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Expression, purification, crystallization and preliminary X-ray diffraction analysis of human uroporphyrinogen decarboxylase

MARTINE LATERRIÈRE,[a] BÉATRICE LANGLOIS D'ESTAINTOT,[a] ALAIN DAUTANT,[a] GILLES PRÉCIGOUX,[a]* ISABELLE HOMBRADOS[b] AND HUBERT DE VERNEUIL[b] *at* [a]*Unité de Biophysique Structurale, EP534-CNRS, Université Bordeaux 1, Talence, France, and* [b]*Laboratoire de Pathologie Moléculaire et Thérapie Génique, Université de Bordeaux 2, Bordeaux, France. E-mail: precigoux@maxime.cristal.u-bordeaux.fr*

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Abstract

A recombinant human uroporphyrinogen decarboxylase (E.C. 4.1.1.37, UROD) has been expressed in *Escherichia coli* and purified to homogeneity. Crystals grew by the hanging-drop vapor-diffusion technique from a starting solution containing 1.5 mg ml^{-1} protein. The crystals belong to the trigonal space group $P3_121$ or its enantiomer $P3_221$ and diffract to 3 Å resolution. The unit-cell parameters are $a = b = 103.4$, $c = 75.7$ Å and $\gamma = 120°$. The asymmetric unit contains one molecule. Preliminary structural predictions suggest for the protein a TIM-barrel type tertiary structure.

1. Introduction

Human uroporphyrinogen decarboxylase (UROD) is a cytosolic enzyme of 367 residues with a calculated molecular weight of 40 831 Da (Roméo *et al.*, 1986). It is the fifth enzyme of the heme biosynthetic pathway which catalyzes decarboxylation of the four carboxylic groups uroporphyrinogen to yield coproporphyrinogen (Smith & Francis, 1979). UROD deficiency is responsible for porphyria cutanea tarda (PCT) and hepatoerythropoïetic porphyria (HEP), the most frequent human porphyrias (Kappas *et al.*, 1995).

Both natural uroporphyrinogen isomers I and III are substrates of UROD and the decarboxylase activity is not associated with the presence of any cofactor (de Verneuil *et al.*, 1983; Straka & Kushner, 1983).

In the N-terminal part of UROD, the hexapeptide Met-Arg-Gln-Ala-Gly-Arg (Met36–Arg41 in the human sequence), appears to be perfectly conserved throughout evolution (Garey *et al.*, 1992) and could be involved in the interaction with the substrate. This hypothesis is supported by the deleterious Gly33→Asp mutation in yeast *Saccharomyces cerevisiae* (equivalent to Gly40 in the human sequence) (Nishimura *et al.*, 1993). At least one thiol group, among the six cysteine residues, could be involved in the decarboxylation process (de Verneuil *et al.*, 1983). Despite extensive studies of the kinetic properties (Tomio *et al.*, 1970; Smith & Francis, 1979; de Verneuil *et al.*, 1980), the mechanism of action remains unclear. A sequential decarboxylation of uroporphyrinogen involving a two-step process with a rapid elimination of one carboxyl group followed by a slower elimination of the three remaining groups has been suggested. In addition it has been postulated that one (Garey *et al.*, 1992) or several (de Verneuil *et al.*, 1980) catalytic sites exist.

Whereas the three-dimensional structures of *Escherichia coli* porphobilinogen deaminase (Louie *et al.*, 1996) and more recently *Bacillus subtilis* ferrochelatase (Hansson & Al-Karadaghi, 1995; Al-Karadaghi *et al.*, 1997), two of the enzymes involved in the heme biosynthetic pathway, have been solved by X-ray diffraction, the three-dimensional structure of UROD remains unsolved.

This work describes overexpression, purification and crystallization of human UROD elongated at the N-terminal end by two residues.

2. Expression and purification

The pGEX-UROD vector (Moran-Jimenez *et al.*, 1996) expresses UROD in *E. coli* as a carboxy-terminal fusion protein to the glutathione S-transferase (GST), allowing its purification by affinity chromatography using glutathione Sepharose 4B. The GST and the protein of interest can be cleaved after purification by thrombin, for which a recognition site (Leu-Val-Pro-Arg-Gly-Ser) is present, at the junction between the two fusion protein moieties.

The fusion protein was expressed from the pGEX-UROD in *E. coli* JM105 cells (Moran-Jimenez *et al.*, 1996). The cells were inoculated in 1 l of LB medium containing 50 µg ml^{-1} ampicillin. The expression of the fusion protein was induced for 3 h by the addition of 0.5 m*M* isopropyl-β-D-thiogalactoside (IPTG) to the medium.

The cells were resuspended in 10 m*M* phosphate buffer, 140 m*M* NaCl, 2.7 m*M* KCl, pH 7.3 supplemented with 5 m*M* dithiothreitol. 0.5%(*w*/*v*) Triton-X100 was used in the first experiments but not in the last ones. The proteins were released from the cells by sonication (4 × 10 s, 273 K) and separated from the bacterial debris by centrifugation (10 000*g*, 15 min, 277 K). After an affinity-chromatography step on a glutathione–Sepharose 4B column, according to the instructions of the manufacturer (Pharmacia), 100 units of bovine plasma thrombin (Sigma) were added directly to the column and incubation was performed during 2 h at 298 K in order to cleave the UROD moiety.

