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Purification and preliminary crystallographic study of *Trametes versicolor* laccase in its native form

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Laccases are multi-copper oxidases that catalyse the oxidation of a wide range of phenols and their use in industrial oxidative processes is increasing. A laccase has been purified from the fungus *Trametes versicolor* and crystallized using the hanging-drop method. Crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 87.7$, $b = 110.5$, $c = 123.2$ Å, $\beta = 103.4^\circ$. A complete data set was collected to 2.4 Å resolution on a Cu $K\alpha$ rotating-anode X-ray source. Molecular replacement was performed and the initial electron-density maps indicate that the four expected Cu atoms are all present.

1. Introduction

Laccases are glycosylated multi-copper oxidases (EC 1.10.3.2). They catalyse the four-electron reduction of dioxygen to water concomitantly with the one-electron oxidation of four reducing substrate molecules (usually phenols or aryl amines). Various fungi, certain plants such as Japanese lacquer tree (*Rhus vernicifera*), bacteria and insects produce laccases. Their biological functions include wound response in plants and lignification in the case of tree enzymes. In contrast, fungal laccases are extracellular enzymes that probably play a role in lignin degradation. The increasing interest in laccases arises from their potential applications in several processes, including the paper (pulp bleaching) and textile industries, the enzymatic conversion of chemical intermediates and the oxidative transformation of environmental pollutants.

Laccases contain four copper ions distributed into three centre types defined according to their spectroscopic properties. The type 1 (T1) Cu, whose tight coordination to a cysteine residue is responsible for an intense absorption band around 600 nm, gives its blue colour to the enzyme. The type 2 (T2) Cu has a characteristic electron paramagnetic resonance (EPR) and the pair of strongly coupled type 3 (T3) Cu ions is EPR silent in the presence of dioxygen. Reduction of molecular oxygen takes place at the trinuclear centre (T2/T3) with the mononuclear (T1) site acting as the primary electron acceptor, extracting electrons from the reducing substrate. The T2 site can bind exogenous compounds such as O_2, OH^- and F^-, the latter being an inhibitor (Messerschmidt, 1997). The redox potential of the active copper centres of laccases is an important physicochemical property: it determines the range of substrates likely to be oxidized, namely, molecules with a redox potential lower than that of the T1 centre. Significant differences were measured in redox potentials among fungal laccases, which are typically between +0.5 and +0.8 V (*versus* normal hydrogen electrode; Xu *et al.*, 1996). For instance, despite 57.9% sequence identity, the redox potential of *Coprinus cinereus* laccase is 0.55 V (Ducros *et al.*, 1998), whereas that of *T. villosa* laccase lcc1 is 0.78 V (Xu *et al.*, 1998). The only laccase with a published crystal structure is that of the fungus *C. cinereus*. However, the authors had to perform an enzymatic deglycosylation to obtain crystals suitable for X-ray diffraction. Moreover, in both the structures refined with data collected at room temperature (PDB code 1a65; Ducros *et al.*, 1998) and at 100 K (1hfu; Ducros *et al.*, 2001), the crystallized enzyme was trapped in a form devoid of the type 2 copper. This depletion of T2 Cu is known to render laccases completely inactive (Reinhammar & Oda, 1979).

We recently cloned a secreted *T. versicolor* laccase (sequence accession number AF414109) inducible by 2,5-xylidine. Several different laccase sequences have been deposited for the white-rot basidiomycete *Trametes* (*Coriolus*, *Polyporus*) *versicolor*. As the enzyme we cloned shares 99.2% sequence identity (only four residues are different) with the laccase III from *T. versicolor* (sequence accession number D13372; Mikuni & Morohoshi, 1997), we name it laccase IIIb (LacIIIb) here. Furthermore, LacIIIb shares 99.2% sequence identity with the lcc1 laccase from *T. villosa* that has a high redox potential (see above). This suggests that the LacIIIb redox potential will have a high similar value. Here, we describe the purification, crystallization and preliminary X-ray diffraction study of LacIIIb from *T. versicolor* in its active form.

2. Materials and methods

2.1. Enzyme production and purification

Laccase was produced from *T. versicolor* (ATCC 32745). The bioreactor was inoculated with pounded mats obtained as described previously (Lesage-Meesen *et al.*, 1996). 5 l of mineral culture medium (as described by Collins *et al.*, 1996, except that maltose replaced glucose) were used. Laccase production was induced with 2,5-xylidine (0.2 m*M*) at the beginning of the culture. 7 d old culture liquid was filtered through glass wool to eliminate the mycelium. Extracellular polysaccharides were precipitated with 10% acetone, frozen and then separated by successive filtrations (final porosity 0.22 m*M*). The filtrate was concentrated by ultrafiltration (Millipore YM10, 10 000 Da cutoff) and laccase was recovered in 20 m*M* citrate–phosphate buffer (CPB) pH 5 by dialysis using the same membrane. The enzyme was then applied to a DEAE 52 anion-exchange column equilibrated in the same buffer. A first fraction containing laccase activity was not retained. A second peak was eluted with a linear NaCl gradient from 0 to 0.75 *M* and concentrated. The sample was then heated (333 K, 5 min) and centrifuged (18 000*g*, 20 min, 277 K) to eliminate the pellet. The supernatant, adjusted to 50 m*M* CPB pH 3.5, was loaded onto phenyl Sepharose (Pharmacia HiTrap, 1 ml bed volume) equilibrated in the same buffer with 1.2 *M* ammonium sulfate. The cartridge was washed and then sequentially eluted with 0.82 and 0.41 *M* ammonium sulfate. Finally, laccase was recovered by dialysis in 50 m*M* Tris–HCl buffer pH 6.8. Laccase activity was assayed as described in Jolivalt *et al.* (1999). The purity was checked by SDS–PAGE. The protein migrated essentially as a unique band at around 60 kDa, in accordance with mass-spectrometry experiments indicating a major isoform of 58.5 kDa.

