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Crystallization and preliminary X-ray diffraction analysis of a dihaem cytochrome *c* peroxidase from *Paracoccus denitrificans*

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Cytochrome *c* peroxidase was isolated from *Paracoccus denitrificans* and purified to homogeneity in three steps prior to crystallization. Two different diffraction-quality crystal forms were obtained by the hanging-drop vapour-diffusion method using a number of screening conditions. The best (needle-shaped) crystal form is suitable for structural studies and was grown from solutions containing 20% PEG 8000, 0.1 *M* Tris pH 8.5 and 0.2 *M* $MgCl_2$. Crystals grew to a maximum length of approximately 0.7 mm and belong to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 78.3, b = 51.0, c = 167.2 Å, β = 97.9°. After a dehydration step and extensive optimization of the cryocooling conditions, a complete data set was collected to 2.2 Å from a native crystal of the fully oxidized form of the enzyme using synchrotron radiation.

1. Introduction

Peroxidases are a large and ubiquitous family of detoxifying enzymes. We are interested in cytochrome *c* peroxidases (CCPs), particularly from Gram-negative bacteria. *Paracoccus denitrificans* is a Gram-negative bacteria that expresses two types of peroxide-removal system: a cytoplasmic catalase and a periplasmic cytochrome *c* peroxidase. At least five bacterial cytochrome *c* peroxidases (CCPs) have been isolated and studied. Most of the bacterial CCPs that have been isolated and characterized are soluble in the periplasm. Very recently, however, *Neisseria gonorrhoeae* CCP has been shown to be linked to the membrane (Turner *et al.*, 2003). The structures of two CCPs have been solved by X-ray crystallography: those of *Pseudomonas aeruginosa* at 2.4 Å resolution (Fülöp *et al.*, 1995; PDB code 1eb7) and *Nitrosomonas europaea* at 1.8 Å resolution (Shimizu *et al.*, 2001; PDB code 1eqc). Almost all bacterial CCPs (except for that from *N. europaea*; Arciero & Hooper, 1994) are isolated in an inactive state called the oxidized or resting state, in which both haem irons are hexacoordinated. They need to be activated by an electron-donor partner, a cytochrome *c* or a copper protein (azurin or pseudoazurin). *N. europaea* CCP binds hydrogen peroxide directly, with no need for an activation step. Most of the bacterial CCPs are strongly dependent on the presence of calcium in their environment. One calcium-binding site has been found in the *P. aeruginosa* cytochrome *c* peroxidase (*Psa* CCP) structure (Fülöp *et al.*, 1995). Two calcium sites have been proposed for the enzyme from *P. denitrificans* (*Pad* CCP). One of these is low affinity and mostly unoccupied in the isolated oxidized enzyme, but the site becomes high affinity in the mixed-valence enzyme and calcium ion is required in this site for the enzyme to be active (Gilmour *et al.*, 1995). In a recent paper, Timóteo *et al.* (2003) also inferred the presence of a second calcium-binding site of lower affinity in *Pseudomonas stutzeri* CCP. Despite the high sequence similarity between bacterial cytochrome *c* peroxidases, they show varying catalytic and biochemical behaviour (Fülöp *et al.*, 2001). Determination of the *Pad* CCP structure will allow a better understanding of the role of calcium in the structure and in the activity of this protein and its relatives.

2. Material and methods

2.1. Sample preparation

Pad CCP was directly isolated from the periplasm of *P. denitrificans* (now known as *P. pantotrophus*; Rainey, 1994) and was purified using anion-exchange, hydroxyapatite and gel-filtration chromatography as previously described in Goodhew *et al.* (1990). After purification, the purity ratio, estimated by the ratio $A_{409\,nm}/A_{280\,nm}$, was about 5.2. The redox state of the protein was checked by an absorbance spectrum from 250 to 750 nm. The CCP concentration was calculated by measurement of the absorbance at 409 nm in the oxidized form ($E_{409\,nm}$ = 250 mM^{-1} cm^{-1}). The purity and integrity of the sample were assessed on an SDS–PAGE gel with Coomassie blue R and haem staining (Goodhew *et al.*, 1990). The weight of the holoprotein measured by electrospray ionization mass spectrometry was

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37 512.3 Da, in very good agreement with the calculated sequence weight of 37 512.8 Da including two haems (Hu *et al.*, 1997). Prior to crystallization trials, the protein was concentrated to 550 µ*M* in 10 m*M* MES pH 6.0 using a Microcon Ultrafree concentrator with a 10 kDa cutoff membrane (Millipore).

