CATALYTIC PROPERTIES OF TRYPTOPHANASE,
A MULTIFUNCTIONAL PYRIDOXAL PHOSPHATE ENZYME

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We described previously\(^1\) a tryptophan-induced tryptophan synthetase (TSase) from *Escherichia coli* which catalyzes reaction (1) but which is not related to the repressible TSase of *E. coli* described by Yanofsky.\(^2\)

\[
\text{Indole} + \text{L-serine} \rightarrow \text{L-tryptophan} + \text{H}_2\text{O} \quad (1)
\]

\[
\text{L-Tryptophan} + \text{H}_2\text{O} \rightarrow \text{indole} + \text{pyruvate} + \text{NH}_3 \quad (2)
\]

This inducible TSase was shown to share a common regulatory mechanism with the enzyme, tryptophanase (TPase), which catalyzes reaction (2). The possibility was suggested that reactions (1) and (2) might be catalyzed by the same enzyme.\(^1\)

Results presented here demonstrate that reactions (1) and (2) are indeed catalyzed by a single enzyme, TPase, and that this same enzyme also catalyzes the related reactions (3), (4), and (5) at appreciable rates. Enzymes that catalyze reactions (3) and (4) have long been known; reaction (5) has not been observed previously. S-Methyl-L-cysteine also serves as a substrate for TPase in reactions (4) and (5) in a manner analogous to cysteine.

\[
\text{L-Serine} \rightarrow \text{pyruvate} + \text{NH}_3 \quad (3)
\]

\[
\text{L-Cysteine} + \text{H}_2\text{O} \rightarrow \text{pyruvate} + \text{NH}_3 + \text{H}_2\text{S} \quad (4)
\]

\[
\text{Indole} + \text{L-cysteine} \rightarrow \text{L-tryptophan} + \text{H}_2\text{S} \quad (5)
\]

The catalysis of these several reactions by a single pyridoxal phosphate-dependent enzyme is a novel situation and one which has important implications
for the correlation of the mechanistic roles of pyridoxal in model and enzymatic systems.

Methods.—(1) Enzyme assays: One unit of each of the enzymatic activities followed is the amount of enzyme catalyzing formation of 0.1 µmole of product in 10 min at 37°. The specific activity is expressed as the number of units of enzyme per milligram of protein. Solutions of substrate amino acids were adjusted to pH 7.8 with KOH before addition to assay solutions. Assays for individual enzymatic activities were conducted as follows:

(a) TPase: The procedure described previously was modified slightly by increasing the potassium phosphate buffer (pH 7.8) from 20 to 40 µmoles, and adding 0.25 mg of bovine serum albumin.

(b) TSase: The assay described previously was used with the following changes: the buffer consisted of 20 µmoles of potassium phosphate, pH 7.8, the reaction was started by addition of 32 µmoles of l-serine, and was allowed to proceed for 10 min. The disappearance of indole and the formation of tryptophan were followed by use of the acid-Ehrlich reagent and by microbiological assay with Leuconostoc mesenteroides P-60, respectively. Synthesis of tryptophan from l-cysteine and S-methyl-l-cysteine was followed by microbiological assay of reaction mixtures containing the appropriate amino acid as substrate in place of l-serine.

(c) Deamination of l-serine, l-cysteine, and S-methyl-l-cysteine: These reactions were followed by measuring pyruvate formation in 0.2 ml of reaction mixture containing 20 µmoles of potassium phosphate, pH 7.8, 20 µmoles of pyridoxal phosphate, 32 µmoles of the amino acid, and enzyme. After 5 min at 37°, the reaction is started by addition of the amino acid and allowed to proceed for 10 min with shaking. The reaction is stopped by addition of 0.1 ml of 2.5 N sodium hydroxide; then 0.5 ml of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid is added. After 5 min, 1.5 ml of absolute ethanol is added and the color developed by the addition of 2.5 ml of 2.5 N sodium hydroxide. After 10 min, the absorbance at 520 µm is determined, and the amount of pyruvate formed is calculated from a standard curve prepared using crystalline sodium pyruvate. The sulfhydryl group interferes with estimation of pyruvate when cysteine is the substrate. This was prevented by addition of 64 µmoles of iodoacetamide in 0.2 ml of water to the reaction mixture immediately after stopping the reaction with alkali and by allowing the mixture to stand for 5 min before adding the 2,4-dinitrophenylhydrazine reagent.

