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Structure of the laccase from *Coprinus cinereus* at 1.68 Å resolution: evidence for different 'type 2 Cu-depleted' isoforms

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Laccases (E.C. 1.10.3.2; benzenediol oxygen oxidoreductases) couple the four-electron reduction of dioxygen to water to four one-electron oxidations of a reducing substrate. The three-dimensional structure of the 'blue' multi-copper oxidase laccase from the fungus *Coprinus cinereus* at 1.68 Å reveals the structural basis for isoforms of the type 2 Cu-depleted species.

1. Introduction

Laccases (E.C. 1.10.3.2; benzenediol oxygen oxidoreductases) are members of the blue multi-copper oxidase family. They catalyse four one-electron oxidations of a reducing substrate and couple this to the four-electron reduction of dioxygen to water and are characterized by their broad specificity for the reducing substrate. They are able to catalyse the oxidation of a number of aromatic (normally phenolic) and inorganic substances (Malmström *et al.*, 1975; Xu, 1996; Xu *et al.*, 1996). Laccases have been isolated from a wide variety of fungal and plant sources and have been extensively studied by spectroscopic and kinetic techniques (for example, Reinhammar, 1984; Solomon *et al.*, 1996; Xu, 1996; Xu *et al.*, 1996). Laccase homologues now also appear to be widespread in bacteria (Alexandre & Zhulin, 2000). The broad substrate specificity of laccases is reflected in a variety of biological roles. They are involved in the wound response in plants (Malmström *et al.*, 1975), both the synthesis and degradation of lignin (Ander & Eriksson, 1976; Bao *et al.*, 1993; Kersten *et al.*, 1990; Thurston, 1994) and are believed to play roles in pathogenesis, sporulation and the development of fruiting bodies (Leatham & Stahmann, 1981; Thurston, 1994). Laccases are increasingly being used in a wide variety of industrial oxidative processes (see, for example, Schneider *et al.*, 1999; Xu, 1996; Xu *et al.*, 1996).

Laccase is the simplest member of the blue multi-copper oxidase family, which includes ascorbate oxidase and mammalian plasma ceruloplasmin (Adman, 1991; Malmström *et al.*, 1975; Messerschmidt, 1997*a*,*b*; Messerschmidt *et al.*, 1992; Zaitseva *et al.*, 1996). The different copper centres in these proteins are characterized by a variety of spectroscopic techniques (reviewed in Holm *et al.*, 1996; Solomon *et al.*, 1996, 1997). They contain a minimum of one type 1 (T1) copper, bound as a mononuclear centre which shows a characteristic intense absorption band at 600 nm which results from the S→Cu(II) charge transfer. The T2 site behaves as a mononuclear site with normal spectral features and is hence EPR active. Together, the T2 and T3 sites form the trinuclear centre which is the site for the reduction of dioxygen.

Laccases readily release a single Cu atom under favourable conditions (Frank & Pecht, 1986; Malkin *et al.*, 1969). It is widely, although not universally (Fraterrigo *et al.*, 1999), assumed that the labile Cu is the EPR-active T2 Cu from the native enzyme. This Cu-depleted species is thus referred to as the T2D laccase and has been the study of much spectroscopic investigation. We have previously described the structure of the T2D *C. cinereus* laccase at 2.2 Å resolution (Ducros *et al.*, 1998). Here, we report the structure of the *C. cinereus* laccase at 1.68 Å resolution with data collected at 100 K. Conformational plasticity in the T2/T3 site reveals the existence of at least two structurally distinct isoforms of the type 2 depleted laccase as a result of two discrete conformations for His399.

2. Experimental methods

Crystals of laccase were grown from polyethylene glycol 8000 as precipitant at pH 5.5 in 100 m*M* sodium acetate buffer, essentially as described previously (Ducros *et al.*, 1997) but without EDTA in the deglycosylation buffer. The catalytic viability of the sample used for crystallization was demonstrated by its ability to polymerize phenolic compounds (data not shown). A single crystal of laccase was mounted in a rayon-fibre loop and placed in a boiling nitrogen stream at 100 K. A cryopro-

tectant solution was produced consisting of 100 m*M* sodium acetate pH 5.5, 30%(*v*/*v*) polyethylene glycol 8000 and ethylene glycol to a final concentration of 20%(*v*/*v*). Data were collected using a MAR Research image-plate system together with a Cu rotating-anode generator and utilizing long focusing-mirror optics (Yale/Molecular Structure Corporation). Data were processed using *DENZO*/*SCALEPACK* (Otwinowski, 1993; Otwinowski & Minor, 1997). All further calculations used the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). The previous cross-validation reflections, between 15 and 2.2 Å, were maintained for the 1.68 Å refinement and extended to the full resolution limit. The structure was refined using *REFMAC* (Murshudov *et al.*, 1997) with solvent added in an automated manner using *ARP* (Lamzin & Wilson, 1993) and verified through manual inspection.

