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PURIFICATION AND CHARACTERIZATION OF PHENYLALANINE 4-MONOOXYGENASE FROM RAT LIVER

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Summary

Phenylalanine 4-monooxygenase (L-phenylalanine, tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1) was purified approx. 600-fold to apparent homogeneity with a 48% yield from rat liver. Two distinct active forms were separable by calcium phosphate gel chromatography and numbered based on their order of elution from the gel column. The predominant form, Form I, had an estimated molecular weight of about 240 000. The enzyme gave a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis, the molecular weight of which was estimated to be approx. 51 000, indicating that the enzyme might be composed of four identical subunits. The molecular properties of Form I were: sedimentation coefficient, 10.1 S; Stokes radius, 55 Å; diffusion coefficient, $3.90 \cdot 10^{-7}$ cm^2/s; frictional ratio, 1.33 and isoelectric point, pH 5.6. The enzyme contained approx. 0.6 mol of iron and 0.3 mol of phosphate/mol of subunit of the enzyme. No significant differences in kinetic properties of the two forms, Form I and Form II, were observed. Amino acid analysis studies revealed that the amino acid composition of Form I was essentially identical with that of Form II, indicating that both forms might be the products of the same gene. There were, however, minor differences in the phosphate content and the isoelectric point between the two forms

Introduction

Phenylalanine 4-monooxygenase (L-phenylalanine, tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1) catalyzes the conversion

Abbreviations: Me_2PteH_4, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; $MePteH_4$, 2-amino-4-hydroxy-6-methyltetrahydropteridine.

of L-phenylalanine to L-tyrosine using tetrahydropterin as a reducing agent and molecular oxygen as an oxidizing agent. Two other enzymes, tyrosine 3-monooxygenase (L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2) and tryptophan 5-monooxygenase (L-tryptophan, tetrahydropteridine:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4), also require tetrahydrobiopterin as an electron source and these three aromatic amino acid monooxygenases are believed to share many properties. Although the most extensive studies have been carried out on phenylalanine 4-monooxygenase because it is available in far greater quantities than the other two monooxygenases, a pure form of mammalian phenylalanine 4-monooxygenase has not been obtained.

In a previous report we described the procedure for complete purification of phenylalanine 4-monooxygenase from *Chromobacterium violaceum* and described some properties of the enzyme [1]. However, a purification as high as 3000-fold was required for obtaining a homogeneous preparation.

This study demonstrates the first purification of mammalian phenylalanine 4-monooxygenase to apparent homogeneity with a very high yield. Some of its physical, chemical and catalytic properties have been examined.

Experimental procedures

Materials

Beef liver catalase and horse spleen ferritin were purchased from Boehringer. DEAE-Sepharose CL-6B and polyacrylamide gradient gels (PAA4/30) were from Pharmacia. Ultrogel AcA 34 and Ampholine were obtained from LKB. Me_2PteH_4 and $MePteH_4$ were purchased from Aldrich and Calbiochem, respectively. Bovine serum albumin, dithiothreitol, and dimethyl suberimidate were the products of Sigma. Cibacron Blue F3GA was obtained as a generous gift from Ciba Geigy, Japan. All other chemicals were reagent grade.

Enzyme assays

The activity of phenylalanine 4-monooxygenase was assayed fluorometrically by measuring the formation of tyrosine according to the nitroso-naphthol procedure of Waalkes and Udenfriend [2] as modified by Woo et al. [3]. The standard assay mixture contained 0.5 μmol of L-phenylalanine, 0.1 μmol of Me_2PteH_4, 1 μmol of dithiothreitol, 25 μmol of potassium phosphate buffer, pH 6.8, 100 μg of catalase and a suitable amount of enzyme in a total volume of 250 μl. The reaction was started by the addition of Me_2PteH_4 and carried out at 24°C for 5 min with shaking. 1 unit of enzyme is defined as the amount that produces 1 μmol of tyrosine per min at 24°C.

Preparation of gels for chromatography

Calcium phosphate gel was prepared by the method of Barranger [4] and uniformly mixed with wet cellulose powder [4]. Blue-Sepharose was prepared by coupling of Cibacron Blue F3GA to Sepharose 6B by the procedure of Heyns and DeMoor [5]. The amount of the coupled dye was determined spectrophotometrically at 610 nm after acid hydrolysis [5]. Approx. 0.9 mg of dye/ml of gel was found to be bound.

Determination

The iron content of enzyme was determined with a Hitachi 508 atomic absorption spectrophotometer with the sensitivity of 0.1 ppm. Prior to determination, the enzyme solution was passed through a Sephadex G-25 column equilibrated with 50 mM Tris-HCl buffer, pH 6.8. Protein-bound phosphate was measured by the method of Ames [6]. The carbohydrate determination was carried out by the phenol-sulfuric acid method [7] with glucose as a standard. Protein was determined by the method of Lowry [8] with bovine serum albumin as a standard.

Sedimentation studies

Sedimentation velocity and sedimentation equilibrium analyses were carried out in a Hitachi Model 282 analytical ultracentrifuge. Prior to centrifugation, samples were dialyzed at 4°C for 3 days against 10 mM Tris-HCl buffer, pH 6.8, containing 200 mM KCl and 0.2 mM dithiothreitol. Sedimentation velocity experiments were carried out at 60 000 rev./min at 4°C or 6°C. The sedimentation coefficient was calculated according to Schachman [9] from the experiments which were carried out at protein concentration of 0.8 mg/ml using absorption optics.

