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FOR THE RECORD

Characterization and crystallization of human uroporphyrinogen decarboxylase

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Abstract: The cytosolic enzyme uroporphyrinogen decarboxylase (URO-D) catalyzes the fifth step in the heme biosynthetic pathway, converting uroporphyrinogen to coproporphyrinogen by decarboxylating the four acetate side chains of the substrate. Recombinant human URO-D has been expressed in *Escherichia coli* with a histidine tag and has been purified to homogeneity. Purified protein was determined to be a monodisperse dimer by dynamic light scattering. Equilibrium sedimentation analysis confirmed that the protein is dimeric, with a dissociation constant of 0.1 μM. URO-D containing an amino-terminal histidine tag was crystallized in space group $P3_121$ or its enantiomer $P3_221$ with unit cell dimensions $a = b = 103.6$ Å, $c = 75.2$ Å. There is one molecule in the asymmetric unit, consistent with generation of the dimer by the twofold axis of this crystallographic operator. Native data have been collected to 3.0 Å resolution.

Keywords: crystallization; monomer–dimer equilibrium; uroporphyrinogen decarboxylase; X-ray diffraction

The formation of tetrapyrroles and their subsequent modification into heme, siroheme, corrins, and chlorophyll, is a multi-step process that has been highly conserved throughout evolution (Beale & Weinstein, 1990). The fifth step in heme biosynthesis is catalyzed by the enzyme uroporphyrinogen decarboxylase (EC 4.1.1.37) (Kappas et al., 1989). Uroporphyrinogen is either converted to coproporphyrinogen and then to heme or chlorophyll, or it is modified to form the corrins, siroheme, and vitamin B_{12} (Beale & Weinstein, 1990).

URO-D catalyzes the stepwise decarboxylation of the four acetate side chains of uroporphyrinogen to generate coproporphyrinogen (Fig. 1). There are no known cofactors or prosthetic groups required for enzymatic activity (de Verneuil et al., 1983; Straka & Kushner, 1983). Two isomers of uroporphyrinogen exist in nature (isomers I and III), and both are URO-D substrates. Porphyrinogens with 7, 6, or 5 carboxyl groups are also substrates for URO-D. The mechanism(s) of decarboxylation is not known, but models involving either single or multiple catalytic sites for URO-D have been suggested (Kappas et al., 1989).

The gene for human URO-D encodes a cytosolic protein of 367 residues with a predicted molecular weight of 40,831 Da (Romeo et al., 1986). The protein does not appear to be posttranslationally modified in vivo. A comparison of URO-D sequences from 12 species including bacteria, plants, yeast, and mammals shows overall 10% identity and 33% similarity, with a highly conserved stretch of residues near the amino terminus and smaller regions of conservation scattered throughout the remainder of the protein (Wyckoff et al., 1996). Mutations in human URO-D have been identified in patients with porphyria cutanea tarda, the most common form of porphyria in humans (Kappas et al., 1989). Previous estimates of URO-D molecular weight, based on size-exclusion chromatography, have suggested that the human, bovine, and bacterial proteins are monomeric (de Verneuil et al., 1983; Elder et al., 1983; Straka & Kushner, 1983; Juknat et al., 1989). In contrast, chicken URO-D was reported to be a dimer (Seki et al., 1986).

We report here the expression and purification of recombinant human URO-D. Analysis by equilibrium sedimentation and dynamic light scattering indicates that the protein is a dimer with $K_d = 0.1$ μM. Recombinant human URO-D has been crystallized in a space group that is consistent with formation of a dimer.

Results and discussion: *Characterization of purified URO-D:* URO-D was expressed to high levels from the inducible T7 promoter (Studier et al., 1990) in the expression vector pAED4 (Wyckoff et al., 1996). The histidine-tagged URO-D (URO-Dt) has a predicted molecular weight of 43,308 Da and was purified to approximately 95% homogeneity in a single step. The protein migrates as a single band on SDS-PAGE, with an estimated molecular weight of 44 kDa. Amino acid analysis confirmed the addition of the 21 amino acids encoded by the histidine tag. Mass spectrom-

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Abbreviations: MES, 2-[N-morpholino]ethanesulfonic acid; MPD, 2-methyl-2-4-pentanediol; β-ME, β-mercaptoethanol; URO-D, uroporphyrinogen decarboxylase; URO-Dt, uroporphyrinogen decarboxylase containing a histidine tag on the amino terminal end; IPTG, isopropylthio-β-galactoside.

