β-Elimination of Indole from L-Tryptophan Catalyzed by Bacterial Tryptophan Synthase: A Comparison between Reactions Catalyzed by Tryptophanase and Tryptophan Synthase

Syed Ashrafuddin Ahmed,[‡] Brian Martin,[§] and Edith Wilson Miles*[,‡]

Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, and Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: Although tryptophan synthase catalyzes a number of pyridoxal phosphate dependent β-elimination and β-replacement reactions that are also catalyzed by tryptophanase, a principal and puzzling difference between the two enzymes lies in the apparent inability of tryptophan synthase to catalyze β-elimination of indole from L-tryptophan. We now demonstrate for the first time that the β_2 subunit and the $\alpha_2\beta_2$ complex of tryptophan synthase from *Escherichia coli* and from *Salmonella typhimurium* do catalyze a slow β-elimination reaction with L-tryptophan to produce indole, pyruvate, and ammonia. The rate of the reaction is about 10-fold higher in the presence of the α subunit. The rate of indole production is increased about 4-fold when the aminoacrylate produced is converted to *S*-(hydroxyethyl)-L-cysteine by a coupled β-replacement reaction with β-mercaptoethanol. The rate of L-tryptophan cleavage is also increased when the indole produced is removed by extraction with toluene or by condensation with D-glyceraldehyde 3-phosphate to form indole-3-glycerol phosphate in a reaction catalyzed by the α subunit of tryptophan synthase. The amount of L-tryptophan cleavage is greatest in the presence of both β-mercaptoethanol and D-glyceraldehyde 3-phosphate, which cause the removal of both products of cleavage. The cleavage reaction is not due to contaminating tryptophanase since the activity is not inhibited by (3*R*)-2,3-dihydro-L-tryptophan, a specific inhibitor of tryptophanase, but is inhibited by (3*S*)-2,3-dihydro-L-tryptophan, a specific inhibitor of tryptophan synthase. The cleavage reaction is also inhibited by D-tryptophan, the product of a slow racemization reaction. Tryptophan synthase also catalyzes the β-elimination of *o*-nitrothiophenol from *S*-(*o*-nitrophenyl)-L-cysteine but at a much lower rate than that catalyzed by tryptophanse. Our results show that tryptophan synthase is similar to tryptophanase in its reaction mechanism and specificity but catalyzes several of the reactions investigated at very different rates.

Bacterial tryptophan synthase (EC 4.2.1.20) is a multienzyme complex formed by interaction of two α subunits and one β_2 dimer [for reviews, see Yanofsky & Crawford (1972) and Miles (1979, 1980, 1986)]. The last two steps in the biosynthesis of L-tryptophan occur at the active sites of the α and β_2 subunits in the $\alpha_2\beta_2$ complex. The α subunit cleaves indole-3-glycerol phosphate to indole and D-glyceraldehyde 3-phosphate (reaction 1, Scheme I legend). The β_2 subunit, which contains pyridoxal phosphate, condenses indole with L-serine to form L-tryptophan (reaction 2). When reactions 1 and 2 are coupled, the net reaction is reaction 3, the physiological reaction. The β_2 subunit alone and the closely related biodegradative enzyme tryptophanase (EC 4.1.99.1) [for a review, see Snell (1975)] also catalyze several pyridoxal phosphate dependent β-elimination reactions, including reaction 4, and β-replacement reactions, including reactions 2 and 5 (Goldberg & Baldwin, 1967; Miles et al., 1968; Kumagai & Miles, 1971). Tryptophanase also catalyzes the β-elimination of the indolyl moiety of L-tryptophan to form indole, pyruvate, and ammonia (reaction 6) and the β-replacement of the indolyl moiety of L-tryptophan by β-mercaptoethanol (reaction 7) (Newton et al., 1965; Watanabe & Snell, 1977). A principal difference between tryptophanase and tryptophan synthase is that tryptophan synthase appears unable to catalyze the elimination of indole from L-tryptophan necessary for reactions 6 and 7 (Kumagai & Miles, 1971). Studies in the accompanying paper (Miles et al., 1986b) using fluorine-19 nuclear magnetic resonance have detected a slow cleavage of 5-fluoro-L-tryptophan by the $\alpha_2\beta_2$ complex of tryptophan synthase (Miles et al., 1986b). The slow cleavage of L-tryptophan (reaction 6) and of *S*-(hydroxyethyl)-L-cysteine (reaction 11) and the slow catalysis of the β-replacement reactions 7–9 are reported here. In reactions 8 and 9, the indole produced in reactions 6 or 7 is condensed with D-glyceraldehyde 3-phosphate at the active site of the α subunit to form indole-3-glycerol phosphate.[1] D-Tryptophan, the product of the slow racemization of L-tryptophan (reaction 10) (Miles et al., 1986b), is an inhibitor of reactions 6–9 catalyzed by tryptophan synthase. Steps in reactions 1–11 are identified in Scheme I and will be elaborated under Discussion.

Experimental Procedures

Materials. Pyridoxal phosphate, NADH, and D- and L-tryptophan were obtained from Sigma or Pharmacia P-L Biochemicals. *p*-(Dimethylamino)benzaldehyde was from Fisher Scientific Co. Lactate dehydrogenase from rabbit muscle was obtained from Boehringer Mannheim Biochemicals as a crystalline suspension in ammonium sulfate. L-[U-*ring*-^{14}C]tryptophan and L-[2-*ring*-^{14}C]tryptophan (49 mCi/mmol)

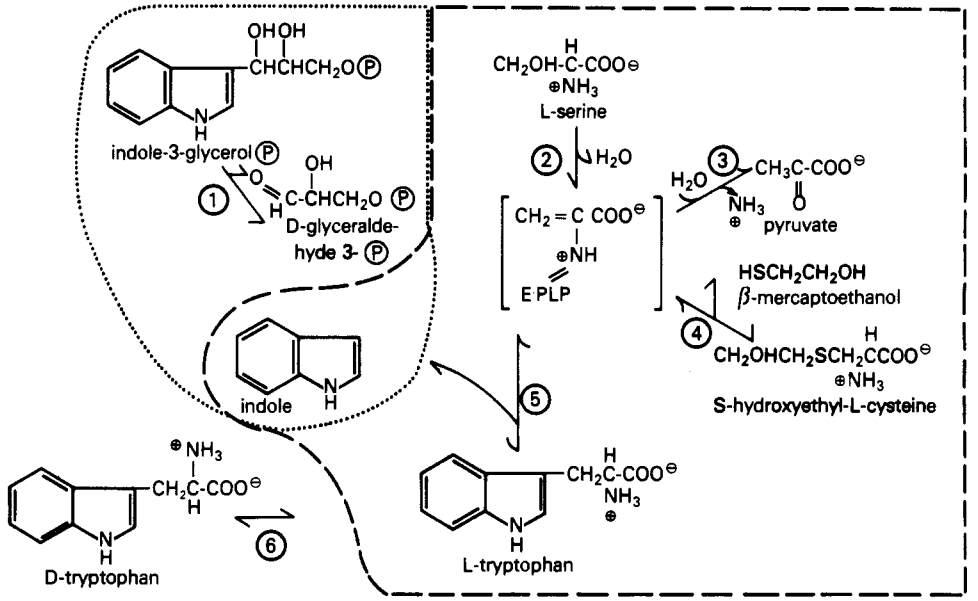
* Author to whom correspondence should be addressed at Building 8, Room 2A09.

