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Crystal structure of the blue multicopper oxidase from the white-rot fungus *Trametes trogii* complexed with *p*-toluate

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Dedicated to Professor Dante Gatteschi.

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ABSTRACT

A multicopper oxidase, the fungal laccase glycoenzyme from the white-rot basidiomycete fungus *Trametes (Funalia) trogii*, was crystallized and its crystal structure was solved at 1.58 Å using molecular replacement techniques.
Model refinement resulted in *R*-factor and *R*-free values of 17.4% and 19.0%, respectively. The *T. trogii* laccase structural model reveals the presence of a ligand bound to the T1 active site which resembles a *p*-toluate molecule, such bound compound is most probably a fungal metabolite. The *p*-toluate is bound into the T1 active site of the laccase forming, with one of the carboxylate oxygens, a H-bond with His455, one of the T1 copper ion ligands, whereas the methyl group presents hydrophobic interactions within a pocket composed by Phe331, Phe336, Pro390 and Val162.
The coordination geometries, the bond distances and the oxidation states of the T1 and T2/T3 copper active sites are analyzed and discussed in terms of the enzymatic mechanism and catalytic functionality.

"My first scientific publication in 1972 was the result of joined work with Dante Gatteschi and Ivano Bertini, and this crucial collaboration resulted in a significant number of studies at the beginning of my career, mainly on single crystal electronic and esr spectra of coordination compounds. Then, our interests set apart, Dante became more and more involved into magnetic characterization of paramagnetic metalloclustered coordination compounds and myself oriented towards the study of metalloenzymes.
It is now a pleasure for my co-workers and myself to present this contribution dedicated to Dante, where, in some way, the metalloenzymes investigated encounter some of the issues pioneered in his studies.
Bioinorganic chemists evidenced that the clustering of paramagnetic metal ions in metalloproteins, beside affecting their magnetic properties, has a real functional role, exploiting a different chemistry compared to that of the single metal ions.
So we can pragmatically say that the study of the magnetic properties of a metal cluster (a topic to which Dante brought many new theoretical contributions) give us essential information on the relative distances between the paramagnetic atoms and the strength of their spin-spin interactions but the original chemical reactivity exhibited by the clustered moieties has to be *de novo* characterized case by case".

Andrea Scozzafava

1. Introduction

Laccases (EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase) are blue-copper proteins distributed in plants, fungi and bacteria, that belong to the so-called multicopper protein family including ascorbate oxidase, ceruloplasmin and bilirubin oxidase that use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism.

Laccases catalyze the reduction of dioxygen to water accompanied by the one electron oxidation of a substrate, typically a *p*-hydroxy phenol or other phenolic compounds, their reducing substrates depending mainly from the redox potential of the laccase which varies largely across the group.

$$O_2 + 4e^- + 4H^+ \rightarrow 4H_2O$$

For the catalytic activity four copper atoms per protein unit are needed. The four copper ions are traditionally distinguished as follows [1] on the basis of their spectroscopic and/or magnetic properties:

Type 1: paramagnetic 'blue' copper, absorbance at 610 nm (oxidized).
Type 2: paramagnetic "normal" copper.
Type 3: diamagnetic spin-coupled copper–copper pair, absorbance at 330 nm (oxidized).

Several crystal structures of laccases of fungal origin from *Lentinus tigrinus*, *Coprinus cinereus*, *Trametes versicolor*, *Melanocarpus*

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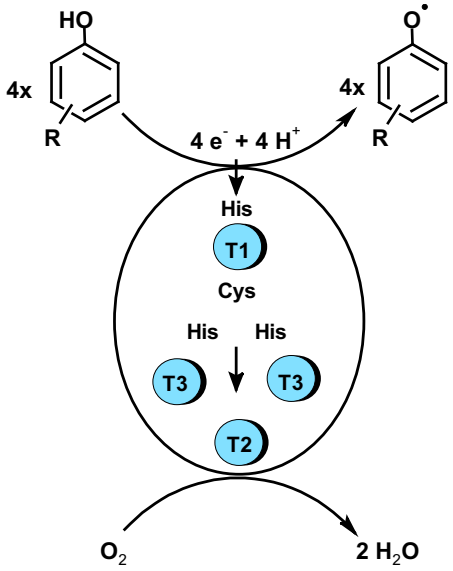
albomyces, *Rigidoporus lignosus* [2–7], and from bacteria: the spore coat protein cotA from *Bacillus subtilis* have been reported [8].

From these structures it results that Type 1 copper has a trigonal coordination, with two histines and a cysteine as conserved equatorial ligands. A further axial ligand at a longer distance is also observed in several multicopper oxidases. It has been widely argued that this axial position ligand strongly influences the oxidation potential of the enzyme, possibly providing the mechanism for regulating its activity. A mutation from phenylalanine to methionine significantly lowered the oxidation potential of a fungal laccase from *Trametes villosa* [9]. Type 1 copper confers the typical blue colour to multicopper proteins, which results from the intense electronic absorption caused by the covalent copper–cysteine bond. Due to its high redox potential of ca. 790 mV, Type 1 copper is the site where substrate oxidation takes place.

