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Hydrostatic Pressure Affects the Conformational Equilibrium of *Salmonella typhimurium* Tryptophan Synthase[†]

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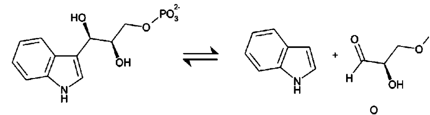
ABSTRACT: The effect of hydrostatic pressure on the tryptophan (Trp) synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* has been investigated. Trp synthase has been shown previously to exhibit low-activity (open) and high-activity (closed) conformations. The equilibrium between the open and closed conformations of Trp synthase has been found to be affected by a wide range of variables, including α-subunit ligands, monovalent cations, organic solvents, pH, and temperature. The absorption spectrum of the Trp synthase−L-Ser complex shows an increase in absorption of the 423 nm band of the external aldimine, which is a characteristic of the open conformation, as hydrostatic pressure is increased from 1 to 2000 bar. The ΔV_o and K_o for the equilibrium between the closed and open conformations of the Trp synthase−L-Ser complex are −126 mL/mol and 0.12 for the Na^+ form and −171 mL/mol and 2.3×10^{-4} for the NH_4^+ form. When the Trp synthase−L-Ser complex is subjected to pressure jumps of 100−400 bar, relaxations are observed, exhibiting an increase in fluorescence emission at wavelengths greater than 455 nm, with 405 nm excitation. The relaxation to the new equilibrium position requires two exponentials to fit the data in the presence of 0.1 M Na^+ and three exponentials to obtain a reasonable fit in the absence of cations and with 0.1 M NH_4^+. Fluorescence emission at 325 nm, with excitation at 280 nm, also increases when the Trp synthase−L-Ser complex is subjected to pressure jump. These data demonstrate that the open conformation of Trp synthase is favored by higher pressure. Thus, the open conformation has a smaller apparent net system volume than the closed conformation. We estimate that there are 35−47 more waters in the solvation shell of the open conformation than in that of the closed conformation.

Tryptophan (Trp) synthase serves as a paradigm in the study of protein−protein interactions and allosteric behavior in enzymes (*1*). Bacterial Trp synthase is an $\alpha_2\beta_2$ tetramer, arranged as individual α subunits attached to the opposite ends of a β_2 dimer (*2*). The α subunit of Trp synthase catalyzes the reversible cleavage of indole-3-glycerol phosphate to indole and D-glyceraldehyde-3-phosphate (α reaction) (eq 1), and the β subunit, containing pyridoxal-5′-phosphate (PLP),[1] catalyzes the condensation reaction of indole with L-serine (β reaction) (eq 2). The physiological reaction is the combination of both the α and β reactions (eq 3); thus, indole is not observed as a free intermediate in solution (*3*−*5*). There is exquisite communication between the α and β sites of Trp synthase to coordinate the progress of the reaction (eq 3) and to avoid release of indole to solution. Indole-3-glycerol phosphate binds to the α site, which closes, activating the β site to bind L-Ser and form an external aldimine complex (E_{EA-Ser}, Scheme 1) in an open conformation. The β site then closes to allow formation of a reactive aminoacrylate intermediate, E_{AA}, which triggers the α site to form indole by cleavage of indole-3-glycerol phosphate. The indole formed at the α site is transferred intramolecularly through a tunnel about 30 Å long to the β site, where it reacts with the aminoacrylate to form L-Trp (*2*, *5*). The α site then opens to release D-glyceraldehye-3-phosphate, which in turn triggers opening of the β site and L-Trp release. The equilibrium between the low-activity (open) and high-activity (closed) conformations of Trp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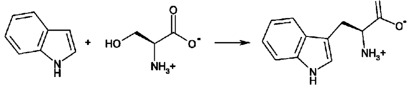
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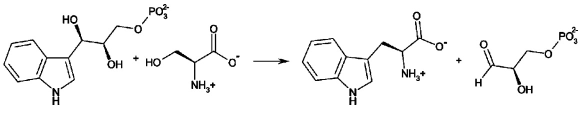
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[1] Abbreviations: PLP, pyridoxal-5′-phosphate; Trp synthase, the $\alpha_2\beta_2$ complex of tryptophan synthase from *Salmonella typhimurium*; E_{GD-Ser}, the *gem*−diamine complex of L-Ser and the $\alpha_2\beta_2$ complex of tryptophan synthase; E_{EA-Ser}, the external aldimine of L-Ser and the $\alpha_2\beta_2$ complex of tryptophan synthase; E_{AA}, the aminoacrylate complex of the $\alpha_2\beta_2$ complex of tryptophan synthase.

