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Phenylalanine Hydroxylase-stimulating Protein/Pterin-4α-carbinolamine Dehydratase from Rat and Human Liver

PURIFICATION, CHARACTERIZATION, AND COMPLETE AMINO ACID SEQUENCE*

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Phenylalanine hydroxylase-stimulating protein, also known as pterin-4α-carbinolamine dehydratase (PHS/PCD), was purified from rat and, for the first time, from human liver. We obtained their complete protein primary sequence using a combination of liquid secondary ionization mass spectrometry/tandem quadrupole mass spectrometry, electrospray ionization mass spectrometry, and Edman microsequence analysis. The amino acid sequences of human and rat PHS/PCD were found to be identical. Surprisingly, the primary structure of PHS/PCD is also essentially identical to a protein of the cell nucleus, named dimerization cofactor of hepatocyte nuclear factor 1α, recently reported to be involved in transcription (Mendel, D. M., Khavari, P. A., Conley, P. B., Graves, M. K., Hansen, L. P., Admon, A., and Crabtree, G. R. (1991) *Science* 254, 1762–1767).

Tetrahydrobiopterin (BH_4)[1] is the cofactor of the aromatic amino acid monooxygenases, enzymes which control the biosynthesis of the neurotransmitters dopamine, serotonin, epinephrin, and norepinephrin (1). Patients with a BH_4 deficiency, which can originate from either a defect in the enzymes involved in its biosynthesis or regeneration, may suffer from mental retardation, severe muscular hypotonia, frequent episodes of hyperthermia, and neurological disorders. The roles of BH_4 and of the enzymes involved in its biosynthesis and regeneration are summarized in Fig. 1. Until recently, genetic defects had only been recognized in the enzymes GTP cyclohydrolase, 6-pyruvoyl-tetrahydropterin synthase, and dihydropteridine reductase.

A new form of hyperphenylalaninemia has been discovered recently that is characterized by the excretion of seven substituted pterins in the urine of affected patients. Normal, biologically relevant pterins found in mammals are substituted at position 6 (2–4). This anomaly is proposed to originate in a defect of pterin-4α-carbinolamine dehydratase (PCD), a factor previously termed "phenylalanine hydroxylase-stimulating protein" (PHS) (5). The formation of 7-pterins was subsequently recognized to arise from 4α-hydroxytetrahydropterin (or pterin-4α-carbinolamine) via chemical rearrangement reactions (6–8) that are minimized in the presence of PHS/PCD. Moreover, it was proposed that PHS/PCD both prevents the formation of seven pterins and accelerates the formation of quinonoid-BH_2. Therefore, in view of the importance of BH_4 in hyperphenylalaninemia, and thus the significance of its enzymatic regeneration from 4α-hydroxytetrahydropterin (9–14), we purified PHS/PCD from rat and human liver and determined its primary structure. Surprisingly, a search of the EMBL data base indicated that PHS/PCD is essentially identical to a cofactor that regulates dimerization of a nuclear homeodomain-containing protein involved in transcription (15).

MATERIALS AND METHODS

Purification of Human PHS/PCD—The protein was purified from autopsy samples of human liver obtained immediately after dissection from kidney transplant donors. Human liver (150 g) was homogenized in buffer A (30 mM Tris/HCl, 20 mM L-phenylalanine, 0.75 M KCl, pH 7.4) and centrifuged (100,000 × *g*, 60 min). The supernatant was applied to a phenyl-Sepharose CL-4B column (3.0 × 20 cm; Pharmacia), previously equilibrated with buffer A, containing 0.1% Tween 80. The column was washed with buffer A, and PCD activity was eluted with buffer B (30 mM Tris/HCl, 10 mM L-phenylalanine, 150 mM KCl, and 4.8% dimethylformamide, pH 7.4). Proteins were precipitated by adding solid ammonium sulfate to 80% saturation. After centrifugation the pellet was dissolved in buffer C (5 mM potassium phosphate, 0.1 M KCl, pH 6.8) and dialyzed against this buffer. Further purification was performed on a hydroxylapatite Bio-Gel HT column (2.5 × 20 cm; Bio-Rad), equilibrated in buffer C. After washing, the enzyme was eluted with 20 mM potassium phosphate, 0.1 M KCl, pH 6.8. Fractions with dehydrating activity were pooled, and the protein was precipitated with ammonium sulfate (60% saturation). Following centrifugation, the pellet was dissolved in 25 mM imidazole buffer, pH 7.4, and dialyzed. The protein was further purified by chromatofocusing (pH gradient from 7.4 to 5.0; using the kit and protocol of Pharmacia). Fractions with dehydrating activity were pooled and concentrated (Centriprep-10 Amicon). Final protein purification was by gel filtration on a Sephacryl S-200 HR column (1.6 × 100 cm; Pharmacia) in buffer C.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) P80095 (the amino acid sequence of human and rat PHS/PCD).

