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Effects of Pressure and Osmolytes on the Allosteric Equilibria of *Salmonella typhimurium* Tryptophan Synthase

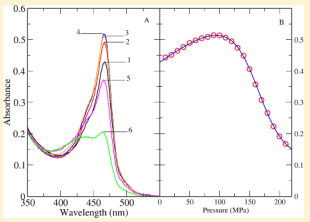
Robert S. Phillips,*[,†,‡] Alexandre Kim Wang,[†] Stephane Marchal,[§] and Reinhard Lange[§]

[†]Department of Chemistry, University of Georgia, Athens, Georgia 30602, United States

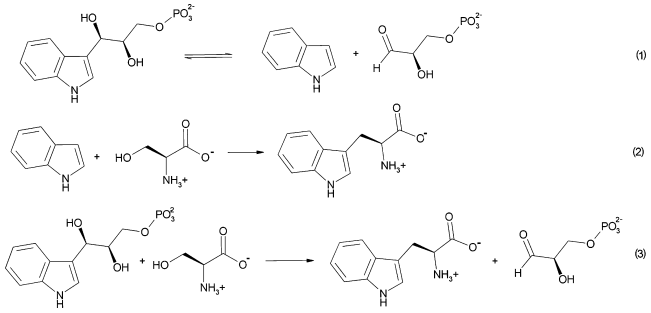
[‡]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602, United States

[§]Inserm U710, Montpellier, F-34095 France, Université de Montpellier 2, Montpellier, F-34095 France, and EPHE, Paris, F-75007 France

ABSTRACT: Osmolytes are common constituents of bacteria that may be produced or accumulate at high concentrations, up to 1 M, when cells are subjected to stresses like ionic strength and temperature. However, the effects of osmolytes on the allosteric properties of bacterial enzymes have rarely been examined. We have studied the effects of osmolytes and hydrostatic pressure on the allosteric equilibria of *Salmonella typhimurium* tryptophan (Trp) synthase. Trp synthase is a well-studied multienzyme complex with activity tightly regulated by allosteric interactions between the α- and β-subunits. Trp synthase activity is affected by a wide range of physical parameters, including monovalent cations, pH, ligands, solvents, and hydrostatic pressure. Osmolytes, including betaine, taurine, sucrose, and polyethylene glycol, activate Trp synthase 2−3-fold in the absence of monovalent cations, indicating that osmolytes can stabilize the active closed conformation. However, in the presence of monovalent cations, osmolytes have only minor effects on activity and allosteric equilibria, but 1 M betaine stabilizes the Trp synthase−Ser−indoline complex against apparent pressure-induced subunit dissociation. Na^+ and K^+ are more effective at shifting the α-aminoacrylate−indoline quinonoid equilibrium toward the quinonoid side, with a K_Q of 8−10, than NH_4^+ ($K_Q \sim 2$). Furthermore, pressure-jump experiments show that the mechanism of indoline reaction to form a quinonoid complex may be different for the NH_4^+ enzyme than the Na^+ and K^+ forms. These results show that osmolytes have subtle but significant effects on the allosteric properties of Trp synthase, and these effects may be important in vivo.



Tryptophan synthase is a multienzyme complex that catalyzes the last two steps in the biosynthesis of L-Trp in bacteria and plants (eqs 1 and 2). The physiological reaction is the sum of eqs 1 and 2 (eq 3). The reactions of the α- and β-subunits are



tightly coordinated by allosteric interactions in the complex to prevent the inefficient loss of indole, because L-Trp is a metabolically expensive amino acid. These allosteric interactions are influenced by a wide range of physical variables, including monovalent cations,[1−6] α- and β-ligands,[7−12] pH,[10] organic solvents,[13,14] temperature,[1,10] and pressure.[15−18] However, the effects of osmolytes on the catalytic and allosteric properties of Trp synthase have not been studied previously.

The mechanism of the β-reaction of Trp synthase begins with formation of an external aldimine of L-Ser, which undergoes subsequent elimination of water in a closed conformation to give an α-aminoacrylate (Scheme 1), activating the α-site to cleave indole-3-glycerol phosphate to indole and D-glyceraldehyde 3-phosphate.[19] The electrophilic α-aminoacrylate Schiff base then reacts with the indole nucleophile, arriving from the active site of the α-subunit through an ~30 Å intramolecular tunnel,[20] to give the quinonoid complex of L-Trp, which is then protonated to give the external aldimine of L-Trp, and finally releases the L-Trp product in the open conformation (Scheme 1). The allosteric properties of the L-Ser external aldimine−α-aminoacrylate equilibrium in the absence of indole have been studied extensively, because the two intermediates have distinct absorbance and fluorescence spectra. The quinonoid species formed by the reaction of indole with the α-aminoacrylate is transient, and thus, its allosteric properties have been more difficult to study. However, the quasi-stable quinonoid complex formed by the reaction of indoline with the α-aminoacrylate intermediate has been crystallized and the structure determined, and the effects of monovalent cations and α-ligands on the α-aminoacrylate−quinonoid equilibrium have been investigated.[21−24] Previously, we showed that hydrostatic pressure could be used to quantify the effects of physical parameters on the L-Ser external aldimine−α-aminoacrylate equilibrium.[15−18] In this work, we have examined and quantified the effects of osmolytes

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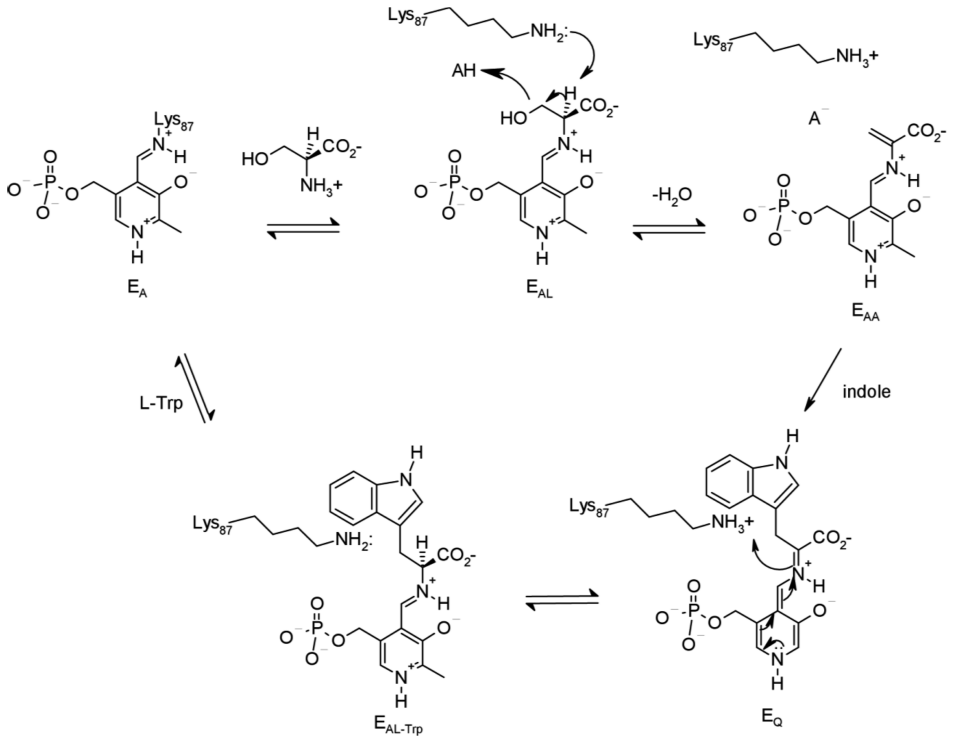
Scheme 1



and hydrostatic pressure on the α-aminoacrylate and quinonoid allosteric equilibria of Trp synthase.