The protein was analyzed by gel electrophoresis in 10% polyacrylamide gels with denaturing conditions (Laemmli, 1970) and Coomassie blue staining. The protein specificity was confirmed by western blotting after transfer to a nitrocellulose membrane. A rabbit antiserum directed against UROD was used together with the combination of an anti-IgG fused to peroxidase in order to allow the detection of the protein by enhanced chemical luminescence (ECL).

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3. Crystallization

The initial screening for the crystallization conditions of UROD was carried out with Crystal Screen and Crystal Screen II sparse-matrix crystallization kits (Hampton Research, Laguna Hills, CA, USA). Some microcrystals were obtained in 0.1 *M* Hepes buffer pH 7.5, 2% PEG 400, 2.0 *M* ammonium sulfate solution. Attempts to grow bigger crystals failed, even when optimizing concentration conditions. A second protein batch was produced, therefore, and purified under modified conditions (without detergent during the last steps) and crystallization conditions were slightly changed. The best crystals were obtained using the hanging-drop vapor-diffusion method from drops containing 2 µl of protein solution (1.5 mg ml^{-1} protein in 2.7 m*M* KCl, 10 m*M* Na_2HPO_4 pH 7.3, 5 m*M* DTT, 2.5 m*M* $CaCl_2$) and 2 µl of reservoir solution (100 m*M* Hepes or 100 m*M* Tris–HCl pH 7.5, 2.0 *M* ammonium sulfate, 1.4–2.2% PEG 550 monomethylether, 3 m*M* NaN_3), equilibrated against 1 ml of reservoir solution, at 293 K. Crystals appear after 2–3 d and grew to a maximum size (0.2 × 0.2 × 0.2 mm) within 4–5 weeks (Fig. 1).

4. Preliminary results and discussion

The crystals belong to the trigonal space group $P3_121$ or its enantiomer $P3_221$ with unit-cell parameters $a = b = 103.43$ and $c = 75.74$ Å. This leads to a calculated V_m value of 2.87 Å^3 Da^{-1} and a solvent content of 57% assuming one protein in the asymmetric unit (Matthews, 1968). These crystals were used for X-ray diffraction at 293 K from a rotating anode (λ Cu *Kα*) and a 300 mm Mar Research image-plate detector (crystal-to-detector distance set to 260 mm). Data corresponding to 33 frames of 2° crystal oscillation with 1200 s exposure time were collected to 3 Å resolution. All images were processed using the *MOSFLM* software (Leslie *et al.*, 1986). Further data analysis was performed with *ROTAVAT/AGROVATA* programs from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). The recorded data set is 95.7% complete from 20 to 3.03 Å. A total of 23 184 reflections were collected and reduced to 8844 independent reflections with an R_{sym} value of 0.136 (Table 1).

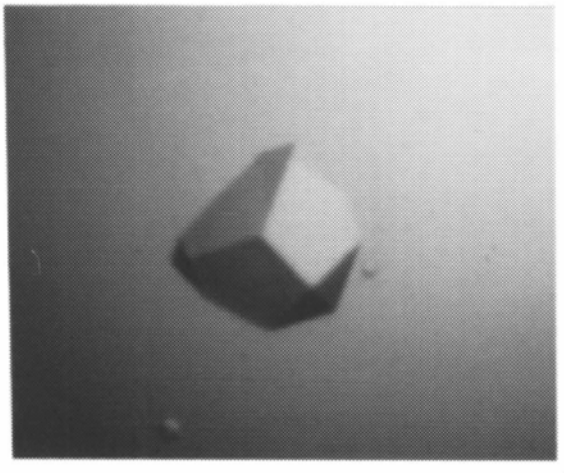

Fig. 1. Photograph of a UROD crystal grown using the hanging-drop vapor-diffusion method. The dimensions are 0.2 × 0.2 × 0.2 mm.

Table 1. *Crystallographic parameters and data-collection statistics*

Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	$a = b = 103.4$, $c = 75.7$
Packing density (Å^3 Da^{-1})	$V_m = 2.87$
Resolution (Å)	3.03
Total observations	23184
Unique reflections	8844
Average $I/\sigma(I)$	5.2
Redundancy	2.8
R_{sym} (I)†	0.136
Completeness for all data (%)	95.7
Completeness in the upper shell (3.19–3.03 Å) (%)	92.7

† $R_{sym} = \sum|I_i - \langle I\rangle|/\sum\langle I\rangle$, where I_i is the intensity of the ith observation, $\langle I\rangle$ is the mean intensity value of the reflection, and the summations are over all reflections.

Since until now no information has been available about any UROD structure, secondary-structure predictions computed by conventional methods were performed on the 13 complete UROD sequences known. The results obtained with the *PHDsec* algorithm (Rost & Sander, 1994) indicate for this enzyme family an α/β type conformation. All UROD's are predicted to have repeated β-strand-turn/loop-α-turn/loop motifs well defined in the C-terminal part of the protein sequence. All β-strands are short and of nearly identical length. Thus, the tertiary structure of UROD should contain an eightfold α/β-barrel topology type (Stultz *et al.*, 1993; White *et al.*, 1994), a fold first observed in triose phosphate isomerase and referred to as a TIM barrel (Banner *et al.*, 1975).

The structure solution is in progress, both by preparation of heavy-atom derivatives, and as stated herein, using molecular replacement with the help of the large structurally known sample of TIM-barrel-containing protein models.

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