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Control of Ionizable Residues in the Catalytic Mechanism of Tryptophan Synthase from *Salmonella typhimurium*[†]

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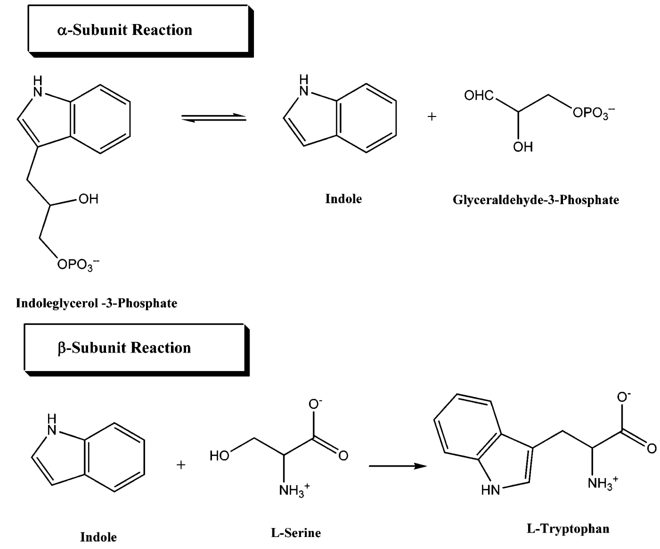
ABSTRACT: The tryptophan synthase $\alpha_2\beta_2$ complex catalyzes the last two steps in the biosynthesis of L-tryptophan in bacteria, plants, and fungi, the conversion of indole-3-glycerol phosphate and L-serine to L-tryptophan, glyceraldehyde 3-phosphate, and water. The β-subunit binds pyridoxal 5′-phosphate and catalyzes the β-replacement reaction with serine and indole. Structural, spectral, and kinetic studies indicate that different monovalent cations stabilize the alternative enzyme conformations and equilibrium distribution of the internal, external, and α-aminoacrylate Schiff base. To improve our understanding of the role of monovalent cations, the pH dependence of steady-state and pre-steady-state kinetic parameters and primary kinetic deuterium isotope effects were measured in the presence of L-serine and [α-^{2}H]-L-serine in the absence and presence of Na^+, K^+, and Cs^+. For the interpretation of the data obtained in this study, it was necessary to re-interpret a number of results published previously. Overall, data suggest that the enzyme exists in two conformers that equilibrate slowly either in the absence of substrates and monovalent cations or in the presence of K^+ or Cs^+, whereas they equilibrate faster in the presence of Na^+. The rate of interconversion of the conformers increases as a group on the enzyme with a pK_a of ∼8 becomes deprotonated. The pH dependence of deuterium isotope effects is suggestive of a mechanism in which a pH-dependent conformational change that closes the active site precedes the chemical steps, likely a result of formation of one or more salt bridges. As the pH increases, the reaction becomes more committed to proceed to products, which causes the deuterium isotope effect to decrease to a value of unity at high pH. The closure of the site is modulated by the different monovalent cations and is fastest in the presence of Na^+, which exhibits the maximum isotope effect of 5.7 (likely the intrinsic effect) on V/K_{serine}, and slowest in the presence of Cs^+, which exhibits the smallest isotope effect of ∼1.5. The isotope effect on V, in all cases, indicates a contribution to rate limitation from steps in the second half of the reaction. Finally, in the presence of Na^+, the steady-state isotope effect on V is greater than that on the pre-steady-state rate constant for decay of the external Schiff base, suggesting that the rate of conversion of the two conformers of the internal aldimine contributes to the pre-steady-state rate, but not the steady-state rate because the high serine concentration traps the enzyme in the active E−serine complex before it can decay to the less active form.

Tryptophan synthase (EC 4.2.1.20) is a heterotetrameric ($\alpha_2\beta_2$) bifunctional pyridoxal 5′-phosphate-dependent enzyme that catalyzes the last two steps in the biosynthesis of L-tryptophan in bacteria, plants, and fungi (*1*−*3*). The physiological reaction is termed the $\alpha\beta$-reaction and involves the conversion of indole-3-glycerol phosphate and L-serine to L-tryptophan, glyceraldehyde 3-phosphate, and water. Two distinct reactions take place in the α- and β-active sites that are physically separated but connected by an intermolecular channel (*4*).



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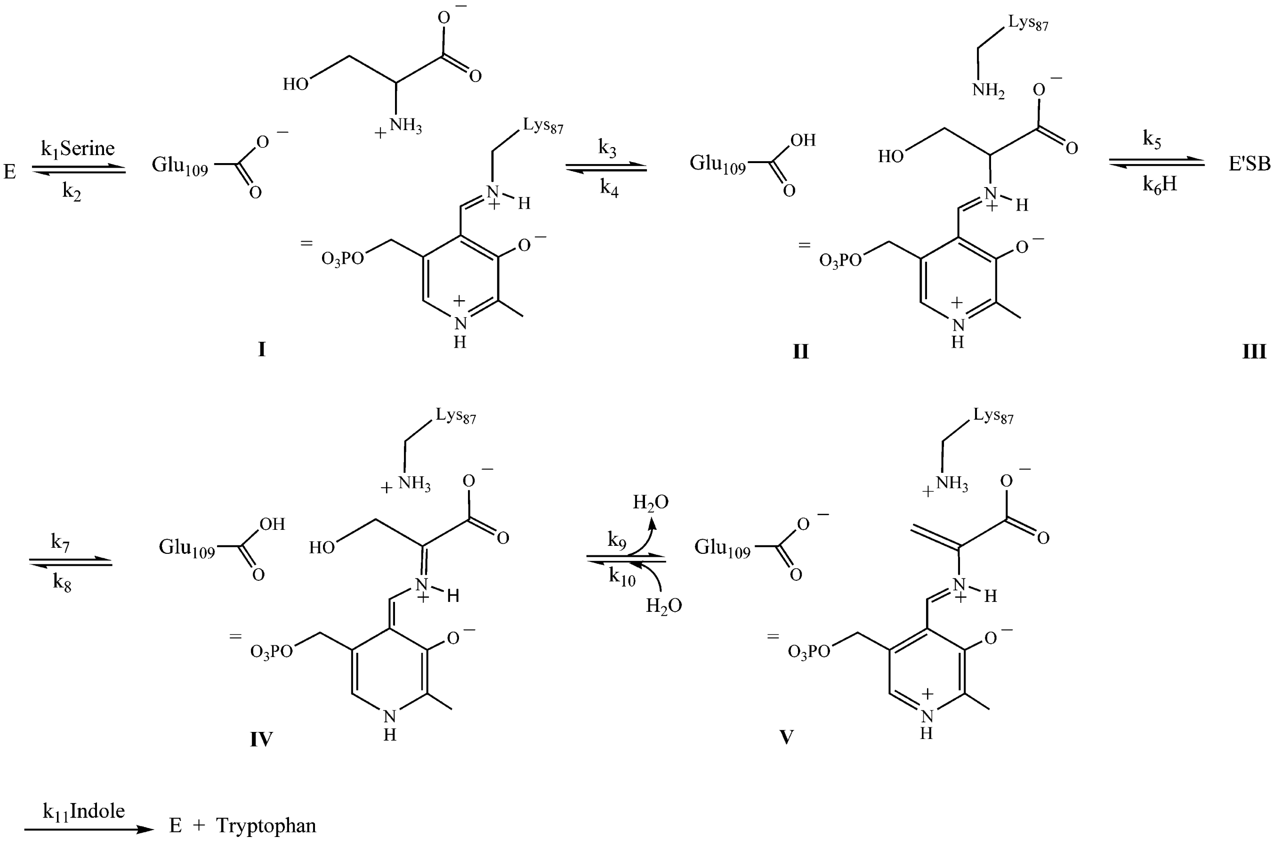
Scheme 1: Proposed Mechanism of the Tryptophan Synthase β-Reaction[a]



[a] Serine is bound to the ISB form of the β-subunit with E109 unprotonated (I). The ESB is formed (II), leaving E109 protonated. Either as the ESB is formed or afterward, the active site closes to give E′SB (III), and K87 acts as a general base to form Q (IV). E109 acts as a general acid to protonate the leaving hydroxide as the AA is formed (V). The indole half of the reaction then occurs to regenerate the ISB.