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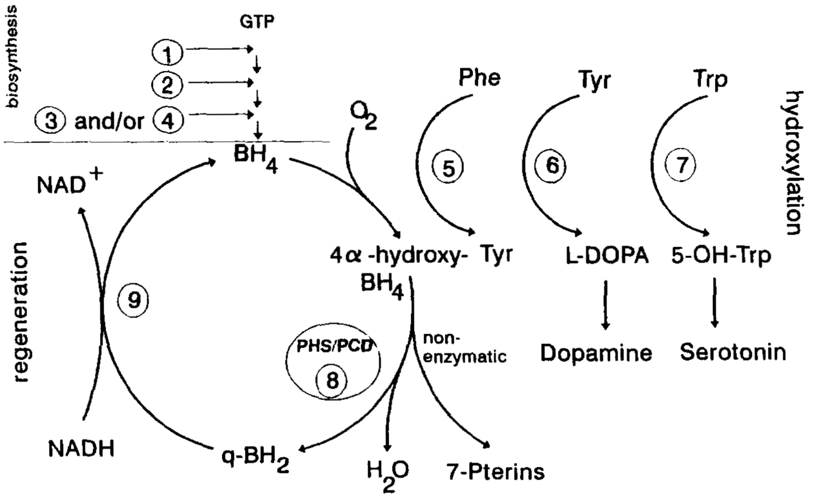
[1] The abbreviations used are: BH_4, tetrahydrobiopterin; DCoH, dimerization cofactor of hepatocyte nuclear factor 1α; ESI-MS, electrospray ionization-mass spectrometry; LSIMS, liquid secondary ionization mass spectrometry; MS/MS, tandem quadrupole mass spectrometry; PHS/PCD, phenylalanine hydroxylase-stimulating protein/pterin-4α-carbinolamine dehydratase; PAH, phenylalanine hydroxylase; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

FIG. 1. **Enzymes involved in the biosynthesis of BH_4 and in the BH_4-dependent hydroxylation of aromatic amino acids.** *BH_4*, tetrahydrobiopterin; *qBH_2*, quinonoid dihydrobiopterin; *4α-hydroxy-BH_4*, 4α-hydroxy 5,6,7,8-tetrahydrobiopterin or 4α-carbinolamine; *1: GTP-CH*, GTP cyclohydrolase I; *2: PTPS*, 6-pyruvoyl-tetrahydropterin synthase; *3: AR*, aldose reductase; *4: SR*, sepiapterin reductase; *5: PAH*, phenylalanine-4-hydroxylase; *6: TH*, tyrosine-3-hydroxylase; *7: TPH*, tryptophan-5-hydroxylase; *8: PHS/PCD*, phenylalanine hydroxylase-stimulating protein/pterin-4α-carbinolamine dehydratase; *9: DHPR*, dihydropteridine reductase.



Purification of Rat PHS/PCD—Protein from rat liver was purified according to the method of Parniak (16). Final purification used chromatofocusing and gel filtration under the conditions described above.

