Purification and Some Physical Properties of Phenylalanine Hydroxylase from Rat Liver

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SUMMARY
Phenylalanine hydroxylase has been obtained from rat liver in a form that is 85 to 95% pure. The enzyme exists as two electrophoretically distinguishable isozymes, each of which is capable of existing as a monomer (51,000 to 55,000 molecular weight), dimer (110,000), and tetramer (210,000). The dimers of each isozyme will dissociate to the monomeric forms by dilution of the enzyme or by increasing the temperature from 0 to 30°C.

Phenylalanine hydroxylase from rat liver is part of a complex system that consists of several enzymes and coenzymes (1). The hydroxylase catalyzes the conversion of phenylalanine to tyrosine according to Equation 1 (2).

\[
\text{Tetrahydropterin + phenylalanine + O}_2 \rightarrow \text{quinonoid dihydropterin + tyrosine + H}_2\text{O} \quad (1)
\]

The partial purification of the hydroxylase from rat liver extracts has been described (3). The present communication describes the further purification of the enzyme from this source. The best preparations of the enzyme obtained by this procedure are 85 to 90% pure. Physical studies indicate that the enzyme exists in multiple forms that differ in their molecular weights and charge.

EXPERIMENTAL PROCEDURES
Materials
Glucose dehydrogenase (4) and dihydropteridine reductase were prepared as previously described. 6,7-Dimethyltetrahydropterin was purchased from Aldrich Chemical Company. Catalase and TPNH were products of Boehringer-Mannheim Corporation. Sephadex G-25 and G-200 were obtained from Pharmacia Fine Chemicals.

Methods
Standard Assay for Phenylalanine Hydroxylase—The complete reaction mixture contains the following components (in micro-

1 The meaning of “pure” can be ambiguous in a system with many different forms of an enzyme. We are using the word “pure” to mean freedom from protein that has no hydroxylase activity.

moles) in a final volume of 1.0 ml: potassium phosphate, pH 6.8, 100; L-phenylalanine, 2.0; TPN\(^+\), 0.25; glucose, 250; 6,7-dimethyltetrahydropterin, 0.1; glucose dehydrogenase, dihydropteridine reductase and catalase (7800 units), in excess, and tyrosine to be assayed. The last two additions were hydroxylase and tetrahydropterin, in that order. Glucose 6-phosphate and glucose 6-phosphate dehydrogenase can be used in place of glucose and glucose dehydrogenase. After a 30-min incubation, the reaction was stopped by addition of 2.0 ml of 12% trichloracetic acid, and tyrosine was determined by the nitrosonaphthol procedure calorimetrically (5) or fluorometrically (6).

Polyacrylamide gel electrophoresis was carried out according to Ornstein (7) and Davis (8), except that 0.0005% riboflavin was used in place of persulfate as the catalyst for the separating gel. Persulfate gels, when added to the reaction mixture, produced 90% inhibition of enzyme activity. Riboflavin gels caused no inhibition. To achieve the necessary temperature control, a constant temperature bath was used to circulate water through a jacketed electrophoresis chamber. Enzymatic activity was localized by the use of a gel fractionator similar to the one described by Maizel (9). Each gel slice was homogenized in the reaction mixture, minus the tetrahydropterin. After a 15-min incubation at 25°C, the reaction was initiated by the addition of the pterin. Gels of differing percentage acrylamide were formed by varying the proportion of Reagent C. To localize the protein bands, the gels were fixed with 12.5% trichloracetic acid for 1 hour, stained overnight with a 0.05% Coomassie brilliant blue in 12.5% trichloracetic acid (10), and destained with 12.5% trichloracetic acid. The gels were photographed, and the negatives were scanned with a Joyce-Loebel densitometer to estimate the relative amounts of protein in each band. The values obtained agreed well with direct ultraviolet scans of the unstained gel but showed better resolution.

Electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate was carried out according to the method of Shapiro, Vinuela, and Maizel (11) with the following exceptions. The concentration of the sodium phosphate bath buffer was reduced from 0.1 M to 0.025 M, thereby decreasing the running time from 8 to 2 hours for a 9-cm gel when run at 4 ma per tube. The gels were fixed in 20% trichloracetic acid for 1 hour followed by overnight staining with 0.1% Coomassie brilliant blue in 12.5% trichloracetic acid (10), and destained with 12.5% trichloracetic acid. The gels were photographed, and the negatives were scanned with a Joyce-Loebel densitometer to estimate the relative amounts of protein in each band. The values obtained agreed well with direct ultraviolet scans of the unstained gel but showed better resolution.
RESULTS

Purification of Phenylalanine Hydroxylase from Rat Liver

All of the steps in the purification procedure were carried out at 2 to 4°C unless specified otherwise. Mechanical stirring was used during all additions. Ammonium sulfate precipitates were collected by centrifugation for 20 min at 18,000 × g and ethanol precipitates were generally obtained by centrifugation for 15 min at 4000 × g. Glass-distilled water was used throughout. The fractionation procedures may be interrupted at any point, the fraction being stored at −15°C, or preferably, at −79°C until ready for the subsequent steps. Rapid freezing with a Dry Ice-acetone bath minimises loss in activity due to freezing.

Step 1: Extraction—Rats, male Osborne-Mendel strain, were killed by a blow on the head, and the livers were quickly removed and placed in cold water. After being blotted to remove the excess water, the livers were weighed, cut up with scissors into small pieces, and blended in a Waring Blender for 30 sec with 1.5 volumes of cold 0.01 M acetic acid. The Blendor was run at one-half to one-third full speed, controlled with a Variac rheostat. Another 1.5 volumes of 0.01 M Tris buffer, pH 7.0, was prepared. The second ammonium sulfate fraction from the previous step was added to the homogenate and the blending was continued at top speeds to severe losses in the activity. The homogenate was centrifuged at 18,000 × g for 45 min, and the sediment was discarded.

Step 2: Ethanol Fractionation—The clear extract was fractionated with ethanol cooled to −30 to −40°C. Two fractions were collected, one between 0 and 10% (temperature, −2°C) and the other between 10 and 21% (temperature, −4°C) ethanol by volume. The bulk of the activity was found in the second fraction. The precipitate from this fraction was dissolved in about one-fourth the original extract volume in 0.033 M potassium phosphate buffer, pH 7.4.

Step 3: First Ammonium Sulfate Fractionation—To each 100 ml of the alcohol fraction, 20.3 g of solid ammonium sulfate were added. The mixture was centrifuged and the precipitate was discarded. Then 5.60 g of ammonium sulfate were added for each 100 ml of original solution. After centrifugation, the precipitate was dissolved in 0.033 M Tris buffer, pH 6.8, equivalent to about one-fourth the volume of the starting ethanol fraction.

Step 4: Adsorption and Elution from Calcium Phosphate Gel—To each 100 ml of the undialyzed ammonium sulfate fraction, 10 ml of cold 0.1 M L-phenylalanine were added, followed by 100 ml of cold water. Then 73 ml of calcium phosphate gel (0.35 volume) containing about 20 mg of solid per milliliter were added over a period of 15 min, and the stirring was continued for another 10 min. The gel was collected by centrifugation and eluted successively with 210 ml of 0.02 M and 160 ml of 0.1 M potassium phosphate buffers, pH 6.8, the latter eluate containing most of the activity.

Step 5: Second Ammonium Sulfate Fractionation—The 0.1 M gel eluate was concentrated and freed of phosphate by reprecipitation with ammonium sulfate. To each 100 ml of eluate, 18.2 g of ammonium sulfate were added. The precipitate was discarded. Another 7.35 g of ammonium sulfate were added for each 100 ml of starting solution. The precipitate obtained after centrifugation was dissolved in one-fourth the volume of 0.01 M Tris buffer, pH 7.0.

A column (1.5 × 23 cm) of Sephadex G-25 suspended in 0.005 M Tris buffer, pH 7.0, was prepared. The second ammonium sulfate fraction from the previous step was added to the column which was then washed with 0.005 M Tris buffer, pH 7.0. Five milliliter fractions were collected. Over 95% of the enzyme activity is separated from the salt by this procedure.

Step 6: Column Chromatography on DEAE-cellulose—The fractions from the Sephadex G-25 step containing a total of