2.2. Crystallization

An initial crystallization screen was performed using Hampton Research Crystal Screens 1 and 2 at 291 K with the hanging-drop vapour-diffusion method. 0.5 µl protein sample and 0.5 µl precipitant were mixed and equilibrated with 0.2 ml precipitant in the well of an XRL plate (Molecular Dimensions). Rectangular crystals appeared after a few days under the conditions 18–20% PEG 5000 MME, 0.1 *M* HEPES (or Tris–HCl) pH 7.5, 10 m*M* $CaCl_2$ (type I; Fig. 1*a*). Further trials were performed using Emerald Biostructures Wizard I and II screens after the addition of 10 m*M* $CaCl_2$ to each well. Very thin needle-shaped crystals appeared within 1 d in Wizard II condition No. 3 (20% PEG 8000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* $MgCl_2$). This condition was further optimized by the addition of 0.5 µl of one of the following solutions directly to the crystallization drops: (i) 0.1 *M* Tris–HCl pH 8.2, 0.05 *M* NaCl, 0.1 *M* urea, (ii) 0.1 *M* HEPES pH 7.5, 0.05 *M* LiCl, 0.1% CHAPS and (iii) 0.1 *M* sodium acetate, 0.1 *M* KCl or 0.1% *n*-octyl-β-D-glucoside. In the presence of any of these additive solutions, needle-shaped crystals appeared in the drops within one to three weeks and reached their final size (0.08 × 0.1 × 0.7 mm) within two to three months (type II; Fig. 1*b*).

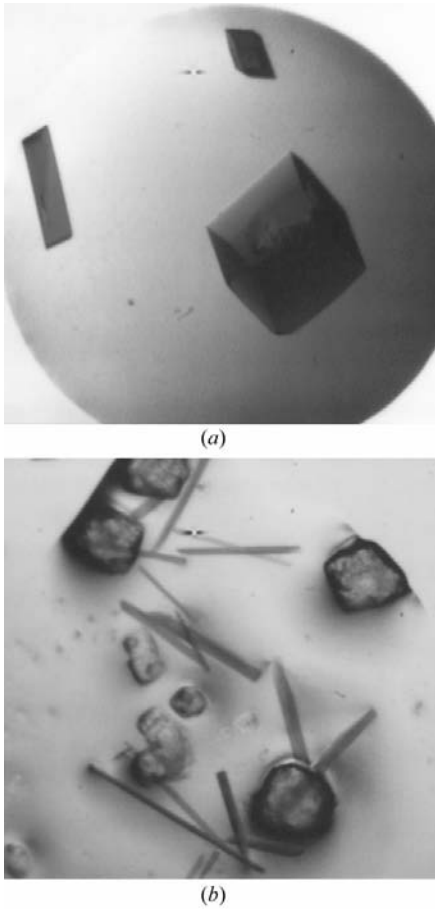

Figure 1
Photographs of *Pad* CCP crystals: (*a*) type I, (*b*) type II. The largest dimension is 0.7 mm for both types.

Table 1
Data-collection and processing statistics.

Values in parentheses correspond to the outer resolution shell.

	Type I	Type II
Synchrotron radiation, detector and wavelength (Å)	DESY BW7B, MAR 345 IP, 1.1037	ESRF ID29, ADSC Q4 CCD, 0.9785
Space group	*P*1	$P2_1$
Unit-cell parameters		
a (Å)	58.6	78.3
b (Å)	79.1	51.0
c (Å)	88.9	167.2
α (°)	103.7	
β (°)	109.2	97.9
γ (°)	99.2	
Matthews coefficient† ($Å^3$ Da^{-1})	2.4	2.2
Molecules per AU	4	4
Solvent content (%)	48	43
Resolution range (Å)	28–2.4 (2.49–2.4)	49–2.2 (2.28–2.2)
Total observations	165250	281141
Unique reflections	53268	65609
Average $I/\sigma(I)$	14.7 (3.4)	10.6 (2.1)
R_{merge} (%)	0.093 (0.142)	0.140 (0.532)
Completeness	96.3 (88.7)	97.7 (96.6)

† Matthews (1968).

