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A near atomic resolution structure of a *Melanocarpus albomyces* laccase ☆

N. Hakulinen [a,*], M. Andberg [b], J. Kallio [a], A. Koivula [b], K. Kruus [b], J. Rouvinen [a]

[a] *Department of Chemistry, University of Joensuu, Yliopistonkatu 7, P.O. Box 111, FIN-80101 Joensuu, Finland*
[b] *VTT Technical Research Center of Finland, P.O. Box 1000, FIN-02044 VTT, Finland*

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Abstract

We have solved a crystal structure from *Melanocarpus albomyces* laccase expressed in the filamentous fungus *Trichoderma reesei* (rMaL) at 1.3 Å resolution by using synchrotron radiation at 100 K. At the moment, this is the highest resolution that has been attained for any multicopper oxidase. The present structure confirmed our earlier proposal regarding the dynamic behaviour of the copper cluster. Thermal ellipsoids of copper atoms indicated movements of trinuclear site coppers. The direction of the type-3 copper motion was perpendicular to the type-2 copper. In addition, the structure at 1.3 Å resolution allowed us to describe important solvent cavities of the enzyme and the structure is also compared with other known multicopper oxidases. T2 and T3 solvent cavities, and a putative SDS-gate, formed by Ser142, Ser510 and the C-terminal Asp556 of rMaL, are described. We also observed a 2-oxohistidine, an oxidized histidine, possibly caused by a metal-catalysed oxidation by the trinuclear site coppers. To our knowledge, this is the first time that 2-oxohistidine has been observed in a protein crystal structure.

Keywords: Laccase; Multicopper oxidase; *Melanocarpus albomyces*; 2-Oxo-histidine; SDS-gate

1. Introduction

Laccase (E.C. 1.10.3.2, *p*-diphenol dioxygen oxidoreductase) belongs to the family of blue multicopper oxidases together with ascorbate oxidase (Messerschmidt et al., 1989), ceruloplasmin (Lindley et al., 1997), CueO (Roberts et al., 2003) and Fet3p (Taylor et al., 2005). For catalytic activity, four copper atoms are needed: one type-1 (T1) copper that forms a mononuclear site, one type-2 (T2) copper and two type-3 (T3 and T3́) coppers that form a trinuclear site. The coppers are classified according to their spectroscopic properties: (1) the T1 copper is a paramagnetic blue copper with an intense absorption at 600 nm. (2) the T2 copper is a paramagnetic normal copper and (3) the T3 and T3′ copper-copper pair is an antiferromagnetically coupled, EPR silent pair with absorption at ~330 nm (Malkin and Malmström, 1970; Solomon et al., 2001).

The first three-dimensional structure of a member of blue multicopper oxidase family, ascorbate oxidase from Zucchini, was solved in 1989 (Messerschmidt et al., 1989). The first laccase structure from *Coprinus cinereus* was reported in 1998, but it lacked the T2 copper (Ducros et al., 1998). Presently, several complete laccase structures from *Trametes versicolor* (Bertrand et al., 2002; Piontek et al., 2002), *M. albomyces* (Hakulinen et al., 2002), *Bacillus subtilis* (Enguita et al., 2003), *Rigidoporus lignosus* (Garavaglia et al., 2004) *and Cerrana maxima* (Lyashenko et al., 2006) are available. The overall structure consists of three domains A, B and C (or 1, 2, 3). All domains have a similar Greek key β-barrel structure, a so-called cuppredoxin fold, which is also found in small blue copper electron transport proteins (Adman, 1991). In blue multicopper oxidases, the

☆ The atomic coordinates and structure factors have been deposited with the Protein Data Bank (Accession Code 2Q9O).

* Corresponding author. Fax: +358 13 2513390.
E-mail address: nina.hakulinen@joensuu.fi (N. Hakulinen).

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mononuclear site is located in the domain C, the trinuclear site is between domains A and C and the hydrophobic pocket for the reducing substrate is between domains B and C. In the mononuclear site, the T1 copper accepts electrons from a substrate and then electrons are further transferred about a 13 Å distance along a Cys–His pathway into the trinuclear site, where dioxygen is reduced to water. As a matter of fact, there are at two potential Cys–His pathways in all known laccase structures, but it is not known whether both of them are used or not. According to theoretical pathway analysis of an ascorbate oxidase, a Cys507–His506 pathway is three times more efficient in electronic coupling than a Cys507–His508 route (Kyritsis et al., 1993). Despite many crystallographic, spectroscopic and quantum chemical calculation studies, electron transfer processes by blue multicopper oxidases are far from being completely understood. Studies of the catalytic cycle of the multicopper oxidases have shown that the reduction of dioxygen involves two $2e^-$ steps (Solomon et al., 2001). The protons have attained much less attention than electrons, although the reduction of dioxygen requires four protons as well.

