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Elucidation of the crystal structure of *Coriolopsis caperata* laccase: restoration of the structure and activity of the native enzyme from the T2-depleted form by copper ions

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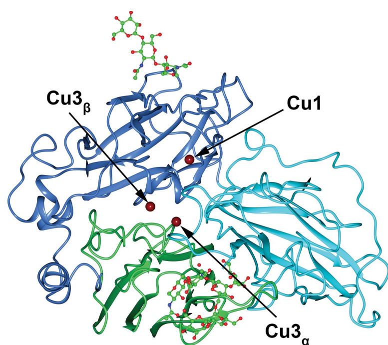
Laccases are members of a large family of multicopper oxidases that catalyze the oxidation of a wide range of organic and inorganic substrates accompanied by the reduction of dioxygen to water. A new laccase was isolated from the basidiomycete *Coriolopsis caperata* strain 0677 and its amino-acid sequence was determined. According to its physicochemical properties and spectroscopic features, the laccase from *C. caperata* is a high redox-potential blue laccase. Attempts to crystallize the native enzyme were unsuccessful. The copper type 2-depleted (T2D) laccase was prepared and crystallized. The structure of T2D laccase from *C. caperata* was solved at 1.6 Å resolution, and attempts to reconstruct the T2 copper centre were performed using Cu^{+} and Cu^{2+} ions. The structure of T2D+Cu^{+} laccase was solved at 1.89 Å resolution. It was shown that the T2D+Cu^{+} laccase structure contained four copper ions in the active site. Reconstruction could not be achieved when the T2D laccase crystals were treated with $CuSO_4$.

1. Introduction

The laccases (EC 1.10.3.2) belong to the multicopper oxidase family and catalyze the four-electron reduction of molecular oxygen to water with the simultaneous oxidation of various phenolic and nonphenolic compounds as well as many environmental pollutants (Baldrian, 2006; Bourbonnais & Paice, 1990; Majeau *et al.*, 2010). The properties of laccases give rise to numerous applications in various branches of biotechnology such as biodegradation and detoxification (Gianfreda *et al.*, 1999; Gianfreda & Rao, 2004; Amitai *et al.*, 1998; Torres *et al.*, 2003), organic synthesis, the production of new pharmaceutical drugs, energy conversion (Barton *et al.*, 2002; Marzorati *et al.*, 2005; Baratto *et al.*, 2006; Riva, 2006), the textile industry, drink preservation and stabilization, and many others (Rodríguez Couto & Toca-Herrera, 2006; Riva, 2006; Shraddha *et al.*, 2011). Therefore, the screening of new fungal strains for production of laccases as well as their catalytic mechanism has been the focus of numerous studies (Rodgers *et al.*, 2010; Dwivedi *et al.*, 2011; Rivera-Hoyos *et al.*, 2013; Viswanath *et al.*, 2014).

The laccase active centre is formed by four copper ions, which are divided into three types depending on their spectral characteristics. The type 1 copper ion (T1) forms a mononuclear centre participating in organic substrate oxidation, and is characterized by optical absorption at 610 nm and



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a characteristic electronic paramagnetic resonance (EPR) signal. The type 2 (T2) copper ion and the pair of type 3 (T3) copper ions form a T2–T3 cluster where oxygen reduction takes place. The T2 copper ion is EPR active, but is not detected in optical absorption spectra. The T3 copper ions are characterized by optical absorption at 340 nm, but they are EPR silent owing to antiferromagnetic fixation (Solomon *et al.*, 1996; Giardina *et al.*, 2010; Rivera-Hoyos *et al.*, 2013). One of the most significant characteristics of laccases is the redox potential of the T1 copper, as it determines the efficiency of enzyme catalysis. Fungal laccases produced by 'white-rot' basidiomycetes commonly have a high redox potential and are of exceptional interest to biotechnologists.

Recently, an extensive analysis of fungal laccases has been performed (Rivera-Hoyos *et al.*, 2013). 11 high redox-potential laccases were structurally characterized, but only two structures were solved, with a resolution of around 1.5 Å. This can be explained by difficulties in laccase crystallization owing to the presence of a number of laccase isoenzymes and their separation and purification. It has been shown for several laccases that elimination of the T2 copper ions facilitated the crystallization of the modified enzymes. Our previous studies showed a significant increase in resolution for crystals of T2-depleted laccases from *Trametes hirsuta* compared with the native enzyme: 1.2 Å (PDB entry 3pxl) and 1.8 Å (PDB entry 3fpx), respectively (Polyakov *et al.*, 2009). For *Coprinus cinereus* (Ducros *et al.*, 2001) and *Botrytis aclada* (PDB entry 3sqr; Osipov *et al.*, 2014) laccases, only structures without the type 2 copper are available. Methods of T2 depletion have been developed for plant, fungal and bacterial laccases (Graziani *et al.*, 1976; Hanna *et al.*, 1988; Klemens & McMillin, 1990; Koroleva *et al.*, 2001; Galli *et al.*, 2004; Durão *et al.*, 2008). Moreover, the possibility of the reconstitution of T2-depleted laccases to give native enzymes with restored catalytic activity by adding copper salts and ascorbic acid to the enzyme solution under anaerobic conditions has been well documented for plant and fungal laccases (Graziani *et al.*, 1976; Hanna *et al.*, 1988; Klemens & McMillin, 1990; Koroleva *et al.*, 2001).