Sedimentation equilibrium was performed according to the method of Yphantis [10]. Centrifugation was carried out at 10 000 rev./min for 30 h at 4°C and scanning was done at 280 nm by absorption optics.

Purification of enzyme

All purification procedures were carried out at 4°C and centrifugations were carried out 27 000 $\times g$ for 15 min unless otherwise specified.

Step 1. Extraction. After adult male Wistar rats were killed by a blow on the head followed by decapitation, the livers, 120 g, were immediately removed, cut into small pieces, and homogenized with 2.5 vols. of 25 mM potassium phosphate buffer, pH 6.8, containing 150 mM KCl and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 39 000 $\times g$ for 30 min, and the sediment was discarded.

Step 2. Protamine treatment. To the supernatant solution (310 ml), 31 ml of 1% protamine sulfate solution were added with stirring. After stirring for 10 min, the precipitate was removed by centrifugation.

Step 3. First $(NH_4)_2SO_4$ fractionation. The supernatant solution (310 ml) was brought to 35% saturation with solid $(NH_4)_2SO_4$, stirred for 20 min, and centrifuged. The resulting supernatant was then brought to 45% saturation with solid $(NH_4)_2SO_4$ and stirred for 20 min. After centrifugation, the precipitate was dissolved in 80 ml of 50 mM Tris-HCl buffer, pH 7.0, containing 10% glycerol.

Step 4. Second $(NH_4)_2SO_4$ fractionation. The enzyme solution (88 ml) was again brought to 45% saturation with solid $(NH_4)_2SO_4$. After stirring for 20 min, the precipitate was collected by centrifugation and dissolved in 45 ml of 50 mM Tris-HCl buffer, pH 7.0, containing 12.5% $(NH_4)_2SO_4$ and 10% glycerol. The enzyme solution was then centrifuged for 10 min at 39 000 $\times g$ to remove insoluble materials.

Step 5. Blue-Sepharose chromatography. The enzyme solution (51 ml) was

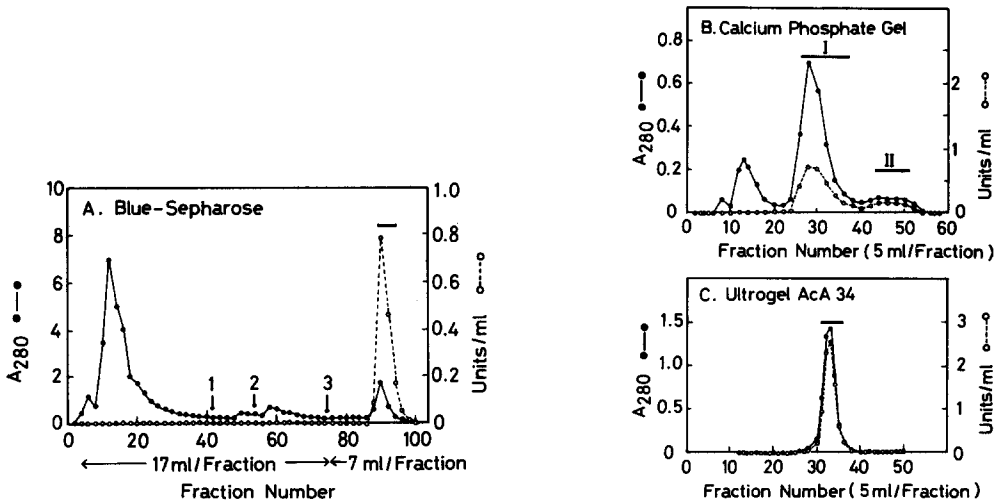

Fig. 1. Column chromatography in the purification of phenylalanine 4-monooxygenase on Blue-Sepharose (A), calcium phosphate gel (B) and Ultrogel AcA 34 (C). ○------○, phenylalanine 4-monooxygenase activity; ●———●, absorbance at 280 nm. The fractions within the bars were pooled for further purification. At the points indicated by the arrows (A), the concentration of $(NH_4)_2SO_4$ in stepwise elution was changed. 1, 10%; 2, 7%, 3, 0%.

applied to a Blue-Sepharose column (3 × 15 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.0, containing 12.5% $(NH_4)_2SO_4$ and 10% glycerol. The column was eluted successively with decreasing concentrations of $(NH_4)_2SO_4$ as shown in Fig. 1A. The phenylalanine 4-monooxygenase activity was recovered in the last elution buffer.

Step 6. Calcium phosphate gel chromatography. The active fractions were pooled (about 42 ml) and precipitated by the addition of solid $(NH_4)_2SO_4$ (60% saturation). The resulting precipitate was dissolved in a minimum volume of 25 mM potassium phosphate buffer, pH 6.8, containing 150 mM KCl and 20 mM L-phenylalanine and dialyzed for 3 h against 500 ml of the same buffer. The dialysate was applied to a column of calcium phosphate gel (1.3 × 25 cm) equilibrated with 25 mM potassium phosphate buffer, pH 6.8, containing 150 mM KCl and 20 mM L-phenylalanine and the column was eluted with a 300-ml linear gradient from 25 to 200 mM potassium phosphate buffer, pH 6.8, containing 150 mM KCl and 20 mM L-phenylalanine. The phenylalanine 4-monooxygenase activity was resolved into two distinct peaks, a predominant form (Form I) and a minor form (Form II) as shown in Fig. 1B, in accord with the observation described by Donlon and Kaufman [11,12]. Active fractions of Form I and Form II were pooled separately, precipitated by $(NH_4)_2SO_4$ (60% saturation), dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 7.0, containing 10% glycerol and 20 mM L-phenylalanine, and stored frozen at −80°C until used for further purification.