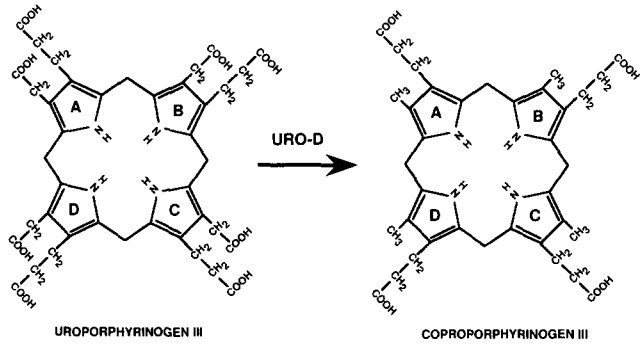

Fig. 1. Enzymatic conversion of uroporphyrinogen III to coproporphyrinogen III. The acetate side chains of uroporphyrinogen III are decarboxylated to methyl groups. In the type I isomer, the acetate and propionate side chains of the D ring are ordered as on the other pyrrole rings, and the molecule is symmetrical.

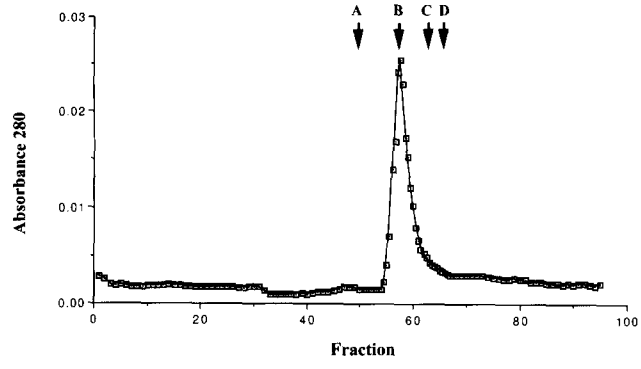

Fig. 2. Characteristic elution pattern of URO-Dt on S-200 matrix. A 200-μL sample of URO-Dt, at 2.0 mg/mL was analyzed on a Pharmacia Superdex 200 16/60 sizing column. The column was calibrated using a MW-GF-200 kit from Sigma Chemical (St. Louis, Missouri), as directed. The peak positions for the standards are indicated at the top of the graph, the estimated molecular weight of each is: A, alcohol dehydrogenase, 150 kDa; B, bovine serum albumin, 66 kDa; C, carbonic anhydrase, 29 kDa; D, cytochrome *c*, 12.4 kDa.

etry indicated that approximately 85% of the URO-Dt has the N-terminal methionine cleaved and the estimated molecular weight is 43,184 ± 6.8 Da. The final recovery of soluble URO-Dt from a 6-L *Escherichia coli* culture, induced for 4 h, was 12–17 mg.

Presence of the histidine tag does not modify enzymatic activity of the recombinant protein. The specific activity of URO-Dt was 8,645 ± 461 nmol/mg/h, using uroporphyrinogen I as a substrate at 30 μM (Straka et al., 1982). This activity is comparable to that of native URO-D purified from human red cells (de Verneuil et al., 1983) and of the recombinant protein expressed without the histidine tag (Wyckoff et al., 1996). The enzymatic activity of URO-Dt was also the same as wild-type URO-D when assayed with the substrates uroporphyrinogen III and pentacarboxyl porphyrinogens of the I and III isomers.

Gel filtration chromatography on URO-D from many sources usually indicates an apparent molecular weight that is 1.5 times the estimate obtained from denaturing SDS-PAGE (Wyckoff & Kushner, 1994). These results generally have been interpreted to indicate that URO-D is a monomeric protein with a nonideal Stokes radius. Our analysis of URO-Dt on a S-200 gel filtration column showed that the protein has an apparent molecular weight of 66 kDa (1.5 times the monomeric molecular weight), consistent with results from native human URO-D (de Verneuil et al., 1983; Elder et al., 1983). Inspection of the chromatogram reveals a pronounced asymmetry, with the URO-Dt peak displaying a sharp leading edge and a tapering trailing edge (Fig. 2). This peak shape is expected for multimers that are dissociating during the gel filtration experiment (Ackers & Thompson, 1965). This observation is consistent with the results from light scattering and equilibrium sedimentation, described below, which show that URO-D exhibits a monomer:dimer dissociation constant of 0.1 μM.

