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Kinetic Studies of Tryptophan Synthetase. Interaction of Substrates with the B Subunit*

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ABSTRACT: The synthesis of L-tryptophan from indole and L-serine, as catalyzed by the B subunit of *Escherichia coli* tryptophan synthetase, has been studied with steady-state and rapid-reaction kinetic techniques. Initial velocity measurements of the reaction have been made utilizing the absorption difference between indole and tryptophan at 289 nm. The results were consistent with both a compulsory sequence of substrate addition and with a random, rapid equilibration between enzyme and substrates. Dissociation constants and/or Michaelis constants for serine and indole were obtained. Temperature-jump, stopped-flow, and combined stopped-flow–temperature-jump measurements made on solutions of B protein combined with indole, L-serine, L-tryptophan, and indole plus L-serine reveal the following mechanistic features. (1) The enzyme exists in two rapidly interconvertible forms; (2) the binding of L-tryptophan can be described as a simple bimolecular reaction; (3) the L-serine binds rapidly and the complex formed undergoes an additional, relatively slow, isomerization which is independent of the absence or presence of indole; (4) only a single first-order (rate-limiting) process can be detected on interaction of the enzyme–serine complex with indole. The mechanistic implications of these findings are considered.

Tryptophan synthetase isolated from *Escherichia coli* is a multisubunit enzyme with two types of subunits, commonly designated as α and β (Crawford and Yanofsky, 1958). The fully associated enzyme complex has the composition $\alpha_2\beta_2$ (Goldberg *et al.*, 1966) and can be readily dissociated into two α subunits (A protein) and a β_2 dimer (B protein) (Wilson and Crawford, 1964; Hathaway *et al.*, 1969). The overall reaction catalyzed by tryptophan synthetase is

$$\text{InGP} + \text{Ser} \rightleftharpoons \text{Trp} + \text{GP} \quad (1)$$

where InGP is indole-3-glycerol phosphate, Ser is L-serine, Trp is L-tryptophan, and GP is glyceraldehyde 3-phosphate. Neither the A nor B protein will catalyze this reaction (*cf.* Crawford and Yanofsky, 1958). Instead the A protein catalyzes the reaction

$$\text{InGP} \rightleftharpoons \text{In} + \text{GP} \quad (2)$$

and the B protein catalyzes the reaction

$$\text{In} + \text{Ser} \rightleftharpoons \text{Trp} \quad (3)$$

with pyridoxal 5′-phosphate being a required cofactor for the reaction given in eq 3. The sum of eq 2 and 3 is eq 1; therefore tryptophan synthetase is a simple example of an organized enzyme system. Moreover addition of A protein to B protein enhances the catalysis of eq 3 by a factor of 30–40 (Wilson and Crawford, 1965), and the addition of B to A enhances the catalysis of eq 2 by a factor of 50–100 (Hatanaka *et al.*, 1962). An understanding of the catalytic mechanism for tryptophan synthetase and of the role the subunit interactions play in the catalysis may provide a basis for consideration of the many complex organized enzymes found in biological systems.

This work reports a detailed kinetic study, using steady-state and rapid-reaction techniques, of the synthesis of L-tryptophan from L-serine and indole (eq 3) by the B protein. The results obtained establish the following mechanistic features. (1) The enzyme exists in two rapidly interconvertible conformational states. (2) The binding of L-tryptophan can be described as a simple bimolecular reaction. (3) The bimolecular reaction of enzyme and serine is too fast to study; however the enzyme–serine complex can isomerize in a manner similar to that of the free enzyme, and the bimolecular reaction is followed by another, relatively slow, isomerization of the enzyme–serine complex. (4) Only a single first-order (rate-limiting) process can be detected on interaction of the enzyme–serine complex with indole. The mechanistic implications of these findings are discussed.

Experimental Section

Materials. The B protein of tryptophan synthetase was purified from a mutant of *E. coli* K-12 (A2/F′ A2), kindly

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supplied to us by Dr. C. Yanofsky and Dr. L. Soll. Some of the bacteria were grown by Mr. A. Tannahill of the Department of Chemical Engineering, Cornell University, and some were supplied by the New England Enzyme Center. The preparative procedure used was a scaled-up version of that described by Wilson and Crawford (1965). Typically, 500 g of bacterial paste was used. After the second heat step, denatured protein was removed by centrifugation at 20,000*g* for 20 min (at 1–3°) followed by Millipore filtration (type HA, 0.45 μ mean pore size). The enzyme was divided into batches of 50–75 mg and quick frozen at −70° in a methanol–Dry Ice bath. Frozen enzyme was stored in the dark at −15°, and showed no loss of activity for at least 2 months.

