Kinetic Characterization of Channel Impaired Mutants of Tryptophan Synthase*

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Tryptophan synthase, an $\alpha_2\beta_2$ tetrameric complex, is a classic example of an enzyme that is thought to "channel" a metabolic intermediate (indole) from the active site of the $\alpha$ subunit to the active site of the $\beta$ subunit. The solution of the three-dimensional structure of the enzyme from Salmonella typhimurium provided physical evidence for a 25-Å hydrophobic tunnel which connects the $\alpha$ and $\beta$ active sites (Hyde et al., 1988). In this case, the metabolic intermediate (indole) would be transferred from the active site of the $\alpha$ subunit to the active site of the $\beta$ subunit through the connecting tunnel. Using rapid reaction kinetics, we have previously established that indole is indeed channeled and have identified three essential kinetic features which govern efficient channeling. Previous rapid kinetic analysis of tryptophan synthase has shown that the product of the $\alpha$ reaction, indole, is transferred to the $\beta$ site where it reacts with serine to form tryptophan (Anderson et al., 1991; Lane and Kirschner, 1991; Brzovic et al., 1992). The transfer of the indole intermediate is highly efficient such that indole cannot be observed in a single enzyme turnover of the $\alpha\beta$ reaction. The efficient channeling of indole is a consequence of three features of the reaction kinetics. 1) The rate of diffusion of indole through the channel is very fast; 2) the reaction of indole to form tryptophan at the $\beta$ site is fast and largely irreversible; and 3) the reaction of serine at the $\beta$ site modulates the formation of indole at the $\alpha$ site or in the tunnel such that indole is not produced until serine has reacted with pyridoxal phosphate to form the highly reactive aminoacrylate (Anderson et al., 1991). This intersubunit communication keeps the $\alpha$ and $\beta$ reactions in phase so that indole does not accumulate at the $\alpha$ site. The combination of these three aspects of the reaction kinetics leads to efficient channeling of indole by maintaining a low concentration of indole bound to the enzyme. Accordingly, this model for channeling of the indole intermediate predicts that blocking or impeding the passage between active sites might allow detection of indole during a single enzyme turnover. One of the residues lining the tunnel is $\beta$C170. Site-directed mutagenesis of the $\beta$C170 to Phe and Trp has been used to construct mutants of tryptophan synthase in which the tunnel is restricted (Schlichting et al., 1994; Ruvinov et al., 1995). In this report we describe the kinetic analysis of the $\beta$C170F and $\beta$C170W mutants by rapid reaction kinetics similar to that previously conducted for wild type tryptophan synthase (Anderson et al., 1991).

MATERIALS AND METHODS

Enzyme Purification—Tryptophan synthase $\alpha_2\beta_2$ complex from S. typhimurium was isolated and purified as described (Miles et al., 1989). The $\beta$C170F and $\beta$C170W mutants of the $\alpha_2\beta_2$ complex tryptophan synthase were produced as described previously (Ruvinov et al., 1995) and purified in a manner analogous to wild type.

Enzyme Assays—The activities of the $\alpha_2\beta_2$ complex in Reactions 1 and 2 (Table I) were measured by spectrophotometric assays at 37 °C (Creighton, 1970). Reaction 3 was measured by a direct spectrophoto-
examined by excitation at 405 nm and monitoring fluorescence at 500 nm. In most experiments an average of four runs was used for data analysis and a minimum of a 5-fold excess of the variable substrate over enzyme was used to allow analysis as a pseudo-first order rate constant. Rate constants were obtained by fitting the data to a single or double exponential by non-linear regression.

Rapid Quench Experiments—The rapid quench experiments were performed using a Kintek RF-Q-3 Rapid-Chemical Quench (Kintek Instruments). The reaction was initiated by mixing the enzyme solution (15 μl) with the radiolabeled substrates (15 μl, approximately 20,000 dpm). In all cases, the concentrations of enzyme and substrates cited in the text are those after mixing and during the enzymatic reaction. The reaction mixture was then quenched by mixing with 67 μl of 0.6 M KOH to give a final concentration of 0.2 M KOH during mixing in the rapid quench apparatus. The quenched reaction solution was collected in a 1.5-ml Eppendorf tube, vortexed, and analyzed by HPLC. The substrate and products were quantified by HPLC as described below. In order to ensure that the base was quenching the enzymatic reaction, a control was included with each experiment to insure that catalysis was being terminated. This involved adding the enzyme to a premixed solution of base and substrates. Control experiments were also done to establish the stability of the radiolabeled substrates under the quench conditions employed.

