Strittmatter, W. J. (1988) *Cell. Mol. Neurobiol. 8*, 19-25.
Tamiya-Koizumi, K., Umekawa, H., Yoshida, S., Ishihara, H., & Kojima, K. (1989) *Biochim. Biophys. Acta 1002*, 182-188.
Terland, O., & Flatmark, T. (1980) *Biochim. Biophys. Acta 597*, 318-330.
Trifaro, J. M., & Duerr, A. C. (1976) *Biochim. Biophys. Acta 421*, 153-167.
Ulevitch, R. J., Watanabe, Y., Sano, M., Lister, M. D., Deems, R. A., & Dennis, E. A. (1988) *J. Biol. Chem. 263*, 3079-3085.
van den Bosch, H. (1980) *Biochim. Biophys. Acta 604*, 191-246.
Volwerk, J. J., Pieterson, W. A., & deHaas, G. H. (1974) *Biochemistry 13*, 1446-1454.
Wilson, S. P., & Kirshner, N. (1983) *J. Biol. Chem. 258*, 4994-5000.
Winkler, H. (1988) in *Handbook of Experimental Pharmacology* (Trendelenburg, U., & Weiner, N., Eds.) Vol. 90/1, pp 43-118, Springer-Verlag, Berlin.
Winkler, H., & Smith, A. D. (1968) *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 261*, 379-388.
Wurtman, R. J., & Axelrod, J. (1963) *Biochem. Pharmacol. 12*, 1439-1440.
Zahler, P., Reist, M., Pilarska, M., & Rosenheck, K. (1986) *Biochim. Biophys. Acta 877*, 372-379.

Reciprocal Communication between the Lyase and Synthase Active Sites of the Tryptophan Synthase Bienzyme Complex†

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ABSTRACT: It is important to understand how the cleavage of indoleglycerol phosphate, which is catalyzed by the α subunits in the $\alpha_2\beta_2$ bienzyme complex of tryptophan synthase, is modulated by the presence of L-serine in the β subunits. Steady-state kinetic data, including the dependence of k_{cat} on pH, allowed values to be assigned to each of the eight rate constants of the minimal catalytic mechanism. An ionizing group having an apparent pK value near 7.5 must be protonated for activity. The α active site ligands indolepropanol phosphate, glyceraldehyde 3-phosphate, and glycerol 3-phosphate increase both the affinity and the molar absorbance of L-serine and L-tryptophan bound to the β active site. These effects prove that the α sites communicate with the β sites over a distance of 30 Å. 6-Nitroindole readily condenses with glyceraldehyde 3-phosphate, but not with L-serine. The turnover numbers for 6-nitroindoleglycerol phosphate and 6-nitroindole increased about 10-fold in both directions in the presence of L-serine bound to the β_2 subunits. These data prove that the α and β active sites communicate reciprocally and explain why the turnover number for the physiological reaction of indoleglycerol phosphate with L-serine greatly exceeds that of the cleavage reaction of indoleglycerol phosphate.

Tryptophan synthase from *Escherichia coli* (EC 4.2.1.20) is a simple bienzyme complex that catalyzes the final two steps in the biosynthesis of L-tryptophan. The enzyme is an $\alpha_2\beta_2$ tetramer, which can be dissociated easily into monomeric α subunits and the dimeric β_2 subunit. The latter contains one molecule of pyridoxal 5′-phosphate (PLP)[1] in each active site (Crawford & Yanofsky, 1958; Miles, 1991). The α subunit catalyzes the reversible cleavage of indoleglycerol phosphate (IGP) to indole (IND) and glyceraldehyde 3-phosphate (GAP); this is the "A" or "lyase" reaction:

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

The β_2 subunit catalyzes the practically irreversible condensation of IND with L-serine to give L-tryptophan. This "B" or "synthase" reaction requires PLP:

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

The intact $\alpha_2\beta_2$ complex catalyzes the physiological AB reaction, which is formally the sum of the A and B reactions:

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

The isolated α and β_2 subunits are poor catalysts of their respective reactions. However, in the $\alpha_2\beta_2$ complex, the subunits become about 100-fold more active. This activation is contingent on conformational changes that occur during the assembly of the mature $\alpha_2\beta_2$ complex (Lane et al., 1984). The three-dimensional structure of tryptophan synthase shows that the α and β active sites are separated by about 30 Å (Hyde et al., 1988), confirming earlier indirect measurements (Heilmann & Holzer, 1981; Lane & Kirschner, 1983c). The

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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; GP, DL-α-glycerol 3-phosphate; 6-nitro-IGP, (6-nitroindol-3-yl)-D-glycerol 3′-phosphate; 6-nitro-IND, 6-nitroindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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active sites are connected by an intramolecular tunnel that is wide enough to allow the transfer of indole during the AB reaction.

The turnover number in the AB reaction is 20 times larger than the turnover number of the A reaction in the $\alpha_2\beta_2$ complex. This interesting discrepancy may reflect an intramolecular information transfer between the lyase (α) and the synthase (β) active sites. Operationally, the A reaction is measured in the absence of serine, which is a substrate in the AB reaction. It is possible that serine bound to the active site of the β_2 subunit increases the catalytic activity of the α subunits (De Moss, 1962; Drewe & Dunn, 1986; Kawasaki et al., 1987; Dunn et al., 1987; Houben & Dunn, 1990). Indeed, the events at one active site influence the properties of the other active site, apparently over a distance of 30 Å. For example, indolepropanol phosphate (IPP), an α-specific analogue of IGP, increases the affinity of the β_2 subunit for tryptophan and decreases the turnover number in the B reaction 3-fold (Lane & Kirschner, 1981). Similarly, serine bound at the active site of β_2 increases the affinity of the α subunits for IPP (Lane & Kirschner, 1983a,b). However, it is not possible to measure the A reaction in the presence of serine, because the nascent indole is immediately converted to tryptophan via the AB reaction (Yanofsky & Rachmeler, 1958).

In this paper we examine the steady-state kinetics of the A reaction. The data are consistent quantitatively with an ordered release mechanism with indole dissociating first and the participation of two proton transfer groups in the bond cleavage step. The use of 6-nitroindoleglycerol phosphate and 6-nitroindole, which does not condense with serine, showed that serine bound to the β active site can activate catalysis by the α active site at a distance of 30 Å.

