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Title

Preliminary crystallographic study of peanut peroxidase

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Journal

Acta Crystallographica Section B: Structural Science, Crystal Engineering and Materials, 48(1)

ISSN

2052-5192

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Publication Date

1992-02-01

DOI

10.1107/s0108768191008807

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Peer reviewed

Biophysical data such as redox potentials and their temperature dependence are currently being measured at the Department of Biochemistry and Biophysics, University of Göteborg, and these data will then be viewed in the light of the structural changes in the mutants.

The authors are grateful to the Department of Biochemistry and Biophysics, University of Göteborg, for the supply of azurin mutant. We are indebted to Dr Anders Svensson for part of the data collection. We also wish to thank Miss Ann-Britt Skånberg for technical assistance.

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Acta Cryst. (1992). **B48**, 109–111

Preliminary crystallographic study of peanut peroxidase. By NENAD BAN, *Department of Biochemistry, University of California at Riverside, Riverside, California 92521, USA*, ROBERT B. VAN HUUSTEE, *The Department of Plant Science, University of Western Ontario, London, Ontario N6A 4B7, Canada*, JOHN DAY, AARON GREENWOOD and STEVE LARSON, *Department of Biochemistry, University of California at Riverside, Riverside, California 92521, USA*, ROBERT ESNAULT, *Institute des Sciences Vegetales, CNRS, 91198 Gif-sur-Yvette CEDEX, France*, and ALEXANDER MCPHERSON,* *Department of Biochemistry, University of California at Riverside, Riverside, California 92521, USA*

(Received 14 June 1991; accepted 16 July 1991)

Abstract

The cationic isozyme of peroxidase isolated from suspension cultures of peanut cells is a heme-containing and calcium-dependent glycoprotein having four covalently attached oligosaccharide chains. Attempts were made to crystallize the glycoprotein for X-ray diffraction analysis, and these have met with some success. Crystals have now been grown that are suitable for a full three-dimensional structural analysis. The crystals are thin plates and we have shown them to be of the orthorhombic space group $P2_12_12_1$ with $a = 48.1$, $b = 97.2$, $c = 146.2$ Å. The crystals diffract to beyond 2.8 Å resolution, appear to be stable to lengthy X-ray exposure, and contain two molecules of 40 000 daltons each in the asymmetric unit.

Introduction

Most higher plants produce a variety of isozymic forms of the enzyme peroxidase (E.C. 1.11.1.7) which has been used as a convenient marker in genetic, physiological and pathological studies (Greppin, Penel & Gaspar, 1986; van Huystee, 1987). In all cases, the enzyme is a glycoprotein that contains a heme prosthetic group responsible for its activity. The pattern of expression in plants is influenced by environmental stimuli, is developmentally regulated, and is tissue specific (Cassab & Varner, 1988). Although the function of peroxidases in plants is still uncertain, it

has been implicated in polysaccharide cross-linkages with extensin monomers, indoleacetic acid oxidation, liquification, wound healing, phenol oxidation, defense against pathogens and regulation of cell elongation (Greppin, Penel & Gaspar, 1986; Cassab & Varner, 1988).

Peroxidases are synthesized by cultured plant cells which then secrete the cationic isozyme into the medium. It provides, thus, a straightforward means for its purification (van Huystee, 1987; Stephan & van Huystee, 1981). Peanut cell peroxidases have been shown to consist predominantly of two cationic and one anionic species (Stephan & van Huystee, 1981). The major cationic isozyme represents 75% of the medium's peroxidase activity. The cDNAs for both cationic forms of peanut peroxidase have now been cloned and sequenced (Buffard, Breda, van Huystee, Asemota, Pierre, Ha & Esnault, 1990), and the four oligosaccharides covalently bound to the protein from the major cationic isozyme have been extensively studied (Hu & van Huystee, 1989; van Huystee, Hu & Sesto, 1990).

The major cationic isozyme of peanut cell peroxidase has a total molecular weight of 40 000 daltons. It consists of a single polypeptide chain 307 residues in length of 32 954 daltons molecular weight. This protein component is covalently attached to four polysaccharide chains of total weight 8500 daltons comprising 21% of the total glycoprotein molecular weight. The isoelectric point of the protein is 8.9. The enzyme contains a single heme group that is essential for its activity and gives it a red color and absorption maximum in the Soret region at 405 nm. The

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activity of the enzyme also depends on the presence of calcium (van Huystee, Hu & Sesto, 1990; Hu, Krol & van Huystee, 1990).