Two preparations of each form, which had been purified and stored as described above, were combined and further purified, separately, in the following steps.

Purification of Form I

Step 7. First Ultrogel AcA 34 chromatography. The active enzyme solution (about 4 ml) of Form I preparation was layered onto a column of Ultrogel AcA 34 (2 × 98 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.0, containing 20 mM L-phenylalanine and 5% glycerol and the column was eluted with the equilibration buffer. The active fractions were pooled and precipitated by $(NH_4)_2SO_4$ (60% saturation). The resulting precipitate was dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 7.0, containing 20 mM L-phenylalanine and 5% glycerol.

Step 8. Second Ultrogel AcA 34 chromatography. The enzyme solution (about 2 ml) was layered onto the same column that was used at step 7. The elution pattern was shown in Fig. 1C. The active fractions were pooled (about 30 ml).

Step 9. DEAE-Sepharose CL-6B chromatography. The pooled enzyme solution was applied to a DEAE-Sepharose CL-6B column (1.5 × 7 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.0, containing 20 mM L-phenylalanine and 5% glycerol. After washing with 25 ml of the equilibration buffer, the column was eluted with a 200-ml linear gradient from 0 to 300 mM KCl in 50 mM Tris-HCl buffer, pH 7.0, containing 20 mM L-phenylalanine and 5% glycerol. Active fractions were pooled and precipitated by the addition of $(NH_4)_2SO_4$ (60% saturation). The precipitate was dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 7.0, containing 10% glycerol and 1 mM 2-mercaptoethanol. The purified enzyme could be stored at −80°C for a month without any appreciable loss of activity.

Purification of Form II

Step 10. Ultrogel AcA 34 chromatography. The active enzyme solution (about 2 ml) of Form II preparation was layered onto a column of Ultrogel AcA 34 (2 × 98 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.0, containing 20 mM L-phenylalanine and 5% glycerol and the column was eluted with the equilibration buffer. The active fractions which emerged near the void volume of the column were pooled and precipitated by $(NH_4)_2SO_4$ (60% saturation). The resulting precipitate was dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 7.0, containing 10% glycerol and 1 mM 2-mercaptoethanol. Storage of the purified enzyme at −80°C for a month led to approx. 30% loss of enzyme activity. Thus, Form II was less stable than Form I.

Results

Purification of enzyme

The results of purification are summarized in Table I. By this procedure, Form I and Form II of phenylalanine 4-monooxygenase were purified approx. 620-fold with a 42% yield and 500-fold with a 6% yield, respectively. Analysis of the enzymes thus obtained on polyacrylamide gel electrophoresis showed two protein bands, each of which had phenylalanine 4-monooxygenase activity, in both Form I and Form II, respectively, as determined by assay of an identical companion gel (Fig. 2).

TABLE I

PURIFICATION OF PHENYLALANINE 4-MONOOXYGENASE FROM 240 g OF RAT LIVER

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
1. Extract	31 000	90.6	0.0029	1	100
2. Protamine	19 500	75.5	0.0039	1	83
3. 1st $(NH_4)_2SO_4$	4 630	48.3	0.0104	4	53
4. 2nd $(NH_4)_2SO_4$	3 150	40.5	0.0129	4	45
5. Blue-Sepharose	94.6	45.9	0.485	167	51
6. Calcium phosphate gel					
Form I	47.4	58.5	1.23	424	65
Form II	6.6	12.8	1.94	666	14
Form I					
7. 1st Ultrogel AcA 34	30.0	42.5	1.42	490	47
8. 2nd Ultrogel AcA 34	23.7	42.0	1.77	610	46
9. DEAE-Sepharose CL-6B	21.0	37.8	1.80	621	42
Form II					
10. Ultrogel AcA 34	3.7	5.40	1.46	503	6

Physical properties

The molecular weight of the purified Form I of phenylalanine 4-monooxygenase was determined by a number of procedures. Ultrogel AcA 34 gel filtration of the enzyme showed a single symmetrical peak of the enzyme activity in a position corresponding to a single peak of protein as shown in Fig. 1C. The molecular weight of the enzyme was calculated from Ultrogel AcA 34 gel filtra-

