Mechanism of the Physiological Reaction Catalyzed by Tryptophan Synthase from *Escherichia coli*†

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ABSTRACT: The physiological synthesis of L-tryptophan from indoleglycerol phosphate and L-serine catalyzed by the $\alpha_2\beta_2$ bienzyme complex of tryptophan synthase requires spatial and dynamic cooperation between the two distant α and β active sites. The carbanion of the adduct of L-tryptophan to pyridoxal phosphate accumulated during the steady state of the catalyzed reaction. Moreover, it was formed transiently and without a lag in single turnovers, and glyceraldehyde 3-phosphate was released only after formation of the carbanion. These and further data prove first that the affinity for indoleglycerol phosphate and its cleavage to indole in the α subunit are enhanced substantially by aminoacrylate bound to the β subunit. This indirect activation explains why the turnover number of the physiological reaction is larger than that of the indoleglycerol phosphate cleavage reaction. Second, reprotonation of nascent tryptophan carbanion is rate limiting for overall tryptophan synthesis. Third, most of the indole generated in the active site of the α subunit is transferred directly to the active site of the β subunit and only insignificant amounts pass through the solvent. Comparison of the single turnover rate constants with the known elementary rate constants of the partial reactions catalyzed by the α and β active sites suggests that the cleavage reaction rather than the transfer of indole or its condensation with aminoacrylate is rate limiting for the formation of nascent tryptophan.

Tryptophan synthase is the type case of a multienzyme complex (Crawford & Yanofsky, 1958; Miles, 1991). It is composed of two α and a tightly associated β_2 subunit and catalyzes the transfer of the indole (IND)[1] moiety from indoleglycerol phosphate (IGP) to L-serine to give L-tryptophan and glyceraldehyde 3-phosphate (GAP):

$$\text{IGP} + \text{Ser} \rightarrow \text{Trp} + \text{GAP} + \text{H}_2\text{O} \qquad \text{(AB)}$$

This physiological (or "AB") reaction consists of a sequence of the following two partial reactions. The reversible IGP lyase (or "A") reaction, which is catalyzed by the α subunit, gives IND and GAP:

$$\text{IGP} \rightleftarrows \text{IND} + \text{GAP} \qquad \text{(A)}$$

In the practically irreversible tryptophan synthase (or "B") reaction, which is catalyzed by the β subunit, indole condenses with L-serine to give tryptophan

$$\text{IND} + \text{Ser} \rightarrow \text{Trp} + \text{H}_2\text{O} \qquad \text{(B)}$$

The B reaction involves cocatalysis by pyridoxal 5′-phosphate (PLP), so that not only serine and tryptophan but also the various identified α amino acid intermediates occur as Schiff's bases with PLP.

The most interesting aspect of tryptophan synthase concerns the functional interaction between the α and β sites. First, substrates and substrate analogues bound to one site affect the events at the other (DeMoss, 1962; Lane & Kirschner, 1981, 1983a,b; Drewe & Dunn, 1986; Kawasaki et al., 1987; Dunn et al., 1987; Houben & Dunn, 1990; Kirschner et al., 1991). Second, indole does not appear as a free intermediate in solution during turnover of the AB reaction (Yanofsky, 1957), suggesting a "channelling" mechanism.

The recent elucidation of the three-dimensional structure of the $\alpha_2\beta_2$ complex from *Salmonella typhimurium* by Hyde et al. (1988) provides a new basis for explaining the large volume of diverse experimental data that has accumulated for the family of homologous microbial tryptophan synthases. In particular, the active sites of the α and β subunits are separated by about 30 Å and are connected by a tunnel that is wide enough to permit the intramolecular transfer of indole.

In this paper we use steady-state and transient kinetics of the AB reactions to derive a mechanism composed of the previously described mechanisms of both the A (Kischner et al., 1991) and the B reactions (Lane & Kirschner, 1983b). The data rule out a transfer of indole through the bulk solvent and require an acceleration of the A reaction by serine binding to the β site.

Materials and Methods

Materials. Tryptophan synthase was isolated as described (Tschopp & Kirschner, 1980). ThioNAD$^+$ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Boehringer Mannheim Corp. and used without further purification. All other reagents were of analytical grade. Unless stated otherwise, the standard buffer (buffer P) was 0.1 M potassium phosphate at pH 7.6, containing 2.5 mM EDTA, 0.2 mM dithioerythritol, and 0.04 mM pyridoxal phosphate. Buffer T was 0.1 M Tris-HCl at pH 7.8, containing the same addiitions as buffer P.