EXPERIMENTAL PROCEDURES

Materials. L-Serine and betaine were obtained from United States Biochemicals Corp. Triethanolamine was a product of J. T. Baker. Monovalent cation chlorides were obtained from Fisher Scientific. Indoline was obtained as the free base from Aldrich and was converted to the HCl salt by addition of a stoichiometric amount of concentrated HCl to a solution in 95% ethanol. Addition of an equal volume of ether resulted in crystallization of indoline hydrochloride as white needles, which were filtered, washed with ether, and dried.

Enzyme. The Trp synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* was prepared from cells of *Escherichia coli* containing plasmid pEBA-10, as described previously.[25] For the pressure experiments, the protein was exchanged with 0.10 M triethanolamine hydrochloride buffer (pH 8.0) on a PD-10 (Amersham) gel filtration column to remove inorganic monovalent cations. Triethanolamine hydrochloride was used as the buffer for hydrostatic pressure measurements, because it has a relatively small ΔpK_a with pressure.[26] For the experiments containing indoline, the buffer was prepared so that the final pH after addition of 20 mM indoline hydrochloride was 8.0. Dilutions of the enzyme were made in the appropriate buffer, containing either 0.1 M NaCl, KCl, NH_4Cl, or CsCl, 0.1 M L-Ser, and 20 mM indoline hydrochloride, if added, immediately prior to collection of the data. For these experiments, the cations were added at concentrations of 0.1 M, as was done in previous papers,[1,15−17] to ensure that the effects of the cations on activity and spectra were maximal. The K_d for binding of the monovalent cations to Trp synthase is in the low millimolar range.[4]

Instrumentation. The absorbance data were collected on a modified Cary 14 instrument (OLIS, Inc., Bogart, GA) with a high-pressure cell from ISS (Champaign, IL), as previously described.[15,16] The samples were enclosed in 1.2 mL quartz bottles with a 9 mm path length, covered with a cap of Teflon tubing. Fluorescence equilibrium and pressure-jump kinetic data were collected with a home-built instrument as previously described.[27,28] The excitation wavelength was 423 nm (4 nm bandwidth), and the emission spectra were recorded 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.

Determination of Equilibrium Parameters. The equilibrium data were fit to a Boltzmann equation (eq 4)

$$A_P = \Delta A[K_o \times \exp(-P\Delta V/RT)] / [1 + K_o \times \exp(-P\Delta V/RT)] + A_o \quad (4)$$

where A_P is the observed absorbance or fluorescence at pressure P, K_o is the pressure-independent value of the equilibrium constant, ΔA is the change in the absorbance or fluorescence amplitude, ΔV is the change in the reaction volume, and A_o is the background absorbance or fluorescence. Where the changes in absorbance were biphasic, for the NH_4^+ enzyme in Figure 3, the data were fit to eq 5 for a two-step process.

$$A_P = \Delta A[K_1 \exp(-P\Delta V_1/RT) \times K_2 \exp(-P\Delta V_2/RT)] / [1 + K_2 \exp(-P\Delta V_2/RT) + K_1 \exp(-P\Delta V_1/RT) \times K_2 \exp(-P\Delta V_2/RT)] + A_o \quad (5)$$

The temperature-dependent experiments were performed as described by Fan et al.,[1] using a Cary 1 UV−vis spectrophotometer with a Peltier temperature-controlled cell holder. Scans were performed from 10 to 45 °C at 5 °C intervals, with a 5 min delay for equilibration between each scan. The K_{eq} at each temperature was calculated from eq 6

$$K_{eq} = A_T - A_{min}/A_{max} - A_T \quad (6)$$

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where A_T is the absorbance at 423 nm and temperature T, A_{max} is the maximal absorbance value at 423 nm, and A_{min} is the minimal absorbance value at 423 nm. The temperature-dependent data were then fitted to eq 7 with a linear regression to obtain the thermodynamic parameters.

$$\ln K = -\Delta H/RT + \Delta S/R \quad (7)$$

Determination of Kinetic Parameters from Relaxation Profiles. After each pressure jump, the relaxation profiles of the reaction were fit to single-exponential and, when necessary, double-exponential (sequential) decays, according to eq 8 or 9

$$I(t) = I_o + A[1 - \exp(-k_{obs}t)] \quad (8)$$

$$I(t) = I_o + A[1 - \exp(-k_{obs1}t)] + B[1 - \exp(-k_{obs2}t)] \quad (9)$$

where $I(t)$ and I_o are the fluorescence intensities at time t and time zero, respectively, A and B are the phase amplitudes, and k_{obs} is the measured rate constant at the final pressure P. The individual apparent forward and reverse rate constants of relaxation, k_o and k_c, respectively, were determined from the values of k_{obs} according to eqs 10 and 11

$$k_{obs} = k_o + k_c \quad (10)$$

$$K(P) = \exp[-(\Delta G_o + P\Delta V_o/RT)] = k_o/k_c \quad (11)$$

where $K(p)$ is the equilibrium constant at pressure P and ΔG_o and ΔV_o are the free energy and the volume change of the transition, respectively. The values of ΔG_o and ΔV_o for eq 11 were obtained from previous equilibrium experiments. It should be noted that P is the final pressure of each pressure jump. Plots of k versus the final pressure of each jump allowed determination of $\Delta V^{\ddagger}$ and $\Delta\beta_o^{\ddagger}$, the activation volume and activation compressibility, respectively, by fitting to eq 12.[29]

$$k_P = k_0 \exp(-P\Delta V_o^{\ddagger}/RT) - \Delta\beta_o^{\ddagger}P^2/2RT \quad (12)$$

RESULTS

Effects of Osmolytes on Trp Synthase Activity. A series of osmolytes was studied to determine their effects on Trp synthase activity. In the absence of monovalent cations, various osmolytes caused a significant 2−3-fold activation of Trp synthase (Table 1). In the presence of 0.1 M Na^+, an activating monovalent cation, smaller 1.3−1.7-fold increases in activity were observed. The effects of osmolytes were concentration-dependent. For the ionic osmolytes, taurine and betaine, the activation showed hyperbolic dependencies, reaching a maximum at 0.5 and 1 M, respectively (data not shown). Thus, 1 M betaine was chosen for the spectroscopic experiments described below because the effects are maximal, and it is an osmolyte commonly found in bacteria. In contrast, the nonionic osmolytes, sucrose and PEG-8000, showed linear dependencies, with maximal effects at the highest concentrations tested, 2 M and 5% (w/v), respectively. Sucrose gave the largest increase in activity both with and without Na^+, while betaine gave the smallest increase both with and without Na^+ (Table 1).