‡ Laboratory of Biochemical Pharmacology.

§ Developmental and Metabolic Neurology Branch.

[1] We thank Dr. Kasper Kirschner for suggesting these experiments.

Scheme I[a]



no.	reaction	steps
1	indole-3-glycerol phosphate ⇄ indole + D-glyceraldehyde 3-phosphate	1
2	indole + L-serine → L-tryptophan + H_2O	2, 5
3	indole-3-glycerol phosphate + L-serine → L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O	1, 2
4	L-serine → pyruvate + ammonia	2, 3
5	β-mercaptoethanol + L-serine → *S*-(hydroxyethyl)-L-cysteine + H_2O	2, 4
6	L-tryptophan → indole + pyruvate + ammonia	5, 3
7	L-tryptophan + β-mercaptoethanol ⇄ *S*-(hydroxyethyl)-L-cysteine + indole	5, 4
8	L-tryptophan + D-glyceraldehyde 3-phosphate → indole-3-glycerol phosphate + pyruvate + ammonia	5, 1
9	L-tryptophan + D-glyceraldehyde 3-phosphate + β-mercaptoethanol → indole-3-glycerol phosphate + *S*-(hydroxyethyl)-L-cysteine	5, 4, 1
10	L-tryptophan ⇄ D-tryptophan	6
11	*S*-(hydroxyethyl)-L-cysteine → β-mercaptoethanol + pyruvate + ammonia	4, 3

[a] Steps in reactions catalyzed by tryptophan synthase and tryptophanase. Step 1, catalyzed by the α subunit of tryptophan synthase, is enclosed by a dotted line. Pyridoxal phosphate dependent steps 2–5, catalyzed by both tryptophanase and either the β_2 subunit of $\alpha_2\beta_2$ complex or tryptophan synthase, are enclosed by a dashed line. The key intermediate in these β-elimination and β-replacement reactions is the enzyme-bound pyridoxal phosphate (E·PLP) Schiff base of aminoacrylic acid, which is formed by the β-elimination in step 2, 4, or 5. The racemization reaction (step 6) has only been found for the $\alpha_2\beta_2$ complex of tryptophan synthase (Miles et al., 1986b).

were purchased from Research Products International Corp., and Aquasol was from New England Nuclear Corp. Indole-3-propanol phosphate was a generous gift from Dr. K. Kirschner (Biozentrum der Universität Basel, Switzerland). *S*-(Hydroxyethyl)-L-cysteine was a gift of Dr. I. P. Crawford and was synthesized by the method of Verderame (1961). The 3*S* and 3*R* diastereoisomers of 2,3-dihydro-L-tryptophan, prepared by reduction of L-tryptophan with pyridine–borane complex (Kikugawa, 1978) and separated by high-performance liquid chromatography (Phillips et al., 1985), were kindly provided by Dr. R. S. Phillips (National Institutes of Health). *S*-(*o*-Nitrophenyl)-L-cysteine (Suelter et al., 1976a) was a gift from Dr. C. H. Suelter (Michigan State University, East Lansing). 5-Fluoro-L-tryptophan was a gift of Dr. R. S. Phillips and was prepared as described (Miles et al., 1986b). D-Glyceraldehyde 3-phosphate was prepared from D-glyceraldehyde 3-phosphoric acid, diethyl acetal di(cyclohexylammonium salt) (Sigma) by the method of Racker et al. (1959), stored frozen at pH 2.5, and neutralized before use. The concentration was determined by a spectrophotometric assay coupled to glyceraldehyde-3-phosphate dehydrogenase (Sigma), which is also used for the assay of reaction 1 (Creighton & Yanofsky, 1970). This assay was used to demonstrate that the half-life of D-glyceraldehyde 3-phosphate was 2.4 h at 37 °C in 0.03 M sodium pyrophosphate buffer, pH 8.5, conditions used in one experiment below, but was much shorter (about 20 min) in the presence of 50 mM β-mercaptoethanol. Indole-3-glycerol phosphate was prepared enzymatically (Hardman & Yanofsky, 1965).

Enzymes and Enzyme Assays. The $\alpha_2\beta_2$ complex of tryptophan synthase was prepared from *Escherichia coli* strain *W3110 trpR cysB⁻ Δtrp LD102 trpB⁺ trpA⁺/F′ colVB cysB⁺ Δtrp LD102 trpB⁺ trpA⁺* (Jackson & Yanofsky, 1973) and from *Salmonella typhimurium* strain *TB1533 trpR782 Δtrp LEDC1682 trpB⁺ trpA⁺* (S. French, L. Hall, and R. Bauerle, unpublished results) as described by Miles et al. (1986a) and Ahmed et al. (1985).

The cleavage of L-tryptophan (reaction 6) catalyzed by the β_2 subunit or by the $\alpha_2\beta_2$ complex of tryptophan synthase at 37 °C was usually followed by measuring the indole produced from the color with *p*-(dimethylamino)benzaldehyde (Miles, 1970) or by measuring [^{14}C]indole derived from L-[U-*ring*-^{14}C]tryptophan or L-[2-*ring*-^{14}C]tryptophan, after its extraction with toluene. Unless otherwise stated, the standard reaction mixture contained in 1 mL 500 nmol of cold L-tryptophan or L-[*ring*-^{14}C]tryptophan, 20 nmol of pyridoxal phosphate, 1 μmol of ethylenediaminetetraacetic acid (EDTA), 50 μmol of sodium *N*,*N*-bis(2-hydroxyethyl)glycine buffer, pH 8.8, and 1 mg of enzyme. The reaction was stopped by adding 0.1 mL of 5 N NaOH to aliquots (0.2 mL) of reaction mixture. Indole was extracted with 0.3 mL of toluene.

