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PURIFICATION AND SOME PROPERTIES OF TRYPTOPHAN-5-MONOOXYGENASE FROM RABBIT HINDBRAIN

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An affinity chromatography procedure for the rapid purification of tryptophan-5-monooxygenase from rabbit hindbrains was developed using ε-aminocaproyl-D-tryptophan methyl ester-Sepharose-4B gels. The precise requirements for the optimal biospecific interaction between the affinity ligand and the ligate (enzyme) was established from a study of the effects of the variation in the length of the "spacer" on the affinity properties of the gel. The enzyme preparation isolated by this procedure was found to be essentially homogeneous and was characterized by a molecular weight of 200,000 ± 20,000. SDS-polyacrylamide gel electrophoresis of the enzyme revealed it to be a dimer, the molecular weight of each subunit being approximately 90,000. The specific activity of the enzyme preparation is approximately 7–10 times that of the crude homogenate, but a further fivefold enhancement in the specific activity could be obtained by limited proteolysis with trypsin. The extreme lability of the enzyme could be circumvented by its immobilization on activated Sepharose or by cross-linking with dimethyl suberimidate. The kinetic properties as well as the advantages of such stabilized enzyme preparations are presented.

INTRODUCTION

Tryptophan-5-monooxygenase (EC 1.14.16.4) catalyzes the initial hydroxylation step in the biosynthesis of the putative neurotransmitter serotonin (1–3).[1] Only a few structural characteristics of this enzyme and details of the reaction it catalyzes have been elucidated (4,5). Kaufman et al. (6,7), besides developing a sensitive fluorometric assay procedure, established (6R,S)-5,6,7,8-tetrahydro-L-biopterin as the naturally occurring cofactor. It remains to be tested if the enzyme preferentially utilizes a particular one of the two diastereomers. Kaufman et al. (6,7), Joh et al. (8), and Youdim et al. (9) employed classical techniques for the purification of this hydroxylase.

[1]Abbreviations: DTT, dithiothreitol; Cbz-benzyloxycarbonyl-; BH_4, (6R, S)-5,6,7,8-tetrahydro-L-biopterin; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindoleacetic acid; D-TrpOMe,D-tryptophan methyl ester; ε-Ahx, ε-amino caproyl-.

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These tedious and time consuming procedures resulted in low yields and provided a 10–70-fold enhancement in specific activity. In light of many successful applications of affinity chromatography to the isolation of proteins, we attempted to develop a rapid, single-step method for the isolation of tryptophan-5-monooxygenase from rabbit hindbrain. Preliminary results were reported some time ago (10), and now we present data on studies undertaken to find optimal conditions for the purification of the monooxygenase by affinity chromatography. Some properties of the enzyme isolated by this procedure are also described.

MATERIALS AND METHODS

Frozen mature rabbit hindbrains were purchased from Pel Freeze Biologicals, Rogers, Ark., U.S.A. Tetrahydrobiopterin · 2HCl(R03-4957) was a generous gift from Hoffmann-LaRoche, Montreal, Canada. 1,4-butane-diol diglycidyl ether and cyanogen bromide were products of Aldrich Chemicals, Milwaukee, Wis., U.S.A. Dextran T40 and Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden. Catalase (57,000 U/mg), chymotrypsinogen (5 × recrystallized) and trypsin (2 × recrystallized) were products of Worthington Biochemicals Co., Freehold, N.J., U.S.A. Bovine serum albumin, aldolase (rabbit muscle), and Coomasie blue were supplied by Sigma Chemicals, St. Louis, Mo., U.S.A. Acrylamide and Fotoflow 200 were products of Eastman Kodak Co., Rochester, N.Y., U.S.A. All other chemicals of reagent grade were obtained from Fisher Chemical Co., Toronto, Canada.

Synthesis of Spacer-Ligand Units

D-Tryptophan was converted to its methyl ester hydrochloride derivative according to the procedure described in Ref. (11). The product was found to be homogeneous by TLC and was characterized by its mp (213°C), which was identical with that earlier recorded (11). The Cbz derivatives of glycine, β-alanine, 4-amino butyric acid, 5-amino pentanoic acid, and 6-amino hexanoic acid were prepared according to the procedure described in Ref. (12). Each of the derivatives was found to be homogeneous by TLC and possessed an mp identical with that recorded in the literature (12–14).

The coupling of the various Cbz-ω-amino acids to D-tryptophan methyl ester and the subsequent recovery of the peptides was accomplished using dicyclohexyl carbodiimide (15). The homogeneity of the isolated peptides was established by TLC in different solvent systems. Removal of the Cbz-protecting group was achieved either by treatment with glacial acetic acid saturated with HBr or by catalytic hydrogenation (15).

TLC was performed on aluminium sheets coated with silica gel. The solvent systems used were (*a*) $CH_2Cl_2/CHCl_3/CH_3OH$, 2:2:4; (*b*) *n*-butanol/glacial acetic acid/7% NH_4OH; (*c*) *n*-butanol/glacial acetic acid/H_2O, 4:1:1. Compounds were detected by iodine saturation, ninhydrin spray, and in the case of amino-protected amino acids and peptides, with Rydon-Smith reagent (16).

Activation of Sepharose and Coupling of Spacer-Ligand Units

Sepharose 4B (~50 ml) was activated by treatment with CNBr (17). After washing of the activated Sepharose with a cold solution of $NaHCO_3$ (100 mM) and cold water to remove traces of unreacted CNBr, the gel was resuspended in 30 ml of $NaHCO_3$ (100 mM) and mixed with an equal volume of ethanol containing 1 mmol of the desired spacer-ligand unit. After stirring the suspension (pH 9.5) for 24 h at 4°C, it was filtered, and the gel was washed successively with 1 liter of 100 mM bicarbonate, 1 liter of 10% NaCl solution and 500 ml Tris-acetate buffer, pH 7.5.