Here, we report the X-ray crystal structures of laccase from *Coriolopsis caperata* in the T2-depleted (T2D) form and in the reconstructed enzyme form with four copper ions obtained by the soaking of T2D laccase crystals in cuprous ion solution.

2. Methods

2.1. Enzyme production, purification and preliminary characterization

The basidiomycete *C. caperata* strain 0677 was obtained from the Komarov Botanical Institute, Russian Academy of Sciences, St Petersburg, Russia. The strain was stored on an agar slant. The medium was prepared by diluting ale wort with water in a 1:4(*v*:*v*) ratio and adding 2% agar. The strain was cultivated in the dark on a shaker at 180 rev min^{-1} for 14 d at 28°C in 750 ml conical flasks containing 25 ml fungal inoculum and 200 ml glucose–peptone medium with the following composition: 10.0 g l^{-1} glucose, 3.0 g l^{-1} peptone, 0.6 g l^{-1} KH_2PO_4, 0.001 g l^{-1} $ZnSO_4.2H_2O$, 0.4 g l^{-1} K_2HPO_4, 0.0005 g l^{-1} $FeSO_4.7H_2O$, 0.05 g l^{-1} $MnSO_4.7H_2O$, 0.5 g l^{-1} $MgSO_4.7H_2O$, 0.5 g l^{-1} $CaCl_2$, with 0.15 g l^{-1} $CuSO_4$ as an inducer of laccase production (Fedorova *et al.*, 2013).

The laccase was isolated from the culture liquid after submerged cultivation of *C. caperata* 0677. At the end of the fermentation period, the culture liquid was separated by filtration (Whatman No. 1 filter paper) and the filtrate was precipitated with ammonium sulfate at 80% saturation. The laccase was purified by ion-exchange chromatography on DEAE Cellulose (batch) and Toyopearl DEAE 650M (Tosoh, Japan) as described in Koroljova-Skorobogat'ko *et al.* (1998). FPLC size-exclusion chromatography was used to purify the enzyme to a homogeneous state. FPLC was performed on a Superdex 75 (26/60) column (GE Healthcare Life Sciences, USA) previously equilibrated with 20 m*M* potassium phosphate buffer pH 6.5.

The molecular weight and homogeneity of the enzyme preparation were determined by SDS–PAGE and FPLC. SDS–PAGE was carried out according to Laemmli (1970) in a Mini-PROTEAN 3 device (Bio-Rad, USA). Protein bands were stained with Coomassie Brilliant Blue R-250 (Sigma, USA). A PageRuler Prestained Protein Ladder (Fermentas, Lithuania) protein mixture with a range of 10–200 kDa was used as a standard. FPLC was performed on a Superdex 200 preparative-grade column (GE Healthcare/Life Sciences, USA) calibrated with a set of standard markers with a range of 30–200 kDa (Molecular Weight Marker Kit, Sigma, USA).

Isoelectrofocusing (IEF) was carried out using Ampholyte 3/5 (Bio-Rad, USA) in a Mini IEF Cell (Bio-Rad, USA). An IEF kit low-range (Amersham) protein mixture with a pI range of 2.5–6.5 was used as a standard.

Determination of the carbohydrate content was performed after acid hydrolysis (incubation in a mixture of 4 *M* HCl and 4 *M* trifluoroacetic acid for 4 h at 100°C) and subsequent analysis of the monosaccharides on a CarboPac PA20 column using an Agilent 1100 Series HPLC system (Agilent, USA) with a Caulochem III detector.

Genomic DNA and RNA were extracted from the *C. caperata* 0677 fungal biomass as described by Fedorova *et al.* (2013). Total RNA was used as a template for the synthesis of the first cDNA strand in the reverse transcription reaction (RT-PCR) using a MINT cDNA kit (Evrogen, Russia) in accordance with the manufacturer's instructions. The obtained cDNA was used for PCR. The full-length cDNA sequence of the laccase was determined using rapid amplification of cDNA ends (RACE) PCR. The Mint RACE cDNA amplification kit (Evrogen, Russia) was used according to the manufacturer's protocol. For amplification of the 3′-end, the gene-specific primers 3′for1 (5′-AACCACACCTTCAGCATCGAC-3′), 3′for2 (5′-CTGTAATCACTCTCGCCGAC-3′) and 3′for3 (5′-AACCACACCTTCAGCATCGAC-3′) were designed and used. For amplification of the 5′-end, the gene-specific primers 5′for1 (5′-CGGAGGATAGCAGAGTTGAC-3′), 5′for2 (5′-GGCGAAGATCTGAATGGAGT-3′) and 5′for3 (5′-GTAGAGGTCCTTGTGGGGGT-3′) were designed and