The first reaction takes place at the α-site with the formation of glyceraldehyde 3-phosphate and indole, which is channelled to the β-site where it condenses with L-serine via a β-replacement reaction. The β-reaction proceeds via a series of intermediates (*5*, *6*) shown in Scheme 1. The internal aldimine (ISB) between PLP and the ϵ-amino group of βK87[1] reacts with L-serine via a *gem*-diamine intermediate to rapidly form an external aldimine (ESB). Abstraction of the α-proton of the substrate ESB yields an unstable quinonoid intermediate (Q) that eliminates hydroxide to give the quasi-stable, external aldimine of α-aminoacrylate (AA), which is a Michael acceptor. Indole, produced in the α-site, reacts in the β-site as a nucleophile at C-3 of the AA and is proposed to generate an indoleninium quinonoid intermediate. Deprotonation of C-3 of this intermediate leads to the quinonoid complex of tryptophan, which is then protonated to form the ESB of the product, L-tryptophan (*2*). Transimination, via a *gem*-diamine intermediate, results in the release of L-tryptophan and regenerates the ISB, completing the catalytic cycle.

Each of the reaction intermediates has a characteristic spectroscopic signature. The internal aldimine absorbs at 412 nm, which is attributed to the ketoenamine tautomer, with a minor band at 330−340 nm, which is attributed to the enolimine tautomer (*7*). The ESB absorbs at 420 nm and is the only species that exhibits significant fluorescence emission. The AA absorbs predominantly at 350−360 nm and has a broad low-intensity band at 470 nm. The 330−360 and 420−470 nm maxima may be attributed to enolimine and ketoenamine tautomers or to a species unprotonated and protonated at the aminoacrylate Schiff base nitrogen (*8*, *9*). The equilibrium between the enolimine and ketoenamine tautomers of the ISB is pH-independent over the pH range of 6.0−10.0, indicating that the microenvironment of the β-site locks the species into a single ionization state, where the Schiff base nitrogen is protonated (i.e., favors the ketoenamine tautomer) (*10*, *11*).

Addition of L-serine in the absence of indole generates an equilibrium mixture of the ESB and AA. Formation of the AA from the ESB causes the β-site to switch from a low-activity open to a high-activity closed conformation. The equilibrium distribution is affected by temperature and pH, with low temperature and high pH favoring the ESB species. Studies of the enzyme in the crystalline state and in solution indicate that different monovalent cations stabilize alternative enzyme conformations and catalytic intermediates (*8*, *12*, *13*), suggesting they may be important effectors of tryptophan synthase. Recent reviews suggest that a large number of enzymes have evolved to use monovalent cations to modulate catalysis and regulation (*14*−*16*). In tryptophan synthase, K^+ and more strongly Na^+ favor the accumulation of the ESB species and stabilize a partially closed conformation of the enzyme, while Cs^+ stabilizes the AA and the closed state. Structures of the native enzyme in complex with Na^+ (*4*),

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[1] Abbreviations: MVC, monovalent cations; PLP, pyridoxal 5′-phosphate; DTT, 1,4-dithiothreitol. The α and β associated with numbered residues refer to the subunit in the $\alpha_2\beta_2$ complex that contains the residue.

K^+ (*17*), and Cs^+ (*17*) have been determined and show that MVC act as allosteric effectors, specifically binding to a site in the β-subunit separate from the catalytic site. Each of the monovalent cations binds to approximately the same site ~8 Å from the 5′-phosphate of PLP and involves a common set of ligands, but with one or more unique to the specific cation. For example, Cs^+ is uniquely coordinated to the backbone carbonyl oxygen of βV231, βG268, and βL304. The monovalent ion-bound structures generally differ with respect to residues involved in interactions at the interface between the α- and β-subunits and residues acting as a molecular gate within the intermolecular tunnel. A structure of the cation-free enzyme has not yet been reported.

An ordered sequential mechanism is generally favored for the β-subunit tryptophan synthase with L-serine binding first; this is supported by the requirement that L-serine binds before indole. L-Serine forms a specific binary complex with the active site of the β-protomer, while indole does not (*18*−*22*). The pH dependence of the steady-state kinetic parameters for the *Salmonella typhimurium* tryptophan synthase has been obtained in the presence of sodium chloride (*23*). Data suggest the requirement for three ionizable groups involved in substrate binding and/or catalysis. A group with a pK_a of ~6.5 was assigned to βD305, another with a pK_a of ~7.3 to βK87, and the third with a pK_a of 8.2−9 to βK167. We measured the pH dependence of the pre-steady-state rate constant for decay of the ESB, as it is converted to the AA, in the absence and presence of MVC to obtain information about functional groups in the enzyme−substrate complex and the effect of cations on the catalytic mechanism (*24*). The formation of the α-aminoacrylate intermediate requires a group protonated with a pK_a of ~9 in the presence of Cs^+, or two groups with pK_a's of ~6 and ~9, which must be unprotonated and protonated in the absence and presence of Na^+ and K^+; pK_a's were assigned to βD305 and βK87. On the basis of the three-dimensional structure (*4*, *25*), there are a number of ionizable residues located in the β-active site that could participate in catalysis and/or substrate binding, including βH86, βK87, βE109, βH115, βK167, and βD305. Ro and Miles (*26*) suggested that the base abstracting the α-proton from the ESB was βK87, while Schiaretti et al. (*24*) suggested this role could be played by βD305, which might also contribute, together with βR141, βK167, and αD56, to allosteric signaling between the α- and β-subunits. βH86 was proposed to interact with the PLP 5′-phosphate group and contribute to lowering the pK_a of βK87 (*23*), while βE109 was thought to activate indole for nucleophilic addition to the α-aminoacrylate in the β-reaction (*27*). No involvement of βH115 in catalysis has ever been experimentally confirmed.

Isotope effects are a versatile probe of enzyme mechanism (*28*), and this kind of study was instrumental in our understanding of the mechanism and proposing a transition-state structure for the elimination reaction catalyzedby *O*-acetylserine sulfhydrylase (*29*), an enzyme that belongs to the same fold type (fold type II) and β-family of tryptophan synthase. In this paper, we measure the pH dependence of steady-state kinetic parameters, the pre-steady-state rate of decay of the ESB, and primary deuterium kinetic isotope effects on the steady-state and pre-steady-state rate constants, in the absence and presence of MVC, in investigating the mechanism of the β-elimination reaction catalyzed by tryptophan synthase.

MATERIALS AND METHODS

Chemicals. L-Serine and BTP were from Sigma, while KOD, DCl, and D_2O (99.8 at. % D) were from Cambridge Isotope Lab. All other reagents and chemicals were obtained from commercially available sources and were of the highest quality available.

Enzyme Preparation. The tryptophan synthase multienzyme complex from *S. typhimurium* was purified as described previously (*30*). The enzyme was stored at −80 °C in 50 mM Bicine and 20 μM PLP (pH 7.8) and dialyzed against MVC-free 25 mM Bis-Tris propane (pH 7.8), before use. Protein concentrations were determined from the absorbance at 278 nm using an $A_{1cm}^{1\%}$ of 6.05 for the $\alpha_2\beta_2$ complex.

[α-^{2}H]-L-Serine. L-Serine was incubated with *O*-acetylserine sulfhydrylase A in D_2O at pD 9.0 (adjusted with KOD) to catalyze exchange of the α-proton (*29*). The progress of incorporation of deuterium into the C-2 position of L-serine was monitored via the disappearance of the signal at 3.80 ppm using a 400 MHz Varian ^{1}H NMR spectrometer. The reaction mixture was allowed to stand overnight, and the reaction was stopped by the removal of the enzyme using Amicon ultrafiltration with a PM10 membrane. Potassium ions were removed by chromatography on a Dowex-50W column in the H^+ form. The sample was then lyophilized to dryness. The level of incorporation of deuterium, measured by ^{1}H NMR, was 97.7%. The product was also analyzed with an electrospray quadrupole ESI-Q_APII50EX Applied Biosystem mass spectrometer, and its mass spectrum was also consistent with deuterium incorporation.

