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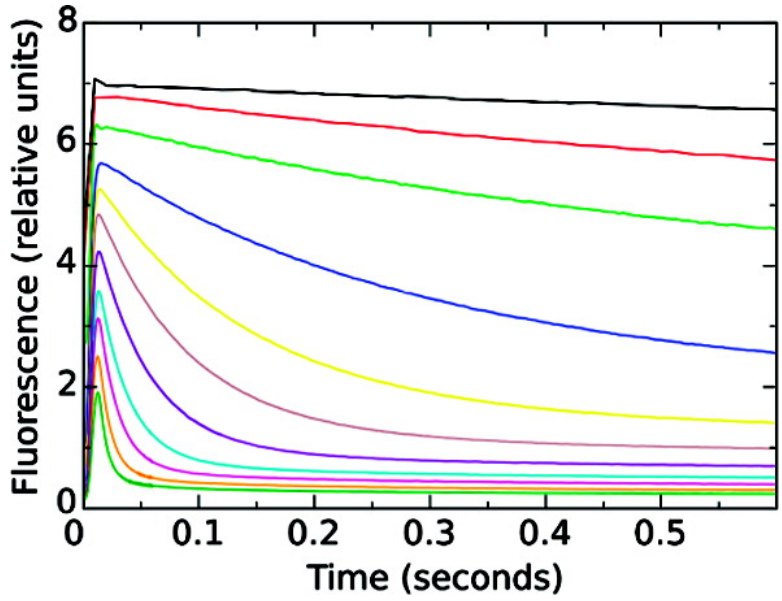

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Pressure and Temperature Jump Relaxation Kinetics of the Conformational Change in *Salmonella typhimurium* Tryptophan Synthase L-Serine Complex: Large Activation Compressibility and Heat Capacity Changes Demonstrate the Contribution of Solvation

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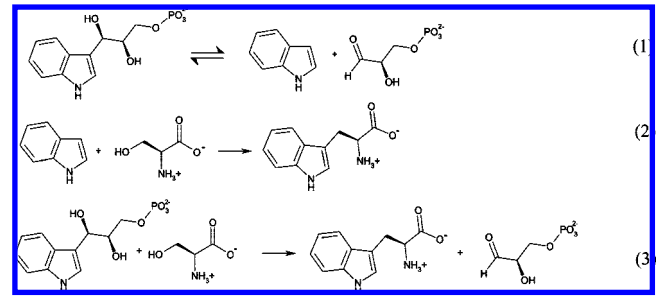
Abstract: Tryptophan synthase is an $\alpha_2\beta_2$ multienzyme complex that exhibits coupling of the α- and β-subunit reactions by tightly controlled allosteric interactions. A wide range of parameters can affect the allosteric interactions, including monovalent cations, pH, α-site and β-site ligands, temperature, and pressure. Rapid changes in hydrostatic pressure (P-jump) and temperature (T-jump) were used to examine the effects of pressure and temperature on the rates of the interconversion of external aldimine and aminoacrylate intermediates in the Tryptophan synthase-L-Ser complex. The intense fluorescence emission of the Tryptophan synthase L-Ser external aldimine complex at 495 nm, with 420 nm excitation, provides a probe of the conformational state of Trp synthase. P-jump measurements allowed the determination of rate constants for the reactions in the presence of Na^+, Na^+ with benzimidazole (BZI), and NH_4^+. The data require a compressibility term, $\beta_o^{\ddagger}$, to obtain good fits, especially for the NH_4^+ and BZI/Na^+ data. The compressibility changes are consistent with changes in solvation in the transition state. The transition state for the relaxation is more similar in volume to the closed aminoacrylate complex in the presence of Na^+, while it is more similar to the open external aldimine in the presence of NH_4^+. Differences between the relaxations for positive and negative P-jumps may arise from changing relative populations of microstates with pressure. T-jump experiments of the Na^+ form of the tryptophan synthase-L-Ser complex show large changes in rate and amplitude over the temperature range from 7 to 45 °C. The Arrhenius plots show strong curvature, and hence require a heat capacity term, $\Delta C_p^{\ddagger}$, to obtain good fits. The values of $\Delta C_p^{\ddagger}$ are very large and negative (−3.6 to −4.4 kJ mol^{-1} K^{-1}). These changes are also consistent with large changes in solvation in the transition state for interconversion of external aldimine and aminoacrylate intermediates in the Tryptophan synthase-L-Ser complex.

Introduction

Tryptophan (Trp) synthase[1] from *Salmonella typhimurium* is an $\alpha_2\beta_2$ tetramer, with the individual α-subunits attached to the opposite ends of a β_2-dimer.[2,3] The α-subunit of Trp synthase catalyzes the reversible cleavage of indole-3-glycerol phosphate to give indole and D-glyceraldehyde-3-phosphate (G3P) (eq 1), and the β-subunit, containing pyridoxal-5′-phosphate (PLP), catalyzes the condensation reaction of indole with L-serine resulting in L-Trp (eq 2). The physiological reaction of Trp synthase (eq 3) is the coupled reaction of both eqs 1 and 2; thus, indole is an obligatory intermediate but is not released into solution.[4−6] Allosteric communication between the α- and β-sites of Trp synthase coordinates the coupled reaction (eq 3) to



avoid the release of indole to solution. The enzyme, initially in an open conformation, binds indole-3-glycerol phosphate (IGP) at the α-site and L-Ser at the β-site, forming an external aldimine complex (E_{AL}, Scheme 1). This complex is in equilibrium with a closed conformation, in which dehydration of E_{AL} results in formation of a electrophilic aminoacrylate intermediate, E_{AA}, in the β-active site, which activates the α-site to cleave IGP to

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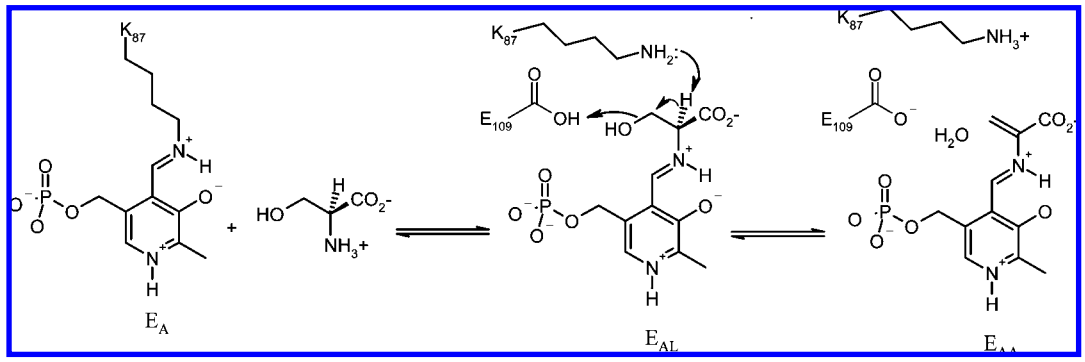
Scheme 1



