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Dioxygen reduction by multi-copper oxidases; a structural perspective†

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The multi-copper oxidases oxidise substrate molecules by accepting electrons at a mononuclear copper centre and transferring them to a trinuclear centre. Dioxygen binds to the trinuclear centre and, following the transfer of four electrons, is reduced to two molecules of water. The precise mechanism of this reduction has been unclear, but recent X-ray structural studies using the CotA endospore coat protein from *Bacillus subtilis* have given further insights into the principal stages. It is proposed that the mechanism involves binding of the dioxygen into the trinuclear centre so that it is sited approximately symmetrically between the two type 3 copper ions with one oxygen atom close to the type 2 copper ion. Further stages involve the formation of a peroxide intermediate and following the splitting of this intermediate, the migration of the hydroxide moieties towards the solvent exit channel. The migration steps are likely to involve a movement of the type 2 copper ion and its environment. Details of a putative mechanism are described herein based both on structures already reported in the literature and on structures of the CotA protein in the oxidised and reduced states and with the addition of peroxide and the inhibitor, azide.

Introduction

The multi-copper oxidases constitute a family of enzymes whose principal members are laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2), ascorbate oxidase (L-ascorbate oxygen oxidoreductase, EC 1.10.3.3) and ceruloplasmin (Fe(II) oxygen oxidoreductase, EC 1.16.3.1); for recent reviews see.[1–3] All the members of the family normally contain at least four copper ions comprising a mononuclear blue type 1 copper centre and a trinuclear centre some 12–13 Å distant. The trinuclear centre has two type 3 copper ions, that can be anti-ferromagnetically coupled through an hydroxyl moiety in between them, and one type 2 copper ion.‡ The mononuclear copper is able to accept an electron from a variety of phenolic substrates and then transmits it to the trinuclear centre. The substrate is thereby oxidised and the electron used to reduce dioxygen bound at the trinuclear centre. The transfer of four electrons results in the reduction of the dioxygen to two molecules of water. X-Ray structural studies over the past decade have enabled the elucidation of a significant number of structural and functional aspects of these enzymes, but the precise mechanism of dioxygen reduction remains one of the outstanding questions.[1–3,5,6]

The laccases have been studied extensively both from the biochemical and spectroscopic viewpoints and the X-ray structure of three fungal laccases have recently been reported, from *Coprinus cinereu*[7,8] (PDB codes: 1A65 and 1HFU), *Trametes versicolor*[9,10] (PDB codes: 1KYA and 1GYC), *Melanocarpus albomyces*[11] (PDB code: 1GE0) and *Rigidoporus lignosus*[12] (PDB code: 1V10). In addition, the bacterial laccase, CotA from *Bacillus subtilis*[13] (PDB code: 1GSK) has been characterized. The CotA enzyme is thermoactive and thermostable and thus a good model for studying the mechanism of dioxygen binding as well as being an interesting enzyme for industrial applications.[14]

X-Ray studies of crystals of the enzyme CotA laccase from *Bacillus subtilis* have been undertaken both in the reduced and oxidized forms and in the presence of peroxide and the inhibitor, azide. In a previous paper we reported the structure of a 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) adduct of CotA[15] which had a dioxygen molecule trapped in the water channel leading towards the trinuclear copper centre, but not bound to it. In this paper we have extended the studies on the CotA enzyme to show a diatomic species bound to the trinuclear centre in a manner similar to that found in the laccase from *Melanocarpus albomyces*[11] and predicted from spectroscopic studies of *Rhus vernicifera*.[16–18] The implications of these structural observations can now be focused on an overall mechanism for dioxygen reduction.

Experimental procedures

Protein purification, crystallization and soaking experiments

A modified purification procedure was adopted from that reported previously[13,19] as follows. The CotA protein was purified by FPLC (AKTA Purifier) at room temperature from *Escherichia coli* overproducing cells by two chromatographic steps, a cationic exchange SP-sepharose column and then a gel filtration column; a second cationic exchange step, used in the previous purification protocol, was omitted because of copper depletion. Active fractions from the first step were pooled and concentrated by ultra-filtration (30 kDa cut-off) and applied to a gel filtration column packed with Superdex-200 (Amersham Pharmacia Biotech.), equilibrated and eluted with 0.2 M NaCl in 20 mM Tris-HCl at pH 7.6. A new step was introduced whereby the active fractions from the Superdex column were pooled, concentrated and equilibrated with 0.1 M NaCl, 10 μM $CuCl_2$ in 20 mM MES buffer at a decreased pH of 6.0.