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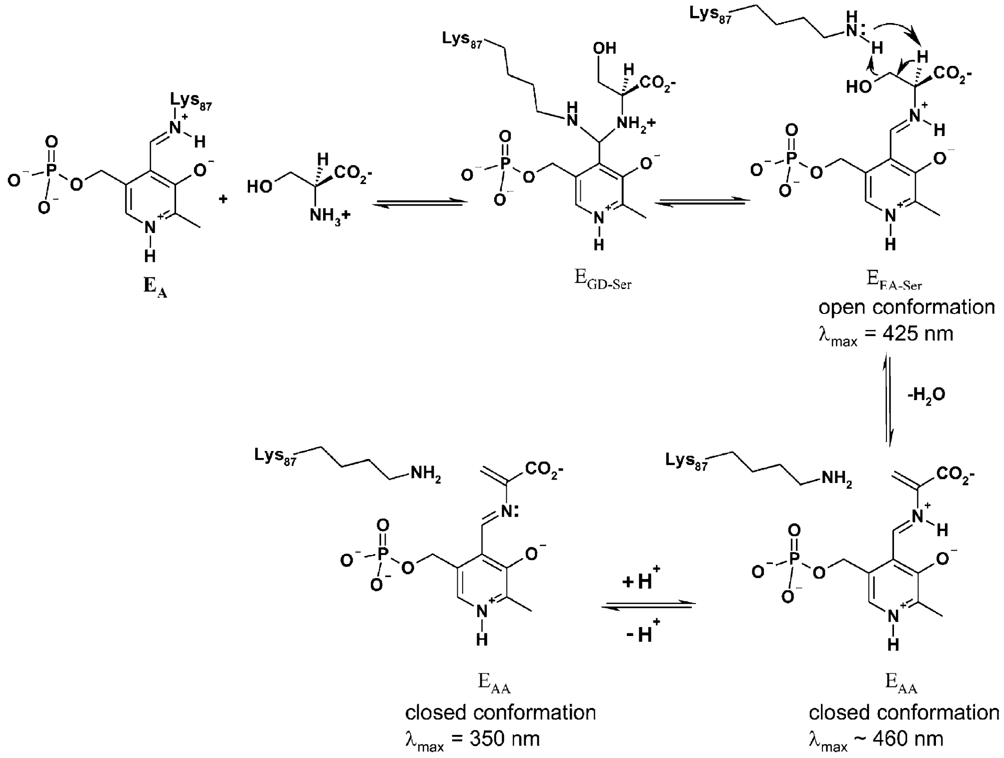
Scheme 1



synthase is known to be affected by a wide range of variables, including α-subunit ligands (*5*−*7*), monovalent cations (*8*−*12*), solvents (*13*, *14*), pH (*15*), and temperature (7, *15*). However, the effects of hydrostatic pressure on the conformational behavior of Trp synthase have not been examined previously. We have now found that hydrostatic pressures of 100−2000 bar exert large effects on the conformational equilibrium in Trp synthase, as monitored by the absorption spectrum of the PLP−serine complex, as well as by fluorescence emission of the serine external aldimine complex and protein βTrp177.

EXPERIMENTAL PROCEDURES

Materials. L-Serine was obtained from Sigma. Triethanolamine was a product of J. T. Baker. NaCl and NH_4Cl were obtained from Fisher Scientific.

Absorption Spectra under Hydrostatic Pressure. The effects of hydrostatic pressure on the absorption spectra were measured using a Cary 14 UV−vis spectrophotometer modified by OLIS, Inc. (Bogart, GA) to contain a high-pressure cell from ISS (Champaign, IL), equipped with a manual pressure pump. The cell was maintained at 25 °C with an external circulating water bath. The enzyme solutions were contained in 1 mL quartz bottles with a 9 mm path length, capped with Teflon tubing and immersed in spectroscopic-grade ethanol as the pressurizing fluid. A buffer blank at 1 bar was used to obtain a baseline reading. The buffer, triethanolamine hydrochloride, at pH 8.0, was chosen because the pK_a is 7.88 and the ΔV_o for ionization is 4.5 ± 0.3 mL/mol (*16*); therefore, the pressure change will not significantly change the pH. The enzyme solutions were scanned at 1 bar and immediately after each incremental increase in pressure. The total time to measure the spectra through the range of pressures was approximately 1 h. The spectra were analyzed by global analysis (*17*) using the GlobalWorks program obtained from OLIS, Inc.

Pressure-Jump (P-Jump) Measurements. These experiments were carried out in a home-built p-jump instrument described previously (*18*, *19*). The pressure perturbations applied ranged from 100 to 400 bar in 50 bar steps. A prepressure of approximately 10 bar was applied to the sample. The pressure change was monitored by a transducer, and typically, the pressure increase was 90% complete within 100 μs and the decrease was 90% complete within 50 μs. For measurement of the L-serine-PLP external aldimine fluorescence, the excitation was performed at 405 nm and the emission was measured with a 455 nm high-pass filter. For the protein tryptophan fluorescence, excitation was at 280 nm and the emission was measured with a 325 nm band-pass filter, to avoid collection of the PLP emission at 500 nm. Typically, 20−100 transients were collected and averaged, with collection of 1000 data points for 1 s following the pressure increase, a short delay (1−2 s) between the end of data collection and the pressure release, and collection of 1000 data points for 1 s following the pressure decrease. The initial fluorescence signal from the sample was adjusted to a PMT output of −1.0 V prior to data collection, and a 1.0 V offset was applied to zero the reading. A 10× amplification was then applied to the output, except for the 405 nm excitation with Na^+, because of the high signal intensity. The temperature of the sample was measured by a Pt 100 sensor in contact with the cell and controlled by a circulating water bath at 25.0 °C. The data were analyzed by fitting to eq 4, where F_t is the fluorescence observed at time t, F_n is the amplitude of phase n, F_o is the initial value of the fluorescence, and k_n is the rate constant of phase n, using the Globalworks program (*17*) obtained from OLIS, Inc.