![Fig. 1. A (left), electrophoresis of phenylalanine hydroxylase on polyacrylamide gel (7%). Purified phenylalanine hydroxylase (0.025 mg) was subjected to electrophoresis at 0-2°C for 2 hours on a 9-cm long gel. The marker dye was at the bottom end of the gel. B, distribution of enzyme activity on a second gel run in parallel to the one depicted in A. Slices of the gel (1.3 mm) were assayed for enzyme activity in the standard assay system. No other portion of the gel had enzyme activity. The optical density scale is actually an inverse scale, because the negative of the photograph in A was scanned with a visible light scanner.](image)
about 1.5 g of protein were added to the top of a column (1.0 × 40 cm) of DEAE-cellulose that had previously been equilibrated with 0.005 M Tris buffer, pH 7.0. A gradient elution device similar to that described by Peterson and Sober (12) was used for elution. Two bottles of equal diameter, the first containing 300 ml of 0.01 M Tris buffer, pH 7.0, the second containing 300 ml of 0.01 M Tris buffer, pH 7.0, 0.22 M with respect to KCI, were connected in series. The collecting tubes contained 0.3 ml of 3 M KCI. Fractions of 6 ml were collected so that the final KCl concentration in each tube (not counting the amount contributed by the eluant) was 0.15 M. The enzyme was usually eluted with 300 to 540 ml of eluate. The fractions in which the enzyme had been purified at least a-fold were combined and concentrated by ultrafiltration with the device manufactured by Amicon Corporation, Cambridge, Massachusetts. The yield of 3-fold purified enzyme from the DEAE-cellulose step is about 30%. An equal amount of enzyme units, purified about 1.5-fold, can also be recovered from the DEAE-cellulose step.

**Step 7: Gel Filtration on Sephadex G-200**—Between 200 and 400 mg of protein from the previous step were added to the top of a column (2.5 × 60 cm) of Sephadex G-200 that had previously been equilibrated with 0.01 M Tris buffer, pH 7.0, 0.1 M with respect to KCl. The same buffer was used to elute the protein from the Sephadex. Most of the hydroxylase activity was usually eluted between 150 and 200 ml of eluate. The specific activity in the peak fractions is about 3 times higher than that of the starting material. About 40% of the enzyme is purified 2-fold and another 25% is purified 1.5-fold by the Sephadex step. The desired fractions were combined and concentrated by ultrafiltration.

Early attempts to purify the hydroxylase on columns of DEAE-cellulose and Sephadex G-200 led to very low recovery of activity. The avoidance of phosphate buffers as eluants and the addition of KCl in the collection tubes, especially at low protein concentration, were the keys to the successful use of DEAE-cellulose. As for the Sephadex step, it was found that the ratio of protein to Sephadex was critical; at least 0.7 mg of protein per ml of Sephadex bed volume was required for high recovery of active enzyme. It was also found that the recovery was increased by pretreatment of the Sephadex column with crude

### Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Units</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
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<tr>
<td>Extract</td>
<td>4,664</td>
<td>496</td>
<td>143,000</td>
<td>0.0034</td>
<td>100</td>
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<td>Ethanol fractionation</td>
<td>1,166</td>
<td>558</td>
<td>51,000</td>
<td>0.011</td>
<td>112</td>
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<tr>
<td>First ammonium sulfate fractionation</td>
<td>290</td>
<td>300</td>
<td>13,800</td>
<td>0.030</td>
<td>101</td>
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<tr>
<td>Calcium phosphate gel eluate</td>
<td>433</td>
<td>254</td>
<td>3,050</td>
<td>0.083</td>
<td>51</td>
</tr>
<tr>
<td>Second ammonium sulfate fractionation and Sephadex G-200</td>
<td>24</td>
<td>150</td>
<td>1,020</td>
<td>0.096</td>
<td>31</td>
</tr>
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<td>50</td>
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<td>0.29</td>
<td>11</td>
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<tr>
<td>Sephadex G-200 peak tubes</td>
<td>51</td>
<td>23</td>
<td>39</td>
<td>0.59</td>
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### Table II

<table>
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<th>$d_{w}$</th>
<th>Phenylalanine hydroxylase $d_{w}$</th>
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<tr>
<td></td>
<td>Slower sedimenting form</td>
<td>Faster sedimenting form</td>
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<tr>
<td>Peroxidase</td>
<td>3.65</td>
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<td>Ovalbumin</td>
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<td>Bovine serum albumin</td>
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<td>5.60</td>
</tr>
<tr>
<td>Average</td>
<td>5.90</td>
<td>8.15</td>
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</tbody>
</table>

**Fig. 2.** Sucrose gradient centrifugation pattern of phenylalanine hydroxylase. Purified phenylalanine hydroxylase (3.2 mg, in 250 μl) was layered on a 5 to 20% sucrose gradient and centrifuged at 2°C at 50,000 rpm for 15 hours in an SW-65 rotor. Of the enzyme activity which was applied to the gradient, 80% was recovered in the fractions---, optical density of blank gradient.
fractions of the hydroxylase, e.g. side-fractions from the previous step. When both of these precautions are observed, the recovery of hydroxylase activity from the Sephadex G-200 step is 85 to 95%.

