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ENZYMIC PROPERTIES OF A MUTANT TRYPTOPHAN SYNTHASE FROM *NEUROSPORA CRASSA**

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SUMMARY

Tryptophan synthase (L-serine hydro-lyase (adding indole), EC 4.2.1.20) obtained from *Neurospora crassa* strain td_{201}, a tryptophan auxotroph, is only capable of catalyzing the conversion of indole and serine to tryptophan. The enzyme is largely resolved from its cofactor, pyridoxal phosphate (pyridoxal-*P*); hence the enzymic activity in the crude extract is practically undetectable unless exogeneous pyridoxal-*P* is added. The stoichiometry of the cofactor has been determined by spectrophotometric titration with purified apoenzyme; about 1.5 moles of pyridoxal-*P* are bound to 1 mole of enzyme. Two types of binding of pyridoxal-*P* are present in the enzyme; one absorbs at 325 nm ($\lambda_{max}^{emission}$ = 385 nm) and the other absorbs at 410 nm ($\lambda_{max}^{emission}$ = 495 nm). These two forms are not interconvertible by varying the pH.

The holoenzyme can be easily resolved by passage through a hydroxylapatite column. The resulting apoenzyme is inactive but it can be reactivated by pyridoxal-*P*. $NaBH_4$ reduction leads to irreversible inactivation of the enzyme.

The enzyme is very sensitive to some endogenous proteases present in the *Neurospora* extract. Phenylmethanesulfonyl fluoride can prevent to some extent the spontaneous inactivation of the enzyme. Highly purified enzyme has been found to be fairly stable. Therefore, the apparent lability of this enzyme is not an intrinsic property.

The catalytic activity of purified enzyme can be stimulated by proteins such as serum albumin. This phenomenon has been interpreted in terms of the "intermolecular cooperativity" which might exist *in vivo* between this enzyme and the other enzymic components of the tryptophan pathway. In this paper, an improved assay method for enzymic activity is also described.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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INTRODUCTION

Tryptophan synthase (L-serine hydro-lyase (adding indole), EC 4.2.1.20) catalyzes the final reaction in tryptophan biosynthesis:

$$\text{Indole-3-glycerol-}P + \text{L-serine} \xrightarrow{\text{pyridoxal-}P} \text{L-tryptophan} + \text{D-glyceraldehyde-3-}P \quad (1)$$

The enzyme is also capable of catalyzing the following two half reactions:

$$\text{Indole} + \text{L-serine} \xrightarrow{\text{pyridoxal-}P} \text{L-tryptophan} \quad (2)$$

$$\text{Indole-3-glycerol-}P \xrightleftharpoons{\text{pyridoxal-}P^*} \text{indole} + \text{D-glyceraldehyde-3-}P \quad (3)$$

In bacteria such as *Escherichia coli*[1], *Salmonella typhimurium*[2,3], *Bacillus subtilis*[4] and *Pseudomonas putida*[5], the enzyme can be readily separated into two distinct protein components designated A and B. The A protein alone can catalyze the reversible Reaction 3, and the B protein alone catalyzes the irreversible Reaction 2. However, the rate of reaction catalyzed by an individual component is, in general, much lower than that catalyzed by an AB complex. The same phenomenon has also been demonstrated with the enzyme obtained from algae[6] and higher plant cells[7]. This behavior, however, has not been observed with tryptophan synthase from fungi such as *Neurospora crassa*[8–10], *Aspergillus nidulans*[3] and *Saccharomyces cerevisiae*[2,11]. Hence, it is generally believed that the tryptophan synthases of fungal type catalyze all three reactions by a single, undissociable protein species[2,6].

In fungi, particularly with *Neurospora*, studies on the effects of mutations on structure and function of the tryptophan synthase have been one of the central themes in biochemical genetics for almost two decades. Vast genetic and biochemical information accumulated in the past indicates that all the mutant types characterized so far can be assorted immunochemically into two groups (CRM^+ and CRM^-) according to the ability of their gene products to neutralize antibodies against the wild type enzyme[2]. The CRM^+ mutants, depending on their enzymic activity, can be further divided into three classes: (a) The indole utilizers which are analogous to the A-type mutants of *E. coli*, with an enzyme having the activity for Reaction 2; (b) the indole accumulators which are analogous to the B-type mutants of *E. coli*, in which the enzyme is capable of catalyzing Reaction 3; (c) the "profound" mutants which have no counterpart in *E. coli* where the enzyme loses the catalytic activity for all of the three reactions.

The present work is concerned with the enzymic properties of a mutant enzyme obtained from a tryptophan auxotroph strain td_{201}, an indole utilizer. This mutant was originally isolated by Ahmad and Catcheside[12]. Subsequent studies by other investigators[13–15] have shown that the mutant enzyme is active only in Reaction 2, namely the conversion of indole to tryptophan, but otherwise is very similar to the wild type enzyme with respect to its physical, chemical and immunological properies.

* Pyridoxal-*P* is also essential to Reaction 3[45], but it may not be directly involved in catalysis as in Reaction 2 and in other classical B_6 enzymes[31]. The non-catalytic role of pyridoxal-*P* coenzyme has also been observed in glycogen phosphorylase[46].

EXPERIMENTAL PROCEDURE

Materials

p-Dimethylaminobenzaldehyde and indole were purchased from Eastman; pyridoxal phosphate was a gift from Hoffmann-La Roche; pyridoxine phosphate, pyridoxamine phosphate and 4-deoxypyridoxine phosphate, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), phenylmethanesulfonyl fluoride and L-serine were obtained from Calbiochem; hydroxylapatite was purchased from Bio-Rad or Clarkson Chemical Co., Williamsport, Pa.