used. PCR conditions depended on the primer-annealing time and temperature. All reactions contained 1.25 units of Encyclo DNA polymerase (Evrogen, Russia) per probe, 1.5–2.5 m*M* $MgCl_2$, 0.2 m*M* of each dNTP, a suitable quantity of DNA and 10 p*M* of each primer in a total volume of 50 µl. The resulting PCR fragments were cloned into pAL-TA vector (Evrogen, Russia) and sequenced using plasmid-specific primers in *Escherichia coli* XL1-Blue competent cells. The nucleotide sequences of the cloned DNA fragments were determined using an ABI PRISM BigDye Terminator v.3.1 reagent kit, with subsequent analysis of the reaction products using an ABI PRISM 3730 automatic DNA sequencer (Applied Biosystems, USA) at the Genome Collective Use Center (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). As a result, a 1551 bp sequence of the *C. caperata* 0677 laccase gene was identified. Sequence alignment was carried out using *ClustalW* (Thompson *et al.*, 1994). The obtained sequence was submitted to GenBank (accession No. KC119400). The amino-acid sequence was translated from the nucleotide sequence.

Protein concentration was measured with the BCA Protein assay kit (Pierce, USA) according to the manufacturer's instructions.

2.2. Enzymatic activity

Laccase activity was measured spectrophotometrically at 410 nm (ε = 740 cm^{-1} M^{-1}) using a PerkinElmer Lambda 25 spectrophotometer (USA) with catechol (Sigma, USA) as a substrate. 10 µl enzyme solution at a concentration of 1 mg ml^{-1} (0.05 *M* potassium phosphate buffer pH 6.5) was added to 2 ml 10 m*M* catechol solution in 0.1 *M* sodium acetate buffer pH 4.5. One unit (U) of activity is defined as the amount of enzyme that produces 1 µmol of product per minute under the assay conditions.

2.3. Redox-potential measurement

Redox potential was measured according to the redox-titration technique (Farver *et al.*, 2011). $Na_2(IrCl_6)$ and $K_4[Fe(CN)_6]$ were used as a redox pair.

2.4. T2 copper depletion and reconstruction

The type 2 copper-depleted (T2D) enzyme was prepared as described previously (Koroleva *et al.*, 2001). The reaction mixture (final volume of 6 ml) consisted of 0.13 m*M* laccase, 28 m*M* ascorbic acid (Sigma, USA), 1 *M* guanidinium chloride (Merck, Germany) and 1.2 m*M* bathocuproinedisulfonic acid disodium salt (Merck, Germany) in 100 m*M* sodium acetate buffer pH 4.5. After incubation for 40 min at room temperature, the reaction mixture was loaded onto a Sephadex G-25 column (2 × 55 cm; Pharmacia, Sweden) equilibrated with 20 m*M* phosphate buffer pH 6.0. The eluted protein solution was concentrated by ultrafiltration using Vivaspin 20 membranes (Sartorius, Germany).

The reconstruction of laccases in solution for activity measurements was carried out using cupric and cuprous ions. The enzyme at a concentration of 1 mg ml^{-1} in 0.05 *M* potassium phosphate buffer pH 6.5 was incubated with a saturated solution of 0.6 m*M* CuCl (Sigma, USA) and with 100 m*M* $CuSO_4.5H_2O$ (Sigma, USA) for up to 24 h at 25°C under aerobic conditions. The enzymatic activity was monitored.

Table 1
Data-collection and refinement statistics.

Values in parentheses are for the outer shell.

Structure	T2D	T2D+Cu^+	T2D+Cu^{2+}
PDB code	4jhv	4jhu	
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	*a* = 62.78, *b* = 84.91, *c* = 116.95	*a* = 62.90, *b* = 84.42, *c* = 116.12	*a* = 62.31, *b* = 84.18, *c* = 115.70
Resolution (Å)	20–1.60 (1.65–1.60)	50–1.89 (2.01–1.89)	20–1.67 (1.78–1.67)
No. of observed reflections	599320	124869	311109
No. of unique reflections	80952	20881	68244
Completeness (%)	97.4 (88.0)	98.2 (91.8)	96.8 (95.4)
$\langle I/\sigma(I)\rangle$	32.6 (5.9)	16.1 (3.3)	22.75 (5.0)
R_{meas}† (%)	4.3 (34.1)	9.2 (59.8)	4.8 (30.0)
B factor (Wilson plot) ($Å^2$)	17.3	25.3	18.6
R factor (%)	15.6	20.2	16.5
R_{free} (%)	17.8	26.2	19.9
Cruickshank's DPI for coordinate error (Å)	0.07	0.12	0.09
No. of atoms			
Protein	3804	3798	3792
Cu	3	14	3
Water	569	341	473
Other	149	67	140
R.m.s.d. from ideal values			
Bond lengths (Å)	0.018	0.021	0.019
Bond angles (°)	1.9	1.8	2.2

† $R_{meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i|I_i(hkl)-\langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th observation with indices *hkl*, $\langle I(hkl)\rangle$ is the mean intensity of the reflection and $N(hkl)$ is the multiplicity of the reflection with indices *hkl* (Diederichs & Karplus, 1997).

