Acta Cryst. (1997). D**53**, 605–607

Crystallization and preliminary X-ray analysis of the laccase from *Coprinus cinereus*

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(*Received 12 March 1997; accepted 27 March 1997*)

Abstract

The laccase from the fungus *Coprinus cinereus* has been prepared and crystallized in a form suitable for X-ray diffraction analysis. Small plate-like crystals of an enzymatically deglycosylated form of the enzyme have been grown by the hanging-drop method using polyethylene glycol as precipitant. These crystals diffract to at least 2.2 Å. They belong to the space group $P2_12_12_1$ with cell dimensions $a = 45.4$, $b = 85.7$, $c = 143.1$ Å with a single molecule of laccase in the asymmetric unit.

1. Introduction

Laccase is a polyphenol oxidase (E.C. 1.10.3.2) which catalyzes the oxidation of a variety of inorganic and aromatic compounds, particularly phenols, with the concomitant reduction of molecular oxygen to water (Adman, 1991; Reinhammar, 1984; Thurston, 1994). Laccase belongs to a family of blue copper-containing oxidases which includes ascorbate oxidase and the mammalian plasma protein ceruloplasmin. All these enzymes are multi-copper proteins. Generally, copper centres are classified on the basis of their distinct spectral features and members of this family contain a combination of all three classes of Cu centres (Solomon & Lowery, 1993).

Laccase is widely distributed in certain trees and fungi. Its biological function is still unclear. One of the proposed functions in woody tissues is a role in lignin biosynthesis (Bao, O'Malley, Whetten & Sederoff, 1993), whilst the biological function in fungi is less obvious. Fungal laccases may participate directly, or indirectly, in lignin degradation (Kersten, Kalyanaraman, Hammel, Reinhammar & Kirk, 1990). In certain fungi, laccase activity has also been suggested to play a role in pathogenesis (Nun, Vel, Harel & Mayer, 1988), development of fruiting bodies (Leatham & Stahmann, 1981) and formation of structural pigments (Clutterbuck, 1972). They have recently become the subject of intensive studies because of their potential in a variety of processes such as delignification, detoxification, and in the textile and dye industries (Xu, 1996).

X-ray crystal structures of blue copper oxidases are now available for the ascorbate oxidase from zucchini (Messerschmidt *et al.*, 1989, 1992) and the human serum ceruloplasmin (Zaitseva *et al.*, 1996). Thus far, no three-dimensional structural information is available for laccase. These enzymes have a modular structure with a number of cupredoxin β-barrel domains (Baker, 1988; Collyer, Guss, Sugimura, Yoshizaki & Freeman, 1990). Ascorbate oxidase and ceruloplasmin have three and six of these β-barrel domains, respectively. In these multi-copper proteins, the Cu atoms are bound as mononuclear and trinuclear species. The mononuclear centre contains the type-1 copper which is coordinated to two histidines and a cysteine and forms a close association with a methionine. This copper constitutes the primary electron acceptor from the reducing substrate. Electrons are transferred from the reduced type-1 copper to the two-electron accepting type-3 copper pair. The trinuclear centre, which is the dioxygen binding site, accepts these electrons with the concomitant reduction of molecular oxygen. Fungal laccases contain the same copper ligands as ascorbate oxidase, with the exception of the type-1 copper in which the methionine ligand may be replaced by a leucine. This methionine residue is also replaced by a leucine residue in two of the three mononuclear centres of ceruloplasmin. In this paper we describe the preparation, crystallization and preliminary X-ray studies on the fungal laccase from *Coprinus cinereus*.

2. Experimental

2.1. *Protein purification*

In order to express the *C. cinereus* laccase in sufficient quantities for crystallization trials, laccase was expressed in and excreted from an *Aspergillus oryzae* expression system. The *C. cinereus lcc*1 gene was inserted behind the *Aspergillus oryzae* α-amylase promoter (Christensen *et al.*, 1988). DNA fragments containing the transcriptional terminator and polyadenylation signals were from the *A. niger* glucoamylase gene. The expression vector pDSY67 also contained the *A. nidulans pyrG* gene for selection of transformants. A pyrg auxotroph of *A. oryzae* A1560 was transformed with pDSY67, and transformants were selected on minimal medium plates with

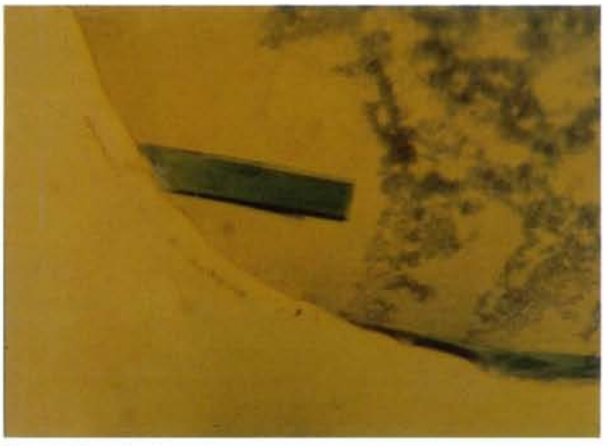

Fig. 1. Crystal of *Coprinus cinereus* laccase. The crystal shown has dimensions 0.2 × 0.02 0.05 × 0.02 mm.

