Genetics of the Mammalian Phenylalanine Hydroxylase System*

STUDIES OF HUMAN LIVER PHENYLALANINE HYDROXYLASE SUBUNIT STRUCTURE AND OF MUTATIONS IN PHENYLKETONURIA

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Phenylalanine hydroxylase was purified from crude extracts of human livers which show enzyme activity by using two different methods: (a) affinity chromatography and (b) immunoprecipitation with an antiserum against highly purified monkey liver phenylalanine hydroxylase. Purified human liver phenylalanine hydroxylase has an estimated mol.wt. of 275 000, and subunit mol.wts. of approx. 50 000 and 49 000. These two molecular-weight forms are designated H and L subunits. On two-dimensional polyacrylamide gel under dissociating conditions, enzyme purified by the two methods revealed at least six subunit species, which were resolved into two size classes. Two of these species have a molecular weight corresponding to that of the H subunit, whereas the other four have a molecular weight corresponding to that of the L subunit. This evidence indicates that active phenylalanine hydroxylase purified from human liver is composed of a mixture of subunits which are different in charge and size. None of the subunit species could be detected in crude extracts of livers from two patients with classical phenylketonuria by either the affinity or the immunoprecipitation method. However, they were present in liver from a patient with malignant hyperphenylalaninaemia with normal activity of dihydropteridine reductase.

Most of the current knowledge on the genetic regulation and molecular structure of mammalian phenylalanine hydroxylase has come from studies on the enzyme in rat liver, human liver and cultured rat hepatoma cells. The enzyme has been isolated and extensively characterized (see reviews by Kaufman & Fisher, 1974; Barranger, 1976; Woolf, 1976; Kaufman, 1976; Cotton, 1977). Studies on purified protein have revealed considerable molecular heterogeneity in mammalian phenylalanine hydroxylase. Such heterogeneity is seen both in the undissociated, active molecules (referred to here as intact molecules), as well as in subunit polypeptides. On SDS/polyacrylamide-gel electrophoresis, two polypeptides of different molecular weights have been demonstrated in enzymes from rat liver (Kaufman & Fisher, 1974) and foetal human and monkey livers (Cotton & Danks, 1976). It is not clear if these two molecular-weight classes represent products of two separate genes, or if one may arise from the other by a post-translational modification such as proteolysis. In addition, charge heterogeneity in phenylalanine hydroxylase has been reported. Gillam *et al.* (1974)

have detected four polypeptide species differing in charge in a preparation of purified rat liver enzyme. Three active forms of intact human and rat liver enzyme from adult animals have been separated on a calcium phosphate column (Barranger, 1976). Other workers have also found three forms of rat liver phenylalanine hydroxylase which are distinguishable immunologically (Tourian *et al.*, 1975), by isoelectric focusing (Tourian, 1976) and by hydroxyapatite-chromatographic separation (Miller & Shiman, 1976; Miller *et al.*, 1976) properties. These three forms of the enzyme are also present in cultured rat hepatoma cells which have been treated with cortisol (Miller & Shiman, 1976; Miller *et al.*, 1976). In rat, the isoenzymes are of a uniform mol.wt. 200 000 (Barranger, 1976). However, it is not possible to infer the number of subunits and their charge and size difference from these studies. This lack of a unified model for the subunit structure of phenylalanine hydroxylase has severely hindered rational investigations of the molecular and genetic mechanisms underlying the phenotypic expression of this enzyme, and of phenylketonuria.

In the present study, we have purified normal human liver phenylalanine hydroxylase using two different methods: (a) affinity chromatography and

Abbreviation used: SDS, sodium dodecyl sulphate.

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(b) immunoprecipitation with a phenylalanine hydroxylase-specific antiserum. We have examined the subunit structure of the purified normal protein with a two-dimensional electrophoretic system, which separates protein according to size and charge independently (O'Farrell, 1975). Additional evidence came from our study of livers from individuals with classical phenylketonuria and malignant hyperphenylalaninaemia. The use of affinity chromatography, immunoprecipitation and two-dimensional electrophoretic techniques for the analysis of subunit structure and molecular defects of liver-specific enzymes is illustrated.

Materials and Methods

Human livers

Livers were obtained at autopsy from children dying of various causes at the Royal Children's Hospital, Melbourne (see Table 1).

Phenylalanine hydroxylase assay

This involved measuring the conversion of [^{14}C]phenylalanine into [^{14}C]tyrosine in a reaction mixture containing dithiothreitol (2 mM; Calbiochem, La Jolla, CA, U.S.A.), lysophosphatidylcholine (1 mM; Sigma Chemical Co., St. Louis, MO, U.S.A.) and tetrahydrobiopterin (0.25 mM; Roche, Basle, Switzerland) (Bartholomé *et al.*, 1975). Reaction was carried out for 60 min at 25°C and terminated with 50 μl of 30% (w/v) trichloroacetic acid. After centrifugation at 2720 g for 10 min, 20 μl of the supernatant was applied on to a cellulose-coated plastic-sheet t.l.c. plate. Development (Choo & Cotton, 1977) of the t.l.c. plate was carried out twice, with drying of plate and a change of fresh solvents in between. The subsequent steps were as described before (Choo & Cotton, 1977).

