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TRYPTOPHANASE FROM *AEROMONAS LIQUEFACIENS*

PURIFICATION, MOLECULAR WEIGHT AND SOME CHEMICAL, CATALYTIC AND IMMUNOCHEMICAL PROPERTIES

JAMES L. COWELL*, KAREN MOSER AND RALPH D. DEMOSS
Department of Microbiology, University of Illinois, Urbana, Illi. 61801 (U.S.A.)

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SUMMARY

Tryptophanase from *Aeromonas liquefaciens* has been purified to homogeniety by employing a heat treatment, $(NH_4)_2SO_4$ fractionations and hydroxyapatite chromatography. The enzyme binds four moles of pyridoxal 5'-phosphate per mole of enzyme, has a $s^0_{20,w}$ value of 9.78 S and a mol. wt of 216 000. These properties and the general catalytic properties of the enzyme are similar to other tryptophanase molecules.

The enzyme requires pyridoxal 5'-phosphate (K_m $2.09 \cdot 10^{-6}$ M), NH_4^+ or K^+ for catalytic activity and besides using tryptophan (K_m $2.21 \cdot 10^{-4}$ M), the enzyme can also utilize L-serine, L-cysteine and *S*-methyl-L-cysteine in catalyzing α,β-elimination and β-replacement reactions. Anthranilic acid (K_i $3.0 \cdot 10^{-4}$ M) alanine (K_i $1.31 \cdot 10^{-3}$ M), and phenylalanine (K_i $7.79 \cdot 10^{-3}$ M) are competitive inhibitors of the tryptophanase reaction.

Immunochemical studies indicate that the *A. liquefaciens* enzyme has some common antigenic determinant sites with the tryptophanases from *Escherichia coli*, *Bacillus alvei*, a marine vibrio (K-7), *Paracolobactrum coliforme*, *Micrococcus aerogenes*, and *Sphaerophorus funduliformis*.

Each tryptophanase studied has a distinct amino acid composition, but as a group they are characterized by a fairly high content (50–56%) of non-polar amino acids and a very similar total basic amino acid content. The *A. liquefaciens* enzyme has the highest non-polar and lowest acidic amino acid content of the group, and may be expected to have an electrophoretic mobility significantly different from the other molecules.

Abbreviations: pyridoxal-*P*, pyridoxal-5'-phosphate; bicine, *N*,*N*-bis-(hydroxyethyl)-glycine.

* Present address: Department of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850.

INTRODUCTION

Tryptophanase* (EC 4.1.99.1) is a pyridoxal 5'-phosphate(pyridoxal-*P*)-dependent enzyme whose primary activity is the cleavage of tryptophan to indole, pyruvate and ammonia. The enzyme is found exclusively in bacteria and occurs in diverse bacterial species[1]. The enzyme has been purified to homogeneity and characterized from *Escherichia coli* B/lt7-A[2-5], *E. coli* K-12[6,7], *Bacillus alvei*[8-11] and *Sphaerophorus varius*[12] and *Sphaerophorus funduliformis*[12]. The study of the enzyme from these sources has indicated that the enzyme has interesting properties for studying structure and function relationships. An understanding of the structure and action of the enzyme has added and could continue to add to the general understanding of such concepts as the mechanism(s) of pyridoxal-*P*-catalyzed reactions[10,13-15] the role of pyridoxal-*P* in the structure and catalytic action of enzymes[9,11,16,17], the role of monovalent cations in the structure and catalytic action of enzymes[14,16,18] and protein–protein interactions as related to subunit enzymes[16,17]. Close examination of the properties of a number of enzymes catalyzing the same activity allows for a much better understanding of the characteristics of the enzyme. Considering these aspects, the present investigation was initiated to further the study of tryptophanase from diverse bacterial species with the ultimate goal of understanding the structure and action of the enzyme.

In this paper a procedure is described for obtaining a homogeneous preparation of the enzyme from *Aeromonas liquefaciens*. The molecular weight and some chemical, catalytic and immunochemical properties of the enzyme are also reported. The subunit structure of the enzyme and the ability of the enzyme to aggregate into enzymatically active polymeric species will be the subject of another communication[31].

MATERIALS AND METHODS

Chemicals

Enzyme grade $(NH_4)_2SO_4$ (Mann) was used throughout the purification. Iodoacetamide was recrystallized twice by suspending the iodoacetamide in light petroleum and freezing this suspension at −20 °C. Hydroxyapatite was prepared as described by Levin[19]. All other reagents were obtained commercially and used without further purification.

Organism and growth

A. liquefaciens (ATCC 14715) was grown under batch conditions in a 200-l fermentation tank (American Sterilizer Co.) containing 150 l of 2% tryptone (Difco), pH 7.2. Growth was at 25 °C with forced aeration. Cells were harvested at early stationary phase of growth before the onset of visible pigmentation of the culture. The cells were stored at −20 °C until preparation of the cell-free extract. Cells have been stored for up to one year without loss of enzyme activity. Cell-free crude extracts prepared from pigmented cells have a lower tryptophanase specific activity than extracts prepared from non-pigmented cells. Also, the purification is not valid for pigmented cells.

* L-tryptophan indole-lyase (deaminating).

Tryptophanase assay

During the purification of the enzyme, activity was routinely measured by the flask assay as described by Hoch *et al.*[8] with the exception that the 1.5-ml reaction mixture contained 200 μmoles *N,N*-bis-(hydroxyethyl)-glycine (bicine) buffer, pH 8.2, 500 nmoles pyridoxal-*P*, 250 μg bovine serum albumin and enzyme. For all the enzymatic assays one unit of enzyme activity is defined as the production of 1 μmole of product per min. Specific activity is expressed as units of activity per mg of protein.

