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Stereochemistry and Mechanism of a New Single-Turnover, Half-Transamination Reaction Catalyzed by the Tryptophan Synthase $\alpha_2\beta_2$ Complex

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ABSTRACT: Tryptophan synthase is a versatile enzyme that catalyzes a wide variety of pyridoxal phosphate dependent reactions that are also catalyzed in model systems. These include β-replacement, β-elimination, racemization, and transamination reactions. We now show that the apo-$\alpha_2\beta_2$ complex of tryptophan synthase will bind two unnatural substrates, pyridoxamine phosphate and indole-3-pyruvic acid, and will convert them by a single-turnover, half-transamination reaction to pyridoxal phosphate and L-tryptophan, the natural coenzyme and a natural product, respectively. This enzyme-catalyzed reaction is more rapid and more stereospecific than an analogous model reaction. The *pro-S* 4′-methylene proton of pyridoxamine phosphate is removed during the reaction, and the product is primarily L-tryptophan. We conclude that pyridoxal phosphate enzymes may be able to catalyze some unnatural reactions involving bound reactants and bound coenzyme since the coenzyme itself has the intrinsic ability to promote a variety of reactions.

We are examining the reaction specificity of the tryptophan synthase $\alpha_2\beta_2$ complex of *Escherichia coli* (EC 4.1.2.20) and the stereochemistry of the reactions catalyzed in order to understand the relative roles of the protein and of the pyridoxal phosphate coenzyme. This study was designed to test whether the apo-$\alpha_2\beta_2$ complex formed by removal of pyridoxal phosphate from the holo-$\alpha_2\beta_2$ complex will catalyze the single-turnover transamination reaction 1. Although pyridoxamine phosphate and indole-3-pyruvic acid are not natural substrates of this enzyme, the products pyridoxal phosphate and L-

pyridoxamine phosphate + indole-3-pyruvic acid → pyridoxal phosphate + L-tryptophan (1)

tryptophan are the natural coenzyme and product, respectively, and bind tightly. Since the pyridoxal phosphate is not released from the holo-$\alpha_2\beta_2$ complex, the reaction is stoichiometric to the apo-$\alpha_2\beta_2$ complex initially added. That is, it is a single-turnover, half-transamination reaction. A reaction similar to reaction 1 is carried out by an artificial enzyme system in which a pyridoxamine derivative is covalently attached to a β-cyclodextrin (Breslow, 1982, 1983, 1986; Breslow et al., 1980; Breslow & Czarnik, 1983). If this reaction occurred at the active site of an enzyme, it would be expected to occur stereospecifically and at a more rapid rate.

This new reaction is best understood in the context of previous studies of the mechanism and stereochemistry of the pyridoxal phosphate dependent reactions of the tryptophan synthase β_2 subunit and $\alpha_2\beta_2$ complex (reactions 2–7) [for

indole + L-serine → L-tryptophan + H_2O (2)

L-serine → pyruvate + ammonia (3)

L-serine + β-mercaptoethanol → *S*-(hydroxyethyl)-L-cysteine (4)

L-serine + β-mercaptoethanol + pyridoxal-P → *S*-pyruvylmercaptoethanol + pyridoxamine phosphate (5)

L-tryptophan → indole + pyruvate + NH_3 (6)

L-tryptophan ↔ D-tryptophan (7)

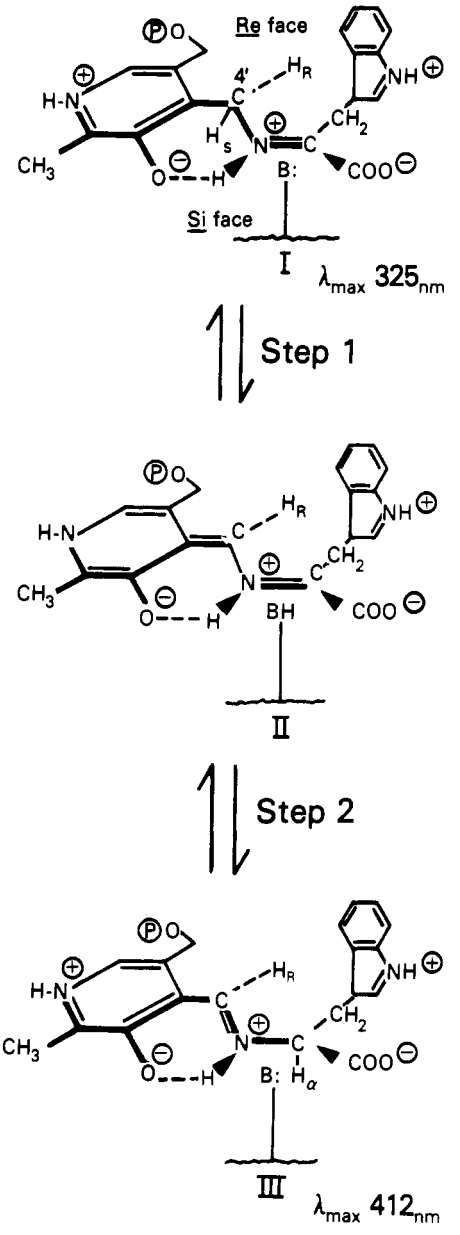
reviews, see Yanofsky and Crawford 1972) and Miles (1979)]. The β_2 subunit of tryptophan synthase contains pyridoxal phosphate and will catalyze a number of pyridoxal phosphate dependent β-elimination and β-replacement reactions, including reactions 2–4 (Kumagai & Miles, 1971) and a thiol-dependent transamination reaction (reaction 5) (Miles et al., 1968). The $\alpha_2\beta_2$ complex catalyzes the β-replacement reactions 2 and 4 but not reactions 3 and 5.