Amino acid analysis

Liver extracts for phenylalanine and tyrosine analysis were prepared by homogenization in a Kontes ground-glass hand homogenizer, 1 g of tissue in 3 ml of 0.1 M-Tris/HCl (pH 7.4). The homogenate was centrifuged at 27000 g for 60 min at 4°C. A portion of the supernatant was deproteinized with sulphosalicylic acid (50 mg of solid acid/ml of supernatant). After centrifugation at 2000 g for 10 min, the resulting supernatant was analysed on a Technicon TSM amino acid analyser. Norleucine was used as an internal standard.

Affinity chromatography

Use of affinity adsorbent for the purification of phenylalanine hydroxylase from crude extracts of

human livers was as reported by Cotton & Danks (1976). Analyses of the concentrated phenylalanine-eluted fractions used Gradipore electrophoresis (Cotton & Jennings, 1978) and SDS/polyacrylamide-gel electrophoresis (see below).

SDS/polyacrylamide-gel electrophoresis

Gels for SDS/polyacrylamide-gel electrophoresis were prepared as described under 'Two-dimensional polyacrylamide-gel electrophoresis' below. Samples were prepared as follows: to 10 μl of sample (freeze-dried and reconstituted in 0.1 M-Tris/HCl, pH 7.4, if necessary) were added 1.5 μl of 20% (w/v) SDS and 0.5 μl of 2-mercaptoethanol. This was placed in a boiling-water bath for 1.5 min, followed by the addition of 12 μl of a solution of 2% (w/v) agarose in a 0.0625 M-Tris/HCl buffer (pH 6.8) containing 2.3% SDS, 5% (v/v) mercaptoethanol and 10% (v/v) glycerol (O'Farrell, 1975). The mixture was boiled for another 1.5 min and then allowed to solidify in glass tubing of 2 mm inside diameter. The solidified sample was then extruded from the tubing and embedded with 1% agarose on to the SDS/polyacrylamide gel as described by O'Farrell (1975).

Analysis of a protein band cut out from a Gradipore gel by SDS/polyacrylamide-gel electrophoresis was as follows. After electrophoretic separation in Gradipore gel, a portion of the gel was cut off and stained (Cotton & Jennings, 1978), and the remaining portion was kept between two glass plates at 4°C. Staining takes less than 1 h. On brief destaining, the phenylalanine hydroxylase position on the untreated portion was identified and cut out. This was then loaded on to SDS/polyacrylamide gel as described above.

Preparation of pure monkey liver phenylalanine hydroxylase for immunization, and immunization schedule

Monkey liver phenylalanine hydroxylase was purified by using an affinity column (Cotton & Grattan, 1975) and a brushite column with a phosphate gradient (Choo & Cotton, 1979). The purified enzyme preparation contained less than 0.3% of any contaminating protein (Choo & Cotton, 1979) (Fig. 2, gel i). Antibody against highly purified monkey liver phenylalanine hydroxylase was raised in rabbit as described (Choo & Cotton, 1979).

Methods of detecting cross-reacting materials

(i) *Immunodiffusion experiments.* Ouchterlony double immunodiffusion (Roiff, 1977) was performed on 0.7% agar (Oxoid, London E.C.4, U.K.) dissolved in 0.28 M-NaCl solution containing 0.5% phenol and 0.003% Methyl Orange. The volume of antiserum

or cell and tissue extract added per well was always 20 μ l, and where necessary homogenization buffer (0.1M-Tris/HCl, pH7.4) was used to make up to this volume. Ouchterlony plates were incubated under humidified conditions at 4°C for 48–72h. Plates were washed with 0.9% NaCl solution for 36–48h at 37°C, followed by water for 4h at 37°C, before they were dried and stained. Staining was for 10min with Nobel Blue stain [0.25% each of Azocarmine, Coomassie Brilliant Blue and Amido Schwarz in acetic acid/ethanol/water (2:9:9, by vol.)]. This latter solution without the stains was used to destain the plates.

Each extract preparation was systematically tested in Ouchterlony plates at various dilutions of the antigen up to 1:32 and at similar dilutions of the antiserum; 0.1M-Tris/HCl buffer (pH7.4) was used for all dilutions. Control serum from a rabbit before immunization was used to test against all extract preparations, and no cross-reaction was observed with any of the preparations.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as standard.