The β-elimination of L-tryptophan (reaction 6) and β-elimination of L-serine (reaction 4) or *S*-(hydroxyethyl)-L-cysteine (reaction 11) were also assayed with coupled reactions in which pyruvate produced is reduced by NADH in the presence of excess lactate dehydrogenase (Morino & Snell, 1970), and the

absorbance change of 340 nm was recorded in a Cary 118 spectrophotometer. The amount of β-mercaptoethanol released in reaction 11 was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

The cleavage of L-tryptophan in the presence of D-glyceraldehyde 3-phosphate (reaction 8) or D-glyceraldehyde 3-phosphate plus β-mercaptoethanol (reaction 9) was also measured in a two-step reaction in which the pyridoxal phosphate dependent cleavage reaction is stopped by addition of hydroxylamine, an inhibitor of pyridoxal phosphate. Indole-3-glycerol phosphate formed in step 1 was converted to indole by reaction 1 catalyzed at the active site of the α subunit; this reaction is not inhibited by hydroxylamine (Miles, 1970). Hydroxylamine shifts the equilibrium of reaction 1 by forming an oxime with D-glyceraldehyde 3-phosphate. The standard reaction mixture contained in 0.1 mL 20 nmol of L-[2-*ring*-^{14}C]tryptophan, 5 μmol of sodium *N*,*N*-bis(2-hydroxyethyl)glycine, pH 7.8, 0.1 μmol of EDTA, 0.1 mg of $\alpha_2\beta_2$ complex, 0.44 μmol of glyceraldehyde 3-phosphate, and, where indicated, 3 μmol of β-mercaptoethanol. The reaction was stopped after incubation for the indicated times at 37 °C by addition of 10 μL of 1 M hydroxylamine hydrochloride in 1 N NaOH, incubated for 5 additional min at 37 °C, treated with 0.1 mL of 1 M NaOH, and extracted with 0.4 mL of toluene. Aliquots (0.2 mL) were counted.

The synthesis of L-tryptophan (reaction 2), the cleavage of L-tryptophan (reaction 6), and the analogous reactions with 5-fluoro-L-tryptophan were also determined by spectrophotometric assays utilizing the difference in absorbance between indole and L-tryptophan at 290 nm (Higgins et al., 1979; Miles et al., 1986a) or between 5-fluoroindole and 5-fluoro-L-tryptophan at 298 nm (this paper). The difference in absorbance at 298 nm between 0.1 mM 5-fluoro-L-tryptophan and 0.1 mM 5-fluoroindole was determined to be 0.18 by incubating 0.1 mM 5-fluoroindole with 1 mM L-serine and 10 μg of *E. coli* tryptophan synthase $\alpha_2\beta_2$ complex in 0.10 M potassium phosphate, pH 7.8, at 37 °C and measuring the difference spectrum after the reaction was complete (about 2 min) between the sample cell and a reference cell from which enzyme was omitted. Reaction mixtures for the cleavage reaction contained 0.1 mM L-tryptophan or 5-fluoro-L-tryptophan, 50 mM β-mercaptoethanol, and 1.0 mg/mL *E. coli* tryptophan synthase $\alpha_2\beta_2$ complex in 0.05 mM sodium pyrophosphate buffer, pH 9.0. The decrease in absorbance at 290 nm or at 298 nm was measured at 37 °C in a Cary 118 spectrophotometer utilizing the 0–0.1 absorbance scale. A decrease in absorbance at 290 nm of 0.00185 or at 298 nm of 0.0018 is equivalent to the cleavage of 1 nmol of L-tryptophan or of 5-fluoro-L-tryptophan, respectively. The cleavage of *S*-(*o*-nitrophenyl)-L-cysteine was followed as described for tryptophanase (Suelter et al., 1976a).

Amino Acid Analysis. Reaction mixtures to be analyzed were deproteinized by addition of one-tenth volume of 5 N HCl, followed by centrifugation to remove the precipitated protein. Aliquots of the supernatant, which was adjusted to pH 8.2 by addition of one-tenth volume of 5 N NaOH, were diluted with 0.2 M citrate buffer, pH 2.2, and used for analyses of tryptophan and *S*-(hydroxyethyl)cysteine on a Beckman 121MB amino acid analyzer equipped with a 4270 dual-channel integrator and norleucine as an internal standard. Other aliquots were treated with L-amino acid oxidase, which specifically oxidizes L-tryptophan and *S*-(hydroxyethyl)-L-cysteine, or D-amino acid oxidase, which specifically oxidizes D-tryptophan, under the conditions described below, and then used for amino acid analyses. One aliquot (0.1 mL) was diluted to 0.2 mL with 50 mM sodium pyrophosphate buffer, pH 8.3, containing 1.25 μg/mL crystalline catalase (Worthington), 0.5 mM FAD, and 2.5 μg of hog kidney D-amino acid oxidase (17.4 units/mg from Sigma), incubated at 37 °C for 4 h, diluted with 0.2 M citrate buffer, pH 2.2, and used for amino acid analyses. Another aliquot (0.1 mL) was diluted to 0.2 mL with 0.2 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)–0.1 M KCl, pH 7.0, containing 1.25 μg/mL crystalline catalase and 50 μg of snake venom L-amino acid oxidase (0.6 unit/mg from Sigma), incubated at 37 °C for 4 h, diluted with 0.2 M citrate buffer, pH 2.2, and used for amino acid analyses. Amino acid analyses of control solutions of L- or D-tryptophan treated under the same conditions showed that each amino acid was absent after treatment with L- or D-amino acid oxidase, respectively, and was completely recovered after treatment with D- or L-amino acid oxidase, respectively.

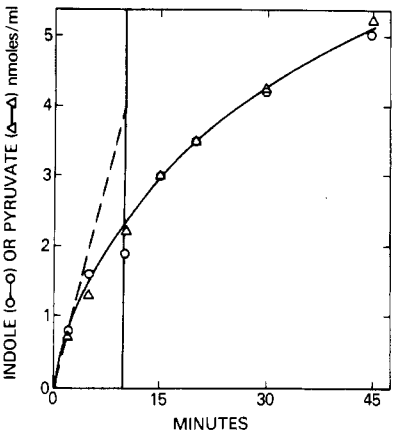


FIGURE 1: Cleavage of L-tryptophan catalyzed by the $\alpha_2\beta_2$ complex of tryptophan synthase from *S. typhimurium*. The reactions were carried out under the conditions described under Experimental Procedures. Indole was determined by its reaction with *p*-(dimethylamino)benzaldehyde (Ehrlich reagent). Pyruvate in an identical reaction mixture was determined spectrophotometrically by its reduction with NADH in the presence of excess lactate dehydrogenase. The initial rate was extrapolated with the dashed line to give the rate per 10 min at intersection with the solid line.

