PURIFICATION AND SOME PROPERTIES OF BOVINE PINEAL TRYPTOPHAN 5-MONOOXYGENASE.

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SUMMARY : Tryptophan 5-monooxygenase was purified approximately 1,000-fold from the bovine pineal gland. The purified enzyme catalyzed the hydroxylations of both L-tryptophan and L-phenylalanine at a comparable rate. Evidence was presented suggesting that the hydroxylations of both amino acids were catalyzed by the single enzyme. The apparent Km values for L-tryptophan and for L-phenylalanine were approximately 16 and 32 μM, respectively, when tetrahydrobiopterin was used as a cofactor. The apparent molecular weight of the enzyme was estimated to be approximately 30,000 by gel filtration on columns of Sephadex G-75 and G-100 and by ultracentrifugation in sucrose density gradients. These properties of bovine pineal tryptophan 5-monooxygenase were distinguishable from those of rat liver phenylalanine hydroxylase, another enzyme which had been shown to catalyze the hydroxylations of both L-tryptophan and L-phenylalanine.

Tryptophan 5-monooxygenase (E.C.1.99.1.4) catalyzes the hydroxylation of L-tryptophan to L-5HTP[+], the initial reaction in the biosynthesis of serotonin in the brain and of melatonin in the pineal gland. The enzyme activity has been detected in various cell-free preparations from these organs (1-7) but the enzyme has not been extensively purified mainly due to low activity in tissue extracts and poor yields upon attempts at purification.

In a previous report from this laboratory (6), tryptophan 5-monooxygenase of the bovine pineal gland was shown to exist largely as an inactive form in tissue extracts. Preincubation of the inactive enzyme under anaerobic conditions in the presence of DTT resulted in an approximately 100-fold activation. During the course of this study, we have observed that the inactive form of the

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+ Abbreviations used are : 5HTP, 5-hydroxytryptophan; BpH_4, 5,6,7,8-tetrahydrobiopterin; $DMPH_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterin; $6MPH_4$, 6-methyl-5,6,7,8-tetrahydropterin; DTT, dithiothreitol; BSA, bovine serum albumin; Tryptophan-1-^{14}C, tryptophan-(side chain-1)^{14}C; Tyrosine-1-^{14}C, tyrosine-(side chain-1)^{14}C.

enzyme was much more stable than the activated enzyme. In the present study, therefore, tryptophan 5-monooxygenase was purified as the inactive form about 1,000-fold from the bovine pineal gland. The enzyme activity was determined each time after the enzyme was activated. This communication describes the procedure for the purification and some properties of the purified enzyme.

MATERIALS AND METHODS

Bovine pineal glands were obtained at a slaughterhouse and stored frozen at -80° until use. L-tryptophan-1-^{14}C and L-tyrosine-1-^{14}C (New England Nuclear), L-phenylalanine-4-T (Radiochemical Centre), $DMPH_4$, $6MPH_4$ and DTT (Calbiochem), hydroxylapatite (Clarkson), P-cellulose and DEAE-cellulose (Brown), Sephadex G-75, G-100 and G-200 and CM-Sephadex C-50 (Pharmacia) were purchased from the sources indicated in parentheses. Biopterin was kindly donated by Drs. H. Hasegawa, T. Fukushima and M. Akino, Tokyo Metropolitan University and reduced to the tetrahydro form by a modification[1] of the method of Bobst and Viscontini (8). Sheep liver dihydropteridine reductase was purified according to the method of Kaufman and Levenberg (9) up to the step of the calcium phosphate gel treatment.

The activation and the assay of tryptophan 5-monooxygenase were performed as described previously (6), except that the activation was carried out at 30° for 30 min unless otherwise stated. Tyrosine and phenylalanine monooxygenase activities were determined under the same conditions as those for the assay of tryptophan 5-monooxygenase, except that L-tryptophan-1-^{14}C was replaced by 200 μM L-tyrosine-1-^{14}C or 200 μM L-phenylalanine-4-T, respectively, and the tyrosine monooxygenase reaction was carried out for 15 min. Tritium released from L-phenylalanine-4-T as THO or migrated to the adjacent position of the benzene ring was determined by a slight modification of the method of Guroff and Abramdwitz (10). L-Dopa-^{14}C formed from L-tyrosine-1-^{14}C was isolated by an alumina column according to the method of Nagatsu et al. (11) and counted. When the enzyme preparation contained L-tryptophan, the enzyme was dialyzed against 5 mM K-phosphate (pH 7.0) containing 5% glycerol, 3% ethanol and 20 mM $(NH_4)_2SO_4$ before the assay. The sucrose density gradient ultracentrifugation and the gel filtration on calibrated columns of Sephadex G-75 or G-100 were carried out according to the method of Martin and Ames (12) and of Andrews (13) or Siegel and Monty (14), respectively. Protein was determined by the method of Lowry et al. (15) with BSA as standard.