(ii) *Absorption of antiserum before immunodiffusion.* Antiserum (10 μ l) was incubated with 20 μ l of an enzyme preparation of protein concentration as specified in the text for 10h at 4°C. At the end of the incubation, 20 μ l of the mixture was loaded on to the centre well of an Ouchterlony plate to test against pure monkey liver phenylalanine hydroxylase or active liver extracts. Incubation of antiserum alone for 10h at 4°C did not significantly alter the potency of the antiserum.

(iii) *Immunoprecipitation of phenylalanine hydroxylase.* Immunoprecipitation of phenylalanine hydroxylase from crude extracts (or affinity-purified preparation where indicated) is a modification of the double-antibody procedure described by Milman *et al.* (1976). All steps are performed at 0–4°C. Liver extracts were prepared in 0.1M-Tris/HCl buffer (pH7.4) as previously described (Cotton & Grattan, 1975), except that the final protein concentrations of supernatant were adjusted to 30–35mg/ml. All samples, including liver extracts, antiserum and non-immune serum, were centrifuged for 15min, 27000g, 4°C, just before use to remove any protein precipitate resulting from storage. Goat antibody to rabbit γ -globulin (25 units/ml) was from Calbiochem, and Triton X-100 and sodium deoxycholate were from BDH, Poole, Dorset, U.K.

Immunoprecipitation was performed as follows: to 100 μ l of extract were added 10 μ l of 5mM-MgCl₂, and 15 μ l of a 1mg/ml solution of deoxyribonuclease. This was kept in ice for 5min before 25 μ l of 10% Triton X-100/10% sodium deoxycholate (McClure *et al.*, 1976) was added. This was followed by 100 μ l of antiserum (or non-immune control serum). The

mixture was left for 10–12h at 4°C, after which 50 μ l of goat anti-(rabbit γ -globulin) serum was added. The mixture was left for an additional 16h. The resulting immunoprecipitate was collected by centrifugation for 15min at 3000g, washed once with 0.5ml of cold 0.5% Triton X-100/0.5% sodium deoxycholate in 0.1M-Tris/HCl (pH7.4), and centrifuged a second time for 15min at 3000g. The immunoprecipitate was then dissolved in 15 μ l of a solution of 0.5mM-MgCl₂, 3% SDS and 5% 2-mercaptoethanol in 0.01M-Tris/HCl (pH7.4). This was boiled for 2min and treated as described below for two-dimensional polyacrylamide-gel electrophoresis before isoelectric focusing and SDS/polyacrylamide-gel electrophoresis.

Two-dimensional polyacrylamide-gel electrophoresis

This was performed as described by O'Farrell (1975) with the following modifications. Nonidet P-40, urea (AristaR grade) and ammonium persulphate were purchased from BDH. *NNN'*-Tetramethylethylenediamine and bovine pancreatic deoxyribonuclease were from Sigma. Acrylamide, *NN'*-methylenebisacrylamide and 2-mercaptoethanol were from Merck, Darmstadt, Germany.

First-dimension isoelectric-focusing gels (100mm in length) were made in glass tubing 130mm long and 2mm inside diameter. The total concentrations of Ampholine in the gel was 2%, with 1.6% at pH5–8 and 0.4% at pH3.5–10. Concentrations of *NNN'*-tetramethylethylenediamine and ammonium persulphate were increased by 25%, as used by O'Farrell (1975).

Samples for isoelectric focusing were prepared as follows. To 10 μ l of a sample were added 1 μ l of 5mM-MgCl₂, which was necessary for activation of deoxyribonuclease, and 1 μ l of a 1mg/ml solution of deoxyribonuclease. After standing in ice for 5–10min 1 μ l of 20% SDS was added to the mixture. This was heated at 100°C for 1.5min. When cool, 7–8mg of solid urea was added to give a concentration of 9.5M-urea. To this final preparation was added 20 μ l of sample-dilution buffer, which contains 9.5M-urea, 2% Ampholines (comprising 1.6% pH5–8, 0.4% pH3.5–10), 5% mercaptoethanol, 8% (v/v) Nonidet P-40 and 1.5mM-dithiothreitol (Calbiochem). Once the urea was completely dissolved, the sample was run without delay. The treatment of sample was therefore slightly different from those described by Ames & Nikaido (1976) and Anderson & Anderson (1977).

After isoelectric focusing, the gel was run in the second dimension immediately. The second-dimension gel was 0.8mm thick, and consisted of a 9cm resolving gel of 9% polyacrylamide and a 4.8cm stacking gel of 4.5% polyacrylamide. Electrophoresis was at 25mA for 25min in a high-SDS (2%) running

buffer (O'Farrell, 1975), followed by SDS (0.1%) running buffer (O'Farrell, 1975) for 5–6 h. The gel was fixed in 3.5% (v/v) HClO_4 for over 5 h at 25°C, stained with 0.04% Coomassie Brilliant Blue G250 in 3.5% HClO_4 for 1 h at 37°C, then destained and stored in 7% acetic acid.