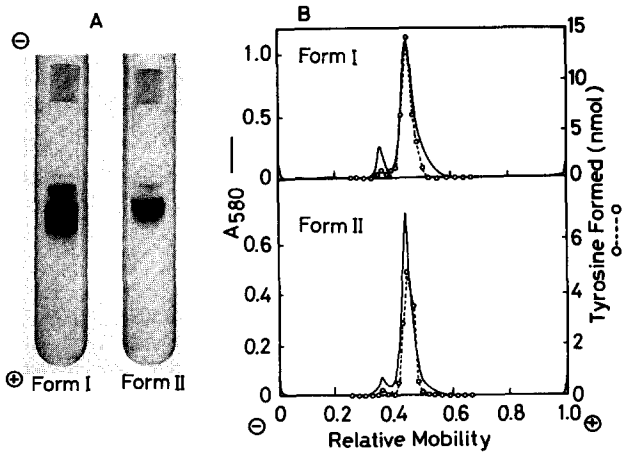

Fig. 2. Polyacrylamide gel electrophoresis of phenylalanine 4-monooxygenase. Approx. 15 μg of purified Form I or 10 μg of purified Form II was applied to each gel. Electrophoresis was carried out with 7% polyacrylamide gels (pH 8.9) essentially according to the method descrbied by Davis [28] and by Ornstein [29] with riboflavin as a catalyst. After electrophoresis and staining for protein with Coomassie brilliant blue R-250, the gels were scanned at 590 nm. Parallel gels were sliced in 1-mm sections and each section was homogeneized in the standard assay mixture and was then assayed, with incubation at 24°C for 30 min.

tion to be 280 000. In addition, gel filtration on Ultrogel AcA 34 revealed a Stokes radius, a, of 55.0 Å and the diffusion coefficient, $D_{20,w}$, was calculated to be $3.90 \cdot 10^{-7}$ cm^2/s from the Stokes radius of 55.0 Å. A molecular weight of 238 000 was obtained from the high speed sedimentation equilibrium plot [10] of the enzyme, taking a partial specific volume of 0.740 cm^3/g estimated from the amino acid composition as described below. The sedimentation velocity of the enzyme was measured by ultracentrifugation. A single, symmetrical Schlieren peak was observed to sediment at $s_{20,w} = 10.1$ S. A molecular weight of the enzyme, as determined from the sedimentation coefficient of 10.1 S, the Stokes radius of 55.0 Å, and the partial specific volume of 0.740 cm^3/g, was 241 000, which was closely in accord with the value of 238 000 obtained from sedimentation equilibrium. A frictional ratio, f/f_0, of the enzyme was calculated to be 1.33 from the Stokes radius and the partial specific volume, when 240 000 was used as molecular weight.

SDS-polyacrylamide gel electrophoresis revealed a single protein band with

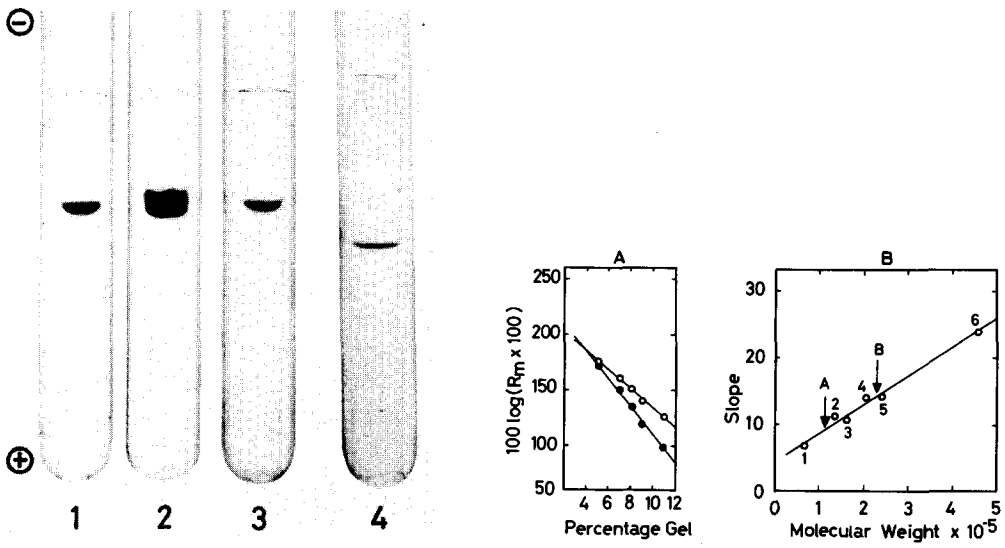

Fig. 3. SDS-polyacrylamide gel electrophoresis of phenylalanine 4-monooxygenase. Electrophoresis was performed on 10% polyacrylamide gel by the method of Weber and Osborn [26] (1, 2, and 3). Protein samples were denatured in the presence of 4% 2-mercaptoethanol and 1% SDS at 100° C for 2 min. Electrophoresis on 11% polyacrylamide gel in the presence of SDS was also performed by the method of Neville [27] (4). Approx. 10 μg of Form I (1), 50 μg of Form I (2), 10 μg of Form II (3), or 5 μg of Form I (4) was applied to each gel. After electrophoresis, gels were stained for protein with Coomassie brilliant blue R-250.

Fig. 4. Determination of molecular weight of phenylalanine 4-monooxygenase by electrophoresis on gels of varying polyacrylamide concentrations. Electrophoresis was carried out at pH 8.9 essentially according to Davis [28] and Ornstein [29]. A, linear relationship between the logarithm of the relative mobility and the polyacrylamide concentration. ○———○, a faster migrating band of the enzyme. ●———●, a slower migrating band of the enzyme. B, linear relationship between molecular weight and the slope determined as described in A. The molecular weight of the protein used as standards were taken to be as follows: 1, bovine serum albumin, 68 000; 2, bovine serum albumin, dimer, 136 000; 3, aldolase, 158 000; 4, bovine serum albumin, trimer, 204 000; 5, catalase, 240 000; 6, ferritin, 460 000. The arrows A and B indicate the positions of a faster migrating band and a slower migrating band of phenylalanine 4-monooxygenase, respectively.

an apparent molecular weight of 51 000 in both forms (Fig. 3), indicating that both Form I and Form II might consist of an identical polypeptide chain and furthermore suggesting that Form I might be a tetramer of identical subunits.