The results from a typical preparation are summarized in Table I. The enzyme is purified about 400-fold by the procedure with a yield of about 5%. The extent of purification shown in the table is less than this because there is an aggregate loss of about 40 to 50% of the activity during the time intervals between each of the steps. The yield can be more than doubled by fractionating the side-fractions from the last two steps on a second, smaller Sephadex G-200 column.

The most active enzyme obtained by this procedure has a specific activity of 0.5 to 0.6 (micromoles of tyrosine formed per min per mg protein) when assayed under standard conditions. At this stage the enzyme can be stored at -80°C for 1 year with a 30% loss in activity.

**Purity of Enzyme**

Analysis of the enzyme by polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate and by sucrose gradient centrifugation reveals that the present procedure yields 85 to 90% pure hydroxylase. In the standard polyacrylamide gel electrophoresis system the enzyme activity is associated with the two major bands which represent 85% of the protein (Fig. 1, A and B). Sucrose gradient centrifugation resolves the hydroxylase into two peaks of activity which comprise 90% of the protein (Fig. 2). Electrophoresis of the enzyme on a sodium dodecyl sulfate polyacrylamide gel gave a major band which was 85% of the total protein. A sodium dodecyl sulfate gel of the sucrose-gradient purified enzyme showed the same major band which was 95% of the protein.

**Molecular Weights of Different Forms of Enzyme**

Sucrose gradient centrifugation indicated that there are two active forms of the hydroxylase which differ in their sedimentation velocities (Fig. 2). The sedimentation constants for these two forms were calculated by the method of Martin and Ames (13). As summarized in Table II, the value for the slower sedimenting form was 5.90 S and for the faster form was 8.15 S.

Chromatography of the enzyme on Sephadex G-200 also revealed two active forms of the enzyme with different sizes. Fig. 3A shows the elution pattern obtained when the fractions that

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**Fig. 3. A.** Sephadex G-200 column chromatography of phenylalanine hydroxylase. Protein (203 mg) eluted prior to the main peak on DEAE-cellulose was applied to a column (60 × 2.5 cm) of Sephadex G-200 (294 ml) and eluted with 0.1 M KCl buffered with 0.01 M Tris-HCl, pH 7.0. The excluded peak determined by blue dextran was at Fraction 41 and the volume of each fraction was 2.2 ml. B, 125 mg of protein eluted after the main DEAE-cellulose peak were chromatographed on the column described in A.
RELATIVE MIGRATION

Fig. 5. A (upper), electrophoresis of phenylalanine hydroxylase on a sodium dodecyl sulfate-containing polyacrylamide gel. A solution (0.025 mg) of purified phenylalanine hydroxylase, in 0.05 ml, 0.01 M sodium phosphate buffer, pH 7.0, 0.1 ml of glycerol, 0.005 ml of bromphenol blue, and 0.005 ml of mercaptoethanol was applied to a 9-cm sodium dodecyl sulfate gel. Electrophoresis was carried out for 2.5 hours at 4 mA per gel. The dye migrated about 8 cm under these conditions. B (lower), determination of the molecular weight of the phenylalanine hydroxylase subunit by sodium dodecyl sulfate polyacrylamide gel electrophoresis. BSA is bovine serum albumin, and OVA is ovalbumin. The catalase and glyceraldehyde phosphate dehydrogenase are subunits of the native enzymes prepared as described under "Methods." Relative migration = (distance of protein migration)/(length of gel before staining) X (length before staining)/(migration of dye).

Fig. 6. The effect of differing acrylamide concentrations on the migration of phenylalanine hydroxylase isozymes during electrophoresis in polyacrylamide gels. When electrophoresis was carried out at 0°C (0-2°C) two closely migrating bands, the same as depicted in Fig. 4A and Fig. 8 (Bands I and II) were observed. The slope of the log relative mobility against percent acrylamide was 10.0 for each of these bands. When electrophoresis was carried out at 30°C, two faster migrating bands were observed. The slope for the fastest of these bands (Band IV, Fig. 8) was 7.1 in the above determination. At 0°C: - - - - - , Band I; O--O, Band II. At 30°C: •—•, Band I; ▲—▲, Band IV.

Fig. 7. Determination of molecular weight of the associated and dissociated forms of phenylalanine hydroxylase by varying gel concentration in polyacrylamide electrophoresis. The slope for each form was determined as described in Fig. 6. For the higher molecular weight form the mean of five values (10, 10, 10, 10, 9.7) was 9.9. For the lower molecular weight form the mean was 6.8 (7.1, 6.8, 6.5). BSA1, BSA2, and BSA3 are bovine serum albumin monomer, dimer, and trimer, respectively. OVA1 and OVA2 are ovalbumin monomer and dimer.