Table 1. Activation of Trp Synthase by Osmolytes

osmolyte	NaCl[a]	*x*-fold activation
betaine (1 M)	no	1.8
betaine (1 M)	yes	1.3
taurine (0.5 M)	no	3.0
taurine (0.5 M)	yes	1.3
PEG-8000 (5%)	no	2.1
PEG-8000 (5%)	yes	1.4
sucrose (2 M)	no	3.3
sucrose (2 M)	yes	1.7

[a]NaCl was added to a concentration of 0.1 M where indicated.

Effect of Betaine on the Trp Synthase Ser−External Aldimine−Aminoacrylate Equilibrium. The Trp synthase external aldimine (E_{AL}), α-aminoacrylate (E_{AA}), and L-Trp quinonoid (E_Q) intermediates have absorbance and fluorescence properties that are ideal for spectroscopic measurements (Scheme 2). The absorbance spectra can in principle provide quantification of all of the Trp synthase reaction intermediates, E_{AL}, E_{AA}, and E_Q. In contrast, the fluorescence spectra specifically detect only E_{AL}. Thus, the absorbance and fluorescence data provide complementary information. The effect of pressure on the spectra of Trp synthase complexed with Ser and monovalent cations (Na^+, K^+, and NH_4^+) in the absence and presence of 1 M betaine was determined using both absorbance and fluorescence spectroscopy (results listed in Table 2). Although Na^+ and K^+ are the most likely physiological cations of Trp synthase, we also studied NH_4^+, because it is known to have different effects on the kinetic and spectroscopic properties of Trp synthase.[1−6] The fluorescence data for the Trp synthase−Na^+−Ser complex in the presence of 1 M betaine are shown in Figure 1A. The fits of the fluorescence data as a function of pressure for all three cations to eq 4 are shown in Figure 1B. The fluorescence values at any given pressure are nearly identical whether they were obtained during compression or decompression (compare filled symbols and empty symbols in Figure 1B), indicating that the system is freely and completely reversible. The values of K_{eq} and ΔV obtained in these experiments in the presence of betaine are very similar to those obtained in its absence, by both absorbance and fluorescence measurements (Table 2). It should be noted that K_{eq} is defined in the direction from right to left of the reaction shown in Scheme 2, and a negative value of ΔV indicates a reaction that is favored by increasing pressure.

The effect of temperature on the Trp synthase−Ser equibrium with Na^+ was examined by absorbance at 423 nm in the absence and presence of 1 M betaine, as shown in Figure 2. In the absence of betaine [Figure 2 (●)], the values of ΔH and ΔS from linear regression of the data in Figure 2 to eq 6 are as follows for the direction of α-aminoacrylate formation: ΔH = 92.1 ± 3.5 kJ/mol, and ΔS = 328 ± 12 J mol^{-1} K^{-1}. This agrees exactly with the results of Fan et al.:[1] ΔH = 92 kJ/mol, and ΔS = 327 J mol^{-1} K^{-1}. In the presence of betaine [Figure 2 (○)], fitting gives the following values: ΔH = 68.7 ± 4.2 kJ/mol, and ΔS = 248 ± 15 J mol^{-1} K^{-1} (both of which are significantly smaller than those without betaine); however, the value of ΔG at 298 K is nonetheless very similar, in agreement with the results from pressure perturbation.

Effect of Pressure on the Spectra of the Trp Synthase−Ser−Indoline Complex. Addition of indoline to Trp synthase−Ser solutions results in an intense absorbance peak at 466 nm, because of the formation of a quasi-stable quinonoid intermediate (Scheme 2). Increasing the hydrostatic pressure on the complex of Trp synthase with L-Ser, indoline, and NH_4^+ results in a gradual increase in the absorbance peak at 466 nm, reaching a maximum at ∼100 MPa, and then a subsequent decrease at higher pressures, with the eventual formation of a much more weakly absorbing aldimine peak at

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Scheme 2

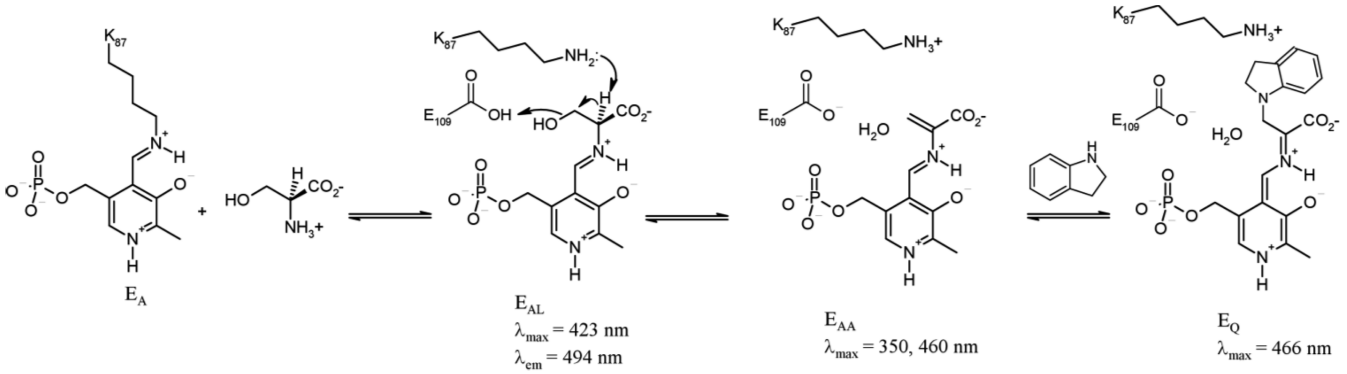

Table 2. Thermodynamic Parameters for Trp Synthase−Ser Complexes

	absorbance				fluorescence			
	K_{eq}		ΔV (mL/mol)		K_{eq}		ΔV (mL/mol)	
cation	with 1 M betaine	without 1 M betaine	with 1 M betaine	without 1 M betaine	with 1 M betaine	without 1 M betaine	with 1 M betaine	without 1 M betaine
Na^+	$(6.9 \pm 1.6) \times 10^{-2}$	$(8.8 \pm 4.0) \times 10^{-2}$	-141 ± 11	-136 ± 16	$(7.2 \pm 0.5) \times 10^{-2}$	$(6.9 \pm 0.5) \times 10^{-2}$	-133 ± 3	-133 ± 4
K^+	$(1.7 \pm 0.3) \times 10^{-2}$	$(5.2 \pm 1.0) \times 10^{-2}$	-141 ± 5	-123 ± 8	$(2.8 \pm 0.1) \times 10^{-2}$	$(3.8 \pm 0.4) \times 10^{-2}$	-123 ± 1	-131 ± 3
NH_4^+	$(1.6 \pm 0.4) \times 10^{-3}$	$(3.6 \pm 0.7) \times 10^{-3}$	-131 ± 6	-129 ± 5	$(1.6 \pm 0.2) \times 10^{-3}$	$(1.6 \pm 0.2) \times 10^{-3}$	-124 ± 2	-135 ± 3