HPLC Analysis—The substrates and products were quantified by HPLC using a radioactivity detector. The HPLC separation was performed using a BDS-Hypersil-C18 reverse phase column (250 mm × 4.6 mm, Keystone Scientific, Bellefonte, PA) with a flow rate of 1 ml/min. The following gradient separation was employed where solvent A is 0.2% sodium bicarbonate and solvent B is methanol. The linear gradient program was as follows: 0–100% B in 20 min, hold at 100% B for 10 min, recycle (100–0% B in 15 min), and then re-equilibrate by holding at 100% A for 5 min. The elution times were as follows: IGP, 7 min; tryptophan, 19 min; and indole, 23 min. The HPLC effluent from the column was mixed with liquid scintillation mixture (Mono-flow V, National Diagnostics) at a flow rate of 4 ml/min. Radioactivity was monitored continuously using a Flo-One radioactivity detector (Radiomatic Instruments, Tampa, FL).

Data Analysis—The KINSIM kinetic simulation program (kindly provided by Carl Frieden and Bruce Barshop, Washington University, St. Louis, MO; Barshop et al., 1983) was used to model all of the kinetic data presented in this paper. The program was modified to allow the input of data from the rapid quench experiments as x/y pairs and to calculate the sum square errors in fitting the data (Anderson et al., 1989). The entire data set was fit to a single model and a single set of rate constants. The data were fit by a trial and error process maintaining the constraints of known dissociation constants and K_m values for IGP, G3P, indole, tryptophan, and serine (Nagata et al., 1989; Kawasaki et al., 1987; Phillips et al., 1984). Not all of the rate constants are known with equal certainty. In the α reaction the rates of IGP binding in the forward direction and of indole and G3P binding in the reverse reaction are rough estimates based upon the concentration dependence of the single turnover kinetics in each direction. The corresponding dissociation rates for IGP, indole, and G3P are then estimated from the approximate dissociation equilibrium constants. Although these constants are not known with certainty, their values do not greatly affect the fitting process or the interpretation. The remaining rate constants involving the chemical interconversion of IGP with indole and G3P were obtained as fits to the single turnover kinetics in each direction and are therefore known with more certainty. Regression analysis in the final stages of the fitting process place the limits of error on the rate constants of approximately ±20%. In the β reaction, the rates of serine reaction to form the external aldimine (E-Ser) and the aminoacrylate (E = AA) were obtained by direct fitting of stopped-flow fluorescence data as a function of serine concentration and are within ±10%. The rates of indole channeling and the reaction of indole with the aminoacrylate to form tryptophan are set at the lower limit sufficient to account for the accumulation of indole in the single turnover (Figs. 1, 3, and 6). The rate of tryptophan release is determined by the presteady state burst experiments as well as the steady state turnover rate analogous to previous analysis of wild type tryptophan synthase. The reverse rate constants governing rebound and reaction of tryptophan are set according to K_m and K_d values for the reverse α reaction for wild type tryptophan synthase (Weischt and Kirshen, 1976; Ahmed et al., 1986), although they do not affect the interpretation of the data presented in this paper.

### Table 1

Comparison of steady state turnover rates of wild type and mutant α,β complexes

| Reaction | Rate (s⁻¹) | Wild type | pH 170F | pH 170W |
|----------|------------|-----------|---------|---------|
| 1. IGP → Ind + G3P | 0.16 | 0.16 | 0.08 |
| 2. IGP + L-Ser → G3P + L-Trp + H₂O | 4.7 | 1.2 | 0.14 |
| 3. Indole + L-Ser → L-Trp + H₂O | 8 | 4.7 | 0.47 |
| 4. L-Ser → Pyr + NH₃ + H₂O | 0.2 | 0.4 | 0.8 |
| K_d for L-serine (mM) | 0.5 | 0.5 | 50 |

*a Assays at 37 °C as described under "Materials and Methods."

*b Binding constants were determined from reciprocal plots of the fluorescent increment (emission at 510 nm with excitation at 420 nm) on addition of L-serine (0.15–62 mM).