Isolation of Indole-3-glycerol Phosphate. Reaction mixtures to be analyzed for [2-*ring*-^{14}C]indole-3-glycerol phosphate formed by the cleavage of L-[2-*ring*-^{14}C]tryptophan in the presence of D-glyceraldehyde 3-phosphate were deproteinized as described above. An aliquot of the neutralized supernatant solution was applied to a 0.9 cm × 17 cm column of DEAE-Sephadex A-25 (Pharmacia) equilibrated with 25 mM ammonium bicarbonate and eluted with a linear gradient between 90 mL of 25 mM ammonium bicarbonate and 90 mL of 1 M ammonium bicarbonate at 6 mL/h collecting 0.6-mL fractions. Standard tryptophan and indole-3-glycerol phosphate elute in sharp peaks centered at fractions 85 and 115 under these conditions and are well separated. This method is adapted from a method used for the isolation of 6-nitroindole-3-glycerol phosphate.[2]

RESULTS

Cleavage of L-Tryptophan by Tryptophan Synthase. Incubation of L-tryptophan with the $\alpha_2\beta_2$ complex of *S. typhimurium* tryptophan synthase results in the stoichiometric formation of indole and pyruvate (Figure 1). Similar results were obtained when indole was measured by its reaction with *p*-(dimethylamino)benzaldehyde (Figure 1) or by analysis of

[2] Dr. Kasper Kirschner, personal communication.

Table I: Effects of Substrates and Inhibitors on Cleavage of L-Tryptophan (Experiments A–C) and of 5-Fluoro-L-tryptophan (Experiment D) Catalyzed by $\alpha_2\beta_2$ Complex of Tryptophan Synthase[a]

expt		addition other than L-tryptophan	product(s) determined	nmol (mg of $\alpha_2\beta_2$)$^{-1}$ (10 min)$^{-1}$	% of control
A	1	none (L-tryptophan only)	[U-*ring*-^{14}C]indole	2.2	[100]
			pyruvate	2.2	[100]
	2	0.1 mM indole-3-propanol phosphate	[U-*ring*-^{14}C]indole	1.2	60
	3	toluene[b]	[U-*ring*-^{14}C]indole	3.7	180
	4	2 mM D-glyceraldehyde phosphate	pyruvate	3.2	140
	5	4 mM D-glyceraldehyde phosphate	pyruvate	4.2	190
	6	12 mM β-mercaptoethanol	[U-*ring*-^{14}C]indole	6.7	320
	7	50 mM β-mercaptoethanol	[U-*ring*-^{14}C]indole	9.2	440
B[c]	1	10 mM β-mercaptoethanol	[2-*ring*-^{14}C]indole	4.9	[100]
	2	10 mM β-mercaptoethanol + 30 μM (3*S*)-2,3-H_2-L-Trp	[2-*ring*-^{14}C]indole	2.4	50
	3	10 mM β-mercaptoethanol + 30 μM (3*R*)-2,3-H_2-L-Trp	[2-*ring*-^{14}C]indole	4.9	100
C	1	50 mM β-mercaptoethanol	[2-*ring*-^{14}C]indole	9.2	[100]
	2	50 mM β-mercaptoethanol + 10 μM D-tryptophan	[2-*ring*-^{14}C]indole	6.7	73
	3	50 mM β-mercaptoethanol + 100 μM D-tryptophan	[2-*ring*-^{14}C]indole	5.2	56
D[d]	1	50 mM β-mercaptoethanol		15, 14, 12	[100]
	2	β-mercaptoethanol + 20 μM D-tryptophan		8, 9	60
	3	β-mercaptoethanol + 40 μM D-tryptophan		5, 6	40
	4	β-mercaptoethanol + 100 μM D-tryptophan		4, 4	30
	5	β-mercaptoethanol + 100 μM (3*S*)-2,3-H_2-L-Trp		4	30
	6	β-mercaptoethanol + 1.0 mM L-serine		0	0

[a] Tryptophan synthase $\alpha_2\beta_2$ complex [1 mg/mL in 0.05 M sodium *N*,*N*-bis(2-hydroxyethyl)glycine buffer, pH 8.8, containing 50 μM pyridoxal phosphate] was incubated with 0.5 mM L-[U-*ring*-^{14}C]tryptophan (experiment A) or L-[2-*ring*-^{14}C]tryptophan (experiments B and C) or 0.1 mM 5-fluoro-L-tryptophan (experiment D) and the indicated additions at 37 °C for 10 min. Products were determined as described under Experimental Procedures. [b] Experiment A-3 was carried out by adding 0.3 mL of toluene to 0.2 mL of reaction mixture and mixing every 30 s in a Vortex mixer. [c] Experiment B was in potassium phosphate, pH 7.8. [d] Since the spectrophotometric assay of 5-fluoro-L-tryptophan cleavage measures rather small changes in absorbance at 298 nm (0–0.03/10 min), the results are subject to some variation, and low levels of activity are hard to measure accurately. The time course of the reaction in experiment D-1 (data not shown) in nonlinear: 1.8 nmol of 5-fluoro-L-tryptophan are cleaved after 1 min, 14 nmol after 10 min, and 34 nmol after 60 min. Addition of L-serine (1.0 mM) after 60 min results in an increase in absorbance of 298 nm equivalent to the synthesis of 32 nmol of 5-fluoro-L-tryptophan from the L-serine and the 5-fluoroindole produced in the cleavage reactions.

Table II: Comparison of Rates of Reactions Catalyzed by β_2 Subunit and $\alpha_2\beta_2$ Complex of *S. typhimurium* Tryptophan Synthase and by Tryptophanase

no.	reaction	*S. typhimurium* tryptophan synthase [nmol (nmol of enzyme)$^{-1}$ min^{-1}][a]		*E. coli* tryptophanase [nmol (nmol of enzyme)$^{-1}$ min^{-1}][a]
		β_2 subunit	$\alpha_2\beta_2$ complex	
2	L-serine + indole → L-tryptophan + H_2O	4[b]	260[b]	570[c]
4	L-serine → pyruvate + NH_3	48[d]	1.0[d]	730[e]
5	β-mercaptoethanol + L-serine → *S*-(hydroxyethyl)-L-cysteine + H_2O	31[f]	97[f]	33[e]
6	L-tryptophan → indole + pyruvate + NH_3	0.004[b]	0.030[b]	1350[e]
7	L-tryptophan + β-mercaptoethanol → *S*-(hydroxyethyl)-L-cysteine + indole	0.007[b]	0.075,[b] 0.11[g]	
9	L-tryptophan + D-glyceraldehyde 3-phosphate + β-mercaptoethanol → indole-3-glycerol phosphate + *S*-(hydroxyethyl)-L-cysteine		0.40[g]	
11	*S*-(hydroxyethyl)-L-cysteine → β-mercaptoethanol + pyruvate + NH_3	0.23[h]	0.23[h]	
12	*S*-(*o*-nitrophenyl)-L-cysteine → *o*-nitrothiophenol + pyruvate + NH_3	3[i]	7.6[i]	7200[j]