The enzyme was prepared for experiments by dialyzing against 1 l. of 0.1 M phosphate buffer containing 1×10^{-5} M pyridoxal 5′-phosphate for 9 hr, with two additional buffer changes, at 2–5°. Enzyme prepared in this manner was used within a period of several hours, since the protein denatures quite rapidly in the absence of 2-mercaptoethanol. The 2-mercaptoethanol was removed for the kinetic studies since it is well known that carbonyl compounds and mercaptans react to form hemithioacetals (Lienhard and Jencks, 1966), which interfere with the study of the enzymatic processes. Furthermore, Goldberg and Baldwin (1967) have shown that A and B protein, L-serine, and 2-mercaptoethanol undergo a reaction to form *S*-hydroxyethyl-L-cysteine.

The L-serine, indole, L-tryptophan, and pyridoxal 5′-phosphate were purchased from Calbiochem. All other materials used were standard reagent grade.

Methods. Protein was estimated by the method of Lowry *et al.* (1951), using human serum albumin as a standard, and multiplying the absorbancy of the B protein by 0.91 to reduce it to an equivalent weight of albumin (Wilson, 1965).

The activity of the enzyme was measured by the method of Smith and Yanofsky (1962). One unit of activity is defined as the conversion of 0.1 μmole of indole into L-tryptophan in 20 min at 37°. The specific activity of the B protein alone was 45–50 units/mg. The specific activity of the B protein in the presence of a threefold excess of A protein was 1600–2000 units/mg.

Initial velocity measurements were made utilizing the difference in absorption between indole and L-tryptophan at 289 nm. A Cary 14 recording spectrophotometer equipped with a 0.0–0.1 absorbancy slidewire and thermostated at 24.5° (±0.5) was used. Each experiment was performed at least in triplicate. The procedure used was as follows: 1.00 ml of solution containing indole, L-serine, 1×10^{-5} M pyridoxal 5′-phosphate, 0.1 M phosphate buffer, pH 7.80, was pipetted into the cuvets; 5 μl of enzyme stock solution was transferred into the sample cell with a micropipet and the absorbance change was recorded at a constant slit width of 0.2 mm. The absorbancy change was recorded for 3 min and the initial velocity was calculated using an experimentally determined difference extinction coefficient of 1.85×10^3 M^{-1} cm^{-1}, and assuming a molecular weight of 45,000 per monomeric B subunit (Hathaway *et al.*, 1969).

A Durrum–Gibson stopped-flow spectrophotometer with a dead time of 3.6 ± 0.8 msec (Hammes and Haslam, 1968) was used for stopped-flow measurements. Enzyme concentrations ranged from 3.3×10^{-5} to 6.7×10^{-5} M.

A combined stopped-flow–temperature-jump apparatus (Faeder, 1970) requiring 0.15 ml of each reactant solution for each stopped-flow–temperature-jump experiment and 0.15-ml total volume for a temperature-jump experiment was used in these studies. The apparatus produced a 7.5° temperature rise with a heating time constant of about 8 μsec, and had a mixing time of about 5 msec when used as a stopped-flow apparatus (Faeder, 1970). Since L-serine is known to undergo reactions other than tryptophan synthesis with B protein (Crawford and Ito, 1964), the temperature jump was applied within 3 min after mixing. The experimental results were independent of the time interval between mixing and application of the jump in the time range 10 msec–3 min. The relaxation times were calculated by semilogarithmic plots of the signal amplitude *vs.* time. The relaxation times reported are averages of at least 4–8 experiments and have an uncertainty of ±10–15%. Enzyme concentrations used in these experiments varied between 4.4×10^{-5} and 1.3×10^{-4} M.

The binding constant for L-serine to B protein was determined spectrophotometrically using a procedure analogous to that of Goldberg *et al.* (1968). A Zeiss PMQ II spectrophotometer was employed.