2.5. Crystallization

Screening of crystallization conditions was performed for the native and the T2D laccase. The protein samples used for crystallization were at a concentration of 10 mg ml^{-1} in 20 m*M* phosphate buffer pH 6.5. Preliminary crystallization trials were performed at 297 K by the hanging-drop vapour-diffusion method (2 µl protein solution and 2 µl reservoir solution was equilibrated against 600 µl reservoir solution) using the Crystal Screen and Crystal Screen 2 kits (Hampton Research, USA). Attempts to obtain native laccase crystals were unsuccessful. Rod-like crystals of T2D laccase were obtained under the condition 0.1 *M* HEPES pH 7.5, 10% PEG 8000, 8% ethylene glycol. The most promising conditions for T2D laccase were optimized. The concentrations of the protein and the precipitant, as well as the buffer pH, were systematically varied in order to grow larger and regularly shaped crystals. The best crystals were grown by mixing 2.0 µl protein solution at a concentration of 24 mg ml^{-1} and an equal volume of reservoir solution consisting of 0.1 *M* HEPES buffer pH 6.0, 20%(*w*/*v*) PEG 8000.

2.6. Data collection

Before data collection, the T2D laccase crystals were soaked for 5–10 s in a cryoprotective solution consisting of reservoir solution supplemented with 20%(*v*/*v*) PEG 600. For the reconstruction of T2D laccase with cuprous and cupric ions, the cryoprotective solution additionally contained CuCl at a concentration of 0.6 m*M* (saturating concentration) and $CuSO_4$ at a concentration of 100 m*M*. The crystal was soaked for 10 min in the solution containing CuCl and for 24 h in the solution containing $CuSO_4$. Sets of diffraction data were collected at 100 K using an Oxford Instruments low-temperature system.

Collection of data from the T2-depleted *C. caperata* (T2D) laccase crystal was carried out at station X13 of the DESY synchrotron at EMBL (Hamburg) to 1.6 Å resolution and at a wavelength of 0.8123 Å using a 165 mm MAR CCD detector.

Collection of data from crystals soaked in CuCl (T2D+Cu^+) and $CuSO_4$ (T2D+Cu^{2+}) was carried out at the K4.4 station of the Kurchatov Center for Synchrotron Radiation and Nanotechnology at a wavelength of 0.99 Å using a MAR CCD 165 detector. The maximum resolution for the crystal soaked in CuCl was 1.89 Å and that for the crystal soaked in $CuSO_4$ was 1.67 Å. Statistical characteristics of the diffraction data sets are given in Table 1.

2.7. Structure solution

The T2D laccase structure resolution was solved by molecular replacement using *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP*4 package (Winn *et al.*, 2011). The *T. hirsuta* laccase structure was used as a starting model (Polyakov *et al.*, 2009; PDB entry 3fpx).

The structures were refined with *REFMAC*5 (Murshudov *et al.*, 2011).

Manual correction of the model was carried out with *Coot* (Emsley *et al.*, 2010). Data-collection and refinement statistics are given in Table 1.

For the T2D and T2D+Cu^{2+} structures, the occupancies of the copper ions were refined in manual mode during the final stage of structure refinement. The atomic temperature factors of the copper ions were considered to be approximately equal to the temperature factors of the copper-coordinating atoms, and the occupancy values of the copper ions were varied until the peaks in the difference electron-density synthesis disappeared. For the T2D+Cu^+ structure occupancy values were not specified for the copper ions in the active centre because of insufficient quality of the diffraction data.

3. Results and discussion

3.1. Characterization of the laccase from *C. caperata* and preparation of the T2-depleted enzyme

Screening of a basidiomycetes collection revealed the strain *C. caperata* 0677, which demonstrates high delignification and detoxification potential (Index Fungorum LSID 445868; Fedorova *et al.*, 2013). Homogeneous laccase from *C. caperata* was obtained. The homogeneity was shown by SDS–PAGE and FPLC (data not shown). The molecular weight of the laccase was estimated to be about 63 kDa according to SDS–PAGE and FPLC, which is in line with the values for fungal laccases. The data obtained made it evident that the laccase was monomeric. Based on the molecular weight calculated from the amino-acid sequence (about 54 kDa), the carbohydrate content should be 14%. The glycosylation level of the laccase was about 16% after the hydrolytic procedure, which is in the range of values reported for basidiomycetes laccases.

