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Crystallization and preliminary structure analysis of the blue laccase from the ligninolytic fungus *Panus tigrinus*

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The blue laccase from the white-rot basidiomycete fungus *Panus tigrinus*, an enzyme involved in lignin biodegradation, has been crystallized. *P. tigrinus* laccase crystals grew within one week at 296 K using the sitting-drop vapour-diffusion method in 22%(*w*/*v*) PEG 4000, 0.2 *M* $CaCl_2$, 100 m*M* Tris–HCl pH 7.5. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 54.2$, $b = 111.6$, $c = 97.1$, $\beta = 97.7^\circ$, and contain 46% solvent. A complete native data set was collected to 1.4 Å resolution at the copper edge. Molecular replacement using the *Coprinus cinereus* laccase structure (PDB code 1hfu) as a starting model was performed and initial electron-density maps revealed the presence of a full complement of copper ions. Model refinement is in progress. The *P. tigrinus* laccase structural model exhibits the highest resolution available to date and will assist in further elucidation of the catalytic mechanism and electron-transfer processes for this class of enzymes.

1. Introduction

White-rot fungi degrade wood lignin using a combination of specialized intracellular and extracellular enzymes (Leonowicz *et al.*, 1999). Lignin, the most common polymer on earth, which provides the structural component of the plant cell wall, is a heterogeneous biopolymer composed of phenyl propanoid units linked by various non-hydrolyzable C—C and C—O bonds (Lewis *et al.*, 1998).

The ligninolytic system of white-rot fungi was thought to be mainly composed of lignin-peroxidase and manganese-peroxidase (Evans, 1985). Laccases, which are incapable of cleaving the non-phenolic bonds of lignin, were not considered to be significant components of the ligninolytic system, despite the secretion of large quantities of laccases by the vast majority of white-rot fungi under ligninolytic conditions.

More recently, it was discovered that white-rot fungi laccases can enlarge their substrate range and are able to oxidize compounds with a redox potential exceeding their own, such as non-phenolic benzylalcohols, in the presence of synthetic or natural mediator molecules (Bourbonnais & Paice, 1990; Johannes & Majcherczyk, 2000; Li *et al.*, 1999). This finding led to the discovery that laccase-mediator systems effectively play a major role in the biodegradation of lignin and recalcitrant aromatic pollutants (Bourbonnais & Paice, 1990; Mayer & Staples, 2002; Murugesan, 2003).

Since their discovery more than a century ago in the Japanese tree *Rhus venicifera* (Yoshida, 1883), laccases have been found to be widely distributed in plants, where they are involved in the wounding response and the synthesis of lignin, and have subsequently been discovered to be present in fungi and bacteria (Claus, 2004).

Laccases (benzenediol oxygen oxidoreductases; EC 1.10.3.2) are polyphenol oxidases belonging to the family of multicopper oxidases. The most prominent representatives of this family include ascorbate oxidase and mammalian plasma ceruloplasmin (Messerschmidt, 1997; Messerschmidt & Huber, 1990; Solomon *et al.*, 1996). These multi-copper enzymes contain four Cu atoms per molecule, which are organized into three different copper sites that catalyze the one-electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water molecules. Blue copper oxidases contain at least one type-1 copper, which is presumably the primary oxidation site, whereas blue multi-

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copper oxidases typically employ at least three additional coppers: one type-2 and two type-3 copper ions arranged in a trinuclear cluster, the latter being the site where the reduction of molecular oxygen occurs.

A broad range of substrates such as polyphenols, methoxy-substituted phenols, diamines and particular inorganic compounds are generally oxidized through the catalytic action of laccases and, as reported above, synthetic or natural mediator molecules can further enlarge their range of action (Xu, 1996).

Biotechnological research on laccases, aiming towards the development of industrial processes such as pulp delignification and removal of environmental pollutants, for example pesticides and textile dyes, from contaminated soil and water, is currently being performed (Aust & Benson, 1993; Murugesan & Kalaichelvan, 2003). In order to optimize these promising processes, complete understanding of the catalytic mechanism of laccases and in particular of their redox potential and substrate-selectivity control are needed; detailed characterization of the high-resolution molecular structure of such enzymes will surely help in achieving these aims.