3. Results and discussion

As part of an industrial screening process for oxidative enzymes, the *C. cinereus* laccase was first identified, expressed and characterized because of its powerful catalytic activity at high pH (Schneider *et al.*, 1999). The three-dimensional structure was reported at 2.2 Å using room-temperature data (Ducros *et al.*, 1998) and is presented here from crystals at 100 K to 1.68 Å resolution (details in Table 1). *C. cinereus* laccase is a monomeric molecule consisting of three tightly associated cupredoxin-like domains, yielding a globular structure with approximate dimensions of 70 × 50 × 45 Å (Fig. 1), similar to other blue multi-copper oxidases (as reviewed in Murphy *et al.*, 1997). The mononuclear T1 copper is located in domain 3, where it is bound by two histidines and a cysteine, whilst the trinuclear centre is located at the interface between domains 1 and 3. The blue T1 copper site is the site binding the primary reducing substrate. In all multi-copper oxidases and cupredoxins of known three-dimensional structure the T1 Cu is coordinated by two histidine N atoms and a cysteine S atom, with the highly covalent Cu–S_{Cys} bond giving rise to the pronounced blue coloration. Some multi-copper oxidases, such as ascorbate oxidase, possess an axial methionine ligand, with the SD atom approximately 2.9 Å from the Cu atom, which modifies the properties of the T1 centre (most recently discussed in Palmer *et al.*, 1999). The 1.68 Å *C. cinereus* laccase structure reveals an essentially identical T1 site to that reported previously, albeit at greater precision (Ducros *et al.*, 1998). The T1 copper is trigonally coordinated to the S atom of Cys452 and the ND1 N atoms of His396 and His457, with bond lengths of 2.19, 2.07 and 2.03 Å, respectively. The Cu atoms lie exactly in the plane of the C and N ligands and the axial position is occupied by Leu462 at a distance of 3.51 Å from the Cu. No electron density is observed for the loop which forms the base of the T1 site (residues 160–164) in the 100 K study.

Table 1
Refinement and structure quality statistics for *C. cinereus* laccase at 1.68 Å resolution.

Values in parentheses refer to the outer resolution shell.

Data quality	
Resolution of data (Å)	15–1.68 (1.74–1.68)
R_{merge}†	0.058 (0.275)
Mean $I/\sigma(I)$	22.2 (3.3)
Completeness (%)	96 (92)
Multiplicity	4.6 (2.3)
Crystal parameters	
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 45.39, b = 85.72, c = 143.07
Refinement	
No. protein atoms	3844
No. solvent waters	461
Resolution used in refinement (Å)	15–1.68
R_{cryst}	0.181
R_{free}	0.219
R.m.s. deviation, 1–2 bonds (Å)	0.011
R.m.s. deviation, 1–3 angles (Å)	0.030
R.m.s. deviation, chiral volumes (Å^3)	0.131
Average main-chain B (Å^2)	19
Average side-chain B (Å^2)	21
Average solvent B (Å^2)	28
Main-chain ΔB, bonded atoms (Å^2)	1.9

† $R_{merge} = \sum_{hkl}\sum_i |I_{hkli} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i \langle I_{hkl}\rangle$.

The most interesting feature of the 1.68 Å structure is the insight it gives into potential T2D isoforms at the trinuclear centre. The T2/T3 site of the *C. cinereus* laccase contains eight histidine ligands as expected for a multi-copper oxidase, but in both the 2.2 Å study and the 1.68 Å 100 K study we observe no electron density corresponding to the putative type 2 Cu atom (Fig. 2*a*). The putative T2 copper is lost completely, whilst the T1 and T3 pair remain at full occupancy. The two T3 Cu atoms are separated by approximately 5.3 Å in the T2D laccase, compared with the 3.7 Å observed in the oxidized form of ascorbate oxidase. The oxygen-containing ligand, possibly water, binds to just one atom of the T3 pair and is asymmetrically disposed between the two Cu atoms, lying approximately 2.17 and 3.26 Å from Cu3(a) and Cu3(b), respectively (Fig. 2*b*). It no longer 'bridges' the T3 pair. One copper of the T3 pair shows a distorted trigonal planar conformation consistent with observed X-ray absorption edge features and its likely reduction to a Cu(I) species (Kau *et al.*, 1987; LuBien *et al.*, 1981). It is coordinated to His66, His109 and His453 and 3.26 Å distant from the bridging ligand. The other Cu shows two distinct different coordinations as a result of the multiple conformations of His399.

