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Crystallization and preliminary X-ray diffraction analysis of a flavoenzyme amine dehydrogenase/oxidase from *Pyrococcus furiosus* DSM 3638

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A flavoprotein amine dehydrogenase/oxidase with subunit molecular weights of 54.8 kDa (α-subunit) and 42.4 kDa (β-subunit) and specificity for L-proline was cloned from the genomic DNA of the hyperthermophilic marine archaeon *Pyrococcus furiosus* DSM 3638. The enzyme was overexpressed in *Escherichia coli* and purified to homogeneity. The enzyme was crystallized using the sitting-drop vapour-diffusion technique. Diffraction data from two different crystal forms were collected to 3.3 and 3.6 Å, respectively, using synchrotron radiation. Both crystals belonged to space group $P1$, with unit-cell parameters a = 91.3, b = 136.3, c = 203.8 Å, α = 94.5, β = 99.4, γ = 102.7° and a = 93.7, b = 116.3, c = 126.9 Å, α = 97.3, β = 99.9, γ = 104.6°.

1. Introduction

The mechanisms of amine oxidation catalysed by the quinoprotein amine dehydrogenase/oxidases are well established. Oxidation occurs through the formation of enzyme–substrate covalent adducts with topaquinone (TPQ), tryptophan tryptophylquinone (TTQ), cysteine tryptophylquinone (CTQ) and lysine tyrosyl quinone (LTQ) redox centres (Mure *et al.*, 2002; Datta *et al.*, 2001; Satoh *et al.*, 2002; Davidson, 2000; Wang *et al.*, 1996) and hydrogen transfer by quantum-mechanical tunnelling has been demonstrated for some quinoprotein enzymes (Masgrau *et al.*, 2004). The mechanism of amine oxidation by flavoproteins is less well understood (Scrutton, 2004). These enzymes catalyse the two-electron oxidation of amine substrates to the corresponding iminium ion. Mechanisms involving (i) proton abstraction by an active-site base to generate a carbanion species (Rohlfs & Hille, 1994), (ii) an aminium radical cation species (Silverman, 1995), (iii) H-atom abstraction by an active-site radical species (Edmondson, 1995) and (iv) nucleophilic attack by the substrate nitrogen on the flavin C4a atom followed by proton abstraction by an active-site base or the flavin N5 atom (Kim *et al.*, 1993) have been considered over the years, but controversy remains in the field. Further structural elucidation of flavoprotein amine dehydrogenase/oxidases coupled with detailed spectroscopic and kinetic studies are required to unravel further the mechanistic details of amine oxidation by this class of enzyme.

We have identified a putative flavoprotein amine dehydrogenase/oxidase from *Pyrococcus furiosus* DSM 3638. The β-subunit of this putative amine oxidase is related to the active-site region of dimethylglycine oxidase (DMGO) of *Arthrobacter globiformis*, for which a crystal structure has been elucidated and a mechanism of substrate oxidation proposed (Leys *et al.*, 2003). The *P. furiosus* enzyme oxidizes sarcosine, L-pipecolic acid and L-proline, but is not reactive with dimethyglycine, glycine or betaine. Kinetic studies suggest that L-proline is the natural substrate, but the enzyme is not related in sequence to the structurally characterized and bifunctional proline dehydrogenase of *Escherichia coli* (Lee *et al.*, 2003). The flavoprotein amine dehydrogenase/oxidase from *P. furiosus* appears to represent a new class of proline-oxidizing flavoprotein. To further our understanding of the mechanism and structure of flavoprotein amine dehydrogenase/oxidases, we have initiated a structural study of this novel flavoprotein amine dehydrogenase/oxidase from *P. furiosus*. We have expressed the recombinant enzyme in *E. coli* and

Table 1
Data-collection and processing statistics for the form 1 and form 2 crystals of recombinant *P. furiosus* flavoprotein amine oxidase/dehydrogenase.

Crystal form	1	2
Synchrotron radiation	Beamline ID 14-3, ESRF	Beamline ID 14-3, ESRF
Space group	*P*1	*P*1
Wavelength (Å)	0.931	0.931
Unit-cell parameters		
a (Å)	93.7	91.3
b (Å)	116.3	136.3
c (Å)	126.9	203.8
α (°)	97.3	94.5
β (°)	99.9	99.4
γ (°)	104.6	102.7
Matthews coefficient (Å^3 Da^{-1})	3.34	3.12
Resolution (Å)	30–3.6 (3.8–3.6)	30–3.3 (3.4–3.3)
$R_{\rm merge}$ (%)	0.120 (0.432)	0.084 (0.357)
Completeness (%)	96.4 (95.0)	96.0 (93.0)
Average $I/\sigma(I)$	6.2 (1.8)	8.2 (2.0)
Average redundancy	2.5 (2.3)	1.9 (1.8)
Unique reflections	55367	132489

purified the protein to homogeneity. Diffraction data are reported to 3.3 and 3.6 Å for two crystal forms both in space group *P*1.