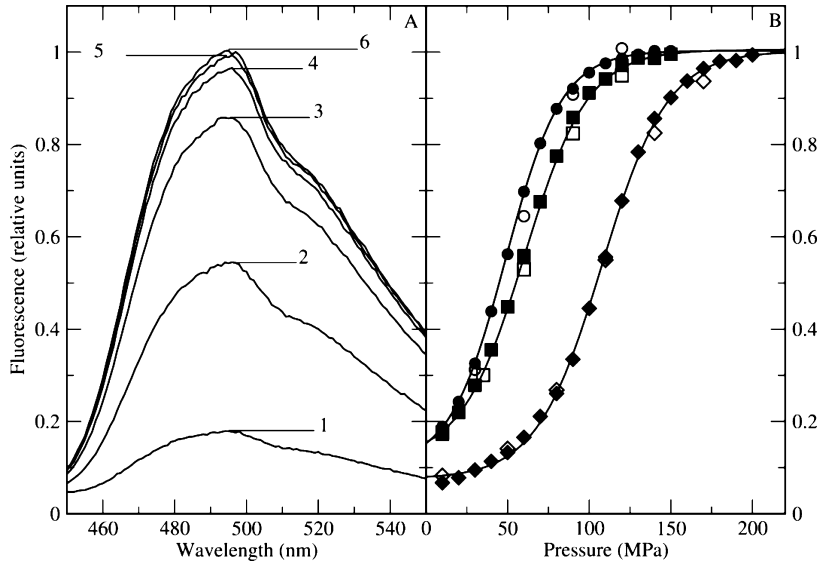

Figure 1. Fluorescence spectra of the Trp synthase−Ser complex in the presence of 1 M betaine. (A) Spectra for the Na^+ enzyme at 10 (1), 50 (2), 80 (3), 110 (4), 140 (5), and 150 MPa (6). (B) Plot of the fluorescence at 494 nm as a function of pressure for the Na^+ (circles), K^+ (squares), and NH_4^+ (diamonds) enzymes. The filled symbols are the results obtained during compression, and the empty symbols are the results obtained during decompression. The lines are the curves obtained from fitting to eq 4, with the parameters listed in Table 1.

423 nm (Figure 3A). These changes in spectra are not fully reversible upon decompression in the absence of 1 M betaine, because the absorbance at 466 nm returns to only ~50% of its initial level (data not shown), but in the presence of 1 M betaine, the absorbance returns to more than 95% of the initial value on decompression. Because of the apparent irreversibility of the pressure effects, the data with indoline in the absence of betaine were not fit to determine K_{eq} and ΔV values. Fitting of the compression data in the presence of indoline and 1 M betaine for the NH_4^+ enzyme in Figure 3B, between 10 and 200 MPa, to eq 5 gives a K_1 of $(1.3 \pm 0.3) \times 10^{-3}$and a ΔV_1 of -138 ± 10 mL/mol, with a K_2 of $(8.0 \pm 2.0) \times 10^{-4}$ and a ΔV_2 of -110 ± 15 mL/mol (Table 3). The values of K_1 and ΔV_1 are in excellent agreement with the values of K_{eq} and ΔV obtained with NH_4^+ in the absence of indoline (Table 2). The fitted curve of the 466 nm absorbance data with those parameters is shown in Figure 3B (diamonds). The absorbance changes for the Na^+ and K^+ form of the enzyme with L-Ser and indoline in 1 M betaine are simpler than that for NH_4^+, in that the absorbance at 466 nm decreases immediately as the pressure increases (Figure 3B). In contrast to the NH_4^+ data, fits of the Na^+ and K^+ data to eq 5 gave very large errors for the second set of K and ΔV parameters. Thus, the Na^+ and K^+ data were fit to eq 4, as shown in Figure 3B, to give the values of K_{eq} and ΔV listed in Table 3.

In this experiment, the observed K_{eq} is the product of two steps, K_{AA} for formation of the α-aminoacrylate, E_{AA}, from the

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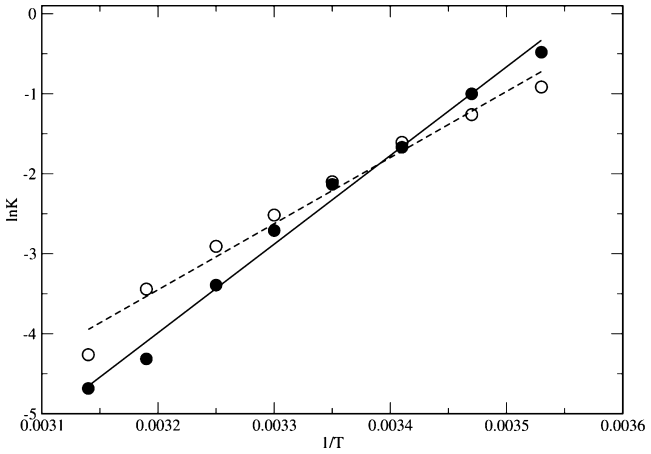

Figure 2. Effect of temperature on the Trp synthase–Ser–Na^+ equilibrium (●) without betaine or (○) with 1 M betaine. The lines are the results of fitting to eq 6.

external aldimine, E_{AL}, and K_Q for formation of the quinonoid intermediate from the aminoacrylate and indoline (eq 13).

$$E_{AL} \overset{K_{AA}}{\Leftrightarrow} E_{AA} \overset{K_Q}{\Leftrightarrow} E_Q \quad (13)$$

The values of K_{eq} obtained in the absence of indoline give us K_{AA}. Thus, $K_{eq} = K_{AA}K_Q$, and on the basis of the value of 1.6×10^{-3} found for K_{eq} for the aminoacrylate–aldimine equilibrium in the direction of formation of E_{AL} (Table 2), and the value of 8.0×10^{-4} found with indoline (Table 3), we calculate the value of K_Q for the quinonoid–aminoacrylate equilibrium with indoline in NH_4^+ to be 2. On the basis of the values of K_{eq} for Na^+ and K^+ obtained previously in Table 2, we can calculate the K_Q to be 9.7 and 7.7, respectively. In contrast to the Na^+, NH_4^+, and K^+ results, the absorbance spectra in the presence of Cs^+ are only partially reversible upon decompression, even in the presence of 1 M betaine, similar to the data without indoline. Despite being nonphysiological, Cs^+ is a cation commonly used in studies of Trp synthase because it is strongly activating and the resulting enzyme complex has allosteric properties different from those of the other alkali metal cations.[1−6,16,34] The $\Delta\Delta V$ values with Na^+ and K^+ are ~30 mL/mol and for NH_4^+ 20 mL/mol in the presence of indoline (Table 3).

