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Crystallization and preliminary X-ray analysis of a four-copper laccase from *Coriolus hirsutus*

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Laccase from the fungus *Coriolus hirsutus* has been purified. Crystals of the enzyme suitable for X-ray structure analysis have been obtained under optimized crystallization conditions using polyethylene glycol as a precipitant. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 50.65, b = 74.01, c = 124.83 Å, and contain 40% solvent and a single molecule of laccase in the asymmetric unit. X-ray data were collected to 1.85 Å at the copper edge and the four copper sites have been located from the anomalous signal. The obtained SAD phases with subsequent density modification produced a promising initial electron-density map.

1. Introduction

Laccase (EC 1.10.3.2) is a blue multi-copper enzyme that catalyses the oxidation of a variety of inorganic and aromatic compounds, particularly phenols, methoxy-substituted phenols and diamines, with the concomitant reduction of molecular oxygen to water (Adman, 1991; Reinhammar, 1984; Thurston, 1994). The enzyme can also catalyse the oxidation of non-phenolic compounds using an appropriate redox mediator. In addition, laccase is capable of performing direct electron transfer between its active site and an electrode (Berezin *et al.*, 1978). The properties of laccase provide a wide applicability of the enzyme for bioremediation, biodetoxification and biosensor technology (Xu, 1996).

Copper centres in blue copper oxidases are classified into three types on the basis of their distinct spectral features. Laccase, in common with other members of the multi-copper oxidase family, contains a combination of all three types of copper centre (Solomon *et al.*, 1996). Generally, the efficiency of laccase catalysis depends on the redox potential of the type 1 (T1) copper in the active site of the enzyme. The T1 copper has intense blue absorption around 600 nm. The T2 copper exhibits weak absorption in the visible region. Both T1 and T2 are EPR-active. The T3 site contains a strongly antiferromagnetically coupled copper pair bridged by a hydroxide and has an absorption band at around 330–340 nm. The T3 copper centre is EPR silent. The T2 and T3 centres form a trinuclear cluster. In spite of the seemingly identical copper coordination geometry, the redox potential of the T1 site varies significantly among fungal laccases.

Several crystal structures of fungal laccases are now available from the PDB. A T2-depleted structure of laccase from *Coprinus cinereus* has been determined at 1.7 Å resolution (PDB code 1hfu; Ducros *et al.*, 2001). Structures of two laccases from *Trametes versicolor* containing a full complement of coppers are also available both at 1.9 Å resolution (PDB code 1gyc; Piontek *et al.*, 2002) and the lower resolution of 2.4 Å (PDB code 1kya; Bertrand *et al.*, 2002). The latter has a high redox potential and was determined in the presence of a reducing substrate. A highly glycosylated four-copper laccase from *Melanocarpus albomyces* has been determined at 2.4 Å resolution (PDB code 1gw0; Hakulinen *et al.*, 2002).

Understanding the mechanism governing the high redox potential of laccase is of major importance for effective biotechnological application in protein-engineering strategies. Therefore, a series of 'high redox potential' laccases from different sources have been isolated and characterized (Koroljova *et al.*, 1998, 1999; Koroleva, Gavrilova *et al.*, 2002). Their redox potentials range from 750 to 820 mV *versus* the normal hydrogen electrode. Here, we present a preliminary crystallographic analysis on a laccase from the fungus *Coriolus hirsutus* with a T1 redox potential of 812 ± 10 mV.

2. Experimental

2.1. Protein purification

A strain producing extracellular laccase, *C. hirsutus* 072, was obtained from the Komarov Botanical Institute, Russian Academy of Sciences. The *C. hirsutus* strain was grown in a 10 l (6 l working volume) glass jar with an ANKUM-2 metal head fermenter (Puschino, Russia) under the conditions described by Koroleva, Stepanova *et al.* (2002).

Table 1
Data collection and phasing statistics.

Values for the outer shell (1.86–1.84 Å) are given in parentheses.

Resolution range (Å)	19.0–1.84
$\langle I/\sigma(I)\rangle$	19.8 (2.7)
Completeness (%)	94.7 (81.3)
Total/unique number of reflections	215595/46382
$R_{\rm merge}$	0.074 (0.58)
Redundancy	4.6
FOM from *SHARP*	0.31
FOM from *DM*	0.72

At the end of the fermentation period, the culture liquid was separated by filtration (Whatman No. 1 filter paper) and the culture filtrate was cooled to 233 K to eliminate polysaccharides and pigments. After cooling, the supernatant was concentrated by ultrafiltration (molecular-weight cutoff 15 kDa) and precipitated with ammonium sulfate at 90% saturation. Laccase was purified by preparative isoelectofocusing in wide-range 3-10 ampholytes (Pharmacia, Sweden) and fractions containing active enzyme were collected. Further purification was carried out using ion-exchange chromatography on a DEAE-Toyopearl 650M (Toyo Soda, Japan) and FPLC on a Sephadex 200 column as described by Koroljova *et al.* (1998). The homogeneity of the enzyme was confirmed by SDS–PAGE (12.6% acrylamide in 0.2 *M* Tris–glycine buffer pH 8.3) under denaturing conditions (Westermeier, 1993). Proteins were stained with Coomassie blue and the laccase activity was localized by staining with ABTS (Palmieri *et al.*, 2000). The amino-acid sequence was obtained from cDNA using the PCR technique (EMBL accession code AY081775).