form indole and D-glyceraldehyde-3-phosphate (G3P).[5−7] The indole then travels from the α-site through a 25−30 Å long tunnel to the β-site, where a Michael-type reaction with the aminoacrylate forms the external aldimine complex of the product, L-Trp.[3,5,8] In this product complex, the equilibrium shifts toward the open conformation,[5] and G3P is then released from the α-site, resulting in opening of the β-site and release of newly formed L-Trp. The equilibrium between the low activity (open) and high activity (closed) conformations of Trp synthase has been investigated extensively and is known to be affected by a wide range of variables, including α-subunit ligands,[6−12] monovalent cations,[9,13−17] solvents,[18,19] pH,[10] temperature,[9,10] and, more recently, hydrostatic pressure.[20,21] Only hydrostatic pressure perturbation allows for the quantitative determination of the internal equilibrium constant, K_{eq}, for the interconversion of $E_{AL}-E_{AA}$ across a wide range of values of K_{eq}.[20,21] We have now used the pressure-jump and temperature-jump techniques to determine the kinetics of relaxation of Trp synthase in the presence of monovalent cations, Na^+ and NH_4^+, and Na^+ together with an allosteric ligand, benzimidazole (BZI). The results indicate that there are significant changes of hydration in the transition state for the interconversion of E_{AL} and E_{AA}, and the degree of hydration of the transition state depends on the nature of the ligands.

Experimental Methods

Materials. L-Serine was obtained from United States Biochemicals Corporation. Triethanolamine was a product of J. T. Baker. Monovalent cation chlorides were obtained from Fisher Scientific. Benzimidazole (BZI) was obtained from Aldrich and was recrystallized from water after decolorization with charcoal before use.

Enzyme. Trp synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* was prepared from cells of *E. coli* containing the plasmid pEBA-10[22] as described previously.[23] For the experiments, the protein was applied to a PD-10 (Pharmacia) column equilibrated with 0.05 M triethanolamine hydrochloride buffer, pH 8.0, to remove inorganic monovalent cations, and then concentrated in an Amicon untrafiltration cell over a YM-30 membrane. Triethanolamine hydrochloride was used as the buffer for hydrostatic pressure measurements since it has a small pK_a change with pressure,[24] while sodium phosphate was used for the temperature experiments, since phosphate has a small temperature coefficient. Dilutions of the enzyme, to a final concentration of 0.7 mg/mL, were made in the same buffer, containing either 0.1 M NaCl or NH_4Cl and 0.1 M L-Ser, immediately prior to collection of the data. For some experiments, 5 mM BZI was added in addition to NaCl. The P-jump experiments were performed at 25 ± 0.1 °C.

Instrumentation. Pressure jumps were performed with an Aminco SLM Bowman2 spectrofluorimeter as previously described.[25,26] The principle of this technique is as follows: Two high pressure cells (the one containing the sample has sapphire windows; the other one being used as a ballast is blind) are independently pressurized. Opening a pneumatic valve between the two cells results in a pressure jump with a dead time of less than 5 ms. The excitation wavelength was 420 nm (4 nm bandwidth), and emission

(1) Trp synthase, the $\alpha_2\beta_2$ dimer of Tryptophan synthase from *Salmonella typhimurium*; E_{AL}, the external aldimine of Trp synthase and L-Serine; E_{AA}, the aminoacrylate intermediate of Trp synthase; IGP, indoleglycerol-3-phosphate; BZI, benzimidazole; G3P, D-glyceraldehyde-3-phosphate; P-jump, rapid pressure changes; T-jump, rapid temperature changes; k_o, the rate constant for opening the closed conformation; k_c, the rate constant for closing of the open conformation.

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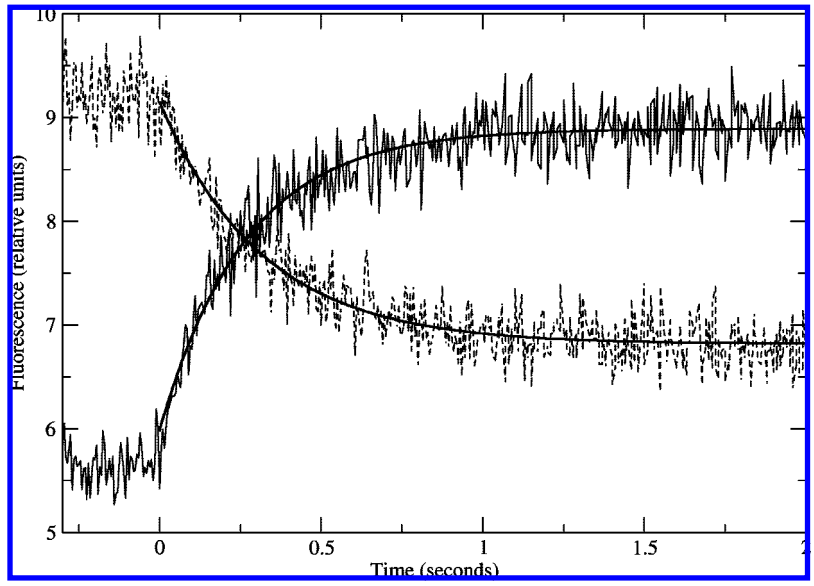

Figure 1. P-jump of Trp synthase−L-Ser complex. The emission at 495 nm was measured with excitation at 420 nm. Solid line, relaxation after jump from 745 to 910 bar. Dashed line, relaxation after jump from 921 to 760 bar. The curves show the results of fitting the data to a single exponential.

was recorded at 495 nm with a bandwidth of 4 nm. The sample (0.6 mL, contained in a cylindrical quartz cuvette) was pressurized in the sample compartment of the spectrofluorimeter according to our standard method.[25,26] Temperature-jump experiments were performed with a stopped-flow SFM3 apparatus from BioLogic, equipped with an mT-jump device. The principle of these experiments is the following: The two injection lines (the one containing the enzyme, the other one containing the buffer), as well as the observation cell, are independently thermostatted by Peltier elements. Before each experimental series, the Peltier elements had to be calibrated to make sure that the temperature resulting from mixing the contents of the two injection lines was identical to the temperature of the observation cell. These calibration experiments were conducted using the temperature dependence of the fluorescence emission intensity of *N*-acetyltryptophanamide.[25] The dead time of the T-jump experiments was estimated at 3.4 ms, as calculated by the driving software provided by the manufacturer. During all the experiments, the enzyme was conserved in the injection syringe, thermostatted at 25 °C. Care was taken that it did not reside more than 30 s inside the injection line.