Materials and Methods

Materials. Buffer T was 0.1 M Tris-HCl, pH 7.8 at 25 °C. Buffer P was 0.1 M potassium phosphate, pH 7.6. Buffer G was 0.1 M sodium DL-3-glycerol phosphate (GP), pH 7.6, treated with acid-washed charcoal to remove colored impurities. All buffers were supplemented with 2.5 mM EDTA, 0.2 mM dithioerythritol, and 0.04 mM PLP. The pH was adjusted without controlling the ionic strength. The effect of ionic strength on the reaction rate was studied separately at pH 7.6. At pH values above 8 and below 6, buffers were supplemented with citrate, pyrophosphate, or GlyGly to increase the buffer capacity.

The $\alpha_2\beta_2$ complex of tryptophan synthase and its subunits were purified as described (Tschopp & Kirschner, 1980). Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was purchased from Böhringer-Mannheim. The concentration of tryptophan synthase was determined by absorbance in 0.1 M sodium hydroxide (Kirschner et al., 1975; Tschopp & Kirschner, 1980).

Indoleglycerol phosphate and indolepropanol phosphate were prepared as the cyclohexylammonium salts (Kirschner et al., 1975). Indole was purchased from Merck and its concentration determined by using an extinction coefficient of 5.54 mM^{-1} cm^{-1} at 278 nm (Weischet & Kirschner, 1976a). 6-Nitroindole (6-nitro-IND) was purchased from Aldrich, and as it gave a single spot on thin-layer chromatography, it was used without further purification. NAD^+ and the dicyclohexylammonium DL-glyceraldehyde 3-phosphate diethyl acetal barium salt were purchased from Böhringer-Mannheim. The acetals were converted to the free aldehydes by exchange with Dowex 50 (H^+) resin (Racker et al., 1959), neutralized with K_2CO_3 (pH = 7.5), and used within 6 h of preparation.

6-Nitroindoleglycerol phosphate (6-nitro-IGP) was prepared enzymatically with tryptophan synthase. 6-Nitroindole, 2.95 mM, and DL-GAP, 4.6 mM, in 50 mM Tris, pH 7.6, 50 μM pyridoxal phosphate, 5 mM EDTA, and 0.2 mM dithioerythritol were treated with 0.05 μM tryptophan synthase at 23 °C in the dark for 17 h. The reaction product was purified by chromatography on DEAE-Sephadex A25 (2.5 × 30 cm column) equilibrated with 25 mM NH_4HCO_3, pH 8.5. The column was eluted with a linear gradient (1 L) from 25 to 500 mM NH_4HCO_3 at 2 mL/min. Fractions were assayed by spectrophotometry, enzymic assay (see below), and TLC on Merck F_{254} silica gel plates developed in 48% ethanol, 2% ammonia, and 5% trichloroacetic acid. The first peak off the column was unreacted 6-nitroindole (R_f = 0.80) and the second peak was 6-nitroindoleglycerol phosphate (R_f = 0.43). The relevant fractions were pooled and lyophilized.

Methods. The steady-state kinetics of the forward A reaction were measured by coupling to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and monitoring the appearance of NADH at 340 nm (Creighton & Yanofsky, 1970). Buffers were supplemented with 10 mM Na_2HAsO_4, 1 mM NAD^+, and excess GAPDH. The kinetics of the reverse reaction were measured as described (Weischet & Kirschner, 1976b), using 2-mm cuvettes for indole (observation at 290 nm) and 10-mm cuvettes for 6-nitroindole (observation at 420 nm). The B reaction was monitored at 291 nm as previously described (Lane & Kirschner, 1981). This method was also used for 5-methyl, 5-hydroxy-, and 5-methoxyindole at the appropriate wavelength (cf. Table II). For bromo- and nitroindoles, the reaction rates are so slow that the disappearance of the indole derivatives was followed spectrophotometrically after extraction of aliquots of the reaction mixture with ethyl acetate (Yanofsky, 1956).

Difference spectra were measured by using tandem cuvettes on a Cary Model 118 spectrophotometer (Kirschner et al., 1975). The absolute difference spectrum for the conversion of 6-nitro-IGP into 6-nitro-IND was determined as follows. The spectrum of a solution of 6-nitro IGP was recorded in buffer P containing 1 mM NAD^+, 12.5 mM L-serine, and 3 μM GAPDH (spectrum A1). The cleavage reaction was initiated by adding tryptophan synthase (final concentration 2 μM) and incubated in the cuvette until no further changes occurred. A second spectrum (A2) was then recorded and corrected for the minor contribution from tryptophan synthase.

Results

Effect of Buffer and pH on the Kinetics of the A Reaction. The steady-state kinetics of the A reaction catalyzed by the $\alpha_2\beta_2$ complex of tryptophan synthase have been measured previously only in Tris buffer (Creighton, 1970; Weischet & Kirschner, 1976b,c), whereas detailed information on the mechanism of both the B reaction (Lane & Kirschner, 1981, 1983a,b) and the AB reaction [see Lane & Kirschner (1991)] has been obtained from measurements made in phosphate buffer. To facilitate comparisons between the different reactions, we have measured the kinetics of the A reaction in phosphate buffer.

