Pteridines
Vol. 3, pp. 55–57

Short Communication

Human Liver Pterin 4a-Carbinolamine Dehydratase. Purification and Characterization

I. Rebrin, L. Petruschka, H.-Ch. Curtius*, C. Adler* and F. H. Herrmann

Institute of Medical Genetics, Ernst-Moritz-Arndt University, Fleischmannstr. 42–44, D/O-2200 Greifswald, Fed. Rep. of Germany
* *Department of Pediatrics, Division of Clinical Chemistry, University of Zürich, Switzerland*

(Received March 1992)

Introduction

The pterin 4a-carbinolamine dehydratase (P4aCD) is an enzyme that catalyzed the dehydration of the 4a-carbinolamine, the tetrahydrobiopterin derived intermediate product by phenylalanine hydroxylase (PAH) catalyzed reaction, to form q-dihydropterin. The dehydratase is of special interest because it is proposed, that a new variant form of hyperphenylalaninemia (primapterinuria) can be caused by absence of this enzyme. Recently, Curtius *et al.* have described patients with characteristic excretion of 7-substituted pterins (primapterin, anapterin and 6-oxo-primapterin) in urine (1). New data on the formation of primapterin during PAH catalyzed reaction in the absence of dehydratase and the proposed detailed chemical mechanism for 7-substituted pterin formation suggest the evidence, that the enzyme P4aCD can be deficient in these patients (2). In order to study the novel aspects of PAH catalyzed reaction and the mechanism of 7-substituted pterin formation, we described purification procedure for dehydratase from human liver, partial characterization of this enzyme and comparison with P4aCD from rat liver.

Materials

Phenyl Sepharose CL-4B, chromatofocussing kit, Sepacryl S-200 HR were purchased from Pharmacia. Hydroxylapatite Biogel HT was from Bio-Rad. The human livers were obtained from kidney transplantation donors and used immediately.

Methods

Purification P4aCD

All procedures were performed at 4 °C, unless otherwise stated.

200 g liver was homogenized in buffer A (0.03 M Tris/HCl, pH 7.4, 0.02 M L-phenylalanine, 0.75 M KCl). The homogenate was centrifuged at 10^5 g for 60 min and supernatant (liver extract) was incubated at 25 °C for 10 min (3). The activated extract was applied to phenyl Sepharose column (5.10 × 10 cm) which was equilibrated with buffer A containing 0.1% Tween 80 (flow rate was 800 ml/h). The column was washed with 250 ml buffer A and then with 1000 ml buffer B (0.03 M Tris/HCl, pH 7.4, 0.01 M L-phenylalanine, 0.15 M KCl and 4.8% dimethylformamide). The first 2.5 bed volumes of the dimethylformamide containing wash were collected and solid $(NH_4)_2SO_4$ was added to obtain 80% saturation. The precipitate was collected by centrifugation, dissolved in a small volume of buffer C (5 mM potasium phosphate buffer, pH 6.8, 0.1 M KCl) and dialyzed against the same buffer. The dialyzed sample was applied to a Biogel HT column (3.2 × 18cm), washed with 250 ml buffer C and eluted with 20 mM potassium phosphate buffer, pH 6.8, 0.1 M KCl. The fractions containing enzyme activity were precipitated with 60% $(NH_4)_2SO_4$. The precipitate was dissolved in a small volume of 0.025 M imidazol buffer, pH 7.4, and dialyzed against the same buffer. The sample was separated on a chromatofocussing column (1.0 × 30 cm) with pH gradient from 7.4 to 5.0 according instructions from Pharmacia.

Table 1. Purification of pterin 4a-carbinolamine dehydratase from human liver.

Purification step	Total Activity (units[a])	Total Protein (mg)	Specific Activity (units/mg)	Purification (-fold)	Yield (%)
Crude extract	2336	14500	0.16	1	100
Phenyl Sepharose/80% $(NH_4)_2SO_4$	1817	790	2.3	14	78
Biogel HT pool	1525	82	18.6	116	65
Chromatofocussing pool	1060.5	10.1	105.0	656	45
Gel filtration pool	940.5	4.5	158.0	990	30

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

P4aCD active fractions were pooled, concentrated to 1.5 ml (Amicon Centriprep-10) and separated on a Sephacryl S-200 HR column (1.6 × 100 cm) with buffer C at flow rate of 25 ml/h. The final dehydratase pool was concentrated and stored at −80 °C.

The dehydratase from rat liver was purified according to the method of Parniak (4), followed by two further purification steps: column chromatofocussing and gel filtration. Dehydratase-free PAH was obtained according to Shiman (3). The activity of enzymes were assayed fluorimetrically (5).