While activation of Sepharose was normally accomplished using solid CNBr, in some experiments designed to promote immobilization of the enzyme, solutions of the reagent in either dimethyl formamide or CH_3CN were employed (18).

In some instances, the affinity gels were acetylated prior to their use according to a procedure described in the literature (19).

For the coupling of the ligand by the oxirane procedure (20), Sepharose 4B (20 ml) was functionalized by stirring with a mixture of 20 ml of 1,4-butanadiol diglicidyl ether and 20 ml NaOH (0.6 M) containing 40 mg $NaBH_4$ for 8 h at 25°C. The suspension was filtered and washed with 3 liter of ice-cold water. The washed gel was finally suspended in 20 ml $NaHCO_3$ (0.3 M, pH 10.0) containing 1 g of D-tryptophan methyl ester. Following stirring for 36 h at 25°C, the gel was washed thoroughly with water.

Preparation of Crude Enzyme Homogenate

Frozen rabbit brains were thawed and cut into small pieces on a glass plate placed on ice. The cuttings from three brains were transferred to a 3×10 cm test tube, mixed with 20 ml of 50 mM Tris-acetate buffer containing 2 mM DTT, and homogenized in a Polytron homogenizer (Kinematica, Switzerland) for 20–30 s at setting 6. The homogenate was centrifuged ($39{,}000 \times g$, 4°C) for 50 min. The supernatant was used as the source of tryptophan-5- monooxygenase.

Affinity Chromotography

All experiments were performed at 4°C using 10 ml of settled affinity gel in a 2 × 12-cm glass column. Affinity gels prepared via CNBr activation of Sepharose were preequilibrated with 60 mM Tris-acetate buffer, pH 7.5, containing 2 mM DTT. Thirty ml of soluble enzyme preparation was applied to the gel. Elution of the gel with the equilibration buffer was continued until the A_{280} of the effluent reached a constant, minimum value. The gel was subsequently eluted with equilibration buffer containing NaCl (1.5 M) until no further release of protein (as indicated by A_{280}) could be detected. The enzyme was finally recovered by eluting the gel with either carbonate buffer, pH 10.5, or 50 mM Tris acetate buffer, pH 7.5, containing 2 mM DTT and 1% Triton X-100. When the former buffer was employed, fractions were collected in tubes containing 1 ml of Tris-acetate buffer, 2.0 M, pH 7.5, so as to rapidly lower the pH of eluted enzyme solution to 7.0.

Affinity gels, prepared by the oxirane method, were equilibrated with 50 mM Tris-acetate buffer, pH 7.5, containing NaCl (2.0 M) and DTT (2 mM). Crude soluble enzyme (30 ml) was treated with enough NaCl to achieve a final concentration of 2.0 M with respect to the salt prior to its use. Following its application, the gel was washed initially with equilibration buffer and subsequently with buffers containing 1.0, 0.5, and 0.0 M NaCl, respectively. Buffer changes were made when the A_{280} of the effluent was minimum and constant. Under these conditions, the enzyme was recovered in the buffer of low ionic strength.

Assay for Enzymatic Activity

A modified version of the fluorometric procedure (6) was used for the assay of enzymatic activity. A typical assay mixture (total volume of 1.8 ml) consisted of the following ingredients (added in sequence shown), the final concentrations or units in the reaction as indicated in parentheses: 50 μl of L-tryptophan (167 μM), 50 μl of catalase (1125 U), 0.5–1.0 ml of enzyme, 100 μl of BH_4 (190 μM), and the required amount of 50 mM Tris–acetate buffer, pH 7.5, containing 2 mM DTT. Based on the known solubility of oxygen under standard conditions (4 mg/100 ml) (21), its concentration was at least 300-fold of molar excess over that of the product formed. Samples were incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.2 ml of 70% $HClO_4$. Following the removal of the precipitate by centrifugation, 1 ml of supernatant was treated with concentrated HCl (0.5 ml) and the fluorescence of the solution recorded at 540 nm (excitation 295 nm) using an Aminco Bowman spectrofluorometer.

Corrections for background fluorescence (blank) were made by using samples identical with the assay mixture except for the replacement of

L-tryptophan by the D-isomer of the amino acid. Assay mixtures containing D-tryptophan and the internal standard, 5-hydroxytryptamine (1.25–2.5 μM), were used to correct for the fluorescence quenching. The absolute amount of the products (5-HTP, 5-HT, and 5-HIAA) formed were calculated in reference to a standard curve constructed with known amounts of 5-HT in the assay media devoid of the amino acid substrate. The relationship used for the determination of the amount of product is given below:

$$RI_n = \frac{X \cdot Y}{Z}$$

Here X is the fluorescence value (taken from the standard curve) corresponding to the concentration of 5-HT used in the internal standard; Y is the fluorescence reading of the desired sample after correction for the blank; Z is the fluorescence of the internal standard corrected for the blank; and ΔRI_n is the normalized fluorescence value used in the estimation of the absolute amount of product formed using the standard curve. A standard fluorescence block purchased from the suppliers of the instrument was used to check the lamp intensity at the beginning and end of a series of measurements.

The specific activity of the enzyme is defined as the number corresponding to n moles of products (5-HTP, 5-HT, and 5-HIAA) formed per min per mg protein. The concentration of protein was routinely assessed by using the procedure of Lowry et al. (22) except in the case of samples containing Triton X-100, when the method of Chandrarajan and Klein (23) was adopted.