(2) Other analytical procedures: Hydrogen sulfide in reaction mixtures was trapped as cadmium sulfide in the center well of a Warburg vessel and determined as described by Smythe. The incubation was carried out for 20 min at 37° with shaking. Ammonia was determined after the reaction was stopped with 2 N hydrochloric acid (0.5 ml added to a reaction volume of 2 ml) by adsorption and elution from permute followed by Nessler's reagent.

Starch gel electrophoresis patterns were obtained by a modification of the procedure of Barrett, Friesen, and Astwood. The TPase preparations were diluted to 10 mg protein per ml and dialyzed overnight against 0.01 M potassium phosphate buffer (pH 7) before applying to the gel.

(3) Cultures and growth media: Mutant strains B/117 and B/117-A of E. coli and media for their culture have been described. Mutant B/117-A, which produces tryptophanase constitutively, was grown at 30° with vigorous aeration in 16-liter carboys. Each carboy, containing 12 liters of broth medium, was inoculated with 4 liters of an overnight culture grown on minimal medium supplemented with 0.1% glucose and 10 µg/ml of indole. The cells were harvested by centrifugation at the end of the logarithmic phase of growth (4–6 hr). Cells grown in this fashion are unusually rich in TPase.

(4) Purification of TPase: Unless specified otherwise, all purification steps are carried out at 0–4°. The dilution buffer used at several points contains potassium phosphate (0.1 M), mercaptoethanol (5 mM), ethylenediamine tetraacetate (2 mM), pyridoxal phosphate (0.04 mM), and ammonium sulfate (0.8 M) at pH 7.0.

(a) Preparation of cell extracts: Freshly harvested cells are suspended in 0.1 M potassium phosphate buffer, pH 7, at a density of 0.2 gm (wet weight) per ml and disrupted in 50-ml portions by treatment in a Raytheon 10-ke ultrasonic oscillator for 7 min. The treated suspension is centrifuged for 40 min at 35,000 × g and the pellet discarded.

(b) Protamine and first ammonium sulfate step: The supernatant solution from (a) is adjusted to pH 6 with cold 1 N acetic acid and diluted with distilled water to a protein concentration of 10 mg per ml. After the solution is brought to 14%, protamine sulfate (7 µl of a 2% solution
(pH 5.0) per mg of protein) is added, and the precipitated nucleic acids are centrifuged out and discarded. The supernatant solution is fractionated by the addition of solid ammonium sulfate without adjusting the pH. The protein precipitated between 45 and 75% saturation usually contains most of the activity. The precipitate is dissolved in sufficient dilution buffer to give a concentration of 10 mg of protein per ml.

(c) Heat step: One hundred-ml portions of the enzyme solution from (b) in 500-ml flasks are heated in a 70° waterbath for 8 min after the temperature of the solution reaches 60°. The temperature is not allowed to exceed 65°. The solution is chilled, and the denatured protein is removed by centrifugation.

(d) Second ammonium sulfate step: Solid ammonium sulfate is added to the solution from step (c) to yield 50% saturation, and the precipitate is discarded. Additional ammonium sulfate is added to yield 70% saturation, and the active precipitate is collected by centrifugation. This precipitate is then back-extracted by gentle stirring for 10 min with convenient volumes (ca. 1 ml per 40 mg of protein) of dilution buffer which is made 57, 55, and 52% saturated with ammonium sulfate and adjusted to pH 7.0. Additional activity can often be recovered by re-extracting with the 52% saturated buffer. The 55 and the 52% ammonium sulfate extracts usually contain the TPase and are pooled for further purification.

(e) Crystallization: The pooled fractions from (d) are saturated by addition of solid ammonium sulfate. The precipitate is collected by centrifugation and is redissolved in a minimal amount of the dilution buffer (ca. 1 ml/80–100 mg of protein). The first crop of crystals generally appears after the solution stands overnight in ice, and is removed by centrifugation. A second crop of crystals is obtained by the addition of saturated ammonium sulfate solution to the supernatant solution until a slight silkiness appears, then allowing it to stand for several days in an ice bath. Tryptophanase can be recrystallized by dissolving the crystals in the dilution buffer and repeating the above procedure.

Materials.—Crystalline bovine serum albumin was obtained from the Pentex Corporation. Sodium pyruvate and the l-amino acids used for assay purposes were obtained from the California Corp. for Biochemical Research.

Results.—Purification of TPase: Cells of E. coli B/17-A are constitutive for TPase and contain a very high level (about 10% of the soluble protein in the crude extract) of the enzyme when grown as described. The purification procedure resembles those of Browder and of Burns and DeMoss, with the exception of the final steps which make use of crystallization of the enzyme rather than column purification.