Results and Treatment of Data

Steady-State Experiments. Since pyridoxal 5′-phosphate is bound quite tightly to tryptophan synthetase B protein, the enzymatic reaction can be regarded as a two substrate reaction producing a single product. Both fluorescence (Goldberg *et al.*, 1968) and absorption measurements fail to detect indole binding to the enzyme in the absence of serine. On the other hand, both fluorescence and absorption studies indicate that L-serine binds to the enzyme. Therefore, a reasonable mechanism to consider is a compulsory binding sequence, with L-serine binding to form an intermediate, which then combines with indole.

$$\begin{aligned} E + Ser &\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \\ X_1 + In &\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2 \\ X_2 &\xrightarrow{k_3} Trp + E \end{aligned} \tag{4}$$

In these equations, the X_i's represent reaction intermediates. The initial steady-state velocity for this mechanism can be written as

$$\frac{(E_0)}{v} = \varphi_1 + \frac{\varphi_2}{(Ser)} + \frac{\varphi_3}{(In)} + \frac{\varphi_4}{(In)(Ser)} \tag{5}$$

where (E_0) is the total concentration of enzyme and $\varphi_1 = 1/k_3$, $\varphi_2 = 1/k_1$, $\varphi_3 = (k_{-2} + k_3)/k_2k_3$, and $\varphi_4 = \varphi_3k_{-1}/k_1$. Figure 1 shows some typical plots of $(E_0)/v$ *vs.* 1/(Ser) for fixed initial concentrations of indole. The lines were fit to the data by a method of weighted least squares (Cleland, 1967), with a weighting factor of v^4, since each series of experiments is run at constant enzyme concentration. Initial velocities were measured for permutations of nine concen-

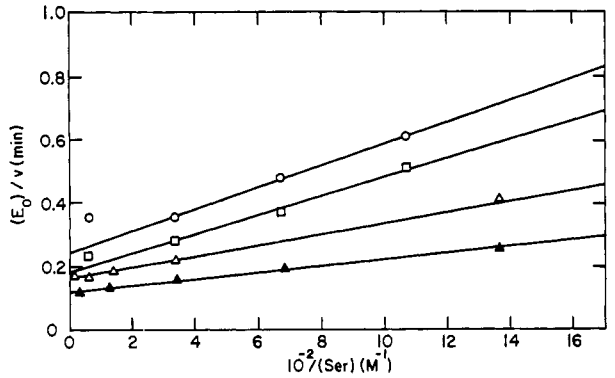


FIGURE 1: Some typical plots of the reciprocal initial steady-state velocity, $(E_0)/v$, *vs.* 1/(Ser) at fixed initial concentrations of indole: (○) 7.36×10^{-6} M, (□) 1.18×10^{-5} M, (△) 2.93×10^{-5} M, and (▲) 1.76×10^{-4} M. The concentration of enzyme, (E_0), was fixed for each concentration of indole, typically, $(E_0) = 1.63 \times 10^{-6}$ M. The solid line represents a weighted least-squares analysis of the data; experimental conditions are given in the text.

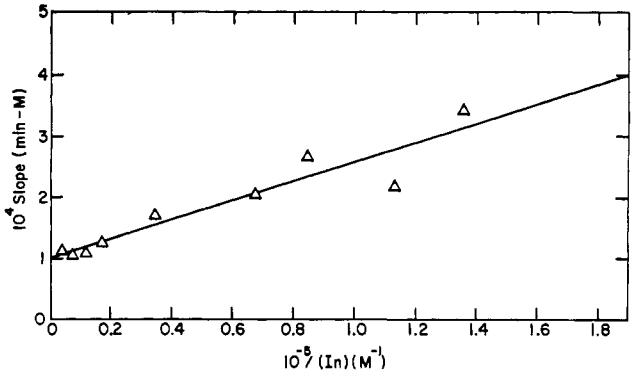


FIGURE 3: Secondary plot of the slopes of double reciprocal plots such as shown in Figure 1 *vs.* 1/(In). The solid line represents a least-squares analysis of the data.

trations of indole (7.36×10^{-6}–2.94×10^{-4} M) and five concentrations of serine (7.32×10^{-4}–2.93×10^{-2} M). Figures 2 and 3 show plots of the intercepts and slopes of the lines in Figure 1 (together with other data) *vs.* 1/(In). The values of the φ's obtained from these secondary plots are summarized in Table I. For the mechanism of eq 4, the dissociation constant for the first substrate, K_{Ser}, is φ_4/φ_3. The Michaelis constant for indole, K_{In}, is φ_3/φ_1. These quantities are also given in Table I.

