution of the open and closed conformers of the tryptophan synthase $\alpha_2\beta_2$ complex provided evidence that a nonpolar region of the β subunit becomes less exposed to solvent in the closed, active conformation. The proposal is also consistent with recent crystallographic results that show that the reaction of L-serine results in movement of a mobile region (β subunit residues 93−183) toward the rest of the β subunit and in partial closure of the indole tunnel (*27*, *30*). The observed ligand-induced domain movement excludes water from the active site and increases the hydrophobicity of the β site. Kinetic and spectroscopic studies also provide evidence that solvent is excluded from the active site of the $\alpha_2\beta_2$ complex in the E-AA intermediate (*61*).

The different effects of temperature on the conformation of tryptophan synthase in the presence of Na^+ or Cs^+ may be related to observed differences in the structure of the $\alpha_2\beta_2$ complex in the presence of these two cations and to the fact that Cs^+ interacts with the peptide carbonyls of six residues whereas Na^+ only interacts with the carbonyls of three residues (*26*). Of special interest is a salt bridge that forms between β subunit Lys167 and α subunit Asp56 in the presence of Cs^+ and is replaced by a salt bridge between β subunit Lys167 and β subunit Asp305 in the presence of Na^+. The salt bridge formed at the interface between the α and β subunits in the presence of Cs^+ may be important for intersubunit communication and for formation of the closed, active conformer of the $\alpha_2\beta_2$ complex (*4*, *17*, *24*, *25*, *62*).

Very large primary kinetic isotope effects (∼6) were observed at low temperatures for the $\alpha_2\beta_2$ complex in the presence of NaCl and for the β_2 subunit under several conditions (Figure 6). These values approach the maximum theoretical value of 7 for cleavage of a C−H bond (*63*), indicating that the C_a proton abstraction is nearly completely rate determining. A primary [C_α-^{2}H] kinetic isotope effect of 6.7 has been reported for the D222A mutant of aspartate aminotransferase (*64*). A primary [C_α-^{2}H] kinetic isotope effect of 6.9 was observed for a model transamination reaction proceeding by general base catalysis (*65*).

SUMMARY

Analysis of the temperature dependence of reactions catalyzed by the tryptophan synthase $\alpha_2\beta_2$ complex and β_2 subunit with L-serine has allowed determination of the thermodynamic parameters for these reactions. This analysis shows that entropic effects play a large role in catalysis. The results suggest that a temperature-dependent conformational change results in activation of the enzyme and in exclusion of water from the active site. Monovalent cations and an allosteric ligand, GP, regulate the conformational transition.