The isoelectric point of the enzyme was 3.5, which is common for fungal laccases. The UV–Vis spectra showed a

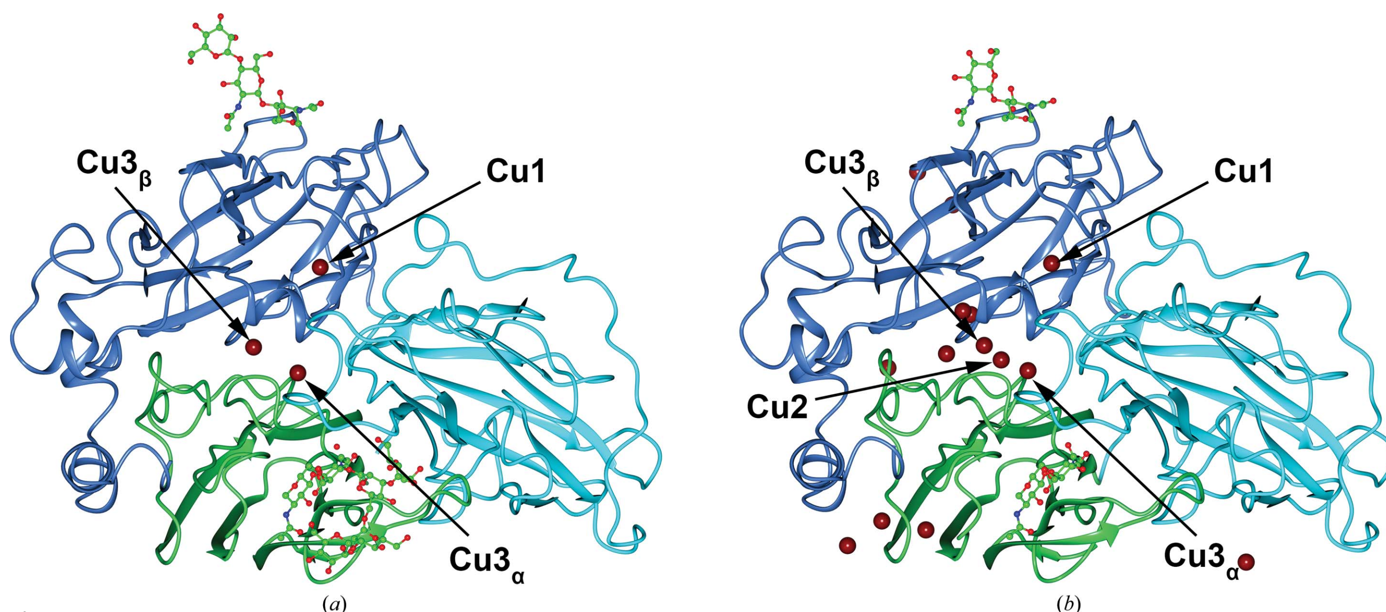

Figure 1
Overall structure of *C. caperata* laccase: (*a*) T2D structure, (*b*) T2D+Cu^+ structure. The first domain is indicated in green, the second domain in cyan and the third domain in blue. Copper ions are indicated as red spheres.

peak in absorption at around 610 nm typical for T1 copper ions and a shoulder at around 340 nm corresponding to the T3 binuclear copper pair. The data obtained allowed the laccase from *C. caperata* to be defined as a typical blue laccase. The enzyme possessed a high redox potential of 780 mV for the T1 centre.

The catalytic activity of the T2D laccase amounted to approximately 4% of that of the native enzyme. The UV–vis spectra of the laccases were similar and contained an absorbance peak at 605–610 nm and a shoulder at 330 nm characteristic of blue laccases. This confirmed that the T1 and T3 centres in T2D remained unmodified. The EPR spectrum of the T2D enzyme did not contain copper T2 peaks, which were clearly detected for the native laccase (data not shown).

The incubation of the T2D laccase with $CuSO_4$ did not result in restoration of activity (which remained at 4% of that of the native enzyme), whereas incubation with CuCl led to complete recovery of enzymatic activity.

3.2. Overall structure of the laccase from *C. caperata*

The T2D, T2D+Cu^{+} and T2D+Cu^{2+} structures were solved. The resolution of the T2D+Cu^{2+} structure did not differ compared with the resolution of the T2D structure. The resolution of the T2D+Cu^{+} structure decreased to 1.89 Å and its mosaicity increased from 0.2 to 0.4°.

