Crystallization and preliminary diffraction analysis of a truncated homodimer of human phenylalanine hydroxylase

Heidi Erlandsen[a], Aurora Martinez[b], Per M. Knappskog[b,c], Jan Haavik[b], Edward Hough[a], Torgeir Flatmark[b,*]

[a] *Protein Crystallography Group, Department of Chemistry, Institute of Mathematical and Physical Sciences, University of Tromsø, Tomsø, Norway*
[b] *Department of Biochemistry and Molecular Biology, University of Bergen, Bergen, Norway*
[c] *Department of Medical Genetics, University of Bergen, Bergen, Norway*

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Abstract A recombinant truncated form (Δ1-102/Δ428-452) of the non-heme iron-dependent metalloenzyme human phenylalanine hydroxylase (hPAH, phenylalanine 4-monooxygenase; EC 1.14.16.1) was expressed in *E. coli*, purified to homogeneity as a homodimer (70 kDa) and crystallized using the hanging drop vapour diffusion method. The crystals are orthorhombic, space group C222 with cell dimensions of $a = 66.6$ Å, $b = 108.4$ Å, $c = 125.7$ Å. The calculated packing parameter (V_m) is 3.24 Å^3/Da with four 2-fold symmetric dimers (or eight momomers) in the unit cell. Data have been collected to 2.0 Å resolution.

Key words: Phenylalanine hydroxylase; Recombinant enzyme; Truncated form; Crystallization; X-ray diffraction analysis

1. Introduction

The non-heme iron-dependent metalloenzyme, mammalian phenylalanine hydroxylase (EC 1.14.16.1, phenylalanine 4-monooxygenase (PAH)) catalyzes the hydroxylation of l-phenylalanine to L-tyrosine in the presence of 6(*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin and dioxygen, the intitial and rate-limiting step in the catabolism of this amino acid [1]. Phenylketonuria (PKU) is an autosomal recessive disease caused by mutations in the *PAH* gene [2], which results in a considerable heterogeneity both in terms of clinical, metabolic and enzymatic phenotypes [3]. More than 250 different PKU mutations have been reported [4]. Structural information on the human enzyme is greatly needed for a correlation between structure and function of this enzyme. PAH purified from rat liver (rPAH) is so far the best studied of all the pterin-dependent hydroxylases, and preliminary data on its crystallization as a phosphorylated protein was reported 5 years ago [5]. Further structural analysis of rPAH has not, however, been reported, probably related to the occurrence of a molecular heterogeneity (tetramer $<-->$ dimer equilibrium and different conformations as a result of a possible variable degree of enzyme phosphorylation). Our recent success in the large-scale production of recombinant human PAH (hPAH) has made it possible to produce the non-phosphorylated forms of both the wild-type and mutant forms in *E. coli*, using the pMAL expression vector [6,7]. In this paper, we report the overexpression, purification and crystallization of the truncated form of hPAH (Δ1-102/Δ428-452), recovered in high yield as a soluble dimeric protein of relative molecular mass 70 kDa and with a specific activity similar to the tetrameric wild-type enzyme (200 kDa), as well as the diffraction analysis of native crystals.

2. Experimental

2.1. Materials

IPTG and factor Xa were purchased from Boehringer-Mannheim (Germany). All other chemicals were obtained from Merck (Darmstadt, Germany). Polyethylene glycol 2000 and 4000 (PEG-2000 and PEG-4000) were obtained from Dow Chemicals (USA). For column chromatography a FPLC system (Pharmica, Uppsala, Sweden) was used [6]. The wild-type cDNA was subcloned into the pMAL-c2 vector (New England Biolabs), and the double deletion mutant form (Δ1-102/Δ428-452)hPAH was prepared by PCR-based site-directed mutagenesis as described [7,8].

