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Crystallization and preliminary crystallographic studies of recombinant dimerization cofactor of transcription factor HNF1/pterin-4α-carbinolamine dehydratase from liver

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Abstract The bi-functional protein dimerization cofactor of HNF1 (DCoH)/pterin-4α-carbinolamine dehydratase (PCD) is found in liver cell nuclei bound to the transcription factor hepatocyte nuclear factor 1 (HNF1) as well as in the cytoplasm acting as an enzyme involved in the phenylalanine hydroxylation system. Deficiency of DCoH/PCD activity in liver causes an atypical hyperphenylalaninemia and deficiency in human epidermis is related to the depigmentation disorder vitiligo. DCoH/PCD from rat liver, which is identical to the human protein, was expressed in *E. coli*, purified to homogeneity and crystallized. The crystals belong to the trigonal space group $P3_121$ (or $P3_221$) with unit cell dimensions of $a = b = 106.2$ Å, $c = 197.1$ Å. Native crystals diffract to a resolution of 2.5 Å.

Key words: Transcription factor; Phenylalanine hydroxylation; Biopterin; Crystallization; X-ray crystallography

1. Introduction

Transcription factors are frequently observed to form homo- or heterodimers as a means to ensure specificity and regulatory control of their interaction with cognate DNA binding sites. The dimeric hepatocyte nuclear factor 1 (HNF1, also called LFB1) belongs to a family of homeodomain proteins and regulates the expression of many liver-specific and liver-enriched genes (for reviews see [1–3]).

DCoH was initially co-purified with the HNF1 from rat liver nuclear extracts. The sequence of the 104 amino acids was determined and the cDNAs from rat, mouse and human were cloned. The rat and human sequences are identical and differ in only one residue from that of mouse [4]. Immunoprecipitation experiments showed that DCoH binds to the dimerization domain of HNF1, which is located in the 32 N-terminal amino acid residues. Stabilization of dimeric HNF1 and enhancement of its transcriptional activity were proposed functions of DCoH [3,4].

The cytoplasmic enzyme PCD, which is involved in the phenylalanine hydroxylation system, was isolated from rat and human liver cells, and the amino acid sequence was determined [5,6]. Surprisingly, the PCD sequence turned out to be identical to DCoH, but no homology to any other protein could be detected [4–6]. The only observed difference between DCoH and PCD concerns their oligomerization state. PCD was found to form a homo-tetramer in solution [5,7], whereas DCoH was observed to bind as a dimer to the dimeric HNF1 [4]. The enzymatic function of PCD/DCoH is to catalyze the dehydration of pterin-4α-carbinolamine, which is formed in the tetrahydrobiopterin dependent conversion of phenylalanine to tyrosine. An atypical mild hyperphenylalaninemia characterized by an increased excretion of 7-biopterin in the urine of affected patients is related to a deficiency of PCD/DCoH activity [8–11]. In addition, patients with the depigmentation disorder vitiligo show a lack of PCD/DCoH activity in the affected epidermis [12].

Recently, a prokaryotic homologue of PCD/DCoH was discovered as part of the phenylalanine hydroxylase operon in *Pseudomonas aeruginosa*, where it apparently controls the expression of phenylalanine hydroxylase [13].

In this paper, we report the overexpression, purification, and crystallization of tetrameric PCD/DCoH from rat as well as preliminary crystallographic results.

2. Experimental

2.1. Materials

Kanamycin, DTT, Nonidet -P40, MES and CHES were obtained from Sigma, Pefabloc and IPTG were purchased from Boehringer and Saxon Biochemicals, respectively. All other chemicals were purchased from Merck. For column chromatography an FPLC system (Pharmacia) was used. A plasmid containing the DCoH/PCD gene from rat liver was a generous gift from Dr. M. Yaniv, Paris.

2.2. Protein purification

DCoH/PCD was overexpressed in *E. coli* strain BL21 (DE3) from a pET9d plasmid. Cells were grown at 37° C in 5 l of LB medium. At an A_{600} of 1.2 the cells were induced with 1 mM IPTG for 2 h and harvested by centrifugation at $6000 \times g$ for 30 min. All subsequent steps with exception of the gel filtration were done at 4°C and all buffers contained 0.02% (w/v) sodium azide. 15 g wet cells were suspended in a lysis buffer containing 30 mM Tris-HCl (pH 7.8), 0.5% (v/v) Nonidet P-40, 2 mM EDTA, 1 mM Pefabloc and 2 mM DTT, and disrupted by sonication. The lysate was clarified by centrifugation at $40,000 \times g$ for 20 min, the supernatant again at $100,000 \times g$ for 1.5 h. The $100,000 \times g$ supernatant was subjected to an ammonium sulphate fractionation step. The pellet of the precipitation with 30% sat. ammonium sulphate was discarded and more ammonium sulphate was added to the supernatant to give a final saturation of 50%. The precipitate was pelleted and dissolved in 25 ml buffer containing 20 mM Tris-HCl (pH 7.8), 1 mM EDTA and 1 mM DTT and dialyzed against 5 l of the same buffer overnight. The protein solution was loaded onto a Q-Sepharose Fastflow column (Pharmacia) equilibrated with buffer described above for the dialysis. The column was eluted with a linear gradient of 0–1.0 M NaCl. The DCoH/PCD containing fractions were desalted and concentrated by