For kinetic assays with pure tryptophanase the flask assay was used as above except the 1.5-ml reaction mixture contained 120 μg albumin. In performing pyridoxal-*P*-activation kinetic assays using apoenzyme, the 1.5-ml reaction mixture contained 120 μg of acetylated albumin. Albumin (Sigma) was acetylated according to the procedure of Epstein and Goldberger[20].

Tryptophan synthetase and dehydratase assays

The tryptophanase-catalyzed formation of tryptophan from indole and L-serine, L-cysteine or *S*-methyl-L-cysteine was measured by a modification of the method of Yanofsky[21] as described by O'Neil[10] and Hoch and DeMoss[32] except that the reaction mixture contained, in a volume of 0.4 ml, 60 μmoles bicine, pH 8.2, 15 nmoles pyridoxal-*P*, 15 μg albumin, 125 nmoles indole, the respective amino acid and enzyme.

The tryptophanase-catalyzed breakdown of L-serine, L-cysteine or *S*-methyl-L-cysteine was measured by a modification of the method of Friedemann and Haugen[22] as described by O'Neil[10] and Hoch and DeMoss[32] except the reaction mixture contained, in a volume of 0.4 ml, 60 μmoles bicine, pH 8.2, 15 nmoles pyridoxal-*P*, 15 μg albumin, the respective amino acid and enzyme.

Analysis of kinetic parameters

K_m and V values were determined on a IBM 360 computer using an iterative program for fitting the data points to a hyperbola. Inhibition patterns were determined graphically from linear plots of $1/v$ *vs* $1/[S]$ and from statistical analysis of intercepts using an iterative program which plots kinetic data as v *vs* v/S. K_i values were determined with three different inhibitor concentrations using Eqn VIII.52 described by Dixon and Webb[23].

Determination of protein

For impure preparations of tryptophanase, protein was determined by the method of Lowry *et al.*[24]. Dithiothreitol when present in concentrations of 0.1–1.0 mM and pyridoxal-*P* (0.01–0.1 mM) appeared to interfere with this method. These concentrations were reduced by dilution with distilled water before the assay of protein. Crystalline albumin (Sigma) was used as the standard. The concentration of purified enzyme was calculated from its absorbance at 278 nm. The absorbance of a 1% solution of holoenzyme at 278 nm ($E^{1\%}_{278\text{ nm}}$) was determined experimentally with the protein concentration based on dry weight and amino acid content (alanine and leucine). In 0.1 M potassium phosphate buffer (pH 6.8), containing 1.0 mM dithiothreitol and 0.1 mM pyridoxal-*P*, the $E^{1\%}_{278\text{ nm}}$ of the holo-enzyme determined by these methods was 9.49 and 9.54, respectively. These values were averaged to give a final

$E^{1\%}_{278\,nm}$ for the holoenzyme of 9.51. The concentration of pure holoenzyme determined by the method of Lowry *et al.*[24] against a standard of albumin must be divided by 1.26 to give the correct value. The $E^{1\%}_{278\,nm}$ of the apoenzyme in 0.1 M potassium phosphate buffer (pH 7.8), containing 0.5 mM dithiothreitol was determined to be 9.29 on the basis of amino acid content. For dry weight determinations tryptophanase was made salt-free as described by Dowhan and Snell[25] and dried to constant weight at 106 °C.

Disc gel electrophoresis

The purity of the enzyme preparations was routinely checked by acrylamide gel electrophoresis as described by Hoch and DeMoss[11].

Amino acid analyses

The amino acid composition of the enzyme was determined by standard procedures as described by Hoch and DeMoss[11].

Preparation of apoenzyme

The procedure described by Dowhan and Snell[25] for resolution of D-serine dehydratase was modified slightly for resolution of holotryptophanase. The modification consisted of replacing the 0.1 M cysteine with 0.1 M hydroxylamine·HCl in the resolving buffer. The apoenzyme, prepared in this manner, had a residual activity of less than 1% and no pyridoxal-*P* absorption peak in the 400–450 nm region. The recovery of protein was 100%. Enzymatic activity (86–100%) was recoverable by incubation of the apoenzyme with pyridoxal-*P*.

Ultracentrifugation analyses

Sedimentation velocity and equilibrium experiments were performed using standard procedures and conditions as described by Hoch and DeMoss[11]. The partial specific volume of the enzyme (0.738 ml/g) was calculated from the amino acid composition[26].

Inhibition of enzyme activity by antibody

Antibody inhibition experiments were performed using antiserum prepared by injecting New Zealand White rabbits in the foot pads and subcutaneously in the back with a 1:1 (v/v) emulsion of Freund's adjuvant (Colorado Serum Co.) and protein. Before injecting the animals they were bled for normal serum. Using pure *A. liquefaciens* tryptophanase as the antigen, 3.96 mg in 1.0 ml was injected followed by a secondary injection of 3.56 mg six weeks later. Ten days after the secondary injection the animals were bled. The antiserum was stored at −20 °C.

Cell-free crude extracts of *E. coli* K-12, a marine vibrio (K-7) isolated by Klug and DeMoss[27], *Paracolobactrum coliforme* 11605, *Photobacterium harveyi* 14126 and *Micrococcus aerogenes* were prepared as described by DeMoss and Moser[1]. Purified preparations of *B. alvei*[8] and *S. funduliformis*[12] tryptophanase were used in these experiments.