2. Materials and methods

2.1. Cloning, overexpression and purification

Two open reading frames [gi|18977617 and gi|18977618; protein-extraction description and analysis tool (PEDANT) database] were identified in the genome of *P. furious* DSM 3638 that encode a putative flavoenzyme amine dehydrogenase/oxidase and were amplified by the polymerase chain reaction. Both genes encoding the α- and β-subunits (492 and 382 amino-acid residues, respectively) were cloned into pET11d (Novagen) and the resultant construct transformed into *E. coli* strain Rosetta(DE3)pLysS for recombinant expression. Details of plasmid construction and propagation will be reported elsewhere. Selected transformed cells were grown in 12 l of 2×YT medium containing chloramphenicol (34 µg ml^{-1}) and ampicillin (50 µg ml^{-1}) at 310 K to an $OD_{600} \simeq 0.8$ and were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 m*M*. Incubation took place for a further 8 h, after which cells were harvested and resuspended in 50 m*M* ice-cold potassium phosphate buffer pH 8.0 containing a Complete protease-inhibitor cocktail tablet (Roche). The cell suspension was incubated with lysozyme (10 µg ml^{-1}) and sonicated to effect cell breakage.

Table 2
Peaks observed in the self-rotation analysis of the form 1 crystals.

The polar angles θ, φ and χ are defined as follows: θ is the angle between the rotation axis and z, φ is the angle in the xy plane between x and the projection of the rotation axis and χ is the rotation angle around the rotation axis.

Peak	θ (°)	φ (°)	χ (°)	Rf/σ
1	86.6	−30.9	180	10.6
2	127.9	57.1	180	10.5
3	38.3	64.3	180	9.7

Deoxyribonuclease I (∼10 µg) was added and the suspension was incubated for a further 30 min at 277 K. Cellular debris was removed by centrifugation and the supernatant was incubated at 353 K for 1 h followed by centrifugation to remove denatured host-cell proteins. The supernatant was dialysed exhaustively against 50 m*M* Tris–HCl pH 8.0 at 277 K, loaded onto a Q-Sepharose column (75 ml bed volume) and eluted using a 0.2–0.4 *M* gradient of NaCl. Fractions containing recombinant protein were pooled, concentrated and applied onto a Superdex 75 column (330 ml bed volume); protein was eluted at a flow rate of 0.75 ml min^{-1}. Purified enzyme was exchanged into 10 m*M* Tris–HCl buffer pH 7.5 containing 100 m*M* KCl, concentrated to 12.2 mg ml^{-1}, sterilized (0.22 µm Millex-GP Acrodisc filter, Millipore) and stored on ice at 277 K. Protein concentration was determined by the method of Bradford (1976). Purity was >95% as judged by SDS–PAGE.

2.2. Crystallization

Crystallization trials were performed using the sitting-drop vapour-diffusion technique. Drops were prepared by mixing 2 µl of 6.1 mg ml^{-1} protein solution with 2 µl reservoir solution. After 3–4 d incubation at 292 K, yellow crystals were observed in two conditions containing 0.2 *M* potassium thiocyanate, 8% PEG 20K, 8% PEG 550 MME pH 7.5, 100 m*M* Tris (crystal form 1; Fig. 1) and 0.2 *M* sodium formate, 8% PEG 20K and 8% PEG 550 MME pH 8.5, 100 m*M* Tris (crystal form 2; Fig. 2) in the reservoir solution. The average dimensions of these crystals were 40 × 250 × 250 µm (form 1) and 50 × 50 × 200 µm (form 2).

Prior to data collection at 100 K, selected crystals were soaked in mother liquor with 10% PEG 200 as cryoprotectant and flash-cooled in liquid nitrogen.

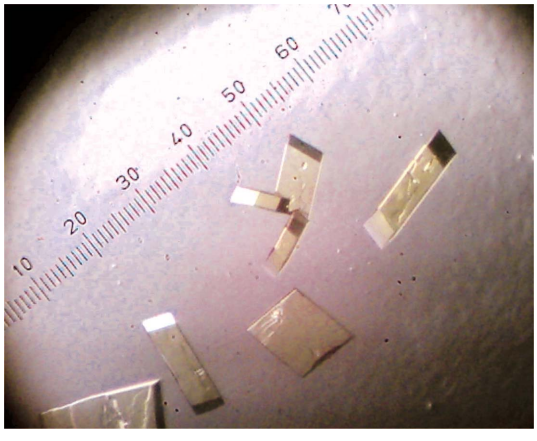

Figure 1
Crystals (form 1) of the recombinant flavoprotein amine oxidase/dehydrogenase from *P. furiosus*.