We undertook the crystallization of the enzyme with the intention of pursuing its three-dimensional atomic structure. We report below our first steps in this direction which we hope may ultimately provide a sound structural basis for the interpretation and correlation of its many biochemical features.

Materials and methods

The major cationic isozyme of cultured peanut cells was isolated and purified to homogeneity from the culture medium according to the procedures of Hu, Krol & van Huystee (1990). These included ion exchange and molecular sieving chromatography and finally lectin affinity chromatography directed at the oligosaccharide moieties. With an initial amount of 40 mg of the purified protein, crystallization conditions were screened and optimized.

A matrix of crystallization conditions was explored using the procedures described by McPherson (1990), concentrating initially on precipitant type and concentration, pH, and temperature. The trials were conducted using 16 μ l droplets of protein solution on nine-well glass depression plates equilibrating against 25 ml of reservoir solution. Once crystals were observed under a specific set of conditions, finer matrices of the variables were examined that were focused on the successful trials.

Crystals of the peroxidase were obtained over a wide range of ammonium sulfate concentrations from 20 to 45% at both 277 and 295 K at pH 6.5 to 7.5. Crystals were also observed when PEG 4000 was employed as the precipitant at pH 6.5 to 7.5. The best crystals we have so far obtained have been from 12% PEG at pH 6.7. The protein droplets used in the trials consisted of 7 μ l of a 15 mg ml⁻¹ stock protein solution, 2 μ l of a 0.1 M sodium phosphate buffer at the appropriate pH, and 7 μ l of the reservoir.

For X-ray diffraction characterization, crystals were sealed in quartz capillaries along with a small amount of mother liquor and photographs of the reciprocal lattice recorded using a Buerger precession camera. The X-rays were nickel-filtered Cu K α produced by an Enraf-Nonius generator fitted with a fine focus tube operated at 45 kV and 32 mA.

Results

The peroxidase crystals, as seen in Fig. 1, are orange-red in color and tend to grow as clusters of very thin plates. Over time, however, the plates increase in thickness and some ultimately reach dimensions suitable for X-ray study. These plates typically have edge lengths of 0.5 \times 0.5 \times 0.1 mm. Crystals appear after a week to ten days but continue to grow for some weeks thereafter.

Photographs of the reciprocal lattice of the crystals demonstrated that the crystals are orthorhombic. Axial reflections *h*00, 0*k*0 and 00*l* were present only when they were of even index. No other systematic absences were present in the reciprocal lattice. The space group is, therefore, *P*2₁2₁2₁ and the corresponding unit-cell parameters are *a* = 48.1, *b* = 97.2, *c* = 146.2 Å.

The volume of the crystallographic unit cell is $V = 6.84 \times 10^5 \text{ \AA}^3$. If one were to assume a single molecule of peroxidase as the asymmetric unit of the crystal, then the volume-to-mass ratio of $V_m = 4.4 \text{ \AA}^3$ dalton would be at the extreme, if not outside, the range observed for most other crystalline proteins (Matthews, 1968). Assumption of two peroxidase molecules of 40 000 daltons each would, however, give an acceptable value of $V_m = 2.2 \text{ \AA}^3$ dalton. Thus, we conclude that there are two molecules of the peroxidase per asymmetric unit of the crystal.

The peroxidase crystals are mechanically and physically stable, and appear to be unusually stable in the X-ray beam. The crystals, though small and very thin, diffract X-rays remarkably well and the diffraction pattern can be seen to extend well beyond 2.8 Å resolution.

Discussion

Rather few glycoproteins having a percentage of carbohydrate as high as 20% have been crystallized, and those that have, in general, produced diffraction patterns of quite limited extent. That is not true of the peroxidase crystals described here, which diffract well to high resolution. This may indicate that in these crystals the oligosaccharides are better ordered and less mobile.

The unit-cell parameters, the extent and quality of the diffraction pattern, and the stability of these crystals make them, we feel, suitable for a full three-dimensional structure analysis, and we are initiating such an investigation using conventional MIR techniques.