Assay of the inhibition of enzyme activity by the secondary antiserum was performed using the flask assay. Antiserum and normal serum were diluted with an equal volume of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.05 mM or

0.5 mM pyridoxal-*P* and incubated at 37 °C for 10 min without shaking. The reaction mixture contained in a volume of 1.5 ml, 200 μmoles bicine, pH 8.2, 120 μg albumin, 50 nmoles or 500 nmoles pyridoxal-*P*, enzyme and various amounts of the pre-incubated antiserum or normal serum. The assay of activity was initiated and measured as previously described[8].

RESULTS

Purification of tryptophanase from A. liquefaciens

(1) Crude extract. 400–500 g (wet wt) of cells were thawed slowly (12–16 h) at 5 °C. A 40% (w/v) cell slurry was then prepared by suspending the cell paste in 0.1 M potassium phosphate buffer, pH 6.8, containing 0.1 mM pyridoxal-*P* and about 1.0 mg of crude deoxyribonuclease I from beef pancreas (Sigma). In the earlier experiments, the cells were ruptured by sonic treatment with a Branson Sonifier. Later in the study, the cells were ruptured by pressure using a Manton–Gaulin Homogenizer. The purification procedure behaved the same regardless of the method of breakage, but homogenization gave a 50% increase in specific activity of the cell-free crude extract relative to sonic treatment. 250-ml batches of the uniform cell suspension at 4 °C were subjected to two 2-min sonic bursts (full intensity) and one 1-min sonic burst. After each sonic burst interval the contents were allowed to cool for 1 min. Alternatively, the cell suspension (4 °C) was passed through a pre-cooled Gaulin Homogenizer twice at 8000 lbs/inch^2. This material was centrifuged in a Model L preparative ultracentrifuge at 39 000 × *g* for 20 min at 0 °C. The pellet was discarded and the supernatant was made 1.0 mM with respect to pyridoxal-*P*.

(2) Heat treatment. Batches of the above supernatant (250 ml) in 1-l flasks were brought to 63 °C in a boiling water bath and maintained at 63 °C for 15 min (with occasional gentle swirling). The heated extract was then cooled rapidly to 25 °C in an acetone–alcohol ice bath. The precipitated, denatured material was removed by centrifugation at 0 °C at 14 600 × *g* for 45 min. Through the remainder of the purification procedure centrifugations were performed in a refrigerated Sorvall at 27 000 × *g* for 15 min at 0 °C. Also, all remaining steps were performed at 0–4 °C. The pellets from the heat-treatment were washed with glass-distilled water (1/3 vol. of the supernatant) by centrifugation. The pellets were discarded and the combined supernatants were made 1.0 mM with respect to dithiothreitol. At this point in the purification procedure the enzyme preparation could be frozen (−20 °C) without loss of activity, if dithiothreitol is present. However, since the purified enzyme aggregates on freezing and thawing, the purification procedure was usually continued to the next step.

(3) 45% $(NH_4)_2SO_4$. Solid $(NH_4)_2SO_4$ was added according to the initial volume using the table provided by Green and Huges[28] and no pH adjustments were made. Final concentrations are expressed as percent saturation at 25 °C and no correction was made for temperature. This applies to the other solid $(NH_4)_2SO_4$ stepwise fractionations in the purification procedure.

Solid $(NH_4)_2SO_4$ was added to the heat-treated preparation to 45% saturation during gentle stirring. The addition was complete after 20 min. The solution was allowed to stir for 20 min. The precipitate was dissolved in 0.01 M potassium phosphate buffer, pH 6.8, containing 10 mM dithiothreitol and 1.0 mM pyridoxal-*P* using

1/10 the volume of the heat-treated preparation. This was then dialyzed against 12 l of 0.005 M potassium phosphate buffer, pH 6.8, for 1 h.

(4) Hydroxyapatite column chromatography. After dialysis, the extract (2–3 g of protein in 100–140 ml) was adsorbed onto a 500-ml (50 mm × 260 nm) or 300-ml (35 mm × 260 mm) column of hydroxyapatite equilibrated with 0.005 M buffer. All buffers in this step and the remainder of the procedure are potassium phosphate, pH 6.8, supplemented with 1.0 mM dithiothreitol and 0.1 mM pyridoxal-*P*. After application of the extract the column was developed first with 0.01 M buffer (1.0–1.5 column volumes) and then with 0.05 M buffer. The fractions of the second elution containing the bulk of the tryptophanase units were pooled. The enzyme can be stored on the column or, after elution, on ice overnight (12–16 h) without loss of activity.

The pooled fractions were precipitated with $(NH_4)_2SO_4$ which was added to 50% saturation over a period of 20 min. This solution was allowed to stir for 15 min. The precipitate was collected by centrifugation.

(5) Back extractions. In the purification procedure, $(NH_4)_2SO_4$ solutions to be used for back extractions were prepared by dissolving the $(NH_4)_2SO_4$ in 0.1 M buffer and adjusting the pH to 7.0 with KOH. The 50% precipitate was suspended in 35% saturated $(NH_4)_2SO_4$ with a ratio of 1 ml/5 mg of protein in the hydroxyapatite-pooled fractions. The suspension was allowed to stir for 15 min. The precipitate was collected by centrifugation.

The 35% precipitate was extracted with 30% $(NH_4)_2SO_4$ exactly as the 35% extraction. The precipitate was collected by centrifugation, dissolved in a minimal amount of 0.1 M buffer and dialyzed against about 100 vol. of the same buffer for 1 h. This solution was then adjusted to a protein concentration of 8 mg/ml.