$$F_t = \sum_n F_n e^{-k_n t} + F_o \quad (4)$$

Enzyme. The Trp synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* was prepared from cells of *Escherichia coli* containing pEBA as described previously (*20*). For the experiments, the protein was applied to a PD-10 (Pharmacia) column equilibrated with 0.05 M triethanolamine hydrochloride buffer at pH 8.0 to remove inorganic monovalent cations and then concentrated to 33 mg/mL in an Amicon untrafiltration cell over a YM-30 membrane. Dilutions of the enzyme, to a final concentration of 1−3 mg/mL, were made in the same buffer, with either no monovalent cation, 0.1 M NaCl or NH_4Cl, and 0.1 M L-Ser, immediately prior to collection of the data.

Effect of Pressure on Absorbance. The effect of pressure on an equilibrium constant is given by eq 5, where K_p is the equilibrium constant at pressure P and K_o is the equilibrium constant at 1 bar. The logarithmic form of eq 5 is given in eq 6. Thus, a plot of ln K_p versus P will give a straight line with a slope equal to $\Delta V_o/RT$ and intercept at ln K_o. For a simple two-step equilibrium, the equilibrium constant can be related to the change in absorbance by eq 7, where A_p is the absorbance at pressure p, A_o is the absorbance at 1 bar, and A_∞ is the absorbance at infinite pressure.

$$K_p = K_o \exp(-P\Delta V_o/RT) \quad (5)$$

$$\ln K_p = \ln K_o - P\Delta V_o/RT \quad (6)$$

$$K = (A_p - A_o)/(A_\infty - A_p) \quad (7)$$

RESULTS

Pressure Dependence of Absorbance of the L-Serine External Aldimine. Figure 1A shows the effect of increasing hydrostatic pressure from 1 to 1500 bar on the absorption spectrum of the complex of Trp synthase with L-Ser in the presence of 0.1 M NaCl. There is a dramatic increase in the 423 nm absorption peak, corresponding to the open conformation of E_{EA-Ser} in Scheme 1, as the pressure is increased. Thus, increasing pressure shifts the equilibrium from favoring the closed conformation of E_{AA} at 1 bar to strongly favoring E_{EA-Ser} at 1500 bar. There are isosbestic points at 376 and 467 nm, indicating that the equilibrium involves only two major species. The changes in the spectra in Figure 1A in the 350 to 550 nm region are fully reversible upon release of pressure. The small increase in absorbance below 350 nm is not reversible and is probably due to slow formation of pyruvate by β elimination of L-serine during the experiment. The spectral data in Figure 1A were fit by global analysis to eqs 5 and 7. The global fit gives a K_o for E_{EA-Ser} formation of 0.12 ± 0.01 at 25 °C, in excellent agreement with the K value of 0.11 at 25 °C calculated from the published thermodynamic parameters obtained from thermal analysis (*7*). Global analysis also provides the ΔV_o for the reaction, −126 ± 2 mL/mol. The calculated spectra of E_{EA-Ser} and E_{AA} from global analysis are shown as the dotted lines in Figure 1A. The equilibrium constant at each pressure, K_p, calculated from eq 7, is logarithmically proportional to the applied pressure (Figure 1B, ●), as predicted by eq 6. The effect of increasing hydrostatic pressure on the spectra of the Trp synthase L-Ser complex with 0.1 M NH_4Cl is similar (Figure 2); however, higher pressures between 1 and 2 kbar are required to see a significant change, because K_o is much smaller. As before, isosbestic points at 378 and 466 nm are observed. These changes are reversible upon release of pressure, as was seen with NaCl. However, the increase in absorbance at 330 nm seen in Figure 1A, ascribed to pyruvate formation, is not seen in Figure 2 in the presence of NH_4Cl. The values of K_o and ΔV_o with NH_4Cl, obtained by global fitting of the data in Figure 2, are (2.34 ± 0.08) × 10^{-4} and −171 ± 2 mL/mol. The logarithmic plot of the equilibrium constant at each pressure, K_p, calculated from eq 7, is linear, as shown in Figure 1B (■). Changes in spectra with pressure similar to those shown in Figure 2 were also seen in the absence of an activating monovalent cation (data not shown).