A mechanism involving rapid, random equilibration between substrates and enzyme, rather than a compulsory sequence of substrate addition to the enzyme, is also consistent with the data. The rate law is identical with eq 5, but in this case φ_2/φ_1 is the dissociation constant for the reaction of serine with the enzyme–indole complex, φ_3/φ_1 is the dissociation constant for the reaction of indole with the enzyme–serine complex, and φ_4/φ_3 is the dissociation constant for the enzyme–serine complex. In this case φ_2/φ_1 (0.90×10^{-3} M) $\approx$ φ_4/φ_3 (1.24×10^{-3} M), which would indicate the binding of the two substrates is essentially independent. In principle the compulsory sequence of substrate addition and the random, rapid equilibration of enzyme and substrate can be distinguished by product inhibition experiments (Alberty, 1958). However, L-tryptophan binds relatively weakly to the enzyme ($K_{Trp} = 2.7 \times 10^{-3}$ M, Goldberg *et al.*, 1968), and its high molar extinction coefficient (4.2×10^3 M^{-1} cm^{-1} at 289 nm) prohibits product inhibition experiments with the assay employed in this work.

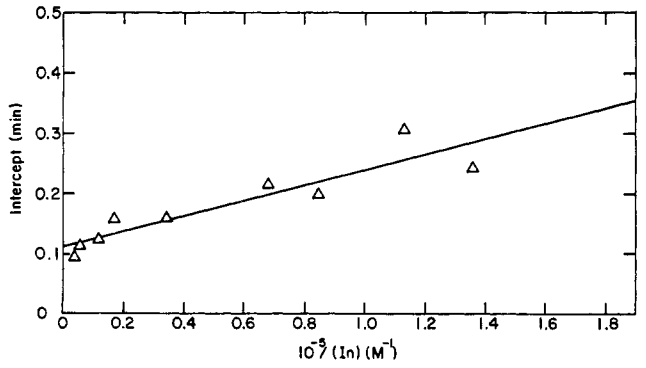


FIGURE 2: Secondary plot of the intercepts of double reciprocal plots such as shown in Figure 1 *vs.* 1/(In). The solid line represents a least-squares analysis of the data.

The value of K_{Ser} can be compared with the value of the steady-state dissociation constant, K'_{Ser}, which is obtained by difference spectroscopy. If B protein is titrated with L-serine, and the absorbance change at either 330 or 420 nm is recorded, the binding constant can be calculated from a plot of the reciprocal of the absorbance change *vs.* the reciprocal of the L-serine concentration (*cf.* Goldberg *et al.*, 1968). This does not represent a true binding constant, since L-serine is deaminated by the enzyme (Crawford and Ito, 1964). The value obtained in this study, 4.0×10^{-3} M, compares favorably with that of 4.2×10^{-3} M obtained using fluorescence measurements (Goldberg *et al.*, 1968). These values are both larger than the steady-state dissociation constant, K_{Ser}, by about a factor of 4.

Fast Reaction Experiments. A single relaxation process is observed in the temperature-jump apparatus with the enzyme itself. Identical relaxation times are measured at 420 and 330 nm (absorption maxima). The value of the reciprocal relaxation time, $1/\tau_1$, is $2.95 \pm 0.46 \times 10^3$ sec^{-1}, and is independent of the enzyme concentration from about 6.7×10^{-5} to 1.2×10^{-4} M. This suggests that the relaxation process is due to an isomerization of the free enzyme, which is known to be dimeric in this concentration range (Wilson

TABLE I: Steady-State Rate Parameters.

φ_1 (sec)	6.78
φ_2 (sec M)	6.12×10^{-3}
φ_3 (sec M)	7.62×10^{-5}
φ_4 (sec M^2)	9.42×10^{-8}
K_{Ser} (φ_4/φ_3) (M)	1.24×10^{-3}
K_{In} (φ_3/φ_1) (M)	1.12×10^{-5}
K'_{Ser} (M)[a]	4.0×10^{-3}

[a] Determined by spectral titration of B protein with serine.

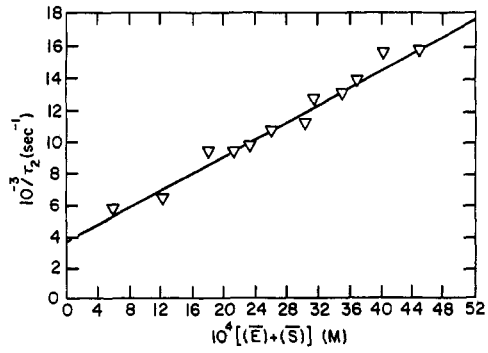


FIGURE 4: Variation of $1/\tau_2$ with the sum of the equilibrium concentrations of enzyme and L-tryptophan. The triangles represent experimental points obtained by the temperature jump method. The solid line is a theoretical line for $1/\tau_2$ calculated from eq 7 and the parameters given in Table II. The enzyme concentration in these studies ranged from 6.7×10^{-5} to 1.1×10^{-4} M.

