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THE TRYPTOPHANASE FROM *PROTEUS RETTGERI*, IMPROVED PURIFICATION AND PROPERTIES OF CRYSTALLINE HOLOTRYPTOPHANASE*

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Summary

The inducible tryptophanase (L-tryptophan indole-lyase (deaminating) EC 4.1.99.1) was crystallized in holoenzyme from the cell extract of *Proteus rettgeri*. The purification procedure included ammonium sulfate fractionation, heat treatment at 60°C, DEAE-Sephadex and hydroxylapatite column chromatographies. Crystallization was performed by the addition of ammonium sulfate to the purified enzyme solution containing 20% (v/v) glycerol, 0.1 mM pyridoxal phosphate and 10 mM mercaptoethanol. The crystallized enzyme was yellow and showed absorption maxima at 340 and 420 nm.

The crystalline holotryptophanase preparation was homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis. The molecular weight of the enzyme was calculated as approx. 222 000. The amount of pyridoxal phosphate bound to the enzyme was determined to be 4 mol per mol of the enzyme.

The enzyme is composed of four subunits of identical molecular size (mol. wt 55 000) and irreversibly dissociates into these subunits in the presence of a high concentration of sodium dodecylsulfate or guanidine hydrochloride. The NH_2-terminal amino acid of the enzyme was identified as alanine.

Introduction

Tryptophanase was first crystallized in its apoenzyme form by Newton and Snell [2] from a mutant of *Escherichia coli* B, which produces this enzyme

* A preliminary account of this work has appeared [1].

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Abbreviations: PLP, pyridoxal phosphate; DNP, 2,4-dinitrophenol.

constitutively. Subsequently, we crystallized an inducible tryptophanase in an apo form from *Proteus rettgeri* grown in a medium supplemented with L-tryptophan [3]. Since these crystalline apotryptophanase preparations are rather unstable [4], many attempts have been made to crystallize the enzyme in the stable holo form. However, no satisfactory method has been reported because of the easy dissociation of PLP from the enzyme. During the investigations of the tryptophanase from *Pr. rettgeri*, we developed a procedure for preparing the crystalline holoenzyme. The crystalline holoenzyme obtained has four times the specific activity and is much more stable than the crystallized apoenzyme. The present paper describes an improved procedure for the purification and crystallization of the tryptophanase from *Pr. rettgeri* which gives crystals of the holoenzyme. Some properties of this crystalline holoenzyme and its subunit structure are also described.

Materials and Methods

Materials. Cytochrome *c*, trypsin, ovalbumin, catalase and serum albumin (combithek) were purchased from Boehringer Mannheim, West Germany. Sodium dodecyl sulfate and guanidine hydrochloride, ultra pure grades, were purchased from Wako Chemicals Co. Ltd, Osaka. DEAE-Sephadex A-50 was purchased from Pharmacia, Uppsala, and hydroxylapatite was from Clarkson Chemical Company Inc., Williamsport, Pennsylvania. All other chemicals used were commercial products.

Microorganism and culture. Proteus rettgeri (Aj 2770) was used throughout this study. The media and culture conditions were the same as those described in previous papers [3,5].

Enzyme assay. Tryptophanase activity was assayed by measuring the amount of pyruvate formed from L-tryptophan as previously described [3]. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of pyruvate per min under standard assay conditions. Specific activity was expressed as units per mg of protein.

Protein determination. The protein concentration of the enzyme was determined spectrophotometrically by measuring the absorbance at 280 nm as previously described [3,6].

Determination of PLP. The crystalline protein, 6.3 mg, was dissolved in a minimum amount of 0.05 M potassium phosphate buffer, pH 8.0, containing 1 mM mercaptoethanol. This enzyme solution was then passed through a column (1 × 10 cm) of Sephadex G-25 equilibrated with the same buffer. Protein fractions were combined and the amount of PLP bound to the protein was determined according to the method of Wada and Snell [7].