Enzyme Assays—PHS/PCD activity was determined by the ability to stimulate PAH at pH 8.0 in the presence of BH_4 (Dr. B. Schircks Laboratory, Jona, Switzerland), according to the method of Huang *et al.* (5). The 250-μl assay reaction mixture contained 50 mM Tris-HCl, pH 8.4, 2000 units of catalase, 0.5 unit of dihydropteridine reductase (sheep liver, Sigma), 0.5 mM NADH, 2 mM L-phenylalanine, and 40 μg (0.025 units) PAH. This mixture was incubated for 2 min at 25 °C, and PCD was added. The amount of tyrosine formed after 30 min was determined fluorimetrically and corrected for the tyrosine formed in controls without dehydratase. One unit is defined as 1 μmol of tyrosine formed per 30 min. PAH (free of PCD activity) was purified from rat and human liver according to Shiman (17), with the addition of a final ion-exchange chromatography (DEAE-Toyopearl 650 M) and gel filtration (Sephacryl S-200 as HR).

Isoelectric Focusing—The LKB Immobiline system was used for preparing immobilized pH gradient (IPG) gels. A linear gradient from pH 4 to pH 10 was cast (Handbook Multiphor II, LKB). The broad pI marker systems from Pharmacia (pI 3.6–9.3) and from Sigma (pI 3.6–9.3) were used.

SDS-PAGE (10%) gels were prepared according to Laemmli (Handbook Multiphor II, LKB). Low molecular weight standards were from Bio-Rad and LKB. Staining was with Coomassie Blue or silver.

Enzymatic Digestion of PHS/PCD—Microbore HPLC (RP-300) repurified native or carboxymethylated PHS/PCD was digested with either pepsin, endoproteinase Asp-N, or chymotrypsin. Proteolysis by pepsin was accomplished by adjusting the native PHS/PCD solution to pH 4 with 5% acetic acid, adding 1 μg of enzyme, and incubating at 37 °C for 4 h. Cleavage with endo Asp-N or chymotrypsin took place in 100 μl of 50 mM ammonium bicarbonate with 1 μg of enzyme at 37 °C for 16 h.

Reverse Phase HPLC—Fractionation of enzymatically cleaved peptides and repurification of whole protein was performed on an Applied Biosystems 130A system. The sample was injected onto a microbore RP-300 (C8) AquaPore column (1 × 25 mm), and components were eluted with a linear gradient (120 min) of 0–70% solvent B. Solvent A: 0.1% trifluoroacetic acid, water. Solvent B: 0.08% trifluoroacetic acid, 80% acetonitrile, 20% water. The combined solvent flow rate was 50 μl/min; the column effluent was monitored at 214 nm. Fractions were collected by hand into 500-μl Eppendorf tubes over 30–90-s intervals to optimize obtained component separation.

Edman Microsequence Analysis—This was performed with an Applied Biosystems model 477A instrument.

Amino Acid Analysis—This was carried out by gas phase HCl hydrolysis, conversion with dabsyl chloride, and evaluation of the derivatized products with a Beckman System Gold HPLC instrument. All samples were previously analyzed by mass spectrometry, and thus expectation values were available for all amino acids except leucine/isoleucine.

Mass Spectrometry—Liquid secondary ionization MS spectra, both for peptide molecular weight and MS/MS sequence analysis, were obtained with a Finnigan TSQ-70 triple quadrupole instrument (Finnigan-MAT, San Jose, CA) equipped with a cesium ion gun (Antek, Palo Alto, CA). Operation of this instrument has been described elsewhere (18, 19). Peptide sample quantities ranged from 5 to 20 pmol for molecular weight spectra and 5 to 50 pmol for MS/MS sequence analysis. The general peptide sequencing methodology used was similar to that of Hunt *et al.* (20). Some peptides were also converted to methyl esters with methanolic 2 N HCl, or NH_2-terminally acetylated with a 3:1 mixture of methanol:acetic anhydride as described previously (20).

Electrospray ionization MS spectra were obtained with a Finnigan TSQ-700 instrument equipped with an Analytica of Branford (Branford, CT) ESI source (21). Protein samples were analyzed in solution as obtained from C8 reverse phase HPLC fractionation (approximately 40% acetonitrile, 0.09% trifluoroacetic acid) at a concentration of approximately 5 pmol/μl. The protein molecular weight was determined from the acquired spectra with ESI deconvolution software from Finnigan.