Determination of Kinetic Parameters from Relaxation Profiles. After each P- or T-jump the relaxation profiles of the reaction were fitted to single-exponential and, when necessary, to double (sequential) exponential decays, according to either eq 4 or 5, where $I(t)$ and I_o are the fluorescence intensities

$$I(t) = I_o + A^*(1 - \exp(-k_{obs}{}^*t)) \quad (4)$$

$$I(t) = I_o + A^*(1 - \exp(-k_{obs1}{}^*t)) + B^*(1 - \exp(-k_{obs2}{}^*t)) \quad (5)$$

at time t and at time zero, A and B are the phase amplitudes, and k_{obs} is the measured rate constant at the final pressure p or temperature T. The individual apparent forward and reverse rate constants of the relaxation, k_o and k_c, were determined from the values of k_{obs} according to eq 6 and to eqs 7 and 8,

$$k_{obs} = k_o + k_c \quad (6)$$

$$K(p) = \exp(-(\Delta G_o + p\Delta V_o/RT)) = k_o/k_c \quad (7)$$

$$K(T) = \exp(\Delta S_o/R - \Delta H_o/RT) = k_o/k_c \quad (8)$$

where $K(p)$ is the equilibrium constant at pressure p; $K(T)$ the equilibrium constant at temperature T; and ΔG_o, ΔV_o, ΔS_o, and ΔH_o are the free energy, the volume, the entropy, and the enthalpy change of the transition. The values of ΔG_o and ΔV_o for eq 7 were obtained from previous equilibrium experiments,[20,21] and ΔS_o and ΔH_o for eq 8 were taken from ref 9. It should be noted that p is the final pressure, and T is the final temperature of each jump. Plots of k versus the final pressure of each jump allowed determination of $\Delta V^{\ddagger}$ and $\beta^{\ddagger}_o$, the volume and compressibility of activation, respectively, by fitting to eq 9. Using a two-state Kramers transition state analysis, thermodynamic parameters of activation ($\Delta H^{\ddagger}_i$, $\Delta S^{\ddagger}_i$, and $\Delta C_p{}^{\ddagger}_i$) were extracted from fitting $\ln(k_i)$ as a function of temperature using eq 10, where $\eta_{(T1/2)}$ and $\eta_{(T)}$ are the viscosity of water at the half-transition temperature and at the final experimental temperature, respectively.[25,27,28] The pre-exponential factor, $\nu_{(T1/2)}$, in eq 10 was set to 10^6 s^{-1}.

$$k_p = k_0 \exp(-p(\Delta V^{\ddagger}/RT) - \beta^{\ddagger}_o p^2) \quad (9)$$

$$\ln k = \ln(\nu_{(T1/2)}) + \ln(\eta_{(T1/2)}/\eta_{(T)}) + (\Delta C^{\ddagger}_p/R - \Delta H^{\ddagger}/R)(1/T) - (\Delta C^{\ddagger}_p/R)\ln(T_0/T) + \Delta S^{\ddagger}/R - \Delta C^{\ddagger}_p/R \quad (10)$$

Results

Effect of Pressure on Relaxation Rate Constants for Trp Synthase−L-Serine Complexes. The Trp synthase−L-Ser complex exists in an equilibrium between external aldimine (E_{AL}) and aminoacrylate (E_{AA}) structures.[4−21] E_{AL} is also highly fluorescent, with a strong emission maximum at 495 nm upon excitation at 420 nm.[29,30] We have shown in previous work that hydrostatic pressure shifts the absorption spectrum of Trp synthase−L-Ser complexes to favor E_{AL}, with λ_{max} at 423 nm, and correspondingly increases fluorescence emission at 495 nm.[20,21] When relatively small pressure changes, about 10 MPa, are performed rapidly, as a "P-jump", relaxations are observed in the fluorescence emission of the Trp synthase-L-Ser external aldimine (Figure 1). Although there is a slow deamination reaction of L-Ser under the reaction conditions, it does not interfere with the fluorescence measurements at 495 nm. Previous experiments had shown that there is no change in the

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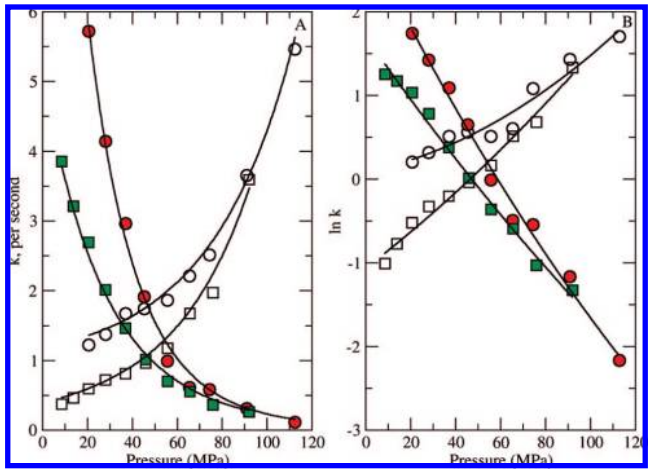

Figure 2. Pressure dependence of individual rate constants, k_o (filled symbols) and k_c (open symbols), determined from upward (filled symbols) and downward (open symbols) P-jumps at 25 °C in the presence of 0.1 M NaCl. The dependence of the individual rate constants on initial conditions is shown for positive (○, ●) and negative P-jumps (□, ■). (A) Plot of the data and of the results of exponential fitting to eq 9. (B) Log plot of the data and fits.

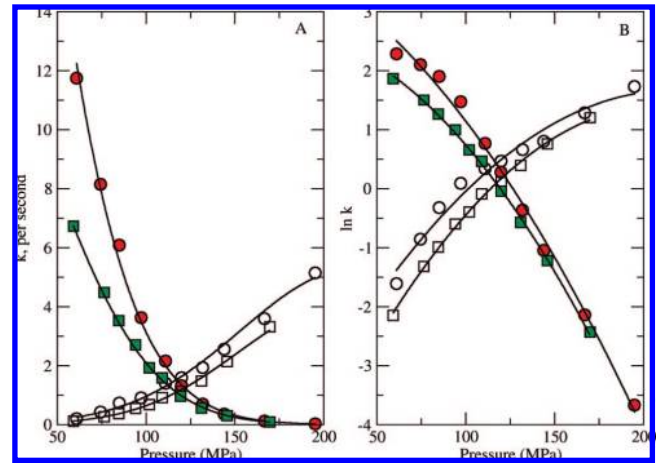

Figure 3. Pressure dependence of individual rate constants, k_o (filled symbols) and k_c (open symbols), determined from upward (filled symbols) and downward (open symbols) P-jumps at 25 °C in the presence of 0.1 M NH_4Cl. The dependence of the individual rate constants on initial conditions is shown for positive (○, ●), and negative P-jumps (□, ■). (A) Plot of the data and of the results of exponential fitting to eq 9. (B) Log plot of the data and fits.