(6) 20% $(NH_4)_2SO_4$–15% extraction. Solid $(NH_4)_2SO_4$ was added to the dissolved 30% precipitate to 20% saturation. The addition was complete after 10 min. This solution was allowed to stir for 15 min.

The 20% precipitate was then suspended in 15% $(NH_4)_2SO_4$, pH 7.5, with a ratio of 1 ml/18 mg of protein in the 30% precipitate. The suspension was allowed to stir for 15 min. The precipitate was collected by centrifugation. The precipitate which appears to be pure tryptophanase was dissolved in a minimal amount of 0.1 M potassium phosphate buffer, pH 6.8, containing 1.0 mM dithiothreitol and 0.1 mM pyridoxal-*P* and dialyzed 16 h against 100 vol. of the same buffer. The homogeneous enzyme obtained after going through the purification procedure is referred to as the native enzyme.

Comments on the purification and stability of the enzyme

The results of a typical purification are shown in Table I. The specific activity of the pure enzyme with protein based on the absorbance at 278 nm varied from 12.4 to 14.7 with most of the values near 14.0. Pyridoxal-*P* contributes to the heat stability of the enzyme, and dithiothreitol enhances the stability of the enzyme if the heat-treated preparation is frozen. However, dithiothreitol does not enhance the stability of the enzyme in the subsequent purification steps, nor the stability of the pure enzyme.

The enzyme was stored at 8–10 mg/ml in 0.1 M potassium phosphate buffer, pH 7.8, containing 0.1 mM pyridoxal-*P* and 1.0 mM dithiothreitol at −20 °C for

TABLE I

PURIFICATION OF *A. liquefaciens* TRYPTOPHANASE

Purification step	*Volume (ml)*	*Activity (units/ml)*	*Protein (mg/ml)*	*Specific activity*	*Yield (%)*
1. Crude extract	900	8.4	47.6	0.18	100
2. Heat: 63 °C, 15 min	1120	6.8	10.2	0.67	99
3. 45% $(NH_4)_2SO_4$	141	50.2	23.6	2.1	93
4. Hydroxyapatite	318	14.7	3.2	4.5	62
5. Back extractions 35%–30%, pH 7.0	33	150.8	16.5	8.96	56
6. 20% $(NH_4)_2SO_4$–15% back extraction	13.4	291.9	21.9	13.3	52

up to a year without loss of activity. The enzyme should be quick-frozen in an acetone–alcohol–solid CO_2 bath (about −70 °C). Repeated (two or more) slow freezing (−20 °C) and subsequent thawing at room temperature causes the enzyme to aggregate. The enzyme can be quick-frozen and thawed at room temperature for at least six times without aggregation or loss in activity.

Homogeneity of preparations

The purity of the final enzyme preparations was routinely checked by analytical polyacrylamide gel electrophoresis and by sedimentation velocity. Homogeneity is indicated by both of these procedures. A disc gel pattern (Fig. 1) shows a single staining zone migrating toward the anode (positive electrode). Sedimentation velocity at protein concentrations ranging from 5.87 to 1.61 mg/ml showed a single, symmetrical peak sedimenting from the top of the cell to the bottom. Homogeneity of the protein preparations was also indicated from the linearity of sedimentation

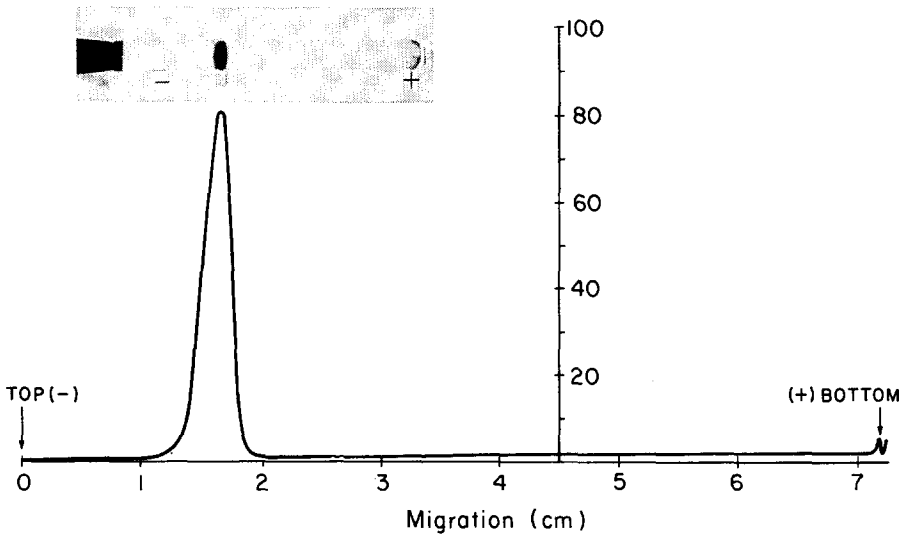

Fig. 1. Analytical acrylamide gel electrophoresis of purified *A. liquefaciens* tryptophanase. 67 μg of the purified enzyme were subjected to electrophoresis in 7.5% acrylamide gels in a bicine–glycine buffer (pH 9.0) for 2.5 h at 6.0 mA/gel as described under Materials and Methods. The stained gel was scanned at 610 nm by means of a recording densiometer (Photovolt Corp).

equilibrium data using three different protein concentrations and two different rotor speeds. The presence of a single precipitin arc after immunoelectrophoresis using pure tryptophanase and antiserum prepared against the crude enzyme extract and a single precipitin arc after immunoelectrophoresis using a crude enzyme preparation and antiserum prepared against the purified protein were also indicative of the homogeneity of the enzyme.