[a] Nanomole of protomer using molecular weights calculated from the amino acid sequences: β promoter = 43 000 and α promoter = 28 700 (Wilhelm et al., 1982) and tryptophanase = 52 200 (Deeley & Yanofsky, 1981). [b] Reactions 2, 6, and 7 were carried out at 37 °C (see Experimental Procedures) with enzymes in 50 mL sodium *N*,*N*-bis(2-hydroxyethyl)glycine buffer, pH 8.8, containing 2 mM EDTA, 50 μM pyridoxal phosphate, and 50 mM β-mercaptoethanol where indicated. The initial rates were calculated from data collection at 2- to 10-min intervals. Reaction 2 was followed spectrophotometrically (Miles et al., 1986a). [c] V_{max}, Newton et al. (1965). [d] Reaction 4 was carried out at 37 °C with *S. typhimurium* enzymes in 0.1 M Tris-HCl buffer, pH 7.8, containing 50 μM pyridoxal phosphate as described under Experimental Procedures. [e] V_{max}, Watanabe & Snell (1977). [f] Miles et al. (1968). [g] Data from Figure 2. [h] Spectrophotometric assay in 0.05 M sodium pyrophosphate buffer, pH 8.8, containing 0.05 mM pyridoxal phosphate, 0.1 mg/mL NADH, and excess lactic dehydrogenase and *E. coli* α and β_2 subunits of tryptophan synthase. The increase in absorbance at 340 nm was followed at 37 °C. [i] Reaction 12 was carried out at 37 °C with enzymes in 50 mM potassium phosphate, pH 8.0, by the method of Suelter et al. (1976a). [j] V_{max}, Seulter et al. (1976b).

[U-*ring*-^{14}C]indole (Table I, experiment A-1). The more sensitive radioactive assay allowed us to show that the β_2 subunit alone catalyzes [U-*ring*-^{14}C]indole production from L-[U-*ring*-^{14}C]tryptophan at an 8-fold lower rate (Table II, reaction 6) and that the α subunit alone does not cleave L-tryptophan (data not shown). The results indicate that L-tryptophan is cleaved by tryptophan synthase into indole, pyruvate, and ammonia by reaction 6. (Ammonia was not determined.) The K_m for L-tryptophan in this reaction catalyzed by the $\alpha_2\beta_2$ complex was determined to be 30 μM from double-reciprocal plots of substrate concentration vs. reaction velocity (data not shown). D-Tryptophan was not cleaved under the conditions used in Figure 1. Similar results were obtained with *E. coli* tryptophan synthase.

Use of Inhibitors to Distinguish between Tryptophan Cleavage by Tryptophan Synthase or by Tryptophanase. In

Table III: Products of L-Tryptophan Cleavage Reactions[a]

	additions to basal test medium	reaction measured	nmol/mL tryptophan cleaved	indole produced	*S*-(hydroxyethyl)cysteine produced	indole-3-glycerol phosphate produced	D-tryptophan produced
1	50 mM β-mercaptoethanol	6	81[b]	76	78	*c*	12[d]
2	0.25 mM D-glyceraldehyde 3-phosphate	8	80[e]	10	0	70	*c*
3	0.25 mM D-glyceraldehyde 3-phosphate + 50 mM β-mercaptoethanol	9	230[e]	30	225	200	*c*

[a] Tryptophan synthase $\alpha_2\beta_2$ complex (1 mg/mL = 13.6 nmol/mL) in 0.05 M sodium pyrophosphate buffer, pH 8.8, containing 2 mM EDTA, 0.5 mM L-[2-*ring*-^{14}C]tryptophan (3500 cpm/nmol), 50 μM pyridoxal phosphate, and the indicated further additions, was incubated 4 h at 37 °C. The reaction mixture was analyzed as described under Experimental Procedures. [b] Decrease in total tryptophan (D + L) measured by amino acid analysis. [c] Not determined. [d] Determined by amino acid analysis after treatment with L-amino acid oxidase to destroy L-tryptophan. [e] Sum of Indole and indole-3-glycerol phosphate.

order to demonstrate that the tryptophan cleavage reaction measured is an intrinsic activity of tryptophan synthase and not due to some contaminating tryptophanase activity, we have tested the two diastereoisomers of 2,3-dihydro-L-tryptophan that are selective and specific inhibitors for each of the two enzymes (Phillips et al., 1984, 1985a). (3*S*)-2,3-Dihydro-L-tryptophan, which is a potent inhibitor of tryptophan synthase (K_I = 6 μM) but a weak inhibitor of tryptophanase (K_I = 1600 μM), inhibits the cleavage reaction (Table I, experiment B-2). In contrast, (3*R*)-2,3-dihydro-L-tryptophan, which is a potent inhibitor of tryptophanase (K_I = 2 μM) but a weak inhibitor of tryptophan synthase (K_I = 940 μM), does not inhibit the cleavage reaction (Table I, experiment B-3). The results indicate that the cleavage activity is a property of tryptophan synthase. This conclusion is also supported by our finding that the cleavage rate with the β_2 subunit is stimulated by addition of the α subunit (Table II, reaction 6) and that the cleavage rate with the $\alpha_2\beta_2$ complex is inhibited by indole-3-propanol phosphate (Table I, experiment A-2). Indole-3-propanol phosphate is a substrate analogue that binds to the α subunit of tryptophan synthase, increases the affinity of the $\alpha_2\beta_2$ complex for L-tryptophan, D-tryptophan, and L-serine, and inhibits the synthesis of L-tryptophan (reaction 1) by affecting the release of bound L-tryptophan (Lane & Kirschner, 1981, 1983). Indole-3-propanol phosphate does not inhibit tryptophanase (unpublished observations).

Stimulation of Cleavage Reaction by Removal of Products. Our finding that the rate of indole and pyruvate formation decreases with time (Figure 1) suggests that tryptophan synthase is inhibited by indole, bound aminoacrylate, or some other product. We tried two methods to remove indole from the reaction mixture: continuous extraction with toluene (Table I, experiment A-3) and conversion to indole-3-glycerol phosphate by a condensation with D-glyceraldehyde 3-phosphate catalyzed by the α subunit of tryptophan synthase (reaction 8, Scheme I legend) (Table I, experiments A-4 and A-5).[1] Both of these methods produced a significant increase in L-tryptophan cleavage. We tried to remove the second product of L-tryptophan cleavage, the Schiff base formed between enzyme-bound pyridoxal phosphate and aminoacrylic acid, by a coupled β-replacement reaction with β-mercaptoethanol (reaction 7, Scheme I legend). The results (Table I, experiments A-6 and A-7) show a large (3–4.4-fold) increase in L-tryptophan cleavage.