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Abbreviations: CHES, cyclohexylaminoethanesulfonic acid; DCoH, dimerization cofactor of HNF1; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; HNF, hepatocyte nuclear factor; IPTG, isopropyl-β-D-thiogalactoside; MES, *N*-morpholinoethanesulfonic acid; MPD, methylpentanediol; PCD, Pterin-4α-carbinolamin dehydratase.

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ultra filtration using Centriprep-10 (Amicon). The protein was applied to a second anion exchange column (MonoQ HR 10/10, Pharmacia) equilibrated with the same buffer as for the Q-Sepharose column, this time at pH 7.0, and eluted with a linear 0–0.3 M NaCl gradient. The DCoH/PCD fraction was concentrated to 10 mg/ml and applied to a Superdex 75 (26/60, Pharmacia) gel filtration column equilibrated in 20 mM Tris/HCl (pH 7.8), 1 mM DTT and 100 mM NaCl. The pure tetrameric DCoH/PCD was finally concentrated to 12 mg/ml in a Centricon-10 (Amicon) and subsequently used for crystallization.

2.3. Crystallization

Crystallization was carried out by the hanging-drop vapour diffusion technique at 18° C. For initial crystallization experiments an automated fast screening crystallization protocol was applied using the described 48 different crystallization conditions [14,15]. All reservoir solutions contained 2 mM DTT, 2 mM EDTA and 0.02% (w/v) sodium azide. Drops were prepared by mixing 2 μl of DCoH solution (12 mg/ml) with 2 μl of the reservoir solution. First small needle like crystals grew in a droplet containing 2.0 M ammonium sulphate, 5% (v/v) MPD and CHES/NaOH (pH 9.5) and they diffracted to a resolution of about 3.5 Å. The pH was screened over a range from 6.4 to 9.5, the concentration of ammonium sulphate optimized and the influence of MPD examined. The best crystals were obtained with a reservoir solution containing 1.44 M ammonium sulphate buffered with 0.1 M MES-NaOH at pH 6.4.

2.4. X-ray diffraction experiments

Crystals were stabilized in 1.7 M ammonium sulphate, 0.1 M MES-NaOH (pH 6.4), 2 mM EDTA, 2 mM DTT and 0.02% (w/v) sodium azide, and mounted in thin walled glass capillary tubes. Diffraction data at 4° C were collected on a 300 mm MAR-research image plate system mounted on a rotating anode X-ray generator and on the prototype 220 mm image plate system mounted on the EMBL beam line X31 at DESY, Hamburg, using synchrotron radiation. Data were processed using the program XDS [16]. Patterson self-rotation functions were calculated using the program POLARRFN [17] which is part of the CCP4 program suite [18].

3. Results and discussion

The overexpression in *E. coli* and purification yielded 45 mg pure DCoH/PCD from 15 g wet cells. Gel filtration chromatography confirmed that DCoH/PCD exclusively exists as a homo-tetramer in solution as previously reported [5,7]. DCoH/PCD crystallizes in the presence of ammonium sulphate as the precipitating agent in the pH interval between pH 6.4 to pH 9.5. The best single crystals are obtained at pH 6.4 from drops containing 2 μl of DCoH/PCD solution (12 mg/ml) mixed with 2 μl of the reservoir solution containing 1.44 M ammonium sulphate. Hexagonal rod like crystals with dimensions up to 0.3 × 0.3 × 0.8 mm grow within 2–3 days (Fig. 1). The crystals diffract to 2.5 Å resolution. Native crystals are resistant to radiation damage and diffract strongly for at least 15 h in the X-ray beam.