Amino acid composition

The amino acid composition of *A. liquefaciens* tryptophanase based on a minimal polypeptide chain mol. wt of 54 000 is given in Table II. The final values

TABLE II

AMINO ACID COMPOSITION OF *A. liquefaciens* TRYPTOPHANASE

Amino acid	*Amino acid residues (g/100 g protein)*	*No. of residues per 54 000 g*	*Assumed no. of residues*	*No. of residues per 216 000 g*
Lysine	3.44	14.5	15	60
Histidine	2.36	9.3	9	36
Arginine	10.97	37.9	38	152
Aspartic acid	4.73	22.2	23	92
Asparagine	3.42	16.2	16	64
Threonine	5.43	29.0	29	116
Serine	2.34	14.5	15	60
Glutamic acid	7.79	32.6	33	132
Glutamine	3.84	16.2	16	64
Proline	4.55	25.3	25	100
Glycine	4.51	42.7	43	172
Alanine	7.96	60.5	61	244
Valine	5.93	32.3	32	128
Methionine	3.97	16.4	16	64
Isoleucine	5.76	27.5	28	112
Leucine	9.66	46.1	46	184
Tyrosine	4.04	13.4	13	52
Phenylalanine	5.67	20.8	21	84
Half-cystine	1.81	9.6	10	40
Tryptophan	1.82	5.3	5	20
Total:	100.00		494	1976

for serine and threonine were obtained by linear extrapolation to zero hydrolysis time. The final value for isoleucine was obtained by averaging the 48 to 72 h hydrolysate values. The half-cystine, tryptophan, and amide content were determined as described under Materials and Methods. It has not yet been determined whether the half-cystine residues exist in disulfide linkages or in free sulfhydryl groups. The amide groups were arbitrarily divided equally between the known aspartic and glutamic residues. The actual recovery of amino acid residues was 95.9% (w/w) based on the g of amino acid residues per 100 g of protein, taking water of hydrolysis into account. The minimal molecular weight calculated from the total 494 residues is 54 069. The average minimal molecular weight computed from the minimal molecular weight, calculated using the assumed number of residues for each amino acid, is 54 112.

Sedimentation coefficient and molecular weight

The sedimentation coefficient of the native enzyme was determined by extrapolation, to infinite dilution, of s values obtained at five different protein concentrations. The buffer used was 0.01 M potassium phosphate (pH 6.8) containing 1.0 mM dithiothreitol and 0.1 mM pyridoxal-*P*. Least squares analysis of these data yielded a $s^0_{20,w}$ of 9.78 S. The $s^0_{20,w}$ at 5.87 mg/ml was 9.09 S and 9.66 S at 1.61 mg/ml. In determining the molecular weight of the native enzyme the sedimentation equilibrium plots were linear indicating that the preparations of tryptophanase were monodisperse. Three concentrations of the enzyme were run simultaneously in a six-channel Yphantis cell at two different rotor speeds. Equilibrium was achieved after

TABLE III

MOLECULAR WEIGHT OF NATIVE *A. liquefaciens* TRYPTOPHANASE

Protein was equilibrated with 0.1 M potassium phosphate buffer, pH 6.8, containing 0.1 mM pyridoxal-*P* and 1.0 mM dithiothreitol by dialysis for 48 h at 4 °C. Sedimentation equilibrium was performed as described under Materials and Methods. Solvent density was taken to be 1.008. Temperature 17.5 °C.

Rotor speed (rev./min)	*Protein (mg/ml)*	*Molecular weight*
16 193	0.16	204 757
	0.37	212 840
	0.56	209 349
14 290	0.16	227 241
	0.37	220 036
	0.56	222 111
Average (± S.D.)		216 055 (± 8.492)

24 h. The results are compiled in Table III. The average molecular weight of *A. liquefaciens* tryptophanase was determined to be 216 055 ± 8492. In view of the standard deviation of less than 4% of the calculated molecular weight, the molecular weight values do not appear to be dependent on either protein concentration or rotor speed. In all subsequent calculations, the mol. wt is assumed to be 216 000.

Stoichiometry of binding pyridoxal-P

The addition of pyridoxal-*P* to the apoenzyme, which shows essentially no absorbance at 330 nm and 425 nm, results in the appearance of absorption maxima at these wavelengths. Absorption maxima near 335 nm and 425 nm are characteristic of all holotryptophanase molecules[9,12,14]. The end point of two spectrophotometric titrations of the *A. liquefaciens* apoenzyme (one of which is shown in Fig. 2) corresponded to the binding of 0.91 and 1.00 mole of pyridoxal-*P* per 54 000 g of enzyme. Colorimetric determination[29] of the amount of pyridoxal-*P* bound to the enzyme after dialysis against three 2000-vol. changes of 0.01 M potassium phosphate buffer (pH 6.8) for 53 h at 4 °C indicated 0.90 moles of pyridoxal-*P* were bound per 54 000 g of enzyme. Without the addition of pyridoxal-*P* the enzyme retained 90% of its activity after this dialysis. These data, in conjunction with the molecular weight data indicate that 4 moles of pyridoxal-*P* are bound per mole of *A. liquefaciens* tryptophanase.