Several crystal structures of laccases have been solved recently. At first, extensive microheterogeneity, which is presumably caused by the variable glycosylation of these enzymes, hindered successful crystallization, but performing deglycosylation in order to enable the production of high-quality diffracting crystals resulted in loss of copper, as in the case of Ducros *et al.* (1998), who reported the crystal structure of a laccase from the fungus *Coprinus cinereus* at 1.68 Å resolution in a form devoid of the type-2 copper and therefore in a catalytically incompetent state.

More recently, the X-ray structures of laccases from the fungi *Trametes versicolor* (1.9 Å resolution), *Melanocarpus albomyces* (2.4 Å resolution) and *Rigidoporus lignosus* (1.7 Å resolution) and the spore-coat laccase from *Bacillus subtilis* (1.7 Å resolution) have been described (Enguita *et al.*, 2003; Garavaglia *et al.*, 2004; Hakulinen *et al.*, 2002; Piontek *et al.*, 2002).

Substrate and dioxygen binding has been investigated for the *B. subtilis* endospore-coat laccase (substrate ABTS) and for the *T. versicolor* laccase (substrate 2,5-xylidine) (Bertrand *et al.*, 2002; Enguita *et al.*, 2004).

Here, we present the crystallization study and preliminary structural analysis at the highest resolution available to date of an active laccase from *Panus tigrinus*. The enzyme is composed of 496 amino acids and exhibits a molecular weight of about 63 kDa (Leontievsky *et al.*, 1997; unpublished results from our laboratory). The preliminary density maps revealed the presence of a full complement of copper ions and several complex carbohydrate moieties.

Figure 1
Microphotography of blue laccase from *P. tigrinus* crystals obtained by the sitting-drop vapour-diffusion method.

2. Experimental procedures

2.1. Protein purification

Enzyme preparations containing the blue laccase from *P. tigrinus* were obtained from fungal cultures grown as described previously (Leontievsky *et al.*, 1997). The blue laccase was purified to apparent electrophoretic homogeneity as reported and was used for subsequent crystallization experiments. Laccase activity was determined quantitatively by monitoring the oxidation of 0.2 m*M* ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] at 420 nm (extinction coefficient 36 000 mM^{-1} cm^{-1}) in the presence of 20 m*M* sodium acetate pH 5.0 at 293 K.

2.2. Crystallization

The blue laccase was washed with 20 m*M* sodium acetate pH 5.0 and concentrated to 20 mg ml^{-1} using a Centricon ultraconcentrator (10 kDa molecular-weight cutoff, Amicon).

Crystallization experiments were performed utilizing the sitting-drop vapour-diffusion methods in 96-well plates (Crystal Clear Strips, Douglas Instruments Ltd, UK). Preliminary crystallization trials were performed utilizing Crystal Screen kits I and II (Hampton Research) and JBScreens 1–5 (Jena Bioscience) at 296 K and the most promising conditions were then optimized. 2 µl protein solution was mixed with 2 µl reservoir solution and equilibrated against 50 µl precipitant solution. A certain number of conditions (B5 of JBScreen-4 and D2 of JBScreen-2) produced crystals with growth defects. The best results were obtained with condition B6 of JBScreen-2 [20%(*w*/*v*) PEG 4000, 0.2 *M* $CaCl_2$, 100 m*M* Tris–HCl pH 8.5] and this was used for optimization. The concentrations of the protein and salts and the pH were systematically varied and trials were also performed to attempt to grow larger and regularly shaped crystals by using additives.

The optimized crystallization buffer contained 22%(*w*/*v*) PEG 4000, 0.2 *M* $CaCl_2$, 100 m*M* Tris–HCl pH 7.5. Drops were prepared using 3 µl protein solution mixed with 2 µl reservoir solution and were equilibrated against 50 µl precipitant solution.

2.3. X-ray data collection

P. tigrinus blue laccase crystals were harvested from mother liquor utilizing cryoloops and soaked for 1–2 min in a solution consisting of the same mother-liquor solution with the addition of 10%(*v*/*v*) glycerol. The crystal mounted in a suitable cryoloop was flash-cooled in a nitrogen-gas stream at 100 K.

Diffraction data were collected at the European Molecular Biology Laboratory (EMBL) beamline BW7A at the DORIS storage ring of the Deutsches Elektronen Synchrotron (DESY), Hamburg using a MAR CCD system at the copper edge (1.377 Å). A total of 340 diffraction images were recorded at a crystal-to-detector distance of 50 mm.