Results

Characterization of liver samples

Table 1 outlines various properties of the human livers used in this study. Phenylalanine/tyrosine analysis of liver extracts, as well as phenylalanine hydroxylase activity assay in sample M, have identified samples Hu and M as being from patients with classical phenylketonuria. It should be noted that sample M was from a prompt autopsy. The presence of phenylalanine hydroxylase activity in sample N was in accordance with the diagnosis of malignant hyperphenylalaninaemia (see Case 2 of Danks *et al.*, 1978). The difference in specific phenylalanine hydroxylase activity between samples A-1, A-2 and F may be due to slightly different conditions of handling of the livers. Delayed autopsy after death has probably resulted in a complete loss of activity in sample C. The lability of phenylalanine hydroxylase after death has been documented elsewhere (Mitoma *et al.*, 1957).

Gradipore analysis of affinity-purified preparations

The characteristics of affinity-purified phenylalanine hydroxylase from different liver samples

were examined on Gradipore gels. In Fig. 1, the solid arrow shows that intact phenylalanine hydroxylase from livers showing enzyme activity (A-1, N and A-2; gels c, e and f, respectively) has an estimated mol.wt. of 275000 (i.e. approximately similar to that of xanthine oxidase). Passage of inactive extracts (M, C, Hu and MM; Fig. 1, gels b, d, g and h, respectively) through the affinity adsorbent yielded no protein entity at 275000 mol.wt., although a species corresponding to 200000 mol.wt. was regularly seen (Fig. 1, broken arrow). This latter species is probably unrelated to phenylalanine hydroxylase, as shown by SDS/polyacrylamide-gel electrophoresis experiments (see below).

Proof that the protein at mol.wt. 275000 on a Gradipore gel (Fig. 1, solid arrow) is phenylalanine hydroxylase has come from the following observations: (i) a portion of the phenylalanine-eluted peak of activity from affinity adsorbent has been further purified through a brushite column equilibrated with potassium phosphate buffer and eluted with a phosphate gradient (Choo & Cotton, 1979); the active fraction from this latter preparation, when concentrated, gives rise to a major band (over 95% of the protein) at 275000 mol.wt. on Gradipore gel (K. H. Choo, unpublished work); (ii) protein from the 275000-mol.wt. band when cut from the gel can be separated into two subunit bands on SDS/polyacrylamide gel (see the results for SDS/polyacrylamide-gel electrophoresis below); (iii) this protein is missing from similar affinity-purified preparations of inactive normal extracts and phenylketonuric-liver extracts (Fig. 1); (iv) the major band

Table 1. *Characterization of liver samples*

Age of patient, interval between death and autopsy, duration of storage at –20 or –70°C, and underlying cause of death are listed for purpose of comparing the condition of the various liver samples. Sample M was from a patient whose sibling has classical phenylketonuria. Sample N was from a patient described to have malignant hyperphenylalaninaemia (Danks <i>et al.</i> , 1978). Sample Hu was from a classical phenylketonuric patient. Sample F was a needle-core biopsy. The phenylalanine and tyrosine values expressed in the ratio were obtained with an amino acid analyser. A unit of phenylalanine hydroxylase activity represented 1 nmol of [^{14}C]tyrosine formed/60 min per mg of extract protein. Abbreviation: n.d., not done.								
Liver sample	A-1	A-2	M	N	Hu	MM	C	F
Age of patient	1 week	1 week	1 week	3½ years	15 years	4½ years	4 years	7½ years
Time after death to autopsy (h)	0.2	0.5	0.5	0.2	10	> 8	25	0
Time of storage (years)	½	½	½	7	8	5	5	0
Cause of death								
	Anen- cephaly	Anen- cephaly	Multiple abnormal- ities	Malignant hyper- phenyl- alaninaemia	Pneumonia	Medullo- blastoma	Spina bifida hydro- cephaly	Intrahepatic biliary atresia
Liver extract ratio phenylalanine/ tyrosine	0.62	0.77	3.53	1.78	3.24	1.43	n.d.	n.d.
Enzyme activity (units)	31.06	9.73	<0.05	>2	<0.05	n.d.	<0.05	23.69

Fig. 1. Photograph (a) and the corresponding drawing (b) of Gradiopore analysis of affinity-purified preparations under non-dissociating conditions

The position of phenylalanine hydroxylase is indicated by the solid arrow. The broken arrow indicates mol.wt. 200000. Gel (a) xanthine oxidase (XO, mol.wt. 275000; Sigma) and catalase (CT, mol.wt. 232000; Calbiochem); gels (b)–(k) are samples M, A-1, C, N, A-2, Hu, MM, A-1, and CT and XO, respectively. The amounts of purified protein from these preparations were insufficient for use in protein determination and this was therefore not done.

on Gradiopore gel from an affinity-purified preparation of monkey liver phenylalanine hydroxylase has been cut out, shown to be active, and to be resolved into two subunit bands on SDS/polyacrylamide gel (Cotton & Grattan, 1975) (see also Fig. 2).