2.2. Crystallization

Prior to crystallization, laccase was washed in a Centricon concentrator (10 kDa cutoff, Amicon) with 50 m*M* phosphate buffer pH 7.0 and concentrated to 30 mg $\rm ml^{-1}$. Crystallization was carried out by vapour-phase diffusion using the hanging-drop technique in Falcon 3047 multiwell plates at 291 K. 1–2 µl protein drops were mixed with equal volumes of reservoir solution. Initial crystallization trials were performed using Crystal Screen kit II (Hampton Research) and the most promising condition was optimized.

2.3. Data collection and processing

X-ray data were collected from a single crystal under cryogenic conditions (100 K) at EMBL beamline BW7A at the copper edge (wavelength of 1.38 Å) using a MAR 165 mm CCD detector. Integration and scaling were carried out with the *HKL* suite (Otwinowski & Minor, 1997). Substructure solution and phasing were carried out with *autoSHARP* (Vonrhein *et al.*, 2003) with subsequent density modification using *DM* (Cowtan, 1994).

3. Results and discussion

Although we have not carried out a systematic full factorial analysis for optimization of the crystal growth as described, for example, by Carter & Yin (1994), we investigated the effect of the pH of the buffer and the concentration of the precipitant. The best crystals grew from 10%(*w*/*v*) PEG 8000, 10%(*w*/*v*) PEG 1000 in 0.05 *M* phosphate buffer at pH 7.0 (Fig. 1). Use of ammonium sulfate at a concentration of 10–500 m*M* has also been tried but resulted in amorphous precipitant. Under optimized conditions, the blue laccase *C. hirsutus* crystals grew after 4–7 d. The largest crystals suitable for data collection had dimensions of approximately 0.7 × 0.14 × 0.02 mm (Fig. 2). The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 50.65, b = 74.01, c = 124.83 Å. Assuming there to be one molecule of approximately 55 kDa per asymmetric unit, the crystal-packing density was determined to be $V_{\rm M}$ = 2.15 $\rm Å^3\,Da^{-1}$, corresponding to a solvent content of approximately 40%. The metal analysis of crystals dissolved in 0.1 *M* acetate buffer pH 4.5 using inductively coupled plasma mass spectrometry has confirmed the presence of four copper ions per laccase molecule.

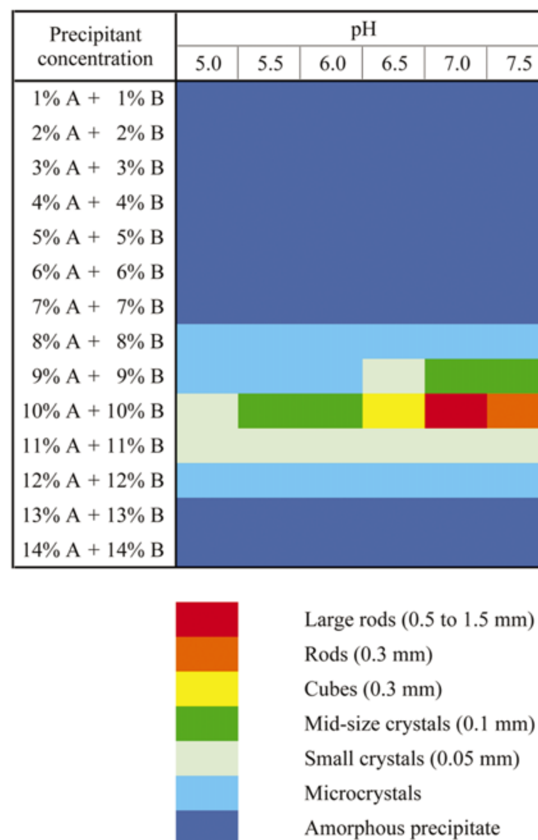

Figure 1
Optimization of crystallization conditions. A, PEG 1000; B, PEG 8000.

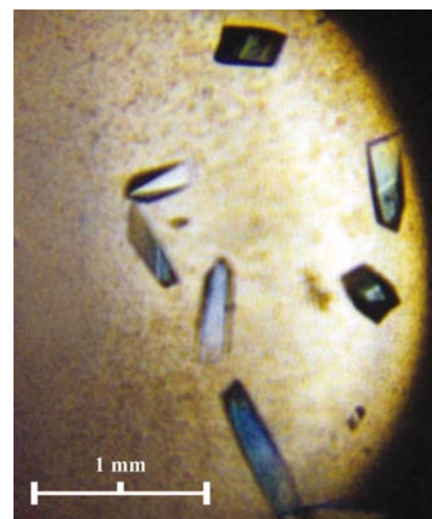

Figure 2
Crystals of *C. hirsutus* laccase.