The kinetics have been measured in both directions, i.e., cleavage and synthesis of IGP. In all cases, initial velocities showed a hyperbolic dependence on the concentrations of the substrates (data not shown). The steady-state parameters are collected in Table I, where they are compared with the values obtained previously in Tris buffer (Weischet & Kirschner, 1976b). In general only small changes in values are obtained by changing the buffer. The K_m values for IGP and GAP are probably increased by the competitive inhibition exerted by

Table I: Steady-State Parameters for the IGP Lyase (A) Reaction Catalyzed by the $\alpha_2\beta_2$ Complex of Tryptophan Synthase at pH 76, 25 °C[a]

directn of reactn	param	dimension	6-H-IND		6-nitro-IND	
			−ser Tris	−ser P_i	−ser P_i	+ser P_i
IGP → IND	k_f	s^{-1}	0.1	0.067	0.0025	0.027
	K_m^{IGP}	mM	0.056	0.14	0.009	0.03
	$k_{f/m}^{IGP}$	mM^{-1} s^{-1}	1.79	0.48	0.28	0.90
IND → IGP	k_r	s^{-1}	122	92	0.5	3.2
	K_m^{IND}	mM	2.2	1.2	0.008	0.2
	K_m^{GAP}	mM	9.4	14.0	2.2	0.7
	k_r/K_m^{IND}	mM^{-1} s^{-1}	55.5	76.7	62.5	16.0
	k_r/K_m^{GAP}	mM^{-1} s^{-1}	13	6.6	0.23	4.6
IGP ⇌ IND	K_e	mM	0.12	0.12	nd	nd

[a] Details of the measurements are given in the text. Values in Tris are taken from Weischet et al. (1976b) recalculated with 0.2 μM total active-site concentration. −Ser (+ser) denotes measurements made in the absence of (presence of 24 mM) serine. k_f and k_r are the turnover numbers in the forward and reverse reactions, respectively. nd: not determined.

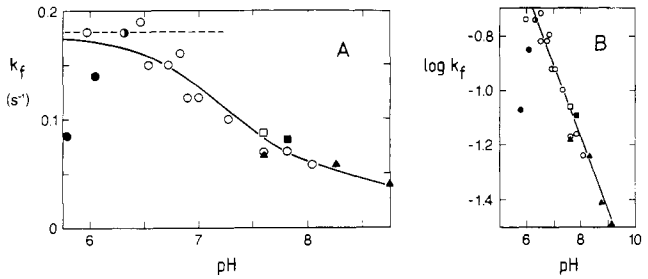

FIGURE 1: Catalysis of IGP lyase (A) reaction by the $\alpha_2\beta_2$ complex depends on pH. k_{cat} values in the cleavage direction were determined at 298 K as a function of pH as described under Materials and Methods. (A) dependence of k_f on pH. (○) 0.1 M phosphate; (●) 0.1 M phosphate + 50 mM citrate; (■) 0.1 M phosphate + 50 mM pyrophosphate; (▲) 0.1 M phosphate + 50 mM GlyGly. (—) best fit to eq 13 using the constants given in the text. (B) log k_f versus pH. (—) linear regression line with slope = −0.28 (correlation coefficient = 0.98 for 17 data points).

phosphate ions of the buffer (Heyn & Weischet, 1975; Houben & Dunn, 1990).

We have also examined the influence of ionic strength on the kinetics of the cleavage reaction. K_m^{IGP} increases with increasing concentration of sodium chloride, presumably reflecting electrostatic interaction between the negatively charged substrate and the active site. By contrast, the value of k_{cat} in the cleavage reaction (k_f) was essentially unaffected by increasing concentrations of sodium phosphate and sodium citrate, as expected for a reaction occurring in a solvent-inaccessible active site (Lane, 1983). However, 190 mM sodium chloride increased k_{cat} about 2-fold, suggesting a relatively specific interaction of the enzyme with chloride ions.

The cleavage and formation of the carbon–carbon bond in IGP is likely to be catalyzed by acid–base groups in the active site of the enzyme (Walsh, 1979). Indeed, using the recently described crystal structure of tryptophan synthase from *Salmonella typhimurium* (Hyde et al., 1988) and kinetic analysis of specific mutants, Miles and co-workers have identified possible active-site residues that can act as general acid–base groups (Nagata et al., 1989). We have therefore measured the dependence of k_f on pH to obtain information about apparent p*K* values of ionizing groups participating in the reaction. Figure 1A shows the dependence of k_f on pH. The maximum value of k_f is about 0.18 s^{-1} (at pH < 6), which decreases only about 5-fold in the range from pH 6 to 8.7. Clearly at least one group must be protonated for maximal catalytic activity in the A reaction. A plot of log (k_f) versus pH is shown in Figure 1B. The plot is linear in the range pH 6–9, with a slope of −0.28. The overlap between points obtained by using different buffers (chloride excepted) also demonstrates the absence of specific ion effects on k_f. Below pH 6, k_f decreases sharply, which may be a consequence of protonation of a group required for catalysis and also may be because of dissociation of the complex. The α subunits have a much smaller k_f than the $\alpha_2\beta_2$ complex (Weischet & Kirschner, 1976b,c). Moreover, the enzyme begins to precipitate at about pH 5.5 in phosphate buffer (unpublished observation). Further, the free α subunit unfolds below pH 5 (Yutani et al., 1984). For these reasons, data collected below pH 5.5 cannot be analyzed. K_m^{IGP} is about 450 μM at pH 6, dropping steeply to a plateau value of 140 μM at pH 7. Analogous results were obtained for the pH dependence of K_m^{IGP} for the isolated α subunit (Smith & Yanofsky, 1962).

Table II: Inhibition of Tryptophan Synthesis from Indole and Serine (B Reaction) by α-Specific Ligands[a]

addition		
compd	concn (mM)	k_{cat} (s^{-1})
NaCl	260	3.9
K phosphate	90	5.3
Na glycerol 3-phosphate	80	2.0
K phosphate	90	
+IPP	0.2	1.9

[a] The maximal velocity was measured in 50 mM Tris·HCl buffer, pH 7.8, at 25 °C as described under Materials and Methods. NaCl was added to the control to adjust the ionic strength to the same value (0.3 M) throughout.

Active-Site-Specific Ligands of the α Subunit Perturb the Spectra of Ligands Bound to the Active Site of the β_2 Subunit. The strictly ordered addition of GAP followed by indole in the synthesis of IGP (Weischet & Kirschner, 1976b,c) implies that bound GAP increases the affinity of the enzyme for indole, which is intrinsically weak (Weischet & Kirschner, 1976a). Indolepropanol phosphate (IPP), a nonreactive analogue of IGP, changes the spectrum of the PLP bound to the active site of the β_2 subunit and also of its complexes with serine (Lane & Kirschner, 1983a) and tryptophan (Lane & Kirschner, 1981). These spectral changes are associated with changes in the kinetics of both catalysis and ligand binding. However, indole has only weak effects on the spectra and kinetics, suggesting that it may be the GAP moiety that is responsible for the intersubunit interactions. We have therefore examined the influence of GAP and glycerol 3-phosphate (GP, which is a nonreactive analogue of GAP; Heyn & Weischet, 1975) on the spectra of tryptophan synthase holoenzyme and its complexes with serine and tryptophan (Kawasaki et al., 1987; Houben & Dunn, 1990).