The fluorescence emission of the Trp synthase–Ser–indoline complex at 494 nm, with 423 nm excitation, increases with pressure. The data for the Na^+, K^+, and NH_4^+ complexes were collected. As observed in the absorbance data, the effects of pressure were not reversible for the fluorescence data in the absence of betaine. The fluorescence data for the Ser–indoline–Trp synthase complex with Na^+ in the presence of 1 M betaine are shown in Figure 4A. Fitting the emission data at 494 nm to eq 4 in Figure 4B gives a K_{eq} of 6×10^{-3} and a ΔV of −106 mL/mol (Table 3), in excellent agreement with the absorbance results. The fluorescence changes in the presence of betaine are completely reversible (compare filled circles and empty circles in Figure 4B). The data and the fitted curves for K^+ and NH_4^+ using eq 4 are also shown in Figure 4B. Although there is an initial slope in the fluorescence data, particularly for NH_4^+, the fits were not improved when eq 5 was used. This slope does not appear to be related to the absorbance increase

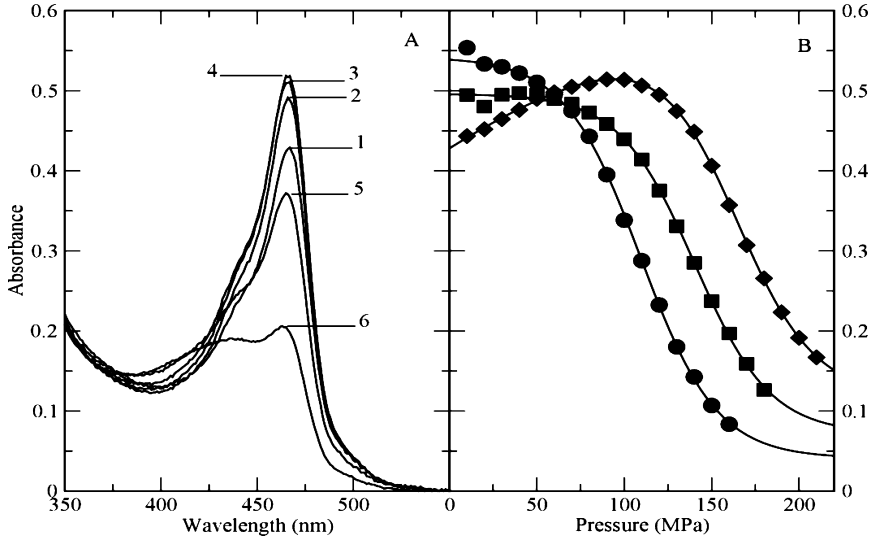

Figure 3. Absorbance spectra of the Trp synthase–Ser–indoline complex with NH_4^+ in the presence of 1 M betaine. (A) Spectra at 10 (1), 40 (2), 80 (3), 120 (4), 160 (5), and 200 MPa (6). (B) Plot of the absorbance at 466 nm as a function of pressure for Na^+ (●), K^+ (■), and NH_4^+ (◆) enzymes. The line for NH_4^+ is the curve obtained from fitting to eq 5, with the parameters listed in Table 1, and the lines for Na^+ and K^+ were obtained from fitting to eq 4.

Table 3. Thermodynamic Parameters for Trp Synthase–Indoline–Quinonoid Complexes[a]

	absorbance				fluorescence			
cation	K_{eq}	ΔV (mL/mol)	K_Q	$\Delta\Delta V$ (mL/mol)	K_{eq}	ΔV (mL/mol)	K_Q	$\Delta\Delta V$ (mL/mol)
Na^+	$(7.1 \pm 2.2) \times 10^{-3}$	-111 ± 8	9.7	30	$(6.0 \pm 0.8) \times 10^{-3}$	-106 ± 2	12	27
K^+	$(2.2 \pm 0.2) \times 10^{-3}$	-108 ± 2	7.7	33	$(3.9 \pm 0.4) \times 10^{-3}$	-97 ± 2	7.2	26
NH_4^+	$(8.0 \pm 2.0) \times 10^{-4}$	-110 ± 15	2.0	21	$(6.3 \pm 1.7) \times 10^{-4}$	-108 ± 4	2.5	16

[a]The solutions contained 1 M betaine, in addition to the indicated cation at 0.1 M, in 0.1 M triethanolamine hydrochloride (pH 8.0).

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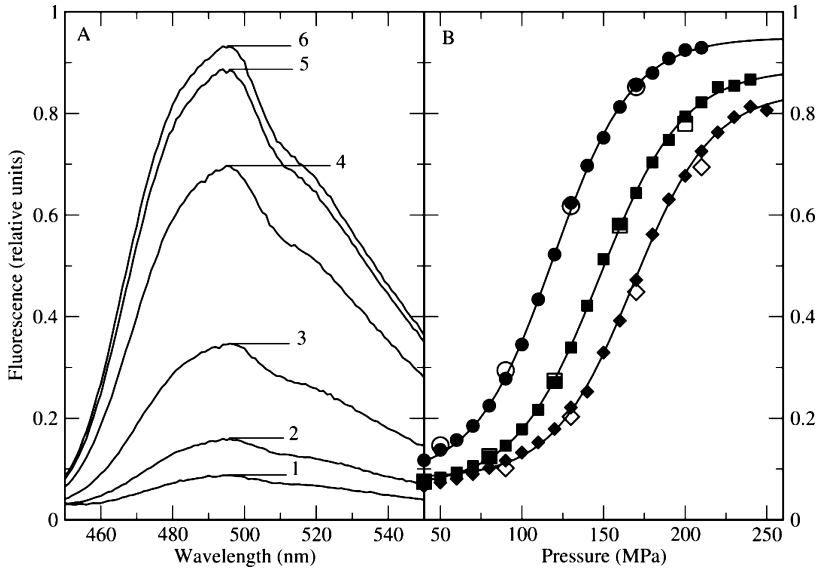

Figure 4. Fluorescence spectra of the Trp synthase–Ser–indoline complex in the presence of 1 M betaine. (A) Spectra for the Na^+ enzyme at 10 (1), 50 (2), 100 (3), 140 (4), 180 (5), and 210 MPa (6). (B) Plot of the fluorescence at 494 nm as a function of pressure for the Na^+, K^+, and NH_4^+ forms of the enzyme. The filled symbols are the results obtained during compression, and the empty symbols are the results obtained during decompression: (circles) Na^+, (squares) K^+, and (diamonds) NH_4^+. The lines are the curves obtained from fitting to eq 4, with the parameters listed in Table 1.

at 466 nm seen in Figure 3, because that would be expected to result in a decrease in fluorescence. The K_Q for the aminoacrylate–quinonoid equilibria can be calculated from these data to be 12, 7.8, and 2.5 for Na^+, K^+, and NH_4^+ enzymes, respectively, in good agreement with the absorbance results (Table 3). The $\Delta\Delta V$ values from the fluorescence determinations are also consistently lower by ~20–30 mL/mol in the presence of indoline (Table 3), in good agreement with the absorbance results.