Ultracentrifugal analysis. Sedimentation coefficients were measured with a Spinco model E ultracentrifuge operated at 56 100 rev./min and 20° C. Diffusion coefficients were measured with the same apparatus operated at 12 590 rev./min and 20° C, with the boundary condition at the meniscus in a sector-shaped centrifuge cell [8]. The partial specific volume of the enzyme was calculated from the results of the amino acid analysis of the enzyme [9] as 0.739 ml/g. The molecular weight was calculated from the sedimentation and

diffusion coefficients, according to the equation of Svedberg and Pederson [10].

Electrophoresis. Disc gel electrophoresis was carried out with acrylamide gel in Tris/glycine buffer, pH 8.3, using the method of Davis [11]. Stacking and running gels were polymerized in Pyrex tube (5 × 65 mm). After the run, the gel was stained with 0.25% Coomassie brilliant blue, then it was destained electrophoretically and stored in 7% acetic acid.

The molecular weight of the subunit was estimated by sodium dodecylsulfate disc gel electrophoresis. The disc gel electrophoresis in 0.1% sodium dodecylsulfate was carried out according to the method of Weber and Osborn [12]. The enzyme was incubated with 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecylsulfate for 2 h at 37° C before the run. The relative migration versus the logarithm of molecular weight were obtained with the following marker proteins: Cytochrome *c* (mol. wt 11 700); trypsin (mol. wt 23 300); ovalbumin (mol. wt 43 000); catalase (mol. wt 60 000) and serum albumin (mol. wt 68 000).

Amino acid analysis. Approx. 0.2 mg of tryptophanase was hydrolyzed with 0.3 ml of twice distilled 6 M HCl in a sealed, evacuated tube for 24 and 68 h at 110° C. The hydrolyzates were dried and analyzed with an amino acid analyzer, Spinco model 120 B, according to Hubbard [13], based on the method of Spackman et al. [14].

Tryptophan was determined according to Matsubara and Sasaki [15]. Cysteine content was analyzed with a sample oxidized by performic acid according to the method of Moore [16].

Determination and identification of the NH_2-terminal amino acid. The NH_2-terminal amino acid was determined using the method of Sanger and Thompson [17,18]. The enzyme protein, 14.7 mg, was treated with 0.4 ml of a 5% (v/v) solution of 1-fluoro-2,4-dinitrobenzene in ethanol and 0.2 ml of a 1% (v/v) triethylamine solution for 2 h at 30° C. The DNP-protein was hydrolyzed in a sealed tube with 6 M HCl for 16 h at 105° C, then it was extracted with ether. The DNP-amino acid in the ether phase was identified by two dimensional paper chromatography using solvent systems of toluene/2-chloroethanol/pyridine (0.8 M)/ammonia (5 : 3 : 1.5 : 3) and 1.5 M sodium phosphate buffer, pH 6.0. The yellow spot of the DNP-amino acid was eluted from the chromatogram with a 1% $NaHCO_3$ solution and was quantitatively determined by measuring the absorbance at 360 nm [19].

Isolation procedure. General conditions for purification were the same as those previously described [3]. The modification made in this work gave a crystalline holoenzyme preparation which has about four times the specific activity of the previously crystallized apoenzyme. Purification was performed at 0—5° C and all the potassium phosphate buffers used contained 1 mM mercaptoethanol and 0.01 mM PLP unless otherwise indicated.

Step 1. Preparation of cell extract. This step was carried out as previously described [3].

Step 2. First ammonium sulfate fractionation and heat treatment. This procedure was the same as previously described [3], except that the concentration of ammonium sulfate after the heat treatment was increased to 0.80 saturation.

