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Crystallization and preliminary X-ray diffraction analysis of two pH-dependent forms of a di-haem cytochrome *c* peroxidase from *Pseudomonas nautica*

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Two crystal forms of cytochrome *c* peroxidase from *Pseudomonas nautica* were obtained, one at pH 4.0 using sodium citrate as precipitant and another at pH 5.3 using ammonium phosphate and sodium citrate as precipitants. The two forms belong to different space groups $P3_121$ (pH 4.0) and $P6_422$ (pH 5.3), with unit-cell parameters $a = b = 114.5$, $c = 90.7$ Å and $a = b = 151.0$, $c = 155.9$ Å, respectively. Several complete data sets were collected using synchrotron radiation at ESRF and Cu $K\alpha$ X-ray radiation from a rotating-anode generator. These results will contribute to clarifying the haem transitions occurring during peroxidatic reaction and the required electron-transfer processes and to elucidating the catalytic mechanism of the enzyme and the role of calcium in the activation process.

1. Introduction

Under micro-aerophilic conditions, the cells of *Pseudomonas nautica* (*Ps. nautica*) express the periplasmic enzyme cytochrome *c* peroxidase (CCP). This enzyme catalyses the reduction of H_2O_2 to H_2O coupled to the oxidation of the monohaem cytochrome c_{552}, which was identified as the physiological electron donor (Alves *et al.*, 1999). This activity is a mechanism of protecting the cells from the toxicity of hydrogen peroxide (Halliwell & Gutteridge, 1984; Cheeseman & Slater, 1993). The CCP isolated from *Ps. nautica* strain 617 is a homodimer of 2 × 36.5 kDa, corresponding to 326 amino-acid residues (unpublished results). Sequence comparison showed that the *Ps. nautica* enzyme exhibits a high similarity to the homologous proteins from *Paracoccus denitrificans* (*Pa. denitrificans*; Goodhew *et al.*, 1990; Pettigrew, 1991) and *Ps. aeruginosa* (Ellfolk & Soininen, 1970; Foote *et al.*, 1983). Like other CCPs, this enzyme contains two haem *c* groups: a high-potential haem that functions as an electron-transfer centre located in the C-terminal domain and a low-potential haem that is the peroxidatic centre located in the N-terminal domain (Fülöp *et al.*, 1995, 2001; Shimizu *et al.*, 2001).

The high-potential haem (electron-transfer centre) accepts electrons from the physiological electron donor (cytochrome c_{552}), which are then transferred to the low-potential haem (peroxidatic centre). Like the CCP isolated from *Pa. denitrificans* (Gilmour *et al.*, 1993; Lopes *et al.*, 1998), the CCP from *Ps. nautica* also requires Ca^{2+} activation to become active. Upon ascorbate reduction, the calcium dependency exhibited by *Ps. nautica* CCP is similar to that observed by *Pa. denitrificans* CCP. After addition of $CaCl_2$ to the reduced enzyme solution, a transition of the peroxidatic ferrihaem from low spin to high spin may be observed in the visible spectra, which corresponds to a drastic change in the peroxidatic haem environment (Foote *et al.*, 1985). It was proposed that the calcium ion should trigger a conformational change that removes the sixth ligand from the peroxidatic haem, which then becomes high spin, being ready to bind the hydrogen peroxide substrate (Alves *et al.*, 1999). This work should provide a structural basis for the mechanism of Ca^{2+} activation in di-haem cytochrome *c* peroxidases, in particular the CCP from *Ps. nautica*.

2. Material and methods

2.1. Protein purification

Ps. nautica CCP was purified in a four-step purification protocol as described by Alves *et al.* (1999). The entire purification was carried out in 1 d in order to minimize proteolysis. An anion-exchange column was first employed, followed by gel filtration and then a hydroxylapatite column. The final chromatographic step consisted of an anion-exchange 6 ml Q-Resource cartridge (Pharmacia) to obtain high-purity samples for protein crystallization. The pure fraction of the enzyme had an A_{407}/A_{280} ratio of 4.5 and showed a single band in SDS–PAGE. Enzyme activity was measured at 298 K following the procedure described by Gilmour *et al.* (1994).

2.2. Crystallization

The initial crystallization conditions were screened using an in-house modified version of the sparse-matrix method of Jancarik (Jancarik & Kim, 1991), with and without the presence of additives. The crystallization screens were produced using the hanging-drop vapour-diffusion method. The first experiments yielded small needles which were unsuitable for X-ray diffraction. Further studies were carried out in order to improve and reproduce some of the crystallization conditions and it was observed that the presence of calcium was absolutely essential. The protein was incubated with 1 m*M* calcium chloride and the screenings were repeated at both 277 and 293 K. Results of these screenings showed that crystals were formed only at 277 K and at different pH values. Crystals grown at pH 4.0 and at pH 5.3 corresponded to different crystal forms: form I and form II, respectively.