### Scheme I

**α Reaction**

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\begin{align*}
\text{IGP} & \rightarrow \text{Ind} + \text{G3P} \\
\text{β Reaction} & \rightarrow \text{L-Trp} + \text{H}_2\text{O} \\
\end{align*}
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**β Reaction**

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\begin{align*}
\text{Indole} + \text{L-Ser} & \rightarrow \text{L-Trp} + \text{H}_2\text{O} \\
\text{L-Ser} & \rightarrow \text{Pyr} + \text{NH}_3 + \text{H}_2\text{O} \\
\end{align*}
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**Ind**

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\begin{align*}
\text{G3P} & \rightarrow \text{NH}_3 \text{PO}_4 \\
\text{H}_2\text{O} & \rightarrow \text{H}_2\text{O} \\
\end{align*}
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RESULTS

The rapid kinetic analysis described in this paper leads to the pathways for the $\beta C170F$ mutant and $\beta C170W$ mutant shown in Schemes II and III, respectively. Key rate constants obtained in Schemes II and III are summarized in Table I and compared with those obtained for the wild type $\alpha\beta 2$ complex (Anderson et al., 1991). The curves shown in Figs. 1-8 were calculated by numerical integration using one set of rate constants for each mutant as summarized in Schemes II and III, with no simplifying assumptions. The rates of indole channeling and the reaction of indole with the aminoaacrylate to form tryptophan are set at the lower limit sufficient to account for the accumulation of indole in the single turnover experiments. In fitting the data, the rate constants for the reaction were further constrained by dissociation constants and $K_m$ values for IGP, G3P, serine, and tryptophan as previously determined (Nagata et al., 1989; Kawasaki et al., 1987; Phillips et al., 1984) and in Table I in this work.

Steady State Kinetic Characterization of Mutants

A comparison of steady state kinetic parameters for wild type and mutant tryptophan synthase is shown in Table I. The steady state enzyme turnover rates of the wild type and mutant $\alpha\beta 2$ complexes are compared in Reactions 1-4. This characterization was important for providing direction for an in-depth transient kinetic analysis. There were several differences which are noteworthy of mention. First, the rate of the $\alpha$ reaction (Reaction 1) in the absence of serine for the $\beta C170F$ mutant is similar to that observed for wild type (0.16 s$^{-1}$) while the rate for the $\beta C170W$ mutant is 2-fold slower (0.08 s$^{-1}$). Second, the $K_m$ for serine was similar for wild type and $\beta C170W$ mutant (0.5 mM) while the $K_m$ for the $\beta C170W$ mutant was almost 100-fold higher (50 mM). Our previous analysis with wild type trypthpan synthase has shown that the binding of indole at the $\alpha$ site and channeling to the $\beta$ site. The steady state rate of the $\alpha\beta$ reaction (Reaction 2) is enhanced 30-fold for wild type (4.7 s$^{-1}$) and 7.5-fold for the $\beta C170F$ mutant (1.2 s$^{-1}$) in the presence of serine (Table I). On the other hand, a less than 2-fold activation was noted with the $\beta C170W$ mutant (0.14 s$^{-1}$). The slower rate of enhancement, particularly in the case of the $\beta C170W$ mutant, suggests that these mutants may be impaired in intersubunit communication as well as channeling.

The transient kinetic experiments described below were designed to probe the channeling of indole and intersubunit communication in the tryptophan synthase mutants. Finally, in the absence of $\alpha$ site ligands (indole or IGP) tryptophan synthase catalyzes a side reaction, the slow hydrolysis of serine to form pyruvate, ammonia, and water (Table I, Reaction 4). The rates in Reaction 4 of the $\beta C170F$ and $\beta C170W$ mutants are 2- and 4-fold higher, respectively, than that of the wild type $\alpha\beta 2$ tryptophan synthase. Since this side reaction may complicate interpretation of results in experiments in which serine is preincubated with enzyme, some of the single turnover experiments were carried out by simultaneously mixing enzyme with a solution of IGP and serine.

Kinetic Characterization of the $\beta C170F$ Mutant

The rapid kinetic analysis of the $\beta C170F$ mutant described below leads to the kinetic pathway summarized in Scheme II and Table I. The rate constants which distinguish wild type and mutant are highlighted in boxes.