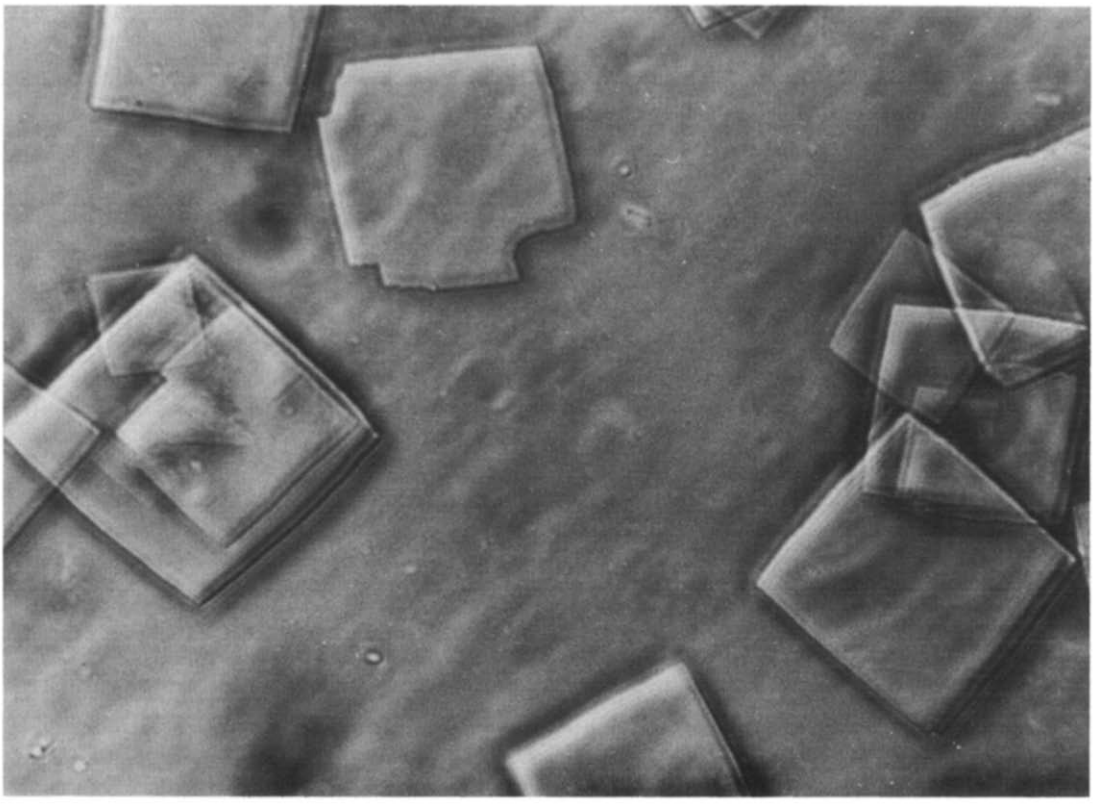


Fig. 1. Photomicrograph of crystalline holotryptophanase from *Proteus rettgeri*. Magnified 150-fold.

Step 3. DEAE-Sephadex column chromatography. The procedure described previously [3] was used.

Step 4. Second ammonium sulfate fractionation. The precipitate formed by ammonium sulfate between 0.60 and 0.70 saturation was collected.

Step 5. Hydroxylapatite column chromatography. This procedure was performed as previosuly described [3], except that the column was equilibrated with 0.05 M potassium phosphate buffer at pH 7.0.

Step 6. Third ammonium sulfate fractionation. The precipitate formed by ammonium sulfate between 0.60 and 0.70 saturation was collected. It was then dissolved in a minimum amount of 0.1 M potassium phosphate buffer, pH 8.0, containin 20% (v/v) glycerol, 0.1 mM PLP and 10 mM mercaptoethanol, after which it was dialyzed for 24 h against the same buffer.

Step 7. Crystallization. Solid ammonium sulfate was cautiously added to the dialyzed enzyme solution until it became slightly turbid. Crystallization began after about 2 h and was completed within a month. Fig. 1 shows a photomicrograph of the crystalline tryptophanase which appears as thin square plates with a bright yellow color. Recrystallization was carried out by repeating the last step. The specific activity of the enzyme did not increase on further crystallization. Approx. a 231-fold purification was achieved with an over-all yield of 7.2%. A summary of the typical purification procedure is shown in Table I.