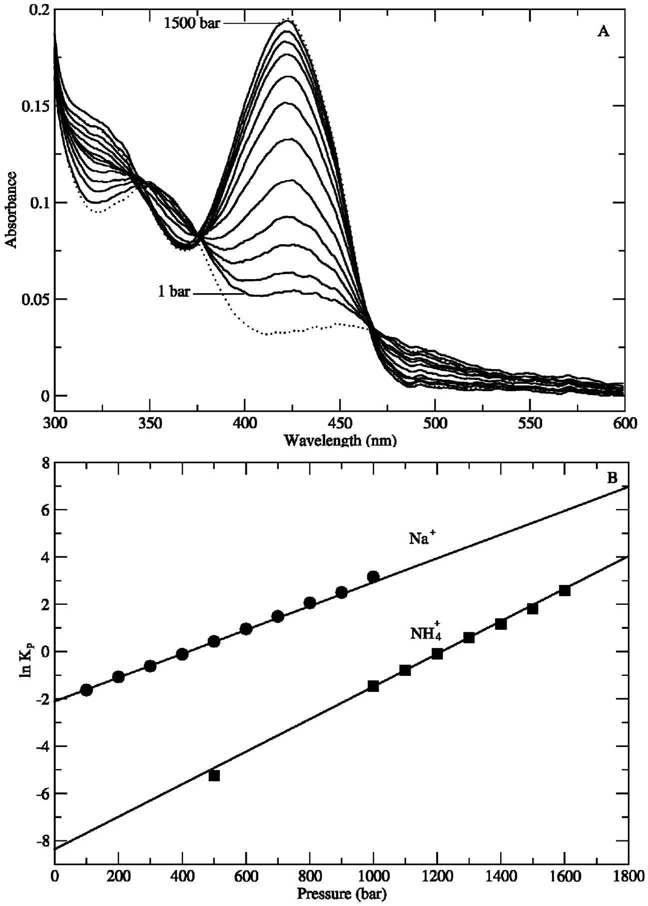


FIGURE 1: (A) Effect of hydrostatic pressure of the absorption spectra of the Trp synthase−Ser complex. The solution contained 21.6 μM Trp synthase ($\alpha\beta$) and 0.1 M L-serine in 0.05 M triethanolamine hydrochloride at pH 8 containing 0.1 M NaCl. Scans were collected at 1, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, and 1500 bar. The dotted lines are the calculated spectra of the two components of the equilibrium obtained from global analysis. (B) Plot of ln K_p, determined from the 423 nm absorbance, versus applied pressure. The lines shown are calculated from the parameters given in the text. (●) Data collected in 0.1 M NaCl. (■) Data collected in 0.1 M NH_4Cl.

Kinetics of Pressure Dependence of Fluorescence of the L-Serine External Aldimine. Previous studies have shown that the L-Ser external aldimine, E_{EA-Ser} in Scheme 1 [“aqua” species of York (*21*)], of Trp synthase is highly fluorescent (*4*, *21*, *22*), with an excitation maximum at 425 nm and

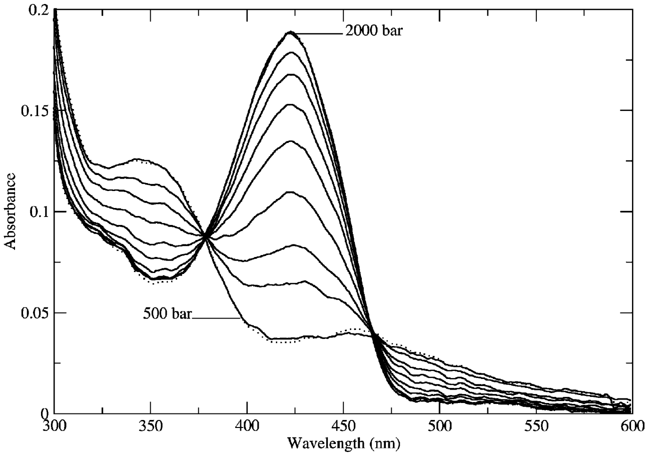


FIGURE 2: Effect of hydrostatic pressure of the absorption spectra of the Trp synthase−Ser complex. The solution contained 21.6 μM Trp synthase ($\alpha\beta$) and 0.1 M L-serine in 0.05 M triethanolamine hydrochloride at pH 8 containing 0.1 M NH_4Cl. Scans were collected at 500, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1800, and 2000 bar. The dotted lines are the calculated spectra of the two components of the equilibrium obtained from global analysis.

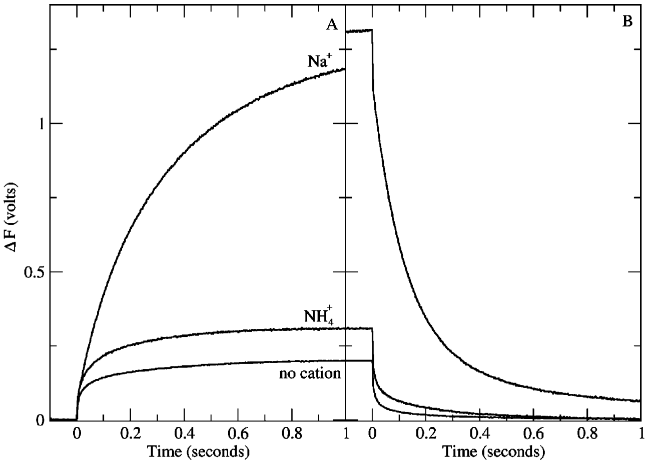


FIGURE 3: Pressure perturbation of fluorescence emission of the Trp synthase−Ser complex with 405 nm excitation. The solution contained 16.2 μM Trp synthase ($\alpha\beta$) and 0.1 M L-serine in 0.05 M triethanolamine hydrochloride at pH 8 without cations or with 0.1 M NH_4Cl or NaCl. (A) Relaxation after pressure push to 400 bar. (B) Relaxation after pressure release to 10 bar.