Organism

The *N. crassa* strain used in these studies was the tryp-3 mutant td_{201} (Fungal Genetics Stock Center, No. 702). The stock was obtained from Dr C. Yanofsky and maintained by serial transfer on agar slants containing Vogel's medium N^{16} supplemented with 150 mg of DL-tryptophan per l.

Preparation of mycelia

The cells were grown at 30 °C in a New Brunswick Fermacell (Model FSI-130) containing 100 l of Vogel's medium N with a supplement of 15 g of DL-tryptophan as described by Meyer *et al.*[17]. The Fermacell was inoculated with about 1 l of a sterile water suspension of conidia collected from 6 to 8 Fernbach flask cultures which were grown at 30 °C for about 10 days prior to inoculation. The mycelia, after growth in the Fermacell for 22 to 24 h with forced aeration (3 cubic feet per min), were harvested by filtration through cheesecloth, washed with distilled water and lyophilized. The lyophilized mycelia were ground into a fine powder in a Waring blender and were stored at −20 °C.

Purification of the enzyme

The enzyme was prepared from lyophilized mycelia by a procedure outlined by Meyer *et al.*[17], with the following modifications in the early steps: (i) The buffer used for preparation of crude extract was 0.01 M potassium phosphate (pH 7.2), containing 3 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride and 0.04 mM pyridoxal-*P*, because the ionic strength and the pH of the buffer must be lower than that used by the original authors when streptomycin is employed for the subsequent step; (ii) protamine sulfate was found to be inhibitory to the enzyme (Table II); therefore, it was replaced by streptomycin sulfate. To every 2 l of acidified crude extract (the pH was adjusted to 5.9 with 2 M acetic acid), 50 ml of a solution of 6% streptomycin sulfate in water were added dropwise with stirring. After 30 min, the pink precipitate was removed by centrifugation and the supernatant solution was used for further fractionation. The purified enzyme was homogeneous by the criteria of acrylamide gel electrophoresis, Ouchterlony immunodiffusion and ultracentrifugal studies as reported previously[17].

Indole reagent

The reagent was modified from a formula described by Yanofsky[18]. The new color reagent consists of 2 M HCl and 0.16 M *p*-dimethylaminobenzaldehyde in 95% ethanol.

Assay of enzymic activity

The most commonly used assay for tryptophan synthase of *N. crassa* is based on the disappearance of indole according to the procedure described by Yanofsky[18] and recently by Meyer *et al.*[17]. The original procedure, which involves extraction of indole with toluene and a series of dilutions with ethanol, has been found to be rather insensitive and laborious. Hence, a direct assay method was developed in the course of this work. Routinely, the assay was performed in a 0.5 ml assay mixture containing 20 μmoles of L-serine (neutralized with NaOH), 0.15 μmole of indole, 0.04 μmole of pyridoxal-*P* and 25 μmoles of HEPES buffer, pH 7.4 (measured at room temperature). The reaction was initiated by 5 to 20 μl of properly diluted enzyme and allowed to progress at 37 °C for 5 to 20 min. The reaction was stopped by the addition of 3 ml of indole color reagent. The absorbance at 540 nm or at 570 nm can be determined after 5 min[19]. Enzymic activity is expressed in terms of international units (I.U.) which is defined as that amount of enzyme which catalyzes the conversion of 1 μmole of indole per min under the assay conditions. Specific activity is expressed as I.U. per mg of protein. Protein was determined by the microbiuret method[20] with crystalline bovine serum albumin as a standard.

Optical studies

Ultraviolet and visible spectra were measured at room temperature in a Cary Model 14 recording spectrophotometer using a 10 mm light path. Fluorometric measurements were made with a spectrophotofluorometer constructed at the Johns Hopkins University[21], using right-angle optics. Suprasil quartz cuvettes with a 10 mm light path were used for all measurements. The temperature of the sample compartment was maintained at 27 °C.

RESULTS

Quantitative determination of indole

Indole either in aqueous solution or in the assay mixture can be directly measured by reaction with the modified indole reagent described in the experimental procedure. The resulting color has a maximum absorption peak at 570 nm and a less intensive peak at 540 nm as shown in Fig. 1A. The absorbance at either 570 or 540 nm is proportional to the concentration of indole (Fig. 1B). The molar extinction coefficients for indole at 570 and 540 nm were determined to be $4.12 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ and $2.85 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$, respectively. The reaction is specific for indole; none of the other components of the reaction mixture employed for the assay of the Reaction 2 activity interfered with this colorimetric reaction. Tryptophan, the product of Reaction 2, does not react.

Effect of buffers on the enzyme activity

Although Tris and phosphate are the most widely used buffer systems for assay of tryptophan synthase[17,22], neither of them is optimal for enzyme activity (Table I). Of the various buffer systems tested in our assay, HEPES was the best buffer system for obtaining maximal catalytic activity of the tryptophan synthase of *Neurospora*. It should be noted that the concentration-dependent inhibition of enzymic activity by some buffers, as shown in Table I, may not be solely attributed to