Figure 2 shows that the effects of GAP and GP on the absorption spectra are very similar to those of IPP. Further, the value of k_{cat} for the β reaction decreases about 3-fold in the presence of either GAP or GP (Table II), analogous to the effects of IPP (Lane & Kirschner, 1981). We conclude that the bound alkyl phosphate moiety of IPP is largely responsible for the interactions between the α and β active sites. Given the structural similarity between IPP and IGP, we expect the substrate to have similar properties.

L-Serine Activates the Synthesis and Cleavage of 6-Nitroindoleglycerol Phosphate. Because it is not possible to measure the effect of serine on the kinetics of the A reaction, we sought analogues of indole that are substrates in the A reaction but that cannot condense with serine. According to Hall et al. (1962), methyl substituents on the benzenoid ring of indole decrease k_{cat} and increase K_m. We have confirmed this result for 5-methylindole (cf. Table III). Several other derivatives have been tested, and they can be grouped into two

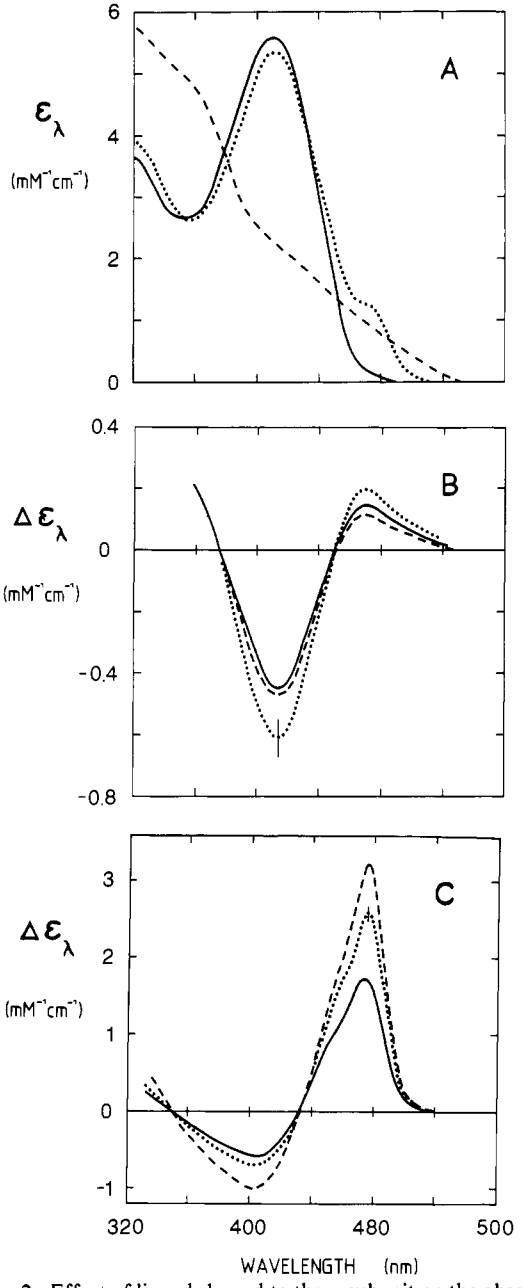

FIGURE 2: Effect of ligands bound to the α subunit on the absorbance spectra of substrate adducts to pyridoxal phosphate in the β subunit. Spectra were recorded as described under Materials and Methods. (A) Absolute spectra: (—) holoenzyme, (---) enzyme + 5 mM L-serine; (···) enzyme + 5 mM L-tryptophan. (B) Difference spectra of the enzyme–L-serine complex generated by addition of (—) 7 mM DL-glyceraldehyde 3-phosphate, (---) 80 mM DL-glycerol 3-phosphate, or (···) 0.4 mM indolepropanol phosphate. (C) Difference spectra of the enzyme–L-tryptophan complex generated by addition of (—) 7 mM DL-glyceraldehyde 3-phosphate, (---) 80 mM DL-glycerol 3-phosphate, or (···) 0.4 mM indolepropanol phosphate.

classes. Derivatives containing electron-donating groups in the 5-position decrease k_{cat} 2–5-fold and increase K_m about 50-fold. Derivatives that have electron-withdrawing groups in the 5-, 6-, or 7-position decrease k_{cat} nearly to zero, while not significantly affecting K_m. The least reactive analogue is 6-nitroindole, which is essentially inactive in the B reaction. It will, however, condense with GAP to form 6-nitro-IGP, which is a substrate for the A reaction.

The cleavage of 6-nitro-IGP to 6-nitroindole generates a difference absorption spectrum as shown in Figure 3. While this difference absorption could be used to monitor the kinetics, we have used the more sensitive coupled assay with NAD^+

Table III: Specificity of the $\alpha_2\beta_2$ Complex of Tryptophan Synthase for the Synthesis of Tryptophan from Indole Derivatives (B Reaction)[a]

substituent	method	λ_{max} (nm)	$10^9 v_0$ (M s^{-1})	k_{cat} (s^{-1})	K_m (mM)
H	A	289	7800	14	0.027
5-CH_3	A	299	180	1.4	1.48
5-HO	A	309	740	4.8	1.2
5-CH_3O	A	311	260	2.0	1.4
5-Br	B	279	0.9	0.007	<0.05
5-NO_2	B	318	0.6	0.005	<0.05
7-NO_2	B	326	0.2	0.002	<0.05
6-NO_2	B	321	<0.02	<0.00002	0.016

[a] The enzyme was assayed at 37 °C in 0.1 M Tris·HCl buffer, pH 7.8. Initial velocities, v_0, were determined in the presence of 0.4 mM indole derivative and 10 mM L-serine, using an enzyme concentration of 0.6 μM. λ_{max} is the wavelength of maximal difference absorbance for measurements by method A (by UV difference) and the wavelength of maximum absorbance for measurements made by method B (solvent extraction, see the Materials and Methods section).