![Fig. 1.—Starch gel electrophoresis patterns of crystalline preparations of TPase and serum albumin. A: bovine serum albumin; B, C, and D: TPase following 1, 2, or 3 crystallizations, respectively. Each sample contained 200 μg of protein and was subjected to electrophoresis at about pH 8 under the conditions described by Barrett et al.](image-url)
chromatography. The instability of purified TPase observed by Burns and De-Moss was particularly pronounced during the final stages of purification. Because of this instability, the activity of crystalline TPase is somewhat variable, and decreases on storage. Specific activities up to 2500 have been achieved by this procedure and are the highest yet reported for TPase. Figure 1 demonstrates the progressive purification achieved by the crystallization procedure, and indicates that the recrystallized product contains only a single protein. A more detailed report on the purification and the physical and chemical properties of the enzyme is in preparation.

Reactions catalyzed by TPase: Crystalline TPase was found to catalyze both synthesis and degradation of tryptophan (reactions (1) and (2)). Trials showed that the crystalline enzyme also catalyzed the dehydration of serine (reaction 3) in the absence of added indole or tryptophan.

Each of these related reactions follows a common postulated course involving elimination of a β-substituent of an α-amino acid (together with a proton) followed either by addition of a new β-substituent (β-replacement, Table 1) or hydrolysis to pyruvate and ammonia (β-elimination, Table 1). Cysteine and S-methylcysteine, which undergo formally similar reactions to yield pyruvate in enzymatic systems were found, like serine, to serve as substrates for both pyruvate formation and tryptophan synthesis (Table 1). The stoichiometry of product formation in reactions (3) and (4) was established (Table 2); the formation of L-tryptophan from serine, cysteine, and S-methylcysteine was established by assay with L. mesenteroides P-60, which responds specifically to this compound. Tryptophan formation was equivalent in each case to indole disappearance.

Since the TPase preparation used appears homogeneous (Fig. 1), a single enzyme must catalyze each of these several reactions. This conclusion is supported by

**TABLE 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction no. and type</th>
<th>Product measured</th>
<th>Relative activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Tryptophan</td>
<td>2, E</td>
<td>Indole</td>
<td>100</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>4, E</td>
<td>Pyruvate</td>
<td>100</td>
</tr>
<tr>
<td>S-Methyl-L-cysteine</td>
<td>E</td>
<td>Pyruvate</td>
<td>35</td>
</tr>
<tr>
<td>L-Serine</td>
<td>3, E</td>
<td>Pyruvate</td>
<td>12</td>
</tr>
<tr>
<td>L-Cysteine + indole</td>
<td>5, R</td>
<td>L-Tryptophan</td>
<td>30</td>
</tr>
<tr>
<td>S-Methyl-L-cysteine + indole</td>
<td>R</td>
<td>L-Tryptophan</td>
<td>26</td>
</tr>
<tr>
<td>L-Serine + indole</td>
<td>1, R</td>
<td>L-Tryptophan</td>
<td>16</td>
</tr>
</tbody>
</table>

* Numbers refer to reactions in text; E = β-elimination, R = β-replacement reaction.
† All products and activities were assayed as described under Methods. Recrystallized TPase having a specific activity of 1800 in the TPase assay was used and assigned a relative rate of 100.

**TABLE 2**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pyruvate</th>
<th>Ammonia</th>
<th>Hydrogen sulfide</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Cysteine</td>
<td>3.1</td>
<td>3.1</td>
<td>3.0</td>
</tr>
<tr>
<td>L-Serine</td>
<td>0.99</td>
<td>0.99</td>
<td>-</td>
</tr>
</tbody>
</table>

Each incubation flask contained 20 μmoles of the appropriate substrate together with 0.8 mg of a dialyzed TPase preparation (D, Fig. 1) when cysteine was the substrate, or 4 mg when serine was the substrate. The large amount of enzyme was required due to the low substrate concentrations employed and the losses in enzymatic activity on dialysis.
the observations that the relative rates of reactions (1) and (2) determined for the crystalline protein (Table 1) are the same as those observed for crude cell extracts, by unpublished genetic observations, and by the unusual ion requirements and inhibition by indole discussed below.