The thiol-dependent transamination reaction (reaction 5) and the recently discovered cleavage of L-tryptophan (reaction 6) (Ahmed et al., 1986), racemization of L-tryptophan (reaction 7) (Miles et al., 1986), and isomerization of (3*S*)-2,3-dihydro-L-tryptophan (Miles et al., 1986) are very slow reactions.

Studies of the stereochemical course of the pyridoxal phosphate dependent reactions of tryptophan synthase have defined the geometry of the coenzyme–substrate complex of reaction intermediates at the active site (Skye et al., 1973; Fuganti et al., 1974; Dunathan & Voet, 1974; Schleicher et al., 1976) as well as the stereochemistry of sodium borohydride reduction of the internal pyridoxal phosphate–lysine Schiff base and the Schiff base formed between pyridoxal phosphate and an α-aminoacrylate intermediate (Miles et al., 1982). All of the enzyme-mediated reactions and sodium borohydride reduction occur on only one face of the coenzyme–substrate complex (the *si* face), suggesting that one face of the coenzyme–substrate complex (the *re* face) is shielded and the other face (the *si* face) is exposed.

In the thiol-dependent transamination, the pyridoxal phosphate Schiff base intermediate is protonated on the *si* face at C-4′. As with other transaminases studied, the *pro-S* hydrogen at C-4′ of the pyridoxamine phosphate is the hydrogen that is introduced in the transamination reaction (Dunathan & Voet, 1974; Floss & Vederas, 1982). The finding that the hydrogen from C_α of L-serine is transferred to the extent of 70% to C-4′ of the cofactor when the reaction is carried out in D_2O indicates that intramolecular proton transfer is catalyzed by a monoprotic base (Tsai et al., 1978). This conclusion is consistent with the results of Miles and Kumagai (1974) implicating a histidyl residue in the abstraction of the α-proton of L-serine.

Scheme I



If the transamination reaction 1 is catalyzed by tryptophan synthase, it would be expected to follow the stereochemical course (Scheme I) similar to that shown for other pyridoxal phosphate dependent transamination reactions (Floss & Vederas, 1982). In step 1, the *pro-S* proton is removed by the base B, from *si* face of I, the Schiff base between pyridoxamine phosphate and indole-3-pyruvic acid, to yield the quinonoid intermediate II. In step 2, base BH protonates C_α to yield III, the Schiff base between pyridoxal phosphate and L-tryptophan. Since intermediates I–III should have very different absorption spectra, reaction 1 could be followed spectroscopically. Since tryptophan synthase must bind pyridoxal phosphate in order to catalyze the β-replacement reaction 2, the rate of formation of pyridoxal phosphate in reaction 1 can also be measured by removing aliquots of enzyme and determining enzyme activity in reaction 2 in the absence of added pyridoxal phosphate.

If reaction 1 does occur, we will determine whether the reaction is stereospecific, has the same stereospecificty as other

transamination reactions, and whether either of the two proton-transfer steps is rate-limiting. The first two questions can be answered by using [4′-^{3}H]pyridoxamine phosphate labeled in the *pro-R* or in the *pro-S* position. These compounds can be prepared from [4′-^{3}H]pyridoxal phosphate or from pyridoxal phosphate in ^3H_2O with the β_2 subunit of tryptophan synthase and the thiol-dependent transamination reaction 5 of known stereochemical course (Dunathan & Voet 1974). The last questions may be answered by comparing the rates of reaction with [4′,4′-$^2H_2$]pyridoxamine phosphate and 4′,4′-$^1H_2$]pyridoxamine phosphate. Finally, we will determine the optical purity of the tryptophan product.

The results obtained show that the $\alpha_2\beta_2$ complex does bind pyridoxamine phosphate and indole-3-pyruvic acid, that reaction 1 does occur, and that the *pro-S* proton is stereospecifically removed from the *si* face of pyridoxamine phosphate. These results and the kinetic results will be discussed in relation to the relative roles of the enzyme and the enzyme-bound pyridoxal phosphate in catalyzing reaction 1.