Effect of Pressure on the Kinetics of Trp Synthase–Ser and Trp Synthase–Ser–Indoline Complexes. We found in previous studies that the effects of pressure on the kinetics of the Trp synthase Ser–aldimine–α-aminoacrylate equilibrium are complex, with chevron-shaped plots of $\ln(k_{obs})$ versus pressure.[17] Because the K_{eq} at each pressure is known from the fluorescence change, we can extract the forward (k_c) and reverse (k_o) rate constants from k_{obs} using eqs 9 and 10. Furthermore, there are different effects on the relaxations depending on the directions of the pressure jump, increasing pressure or decreasing pressure, as we observed previously. In this work, we performed similar pressure-jump experiments in the presence of 1 M betaine with Na^+, K^+, and NH_4^+, and also with indoline. Although we found previously that the relaxations could be fit with only a single phase, in these experiments a very slow second phase was sometimes observed. Because the rate constants of the slow phases are significantly lower than rates of turnover for Na^+ and K^+, they are not relevant to catalysis and will not be discussed further. The results of the fast phase of the pressure-jump experiments for the Trp synthase–Ser complex with K^+ are shown in Figure 5 and for the Trp synthase–Ser–indoline complex with Na^+ in Figure 6. It is interesting that the Na^+ and NH_4^+ forms of the enzyme show nonlinear plots of $\ln(k)$ versus pressure without betaine, but they show linear plots in the presence of betaine, similar to Figure 5. The kinetic parameters for the relaxations are generally similar with or without betaine (Table 4), although in some cases they differ by nearly an order of magnitude. Interestingly, addition of indoline

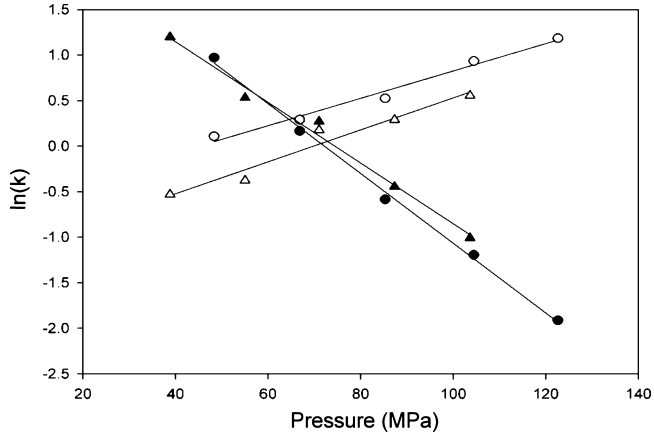

Figure 5. Effect of pressure on the rate constants for the Trp synthase–Ser complex in the presence of 0.1 M K^+ and 1 M betaine: (●) k_o, positive pressure jump; (○) k_o, negative pressure jump; (▲) k_c, positive pressure jump; (△) k_c, negative pressure jump. The lines are calculated with the parameters listed in Table 4.

slows the relaxations for the Na^+ and K^+ forms of the enzyme but accelerates the relaxation for the NH_4^+ enzyme (Table 4).

DISCUSSION

Effect of Osmolytes on Trp Synthase Activity. Bacterial cells contain high concentrations of osmolytes, such as betaine, proline, and trehalose,[30] in response to stresses such as changes in temperature or ionic strength in the medium. Intracellular concentrations of these osmolytes in bacteria under stress can be as high as 1 M,[30] yet they are rarely included in enzyme assays in vitro. The effect of osmolytes is to stabilize folded states of proteins, although the reasons for this stabilization are still not fully understood.[31,32] In the case of Trp synthase, formation of the α-aminoacrylate is largely due to hydrophobic effects, because there is a large increase in entropy with the formation of the closed conformation (326 J mol^{-1} K^{-1} for the

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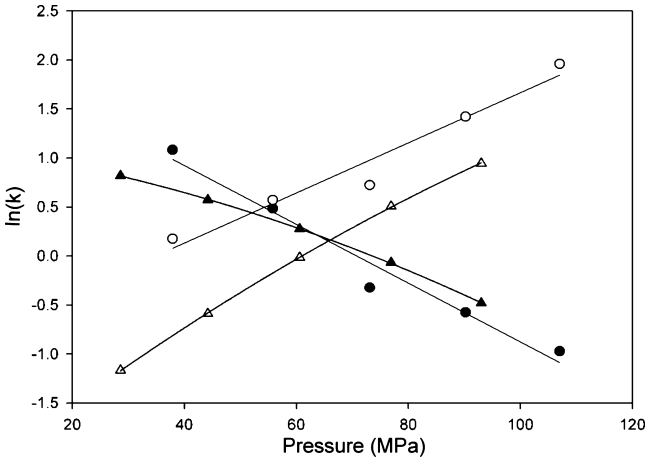

Figure 6. Effect of pressure on the rate constants for the Trp synthase–Ser–indoline complex in the presence of 0.1 M Na^+ and 1 M betaine: (●) k_o, positive pressure jump; (○) k_o, negative pressure jump; (▲) k_c, positive pressure jump; (△) k_c, negative pressure jump. The lines are calculated with the parameters listed in Table 4.

Na^+ enzyme), and indeed, the corresponding enthalpy change is positive.[1] We found that a wide range of osmolytes can increase Trp synthase activity up to 2–3-fold in the absence of monovalent cations (Table 1), consistent with osmolyte-dependent stabilization of the active, closed conformation. In the presence of Na^+, the osmolytes have much weaker effects on activity, 1.3–1.7-fold, because the monovalent cation already has a stimulatory effect on the allosteric equilibrium. It is also possible that 1 M betaine could affect Trp synthase activity by increasing the dielectric constant, especially in the absence of a monovalent cation. However, the stabilizing effects against hydrostatic pressure discussed below are more consistent with an osmotic effect. Furthermore, because the activation by osmolytes and monovalent cations is not independent (Table 1), it would suggest a common mechanism for activation, and that implies that dielectric effects are unlikely to play a major role in the effects of betaine. We selected 1 M betaine for further study under hydrostatic pressure, because it is commonly found as an osmolyte at comparable concentrations in bacterial cells,[30] and it does not increase viscosity, as some common osmolytes, such as sucrose or PEG, would.

