Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction analysis of cytochrome *c* peroxidase from the purple phototrophic bacterium *Rhodobacter capsulatus*

Lina De Smet, David Leys and Jozef J. Van Beeumen*

Department of Biochemistry, Physiology and Microbiology, Laboratory for Protein Biochemistry and Protein Engineering, University of Gent, 9000 Gent, Belgium

Correspondence e-mail: jozef.vanbeeumen@rug.ac.be

Received 13 July 2001
Accepted 18 December 2001

Bacterial cytochrome *c* peroxidase (BCCP) from *Rhodobacter capsulatus* was heterologously expressed in *Escherichia coli*. It was purified to homogeneity and crystallized using the hanging-drop vapour-diffusion method. Diffraction-quality crystals of the enzyme were obtained under two conditions. The first crystal belonged to space group $P2_1$, with unit-cell parameters a = 99.2, b = 224.7, c = 167.9 Å, β = 105°, and diffracted to 3.5 Å resolution. The crystallographic asymmetric unit of these crystals contained ten peroxidase molecules. *R. capsulatus* BCCP also crystallized in space group $P2_12_12_1$, with unit-cell parameters a = 67.2, b = 134.4, c = 167.9 Å. These crystals diffracted to 2.7 Å resolution and contained four peroxidase molecules per crystallographic asymmetric unit.

1. Introduction

Bacterial cytochrome *c* peroxidase (BCCP) from *R. capsulatus* catalyzes the reduction of hydrogen peroxide using ferrocytochrome c_2 as electron donor. The enzyme belongs to the greater family of the BCCPs, which have been found in diverse bacterial species but not in plants or animals (Ellfolk & Soininen, 1970; Goodhew *et al.*, 1990; Arciero & Hooper, 1994; Zahn *et al.*, 1997; Villalain *et al.*, 1984; Alves *et al.*, 1999). The most studied BCCPs are those from *Paracoccus denitrificans* and *Pseudomonas aeruginosa*. The bacterial cytochrome *c* peroxidases differ from the well known horse radish and yeast cytochrome *c* peroxidases in binding two haem groups covalently instead of one haem group non-covalently. One haem group of BCCP is high potential (+270 mV), while the other is low potential (between −190 and −310 mV).

At one point in the reaction mechanism, the two oxidizing equivalents of hydrogen peroxide are stored in the two haem groups of BCCP, with the formation of a high-energy completely oxidized form of the enzyme. The monohaem peroxidases, such as yeast cytochrome *c* peroxidase, form an oxy-ferryl centre and a non metal-based radical. The property of having two haem groups makes the formation of a radical in BCCP unnecessary.

The completely oxidized form of *R. capsulatus* BCCP is inactive. Reduction of the high-potential haem with ascorbate and activation of the enzyme with calcium results in opening of the low-potential haem to solvent by removal of ligand. This mixed-valence state of the enzyme can react with hydrogen peroxide with the formation of a stable oxy-ferryl centre at the peroxidatic haem and an oxidized high-potential haem. Both haem Fe atoms become reduced through electron transfer from ferrocytochrome *c*, so that the reaction cycle can start again. The three-dimensional structure of the completely oxidized and inactive form of *P. aeruginosa* BCCP has been solved to 2.4 Å resolution (Fülöp *et al.*, 1995).

Here, we report the crystallization and preliminary X-ray characterization of the oxidized *R. capsulatus* BCCP. This should form the basis of the structural study of this enzyme in different valence states with the aim to gain an insight into the catalytic cycle of the bacterial cytochrome *c* peroxidases in general.

2. Methods and results

2.1. Sample preparation

The *R. capsulatus* BCCP was heterologously expressed in *E. coli* and was purified to homogeneity using a four-step procedure (De Smet *et al.*, 2001). Briefly, the periplasmic fraction was loaded onto a Q-Sepharose column and eluted with a step gradient of 0–500 m*M* NaCl. The fractions between 0.2 and 0.3 *M* NaCl were pooled and concentrated using ammonium sulfate precipation. The BCCP-containing fractions were separated on an octyl-Sepharose hydrophobic interaction column. As a polishing step, the BCCP was subjected to anion-exchange chromatography. The enzyme fractions were pure according to SDS–PAGE and mass-spectrometric analysis.