The rate of L-tryptophan cleavage in the presence of β-mercaptoethanol with (reaction 9) or without (reaction 7) D-glyceraldehyde 3-phosphate was also determined (Figure 2 and Table II). The extrapolated initial rate of reaction 9 [55 nmol mg^{-1} (10 min)$^{-1}$] is about 4-fold greater than the extrapolated initial rate of reaction 7 [15 nmol mg^{-1} (10 min)$^{-1}$] and about 14-fold greater than the extrapolated initial

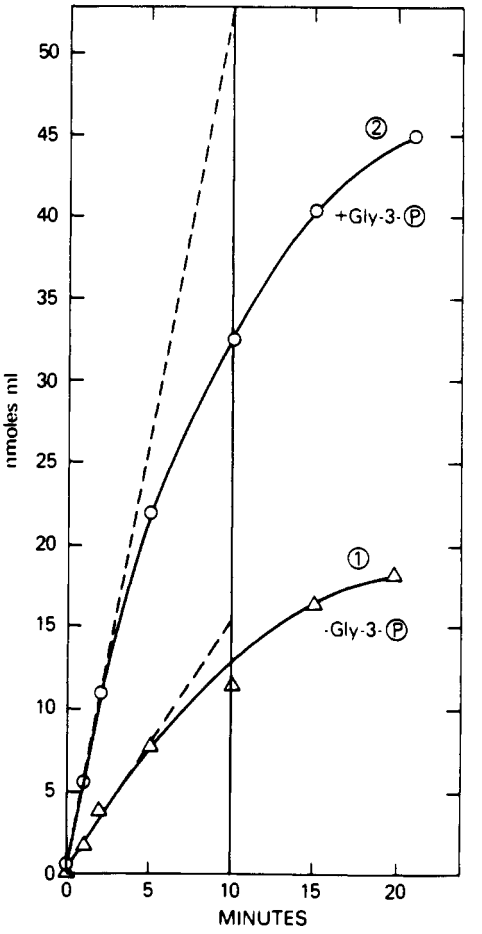


FIGURE 2: Cleavage of L-tryptophan catalyzed by the $\alpha_2\beta_2$ complex of tryptophan synthase from *E. coli* in the presence of 30 mM β-mercaptoethanol together with 4.4 mM D-glyceraldehyde 3-phosphate (curve 2, reaction 9) and with no further addition (curve 1, reaction 8). The reactions were carried out by the two-step assay described under Experimental Procedures. The initial rates were extrapolated with the dashed lines to give the rates per 10 min at the intersections with the solid line.

rate of reaction 6 [4 nmol mg^{-1} (10 min)$^{-1}$] (Figure 1). The rapid decrease in the rate of reaction 9 is probably a result of the formation of a thiohemiacetal between β-mercaptoethanol and D-glyceraldehyde 3-phosphate, which rapidly decreases the effective concentration of D-glyceraldehyde 3-phosphate (see Experimental Procedures). The rate observed for reaction 9 is significantly lower than V_{max} since D-glyceraldehyde 3-phosphate is not saturating under these conditions (see Discussion).

Identification of S-(Hydroxyethyl)-L-cysteine as a Product of L-Tryptophan Cleavage in the Presence of β-Mercaptoethanol. A reaction mixture of tryptophan synthase with

L-tryptophan and β-mercaptoethanol was incubated for 4 h at 37 °C, deproteinized, and analyzed for indole and amino acids as described in Table III, experiment 1, and under Experimental Procedures. Amino acid analysis showed only two ninhydrin-positive products with retention times identical with those of authentic *S*-(hydroxyethyl)-L-cysteine and of L- or D-tryptophan. The *S*-(hydroxyethyl)cysteine was identified as the L isomer since it was oxidized by L-amino acid oxidase but not by D-amino acid oxidase (see Experimental Procedures). Nearly stoichiometric amounts of indole (76 nmol/mL) and of *S*-(hydroxyethyl)-L-cysteine (78 nmol/mL) were formed from L-tryptophan (500 nmol/mL) by the $\alpha_2\beta_2$ complex (14 nmol/mL) under these conditions (Table III); no pyruvate was formed (data not shown). These results show that tryptophan synthase catalyzes the β-replacement reaction 7 (Scheme I).

The fact that we obtained stoichiometric amounts of *S*-(hydroxyethyl)-L-cysteine and indole as products of reaction 7 (Table III, experiment 1) and stoichiometric amounts of indole-3-glycerol phosphate and *S*-(hydroxyethyl)-L-cysteine as products of reaction 9 (see below and Table III, experiment 3) indicates that *S*-(hydroxyethyl)-L-cysteine is not degraded by the possible β-elimination reaction 11 or converted to L-serine by a β-replacement reaction. The latter possibility is also made improbable by the absence of serine in amino acid analyses of reaction mixtures from experiments in Table III. The previously unidentified β-elimination reaction of *S*-(hydroxyethyl)-L-cysteine (reaction 11) was measured by a coupled spectrophotometric assay and demonstrated to be catalyzed at a low rate by both the β_2 subunit and the $\alpha_2\beta_2$ complex (Table II).[1] The stoichiometric formation of pyruvate and β-mercaptoethanol from *S*-(hydroxyethyl)-L-cysteine was demonstrated (data not shown; see Experimental Procedures for methods). Since L-tryptophan is a strong competitive inhibitor (K_I = 34 μM) of *S*-(hydroxyethyl)-L-cysteine cleavage (K_m = 500 μM) (data not shown), the 500 μM L-tryptophan present in the reaction mixtures in experiments in Table III should completely displace the *S*-(hydroxyethyl)-L-cysteine formed (80–225 μM) and prevent the cleavage of *S*-(hydroxyethyl)-L-cysteine.

Identification of Indole-3-glycerol Phosphate as a Product of L-Tryptophan Cleavage in the Presence of D-Glyceraldehyde 3-Phosphate. Reaction mixtures of tryptophan synthase $\alpha_2\beta_2$ complex with L-[2-*ring*-^{14}C]tryptophan and D-glyceraldehyde 3-phosphate were incubated with or without β-mercaptoethanol for 4 h at 37 °C and analyzed for indole, indole-3-glycerol phosphate, and *S*-(hydroxyethyl)-L-cysteine as described in Table III, experiments 2 and 3, and under Experimental Procedures.

[2-*ring*-^{14}C]Indole-3-glycerol phosphate, a product in both experiments, was identified by its position on elution from a DEAE-Sephadex A-25 column (see Experimental Procedures) and by its absorption spectrum (Kirschner et al., 1975). The amount of L-tryptophan cleaved in the presence of both D-glyceraldehyde 3-phosphate and β-mercaptoethanol (Table III, experiment 3) is much greater than the amount cleaved in the presence of β-mercaptoethanol alone (Table III, experiment 1) or in the presence of D-glyceraldehyde 3-phosphate alone (Table III, experiment 2). Thus, the cleavage of L-tryptophan is facilitated by the simultaneous removal of both products: indole and aminoacrylic acid by reaction 9, Scheme I legend.