Determination of the K_m values of the enzyme for L-tryptophan and BH_4 was accomplished according to the method of Lineweaver and Burke. Care was taken to bracket apparent K_m values and to select concentrations of substrate or cosubstrate so as to yield nearly equally spaced points in a double-reciprocal plot. Data were fitted by the linear least-square method using a Hewlett–Packard plotter Model-9830A.

Molecular Weight Determination

Assessment of the molecular weight of the native enzyme was accomplished by electrophoresis on polyacrylamide gels differing in the extent of cross-linking, and subsequent analysis of the data was carried out according to the procedure of Hedrick and Smith (24). Molecular weight of the subunits was determined by SDS-disk gel electrophoresis of the enzyme (25).

Modification of the Enzyme

The reaction of tryptophan-5-monooxygenase with (*a*) dimethyl suberimidate, (*b*) glutaraldehyde, (*c*) polyaldehyde-dextran T40, and (*d*) unmodified dextran T40 was accomplished as follows. Five ml of enzyme (1 mg/ml of H_2O) at 4°C was treated with polyaldehyde dextran T40 (10 mg), unmodified dextran T40 (10 mg), dimethyl suberimidate (1.5 mg), or glutaraldehyde (0.5 ml of 0.25% solution in water). The molar ratios of the reagent to the enzyme in the above-mentioned cases were 10 : 1, 10 : 1, 250 : 1, and 550 : 1, respectively. Aliquots were drawn at regular intervals for monitoring the enzymatic activity. The conversion of dextran T40 to the polyaldehyde derivative was accomplished by periodate oxidation as described in ref. (26).

RESULTS

Sepharose 4B gels coupled to Trp-OMe through spacers of different length (prepared as described above) were tested for their ability to bind tryptophan-5-monooxygenase so as to establish optimal conditions for the purification of the enzyme. None of the gel preparations with less than six carbons in the spacer was found to retain the enzyme. Thus ε-aminohexanoyl-D-trp-OMe-Sepharose was found to be the most suitable gel for the purification of the enzyme. Results obtained are illustrated in Fig. 1.

The recovery of the enzyme from the affinity gel could only be accomplished by elution with buffer or high pH (carbonate, pH 10.5). In an attempt to find mild conditions for elution of the enzyme, a variety of reagents and media (Table 1) were employed. Data recorded in Table 1 demonstrate that the conditions employed usually resulted in the recovery of protein devoid of catalytic activity. Inclusion of a surfactant (1% Triton X-100) in the buffer medium permitted the elution of the enzyme at neutral pH. However, the specific activity of the enzyme recovered by this procedure was approximately the same as that obtained by elution with carbonate buffer, pH 10.5. The details of the experiments using various gels and the results obtained are presented in Table 2.

The homogeneity of the enzyme preparation isolated by chromatography on ε-aminohexanoyl-D-trp-OMe-Sepharose was assessed by polyacrylamide gel electrophoresis. Approximately 90% of the material was found to migrate as a major component, with the remaining being distributed between two minor components. Thus the affinity procedure was effective in removing most of the extraneous proteins from the enzyme. For determination of the molecular weight of the native enzyme, electrophoresis was performed on polyacrylamide gels of varying cross-links, with aldolase, monomer and dimer of bovine serum albumin, and chymotrypsinogen

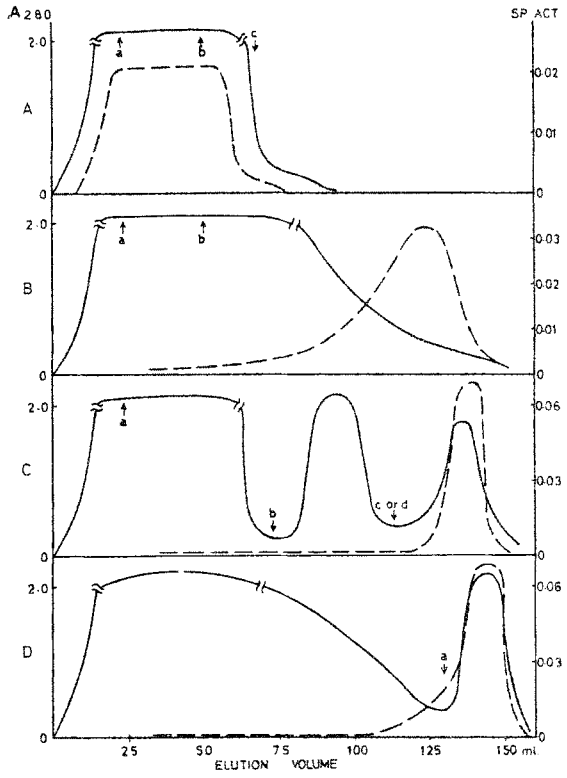

FIG. 1. Chromatography of tryptophan-5-monooxygenase on various gels. (A) Sepharose-4B; CNBr-activated Sepharose-4B; ε-Ahx-Sepharose-4B. (B) D-TrpOMe-Sepharose-4B; NH_2-$[CH_2]_n$-CO-D-TrpOME-Sepharose-4B ($n = 2$–4). The tail in the elution profile becomes longer as n increases, but no clear resolution is achieved with any of these gels. (C) ε-AHx-D-TrpOMe-Sepharose-4B. (D) 1,4-butanedioldiglycidyl-D-TrpOMe-Sepharose-4B (oxirane gel). Buffers used in elution were: (a) equilibration buffer, 50 mM Tris-acetate, pH 7.5; 2 mM DTT (in certain experiments 50 mM maleate, pH 7.2, 2 mM DTT was used); (b) as in (a), but containing 1.5 M NaCl; (c) 25 mM carbonate, pH 10.5; (d) as in (a), but containing 1% Triton X-100. With gel (D), crude homogenate was made with 2.0 M NaCl prior to application and was then eluted with buffer of decreasing ionic strength (cf. text under methods); active enzyme was finally eluted with buffer (a); some active enzyme did come off at 0.5 M NaCl. Dashed line activity; solid line, protein. Elution of protein was monitored at A_{280}, except for maleate buffer and buffer (d), which were scanned at A_{300} to avoid interference due to surfactant and maleic acid. Flow rate; 100–200 ml/h for application and washing, and 30–40 ml/h for the final elution.