© 2002 International Union of Crystallography
Printed in Denmark – all rights reserved

The molecular mass of the BCCP was 36 202 Da.

2.2. Crystallization

Protein samples of BCCP were concentrated to approximately 20 mg ml^{-1} using an Amicon ultrafiltration unit with a YM10 membrane of molecular-weight cutoff 10 kDa (Millipore, Bedford, Massachusetts). At the same time, the buffer was changed to 10 m*M* Tris–HCl pH 8.0. Protein concentrations were estimated by the method of Bradford, using BSA as standard (Bradford, 1976). Initial crystallization conditions were found using the screens I and II from Molecular Dimensions Limited (Stratech Scientific Limited, Luton) at 294 K. From structure screen I, condition 14 was successful [0.2 *M* ammonium sulfate, 0.1 *M* sodium cacodylate pH6. 5, 30%(*w*/*v*) PEG 8000 and 0.2 *M* sodium acetate trihydrate] as well as condition 18 [0.1 *M* sodium cacodylate pH 6.5, 18%(*w*/*v*) PEG 8000]. Condition 27 from structure screen II [0.01 *M* zinc sulfate heptahydrate, 0.1 *M* MES pH 6.5, 25%(*v*/*v*) PEG monomethylether 550] resulted in crystals that were used for further analysis. Crystals were grown initially by hanging-drop vapour diffusion using 2 µl drops containing equal volumes of protein and precipitant solution. The reservoir itself contained 0.5 ml of precipitant solution.

The crystallization was further optimized by a systematic search around the initial conditions. Crystals suitable for X-ray diffraction analysis were obtained from 27%(*w*/*v*) polyethyleneglycol (PEG) 6000, 100 m*M* MES pH 6.0 and appeared after 3–4 d at 294 K (Fig. 1*a*). A second condition, using 100 m*M* MES pH 6.5, 25%(*v*/*v*) PEG monomethylether 550, 0.01 *M* $ZnSO_4$, resulted in crystals that appeared after 2–3 weeks at 294 K (Fig. 1*b*). The last screenings were carried out with 1 ml of precipitant solution in the reservoir; in some conditions drops of 4 and 6 µl were used with equal volumes of protein and precipitant solution. In the second condition, the protein solution was diluted to 5 mg ml^{-1}.

2.3. Data collection and analysis

For X-ray diffraction analysis, crystals from the first condition were mounted in a loop containing mother liquor plus 10% glycerol and they were flash-cooled (Hope, 1988) to 90 K in a nitrogen-gas cold stream. The crystals belong to the monoclinic space group $P2_1$, having unit-cell parameters a = 99.2, b = 224.7, c = 167.9 Å, β = 105°.

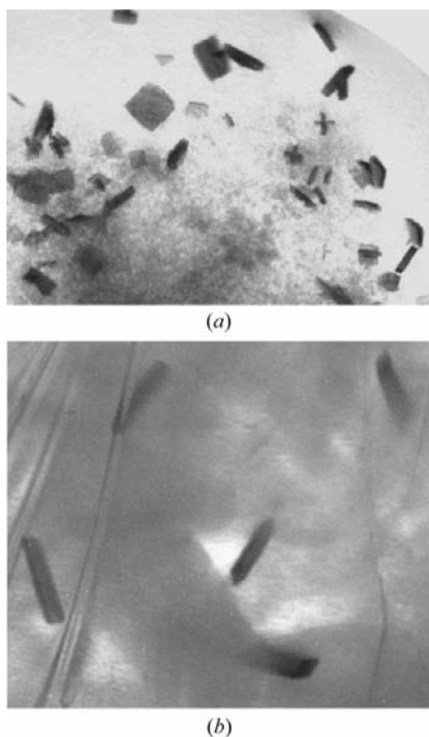

Figure 1
Crystals of *R. capsulatus* cytochrome *c* peroxidase. (*a*) Crystals belonging to space group $P2_1$ were very irregular. (*b*) Crystals belonging to space group $P2_12_12_1$. The approximate dimensions of the crystal are 0.5 × 0.1 × 0.1 mm.

