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Crystallization and preliminary X-ray studies of recombinant horseradish peroxidase.

By ANETTE HENRIKSEN and MICHAEL GAJHEDE, *Centre for Crystallographic Studies, Chemical Institute, Copenhagen University, DK 2100 Copenhagen, Denmark,* PATRICK BAKER, *Department of Molecular Biology and Biotechnology, University of Sheffield, PO Box 594, Firth Court, Western Bank, Sheffield S10 2UH, England,* and ANDREW T. SMITH and JULIAN F. BURKE, *Biochemistry Laboratory, School of Biological Science, University of Sussex, Brighton BN1 9QG, England*

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Abstract

A non-glycosylated form of horseradish peroxidase *c* extracted from *Escherichia coli* inclusion bodies and refolded in the presence of haem and Ca^{2+} ions has been used to grow protein crystals suitable for X-ray diffraction analysis. The crystals are prisms in the trigonal space group $P3_112$ or $P3_212$ with $a = b = 158.9$ and $c = 114.3$ Å and diffract to 1.9 Å. There are four molecules, each of 34 kDa, in the asymmetric unit. The molecules of the asymmetric unit are related by approximate translational symmetry, resulting in pseudo-centerings. Data to approximately 15 Å can thus be described by a lattice of $a' = b' = 91.7$ Å and $c' = 57.1$ Å, $\alpha = \beta = 90°$ and $\gamma = 120°$, including four molecules.

Introduction

Horseradish peroxidase *c* (HRPC) (donor: hydrogen peroxide oxidoreductase, E.C. 1.11.1.7) is the major peroxidase isoenzyme of horseradish roots. It is a monomeric haem protein of 308 amino acids, containing structural calcium and eight *N*-linked neutral glycans (Welinder, 1979). Biochemically, it is the most thoroughly studied member of the plant peroxidase superfamily. This group of enzymes oxidizes a wide range of aromatic substrates to free radicals (Dunford, 1990; Gaspar, Penel, Thorpe & Greppin, 1982), but their diverse *in vivo* functions are still a subject of discussion. Owing to the wealth of biochemical information on HRPC, the determination of the structure of this archetypal plant peroxidase will contribute greatly to the understanding of the structure–function relationships of the plant peroxidase superfamily.

The enzyme was initially crystallized by Braithwaite (1976), but since the crystals were tetragonal with approximately 32 molecules in the unit cell, detailed X-ray analysis was not pursued. The inability to obtain good crystals with the plant enzyme could be related to the marked microheterogeneity of samples of plant HRPC, which have been shown by spray mass spectroscopy to contain some 21 components, most of which are glycovariants (Green & Olivier, 1991). In contrast, the recombinant enzyme from *E. coli* (Smith *et al.*, 1990) contains only one principal species (M. J. Geisow & T. Hutton, personal communication). Although other plant peroxidases have been crystallized (Aibara, Kobayashi & Morita, 1981; Ban *et al.*, 1992; Henriksen *et al.*, 1992; Henriksen *et al.*, 1993; Morita, Kameda & Mizuno, 1961; Morita *et al.*, 1991), very few of the crystals have been of sufficient quality for X-ray diffraction analysis and there is still no high-resolution structural information available for any plant peroxidase. This paper describes the crystallization and preliminary X-ray diffraction studies of crystals of recombinant, non-glycosylated HRPC (HRPC*) (Smith *et al.*, 1990).

Crystallization and X-ray diffraction analysis

Fully active recombinant HRPC expressed in *E. coli* was used in the present studies. Apoperoxidase was extracted from inclusion bodies, folded in the presence of haem and Ca^{2+} ions and purified to an RZ (A_{403}/A_{280}) greater than 3.2 (Smith *et al.*, 1990; Smith, Saunders, Thorneley, Burke & Brady, 1992).

The procedure by Jancarik & Kim (1991) was used for the initial screening for crystallization conditions. These conditions were further refined by exploring a matrix of conditions, including various molecular weights of PEG, pH, temperature and additives, as well as the concentration of PEG, the additives and the protein. The best results were achieved with the hanging-drop vapour diffusion method, using protein droplets of 6 µl (3 µl, 6.0 mg ml^{-1} of HRPC* and 3 µl reservoir solution) which were left to equilibrate against a 500 µl reservoir solution consisting of 16%(*w*/*v*) PEG 4000, 0.2 *M* zinc acetate and 0.1 *M* cacodylate buffer, pH 6.5 (Fig. 1). Crystallization was carried out in the dark with a crystallization time of approximately 2 weeks. Crystals with the same morphology have been found in both PEG4000 and PEG6000, in the concentration range 16–30%(*w*/*v*). The presence of zinc acetate proved to be essential for crystal formation. Crystals formed at 283 K as well as at 294 K under slightly different conditions. If transferred to 277 K, the crystals crumble.