Steady-State Measurements. The activity of the tryptophan synthase β-replacement reaction with L-serine and indole was measured by a direct spectrophotometric assay (*31*). Initial rates were measured using a Cary 219 spectrophotometer equipped with a temperature-controlled cuvette holder. Reaction mixtures were equilibrated at 20 °C in a 1 cm cuvette for at least 5 min at the desired temperature prior to the addition of enzyme. Reaction mixtures contained 25 mM BTP at the appropriate pH, 0.04 mM PLP, 0.1 mM DTT, 0.18 mM indole, and variable concentrations of L-serine and [α-^{2}H]-L-serine (0.25−10 × K_{serine}) in the absence and presence of MVC. At pH 7.8, values of K_{indole} of 13 (*19*), 50 (*32*), and 7.4 μM (*33*) are reported for the *Escherichia coli* enzyme, 18 μM for the *S. typhimurium* enzyme in the presence of Na^+, and 590 μM in the absence of MVC (*8*). Thus, 0.18 mM indole is greater than the reported K_{indole} values in the presence of MVC, and the reported $^{\text{app}}k_{\text{cat}}$ will be close to the absolute value. In the absence of MVC, the absolute value of k_{cat} will be at least 4-fold greater than the values reported in Table 1. When present, the MVC concentrations were as follows: 250 mM NaCl, 100 mM KCl, or 100 mM CsCl. All of the initial rates were measured in at least duplicate. The final enzyme concentration was 118 nM. Under some conditions, the protein concentration was doubled to increase the quality of the signal, and the initial rate was corrected for enzyme concentration.

Pre-Steady-State Measurements. Single-wavelength kinetic experiments were carried out with a temperature-controlled Olis RSM 1000 stopped-flow apparatus. One syringe con-

Table 1: Kinetic Parameters for the β-Replacement Reaction Catalyzed by the TS $\alpha_2\beta_2$ Complex in the Absence and Presence of Monovalent Cations as a Function of pH[a]

pH	${}^{app}k_{cat}$ (s^{-1})	${}^{app}K_{serine}$ (mM)	${}^{app}(k_{cat}/K_{serine})$ (M^{-1} s^{-1})
	Without Monovalent Cations		
6.5	0.24 ± 0.01	1.60 ± 0.50	160 ± 10
7.8	0.91 ± 0.03	1.60 ± 0.10	570 ± 60
8.5	1.37 ± 0.01	2.70 ± 0.30	520 ± 50
	With CsCl		
6.5	6.00 ± 0.25	9.00 ± 0.40	670 ± 4
7.8	8.70 ± 0.10	5.60 ± 0.60	1590 ± 160
8.5	6.15 ± 0.02	1.90 ± 0.20	3310 ± 360
	With NaCl		
6.5	1.90 ± 0.10	4.78 ± 0.03	410 ± 10
7.8	2.80 ± 0.20	0.52 ± 0.02	4900 ± 390
8.5	1.90 ± 0.10	0.14 ± 0.01	13660 ± 890
	With KCl		
6.5	2.08 ± 0.06	1.89 ± 0.09	1110 ± 20
7.8	4.80 ± 0.20	0.58 ± 0.03	8420 ± 80
8.5	4.00 ± 0.20	0.27 ± 0.01	14760 ± 1250

[a] Parameters were obtained by fitting the data points with the Michaelis−Menten equation with SigmaPlot. The reported values are the average between two sets of experiments on the same solutions (see Table 2).

tained 17 μM $\alpha_2\beta_2$ in 25 mM Bis-Tris propane at the appropriate pH, in the absence or presence of MVC. The second syringe contained 25 mM Bis-Tris propane, 100 mM L-serine, or [α-^{2}H]-L-serine, at a pH value and MVC concentration identical to those in the first syringe. Experiments were conducted with the pH maintained at 6.5, 7.8, or 8.5, at 20 °C. The pH of the final reaction mixture in the stopping syringe was always determined at the end of the experiment. The formation and decay of the ESB of L-serine were followed by monitoring fluorescence emission at 500 nm, upon excitation at 420 nm. Kinetic traces at a single wavelength were recorded by collecting 1000 data points in 10 s.

Nomenclature. Isotope effects are expressed using the nomenclature developed by Northrop (*34*) and Cook and Cleland (*35*−*38*). Deuterium isotope effects are written with a leading superscript D; e.g., a primary deuterium isotope effect on V/K_{serine} is written ${}^{D}(V/K_{serine})$.

Data Analysis. Reciprocal initial rates were plotted against reciprocal substrate concentration to assess the quality of the data. Kinetic parameters, V and V/K_{serine}, were then estimated by fitting the initial rate data to the Michaelis−Menten equation using SigmaPlot (Systat Software, Inc.).

Pre-steady-state time courses were fitted using eq 1 describing a biphasic decay:

$$y = y_o + a\mathrm{e}^{-k_1 x} + b\mathrm{e}^{-k_2 x} \qquad (1)$$

where y_0 is the horizontal offset, a and b are the amplitudes of the two phases, and k_1 and k_2 are the corresponding first-order rate constants.

Standard errors of the isotope effects were calculated according to the following equation:

$$\mathrm{SE}\frac{k_H}{k_D} = \frac{k_H}{k_D}\sqrt{\left(\frac{\mathrm{SE}k_H}{k_H}\right)^2 + \left(\frac{\mathrm{SE}k_D}{k_D}\right)^2} \qquad (2)$$

RESULTS

Steady-State Studies. The pH dependence of steady-state kinetic parameters for the *S. typhimurium* tryptophan synthase has only been realized in the presence of NaCl (*26*), and thus, very little is known of the reaction in the absence of MVC and in the presence of K$^+$ and Cs$^+$. Steady-state kinetic parameters were measured at three pH values over the pH range they were reported to change (*26*), and the data are summarized in Table 1. The value of k_{cat} is increased in the presence of MVC at all pH values, in agreement with the work of Peracchi et al. (*8*), who used a saturating concentration of L-serine and varied the concentration of indole. The order of activation does not change with pH and was as follows: Cs$^+$ > K$^+$ > Na$^+$. However, it should be pointed out that although the k_{cat} values measured in the presence of MVC are close to the true values, those in the absence of MVC are not and will be significantly higher (see Steady-State Measurements in Materials and Methods). In fact, correction to saturating indole concentration will generate a value at pH 7.8 that is close to that measured in the presence of Na$^+$.

The presence of MVC also affects K_{serine}. At pH >8, ${}^{app}K_{serine}$ is lowest in the presence of MVC (it is not possible to accurately interpret the data in the absence of MVC). The value is approximately the same in the presence of Na$^+$ and K$^+$ (0.14−0.27 mM), and 1 order of magnitude lower than that measured in the presence of Cs$^+$. Because k_{cat} is relatively insensitive to pH in the presence of MVC, the changes in K_{serine} are also reflected in the second-order rate constant, k_{cat}/K_{serine}. Previously published data indicate MVC have a relatively small effect on L-serine affinity with a K_d value of ∼90 μM in the absence of MVC and ∼60 and ∼20 μM in the presence of Na$^+$ and Cs$^+$, respectively (*8*). However, the dependence of the relaxation rate constants for formation and decay of the serine ESB on the concentration of L-serine suggested an increase of ∼5-fold in the affinity for L-serine upon addition of Na$^+$ (*39*). In the presence of MVC, the variations in K_{serine} (Table 1) are even greater than 5-fold in some cases and, thus, do not reflect changes in affinity alone; rather, it likely reflects changes in the relative rates of steps along the reaction pathway, perhaps including the net off-rate constant for serine from the ESB (see below).

Although data have only been obtained at three pH values, they serve, along with the isotope effects discussed below, to provide a rough indication of the pH−rate profiles for k_{cat} and k_{cat}/K_{serine} in the presence of MVC and for k_{cat}/K_{serine} in the absence of MVC. As suggested above, k_{cat} is relatively insensitive to pH over the pH range studied (we will not consider the data in the absence of MVC). The k_{cat}/K_{serine} value, however, does change with pH, and all data are consistent with the requirement of a group with a pK_a of 7.5−8 that must be unprotonated for optimum binding of serine and/or catalysis. Data are consistent with those obtained previously (*26*).