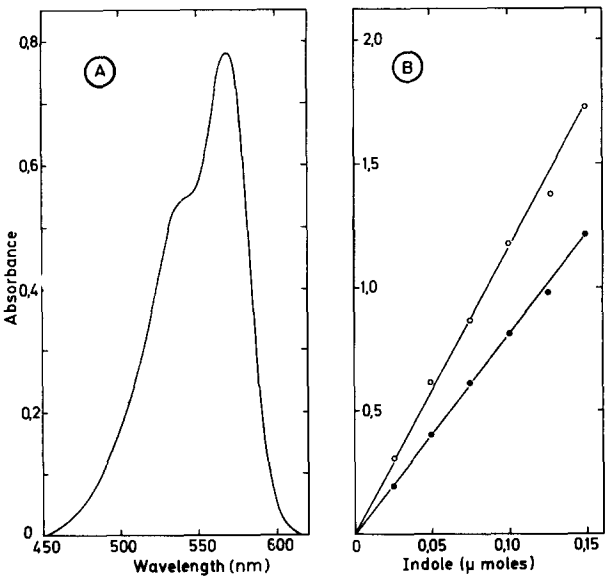

Fig. 1. Colorimetric determination of indole. A. The difference absorbance spectrum of the adduct of indole and *p*-dimethylaminobenzaldehyde in 95% ethanol containing 2 M HCl. The spectrum was measured in a Cary Model 14 recording spectrophotometer with indole reagent in the reference compartment. B. The proportionality of absorbance at 570 nm (○—○) and at 540 nm (●—●) with respect to the quantity of indole. To 0.5 ml of an aqueous solution containing variable amounts of indole as indicated, 3 ml of the indole reagent were added. Adsorbance was measured 1 h after mixing. Points are the average of duplicate experiments.

TABLE I

EFFECT OF BUFFER ON ENZYMIC ACTIVITY

The enzymic activity was assayed under otherwise similar conditions as described in Experimental Procedure, except for the buffer used. The pH of all buffers was adjusted to 7.8 at 37 °C. Two different concentrations (*i.e.*, final concentration in the reaction mixture) of each buffer were compared. The results are expressed as relative activity taking the enzymic activity in 0.05 M HEPES buffer as 100%.

Buffer species	*Concentration (M)*	*Relative activity (%)*
HEPES[a]	0.05	100
	0.10	95
Triethanolamine[b]	0.05	91
	0.10	88
Tris–HCl	0.05	76
	0.10	60
Tris–citrate	0.05	79
	0.10	69
Potassium phosphate	0.05	75
	0.10	67
Sodium pyrophosphate[b]	0.05	84
	0.10	84

[a] pH was adjusted with NaOH.
[b] pH was adjusted with HCl.

the effect of ionic strength; it may also depend on the nature of buffer ions. For instance, with pyrophosphate buffer, the concentration-dependent inhibition was not appreciable. The effect of various buffers on the properties of other pyridoxal-*P* enzymes has been studied by Jenkins *et al.*[23,24]. They showed that this effect is due to the binding of buffer ions to the enzyme.

Inhibition and activation by proteins

In the course of the purification of this enzyme, we have frequently experienced that the enzymic activity is partially or completely lost after treating the crude extract with protamine sulfate. The extent of the loss of activity was dependent on the amount of protamine sulfate. When the enzyme was precipitated from the protamine-treated solution by ammonium sulfate, the activity could be partially recovered. This suggested to us that the inhibition of enzymic activity by protamine must be reversible. This was further proved by the demonstration that protamine acts as an inhibitor of the purified enzyme and that this inhibition is readily reversed by serum albumin (Table II). Other proteins such as pancreatic ribonuclease did not have any effect on the activity of tryptophan synthase.

TABLE II

INACTIVATION OF TRYPTOPHAN SYNTHASE BY PROTAMINE SULFATE

To the standard assay mixture (0.5 ml), the indicated amount of protamine and bovine serum albumin were added. The final volume of reaction mixture was 0.62 ml. The reaction was initiated with about 18 μg enzyme (specific activity = 0.47 I.U./mg) and proceeded for 5 min at 37 °C.

Addition	*Relative activity (%)*
None	100
Protamine (5 μg)	57
(10 μg)	16
Albumin (1 mg)	106
(2 mg)	100
Protamine (5 μg) + albumin (2 mg)	100
(10 μg) + albumin (2 mg)	97

The catalytic activity of the enzyme when it is highly purified can be stimulated up to about 40% by serum albumin or ovalbumin, although such stimulation was not appreciable with partially purified enzyme. The effect of serum albumin on enzymic activity was previously thought to be due to the protection of the enzyme from inactivation upon dilution[9]; however, this explanation did not apply to the present observations. When the enzyme was diluted to less than 0.3 mg protein per ml in the absence of serum albumin, kept at 5 °C for two to three days and then assayed in the presence of serum albumin, full activity was recovered. In the control experiment, the enzyme was diluted in the presence of 5 mg albumin per ml (Table III).

Intrinsic instability of the enzyme?

In the course of this work, we found that the partially purified enzyme, *e.g.* the preparation obtained from Step 5 (*cf.* ref. 17), could be separated into a very stable fraction (S) and a very labile fraction (L). Fraction S which precipitated at 25–

TABLE III

ACTIVATION OF PURIFIED ENZYME BY ALBUMIN

The enzyme preparation obtained from the second ammonium sulfate fraction[17] was diluted to 0.26 mg/ml in 0,025 M potassium phosphate buffer (pH 7.2) or in the same buffer supplemented with bovine serum albumin (5 mg/ml). The diluted enzyme solutions were kept at 5 °C for 3 days. The enzymic activity was then assayed with the standard mixture ± albumin (1 mg/ml). The reaction was initiated with 10 μl of the dilute enzyme solution and continued for 10 min at 37 °C.