Experimental Procedures

Materials

Sodium pyruvate, sodium phenylpyruvate, indole-3-pyruvic acid, pyridoxal phosphate, pyridoxamine phosphate, and L-tryptophan were products of Sigma Chemical Co. A stock solution of indole-3-pyruvic acid (0.1 M in ethanol) was stored at −20 °C and was diluted to 1 mM in 0.05 M sodium *N*,*N*-bis(2-hydroxyethyl)glycine, pH 7.8, before use. (Hydroxyethyl)-L-cysteine was a gift of Dr. Irving P. Crawford and was synthesized by the method of Verderame (1961). *S*-(Hydroxyethyl)thiopyruvic acid was prepared from *S*-(hydroxyethyl)-L-cysteine by reaction with L-amino acid oxidase (Miles et al., 1968). [4′-^{3}H]Pyridoxal phosphate (≃9600 cpm/nmol) was synthesized by a modification (Raibaud & Goldberg, 1974) of the method of Stock et al. (1966).

Enzymes and Enzyme Assays. The apo- and holo-β_2 subunit and the $\alpha_2\beta_2$ complex of tryptophan synthase of *Escherichia coli* were prepared as described by Higgins et al. (1979). Apo-$\alpha_2\beta_2$ complex was prepared from holo-$\alpha_2\beta_2$ complex as described previously (Miles et al., 1982). Tryptophan synthase activity in reaction 2 was determined by the spectrophotometric assay (Higgins et al., 1979) in the presence or absence of added pyridoxal phosphate. Tryptophanase was prepared from *E. coli* B/lt7-A by a modification of the method of Suelter et al. (1976).

Methods

Synthesis of (4′R)- and (4′S)-[4′-^{3}H]Pyridoxamine Phosphate. (4′*R*)-[^{3}H]Pyridoxamine phosphate was produced by the thiol-dependent transamination reaction catalyzed by the apo-β_2 subunit of tryptophan synthase (Miles et al., 1968; Dunathan & Voet, 1974). Apo-β_2 subunit [0.013 mM in 1.0 mL of 0.05 M potassium phosphate buffer, pH 7.8, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 25 mM L-serine, and 25 mM dithioerythritol at 37 °C] was treated with 10 aliquots (14.5 nmol per aliquot) of [4′-^{3}H]pyridoxal phosphate (sp act. ≃9600 cpm/nmol) over a period of 40 min. After 46 min, the reaction was applied to a 0.9 cm × 32 cm column of Sephadex G-25 fine in water. The combined radioactive fractions were fractionated on a 1-mL column of Dowex AG 1X8 acetate (Miles et al., 1982). Fractions containing pyridoxamine phosphate were identified by their absorption spectra (Peterson & Sober, 1954) and lyophilized. The specific activity of the (4′*R*)-[4′-^{3}H]pyridoxamine phosphate (8600 cpm/nmol) was determined from the absorbance (Peterson & Sober, 1954) and by measurement of radioactivity in a Beckman LS-345 scintillation counter with Aquasol (New England Nuclear Corp.) as the scintillator solution. The yield on the basis of radioactivity was 90%.

(4′*S*)-[^{3}H]Pyridoxamine phosphate was prepared by the same thiol-dependent transamination reaction. Three tubes containing (i) 0.6 mg of holo-β_2 subunit and 5 μmol of potassium phosphate, pH 7.8, (ii) 50 μmol of L-serine and 50 μmol of dithioerythritol, and (iii) 2000 nmol of pyridoxal phosphate were lyophilized. The contents of these tubes were dissolved in 1 mL of ^3H_2O (25 mCi/mL) (New England Nuclear) and combined, incubated at 37 °C for 3 h, and applied to a PD-10 column (Pharmacia). The combined radioactive protein-free fractions were applied to a 1.0-mL column of Dowex 1 acetate. The column was washed with 40 mL of H_2O and eluted with 0.2 N HOAc. The eluted radioactive fractions were lyophilized and dissolved in 0.5 mL of H_2O. The specific activity of the (4′*S*)-[^{3}H]pyridoxamine phosphate (107 cpm/nmol) was determined as described above.

Preparation of [4′,4′-$^2H_2$]Pyridoxamine Phosphate. [4′,4′-$^2H_2$]Pyridoxamine phosphate was prepared by a nonenzymatic transamination reaction in ^2H_2O (Benecky et al., 1985), isolated by ion-exchange chromatography on a 0.9 cm × 23 cm column of Dowex AG 1X8 [scaled-up method of Miles et al. (1982)], and characterized by proton NMR (Benecky et al., 1985).

Results

Spectroscopic Evidence for Transamination between Pyridoxamine Phosphate and Indole-3-pyruvic Acid. Incubation of apo-$\alpha_2\beta_2$ complex with stoichiometric amounts of pyridoxamine phosphate and indole-3-pyruvic acid results in a time-dependent increase in the absorbance at 412 nm characteristic of the enzyme-bound pyridoxal phosphate in the holoenzyme (Figure 1). No changes in absorbance were observed when apo-$\alpha_2\beta_2$ was omitted (data not shown). The extent of reconstitution of the apo-$\alpha_2\beta_2$ complex with pyridoxal phosphate determined by measuring the activity of the enzyme in reaction 2 in the absence of pyridoxal phosphate shows a time-dependent increase proportional to the increase in absorbance at 412 nm (inset, Figure 1).