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RESULTS AND DISCUSSION

Purification --- All procedures for the purification of tryptophan 5-monooxygenase were carried out at 4°. The extraction of the enzyme from bovine pineal glands, the ammonium sulfate fractionation and the chromatography on a hydroxylapatite column were carried out as already described (6). All buffers used for the subsequent purification procedures contained 5% glycerol, 3% ethanol, 20 mM $(NH_4)_2SO_4$ and 0.1 mM L-tryptophan unless otherwise stated.

The concentrated hydroxylapatite fraction (6) obtained from 400 g of bovine pineal glands was applied on a column of P-cellulose (3.2 x 24 cm) which had been equilibrated with 5 mM K-phosphate (pH 7.0) and the enzyme was eluted by a linear gradient between 1.2 l each of 5 mM and 300 mM K-phosphate (pH 7.0). The active fractions (1-1.4 l) were combined, concentrated to 50 ml by ultrafiltration and dialyzed against 5 mM K-phosphate (pH 7.3)[2]. The dialyzed enzyme was then applied on a DEAE-cellulose column (2 x 36 cm) which had been equilibrated with 5 mM K-phosphate (pH 7.3). After the column was washed with 150 ml of the same buffer, a linear gradient elution was carried out between 500 ml each of 5 mM and 50 mM K-phosphate (pH 7.3). Fractions (12 ml each) were collected at a flow rate of 60 ml per hour. The enzyme was eluted into 2 peaks; a small peak in tubes No.9-20 was followed by a main activity peak in tubes No.30-55. The second active fractions were combined and made 80% saturated with respect to $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in a small amount of 20 mM K-phosphate (pH 7.0). The enzyme solution was then applied on a column of Sephadex G-200 (2 x 81 cm) which had been equilibrated with 20 mM K-phosphate (pH 7.0). Elution was carried out to the ascending direction at a flow rate of 10 ml per hour and 2 ml-fractions were collected. Active fractions (tubes No.76-88) were combined and concentrated to about 5 ml with the aid of a collodion bag. A summary of the purification is given in Table 1. The overall purification achieved was about 1,000-fold with a yield of 3%.

Properties of the enzyme --- Throughout the purification procedure phenylalanine monooxygenase activity remained associated with tryptophan 5-monooxygenase. Furthermore, when the purified enzyme was subjected to Sephadex G-100 column chromatography, both enzyme activities were eluted in the same single symmetrical peak and upon sucrose density gradient ultracentrifugation they moved down to an identical position. As shown in Fig. 1, both enzyme activities increased approximately 100-fold on the anaerobic preincubation in the presence of DTT and the time course of the activation was in parallel with each other both in the presence and absence of Fe^{2+}. When the enzyme was subjected to the

2. 5 mM K-phosphate (pH 7.3) used in the DEAE-cellulose column chromatography contained no $(NH_4)_2SO_4$.

Table 1. Purification of tryptophan 5-monooxygenase.

Enzyme preparations	Volume	Enzyme activity	Yield	Protein	Specific activity
	(ml)	(units)*	(%)	(mg)	(units/mg)
Homogenate	1,300	11,040	100	50,700	0.22
Digitonin Sup.	1,820	9,397	85	24,600	0.38
Amm. sulfate (25-57%)	270	7,171	65	5,990	1.20
Hydroxylapatite	125	3,548	32	1,100	3.23
P-Cellulose	46.5	2,521	23	393	6.42
DEAE-Cellulose	5.3	748.5	6.8	33.8	22.14
Sephadex G-200	4.6	400.2	3.6	1.7	235.40