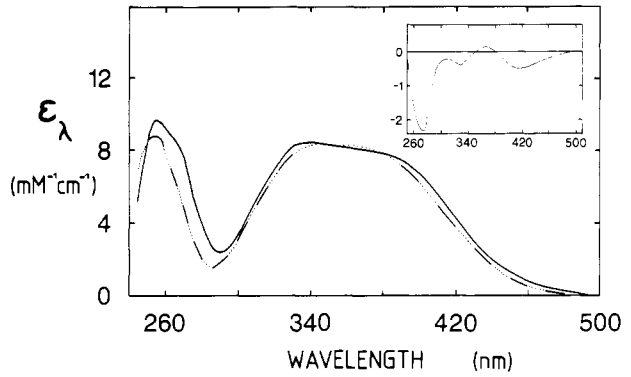

FIGURE 3: Spectra of 6-nitroindole and 6-nitroindoleglycerol phosphate. Spectra were recorded in 0.1 M Tris HCl, pH 7.8, at 298 K and normalized as described under Materials and Methods. (—) 6-Nitroindole; (···) 6-nitroindoleglycerol phosphate. Inset shows the difference spectrum 6-nitroindole minus 6-nitroindoleglycerol phosphate.

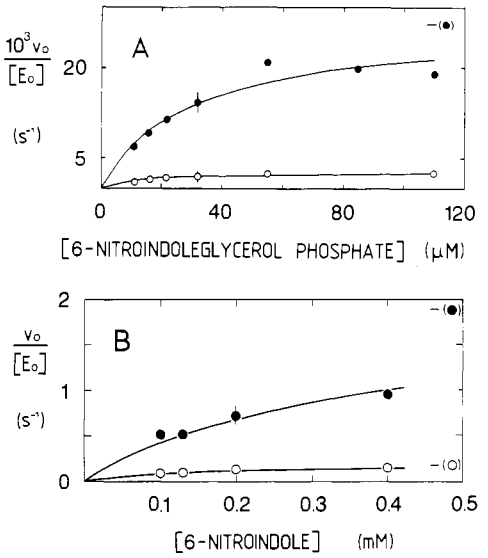

FIGURE 4: Acceleration by L-serine of the synthesis and cleavage of 6-nitroindoleglycerol phosphate as catalyzed by the $\alpha_2\beta_2$ complex. Initial velocities were measured in 0.1 M phosphate buffer at 298 K as described under Materials and Methods. (—) Nonlinear regression lines to the Michaelis–Menten equation. (A) Cleavage reaction: (○) no L-serine; (●) +24 mM L-serine. (B) Synthesis reaction: (○) no L-serine; (●) +24 mM L-serine.

and GAPDH (Stein et al., 1986). The dependence of the initial velocities on substrate concentration in the absence and presence of serine is shown in Figure 4. Clearly, serine in-

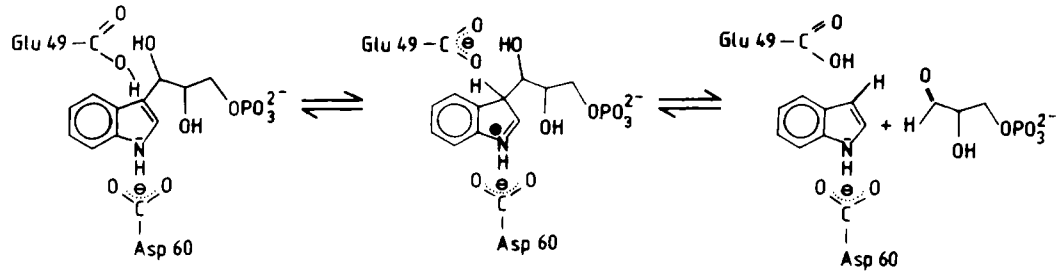

FIGURE 5: Proposed mechanism of the proton-transfer steps during cleavage and synthesis of indoleglycerol phosphate in the active site of the α subunit. The mechanism is adapted from Nagata et al. (1989) as described in the text.

creases the rate in both directions about 10-fold (cf. Table I).

Discussion

Mechanism of the Indoleglycerol Phosphate Lyase (A) Reaction. The mechanism of the A reaction has been shown to be ordered Uni Bi Bi (Weischet & Kirschner, 1976 b,c) with indole being released first. Hence the minimal mechanism is as follows:

$$\text{IGP} + \text{A} \underset{k_{-11}}{\overset{k_{11}}{\rightleftharpoons}} {}_{\text{IGP}}\text{A} \underset{k_{-12}}{\overset{k_{12}}{\rightleftharpoons}} {}^{\text{IND}}_{\text{GAP}}\text{A} \underset{k_{-13}}{\overset{k_{13}}{\rightleftharpoons}} \text{IND} + {}_{\text{GAP}}\text{A} \underset{k_{-14}}{\overset{k_{14}}{\rightleftharpoons}} \text{GAP} + \text{IND} + \text{A} \quad (1)$$

The subscripts IGP and GAP and the superscript IND to the left of the symbol A indicate ligands bound to the corresponding subsites in the α active site of the $\alpha_2\beta_2$ complex of tryptophan synthase. The expressions for k_{cat} in the forward (k_f) and reverse (k_r) A reactions and the K_m and k_{cat}/K_m values for this mechanism are

$$k_f = k_{12}k_{13}k_{14}/[k_{12}(k_{13} + k_{14}) + k_{14}(k_{-12} + k_{12})] \quad (2)$$

$$K_m^{IGP} = k_{14}[k_{13}(k_{-11} + k_{12}) + k_{-11}k_{-12}]/k_{11}[k_{12}(k_{13} + k_{14}) + k_{14}(k_{-12} + k_{13})] \quad (3)$$

$$k_f/K_m^{IGP} = k_{11}k_{12}k_{13}/[k_{-11}(k_{-12} + k_{13}) + k_{12}k_{13}] \quad (4)$$

$$k_r = k_{-11}k_{-12}/[k_{-11} + k_{-12} + k_{12}] \quad (5)$$

$$K_m^{IND} = [k_{-11}k_{-12} + k_{13}(k_{-11} + k_{12})]/k_{-13}(k_{-11} + k_{-12} + k_{12}) \quad (6)$$