Dry paraffin oil was used as a cryoprotectant for data collection under cryogenic conditions. Attempts were also made to use glycerol; however, this led to a considerable increase in crystal mosaicity. A 95% overall complete X-ray data set to 1.85 Å resolution was collected in two passes to ensure reliable measurement of strong low-angle reflections. The initial model could be obtained with molecular replacement using the 1kya structure as a search model. However, the data were of sufficient quality that the positions of the four copper sites could be obtained and refined from the anomalous signal alone. This will be used as an unbiased validation tool for interpretation of the laccase active site. The SAD phases were subsequently improved using density modification. This has increased the figure of merit from 0.31 to 0.72 and clearly showed the contrast between the protein and solvent regions (Fig. 3). Data-collection and phasing statistics are given in Table 1.

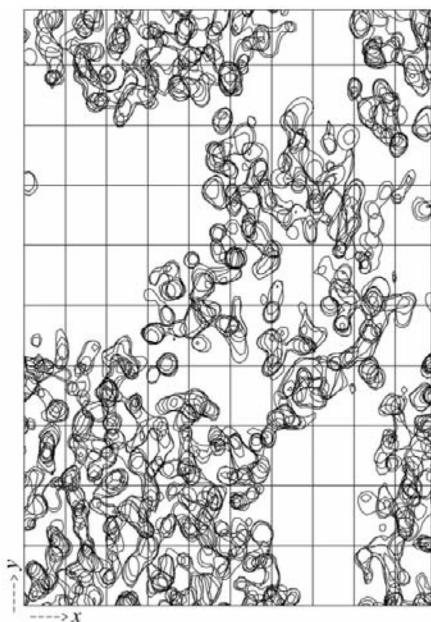

Figure 3
Ten sections (with 0.6 Å spacing) along the z axis of the solvent-flattened SAD map showing a clear protein–solvent contrast. The contour levels correspond to 1, 2 *etc.* standard deviations above the mean (corresponding to 0.25, 0.50 e Å^{-3} *etc.*).

Map interpretation and model refinement is currently in progress. It is hoped that the *C. hirsutus* laccase structural model will assist in elucidation of the catalytic mechanism and electron-transfer processes in this important family of proteins.

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References

Adman, E. T. (1991). *Curr. Opin. Struct. Biol.* **1**, 895–904.

Berezin, I. V., Bogdanovskaya, V. A., Varfolomeev, S. D., Tarasevich, M. R. & Yaropolov, A. I. (1978). *Dokl. Akad. Nauk SSSR*, **240**, 615–618.

Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C. & Mougin, C. (2002). *Biochemistry*, **41**, 7325–7333.

Carter, C. W. Jr & Yin, Y. (1994). *Acta Cryst.* D**50**, 572–590.

Cowtan, K. (1994). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **31**, 34–38.

Ducros, V., Brzozowski, A. M., Wilson, K. S., Ostergaard, P., Schneider, P. & Svendson, A., Davies, G. J. (2001). *Acta Cryst.* D**57**, 333–336.

Hakulinen, N., Kiiskinen, L.-L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A. & Rouvinen, J. (2002). *Nature Struct. Biol.* **9**, 601–605.

Koroleva, O. V., Gavrilova, V. P., Stepanova, E. V., Lebedeva, V. I., Sverdlova, N. I., Landesman, E. O., Yavmetdinov, I. S. & Yaropolov, A. I. (2002). *Enzyme Microb. Technol.* **30**, 573–580.

Koroleva, O. V., Stepanova, E. V., Gavrilova, V. P., Yakovleva, N. S., Landesman, E. O., Yavmetdinov, I. S. & Yaropolov, A. I. (2002). *J. Biosci. Bioeng.* **93**, 449–455.

Koroljova, O. V., Stepanova, E. V., Gavrilova, V. P., Biniukov, V. I., Jaropolov, A. I., Varfolomeyev, S. D., Scheller, F., Makower, A. & Otto, A. (1999). *Appl. Biochem. Biotechnol.* **76,** 115–128.

Koroljova (Skorobogat'ko), O., Stepanova, E., Gavrilova, V., Morozova, O., Lubimova, N., Dzchafarova, A., Jaropolov, A. & Makower, A. (1998). *Biotechnol. Appl. Biochem.* **28**, 47–54.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Palmieri, G., Giardina, P., Bianca, C., Fontanella, B. & Sannia, G. (2000). *Appl. Environ. Microbiol.* **66**, 920–924.

Piontek, K., Antorini, M. & Choinowski, T. (2002). *J. Biol. Chem.* **277**, 37663–37669.

Reinhammar, B. (1984). *Copper Proteins and Copper Enzymes*, edited by R. Lontie, pp. 1–36. Boca Raton, FL, USA: CRC Press.

Solomon, E. I., Sundraham, U. M. & Machonkin, T. E. (1996). *Chem. Rev.* **96**, 2563–2606.

Thurston, C. F. (1994). *Microbiology*, **140**, 19–26.

Vonrhein, C., Blanc, E., Roversi, P. & Bricogne, G. (2003). In preparation.

Westermeier, R. (1993). *Electrophoresis in Practice*, pp. 1–36. New York/Weinheim: VCH.

Xu, F. (1996). *Biochemistry*, **35**, 7608–7614.