Kinetcs of a Single Turnover: The $\alpha\beta$ Reaction—We have previously found that the examination of a single enzyme turnover of the $\alpha\beta$ reaction is an important diagnostic experiment to evaluate channel impairment. One of the essential features of efficient channeling of indole is that the transfer of indole from the $\alpha$ to the $\beta$ subunit should be rapid such that no indole should accumulate during a single enzyme turnover. If passage of indole is blocked or impeded then it is likely to be observed during this type of experiment. (In this experiment, excess enzyme (20 $\mu$M) was mixed with a solution of serine (10 mM) containing a limiting concentration of $[^{14}C]$IGP (8.7 $\mu$M), and the disappearance of IGP and production $[^{14}C]$indole and tryptophan were monitored.) The observed production of indole (Fig. 1) provides the first kinetic evidence that the channeling is impaired with the $\beta C170F$ $\alpha\beta 2$ complex.

Kinetcs of Reaction of Indole from Solution—In order to establish that the indole is channelled from the $\alpha$ site to the $\beta$
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Table II

| Reaction | Rate (s⁻¹) |
|----------|------------|
| IGP → IGP-E⁺ (s⁻¹) | 0.16 | 0.16 | 0.08 |
| IGP-E⁺ → indole-G3P-E⁺ (s⁻¹) | 24 | 24 | 0.12 |
| Activation by Ser | 150-fold | 150-fold | 1.5-fold |
| Indole channeling (s⁻¹) | >1000 | 100 | 0.2 |
| E + Ser → E-Ser (µM⁻¹ s⁻¹) | 0.135 | 0.03 | 0.002 |
| E-Ser → E → AA (s⁻¹) | 45 | 12 | 12 |
| E → AA-indole → E → AA-indole (µM⁻¹ s⁻¹) | 0.03 | 0.03 |
| E → AA-indole → E* · Trp (s⁻¹) | >1000 | 250 | 500 |
| E* · Trp → E + Trp (s⁻¹) | 8 | 3 | 1 |

**a** Data from Anderson et al. (1991).
**b** Scheme II, Figs. 1-4.
**c** Scheme III, Figs. 6-8.

Figure 1. A single turnover of βC170F mutant tryptophan synthase αβ₂ complex in the αβ reaction. A solution of serine and [¹⁴C]IGP was mixed with enzyme to initiate the reaction. The final concentrations of reactants were as follows: 10 mM serine, 20 µM enzyme, and 8.77 µM [¹⁴C]IGP. The concentrations shown refer to final concentrations after mixing. The disappearance and formation of radiolabeled IGP (○), indole (▲), and tryptophan (▼) were quantified by HPLC as described under “Materials and Methods.” Each curve was calculated by computer simulation of the kinetics using the rate constants summarized in Scheme II.

Figure 2. A single turnover βC170F mutant tryptophan synthase αβ₂ complex in the β reaction. The β reaction of indole from solution: A solution of serine and enzyme was mixed with a solution of [¹³C]indole to initiate the reaction. The final concentrations were serine (10 mM), enzyme (40 µM), and [¹³C]indole (6 µM). The disappearance and formation of indole (▲) and tryptophan (▼) were monitored. B, the curves were simulated using a rate of indole binding from solution of 0.03 µM⁻¹ s⁻¹ (0.6 s⁻¹ at 20 µM enzyme). The rate of reaction of indole from solution is too slow to account for the amount of indole observed in the αβ reaction above.

However, the rate of channeling from the α to β site must have slowed from >1000 s⁻¹ in wild type to 100 s⁻¹ in order to account for the observation of any indole. Therefore, based upon this computer simulation of the reaction kinetics, we conclude that indole must pass through the channel and it must do so more slowly than in the wild type enzyme. Kinetics of the αβ Presteady State Burst Reaction—We sought to provide additional information which would discern whether the indole observed in the single turnover experiment...
reaction was quenched by the addition of 0.2M KOH (final concentrations after mixing were 20 µM serine, and 200 µM indole) to enzyme. 

The rate of the chemical reaction observed here is consistent with the rate of IGP cleavage observed in the forward αβ reaction (with serine), suggesting that only the rate of the conformational change which limits the α reaction (IGP-E ↔ IGP-E⁺) is affected by the presence of serine. 