Table 1: Rate Constants for Relaxations of the Trp Synthase−L-Ser Complex Observed with 405 nm Excitation

cation	*P* (bar)	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})	$1/\tau_3$ (s^{-1})
none	400	179 ± 16	23.4 ± 1.2	2.7 ± 0.1
none	10	152 ± 15	23.5 ± 0.7	3.5 ± 0.1
NH_4^+	400	381 ± 14	28.9 ± 1.3	4.0 ± 0.2
NH_4^+	10	555 ± 14	41.8 ± 1.2	4.0 ± 0.2
Na^+	400		10.2 ± 0.6	2.4 ± 0.1
Na^+	10		8.9 ± 0.1	2.4 ± 0.1

emission maximum at 500 nm. In contrast, the next intermediate in the reaction mechanism, the Schiff's base of α aminoacrylate, E_{AA} ["pale" species of York (*21*)], which absorbs at 350 and 460 nm, is only weakly fluorescent (*21, 23*). When the Trp synthase-L-Ser complex is subjected to rapid pressure increases, ranging from 100 to 400 bar, in the p-jump instrument, subsequent relaxations, with an increase in fluorescence emission at 500 nm, with 405 nm excitation, are observed (Figure 3A). The relaxation to the new equilibrium is complex, requiring two exponentials to fit the data in the presence of Na^+ and three exponentials to obtain a reasonable fit in the absence of cations and with NH_4^+. The apparent rate constants obtained at 400 bar are presented in Table 1. There is a fast relaxation, with $1/\tau$ = 150−500 s^{-1}, without cations, and in the presence of NH_4^+, but which could not be fit in the presence of Na^+, because it occurs during the push time of the p-jump instrument (this is most prominent in Figure 3B). Thus, this process in the presence of Na^+ must proceed with a much faster rate constant. The rate constant of the fast process in the presence of NH_4^+ is somewhat faster for the jump from 400 back to 10 bar (555 s^{-1}) than for the jump from 10 to 400 bar (381 s^{-1}) (Table 1), but this difference was not consistently observed at other pressures. The second process proceeds with $1/\tau$ = 10−40 s^{-1}, with the slowest relaxation seen in the presence of Na^+. The third phase of relaxation proceeds at 2.4−4 s^{-1}, again with the slowest rate constant observed in the presence of Na^+. These results are similar to stopped-flow studies of the binding of L-Ser to form the external aldimine and aminoacrylate species, which also exhibit two or three phases (*24−27*). It should be noted that the perturbation seen when pressure is applied is fully reversible upon pressure release (compare parts A and B of Figure 3) for at least 100 cycles.

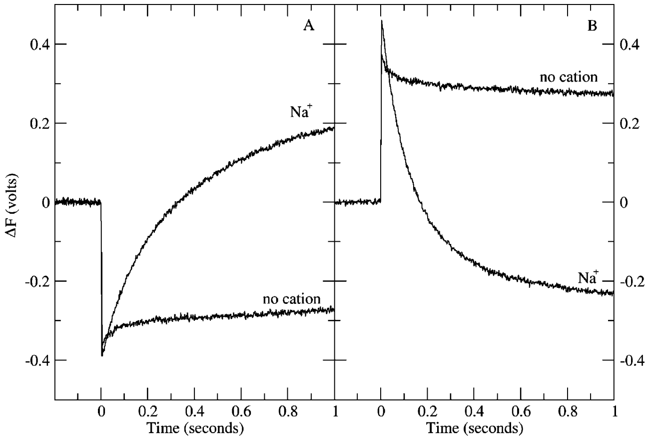


FIGURE 4: Pressure perturbation of fluorescence emission of the Trp synthase−Ser complex with excitation at 280 nm. The solution contained 48.6 μM Trp synthase ($\alpha\beta$) and 0.1 M L-serine in 0.05 M triethanolamine hydrochloride at pH 8 without cations or with 0.1 M NaCl. (A) Relaxation after pressure push to 400 bar. (B) Relaxation after pressure release to 10 bar.

Kinetics of Pressure Dependence of Protein Fluorescence. The open and closed conformations of Trp synthase exhibit different fluorescence properties for the single tryptophan residue, βTrp177, with the closed conformation exhibiting lower emission intensity at 330 nm (*4*). Thus, we also examined the effect of pressure on the emission of the Trp synthase−L-serine complex with 280 nm excitation, without a cation, and in the presence of Na^+. There is a relaxation resulting in an increase in the tryptophan fluorescence emission at 325 nm when pressure is increased (Figure 4). In contrast to the data collected with 405 nm excitation, the fastest phase of the relaxation is not observed in the emission at 325 nm, but there is a very fast decrease in fluorescence during the pressure push. The 325 nm emission data at 400 bar can be fitted well with two exponential processes (Table 2), the rate constants of which correspond in value to the second and third relaxations observed with 405 nm excitation. There is excellent agreement of the rate constants for the

Table 2: Rate Constants for Relaxations of the Trp Synthase−L-Ser Complex Observed with 280 nm Excitation

cation	P (bar)	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})
none	400	24.0 ± 7.0	1.2 ± 0.2
none	10	25.7 ± 1.4	1.2 ± 0.1
Na^+	400	9.3 ± 0.8	2.0 ± 0.1
Na^+	10	9.0 ± 0.4	1.9 ± 0.1

reactions without monovalent cations (24 s^{-1}) and with Na^+ (9−10 s^{-1}) obtained with both 280 and 405 nm excitation and reasonable agreement for the slowest phase (1−3 s^{-1}).