A low-resolution data set was collected on a HRPC* crystal of approximately 0.3 × 0.3 × 0.2 mm by 1.2° oscillation at a distance of 160 mm on an R-Axis II imaging plate. The X-ray source was Cu $K\alpha$, supplied by a Rigaku RU200HB rotating anode (normal focus) with a graphite monochromator and a 0.5 mm collimator. The anode was operated at 180 mA, 50 kV and each frame was exposed for 20 min. Diffraction was recorded over 180°. The purpose of this data set was to provide us with a complete data set from one crystal for space-group determination without the merging of symmetry-equivalent reflections. A data set to higher resolution was collected on a crystal of 0.4 × 0.4 × 0.25 mm, with a crystal-to-image plate distance of 140 mm and a 2θ setting of 20°. The oscillation range was 1.5° and the time of exposure was 30 min for each frame for this data set.

A range of oscillation frames from the first data set were used in the *Amethyst* program (Schafmeister, 1993) to determine unit-cell parameters and crystal orientation by semi-automatic indexing. The refined parameters were entered into the R-Axis II data-processing software and further refined. The *DENZO* program (Otwinowski, 1986) was used for integration of intensities, and processing of the data in space group *P*1 was performed with the *CCP*4 program package (SERC Daresbury Laboratory, 1979).

Space-group determination, including data to 3 Å *resolution*

Determination of the point group was achieved from pictures made by the *PRECESS* program of the *PHASES* program

package (Furey, 1992). The *hk*0 plane shows sixfold symmetry with mirror planes for every 30°. The *hk*1 plane shows threefold symmetry and mirror planes between the reciprocal axis (Fig. 2). This is consistent only with point group 312. The 0*kl* plane shows *mm* symmetry, in agreement with 312. Pseudo-systematic extinctions: *hkl*, $l = 2n+1$, $h-k = 3n$ can be observed in the low-order data. The reflections on the **c*** axis show the apparent condition $l = 6n$. Only the (009) reflection is present, but weak, giving the true condition of $l = 3n$, consistent with a 3_1 or 3_2 axis. An approximate sixfold symmetry, as seen in *hk*2, indicates the presence of pseudo-hexagonal symmetry.

Approximate space-group assignment using data to 15 Å *resolution*

A larger rhombohedron rotated 30° relative to that spanned by the **a*** and **b*** vectors can describe a reciprocal unit cell at this resolution (Fig. 3), and we can determine a transformation of the reciprocal unit cell,

$$\begin{pmatrix} a^{*\prime} \\ b^{*\prime} \\ c^{*\prime} \end{pmatrix} = \begin{pmatrix} 2 & -1 & 0 \\ 1 & 1 & 0 \\ 0 & 0 & 2 \end{pmatrix} \begin{pmatrix} a^{*} \\ b^{*} \\ c^{*} \end{pmatrix}$$

The relationship between the real and the approximate reciprocal unit cells at low resolution is thus as shown in Fig. 3. Consistently, the calculated Patterson map using all data in space group $P3_112$ shows large peaks at $(\frac{2}{3},\frac{1}{3},\frac{1}{2})$, and $(0,0,\frac{1}{2})$. If only low-resolution data is included (> 15 Å), the height of the peaks increases to the level of the origo. As the resolution is increased, the peak height of the Patterson map decreases, with $(\frac{2}{3},\frac{1}{3},\frac{1}{2})$ being the most constant peak. The dimensions of the smaller real cell are $a' = b' = 91.7$ and $c' = 57.1$ Å. The pseudo-centerings are also confirmed by peaks of the self-rotation function.

A data set of 165 509 observations with 77 118 unique reflections in $P3_112$ was collected from a single crystal, giving a completeness of the data set of 93.1% at 2.20 Å. The R_{merge} of the data set is 7.0% when processed in the space group $P3_112$. The crystals showed no significant decay upon exposure and

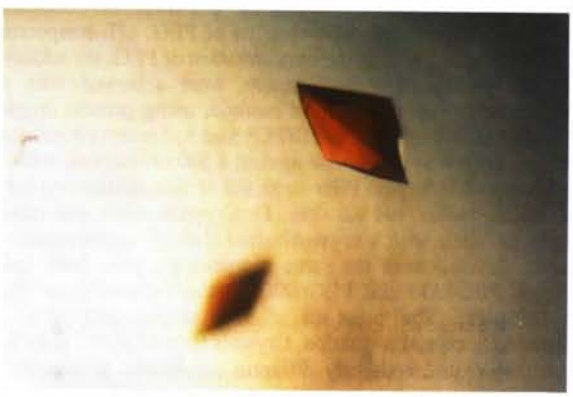

Fig. 1. HRPC* prisms of 0.3 × 0.3 × 0.2 mm grown from 16% PEG4000, 0.2 *M* zinc acetate and 0.1 *M* cacodylate buffer of pH 6.5.