Ion requirement for TPase activity: Crude preparations of TPase require K⁺ or NH₄⁺ for degradation of tryptophan.¹⁰ ¹¹ The crystalline enzyme shows a similar requirement for maximal activity in the catalysis of reactions (1), (2), and (3); Na⁺ is inactive (Fig. 2). The ion requirement of the other reactions was not investigated. It is not clear why the optimum K⁺ or NH₄⁺ concentration for the different reactions should show such variation. K⁺ and NH₄⁺ also activate tryptophan synthesis by the B protein of the repressible TSase from E. coli.¹²

Inhibition of TPase activities by indole: The inhibitory action of indole on degradation of tryptophan by TPase is well known. If degradation of serine and cysteine to pyruvate (reactions (3) and (4)) is catalyzed by TPase, these reactions should also be inhibited by indole. As shown in Table 3, this is indeed the case, and the inhibitory effect of indole appears to result primarily from its action as a substrate leading to tryptophan formation. The inhibitory action of indole on degradation of tryptophan may have a similar mechanistic basis.
TABLE 3
INHIBITION BY INDOLE OF THE TPASE-CATALYZED DEGRADATION OF SERINE AND CYSTEINE

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Product</th>
<th>Indole Added (mMoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L-Serine</td>
<td>Pyruvate</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
<td>0</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>Pyruvate</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
<td>0</td>
</tr>
</tbody>
</table>

* When serine was the substrate, 20 μg of purified TPase (D, Fig. 1) per reaction vessel was used; with cysteine as substrate 3 μg of the same enzyme preparation was employed. Standard deaminase mixtures (see Methods) were employed with indole added as indicated. Replicate tubes were set up at each concentration, and pyruvate and tryptophan determinations were conducted on separate reaction mixtures.

TABLE 4
COMPARATIVE EFFECT OF INDOLE ON PYRUVATE FORMATION FROM SERINE BY CRUDE EXTRACTS OF E. coli CULTURES

<table>
<thead>
<tr>
<th>Source of crude extract</th>
<th>Pyruvate formed (mMoles)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>E. coli B/1t7-A†</td>
<td>150</td>
</tr>
<tr>
<td>E. coli B/1t7 (serine-induced)‡</td>
<td>90</td>
</tr>
</tbody>
</table>

* Assay for serine dehydrase as described in Methods, but 1.0 ml volumes and 9.5 mM serine.
† Cells grown in minimal medium + glucose (0.15%) supplemented with 20 μg of l-tryptophan per ml. Each assay tube contained 1.5 mg of protein (specific activity, TPase = 32).
‡ Cells grown as in the above footnote, but with 5 μg per ml of n-o-serine added. Each assay tube contained 0.42 mg of protein (specific activity, TPase = 0).

Added indole also inhibits the serine dehydrase activity of a cell-free extract of E. coli B/1t7-A (Table 4) indicating that the serine dehydrase activity of these extracts is due to the constitutive TPase they contain. In contrast, the serine dehydrase activity of the serine-induced parent strain, B/1t7, which does not contain TPase, is not inhibited by indole (Table 4). Thus, the TPase-associated L-serine dehydrase activity is distinct from the previously studied\textsuperscript{18} serine-induced L-serine dehydrase of E. coli, and the latter enzyme appears to have no TPase activity.

Discussion.—The broad substrate specificity shown by TPase is strikingly reminiscent of previously studied model systems in which pyridoxal plus metal ions catalyze pyruvate formation from serine,\textsuperscript{19} cysteine,\textsuperscript{14} cystine,\textsuperscript{8} and many other β-substituted amino acids,\textsuperscript{8} \textsuperscript{13} and also the synthesis of tryptophan from indole and serine.\textsuperscript{8} While many additional reactions (e.g., transamination, racemization, etc.) can occur in model systems,\textsuperscript{8} \textsuperscript{16} these have not so far been observed in the enzymatic system, which appears to show reaction specificity for catalysis of α,β-elimination and replacement reactions. The rate of the nonenzymatic reactions increases with increasing electronegativity of the β-substituent,\textsuperscript{18} and the same general effect is evident for the enzymatic reaction (Table 1), where the rate of reaction increases for both pyruvate and tryptophan formation as the β-group is changed from —OH to —SCH\textsubscript{3} to —SH. Because of the effect of the β-substituent on affinity of the substrate for the enzyme, however, such a relationship need not hold for an extended series of potential substrates.