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REFERENCES

1. Miles, E. W. (1979) *Adv. Enzymol. 49*, 127−186.
2. Miles, E. W. (1991) *Adv. Enzymol. Relat. Areas Mol. Biol. 64*, 93−172.
3. Miles, E. W. (1995) Subcellular Biochemistry. In *Proteins: Structure, Function, and Protein Engineering* (Biswas, B. B., and Roy, S., Eds.) Vol. 24, pp 207−254, Plenum Press, New York.
4. Pan, P., Woehl, E., and Dunn, M. F. (1997) *Trends Biochem. Sci. 22*, 22−27.
5. Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., and Davies, D. R. (1988) *J. Biol. Chem. 263* (33), 17857−17871.
6. Miles, E. W., Rhee, S., and Davies, D. R. (1999) *J. Biol. Chem. 274* (18), 12193−12196.
7. Goldberg, M. E., York, S., and Stryer, L. (1968) *Biochemistry 7* (10), 3662−3667.
8. Miles, E. W. (1986) in *Pyridoxal Phosphate: Chemical, Biochemical and Medical Aspects* (Dolphin, D., Poulson, D., and Avramovic, O., Eds.) Vol. 1B, Part B, pp 253−310, John Wiley and Sons, New York.
9. Drewe, W. J., and Dunn, M. F. (1985) *Biochemistry 24* (15), 3977−3987.
10. Drewe, W. J., and Dunn, M. F. (1986) *Biochemistry 25* (9), 2494−2501.
11. Ahmed, S. A., Ruvinov, S. B., Kayastha, A. M., and Miles, E. W. (1991) *J. Biol. Chem. 266*, 21540−21557.
12. Ahmed, S. A., McPhie, P., and Miles, E. W. (1996) *J. Biol. Chem. 271*, 29100−29106.
13. Ahmed, S. A., and Miles, E. W. (1994) *J. Biol. Chem. 269*, 16486−16492.
14. Fan, Y. X., McPhie, P., and Miles, E. W. (1999) *Biochemistry 38* (24), 7881−7890.
15. Peracchi, A., Bettati, S., Mozzarelli, A., Rossi, G. L., Miles, E. W., and Dunn, M. F. (1996) *Biochemistry 35* (6), 1872−1880.
16. Ruvinov, S. B., Ahmed, S. A., McPhie, P., and Miles, E. W. (1995) *J. Biol. Chem. 270*, 17333−17338.
17. Rowlett, R., Yang, L.-h., Ahmed, S. A., McPhie, P., Jhee, K.-H., and Miles, E. W. (1998) *Biochemistry 37*, 2961−2968.
18. Yang, L.-h., Ahmed, S. A., Rhee, S., and Miles, E. W. (1997) *J. Biol. Chem. 272*, 7859−7866.
19. Peracchi, A., Mozzarelli, A., and Rossi, G. L. (1995) *Biochemistry 34* (29), 9459−9465.
20. Brzović, P. S., Ngo, K., and Dunn, M. F. (1992) *Biochemistry 31*, 3831−3839.
21. Brzović, P. S., Sawa, Y., Hyde, C. C., Miles, E. W., and Dunn, M. F. (1992) *J. Biol. Chem. 267*, 13028−13038.
22. Pan, P., and Dunn, M. F. (1996) *Biochemistry 35*, 5002−5013.
23. Leja, C. A., Woehl, E. U., and Dunn, M. F. (1995) *Biochemistry 34*, 6552−6561.
24. Woehl, E., and Dunn, M. F. (1999) *Biochemistry 38* (22), 7118−7130.
25. Woehl, E., and Dunn, M. F. (1999) *Biochemistry 38* (22), 7131−7140.
26. Rhee, S., Parris, K. D., Ahmed, S. A., Miles, E. W., and Davies, D. R. (1996) *Biochemistry 35* (13), 4211−4221.
27. Rhee, S., Parris, K. D., Hyde, C. C., Ahmed, S. A., Miles, E. W., and Davies, D. R. (1997) *Biochemistry 36* (25), 7664−7680.
28. Rhee, S., Miles, E. W., and Davies, D. R. (1998) *J. Biol. Chem. 273* (15), 8553−8555.
29. Rhee, S., Miles, E. W., Mozzarelli, A., and Davies, D. R. (1998) *Biochemistry 37* (30), 10653−10659.
30. Schneider, T. R., Gerhardt, E., Lee, M., Liang, P.-H., Anderson, K. S., and Schlichting, I. (1998) *Biochemistry 37* (16), 5394−5406.
31. Hatanaka, M., White, E. A., Horibata, K., and Crawford, I. P. (1962) *Arch. Biochem. Biophys. 97*, 596−606.
32. Woehl, E. U., and Dunn, M. F. (1995) *Biochemistry 34* (29), 9466−9476.
33. Miles, E. W., and McPhie, P. (1974) *J. Biol. Chem. 249*, 2852−2857.