Effect of Betaine on Trp Synthase Conformational Equilibria. We measured the thermodynamic parameters for the Trp synthase aldimine–aminoacrylate equilibrium with three monovalent cations, Na^+, K^+, and NH_4^+, in the presence and absence of 1 M betaine (results listed in Table 2). Within experimental error, there is no significant difference in the values of K_{eq} and ΔV obtained for any particular cation with or without betaine. This is consistent with the data in Table 1, which showed an only 30% increase in activity when betaine was added together with Na^+. It is interesting that the temperature dependence data show a decrease in both ΔH and ΔS in the presence of betaine (Figure 2). However, there is no significant change in ΔG at 25 °C, in agreement with the results from the pressure perturbation. We used the values of ΔS and ΔV in a previous report to calculate that each water molecule released from the protein solvation shell for the Trp synthase conformational transition contributes approximately −2.8 kJ/mol at 298 K for the Na^+ enzyme in the absence of betaine.[15] This value was found to be in excellent agreement with other measurements of the molecular contribution of

Table 4. Kinetic Parameters for Trp Synthase–L-Ser Complexes from Pressure-Jump Measurements[a]

	positive pressure jump					negative pressure jump				
cation, ligand, osmolyte	k_o (s^{-1})	$\Delta V_o^{\ddagger}$ (mL/mol)	$\Delta\beta_o^{\ddagger}$ (mL/MPa)	k_c (s^{-1})	$\Delta V_c^{\ddagger}$ (mL/mol)	$\Delta\beta_o^{\ddagger}$ (mL/MPa)	k_o (s^{-1})	$\Delta V_o^{\ddagger}$ (mL/mol)	$\Delta\beta_o^{\ddagger}$ (mL/MPa)	k_c (s^{-1})	$\Delta V_c^{\ddagger}$ (mL/mol)	$\Delta\beta_o^{\ddagger}$ (mL/MPa)
Na^+	1.09 ± 0.09	−12 ± 1	0.397 ± 0.030	17.3 ± 2	125 ± 10	0.397 ± 0.030	0.34 ± 0.03	−48 ± 3	0.230 ± 0.021	5.45 ± 0.04	89 ± 7	0.229 ± 0.021
Na^+, betaine	0.41 ± 0.05	−62.5 ± 5	–	8.36 ± 9	73.3 ± 7.4	–	0.093 ± 0.008	−119.5 ± 12	–	3.08 ± 0.21	21.0 ± 1.1	–
Na^+, indoline, betaine	0.047 ± 0.005	−31.8 ± 2	–	2.05 ± 0.3	56.6 ± 4	–	0.13 ± 0.03	1.85 ± 0.2	–	8.39 ± 1	93.9 ± 6	–
NH_4^+	0.014 ± 0.002	−127 ± 10	−0.588 ± 0.032	47.5 ± 3	36 ± 2	−0.630 ± 0.032	0.0043 ± 0.001	−153 ± 8	−0.736 ± 0.006	14.4 ± 3	8 ± 0.5	−0.736 ± 0.032
NH_4^+, betaine	0.12 ± 0.01	−43.1 ± 3.5	–	86.1 ± 6	71.3 ± 6	–	0.56 ± 0.04	−14.1 ± 2	–	87.9 ± 10	92.9 ± 6	–
NH_4^+, indoline, betaine, fast	127 ± 10	230 ± 17	1.66 ± 0.02	2913 ± 119	324 ± 25	1.66 ± 0.09	0.21 ± 0.01	−6.26 ± 0.81	–	98.7 ± 20	88.8 ± 7.2	–
NH_4^+, indoline, betaine, slow	5.71 ± 0.32	218 ± 15	1.67 ± 0.065	721 ± 79	304 ± 31	1.67 ± 0.8	6×10^{-6}	287.6	2.01 ± 0.12	0.003 ± 0.0007	−184.6 ± 20	−2.01 ± 0.15
K^+, betaine	1.96 ± 0.02	−37.1 ± 2.3	–	11.9 ± 1.7	93.6 ± 9.3	–	0.29 ± 0.02	−42.9 ± 4	–	11.9 ± 2.1	81.5 ± 6.14	–
K^+, indoline, betaine	0.044 ± 0.004	−27.9 ± 0.015	–	5.05 ± 0.03	51.0 ± 4	–	0.027 ± 0.007	−30.8 ± 2.8	–	3.15 ± 1.98	48.3 ± 0.9	–

[a]The solutions contained the indicated cation at 0.1 M, 10 mM indoline, and 1 M betaine, in 0.1 M triethanolamine hydrochloride (pH 8.0).

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water to protein conformational transitions,[33] suggesting that solvation is in fact the major contributor to ΔV. From these results, that contribution is reduced to −2.0 kJ/mol for the Na^+ enzyme in the presence of 1 M betaine, assuming that the number of waters of solvation is constant in the presence of the osmolyte.

The effect of betaine on the relaxation kinetics is also modest, with the most striking effect being the changes in compressibility. This is consistent with our previous conclusion that solvation contributes to the transition state of the conformational change.[17] In the experiments on the effects of pressure on the Trp synthase−Ser−indoline complex (Figure 4), addition of betaine was necessary to obtain complete reversibility of the absorbance and fluorescence spectra of the Na^+, K^+, and NH_4^+ enzymes upon decompression, whereas the corresponding Trp synthase−Ser complexes show full reversibility without the addition of betaine.[16,17] This suggests that osmolytes stabilize the $\alpha_2\beta_2$ complex against dissociation at high pressures. We believe that the irreversibility results from the dissociation of the $\alpha_2\beta_2$ complex into subunits under pressure. It was shown previously that both holo and apo Trp synthase readily dissociate into isolated α- and β-subunits under pressures of >100 MPa and only slowly reassociate when decompressed.[34] In those studies, sucrose was found to stabilize against pressure-induced dissociation.[34] In our previous work, we found no evidence of dissociation of the Trp synthase−Ser complex up to 200 MPa, based on the reversibility of spectroscopic changes.[15−17]

However, while betaine protected the Na^+, K^+, and NH_4^+ enzymes, it did not prevent the lack of complete reversibility of the Trp synthase−Ser−indoline−Cs^+ complex on decompression. It is known that Cs^+ binds differently to the monovalent cation site, and to an additional site, in the structure,[35] and this appears to make the enzyme more labile to pressure-induced dissociation. It is not clear why all the quinonoid complexes formed with indoline should be more susceptible to pressure-induced dissociation in the absence of an osmolyte.

Effect on Indoline on Conformational Changes in Trp Synthase. Indoline undergoes nucleophilic addition to the Trp synthase aminoacrylate intermediate, E_{AA}, to form a quasi-stable quinonoid complex, E_Q,[20−24,36,37] thus shifting the overall equilibrium away from the external aldimine, E_{AL} (Scheme 2). In our spectroscopic experiments, under these conditions we measured the overall equilibrium, K_{eq}, between the aldimine and the quinonoid intermediate (results listed in Table 3). By comparison of the K_{eq} values with and without indoline, we can calculate the K_Q in the presence of each cation, Na^+, K^+, and NH_4^+, because $K_{eq} = K_{AA}K_Q$. Thus, Na^+ has the largest K_Q, ~10; K^+ has a K_Q of ~7−8, and NH_4^+ has the smallest K_Q, only ~2 (Table 3). The different monovalent cation complexes of Trp synthase exhibit small but significant structural differences that are responsible for the observed changes in the accumulation of reaction intermediates.[35,38] Furthermore, indoline also affects the magnitude of $\Delta\Delta V$, reducing it by ~20−30 mL/mol. The diminished effect of NH_4^+ on the aminoacrylate−quinonoid equilibrium can also be seen in the biphasic nature of the pressure effect, initially increasing absorbance at 466 nm with pressure, up to 100 MPa, and then decreasing above 100 MPa (Figure 3B). A similar effect of pressure was seen on the spectra of the Trp synthase−Ser−indoline−Cs^+ complex, but we did not analyze the data because of the apparent irreversibility of the spectra upon decompression. These data demonstrate that the Trp synthase−indoline−quinonoid complex has a net volume slightly smaller than that of the α-aminoacrylate complex, but larger than that of the Ser−aldimine complex. Assuming that solvation is the major contribution to ΔV, as discussed above, the difference in $\Delta\Delta V$ for the indoline complexes of 20−30 mL/mol corresponds to a difference in solvation of six to eight waters, based on the difference in density of the protein-bound and free water, as described in our previous paper.[15] Thus, formation of the quinonoid intermediate from the reaction of indoline with the α-aminoacrylate is accompanied by the uptake of six to eight water molecules.