Kinetics of Transamination Reaction. As shown in the inset in Figure 1, the rate of the transamination reaction can conveniently be followed by measuring the rate of reconstitution or reactivation of the apo-$\alpha_2\beta_2$ complex (i.e., tryptophan synthase activity in the absence of added pyridoxal phosphate). The rate of reaction of the apo-$\alpha_2\beta_2$ complex follows pseudo-first-order kinetics when the apo-$\alpha_2\beta_2$ complex is incubated with excess pyridoxamine phosphate and indole-3-pyruvic acid and is dependent on the concentration of pyridoxamine phosphate and indole-3-pyruvic acid (Figures 2). The apparent dissociation constants for pyridoxamine phosphate (0.2 mM) and indole-3-pyruvic acid (0.1 mM) are calculated from the plot of the half-times of reactivation ($t_{1/2}$) vs. 1/[substrate] (Figure 2B). The apparent dissociation constants are not true K_d values since neither one of the constant substrates is present at saturating levels in Figure 2. Addition of 0.01 M potassium phosphate strongly inhibits the transamination reaction (Figure 2A), presumably by inhibiting binding of pyridoxamine phosphate (see Discussion).

Transamination with Other α-Keto Acid Substrates. Several other α-keto acids [pyruvic acid, phenylpyruvic acid, *S*-(hydroxyethyl)thiopyruvic acid, and α-ketoglutaric acid] show some activity as substrates at 0.2 mM concentration but are much less reactive than indole-3-pyruvic acid at the same concentration (Table I).

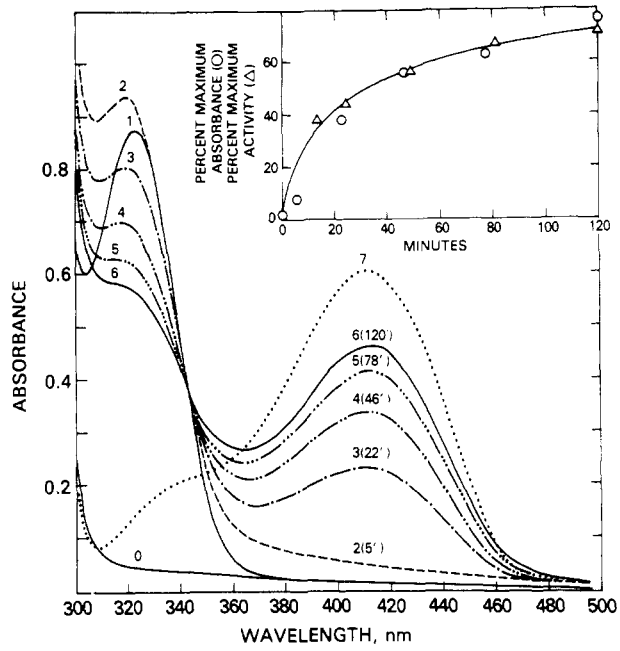


FIGURE 1: Spectroscopic evidence for transamination between pyridoxamine phosphate and indole-3-pyruvic acid. Absorption of the apo-$\alpha_2\beta_2$ complex [0.1 mM $\alpha\beta$ protomer in 0.05 M sodium *N,N*-bis(2-hydroxyethyl)glycine buffer, pH 7.8, containing 1 mM EDTA] were recorded at 25 °C before (curve 0) and after addition of 0.1 mM pyridoxamine phosphate (curve 1) and at 5, 22, 46, 78, and 120 min after addition of 0.1 mM indole-3-pyruvic acid (curves 2–6). The absorption spectrum of holo-$\alpha_2\beta_2$ complex (0.1 mM $\alpha\beta$ protomer) is shown in curve 7. The activity in reaction 2 of aliquots of the enzyme reaction mixture was assayed at intervals in the presence or absence of pyridoxal phosphate. The percent activity [(activity − pyridoxal phosphate)/(activity + pyridoxal phosphate) × 100] is plotted vs. time (Δ) in the inset. The percent maximum absorbance at 412 nm (i.e., percent of absorbance at 412 nm = 0.6 in curve 7) is plotted vs. time (O) in the inset.

Table I: Kinetics of Transamination of Pyridoxamine Phosphate and α-Keto Acid Substrates[a]

α-keto acid	$t_{1/2}$ (min)
indole-3-pyruvic acid	12
pyruvic acid	40
phenylpyruvic acid	48
S-(hydroxyethyl)thiopyruvic acid	67
α-ketoglutaric acid	≃180

[a] Apo-$\alpha_2\beta_2$ complex (0.02 mM) was incubated with 0.2 mM pyridoxamine phosphate and 0.2 mM α-keto acid and assayed as described in Figure 2. Half-times of reaction ($t_{1/2}$) are calculated from the kinetic data plotted as in Figure 2A.