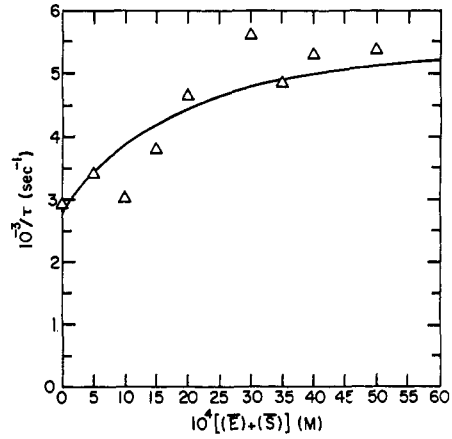


FIGURE 5: Variation of $1/\tau_3$ with L-serine concentration. The points were determined with the stopped-flow–temperature-jump method, from data taken within 3 min after mixing L-serine with enzyme. Measurements were made at wavelengths of 330 and 420 nm. The solid line is a theoretical line for $1/\tau_3$ calculated with eq 9 and the parameters given in Table II. The enzyme concentration ranged from 5.6×10^{-5} to 6.7×10^{-5} M.

and Crawford, 1964). The dimer is dissociable into monomers only with severe chemical treatments (Hathaway *et al.*, 1969).

The interaction of L-tryptophan with the enzyme was studied in the temperature-jump apparatus over a concentration range of tryptophan of 5×10^{-4} to 4.5×10^{-3} M at 420 nm. A single relaxation process was observed, and the dependence of the reciprocal relaxation time ($1/\tau_2$) on the equilibrium concentration of enzyme and substrate is shown in Figure 4. The equilibrium concentrations were calculated from the binding constant determined by fluorescence inhibition studies (Goldberg *et al.*, 1968).

The simplest mechanism consistent with the data is a simple bimolecular reaction between both isomeric forms of the free enzyme and tryptophan

$$E + Trp \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ETrp \qquad (6)$$

The associated reciprocal relaxation time, $1/\tau_2$, is

$$1/\tau_2 = k_2[(\bar{E}) + (\overline{Trp})] + k_{-2} \qquad (7)$$

where the overbars designate equilibrium concentrations. The values of the rate constants have been determined by a least squares analysis of the data and are given in Table II.

TABLE II: Rate Constants Associated with Enzyme–Substrate Reactions.

$k_1 + k_{-1}$ (sec^{-1})	2.95×10^3
k_2 (M^{-1} sec^{-1})	2.8×10^6
k_{-2} (sec^{-1})	3.7×10^3
$k_1' + k_{-1}'$ (sec^{-1})	6.0×10^3
K (M)	2.7×10^{-3},[a] 1.7×10^{-3}[b]
k_3 (sec^{-1})	13
k_{-3} (sec^{-1})	27

[a] Stopped-flow experiments. [b] Stopped-flow–temperature-jump experiments.

The equilibrium dissociation constant calculated from the data, 1.3×10^{-3} M, is in reasonable agreement with that determined from fluorescence studies, 2.7×10^{-3} M (Goldberg *et al.*, 1968). The implicit assumption has been made that the amplitude of the relaxation process associated with τ_1 is effectively reduced to zero when tryptophan is added to the enzyme. An alternative explanation for the data is that one of the isomeric forms binds tryptophan preferentially, and that the coupled relaxation times for the isomerization and tryptophan binding must be considered. One of the coupled relaxation times has the correct concentration dependence; the value of k_2 obtained is virtually identical with that given in Table II, but k_{-2} is decreased by about a factor of 5 and the ratio of the two isomeric forms of the enzyme is about 10, with the least prevalent form of the enzyme binding tryptophan preferentially. At the present time, either of the above analyses provides an adequate explanation of the data.

The interaction of tryptophan synthetase B with L-serine was studied over a concentration range of L-serine from 5×10^{-4} to 5×10^{-3} M in the stopped-flow–temperature-jump apparatus, and over a concentration range of 5×10^{-4} to 1×10^{-2} M in the stopped-flow apparatus. Two relaxation processes were detected. The faster process could only be observed with the tempreature-jump method, while the slower effect could be seen with both stopped-flow and temperature-jump methods. However, most of the data for the slower process were obtained by stopped-flow experiments. The faster process, characterized by τ_3, was observed at 420 nm and 330 nm. No effects were observed at 340 and 380 nm. The magnitude of the relaxation time was independent of wavelength; the absorbancy decreased at 420 nm and increased at 330 nm. The concentration dependence of the relaxation time is shown in Figure 5. Although some scatter occurs in the data, the reciprocal relaxation time appears to approach a limiting value at high substrate concentrations, which indicates an isomerization process is being observed. If the isomerization of the

free enzyme is considered, the simplest mechanism which can account for the data is

$$\begin{array}{ccc} E + S & \overset{K}{\rightleftharpoons} & ES \\ {\scriptstyle k_{-1}}\uparrow\downarrow{\scriptstyle k_1} & & {\scriptstyle k_{-1}'}\uparrow\downarrow{\scriptstyle k_1'} \\ E' + S & \underset{K}{\rightleftharpoons} & ES' \end{array} \qquad (8)$$