Methods. Steady-state kinetics of the AB reaction were measured as described for the A reaction (Kirschner et al.,

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; IGP, 3-indole-D-glycerol 3′-phosphate; IND, indole; GAP, D-glyceraldehyde 3-phosphate; Ser, L-serine; Trp, L-tryptophan; Trp$^-$, α-carbanion of tryptophan; Acr, α-aminoacrylate; GP, DL-α-glycerol 3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAD$^+$, nicotinamide adenine dinucleotide; NADH, reduced NAD$^+$; thioNAD$^+$, (thiocarbamoyl)pyridine adenine dinucleotide.

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Table I: Physiological and Partial Reactions of the $\alpha_2\beta_2$ Complex of Tryptophan Synthase[a]

reaction	parameter	dimension	value	ref
AB (IGP → Trp)	k_{cat}	s^{-1}	1.4	this work
	K_m^{IGP}	mM	0.069	this work
	K_m^{Ser}	mM	0.34	this work
	k_{cat}/K_m^{IGP}	$mM^{-1}\ s^{-1}$	20	this work
	k_{cat}/K_m^{Ser}	$mM^{-1}\ s^{-1}$	4.1	this work
A (IGP → IND)	k_{cat}	s^{-1}	0.067	Kirschner et al. (1991)
	K_m^{IGP}	mM	0.14	Kirschner et al. (1991)
	k_{cat}/K_m^{IGP}	$mM^{-1}\ s^{-1}$	0.48	Kirschner et al. (1991)
B (IND → Trp)	k_{cat}	s^{-1}	5.3	Lane & Kirschner (1983)
	K_m^{Ser}	mM	0.43	Lane & Kirschner (1983)
	K_m^{IND}	mM	0.013	Lane & Kirschner (1983)
	k_{cat}/K_m^{Ser}	$mM^{-1}\ s^{-1}$	12.3	Lane & Kirschner (1983)
	k_{cat}/K_m^{IND}	$mM^{-1}\ s^{-1}$	408	Lane & Kirschner (1983)

[a] Steady-state parameters valid for 0.1 M phosphate buffer, pH 7.6, 25 °C.

1991). Steady-state spectra were recorded as follows. Tryptophan synthase (16.7 μM, concentration of active sites) was preincubated with 23.8 mM L-serine in buffer P at 22 °C. The reaction was started by adding IGP to a final concentration of 5 mM, and the spectrum was recorded at a rate of 100 nm min^{-1}. The duration of the steady state calculated from V_{max} = 23.4 μM s^{-1} is more than 3 min under these conditions.

Rapid mixing experiments were performed on a modified Durrum stopped-flow instrument as previously described (Paul et al., 1980). All concentrations are given as final concentrations. Typically five to six progress curves were averaged, and the data were analyzed as a sum of exponentials by using the program DIALOG (Labhardt, 1982). The kinetic absorption spectrum at intermediate times was determined by mixing 61.4 μM tryptophan synthase preincubated with 2.5 mM L-serine with 27.4 μM IGP and monitoring the change in absorption with respect to the final values as a function of wavelength.

Single-turnover experiments were conducted either with L-serine or IGP as the limiting substrate (Lane & Kirschner, 1983b). In the latter condition, 40–60 μM enzyme was preincubated with 2.5 mM L-serine to form the aminoacrylate complex (Lane & Kirschner, 1983a,b), which was then rapidly mixed with 10–20 μM IGP in the stopped-flow apparatus.

Release of the product GAP was monitored by coupling to the reduction of NAD^+ or thioNAD^+ with GAPDH, under single-turnover conditions. Given a K_m for unhydrated GAP of about 4 μM (Trentham et al., 1969; Scheek & Slater, 1972) and a V_{max} at 29 μM enzyme of 3200 μM s^{-1}, the capacity of the coupling enzyme far exceeds the maximum possible rate of release of GAP from tryptophan synthase under the single-turnover conditions. Similar conditions were valid for thioNAD^+. Indeed the observed rates of formation of NADH and thioNADH were esssentially identical and were unaffected by doubling the concentration of GAPDH. Hence, the observed rate of formation of NADH closely follows the kinetics of release of GAP from tryptophan synthase under these conditions.