The affinity preparations of active human phenylalanine hydroxylase used in the present study carry a considerable amount (an estimated 40–50%) of contaminating proteins (Fig. 1, gels c, f and i). Further purification steps have not been performed for the following reasons: (i) scarcity of active liver samples; (ii) highly purified enzyme preparations were not necessary for the purpose of this study, as two-dimensional polyacrylamide-gel electrophoresis gives a high resolution of protein subunits, and only those at molecular weights corresponding to the H and L subunits are relevant to phenylalanine hydroxylase (see results for SDS/polyacrylamide-gel electrophoresis below); (iii) the subunit structure of

phenylalanine hydroxylase purified by this method has been compared and found to be the same as that of enzyme purified by a second method (immuno-precipitation, see below).

SDS/polyacrylamide-gel electrophoresis of affinity-purified preparations

Purified phenylalanine hydroxylase from active human liver extracts (Fig. 2, gels c, e and f) showed two different subunits with estimated mol.wts. of 50000 and 49000 when analysed on SDS/polyacrylamide gels. These are designated H and L subunits. For comparison, purified monkey liver enzyme (Fig. 2, gel i) and rat liver enzyme (Fig. 2, gel k) also showed two different subunits, as published [Cotton & Danks (1976) and Kaufman & Fisher (1974) respectively]. It is noteworthy that a similar affinity-purified preparation of cultured rat hepatoma phenylalanine hydroxylase showed only one prominent subunit species corresponding to the higher-molecular-weight band of rat liver enzyme (Choo & Cotton, 1979) (Fig. 2, gel l).

In normal human livers obtained at delayed autopsy, which are devoid of phenylalanine hydroxylase activity, only the L subunit was seen (Fig. 2, gels d and h). The position of the L-subunit polypeptide has not been identified on the Gradiopore gel. Kaufman & Fisher (1974) have produced phenylalanine hydroxylase species which are smaller than normal enzyme and which have retained their ability to recognize the pteridine cofactor, by proteolytic digestion of purified rat liver enzyme. This type of molecule may be responsible for the L subunit seen in affinity preparations of inactive normal livers, and it may be represented among some of the smaller species seen in the Gradiopore gel (Fig. 1).

The identity of the L-subunit and the H-subunit species as arising from phenylalanine hydroxylase has been established by three criteria. (i) When active (275000-mol.wt.) human protein was cut out of Gradiopore gel (Fig. 1, solid arrow) and run on SDS/polyacrylamide-gel electrophoresis, only H- and L-subunit species were observed (Fig. 2, gel j). A similar treatment of the 200000-mol.wt. protein (Fig. 1) did not yield any band corresponding to either H or L subunit, suggesting that this protein was not related to phenylalanine hydroxylase. (ii) Two subunit species were observed with highly purified monkey liver phenylalanine hydroxylase which contained less than 0.3% of any contaminating protein (Choo & Cotton, 1979) (Fig. 2, gel i). When the undissociated monkey liver phenylalanine hydroxylase band was cut out of Gradiopore gel and run on SDS/polyacrylamide gel it also yielded two subunits (results not shown). (iii) Both species were absent from materials purified by affinity chromatography

Fig. 2. Photograph (a) and the corresponding drawing (b) of SDS/polyacrylamide-gel-electrophoretic analysis of affinity-purified preparations

Gels (a) and (m), catalase (CT, mol.wt. 57000) and ovalbumin (OA, mol.wt. 45000; Sigma); gels (b)–(h), (k) and (l) are samples M, A-2, MM, N, A-1, Hu, C, rat liver phenylalanine hydroxylase and cultured rat hepatoma phenylalanine hydroxylase respectively; gel (i), monkey liver phenylalanine hydroxylase that has been purified through an affinity column and a brushite column with a phosphate gradient; gel (j), intact phenylalanine hydroxylase of an affinity-purified preparation from human sample A-2, which has been cut out of Gradipore gel. Human phenylalanine hydroxylase subunits (H and L) are indicated by arrows. Protein determinations were not performed on these samples for reasons given in the legend of Fig. 1.

from liver extracts of patients with phenylketonuria (Fig. 2, gels b, g). It is noteworthy that the ratio of intensities of the H band to the L band on SDS/polyacrylamide gel parallels the phenylalanine hydroxylase activity in liver extracts (Fig. 2).