1 *M* sucrose (Christensen *et al.*, 1988). Transformants were screened for laccase production as described previously (Yaver *et al.*, 1996). The highest producing transformant was grown in a 2 l fed-batch fermentor for 6 d. The fungal mycelia were removed by filtration through Miracloth. 'Filter aid' was added to the culture supernatant and the resulting slurry was filtered through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear solution. The filtrate was concentrated by ultrafiltration on 20 kDa cut-off polysulfone membranes and refiltered on a germ filter plate. The protein was purified on a Phenyl Toyopearl 650S column equilibrated with 1.6 *M* $(NH_4)_2SO_4$, 10 m*M* succinic acid–NaOH pH 6.0 with a decreasing linear gradient from 1.6 *M* ammonium sulfate. The laccase-containing fractions were pooled and buffer exchanged with 20 m*M* acetate–HCl pH 6.0 on a Sephadex G25 column. Laccase was further purified on a fast-flow Q-Sepharose column equilibrated with 20 m*M* acetate buffer pH 6.0, and eluted with a linear NaCl gradient from 0 to 0.4 *M* in the same buffer. This purification resulted in a pure laccase preparation as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

2.2. *Crystallization*

Pure recombinant *C. cinereus* laccase was purified as above. Prior to crystallization, the laccase was washed in a Centricon concentrator (30 kDa cut-off, Amicon) with 10 m*M* acetate buffer pH 5.5 and concentrated to 30 mg ml^{-1}. All crystallizations were performed 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 the reservoir solutions. Optimized crystallization trials of *C. cinereus* laccase usually produced blue plate-like crystals with irregular edges after 4–7 d, the largest having dimensions of approximately 0.5 × 0.5 × 0.02 mm, Fig. 1. Crystals grew from polyethylene glycol 8000 [26–30%(*v*/*v*) of 50%(*w*/*v*) stock solution], 0.1 *M* acetate buffer pH 5.5. The resulting crystals were extremely fragile and sensitive to slight changes in temperature and mother liquor and decomposed quickly even upon gentle manipulation. All these properties made these crystals very difficult objects for use in the X-ray experiments. The best crystals diffracted well, to 2.2 Å using a Cu rotating-anode source, but were disordered and displayed extremely high mosaicity preventing further X-ray studies. All attempts of stabilization of these crystals proved unsuccessful and, therefore, enzymatic deglycosylation of laccase was undertaken to improve crystal quality. Laccase was deglycosylated using a mixture of endoglycosidase F and *N*-glycosidase F (Boehringer Mannheim Biochemica): 3 units of enzymes were added per 3 mg of laccase in 0.1 *M* acetate pH 5.0, 20 m*M* EDTA, and followed by overnight incubation at room temperature.

2.3. *Data collection and processing*

X-ray diffraction data from a single native crystal were measured, at room temperature, to 2.2 Å using a 300 mm MAR Research imaging-plate detector on a Rigaku rotating-anode RU-200 X-ray generator with Cu target, operating at 50 kV and 100 mA, with the use of focusing X-ray optics (MSC). 144° of data, to a resolution of 2.2 Å, were collected with a oscillation range of 1° and exposure time of 30 min per image. Data were processed with the *DENZO* program (Otwinowski, 1993) and scaled and reduced using the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Enzymatic deglycosylation resulted in a laccase sample that gave reproducible crystals. Although the degree of the deglycosylation was difficult to monitor on SDS–PAGE gels, perhaps indicating some remaining *O*-linked glycosylation, morphologically well defined crystals were obtained. These plate-like crystals could be mounted in capillaries and were sufficiently stable for data collection. Additional stabilization of the deglycosylated crystal form was achieved by the presence of 200 m*M* NaCl in the mother liquor during crystallizations. *C. cinereus* laccase crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell dimensions $a = 45.4$, $b = 85.7$, $c = 143.1$ Å. The final data are 99.6% complete to 2.23 Å resolution with an overall $R_{\rm merge}$ $(\sum_{hkl}\sum_i |I_{hkl} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i \langle I_{hkl}\rangle)$ of 0.117, a mean $I/\sigma(I)$ of 8.4 and a multiplicity of 5.4 observations per reflection. In the outer resolution bin (2.35–2.23 Å) the $R_{\rm merge}$ is 0.27 with an $I/\sigma(I)$ of 4.8, completeness of 98.5% and multiplicity of 5.2. Optimal $I/\sigma(I)$ was achieved with the highest redundancy data, although 'better' $R_{\rm merge}$ statistics could be achieved through harsher rejection criteria. Assuming that there is one molecule of approximately 52 kDa per asymmetric unit, the crystal packing density was determined to be $V_M = 2.7$ Å^3 Da^{-1}, corresponding to a solvent content of approximately 54% (Matthews, 1968). Heavy-metal derivative screening, and molecular replacement studies are currently in progress to assist with rapid structural determination. Laccase is the simplest of the blue multi-copper oxidases, containing one of each class of Cu centre for a total of four Cu atoms. This relative simplicity makes laccase a good model to study structure–function relationships within this family. The structure determination should assist in the elucidation of the catalytic mechanism and electron-transfer processes in this important family of proteins. It is hoped that the *C. cinereus* laccase may be interesting for various industrial applications since it shows relatively high activity at alkaline pH compared with other fungal laccases (Damhus, Schneider, Bech & Heinzkill, 1996).

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