Serine Modulates the α Reaction—In the kinetic pathway of wild type tryptophan synthase, it has been shown that a protein conformational change occurs prior to and limits the rate of IGP cleavage. The rate of tryptophan formation (in the presence of serine) is much faster than the cleavage of IGP in the absence of serine, since serine increases the rate of this conformational change occurring at the α subunit. According to this model, the enzyme is converted from a less active state to a more active state upon reaction with serine at the α site. Support for this model was provided in earlier studies with wild type enzyme by comparison of a single turnover αβ reaction in which serine is preincubated with enzyme versus the simultaneous addition of IGP and serine to enzyme. A lag in tryptophan production was observed in the experiment in which there was no preincubation, indicating that IGP cleavage at the α site is slow until serine binds to the β site. A similar comparison could not be made with the β₁₇₀F mutant since the faster rate of serine hydrolysis complicated the αβ single turnover experiment in which enzyme is preincubated with serine. Nonetheless, this serine activation step appears to be present in the kinetic pathway of the β₁₇₀F mutant as evidenced by the slow rate of IGP cleavage in the absence of serine and the much faster IGP cleavage during the single turnover of the αβ reaction in the presence of serine. Our estimated rate of IGP cleavage in the presence of serine (24 s⁻¹ in Scheme II and Table II) is 150-fold lower than the rate in the absence of serine (0.16 s⁻¹ in Scheme II and Table II). This 150-fold activation by serine is similar to that observed with the wild type enzyme (Anderson et al. (1991)) and Table II). The magnitude of this activation step is masked in the steady state comparison since product release is the rate-limiting step.

Kinetics of Serine Reaction Measured by Stopped-flow Fluorescence Methods—To further define the activation step we...
examined the kinetics of serine binding. It has previously been shown that there are absorbance and fluorescence changes exhibited by the pyridoxal phosphate upon reaction with serine and chemical nature of the serine-pyridoxal phosphate complexes have been defined (Faeder and Hammes, 1971; York, 1972; Lane and Kirschner, 1983; Lane et al., 1984; Drewe and Dunn, 1985). In the first step, serine displaces the internal aldimine formed between Lys-87 and the pyridoxal phosphate at the active site of the β subunit to form the external aldimine (E·Ser in Schemes II and III), which is highly fluorescent, with emission at 500 nm upon excitation at 405 nm. The α proton of serine is then removed to form a quinonoid species which is subsequently dehydrated to form a reactive aminoacrylate (E·AA in Schemes II and III). The electrophilic aminoacrylate is then poised to react with the incoming nucleophile, indole, in the physiological reaction to form tryptophan. If no indole is present the aminoacrylate is slowly hydrolyzed to form pyridoxal phosphate, pyruvate, and ammonia (Reaction 4 in Table I).

In the current study, we focused on measuring the rate of formation of the external aldimine and of its decay to the aminoacrylate by stopped-flow fluorescence because of the increased sensitivity of these methods. A representative fluorescence trace is shown in Fig. 4A. The data were fit to a double exponential to measure the rates of formation and decay of the external aldimine. The dependence of the rates of the fast and slow phases on serine concentration are shown in Fig. 4, B and C. The data for the faster phase (formation of the external aldimine) were fit to a straight line giving a second-order rate constant of 0.03 μM⁻¹ s⁻¹ and showed no signs of leveling off at the higher serine concentrations (Fig. 4B). The concentration dependence of the rate of the slower phase (decay to the aminoacrylate) was fit to a hyperbola which reached an observed maximum with a rate of 12 s⁻¹. Complete solution for the two-step reaction kinetics (Johnson, 1992) yielded all four rate constants for the reaction as shown below and summarized in Scheme II:

\[
E + \text{Ser} \stackrel{k_1}{\rightarrow} E \cdot \text{Ser} \stackrel{k_2}{\rightarrow} E \sim \text{AA}
\]  

(Eq. 1)

with \(k_1 = 0.03 \mu M^{-1} s^{-1}\), \(k_{-1} = 31 s^{-1}\), \(k_2 = 5 s^{-1}\), and \(k_{-2} = 7 s^{-1}\). Thus the rate of aminoacrylate formation for the βC170F mutant (12 s⁻¹) is approximately 4-fold slower than wild type (45 s⁻¹, Table II).

Changes in Protein Conformation Upon Serine Binding—Our earlier studies have indicated that the activation at the α site is mediated by a change in protein conformation. A change in intrinsic protein fluorescence was observed when wild type tryptophan synthase was mixed with serine. It was found that the rate of the protein conformational change was coincident with the rate of formation of aminoacrylate. These data suggest that the formation of the aminoacrylate at the β site elicits a protein conformational change which is transmitted to the α site where it triggers the cleavage of IGP to indole and G3P (Anderson et al., 1991). Similar results were obtained with the βC170F mutant. A representative trace illustrating the protein conformation is shown in Fig. 5A. The concentration dependence of the rate of the fluorescence change (Fig. 5B) was fit to a hyperbola giving a maximum rate of 15 s⁻¹. In conclusion, the kinetics of serine binding occur at a slower rate than wild type. The rate of the aminoacrylate formation (12 s⁻¹) is accompanied by a protein conformational change occurring at a similar rate (15 s⁻¹).