If the bimolecular steps are assumed to be rapid relative to the isomerization, the slowest relaxation time, $1/\tau_3$, is given by

$$1/\tau_3 = \frac{k_{-1} + k_1}{1 + (S)/K} + \frac{k_{-1}' + k_1'}{1 + K/(S)} \qquad (9)$$

Since $k_1 + k_{-1}$ is equal to $1/\tau_1$, two adjustable parameters (K and $k_1' + k_{-1}'$) are available to fit the data of Figure 5. The solid line in Figure 5 has been calculated with the parameters given in Table II, and describes the data quite well. Alternatively the data in Figure 5 might be construed to represent a straight line, which would imply a bimolecular reaction is being observed. However, since $\tau_1 \approx \tau_3$ and τ_3 approaches τ_1 at zero concentration of serine, the association of τ_3 with an isomerization process is much more rational.

The slower process was observed in the stopped-flow apparatus at 330, 420, and 470 nm. The absorbance increases at 420 and 470 nm, and decreases at 330 nm. The relaxation time is independent of wavelength (Figure 6), and the concentration dependence of the relaxation time is shown in Figure 6. Again, the concentration dependence suggests that an isomerization is being observed. A possible mechanism involves the formation of a new enzyme-substrate complex.

$$\left.\begin{array}{ccc} E + S & \overset{K}{\rightleftharpoons} & ES \\ {\scriptstyle k_{-1}}\uparrow\downarrow{\scriptstyle k_1} & & {\scriptstyle k_{-1}'}\uparrow\downarrow{\scriptstyle k_1'} \\ E' + S & \underset{K}{\rightleftharpoons} & ES' \end{array}\right) \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} ES'' \qquad (10)$$

(This mechanism is kinetically equivalent to a rapid bimolecular reaction followed by a relatively slow isomerization of the enzyme-substrate complex.) Since τ_3 and τ_4 are widely separated, all steps to the left of ES'' can be assumed to equilibrate relatively rapidly, and the slowest relaxation time is (*cf.* Amdur and Hammes, 1966)

$$1/\tau_4 = k_{-3} + \frac{k_3}{1 + K/[(\bar{E}) + (\bar{S})]} \qquad (11)$$

where K is the apparent dissociation constant, which is related to the overall dissociation constant, K_{Ser}, as follows.

$$K_{Ser} = \frac{[(E) + (E')](S)}{[(ES) + (ES') + (ES'')]} = \frac{K}{1 + k_3/k_{-3}}$$

The kinetic data can be fit quite well with the values of k_3, k_{-3}, and K given in Table II, and were used to calculate the curve in Figure 6. The calculated value of K_{Ser} is 0.85×10^{-3} M, which is in reasonable agreement with the steady-state value of the enzyme-serine dissociation constant, 1.2×10^{-3} M.

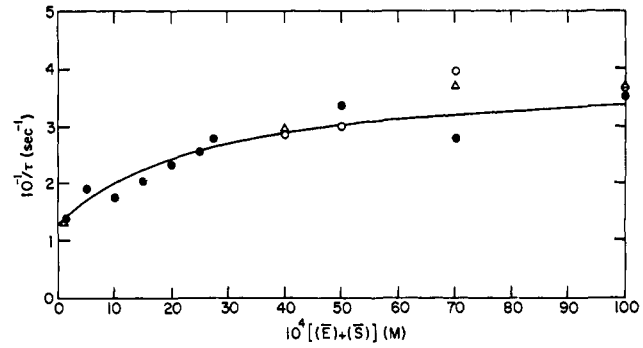


FIGURE 6: Variation of $1/\tau_4$ with L-serine concentration. The data points were obtained with the stopped-flow technique at 470 (△), 420 (●), and 330 (○) nm. The solid line has been calculated with eq 11 and the parameters given in Table II. The enzyme concentration ranged from 2.9×10^{-5} to 5.6×10^{-5} M.