Results

Steady-State Kinetics of the AB Reaction. We have redetermined the steady-state kinetics of the AB reaction in standard phosphate buffer to allow comparison with the steady-state constants for the A and B reactions, which have been measured previously in this buffer (Lane & Kirschner, 1981, 1983 a,b; Kirschner et al., 1991). Figure 1 shows the dependence of the reduced initial velocity (i.e., $v_0/[E_0]$, where $[E_0]$ is the total enzyme concentration) on the concentrations of IGP and L-serine at saturating concentrations of the other substrate. Nonlinear regression of the Michaelis–Menten

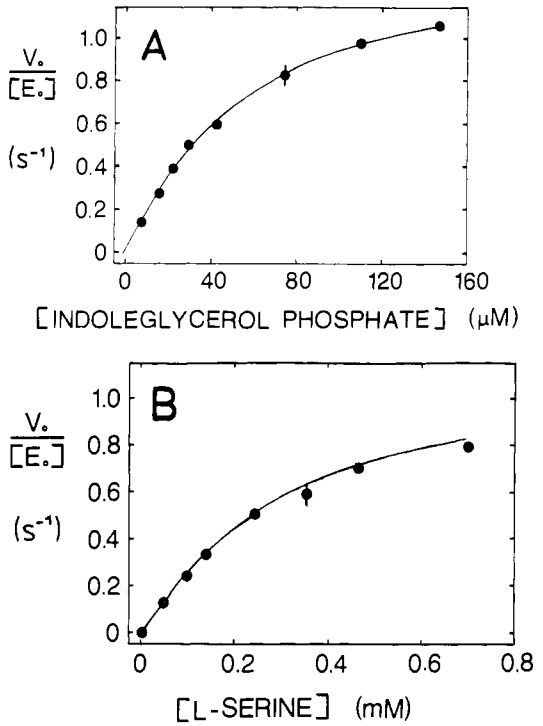

FIGURE 1: Steady-state kinetics of the AB reaction catalyzed by tryptophan synthase. Initial velocities (v_0) were measured at 298 K in phosphate buffer as described under Materials and Methods. $[E_0]$, total concentration of active sites. Continuous curves are regression lines to the Michaelis–Menten equation. (A) Initial velocity versus concentration of IGP at a fixed concentration of L-serine of 24 mM. (B) Initial velocity versus concentration of L-serine at a fixed concentration of IGP of 0.92 mM.

equation to these data yields the values of k_{cat}, K_m^{Ser}, and K_m^{IGP}. The values are given in Table I along with the steady-state parameters for the A and B reactions obtained under identical conditions.

As Table I shows, the value of k_{cat} in the AB reaction is about 21 times larger than that k_{cat} of the A reaction. Similarly, the value of k_{cat}/K_m^{IGP} is about 42 times higher in the AB reaction. These data confirm the discrepancy observed previously for tryptophan synthase from various organisms and under different assay conditions (DeMoss, 1962; Miles, 1991).

During the steady state of the B reaction with serine and indole, an intermediate accumulates that has an absorption band at 473 nm (Goldberg & Baldwin, 1967). This intermediate is the carbanion of nascent tryptophan ("Trp^-"; Tsai et al., 1978; Miles, 1980; Lane & Kirschner, 1981). To determine which intermediate accumulates during the AB reaction, we have recorded its steady-state spectrum as follows.

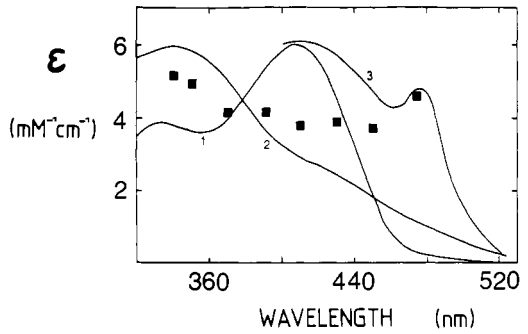

FIGURE 2: Steady-state and kinetic difference spectra of the AB reaction. Spectra were recorded as described in the text. Spectrum 1 is free holoenzyme; spectrum 2 is the enzyme–aminoacrylate complex; spectrum 3 is the intermediate formed during the steady state from excess serine and IGP. (■) Difference absorption spectrum derived from the first phase of the reaction of the aminoacrylate enzyme complex with excess IGP as described under Materials and Methods (Figure 3A).

First, addition of serine to the holoenzyme (Figure 2, spectrum 1) results in its dehydration to the aminoacrylate (Acr), spectrum 2 (York, 1972):

$$E + Ser \rightleftarrows E_{Ser} \rightleftarrows \rightleftarrows E_{Acr} + H_2O$$

Addition of IGP to this complex initiates the AB reaction which, during the steady-state period, is characterized by a new absorption band at 473 nm (spectrum 3):

$$E_{Acr} + IGP \rightleftarrows {}_{IGP}E_{Acr} \rightarrow\rightarrow {}_{GAP}E_{Trp^-} \rightleftarrows \rightleftarrows E + Trp + GAP$$

This observation suggests that the rate-limiting step in the AB reaction occurs after the formation of the same intermediate that accumulates during the B reaction, namely, the carbanion of tryptophan (Trp^-). The extinction coefficient is about four times larger than observed during the B reaction but is comparable to the spectrum of bound tryptophan in the presence of either GAP, glycerol 3-phosphate (GP), or indolepropanol phosphate (IPP) (Lane & Kirschner, 1981; Kawasaki et al., 1987; Houben & Dunn, 1990; Kirschner et al., 1991).