The structure of *C. caperata* laccase is similar to previously solved structures of basidiomycetes laccases (Polyakov *et al.*, 2009; Giardina *et al.*, 2010; Dwivedi *et al.*, 2011). The overall structure of the *C. caperata* laccase is shown in Fig. 1. The T2D+Cu^{2+} and T2D+Cu^{+} structures may be superposed with the T2D structure on the coordinates of equivalent C^{α} atoms with r.m.s.d.s of 0.18 and 0.24 Å, respectively. The T2D and T2D+Cu^{2+} structures were almost identical (Fig. 1*a*). Therefore, the T2D+Cu^{2+} structure was not deposited in the PDB. In the T2D and T2D+Cu^{2+} structures copper ions were detected in the T1 and T3 centres. The differences in the T2D+Cu^{+} structure were mainly associated with the detection of additional binding sites for copper ions. In this structure additional copper ions were detected in the T2 centre and in ten places on the surface of enzyme far from the active sites (Fig. 1*b*). These ten ions seem unlikely to be of functional importance.

The laccase from *C. caperata* is a monomer consisting of three cupredoxin-like domains located sequentially in the amino-acid chain and forming a globule with dimensions of 65 × 53 × 40 Å. The first domain (residues 1–128) contained three α-helices and two β-sheets.

The second domain (residues 129–308) was composed of four short α-helices and two β-sheets of mixed type consisting of five and six β-strands.

The third domain (residues 309–496) was formed of six α-helices and two β-sheets, each consisting of five β-strands.

The structure contained two disulfide bridges. One was formed by Cys117 and Cys204 and was located in the first domain and the second connected the first domain (Cys85) and the α-helix of the third domain (Cys485). The first bridge had two conformations in the T2D+Cu^{+} structure, but the occupancy of the residues in the second (open) conformation was only 0.1.

The mononuclear T1 centre was located in the third domain and the T2–T3 cluster was located between the first and third domains. In the T2D+Cu^{+} structure a T2 copper ion with full occupancy was localized and ten additional copper ions with partial occupancy were localized on the surface of the globule in different parts of the molecule (Fig. 1). Additional copper ions were coordinated mainly by aspartic acid, histidine and glutamic acid residues, which are common ligands for copper-binding sites (Bertini *et al.*, 2010).

Analysis of the *C. caperata* amino-acid sequence showed three potential glycosylation sites (N*X*S/T motifs). Sugar residues were localized at two of them (Asn54 and Asn433). The T2D laccase structure contained nine sugar residues. The branched chain linked to Asn54 consisted of two *N*-acetylglucosamine residues and four mannose residues. The *N*-acetylglucosamine residues and β-D-mannose residue formed $\beta(1\rightarrow4)$ bonds. The O3 atom of the β-D-mannose residue was attached to an α-D-mannose and the O6 atom was attached to two α-D-mannose residues [constrained $\alpha(1\rightarrow3)$; Fig. 2].

The Asn433 chain was linear and consisted of two *N*-acetylglucosamine residues [$\beta(1\rightarrow4)$ linkage] and a β-D-mannose residue with a $\beta(1\rightarrow3)$ bond.

In the structure of T2D+Cu^{2+} the glycosylation was identical to that for the T2D structure. In the T2D+Cu^{+} structure only *N*-acetylglucosamine residues and one β-D-mannose residue in the chain at Asn54 were localized in the electron-density maps; this may be caused by the reduced resolution of the structure owing to crystal soaking in a CuCl-saturated solution.

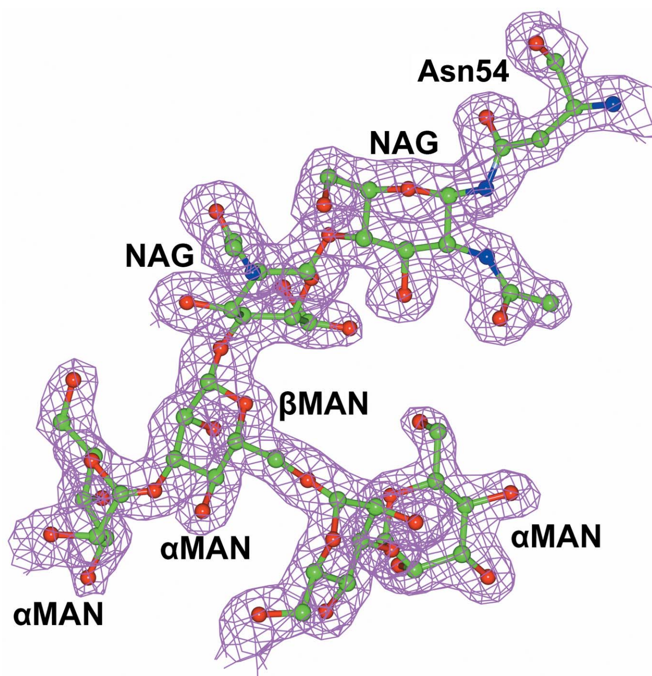

Figure 2
Sugar residues bound to Asn54 in the T2D structure. The electron density ($2F_o - F_c$, 1σ) is shown.

Table 2
Interatomic distances in the active site in the T2D, T2D+Cu^+ and T2D+Cu^{2+} laccase structures.