TABLE 1. **Reagents Used in an Attempt to Elute the Enzyme at Neutral pH from the Affinity gel**[a]

Concentration of reagents added to 50 mM Tris-acetate, pH 7.5[b], 2 mM DTT	Protein eluted	Activity
0.7 M and 1 mg L-Trp/ml buffer	–	–
As above, plus 20% glycerol	–	–
30% glycerol	–	–
30% ethylenglycol	–	–
2 M urea[d,e]	+	–[c]
2 M NaSCN[d,e]	+	–[c]
2 M NH_4SCN[d,e]	+	–[c]
2 M LiBr[d,e]	+	–[c]
Any of above four, plus 20% glycerol	+	–[c]
25%Saccharose	–	–
4 M imidazole	+	–
0.1% deoxycholate	–	–
0.5% deoxycholate	+	–
0.5% sodium dodecylsulfate	+	–
1% Triton X-100	+	+[f]
1 mM phosphatidylcholine (PC)	–[g]	–

[a] In these experiments, 10 ml crude homogenate was applied to 5 ml of affinity gel. Following removal of extraneous protein by washing the gel with equilibration buffer, the gel was eluted with the media shown.

[b] In view of possible inactivation of enzyme due to *N*-carboxylation by the carbonate buffer (pH 10–11), the following noncarbonate buffer media (pH 10.5) were examined as eluants: 50 mM lysine, borate, Tris, pyrophosphate and 1 mM KOH. The enzyme eluted by these buffers has a sp. act. similar to that observed with carbonate buffer. Hence, possible *N*-carboxylation of amino group(s) of the enzyme did not appear to cause low specific activity.

[c] Dialysis of these samples did not result in active preparations.

[d] Little protein was found to elute at lower concentrations.

[e] Choatropic ions have been successfully used as eluants in several affinity methods (27,28).

[f] The Triton X-100 can be removed from the protein by adsorbing the latter on calcium phsophate gel, washing with Tris/DTT buffer, and eluting with 0.125 M phosphate buffer (pH 6.8) containing 2 mM DDT. As noted earlier (8), this treatment results in loss of enzyme protein.

[g] Enzyme was then eluted with 1% Triton X-100; this eluate contained residual PC and has a sp. act. approx. 12 times higher than the crude.

serving as reference proteins. The molecular weight of native protein estimated from a secondary plot of slopes vs molecular weight (Fig. 2) was found to be approximately 200,000 ± 20,000. The subunit molecular weight estimated by SDS-polyacryl-amide gel electrophoresis was found to be approximately 90,000 (Fig. 3).

During the course of these investigations, the affinity gels that had been used repeatedly in the isolation of enzyme, or that had been left standing (unused) at 4°C in equilibration buffer for several days, were found to bind the enzyme very tightly. Enzyme recovery could not be accomplished either

TABLE 2. **Purification of Tryptophan Hydroxylase on Affinity Gels**

Sepharose-4B derivative	Protein applied (mg)[a]	Protein in active peak[b]	Total units applied	Total units recovered	Sp. act. of appl. sample[c]	Sp. act. of active peak	Purification
No ligand	225	170	3.4	2.9(85%)	0.015	0.017	
$NH_2-[CH_2]_n-CO$-D-TrpOMe[d]	345	135	5.0	4.7(93%)	0.015	0.035	2.3
ε-Ahx-D-TrpOMe[e]	200	8	4.5	1.5(35%)	0.018	0.138	7.7
	240	20	2.2	1.0(46%)	0.009	0.057	6.3
1,4-butanedioldiglycidyl-D-TrpOMe	230	14	2.3	0.83(36%)	0.008	0.060	7.5

[a] Crude homogenate had on the average 10–12 mg protein/ml.
[b] Purified fractions contained 0.5–1.0 mg protein/ml.
[c] Sp. act. of crude homogenate varied greatly with the age and batch of the frozen brains.
[d] $n = 0, 2,3,4$. No clear resolution is achieved with any of these gels (cf. Fig. 1B). The numbers given here are for a gel with $n = 3$.
[e] Upper line, for enzyme eluted from acetylated gel at pH 10.5; lower line, for enzyme eluted from acetylated gel with Tris-buffer containing 1% Triton X-100.
Note. Unit: nmol product formed per min under standard assay conditions. Sp. act.: units per mg protein.