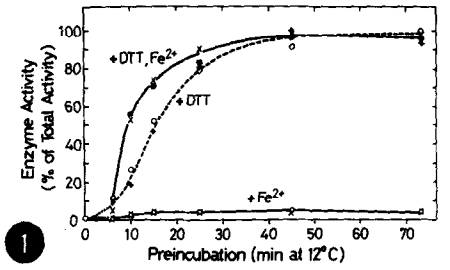
* One unit of enzyme activity was defined as the amount which catalyzed the hydroxylation of 1 nmole of L-tryptophan per min under the standard assay conditions.

heat treatment at various temperatures (45-65°) for 2 min or at 53° for various periods of time (1-10 min), both monooxygenase activities decreased to nearly the same extent. In good agreement with Lovenberg et al.(3), L-phenylalanine inhibited tryptophan 5-monooxygenase activity competitively with respect to L-tryptophan and vice versa. All experimental evidence described above thus suggested that hydroxylations of both L-tryptophan and L-phenylalanine were catalyzed by the single enzyme. On the other hand, the hydroxylation of L-tyrosine by a purified enzyme preparation[3] occurred at less than one-hundredth the velocity of the hydroxylation of L-tryptophan. Furthermore, L-tyrosine inhibited tryptophan and phenylalanine monooxygenase activities appreciably only at a high concentration with an approximate Ki value of 500 to 600 μM, suggesting that L-tyrosine is a much poorer substrate of this enzyme, if at all[4].

The apparent Km values of the enzyme for L-tryptophan and for L-phenyl-

3. The enzyme preparation was purified with the following modifications of the procedure: 1. The DEAE-cellulose step was omitted, 2. P-Cellulose and Sephadex G-200 were replaced by CM-Sephadex C-50 and Sephadex G-100, respectively. The specific activity of this preparation was 140 and 310 nmoles of tryptophan and phenylalanine hydroxylated, respectively, per min per mg of protein.

4. The trace activity of tyrosine hydroxylase in the purified preparation was first detected by Dr. S. Nagatsu, Aichi Gakuin University. The authers are grateful for his help. The possibility has not been excluded that the tyrosine hydroxylase activity was due to the contamination of other enzyme.



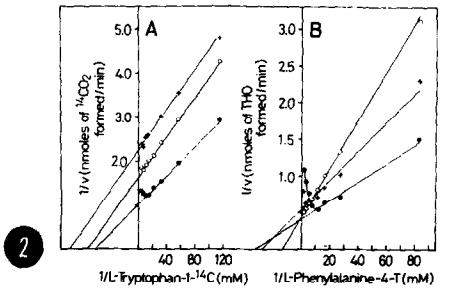


Fig. 1. Effect of preincubation on tryptophan and phenylalanine monooxygenase activities. The preincubation of the enzyme was carried out at 12° in the presence of either Fe^{2+}, DTT or Fe^{2+} plus DTT as described previously (6). At various time intervals an aliquot (50 µl) of the preincubated enzyme was removed and assayed for tryptophan (• and o) and phenylalanine (x and +) monooxygenase activities as described under "Methods".

Fig. 2. Tryptophan (A) and phenylalanine (B) monooxygenase activities as a function of substrate concentration. Enzyme activities were determined as described under "Methods" with the following modifications; 1. Reactions were carried out for 1 min instead of 3 min, 2. The concentration of DTT in the reaction mixture was reduced to 1.67 mM and an excess amount of dihydropteridine reductase and 0.1 mM NADPH were included, 3. Various concentrations of L-tryptophan-1-^{14}C (A) or L-phenylalanine-4-T (B) were used as substrate, 4. $DMPH_4$ (o, 0.89 mM), $6MPH_4$ (+, 0.36 mM) or BpH_4 (•, 0.77 mM) was used as a cofactor. The apparent Km values for L-tryptophan and L-phenylalanine estimated from this experiment were 14 and 70 µM (cofactor: $DMPH_4$), 11 and 37 µM (cofactor: $6MPH_4$) and 16 and 32 µM (cofactor: BpH_4), respectively.

alanine were approximately 16 and 32 µM, respectively, when 0.77 mM BpH_4 was used as a cofactor (Fig. 2). The Km for tryptophan did not change significantly with the structure of the pterin cofactor used but the value for phenylalanine increased to approximately 70 µM when BpH_4 was replaced by $DMPH_4$. The Vmax of phenylalanine hydroxylation was 2- to 3-fold higher than that of tryptophan hydroxylation. The apparent molecular weight and the Stokes radius of the enzyme were estimated to be approximately 30,000 and 25 A, respectively, by gel filtration on calibrated columns of Sephadex G-75 and G-100 and by ultracentrifugation in sucrose density gradients (Fig. 3).