Transient Kinetics of Formation of Trp^- from IGP. Stopped-flow studies of the B reaction under single-turnover conditions have shown that indole condenses rapidly with the enzyme–Acr complex to generate the intermediate absorbing at 473 nm, which then decays slowly with a rate constant close to k_{cat} (Lane & Kirschner, 1983b; Drewe & Dunn, 1986). The initial step involves rapid binding of indole followed by the formation of the C–C bond between carbon 3 of indole and the β position of Acr to yield Trp^-. Similar single-turnover experiments of the AB reaction, in which IGP replaces indole, should reveal the coupling between formation of indole in the α site, its transfer to the β site, and its subsequent condensation with Acr.

First, 22 μM serine was preincubated with 75 μM enzyme to generate the enzyme–Acr complex. Under these conditions, about 85% of the serine is bound (K_d^{Ser} = 10 μM; Lane & Kirschner, 1983a). Rapid mixing of this complex with an excess (230 μM) of IGP led to a single turnover, which was monitored at 473 nm (Figure 3A). The absorbance first increased and then decreased, and each event was fitted to a single exponential. Most importantly, there was no observable lag in the first phase of the reaction (note that the time scale is logarithmic).

We have also measured the kinetics of formation of the transient intermediate as a function of wavelength. The amplitude of the fast phase (cf. Figure 3A, ΔA_1) was added to the spectrum of the enzyme–Acr complex (spectrum 2, Figure 2) to give the kinetic difference spectrum, as shown in Figure 2. This spectrum is similar to that of the enzyme–Trp^- complex (Lane & Kirschner, 1981; Kawasaki et al., 1987; Houben & Dunn, 1990; Kirschner et al., 1991). However, we cannot exclude the presence of other intermediates.

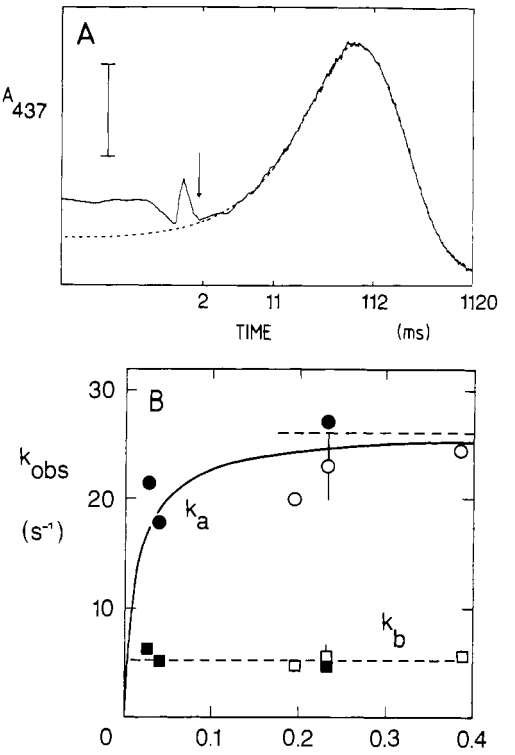

FIGURE 3: Reaction of the enzyme–aminoacrylate complex with IGP under single-turnover conditions. The reaction was monitored by absorbance changes at 473 nm in the stopped-flow apparatus as described in the text. (A) Stopped-flow trace. The arrow denotes the time at which flow stops. Note the logarithmic time scale. (---) Best fit to k_a = 23 s^{-1} and k_b = 5.4 s^{-1}. The bar corresponds to ΔA_{473} = 0.03. (B) Dependence of the rate constants k_a (increase of absorbance) and k_b (decrease) on the concentration of limiting substrate. Filled symbols denote variation of L-serine with limiting IGP; open symbols denote variation of IGP with limiting L-serine. (---) Asymptotes to k_a and k_b.