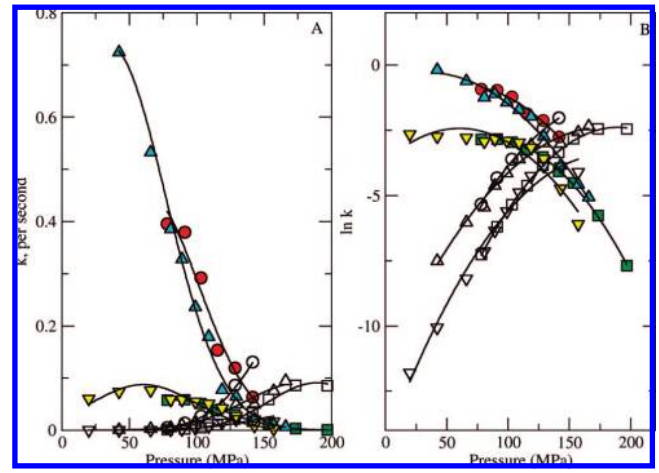

Figure 4. Pressure dependence of individual rate constants, k_o (filled symbols) and k_c (open symbols), determined from upward (filled symbols) and downward (open symbols) P-jumps at 25 °C in the presence of 0.1 M NaCl and 5 mM BZI. The dependence of the individual rate constants on initial conditions is shown for positive (●,○, ■, □), and negative P-jumps (▲, Δ, ▼, □). (A) Plot of the data and of the results of exponential fitting to eq 9. (B) Log plot of the data and fits.

equilbrium position at any given pressure over the period of data collection, 1−2 h.[21] In previous studies, we performed P-jumps from 1 MPa to relatively low final hydrostatic pressures (10−40 MPa) and back, with a dead time of about 0.1 ms, and we observed relaxations with fast (>200 s^{-1}), intermediate (10−20 s^{-1}), and slow (1−2 s^{-1}) phases.[20] These relaxations are similar to those observed in stopped flow experiments of the reaction of Trp synthase with L-Ser.[4,16,17,31−34] At those pressures, we did not see consistent pressure dependence of the rate constant for the fastest phase, so we concluded that it must have a small activation volume. In the present work, we were able to perform P-jumps in approximately 10 MPa steps reaching final pressures of up to 200 MPa, but with a significantly longer dead time of about 5 ms, thus precluding observation of the fast phase. In contrast to the previous data,[20] the present relaxation data fit well to a single exponential, so the data were analyzed using eq 4. The apparent relaxation rate constants, k_{obs}, decrease with increasing pressure up to 60 MPa in the presence of Na^+, or 140 MPa in the presence of NH_4^+, then increase as pressure is raised further. These chevron-shaped plots result from differences in the activation volumes of k_o and k_c, the apparent relaxation rate constants in the forward and reverse directions, respectively. The values of k_{obs} can be separated into the apparent forward and reverse rate constants, k_o and k_c, for forming the open and closed conformations, respectively, as described in the Experimental Section. The effects of pressure on k_o and k_c for the Trp synthase−L-Ser complex in the presence of Na^+, NH_4^+, and Na^+ with BZI are shown in Figures 2−4, respectively. Intriguingly, the rate constant for the relaxation at any given pressure was found to be dependent on whether it was reached by a positive or negative p-jump (compare circles and squares in Figures 2, 3, and 4). Although the plots of k versus pressure in the presence of Na^+ in Figure 2 can be fitted reasonably well with simple exponential regressions, the data in the presence of NH_4^+ in Figure 3 are strongly curved, indicating that the apparent activation volume depends on pressure. Indeed, a significantly better fit was obtained for the data in Figure 2 by including the activation compressibility, $\beta^{\ddagger}_o$ ($= \delta(\Delta V^{\ddagger})/\delta p$) as a fitting parameter. Fitting of the pressure dependent kinetic data in Figures 2 and 3 to eq 9 allows us to obtain the activation volumes and $\beta^{\ddagger}_o$ values for k_o and k_c, with the results given in Table 1. In the presence of both Na^+ and BZI (Figure 4), the relaxations are much slower and distinctly biphasic, so the data were fit with eq 5. The pressure dependencies for these data also show strong curvature, and hence both phases were also fitted to eq 9 to obtain the $\Delta V^{\ddagger}_o$ and $\beta^{\ddagger}_o$ values given in Table 1. There are striking differences in the parameters for the relaxations of the Na^+ complex with those of the NH_4^+ and Na^+/BZI systems. In particular, the signs of the $\beta^{\ddagger}_o$ values are positive for measurements in Na^+ and negative for the others (Table 1), suggesting that the transition states are structurally very different.

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Table 1. Kinetic Parameters for Trp Synthase−L-Ser Complex from P-Jump Measurements

cation, ligand	positive p-jump						negative p-jump					
	k_o, s^{-1}	$\Delta V_o^{\ddagger}$ mL mol^{-1}	$\beta_o^{\ddagger}$, mL MPa^{-1}	k_c, s^{-1}	$\Delta V_c^{\ddagger}$ mL mol^{-1}	$\beta_o^{\ddagger}$, mL MPa^{-1}	k_o, s^{-1}	$\Delta V_o^{\ddagger}$ mL mol^{-1}	$\beta_o^{\ddagger}$, mL MPa^{-1}	k_c, s^{-1}	$\Delta V_c^{\ddagger}$ mL mol^{-1}	$\beta_o^{\ddagger}$, mL MPa^{-1}
Na^+	1.09	−12	0.397	17.3	125	0.397	0.34	−48	0.230	5.45	89	0.229
NH_4^+	0.014	−127	−0.588	47.5	36	−0.630	0.0043	−153	−0.736	14.4	8	−0.736
Na^+, BZI	0 (1.8×10^{-6})[a]	−279	−1.46	0.139	−89	−1.46	0 (2.0×10^{-8})[a]	−235	−1.32	0.0015	−46	−1.32
	0 (3.9×10^{-8})[a]	−349	−1.84	0.003	−160	−1.84	0 (3.2×10^{-7})[a]	−285	−1.59	0.0247	−96	−1.59

[a] Calculated from the value of $K_{eq} = k_o/k_c = 1.3 \times 10^{-5}$, from ref 21.

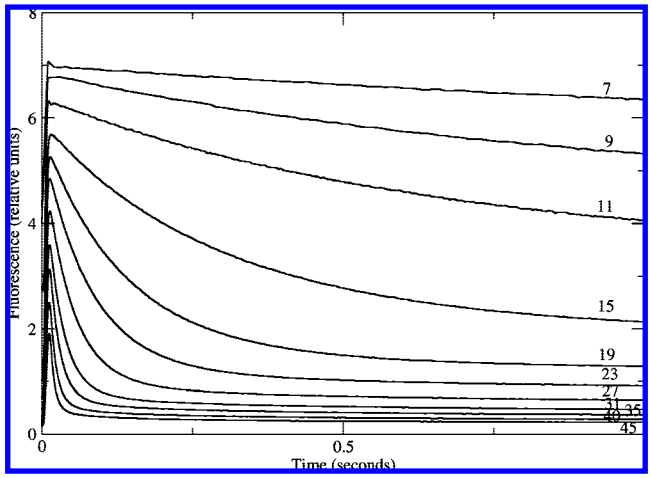


Figure 5. T-jump of Trp synthase−L-Ser complex. Solutions contained Trp synthase (1 mg/mL, 13.5 μM in $\alpha\beta$) in 0.05 M TEA-HCl, pH 8.0, with 0.1 M L-Ser and 0.1 M NaCl. The enzyme was incubated at 5 °C and mixed with 0.05 M TEA-HCl, pH 8.0, with 0.1 M L-Ser and 0.1 M NaCl maintained at another temperature to achieve the final temperatures (°C) indicated.