Crystals of form I obtained in the presence of sodium citrate at pH 4.0 are red–orange hexagonal bipyramids that grow to maximum dimensions of about 0.2 × 0.2 × 0.1 mm within one week (Fig. 1*a*). The best crystals were obtained using 2 µl of a 10 mg ml^{-1} protein solution in 5 m*M* HEPES buffer pH 7.5 and 2 µl of a reservoir solution containing 0.8 *M* sodium citrate pH 4.0 at 277 K. Optimized crystallization conditions for crystal form II were obtained in the presence of 1.0 *M* ammonium dihydrogen phosphate as precipitant and 0.1 *M* sodium citrate buffer pH 5.3 at 277 K. After approximately three to four weeks, small red–orange hexagonal bipyramids appear in the drops and continue to grow to approximately 0.14 × 0.14 × 0.14 mm (Fig. 1*b*) within two months. Different approaches with microseeding and macroseeding techniques were used to try to improve the crystal size, but without success.

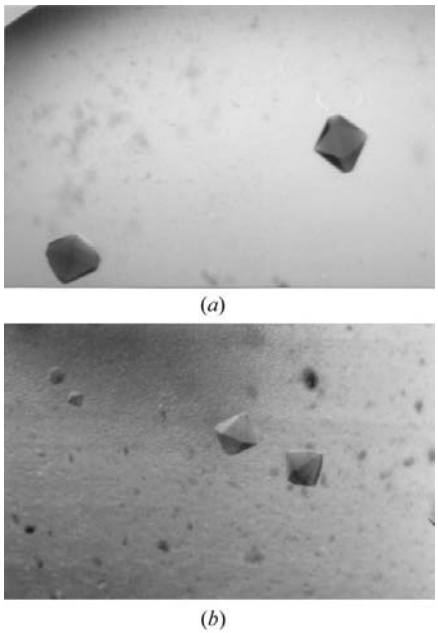

Figure 1
(*a*) Form I crystals (pH 4.0) of cytochrome *c* peroxidase from *Ps. nautica* grown at 277 K. Maximum dimensions are ∼0.2 × 0.2 × 0.1 mm. (*b*) Form II crystals (pH 5.3) of cytochrome *c* peroxidase from *Ps. nautica* grown at 277 K. Maximum dimensions are ∼0.14 × 0.14 × 0.14 mm.

2.3. Data collection and processing

Cryoprotection of the *Ps. nautica* CCP crystals was necessary for complete data collection in order to eliminate radiation damage of these crystals. They were cryocooled with a cryoprotectant solution containing 30% glycerol. The most efficient protocol was to first add the harvesting buffer (of similar composition to the cryoprotectant solution, but without glycerol) to the crystallization drop and then to transfer the crystal to a new drop of harvesting buffer. The cryoprotectant solution was then slowly added to this drop and, after equilibration, the crystal was transferred to a new drop of cryoprotectant and then rapidly mounted in a cryoloop followed by flash-freezing in a stream of cooled nitrogen gas maintained at 100 K throughout data collection.

X-ray diffraction data of form I (pH 4.0) crystals were first collected in-house on an X-ray imaging-plate system (MAR Research, Hamburg, Germany) using graphite-monochromated Cu $K\alpha$ radiation (1.5418 Å) from an Enraf–Nonius FR-591 generator operated at 4.5 kW. Under these conditions the crystals diffracted to only about 3.45 Å, with an overall completeness of 96.4% in the resolution range 23.0–3.45 Å, an overall $R_{\rm sym}$ of 21.6% and an $R_{\rm sym}$ of 58.0% for the last shell (3.57–3.45 Å). A second data set for form I crystals was collected at the European Synchrotron Radiation Facility (ESRF) beamline ID14-2, where the crystals diffracted beyond 2.0 Å. The statistics for this crystal form showed an overall completeness of 99.9% in the resolution range 20.0–2.20 Å, with an overall $R_{\rm sym}$ of 5.5% and an $R_{\rm sym}$ of 53.5% for the last shell (2.28–2.20 Å). Crystals of form I belong to space group $P3_{1,2}21$, with unit-cell parameters $a = b = 114.5$, $c = 90.7$ Å.