TABLE I
Purification scheme of PHS/PCD from human liver

Purification step	Total activity[a]	Total protein	Specific activity	Purification	Yield
	units	*mg*	*units/mg*	*-fold*	*%*
Crude extract	2,336	14,600	0.16	1	100
Phenyl-Sepharose, 80% $(NH_4)_2SO_4$	1,817	790	2.3	14	78
Bio-Gel HT chromatography, 60% $(NH_4)_2SO_4$	1,525	82	18.6	116	65
Chromatofocussing	1,060.5	10.1	105.0	656	45
Sephacryl S-200 HR	940.5	4.5	158.0	990	40

[a] 1 unit is defined as 1 μmol of tyrosine formed per 30 min.

RESULTS AND DISCUSSION

Purification of PHS/PCD—PHS/PCD was isolated from the cytosol of both rat and human liver. Final purification that maintained pterin-4α-carbinolamine dehydrating activity was by gel filtration, where activity eluted in a single peak. The elution profile from gel filtration indicated an apparent molecular mass of 45 kDa, indicating that PHS/PCD can exist as a homotetramer (data not shown). The purification scheme of the human protein is shown in Table I. The overall purification factor was 990-fold, with a yield of 40%. Analysis of this preparation by C8 reverse phase HPLC found more than 90% of the total protein eluted as a single symmetric peak corresponding to PHS/PCD. Thus, extrapolated from the data in Table I, PHS/PCD abundance in human liver is (0.9)(4.5 mg)(2336/940) = 10.1 mg/150 g of tissue or approximately 6.7 mg/100 g of human liver.

Protein purity and physical properties were assessed by SDS-PAGE, microbore HPLC and isoelectric focusing gel electrophoresis. Fig. 2 depicts the SDS-PAGE results; both the human and rat monomeric PHS/PCD migrate identically at ~12 kDa. Fig. 2 also shows the isoelectric focusing gel results; both human and rat native PHS/PCD exhibit a primary signal at pI 5.8, with two weak intensity signals at slightly lower pI values. These weak signals ostensibly correspond to the sulfone and sulfate oxidation products of the sulfhydryl group of the lone cysteine.

Primary Structure of Rat and Human PHS/PCD—The primary protein structure was determined by a combination of tandem quadrupole mass spectrometry, electrospray ionization mass spectrometry, and classical Edman microsequence methods. The complete amino acid structure of human PHS/PCD, assembled mostly from peptic peptides, is presented in Fig. 3. The peptide sequences used to assemble this structure are presented in Table II. A similar set of peptides was obtained from enzymatic cleavage of rat PHS/PCD. Surprisingly, the amino acid sequence of human and rat proteins was found to be completely identical. Although no overlapping peptide was obtained between residues 94 and 95, the verity of the overall structure was demonstrated by the ESI-MS results.

Assignment of the blocked NH_2-terminal residue as acetyl-alanine was determined by mass spectrometry. The MS/MS spectrum of peptide d2 (see Table II) indicated that the amine-terminal residue has incremental mass of 113 Da, and thus could be Leu, Ile, or *N*-acetyl-Ala. Acetylation, under acidic conditions, of peptide d2 produced no mass shift, demonstrating the lack of an amino-terminal $-NH_2$ group. Absence of a free amino terminus leads to the assignment of acetyl-alanine as the NH_2-terminal residue.

Analysis by ESI-MS of both rat and human PHS/PCD produced identical spectra corresponding to a protein molecular mass of 11,909 Da. This result coincides with the calculated average isotopic mass of the protein sequence assembled here and demonstrates the absence of an intervening sequence between residue sites 94 and 95. No signals corresponding to other molecular mass values were observed in the ESI-MS spectra, indicating that PHS/PCD has a homogeneous amino acid sequence and is not posttranslationally modified other than the acetylation of the amino terminus.