Atom *I*	Atom *J*	*I*–*J* distance (Å)		
		T2D	T2D+Cu^+	T2D+Cu^{2+}
Cu1	His394 ND1	2.0	2.0	2.0
	His455 ND1	2.0	2.0	2.0
	Cys450 S	2.2	2.2	2.2
Cu2	$Cu3_\alpha$	—	4.6	—
	$Cu3_\beta$	—	4.1	—
	His64 NE2	—	1.9	—
	His397 NE2	—	1.9	—
	W2	—	2.5	—
$Cu3_\alpha$	$Cu3_\beta$	5.2	5.0	5.1
	His111 NE2	2.0	2.0	2.0
	His399 NE	2.0	2.0	2.0
	His449 NE2	2.0	2.0	2.0
	W1	2.4	2.7	2.9
$Cu3_\beta$	His66 ND1	2.0	2.0	2.0
	His109 NE2	2.0	2.0	2.0
	His451 NE2	2.1	2.0	2.0
	W1	2.8	2.4	2.3

Table 3
Occupancies and *B* factors of copper ions in the active site.

	T2D		T2D+Cu^+		T2D+Cu^{2+}	
	Occupancy	*B* ($Å^2$)	Occupancy	*B* ($Å^2$)	Occupancy	*B* ($Å^2$)
Cu1	0.8	9.5	1.0	20.5	0.8	11.3
Cu2	0.1	16.1	1.0	19.8	—	—
$Cu3_\alpha$	0.8	10.7	1.0	19.1	0.8	11.5
$Cu3_\beta$	0.8	9.6	1.0	17.5	0.8	10.7

3.3. Active sites

The interatomic distances in the active sites of the *C. caperata* laccase structures are given in Table 2.

The mononuclear T1 copper centre was located about 5 Å from the surface of the protein globule. The Cu1 copper ion was coordinated by the ND1 atoms of His394 and His355 and the SG atom of Cys450. The Cu1 copper ion was in a planar coordination and was covered with hydrophobic side chains from Ile452 and Phe460 on both sides of the plane. The structure of the T1 centre was identical in all of the studied structures. It should be noted that the occupancy of the copper ion (Cu1) in the T2D and T2D+Cu^{2+} structures was refined and was 0.8; in the T2D+Cu^+ structure it was 1.0 and was not refined.

The T2–T3 cluster consisted of the T2 copper ion (Cu2) and two T3 copper ions ($Cu3_\alpha$ and $Cu3_\beta$). Cu2 was coordinated by the NE2 atoms of His64 and His397 and an oxygen ligand. $Cu3_\alpha$ was coordinated by the NE2 atoms of His111, His399 and His449 and $Cu3_\beta$ was coordinated by the NE2 atoms of His109 and His451 and the ND1 atom of His66. Both $Cu3_\alpha$ and $Cu3_\beta$ in the T2–T3 cluster were coordinated by the bridging oxygen ligand. The T2–T3 clusters in the T2D and the T2D+Cu^+ laccase structures are shown in ball-and-stick representation in Fig. 3.

The occupancies and *B* factors of copper ions in the active site are given in Table 3. In the T2D structure electron density for Cu2 was almost absent and the occupancy of the T2 copper ion was 0.1. In the T2D and T2D+Cu^{2+} structures the occupancy of Cu1, $Cu3_\alpha$ and $Cu3_\beta$ (T3 copper ions) was 0.8. In the T2D+Cu^+ structure the occupancy of Cu2 was 1.0. These data indicated that copper reconstitution only took place on soaking in CuCl solution in the case of the T2D laccase crystal; there was no T2-site reconstitution in T2D laccase soaked in $CuSO_4$.