In principle, the value of the equilibrium constant, K, obtained from stopped-flow and combined stopped-flow–temperature-jump experiments should be identical. However, the fits of the data shown in Figure 5 and 6 give slightly different values, 1.7×10^{-3} M *vs.* 2.7×10^{-3} M, respectively. This difference may be a reflection of experimental uncertainty, but more likely is due to the approximation made in treating the mechanism of eq 8 that the serine binding constant is identical for both E and E'. Unfortunately the data do not warrant a more complex interpretation at this time.

The relaxation spectrum of B protein was unchanged by the addition of indole over the concentration range 9.4×10^{-5}–1.7×10^{-3} M. This is consistent with the previously noted observations that no absorption or fluorescence changes can be noted when enzyme and indole are mixed, and further suggests a compulsory sequence of substrate additions.

Indole and L-serine were simultaneously mixed with enzyme and observed by stopped-flow–temperature-jump and stopped-flow methods. The L-serine concentration was fixed at 5.0×10^{-3} M, essentially saturating the enzyme, and the indole concentration varied from 5×10^{-5} to 8×10^{-4} M. The results are summarized in Tables III and IV. The relaxation time, τ_3, changes very little over the concentration range of indole employed, but differs somewhat

TABLE III: Stopped-Flow–Temperature-Jump Data for B Protein–Indole–L-Serine.[a]

Indole (M)	τ_3 (μsec)
	188 ± 21
5.13×10^{-5}	294 ± 59
1.03×10^{-4}	339 ± 80
2.05×10^{-4}	384 ± 52
4.10×10^{-4}	349 ± 43

[a] (Ser) = 5.00×10^{-3} M, (E_0) = 5.3×10^{-5} M, λ 420 nm.

TABLE IV: Stopped-Flow Data for B Protein–Indole–L-Serine.

Indole (M)	τ_4 (msec) 470 nm[a]	420 nm[b]	330 nm[a]
	31.1	29.3	31.6
2.0×10^{-5}	31.3	33.5	28.9
1.0×10^{-4}	28.5	36.1	27.2
2.0×10^{-4}	27.0	33.1	27.2
8.0×10^{-4}	27.0	26.9	24.6

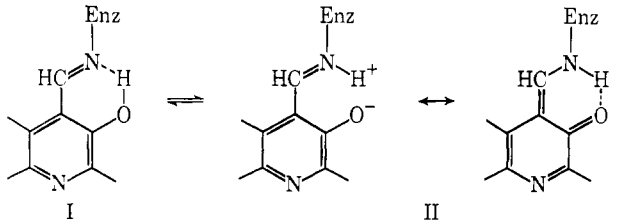
[a] (Ser) = 5.00×10^{-3} M; (E_0) = 4.2×10^{-5} M. [b] (Ser) = 1.00×10^{-2} M; (E_0) = 2.9×10^{-5} M.

from the relaxation time observed with L-serine alone. The isomerization step associated with this process is apparently influenced by the binding of indole with a mechanism similar to eq 8. The relaxation time, τ_4, is also invariant over the concentration range of indole used; moreover, it is identical with that observed with enzyme and L-serine alone. No new relaxation processes can be detected accompanying indole binding, although the overall reaction can be readily observed by stopped-flow measurements. It is very much slower than the processes discussed above, and is consistent with the turnover number obtained from the steady-state kinetics.

Discussion

A comparison of the steady-state parameters reported here with those in the literature (*cf.* Hatanaka *et al.*, 1962) is not possible since only apparent Michaelis constants have been reported at 37°. Although a compulsory sequence of substrate addition has not been proven, this mechanism seems probable and is consistent with all available data, such as independently determined binding constants. However, on the basis of the steady-state data presented here, a distinction cannot be made between a compulsory sequence of addition of substrates and a random, rapid equilibration between enzyme and substrates.

The relaxation process observed with the free enzyme can be attributed to an isomerization. This could involve conformational changes affecting the environment of pyridoxal phosphate and associated with an equilibrium between different forms of the pyridoxal phosphate. For example, a possible equilibrium might be between two different protonated Schiff bases (*cf.* Jencks, 1969)



I II

Work on model compounds (Heinert and Martell, 1963) suggests that I has an absorption maximum around 330 nm and II has one around 410 nm. This is consistent with the observed absorption spectrum. Since the actual proton transfer reactions involved in the interconversion I $\rightleftharpoons$ II would be considerably more rapid than the observed rates, some type of conformational change is probably involved.

The reaction of L-tryptophan with the B subunit appears to be due to a simple bimolecular association reaction, with possible coupling to the isomerization of the free enzyme. The observed rate constants (Table II) are similar in magnitude to those observed with other enzyme–substrate complexes (Hammes, 1968). Moreover, the kinetic results are consistent with the independently determined binding constant.