TABLE I

PURIFICATION OF HOLOTRYPTOPHANASE FROM *PROTEUS RETTGERI*

Step	Fraction	Total protein (mg)	Total units	Specific activity
1	Crude extract	107,993	4,258	0.039
2	First ammonium sulfate and heat	60,158	3,321	0.055
3	DEAE-Sephadex	765	601	0.79
4	Second ammonium sulfate	533	595	1.11
5	Hydroxylapatite	210	592	2.81
6	Third ammonium sulfate	83	460	5.52
7	First crystals	40	350	8.75
8	Second crystals	34	310	9.01

Results

Stability. Solutions of the purified enzyme were relatively unstable, particularly in preparations with a specific activity greater than 3.0. In the presence of mercaptoethanol and PLP, high concentrations of ammonium sulfate plus glycerol markedly stabilize the enzyme from denaturation (Table II). The crystalline enzyme prepared by the above procedure was much more stable than the previously crystallized apoenzyme. This enzyme could be stored at 5° C as a suspension in 0.1 M potassium phosphate buffer, pH 8.0, containing 20% (v/v) glycerol, 0.1 mM PLP, 10 mM mercaptoethanol and 60% saturated ammonium sulfate for a period of more than five months without loss of activity.

Homogeneity. The crystalline enzyme preparation gave a single band on disc gel electrophoresis carried out at pH 8.3, and sedimented as a single symmetrical peak under ultracentrifugation in 0.01 M potassium phosphate buffer pH 7.0, containing 0.1 M ammonium sulfate, 1 mM mercaptoethanol and 0.1 mM PLP (Fig. 2).

TABLE II

EFFECT OF AMMONIUM SULFATE AND GLYCEROL ON STABILITY OF THE TRYPTOPHANASE

The solution contained 0.44 units of the enzyme (spec. act. 3.0) per ml in 1 mM mercaptoethanol, 0.01 mM PLP, 0.1 M potassium phosphate buffer, pH 7.0, and the additions listed and stored for 21 days at 4 °C.

	Condition	Additions	Inactivation (%)
1	4 °C, 21 days	None	67
2	4 °C, 21 days	$(NH_4)_2SO_4$, 11.4%	29
3	4 °C, 21 days	Glycerol, 20%	14
4	4 °C, 21 days	$(NH_4)_2SO_4$, 11.4% + glycerol, 20%	9