Although both Form I and Form II, when electrophoresed on SDS-polyacrylamide gels, gave a single protein staining band (Fig. 3), each of the forms resolved into two active species, a predominant faster migrating species and a minor slower migrating species, on polyacrylamide gel electrophoresis under nondenaturing conditions in the absence of added detergent (Fig. 2). In order to estimate the molecular weights of these forms, purified enzyme preparations were analyzed by electrophoresis on polyacrylamide gels of different concentration according to Hedrick and Smith [13] as shown in Fig. 4. The slope of the logarithm of the relative migration of the protein against the gel concentration was proportional to the molecular weight of the protein and the slopes for a faster migrating species and a slower migrating species of both forms, Form I and Form II, of phenylalanine 4-monooxygenase corresponded to molecular weights of approx. 110 000 and 230 000, respectively. The latter value is in good agreement with the value of 238 000 obtained from sedimentation equilibrium and with the value of 241 000 estimated from the sedimentation coefficient, the Stokes radius and the partial specific volume. These results, taken together with the fact that SDS-polyacrylamide gel electrophoresis of the enzyme showed a single band with the molecular weight of 51 000, indicate that the predominant faster migrating species and the minor slower migrating species may be a dimer and a tetramer, respectively, composed of identical subunits. Both gel filtration study and sedimentation studies demonstrated that native Form I of phenylalanine 4-monooxygenase was a tetramer, whereas polyacrylamide gel electrophoresis revealed that the dimer was a predominant species. Thus, a tetrameric species appears to dissociate to dimeric species during the course of polyacrylamide gel electrophoresis.

Electrophoresis of phenylalanine 4-monooxygenase on a 4—30% polyacrylamide gradient gel showed additional species as shown in Fig. 5. When electrophoresis was performed at pH 8.35 (Fig. 5A), one major protein band and two broad bands were observed, the molecular weights of which were calculated to be approx. 300 000, 120 000 and 67 000, respectively, from the linear relationship between the relative mobility and the logarithm of the molecular weight of the standard proteins. The species of the molecular weights 300 000 and 120 000 had phenylalanine 4-monooxygenase activity as determined by assay of identical companion gel, whereas the species of the molecular weight of 67 000 had not detectable activity. When electrophoresis was performed at pH 7.3 (Fig. 5B), three protein bands of molecular weights of 290 000, 210 000 and 140 000, respectively, and one minor broad band of molecular weight of approx. 80 000, were observed. When assays were performed after electrophoresis, the species of the molecular weights 290 000, 210 000 and 140 000 were found to be active and the species of the molecular weight 80 000 were inactive. These results, taken together, suggest that phenylalanine 4-monooxygenase can exist as a monomer, a dimer, even a trimer as well as a tetramer of identical subunits and all species other than a monomer are active. Additional confirmation that native phenylalanine 4-monooxygenase was a tetramer was obtained from the cross-linking analysis of the enzyme. When cross-linked

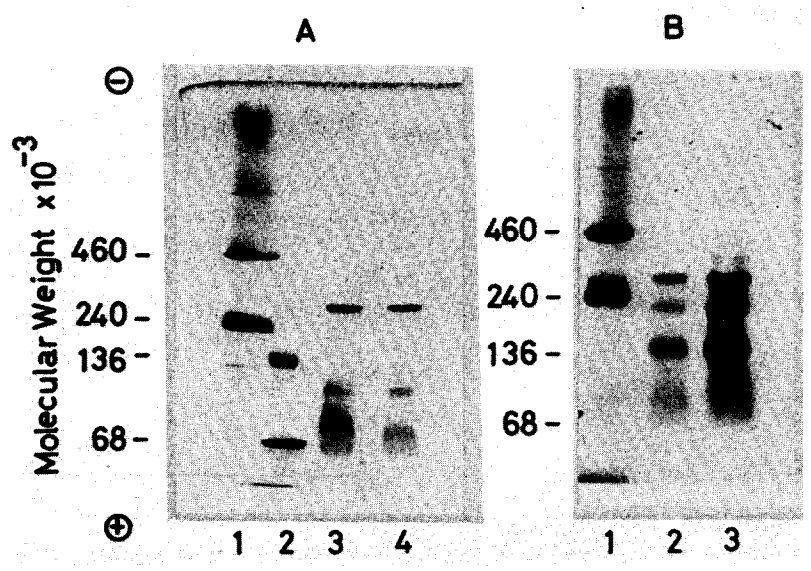

Fig. 5. Electrophoresis of phenylalanine 4-monooxygenase on a 4—30% polyacrylamide gradient gel. A, electrophoresis was carried out at pH 8.35 for 18 h, 200 V at 13°C in the running buffer consisting of 25 mM Tris, 192 mM glycine, 0.1 mM EDTA, and 0.1 mM dithiothreitol. 1, ferritin (upper) and catalase (lower); 2, bovine serum albumin, dimer (upper) and monomer (lower); 3, Form I of phenylalanine 4-monooxygenase (20 μg); 4, Form II of phenylalanine 4-monooxygenase (10 μg). B, electrophoresis was carried out at pH 7.3 for 18 h, 200 V at 13°C in the running buffer consisting of 10 mM Tris, 80 mM glycine, 10 mM HCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol. 1, ferritin (upper) and catalase (lower); 2, Form II (10 μg); 3, Form I (20 μg). Gels were stained for protein with Coomassie brilliant blue R-250.

enzyme protein was analyzed on SDS-polyacrylamide gel electrophoresis, four protein species were observed (Fig. 6), the molecular weights of which were calculated to be 51 000, 107 000, 160 000 and 230 000, respectively, from the linear relationship between the relative migration and the logarithm of molecular weight of protein using cross-linked ovalbumin as a standard [14]. Based on the molecular weights of these species, a monomer, a dimer, a trimer, and a tetramer were suggested.