All CotA crystals were obtained at 277 K, from a crystallisation solution containing 6–8% of PEG 4000, 6–8% of

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‡ The type of copper ion is based on the spectroscopic properties according to Malmström[4] (B. G. Malmström. "Enzymology of oxygen". *Annu. Rev. Biochem*., 1982, **51**, 21–59).

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isopropanol, and 100 mM of sodium citrate at pH 5.5, using the vapour diffusion method and a protein concentration of 5–6 mg ml^{-1}. These conditions differ slightly from those described previously by Enguita *et al.*[13] where the crystals were obtained at 298 K, using a protein concentration of 10–15 mg ml^{-1} and a crystallisation solution containing 12–15% of PEG 4000, 12–15% isopropanol, 15% glycerol and 100 mM of sodium citrate at pH 5.5.

All the crystal soaking experiments were performed at 277 K except for the reduction process. In this case the crystals were transferred to an anaerobic chamber where they were kept for two days before they were transferred to the cryo solution containing an excess of sodium dithionite. The change in crystal colour from blue to colourless, indicating reduction of the type I copper ion, was observed after a few minutes. However the crystals were kept under these conditions for 5 h and then frozen under liquid nitrogen. In the other soaking experiments crystals, which retained their blue colour, were transferred to a cryo solution of the mother-liquor containing 25% glycerol and soaked with 10 mM of hydrogen peroxide for 2 h, 10 mM of $CuCl_2$ for 1 h and 10 mM sodium azide for seven days.

Data collection, model building and refinement

Data collection was performed at 100 K using synchrotron radiation at the ESRF, Grenoble. Data collection and refinement statistics are also shown in Table 1. Data sets for the $CuCl_2$ and azide soaked crystals were processed with MOSFLM,[20] and scaled with SCALA from the CCP4 program suite.[21] The remaining data sets were processed with DENZO and SCALEPACK.[22] The structures were elucidated by molecular replacement using MOLREP.[23] The starting model was the CotA native structure (PDB code: 1GSK)[13] from which all the copper ions and solvent atoms had been removed. In each case only one solution was evident. Subsequent electron density syntheses enabled the four copper ions in the molecule to be located and details of the solvent structure to be modelled; small adjustments were also made to some side chains. Refinement was performed using the maximum likelihood functions implemented in REFMAC5.[24] Rounds of conjugate-gradient sparse-matrix refinement with bulk-solvent modelling according to the Babinet principle[25] were alternated with model building using the O program suite[26] in combination with SigmaA weighted $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ maps.[27] After the first rounds of refinement, solvent molecules were added to the models based on standard geometrical and chemical restraints; molecules of glycol, used as a cryo-protectant, were also located. In all cases the loop region between residues 89 and 97 was very poorly defined.

The occupancies of the copper ions in the trinuclear clusters were adjusted so that their isotropic thermal vibration parameters refined approximately to the values of their local environment; assignment of full occupancies led to thermal vibration coefficients significantly higher than the local average and significant features in difference Fourier syntheses. Careful use of omit and standard difference Fourier syntheses, as well as monitoring of thermal vibration coefficients during refinement, enabled the identification of a diatomic species between the type 3 copper sites in both the cases of the crystals soaked with $CuCl_2$ and peroxide. With the $CuCl_2$ soaked crystals an ovoid shaped peak of positive electron density was found in a difference Fourier synthesis with its long axis almost perpendicular to the vector between the type 3 copper ions and one end overlapping the position of the bridging hydroxide found in ascorbate oxidase. Initially it was modelled and refined as a single oxygen atom at this position. However, this resulted in a further positive electron density peak being observed in a difference Fourier synthesis, roughly equidistant from the three copper ions of the trinuclear centre. Placing an oxygen atom in this position and removing the first followed by refinement, resulted in a positive peak in a difference Fourier synthesis corresponding to the atom that had been removed. These results were interpreted in terms of the presence of a diatomic species as indicated in Fig. 2(a). However, in addition since the crystal had been soaked with excess of $CuCl_2$, a single chloride anion was also modelled into the ovoid shaped peak and refined. After refinement it showed an high thermal vibration coefficient indicating an excess of scattering power and, again, a positive electron density in a difference Fourier synthesis adjacent to its position, indicative of a diatomic species. In the case of the peroxide adduct the ovoid shaped density was inclined to the vector between the two type 3 copper ions as shown in Fig. 2(b). Unconstrained refinement of the diatomic species indicated that the inter-atomic separation of the atoms was slightly less in the case of the $CuCl_2$ adduct than for that observed in the peroxide soaked crystals. It was therefore assumed that these species were dioxygen and peroxide, respectively, and refinement proceeded constraining the O–O distances to target values of 1.26 Å for the dioxygen and 1.45 Å for the peroxide. The dioxygen species found in the $CuCl_2$ soaked crystals was also observed in the refinements of several other data sets collected from crystals that had been used for soaking experiments with various substrates and mediators. It appears to represent the "as-isolated" state of the CotA molecule in the crystals prepared as indicated in the Experimental section.