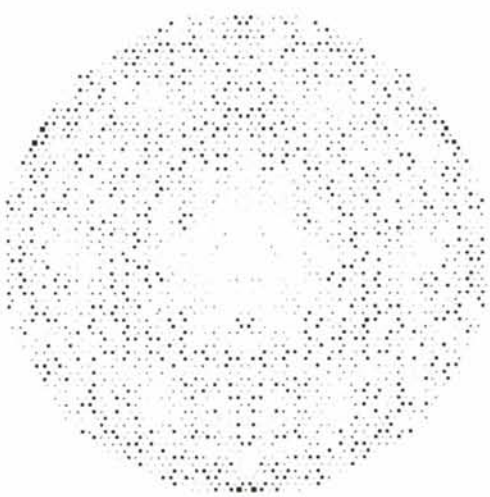

Fig. 2. Diagram of the intensities at reciprocal lattice points in the *hk*1 plane for data processed in space group *P*1. **c*** is normal to the plane and **b*** is running horizontally from left to right. The diagram shows threefold symmetry and mirror planes between the reciprocal axis.

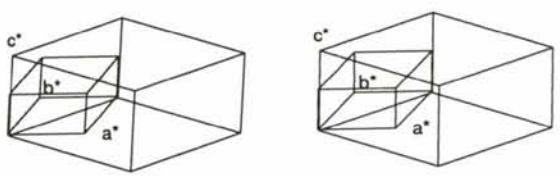

Fig. 3. The relationship between the reciprocal unit cell spanned by **a***, **b*** and **c*** and that caused by the pseudo-centrings at $(\frac{2}{3},\frac{1}{3},\frac{1}{2})$, $(\frac{2}{3},\frac{1}{3},0)$ and $(0,0,\frac{1}{2})$.

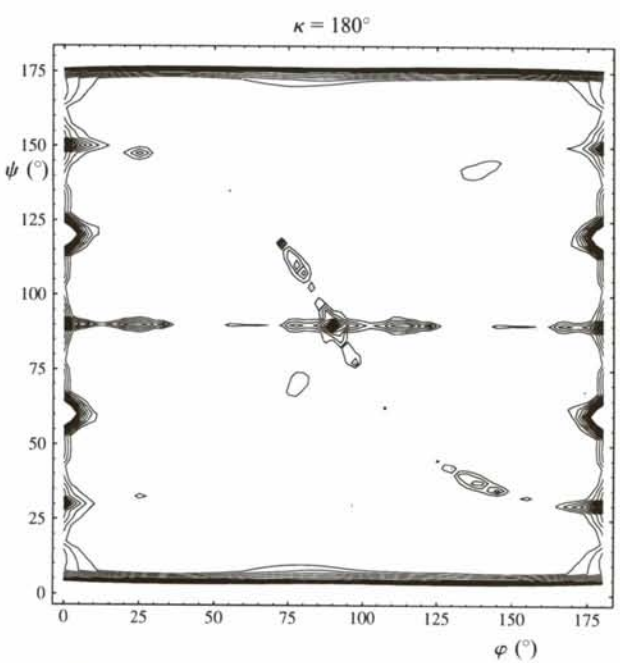

Fig. 4. Plot of the rotation function for $\kappa = 180°$. Peaks at (0,60) and (0,120) represent the twofold axis of $P3_112$, while the peaks at (0,30), (0,90), (0,150) and (90,90) represent pseudo-twofold axes.

diffracted to approximately 1.9 Å resolution. The refined unit-cell dimensions are $a = b = 158.9$ and $c = 114.3$ Å, and the unit-cell volume is 2.5×10^6 Å^3. This gives a range of possible numbers of molecules in the asymmetric unit from 4 to 6. However, the four Patterson peaks of origo height at low resolution, (0,0,0), $(0,0,\frac{1}{2})$,$(\frac{2}{3},\frac{1}{3},0)$, $(\frac{2}{3},\frac{1}{3},\frac{1}{2})$, implies that the number of molecules in the asymmetric unit is four. The volume-to-mass ratio (V_m) for this number of molecules is 3.05 Å^3 Da^{-1} and the solvent content is 60%. This agrees with the V_m and solvent content normally found (Matthews, 1968).

At low resolution, a cell with $a = b = 91.7$ and $c = 57.15$ Å, $\alpha = \beta = 90°$ and $\gamma = 120°$ describes the data. This cell can be either monoclinic with one pseudo-twofold axis or triclinic with two pseudo-twofold axes, as seen from the self-rotation function (Fig. 4). The small cell describes the asymmetric unit of the true unit cell.

In conclusion, a high-resolution structure for the classic horseradish peroxidase may be obtained either by the preparation of isomorphous heavy-atom derivatives or by molecular replacement of the distantly related structures (Welinder & Gajhede, 1993) yeast cytochrome *c* peroxidase (Finzel, Poulos & Kraut, 1984), lignin peroxidase (Poulos, Edwards, Wariishi & Gold, 1993) and *coprinus cinerius* peroxidase (Petersen, Kadziola & Larsen, 1994). Hopefully, our knowledge of the approximate symmetry, which reduces the crystallographic problem of four independent molecules in the asymmetric unit to a problem of four molecules related by a known pseudo-symmetry, can be utilized in the structure-solution procedure.

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