Steady-State Kinetic Isotopic Effects. Primary kinetic deuterium isotope effects were measured using L-serine or [α-^{2}H]-L-serine as a function of pH, in the absence and presence of MVC, and the data are summarized in Table 2. In the absence of MVC, ${}^{D}(V/K_{serine})$ may increase slightly as the pH is either increased or decreased from 7.8 but is

Table 2: pH Dependence of Steady-State Primary Kinetic Deuterium Isotope Effects in the Absence and Presence of Monovalent Cations[a]

pH	${}^{D}V$	${}^{D}(V/K_{serine})$
Without Monovalent Cations		
6.5	1.3 ± 0.1	2.1 ± 0.2
7.8	1.4 ± 0.1	1.6 ± 0.1
8.5	2.0 ± 0.1	1.9 ± 0.1
With CsCl		
6.5	1.1 ± 0.1	1.5 ± 0.2
7.8	1.2 ± 0.1	1.0 ± 0.1
8.5	1.2 ± 0.1	1.0 ± 0.1
With NaCl		
6.5	4.9 ± 0.3	5.7 ± 0.2
7.8	4.8 ± 0.5	4.0 ± 0.3
8.5	4.1 ± 0.2	2.6 ± 0.2
With KCl		
6.5	2.4 ± 0.2	3.5 ± 0.2
7.8	2.9 ± 0.2	2.3 ± 0.2
8.5	3.3 ± 0.2	1.7 ± 0.2

[a] Values of ${}^{D}V$ and ${}^{D}(V/K_{serine})$ are estimated from the ratio of the kinetic parameters obtained as described in Table 1 with serine and [α-^{2}H]-L-serine. Standard errors of the mean are given.

Table 3: Pre-Steady-State Kinetic Parameters and Primary Deuterium Kinetic Isotope Effects on the Rate Constant for External Aldimine Decay[a]

pH	k_1 (s^{-1}) L-serine	k_1 (s^{-1}) [α-^{2}H]-L-serine	${}^{D}k_1$
Without Monovalent Cations			
6.5	10.25 ± 0.38	3.15 ± 0.06	3.2 ± 0.1
7.8	12.05 ± 0.16	3.90 ± 0.03	3.1 ± 0.1
8.5	7.34 ± 0.08	2.02 ± 0.02	3.6 ± 0.1
With CsCl			
6.5	24.25 ± 1.72	17.72 ± 0.48	1.4 ± 0.1
8.5	10.13 ± 0.21	8.09 ± 0.19	1.2 ± 0.1
With NaCl			
6.5	5.01 ± 0.05	2.42 ± 0.11	2.1 ± 0.1
7.8	4.28 ± 0.03	1.64 ± 0.04	2.6 ± 0.1
8.5	3.10 ± 0.04	1.13 ± 0.04	2.7 ± 0.1
With KCl			
6.5	11.49 ± 0.09	3.46 ± 0.05	3.3 ± 0.1
7.8	9.68 ± 0.08	2.55 ± 0.03	3.8 ± 0.1
8.5	5.80 ± 0.05	1.37 ± 0.02	4.2 ± 0.1

[a] Standard errors on the isotope effect were calculated as discussed in Materials and Methods.

relatively constant. Note that a value of ~2 observed for ${}^{D}V$ at pH 8.5 is identical to that on V/K. In the presence of Na^{+} and Cs^{+}, the isotope effect is smallest at high pH and increases as the pH is decreased over the same range where the value of k_{cat}/K_{serine} increases, whereas it is the opposite for K^{+}. The pH value at which the isotope effect changes by half [the effect goes to unity at high pH (see Discussion)] provides a rough estimate of the pK_a of the group titrated; a value of ~7.5−8 is obtained for all of the MVC, consistent with the pH dependence of k_{cat}/K_{serine} above. The largest kinetic isotope effect (~6) was observed in the presence of NaCl, while the smallest is observed in the presence of Cs^{+} with values that are not significantly different from unity with the exception of the ${}^{D}(V/K)$ value measured at pH 6.5. Note that, in all cases, at low pH the isotope effect on V is smaller than that measured on V/K, suggesting a difference in the contribution to rate limitation of the indole half of the reaction, which will be discussed below.

Data measured for ${}^{D}V$ are consistent with the limited available literature (*39*). Isotope effects estimated for the *S. typhimurium* $\alpha_2\beta_2$ complex at pH 7.8 in the presence of Na^{+} and K^{+} were 5.3 and 3.0, respectively, while that observed in the absence of MVC was 1.28.

Pre-Steady-State Kinetic Studies. The reaction of L-serine with the $\alpha_2\beta_2$ complex leads to the formation of an equilibrium distribution of intermediates (*8*, *12*, *13*). The distribution is affected by temperature, pH, α-subunit ligands, and monovalent cations. In the absence of MVC, at pH 7.9 and 25 °C, the predominant species is the AA. Binding of Na^{+} or K^{+} favors the accumulation of the substrate ESB, while Cs^{+} shifts the equilibrium toward the AA (*8*, *12*). To determine the kinetic isotope effect for α-proton abstraction in the first half-reaction, rate constants were measured with protonated and deuterated L-serine using a single-wavelength stopped-flow method, in the absence and presence of MVC as a function of pH. Stopped-flow time courses measured the fluorescence change at 500 nm upon excitation at 420 nm, reflecting the formation and decay of the ESB for the reaction of L-serine with $\alpha_2\beta_2$ in the first half of the reaction. The ESB is the only significantly fluorescent species along the tryptophan synthase reaction pathway (*2*).

When enzyme is mixed with serine, a rapid initial fluorescence increase is observed upon formation of the ESB, followed by a decrease in the intensity of the signal as the external aldimine is converted to the AA. The formation of the ESB occurred largely in the dead time of the instrument and precluded a detailed analysis of the pH dependence of this process. The decay in fluorescence of the ESB is comprised of two kinetic phases defined by first-order rate constants k_1 and k_2 (Table 3) (*5*, *6*, *24*). The value of k_1 accounts for >70% of the decay process, decreasing as the pH increases, and is dependent on MVC (Table 2) (*24*). The rate constant changes according to the following: Cs^{+} > without MVC > K^{+} > Na^{+}. On the other hand, k_2 accounts for ~10−30% of the decay process with rates that are ~1 order of magnitude lower than k_1 (data not shown). The rate of the slow process is essentially unaffected by pH and MVC (*24*). As observed in the low-β-activity mutant βD305A (*40*), this slower phase might represent a "survival" pathway, which allows the formation of L-tryptophan even in the presence of some mutations that affect conformational transitions (*24*). Rate constants measured using L-serine at pH 7.8 in the absence and presence of Na^{+} or K^{+} are in good agreement with those previously published (*12*, *24*, *39*, *41*).

Isotope Effects on the Decay of the ESB. The primary kinetic deuterium isotope effect on rate constants for formation of the AA was examined using L-serine or [α-^{2}H]-L-serine, in the absence of MVC and in the presence of K^{+}, Na^{+}, or Cs^{+}, as a function of pH (Table 3). In the absence of MVC, k_1 with L-serine is ~3−4 times faster than that for [α-^{2}H]-L-serine; i.e., an isotope effect of ~3.1−3.6 is observed. A slightly larger isotope effect is observed in the presence of K^{+} (3.3−4.2), while it is smaller in the presence of Na^{+} (2.1−2.7) and quite small in the presence of Cs^{+} (1.4). In all cases, with the exception of the presence of Cs^{+}, the isotope effect increases with an increase in pH. Note that the isotope effects do not correlate with those measured in the steady state.

Findings reported here agree well with previously published data (*42*). The primary deuterium isotope effect estimated for the fast decay phase in the presence of 100 mM NaCl at pH 7.8 and 25 °C was 2.95, in agreement with the value of 2.6 given in Table 3.