2.3. X-ray data collection and analysis

Initial diffraction experiments were carried out using Cu $K\alpha$ radiation from a rotating-anode generator (Nonius FR591) or the SRS, Daresbury. Type I crystals were extremely fragile and sensitive to manipulation; rapid cooling conditions were therefore established by immersing them in liquid propane using standard nylon loops without the need for cryoprotection. A native data set from the fully oxidized form of the enzyme was collected at DESY, Hamburg. All data were indexed, integrated and scaled using the *HKL* suite of programs (Otwinowski & Minor, 1997) and statistics are given in Table 1. A self-rotation function was calculated using *POLARRFN* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) and revealed non-crystallographic 222 symmetry in the triclinic cell. Subsequently, a molecular-replacement solution (*AMoRe*; Navaza, 1994) using the *Psa* CCP (61% sequence identity) coordinates (Fülöp *et al.*, 1995; PDB code 1eb7) revealed four molecules in the asymmetric unit. These molecules gave good crystal packing, but unfortunately there were long insertion loops compared with the *Psa* CCP structure and fine details could not be resolved. The *R* and free *R* values failed to decrease beyond 0.268 and 0.294, respectively; further refinement and model building were therefore abandoned.

The first type II crystals (grown in the presence of 0.1 *M* Tris–HCl pH 8.2, 0.05 *M* NaCl, 0.1 *M* urea additives) were tested in-house at room temperature. They initially diffracted X-rays to 3.5 Å, but diffraction severely decayed within 10 h of exposure. These crystals belong to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 75.0, b = 57.4, c = 172.7 Å, β = 99.0°. Routinely used procedures for cryogenic data collection, such as crystal soaking or co-crystallization in mother liquor containing cryoprotectant or propane freezing, were not successful and gave very smudgy diffraction patterns. The resolution was limited to 4 Å and none of the diffraction patterns could be indexed; therefore, a more systematic approach had to be performed. Firstly, a dehydration step using paraffin or Paratone-N improved the resolution limit, but the diffraction spots were still smudgy. In the second experiment, concentrated NaCl (1.5–1.3 *M*) was added to the wells after crystal formation and crystals were then extensively soaked in paraffin oil. This procedure dramatically improved the diffraction pattern, but was very difficult to reproduce and a number of crystals suffered

severe cracks during the dehydration process. In the third experiment, crystallization plates were left for up to eight months or until the mother liquor in the well had completely dried out and salt crystals started to appear in the drops (Fig. 1*b*). The diffraction was considerably improved by this method and the improvement proved to be more reproducible than in the previous methods. The unit-cell parameters of the dehydrated crystals showed variations compared with those of the non-dehydrated crystals determined at room temperature. The *a* unit-cell parameter increased by 4.4%, while *b* decreased by 11.1% and *c* also decreased by 3.2% (Table 1). This experiment suggested that a small rearrangement of the molecules in the unit cell takes place during the dehydration process which improves the diffraction quality upon flash-cooling. A complete data set was collected at 100 K using an eight-month-old crystal at two positions along the needle on beamline ID29, ESRF, France (Table 1). Molecular replacement (*AMoRe*; Navaza, 1994) using the coordinates of *Psa* CCP failed to give a correct solution; therefore, in subsequent calculations the partial model from the type I *Pad* CCP molecular-replacement solution was used as a search model. By this method, a convincing solution was obtained with the asymmetric unit containing two dimers. Initial refinement with *CNS* (Brünger *et al.*, 1998) showed promising results and model rebuilding using *O* (Jones *et al.*, 1991) and further refinement are in progress.

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