Conformational changes involving His399 (whose equivalent is a T2 copper ligand in ascorbate oxidase and ceruloplasmin)

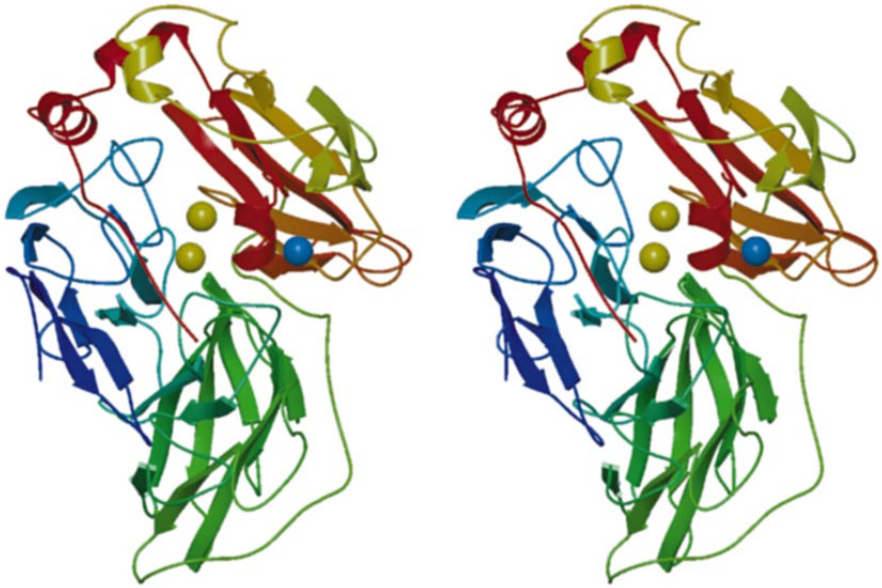

Figure 1
Cartoon representation of the three-dimensional structure of the *C. cinereus* laccase. The figure is colour-ramped from the N-terminus (blue) to the C-terminus (red). The Cu atoms are shown as shaded spheres, with the T1 site in blue and the T3 pair in yellow. This figure was produced using *MOLSCRIPT*/*BOBSCRIPT* (Esnouf, 1997; Kraulis, 1991) and rendered using *Raster3D* (Merritt & Bacon, 1997; Merritt & Murphy, 1994). The figure is in divergent (wall-eyed) stereo.

accompany T2 Cu depletion and it shows two distinct conformations. In the first conformation it remains in the same position observed in ascorbate oxidase and ceruloplasmin; thus Cu3(a) has four ligands, three N atoms from His111, His401, His451 and the oxygen-containing ligand, at distances of 2.01, 2.04, 1.98 and 2.17 Å, respectively. This coordination is best described as distorted tetrahedral. In the second position, His399 has swung into the coordination shell of Cu3(a) which becomes pentacoordinate with a 2.5 Å bond to His399 (Fig. 2*b*). The relative occupancies of the two positions for His399 are different in the 100 and 293 K structures, leaving one to believe that the two species clearly lie in an equilibrium which is sufficiently well poised so as to be influenced by changes in temperature, crystallization conditions *etc.*

4. Conclusions

The most commonly studied derivative of blue multi-copper oxidases is the type 2 depleted form in which the labile Cu has been selectively removed. It is significant that there is strong evidence as to the existence of a number of different T2D isoforms (for example, Frank & Pecht, 1986) and the crystal structure discussed here indeed reveals at least two T2D isoforms. Furthermore, whilst it is widely believed that the loss of the EPR signal from the T2D laccase results from the depletion of the labile T2 copper, McMillin and colleagues (Fraterrigo *et al.*, 1999) have recently questioned the evidence that the depleted copper is actually the EPR-active species. Instead they propose that the loss of the EPR signal may simply reflect conformational changes upon loss of copper, such as observed here. Such proposals could have profound implications for spectroscopic interpretation and mechanistic schemes.