2.2. Protein purification

The fusion proteins MBP-(pepIEGR)$_{Xa}$-hPAH and MBP-(pepIEGR)$_{Xa}$-(Δ1-102/Δ428-452)hPAH were expressed in the pMAL system of *E. coli* and purified as described [6]. The fusion proteins were cleaved by the restriction protease factor Xa and obtained in a highly purified form by high-performance size exclusion liquid chromatography. N-terminal amino acid sequence analysis was carried out using an ABI Model 477A automated sequencer following the manufacturer's instructions. The protein was concentrated to 40–50 mg/ml by ultrafiltration (PLGC11K25 from Millipore Co., MA), gel-filtrated (prepacked NAP-5 column containing Sephadex G-25 Medium from Pharmica, Sweden) for solvent equilibration (see Section 3), and further concentration to 15–25 mg/ml by ultrafiltration and subsequently used for crystallization.

2.3. Crystallization

For initial crystallization experiments an automated fast screening protocol was applied using the Imperial College automated crystallization device (IMPAX I-5) [9,10] designed by Leslie Lloyd and Peter Brick (private communication). The microbatch droplet volumes were 2.5 μl. Crystals were grown at 4°C, and the conditions that gave the best results were optimized. Final crystallization was carried out by the hanging-drop vapour diffusion technique at 4°C [11]. Droplets of 10–12 μl initial volume were prepared on siliconized coverslips by mixing 5–6 μl protein solution and an equal volume of the reservoir solution and sealed against 0.8 ml of this solution.

2.4. X-ray diffraction experiments

Crystals were mounted in thin-walled glass capillaries with a small volume of reservoir solution. X-ray data were collected on the Swiss-Norwegian beamline (BM1, Station A) at ESRF (Grenoble, France) using a 30 cm MAR image plate system (Mar Research). The data were collected at a wavelength of 0.873 Å and crystal-to-detector distance of 25 cm. The exposure time was 60 s/1° frame and a total of 90 frames were collected. The intensities were indexed and inte-

*Corresponding author. Fax: (47) 55-586400

Abbreviations: pPAH, human phenylalanine hydroxylase; wt-hPAH, wild-type human phenylalanine hydroxylase; (Δ1-102/Δ428-452)hPAH, a truncated form of hPAH with a 102 amino-terminal deletion and a 24 carboxyl-terminal deletion; 6-BH$_4$, 6(*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin; PEG, polythylene glycol; MBP, maltose binding protein; PKU, phenylketonuria