Conditions	*Assay in the presence (+) or absence (−) of albumin*	*Spec. act. (I.U./mg)*	*Relative activity (%)*
Enzyme diluted in	+	2.08	100
buffer alone	−	1.23	59
Enzyme diluted in	+	2.08	100
buffer + albumin	−	1.62	78

30% saturation of ammonium sulfate could be kept at 5 °C for a long period without significant loss of activity, whereas Fraction L, precipitating at 30–50% saturation of ammonium sulfate, lost more than 90% of its original activity within three days. Later experiments showed that when an aliquot of Fraction L was added to an equal volume of Fraction S, the rate of inactivation of the resulting mixture was comparable to that of the unstable fraction. On the other hand, when Fraction L was boiled prior to addition to the Fraction S, no enhancement of inactivation of the latter was observed. These observations suggested that the instability is not an intrinsic property of *Neurospora* tryptophan synthase, but rather that the enzyme is inactivated by some contaminating heat-labile material, for example, proteases. This contention was supported by the demonstration that the proteolytic inhibitor, phenylmethanesulfonyl fluoride[25], could protect the enzyme from inactivation. Results of this experiment are shown in Table IV. It should be noted, however, that the inhibition of protease by phenylmethanesulfonyl fluoride is reversible[26]. For effective protection of enzyme from proteolytic digestion during storage, the buffer solution in which the enzyme is dissolved should be saturated with phenylmenthanesulfonyl fluoride.

Thermal stability

Despite the fact that both pyridoxal-*P* and serine were required to protect the partially purified enzyme against heat inactivation at 55–56 °C (ref. 9), when purified apoenzyme was heated at 65 °C for a period of 10 min, no significant loss of activity was observed (Fig. 2) no matter whether pyridoxal-*P* and serine were added or not. Whereas the enzyme appears to be stable at 65 °C, it was quickly inactivated at 67 °C (Fig. 2, Curve 3). The exact reason for the improvement in thermal stability of the purified enzyme as compared to the enzyme in the crude extract is not yet clear. The effect of pyridoxal-*P* and serine against heat inactivation of partially purified enzyme may be partly due to the protection of enzyme from proteolytic digestion. For instance, if the contaminating protease has a trypsin-like specificity, binding of pyridoxal-*P* to the ε-NH_2 group of lysines in the active site(s) or elsewhere on the surface of the enzyme molecule could protect the enzyme from attack by such a protease.

Furthermore, it is worthwhile to mention that although we have included

TABLE IV

PROTECTION OF THE ENZYME FROM INACTIVATION BY PHENYLMETHANESULFONYL FLUORIDE

The enzyme preparation was the same as described in the legend to Table III. It was dissolved (2 mg/ml) in 0.1 M potassium phosphate buffer (pH 7.2) containing 2.5 mM of EDTA, 10 mM of DL-serine and 50 μg/ml of pyridoxal-*P*. Phenylmethanesulfonyl fluoride (PMSF) (0.1 M stock in 95% ethanol) was added to the final concentration as indicated. The final concentration of ethanol in each sample was about 10%. Enzymic activity was assayed according to the standard procedure. Results were the average of duplicate assays.

Assay interval (days)	*Concentration of PMSF (mM)*	*Activity (I.U./ml)*	*% original activity*
0	0.0	2.60	100
	0.1	—	—
	1.0	—	—
1	0.0	1.55	60
	0.1	2.55	98
	1.0	2.70	104
3	0.0	0.10	4
	0.1	2.10	81
	1.0	2.35	91
6	0.0	—	—
	0.1	2.25	86
	1.0	2.35	91
13	0.0	—	—
	0.1	0.65	25
	1.0	1.75	68
18	0.0	—	—
	0.1	0.5	22
	1.0	1.75	68
25	0.0	—	—
	0.1	0.13	5
	1.0	1.75	68

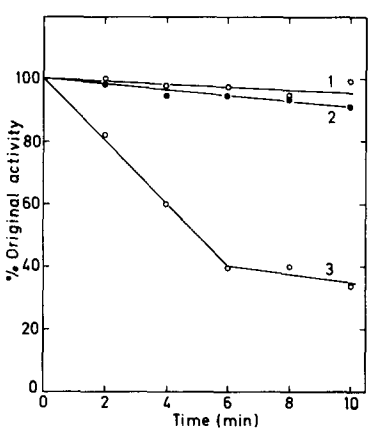

Fig. 2. Heat stability of the purified apotryptophan synthase. Each 0.05 ml apoenzyme stock (3 mg per ml in 0.75 M ammonium sulfate and 2 mM EDTA) was mixed with an equal volume of 0.4M potassium phosphate buffer, pH 7.2. The mixture was heated in the presence (●—●) and absence (○—○) of 0.01 M DL-serine and 100 μg/ml of pyridoxal-*P*. After heating, the residual enzyme activity was assayed according to the standard method as described in Experimental Procedure. For Curves 1 and 2, the enzyme was heated at 65 °C, Curve 3 at 67 °C.

pyridoxal-*P* in all buffers used for the purification of the enzyme, to freeze the enzyme in buffer solutions supplemented with pyridoxal-*P* and 2-mercaptoethanol must be avoided because this tends to inactivate the enzyme.

Relationship between the enzyme and the cofactor

(a) Effect of pyridoxal-P on structure and activity. When crude extracts of mycelia were made in buffer solutions without pyridoxal-*P* and subsequently assayed in the absence of pyridoxal-*P*, virtually no Reaction 2 activity was detectable. This finding, however, is not a peculiarity of the td_{201} mutant enzyme. It rather seems a property of all *Neurospora* tryptophan synthases, since similar observations have been made with enzyme preparations from wild type and other mutant strains[27–29]. We have also found that with purified holoenzyme maximum activity could not be obtained unless pyridoxal-*P* is added to the assay mixture, even when the enzyme has previously been kept in pyridoxal-*P* buffer. This suggests that the cofactor of *Neurospora* tryptophan synthase is not firmly bound to the enzyme.

It has been known for some time that pyridoxal-*P*, as well as serine, are required for the association of A and B proteins of the tryptophan synthase of *E. coli*[30]. This seems very unlikely for the *Neurospora* enzyme, for sedimentation studies with the holo- and apoenzyme failed to show any substrate or cofactor-dependent dissociation–association (H. Tsai and S. R. Suskind, unpublished results). However, these substances may still cause some changes in the shape of the protein molecule[15].