Two-dimensional polyacrylamide-gel electrophoresis of affinity-purified preparations

On two-dimensional polyacrylamide gels, human liver phenylalanine hydroxylase (sample A-1) purified from affinity adsorbent displayed at least six spots (Fig. 3). Two of these spots (numbered H1 and H2 in Fig. 3) have an estimated mol.wt. of 50000, whereas the other spots (numbered L1–L4 in Fig. 3) have an estimated mol.wt. of 49000. Purified phenylalanine hydroxylase from liver sample A-2 exhibited the same pattern on two-dimensional polyacrylamide gels, except that the ratio of intensities of the H spots to the L spots was lower for sample A-2 (results not shown).

Immunological detection of phenylalanine hydroxylase-related cross-reacting material

The antiserum used in this study is highly specific for mammalian phenylalanine hydroxylase. It has been shown to cross-react with active phenylalanine hydroxylase from monkey, human, rat, a rat hepatoma cell line (Choo & Cotton, 1979) and a human hepatoma cell line which exhibited phenylalanine hydroxylase activity (K. H. Choo, I. G. Jennings & K. Fowler, unpublished work).

In the present study, the presence of cross-reacting material in active human liver extract (A-1) was shown by four methods: (i) double immunodiffusion, which showed a precipitin line between wells containing the undiluted antiserum and the extract at concentrations as low as 80 µg of protein/well; (ii) preincubation with the extract abolished antiserum activity, as shown by the lack of cross-reaction with active mammalian phenylalanine hydroxylase in double-immunodiffusion reactions; (iii) activities in

Fig. 3. Two-dimensional polyacrylamide gel of purified human phenylalanine hydroxylase from a normal liver

(a) Close-up of the phenylalanine hydroxylase region on a two-dimensional polyacrylamide gel of affinity-purified phenylalanine hydroxylase from sample A-1. (b) Drawing of (a). Spots were designated H1 and H2 and L1–L4 as explained in the text. The basic end of the first-dimensional isoelectric-focusing (IEF) gel where sample was loaded is on the left. The second dimension was carried out in SDS/polyacrylamide gel.

the crude extract were inhibited (up to 95% with the amounts of antiserum used) by incubation with antiserum in enzyme-inhibition experiments; and (iv) the immunoprecipitation results below. Similar results were obtained for sample A-2, except that twice the amount of extract protein was required, which is in agreement with the lower specific enzyme activity of this sample (Table 1). The antiserum has also detected cross-reacting material in liver from the malignant-hyperphenylalaninaemia patient (sample N) (see immunoprecipitation results below).

No cross-reacting material was found in inactive extracts of samples M, Hu, MM and C by three different methods: (i) double immunodiffusion showed no precipitin line between the antiserum well and wells containing various dilutions (25–700 µg of protein/well) of extracts; (ii) activity of antiserum was not abolished when it was preincubated with various dilutions of these extracts (25–700 µg of protein/20 µl of antiserum), as shown by the persistence of the precipitin line between wells containing extract-treated antiserum and active mammalian phenylalanine hydroxylase in the double-immunodiffusion reaction; and (iii) immunoprecipitation results below.

Immunoprecipitation and two-dimensional polyacrylamide-gel electrophoresis

Immunoprecipitated phenylalanine hydroxylase from active liver crude extract (sample A-1) separates into at least six spots on two-dimensional polyacrylamide gels (Figs. 4a and 4b). Two of these spots (labelled H1 and H2 in Figs. 4a and 4b) have mol.wt. 50 000, and the other spots (labelled L1 to L4 in Figs. 4a and 4b) have mol.wt. 49 000. When phenylalanine hydroxylase that had been purified by using affinity adsorbent was similarly immunoprecipitated and electrophoresed on two-dimensional polyacrylamide gel, the resulting pattern and positions of the spots were identical with that shown in Figs. 4(a) and 4(b). This indicates that the immunoprecipitated subunit species seen in Figs. 4(a) and 4(b) are the same as those seen in Fig. 3. Pictures similar to Figs. 4(a) and 4(b) have also been obtained for liver samples A-2 and N. A material 'peak 1' (Cotton & Grattan, 1975; Cotton & Jennings, 1978) containing phenylalanine hydroxylase activity which was eluted prematurely from the affinity adsorbent with column-washing buffers gave the same two-dimensional gel picture (results not shown). Thus the phenylalanine hydroxylase activities in samples A-1, A-2, N and peak 1 and the affinity-purified enzyme are probably due to the same population of enzyme species.

When this procedure was applied to extracts M (Fig. 4c), Hu and MM (results not shown), no protein species with molecular weight corresponding to those of H and L subunits could be detected on two-dimensional polyacrylamide gels. These protein subunits were also not observed when non-immune control serum instead of antiserum was incubated with various active or inactive extracts (results not shown).