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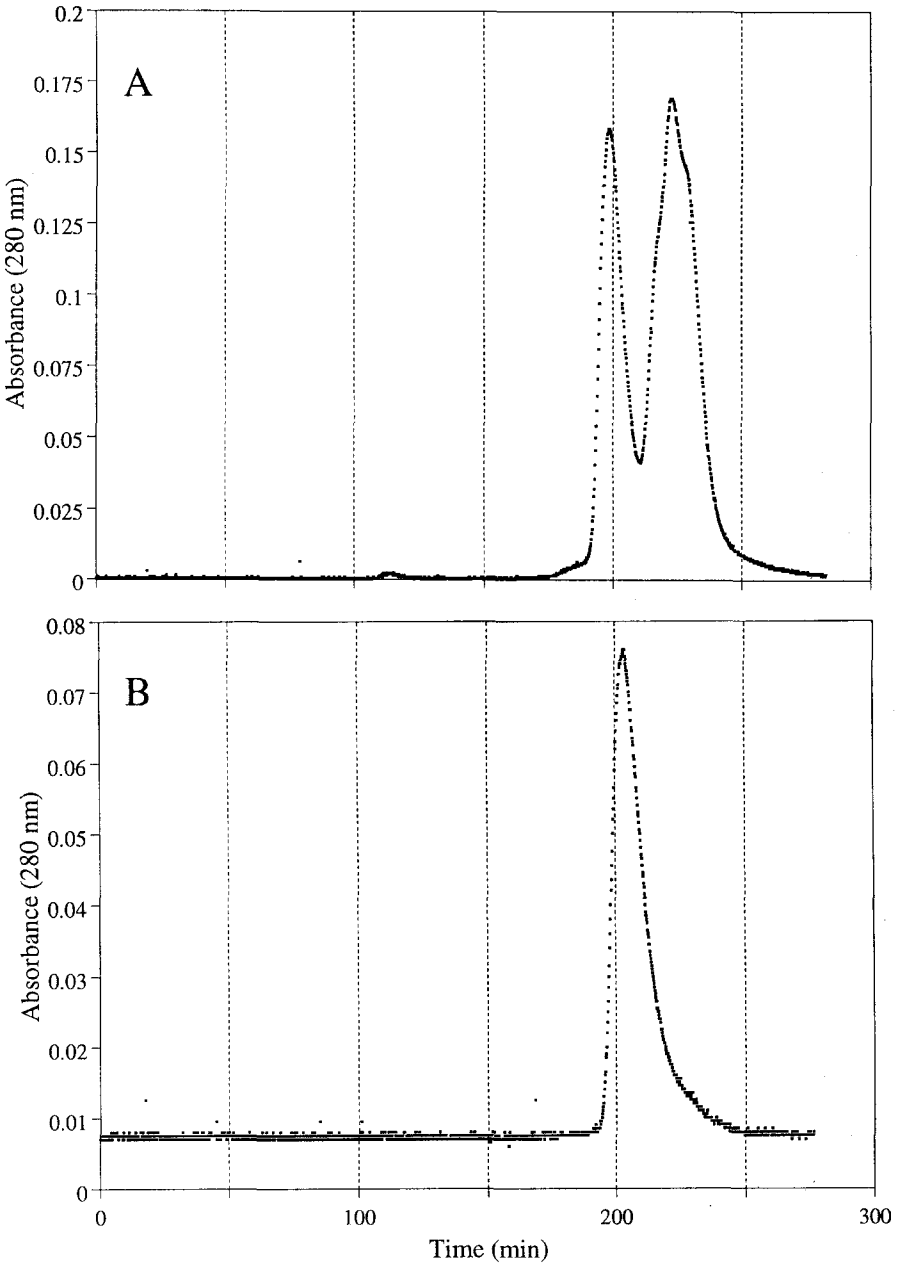

Fig. 1. A: Size-exclusion chromatography on a HiLoad Superdex column (1.6×60 cm) of the fusion protein of the double deletion mutant (Δ1-102/Δ428-452) of hPAH (5.2 mg) following cleavage with the restriction protease factor Xa. The column was equilibrated and eluted with 20 mM Na-Hepes and 0.15 M NaCl, pH 7.0, at a flow rate of 0.38 ml/min. Detection at 280 nm. Peak positions: 196 min, homodimer of (Δ1-102/Δ428-452)hPAH (70 kDa); 217 min, monomer of MBP (42.5 kDa). B: Rechromatography of (Δ1-102/Δ428-452)hPAH (1.8 mg) isolated in (A). The apparent molecular masses of the proteins were estimated using the elution position of the standard molecular mass proteins as a reference (see ref. [6]).

grated using the program DENZO [12]. Scaling and merging were performed using the programs: ROTAVATA and SCALA from the CCP4 program package [13]. Structure factor amplitudes were calculated using the program TRUNCATE [13].

3. Results and discussion

The overexpression in *E. coli* and purification yielded 100–150 mg of pure enzyme from 45 g wet cells. Size-exclusion chromatography revealed (Fig. 1) that the double deletion mutant exclusively exists as a homodimer of relative molecular mass 70 kDa in solution, whereas the wt-hPAH is mainly a tetramer/dimer in equilibrium (data not shown) as reported previously [6,8]. N-terminal amino acid sequence analysis revealed the expected sequence for both enzyme forms [6,8]. Their specific activity, when assayed with 100 μM 6(*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin as the cofactor after preincubation with 1 mM L-phenylalanine [6,8], was 77.0 and 80.0 nmol tyrosine min^{-1}·nmol $subunit^{-1}$ for the wt-hPAH and the (Δ1-102/Δ428-452)hPAH truncated form, respectively. The two enzyme forms (as isolated) contained 0.45 atoms of iron/subunit. The value is comparable to the ≈0.5 atom of iron/subunit found for recombinant rat PAH [14] and catalytically active iron in rat liver PAH [15,16].