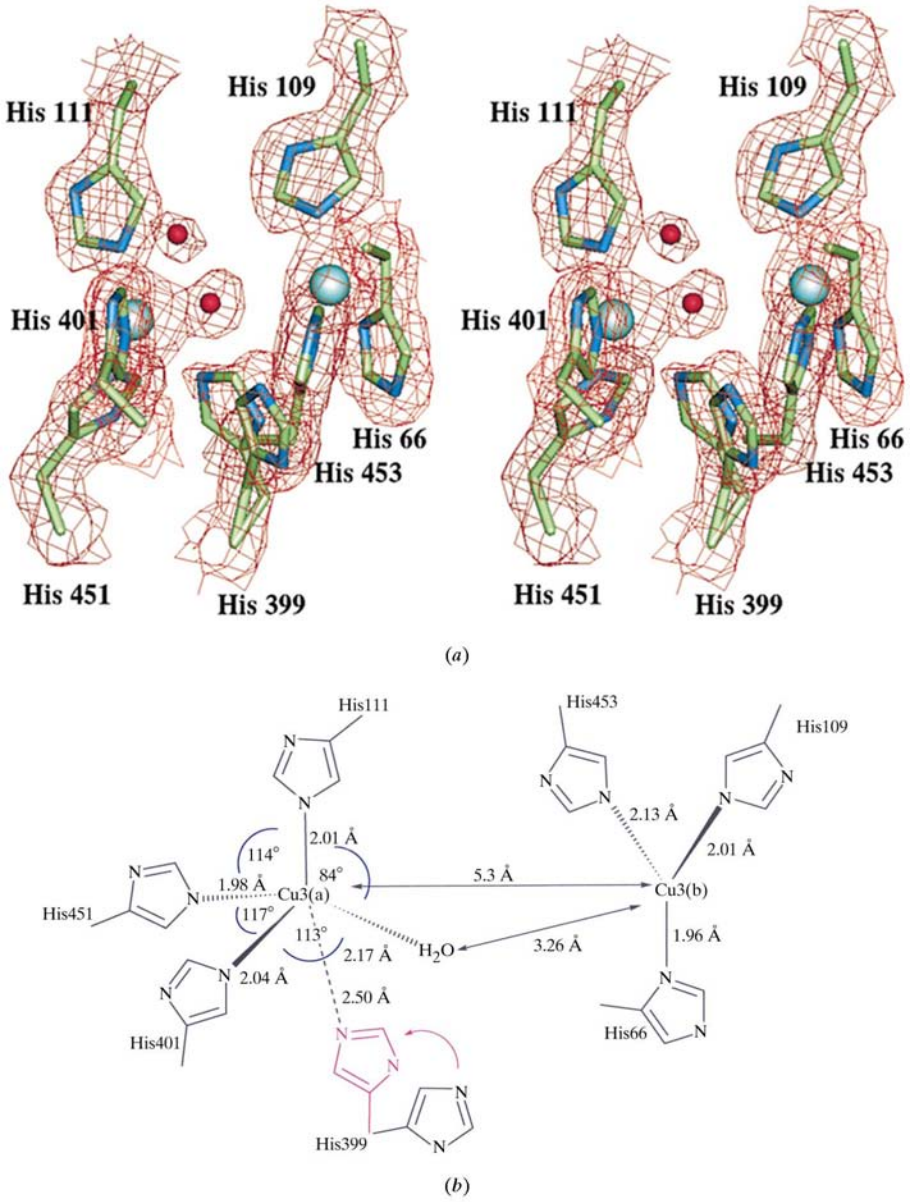

Figure 2
(*a*) Observed electron density for the T3 Cu site in the 1.68 Å *C. cinereus* laccase structure. The map shown is a $2F_{obs} - F_{calc}$ synthesis calculated with σ_A and maximum-likelihood weights and is contoured at 0.44 e Å^{-3}. The figure is in divergent stereo. (*b*) Schematic representation of the T3 Cu site. Coordination distances are shown in Å. Conformational flexibility around atom Cu3(a) (corresponding to Cu3 in ascorbate oxidase nomenclature) is reflected in an equilibrium between two different coordination geometries best described as distorted tetrahedral and pentacoordinate.