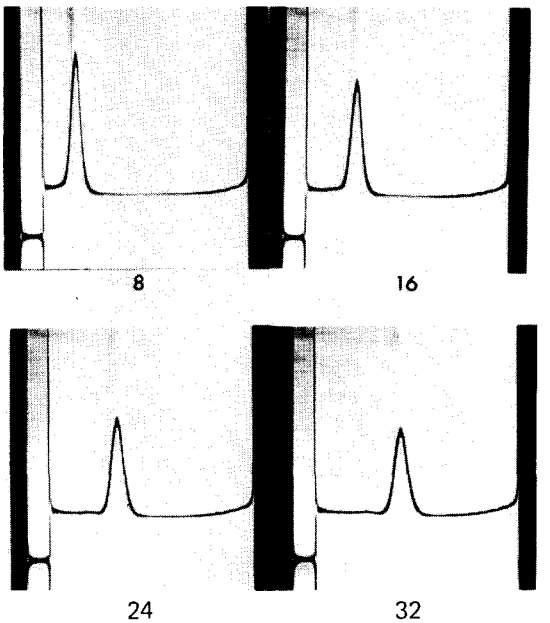


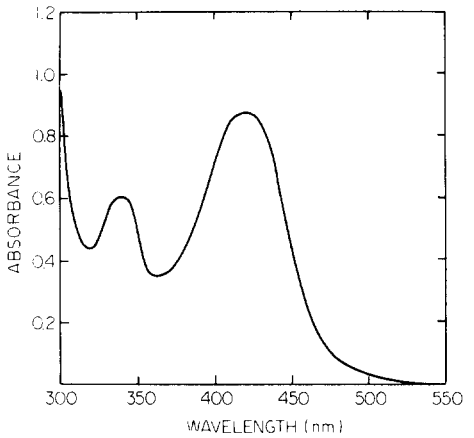
Fig. 2. Sedimentation patterns of the tryptophanase. Recrystallized enzyme was used at a concentration of 7.96 mg/ml in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M ammonium sulfate, 1 mM mercaptoethanol and 0.1 mM PLP. Photographs were taken 8, 16, 24 and 32 min after reaching 56 100 rev./min. The sedimentation is left to right.

Sedimentation and diffusion coefficients. The sedimentation coefficient ($s^0_{20,w}$) was determined to be 9.80 S when the protein concentration was varied from 3.5 to 7.1 mg per ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M ammonium sulfate, 1 mM mercaptoethanol and 0.1 mM PLP. A diffusion coefficient, $D_{20,w}$, of $4.1 \cdot 10^{-7}$ cm^2/s was determined for 7.96 mg per ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M ammonium sulfate, 1 mM mercaptoethanol and 0.1 mM PLP.

Molecular weight. The molecular weight of the enzyme was calculated as 222 000 from the sedimentation and diffusion coefficients values, according to the equation of Svedberg and Pederson [10]. A partial specific volume of 0.739, which was determined from the amino acid composition of the enzyme [9], was used for the calculation.

PLP content. The crystalline enzyme showed its full activity even when diluted and assayed in the absence of added PLP. The crystalline enzyme was yellow and showed absorbance maxima at 340 and 420 nm, at pH 8.0 (Fig. 3). These results indicate that the crystalline enzyme prepared by the above procedure is entirely in the holoenzyme form and that the formyl group of the bound PLP may form an azomethine linkage to an amino group of the protein, as reported for other PLP enzymes. The amount of PLP bound to the enzyme was determined to be 0.0183 μmol per mg of protein, corresponding to the binding of 4 mol of PLP per 220 000 g of the protein.

Dissociation by sodium dodecylsulfate. The enzyme was irreversibly inactivated by incubation with an sodium dodecylsulfate solution at 37°C for 2 h. The rate of inactivation was dependent upon the concentration of sodium



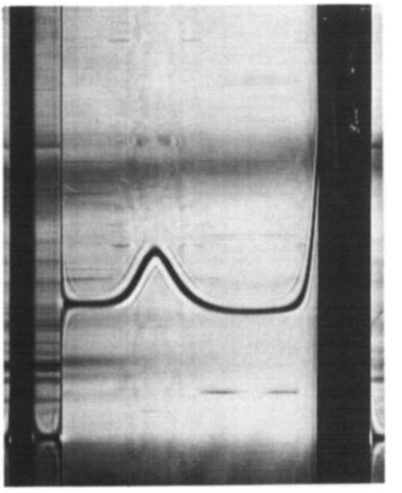


Fig. 3. Absorption spectrum of the tryptophanase. Recrystallized enzyme, 16.5 mg/ml, was dissolved in 1 ml of 0.1 M potassium phosphate buffer, pH 8.0, containing 1 mM mercaptoethanol. The spectrum was taken with a Hitachi model 124 recording spectrophotometer.

Fig. 4. Sedimentation pattern of the tryptophanase in 6 M guanidine hydrochloride. Recrystallized enzyme was dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, containing 6 M guanidine hydrochloride at 20° C for 16 h. The dialyzed enzyme was analyzed by a Spinco model E ultracentrifuge. The photograph was taken 7 h after reaching 56 100 rev./min. The sedimentation is from left to right.

dodecylsulfate, and complete inactivation was observed at the concentration of 0.2%.