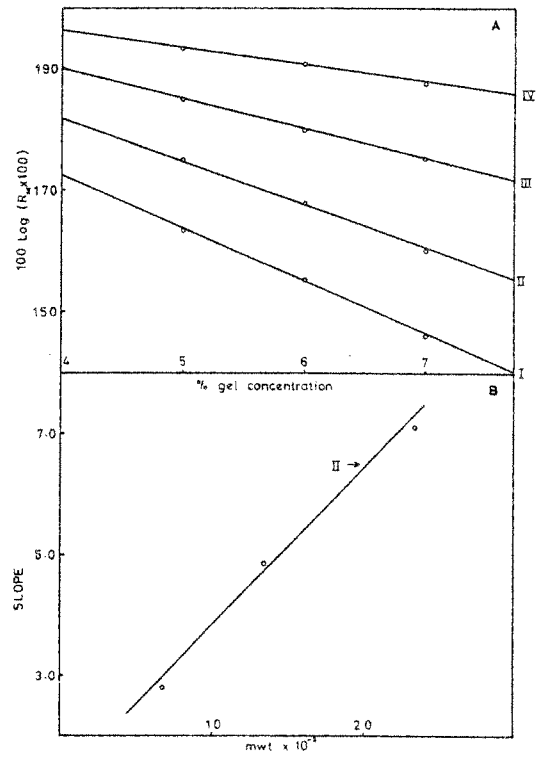

FIG. 2. Standard curves for the determination of the molecular weight of the native enzyme. The method of Hedrick and Smith (24) was employed. (I) catalase; (II) tryptophan-5-monooxygenase; (III) and bovine serum albumin (dimer); (IV) bovine serum albumin (monomer).

by carbonate buffer (pH 10.5) or by neutral buffer containing Triton X-100. Although the protein could be eluted with dilute NH_4OH, the material obtained was devoid of enzymatic activity. However, gels with tightly bound enzyme were found to be capable of catalyzing the hydroxylation of L-tryptophan when used in the normal assay. Such fortuitous immobilization of the enzyme prompted an investigation of the stability and kinetic properties of the immobilized protein preparations.

Enzyme bound to gel was very stable (activity being maintained for several days at 4°C) and could be reused in several experiments after being recovered from the reaction mixture. The pH optimum for its function was found to be approximately 7.5, similar to that noted with purified soluble enzyme preparations (29). Studies on the dependence of rate of hy-

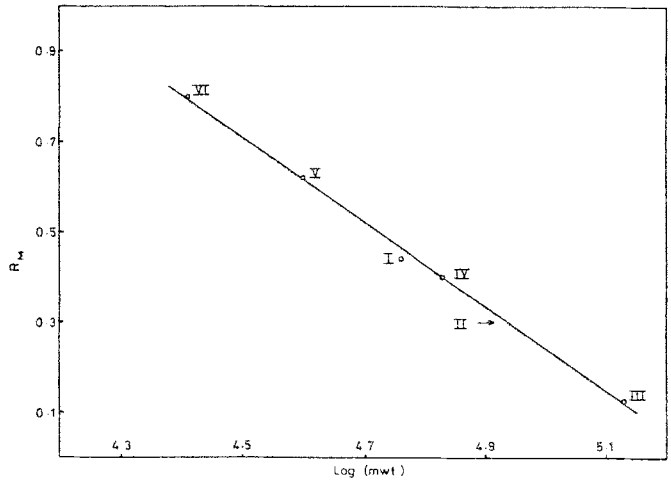

FIG. 3. Determination of the subunit molecular weight. SDS-gel electrophoresis was performed according to the method of Weber and Osborn (25) on 7.5% gels. (I) Catalase (60,000) (II) tryptophan-5-monooxygenase (90,000); (III) BSA-dimer (134,000); (IV) BSA-monomer (67,000); (V) aldolase (40,000); (VI) chymotrypsinogen (26,000).

droxylation on substrate concentration (Fig. 4) provided an estimate of the K_m value (270 μM), which is considerably higher than that (62 μM) noted with a soluble enzyme preparation (29). This feature could readily be attributed to constraints on the availability of the substrate imposed by diffusional limitations (30,31) and also to the possibility of ligand competing with the free substrate. The phenomenon of immobilization of the enzyme was also noted with fresh affinity gel preparations that involved the use of solutions of CNBr (in acetonitrile or dimethyl formamide) instead of the solid reagent for the activation of Sepharose.

The beneficial properties conferred by immobilization of the enzyme provided an impetus to further investigations on the possibility of achieving similar results by other means. Coupling of the enzyme to periodate-treated dextran T40 was attempted. However, the enzyme was found to lose all its activity. Likewise, cross-linking of the enzyme with glutaraldehyde (32) caused its inactivation. However, treatment of the protein with unoxidized dextran T40 or cross-linking with suberimidate (32) produced considerable stabilization of the enzymatic activity. Such treated preparations maintained their activity at 4°C for several days, in contrast to the untreated enzyme samples, which lost all their activity within 24 h under similar conditions.

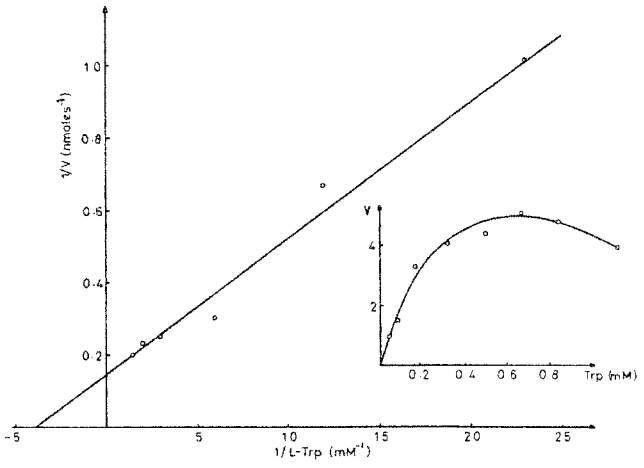

FIG. 4. Determination of the K_m of immobilized enzyme for the substrate L-tryptophan. One ml of immobilized enzyme was assayed as described in the text. Velocities are expressed in nmol product formed per assay. The insert shows a plot of the rate vs the substrate concentration. The substrate becomes inhibitory at concentrations above 0.7 mM. The K_m for L-tryptophan was found to be 270 μM. The cofactor used was $6MPH_4$ at 0.6 mM, twice the standard concentration given in the text under materials and methods.