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References

Adman, E. T. (1991). *Adv. Protein Chem.* **42**, 145–197.
Alexandre, G. & Zhulin, I. B. (2000). *Trends Biochem. Sci.* **18**, 41–84.
Ander, P. & Eriksson, K. E. (1976). *Arch. Microbiol.* **109**, 1–8.
Bao, W., O'Malley, D. M., Whetten, R. & Sederoff, R. R. (1993). *Science*, **260**, 672–674.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Ducros, V., Brzozowski, A. M., Wilson, K. S., Brown, S. H., Østergaard, P., Schneider, P., Pedersen, A. H. & Davies, G. J. (1998). *Nature Struct. Biol.* **5**, 310–316.
Ducros, V., Davies, G. J., Lawson, D. M., Wilson, K. S., Østergaard, P., Schneider, P., Pedersen, A. H. & Brzozowski, A. M. (1997). *Acta Cryst.* D**53**, 605–607.
Esnouf, R. M. (1997). *J. Mol. Graph.* **15**, 133–138.
Frank, P. & Pecht, I. (1986). *J. Phys. Chem.* **90**, 3809–3814.
Fraterrigo, T. L., Miller, C., Reinhammar, B. & McMillin, D. R. (1999). *J. Biol. Inorg. Chem.* **4**(2), 183–187.
Holm, R. H., Kennepohl, P. & Solomon, E. I. (1996). *Chem. Rev.* **96**, 2239–2314.
Kau, L.-S., Spira-Solomon, D. J., Penner-Hahn, J. E., Hodgson, K. O. & Solomon, E. I. (1987). *J. Am. Chem. Soc.* **109**, 6433–6442.
Kersten, P. J., Kalyanaraman, B., Hammel, K. E., Reinhammar, B. & Kirk, T. K. (1990). *Biochem. J.* **26**8, 475–480.
Kraulis, P. J. (1991). *J. Appl. Cryst.* **24**, 946–950.
Lamzin, V. S. & Wilson, K. S. (1993). *Acta Cryst.* D**49**, 129–147.
Leatham, G. F. & Stahmann, M. A. (1981). *J. Gen. Microbiol.* **125**, 147–157.
LuBien, C. D., Winkler, M. E., Thamann, T. J., Scott, R. A., Co, M. S., Hodgson, K. O. & Solomon, E. I. (1981). *J. Am. Chem. Soc.* **103**, 7014–7016.
Malkin, R., Malmström, B. G. & Vänngård, T. (1969). *Eur. J. Biochem.* **10**, 253–259.

Malmström, B. G., Andréasson, L. E. & Reinhammar, R. (1975). *The Enzymes*, edited by P. Boyer, Vol. 12, pp. 507–578. New York: Academic Press.
Merritt, E. A. & Bacon, D. J. (1997). *Methods Enzymol.* **277**, 505–524.
Merritt, E. A. & Murphy, M. E. P. (1994). *Acta Cryst.* D**50**, 869–873.
Messerschmidt, A. (1997*a*). *Comprehensive Biological Catalysis*, edited by M. Sinnott, Vol. III, pp. 401–426. London: Academic Press.
Messerschmidt, A. (1997*b*). Editor. *Multi-Copper Oxidases*. Singapore: World Scientific.
Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Gatti, G., Petruzzelli, R., Rossi, A. & Finazzi-Agró, A. (1992). *J. Mol. Biol.* **224**, 179–205.
Murphy, M. E. P., Lindley, P. F. & Adman, E. T. (1997). *Protein Sci.* **6**, 761–770.
Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). *Acta Cryst.* D**53**, 240–255.
Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 56–62. Warrington: Daresbury Laboratory.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Palmer, A. E., Randall, D. W., Xu, F. & Solomon, E. I. (1999). *J. Am. Chem. Soc.* **121**(30), 7138–7149.
Reinhammar, B. (1984). *Copper Proteins and Copper Enzymes*, edited by R. Lontie, Vol. 3, pp. 1–36. Boca Raton, Florida: CRC Press.
Schneider, P., Caspersen, M. B., Mondorf, K., Halkier, T., Skov, L. K., Ostergaard, P. R., Brown, K. M., Brown, S. H. & Xu, F. (1999). *Enzyme Microb. Technol.* **25**(6), 502–508.
Solomon, E. I., Machonkin, T. E. & Sundaram, U. .M. (1997). *Multi-Copper Oxidases*, edited by A. Messerschmidt, pp. 103–127. Singapore: World Scientific.
Solomon, E. I., Sundaram, U. M. & Machonkin, T. E. (1996). *Chem. Rev.* **96**, 2536–2605.
Thurston, C. F. (1994). *Microbiology*, **140**, 19–26.
Xu, F. (1996). *Biochemistry*, **35**, 7608–7614.
Xu, F., Shin, W., Brown, S. H., Wahleithner, J. A., Sundaram, U. M. & Solomon, E. I. (1996). *Biochim. Biophys. Acta*, **1292**, 303–311.
Zaitseva, I., Zaitsev, V., Card, G., Moshkov, K., Bax, B., Ralph, A. & Lindley, P. (1996). *J. Biol. Inorg. Chem.* **1**, 15–23.