The time course of the absorbance changes in Figure 3A is described by

$$\Delta A(t) = \Delta A_1^0 \exp(-k_a t) + \Delta A_2^0(-k_b t) \qquad (1)$$

where $\Delta A(t)$ is the deviation of the absorbance at time t from the final equilibrium value, ΔA_1^0 and ΔA_2^0 are the absorbance amplitudes, and k_a and k_b are the rate constants of the two exponential phases. ΔA_2^0 is larger than ΔA_1^0 because the spectrum at t = 0 corresponds to that of the aminoacrylate species, whereas at t = ∞ the spectrum corresponds to the enzyme–IGP complex.

Similar results were obtained under the conditions [L-Ser] $\gg$ [enzyme] > [IGP], which is an alternative means of obtaining single-turnover conditions. The observed rate constants for the two phases were plotted as a function of the concentration of the excess substrate in Figure 3B. The value of k_b is independent of the concentration of both IGP and serine, while the value of k_a increases somewhat with increasing IGP concentration.

The reaction of indole with serine is an almost irreversible reaction (Miles, 1991). The reaction of IGP must also be almost irreversible because it consists of a sequence of the IGP lyase (A) and tryptophan synthase (B) reactions. Therefore k_a, which reflects the condensation of indole with Acr, must be practically zero at zero IGP concentration.

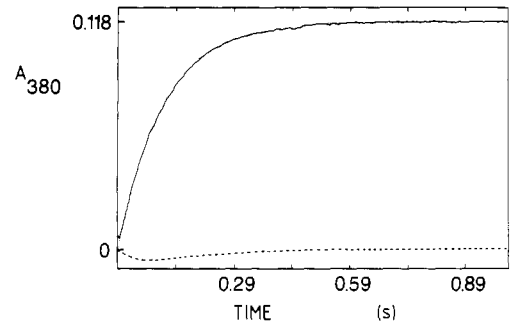

FIGURE 4: Kinetics of release of glyceraldehyde 3-phosphate under single-turnover conditions. The time course of release of GAP was monitored by coupling to excess GAPDH and thioNAD$^+$ as described under Materials and Methods. (---) Reaction monitored at 380 nm in the absence of thioNAD$^+$. (—) Reaction monitored in the presence of thioNAD$^+$.

If it were determined solely by the rate constants of IGP binding, k_a would increase linearly with IGP concentration (Bernasconi, 1976). Since k_a approaches a plateau value, the enzyme–Acr complex is almost saturated with IGP at the lowest concentrations used ($K_d^{IGP} \sim 10\ \mu M$). This is much smaller than the value of K_d^{IGP} found in the absence of serine, that is, in the A reaction ($K_d^{IGP} \geq K_M = 140\ \mu M$; Kirschner et al., 1990).

The value of k_b (5 s^{-1}) is identical with the value of the slower of the two rate constants observed for the decay of Trp$^-$ formed biosynthetically in the B reaction from indole and serine (Lane & Kirschner, 1983b) or by release of bound tryptophan (Lane & Kirschner, 1981). With IGP as the substrate, only a single exponential is needed to describe the decay process, even though the enzyme–Acr complex consists of two slowly interconverting forms (Lane & Kirschner, 1983a).

Release of Glyceraldehyde 3-Phosphate in a Single Turnover. Because the kinetics of release of Trp formed from IGP differ from those of release of Trp formed from indole, it is likely that the second product GAP remains bound until late in the AB reaction. We have therefore measured the release of GAP by coupling it to the reduction of NAD$^+$ with GAPDH.

When the single-turnover experiments were repeated in the presence of a large excess of NAD$^+$ and GAPDH, there was a large increase in absorbance of NADH at 344 nm. It was characterized by a single exponential, with k_{obs} = 9.4 s^{-1} (data not shown). Doubling the concentration of GAPDH had no effect on the rate constant, showing that the reduction of NAD$^+$ to NADH by GAP was not rate limiting. Although the extinction coefficient for NADH at this wavelength (6.22 mM^{-1} cm^{-1}) is much larger than the difference extinction coefficients between the various enzyme–substrate complexes (cf. Figure 2), it is possible that there is some interference from absorbance changes in tryptophan synthase.

We therefore repeated the experiments with thioNAD$^+$, which absorbs maximally at about 380 nm, where the various enzyme complexes have similar extinction coefficients (Figure 2). The time course of the synthesis of thioNADH is shown in Figure 4. The dotted line shows the absorbance changes due to the reaction of tryptophan synthase with its substrates in the absence of thioNAD$^+$; these absorbance changes contribute insignificantly to the absorbance changes in the presence of thioNAD$^+$. The rate constant obtained from the single-exponential process was 11 s^{-1}, close to that obtained for NAD$^+$. Hence, GAP is released after the condensation step (k_a = 26 s^{-1}; cf. Figure 3B) and can influence events in the active site of the β subunits at least up to and including the condensation step.