DISCUSSION

Hydrostatic pressure has been utilized as a tool to study protein folding and subunit dissociation at elevated pressure (*28*). However, there are relatively few reports of the use of pressure to study conformational equilibria of proteins at more modest pressures. Conformational changes between low-activity (open) and high-activity (closed) conformations play a key role in catalysis in some enzymes. Evidence has been obtained for open and closed conformations in crystal structures of both the α family [e.g., aspartate aminotransferase (*29*)] and β family [e.g., Trp synthase (*30*)] of PLP-dependent enzymes. The pressures used in our studies are less than those which have been shown to cause β_2-subunit dissociation and concomitant inactivation of the isolated β_2 dimer of Trp synthase (*31*−*36*). In our measurements, the changes in the enzyme absorption and fluorescence properties can be used to monitor the conformational equilibrium of Trp synthase. The absorption spectra are fully reversible during the period of measurement (ca. 1 h), and no degradation of the fluorescence signal was detected in p-jump experiments during data collection periods of 5−15 min with hundreds of pressure cycles.

The data presented in Figures 1 and 2 demonstrate that the low-activity open conformation of the L-Ser external aldimine, E_{EA-Ser}, is favored by higher hydrostatic pressure, and thus, the open conformation has a smaller apparent net system volume than the closed conformation of the aminoacrylate, E_{AA}, because applied pressure will tend to shift the equilibrium to the side with the smallest system volume or highest density, according to Le Chatelier's principle. A similar effect of pressure was reported with plasminogen, where pressures in the range of 50−350 bar were found also to shift a conformational equilibrium toward an open conformation (*37*). The net volume change in conformational changes in proteins can be due to changes in the volume of the protein (compressibility) and differences in solvation. However, volume changes in proteins are often interpreted as being mainly due to solvation differences. Thus, it is reasonable to assume that solvation is the major contributor to the volume change for the conformational equilibrium of Trp synthase. This interpretation is consistent with the large negative ΔS (−327 J deg^{-1} mol^{-1}) for the closed to open conformational equilibrium of the Trp synthase serine complex in Na^+ found in previous temperature-dependent experiments (*7*), which was also interpreted as evidence for significant changes in solvation between the closed and open conformations.

Evidence has been presented that water in the first solvation shell of proteins has a higher density (estimated to be 15−25% higher) than the bulk solvent (*38*, *39*). Thus, the binding of this structured water to the open conformation upon conformational change can result in a net decrease in system volume. Our results support this proposition, because we find a large negative ΔV_o of −126 mL/mol for the closed to open transition in the presence of Na^+. Assuming a 20 ± 5% higher average density for bound water (*38*, *39*) and assuming that the compressibility of the protein is negligible, this value of ΔV_o corresponds to a net increase in solvation of 35 ± 9 water molecules/mol of αβ dimer for the open conformation of Trp synthase.[2] The ΔV_o is even more negative in the presence of NH_4^+; the value of −171 mL/mol corresponds to a solvation increase of 47 ± 12 water molecules. These estimates are comparable to the solvation changes of 20−65 waters estimated for the conformational change between R and T states in hemoglobin (*40*). It is interesting to note that the conformational change in Trp synthase is entropy-driven, because the ΔH for the equilibrium in the direction of the closed conformation is +92 kJ/mol, while ΔS is +327 J mol^{-1} deg^{-1} (*7*). It has been suggested recently that rapidly reversible protein−protein interactions are entropy-driven, by release of bound water to the bulk solvent (*41*). We can estimate the entropy gain per bound H_2O released to the solvent from our data and the previous entropy measurement to be 9.3 ± 2.4 J mol^{-1} deg^{-1}, corresponding to 0.67 ± 0.17 kcal/mol at 298 K, in excellent agreement with Habermann and Murphy, who reported a value of 0.69 ± 0.48 kcal/mol (*42*).

The rate constants of the relaxations observed after p-jump (Figures 3 and 4) do not show consistent changes with pressure greater than the experimental error; hence, the value of the activation volume, $\Delta V^{\ddagger}$, for the reactions must be less than about ±20 mL/mol for Trp synthase, because that is the minimum change that could be reliably detected over experimental error at 400 bar. The fluorescence emission at 500 nm with 405 nm excitation arises from the external aldimine complex, E_{EA-Ser}. The requirement of three exponentials to fit the relaxations seen with 405 nm excitation requires a minimum of four species in the mechanism. The mechanism of aminoacrylate formation from L-Ser is expected to take place in two steps (Scheme 1), with initial formation of a *gem*−diamine complex (E_{GD-Ser}), followed by the external aldimine (E_{EA-Ser}), and the aminoacrylate (E_{AA}). Although a quinonoid carbanion intermediate is likely to exist between E_{EA-Ser} and E_{AA}, it does not accumulate enough to be detected under normal conditions. E_{AA} can exist in protonated and unprotonated forms, with λ_{max} at 460 and 350 nm, respectively (Scheme 1) (*43*), and Trp synthase favors the unprotonated 350 nm form of E_{AA}. The two aminoacrylate absorption peaks can be clearly seen in the fitted spectra shown as dotted lines in Figures 1 and 2. All of the relaxations observed after p-jump result in an increase in the concentration of external aldimine, E_{EA-Ser}. Because the fastest phase, $1/\tau_1$, is observed in the fluorescence signal