Identity of PHS/PCD with a Dimerization Cofactor—A search of the EMBL sequence library indicated that the primary structure of human and rat PHS/PCD is essentially identical to a nuclear protein dimerization cofactor reported recently by Mendel *et al.* (15) to be involved in the regulation of transcription. They identified a dimerization cofactor of the homeodomain-containing protein hepatocyte nuclear factor-1α (HNF-1α). This HNF-1α cofactor, named DCoH, was cloned from rat, mouse, and human cDNA libraries and found to code for essentially identical protein sequences. Their longest human clone codes for the same protein as rat, but extended only to the amino terminal alanine residue; also, the mouse cDNA encodes a valine at amino acid position 28 in

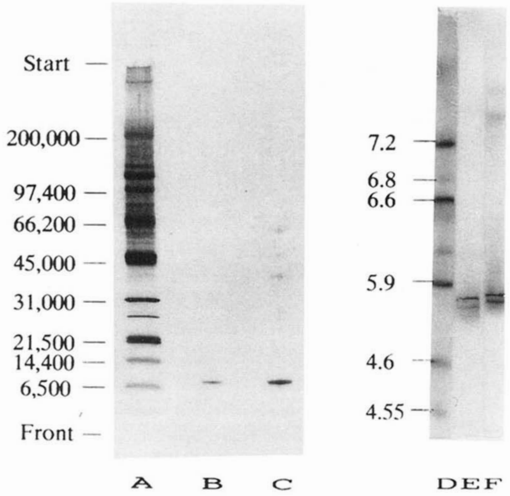
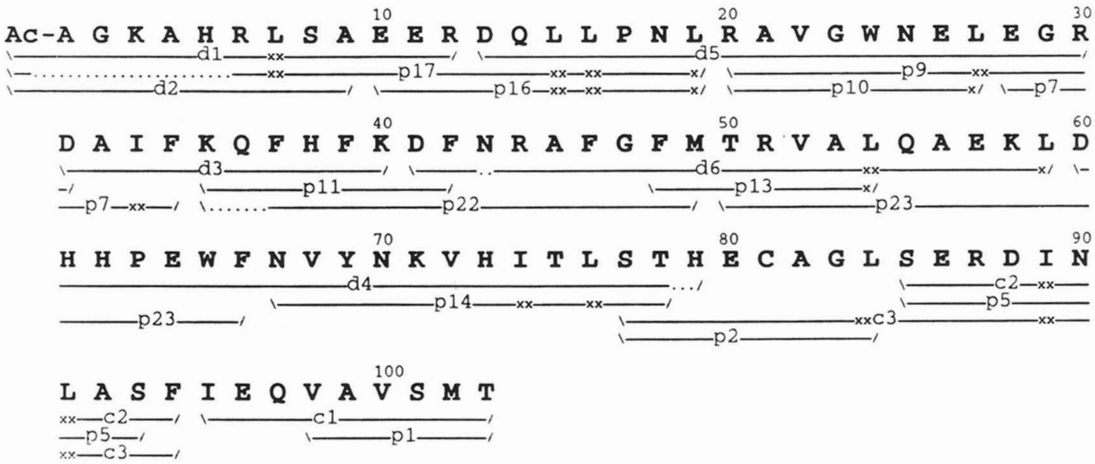


FIG. 2. **SDS-PAGE (*A–C*) and isoelectric focusing (*D–F*) of rat (*B* and *E*) and human (*C* and *F*) PHS/PCD.** *Lanes A* and *D* contain marker proteins (see "Materials and Methods"). In each lane, 2 μg of protein were applied. Visualization was with silver staining (*a–c*) or with Coomassie Blue (*d–f*).



KEY

... Indicates sequence corresponding to deduced incremental mass difference.
xx Assignment of I or L in MS/MS spectrum.
d = Asp-N cleavage, p = pepsin cleavage, c = chymotrypsin cleavage.

FIG. 3. **Protein primary structure of human PHS/PCD assembled from sequenced peptide fragments.** Refer to Table II for peptide description.

TABLE II
Peptide fragments from human PHS/PCD characterized by tandem mass spectrometry or Edman microsequence analysis