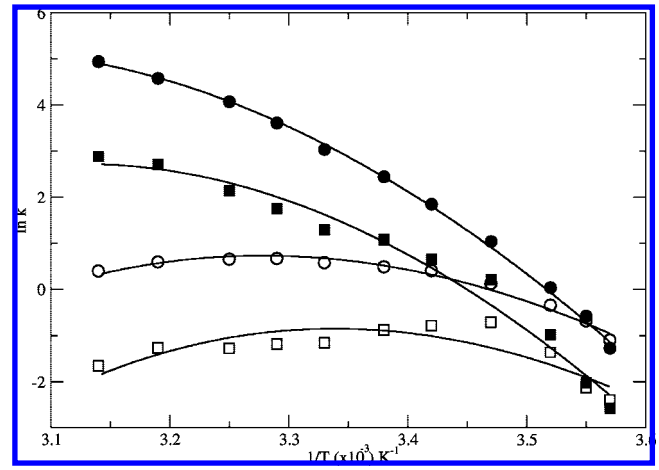


Figure 6. Arrhenius plot of upward T-jump induced kinetics. The fast phase is presented by circles, and the slow phase by squares; k_o is presented by open symbols, and k_c by closed symbols.

Table 2. Activation parameters for Trp Synthase−L-Ser Complex from T-Jump Measurements[a]

	$\Delta C_p^{\ddagger}$ (kJ mol^{-1} K^{-1})	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta G^{\ddagger}$ (kJ mol^{-1})
k_c (fast)	-3.6 ± 0.3	27.1 ± 2.5	-21.7 ± 0.8	29.7
k_o (fast)	-3.6 ± 0.3	119.2 ± 2.4	305.2 ± 8.5	33.5
k_c (slow)	-4.4 ± 0.5	11.6 ± 1.5	-85.8 ± 9.8	32.9
k_o (slow)	-4.4 ± 0.5	103.6 ± 7.8	241.1 ± 20.1	36.8

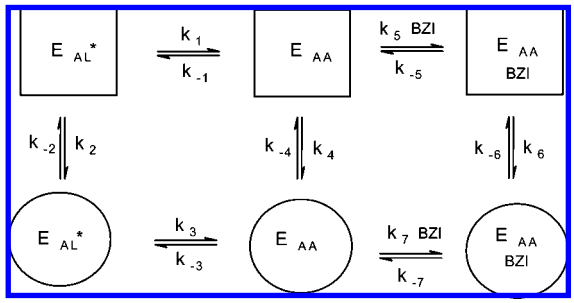
[a] Parameters are calculated for $T = 20$ °C.

Effect of Temperature on Relaxation Rate Constants for Trp Synthase−L-Serine Complexes. The absorption spectra of the Trp synthase−L-Ser complex are temperature dependent in the presence of Na^+,[7,8] whereas in the presence of NH_4^+ or Na^+ and BZI, there is no significant change with temperature due to the position of the equilibrium far toward E_{AA}.[20,21] Thus, the fluorescence emission of the Trp synthase−L-Ser complex in the presence of Na^+ is temperature dependent. Early studies by Faeder and Hammes showed that relaxations can be observed when Trp synthase is subjected to rapid increases in temperature (T-jump).[31] When the temperature of the Trp synthase−L-Ser complex in the presence of Na^+ is rapidly changed by mixing of two solutions at different temperatures in a stopped-flow T-jump experiment, relaxations are observed (Figure 5). These data exhibit two exponential phases, as has been observed commonly in stopped-flow studies with Trp synthase and L-Ser in the presence of Na^+.[4,16,17,31−34] The values of our observed rate constants, obtained by fitting to eq 5, are in good agreement with those obtained from stopped-flow results. Both the rates and the amplitudes of the relaxations are strongly temperature dependent, as can be seen from Figure 5, as k_{obs} for the fast phase increases more than 150-fold over the temperature range from 7 to 45 °C. As described in the Experimental Section, we can extract the individual component rate constants from the observed rate constants. The Arrhenius plots for the individual rate constants, k_o and k_c, show strong curvature (Figure 6), so a simple linear fit is not adequate, but the data fit quite well when a term containing a change in heat capacity in the transition state, $\Delta C_p^{\ddagger}$, is included, as in eq 10. The activation parameters obtained from fitting the data in Figure 6 to eq 10 are given in Table 2, and the lines shown in Figure 6 are the fitted curves. Since previous studies did not find a ΔC_p for the equilibrium reaction,[9] we assumed that ΔC_p was negligible to calculate K_{eq} at each temperature to separate the rate constants. The results in Table 2 show that there is quite a large negative $\Delta C_p^{\ddagger}$, about −4 kJ/mol K, for the relaxation. The values of $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ of k_o (closed to open) and k_c (open to closed) are also quite large, and their combination gives the expected equilibrium thermodynamic values of ΔS and ΔH.[9]

Discussion

Trp synthase is one of the best characterized examples of a multienzyme complex with stringent allosteric control of the overall reaction. The enzyme has been proposed to cycle between a high activity closed conformation and a low activity open conformation during catalysis. The transition between the open and closed conformations is controlled by the allosteric interactions of the α-subunit and the β-subunit in response to the binding of substrates and effectors. These allosteric interactions are influenced by the presence of α- and β-site ligands,[5−7,10−12] monovalent cations,[9−17] temperature,[9,10] solvents,[18,19] pH,[10] and hydrostatic pressure.[20,21] In the open conformation, the internal equilibrium between L-Ser external aldimine (E_{AL}) and aminoacrylate (E_{AA}) forms of the enzyme

Scheme 2



favors the highly fluorescent E_{AL} (Scheme 1). In contrast, in the closed conformation, the equilibrium position favors E_{AA}, which is only weakly fluorescent.[29,30] Thus, the fluorescence of E_{AL} serves as a highly sensitive probe for the conformational state of Trp synthase. As pressure is applied to solutions of the Trp synthase−L-Ser complex, we previously observed increased fluorescence emission resulting from a greater relative concentration of E_{AL}.[20,21] These data also show that the conformational transition is fully reversible.[20,21] The rapid kinetics of the relaxations (Figure 1) with both positive and negative P-jumps show that these results are not due to subunit dissociation of the $\alpha_2\beta_2$ tetramer under pressure, since subunit dissociation and reassociation occur with a $t_{1/2}$ of about 10 min.[35,36]