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[2] The ΔV_o, −126 ± 2 mL/mol, divided by the density difference, 0.2 ± 0.05 g/mL, gives −630 ± 158 g/mol as the difference in mass. Dividing −630 ± 158 g/mol by the molecular weight of water, 18 g/mol, gives a difference of −35 ± 9 mol of water for the open to closed conformational change. Because the measured equilibrium constant involves the communication between the α and β subunits in an αβ dimer and there is no evidence for allosteric communication between αβ dimers in the $\alpha_2\beta_2$ tetramer, this figure represents the solvation difference between the open and closed states of an αβ dimer.

with 405 nm excitation without cations present but is not observed without cations present in the fluorescence signal with 280 nm excitation, this relaxation does not appear to be associated with the equilibrium between E_{EA-Ser} and E_{AA}. Thus, the fast relaxation is probably associated with an equilibrium that precedes E_{EA-Ser} in the reaction. This equilibrium is not likely to be due to the external binding equilibrium, because the reactions were performed at 0.1 M L-serine and the K_d for L-serine binding is about 2×10^{-5} M (*25*, *26*), but it could be a result of the internal *gem*-diamine-external aldimine equilibrium (E_{GD-Ser} and E_{EA-Ser} in Scheme 1), if it shifts toward the external aldimine with increased pressure.

The second relaxation observed with 405 nm excitation, $1/\tau_2$, exhibits rate constants that range in value from about 10 to 40 s^{-1} (Table 1), depending on the presence and the identity of monovalent cations. Previous studies of the "aqua" form of Trp synthase from *E. coli* and *S. typhimurium* followed by absorbance at 420 nm or fluorescence emission at 500 nm using stopped-flow and temperature jump techniques have reported rate constants between 10−90 s^{-1} for the decay of E_{EA-Ser} to form E_{AA} (*4, 11, 12, 21, 22, 24*−*27*). In our experiments, we are observing the reverse of that process, the formation of E_{EA-Ser} from E_{AA}, which will relax with the same apparent rate constant. This rate constant is quite sensitive to the presence or absence of monovalent cations (Table 1); therefore, the variability in the reported values may be due to variation in the buffers used for measurements. The relative order of the rate constants in Table 1 is cation-dependent, with $Na^+ <$ no cation $< NH_4^+$, in agreement with previous results (*11*, *12*, *27*). Furthermore, the decay of the 340 nm emission of Trp synthase in the presence of L-serine was previously found to exhibit a rate constant of 48 s^{-1}, very similar to the rate constant observed for the fluorescence decay at 500 nm (*4*). These results were proposed as evidence for a conformational change associated with formation of E_{AA} (*4*). Our results, which show that the conversion of E_{AA} to E_{EA-Ser}, observed from the emission of E_{EA-Ser} with 405 nm excitation, occurs with an identical rate constant as the protein fluorescence change, observed from the 325 nm emission, either without monovalent cations (24 s^{-1}) or with Na^+ (9−10 s^{-1}), are in agreement with the previous data. It should be noted that the fluorescence emission at 325 nm with 280 nm excitation does not arise solely from the single tryptophan residue, βW177, because βW177F Trp synthase exhibits weaker but still significant fluorescence (*44*). Furthermore, the fluorescence change at 325 nm may be due, at least in part, to quenching of the protein tryptophan emission by Förster-type energy transfer to E_{AA}, which absorbs at 350 nm (Figures 1 and 2).