Polyacrylamide gel electrofocusing showed a single protein band and the isoelectric points for both forms were estimated to be approx. 5.6 as shown in Fig. 7. As judged by the position of the individual protein bands, the isoelectric point of Form I appears to be slightly more acidic than that of Form II.

The physical parameters of Form I of phenylalanine 4-monooxygenase are summarized in Table II.

Chemical properties

The results of amino acid analyses are summarized in Table III. The results were essentially similar to those described by Fisher et al. [15] with the striking exception of the contents of aspartic acid and methionine. There were no significant differences between Form I and Form II, indicating that both forms might be the same gene products. Since 8 mol of half-cystine/mol of subunit of the enzyme were determined as cysteic acid after performic acid oxida-

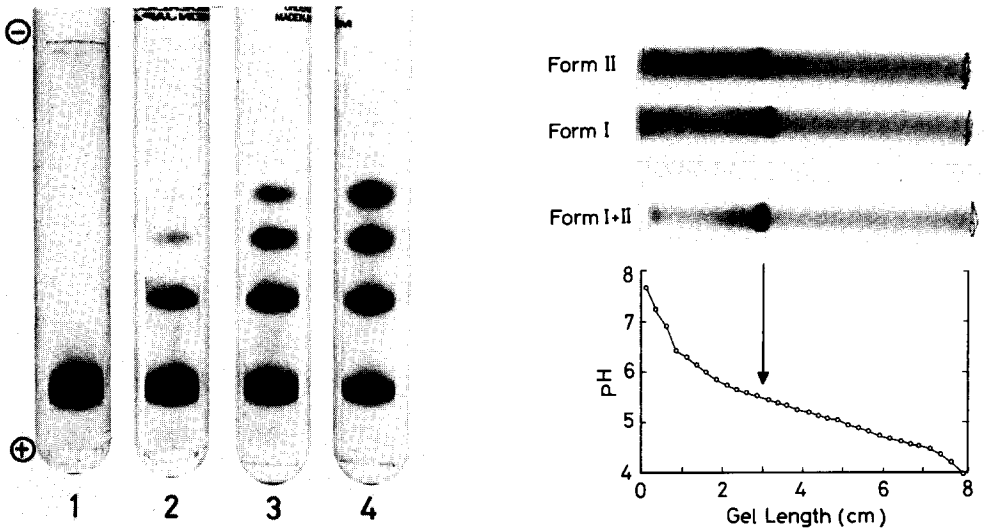

Fig. 6. SDS-polyacrylamide gel electrophoresis of cross-linked phenylalanine 4-monooxygenase. Cross-linking and gel analysis were carried out essentially according to the procedure of Davies and Stark [35]. Form I of phenylalanine 4-monooxygenase, 0.6 mg/ml, was cross-linked with dimethyl suberimidate of 0.5 mg/ml (2), 2 mg/ml (3), and 5 mg/ml (4) at room temperature for 1 h in 0.2 M triethanolamine-HCl buffer, pH 8.5. Control experiment (1) was without treatment with dimethyl suberimidate.

Fig. 7. Analytical isoelectrofocusing of Form I and Form II of phenylalanine 4-monooxygenas. Approx. 10 μg of each was focused on 5% polyacrylamide gels containing 2% Ampholine (pH 4.0 to 6.0) and 8% sucrose essentially according to the procedure described by Wrigley [36]. Electrofocusing was carried out at 200 v for 5 h at 4°C. After electrofocusing, gels were stained for protein with Coomassie brilliant blue G-250 [37]. A parallel gel was sliced into 2.5-mm sections and each section was placed into 0.5 ml of water. After standing for 2 h with shaking, the pH of water extract of each piece was measured at 4°C.

TABLE II

SUMMARY OF THE PHYSICAL PARAMETERS OF PHENYLALANINE 4-MONOOXYGENASE (FORM I)

Parameters	Value
Stokes radius, a *	55.0 Å
Sedimentation coefficient, $s_{20,w}$	10.1 S
Partial specific volume, $\bar{v}$ **	0.740 cm^3/g
Diffusion coefficient, $D_{20,w}$ ***	$3.90 \cdot 10^{-7}$ cm^2/s
Isoelectric point	pH 5.6
Molecular weight	
Sedimentation equilibrium	238 000
Sedimentation velocity	241 000
Ultrogel AcA 34 gel filtration	280 000
SDS-polyacrylamide gel electrophoresis	51 000
Frictional ratio, f/f_0 ***	1.33

* Estimated by the gel filtration on Ultrogel AcA 34 by the method of Siegel and Monty [34].