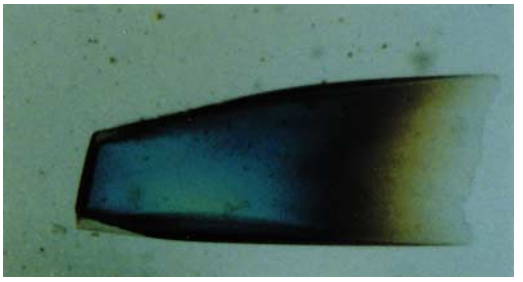

Fig. 2. Orthorombic crystal of the (Δ1-102/Δ428-452)pPAH homodimer grown in 15-20% (w/v) PEG-2000, 0.06-0.08 M PIPES, pH 6.8, at the estimated final protein concentration of 10-12 mg/ml. Crystal size is $2.0\times0.7\times0.2$ mm^3.

Both proteins crystallize in the presence of PEG as the precipitating agent, and the best crystals were obtained at pH 6.5–7.2 (wild-type) and at pH 6.8 for the double deletion mutant, but the wild-type formed only microcrystals. Initially the screening conditions that gave small crystals of the truncated form contained 25% (w/v) PEG-4000 buffered with 0.1 M MES, pH 6.2 or 15–25% PEG-4000 buffered with 0.1 M PIPES, pH 6.8 at a protein concentration of 4 mg/ml. Larger crystals of the truncated form (thin plates of maximum size $1.0\times0.4\times0.05$ mm grew in 1 week) were obtained using PEG-2000 (15–20%, w/v) buffered with 0.06–0.08 M PIPES, pH 6.8 at a protein concentration of 3–6 mg/ml. Finally, orthorhombic crystals with the same appearance as those produced by the microbath method, but dimensions up to $2.0\times0.7\times0.2$ mm^3, were reproducibly grown in hanging drops within 2–3 days using PEG-2000 (12–17%, w/v) buffered with 0.05–0.08 M PIPES, pH 6.8 at a protein concentration of 10–12 mg/ml (Fig. 2).

The crystals belong to the orthorhombic space group C222 or C222$_1$ with $a=66.6$ Å, $b=108.4$ Å, $c=125.7$ Å. The Matthews coefficient, V_m, assuming one monomer (35 kDa) in the asymmetric unit is calculated to be 3.24 Å^3/Da which corresponds to a solvent content of 62% [17]. Combined with biochemical evidence this suggest that the protein has crystallized as a perfect 2-fold symmetric dimer. The present dataset was collected to 2.0 Å at room temperature and from one crystal, but diffraction was observed to about 1.8 Å at a longer exposure time than 60 s. Data collection and statistics are summarized in Table 1. The dataset has a completeness of 97.3% between 28.37 and 2.04 Å, with a total of 30 523 independent reflections.

The determination of the crystal structure of hPAH will require the method of multiple isomorphous replacement, since our sequence alignment search with the hPAH sequence suggests no homology to any protein of known three-dimensional structure. A number of potential heavy atom derivatives have been investigated, but they are not fully isomorphous with the native protein and the search is continuing. Furthermore, we have produced a catalytically active selenomethionine-substituted form of the double deletion mutant, which has two methionines of the total 325 amino acids.The X-ray structure analysis of hPAH should provide insight into the catalytic mechanism of its enzymatic function as well as the structurally and functionally homologous enzymes tyrosine hydroxylase and tryptophan hydroxylase (for review, see [18]). Furthermore, it will represent an important frame of reference in the molecular characterization of the enzymatic phenotypes of the observed mutations in the hPAH [2,7,8] and human tyrosine hydroxylase [19,20] genes.