After collecting the diffraction data, some of the crystals were dissolved in 20 m*M* sodium acetate buffer pH 5.0 and tested for activity as described above. They showed complete retention of the initial activity.

The data were processed and scaled with *DENZO* and *SCALEPACK* from the *HKL* suite (Otwinowski & Minor, 1997).

3. Results

Under the optimal conditions (see §2), crystals of laccase from *P. tigrinus* grew within one week at 296 K using the sitting-drop vapour-diffusion method to approximate dimensions of

Table 1
Crystal parameters and data-collection statistics.

Values in parentheses correspond to the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	54.2
b (Å)	111.6
c (Å)	97.1
β (°)	97.7
Asymmetric unit contents	2 molecules
Limiting resolution (Å)	1.4 (1.44–1.42)
Total reflections measured	1194919
No. unique reflections	210855
R_{sym}†	0.064 (0.43)
Completeness (%)	98.1 (86.8)
$\langle I/\sigma(I)\rangle$‡	19.7 (1.56)
Redundancy	5.7

† $R_{sym} = \sum |I_i - \langle I \rangle| / \sum \langle I \rangle$, where I_i is an individual intensity measurement and $\langle I \rangle$ is the average intensity for this reflection with summation over all data. ‡ $\langle I/\sigma(I)\rangle$ crosses the value 2 at 1.44 Å resolution.

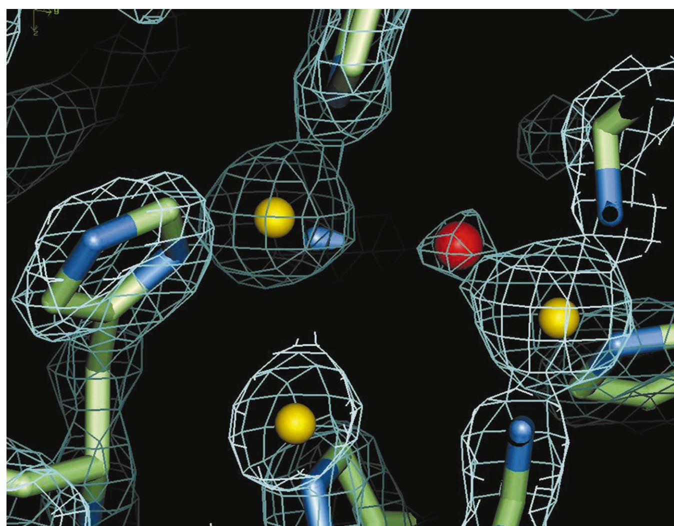

Figure 2
Detail of the modelled $2F_o - F_c$ electron-density map for the trinuclear copper centre in the blue laccase from *P. tigrinus*. The copper ions are depicted in yellow. The electron density is contoured at the 3σ level.

0.2 × 0.2 × 0.8 mm (see Fig. 1). Table 1 gives a summary of data collection and processing.

Assuming the presence of two molecules in the asymmetric unit, the solvent content is 46% (V_M = 2.31 Å^3 Da^{-1}). Data collected at 100 K with the addition of 10%(v/v) glycerol to the mother liquor as a cryoprotectant reached a maximum resolution of 1.4 Å. Data processing with *DENZO* and *SCALEPACK* gave 210 855 unique reflections, an overall completeness of 98.1% and an R_{sym} of 0.064 (Otwinowski & Minor, 1997).

Structure determination was carried out by the molecular-replacement technique using the laccase from *C. cinereus* (PDB code 1hfu) as a search model. This enzyme shares 58% sequence identity with the *P. tigrinus* laccase. Water molecules, Cu atoms and sugars were omitted from the standard model, which was used within the program *MOLREP* to calculate cross-rotation and translation function in the 10–3 Å resolution range. Two clear solutions were evident, giving an R factor and correlation coefficient of 0.469 and 0.436, respectively. The initial electron-density maps are very clear and show the presence of all four Cu atoms (see Fig. 2) and of several carbohydrate moieties.

The *P. tigrinus* blue laccase structural model will assist in the further elucidation of the catalytic mechanism and electron-transfer processes for this class of enzymes.

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