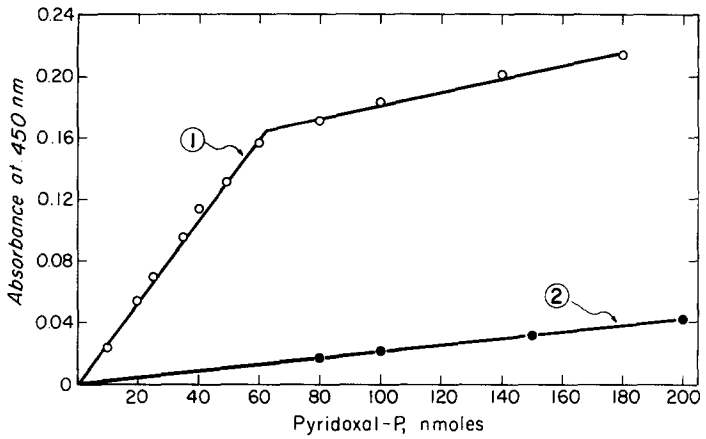

Fig. 2. Spectrophotometric titration of *A. liquefaciens* tryptophanase with pyridoxal-*P*. In Curve 1, pyridoxal-*P* was added to 3.42 mg of apoenzyme in 1.0 ml of 0.1 M potassium phosphate–0.5 mM dithiothreitol buffer, pH 7.8; in Curve 2 no enzyme was present. The absorbance was read 15 min after each addition and corrected for dilution.

Cation requirement for catalytic activity

A. liquefaciens tryptophanase showed an absolute requirement for either NH_4^+ or K^+, whereas Na^+ and imidazole did not activate the enzyme. This monovalent cation requirement is similar to that observed with the enzyme from *E. coli*[30], *B. alvei*[8], *S. varius* and *S. funculiformis*[12].

Affinity for tryptophan and pyridoxal-P

The calculated K_m of $2.21 \cdot 10^{-4}$ M for tryptophan (Table IV) is similar to that reported for other tryptophanase molecules, except for the *S. funduliformis* tryptophanase[12] which has a K_m of $4.1 \cdot 10^{-4}$. Double reciprocal plots of the reaction velocity as a function of pyridoxal-*P* concentration are linear and yield a K_m for pyridoxal-*P* of $2.09 \cdot 10^{-6}$ M (Table IV). This value is similar to those reported for other tryptophanase molecules[2,12].

TABLE IV

KINETIC CONSTANTS FOR THE TRYPTOPHANASE-CATALYZED REACTIONS

Kinetic data were obtained using the assays as described under Materials and Methods. Enzyme was used to initiate the reaction. However, tryptophan was used to initiate the reaction when assaying the activation of the enzyme by pyridoxal-*P*. Purified, homogeneous enzyme (spec. act. 14.3) was used in all cases. *V* is expressed as units per mg of protein. Values in parentheses refer to percent activity relative to that obtained with L-tryptophan as the substrate.

Compound	*Role*	*Reaction followed*	$K_m \pm$ S.D. (*M*)	*V* (%)
L-Tryptophan	Substrate	Tryptophanase	$(2.21 \pm 0.02) \cdot 10^{-4}$	14.3 (100)
Pyridoxal-*P*	Cofactor	Tryptophanase	$(2.09 \pm 0.17) \cdot 10^{-4}$	
L-Serine	Cosubstrate	Tryptophan synthetase	$(1.09 \pm 0.06) \cdot 10^{-1}$	3.4 (24)
	Substrate	Serine dehydratase	$(0.99 \pm 0.05) \cdot 10^{-1}$	4.0 (28)
S-Methyl-L-Cysteine	Cosubstrate	Tryptophan synthetase	$(0.96 \pm 0.11) \cdot 10^{-2}$	5.8 (41)
	Substrate	*S*-Alkyl-cysteine lyase	$(1.08 \pm 0.08) \cdot 10^{-2}$	9.9 (69)
L-Cysteine	Cosubstrate	Tryptophan synthetase	$(7.65 \pm 0.96) \cdot 10^{-3}$	8.6 (60)
	Substrate	Cysteine desulfhydrase	$(6.89 \pm 0.65) \cdot 10^{-3}$	10.4 (73)

Substrate specificity and subsidiary reactions

L-Tryptophans substituted in the 4-, 5- or 6-positions of indole are acceptable as substrates, whereas no activity is observed with side chain (alanine moiety) substituted analogues (data not shown). The amino acids L-serine, L-cysteine and *S*-methyl-L-cysteine can also serve as substrates for the enzyme in α,β-elimination and β-replacement reactions. The kinetic constants for these substrates are shown in Table IV. In the discussion of these activities, serine dehydratase, *S*-alkali-cysteine lyase and cysteine desulfhydrase will be referred to by the common title of dehydratase. The K_m for the amino acid substrate in the synthetase reaction is an apparent value because it was determined only in the presence of a large excess of indole (125 nmoles). The level of cysteine required for maximal activity of this synthetase reaction is in fact inhibitory to the cysteine desulfhydrase reaction. Therefore, the apparent K_m for cysteine in the desulfhydrase reaction was obtained by extrapolation. The Michaelis–Menten constants obtained for the reactions catalyzed by the tryptophanase from *A. liquefaciens* (Table IV) show similar trends as with the enzymes from *E. coli*[2], *S. varius* and *S. funduliformis*[12]. The Michaelis–Menten constants for the amino acid substrates for the dehydratase and synthetase reactions appear to be equal, within experimental error. The K_m values for the substrates tested increase in the same order: L-tryptophan, $<$ L-cysteine, $<$ *S*-methyl-L-cysteine, $<$ L-serine. The five enzymes, however, do appear to differ significantly with regard to the efficiency (V values) with which the various substrates are acted upon.