Currently, we have collected X-ray diffraction data to 2.8 Å resolution from three separate peroxidase crystals and have merged the data, with a conventional residual of *R* = 0.08, into a single set of 34 000 independent reflections having an intensity to estimated standard deviation ratio greater than three. We anticipate that the presence of two copies of the protein in the crystallographic asymmetric unit and the attendant possibility for molecular averaging may ultimately assist us in producing a detailed and clear image of the protein structure. We are carrying out experiments now with the rotation function as programmed by Fitzgerald (1989) to deduce the relationship between the two molecules within the asymmetric unit.

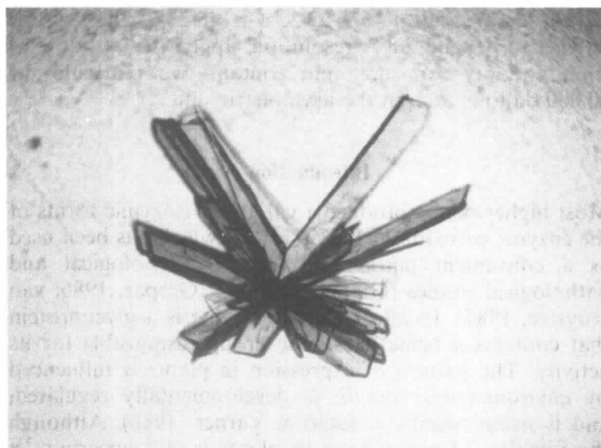


Fig. 1. Thin plate crystals of peanut peroxidase grown from PEG 8000.

This work was supported by grants from the NIH (No. GM40706) and from NASA (No. NAG8-804). The authors would like to recognize the efforts of J. Hendrick in the initial stages of the crystallization.

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Acta Cryst. (1992). **B48**, 111–113

Conformational similarities between crystallographically independent molecules in organic crystals. By

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(Received 23 May 1991; accepted 17 July 1991)

Abstract

The extent of conformational similarity between crystallographically independent molecules in organic crystals was quantified using the r.m.s. deviation of the atoms from a least-squares superposition. This analysis was carried out for a total of 399 structures taken from the Cambridge Structural Database. The structures are distributed among 65 chiral space groups. The analysis shows that in most cases the conformations of the crystallographically independent molecules are very similar.

Introduction

The availability of the vast amount of crystallographic data in an easily accessible form *viz.*, the Cambridge Structural Database (CSD, Allen *et al.*, 1979; Allen, Kennard & Taylor, 1983), has given a new impetus to the statistical study of the conformation and packing of organic molecules in crystals. Many of these studies, old (Kitaigorodskii, 1961, 1973) and new (Wilson, 1988, 1990) have been aimed at rationalizing the distribution of reported structures among the various space groups (Mighell, Himes & Rodgers, 1983). Others have sought to exploit the database to obtain new insights into molecular conformation and energetics (Allen, Kennard & Taylor, 1983; Bürgi & Dunitz, 1983; Allen, 1986) and to analyse intermolecular environments (Gavezzotti & Desiraju, 1988; Ramasubbu, Parthasarathy & Murray-Rust, 1986). We have been interested in the packing of organic molecules from the point of view of intermolecular interactions. A

specific type of crystallographic arrangement of molecules which lends itself to such a study is that in which there is more than one molecule in the asymmetric unit. A recent systematic study of such crystallographic non-equivalence in the unit cell using the CSD (Padmaja, Ramakumar & Viswamitra, 1990) has shown that about 8.3% of crystal structures solved have a *Z* value greater than the crystallographic multiplicity. Some of these [typically 3–12% according to Mighell, Himes & Rodgers (1983)], however, may be attributed to an incorrect choice of space group.

We undertook a comparison of the conformations of the crystallographically independent molecules in 399 structures distributed over 65 space groups. The results are presented here.

Method

Out of a total of 1576 structures in the 1987 release of the CSD with two or more molecules per asymmetric unit in the 65 chiral space groups, all but 399 have been eliminated according to the following criteria: (a) Structures with *R* factors greater than 0.10 were removed. (b) Structures with more than two molecules in the asymmetric unit were eliminated. (c) A computer program was used to separate the coordinates of each entry into covalently bound clusters. The maximum bonding distance was taken to be 2.2 Å in order to include coordination bonds. When more than two such clusters or clusters with unequal numbers of atoms emerged, that structure was eliminated. (d) The order of arrangement of the atoms in the two molecules was checked by comparing the two-dimensional connectivity pattern of each atom in the two molecules. If the order was not the same or the atom list could not be

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† Contribution No. 776.