Rat liver phenylalanine hydroxylase (E.C.1.14.3.1) has been shown to catalyze tryptophan hydroxylation as well (16) and the tryptophan 5-monooxygenase activity of that enzyme was shown to be stimulated by lysolecithin (17). However, the Km value for L-tryptophan and the Vmax of the tryptophan hydroxylation of rat liver phenylalanine hydroxylase were shown to be 1 mM and 0.1% of that of phenylalanine hydroxylation, respectively, even in the presence of lysolecithin (17). The molecular weights for the two major forms of phenylalanine hydroxylase have been reported to be 110,000 and 210,000 (18). In a preliminary experiment, phenylalanine hydroxylase was purified from the rat liver according to the method of Kaufman and Fisher (18) up to the step of second am-

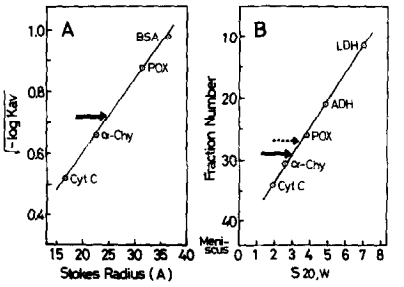


Fig. 3. Gel filtration on a Sephadex G-75 column (A) and sucrose density gradient ultracentrifugation (B) of tryptophan 5-monooxygenase. Analytical gel chromatography was carried out on a calibrated column (3.5 x 85 cm) of Sephadex G-75 equilibrated with 5 mM K-phosphate (pH 7.0) containing 5% glycerol, 3% ethanol, 20 mM $(NH_4)_2SO_4$ and 0.1 mM L-tryptophan. Six ml-fractions were collected at 4° at a flow rate of 12 ml per hour. Proteins with the following molecular weights and Stokes radii were employed as standards: BSA (Fraction V), 68,000, 36.4 A; horse radish peroxidase (POX), 44,000, 31.4 A; beef pancreatic α-chymotrypsinogen (α-Chy), 23,500, 22.6 A; and horse heart cytochrome C (Cyt C), 13,400, 16.5 A. The ultracentrifugation was carried out at 4° and 40,000 rpm for 42 hours in a sucrose gradient (5 to 16%) containing 50 mM K-phosphate (pH 7.0), 5% glycerol, 3% ethanol, 20 mM $(NH_4)_2SO_4$ and 0.1 mM L-tryptophan. After centrifugation 44 fractions (120 µl each) were collected from the bottom of the tube. Standard proteins used were rabbit skeletal muscle lactate dehydrogenase (LDH, S20,w = 7.0-7.3), horse liver alcohol dehydrogenase (ADH, S20,w = 4.82), horse radish peroxidase (POX, S20,w = 3.85), beef pancreatic α-chymotrypsinogen (α-Chy, S20,w = 2.58) and horse heart cytochrome C (Cyt C, S20,w = 1.9-2.1). The arrows indicate the peak of tryptophan 5-monooxygenase activity. The dotted arrow represents the presence of s smaller peak or a shoulder of the enzyme activity.

monium sulfate fractionation and this enzyme was shown to be activated only less than 2-fold on the anaerobic preincubation in the presence of DTT and Fe^{2+}. 6-Fluorotryptophan at 1 mM was found, in the present study, to produce greater than 90% inhibition of bovine pineal tryptophan 5-monooxygenase whereas the inhibitory effect on rat liver phenylalanine hydroxylase has been reported to be no more than 5% (19). The experimental evidence thus indicated that bovine pineal tryptophan 5-monooxygenase and rat liver phenylalanine hydroxylase are distinguishable by a number of criteria. The high activity of the pineal enzyme for tryptophan may suggest that the hydroxylation of tryptophan in the pineal gland is an important function of this enzyme.

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