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References

[1] Kaufman S. Adv Enzymol 1993;67:77.
[2] Scriver CR, Kaufman S, Woo SLC. In: The metabolic basis of inherited disease, New York: McGraw-Hill, 1989:495–546.
[3] Okano Y, Eisensmith RC, Güttler F, Lichter-Konecki U, Konecki DS, Trefz FK, Dasovich M, Wang T, Henriksen K, Lou H, Woo SLC. N Engl J Med 1991;324:1232–38.
[4] Hoang L, Byck S, Prevost, L, Schriver, CR. Nucl Acid Res 1996;24:127–31.
[5] Celikel R, Davis MD, Dai X, Kaufman S, Xuong N. J Mol Biol 1991;218:495–98.
[6] Martínez A, Knappskog PM, Olafsdottir S, Døskeland AP, Eiken HG, Svebak RM, Bozzini ML, Apold J, Flatmark T. Biochem J 1995;306:589–97.
[7] Knappskog PM, Eiken HG, Martínez A, Bruland O, Apold J, Flatmark T. Hum Mut 1996;8:236–46.
[8] Knappskog PM, Flatmark T, Aarden JM, Haavik J, Martínez A.

Table 1
Data collection statistics for the native data

Resolution (Å)	Observations (*n*)	Completeness (%)	F > 2σF (%)	F > 4σF (%)	F > 6σF (%)	< F/σF >
28.37-4.91	2173	98	96	95	94	72.52
4.91-3.90	2131	99	98	96	95	77.12
3.90-3.41	2113	99	98	94	92	55.90
3.41-3.10	2077	99	97	88	88	37.86
3.10-2.88	2039	99	97	83	76	28.52
2.88-2.71	2048	98	96	79	71	20.90
2.71-2.57	2019	97	95	73	63	17.46
2.57-2.46	2009	97	94	67	57	14.94
2.46-2.36	2015	98	96	63	52	11.99
2.36-2.28	2029	97	95	60	48	11.08
2.28-2.21	2014	97	95	53	40	9.03
2.21-2.15	2018	98	94	44	34	7.57
2.15-2.09	2021	98	95	39	28	6.43
2.09-2.04	2026	98	94	34	23	5.43
2.04-1.99	1791	87	84	26	17	4.83
28.37-1.99	30523	97.3	97.7	68.4	60.3	26.13

Eur. J Biochem 1996;242:813–21.
[9] Chayen NE, Shaw Stewart PD, Maeder DL, Blow DM. J Appl Cryst 1990;23:297–302.
[10] Chayen NE, Shaw Steward PD, Blow DM. J Cryst Growth 1992;122:176–80.
[11] McPherson, A. In: The preparation and analysis of protein crystals, New York, John Wiley, 1982.
[12] Otwinowski Z. DENZO. A Film Processing Program for Macromolecular Crystallography. New Haven:Yale University, 1991.
[13] Collaborative Computing Project, Number 4. Acta Cryst 1994;D50:760–63.
[14] Citron BA, Davis MD, Kaufman S. Prot Expr Purif 1992;3:93–100.
[15] Bloom LM, Benkovic SJ, Gaffney BJ, Biochemistry 1986;25:4204–10.
[16] Martínez A, Andersson KK, Haavik J, Flatmark T. Eur J Biochem 1991;198:675–82.
[17] Matthews BW. J Mol Biol 1968;33:491–97.
[18] Kappock TJ, Caradonna JP. Chem Rev 1996;96:2659–56.
[19] Knappskog M, Flatmark T, Mallet J, Lüdecke B, Bartholomé K. Hum Mol Gen 1995;4:1209–12.
[20] Lüdecke B, Knappskog M, Clayton PT, Surtees RAH, Clelland J, Heales SJR, Brand MP, Bartholomé K, Flatmark T. Hum Mol Gen 1996;5:1023–28.