It is interesting that, when the L subunit only is detectable in affinity preparations from inactive normal livers, it is not possible to precipitate any H or L subunit. Thus the L chains in the immunoprecipitates of active extracts must be brought down because of their association with the H chains. This is further evidence for the co-existence of H and L subunits in the intact enzyme (see the results of SDS/polyacrylamide-gel electrophoresis for other evidence).

Discussion

Subunit structure of normal liver phenylalanine hydroxylase

Previous studies (see the introduction) have shown considerable molecular heterogeneity in mammalian liver phenylalanine hydroxylase. However, the number of subunits and their charge and size

Fig. 4. Immunoprecipitation and two-dimensional polyacrylamide-gel electrophoresis of human phenylalanine hydroxylase from crude liver extracts

(a) Close-up of the phenylalanine hydroxylase region on a two-dimensional polyacrylamide gel of immunoprecipitated active normal crude liver extract (sample A-1). (b) Drawing of (a) showing the phenylalanine hydroxylase subunits (designated H1, H2, L1-L4). (c) Close-up of the phenylalanine hydroxylase region of immunoprecipitated phenylketonuric liver extract (sample M) showing the position of immunoglobulin proteins, and absence of phenylalanine hydroxylase subunit spots. The basic end of the first-dimensional isoelectric-focusing (IEF) gel where sample was loaded is on the left. The second dimension was carried out in SDS/polyacrylamide-gel.

difference are not clear from these studies. In the present study, we have examined the subunit structure of normal human phenylalanine hydroxylase using a two-dimensional gel-electrophoretic system which separates protein subunits on the basis of both charge (first dimension) and size (second dimension) independently. This method has resolved at least six subunit species in active enzyme purified by both the affinity and the immunoprecipitation methods. Two of these species have molecular weights corresponding to that of the H subunit, whereas the other four have molecular weights corresponding to that of the L subunit (Figs. 3 and 4). With the use of two different methods of enzyme purification, both of which are highly specific for phenylalanine hydroxylase, it is very unlikely that another major active phenylalanine hydroxylase species exists in liver extracts that has not been detected. This is supported by the disappearance of all phenylalanine hydroxylase activity with a single mutation in phenylketonuria. The subunit structure shown by Figs. 3 and 4 should therefore represent that which is responsible for the major (if not the entire) phenylalanine-hydroxylating activity observed in an extract.

It is quite unlikely that the different charged species at each of the two subunit molecular weights (Figs. 3 and 4) are artifacts caused, for example, by interaction of proteins with cyanate groups which may be present in the urea (Stark, 1965; Gerding *et al.*, 1971). Precautions have been taken to avoid

unnecessary heating of urea solution and long contact of proteins with urea. Further, cultured rat hepatoma phenylalanine hydroxylase, in its purified form or in crude extracts, gives rise to only a single species on two-dimensional polyacrylamide gel (Choo & Cotton, 1979) under conditions of treatment similar to those used in the present study.

The origin of the different charged species is not certain. Some of the species may represent products of different structural genes; however, they may be the result of post-translational modification of a parent protein. There are several lines of evidence which seem to favour the second explanation. Studies have now shown that phenylalanine hydroxylase is a phosphoprotein and that the extent of phosphorylation is a structural determinant which may be responsible for the charge heterogeneity seen in this enzyme (Abita *et al.*, 1976; Donlon & Kaufman, 1977, 1978).

The origin of the two subunit molecular-weight (H and L) forms is also not clearly resolved. However, the present results are compatible with a suggestion that the L subunit may represent a degradative product of the H subunit. First, our results showed that the H subunit is essential for three properties of the enzyme, namely tertiary structure (i.e. 275000 mol.wt. on Gradipore gel), hydroxylating activity and antigenic activity. The L subunit therefore seems to represent an inactive (degraded) molecule. Second, decreasing specific phenylalanine hydroxylase activity in crude extracts is directly proportional

to increasing intensity of the L subunit relative to the H subunit band in affinity-purified preparations. (The ratio of the two subunits does not alter significantly through repeated freeze-thawing of purified enzyme. This suggests that any conversion between the two species may not be spontaneous.) A third line of evidence has come from studies which compared phenylalanine hydroxylase from rat liver and cultured rat hepatoma cells. Earlier studies (Miller & Shiman, 1976; Miller *et al.*, 1976) have indicated that the enzyme from these two sources is identical. In the present study we have evidence that active rat hepatoma phenylalanine hydroxylase is composed of subunits with molecular weight similar to that of the H subunit in rat liver enzyme (Fig. 2). The simplest explanation for the latter observation is that the presumed degradative process present in the liver tissue does not exist (or is less active) in the cultured rat hepatoma cells. However, until direct evidence shows sequence homology between the subunits, or derivation of the L subunit from the H subunit *in vitro* (for example, by limited proteolysis of affinity-purified active human enzyme), the proposed relationship between the different subunits remains rather speculative.