The relaxations observed after P-jumps (Figure 1) can be fitted to obtain the rate constants for relaxation between E_{AL} and E_{AA}. Stopped-flow and temperature-jump measurements of the formation of E_{AA} from Trp synthase and L-Ser have been performed previously,[4,16,17,31−34] and k_{obs} values determined. These reactions have frequently been found to be biphasic. However the slow phase is of low amplitude and slower than k_{cat}, so its significance for catalysis is unclear. Although we have suggested that the slow phase corresponds to the reaction of the open, low activity conformation,[20] it may also represent an alternative low activity conformation. In our present P-jump experiments with the Na^+ enzyme, we observed only a single exponential process, but in the T-jumps, the relaxations were biphasic. The values of k_{obs} for E_{AA} formation are strongly influenced by pH, temperature, ligands, and monovalent cations,[4,16,17,31−34] and our results are in good agreement with these previous results. The values of k_{obs} obtained from any relaxation to equilibrium are a composite of two rate constants, corresponding to the apparent forward and reverse rate constants, and it is sometimes difficult to extract these individual components from k_{obs}. In our case, however, analysis of the variation of k_{obs} and K_{eq} with pressure and temperature allows us to obtain the individual values of k_o, the apparent rate constant for formation of the open conformation from the closed conformation, and k_c, the apparent rate constants for formation of the closed conformation from the open conformation (Table 1).

A minimal mechanism for the reactions is given in Scheme 2, where the squares respresent the open conformation and the circles the closed conformation. In the absence of BZI, we have a cyclic mechanism with four species. In the general case, the differential equations for this mechanism do not have an exact solution,[37] but there are simplifying conditions which allow solution. It has been shown that the interconversion of E_{AA} from open to closed conformations does not occur ($k_4 + k_{-4} \approx 0$) and the conformational change is faster than the chemistry of conversion of E_{AL} to E_{AA} ($k_2 + k_{-2} \gg k_3 + k_{-3}$) for the Na^+ form of Trp synthase.[16] In addition, the formation of E_{AA} from E_{AL} for the open, inactive form of enzyme must be slower than that for the active enzyme ($k_3 + k_{-3} \gg k_1 + k_{-1}$). Thus, with these assumptions, the two possible relaxations are given by eqs 11 and 12, where $K_2 = k_2/k_{-2}$. In these equations, k_o corresponds to steps in the closed to open direction and k_c to steps in the open to closed direction (eqs 11a, b and 12a, b). The other simplifying case would be that where

$$1/\tau_1 = k_o + k_c = k_3K_2/(1 + K_2) + k_{-3} \quad (11)$$

$$k_o = k_{-3} \quad (11a)$$

$$k_c = k_3K_2/(1 + K_2) \quad (11b)$$

$$1/\tau_2 = k_o + k_c = k_1/(1 + K_2)(1 + K_3) + k_{-1} \quad (12)$$

$$k_o = k_1/(1 + K_2)(1 + K_3) \quad (12a)$$

$$k_c = k_{-1} \quad (12b)$$

$$1/\tau = k_o + k_c = k_2/(1 + K_1) + k_{-2}/(1 + K_3) \quad (13)$$

$$k_o = k_{-2}/(1 + K_3) \quad (13a)$$

$$k_c = k_2/(1 + K_1) \quad (13b)$$

the conformational change is slower than the chemical steps ($k_2 + k_{-2} \ll k_3 + k_{-3}$ and $k_1 + k_{-1}$). In this case the relaxation is given by eq 13, where $K_1 = k_1/k_{-1}$ and $K_3 = k_3/k_{-3}$, and only a single relaxation is expected, assuming that $k_4 + k_{-4} \approx 0$. The corresponding equations for k_o and k_c are given by eqs 13a and 13b, respectively.

In the presence of saturating BZI, which shifts the equilibrium toward the closed form by a factor of about 10 000,[21] the mechanism has at least six possible species, with the two new species present in the BZI bound states, E_{AA}−BZI, which can exist as either open or closed complexes. Since the four other species in the mechanism are identical to the reaction without BZI, and the relaxations without BZI are at least an order of magnitude faster than that with BZI (Table 1), the relaxations in the presence of BZI would be expected to involve only the BZI complexes. Thus, there are four steps which may be involved in the P-jumps with BZI, $k_4 + k_{-4}$, $k_5 + k_{-5}$, $k_6 + k_{-6}$, and $k_7 + k_{-7}$. The mechanism is cyclic, as above, but limiting cases are possible. One limiting case is if the binding of BZI is fast. In this case, due to the loss of 1 degree of freedom in the cyclic mechanism, only one relaxation is predicted. On the other hand, $k_6 + k_{-6}$ may be negligible, which is reasonable since the corresponding opening of the closed aminoacrylate ($k_4 + k_{-4}$) is not observed.[16] In this case, two relaxations are expected, corresponding to $k_5 + k_{-5}$ and $k_{-7} + k_{-7}$, and these relaxations are observed to be distinctly biphasic. Thus, the two relaxations in the presence of BZI are given by eqs 14 and 15, where $K_1 = k_1/k_{-1}$, and $K_3 = k_3/k_{-3}$. The corresponding equations for k_o and k_c are given by eqs 14a, 14b, 15a, and 15b. It is also possible that there are two different conformational states in the equilibrium for the E_{AA}−BZI complex, since BZI has been shown to bind to both α- and β-active sites of Trp synthase.[38,39]

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$$1/\tau_1 = k_o + k_c = k_7K_3[BZI]/(1 + K_3) + k_{-7} \quad (14)$$