$$K_m^{GAP} = k_{-11}k_{-12}/k_{-14}(k_{-11} + k_{-12} + k_{12}) \quad (7)$$

$$k_r/K_m^{IND} = k_{-11}k_{-12}k_{-13}/[k_{-11}(k_{-12} + k_{13}) + k_{12}k_{13}] \quad (8)$$

$$k_r/K_m^{GAP} = k_{-14} \quad (9)$$

The expression for the experimentally determined equilibrium constant of the IGP lyase reaction, K_e (=0.12 mM; Weischet & Kirschner, 1976b), is given by

$$K_e = [\text{IND}][\text{GAP}]/[\text{IGP}] = k_{11}k_{12}k_{13}k_{14}/k_{-11}k_{-12}k_{-13}k_{-14} = k_fK_m^{IND}K_d^{GAP}/k_rK_m^{IGP} \quad (10)$$

where K_d^{GAP} is the dissociation constant of ${}_{GAP}$A. As there are only six independently determined parameters, it is clear that not all eight rate constants can be calculated from the steady-state data alone. However, k_{-14} is simply equal to the value of k_r/K_m^{GAP} = 6.6 mM^{-1} s^{-1} (eq 9). From eq 10 and the data in Table I, we calculate the value of K_d^{GAP} as 19.2 mM, with which the value for k_{-14} gives k_{14} as 126 s^{-1}. Therefore, in the cleavage reaction catalyzed by the α subunit, the release of the second product (GAP) is not rate determining for k_{cat} (k_f = 0.067 s^{-1}). It is shown later that, depending on the pH value, either the cleavage of IGP or the release of IND or both are rate limiting.

The data in Figure 1 clearly show that at least one protonated group is required to catalyze the cleavage reaction. In the crystal structure (Hyde et al., 1988), three potential acid–base groups have been identified in the active site of the α subunit, Asp 60, Glu 49, and Tyr 175. Subsequent kinetic analysis of site-specific mutants showed that only Asp 60 and Glu 49 had any role in catalysis (Nagata et al., 1989), and the authors have proposed a detailed chemical mechanism of the cleavage step involving three acid–base groups. It may not be necessary for Asp 60 to remove the proton from N_1 of indole, but Asp 60 could function by forming a strong hydrogen bond to polarize that nitrogen atom. Glu 49 was proposed as the proton acceptor for removing the hydroxyl proton, while the proton donor for the indolic C_3 position was not identified. One possibility is that Glu 49 performs two tasks, first protonating the C_3 position of the indole moiety, and subsequently accepting the hydroxyl proton of the glycerophosphate moiety, by suitable rotation within the active site. It is notable that the substitution E49D destroys the activity (Yutani et al., 1987; Miles et al., 1988), perhaps because of the lower rotational freedom of the shorter aspartyl side chain. Figure 5 shows a mechanism based on that of Nagata et al. (1989) that is consistent with the pH dependence of k_f (Figure 1).

The pH dependence of k_f is incompatible with a single ionization event (otherwise the slope of the log plot would approach −1 at high pH). However, the pH profile can be rationalized first by assuming that Asp 60 has a low pK value (e.g., pK < 5), while Glu 49 has a higher pK value that is not the same in the states ${}_{IGP}$A and ${}^{IND}_{GAP}$A (eq 1). The pK value of Glu 49 in the free α subunit has been estimated at about 7.5 (Yutani et al., 1984). The negative charge on Asp 60 would stabilize the positive charge that develops on N_1 of the indolenine intermediate (see Figure 5). For this mechanism, the pH dependencies of k_{12} and k_{-12} are given by

$$k_{12} = k_{12}^0h/(h + K) \quad (11)$$

$$k_{-12} = k_{-12}^0h/(h + K) \quad (12)$$

where the superscript 0 denotes the pH-independent rate constant, K and K' are dissociation constants of ${}_{IGP}$A and ${}^{IND}_{GAP}$A, respectively, and $h = 10^{-pH}$. The dependence of k_f on pH is then formally given by

$$k_f(h) = (\alpha h^2 + \beta h)/(h^2 + \gamma h + \delta) \quad (13)$$

where α, β, γ, and δ are constants given by

$$\alpha = k_{12}^0k_{13}k_{14}/D \quad (14)$$

$$\beta = k_{12}^0k_{13}k_{14}K'/D \quad (15)$$

$$\gamma = [k_{-12}^0(k_{13} + k_{14})K' + k_{13}k_{14}(K + K') + k_{-12}^0k_{14}K]/D \quad (16)$$

$$\delta = k_{13}k_{14}KK'/D \quad (17)$$

and

$$D = k_{14}(k_{-12}^0 + k_{13}) + k_{12}^0(k_{13} + k_{14})$$

From a nonlinear least-squares fit of eq 13 to the data in Figure 1, we obtain α = 0.18 s^{-1}, β = 7.1 × 10^{-9} M s^{-1}, γ =

Table IV: Comparison of Rate and Dissociation Constants for the α Subunit and the $\alpha_2\beta_2$ Complex Catalyzing the IGP Lyase (A) Reaction[a]

constant subunit	dimension	value	
		$\alpha_2\beta_2$ complex	α
k_{-11}	s^{-1}	>200	nd
k_{11}	$mM^{-1}\ s^{-1}$	>1400	nd
$K_d^{IGP} = k_{-11}/k_{11}$	mM	0.14	0.48
k_{14}	s^{-1}	126	10
k_{-14}	$mM^{-1}\ s^{-1}$	6.6	0.35
$K_d^{GAP} = k_{14}/k_{-14}$	mM	19.2	28.5
k_{12}	s^{-1}	2.4–7.5	>0.0016
k_{-12} ($=k_{-12}^0$)	s^{-1}	92–170 (340–440)	>0.5
$K_{12} = k_{12}/k_{-12}$		0.02–0.05	0.003
k_{13}	s^{-1}	6.3–44	nd
k_{-13}	$mM^{-1}\ s^{-1}$	76.7	nd
$K_{13} = k_{13}/k_{-13}$	mM	0.08–0.57	~25

[a] Values were calculated as described in the text by using the data for the α subunit from Weischet & Kirschner (1976b) and for the $\alpha_2\beta_2$ complex the data in Table I. Values refer to 0.1 M phosphate buffer, pH 7.6, 298 K. nd: not determined.