** Calculated from the amino acid composition [16].

*** These values were calculated by the following equations [34]:

$$D = RT/N\pi 6a\eta$$

$$f/f_0 = a/(3\bar{v}M_r/4\pi N)^{1/3}.$$

TABLE III

AMINO ACID COMPOSITION OF PHENYLALANINE 4-MONOOXYGENASE OF RAT LIVER

Amino acid analysis was performed on a Hitachi KLA-5 automatic amino acid analyzer as described by Spackman et al. [30]. Samples were dialyzed against distilled water and then hydrolyzed in vacuo in 6 N HCl for 24, 48 and 72 h at 110°C. Except where noted, each value is the average of 24, 48, and 72-h hydrolysis. Residues per molecule are based on a subunit molecular weight of 51 000. Mole percentage of Form I is from this work but the partially purified mole percentage is calculated from the data reported by Fisher et al. [15].

Amino acid	Residues/molecule		Mol%	
	Form I	Form II	Form I	Partially purified
Lysine	28.5	29.8	6.4	5.1
Histidine	10.5	10.6	2.4	1.8
Arginine	27.2	26.6	6.1	4.7
Aspartic acid	39.2	40.6	8.8	15.8
Threonine [a]	22.4	22.6	5.0	4.5
Serine [a]	30.0	31.0	6.7	6.7
Glutamic acid	59.4	60.0	13.3	12.5
Proline	23.7	22.6	5.3	6.3
Glycine	24.9	25.4	5.6	5.8
Alanine	28.4	29.0	6.4	6.9
Cysteic acid [b]	7.9	N.D.	1.8	1.6
Cysteine [c]	5.6	N.D.	1.3	1.1
Valine	22.5	23.8	5.1	4.7
Methionine	0.9	0.9	0.2	0.9
Isoleucine	24.0	24.4	5.4	4.2
Leucine	46.8	47.6	10.5	10.3
Tyrosine	21.5	20.5	4.8	3.1
Phenylalanine	25.0	26.5	5.6	4.7
Tryptophan [d]	2.8	3.3	0.6	0.4

[a] Estimated by extrapolation to zero time of hydrolysis.
[b] Determined by performic acid oxidation [31].
[c] Determined by the use of 5,5′-dithiobis(2-nitrobenzoic acid) in 2% SDS [32].
[d] Determined spectrophotometrically [33].
N.D., not determined.

tion and about six sulfhydryl groups/mol of subunit were determined by the use of 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of 2% SDS, there appeared to be one disulfide linkage between the cysteine residues. When the native enzyme was titrated with 5,5′-dithiobis(2-nitrobenzoic acid), only 1.5 mol of sulfhydryl residues/mol of subunit reacted with the reagent. Therefore, it appeared that four or more cysteine residues per subunit were buried inside the enzyme molecule. A partial specific volume of 0.740 cm^3/g was obtained from the amino acid composition [16]. No carbohydrate was detected by the phenol-sulfuric acid method [7] under the experimental conditions that the quantity of 0.05 mol of sugar/mol of subunit of the enzyme could be measured. The iron content of Form I was determined by the atomic absorption spectrophotometer. Based on a molecular weight of 51 000, 0.6 mol of iron was found per mol of subunit of the enzyme. The protein-bound phosphate contents per mol of subunit of Form I and Form II were determined to be 0.3 mol and 0.4 mol, respectively.

Catalytic properties

The purified Form I and Form II exhibited their respective broad pH optima from pH 6.5 to 8.5. The apparent K_m values for L-phenylalanine, Me_2PteH_4, and $MePteH_4$ and the V values of both forms of phenylalanine 4-monooxygenase, which were obtained from the linear double-reciprocal plots [17], are listed in Table IV. When Me_2PteH_4 was used as an electron donor, a turnover number of 545 min^{-1} was calculated from the V values, based on the molecular weight of 240 000. There was essentially no difference in these kinetic values between Form I and Form II. As an electron donor, $MePteH_4$ was more active than Me_2PteH_4.

Partially purified rat liver phenylalanine 4-monooxygenase [18,19] and highly purified bacterial phenylalanine 4-monooxygenase [1] have been reported to catalyze the hydroxylation of tryptophan. A completely purified preparation of rat liver phenylalanine 4-monooxygenase also catalyzed the hydroxylation of tryptophan at 0.2% of the rate of the hydroxylation of phenylalanine under the standard assay conditions. A double-reciprocal plot of initial velocity vs. tryptophan concentration did not give a straight line. When $\log v/(V-v)$ was plotted against logarithm of the concentration of tryptophan, a Hill coefficient of 1.5 was obtained in the presence of either electron donor, $MePteH_4$ or Me_2PteH_4, indicating some positive cooperativity. The apparent K_m values for $MePteH_4$ and Me_2PteH_4 and the V values which were obtained from the linear double-reciprocal plots, are listed in Table IV.

TABLE IV

KINETIC PARAMETERS OF PHENYLALANINE 4-MONOOXYGENASE OF RAT LIVER

Tryptophan 5-monooxygenase activity was assayed fluorimetrically by measuring the formation of 5-hydroxytryptophan by the method of Friedman et al. [38]. The standard assay system contained 0.5 μmol of L-tryptophan, 0.1 μmol of Me_2PteH_4 or $MePteH_4$, 1 μmol of dithiothreitol, 25 μmol of potassium phosphate buffer, pH 6.8, 100 μg of catalase and a suitable amount of enzyme in a total volume of 250 μl. The reaction was carried out at 24°C for 30 min with shaking.