The initial association of enzyme with serine is apparently very rapid since it is not observed in these measurements. If the conservative assumption is made that the associated relaxation time is <100 μsec, then the bimolecular rate constant is $>10^7$ M^{-1} sec^{-1}. The intramolecular process observed with the free enzyme also occurs here, but with slightly altered rate constants (eq 9 and Table II). The specific processes discussed above, therefore, also apply to the enzyme–serine complex. The relatively slow process which also accompanies serine binding might be due to actual Schiff base formation or to an isomerization after the Schiff base is formed. The same relaxation time is observed at 330, 420, and 470 nm, so only a single kinetic process is being observed. The formation of ES'' probably corresponds to the formation of the "aqua complex" observed by fluorescence measurements (Goldberg *et al.*, 1968). The concentration dependence of the measured relaxation time (Figure 6) is consistent with the sequential mechanism postulated (eq 10). Moreover, the steady-state binding constants are also consistent with this mechanism. The associated spectral change (an absorbancy increase at 420 nm and a decrease at 330 nm) may be associated with exposure of the imine to a more polar environment, which would favor a dipolar structure similar to II.

No kinetic processes associated with indole binding to the enzyme–serine complex can be observed, other than the overall reaction. This implies that either the binding processes are very rapid or that significant spectral changes are not associated with the indole interactions. Indole has a very little effect on the rate processes associated with serine binding: τ_3 changes somewhat, while τ_4 is unchanged.

The results presented above indicate that the B protein and its enzyme–substrate complexes can exist in several different conformational states, and that the rates of interconversion of these states are quite rapid. Similar results have been found with many other enzymes (Hammes, 1968). The two processes which are most likely rate controlling for the overall reaction are the formation of an aminoacrylic intermediate or the addition of indole to this intermediate. An aminoacrylic intermediate has been identified with an absorption peak around 470 nm found in the presence of A and B proteins, serine, and indole (Goldberg and Baldwin, 1967), but not in the absence of A protein. [However, a similar absorption peak is observed in the presence of A and B protein, serine, and mercaptoethanol (Goldberg and Baldwin, 1967).] A general discussion of the enzymatic mechanism, including this intermediate, and the role of A protein in the mechanism will be presented in a subsequent paper.

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Purification and Properties of an Acid Nucleoside Triphosphatase from Rat Liver Mitochondria*

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ABSTRACT: A nucleoside triphosphatase with an optimal pH of 5.5 has been partially purified from rat liver mitochondria. The enzyme shows phosphohydrolytic activity toward ribo- and deoxyribonucleoside di- and triphosphates and *p*-nitrophenyl phosphate but does not hydrolyze 5′-nucleoside monophosphates or other monophosphate esters. Activity is inhibited by low levels (1 mM) of Ca^{2+}, Mg^{2+}, and Fe^{3+} and a number of other cations but is stimulated by Fe^{2+}. The activity is virtually unaffected by organic compounds such as EDTA and L(+)-tartrate.

2-Mercaptoethanol and other sulfhydryl compounds stimulate activity. Fluoride is a potent inhibitor. Evidence obtained from column chromatography, polyacrylamide disc gel electrophoresis, and a heat study suggests that the broad substrate specificity results from the action of a single enzyme.

In this paper, we report on the purification and properties of a soluble nucleoside triphosphatase from rat liver mitochondria which has a pH optimum of 5.5. Nucleoside triphosphatases with somewhat similar properties have been reported to be present in normal and regenerating rat liver nuclei (Fischer *et al.*, 1959; Siebert and Humphrey, 1965) and pea leaves (Forti *et al.*, 1962), and an acid nucleotidase has also been purified from rat liver lysosomes (Arsenis and Touster, 1968). It is also of considerable interest that chloroplasts of *Euglena gracilis* contain a Mg^{2+}-dependent ATPase with a pH optimum of 5.5, which hydrolyzes dATP more rapidly than ATP and shows activity on other nucleoside triphosphates (Carell and Kahn, 1967). Spinach chloroplasts, on the other hand, contain a similar enzyme which does not require Mg^{2+} (Young and Packer, 1966).

Experimental Procedure

Materials. Nucleotides were supplied by either Sigma or Schwarz BioResearch. Glucose 6-phosphate, glucose 1-phosphate, α- and β-glycerol phosphate, D-3-phosphoglyceric acid, phosphoenol pyruvate, NAD, NADP, *p*-nitrophenyl phos-

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