DISCUSSION

Kinetic Parameters

In the presteady state, the kinetics of the disappearance of the ESB are biphasic, thus suggesting that the enzyme possesses two routes for formation of the AA intermediate. The parallel processes have been explained on the basis of catalysis associated with the low-activity open conformation and the high-activity closed state of the β-subunit (*39*). Production of the AA is the first step along the tryptophan synthase reaction pathway to exhibit slow interconversion of open and closed enzyme conformations. Other intermediates along the reaction pathway prior to formation of the AA likely also exist as an equilibrium between open and closed conformations, but their interconversion is thought to be rapid; as a result, the kinetic behavior of these species is indistinguishable from that of a single conformation. In steady-state experiments, only the faster of the two pathways is observed, since the first-order rate constants estimated in the presteady state differ by 1 order of magnitude.

Analysis of the dependencies of relaxation rates with respect to variation of the metal ion concentration together with the dependencies of relaxation rates on serine concentration in the presence of NaCl suggests that MVC binding changes the ground-state energy surface of the first half-reaction and yields altered equilibria between active and less active states of the AA (*39*). The ESB species do not appear to be significantly affected since the affinity of the enzyme for serine is unaffected by MVC (*8*). The dependency of the ESB relaxation rate (formation phase) on the metal ion concentration follows a hyperbolic curve, reflecting binding of metal ion to the enzyme at an earlier step in the pathway (*39*).

Re-Interpretation of the Literature Data

pH Dependence of Kinetic Parameters. There is an extensive literature about the steady-state kinetics of the tryptophan synthase β-reaction. However, there is a lack of consensus concerning the interpretation of the data, as well as errors in interpretation. To more accurately interpret the pH−rate profiles and isotope effects obtained in these studies and those present in the literature, we need a frame of reference. Below we generate a frame of reference by providing a general mechanism taking into account experimental data and structures available for tryptophan synthase and maintaining an inventory of protons throughout the reaction.

The first portion of the β-reaction involves binding of the amino acid substrate to the ISB form of the enzyme and conversion to the serine ESB. There are three possibilities with respect to the protonation state of the α-amine for binding the amino acid. The enzyme can selectively bind the amino acid with a neutral amine, the protonated amine, or both. An example of the former is observed for *O*-acetylserine sulfhydrylase binding *O*-acetyl-L-serine (*43*), while the aspartate aminotransferase reaction provides an example of binding the amino acid with a protonated amine (*44*); the tyrosine phenol-lyase binds the amino acid with protonated or unprotonated amine (*45*). To form the external aldimine, the α-amine of the substrate must be neutral to allow nucleophilic attack on C-4′ of the internal aldimine. If the enzyme binds the amino acid with the α-amine protonated, there are generally two ways utilized to deprotonate it: the internal aldimine nitrogen can act as a base as in the case of aspartate aminotransferase (*44*), or an enzyme side chain can serve as the proton acceptor. The resting form of tryptophan synthase absorbs maximally at 412 nm, indicating that the ketoenamine tautomer of the enzyme predominates with the internal aldimine nitrogen protonated, and there is no evidence that the amino acid with a neutral amine is selectively bound by enzyme. As a result, there must be an enzyme side chain responsible for deprotonating the α-amine of L-serine once it has bound to the enzyme. There are two residues that are positioned to accept the α-amine proton, βD305 and βE109. Closure of the β-active site is aided by salt bridge linkages between βD305 and βR141 and between βK167 and αD56 as the ESB is formed (*25*, *46*). βD305 is at the junction between the β-active site and the MVC site, and formation of the βD305−βR141 salt bridge blocks entry into the β-site and appears to be important for the transition to the closed conformation (*40*). The D305A mutation results in a destabilization of the enzyme-bound AA and Q species, giving a buildup of the open conformation of the serine ESB, a decreased affinity of the β-site for L-serine, and a change in the nucleophile specificity of the β-reaction, consistent with its role in site closure. As a result, βD305 must be ionized as the external aldimine is formed and cannot donate a proton to the serine hydroxyl as it is eliminated. On the other hand, there is good evidence, including a characterization of the Glu to Asp mutation, to suggest that βE109 acts as the base to accept a proton from the α-amine of L-serine and donate it to the leaving hydroxide (*27*). A mechanism consistent with the data obtained to date is shown in Scheme 1.

Once the ESB is formed, the side chain that accepted the α-amine proton is protonated, and the ISB lysine (βK87) is unprotonated. Comparison of the structure of the ESB and ISB (*17*, *46*) shows that βK87 is directed toward the *si* face of the imine (it is pointed toward the 5′-phosphate but is highly flexible and could with a minor structural rearrangement move toward C_α of the bound serine), and with adjustment of the serine hydroxyl group, both βD305 and βE109 are within hydrogen bonding distance of the serine hydroxyl. Given that βD305 must be ionized to form a salt bridge with βR141, it is unlikely that this side chain can serve as an acid−base catalyst. It is thus likely that βE109 plays a role in accepting a proton from the α-amine of L-serine and donating a proton to the hydroxyl as it is eliminated. It is then anticipated that βK87 is the catalytic base that accepts the α-proton to generate Q, which then rearranges to expel the hydroxide that is protonated by βE109 (Scheme 1).

The mechanism proposed in Scheme 1 makes predictions concerning the pH dependence of V/K_{serine}. Specifically, since free enzyme and free serine predominate under V/K conditions, the protonation state of only the α-amine of serine and the carboxylate of βE109 should be observed, that is, a bell-shaped pH−rate profile. The group seen on the low-pH side of the profile could be unprotonated for optimal activity, while that seen on the high-pH side would then be protonated for optimal activity. However, it is equally possible that the two groups could have reverse protonation states; i.e., the one on the acid side could be protonated and the one on the basic side unprotonated for optimal activity (*28*). The *V* pH−rate profile is more difficult to interpret, since it will include steps from both halves of the reaction depending on whether one, the other, or both half-reactions contribute to rate limitation. With this frame of reference, the pH−rate profiles obtained previously for the tryptophan synthase β-reaction can be interpreted.

A study of the pH dependence of the kinetic parameters of the β-replacement reaction catalyzed by the tryptophan synthase $\alpha_2\beta_2$ complex from *S. typhimurium* was carried out previously (*26*). V_{max} and V/K_{serine}, in the presence of NaCl, exhibited bell-shaped pH−rate profiles, and we will first consider the V/K_{serine} pH−rate profile. The *V*/*K* for serine decreases below pK_a values of 6.5 and 7.3 and above a pK_a of ∼9. The observed pK_a of 7.3 was erroneously assigned to βK87, which cannot be titrated in the ISB, the predominant enzyme form under *V*/*K* (limiting substrate) conditions. According to the proposed mechanism (Scheme 1), the pK_a value of 6.5 likely reflects βE109, responsible for accepting a proton from serine as it binds and delivering the proton to hydroxide as it leaves. This interpretation is consistent with previous proposals, which have suggested a number of functions for this residue, including deprotonation of the incoming serine to protonation of the β-hydroxyl leaving group and enzymatic activation of indole as a nucleophile. Conversion of βE109 to aspartate results in a stabilization of the ESB, consistent with its proposed role as a general acid catalyst in the elimination reaction, and this will be further considered below (*27*). The group with a p*K* of ∼9 is almost certainly the α-amine of L-serine, which has a pK_a of 9.25, and must be protonated for optimal binding (*47*). The group with a pK_a of ∼7.3 may reflect a group involved in a structural change important for closing the site. Schiaretti et al. (*24*) suggest this group may be βK167, which forms a salt bridge required to close the site. Although this is unlikely since the group must be unprotonated, according to the pH−rate profile, for optimal activity, it could certainly be the partner of βK167 in the salt bridge, αD56, which would be required to be unprotonated. At any rate, an enzyme residue is required to be unprotonated for optimal catalysis at a limiting serine concentration.

The *V* pH−rate profile decreases below a pK_a of 6.5 and above a pK_a of 8.2. Groups observed in the *V* pH−rate profile may reflect groups in both half-reactions that are important for catalysis if both reactions contribute to rate limitation. A significant primary kinetic deuterium isotope effect on *V* has been observed with [α-^{2}H]-L-serine, suggesting that the first half of the β-reaction contributes to rate limitation (*26*), while release of tryptophan is reported to limit the *E. coli* enzyme at pH >7.8 (*21*). Thus, the groups with pK_a's of 6.5 and 8.2, important for catalysis, likely reflect both half-reactions. The group with a pK_a of 6.5, most likely βE109, functions as discussed above, while the group with a pK_a of 8.2 that must be protonated for optimal activity is likely βK87, which must protonate the tryptophan quinonoid intermediate prior to release of L-tryptophan from its ESB.