(b) Resolution and reconstitution of the holoenzyme. The cofactor pyridoxal-*P* has been found to be remarkably easy to dissociate from the enzyme by either extensive dialysis or simply by passage through a hydroxylapatite column equilibrated with 0.02 M potassium phosphate buffer (pH 6.8). The enzyme solution was usually dialyzed against the same buffer prior to loading onto the column. As soon as the enzyme solution was absorbed into the column, it was immediately washed with several column volumes of buffer. The degree of cofactor dissociation was found to be dependent on the thoroughness of this wash. The apoenzyme was eluted with 0.04 M potassium phosphate buffer (pH 6.8). The fractions under the symmetrical protein peak were pooled and concentrated either by ultrafiltration through a Sartoirus membrane (Carl Schleicher and Schuell, Keene, N. H.) in 0.2 M potassium phosphate buffer (pH 7.2), or by precipitation with 50% saturation of ammonium sulfate. The precipitate was redissolved in 0.75 M ammonium sulfate (pH 7.2, adjusted with NH_4OH) containing 2 mM EDTA. This apoenzyme solution can be stored at 5 °C as it is not desirable to freeze and thaw the enzyme frequently.

As it can be seen in Table V, the specific activity of the apoenzyme was less than 3% of that of the original holoenzyme when the enzyme activity was assayed in the absence of the added pyridoxal-*P*. However, the enzymic activity of the apoenzyme could be completely regenerated when pyridoxal-*P* was added to the reaction mixture. Some increase in the specific activity of the reconstituted enzyme was occasionally observed, especially when aged holoenzyme was used for resolution. Neither pyridoxine phosphate nor 4-deoxypyridoxine phosphate were active in the reconstitution of apotryptophan synthase. Pyridoxamine phosphate, however, could replace pyridoxal-*P* in the reactivation of the apoenzyme (H. Tsai and S. R. Suskind, unpublished results).

(c) Absorption and fluorescence spectra. Fig. 3A depicts the ultraviolet and

TABLE V

RECONSTITUTION OF THE APOTRYPTOPHAN SYNTHASE WITH PYRIDOXAL 5'-PHOSPHATE

The composition of the assay mixture is the same as described in Experimental Procedure except for pyridoxal-*P*. The apoenzyme was prepared as described in the text and was dissolved (0.75 mg/ml) in 0.75 M of ammonium sulfate (pH 7.2) containing 2 mM EDTA. The reaction (+ pyridoxal-*P*) was initiated with 3.75 μg apoenzyme and proceeded for 5 min at 37 °C. When the reaction was carried out in the absence of pyridoxal-*P*, a 3-fold excess of apoenzyme was used and the reaction time was extended to 60 min.

Enzyme	*Assay in the presence (+) and absence (−) of pyridoxal-P*	*Spec. act. (I.U./mg)*
Before resolution	+	1.92
After resolution	−	0.08
After resolution	+	3.08

visible absorbance spectra of apoenzyme and of holoenzyme reconstituted by adding pyridoxal-*P* immediately before the measurement. The apoenzyme shows a single protein absorbance peak at 280 nm (Fig. 3A, Curve 1). When pyridoxal-*P* is added to the apoenzyme, an absorbance band at 430 nm appears (Fig. 3A, Curve 2). Absorption in the 410 to 430 nm region is a characteristic feature of many pyridoxal-*P* enzymes and has been ascribed to the formation of an azomethine bond between the pyridoxal-*P* and the protein. In other pyridoxal-*P* enzymes[31], such azomethine bonds involve the formyl group of the pyridoxal-*P* and the ε-NH_2 group of a lysine residue in the protein moiety. However, upon aging or when the apotryptophan synthase was reconstituted by equilibrium dialysis against buffered pyridoxal-*P* solution, the spectrum of the resulting enzyme was different from that of freshly reconstituted enzyme as above. It became indistinguishable from the spectrum of purified holoenzyme (Fig. 3B, Curve 1). It can be seen that together with the appearance of an absorbance maximum at 325 nm, there also appears to be a blue-shift (from 430 to 410 nm) of the azomethine absorbance band. Unlike with several other pyridoxal-*P* enzymes[32], the absorbance bands at 325 and 410 nm are not interconvertible by varying the pH from 6.2 to 8.0. When L-serine was added to the holoenzyme, the intensity of the absorbance maximum at 410 nm decreased with a concomitant increase of the absorption maximum at 325 nm. A thiol-dependent substrate induced absorbance band at 468 nm which was previously observed in *E. coli* tryptophan synthase systems[33,34] was not detected with this enzyme.

Reduction with sodium borohydride eliminates the absorbance band at 410 nm (Fig. 3B, Curve 2), together with a complete loss of the catalytic activity for Reaction 2. This would be expected if the intrinsic azomethine bond of the enzyme is involved in catalyzing the synthesis of tryptophan from indole and serine, according to the general mechanism proposed by Metzler *et al.*[35], and by Braunstein and Shemyakin[36].

The different modes of binding of pyridoxal-*P* to the holoenzyme were further characterized by fluorescence excitation-emission spectroscopy. These results are summarized in Table VI.