Products and Stereochemical Course of Transamination Reaction. Reaction mixtures of apo-$\alpha_2\beta_2$ complex with pyridoxamine phosphate and indole-3-pyruvic acid were analyzed for tryptophan and enzyme-bound pyridoxal phosphate as described in Table II, experiment 1. The yields of pyridoxal phosphate (70%) and tryptophan (48%) have not been corrected for losses. Most of the tryptophan was in the L form (see Discussion).

The use of pyridoxamine phosphate stereospecifically tritiated at C-4′ as substrate enabled us to determine the stereochemistry of proton transfer. Since all of the label was retained when (4′*R*)-[^{3}H]pyridoxamine phosphate was converted to pyridoxal phosphate (Table II, experiment 2) and since all of the label was lost when (4′*S*)-[^{3}H]pyridoxamine phosphate was converted to pyridoxal phosphate, the *pro-S* 4′-hydrogen was stereospecifically removed in the transamination reaction and proton removal must occur on the *si* face. The absence of label in the isolated tryptophan indicates

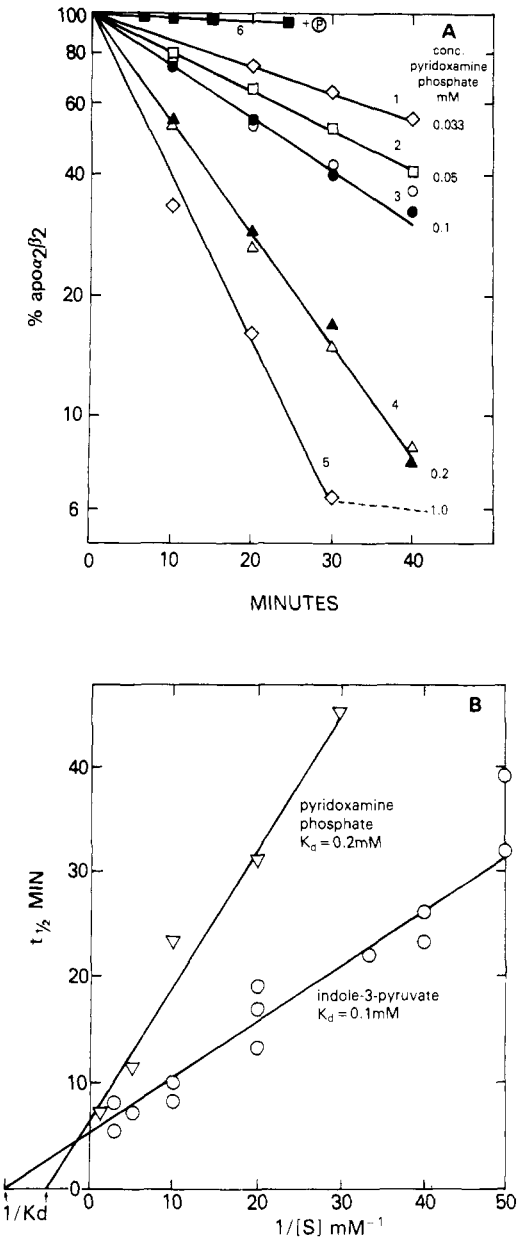


FIGURE 2: Effect of concentration of indole-3-pyruvic acid and of pyridoxamine phosphate upon the rate of transamination. Apo-$\alpha_2\beta_2$ complex [0.02 mM $\alpha\beta$ protomer in 0.05 M sodium *N,N*-bis(2-hydroxyethyl)glycine buffer, pH 7.8, containing 1 mM EDTA] was incubated at 37 °C with pyridoxamine phosphate (0.033–1.0 mM) and indole-3-pyruvic acid (0.02–0.5 mM). Aliquots (0.01 mL) were removed at intervals and assayed for activity in reaction 2 in the presence or absence of pyridoxal phosphate. (A) The percent apo-$\alpha_2\beta_2$ [(activity + pyridoxal phosphate) − (activity −pyridoxal phosphate)/(activity + pyridoxal phosphate) × 100] is plotted vs. time on a semilog scale. Curves 1–5 were at 0.4 mM indole-3-pyruvic acid and 0.033, 0.05, 0.1, 0.2, and 1.0 mM pyridoxamine phosphate, respectively. Curve 6 was at 0.2 mM pyridoxamine phosphate and 0.1 mM indole-3-pyruvic acid in 0.02 M potassium phosphate, pH 7.8. Curves 3 and 4 represent data with [4′,4′-$^1H_2$]pyridoxamine phosphate (open circles) or [4′,4′-$^2H_2$]pyridoxamine phosphate (closed symbols). (B) Plots of the half-times of reactivation ($t_{1/2}$) vs. reciprocal concentration of pyridoxamine phosphate at constant indole-3-pyruvic acid (0.4 mM) [data from (A)] or of indole-3-pyruvic acid at constant pyridoxamine phosphate (1 mM) (data not shown). Apparent dissociation constants (K_d) are calculated from the intersection with the ordinant of the straight line in (B) ($1/K_d$). These are not true K_d values, since neither one of the constant substrates is present at saturating levels in Figure 2. As a control experiment, 0.5 mM indole-3-pyruvic acid and 1 mM pyridoxamine were incubated for 50 min as above and then mixed with apo-$\alpha_2\beta_2$ complex. The absence of enzymatic activity indicates that no pyridoxal phosphate was formed in the control.