In agreement with the suggested pH-dependent conformational change reflected by the pK_a of 7.3 are the ^{31}P data obtained previously (*48*). In the absence of MVC, the ISB exists as a mixture of conformers that interconvert slowly, signalled by two distinct chemical shifts of 3.71 and 4.11 ppm in a ratio of ∼2:1; line widths of 10 and 12 Hz, respectively, are obtained for the two peaks. These two conformers likely reflect the open and closed form, respectively, of the β-subunit as suggested previously (*49*). At pH 9.5, only the lower field peak is observed at 3.73 ppm with a line width of 12 Hz. The presence of Cs$^+$ has no significant effect on this equilibrium mixture. In the presence of Na$^+$, however, only a single resonance at 3.97 ppm is observed, but with a large increase in the line width to 35 Hz. The chemical shift is intermediate between the two resonances observed in the absence of Na$^+$, likely reflecting an increase in the rate of interconversion of the two conformers into the intermediate exchange domain, with identical results at pH 8 and 9.5 (*50*). Results are identical in terms of the chemical shift of the ESB at pH 9.5 in the presence of Na$^+$, but the line width decreases from 35 to 5 Hz, suggesting a further increase in the rate of interconversion of the two conformers into the fast exchange domain. In the presence of Na$^+$, the Q exhibits a chemical shift of 4.2 ppm, the highest observed for the tryptophan synthase β-subunit, with a line width of ∼10 Hz. Although the AA does not build up in the presence of Na$^+$, it does in the presence of Cs$^+$ and gives a chemical shift of 3.72 (likely representing the closed form) at pH 7.3 with a line width of 5 Hz. The narrow line width suggests a more freely rotating 5′-phosphate in this enzyme form. The open to closed transition is concomitant with formation of salt bridges between βK167 and αD56 and between βD305 and βR141.

The exchange rates can be calculated using the line width in hertz and the resonance frequency of the two conformers according to

$$\Delta\nu = \pi(\nu_A - \nu_B)^2/2k \qquad (3)$$

where $\Delta\nu$ is the line width in hertz, ν_A and ν_B are resonance frequencies for the species in the exchange reaction, and *k* is the first-order rate constant. In the presence of Na$^+$, the rate constant for interconversion of the two conformers of the ISB at pH 7.3 is 95 s^{-1}, while the rate increases to ∼670 s^{-1} at pH 9.5, consistent with the increase in k_{cat}/K_{serine} as the pH is increased. On the other hand, Cs$^+$ stabilizes the closed conformation, and at high pH, only the resonance at 3.72 is observed with the one at 4.1 absent. Data are consistent with the pH-dependent conformational change discussed above, controlled by the ionization state of the enzyme group with a pK_a of ∼8 (estimated as the pH that would give a rate constant of 335 s^{-1}, half of the value observed at pH 9.5).

pH Dependence of Isotope Effects. The pH dependence of ^DV and $^D(V/K_{serine})$ has previously been measured, and the data provide insight into the mechanism of the β-reaction and its interplay with the α-reaction of tryptophan synthase (*26*). The measured deuterium isotope effects, ^DV and $^D(V/K_{serine})$, decrease from a value of ∼3.5 at pH 6 to a value of unity at high pH (>9). In addition, although the apparent pK_a values obtained from these studies, 8.6 for ^DV and 7.6 for $^D(V/K_{serine})$, are close to values observed in the pH−rate profiles, they do not reflect the same groups (see below) in contrast to the interpretation in the literature.

Theory for the pH dependence of isotope effects has been developed for cases in which the isotope- and pH-dependent steps are the same (*36*) and not the same (*35*). In the case where the pH- and isotope-dependent steps are the same (assuming a bell-shaped pH−rate profile as for tryptophan synthase), the isotope effects on V and V/K will be lowest at the pH-independent region of the profile (highest value of the parameter). The isotope effects will increase as the pH is decreased below or increased above the observed pK_a's and will eventually become equal to one another. This would, at first glance, appear to be the case for tryptophan synthase, given the equal isotope effects on the two parameters at low pH. However, as the pH is increased above the pK on the basic side of the pH−rate profiles, the isotope effect on V and V/K_{serine} continues to decrease to a value of unity. Data are consistent only with a mechanism in which the pH- and isotope-dependent steps differ, and given the limiting value of unity at high pH, this suggests the pH-dependent step precedes the isotope-dependent step. A likely possibility for the pH-dependent step is a pH-dependent structural change accompanied by deprotonation of one or more enzyme side chains. A reasonable possibility, as suggested above, is formation of the salt bridges required to close, at least partially, the β-site and perhaps properly orient βK87 and βE109 for the subsequent proton abstraction and elimination of water from the serine ESB.

The mechanism, including the pH-dependent conformational change prior to formation of the quinonoid intermediate, is shown in Scheme 1. On the basis of the mechanism, the following equations for kinetic parameters and deuterium isotope effects are obtained:

$$V = \{k_7/[1 + (H/K_2)(1 + k_4/k_3)]\}/(1 + \{k_7[(1/k_5)(1 + k_4/k_3) + 1/k_3 + (1/k_9)(1 + k_{10}/k_{11}) + 1/k_{11}]\}/[1 + (H/K_2)(1 + k_4/k_3)] + (k_8/k_9)(1 + k_{10}/k_{11})) \quad (4)$$

$$V/K_{serine} = [(k_1k_3k_5k_7A)/(k_2k_4k_6H)]/((1 + H/K_1)\{1 + (k_7/k_6H)[1 + (k_5/k_4)(1 + k_3/k_2)] + (k_8/k_9)(1 + k_{10}/k_{11})\}) \quad (5)$$

$$^DV = (^Dk_7 + \{k_7[(1/k_5)(1 + k_4/k_3) + 1/k_3 + (1/k_9)(1 + k_{10}/k_{11}) + 1/k_{11}]\}/[1 + (H/K_2)(1 + k_4/k_3)] + {}^DK_{eq}(k_8/k_9)(1 + k_{10}/k_{11}))/(1 + \{k_7[(1/k_5)(1 + k_4/k_3) + 1/k_3 + (1/k_9)(1 + k_{10}/k_{11}) + 1/k_{11}]\}/[1 + (H/K_2)(1 + k_4/k_3)] + (k_8/k_9)(1 + k_{10}/k_{11})) \quad (6)$$

$$^D(V/K_{serine}) = \{^Dk_7 + (k_7/k_6H)[1 + (k_5/k_4)(1 + k_3/k_2)] + {}^DK_{eq}(k_8/k_9)(1 + k_{10}/k_{11})\}/\{1 + (k_7/k_6H)[1 + (k_5/k_4)(1 + k_3/k_2)] + (k_8/k_9)(1 + k_{10}/k_{11})\} \quad (7)$$

In eqs 4 and 6, K_2 is equal to k_5/k_6, while K_1 likely reflects the group with a pK_a of ∼7.3 that must be unprotonated for optimal binding of L-serine. Equations 6 and 7 adhere to the general equations for isotope effect: $^DV = [^Dk_7 + c_{Vf} + {}^DK_{eq}(c_r)]/(1 + c_{Vf} + c_r)$ and $^D(V/K_{serine}) = [^Dk_7 + c_f + {}^DK_{eq}(c_r)]/(1 + c_f + c_r)$, respectively. The isotope effects on V and V/K_{serine} change from a value of 1 at high pH as c_f and c_{Vf} approach infinity as a result of the irreversibility of the deprotonation of βD305 prior to ESB formation to a value of $[^Dk_7 + {}^DK_{eq}(c_r)]/(1 + c_r)$ at low pH where the amount of the correctly protonated ESB is very small (the protonation state of βD305 may be the group that helps to determines the open and closed states of the enzyme). Assuming the value of ∼6 is the intrinsic deuterium isotope effect, the value of c_r at low pH can be calculated in the absence and presence of MVC.