Melanocarpus albomyces is an ascomycete fungus, which produces a laccase that has a rather high thermostability and a neutral pH optimum for phenolic substrates (Kiiskinen et al., 2002). The gene encodes a protein of 623 amino acids, but a signal sequence, a propeptide and a C-terminal extension are removed during maturation (Kiiskinen and Saloheimo, 2004). The mature protein consists of 559 amino acids. The three-dimensional structure of *M. albomyces* laccase (MaL) has been solved at 2.4 Å resolution (Hakulinen et al., 2002). This was the first multicopper oxidase structure to show a dioxygen molecule within the trinuclear site. Recently, *M. albomyces* laccase was produced in *T. reesei* (rMaL)[1] and the structure was solved at 2.0 Å (Hakulinen et al., 2006). Here, we report this rMaL structure at 1.3 Å resolution, which is the highest resolution observed for any multicopper oxidase.

2. Results and discussion

2.1. Overall structure

rMaL crystallized in the space group C2 with two molecules, designed as molecules A and B, in the asymmetric unit. The space group was identical and the unit cell dimensions were very similar than those of previously reported for rMaL (Hakulinen et al., 2006). MaL, instead, crystallized in space group P1 (Hakulinen et al., 2002). Two monomers in the asymmetric unit in the crystal have rather extensive interactions with each other (Fig. 1). This kind of "weak dimer" has been observed both with the rMaL in space group C2 and with the MaL in space group P1. There is a two-fold non-crystallographic symmetry axis between molecules A and B and about 4.5% (525 $Å^2$) of the monomer surface is buried on the dimer interface. Molecules A and B are very similar with only 0.2 Å rmsd for the Cα atoms.

Whether the weak dimer of rMaL/MaL has a biological role or not is not known. In solution, the enzyme is a monomer according dynamic light scattering measurements and therefore, it is possible that the weak dimer is only a crystallographic artefact. In a comparison, an ascorbate oxidase is reported to exist as a dimer in solution and as a homo-tetramer in crystal (Messerschmidt et al., 1992). However, it has been suggested very recently that in a high protein concentration the autoreduction of a T1 copper occurs through aggregation, which brings T1 sites into close proximity (Shleev et al., 2006). T1 sites of molecules A and B are near the interface of the dimer in the crystal structure of rMaL/MaL (Fig. 1a). Therefore, the dimerization brings the T1 sites 27 Å apart from each other probably enabling the autoreduction. The distance is rather long for electron transfer, but mononuclear sites are obviously connected by water and glycerol molecules. On the other hand, the fresh unexposed crystals are blue in colour indicating that the T1 site is in an oxidized, not in reduced, form. Crystals decolorize during X-ray measurements indicating the reduction of T1 sites by X-rays (Hakulinen et al., 2006).

Based on the molecular mass of the enzyme, determined by FT-IR ESI mass spectrometer (main peak 71664 Da, data is not shown), the carbohydrate content of rMaL is 13% (9625 Da). In the present structure of rMaL, there are 18 (3449 Da) and 16 (3084 Da) glycan moieties in molecules A and B, respectively. The rest of the glycans may be disordered and not visible in the electron density map. Glycans are normally thought to be flexible and even interfere with the formation of crystal contacts. Therefore, it is rather surprising how well ordered glycan moieties are in *M. albomyces* laccase and how well these crystals with large glycans diffract. Glycan moieties are also well ordered in *C. maxima* laccase (Lyashenko et al., 2006), where glycans were reported to adhere to neighbouring molecules in the lattice in an ordered manner probably assisting the crystallization. In *C. maxima* laccase, glycans were obtained at Asn54, Asn217, Asn333 and Asn436. In the present structure of rMaL, glycans were obtained at Asn39, Asn88, Asn201, Asn216, Asn244, Asn289, Asn376 and Asn396. The positions of glycans are rather different than those of observed in *C. maxima* laccase, except that the glycan at Asn88 is almost in the same place than glycan at Asn54 in *C.maxima* laccase. Glycosylation pattern of rMaL in the space group C2 also differ the glycosylation pattern of MaL in space group P1. The *N*-glycosylation sites between the recombinant and the native form are the same, but the number of carbohydrate units in the glycan trees may differ. Many of the carbohydrate rings in rMaL/MaL are hydrogen-bonded to the parent molecule and also

[1] *Abbreviations used:* ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); BsL, *Bacillus subtilis* laccase; MaL, *Melanocarpus albomyces* laccase; rMaL, recombinant *Melanocarpus albomyces* laccase; RlL, *Rigidiporus lignosus* laccase; TvL, *Trametes versicolor* laccase.