Form II (pH 5.3) crystals did not diffract enough using an in-house rotating-anode source to be characterized. The smaller size and lower quality of the crystals produced good diffraction spots to only 5–6 Å resolution. Native diffraction data from this crystal form were collected using synchrotron radiation at the ESRF beamline ID14-EH1 using a wavelength of 0.934 Å. A crystal-to-detector distance of 200 mm was chosen in order to avoid overlap of reflections. The crystals were cryocooled using 20% glycerol and diffracted to 2.60 Å resolution. The statistics for this second crystal form showed an overall completeness of 99.9% in the resolution range 40.0–2.60 Å, with an overall $R_{\rm sym}$ of 8.7% and an $R_{\rm sym}$ of 44.7% for the last shell (2.69–2.60 Å). Form II crystals belong to space group $P6_{2,4}22$, with unit-cell parameters $a = b = 151.0$, $c = 155.9$ Å. All data were processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and a summary of the data-collection and final processing statistics is shown in Table 1.

3. Results and discussion

Molecular-replacement calculations were performed using the crystal structure of cytochrome *c* peroxidase from *Ps. aeruginosa* as a search model (kindly provided by Vilmos Fülöp; Fülöp *et al.*, 1995). This model was edited according to sequence homology. The amino-acid sequence of *Ps. aeruginosa* CCP is 64% identical to that of *Ps. nautica* CCP, with 80% homologous amino-acid residues. Calculations were carried out using all reflections in the resolution range 20–4.0 Å. The correct orientation could easily be found using the program *EPMR* (Kissinger *et al.*, 1999). For crystal form I, the space group $P3_121$ was confirmed to be the only one giving a correct solution, emerging as a clear peak with a correlation coefficient (CC) of 0.481 and an *R* factor of 0.477, while the values for the next highest peak were CC = 0.328 and *R* = 0.539. For crystal form II, the space group $P6_422$ was found to be the correct one and not its enantiomorph, which gave no solution. The correct solution presented a CC of 0.446 and an *R* factor of 0.507, a clear solution when compared with the values for the next peak (CC = 0.310 and *R* = 0.586).

Biochemical studies suggest that the active enzyme is a homodimer of 2 × 36.5 kDa as found in other homologous di-haem peroxidases (Alves *et al.*, 1999; Fülöp *et al.*, 1995; Shimizu *et al.*, 2001). However, the self-rotation function calculated with *POLARRFN* and *AMoRe* (from the *CCP*4 package; Collaborative Computational Project, Number 4, 1994) for both crystal forms I and II does not support the existence of more than one molecule per asymmetric unit, suggesting the presence of a crystallo-

Table 1
Crystallographic data and data-collection statistics.

Values in parentheses refer to the highest resolution shell.

	Crystal form I	Crystal form II
pH	4.0	5.3
X-ray source	ID14-2, ESRF	ID14-1, ESRF
Wavelength (Å)	0.933	0.934
Crystal data		
Space group	$P3_121$	$P6_422$
Unit-cell parameters (Å)	$a = b = 114.5$, $c = 90.7$	$a = b = 151.0$, $c = 155.9$
V_M (Å^3 Da^{-1})	4.57 (1 mol per a.u.)	7.02 (1 mol per a.u.)
Solvent content (%)	72.9	82.4
Mosaicity	0.29	0.36
Data collection		
Resolution (Å)	20.0–2.20 (2.28–2.20)	40.0–2.60 (2.69–2.60)
No. of observations	473508	811938
No. unique reflections	35072	32831
R_{sym} (%)	5.5 (53.5)	8.7 (44.7)
Completeness (%)	99.9 (100.0)	99.9 (100.0)
$I/\sigma(I)$	26.5 (4.0)	21.3 (7.0)

graphic homodimer as found in the *Ps. aeruginosa* CCP.

According to the cell size and symmetry, calculation of the solvent content (Matthews, 1968) indicates both crystal forms to have an unusually high solvent content, which may explain the poor diffraction power. Considering only one molecule per asymmetric unit, crystal form I has a solvent content of 73%, based on a V_M of 4.57 Å^3 Da^{-1}, and crystal form II a solvent content of 82%, based on a V_M of 7.02 Å^3 Da^{-1}. In the *Ps. aeruginosa* CCP (same space group as form I, $P3_121$, and similar unit-cell parameters, $a = b = 113.9$, $c = 72.0$ Å), the solvent content is also high (61%; Fülöp *et al.*, 1993, 1995).

The solutions found are a starting point for further refinement of the cytochrome *c* peroxidase of *Ps. nautica*, which is in progress, revealing preliminary but significant differences at the two different pH values. These results will contribute to answering some questions raised by biochemical and spectroscopic studies as well as to elucidating the catalytic mechanism of the enzyme and the role of calcium in its activation. In addition, it will help to clarify the haem transitions occurring during the peroxidatic reaction and the required electron-transfer processes. The resulting structures and their comparison will be described in detail in a subsequent paper.

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