Fig. 8. Effect of temperature and protein concentration on the phenylalanine hydroxylase migration during polyacrylamide gel electrophoresis. The direction of migration was from the top to the bottom of the gel and the lowest band is the marker dye.
Phenylalanine Hydroxylase

Vol. 245, No. 18

The Sephadex procedure. Therefore, phenylalanine hydroxylase and 7) which corresponds favorably with the value obtained by calculations of molecular weight based on the sedimentation constants as well as the Stokes radii are more accurate.

Results similar to those shown in Fig. 3, A and B, have been obtained with a different preparation of the hydroxylase. For both preparations, elution volumes for the two major forms of the enzyme have been determined and these values have been used to calculate their Stokes radii by the method of Ackers (14). Values of 5.9 and 6.7 mm were obtained for the more excluded peak and values of 4.6 and 4.4 mm for the more included peak. The average of these values for the Stokes radii were used to calculate a diffusion constant. The diffusion constant and the previously mentioned sedimentation constants (determined by the sucrose gradient centrifugation technique) have been used to calculate molecular weights of 110,000 and 210,000 for the two major forms of the enzyme from the Svedberg (assuming a partial specific volume of 0.72).

Electrophoresis on Sodium Dodecyl Sulfate
Polyacrylamide Gels

It has been shown that reliable molecular weights of polypeptide chains of proteins can be determined by disc gel electrophoresis carried out in the presence of sodium dodecyl sulfate (13). When the hydroxylase was subjected to electrophoresis under these conditions and the gel stained with Coomassie blue, a single major band was evident (Fig. 6A). The electrophoretic mobility of the hydroxylase and those of marker proteins with polypeptide chains of known molecular weights (Fig. 5B) were used to calculate a molecular weight of 51,000 (with a standard deviation of ±1% based on three determinations) for the polypeptide chains of the hydroxylase.

Evidence for Isozymes and for Their Polymeric Form

It was demonstrated above that enzyme activity is associated with both of the major protein bands obtained by polyacrylamide gel electrophoresis at 0° (Fig. 1). The molecular weights of these enzyme forms may be determined by a study of their migration on gels of varying acrylamide concentration (15). The slope of the logarithm of the relative migration of the protein against the gel concentration is proportional to the molecular weight of the protein. When electrophoresis was performed at 0° on gels of differing percent acrylamide, the slopes for the two protein bands were identical. The slopes for these bands correspond to a molecular weight of 110,000 (±3%, N = 5, Figs. 6 and 7) which corresponds favorably with the value obtained by the Sephadex procedure. Therefore, phenylalanine hydroxylase exists as two isozymes which differ in charge but not molecular weight.

When polyacrylamide electrophoresis is performed at 30° (which decreases the running pH from 10.2 to about 9.5), the pattern observed depends upon the amount of protein applied to the gel. Application of 40 μg of enzyme to gels at 30° results in the same two major bands observed at 0° (relative mobility, 0.4). However, two new, faster migrating bands appear at lower protein concentrations (Fig. 8). When the relative migration was studied at different gel concentrations, the results indicated that the molecular weight of the new, faster migrating form is 55,000 (±7%, N = 3). It is probable that the other fast migrating band is of the same molecular weight.

Discussion

Phenylalanine hydroxylase prepared by the present procedure is 85 to 90% pure. Evidence has been presented that the hydroxylase exists in multiple forms. Indeed, the presence of various forms has complicated attempts to determine purity. Based on sedimentation behavior in sucrose gradients and elution patterns from Sephadex G-200, it has been estimated that the two major forms have molecular weights of 110,000 and 210,000. Polyacrylamide electrophoresis on gels of differing acrylamide concentration reveals that the enzyme exists as two isozymes each having a molecular weight of 110,000. Enzyme dilution and increased temperature are capable of dissociating these isozymes into two nonidentical subunits of 55,000 each. This value is close to the molecular weight of 51,000 obtained by the sodium dodecyl sulfate technique. We may conclude that phenylalanine hydroxylase consists of two isozymes, each of which is capable of existing as a monomer (51,000 to 55,000), a dimer (110,000), and a tetramer (210,000). It remains to be established by a nontransport technique, such as light scattering, which of these forms exist under assay conditions.

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References


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