Discussion

Mutual Interactions between the α and β Sites. The interpretation of the single-turnover transient of absorbance at 473 nm is based on the assumption that it reflects concentration changes of a single defined intermediate on the reaction pathway, namely, the carbanion of tryptophan bound as the Schiff's base with PLP (Trp$^-$) to the β active site. This assumption is supported by previous equilibrium binding studies of tryptophan to the $\alpha_2\beta_2$ complex (Lane & Kirschner, 1981; Kawasaki et al., 1987; Houben & Dunn, 1990) and transient kinetic studies (Lane & Kirschner, 1981, 1983b), including primary isotope effects, which give a consistent picture of the role of enzyme–Trp$^-$ in the mechanisms of both the B reaction and the exchange of the C_α proton of tryptophan (Tsai et al., 1978). The rapid increase of A_{473} in Figure 3A then means that IGP has been cleaved to GAP and IND in the α site and that indole has been transferred to the β site and has condensed Acr to form Trp$^-$ (Figure 3A). The decrease of A_{473}, which occurs more slowly (k_b = 5 s^{-1}) than the release of GAP (Figure 4), must then reflect the same step that limits the rate of release of tryptophan during the turnover of the B reaction (Lane & Kirschner, 1983a; Houben & Dunn, 1990).

The maximum value of the overall rate constant of formation of the enzyme–Trp$^-$ complex (k_a^0 = 26 s^{-1}, Figure 3B) requires that the rate constant of IGP cleavage must be more rapid in the AB reaction than in the A reaction ($k_{12} \sim 5\ s^{-1}$; Kirschner et al., 1991). Because the enzyme–Acr complex cleaves 6-nitroindoleglycerol phosphate 10 times more rapidly than does the free $\alpha_2\beta_2$ complex, we conclude that the acceleration of both cleavage reactions is due to similar indirect interactions. These are induced by Acr in the β site and are transmitted over a distance of 30 Å to the α site.

The $K_{0.5}$ value that characterizes the hyperbolic increase of k_a with increasing [IGP] (Figure 3B) indicates that K_d^{IGP} (=10 μM), the dissociation constant of the IGP–enzyme–Acr complex, is much smaller than that of the IGP–enzyme complex (K_d^{IGP} = 140 μM; Kirschner et al., 1991). Because it is also known that the enzyme–Acr complex binds indolepropanol phosphate (IPP) 10 times more strongly than does the free $\alpha_2\beta_2$ complex, we conclude that this increase of affinity is due to the same indirect interactions that are exerted by Acr in the β site on the rate of IGP cleavage in the α site.

GAP is the first product of the AB reaction to be released from the ternary GAP–enzyme–Trp$^-$ complex. Thus the same order of release of products is observed in both the AB and the A reactions (Weischet & Kirschner, 1976a,b; Kirschner et al., 1991). During a single turnover, both the transfer of indole from α to β and the condensation of indole with Acr occur with GAP still bound to the α site. Not only do both GAP and its analogue glycerol 3-phosphate (GP), as well as IPP, increase the absorbance of the enzyme–Trp$^-$ complex (Kirschner et al., 1991), but both also IPP (Lane & Kirschner, 1981) and GP (Kawasaki et al., 1987; Houben & Dunn, 1990) decrease its dissociation constant about 10-fold and decrease k_{cat} of the B reaction. Similarly, the dissociation rate constant of the GAP–enzyme complex is decreased from 126 s^{-1} in the absence of Trp$^-$ to 10 s^{-1} in the presence of Trp$^-$ (cf. Figure 4). These effects must be due to reciprocal and indirect interactions between ligands in the α site and ligands in the β site across a distance of 30 Å.

The plateau value of k_a (26 s^{-1}; Figure 3B) is 10 times smaller than that observed after mixing indole with the enzyme–Acr complex (k_{obs} = 250 s^{-1}) but is comparable to the