Physical properties of URO-Dt: Dynamic light scattering analysis indicated that purified URO-Dt was monodisperse in solution with an estimated hydrodynamic radius of 4.1 nm and a polydispersity distance of 1.1 nm. The predicted molecular weight is 87–92 kDa, suggesting that URO-Dt is dimeric under these conditions. A parallel experiment with wild-type URO-D, purified as described by Wyckoff et al. (1996), also indicated an apparent molecular weight of 92–98 kDa, with a hydrodynamic radius of 4.0 nm and a polydispersity of 0.9 nm. These data indicate that the histidine tag does not effect dimerization.

To determine the associative properties of URO-D more precisely, equilibrium sedimentation analysis was performed on samples of both wild-type URO-D and URO-Dt. This method provides estimates of the molecular weight that are independent of the shape of the protein. Samples of protein at 1.2, 2.3, and 11.5 μM in 100 mM NaCl, 50 mM Tris, pH 7.5, 1 mM β-ME were analyzed. Samples were spun to equilibrium in a Beckman Optima XL-A analytical ultracentrifuge at 20 °C. The data were in good agreement with a self-associating monomer:dimer model possessing an estimated equilibrium constant of 0.11 ± 0.06 μM (Fig. 3).

Crystallization of recombinant URO-Dt: Crystals of URO-Dt were grown by sitting drop vapor diffusion at 21 °C. Crystals grew in 48 h to approximate dimensions of 0.1 × 0.2 × 0.2 mm. A complete data set was collected to 3.0 Å resolution and data were processed with DENZO and SCALEPACK (Otwinowski, 1993) (Wladek Minor) (see Table 1). The space group was determined to be $P3_121$, or its enantiomer $P3_221$, with $a = b = 103.6$ Å, $c = 75.2$ Å. Weak reflections were visible to 1.9 Å resolution. The Matthew's coefficient is 2.6 Å^3/Da, suggesting that there is a monomer in the asymmetric unit (Matthews, 1968). The URO-D dimer is presumably generated by the crystallographic twofold axis.

Knowledge that URO-D is a dimer, together with the crystal structure, should clarify some vexing questions about the enzyme. For example, URO-D can utilize any of eight substrates, (8-, 7-, 6-, and 5-COOH) porphyrinogens of either the I or III isomer series. Also, kinetic analyses of URO-D have suggested that the enzyme might have multiple active sites, although no repetitive domains have been recognized in the amino acid sequence. In addition, the kinetic effects of naturally occurring point mutations in the autosomal dominant form of porphyria cutanea tarda generally have been studied using recombinant mutant URO-Ds, meaning that only mutant homodimers have been studied. However, in vivo, normal-mutant heterodimers might exist and the enzymatic activity and the stability of such heterodimers might differ from normal homodimers.

URO-D likely possesses a novel catalytic mechanism because, in contrast to most characterized decarboxylases, it appears to be devoid of cofactors (Straka & Kushner, 1983). A detailed three-