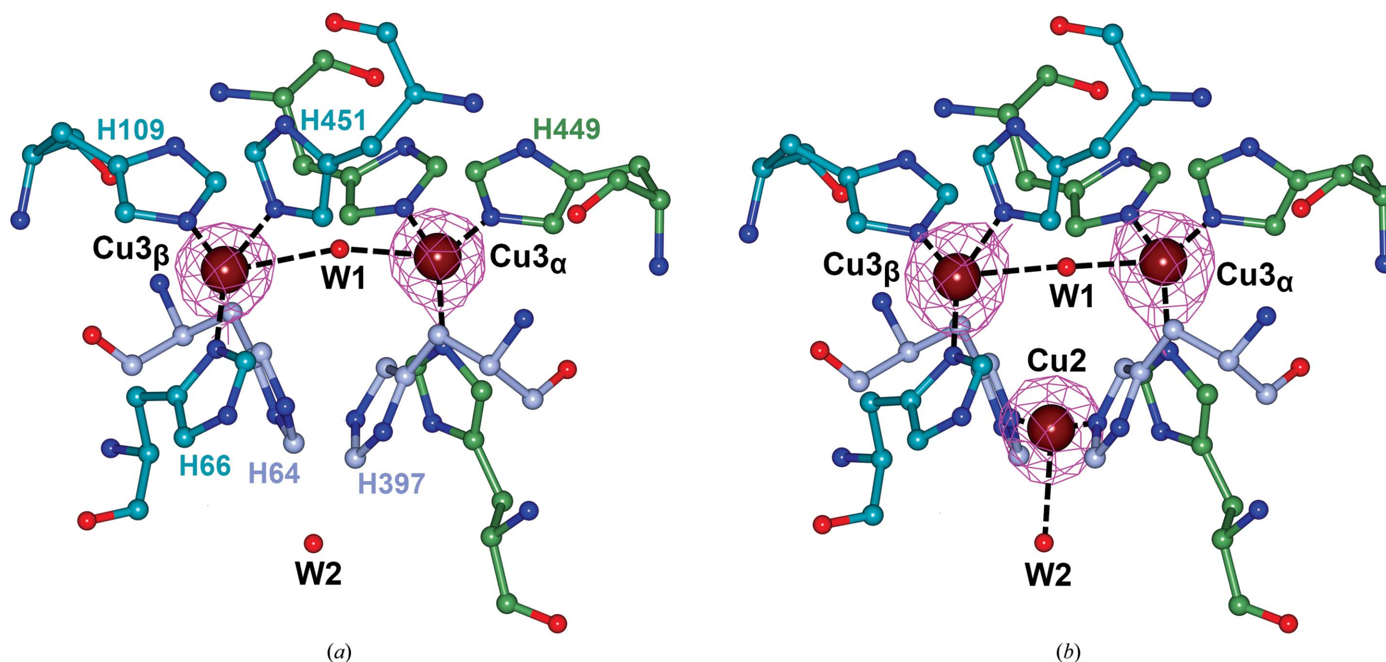

Figure 3
Ball-and-stick models of the laccase T2–T3 cluster: (*a*) T2D structure, (*b*) T2D+Cu^+ structure. Atoms are coloured in accordance with atom type. C atoms of histidine residues coordinating Cu2, $Cu3_\alpha$ and $Cu3_\beta$ are coloured light violet, light green and marine, respectively. Electron density for copper ions ($2F_o - F_c$, 1σ) is shown.

Two conformations of the side chain of the histidine residue coordinating Cu2 have been observed in the structures of laccases from *T. hirsuta*, *C. gallica*, *C. cinereus* and *B. aclada* (Polyakov *et al.*, 2009; De la Mora *et al.*, 2012; Ducros *et al.*, 2001; Osipov *et al.*, 2014). This residue also coordinated $Cu3_{\alpha}$ in several structures. However, the second conformation of His397 was not found in the T2D structure of laccase from *C. caperata*.

To date, attempts have been made to reconstruct T2D laccases from plants, fungi and bacteria in solution. It has been shown that for plant and fungal laccases this can only be carried out with Cu^+, whereas for CotA laccase from *B. subtilis* reconstruction can be achieved with both cupric and cuprous salts; however, treatment with cuprous salts was more effective (Durão *et al.*, 2008). Complete reconstitution was shown for the CueO laccase from *E. coli* on treatment with $CuSO_4$ (Galli *et al.*, 2004). However, structural study of CotA laccase and CueO has shown that full T2 copper-centre reconstruction was not achieved on soaking the laccase crystals in solutions containing cupric ions (Li *et al.*, 2007; Bento *et al.*, 2005). The occupancy of Cu2 in the CotA laccase structure reached 0.3–0.5 when CotA laccase crystals were grown in a solution with elevated $CuCl_2$ concentrations and 0.67 when the crystals were soaked in $CuCl_2$.

In Singh *et al.* (2011), the binding of Cu^+ and Cu^{2+} ions in crystals of the bacterial laccase CueO was studied. The main aim of the investigation was to study the metal ions binding in the methionine-rich region (this region is absent in fungal laccases). The trinuclear copper centres in the wild-type enzyme structure and the structures of complexes with Cu^+ and Cu^{2+} were similar. However, the occupancies of Cu2 in the wild type and in complexes with Cu^+ and Cu^{2+} (PDB entries 3nsc and 3nt0) were 0.75 and 1.0, respectively. However, a significant negative peak was observed in the region of Cu2 in the difference electron-density map. This could result from overestimation of the Cu2 occupancy. These observations are in agreement with our data, considering the ability of Cu^+ ions to restore the T2 laccase centre.

Thus, it has been shown that copper ions can only be incorporated into the T2 site of *C. caperata* laccase crystals in the cuprous form. This is in line with the fact that the activity of the copper-depleted enzyme was restored when the enzyme was treated with CuCl but was not restored when using $CuSO_4$. Moreover, the effective incorporation of copper ions in the +1 oxidation state on the surface of the protein globule suggested that the use of metal ions in unsaturated oxidation states could be an effective method of obtaining heavy-atom derivatives.

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