Inhibition of the Cleavage Reaction by D-Tryptophan, a Second Reaction Product. Since some D-tryptophan is formed from L-tryptophan concurrently with the cleavage of L-tryptophan (Table III) and since D-tryptophan is known to bind to the $\alpha_2\beta_2$ complex more tightly than L-tryptophan (Miles, 1980; Lane & Kirschner, 1981; Miles et al., 1986b), the formation of D-tryptophan may contribute to the observed decrease in the rate of the cleavage reaction with time. Our results (Table I, experiment C) show that D-tryptophan does produce some inhibition of the cleavage reaction 7.

Cleavage of 5-Fluoro-L-tryptophan. A spectrophotometric assay can also be used to measure the rate of cleavage of 5-fluoro-L-tryptophan (Table I, experiment D) and of L-tryptophan (data not shown) (see Experimental Procedures). Although the $\alpha_2\beta_2$ complex cleaves these two substrates at very similar rates (data not shown), the assay of 5-fluoro-L-tryptophan cleavage at 298 nm is preferable to the assay of L-tryptophan cleavage at 290 nm, since there is less absorbance at 298 nm from protein, β-mercaptoethanol, and added tryptophan inhibitors. D-Tryptophan, (3*S*)-2,3-dihydro-L-tryptophan, and L-serine all inhibit this cleavage reaction.

Comparison of Rates of β-Elimination Reaction with S-(o-Nitrophenyl)-L-cysteine and of Other Reactions Catalyzed by both Tryptophan Synthase and Tryptophanase. *S*-(*o*-Nitrophenyl)-L-cysteine is cleaved by tryptophanase according to reaction 12 (Seulter, 1976a). The reaction is conveniently

S-(*o*-nitrophenyl)-L-cysteine → *o*-nitrothiophenol + pyruvate + ammonia (12)

followed by measuring the decrease in absorbance of the substrate at 370 nm. The results (Table II) show that both the β_2 subunit and the $\alpha_2\beta_2$ complex catalyze the cleavage of *S*-(*o*-nitrophenyl)-L-cysteine but at rates much slower than those reported for tryptophanase (see Discussion). Pyruvate was also produced in the reaction mixture in stoichiometric amounts (data not shown; see Experimental Procedures). The initial rates of reactions 2, 6, and 7 at pH 8.8 and of reaction 4 at pH 7.8 are also reported. pH 8.8 is optimal for the cleavage of L-tryptophan, although the rate of cleavage at pH 7.8 is only 10% lower. In contrast, the rate of synthesis of L-tryptophan (reaction 2) is lower at pH 8.8 than at pH 7.8 (data not shown).

Discussion

Previous comparisons of tryptophan synthase and tryptophanase have concluded that these enzymes carry out β-elimination reactions and β-replacement reactions by similar mechanisms that involve a key intermediate, the Schiff base between enzyme-bound pyridoxal phosphate and aminoacrylate (Scheme I) (Kumagai & Miles, 1971; Phillips et al., 1984, 1985; Miles et al., 1986a). The β_2 subunit of tryptophan synthase is more active than the $\alpha_2\beta_2$ complex in the β-elimination reaction of L-serine (reaction 4, Scheme I legend) and is thus more similar to tryptophanase in its reaction specificity than is the $\alpha_2\beta_2$ complex. The failure of tryptophan synthase to catalyze the cleavage of L-tryptophan (reaction 6, Scheme I legend) has been puzzling, since the cleavage and synthesis are thought to occur by the same mechanism involving several reversible steps and an activated intermediate, the indolenine tautomer of L-tryptophan (Phillips et al., 1984, 1985).

We now report that tryptophan synthase can indeed cleave L-tryptophan slowly to indole, pyruvate, and ammonia (reaction 6). The reaction can be followed by estimating indole by its color reaction with Ehrlich reagent (Figure 1), by isolation of [U-*ring*-^{14}C]indole from the reaction mixture with L-[U-*ring*-^{14}C]tryptophan by toluene extraction (Table I, experiment A), by estimating the stoichiometric pyruvate formation (Figure 1), and by following the difference in absorbance between L-tryptophan and indole at 290 nm (data not shown) or between 5-fluoro-L-tryptophan and 5-fluoroindole

at 298 nm (Table I, experiment D). Since the reaction is inhibited by known inhibitors of tryptophan synthase [(3*S*)-2,3-dihydro-L-tryptophan, D-tryptophan, and indole-3-propanol phosphate] but not by an inhibitor of tryptophanase [(3*R*)-2,3-dihydro-L-tryptophan], the cleavage reaction appears to be an intrinsic activity of tryptophan synthase and not due to a contaminating tryptophanase activity.

L-Tryptophan cleavage has not been detected previously because it is very slow and is not linear with time. While this work was in progress, the analogous cleavage of 5-fluoro-L-tryptophan was also detected in fluorine-19 magnetic resonance experiments that used high concentrations of $\alpha_2\beta_2$ complex and long reaction times (Miles et al., 1986b). The nonlinearity of L-tryptophan cleavage could be due to the reversibility of step 5 (Scheme I) with the equilibrium favoring L-tryptophan synthesis. The hydrolysis of the aminoacrylate intermediates to pyruvate and ammonia (step 3, Scheme I) is thought to be very slow, since the $\alpha_2\beta_2$ complex catalyzed conversion of L-serine to pyruvate and ammonia (reaction 4, steps 2 and 3, Scheme I) under these conditions is also a very slow reaction (Table II). Our attempts to increase the rate of L-tryptophan cleavage by removing indole by a coupled reaction with D-glyceraldehyde 3-phosphate (reaction 8, steps 5 and 1, Scheme I), by removing the aminoacrylic intermediate by a coupled β-replacement reaction with β-mercaptoethanol (reaction 7, steps 5 and 4, Scheme I), or by removing both products (reaction 9, steps 5, 4, and 1) have increased both the rate and the extent of L-tryptophan cleavage significantly. The maximal velocity of reaction 9 must be higher than the value shown in Figure 2 and Table II [0.4 nmol (nmol of $\alpha\beta)^{-1}$ min^{-1}] since the enzyme is not saturated by the concentration of D-glyceraldehyde 3-phosphate used (4.4 mM). Weischet and Kirschner (1976) reported that the apparent K_m for D-glyceraldehyde 3-phosphate is very high (4 mM) because only 4% of the compound is in the active form and the remaining 96% is hydrated. However, even with this consideration, the maximal rate of L-tryptophan cleavage by tryptophan synthase is still about 10^4 times lower than the rate of L-tryptophan cleavage by tryptophanase (Table II). We also find that tryptophan synthase catalyzes the cleavage of *S*-(*o*-nitrophenyl)-L-cysteine to *o*-nitrothiophenol, pyruvate, and ammonia (reaction 12, Table III) but at a rate about 10^3 times lower than the corresponding rate with tryptophanase. The β-elimination reactions 6 and 12 with the substrates L-tryptophan and *S*-(*o*-nitrophenyl)-L-cysteine, respectively, are 2–8-fold faster with the $\alpha_2\beta_2$ complex than with the β_2 subunit whereas the β-elimination reaction 11 with *S*-(hydroxyethyl)cysteine is the same with the β_2 subunit and with the $\alpha_2\beta_2$ complex. In contrast, the β-elimination reaction 4 with L-serine is much slower with the $\alpha_2\beta_2$ complex than with the β_2 subunit (Table II). These results suggest that different steps may be rate limiting in different reactions and with the different enzyme species. The hydrolysis of aminoacrylate to pyruvate and ammonia (step 3) may be rate limiting in cleavage of L-serine catalyzed by the $\alpha_2\beta_2$ complex, the reaction that is much faster with the β_2 subunit. The $\alpha_2\beta_2$ complex is a more efficient catalyst than the β_2 subunit in reactions 2, 5, and 7, which do not involve step 3. In the case of L-tryptophan cleavage, reaction 6, the cleavage step 5 must be slower than the hydrolysis step 3 and rate limiting with both the $\alpha_2\beta_2$ complex and the less efficient β_2 subunit.