With the equilibrium and kinetic data, we can construct reaction profiles for ΔV and $\Delta V^{\ddagger}$ for the indoline complexes (Figure 7). It should be noted that the relaxations studied here result from a two-step mechanism; therefore, the observed rate constants are apparent values, made of an algebraic combination of rate constants, and the calculated energies are thus of the virtual transition states. Remarkably, the NH_4^+ form of the enzyme shows very different $\Delta V^{\ddagger}$ values for positive and negative pressure jumps (Figure 7A). The positive pressure-jump activation volume is much greater than the volume of the α-aminoacrylate, suggesting

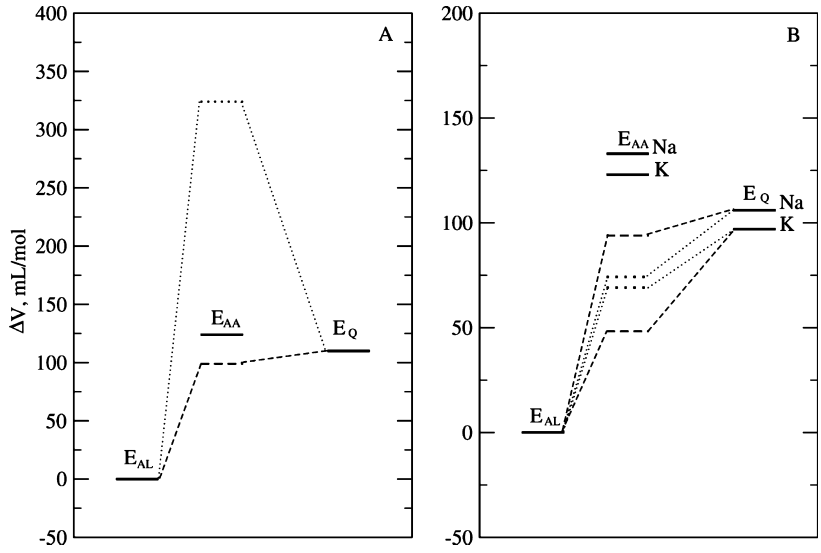

Figure 7. Reaction profiles for ΔV of the Trp synthase−Ser−indoline complex in the presence of cations and 1 M betaine. (A) NH_4^+, fast phase: (···) positive pressure jump and (---) negative pressure jump. (B) Na^+ and K^+: (···) positive pressure jump and (---) negative pressure jump.

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that the rate-limiting step in this relaxation does not simply involve indoline dissociation to form the α-aminoacrylate. In contrast, the transition state volume for the negative pressure jump is between the quinonoid and aldimine in volume, but closer to the quinonoid and α-aminoacrylate, so it is likely to be the indoline dissociation that is at least partly rate-limiting. Thus, the relaxation appears to follow a different mechanism for upward and downward pressure jumps. In the case of Na^+ and K^+, the profiles are much more similar for both positive and negative pressure jumps (Figure 7B), with $\Delta V^{\ddagger}$ values between those of the quinonoid and aldimine.

Conclusions. Osmolytes can activate Trp sythase in the absence of monovalent cations. In the presence of monovalent cations, osmolytes have only minor effects on activity and allosteric parameters but stabilize the Trp synthase−Ser−indoline complex against apparent pressure-induced dissociation. Na^+ and K^+ are more effective than NH_4^+ in shifting the α-aminoacrylate−quinonoid equilibrium toward the quinonoid complex. Furthermore, the pressure-jump experiments show that the mechanism of indoline reaction may be different for the NH_4^+ enzyme than the Na^+ and K^+ forms.

APPENDIX

Derivation of eq 5. Fitting the equilibrium pressure data to a two-step model.

$$A \overset{K_1}{\Leftrightarrow} B \overset{K_2}{\Leftrightarrow} C$$

$$F_A = [A]/([A] + [B] + [C])$$

$$K_1 = [A]/[B]$$

$$K_2 = [B]/[C]$$

$$[B] = [A]/K_1$$

$$[C] = [B]/K_2$$

where F_A is the fraction of species A in the equilibrium.

$$F_A = [A]/([A] + [A]/K_1 + [B]/K_2)$$

$$= [A]/([A] + [A]/K_1 + [A]/K_1K_2)$$

$$= 1/(1 + K_1 + K_1K_2)$$

Multiply through by K_1

$$F_A = K_1/(K_1 + 1 + 1/K_2)$$

Multiply through by K_2

$$F_A = K_1K_2/(1 + K_2 + K_1K_2)$$

Because $K_P = K \exp(-P\Delta V/RT)$, $F_A = [K_1 \exp(-P\Delta V_1/RT) \times K_2 \exp(-P\Delta V_2/RT)]/[1 + K_2 \exp(-P\Delta V_2/RT) + K_1 \exp(-P\Delta V_1/RT) \times K_2 \exp(-P\Delta V_2/RT)]$.

Because $A_{obs} = \Delta AF_A + A_o$, where ΔA is the signal intensity of the absorbance or fluorescence change and A_o is the background signal

$$A_{obs} = \Delta A[K_1 \exp(-P\Delta V_1/RT) \times K_2 \exp(-P\Delta V_2/RT)]$$
$$/[1 + K_2 \exp(-P\Delta V_2/RT) + K_1 \exp(-P\Delta V_1/RT)$$
$$\times K_2 \exp(-P\Delta V_2/RT)] + A_o$$

Abbreviations

Trp synthase, $\alpha_2\beta_2$ complex of tryptophan synthase from *S. typhimurium*; E_{AL}, external aldimine of L-Ser and the $\alpha_2\beta_2$ complex of Trp synthase; E_{AA}, aminoacrylate complex of the $\alpha_2\beta_2$ complex of Trp synthase; E_Q, quinonoid intermediate of indoline, L-Ser, and the $\alpha_2\beta_2$ complex of Trp synthase; PLP, pyridoxal 5′-phosphate.

AUTHOR INFORMATION

Corresponding Author

*Department of Chemistry, University of Georgia, Athens, GA 30602-2556. E-mail: plp@uga.edu. Phone: (706) 542-1996. Fax: (706) 542-9454.

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The authors declare no competing financial interest.

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