**Kinetic Characterization of the βC170W Mutant**

The effects on efficient channeling of indole proved much more pronounced with the βC170W mutation. Earlier studies on the βC170W mutant provided kinetic and structural evi-
The steady state kinetic data as well as the transient kinetic data presented below indicate that the mutation has not only impaired the channel but also has affected a/b intersubunit communication. The rapid kinetic analysis of the βC170W mutant described below leads to the kinetic pathway summarized in Scheme III and Table II. This in-depth analysis provided results in reasonable agreement with a previous preliminary report (Schlichting et al., 1994). The rate constants which distinguish the wild type from βC170W are highlighted in boxes.

Kinetics of a Single Turnover: The a/b Reaction—A single enzyme turnover of the a/b reaction for the βC170W mutant is shown in Fig. 6. In this experiment, excess enzyme (20 μM) was mixed with a solution of serine (100 mM) containing a limiting concentration of [14C]IGP (6 μM), and the disappearance of IGP and production of [14C]tryptophan were monitored. As shown in the figure, a small amount of indole is observed during the conversion of substrate to product. The rate of IGP cleavage to indole is similar in the presence (0.12 s⁻¹) or absence (0.08 s⁻¹) of serine. When the experiment was repeated at higher enzyme concentration there was no change in the rate of indole or tryptophan formation indicating that the binding of IGP is not limiting the reaction. Since the rate of IGP cleavage is so slow and there is little or no activation of the a reaction by the presence of serine, we do not know if the indole we observe is in solution or trapped in the tunnel.

Kinetics of Reaction of Indole from Solution—The rate of reaction of indole from solution was measured in a single turnover experiment using excess enzyme (20 μM) preincubated with serine (100 mM). The enzyme-serine complex was mixed with a limiting concentration of [14C]indole (2.4 μM) and the production of [14C]tryptophan was monitored. The disappearance of indole and the formation of tryptophan are shown in Fig. 7. A comparison of the time scale for Figs. 6 and 7 shows that the a/b reaction is at least 20-fold faster. The observed rate of indole reaction from solution is approximately 0.35 s⁻¹. The rate of the a/b single turnover reaction is limited by the rate of indole binding to the enzyme as confirmed by experiments performed at different enzyme concentrations (data not shown). These data provided a second-order rate constant of 0.03 μM⁻¹ s⁻¹ for the binding of indole to the enzyme. This was similar to the rate determined for the βC170F mutant and approximately 60-fold slower than wild type (Table II). Since the rate of reaction of indole from solution is similar to the rate of IGP cleavage in the single turnover experiment (0.35 s⁻¹ versus 0.12 s⁻¹) it is difficult to distinguish whether the indole we observe is in solution or is trapped in the tunnel. However, computer simulations suggest that if the channeling step is eliminated no indole would be observed. Therefore, the indole we observe in the single turnover experiment may actually be trapped in the tunnel.

Kinetics of the β Presteady State Burst Reaction—A presteady state burst experiment to examine the a/b reaction for the βC170W mutant will not be informative since the reaction will be limited by the rate of IGP cleavage to indole and therefore no burst of product will be observed. On the other hand, a presteady state analysis of the β reaction did show a burst of product formation. The time course for the forward reaction was determined by mixing a solution containing enzyme and serine (pre-mixed) with a solution containing [14C]indole to initiate the reaction. The final concentrations after mixing were 40 μM enzyme, 100 mM serine, and 200 μM [14C]Indole. At various times after mixing, the reaction was quenched by the addition of 0.2 M KOH (final concentration) and the quenched reaction mixture was then analyzed by HPLC as described under "Materials and Methods." Each curve was calculated by computer simulation of the kinetics using the rate constants summarized in Scheme III.
under “Materials and Methods.” The time dependent disappearance of indole and formation of radiolabeled tryptophan were fit to a double exponential to measure the rates of formation of indole (m) and tryptophan (c) were monitored. The curves were calculated by the numerical integration using the rate constants summarized in Scheme III.