Type 2 or normal Cu(II) site is characterized by the lack of strong absorption features in the visible region and reveals usual EPR spectra. It is strategically positioned close to the Type 3 copper, a binuclear center spectroscopically characterized by an electron adsorption at 330 nm (oxidized form) and by the absence of an EPR signal as the result of the anti-ferromagnetic coupling of the copper pair.

The Type 3 copper center is also the common feature of another protein superfamily including the tyrosinases and haemocyanins [10]. Type 2 and Type 3 copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place (Scheme 1). Type 2 copper is coordinated by two histidines and Type 3 copper atoms by six histidines. The strong anti-ferromagnetical coupling between the two Type 3 copper atoms, is related to the presence of a hydroxyl bridge.

The chemistry, function and biotechnological use of laccases have recently been reviewed. The basic aspects of laccase structure and function were reviewed by Thurston [11], Leonowicz et al. [12] focused on the functional properties of fungal laccases and their involvement in lignin transformation and Mayer and Staples [13] dealt with the latest results about the roles of laccases in vivo and its biotechnological applications. The physico-chemical properties of multicopper oxidases have been comprehensively reviewed by several authors [14–18]. An overview of technological applications of oxidases including laccase was published by Duran et al. [19,20]. Recently our group reported the X-ray structure of some oxygen intermediates in the active site of a laccase from *L. tigrinus* [4].



Scheme 1. Catalytic cycle of laccases.

From a mechanistic point of view, the simplest reactions catalyzed by laccases are those in which the substrate molecules are oxidized to the corresponding radicals by direct interaction with the copper cluster. Frequently, however, the substrates are not oxidized directly by laccases being to large to penetrate into the enzyme active site. Nature overcomes this limitation with the utilization of mediators, which are suitable compounds that act as intermediate substrates for laccases, whose oxidized radical forms are able to interact with the bulky substrate or enzyme targets. Indeed, laccase is considered to play a major role in the degradation of lignin by white-rot fungi. Degradation of the high M.W. lignin polymer is expected to occur through the involvement of natural mediators, although many aspects of the process still remain unclear. Indeed, owing to their high non-specific oxidation capacity, and the use of readily available molecular oxygen as an electron acceptor, laccases are useful biocatalysts for diverse biotechnological applications. Redox biocatalysts are highly desirable because of the selectivity, controllability and economy of their reactions, in comparison with conventional chemical reactions. Moreover, biocatalyst-based processes require less energy and minimize the amount of waste produced, whilst at the same time being able to improve the quality and functional specifications of products.

In this paper we report the X-ray crystal structure of the fungal laccase isolated from *Trametes trogii* 201, a white-rot basidiomycete involved in wood decay worldwide, good producer of laccases and other ligninolytic enzymes [21–23]. This fungus was shown to be efficient in the degradation of several organic pollutants including PCB and PAH mixtures as well as textile dyes [24–30].

The X-ray structure of this metalloprotein, beside the well known structural organization of the four copper atoms, shows the unusual presence of a small aromatic molecule bound to the T1 copper. This finding will be discussed in the light of reaching a deeper understanding of the interaction between laccases and small organic molecules to be used as mediators.

2. Materials and methods

2.1. Organism and culture conditions

The white-rot fungus *T. Trogii* 201 (DSM 11919) was maintained on BRM agar plates at 4 °C and periodically transferred onto fresh BRM agar plates and grown at 28 °C [31]. After 4–6 days 500 ml shaken flask cultures containing 150 ml liquid BRM were prepared by inoculating with 10 plugs of fungal mycelia (5 mm) and grown in the dark at 28°C with continuous agitation (130 rpm).

After 4 days the grown mycelia were transferred (10%, v/v) in baffled 2000-ml Erlenmeyer flasks containing 1000 ml of fresh BRM liquid medium and grown under the same conditions. The laccase expression was further induced by addition of 150 μM $CuSO_4$. When the extracellular laccase activity reached a maximum about on day 7, the culture supernatant was collected by filtration through Whatman No. 1 paper and concentrated using an ultrafiltration device with a 30,000 Da cut-off membrane.

2.2. Enzyme assay

Laccase activity was determined spectrophotometrically based on the capacity of this enzyme to oxidize the non-phenolic compound 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) [32]. Oxidation of ABTS by laccase results in the production of a green–blue coloured radical cation ($ABTS^{+\cdot}$) measurable at 420 nm ($\varepsilon_{420} = 36000\ M^{-1}\ cm^{-1}$). The assay mixture consists of 2 mM ABTS (final concentration) and 0.1 M Na-citrate buffer, pH 3, and the fungal extracellular medium containing the laccase activity to be measured at 25 °C.