They diffracted to 3.5 Å resolution. Data collection was performed at the ESRF synchrotron in Grenoble (France) on the microfocusing beamline ID13.

The data were indexed, integrated and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). A total of 364 430 measurements were collected from which 54 816 unique reflections were recorded with a completeness of 96%. Assuming ten molecules in the asymmetric unit, the V_M value is 4.99 $Å^3 Da^{-1}$, corresponding to a solvent content of 75%, which is within the expected range (Matthews, 1968). A molecular-replacement strategy (Navaza, 1994) using the cytochrome *c* peroxidase from *P. aeruginosa* (Fülöp *et al.*, 1995) as the search model has been used. The solution confirmed that there are five dimers in the asymmetric unit.

Crystals from the second condition were mounted in a loop containing mother liquor and flash-cooled to 90 K in a nitrogen-gas cold stream. No additional cryoprotectant was required to stabilize the crystal. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 67.2, b = 134.4, c = 167.9 Å. The crystals diffracted to 2.7 Å on beamline X11 at the

Table 1
Data collection and processing statistics.

Values in parentheses refer to the last resolution shell.

	Crystal I	Crystal II
Space group	$P2_1$	$P2_1\,2_12_1$
Molecules per asymmetric unit	10	4
Resolution (Å)	3.5 (3.62–3.5)	2.7 (2.8–2.7)
No. of observations	364430	414706
No. of unique observations	54816	39539
Completeness	96 (86.8)	95.2 (95.7)
$R_{\rm sym}$†	0.282 (0.410)	0.125 (0.394)
Overall mean $I/\sigma(I)$	4.7 (1.4)	9.2 (3.1)

† $R_{\rm sym} = \sum I_i - \langle I \rangle / \sum I_i$, where I is the intensity of observation i and $\langle I \rangle$ is the mean intensity of the reflection.

synchrotron DESY (Hamburg, Germany). The data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The V_M value is 2.62 $Å^3 Da^{-1}$, which reflects a solvent content of 52.64% assuming four molecules per asymmetric unit cell (Matthews, 1968). The data-collection parameters and data-processing results are summarized in Table 1.

The corresponding author is indebted to the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen for research project G.0054.97. We thank Bart Devreese for the mass-spectrometry experiments. We gratefully acknowledge the ESRF, Grenoble, France and DESY, Hamburg, Germany for the provision of synchrotron radiation.

References

Alves, T., Besson, S., Duarte, L.C., Pettigrew, P. W., Girio, F. M. F., Devreese, B., Vandenberghe, I., Van Beeumen, J., Fauque, G. & Moura, I. (1999). *Biochim. Biophys. Acta*, **1434**, 248–259.
Arciero, D. M. & Hooper, A. B. (1994). *J. Biol. Chem.* **269**, 11878–11886.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
De Smet, L., Pettigrew, E. W. & Van Beeumen, J. J. (2001). *Eur. J. Biochem.* **268**, 6559–6568.
Ellfolk, N. & Soininen, R. (1970). *Acta Chem. Scand.* **24**, 2126–2136.
Fülöp, V., Ridout, C. J., Greenwood, C. & Hajdu, J. (1995). *Structure*, **3**, 1225–1233.
Goodhew, C. F., Wilson, I. B. H., Hunter, D. J. B. & Pettigrew, G. W. (1990). *Biochem. J.* **271**, 707–712.
Hope, H. (1988). *Acta Cryst.* B**44**, 22–26.
Matthews, B. W. (1968). *J. Biol. Chem.* **33**, 491–497.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Villalain, J., Moura, I., Liu, M. C., Payne, W. J., LeGall, J., Xavier, A. V. & Moura, J. G. (1984). *Eur. J. Biochem.* **141**, 305–312.
Zahn, J. A., Arciero, D. M., Hooper, A. B., Coasts, J. R. & Dispirito, A. A. (1997). *Arch. Microbiol.* **168**, 363–372.