L-Tryptophan is also converted to D-tryptophan by a slow racemization reaction (reaction 10, Scheme I, step 6) (Miles et al., 1986b). The racemization reaction predominates over the cleavage reaction (ratio = 3:1) when a small molar excess of L-tryptophan to enzyme is used, because the product D-tryptophan is more tightly bound to the enzyme than is L-tryptophan and is slowly released. D-Tryptophan formed from L-tryptophan has been identified both by the characteristic difference absorption spectrum when bound to the $\alpha_2\beta_2$ complex (Miles et al., 1986b) and by amino acid analysis after destruction of residual L-tryptophan with L-amino acid oxidase (Table III). The ratio of racemization to cleavage is low in the presence of β-mercaptoethanol, which stimulates the cleavage of L-tryptophan (ratio = 1:6.3, Table III, experiment 1). The concomitant formation of D-tryptophan may partially inhibit the cleavage of L-tryptophan, especially at low L-tryptophan concentrations.

In conclusion, our finding that tryptophan synthase, like tryptophanase, can catalyze both the synthesis and cleavage of L-tryptophan (though at vastly different rates) is consistent with the proposal that both reactions occur through a common pathway that is used by both enzymes (Phillips et al., 1984). The key intermediate in this proposed pathway, the indolenine tautomer of L-tryptophan, is thought to be formed by the catalytic action of basic groups on the enzyme, which thus facilitate the β-elimination of indole, a very poor leaving group (Phillips et al., 1984). Thus, the cleavage of L-tryptophan by tryptophan synthase, though very slow, is most probably enzyme catalyzed and does not result solely from the intrinsic catalytic activity of the coenzyme; pyridoxal itself has not been reported to cleave L-tryptophan in model systems. In contrast, it has been suggested that some other very slow reactions observed with both pyridoxal phosphate enzymes and model systems with pyridoxal may result from the intrinsic activity of the coenzyme itself rather than from the participation of catalytic groups on the enzyme[3] (Miles et al., 1986b). The differences in rates of reaction catalyzed by tryptophanase and tryptophan synthase may be due to the exact stereochemistry of the reaction intermediates bound to the two enzymes (Lane & Kirschner, 1983). We have recently provided evidence that these two enzymes catalyze their respective reactions via *enantiomeric* indolenine intermediates and thus differ in the stereochemistry of their active sites (Phillips et al., 1985).

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Isomerization of (3*S*)-2,3-Dihydro-5-fluoro-L-tryptophan and of 5-Fluoro-L-tryptophan Catalyzed by Tryptophan Synthase: Studies Using Fluorine-19 Nuclear Magnetic Resonance and Difference Spectroscopy

Edith Wilson Miles,*,‡ Robert S. Phillips,§,‖ Herman J. C. Yeh,⊥ and Louis A. Cohen§

Laboratory of Biochemical Pharmacology, Laboratory of Chemistry, and Laboratory of Analytical Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: We are exploring the active site and the mechanism of the pyridoxal phosphate dependent reactions of the bacterial tryptophan synthase $\alpha_2\beta_2$ complex by use of substrate analogues and of reaction intermediate analogues. Fluorine-19 nuclear magnetic resonance studies and absorption spectroscopy are used to study the binding and reactions of the D and L isomers of 5-fluorotryptophan, of tryptophan, and of (3*S*)- and (3*R*)-2,3-dihydro-5-fluorotryptophan. Tryptophan synthase specifically and tightly binds the 3*S* diastereoisomer of both 2,3-dihydro-5-fluoro-D-tryptophan and 2,3-dihydro-5-fluoro-L-tryptophan, whereas it binds 5-fluoro-D-tryptophan more tightly than 5-fluoro-L-tryptophan. Unexpectedly, we find that the D and L isomers of 5-fluorotryptophan, of tryptophan, and of (3*S*)-2,3-dihydro-5-fluorotryptophan are slowly interconverted by isomerization reactions. Since these isomerization reactions are 10^3–10^5 times slower than the β-replacement and β-elimination reactions catalyzed by tryptophan synthase, they have no biochemical significance in vivo. However, the occurrence of these slow reactions does throw some light on the nature of the active site of tryptophan synthase and its requirements for substrate binding. Our results raise the interesting question of whether tryptophan synthase itself serves a catalytic role in these slow reactions or whether the enzyme simply binds the substrate and pyridoxal phosphate stereospecifically and thus promotes the intrinsic catalytic activity of pyridoxal phosphate.

We are investigating the interaction of tryptophan synthase of *Escherichia coli* and of *Salmonella typhimurium* with substrates, products, and analogues of reaction intermediates to increase our understanding of the specificity, mechanism, and stereochemistry of the pyridoxal phosphate dependent reactions of this enzyme. The $\alpha_2\beta_2$ complex of tryptophan synthase (EC 4.1.2.20) catalyzes the synthesis of L-tryptophan from L-serine and indole (eq 1) as well as a number of other

$$\text{L-serine} + \text{indole} \rightarrow \text{L-tryptophan} \quad (1)$$

pyridoxal phosphate dependent β-addition and β-elimination reactions that occur at the active site of the β_2 subunit. The cleavage of indole-3-glycerol phosphate occurs at the active site of the α subunit [for review, see Miles (1979 and 1986)]. The pyridoxal phosphate Schiff base of the indolenine tautomer

* Author to whom correspondence should be addressed at Building 8, Room 2A09.
‡ Laboratory of Biochemical Pharmacology.
§ Laboratory of Chemistry.
‖ Present address: Department of Chemistry, University of Georgia, Athens, GA 30602.
⊥ Laboratory of Analytical Chemistry.