Table II: Products and Stereochemical Course of Transamination of Pyridoxamine Phosphate and Indole-3-pyruvic Acid

expt	reactants[a,c]	pyridoxal phosphate[b,c] nmol	pyridoxal phosphate[b,c] cpm/nmol	% ^{3}H lost	attack at C′-4	tryptophan (nmol)	L-tryptophan (nmol)	tryptophan (cpm/nmol)
1	apo-$\alpha_2\beta_2$ complex (230 nmol), pyridoxamine-P (200 nmol), indole-3-pyruvic acid (400 nmol)	140				95	70	
2	apo-$\alpha_2\beta_2$ complex (20 nmol), (4′*R*)-[^{3}H]pyridoxamine-P (20 nmol, 8600 cpm/nmol), indole-3-pyruvic acid (100 nmol)	13	8600	0	*si*			
3	apo-$\alpha_2\beta_2$ complex (115 nmol), (4′*S*)-[4′-^{3}H]pyridoxamine-P (100 nmol, 107 cpm/nmol), indole-3-pyruvic acid (400 nmol), (3*S*)-2,3-dihydro-5-fluoro-L-tryptophan (100 nmol)	57	>9	>91	*si*	39		<1

[a] The indicated reactants were incubated at 37 °C in 1.0 mL of 0.05 M sodium *N*,*N*-bis(2-hydroxyethyl)glycine buffer, pH 7.8, containing 1 mM EDTA for 100 (experiment 1), 150 (experiment 2), or 35 min (experiment 3). [b] Enzyme-bound pyridoxal phosphate was separated from the other reactants either by centrifugation of the reaction mixture in an Amicon Centriflo 25 cone for 30 min at 3000 rpm followed by resuspension in 1.0 mL of buffer and recentrifugation (experiments 1 and 3) or by gel filtration on a PD-10 column in water (experiment 2). The protein from a solution in H_2O was precipitated by addition of $^1/_{10}$ volume 12 N HCl followed by centrifugation in a Beckman microfuge for 2 min. Aliquots of the supernatant solution were counted, and the pyridoxal phosphate content was determined from the absorbance at 295 nm (Peterson & Sober, 1954), from the absorbance at 412 nm of the phenylhydrazone (Wada & Snell, 1961), or from both. [c] The protein-free fractions from footnotes *a* and *b* were applied to a 1.0-mL column of Dowex 1 acetate. The column was washed with 8 mL of H_2O followed by 5 mL of 0.2 N acetic acid. The acidic fractions were lyophilized and used for the determination of total tryptophan by absorbance at 278 nm and L-tryptophan by difference spectroscopy before and after treatment with tryptophanase (Miles et al., 1986) and of radioactivity. Some (4′*S*)-[^{3}H]pyridoxamine phosphate was present in this fraction in experiment 3 and was separated from the tryptophan by chromatography on Whatman 3MM paper in 1-butanol–12 N HCl–H_2O, 5:1:2. Essentially all counts were in the pyridoxamine phosphate and none in the tryptophan.

that intramolecular proton transfer does not occur or that the α-proton of tryptophan is rapidly lost by an exchange reaction (experiment 3). (3*S*)-2,3-Dihydro-5-fluoro-L-tryptophan, a competitive inhibitor (Miles et al., 1986), was added in this experiment in an attempt to displace any L-tryptophan formed and to thus decrease the amount of α-proton exchange with L-tryptophan.

Absence of Kinetic Isotope Effects. No kinetic isotope effect was observed in the transamination of [4′,4′-$^2H_2$]pyridoxamine phosphate (Figure 2A, curves 3 and 4).