$$k_o = k_{-7} \quad (14a)$$

$$k_c = k_{-7}K_3[BZI]/(1 + K_3) \quad (14b)$$

$$1/\tau_2 = k_o + k_c = k_5K_1[BZI]/(1 + K_1) + k_{-5} \quad (15)$$

$$k_o = k_{-5} \quad (15a)$$

$$k_c = k_5K_1[BZI]/(1 + K_1) \quad (15b)$$

For the reaction of L-Ser with Trp synthase in the presence of Na^+, it has been concluded that the conformational change is fast, and the chemical conversion of E_{AL} to E_{AA} is slow, based on the observation of a significant isotope effect on formation of E_{AA} with [α-^{2}H]-Ser in stopped-flow experiments.[16,32,33,40] Thus, the relaxations should be given by eqs 11 and 12, but we observed only one phase, possibly because the amplitude of the second slow phase is small. In our experiments, we measured the relaxation between E_{AL} and E_{AA} after both positive and negative P-jumps (Figure 1). The plots of k and ln(k) versus pressure are modestly curved in the case of the Na^+ enzyme (Figure 2) and strongly curved with the NH_4^+ form (Figure 3) and the Na^+ form complexed with BZI (Figure 4). Nonlinear effects of pressure on rates and equilibria can be interpreted either as the result of kinetic complexity[41] or as a result of compressibility. Since we see no evidence for nonlinearity in the equilibrium data,[20,21] and the logarithmic plots in Figures 2−4 show clear curvature rather than sharp breaks, we analyzed the kinetic data as arising from transition state compressibility, with the $\beta^{\ddagger}_o$ parameters given in Table 2. The expressions of k_o and k_c, given above, generally contain a rate constant and an equilibrium constant. Compressibility changes can arise from differences in solvation, as well as changes in the packing of the protein.[42] The negative values of $\beta^{\ddagger}_o$, observed for the NH_4^+ form and the Na^+ form with BZI, indicate that the transition state for interconversion of E_{AL} and E_{AA} is less compressible than the reactant state and are consistent with a transition state which is more highly solvated and/or is more tightly packed than the ground state. In contrast, the value of $\beta^{\ddagger}_o$ for the Na^+ form of the enzyme is smaller and positive, indicating that the transition state in that case is less solvated than the ground state and/or the protein is more loosely packed. These dramatic differences between the effects of pressure on the Na^+ and NH_4^+ forms of Trp synthase (Figures 2 and 3, Table 1) suggest that the rate-limiting step in the relaxations may be different and the conformational change between open and closed states may be slow, and at least partially rate-limiting, in the NH_4^+ enzyme but fast with respect to chemistry for the Na^+ form of enzyme. In agreement with this interpretation, a recent paper has shown that the magnitude of the kinetic isotope effect on formation of E_{AA} with Trp synthase and α-[^{2}H]-Ser in a stopped-flow experiment is dependent on the monovalent cation, nearly 6 in the presence of Na^+ and close to 1 for NH_4^+.[40] Furthermore, the steady state kinetic isotope effect with α-[^{2}H]-Ser in the presence of Na^+ decreases from about 5 at 4 °C to less than 2 at 43 °C. In the case of the Na^+/BZI system, as discussed above, the very slow reaction is probably due to reaction of E_{AA} with BZI. Since the binding of BZI is a second-order process, it might be expected to be diffusion controlled. The extremely slow relaxation kinetics suggest that BZI binding to E_{AA} requires a slow conformational change. BZI is thought to bind to E_{AA} as an analogue of indole in proximity to the aminoacrylate, and it may interact by hydrogen-bonding either with βAsp-305, which was suggested be an acid−base catalyst in the interconversion of E_{AL} and E_{AA},[43] or with βGlu-109, which interacts with the indole NH in the crystal structure of the βK87T−L-Trp complex[44] and is near the indoline ring of dihydroisotryptophan in the quinonoid complex of that amino acid with wild-type Trp synthase.[45] Recently, the structure of E_{AA} was determined, and βGlu-109 is H-bonded to a bound water near the β-carbon of the aminoacrylate, so it is likely that βGlu-109 is a general acid catalyst for the dehydration.[12]

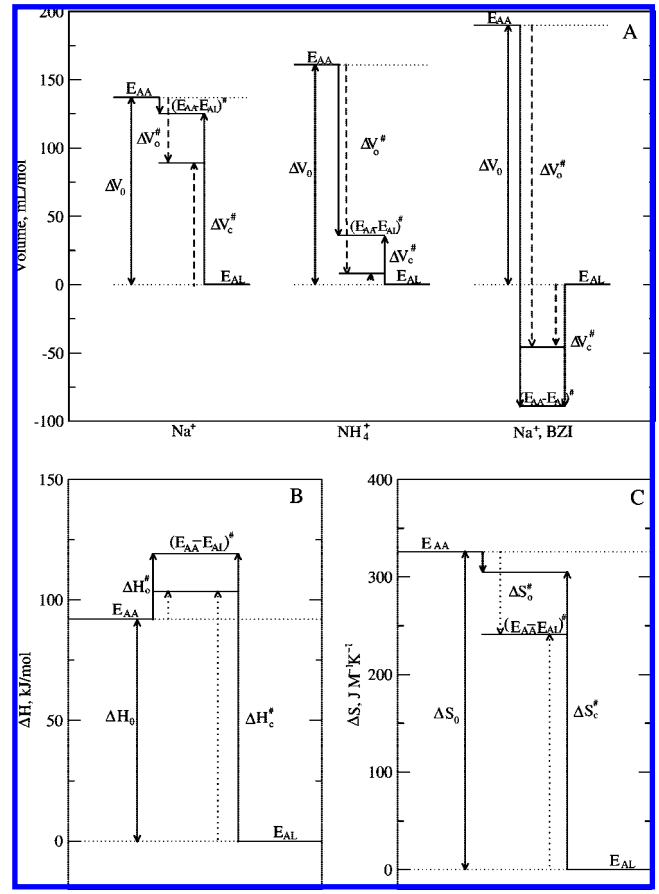

Figure 7. Reaction profiles for the conformational change in Trp synthase−L-Ser complex. (A) Volume changes. The double-headed arrows show the equilibrium reaction volumes. The solid arrows show the activation volumes for the positive P-jumps, and the dashed arrows show the activation volumes for the negative P-jumps. (B) Enthalpy changes. The double-headed arrows show the equilibrium enthalpy changes. The solid arrows show the enthalpy changes for the fast phase, and the dotted arrows show the enthalpy changes for the slow phase. (C) Entropy changes. The double-headed arrows show the equilibrium entropy changes. The solid arrows show the entropy changes for the fast phase, and the dotted arrows show the entropy changes for the slow phase.

The activation volumes for the interconversion of the open and closed conformations of Trp synthase are given in Table 1, and the reaction volume profile is shown in Figure 7A. There

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is excellent agreement between ΔV_o obtained from the equilibrium analysis[20,21] and ΔV_o obtained by combining the values of the activation volumes for k_o and k_c. It is interesting that the transition state position is closer to E_{AL} with negative jumps in Na^+ but lies toward E_{AA} with negative jumps in NH_4^+ (Figure 7A). This may be a reflection of the difference in the rate-determining step suggested above. For the positive P-jumps (solid arrows in Figure 7), the transition state is 72% toward E_{AA} in Na^+ but only 23% in NH_4^+. For the negative P-jumps (dashed arrows in Figure 7), the transition state is 57% and 34% toward E_{AA} in Na^+ and NH_4^+, respectively. Thus, much less volume change is required to reach the transition state in NH_4^+, despite the larger overall equilibrium ΔV_o in NH_4^+. As with the compressibility, discussed above, these effects indicate significant differences between the Na^+ and NH_4^+ forms of the enzyme. This could be another result of the change in the rate-limiting step or be the result of changes in structure. Although a structure of the NH_4^+ form of Trp synthase is not available, it is likely that it is similar to the Cs^+ form, which is known,[46] since Cs^+ and NH_4^+ have more similar effects on the conformational equilibrium than Na^+.[9,21] There are clear structural differences between the Na^+ and Cs^+ forms of the enzyme, including differences in ion pairs formed in the closed conformation.[44,46]

In the case of the BZI complex with E_{AA} in the Na^+ enzyme, the activation volume does not lie between that of the two reactants, but rather it is smaller in volume than either the reactant or product states, E_{AL} or E_{AA} (Figure 7A). This suggests a major structural change is required to reach the transition state for the conformational change when BZI is bound. The reaction volume for the conformational transition of Trp synthase is mainly contributed by solvation of the protein by the aqueous environment.[20] The open conformation, corresponding to E_{AL}, is more highly solvated, and the protein-bound solvent has a smaller volume by about 20% than the bulk solvent;[47,48] hence, the open conformation has the smaller net system volume. The activation volumes reflect the amount of solvation (or desolvation) required to reach the apparent transition states between E_{AL} and E_{AA}, and thus for the Trp synthase Na^+/BZI system it shows that the transition state is more highly solvated than either reactant state. In the case of the BZI complex, the mechanism in Scheme 2 suggests that BZI must dissociate from the enzyme for the reaction to proceed. This would require additional solvation of the ligand and the binding site(s) in the transition state, resulting in an additional decrease in system volume.