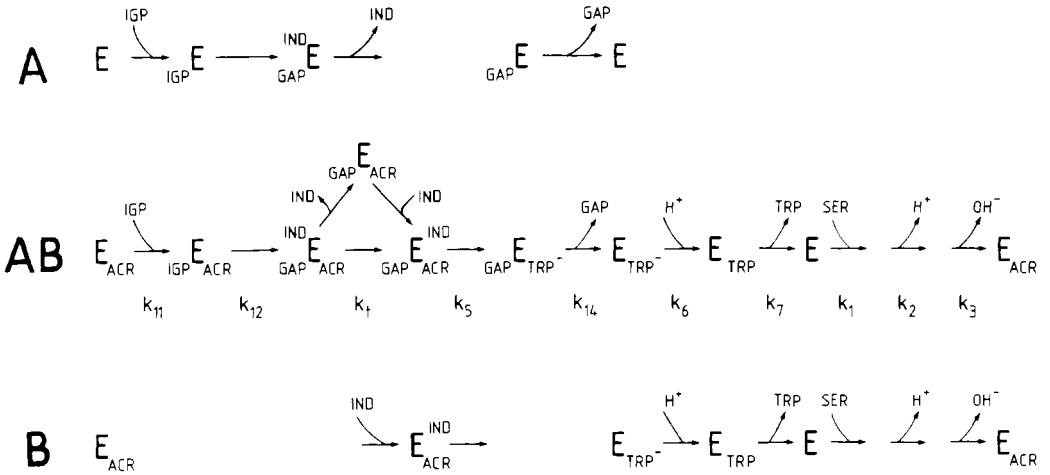

FIGURE 5: Mechanism of the physiological (AB) reaction of IGP with L-serine catalyzed by the $\alpha_2\beta_2$ complex of tryptophan synthase. Comparison to the mechanism of cleavage of IGP to IND (A reaction) catalyzed by the α subunit and the mechanism of condensation of IND with Ser (B reaction) catalyzed by the β subunit. Super- and subscripts on the left-hand side of the symbol for the $\alpha\beta$ heterodimer (E) indicate ligands bound to the α active site; super- and subscripts on the right-hand side are ligands bound to the β active site. Only the forward rate constants are given. The gaps in both A and B mechanisms arise from alignment with the central AB mechanism. Except for k_{-5}, most reverse rate constants are significant.

rate constant observed when the latter process is monitored in the presence of IPP (k_{obs} = 50 s^{-1}; Lane & Kirschner, 1983b). We conclude that the decrease of the rate constant of condensation of indole with the enzyme–Acr complex is due to the same indirect interactions that are exerted by GAP in the α site on the events at the β site.

The rate-limiting steps of both the AB and B reactions are the same, namely, the protonation of the enzyme Trp^- complex. This step must be associated with a conformational change in the β site, because the binding kinetics of α-^{2}H-Trp does not reveal a primary isotope effect (Lane & Kirschner, 1983a). The dissociation of Trp^- occurs with a single phase in the AB reaction k_b (=5 s^{-1}, Figure 3B) and is only about 3 times larger than k_{cat} (=1.5 s^{-1}, Table I). Because bound GAP increases the extinction coefficient of the enzyme–Trp^- complex, the decay of A_{473} observed in Figure 3A should be determined at least partially by the rate constant of GAP dissociation (k_{14} = 10 s^{-1}, Figure 2). It is plausible to regard the decrease of A_{473} in Figure 3A as a superposition of two exponential processes with similar first-order rate constants: decrease of A_{473} of bound Trp^- due to dissociation of GAP with k_{14} = 10 s^{-1} followed by protonation of Trp^- with $k_5 < 5$ s^{-1}. It is possible that the coupling of release of GAP and Trp^- also leads to suppression of the second species of Trp^- observed during the B reaction (Lane & Kirschner, 1983a).

In summary the data are consistent with the view (Drewe & Dunn, 1986; Houben & Dunn, 1990) that the AB reaction is the sequence of the A followed by the B reaction but that reciprocal interactions between ligands bound to the widely separated α and β sites modulate the intrinsic rate and equilibrium constants of each partial reaction.

The Mechanism of the AB Reaction. Figure 5 presents a plausible mechanism of the AB reaction that is consistent with the data. It is composed of the known steps of the partial A and B reactions. These are displayed for comparison above and below the AB mechanism. The central section is novel. It describes two alternative possibilities for the transfer of indole from the α to the β sites: (1) the diffusion mode via release of indole into the medium followed by binding to the β site and (2) the channeling mode via intramolecular transfer of indole.

Channeling is well established experimentally for the tryptophan synthases both from *Neurospora crassa* (Yanofsky & Rachmeler, 1959; De Moss, 1962; Matchett, 1974) and from *Escherichia coli* (Yanofsky, 1959; Crawford & Yanofsky, 1958). By contrast, when experimental conditions were adjusted to make the diffusion mode obligatory (Yanofsky, 1957; Yanofsky & Rachmeler, 1959), IGP was converted to tryptophan only slowly. Moreover, if the diffusion mode were to prevail, the progress curve of formation of enzyme–Trp^- would begin with a lag (and not with the observed single exponential, see Figure 3A). The lag would arise from the time required for indole to accumulate in the medium to a concentration that saturates the β active site. Therefore the channeling mode that is characterized by the transfer rate constant k_t (Figure 5) appears to dominate the catalytic mechanism of the AB reaction. The preference of the channeling over the free diffusion mode appears to be determined solely by the relative rates of the two competing pathways.