(d) Number of pyridoxal-P binding sites and binding constants. Determination of the pyridoxal-*P* content of the holoenzyme by some conventional methods[37] was not possible for the following reasons: (a) The enzyme has been exposed to high con-

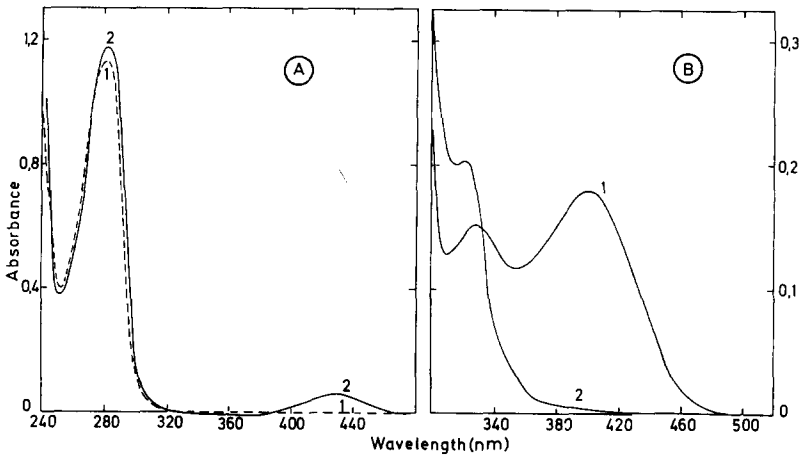

Fig. 3. A. Ultraviolet and visible absorbance spectra of the apoenzyme (Curve 1) and the newly reconstituted holoenzyme (Curve 2) in 0.05 M potassium phosphate buffer, pH 7.2. Protein concentration was about 1.3 mg per ml. B. Spectra of an aged holoenzyme before (Curve 1) and after (Curve 2) $NaBH_4$ reduction. Protein concentration was 2.5 mg/ml in 0.05 M potassium phosphate buffer, pH 7.2. $NaBH_4$ treatment was carried out by dialyzing the holoenzyme solution for 30 min against 0.05 M $NaBH_4$ in 0.2 M potassium phosphate buffer, pH 7.0. Spectra were taken after removing excess $NaBH_4$ by dialysis against 0.05 M potassium phosphate buffer, pH 7.0.

centrations of pyridoxal-*P* in the course of purification. Therefore, the possibility of non-specific binding of pyridoxal-*P* to the enzyme cannot be excluded; (b) one form of the bound pyridoxal-*P*, *i.e.*, the one which absorbs at 410 nm, is extremely easy to remove from the enzyme even by brief dialysis against phosphate buffer or by precipitation with ammonium sulfate. Because of these complications, the quantitative relationship of cofactor to enzyme was only studied by spectrophotometric titration with apoenzyme, based on the unique spectral properties of the newly formed enzyme–pyridoxal-*P* complex. For these studies, the assumption had to be made that the affinity constant in the active-site-directed pyridoxal-*P* binding is much greater than that of the non-specific pyridoxal-*P* binding.

TABLE VI

WAVELENGTH OF THE MAXIMUM FLUORESCENCE EMISSION OF TRYPTOPHAN SYNTHASE

All the enzyme preparations were dissolved in 0.05 M potassium phosphate (pH 7.2). The absorbance of the enzyme solution at the wavelength of excitation was usually less than 0.1 absorbance unit. The emission spectra were measured as described in Experimental Procedure.

Enzyme preparation	λ *excitation* *(nm)*	λ *emission max* *(nm)*
Holoenzyme	280	335
	325	385
	410	495
Apoenzyme	280	335
$NaBH_4$-reduced holoenzyme	340	390
Pyridoxal-*P* + glycine[a]	420	500

[a] Taken from Klein and Sagers[47].

The titration was carried out at room temperature in a Gilford Model 2000 or a Cary Model 14 spectrophotometer with expanded scale. The apoenzyme solution to be titrated contained 0.6 to 2.5 mg of protein in 1 ml of 0.05 M potassium phosphate buffer (pH 7.2). The reference cuvette contained 1.0 ml of the same phosphate buffer without protein. Equal aliquots (5 μl) of pyridoxal-*P* solution (0.4 to 0.8 mM) were added to both sample and reference cuvettes. Difference absorbance at 430 nm was read 10 min after mixing, and was then plotted against the total pyridoxal-*P* concentration as shown in Fig. 4. The resulting hyperbolic curve indicates that the titrable binding sites of pyridoxal-*P* are independent and that the non-specific binding, if any, is insignificant under these experimental conditions. From the coordinates of the intersection of the initial tangent and asymptote of the titration curve, a molar ratio of pyridoxal-*P* to apoprotein was determined to be about 1.47, assuming a molecular weight of 149 000 for the enzyme[15]. Since the protein concentration was determined by comparison with serum albumin as a standard, this value may have to be adjusted when the precise molar extinction coefficient of the enzyme has been determined.

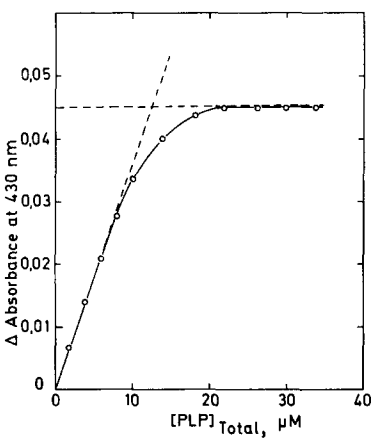

Fig. 4. Spectrophotometric titration of apotryptophan synthase with pyridoxal-*P* (PLP). To 1.0 ml of apoenzyme solution (concn 8.4 μM) in 0.05 M potassium phosphate buffer, pH 7.2, 5 μl each of 0.4 to 0.8 mM pyridoxal-*P* solution in the same buffer was added. The change in absorbance at 430 nm as a result of the formation of the enzyme–coenzyme complex under equilibrium conditions were read against a buffer blank containing an equal concentration of pyridoxal-*P*. The data are plotted against the total concentration of pyridoxal-*P* in the mixture.