34. Ro, H.-S., and Miles, E. W. (1999) *J. Biol. Chem. 274*, 31189−31194.
35. Yang, L.-h., Ahmed, S. A., and Miles, E. W. (1996) *Protein Expression Purif. 8*, 126−136.
36. Kawasaki, H., Bauerle, R., Zon, G., Ahmed, S. A., and Miles, E. W. (1987) *J. Biol. Chem. 262* (22), 10678−10683.
37. Miles, E. W., Kawasaki, H., Ahmed, S. A., Morita, H., Morita, H., and Nagata, S. (1989) *J. Biol. Chem. 264* (11), 6280−6287.
38. Yang, X.-J., Ruvinov, S. B., and Miles, E. M. (1992) *Protein Expression Purif. 3*, 347−354.
39. Miles, E. W., Bauerle, R., and Ahmed, S. A. (1987) *Methods Enzymol. 142*, 398−414.
40. Ruvinov, S. B., and Miles, E. W. (1994) *J. Biol. Chem. 269*, 11703−11706.
41. Ahmed, S. A., McPhie, P., and Miles, E. W. (1996) *J. Biol. Chem. 271* (15), 8612−8617.
42. Remeta, D. P., Miles, E. W., and Ginsburg, A. (1995) *Pure Applied Chem. 67*, 1859−1866.
43. Gutfreund, H. (1995) *Kinetics for the Life Sciences*, Cambridge University Press, Cambridge.
44. Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74* (6), 2236−2240.
45. Segel, I. H. (1975) *Enzyme Kinetics. Behavior and analysis of rapid equilibrium and steady-state enzyme systems*, John Wiley & Sons, New York.
46. Massey, V., Curti, B., and Ganther, H. (1966) *J. Biol. Chem. 241* (10), 2347−2357.
47. Allen, B., Blum, M., Cunningham, A., Tu, G. C., and Hofmann, T. (1990) *J. Biol. Chem. 265* (9), 5060−5065.
48. Lakomek, M., Scharnetzky, M., Tillmann, W., Schroter, W., and Winkler, H. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364* (7), 787−792.
49. Wrba, A., Schweiger, A., Schultes, V., Jaenicke, R., and Zavodsky, P. (1990) *Biochemistry 29* (33), 7584−7592.
50. Shrake, A., Fisher, M. T., McFarland, P. J., and Ginsburg, A. (1989) *Biochemistry 28* (15), 6281−6294.
51. Anderson, K. S., Miles, E. W., and Johnson, K. A. (1991) *J. Biol. Chem. 266*, 8020−8033.
52. Banik, U., Zhu, D.-M., Chock, P. B., and Miles, E. W. (1995) *Biochemistry 34* (39), 12704−12711.
53. Lane, A. N., and Kirschner, K. (1983) *Eur. J. Biochem. 129* (3), 571−582.
54. Buddenbaum, W. E., and Shiner, V. J. (1977) in *Isotope Effects on Enzyme Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., and Northrop, D. B., Eds.) pp 1−36, University Park Press, Baltimore, MD.
55. Cook, P. F., Blanchard, J. S., and Cleland, W. W. (1980) *Biochemistry 19*, 4853−4858.
56. Vogel, P. C., and Stern, M. J. (1971) *J. Chem. Phys. 54* (2), 779−796.
57. Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., and Northrop, D. B., Eds.) pp 122−152, University Park Press, Baltimore, MD.
58. Wiesinger, H., and Hinz, H. J. (1984) *Biochemistry 23* (21), 4928−4934.
59. Dunn, M. F., Aguilar, V., Brzović, P. S., Drewe, W. F. J., Houben, K. F., Leja, C. A., and Roy, M. (1990) *Biochemistry 29*, 8598−8607.
60. Licht, S. S., Lawrence, C. C., and Stubbe, J. (1999) *Biochemistry 38* (4), 1234−1242.
61. Hur, O., Leja, C., and Dunn, M. F. (1996) *Biochemistry 35* (23), 7378−7386.
62. Yang, X.-J., and Miles, E. W. (1993) *J. Biol. Chem. 268*, 22269−22272.
63. Fersht, A. (1985) *Enzyme Structure and Mechanism*, p 93, W. H. Freeman and Company, New York.
64. Onuffer, J. J., and Kirsch, J. F. (1994) *Protein Eng. 7* (3), 413−424.
65. Auld, D. S., and Bruice, T. C. (1967) *J. Am. Chem. Soc. 89*, 2098−2106.

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