In summary, the present evidence indicates that active phenylalanine hydroxylase purified from human liver is composed of a mixture of subunits which are different in charge and size. Further, the ratio of the H and L subunits appears to be variable from multimer to multimer depending on the specific activities of the intact enzymes. It is not clear, however, whether such a mixture of subunits exists *in vivo*. Monkey liver phenylalanine hydroxylase which has been purified to over 99% homogeneity shows a pattern of subunit heterogeneity on two-dimensional polyacrylamide gels similar to that seen with purified human enzyme (R. G. H. Cotton & I. G. Jennings, unpublished work). This suggests that the subunit structure shown in the present study for the human enzyme may be a general one for all mammalian hepatic phenylalanine hydroxylases.

It is noteworthy that the subunit structure of phenylalanine hydroxylase purified by the affinity method is the same as that of enzyme purified by the immunoprecipitation method. In another study (Choo & Cotton, 1979) the affinity-purified phenylalanine hydroxylase from cultured rat hepatoma cells has been shown to be identical with the enzyme species that exists in a crude cell extract. This is shown by an exact 'mapping' of the purified protein on to a region of a two-dimensional polyacrylamide gel of a crude extract which has been independently proved to be the phenylalanine hydroxylase region by studies on phenylalanine hydroxylase-deficient mutants isolated from this cell line (Choo & Cotton, 1979). This suggests a property of the affinity-

purification method which preserves structural integrity of the enzyme, an advantage which may not be offered by other methods of purification (Kaufman & Fisher, 1974; Barranger, 1976; Woelf, 1976).

Molecular defects in phenylketonuria and malignant hyperphenylalaninaemia

In this study, we have examined and compared livers from prompt autopsies (samples A-1, A-2, M and N), as well as delayed autopsies (samples Hu, MM and C). Samples M and Hu were identified as from patients with classical phenylketonuria (Table 1), whereas sample N was from a patient with malignant hyperphenylalaninaemia (Danks *et al.*, 1978) (Table 1).

The results of Gradipore (Fig. 1) and SDS/polyacrylamide-gel-electrophoretic (Fig. 2) analyses have revealed that no protein corresponding to either the intact phenylalanine hydroxylase (mol.wt. 275000) or the subunits (mol.wts. 50000 and 49000) could be purified from extracts of samples M and Hu with the affinity method. This is in disagreement with our earlier report of finding inactive phenylalanine hydroxylase protein from a liver in classical phenylketonuria by using the affinity method (Cotton & Danks, 1976). This discrepancy is attributed to the method of analysis used in the previous study (Cotton & Danks, 1976), which is superseded by that used in the present study. The absence of phenylalanine hydroxylase protein from the affinity-purified preparations indicates that, if a mutant protein exists in the phenylketonuric livers, it has lost its ability to recognize the synthetic cofactor (2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine). Alternatively the mutation involved may have resulted in a greatly decreased or zero rate of production of normal enzyme protein.

These two possibilities were not resolved by the use of phenylalanine hydroxylase-specific antiserum. No cross-reacting material could be detected in either of the phenylketonuric-liver extracts, whereas the method could detect 10% or more of an antigen identical with that found in an active liver extract. With an antiserum to rat liver phenylalanine hydroxylase raised in sheep, Friedman *et al.* (1972) have similarly found no cross-reacting material in two classical phenylketonuric livers. In a preliminary communication, Bartholomé & Ertel (1978) have claimed detection of enzyme protein in phenylketonuria liver by using an antiserum to monkey phenylalanine hydroxylase.

Friedman *et al.* (1973) reported the presence of a structurally altered phenylalanine hydroxylase protein in a phenylketonuric liver with residual activity 0.27% of normal. Choo & Cotton (1977, 1979) have isolated a population of phenylalanine hydroxylase-deficient mutants from cultured rat hepatoma cells

and have shown that the mutants were defective in the regulation of the production of structurally normal phenylalanine hydroxylase. Thus the possibility of regulatory mutation in phenylketonuria cannot be excluded.

The detection of phenylalanine hydroxylase activity and subunit structure in sample N, which are identical with those of normal enzyme, is consistent with expectation in this patient, whose defect was in bipterin metabolism rather than in phenylalanine hydroxylase (Danks *et al.*, 1978).

Human phenylalanine hydroxylase and its associated disease phenylketonuria are very difficult to study because of the lability of the enzyme after death (Mitoma *et al.*, 1957), scarcity of liver tissues from normal individuals and from patients, and the purification procedures, which are non-specific and/or require large amounts of materials. The methods reported in the present study, in particular the combined use of immunoprecipitation (and affinity chromatography) with two-dimensional gel electrophoresis, offer speed and specificity for enzyme purification, plus ability to examine directly the subunit structure of phenylalanine hydroxylase by using small amounts of liver tissue.

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