Kinetics of the α Reaction—The examination of the α reaction for the βC170W mutant revealed a slow rate of IGP cleavage (0.08 s⁻¹) similar to βC170F and wild type (data not shown). As anticipated there was no burst of product indole formation and the rate did not increase with higher enzyme concentration indicating the binding to the enzyme was not rate-limiting. Again, these data indicate that there is a rate-limiting conformational change which must occur after the initial binding of IGP and limits the rate of the chemical reaction.

Serine Does Not Modulate the α Reaction—One of the most surprising results with the βC170W mutant was the lack of substantial activation of the α reaction in the presence of serine, especially since the βC170F mutant behaved very similar to wild type. In order to understand this lack of stimulation by serine we wanted to take a closer look at the interaction of serine with the enzyme. There are several possibilities which may have lead to the observed results. First the aminoacrylate (m) and protein communication. A solution containing enzyme (40 μM) and serine (100 mM) (pre-mixed) was added to a solution of [14C]indole (200 μM). The disappearance and formation of indole (m) and tryptophan (c) were monitored. The curves were calculated by the numerical integration using the rate constants summarized in Scheme III.

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FIG. 7. A single turnover βC170W mutant tryptophan synthase αβ2 complex in the β reaction. The β reaction of indole from solution. A solution of serine and enzyme was mixed with a solution of [14C]indole to initiate the reaction. The final concentrations were 100 mM serine, 40 μM enzyme, and 2.4 μM [14C]indole. The disappearance and formation of indole (m) and tryptophan (c) were monitored. The rates of the fast and slow phases on serine concentration was determined. The data for the faster phase (formation of the external aldimine) were fit to a straight line giving a second-order rate constant of 0.002 μM⁻¹ s⁻¹ and showed no sign of leveling off at the higher serine concentrations. The concentration dependence of the rate of the slower phase (decay to the aminoacrylate) was fit to a hyperbola which reached an observed maximum with a rate of 12 s⁻¹. Complete solution for the two-step reaction kinetics (Johnson, 1992) yielded all four rate constants for the reaction: with k₁ = 0.002 μM⁻¹ s⁻¹, k⁻¹₂ = 156 s⁻¹, k₂ = 9 s⁻¹, and k⁻¹₂ = 3 s⁻¹ as summarized in Scheme III. These results indicate that the equilibrium between external aldimine and aminoacrylate is substantially changed such that a very high concentration of serine is required to shift the equilibrium toward aminoacrylate.

Changes in Protein Conformation Upon Serine Binding—

When we examined the intrinsic protein fluorescence upon binding serine, a protein conformational change was not observed even at higher serine concentrations. It is possible that the protein conformational change necessary to activate the α reaction has now become uncoupled from the formation of the aminoacrylate. An alternate explanation involves interference or fluorescence quenching due to the proximity between the β170 and β177 tryptophans. The β177 tryptophan is located approximately 7 Å from β170 tryptophan² and thus may interfere with the detection of a change in protein fluorescence although similar effects might have been expected to occur with the phenylalanine of the βC170F mutant.

DISCUSSION

The use of rapid chemical quench-flow methods to provide a direct kinetic analysis of mutant forms of tryptophan synthase has allowed comparison with wild type and has given us an opportunity to test our current model of channeling and the kinetic consequences of the channel mutations. Several surprising results were obtained related to substrate channeling and protein communication.

According to our model for the kinetics of efficient channeling of indole, we have established as predicted that indole can be observed in a single enzyme turnover of the αβ reaction if the

² E. Gerhardt, K. S. Anderson, and I. Schlichting, unpublished observations.
rate of passage of indole and/or the rate of chemistry at the β subunit are decreased (Schemes II and III and Table II). The phenylalanine mutation slows the rate of channeling approximately 10-fold compared with wild type, whereas the tryptophan mutation reduces the rate >1000-fold. Although this difference in the two mutations may be related to the size of the obstructing residue, it could also be related indirectly to the difference in the two mutations may be related to the size of the obstructing residue. We speculate that the activation of IGP cleavage at the α site is prevented by the presence of the indole ring of bTrp-170 which may mimic the indole intermediate in the tunnel. These results are supported by our observations that a protein conformational change in intrinsic protein fluorescence is not detected with this mutant. In addition, preliminary structural analysis indicates that the C170W mutation not only restricts the tunnel but also results in the movement of residues which may be important in transmitting the activation or trigger from the β subunit to the α subunit.