For the Na^+ form of Trp synthase−L-Ser, the equilibrium lies much closer to E_{AL} at ambient temperature and pressure than for the NH_4^+ form or for the BZI complex. Thus, it is feasible to perform temperature perturbations of the system. Previous studies have measured thermodynamic parameters for the interconversion of E_{AL} and E_{AA},[9] and T-jumps have been performed at a single temperature.[29] However, the effects of variation of temperature on the kinetics of interconversion of E_{AL} and E_{AA} have not been analyzed before. We performed T-jumps between 5 °C and temperatures ranging from 7 to 45 °C, and we observed relaxations which show quite dramatic changes in amplitude and rate with temperature (Figure 5). Since the thermodynamic parameters, ΔH and ΔS, have been measured for the Na^+ form of Trp synthase,[9] it is possible to calculate K_{eq} at each temperature and separate k_{obs} into the component rate constants, k_o and k_c, as described in the Experimental Section. The Arrhenius plots of these rate constants are strongly curved (Figure 6). The curvature of Arrhenius plots can be often attributed to the temperature dependence of the equilibrium of a conformational change underlying the kinetic events. Previous studies of the temperature dependence of the steady state kinetics of Trp synthase showed nonlinear Arrhenius plots that were explained as resulting from the relatively fast conformational change.[9] However, in our case we are directly observing the conformational change itself, so that is not an explanation for the curvature that we observe. Furthermore, we see distinct curvature in the plots rather than sharp breaks. Thus, the curvature in the Arrhenius plots was treated as arising from transition state heat capacity changes, $\Delta C_p^{\ddagger}$, and excellent fits were obtained (Figure 6). We note that the previous studies of the temperature dependence of the steady state kinetics did not show any evidence for a ΔC_p on the conformational equilibrium.[9] The values of $\Delta C_p^{\ddagger}$ observed in the present work are remarkably large and negative (Table 2), showing that the transition state of the reaction is much less structured than the ground state. Similar negative values of $\Delta C_p^{\ddagger}$ were observed in the folding of barley chymotrypsin inhibitor 2, and this was suggested to arise from a loss in exposed hydrophobic surface as the protein folded.[49] Similarly, the exposed hydrophobic surface in the open and closed conformational states of Trp synthase would have ice-like water ("icebergs") associated with it. These appear to be molten in the T-jump induced kinetic transition state, resulting in a strongly negative activation heat capacity. This interpretation is also consistent with the large activation entropy (Table 2) and with the positive compressibility (Table 1) discussed above. Furthermore, although the conformational change appears to be faster than chemistry at 25 °C, it may be more rate-determining at higher temperatures, since the steady state kinetic isotope effect with α-[^{2}H]-Ser decreases with increasing T.[9] In contrast to the P-jumps, the T-jump reactions are biphasic, and both phases show similar activation parameters, indicating that they arise from similar chemical processes (Table 2). The corresponding reaction profiles are shown in Figure 7B and 7C. A relatively large positive $\Delta H^{\ddagger}$ is offset by the large positive $\Delta S^{\ddagger}$, so the reaction rate constant is favorable as a result of the activation entropy. The large value of $\Delta H^{\ddagger}$ in the direction of the formation of E_{AA} is most likely the result of the formation of ion pairs in the closed conformation, which must be desolvated, while the favorable $\Delta S^{\ddagger}$ results from the desolvation of the ions and the release of water from the hydrophobic surface.

It is interesting that the rate constants we observe are different for the relaxations obtained from positive and negative P-jumps (Figures 2, 3, and 4, compare circles and squares). In addition, the activation volumes for the negative and positive P-jumps are different (Table 1), and thus the positions of the transition state differ (Figure 7A, solid arrows and dashed arrows). Normally, thermodynamics would dictate for a simple reaction that the relaxation kinetics would be independent of the direction of the P-jump and depend only on the final pressure, not on the initial state. Thus, this observation of different kinetics depending on the initial state implies that the relaxation for negative P-jumps takes place via a different manifold of states than the

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positive P-jumps. Similar results have been reported for the folding and unfolding of a 24 kDa protein from photosystem II, RsGFP, and Y115W RnaseA.[26] In the case of Trp synthase, the positive P-jumps relax in the direction of E_{AA} to E_{AL}, resulting in an increase in fluorescence, while the negative P-jumps relax from E_{AL} to E_{AA}, resulting in a decrease in fluorescence (Figure 1). The difference in the relaxation kinetics for the negative and positive P-jumps is likely a reflection of pressure-dependent dynamics in the population of microstates of E_{AL} and E_{AA}

Protein conformational changes are local changes in protein folding which are driven by the same physical forces as global protein folding. Thus, a conformational change can be considered as one step in the global folding or unfolding of a protein. Our results show large transition state compressibility and negative heat capacity changes associated with the conformational change between open and closed forms of the L-Ser complex of Trp synthase. The $\Delta C_p^{\ddagger}$ that we observe, about -4 kJ/mol, is similar to, but larger in magnitude than, that observed in the unfolding of barley chymotrypsin inhibitor 2 and barnase.[49] This is likely due to the larger size of the Trp synthase complex. To our knowledge, this is the largest negative $\Delta C_p^{\ddagger}$ yet reported for a protein folding transition.

Conclusions

P-jumps of Trp synthase−L-Ser complexes show relaxations which exhibit nonlinear plots of P versus ln k, suggesting that there is a significant compressibility of the transition state for interconversion of $E_{AL\text{-}Ser}$ and E_{AA}. The compressibility may be due to solvent reorganization in the transition state due to the conformational change. T-jumps of the Na^+ form of the Trp synthase−L-Ser complex show nonlinear Arrhenius plots, indicating a large heat capacity change, $\Delta C_p^{\ddagger}$, in the transition state. This also is consistent with large solvation changes in the transition state for interconversion of $E_{AL\text{-}Ser}$ and E_{AA}.

Acknowledgment. This research was supported in part by the intramural research program of the NIH, NIDDK (EWM, PM).

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