Discussion

The results show that a single-turnover, half-transamination reaction (reaction 1) occurs when the apo-$\alpha_2\beta_2$ complex of tryptophan synthase is incubated with pyridoxamine phosphate and indole-3-pyruvic acid. The conversion of pyridoxamine phosphate to pyridoxal phosphate is demonstrated by a change in the absorbance maximum from 325 to 412 nm (Figure 1), by the concomitant restoration of the catalytic activity of the apo-$\alpha_2\beta_2$ complex in a β-replacement reaction (Figure 1 inset, Figure 2, and Table I), and by the isolation and quantitation of pyridoxal phosphate (Table II). The formation of L-tryptophan is demonstrated by its isolation and quantitation by its absorbance and its reaction with tryptophanase (Table II). Tryptophanase is highly specific for the L isomer of tryptophan and has proved to be useful in the quantitative analysis of mixtures of D- and L-tryptophan (Miles et al., 1986). Since indole-3-pyruvic acid and pyridoxamine phosphate exhibit saturation kinetics (Figure 2B), these substrates must bind to the active site of the apo-$\alpha_2\beta_2$ complex, presumably to the sites subsequently occupied by the products, L-tryptophan and pyridoxal phosphate. The apparent dissociation constants for pyridoxamine phosphate (0.2 mM, Figure 2) are much weaker than the binding constant for pyridoxal phosphate (K_d = 1.4 μM) (Tschopp & Kirschner, 1980) but comparable to that reported for pyridoxine phosphate (K_d = 0.12 mM) (Tschopp & Kirschner, 1980). The weak binding of analogues lacking the carbonyl group shows the importance of the Schiff base formed between the carbonyl group and the lysine amino group for the tight binding of pyridoxal phosphate (Tschopp & Kirschner, 1980; Miles & Moriguchi, 1977). Pyridoxamine phosphate was reported not to bind to apo-$\alpha_2\beta_2$ complex in potassium phosphate buffer; binding was measured by competition with pyridoxal phosphate binding (Tschopp & Kirschner, 1980). This result is consistent with our results in Figure 2 and indicates that the interaction of the phosphate group of pyridoxamine phosphate with positively charged group(s) on the enzyme that can also bind phosphate ions is an important mode of binding. In contrast, phosphate ions do not inhibit the binding of pyridoxal phosphate (Miles, 1979) for which Schiff base formation is a more important mode of binding. Although the transamination reaction also occurs with some other α-keto acids (Table I), the rate with indole-3-pyruvic acid is most rapid at the concentration of α-keto acid tested (0.2 mM); this is probably due to its specific binding at the indole binding site. *S*-(Hydroxyethyl)thiopyruvic acid was tested since it had previously been shown to undergo a reverse transamination reaction with the apo-β_2 subunit and pyridoxamine phosphate (Miles et al., 1968). The weak substrate activity of *S*-(hydroxyethyl)thiopyruvic acid demonstrates that it has no special advantage. In contrast to the holo-β_2 subunit, the holo-$\alpha_2\beta_2$ complex does not catalyze a forward transamination reaction to any significant extent with L-serine and β-mercaptoethanol (reaction 5) (Miles et al., 1968) or with L-tryptophan.[1] Thus reaction 1 is essentially irreversible.

Questions may be asked about the relative order of binding pyridoxamine phosphate and indole-3-pyruvic acid or whether the enzyme binds ketimine Schiff base formed nonenzymatically from pyridoxamine phosphate and indole-3-pyruvic acid. The latter possibility seems unlikely since the K_d value for formation of the aldimine Schiff base from pyridoxal phosphate and glutamate is about 100 M^{-1} at pH 8 (Metzler et al., 1980) and the K_d value for formation of the ketimine Schiff base from pyridoxamine phosphate and pyruvate is in the same range (Felty & Leussing, 1974). Thus, the amount of Schiff base in equilibrium with 0.1 mM substrates (Figure 1) would be about 0.001 mM or 1% of the substrates and enzyme. Difference spectra between pyridoxamine phosphate and apo-$\alpha_2\beta_2$ complex before and after mixing indicate that pyridoxamine phosphate does bind to the enzyme in the absence of indole-3-pyruvic acid.[1] Similar experiments with indole-3-pyruvic acid and apo-$\alpha_2\beta_2$ suggest that this substrate binds weakly in the absence of pyridoxamine phosphate.[1] Thus, apo-$\alpha_2\beta_2$ probably binds pyridoxamine phosphate first, fol-

[1] Unpublished results.

lowed by indole-3-pyruvic acid.

Our results are consistent with the stereochemical course of the reaction shown in Scheme I. Since the *pro-S* proton and not the *pro-R* proton at C-4′ of pyridoxamine phosphate is specifically removed (Table II), the reaction is stereospecific and follows the same steric course as the several other transamination reactions that have been studied (Floss & Vederas, 1982). In all of these reactions, the *pro-S* proton is removed from the *si* face of the pyridoxamine phosphate Schiff base (I, Scheme I) and added back to the *si* face of the quinoid intermediate II to yield the L-amino acid–pyridoxal phosphate Schiff base III. Our finding of a large enantiomeric excess of L-tryptophan (Table II) is consistent with Scheme I. The possible formation of some D-tryptophan may result from a slow, secondary racemization reaction (Miles et al., 1986). The absence of a kinetic isotope effect in the transamination of [4′,4′-$^2H_2$]pyridoxamine phosphate (Figure 2A) demonstrates that removal of the 4′-proton is not rate determining.