Inhibitors

Benzoic acid derivatives, such as anthranilic acid and kynurenine, and the amino acids, alanine and phenylalanine are competitive inhibitors for tryptophanases. Using different concentrations of inhibitor (0, 0.2, 0.5 and 1.0 mM) anthranilic acid exhibited competitive inhibition with the *A. liquefaciens* enzyme. The average K_i for this inhibition was $(0.301 \pm 0.05) \cdot 10^{-3}$ M. At different concentrations of alanine (0, 10, 30 and 60 mM) and phenylalanine (0, 10, 30 and 50 mM), competitive inhibition of the enzyme was observed. The average K_i values for alanine and phenylalanine were $(13.14 \pm 0.30) \cdot 10^{-3}$ M and $(7.79 \pm 0.14) \cdot 10^{-3}$ M, respectively.

Inhibition of activity by antibody

Antiserum prepared against purified homogeneous *A. liquefaciens* tryptophanase inhibited the enzyme activity most effectively with the homologous enzyme and in order of decreasing effectiveness inhibited the activity of the tryptophanases from *B. alvei*, *E. coli*, *P. coliforme*, a marine vibrio (K-7), *M. aerogenes* and *S. funduliformis* (Fig. 3). The percent inhibition was determined relative to the activity obtained with the same amount of normal serum. The activity of each enzyme in the presence of normal serum was the same as with no serum added. There is the possibility of the enzymes being inactivated by antibodies elicited against the cofactor, pyridoxal-*P*. This possibility was eliminated since the inhibition pattern was essentially the same using 0.05 mM pyridoxal-*P* and 0.5 mM pyridoxal-*P*. If the inhibition of enzyme activity were due to the presence of anti-pyridoxal-*P* molecules in the antiserum, increasing the pyridoxal-*P* molecule concentration should decrease the effective anti-pyridoxal-*P* molecule concentration, a result that was not observed.

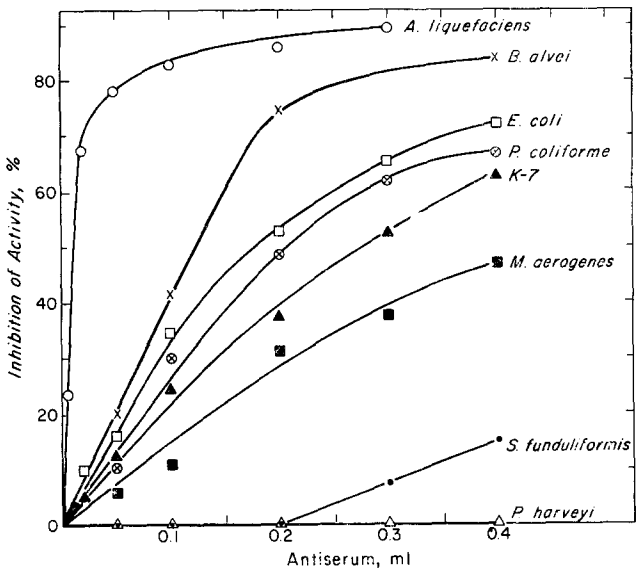

Fig. 3. Immunochemical comparison of tryptophanases using inhibition of activity by antiserum prepared against homogeneous *A. liquefaciens* tryptophanase. Inhibition of enzyme activity by antiserum was tested as described under Materials and Methods. ○—○, *A. liquefaciens* enzyme (9.24 mg/ml; spec. act. 14.0); ×—×, *B. alvei* enzyme (3.0 mg/ml; spec. act. 8.52); □—□, *E. coli* T-3 enzyme (23.8 mg/ml; spec. act. 0.6); ⊗—⊗, *P. coliforme* enzyme (43.2 mg/ml; spec. act. 0.14); ▲—▲, K-7 (52.1 mg/ml; spec. act. 0.84); ■—■, *M. aerogenes* enzyme (38.8 mg/ml; spec. act. 0.012); ●—●, *S. funduliformis* enzyme (4.5 mg/ml; spec. act. 12.0); and △—△, *P. harveyi* enzyme (13.6 mg/ml; spec. act. 0.006).

Ouchterlony double diffusion experiments using antiserum prepared against homogeneous *A. liquefaciens* enzyme showed cross-reacting precipitin bands of low intensity with crude tryptophanase preparations from *E. coli*, *P. coliforme*, a marine vibrio (K-7), and a homogeneous preparation of tryptophanase from *B. alvei* (data not shown). In each case lines of partial indentity with the precipitin band of the homologous *A. liquefaciens* enzyme were obtained.

DISCUSSION

Tryptophanase from *A. liquefaciens* was purified to homogeneity using fractionation procedures which have proven successful in purifying other tryptophanase molecules[2,6,8,12]. The successful application of similar protein fractionation procedures probably is due to the high resolving power of the procedures, since the enzymes behave differently in similar fractionation steps. However, the enzymes have the common property of heat stability. This common property probably reflects similar secondary, tertiary and quaternary stabilizing forces and may reflect similar secondary, tertiary and quaternary structures. As will be discussed below some degree of structural similarity can be found in all tryptophanase molecules.

Studies with the tryptophanase from *E. coli*[16], *B. alvei*[11] and *S. varius* and *S. funduliformis* (Cecchini, G. L., and Bojanowski, R. J., unpublished) indicate that the size and mass of these enzymes are similar to that of the *A. liquefaciens* enzyme with the exception of the *S. funduliformis* enzyme which has a sedimentation coefficient

of 11.2 S and a mol. wt of 245 000. The other tryptophanase molecules are composed of four apparently identical subunits held together by noncovalent interactions. With the exception of the *S. funduliformis* enzyme, there is apparently one mole of pyridoxal-*P* bound per subunit molecular weight. This general tetrameric subunit structure is also characteristic of the *A. liquefaciens* tryptophanase.