Results

The purification of P4aCD was 990-fold as determined by the enzyme assay, based on stimulation of PAH activity (5), and represents a 30% yield (Table 1). Absorption chromatography and column chromatofocussing were the main purification steps in our procedure (Table 1). The isoelectric point for human liver enzyme upon chromatofocussing was estimated as 5.25. The last purification step resulted in a single symmetrical peak of activity, corresponding to an Mr of 45 000. SDS/polyacrylamide-gel electrophoresis of the enzyme activity peak eluted from the size column demonstrated the presence of a single 11 000-Mr component. The enzymes were separated by SDS gel electrophoresis and after entrapment of liberated phosphate the gel was stained with methyl green. Both enzymes (from rat and from human liver) showed a bright green colored band specifically for phosphoproteins (data not shown). The amino acid composition of purified enzymes was also determined and high level of glutamic acid, aspartic acid, alanine and leucine were detected (Table 2).

Table 2. Comparison of amino acid composition of rat and human pterin 4a-carbinolamine dehydratase.

Amino acid	Residues/mol of subunit P4aCD		
	Human liver	Rat liver	Rat liver[1)]
Lysine	5.4	4.7	6.1
Histidine	4.2	4.6	5.4
Arginine	5.9	5.9	7.6
Cysteine &	1.2	1.1	1.4
Cystic acid	1.2	1.1	1.4
Aspartic acid	9.9	9.9	12.1
Threonine	3.9	3.9	4.4
Serine	3.7	4.3	5.8
Glutamine acid	12.9	12.6	14.8
Proline	3.1	2.7	3.3
Glycine	5.7	6.3	6.6
Alanine	9.8	9.3	10.8
Valine	5.4	5.2	6.6
Methionine	0.9	0.9	1.9
Isoleucine	3.6	3.8	4.7
Leucine	9.3	9.1	11.4
Tyrosine	0.8	0.8	1.5
Phenylalanine	6.6	6.7	7.6
Tryptophan	ND	ND	1.0
Mr	11 000	11 000	12 900

[1)] Values from Huang *et al.* (5)

Discussion

We have described the purification of P4aCD from human liver by 990-fold with a recovery of 30%. This is a simple purification protocol utilizing a four column procedure that includes a phenyl Sepharose step, absorption chromatography, column chromatofocussing and gel filtration chromatography. Approximately 80% of the dehydratase activity was bound and eluted from phenyl Sepharose column under the conditions employed (use of 0.75 M KCl in homogenization buffer). This step in the purification was based on the previous finding that rat liver P4aCD bound strongly to hydrophobic support in the buffer with high ionic strength (0.5 M KCl) and could be eluted by decreasing it (4). Remarkably, the PAH bind strongly to phenyl Sepharose by substrate induced interaction (3). Our purification procedure has the additional advantage that both enzymes involved in phenylalanine hydroxylation can be conveniently obtained from the same human liver sample, similar as described for rat enzymes (4).

It is likely that the native human liver dehydratase (Mr 45 000) consist of four subunits with equal Mr

of 11 000, as it has been shown for rat liver enzyme (5). The acidic properties of P4aCD (pI = 5.25) are confirmed by estimated high level of dicarbon amino acid after amino acid analysis (Table 2). Physicochemical studies suggest that the enzyme from human liver is very similar to that from rat liver: Mr, subunit number, pI, amino acid composition, both enzyme are might be phosphoproteins. However, further studies are required to start the characterization of primapterinuric patients by means of biochemical, immunological and molecular-biological methods.

References

1. Curtius, H.-Ch., Kuster, Th., Matasovic, A., Blau, N. & Dhondt, J. L. (1988) Biochem. Biophys. Res. Commun. *153*, 715–721.
2. Curtius, C.-Ch., Adler, C., Rebrin, I., Heizmann, C. & Ghisla, S. (1990) Biochem. Biophys. Res. Commun. *172*, 1060–1066.
3. Shiman, R. (1987) in: *Methods in Enzymology 1987* (Kaufman, S., ed.) pp. 17–27, Academic Press, Inc., Orlando.
4. Parniak, M. A. (1990) in: *Chemistry and Biology of Pteridines 1989* (Curtius, H.-Ch., Ghisla, S. & Blau, N., eds.) pp. 656–659, Walter de Gruyter, Berlin.
5. Huang, C. Y., Max, E. & Kaufman, S. (1973) J. Biol. Chem. *248*, 4235–4241.