Peptide #	Cleavage method	(M + H)+ [a]	Sequence[b]
MS/MS analysis			
p1	Pepsin	607	VAVSMT
p2[c]	Pepsin	817	STHECAGL
p3	Pepsin	559	TRVA*X*
p4	Pepsin	758	TRVA*X*QA
p5	Pepsin	1004	SERD*X*N*X*AS
p6	Pepsin	846	SERD*X*N*X*
p7	Pepsin	690	MTRVA*X*
p8	Pepsin	807	EGRDA*X*F
p9	Pepsin	920	*X*EGRDA*X*F
p10	Pepsin	1401	RAVGWNE*X*EGRD
p11	Pepsin	944	RAVGWNE*X*
p12	Pepsin	1096	KQFHFKDF
p13	Pepsin	837	FMTRVA*X*
p14	Pepsin	1388	NVYNKVH*X*T*X*ST
p15	Pepsin	968	RDQ*XX*PN*X*
p16	Pepsin	1097	ERDQ*XX*PN*X*
p17	Pepsin	1226	EERDQ*XX*PN*X*
p18	Pepsin	2160	Ac-agkahR*X*SAEERDQ*XX*PN*X*
p19	Pepsin	1999	nvYNkVH*X*T*X*STHECAG*X*
p20	Pepsin	1789	kqFHFkDFNRAFGF
p21	Pepsin	1733	RAVGWNEXEGRDA*X*F
p22	Pepsin	1920	kqFHFkDFNRAFGFM
d1	Asp-N	1366	Ac-AGKAHR*X*SAEER
d2[c]	Asp-N	952	Ac-AGKAHRLSA
d3[c]	Asp-N	1280	DAIFKQFHFK
c1[c]	Chymo	977	IEQVAVSMT
c2	Chymo	1151	SERD*X*N*X*ASF
c3[d]	Chymo	2007	STHECAG*X*SERD*X*N*X*ASF
MS/MS and Edman microsequence analysis			
p5	Pepsin	846	SERDINL
p23	Pepsin	2077	TrVALQAEKLDHhPE?F
d4	Asp-N	2475	DHHPEWFNVYNKVhITLST?
d5	Asp-N	2080	DQLLPNLRAVGWNELEGR

[a] Nominal mass values.
[b] Small case letters in peptide sequences indicate residues that could not be unambiguously assigned but were verified from other sequenced peptides. "*X*" indicates the presence of either leucine or isoleucine.
[c] I or L assignment by amino acid analysis.
[d] Carboxymethylated cysteine present.

place of a leucine. This cDNA-deduced protein sequence of rat and human DCoH is identical with the sequence of PHS/PCD found here. The sole difference is in the amino terminus of the proteins; the cDNA-deduced DCoH structure commences with Met-Ala-Gly-..., etc., and PHS/PCD was found as Ac-Ala-Gly-..., etc. Removal of methionine and acetylation of alanine are reasonable, and probable, cotranslational modifications for a eukaryotic protein with a Met-Ala amino terminus (22, 23). Thus, the processed forms of DCoH and PHS/PCD from both human and rat are almost certainly one and the same protein.

The 45-kDa apparent molecular mass value found here by gel filtration agrees with a previous report that native PHS/PCD exists as a tetramer (5, 6). In contrast, native DCoH was observed as a dimer (15). PHS/PCD isolated by reverse phase HPLC (dissolved in 0.1% trifluoroacetic acid, 35% acetonitrile, nonreducing conditions) is observed solely as monomer when analyzed by ESI-MS. Although the protein contains a single free cysteine residue, no disulfide bond is found in this mildly denaturing solvent. Thus, dimers or tetramers are noncovalently bound species and apparently contain free cysteine sulfhydryl groups.

Conclusions—Our results demonstrate that human and rat PHS/PCD have identical protein primary structure. Moreover, human and rat PHS/PCD are likely to be identical in primary structure to DCoH. This suggests that PHS/PCD could possess distinct functions in the cell nucleus and cytosol. Here, PHS/PCD was purified from the cytosolic fraction. The pellet fraction did not contain detectable PHS/PCD activity. Cytosolic PHS/PCD is apparently involved in the regeneration of BH_4, the cofactor of aromatic amino acid monooxygenases. Patients with the new form of hyperphenylalaninemia may have a defect in PHS/PCD. From the protein sequence data reported here we will be able to isolate the corresponding cDNA, analyze the normal and mutated genes, and test the recombinant proteins for enzyme activities. Such information could allow prenatal diagnosis of this inborn error of metabolism. Additionally, the presence of DCoH (PHS/PCD) in the nucleus raises the question if pterins might also be involved in the regulation of eukaryotic gene expression.

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