Each of the reactions catalyzed by TPase is pyridoxal phosphate-dependent, and can be partially explained by adopting a reaction scheme (Fig. 3) analogous to that previously proposed\textsuperscript{8} \textsuperscript{16} to explain the catalytic role of pyridoxal in the corresponding nonenzymatic reactions. In catalysis of α,β-elimination reactions,
the amino acid, I (R = —OH, —SH, —SCH₃ or indolyl radical), interacts with TPase, II, to form reversibly by elimination of H⁺ and R⁻ (H₂O, H₂S, CH₃SH, or indole), III, IV, and V. The latter hydrolyzes irreversibly to yield pyruvate and ammonia and regenerate TPase. In the presence of indole, addition to V,¹⁷ rather than hydrolysis, can occur to yield tryptophan by reversal of these reactions. More generally, however, the scheme predicts that addition of R groups other than indole to V might also be catalyzed by TPase to yield the corresponding amino acid, I; the rate and extent of such additional replacement reactions would depend in part, of course, upon the affinity of TPase for the attacking R group.

The broad substrate specificity found for purified TPase is similar in some respects to that reported for cystathionase¹⁸ and for purified cystathionine synthetase.¹⁹ The degree of substrate specificity imposed by the protein varies, however. Thus, Crawford²⁰ finds that the B protein of the TSase of E. coli also catalyzes the serine dehydrase reaction, but not the tryptophanase reaction,²⁰ and crude TSase from N. crassa reportedly will not catalyze synthesis of tryptophan from cysteine.²¹ However, conditions of assay greatly influence the detection of reactions potentially catalyzed by a given enzyme,²² and it may be that specificity even in well-known enzymes is not as rigid as sometimes thought. Such considerations may explain in part previous failures to detect the TSase and serine dehydrase²³ activities of TPase both in crude extracts and in purified preparations.

Summary.—The purification and crystallization of tryptophanase from a partially derepressed mutant strain of E. coli that produces this enzyme constitutively is described. The crystalline enzyme catalyzes a series of α,β-elimination reactions (b–d) in addition to the tryptophanase reaction (a):

(a) \[ \text{L-Tryptophan} \overset{\text{H}_2\text{O}}{\rightarrow} \text{Pyruvate} + \text{NH}_3 + \text{Indole} \]

(b) \[ \text{L-Serine} \overset{\text{H}_2\text{O}}{\rightarrow} \text{Pyruvate} + \text{NH}_3 \]

(c) \[ \text{L-Cysteine} \overset{\text{H}_2\text{O}}{\rightarrow} \text{Pyruvate} + \text{NH}_3 + \text{H}_2\text{S} \]

(d) \[ \text{S-Methyl-L-cysteine} \overset{\text{H}_2\text{O}}{\rightarrow} \text{Pyruvate} + \text{NH}_3 + \text{CH}_3\text{SH}. \]

In the presence of indole, tryptophanase also catalyzes synthesis of L-tryptophan from serine, cysteine, and S-methylcysteine with elimination of the β-substituent of these amino acids. Each of the reactions investigated is activated by K⁺ or NH₄⁺. A possible mechanism for these several related reactions is discussed briefly.
* Supported in part by a grant (A-1448) from the U.S. Public Health Service.
9 It has been suggested that for some desulphhydrases the actual substrate is the disulfide rather than the sulfhydryl compound (cf. Cavallini, D., B. Mondovi, and C. DeMarco, in Chemical and Biological Aspects of Pyridoxal Catalysis, ed. E. E. Snell et al. (Oxford: Pergamon Press, 1963), p. 361. This possibility has not been excluded for the reactions catalyzed by TPase.
17 Tryptophan synthesis is considered by Braunstein (Braunstein, A. E., in The Enzymes, ed. P. D. Boyer, H. Lardy, and K. Myrbäck (New York: Academic Press, 1960), vol. 2, p. 113) to involve an S\(_N\) reaction in which the \(\beta\)-hydroxyl group of serine is displaced by indole. If applied to the TPase-catalyzed synthesis studied here, this proposal would eliminate the bound \(\alpha\)-aminoacrylic acid (V), required as an intermediate in pyruvate formation, as an obligatory intermediate in tryptophan synthesis. In fact, the two proposals are fundamentally similar; whether an attacking \(R'\) group displaces the partially polarized \(R\) group present in III or IV before its elimination to yield V, or adds only after formation of V as formulated in Fig. 3, cannot yet be decided.
20 Crawford, I. P., private communication.
22 For example, the rates of the TSase and the serine dehydrase reactions catalyzed by TPase are greatly enhanced by the abnormally high \(t\)-serine concentration in the assay mixtures. The \(K_m\) for this amino acid is on the order of 0.1 mole per l.