Our results thus show that reaction 1 occurs at the active site of the apo-$\alpha_2\beta_2$ complex through bound intermediates and by a stereospecific course on one side of the enzyme–substrate complex. The question now arises whether this slow transamination reaction is actually catalyzed by a basic residue B (Scheme I) or whether the enzyme merely binds the substrates and thus facilitates the inherent chemical reactivity of pyridoxamine phosphate. This activity has been demonstrated in very slow, model transamination reactions (Metzler et al., 1954). Breslow et al. (1980) have demonstrated that an artificial enzyme consisting of pyridoxamine attached to a β-cyclodextrin can bind indole-3-pyruvic acid and accelerate the rate of the model transamination reaction about 200-fold, producing tryptophan with a 2:1 preference for L-tryptophan. A different model shows a 1.8:1 preference for D-tryptophan (Breslow & Czarnik, 1983). Although the rate of reaction 1 with tryptophan synthase is slow, it is somewhat faster than the rate achieved with the artificial enzyme,[2] suggesting that it may be enzyme-catalyzed. The best evidence for the catalytic role of a base B would be the demonstration of intramolecular transfer of the *pro-S* proton from (4′*S*)-[4′-^{3}H]-pyridoxamine phosphate to the α-carbon of L-tryptophan. Our failure to observe such transfer (Table II, experiment 3) is probably due to the known ability of the holo-$\alpha_2\beta_2$ complex to exchange the α-proton of L-tryptophan with solvent (Tsai et al., 1978; Miles, 1980; Turner et al., 1985) at a rate faster than the rate of the slow transamination reaction. Alternatively, the failure to observe intramolecular proton transfer and an isotope effect may be due to equilibration of the proton with solvent after transfer to the base B. Tobler et al. (1986) have recently reported that apo-aspartate aminotransferase catalyzes the slow and stereospecific exchange of the *pro-S* C-4′ hydrogen of pyridoxamine phosphate with solvent in the absence of α-keto acid substrate. Intramolecular proton transfer has been demonstrated in several other reactions catalyzed by tryptophan synthase, including the thiol-dependent transamination reaction catalyzed by the β_2 subunit of tryptophan synthase (Floss & Vederas, 1982; Tsai et al., 1978; Miles et al., 1982). There is good evidence that His-86 is the monoprotic base B involved in these proton-transfer reactions (Miles & Kumagai, 1974; Miles & McPhie, 1974; Miles, 1974, 1980). Enzyme in which His-86 has been altered by mutation or modification could be used to test whether reaction 1 is indeed enzyme catalyzed or merely enzyme facilitated.

If the transamination reaction is not catalyzed by a base on the protein but instead by solvent, the rate might be expected to be enhanced by the presence of imidazole or by an increase in pH (Bruice & Topping, 1963a,b). Preliminary experiments[1] show that neither addition of 0.3 M imidazole hydrochloride, pH 7.8, or increasing the pH from 7.0 to 8.5 has a significant effect on the rate of the transamination reaction. Thus, the pH dependence of the rate of reaction 1 is similar to that of reaction 2, which has a plateau above pH 7 (Lane & Kirschner, 1981), suggesting that reaction 1 is enzyme-catalyzed.

In conclusion, our results emphasize the versatility of the coenzymes pyridoxamine phosphate and pyridoxal phosphate in promoting a variety of reactions both in model systems and when bound to enzymes. Several enzymes, including tryptophan synthase, tryptophanase, and tyrosine β-lyase, have been demonstrated to catalyze a variety of classes of these pyridoxal phosphate dependent reactions. We have shown here that tryptophan synthase can be pushed to carry out a new transamination reaction by adding substrate and coenzyme analogues for which it has some binding specificity. The reaction at the active site of the enzyme proceeds stereospecifically and more rapidly than a model reaction in solution. However, the rate of the transamination reaction estimated from the plot in Figure 2B (k_{cat} = 0.002 s^{-1}) is about 3 orders of magnitude slower than the rate of tryptophan synthesis (k_{cat} = 4.3 s^{-1}; Ahmed et al., 1986). Nevertheless, the rate of transamination is similar to the rate of other slow reactions of the enzyme, including the cleavage of L-tryptophan (k_{cat} = 0.0005 s^{-1}; Ahmed et al., 1986) and the epimerization of 2,3-dihydro-5-fluoro-L-tryptophan (k_{cat} = 0.0005 s^{-1}; Miles et al., 1986). These very slow reactions and the slow transamination reactions catalyzed by a number of other phosphopyridoxal enzymes (Miles, 1985) probably have no physiological importance. However, studies of these reactions have been useful in clarifying the reaction mechanism and substrate specificity of several phosphopyridoxal enzymes, including tryptophan synthase.

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Registry No. $HO(CH_2)_2SCH_2COCO_2H$, 105930-97-0; tryptophan synthase, 9014-52-2; indole-3-pyruvic acid, 392-12-1; pyruvic acid, 127-17-3; phenylpyruvic acid, 156-06-9; α-ketoglutaric acid, 328-50-7; (4′*R*)-[^{3}H]pyridoxamine-P, 98618-73-6; (4′*S*)-[^{3}H]pyridoxamine-P, 105930-98-1; [4′,4′-$^2H_2$]pyridoxamine-P, 83961-76-6; pyridoxamine-P, 529-96-4.

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[2] Whereas incubation of 0.1 mM apoenzyme, pyridoxamine phosphate, and indole-3-pyruvic acid at 25 °C for 10 min led to a 15% conversion (Figure 1), incubation of 0.05–5 mM indole-3-pyruvic acid and β-cyclodextrin–pyridoxamine artificial enzyme at room temperature for 10 min led to a 1–5% conversion, depending on pH and concentration (Breslow et al., 1980).

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