1.49×10^{-7} M, and $\delta = 9.5 \times 10^{-17}$ M^2. From the ratio β/α, we obtain $pK' = 7.4$. Also, the details of the pH dependence imply a value of pK in the range 7–7.5, which from the value of β/δ implies that k_{12}^0 is in the range 2.4–7.5 s^{-1}.

Because the value of k_{14} is known unequivocally (see above) we can use the expression for α to obtain a relationship between k_{13} and k_{-12}^0. Thus the minimum value of k_{-12}^0 is equal to k_r (about 92 s^{-1}), which gives a lower limit to k_{13} of about 2.5 s^{-1}. Also, for k_{12}^0 in the range 2.4–7.5 s^{-1}, the ratio k_{13}/k_{-12}^0 must remain less than 0.03 to obtain $\alpha = 0.18$ for $k_{12}^0 > 92$ s^{-1}. An upper limit to the value of k_{12}^0 can be obtained from eq 8 as follows: As $k_{12}^0 > 33k_{13}$, then at pH 7.6, $k_{-12} > 13k_{13}$ (using $pK' = 7.4$). Also, $k_{-11} > k_r = 92$ s^{-1}, and $k_{12} < 7.5$ s^{-1} at pH 7.6. Therefore $k_{-11}k_{-12} \gg k_{12}k_{13}$, and hence

$$k_r/K_m^{IND} \approx k_{-13} = 76.7\ mM^{-1}\ s^{-1}$$

With the empirical relationships $k_{-12} \gg k_{13}$ and $k_{-11} \gg k_{12}$, the expression for K_m^{IGP} (eq 3) simplifies to $K_m^{IGP} = k_{-12}k_{14}K_{11}/(k_{-12}k_{14} + k_{12}k_{13})$. Further, since $k_{14}/k_{12} > 17$ and $k_{-12}/k_{13} > 13$, $K_m^{IGP} \sim K_{11} = 0.14$ mM.

In stopped flow experiments (data not shown), we found that the binding of IGP to the enzyme was complete within the dead time of the stopped-flow instrument (2 ms) at a concentration of IGP of 100 μM. This rapid process is probably uncoupled from subsequent events, and therefore (cf. the mechanism of eq 1)

$$k_{obs} = k_{-11} + k_{11}[IGP] \geq 300\ s^{-1} \qquad (18)$$

However, K_{11} is about 140 μM, and we can write $k_{11} = k_{-11}/K_{11}$ or $k_{-11}(1 + [IGP]/K_{11}) \geq 300$. The absolute lower limit to k_{-11} is then about 200 s^{-1} (and the upper limit must be about 14000 s^{-1} for $k_{11} = 10^8$ M^{-1} s^{-1}). From the expression for k_r (eq 5), we can set an upper limit for k_{-12} (corresponding to the lower limit of k_{-11}) of 170 s^{-1}. Obviously the lower limit to k_{-12} is 92 s^{-1} at pH 7.6 (which would require k_{-11} to be at its maximum value). Even if $k_{-11} > 300$ s^{-1}, $k_{-12}(max) = 133$ s^{-1}. The range of possible values of k_{-12}^0 then follows, by using $pK' = 7.4$, as 238 $s^{-1} < k_{-12}^0 < 440$ s^{-1}. Substituting back into the expression for α (eq 14) leads to k_{13} in the range 6.3–44 s^{-1}.

The values of the rate constants of the $\alpha_2\beta_2$ complex are collected in Table IV, where they are compared with those for the isolated α subunit (Weischet & Kirschner, 1976b). Although only estimates exist for some constants and others are still unknown, the general impression is that assembly of the α subunit into the $\alpha_2\beta_2$ complex enhances the binding affinities and rate constants. For example, K_d^{IGP} decreases about 3-fold when the α subunit is incorporated into the $\alpha_2\beta_2$ complex. By contrast, K_d^{GAP} remains the same, but the on- and off-rate constants are increased at least 10-fold, reflecting a more efficient organization of the α site. The equilibrium constant for the cleavage reaction in the active site is in the range 0.02–0.05, favoring $_{IGP}$A. This equilibrium, which is even more unfavorable with the α subunit, and the relatively slow release of indole can account for the low turnover number of the cleavage reaction ($k_f = 0.067$ s^{-1}, cf. Table I). The affinity of the α subunit for indole (i.e., $1/K_{13}$) also increases substantially in the presence of GAP, as expected for an ordered sequential addition of substrates (Weischet & Kirschner, 1976b). The apparent second-order rate constant for binding GAP, when corrected for the hydration, is only 1.6×10^5 M^{-1} s^{-1}. It is therefore probable that GAP binding is followed by an isomerization that generates a conformation with higher affinity for indole.

At low pH, the forward IGP lyase reaction of the $\alpha_2\beta_2$ complex is limited both by the cleavage step (k_{12}) and release of the leading product indole (k_{13}), whereas at high pH the reaction is increasingly limited by the chemical cleavage step. For the isolated α subunit, where the affinity for indole is exceedingly low, it seems likely that the lyase reaction is limited by bond cleavage of IGP rather than release of indole. This notion is supported by the X-ray structure (Hyde et al., 1988), where release of indole into solution appears to be sterically hampered by the association with the β subunit in the $\alpha\beta$ heterodimer, which cannot happen in the isolated α subunit.

Activation of the A Reaction by L-*Serine.* The data in Figure 2 show that GAP can perturb the spectra of both serine and tryptophan bound to the active site of the β subunit. This communication is reciprocal as shown by the effects of serine on the steady-state parameters for the cleavage and synthesis 6-nitro-IGP (Table I). In general, the 6-nitro derivative is a poor substrate in the A reaction, though $k_f/K_m^{6\text{-nitro-IGP}}$ is comparable to that of the natural substrate. Serine increases k_{cat} 10-fold in both directions. Because the equilibrium constant for the 6-nitro derivative is not known, we cannot calculate the individual rate constants. However, the simplest explanation of the 10-fold increase in k_{cat} and smaller changes in the K_m values is a preferential stabilization of the transition state between E·IGP ⇄ E·GAP·IND, a smaller destabilization of the states E·IGP and E·GAP·IND, and a stabilization of the state E·GAP.