Dextran T40 and suberimidate treatment were also found to confer stability against thermal and urea denaturation. The only change in these modified enzyme preparations was the decrease in affinity for substrate as indicated by high K_m (values similar to that found for the immobilized enzyme preparation) for tryptophan. The beneficial effects of dextran T40 and suberimidate could well be due to their ability to minimize the intermolecular interactions between enzyme molecules and thus prevent aggregation. Since suberimidate effects cross-linking between amino groups (32), the inactivation caused by the oxidized dextran T40 and glutaraldehyde could arise from the interaction of their carbonyl functions with essential thiol(s) of the enzyme. The dependence of the activity of this enzyme on thiols has been noted before (33, 34).

DISCUSSION

Earlier studies on the purification of tryptophan-5-monooxygenase from pineal glands (34) as well as from rabbit brains (6–8) involved the use of multistep and time-consuming conventional procedures. The affinity

chromatography procedure, first introduced in 1968 (35), has proven extremely resourceful in the isolation of biologically active substances (36–39). Consequently, development of a rapid affinity chromatography procedure for the isolation of tryptophan-5-monooxygenase appeared to be a logical step in our investigation of this enzyme.

The initial phase of these investigations concerned the synthesis of the ligand and the choice of an appropriate spacer, separating the ligand from the insoluble matrix. D-tryptophan methyl ester was chosen as the ligand primarily because of the known ability of antipodes of substrates to serve this function. Although D-tryptophan would be the appropriate choice, its use was deemed undesirable in view of the ion-exchange properties that its carboxyl function would confer on the gel.

In an attempt to establish optimal conditions for the retention of the enzyme and its subsequent recovery from the affinity gel, the length of the spacer unit attached to the ligand was varied. This was accomplished by the attachment of different ε-amino acids to the amino group of the ligand. Thus the spacer-ligand units employed were of the type, H_2N–$(CH_2)_n$–CO–NH-Trp-OMe, with n varying from 1 to 5. Synthesis of such spacer-ligand units was accomplished by condensing the Cbz-derivative of the appropriate ω-amino acid with D-tryptophan methyl ester. The removal of the protecting Cbz-group was achieved either by treatment with glacial acetic acid saturated with HBr or by catalytic hydrogenation. The spacer-ligand unit prepared by either procedure of deprotection displayed indentical properties when tested(following its coupling to activated Sepharose) for its efficiency as an affinity ligand.

Of all the gel preparations only that bearing ε-aminohexanoyl-D-Trp-OMe proved effective in the retention of tryptophan-5- monooxygenase activity. All other gels were either ineffective or failed to retard the enzyme sufficiently enough to permit a high degree of purification. Reduction of the spacer length by just one methylene carbon, as in the case of ε-aminopentanoyl-D-Trp-OMe Sepharose, virtually abolished the ability to selectively sequester the enzyme. This observation emphasizes the precise requirements needed for the optimal ligand-ligate interactions.

The high affinity of ε-aminohexanoyl-D-Trp-OMe Sepharose for tryptophan-5-monooxygenase was such that the enzyme could only be recovered by elution with buffer of high pH (carbonate, pH 10.5) or neutral buffer containing Triton X-100, conditions that could be expected to destabilize the native organization of the enzyme. The enzyme preparations obtained by such elution procedures were characterized by a specific activity of approximately seven times that of the starting material. Elution of gel-bound enzyme with a variety of chaotropic agents resulted in the recovery of the protein devoid of catalytic activity.

The failure of Sepharose, of activated Sepharose (washed extensively with 100 mM $NaHCO_3$ prior to use), and of the gel preparations with just the spacer units to retard the enzyme established the biospecificity features of D-Trp-OMe toward tryptophan-5-monooxygenase. The features contributing to the biospecific interactions between the ligand and the ligate in affinity chromatography procedures have been thoroughly discussed (40,41). To minimize the importance of electrostatic interactions, the affinity gel was acetylated so as to abolish the positive charge associated with the iso-urea linkage (19). However, the enzyme was found to bind the gel tightly, even though other extraneous proteins could be eluted at lower ionic strength than normally required. The enzyme, as with nonacetylated gels, could only be recovered by elution with carbonate buffer or a medium containing Triton X-100. Thus contributions from electrostatic forces to the biospecific interaction appear to be minimal. Replacement of the spacer by the relatively more hydrophilic 1,4-butanedioldiglycidyl ether resulted in a requirement of high ionic strength medium for the retention of the enzyme. These observations suggest that hydrophobic interactions play a dominant role (42) in the biospecific interaction between the ligand and the enzyme, a feature not too surprising in view of the nature of the substrate.

Regardless of the nature of the interactions involved, ε-amino-hexanoyl-D-Trp-OMe Sepharose gels provide a useful means for the isolation of tryptophan-5-monooxygenase. The advantages of this procedure over that of conventional methods becomes apparent from a study of the properties of the isolated enzyme discussed below.