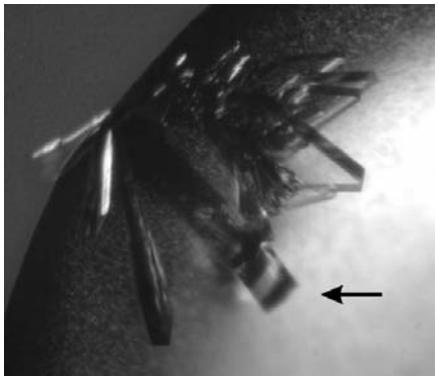

Figure 1
Crystals of *T. versicolor* laccase. The arrow points towards the widest crystal (dimensions 0.3 × 0.15 × 0.05 mm), which was used for X-ray diffraction.

2.2. Crystallization

Prior to crystallization, the protein was concentrated to 24 mg ml^{-1}. Crystals were grown at 293 K by vapour-phase diffusion using the hanging-drop method. 2 µl protein was mixed with 4 µl of the reservoir solution containing 18%(*w*/*v*) polyethylene glycol 8000 and 0.2 *M* zinc acetate in 0.1 *M* sodium cacodylate buffer pH 6.5 (volume of the well 0.7 ml). A bundle of crystals appeared after one week.

2.3. Data collection and processing

After 5 min soaking in a cryoprotectant solution [14.4%(*w*/*v*) PEG 8000, 0.16 *M* zinc acetate and 20%(*v*/*v*) 0.08 *M* glycerol in 0.1 *M* sodium cacodylate buffer pH 6.5], the crystal was flash-frozen in nitrogen gas at 100 K (Oxford Cryosystems Cryostream Cooler). Data were collected using a 300 mm MAR Research imaging-plate detector on a Rigaku rotating-anode RTP 300 RC X-ray generator operating at 50 kV and 100 mA, with the use of Osmic focusing optics. 175° of data were collected in frames of 0.5°, with 3 min exposure per frame. The raw data were indexed, processed and scaled using the *HKL* package (Otwinoski & Minor, 1997).

3. Results and discussion

Crystals appeared to be highly mosaic, probably because the protein was not deglycosylated. In the previously described case of *C. cinereus* laccase, the extremely high mosaicity of crystals grown from the native enzyme prevented X-ray studies. Therefore, an enzymatic deglycosylation was necessary in order to obtain crystals suitable for data collection (Ducros *et al.*, 1997). However, with *T. versicolor* LacIIIb a complete data set could be collected at 2.4 Å with the native enzyme (Table 1). The purified LacIIIb used for crystallization was fully active with different substrates (manuscript in preparation). Crystals (Fig. 1) were blue coloured owing to the presence of Cu atoms in the protein. They belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 87.7, b = 110.5, c = 123.2 Å, β = 103.4°. Assuming that the unit cell contains four molecules per asymmetric unit, the calculated Matthews coefficient (V_M) is 2.48 Å^3 Da^{-1} and the corresponding solvent content is 50.4%. These values are typical of protein crystals (Matthews, 1968).

Table 1
Data-collection statistics.

Values for the highest resolution shell (2.49–2.40 Å) are given in parentheses (ten shells).

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 87.7, b = 110.5, c = 123.2, β = 103.4
Resolution (Å)	35.0–2.4
Observed reflections	1879058
Unique reflections	83644
Multiplicity	22
Data completeness (%)	93.6 (71.6)
R_{sym}† (%)	11.4 (26,6)
$I/\sigma(I)$	9.7 (3.0)

† $R_{sym} = \sum_{hkl}[(\sum_i |I_i - \langle I\rangle|)/\sum_i I_i]$ for equivalent observations.

T. versicolor laccase IIIb shares 57.7% identity in its sequence with the *C. cinereus* enzyme. Molecular replacement was performed with the *AMoRe* program (Navaza, 1994) in the resolution range 15.0–3.5 Å, using the protein part of the laccase from *C. cinereus* (PDB code 1a65) as the search model. After rigid-body refinement, the correlation coefficient was 0.41, giving an initial *R* factor of 45.7%. The initial electron-density maps are very clear and unambiguously show the presence of all four Cu atoms. The structure is currently under refinement. This should give the first crystal structure of a laccase in its native active form, including the T2-site Cu atom which can bind dioxygen. *T. versicolor* LacIIIb shares more than 99% sequence identity with laccase lcc1 from *T. villosa*. Therefore, the LacIIIB structure could also give clues to understanding why, despite their high sequence similarity, laccase lcc1 from *T. villosa* has a higher redox potential than that of *C. cinereus*.

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