Interpretation of Data Obtained in These Studies

pH Dependence of Kinetic Parameters. The pH range covered in these studies, 6.5−8.5, would really only define the pK_a of ∼7.3 in the V/K_{serine} pH−rate profile, although data in the presence of Na$^+$ in Table 1 are consistent with the published data since the values at 6.5 and 8.5 are significantly lower than that measured at 7.8. The pK_a of 7.5−8 observed in the V/K_{serine} pH−rate profile is in the same range as the one around 7.3 (*26*), especially given the limited data obtained in this study. A pK_a of ∼7.5 is observed in the presence of all MVC and likely reflects the pH-dependent conformational change between open and closed forms of the enzyme.

As stated in the Results, the kinetic parameters obtained in the presteady state, monitoring the decay of the external aldimine, are in good agreement with those obtained previously (*12*, *24*, *39*, *41*). With the exception of the enzyme in the presence of Cs$^+$, the pH dependence of k_1 exhibits two pK_a values. In the absence or presence of MVC, the pK_a observed on the basic side of the profile is ∼9, while that on the acid side varies with monovalent cation. The value is ∼6.4 in the absence of MVC and 5.8 and 5.9 in the presence of Na$^+$ and K$^+$, respectively.

Since it is the decay of the external aldimine that is monitored in these studies, the species involved are the external aldimines (II and III in Scheme 1), the quinonoid intermediate (IV in Scheme 1), and the α-aminoacrylate intermediate (V in Scheme 1). Decay of species II and III requires, according to Scheme 1, the unprotonated form of βK87 and the protonated form of βE109. The pK_a's observed in the pH−rate profiles for decay of the external Schiff bases, k_1 (*24*), thus likely reflect these groups in reverse protonation state; that is, although the lysine must be unprotonated, its pK is seen on the basic side of the profile, while the pK for the glutamate, which must be protonated, is seen on the acid side of the profile. The variation in the pK_a for βE109 likely reflects local environmental changes caused by occupancy of the MVC site. The exception to the above is Cs$^+$, for which a pK_a of only ∼9 is observed, and this will be discussed below under isotope effects.

Steady-State Kinetic Isotopic Effects. Interpretation of the isotope effects on V and V/K_{serine} is based on the mechanism shown in Scheme 1. The macroscopic rate constant V/K_{serine} includes all steps from binding serine to formation of the AA, while V includes any isomerization of the enzyme prior to serine binding and all steps from the E−serine Michaelis complex to regeneration of free enzyme (this includes steps in the second half of the reaction). A value of ^DV that is lower than $^D(V/K_{serine})$ thus reflects limitation by isomerization of free enzyme and/or steps in the second half of the reaction.

Isotope Effect on V/K$_{serine}$. In all cases, isotope effects are pH-dependent and decrease in magnitude as the pH is increased (the exception is in the presence of Cs^+ where there is no significant isotope effect). Data are roughly in agreement with those obtained previously in the presence of Na^+ (*26*) and in agreement with the proposed pH-dependent conformational change that precedes chemistry. However, the decrease in the magnitude of the deuterium isotope effect is less pronounced in the present case than in the results reported previously with ^{D}V and $^{D}(V/K)$ decreasing from 3.5 at low pH to unity above pH 9 (*26*). We find higher values of the isotope effects at low pH [~4.9 and ~5.7 for ^{D}V and $^{D}(V/K)$, respectively], which decrease to 4.1 and 2.6, respectively, at pH 8.5. The difference in pH dependence observed in this study and the previous study is likely due to the difference in the temperature used to measure the primary isotope effect, 37 °C (*26*), versus 20 °C in this study. Data suggest a perturbation of the pK_a derived from the pH dependence of $^{D}(V/K_{serine})$ from the value of ~7.6 observed at 37 °C to ~8.2 at 20 °C, while data for ^{D}V suggest an increase in the observed pK_a from 8.6 to ~9.3; the estimated ΔpK_a for ^{D}V and $^{D}(V/K)$ is 0.6−0.7. To test this hypothesis, the isotope effects on V and V/K_{serine} were measured at pH 9.4 in the presence of Na^+ and were 3.8 and 1.8 (data not shown), respectively, consistent with the pK_a of 8.2 calculated above. The calculated pK_a values should be compared to the value of 8 estimated for the pH dependence of the equilibrium between conformers observed via ^{31}P NMR (see above). Thus, there is almost certainly a temperature dependence to the contribution of C_α proton abstraction to rate limitation. Indeed, the primary kinetic isotope effect on V in the presence of NaCl is temperature-dependent and decreases from 5.5 at 5 °C to 1.5 at 45 °C (*51*). Data obtained in these studies are consistent with these values, within error.

The largest isotope effects for the tryptophan synthase β-reaction are observed in the presence of Na^+ with a maximal value of 5.7 at pH 6.5. Using this as the intrinsic isotope effect and assuming no change in transition-state structure for C_α proton abstraction, one can obtain an estimate of the contribution to rate limitation of the bond-breaking step in the absence of MVC and in the presence of K^+ (this step does not contribute in the presence of Cs^+). An isotope effect of 1 indicates no contribution (no effect of isotopic substitution), and thus, the ratio of the isotope effects minus 1 provides a rough estimate of the fractional contribution of C_α proton abstraction. At pH 6.5, where the maximal isotope effect is observed, the fractional contribution is 0.23 ± 0.02 $[(2.1 - 1)/(5.7 - 1)]$ in the absence of MVC and 0.53 ± 0.04 $[(3.5 - 1)/(5.7 - 1)]$ in the presence of K^+.

The magnitude of the observed isotope effect varies dramatically with the monovalent cation present. In the absence of MVC, the maximum isotope effect is ~2 (pH 6.5), suggesting the contribution of steps other than C_α proton abstraction to rate limitation. On the other hand, MVC that stabilize the external aldimine, Na^+ and K^+ to differing extents, give the largest primary isotope effects, 5.7 and 3.5, respectively. These MVC also give the largest increase in V/K with values nearly 1 order of magnitude higher than in the absence of MVC (corrected to k_{cat} as discussed in the Results) (Table 1). However, as indicated above, these effects are pH-dependent, and to provide an overall interpretation, we will first consider data obtained with Na^+. The steady-state deuterium isotope effect on V/K_{serine} indicates that C_α proton abstraction completely limits the β-reaction at pH 6.5, but the effect decreases as the pH increases and the closed form of the enzyme is stabilized, committing the reaction to proceed to the AA, consistent with the interpretation provided above. The decrease in the observed isotope effect as the pH is increased is a result of the increase in the c_{Vf} and c_f terms in eqs 6 and 7. Specifically, the k_6H term decreases compared to C_α proton abstraction, committing the reaction to proceed toward products. The large isotope effect at pH 6.5 thus largely reflects rate-limiting catalysis beginning with the open form of the enzyme. The decrease in the isotope effect as the pH increases is observed in the presence of all MVC.

In the absence of MVC or presence of other MVC, the observed isotope effect is smaller. Data suggest either a change in the intrinsic isotope effect reflecting a change in transition-state structure or the contribution of steps other than C_α proton abstraction to rate limitation. We consider a change in transition-state structure unlikely and believe the smaller isotope effects reflect kinetic complexity. In the presence of K^+ and Cs^+, $^{D}(V/K_{serine})$ is 3.5 and at most 1.5 at low pH, suggesting an increasing contribution from the conformational change to close the active site, with this step being almost completely rate-limiting in the presence of Cs^+. Another possibility for the difference in $^{D}(V/K_{serine})$ at pH 6.5 is a change in the reverse commitment factor, $(k_8/k_9)(1 + k_{10}/k_{11}\text{indole})$. The term that contains indole is unlikely to contribute since binding of indole to AA should be rapid at saturating substrate levels. It is possible, but not likely, that the equilibrium between Q and AA may change depending on the MVC present, since different MVC stabilize different species. Stabilization of the different species is a thermodynamic phenomenon and does not necessarily reflect the kinetics of the reaction, but a contribution by c_r cannot be completely ruled out. It is more likely the structural change to close the site is regulated differentially by the different MVC.