Immunochemical studies indicate that tryptophanase from *A. liquefaciens* has some common antigenic determinant sites with the tryptophanases from *E. coli*, *B. alvei*, a marine vibrio (K-7), *P. coliforme*, *M. aerogenes* and *S. funduliformis*. No antigen–antibody interaction was detected between the *A. liquefaciens* enzyme and the enzyme from *P. harveyi*. Since the amount of tryptophanase in the *P. harveyi* preparation was low and because of the possibility of having an antigen–antibody interaction having no effect on catalytic activity, one can only suggest that there may be no antigenic similarity between the *A. liquefaciens* and *P. harveyi* tryptophanases.

The common antigenic determinant sites could represent similar areas in the secondary, tertiary and quaternary structures of the proteins and/or similarities in

TABLE V

COMPARISON OF THE AMINO ACID COMPOSITIONS OF THE TRYPTOPHANASES FROM FIVE DIFFERENT BACTERIAL SPECIES ON THE BASIS OF MOLES PERCENT OF EACH AMINO ACID RESIDUE

Moles % of each amino acid residue is expressed as the number of residues of that amino acid present per mole of enzyme divided by the total number of amino acid residues per mole of enzyme.

Amino acid	*Tryptophanase*				
	*E. coli**	*B. alvei***	*S. varius****	*S. funduliformis****	*A. liquefaciens*
Lysine	6.12	4.30	7.13	6.08	3.03
Histidine	1.69	2.15	1.78	2.28	1.82
Arginine	5.06	6.45	2.45	4.94	7.66
Aspartic acid	8.44	6.88	6.24	7.61	4.66
Asparagine	unknown	1.94	4.01	3.04	3.24
Threonine	5.69	6.45	4.01	3.80	5.87
Serine	4.01	4.95	4.90	4.56	3.04
Glutamic acid	11.81	10.11	7.14	8.75	6.68
Glutamine	unknown	1.93	4.01	3.04	3.24
Proline	4.01	4.73	4.45	3.80	5.06
Glycine	7.81	9.03	8.46	8.37	8.71
Alanine	9.70	8.82	9.35	7.61	12.35
Valine	6.54	5.37	4.45	6.08	6.48
Methionine	3.38	2.58	3.56	4.56	3.24
Isoleucine	5.91	6.24	9.35	5.71	5.67
Leucine	7.81	8.82	6.68	6.85	9.31
Tyrosine	5.06	3.87	4.90	5.32	2.63
Phenylalanine	5.27	4.09	4.90	5.32	4.25
Half-cystine	1.27	0.43	1.78	1.52	2.02
Tryptophan	0.42	0.86	0.45	0.76	1.01
Totals	100.0	100.00	100.00	100.00	100.00

* Data from Kagamiyama *et al.*[4].
** Data from Hoch and DeMoss[11].
*** Data from Bojanowski[12].

the structure of the catalytic sites. Considering the general catalytic similarities of the tryptophanases it is quite possible that there are enough similarities in the catalytic sites to elicit cross-reacting antibody activity.

In support of similar antigenic sites (possibly the catalytic sites) on tryptophanase molecules, it has been observed (DeMoss, R. D., unpublished) that antiserum prepared against purified *B. alvei* tryptophanase partially inhibited the activity of the tryptophanase from *E. coli*. This antiserum did not precipitate the *E. coli* enzyme[8]. Bojanowski[12] found that antisera prepared against homogeneous enzyme from *S. varius* and *S. funduliformis* only inhibited the activity of the homologous enzymes and neither antiserum inhibited the activity of the tryptophanase from *B. alvei*. The fact that the antiserum prepared against the *A. liquefaciens* enzyme had more and stronger cross-reacting antigenic activity may indicate that this enzyme has antigenic sites that are more effective in eliciting antibody production. It could also be due to the fact that in this study the injection and bleeding schedule was more conducive to obtaining serum which contained substantial secondary antibody.

Because an antigen–antibody interaction can have a varying effect on catalytic activity, evolutionary relationships based on the inhibition of enzyme activity alone cannot be determined. However, the results obtained in this paper indicate that additional comparative immunochemical experiments are possible.

Comparison of the amino acid composition of the *A. liquefaciens* enzyme with the other tryptophanases[4,11,12] (Table V) indicate that each enzyme has a distinct amino acid composition. As a group, however, the enzymes are characterized by a fairly high content (50–56%) of non-polar amino acids. Also, the histidine content of the five enzymes is similar while the lysine and arginine content vary considerably keeping the total content of basic residues very similar in each enzyme. When compared to the other tryptophanases, the *A. liquefaciens* enzyme has a slightly higher non-polar residue content. This is primarily due to a larger alanine content (a relative increase of 22–38%). There are also fewer acidic residues in the *A. liquefaciens* enzyme. From comparison of the total basic and acidic residue content of the tryptophanases, electrophoretic mobility differences might be expected especially comparing the *A. liquefaciens* enzyme with the other molecules. The less acidic and more non-polar amino acid composition of the *A. liquefaciens* enzyme could be responsible for its ability to readily aggregate into higher molecular weight, catalytically active species. Evidence suggests that it is the interaction of non-polar amino acid residues that stabilize the polymeric species[31]. This aggregation phenomenon has not been reported to occur with other tryptophanase molecules.

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