Substrate	Form I		Form II	
	K_m (mM)	V (nmol/min per mg)	K_m (mM)	V (nmol/min per mg)
L-Phenylalanine [a]	1.92	2270	1.43	1590
L-Phenylalanine [b]	0.720	5260	0.454	3700
Me_2PteH_4 [c]	0.0444	1920	0.0344	1110
$MePteH_4$ [c]	0.0370	5560	0.0455	4170
L-Tryptophan [a]	8.50 [e]	33.0		
L-Tryptophan [b]	4.90 [e]	54.0		
Me_2PteH_4 [d]	0.0714	29.4		
$MePteH_4$ [d]	0.0769	52.6		

[a] With 0.4 mM Me_2PteH_4 as a cofactor.
[b] With 0.4 mM $MePteH_4$ as a cofactor.
[c] With 5 mM L-phenylalanine.
[d] With 20 mM L-tryptophan.
[e] Values represent the concentrations of L-tryptophan required for half-maximal velocity, which were obtained from substrate saturation plots.

Discussion

Previous attempts to purify phenylalanine 4-monooxygenase from mammalian sources by conventional protein purification techniques were only partially successful [20]. In the present studies, using a Blue-Sepharose column the enzyme was purified approx. 600-fold to apparent homogeneity with a 48% recovery from rat liver. Judging from the behavior on Blue-Sepharose chromatography (Fig. 1A), Blue-Sepharose appears to act as a hydrophobic adsorbent.

The existence of two or three isozymes of mammalian liver phenylalanine 4-monooxygenase, which are separable by chromatography on calcium phosphate gel [11,21] or on hydroxyapatite [22], has been reported. Recently, Donlon and Kaufman [11,12] have suggested that these two species of the enzyme are due to different degrees of phosphorylation of the enzyme protein. The two species, tentatively designated Form I and Form II in this report, showed essentially the same character in the amino acid composition and the same behavior on polyacrylamide gel electrophoresis, indicating that these species might be the same gene product. In the phosphate content and the isoelectric point, slight differences between the two forms were observed. Although the purified Form I gave a single symmetrical peak with an apparent molecular weight of approx. 280 000 on Ultrogel AcA 34 gel filtration, the purified Form II was eluted in the void volume of the column. However, gel filtration of crude extract on Ultrogel AcA 34 revealed a single peak of the enzyme activity corresponding in elution position to a molecular weight of 280 000 but not detectable enzyme activity in the void volume of the column. The behavior of the purified Form II appeared to be that of a heterogenous mixture of large molecular weight aggregates. There appeared to be a significant difference between the two forms in their stability. Purified Form II was much more unstable than purified Form I under the storage conditions at −80°C. The results described above suggest that the multiplicity of the enzyme may be attributable to posttranslational conformation alteration of the enzyme rather than differential gene expression. Although the conformation alteration of the enzyme might be due to phosphorylation associated with the regulation of the enzyme as suggested by Donlon and Kaufman [11,12], no significant difference between the two purified forms in their kinetic properties was observed under the experimental conditions.

Kaufman and Fisher [23] and Gillam et al. [24] have demonstrated several different polymeric forms of rat liver phenylalanine 4-monooxygenase. Although polyacrylamide gel electrophoresis showed that the enzyme was capable of existing as an inactive monomer, an active dimer, even an active trimer and an active tetramer, both the sedimentation behavior and the elution pattern from Ultrogel AcA 34 revealed that the enzyme existed only as an active tetramer, suggesting that the native tetrameric form might dissociate to monomeric, dimeric and trimeric forms during the course of polyacrylamide gel electrophoresis. Tourian [25] has reported that incubation of the enzyme with L-phenylalanine at room temperature caused the shift of the equilibrium from a dimer to a tetramer of the enzyme. However, the elution pattern from Ultrogel AcA 34 was not affected by the presence or absence of L-phenylalanine in the elution buffer. Addition of dithiothreitol (0.1 mM) also did not affect the elu-

tion pattern. It was, therefore, presumed that native rat liver phenylalanine 4-monooxygenase was a tetramer.

Kaufman and Fisher [19,20] reported the phenylalanine 4-monooxygenase was composed of equal amounts of two different subunits, one with a molecular weight of 49 000 and the other with a molecular weight of 50 000. However, our vigorous attempts to separate the subunits on polyacrylamide gel electrophoresis in a variety of systems, including SDS-gel electrophoresis on 7 or 10% polyacrylamide gel by the method of Weber and Osborn [26], SDS-gel electrophoresis by the method of Neville [27], SDS-gradient gel electrophoresis on 7.5—10% or 4—30% polyacrylamide gel or gel electrophoresis on 7% polyacrylamide gel in the presence of 7 M urea at pH 8.4, were all unsuccessful, indicating only a single polypeptide in our preparation of phenylalanine 4-monooxygenase.

Kaufman and his coworkers [15] demonstrated that 95% pure phenylalanine 4-monooxygenase from rat liver contained 0.5—1 mol of iron per 50 000 molecular weight subunit and the metal might be involved in the enzyme reaction. Our pure preparation of the enzyme contained 0.6 mol of iron per 51 000 molecular weight subunit.

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