The space group and cell dimensions were determined to be

Table 1
Data collection statistics and completeness of the data set

Number of measured reflections	97383	
Number of unique reflections	34118	
R-sym	8.9%	
Completeness	$I > 10\ \sigma(I)$	$I > 2\ \sigma(I)$
for data 15.–2.7 Å	87.8%	81.5%
for data 2.8–2.7 Å	81.7%	68.6%

$R\text{-sym} = \sum_h \sum_i | I_{ih} - < I_h > | / \sum_h \sum_i < I_h >$, where $< I_h >$ is the mean intensity of the i observations of reflection h.

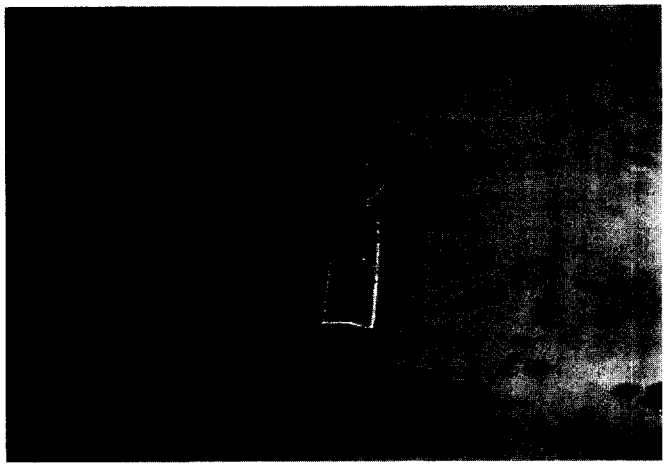

Fig. 1. Crystal of dimerization cofactor of HNF1/pterin-4α-carbinolamin dehydratase. The crystal size is approximately 0.25 × 0.25 × 0.5 mm.

$P3_121$ (or the enantiomorph $P3_221$) with $a = b = 106.2$ Å and $c = 197.1$ Å ($\alpha = \beta = 90°$, $\gamma = 120°$). The Matthews coefficient V_m assuming two tetramers or eight monomers (8 × 12 kDa) in the asymmetric unit is calculated to be 3.3 Å^3/Da which corresponds to a solvent content of 65% [19]. Equally consistent with the unit cell dimensions and space group symmetry is the presence of three tetramers in the asymmetric unit corresponding to a V_m of 2.2 Å^3/Da and 45% solvent content.

A complete native data set was collected from one crystal using synchrotron radiation at a wavelength of 1.0 Å. To prevent overlap of reflections the crystal-to-detector distance had to be adjusted to 270 mm which limits the resolution to 2.6 Å at the edge of the detector. Table 1 lists the data collection statistics and completeness of the processed data set.

Different symmetries are possible for a tetrameric protein. 222 or 2 symmetry are most frequently observed, but proteins with fourfold symmetry, like neuramidase [20], are also known. To analyse local symmetry elements of the DCoH/PCD diffraction data a self-rotation function was calculated in the resolution range 10 to 3 Å using an integration radius in Patterson space from 5 to 15 Å. Several peaks for non-crystallographic twofold ($\kappa = 180°$), threefold ($\kappa = 120°$), and fourfold

Table 2
Self-rotation peaks corresponding to two-, three- and four-fold axes

κ	Ω	Φ	Height*	
180	90.0	0.0	100.0	(7.0 σ)**
180	90.0	90.0	58.8	(4.1 σ)
180	0.0	0.0	58.8	(4.1 σ)
180	34.8	90.0	27.4	(1.9 σ)
180	55.2	30.0	27.4	(1.9 σ)
120	0.0	0.0	100.0	(7.0 σ)**
120	70.5	90.0	27.2	(1.9 σ)
90	0.0	0.0	56.9	(4.0 σ)
90	54.3	30.0	27.3	(1.9 σ)

Polar angles used by the program POLARRFN are defined as Ω: angle from pole, Φ: angle around equator, κ: rotational angle.
*Self-rotation peaks are scaled to a maximum value of 100. The RMS deviation of the mean density (σ) is 14.2.
**These peaks represent crystallographic symmetry axes.

($\kappa = 90°$) axes are found (Table 2), but the exact molecular symmetry still remains ambiguous. For space group $P3_121$ self-rotation peaks corresponding to local two-, three- and fourfold axes are consistent with tetramers having a symmetry of either 2 or 222 or 4 [21].

The determination of the crystal structure of DCoH/PCD will require the method of multiple isomorphous replacement, since a recent sequence alignment search with the rat DCoH/PCD sequence yielded no homology to any protein of known three-dimensional structure.

The X-ray structure analysis of DCoH/PCD should provide insight into the catalytic mechanism of its enzymatic function, the possible structural basis for hyperphenylalaninemia and vitiligo related to DCoH/PCD deficiency, and the mode of interaction with the transcription factor HNF1.

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