The enzyme which had been dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecylsulfate at 20° C for 16 h, was analyzed using the ultracentrifuge. The sedimentation pattern of the enzyme in 1% sodium dodecylsulfate showed a symmetric single peak. The enzyme incubated with 1% sodium dodecylsulfate for 2 h at 37° C also showed a single band on sodium dodecylsulfate disc gel electrophoresis. These results indicate that the native enzyme dissociated into subunits of identical molecular size during inactivation of the enzyme with 1% sodium dodecylsulfate. The molecular weight of the subunit was estimated using sodium dodecylsulfate disc gel electrophoresis [12] with five marker proteins, as 55 000.

Dissociation by guanidine hydrochloride. The enzyme was also inactivated by incubation at 37° C for 2 h with guanidine hydrochloride. Almost complete inactivation was observed at the concentration of 3 M. This inactivation was irreversible and no activity was recovered by dialyzing the inactivated enzyme against 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 mM PLP and 1 mM mercaptoethanol.

The enzyme solution which had been dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, containing 6 M guanidine hydrochloride for 16 h at 20° C, was analyzed using the ultracentrifuge. The sedimentation pattern of the enzyme in 6 M guanidine hydrochloride showed a symmetrical single peak (Fig. 4), from which the sedimentation coefficient ($s_{20,w}$) was determined [20] to be 3.1 S.

TABLE III

COMPARATIVE AMINO ACID COMPOSITION OF TRYPTOPHANASES FROM *PROTEUS RETTGERI* AND *E. COLI* B

Amino acid	Assumed number of residues per 55 000 g of enzyme	
	Proteus rettgeri	*Escherichia coli* B[a]
Lysine	36	29
Histidine	12	8
Arginine	21	24
Tryptophan	2	2
Aspartic acid	57	40
Threonine	21	27
Serine	22	19
Glutamic acid	51	56
Proline	22	19
Glycine	37	37
Alanine	48	46
Half-cystine	6	6
Valine	35	31
Methionine	15	16
Isoleucine	33	28
Leucine	36	37
Tyrosine	22	24
Phenylalanine	21	25
Total	497	474

[a]The *E. coli* B data were taken from Kagamiyama et al. [21].

Amino acid composition. The amino acid composition of the tryptophanase from *Pr. rettgeri*, based on a minimal polypeptide chain mol. wt of 55 000, is given in Table III and is compared with that of the tryptophanase from *E. coli* B [21]. Tryptophan and half-cystine contents were determined as described in Materials and Methods. The amino acid analysis for these two tryptophanases shows a somewhat similar composition. The major differences are in the contents of the amino acid residues of lysine, aspartic acid, threonine, glutamic acid and isoleucine. It has not yet been determined whether the half-cystine residues exist in disulfide linkages or in free sulfhydryl groups.

NH_2-terminal amino acid analysis. The enzyme (14.7 mg) was incubated with 1-fluoro-2,4-dinitrobenzene for 2 h at 30°C under the conditions described by Sanger [17,18]. The DNP-protein was hydrolyzed in 6 M HCl for 16 h at 105°C. The DNP-amino acid in the hydrolyzate was identified and quantitatively determined with two-dimensional paper chromatography. Alanine was detected in the ether phase as the NH_2-terminal amino acid. The amount of DNP-alanine separated from the DNP-protein was determined [19] to be 3.5 mol per mol of protein. It is evident that the enzyme has a single NH_2-terminal amino acid; alanine, and that the amount of DNP-alanine recovered agrees reasonably well with the idea that the enzyme is composed of four apparently identical subunits.

Discussion

The tryptophanase from *Pr. rettgeri* was first crystallized in the apoenzyme form. The removal of PLP from the enzyme occurs during the crystallization step and has been observed as a pronounced decrease in the yellow color. This decrease was observed even when crystallization was carried out in the presence of 0.1 mM PLP. Since this concentration of PLP was more than sufficient to reconstitute the holoenzyme in solution [6], and since high concentrations of ammonium sulfate did not resolve the enzyme during the purification steps, it has been assumed that crystallization is accompanied by conformational changes in the protein that significantly reduce its affinity for the coenzyme. A similar phenomenon has been reported by Newton, Morino and Snell [22] in the crystallization step of the tryptophanase from *E. coli* B.