Figure 2
Crystals (form 2) of the recombinant flavoprotein amine oxidase/dehydrogenase from *P. furiosus*.

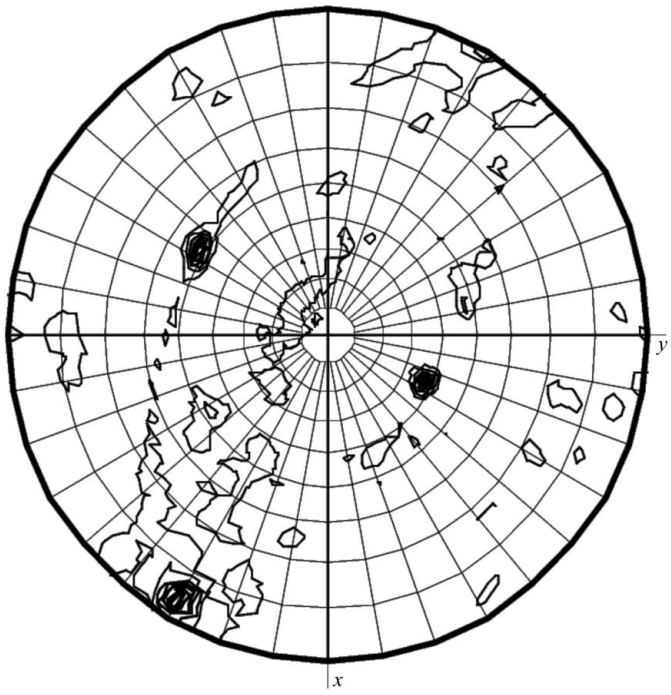

Figure 3
Self-rotation function calculated using crystal form 1 data between 30 and 3.6 Å with a 50.0 Å radius of integration. Twofold symmetry is evident from the very strong peaks in the $\chi = 180^\circ$ plot.

2.3. X-ray diffraction analysis

Diffraction data were collected from cryocooled crystals (100 K) using a Quantum ADSC (Area Dectector Systems Corporation) CCD detector on beamline ID 14-3 at the European Synchrotron Radiation Facility, Grenoble, France. Data were collected using 1° oscillations with the crystal-to-detector distance set to 230 mm. All data were indexed, integrated and scaled using the programs *DENZO* and *SCALEPACK* from the *HKL* software package (Otwinowski & Minor, 1997). Crystals of form 1 diffracted to a resolution of 3.6 Å and belonged to space group *P*1, with unit-cell parameters $a = 93.7, b = 116.3, c = 126.9$ Å, $\alpha = 97.3, \beta = 99.9, \gamma = 104.6^\circ$. Crystals of form 2 diffracted to a resolution of 3.3 Å and belonged to space group *P*1, with unit-cell parameters a = 91.3, b = 136.3, c = 203.8 Å, α = 94.5, β = 99.4, γ = 102.7°. Diffraction data-collection statistics are given in Table 1. Using the program *MOLREP* (Vagin, 1997), a general self-rotation function was computed for a number of κ angles (60, 90, 120 and 180°) to test for the presence of non-crystallographic twofold, threefold, fourfold and sixfold axes. A self-rotation function of the form 1 data set indicates the presence of three perpendicular twofold axes in the unit cell, which strongly suggests the protein is a heterooctamer, with heterodimers arranged in 222 non-crystallographic symmetry (Table 2, Fig. 3). Four heterodimers (one heterooctamer) per unit cell, totalling 388.8 kDa, corresponds to a Matthews coefficient of 3.34 Å^3 Da^{-1} and a solvent content of ∼62.8% (Matthews, 1968). Self-rotation functions of data from form 2 did not reveal any clear indication of the presence of non-crystallographic symmetry. However, two heterooctamers per unit cell, totalling 777.6 kDa, corresponds to a Matthews coefficient of 3.12 Å^3 Da^{-1} and a solvent content of ∼60.2%.

Molecular-replacement trials performed with the available structures of similar flavin oxidases as the starting model failed to identify a solution. Therefore, MIR (multiple isomorphous replacement)/MAD (multiwavelength anomalous diffraction) experiments are planned to provide sufficient experimental phasing information. A search for heavy-atom derivatives and the production of selenomethionine-substituted protein are in progress.

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