The apparent association constant (K'_{assoc}) of the enzyme for pyridoxal-*P* was estimated from the titration curve, taking no account of the absolute number of binding sites, according to the following equation:

$$K'_{assoc} = \left(\frac{a}{1-a}\right) \cdot [S]^{-1} \qquad \text{if } [S] \neq 0;\ a < 1$$

where a is defined as the fraction of the enzyme carrying bound pyridoxal-*P*. Assuming the change in absorbance at 430 nm is strictly proportional to the concentration of the intrinsic Schiff base of the enzyme–coenzyme complex, the value of a is then given by the ratio of ΔA_{430} to ΔA_{max} (the absorbance change at the completion of

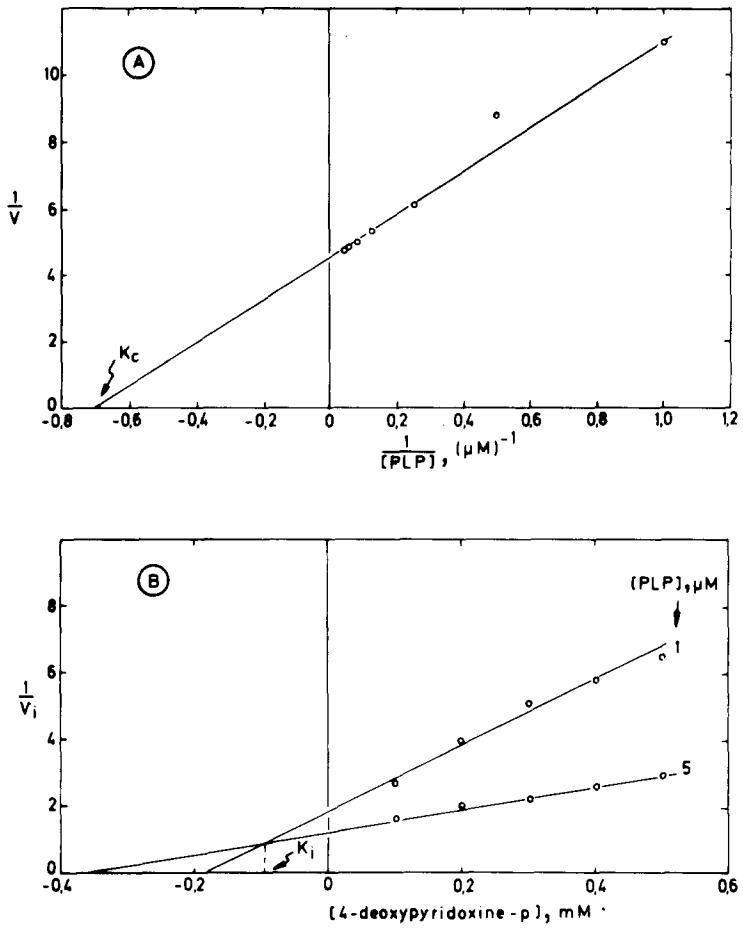

Fig. 5. A. Double reciprocal plot of the initial velocity against the concentration of pyridoxal-*P* (PLP). Pyridoxal-*P* at various concentrations as indicated was directly incorporated into the enzyme–substrate mixture (without serine). The mixture was then incubated at 37 °C for 15 min before the reaction was initiated with 0.2 ml of 0.1 M of L-serine, the final volume was 0.5 ml. About 5.5 μg apoenzyme was present in each assay mixture. *V* is expressed in terms of μmoles of indole consumed per min per mg enzyme. B. Dixon plot for the determination of the inhibitor constant (K_i) for 4-deoxypyridoxine phosphate. 4-Deoxypyridoxine phosphate at various concentrations was directly incorporated into the assay mixture containing a constant amount of pyridoxal-*P* as indicated. V_i is expressed in arbitrary units.

titration). [*S*] represents the free pyridoxal-*P* concentration which can be calculated from the total pyridoxal-*P* concentration, [S_0], by the equation:

$$[S] = (1 - b)\,[S_0]$$

where *b* is defined as the ratio of the ΔA_{obsd} to ΔA_{expd} at 430 nm. The latter is the expected change in absorbance at 430 nm if all the pyridoxal-*P* in the system is specifically bound to the enzyme. It is determined from the theoretical linearity of a line depicting the initial slope of the titration curve when the enzyme concentration is much greater than the dissociation constant for the ligand. The value of K'_{assoc} for

pyridoxal-*P* thus obtained is about $6.3 \cdot 10^5$ M^{-1}, which is in close agreement with the value obtained kinetically.

Kinetic studies

Kinetic parameters for the binding of cofactor or its analogues to the apoenzyme were determined by conventional techniques. The plot of $1/v$ against the reciprocal concentration of pyridoxal-*P* is shown in Fig. 5A. From the intercept on the negative abscissa, the concentration of pyridoxal-*P* required to attain half-maximum velocity, namely the cofactor constant (K_c), was determined to be about 1.4 μM. Pyridoxine phosphate and 4-deoxypyridoxine phosphate were found to inhibit competitively the binding of pyridoxal-*P* to the enzyme. The inhibitor constant (K_i = 0.1 mM) for 4-deoxypyridoxine phosphate was determined graphically by the method of Dixon[38] as shown in Fig. 5B. The K_i for pyridoxine phosphate was determined by the same method; it was found to be 0.03 mM.

Studies on the dependence of the reaction rate on substrate concentration have not yet been made in detail. Preliminary results showed that the apparent Michaelis constant (K'_m) for L-serine is about 2 mM. No attempt to determine the true Michaelis constant for serine was made in this study because of the limitation of the colorimetric assay. The K'_m for indole is too low (approximately in the order of 10^{-5} M) to be determined precisely by our assay method. The K_m value for indole of the td_{201} enzyme found in the literature was in the range of $1.8 \cdot 10^{-5}$ M (ref. 13) to $6 \cdot 10^{-5}$ M (ref. 39). However, these values can only be considered as the apparent Michaelis constant for indole. The determination of the true Michaelis constant should await development of a more sensitive assay method, for instance by using isotopically labelled substrates[19].