Isotope Effect on V. The isotope effect on V in the presence of Cs^+ is near unity which is also observed on V/K_{serine}, suggesting a step common to V and V/K_{serine}, likely the rate of closure of the active site prior to the catalytic steps; pre-steady-state kinetic isotope effects are consistent with this interpretation (see below). On the other hand, values of ^{D}V measured in the presence of Na^+ and K^+ are large, 4.9 and 2.4 at pH 6.5, respectively. Differences in the values at pH 6.5 can be interpreted on the same basis as that used for $^{D}(V/K_{serine})$ above. However, for values of ^{D}V smaller than $^{D}(V/K_{serine})$, data suggest there is rate limitation by isomerization of free enzyme and/or steps in the second half of the reaction at saturating L-serine concentrations. Recently, Cash et al. (*52*) showed that, at pH 8 and 25 °C, deprotonation of the indolenine quinonoid intermediate is rate-limiting for formation of the quinonoid in the absence and presence of MVC. In addition, release of the product tryptophan is slow at pH >7.8 (*21*).

Pre-Steady-State Kinetic Isotope Effects. It is informative to measure isotope effects in the presteady state since one has the possibility of measuring intrinsic isotope effects on elementary steps if they are kinetically isolated. In the case of tryptophan synthase, however, it is clear from the discussion given above that in most cases single elementary steps do not limit the overall reaction. As a result, an analysis of time courses monitoring the appearance and decay of each of the intermediates along the reaction pathway would have to be carried out to estimate values for the intrinsic isotope effects. These experiments are not possible in this case since a number of the intermediates appear and decay too rapidly (for example, *gem*-diamine intermediates) and/or are spectrally invisible (active site closure). As a result, data would be difficult to interpret. However, the combination of steady-state and pre-steady-state isotope effects allows an accurate, at least qualitative, interpretation of the data.

The isotope effect on the rate constant for decay of the ESB, k_1, in the absence of MVC is 3.1−3.6, and larger than the steady-state effect of ∼1.6−2 on V/K_{serine} (it is also greater than the apparent ${}^{D}V$, but this isotope effect likely reflects slow steps in the second half of the reaction). ^{31}P NMR spectra indicate the β-active site in the internal aldimine exists as a mixture of open and closed forms that interconvert slowly at pH 7.3. Data thus suggest that only the open form is observed in the presteady state and the conversion of the closed to the open form likely contributes to the steady-state rate giving a smaller deuterium isotope effect. The isotope effect of 3.1−3.6 in the presteady state is still lower than the maximal effect of 5.7 observed in the presence of Na$^+$, consistent with a contribution from closure of the active site prior to formation of the ESB and subsequently the AA. This explanation is also likely in the presence of K$^+$. The isotope effect on k_1 is 3.3−4.2, greater than the values of 2.9−3.3 for ${}^{D}V$ (the higher value observed at pH 8.5, and the lower value observed at pH 6.5).

Data can be compared with those obtained in the presence of Cs$^+$, which gives a small isotope effect of ∼1.4. The internal aldimine also exists as two conformers in the presence of Cs$^+$ that interconvert slowly. Unlike the case in the absence of MVC, the value of the steady-state isotope effect is also small, 1−1.4. Data suggest a rate-limiting closure of the active site at limiting and saturating serine concentrations (see above). The presteady state thus reflects slow closure of the active site followed by rapid generation of the ESB and AA.

The isotope effects on k_1 in the presence of Na$^+$ are 2.1−2.7. These values are lower than the measured values of ${}^{D}V$, 4.9−4.1. As discussed above (see the discussion of ^{31}P NMR data), the first-order rate constant for interconversion of open and closed forms of the enzyme is faster in the presence of Na$^+$ than in the absence of MVC or in the presence of Cs$^+$ or K$^+$. The most likely explanation of the decrease in the isotope effect on k_1 compared to that on ${}^{D}V$ is a contribution of the rate of interconversion of the open and closed form in the presteady state, resulting in a smaller observed isotope effect. In the steady state, this rate process is not observed at saturating L-serine levels, suggesting the enzyme stays in the active conformation, being trapped by high substrate levels as the ESB before it can relax slowly to the less active conformation.

Isotope effects on microscopic rate constants have been obtained previously by fitting the dependence of the relaxation rate constant for the decay of the ESB on the concentration of L-serine in the absence and presence of Na$^+$ to a simplified three-step mechanism with rapid formation of the Michaelis complex and rapid conversion to the ESB, followed by formation of the AA (*39*). According to relaxation kinetic theory, data gave a kinetic isotope effect of 5.91 on the rate constant for decay of the ESB in the presence of Na$^+$ and 3.96 in the absence of MVC for the system of three equilibrating steps according to Bernasconi with the assumption that the first step equilibrates much faster than the second and third steps. The value of 5.91 is identical within error to the value of 5.97 for ${}^{D}(V/K_{\text{serine}})$ obtained in these studies at pH 6.5. However, as discussed below, with the exception of the value of 5.97, observed values are not necessarily intrinsic. Single-wavelength stopped-flow time courses (*39*) monitoring the appearance of the AA at 350 nm, with D,L-serine and [α-^{2}H,β-^{2}H$_2$]-D,L-serine in the absence of MVC or in the presence of Na$^+$, gave isotope effects of 3 and 2.2, respectively, in agreement with the effects measured in these studies monitoring decay of the ESB.

Rapid-scanning stopped-flow studies with L-serine and [α-^{2}H]-L-serine showed that the primary kinetic isotopic effect on C$_\alpha$ proton abstraction alters the time course for the approach to the equilibrium between the ESB and AA, resulting in a greater transient accumulation of the ESB (*42*).

Conclusions

On the basis of a re-interpretation of existing literature data and the data obtained in these studies, a number of conclusions can be drawn and are outlined below.

(1) On the basis of ^{31}P NMR, the β-subunit exists as two conformers in slow equilibrium in the absence of substrate on the NMR time scale. An exception is noted in the presence of Na$^+$, which brings the equilibrium into the intermediate time scale at pH 7.3 and into the fast time scale at pH 9.5. Data translate into a pH-dependent rate of isomerization that ranges from 670 s^{-1} at pH 9.5 to 95 s^{-1} at pH 7.3, allowing calculation of a pK of ∼8 for a group that must be unprotonated to facilitate conversion of the enzyme to the active open state.

(2) The pH dependence of kinetic parameters reveals the presence of two groups that must be unprotonated and one that must be protonated in the V/K_{serine} pH−rate profile. In the presence of Na$^+$, a group with a pK_a of 6.5 must be unprotonated to accept a proton from the α-amine of L-serine (likely βE109) and a group with a pK_a of ∼9 is attributed to the α-amine of L-serine, which must be protonated to bind to enzyme. The third pK_a is 7.3 and is attributed to a group that must be unprotonated to facilitate active site closure (see the previous paragraph). The V profile exhibits pK_a values of 6.5 and 8.2, attributed to groups which must be unprotonated and protonated, respectively. The group with a pK_a of 6.5 is likely the same one observed in the V/K_{serine} pH−rate profile, while the group with a pK_a of 8.2 likely reflects βK87 in the second half-reaction, which must be protonated to form the tryptophan ESB.

(3) The pH dependence of deuterium isotope effects is indicative of a mechanism in which a pH-dependent step precedes the chemical steps. As a result, as the pH increases, the reaction becomes more committed to proceed to products, which causes the deuterium isotope effect to decrease to a value of unity at high pH. The pH-dependent step is likely associated with the conformational change required to close the site as a result of formation of one or more salt bridges.

(4) The primary kinetic deuterium isotope effect on V/K_{serine} in the presence of Na$^+$ is likely the intrinsic isotope effect, while in the absence of MVC or in the presence of

K^+, active site closure contributes to rate limitation. In the presence of Cs^+, closure of the active site completely limits the reaction. The isotope effect on V, in all cases, indicates a contribution from steps in the indole half of the reaction to rate limitation.

(5) In the presence of Na^+, the steady-state isotope effect on V is greater than that on the pre-steady-state rate constant for decay of the ESB. Data further suggest that the rate of conversion of the two free enzyme conformers contributes to the pre-steady-state rate, but not the steady-state rate because the high serine concentration traps the enzyme in the active E−serine complex before it can decay to the less active form.

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