The molecular weight of the native enzyme, as determined by its mobility on polyacrylamide gels of varying cross-links (24), was found to be approximately 200,000. The subunit molecular weight obtained by SDS-polyacrylamide electrophoresis (25) corresponded approximately to 90,000. Thus the enzyme isolated by affinity chromatography appears to be a dimer with each subunit possessing a molecular weight of approximately 90,000. In contrast, the enzyme isolated from rabbit hindbrain by a conventional procedure (6,7) has been reported to be a tetrameric protein (molecular weight, 240,000) each of the subunits being 60,000 in molecular weight. Such differences in the molecular weight and subunit composition of enzyme preparations obtained by different isolation procedures are indeed noteworthy. It is not inconceivable that the isolation of the enzyme by the rapid affinity procedure would result in the elimination or minimization of alterations in the protein, e.g., changes in size due to the action of contaminant proteases, which could occur under the time-consuming, multistep conventional procedures. Thus, the possibility of the enzyme isolated by the affinity procedure being a precursor of that isolated by conventional methods presents itself for serious consideration. Proteolytic

reduction of larger subunits (molecular weight, 90,000) may well facilitate their association to an active tetrameric species observed under conditions of conventional isolation procedures. Enhancement in the activity of crude preparations of tryptophan-5-monooxygenase upon treatment with proteolytic enzymes has been recently reported (43). This observation is compatible with the proposal regarding the occurrence of the enzyme in a less active precursor form under *in vivo* conditions. An alternative explanation could simply be that the hydroxylase from rabbit hindbrain exists in multiple forms as was reported for the hydroxylase from rat midbrain (8).

The specific activity of the enzyme isolated by the affinity procedure was only seven times that of the starting material. At first glance, the procedure may seem inefficient, especially in view of the 60–70-fold increase in specific activity reported for the enzyme obtained by conventional isolation procedures (7). These differences in the specific activities of enzyme preparations obtained by different approaches may be related to the considerations discussed above, viz., affinity chromatography permits the isolation of an active enzyme precursor. Indeed, the specific activity of the enzyme isolated in the current study could be enhanced 5–6-fold by limited exposure to trypsin (44). The specific activity of such trypsin-treated preparations becomes comparable to those reported for the enzyme obtained by conventional procedure. These observations lend support to the proposals presented above. Thus, the findings of the current investigations point to the added utility of the affinity procedure for the isolation of enzymes, i.e., its ability to demonstrate the presence of precursor enzymes that may go undetected by conventional isolation techniques. Evidently, further work is needed to accomodate the differences in the molecular weights and subunit composition of tryptophan-5-monooxygenase preparations obtained by the two different methods.

Yet another unexpected development in these studies was the facile immobilization of the enzyme on gels (*a*) that had been standing unused for several days, (*b*) that had been repeatedly used in the purification of enzyme, and (*c*) that involved the use of dissolved CNBr in the activation of Sepharose. Such immobilization could arise due to the interaction between reactive groups on the gel and functional groups of the protein. Attachment of spacer-ligand units has been shown to involve interaction between the reactive cyanate ester functions of the activated gel and the amino groups (45). As a possible explanation for the observed immobilization of the enzyme, the following may be visualized. Some of the cyanate ester functions on the activated Sepharose may escape coupling and gain protection from the usual modes of decay due to the hydrophobic environment generated by the interactions between the various spacer-ligand units attached to the gel matrix. However, repeated use of the affinity gel or its

storage can be expected to result in the hydrolysis of the ligand ester functions. The production of negatively charged carboxylate ions can be expected to result in the exposure of the reactive cyanate functions. These groups could serve as the focal points for the immobilization of the enzyme. The abundance of cyanate ester functions in gels activated by solutions of CNBr relative to that found in preparations involving the use of solid reagent (45) can likewise contribute to the immobilization of the enzyme. The preferential attachment of tryptophan-5-monooxygenase may be anticipated in view of the affinity features inherent in the intact spacer-ligand units still present on the gel matrix. Regardless of the mechanism operative in the immobilization phenomenon, the presence of catalytic activity in such preparations demands that the attachment to the gel matrix involve functional groups other than those essential for enzymatic function.

Such fortuitous immobilization, apart from conferring stability to the otherwise labile enzyme, provides the added advantage of repeated use of the same enzyme preparation in several experiments. Following completion of an experiment, the gel-bound enzyme can be recovered, washed free of the ingredients of the reaction medium and reused in subsequent study. Immobilized enzyme preparations could prove extremely resourceful in the elucidation of the structure-function relationship, in the clarification of the stereochemistry of the preferred cofactor, and in the identification of intermediates involved in the hydroxylation process.

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REFERENCES

1. UNDENFRIEND, S., TITUS, E., WEISSBACH, H., and PETERSON, R. E., (1956) J. Biol. Chem 219 : 335.
2. GRAHAME-SMITH, D. G. (1967) Biochem. J. 105 : 351.
3. ICHIYAMA, A., NAKAMURA, D., NISHIZUKA, Y., and HAYASHI, O. (1970) J. Biol. Chem. 245 : 1699.
4. LOVENBERG, W. (1973) Pharmacology and the Future of Man, Proc. 5th Int. Congr. Pharmacology, San Francisco, 1972, Vol. 4, Karger, Basel, pp. 232–244.
5. USDIN, E., WEINER, N., and YOUDIM, M. B. H. (eds.) (1977) Structure and Function of Monoamine Enzymes, Marcel Dekker, New York.
6. FRIEDMAN, P. A., KAPPELMAN, A. H., and KAUFMAN, S. (1972) J. Biol. Chem. 247 : 4165.
7. TONG, J. J., and KAUFMAN, S. (1975) J. Biol. Chem. 250 : 4152.