During further investigations of the tryptophanase from *Pr. rettgeri*, we found that high concentrations of ammonium sulfate plus glycerol stabilize the enzyme from the denaturation which occurs during long storage at 4° C. In the presence of 20% glycerol, the enzyme was crystallized in the holoenzyme form, which is more stable and has about 4 times the specific activity of the apoenzyme. Glycerol may protect the enzyme against changes in the native conformation of the protein caused by long storage in the cold and by crystallization with ammonium sulfate. Glycerol, together with other polyhydric alcohols, is known to protect many enzyme proteins against cold, heat and urea inactivations [23,24]. The mechanism of this protective effect of glycerol is not clear at present, but it has been suggested that glycerol may have the property of stabilizing networks of "structured" water molecules which are essential for maintaining the proper configuration of the protein in the native state [23].

Some physicochemical and enzymatic properties of the tryptophanase

TABLE IV

COMPARISON OF THE PROPERTIES OF TRYPTOPHANASE FROM *PROTEUS RETTGERI* WITH THOSE OF *ESCHERICHIA COLI* B

Property	Tryptophanase	
	Proteus rettgeri	*Escherichia coli* B/1t7A[a]
Type	inducible	constitutive
$S^0_{20,w}$ (S)	9.8	9.5–10.5
$D_{20,w}$ (cm^2/s)	4.1×10^{-7}	—
Molecular weight	222 000	220 000
Number of subunits	4	4
Molecular weight of subunits	55 000	55 000
NH_2-terminal amino acid	alanine	methionine
$A^{1\%}_{1cm}$ at 280 nm	11.0	—
Absorbance maxima at pH 8.0 (nm)	340, 420	337, 420[b]
V (μmol/min/mg of protein)[c]	9.0[d]	26.0[e]
K_m (mM)[c]	0.26	0.33

[a] The data of *E. coli* B were taken from refs 21, 22, 25–27.
[b] Determined after reconstitution of the enzyme with PLP.
[c] The values were determined in the degradative reaction of L-tryptophan.
[d] Assayed at 30 °C in the absence of added PLP.
[e] Assayed at 37 °C in the presence of added PLP.

from *Pr. rettgeri* are summarized and compared with those of *E. coli* B [21,22,25—27] (Table IV). There are several points of similarity between the structures of these tryptophanases. Both tryptophanases have a mol. wt of approx. 220 000 and are composed of four apparently identical subunits with a mol. wt of 55 000. One mol of PLP is bound per subunit. These subunits may be held together by noncovalent interactions and dissociate from each other in the presence of a high concentration of sodium dodecylsulfate or guanidine hydrochloride. A similar tetrametric subunit structure has been reported with other tryptophanase preparations from *E. coli* K-12 [4,28], *Bacillus alvei* [29] and *Aeromonas liquefaciens* [30,31].

A comparison of the amino acid composition of the tryptophanase from *Pr. rettgeri* with that of *E. coli* B [21] indicates that both enzymes have a similar amino acid composition. Similar contents were observed in the residues of tryptophan, glycine, half-cystine, methionine and leucine. The major differences are in lysine, aspartic acid, threonine, glutamic acid and isoleucine. There are also less basic and more acidic residues in the total residue content of the *Pr. rettgeri* enzyme. Analysis of the NH_2-terminal amino acid shows that the tryptophanase from *Pr. rettgeri* has a single NH_2-amino acid, alanine, and that the amount of DNP-alanine recovered from the enzyme is in agreement with the conclusion that the enzyme contains four apparently identical subunits. The NH_2-terminal amino acid of methionine has been reported for the tryptophanase from *E. coli* B [26].

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