DISCUSSION

For comparative studies of the properties of wild-type and mutant enzymes, a sensitive and precise assay method is certainly desirable. Generally, the catalytic activity of a mutationally altered enzyme is much inferior to that of the wild type enzyme; accordingly, important biochemical information on the mutant enzyme could have been missed or overlooked with less sensitive assay methods. For instance, the cross-reacting material obtained from a tryptophan auxotroph strain td_2 (an indole accumulator) was originally claimed only capable of catalyzing Reaction 3[27]. With assay methods described in this paper, we were able to show that it also catalyzes Reaction 2 (H. Tsai and S. R. Suskind, unpublished results), although the catalytic efficiency is much lower (<0.3%) than that of the wild type enzyme.

Like any colorimetric assay, the present modified method certainly still has its limitations. Although it is sensitive and convenient for the routine assay of Reaction 2 activity, it is still not sensitive enough for kinetic studies with subsaturation levels of indole. It also cannot be applied for the assay of Reaction 3 because of the interference of indole-3-glycerol phosphate with the indole reaction.

In the past decade, biochemical and enzymological studies of *Neurospora* tryptophan synthase at the molecular level have been severely handicapped by the apparent lability of the enzyme[9,10]. Although a considerable amount of effort in searching for means to prevent the enzyme from decay has been made, no successful method

has yet been developed. Consequently, many published studies and conclusions which were made solely on the basis of the stability of this enzyme should be reconsidered. For example, Wust and Bonner[40] compared the kinetics of inactivation of the enzyme in various ammonium sulfate fractions and concluded that the enzyme consists of a heterogeneous population of molecules because the rate of inactivation of some enzyme fractions is much faster than that of others. However, we have shown here that the lability of tryptophan synthase is not an intrinsic characteristic of the enzyme. The difference in the rate of inactivation among various ammonium sulfate fractions is due to the unequal distribution of inhibitory material in different fractions. The protection of enzyme from inactivation by phenylmethanesulfonyl fluoride as shown in Table IV strongly supports the notion that proteolytic digestion may be the primary cause for the spontaneous inactivation of the tryptophan synthase of *Neurospora*.

Recently, Gaertner and co-workers[41] have demonstrated in *N. crassa* that the enzymes involved in the tryptophan pathway are in the form of at least two multienzyme complexes, and that the catalytic efficiencies of the different enzymes are increased in the complex. They suggested that such improvement in the catalytic ability of the individual enzymes is either due to facilitated metabolic channeling[42] or to direct activation of the enzymes. Tryptophan synthase, the terminal enzyme of the tryptophan pathway, has not been shown *in vitro* to be associated with these complexes. This does not exclude the possibility that the enzyme *in vivo* interacts with the other components of the pathway. The *in vitro* activation of the purified tryptophan synthase by proteins such as serum albumin may be explained by the molecular interactions between the enzyme and serum albumin.

Unlike with the tryptophan synthase of *E. coli*, dissociation of the *Neurospora* enzyme into enzymically active components A and B has not been possible. Hence, the intramolecular cooperative effect on the catalytic efficiency by complexing the A and B proteins as with the *E. coli* enzyme is not demonstrable in *Neurospora*. Therefore, the possibility of such effects has generally not been considered. On the other hand, intermolecular cooperative effects between tryptophan synthase and the other components of the tryptophan pathway may be present and such effects may be easily detected by the enhancement of enzymic activity upon formation of a multienzyme complex.

It has been shown by Creighton and Yanofsky[30] that pyridoxal-*P* and serine are required for the complexing of the A and B proteins of the *E. coli* enzyme. Comparative studies with purified apo- and holotryptophan synthase of *Neurospora* have not shown any significant differences in quaternary structures between these different forms of the enzyme (H. Tsai and S. R. Suskind, unpublished results). This again supports the notion that the *Neurospora* enzyme is a single undissociable protein with two pairs of polypeptide chains[43]. Previous evidence indicates that, besides its classical function in catalysis[35,36], the pyridoxal-*P* coenzyme may also play a role in maintaining the native conformation giving maximal catalytic activity of the *Neurospora* tryptophan synthase[27]. Thus, Carsiotis and Suskind[44] showed that pyridoxal-*P* and its analogues such as pyridoxine phosphate and 4-deoxypyridoxine phosphate can stimulate Reaction 3 with enzymes obtained from a histidine-requiring mutant strain C_{84} and the cross-reacting material prepared from a tryptophan auxotroph strain td_2. Recently, Tsai[45] showed that wild type apotryptophan synthase was virtually in-

active for all of the three reactions. The synthetic activity (Reaction 2), where pyridoxal-*P* acts directly in catalysis, of the holoenzyme could be selectively inactivated by reduction with $NaBH_4$, whereas the aldolytic activity (Reaction 3) in which pyridoxal-*P* is not directly involved in catalysis was unaffected. On the basis of these findings, two functions, a catalytic as well as a structural one, were postulated for the pyridoxal-*P* coenzyme in tryptophan synthase[45].

Spectral evidence, as shown in Fig. 3B and Table VI, indicates that two types of binding of pyridoxal-*P* exist in the enzyme, one absorbing at 325 nm ($\lambda_{max}^{emission}$ = 385 nm) and the other at 410 nm ($\lambda_{max}^{emission}$ = 495 nm). Although nearly two pyridoxal-*P* binding sites are spectrophotometrically titrable, the results presented in this paper do not permit us to distinguish whether the binding environments for the two coenzyme molecules are identical. They also do not tell us how labors are divided between these two coenzyme molecules. Questions such as these remain for future investigations.

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