8. JOH, T. H., SHIKIMI, T., PICKEL, V. M. and REIS, D. J. (1975) Proc. Natl. Acad. Sci. 72 : 3575.
9. YOUDIM, M. B. H., HOMAN, M., and BOURGOIN, S. (1975) J. Neurochem. 25 : 407.
10. WIDMER, F., MUTUS, B., RAMAMURTHY, J., SNIECKUS, V. A., and VISWANATHA, T. (1975) Life Sci. 17 : 1297.
11. PETER, H., BRUGGER, M., SCHREIBER, J., and ESCHENMOSER, A., Helv. Chem. Acta 46 : 577.
12. SEELA, F., and WESEMAN, W. Z. (1974) Naturforsch. 29C : 248.
13. DAVIES, J. A., HASSALL, C. H., and ROGERS, I. H. (1969) J. Chem. Soc. (C), 1358.
14. DAVIES, N. C., and SMITH, E. L. (1955) *In* Biochem. Prep., Vol. 4, WESTERFIELD, (ed.), pp. 38–45.
15. MÜLLER, E. (ed.) (1974) Houben–Weyl: Methoden der Organischen Chemie, Vol. 15, Parts 1 and 2, Georg Thieme, Stuttgart.
16. RYDON, H. N., and SMITH, P. W., Nature, 169 : 922.
17. AXEL, R., PORATH, J., and ERNBACK, S. (1967) Nature 214 : 1302.
18. NISHIKAWA, A. H., and BAILON, P. (1975) Anal. Biochem. 64 : 268.
19. WILCHECK, M., and MIRON, T. (1976) Biochem. Biophys. Res. Commun. 72 : 108.
20. SUNDBERG, L., and PORATH, J. (1974) J. Chrom. 90 : 87.
21. GORDON, A. J., and FORD, R. A. (1972) The Chemist's Companion, Wiley, New York, p. 444.
22. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951) J. Biol. Chem. 193 : 265.
23. CHANDRARAJAN J., and KLEIN, L. (1975) Anal. Biochem. 69 : 632.
24. HEDRICK, J. L. and SMITH, A. J. (1968) Arch. Biochem. Biophys. 126 : 155 (1968).
25. WEBER, K., and OSBORN, M. (1975) The Proteins, 3rd edn., Vol. 1, NEURATH, H. (ed.), Academic, New York, pp. 180–223.
26. FOSTER, R. L., Experienta 31 : 772.
27. POHLMAN, S., ROSENGREN, J., and HJERTEN, S. (1977) J. Chrom. 131 : 99.
28. ANDERSEN, N. G., WILLIS, D. D., HOLLADAY, D. W., CATON, J. E., HOLLEMANN, J. W., EVELEIGH, J. W., ATTRIL, J. E., BALL, F. L., and ANDERSON, N. L. (1975) Anal. Biochem. 68 : 371.
29. MUTUS, B. (1977) M.Sc. thesis, University of Waterloo, Waterloo, Ont., Canada.
30. ROYER, G. P. (1975) Immobilized Enzymes, Antigens, Antibodies and Peptides, WEETALL, H. H. (ed.), Marcel Dekker, New York, pp. 49–91.
31. MOSBACH, K. (1976) *In* Techniques of Chemistry, WEISSBERGER, A. (ed.), Vol. X/2, Applications of Biochemical Systems in Organic Chemistry Wiley, New York, pp. 969–994.
32. GLAZER, A. N., DELANGE, R. J., and SIGMAN, D. S. (1975) Chemical Modifications of Proteins, *In* Laboratory techniques in Biochemistry and Molecular Biology, Vol. 4, part I, WORK, T. S., and WORK, E., (eds.), North-Holland, Amstedam.
33. SORIMACHI, M., KATAOKA, K., HORI, S., and FUJISAWA, H. (1973) Eur. J. Biochem. 33 : 486.
34. HORI, S. (1975) Biochem. Biophys. Acta 384 : 58.
35. CUATRECASES, P., WILCHEK, M., and ANFINSEN, C. B. (1968) Proc. Natl. Acad. Sci. 61 : 636.
36. PORATH, J., and KRISTIANSEN, T. (1975), *In* The Proteins, 3rd edn., Vol. I, NEURATH, H. (ed.), Academic, New York, pp. 95–178.
37. WHITESIDES, G. M., and NISHIKAWA, A. H. (1976), *In* Techniques in Chemistry, WEISSBERGER, A. (ed.), Vol X/2, Applications of Biochemical Systems in Organic Chemistry, Wiley, New York, pp. 930–968.

38. JACOBY, W. B., and WILCHEK, M. (eds.) (1974) Methods Enzymol., Vol. 34B, Academic, New York.
39. LOWE, C. R. (1977) Int. J. Biochem. 8 : 177.
40. TOMLINSON, G., SCHELLENBERG, W. C., and VISWANTHA, T. (1978) J. Solid-Phase Biochem. 3 : 57.
41. WILCHEK, M., TOMLINSON, G., SCHELLENBERG, W., and VISWANTHA, T. (1978) Physical Aspects of Protein Interactions, CATSIMPOOLAS, N.(ed.), Elsevier, Amsterdam, pp. 209–128.
42. ROSENGREN, J., PAHLMAN, S., GLAD, M., and HJERTEN, S. (1975) Biochem. Biophys. Acta 412 : 51.
43. HAMON, M., and BOURGOIN, S. (1979) J. Neurochem. 32 : 1837.
44. WIDMER, F. (1977) Ph.D. thesis, University of Waterloo, Waterloo, Ont., Canada.
45. KOHN, J., and WILCHEK, M. (1978) Biochem. Biophys. Res. Commun. 84 : 7.