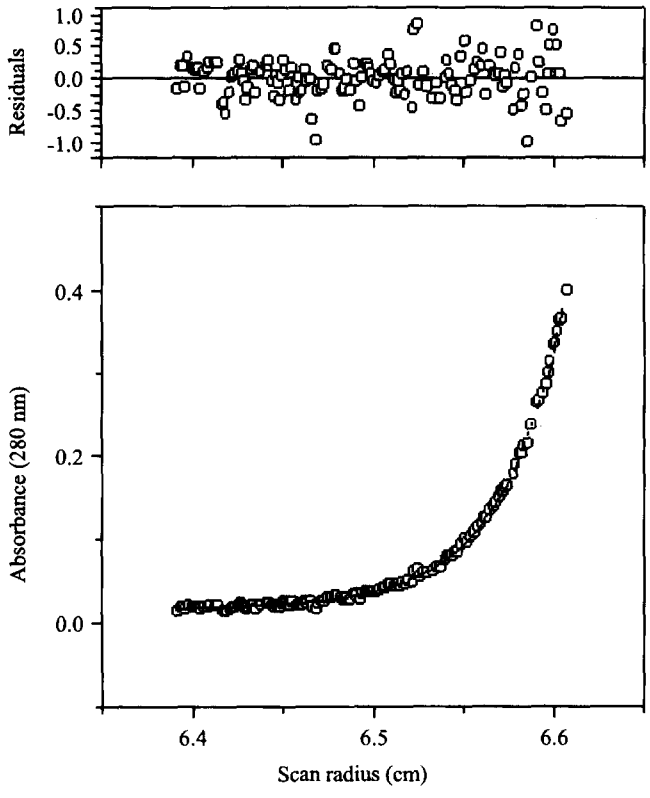

Fig. 3. Analysis of URO-Dt by equilibrium sedimentation. Data are shown for a sample of URO-Dt at 2.3 μM, spun at 17,000 RPM for 42 h and scanned at 280 nm. The equilibrium sedimentation data are fit to a self-associating monomer–dimer equilibrium model using a nonlinear least-squares curve fitting algorithm contained in the XL-A Data Analysis Software. Residuals are shown in the top panel for the best fit curve.

dimensional structure will provide a framework for understanding the basis of substrate specificity and catalytic mechanisms. Efforts to solve the structure by multiple isomorphous replacement are underway.

Materials and methods: *Expression and purification of URO-Dt:* A cDNA encoding human URO-D was amplified, using PCR with primers containing an *Nde* I and *EcoR* I restriction site at the 5′ and 3′ ends, respectively. The product was cloned into the inducible pAED4 expression vector using the engineered restriction sites. A His 10 tag was cut from pET16b (Novagen, Madison, Wisconsin) on an *Xba* I/*Nde* I fragment and cloned in frame to the N-terminus of the URO-D coding region by digestion with *Xba* I and *Nde* I to make the expression vector pHT#77. The complete open reading frame was confirmed by sequencing. The expression vector was transformed into the *E. coli* strain BL21(DE3)plysS (Novagen, Madison, Wisconsin) for protein production. A 6-L culture of *E. coli* was grown to an OD_{600} of 0.5 in LB media and then induced with 400 μM IPTG for 4 h. The cell pellet was harvested by centrifugation and stored at −80 °C. Cells were thawed and resuspended in 30 mL sonication buffer (300 mM NaCl, 50 mM $NaPO_4$, pH 6.8, 10% glycerol) and disrupted by sonication. The membrane fraction was removed by centrifugation at 12,000 × *g* for 30 min. The supernatant was loaded onto a 1 mL Ni^{2+}-NTA (Qiagen, Chatsworth, California) column connected to an FPLC system (Pharmacia). The column was then washed with 40 mL of buffer A (300 mM NaCl, 50 mM $NaPO_4$, pH 6.0, 10% glycerol, 1 mM β-ME), followed by 30 mL of buffer A containing 70 mM imidazole. The protein was eluted with 25 mL of buffer A containing 250 mM imidazole. Fractions containing URO-Dt were concentrated to approximately 6 mg/mL using Centriprep concentrators (Amicon, Beverly, Massachusetts) and then dialyzed three times against 500 volumes of 50 mM Tris, pH 7.5, 10% glycerol, 1 mM β-mercaptoethanol, and stored at 4 °C.

Analysis of URO-Dt by dynamic light scattering: Samples of URO-D and URO-Dt were diluted to approximately 3 mg/mL (70.0 μM) in 50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM β-ME, and then filtered (0.1 μm) prior to analysis. Twenty readings were taken for each protein sample at 20 °C in a DynaPro-801TC (Protein Solutions, Charlottesville, Virginia).

Crystallization of URO-Dt: The protein solution contained URO-Dt at 6 mg/mL in 50 mM Tris, pH 7.5, 10% glycerol, 1 mM β-ME. The reservoir solution contained 16% MPD in 0.1 MES, pH 6.0. The drops contained 5 μL of protein solution, mixed with 2 μL of reservoir solution. For data collection, crystals were suspended in rayon loops and cryo-cooled by plunging into liquid nitrogen, without need for an additional cryoprotectant solution. Data were collected at 120 K on an RAXIS-II image plate detector mounted on a rotating anode generator.

Table 1. *Data collection statistics*[a]

	30.00–3.00 Å	3.05–3.00 Å
Observations	43,953	14,528
Unique reflections	9,385	465
Average $I/\sigma(I)$	9.0	2.9
Completeness	99.2%	99.6%
$I/\sigma(I) > 3$	74.9%	48.0%
$R_{merge}(\Sigma\|I_i - \langle I\rangle\|\Sigma i_i)$	11.4%	32.6%
Mosaicity	0.497°	

[a]Data were collected at 120 K on a rotating-anode generator, using an RAXIS-II image plate detector. Ninety contiguous 1° rotation images were collected.

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