Quantitative Considerations. If the AB reaction were to proceed via the diffusion mode, the steady-state concentration of indole would have to support a k_{cat} of 1.5 s^{-1}. Moreover, in the single-turnover experiments, sufficient indole would have to be released rapidly to allow the enzyme–Trp^- complex to be formed with k_a^0 = 26 s^{-1} (Figure 3B). However, calculations show that the concentration of free indole required to fulfill these requirements must exceed either the observed low values in steady-state experiments or the total concentration of the limiting substrate used in the single-turnover experiments (Figure 3). Hence, on the basis of kinetic data alone, the diffusion mode cannot be the dominant route for the transfer of indole.

In the channeling mode, the rate constant of Trp^- formation (k_a) could in principle be limited by one of three elementary steps in the mechanism (Figure 5): (1) the cleavage of IGP (k_{12}), (2) the transfer of indole (k_t), and (3) the condensation of indole with Acr (k_5). In each case the dependence of k_a on [IGP] is given by

$$k_a = k_a^0[\text{IGP}]/([\text{IGP}] + K_{0.5}) \quad (2)$$

where k_a^0 is the maximal value of k_a and $K_{0.5}$ the half-saturation value of [IGP]. This hyperbolic relationship describes adequately the observed concentration dependence of k_a (Figure 3B).

The available data do not distinguish unequivocally between these possibilities. However, the reciprocal transit time for

the free diffusion of indole over 25 Å ($k_t \sim 10^7$ s^{-1}) is much larger than k_a^0. It appears unlikely that the intramolecular transfer should be decreased by 5 orders of magnitude, especially since the tunnel is wide enough to allow for the passage of indole (Hyde et al., 1988). Moreover, by use of the known rate constants for the steps measured previously for the A and B reactions and with the modulating effects of bound indolepropanol phosphate taken into account, the assumption of rate-limiting indole transfer leads only to unacceptable discrepancies between assumed elementary and observed overall rate constants. Therefore transfer is not likely to be rate-limiting.

It is reasonable to assume that $k_5 \sim 50$ s^{-1} as observed for the condensation of indole with the IPP–enzyme–Acr complex (Lane & Kirschner, 1983b). Therefore, also the condensation step is not likely to be solely rate-limiting for k_a. By contrast, it is plausible that the rate constant of IGP cleavage is predominantly limiting, for the following reasons. $k_{12} \sim 5$ s^{-1} in the A reaction (Kirschner et al., 1991). A 5-fold increase to $k_a^0 = k_{12} = 26$ s^{-1} in the presence of Acr in the β site is supported by the independently observed 10-fold increase of k_{cat} of 6-nitroindoleglycerol phosphate cleavage in the presence of serine. Under these conditions eq 2 becomes

$$k_a = k_{12}[\text{IGP}]/([\text{IGP}] + K_{11}) \quad (3)$$

with $K_{11} = K_d^{IGP} \sim 10$ μM. Thus, the presence of Acr in the β site decreases K_{11} determined for the A reaction (=140 μM) 14-fold. This interpretation is supported by the 10-fold decrease of the dissociation constant of the IGP analogue IPP in the presence of serine (Lane & Kirschner, 1983a).

Conclusions

Tryptophan synthase is an excellent example of many of the proposed benefits of enzyme–enzyme interaction in multienzyme complexes (Welch, 1985; Srere, 1987). The presence of Acr in the β active site accelerates the sluggish A reaction by several measures. First, the rate of IGP cleavage is increased. The concomitant decrease of the rate of indole condensation with Acr, caused by GAP in the α active site, has no effect on the overall rate of tryptophan synthesis. Second, the pathway of indole is switched from release into the solvent to channeling toward the β active site and to its trapping by reaction with Acr. Thus indole is prevented from escaping through the cell membranes (Manney, 1970).

Strictly speaking, our data do not prove that indole actually uses the existing molecular tunnel between the α and β active sites (Hyde et al., 1988). Nevertheless it remains the only plausible alternative to the free diffusion mode.

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Registry No. IGP, 4220-97-7; Ind, 120-72-9; GAP, 591-57-1; L-Ser, 56-45-1; Trp$^-$, 130199-41-6; Acr, 1948-56-7; Trp synthase, 9014-52-2.

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