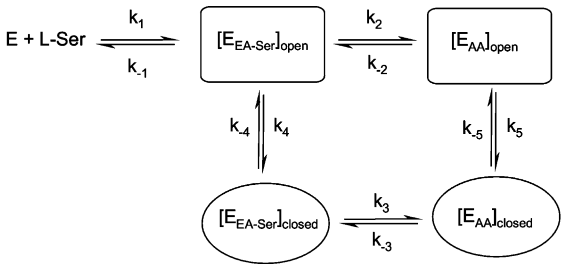
The slowest phase of the relaxations exhibits rate constants ranging from 1 to 4 s^{-1} (Tables 1 and 2). Lane and Kirshner observed a similar slow process, with $1/\tau \approx 1$ s^{-1} at pH 6.5, in stopped-flow studies of the reaction of Trp synthase with L-serine, and they proposed that it was due to a branched mechanism, leading to a species off the main reaction path (*26*), because k_{obs} is slower than the steady-state k_{cat}. Similar slow rate constants for formation of E_{AA} from E_{EA-Ser} have been reported by Woehl and Dunn (*12*). The most likely explanation for our results is a cyclic mechanism, as shown in eq 8. The binding step ($k_1 + k_{-1}$) is fast and at equilibrium; therefore, only three observed relaxations involving E_{EA-Ser} and E_{AA} are expected for this mechanism (*45*). However, only two relaxations are observed, which implies that one of the steps in the cycle must equilibrate rapidly compared to the others. The external aldimine-aminoacrylate interconversion exhibits a primary isotope effect with α-[^{2}H]-serine (*12*, *22*, *24*, *26*); therefore, these steps ($k_2 + k_{-2}$, $k_3 + k_{-3}$) cannot be fast. Thus, the most likely step to equilibrate rapidly is the conformational change between the open and closed states ($k_4 + k_{-4}$ and $k_5 + k_{-5}$), which is affected by pressure, as discussed above. However, it was shown previously that the open and closed conformations of E_{AA} do not directly interconvert; therefore, $k_5 + k_{-5} \approx 0$ (*11*). Under these conditions, the rate equations for the second and third relaxations after p-jump are given by eqs 9 and 10 (*45*), where $K_3 = k_3/k_{-3}$ and $K_4 = k_4/k_{-4}$. Although the major species in the coupled equilibria in eq 8 are expected to be $(E_{EA-Ser})_{open}$ and $(E_{AA})_{closed}$, small concentrations of the minor species, $(E_{EA-Ser})_{closed}$ and $(E_{AA})_{open}$, must

$$1/\tau_2 = k_3K_4/(1 + K_4) + k_{-3} \qquad (9)$$

$$1/\tau_3 = k_2/(1 + K_4 + K_3K_4) + k_{-2} \qquad (10)$$

exist. Thus, the slowest relaxation may be associated with the interconversion of $(E_{EA-Ser})_{open}$ and $(E_{AA})_{open}$ complexes. The apparent equilibrium constant for E_{EA-Ser} and E_{AA}, as measured by the effect of pressure on the absorption spectra, is given in eq 11, where $K_2 = k_2/k_{-2}$.

$$K_{app} = (1 + K_4)/(1 + K_2 + K_4 + K_3K_4) \qquad (11)$$



(8)

The X-ray structure of the closed conformation of *S. typhimurium* Trp synthase in the presence of Na^+ shows the formation of additional hydrogen bonds and salt bridges, between βArg175 and αloop2, between βLys167 and αAsp56, and between βArg141 and βAsp305 (*30*). There is a net increase of one salt bridge and one hydrogen bond in the closed conformation, compared with the open conformation. Mutagenesis has shown that the salt bridge between αAsp56 and βLys167 is the most critical for intersubunit communication (*7*, *44*, *46*). Hydrogen bonds changing from solvated to internal protein states contribute relatively little to ΔV_o, but formation of salt bridges from solvated ions can contribute significantly to ΔV_o because of electrostriction (*47*). Electrostriction causes water molecules solvating ionic groups to be tightly bound and have a reduced molar volume relative to free water. Ionization of neutral acids in aqueous solution to form a solvated cation/anion pair is associated with ΔV_o values from −10 to −30 mL/mol (*47*). Thus, it is possible that the formation of the new solvated ion pair in the open conformation of Trp synthase contributes to the ΔV_o, in addition to solvation of the newly exposed hydrophobic surface. Solvation of a cation−anion pair would likely require about 7−8 water molecules to fill all hydrogen bonds or dipolar interactions. If electrostriction contributes to ΔV_o, the βK167T mutant Trp synthase (*44*), which shows an impaired ability to form the closed conformation, because it cannot form the ion pair, will show a reduced pressure effect and a less negative value of ΔV_o. It is also interesting that a more negative value of ΔV_o was observed for the NH_4^+ complex than the Na^+ complex, because of a larger solvation, about 12 water molecules, suggesting significant differences in either hydrophobic surface or ion pairs exposed in the open conformation. There is also a $\Delta\Delta G$ of 15.4 kJ/mol for the conformational equilibria in Na^+ and NH_4^+, suggesting additional stabilizing interactions for the closed conformation in the NH_4^+ complex. Unfortunately, there are no structural data available for the NH_4^+ complex with L-Ser to allow comparison with the Na^+ complex. However, the structures of the K^+ and Cs^+ enzyme complexes show significant differences with the Na^+ enzyme in the positions of αAsp56, βLys167, and βAsp305 (*30*, *48*). In the presence of Na^+, Trp synthase preferentially forms an ion pair between βLys167 and βAsp305, while the K^+ and Cs^+ enzymes form an ion pair between βLys167 and αAsp56. Our results suggest that the NH_4^+ form of Trp synthase may also form the βLys167−αAsp56 ion pair.

Conclusions. Hydrostatic pressure has been used extensively to study protein folding and subunit association in Trp synthase but has not been previously applied to study the conformational equilibrium between the external aldimine and aminoacrylate complexes. The data presented herein demonstrate that hydrostatic pressure is a useful tool to study rates and equilibria of conformational changes in Trp synthase and likely of other allosteric enzymes. The pressure measurements also allow for convenient estimate of solvation differences, which are sensitive to structural changes, between conformational states of enzymes. Further studies of equilibria and kinetics of Trp synthase and other PLP-dependent enzymes using pressure are in progress.

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