Transmission of Conformation Changes between the α and β Protomers. We have previously shown that IPP affects the relative populations of intermediates in the β protomer at equilibrium, during the steady state, and under transient conditions (Lane & Kirschner, 1981, 1983a,b). In this work, we have shown that it is the alkyl phosphate moiety of IPP that is largely responsible for perturbing the spectra of bound L-serine and bound L-tryptophan. The affinity of the free α subunit for indole is low (≈20–30 mM; Weischet & Kirschner, 1976a) but is much higher in the presence of GAP (see Table IV), apparently because GAP induces a conformational change in the α subunit. As IPP, GP, and GAP had similar effects on the β subunit, we conclude that they induce similar conformation states. Hence, the conformation change of the α subunit is sensed by the β subunit, even though the active sites are about 30 Å apart (Heilmann & Holzner, 1981; Lane & Kirschner, 1983c; Hyde et al., 1988), presumably by the transmission of conformational changes between the distant active sites.

The effects of ligands bound to the α subunit on ligands bound to the β subunit are reciprocal; L-serine increases the affinity for IPP 10-fold (Lane & Kirschner, 1983a). We have shown here that L-serine increases the rate of the A reaction for 6-nitro-IGP also about 10-fold. Presumably, a similar enhancement would be obtained for the natural substrate, namely, IGP.

We note that a 10-fold increase in k_f would not account fully for the turnover number in the AB reaction (k_{cat} = 1.5 s^{-1}). This is because, at pH 7.6, the reaction is partly limited by release of indole. Either serine also increases k_{13} (which would tend to increase K_d^{IND}), or the release of indole into solution is bypassed by channeling (Hyde et al., 1988). The details of the kinetics of the AB reaction, which provide insight into this question, are presented in the accompanying paper (Lane & Kirschner, 1991).

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REFERENCES

Crawford, I. P., & Yanofsky, C. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 1161–1170.

Creighton, T. E. (1970) *Eur. J. Biochem. 13*, 1–10.

Creighton, T. E., & Yanofsky, C. (1970) *Methods Enzymol. 18A* 365–380.

DeMoss, J. A. (1962) *Biochim. Biophys. Acta 62*, 279–293.

Drewe, W. F., Jr., & Dunn, M. F. (1986) *Biochemistry 25*, 2494–2501.

Dunn, M. F., Roy, M., Robustell, B., & Aguilar, V. (1987) in *Biochemistry of Vitamin B_6* (Korpela, A., & Christen, P., Eds.) pp 171–180, Birkhäuser Verlag, Basel.

Hall, A. N., Lea, D. J., & Rydon, H. N. (1962) *Biochem. J. 84*, 12–16.

Heilmann, H. D., & Holzner, M. (1981) *Biochem. Biophys. Res. Commun. 99*, 1146–1152.

Heyn, M. P., & Weischet, W. O. (1975) *Biochemistry 14*, 2962–2968.

Houben, K. F., & Dunn, M. F. (1990) *Biochemistry 29*, 2421–2429.

Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., & Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857–17871.

Kawasaki, H., Bauerle, R., Zon, G., Ahmed, S. A., & Miles, E. W. (1987) *J. Biol. Chem. 262*, 10678–10683.

Kirschner, K., Wiskocil, R., Foehn, M., & Rezeau, L. (1975) *Eur. J. Biochem. 60*, 513–523.

Lane, A. N. (1983) *Eur. J. Biochem. 133*, 531–538.

Lane, A. N., & Kirschner, K. (1981) *Eur. J. Biochem. 120*, 379–387.

Lane, A. N., & Kirschner, K. (1983a) *Eur. J. Biochem. 129*, 561–570.

Lane, A. N., & Kirschner, K. (1983b) *Eur. J. Biochem. 129*, 571–582.

Lane, A. N., & Kirschner, K. (1983c) *Eur. J. Biochem. 129*, 675–684.

Lane, A. N., & Kirschner, K. (1991) *Biochemistry* (following paper in this issue).

Lane, A. N., Paul, C. H., & Kirschner, K. (1984) *EMBO J. 3*, 279–287.

Miles, E. W. (1991) *Adv. Enzymol. Relat. Areas Mol. Biol. 104*, 93–172.

Miles, E. W., McPhie, P., & Yutani, K. (1988) *J. Biol. Chem. 263*, 8611–8614.

Nagata, S., Hyde, C. C., & Miles, E. W. (1989) *J. Biol. Chem. 264*, 6288–6296.

Racker, E., Klybas, V., & Schramm, M. (1959) *J. Biol. Chem. 234*, 2510–2516.

Smith, O. H., & Yanofsky, C. (1962) *Methods Enzymol. 5*, 794–806.

Stein, A. M., Lee, J. K., Anderson, C. D., & Anderson, B. M. (1963) *Biochemistry 2*, 1015–1017.

Tschopp, J., & Kirschner, K. (1980) *Biochemistry 18*, 4514–4521.

Walsh, C. (1979) in *Enzymatic reaction mechanisms*, pp 817–821, W. H. Freeman & Co., San Francisco.

Weischet, W. O., & Kirschner, K. (1976a) *Eur. J. Biochem. 64*, 313–320.

Weischet, W. O., & Kirschner, K. (1976b) *Eur. J. Biochem. 65*, 365–374.

Weischet, W. O., & Kirschner, K. (1976c) *Eur. J. Biochem. 65*, 375–385.

Yanofsky, C. (1956) *J. Biol. Chem. 233*, 171–176.

Yanofsky, C., & Rachmeler, M. (1958) *Biochim. Biophys. Acta 28*, 640–641.

Yutani, K., Ogasahara, K., Aoki, K., Kakuno, T., & Sugino, Y. (1984) *J. Biol. Chem. 259* 14076–14081.

Yutani, K., Ogasahara, K., Tsujita, T., Kanemoto, K., Matsumoto, M., Tanaka, S., Mijashita, T., Matsushiro, A., Sugino, Y., & Miles, E. W. (1987) *J. Biol. Chem. 262*, 13429–13433.