For instance, in the presence of 5-fluoroindole propionol phosphate (an inhibitor of the α reaction) there is a 10-Å movement of the guanidinium group of Arg-175 from the surface of the protein in the native wild type tryptophan synthase without ligand to the αβ interface in the C170W mutant structure. The combination of kinetic results presented here as well as more complete structural analysis of this mutant may provide important clues concerning αβ intersubunit communication.

In summary, the kinetic analysis of the mutant enzymes presented in this report reaffirms the essential kinetic features required for efficient channeling. Slowing the rate of passage of indole and/or the rate of reaction of indole at the β site (Anderson et al., 1991) leads to the detection of the indole in a single enzyme turnover of the physiological reaction. Even though the C170F mutant intersubunit communication maintains coupling of the α and β reactions, the efficiency of channeling is decreased. Efforts to define the structural basis for this conformational coupling by x-ray crystallography, solid-state nmr, and site-directed mutagenesis are currently underway.

REFERENCES

Ahmed, S. A., Martin, B., and Miles, E. W. (1986) Biochemistry 25, 4233–4240
Anderson, K. S., Sikorski, J. A., and Johnson, K. A. (1988) Biochemistry 27, 7395–7406
Anderson, K. S., Miles, E. W., and Johnson, K. A. (1991) J. Biol. Chem. 266, 8020–8033
Barshop, B. A., Wrenn, R. F., and Frieden, C. (1983) Anal. Biochem. 130, 134–145
Birzovic, P., Nigo, K., and Dunn, M. F. (1992) Biochemistry 31, 8831–8839
Crawford, I. P., and Ito. J. (1964) Proc. Natl. Acad. Sci. U. S. A. 51, 390–397
Creighton, T. E. (1970) Eur. J. Biochem. 13, 1–10
Demoss, J. A. (1962) Biochim. Biophys. Acta 62, 279–293
Drew, W. F., and Dunn, M. F. (1985) Biochemistry 24, 3977–3987
Faeder, E. I., and Hannmes, G. G. (1971) Biochemistry 10, 4043–4049
Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., and Davies, D. R. (1988) J. Biol. Chem. 263, 17857–17871
Johnson, K. A. (1992) The Enzymes 20, 1–61
Kawasaki, H., Bauerle, R., Zon, G., Ahmed, S. A., and Miles, E. W. (1987) J. Biol. Chem. 262, 10678–10683
Lane, A. N., and Kirschner, K. (1983) Eur. J. Biochem. 129, 561–570
Lane, A., and Kirschner, K. (1991) Biochemistry 30, 479–484
Lane, A., Paul, C., and Kirschner, K. (1984) EMBO J. 3, 279–287
Matchett, W. H. (1974) J. Biol. Chem. 249, 4041–4049
Miles, E. W. (1979) Adv. Enzymol. 49, 127–86
Miles, E. W. (1991) Adv. Enzymol. 64, 93–172
Miles, E. W. (1995) In Subcellular Biochemistry: Proteins: Structure, Function, and Protein Engineering (Biswas, B. B., and Roy, S., eds) Vol. 24, pp. 207–254, Plenum Press, New York
Miles, E. W., Bauerle, R., and Ahmed, S. A. (1987) Methods Enzymol. 142, 398–414
Miles, E. W., Kawasaki, H., Ahmed, S. A., Morita, H., and Nagata, S. (1989) J. Biol. Chem. 264, 6228–6237
Nagata, S., Hyde, C., and Miles, E. W. (1989) J. Biol. Chem. 264, 6288–6296
Phillips, R. S., Miles, E. W., and Cohen, L. A. (1984) Biochemistry 23, 6228–6234
Racker, E., Klybas, V., and Schramm, M. (1959) J. Biol. Chem. 234, 2510
Ruvniov, S. B., Yang, X., Parris, K., Banik, U., Ahmed, A., Miles, E., and Sackett, D. (1995) J. Biol. Chem. 11, 6357–6369
Schlichting, I., Yang, W. X., Miles, E. W., Kim, A. Y., and Anderson, K. S. (1994) J. Biol. Chem. 269, 26591–26593
Weischet, W., and Kirschner, K. (1976) Eur. J. Biochem. 65, 365–373
York, S. (1972) Biochemistry 10, 2733–2740
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