Details of the overall refinement and final quality of the models are shown in Table 2. All the figures were prepared using PyMol.[28]

Table 1 Data collection

	$CuCl_2$	Peroxide	Reduced	Azide
Beam line at ESRF	ID14–EH3	ID14–EH3	ID14–EH1	ID14–EH3
Wavelength/Å	0.931	0.931	0.934	0.931
Detector distance/mm	Mar CCD 150	Mar CCD 150	Mar CCD 235	Mar CCD 150
Resolution/Å	2.0	2.2	2.5	2.2
Space group	$P3_121$	$P3_121$	$P3_121$	$P3_121$
Cell parameters *a*, *c*/Å	102.07, 137.97	102.72, 137.35	102.10, 136.26	101.83, 137.36
Mosaicity/°	0.5	0.3	0.4	0.25
Oscillation range/°	90	70	60	70
Oscillation angle/°	0.5	0.5	0.5	0.5
No. of unique *hkl*	56356 (7783)	42921 (4260)	28950 (2813)	42452 (6111)
Completeness (%)	99.4 (96.0)	99.9(100.0)	99.5(98.9)	99.9 (99.5)
$I/\sigma(I)$	9.76 (1.91)	21.7 (3.89)	28.2 (3.44)	8.98 (3.3)
R_{sym}	0.063 (0.340)	0.058 (0.316)	0.041(0.309)	0.062 (0.220)
Multiplicity	5.49 (5.35)	4.3 (4.3)	3.63 (3.69)	4.3 (4.3)

[a] Values in parentheses refer to the highest resolution shells as follows: $CuCl_2$, 2.11–2.00 Å; Peroxide, 2.28–2.20 Å; Reduced, 2.59–2.50 Å; Azide, 2.32–2.20 Å.

Table 2 Refinement and quality of refined models

	$CuCl_2$	Peroxide	Reduced	Azide
Refinement				
No. of protein atoms	4055	4059	4059	4046
No. of solvent atoms	576	485	350	477
No. of hetero atoms	4Cu + 2O + 4GOL	4Cu + 2O + 3GOL	4Cu	4Cu + 3N
Final *R*-factor	0.161	0.148	0.163	0.162
Final free *R*-factor	0.183 (5.1%)	0.188 (5.0%)	0.216 (5.1%)	0.197 (5.0%)
Mean *B* values/Å^2				
Protein	22.1	30.2	45.3	25.3
Solvent	34.4	44.5	47.5	38.5
Overall	24.0	32.9	46.4	26.8
Estimated overall coordinate uncertainty[a]/Å	0.07	0.09	0.15	0.09
Deviations[b]				
Bond distances/Å	0.013	0.025	0.011	0.017
Bond angles/°	1.240	1.904	1.287	1.549
Planar groups/Å	0.010	0.014	0.005	0.010
Chiral volume/Å^3	0.093	0.125	0.087	0.096
Quality of models[c]				
Overall *G* factor	−0.06	−0.20	0.00	−0.11
Ramachandran analysis (%) (no.)				
Favourable	86.5 (365)	86.8 (367)	86.3 (365)	87.2 (367)
Additional	13.0 (55)	12.3 (52)	12.8 (54)	11.9 (50)
Generous	0.2 (1)	0.5 (2)	0.7 (3)	0.5 (2)
Disallowed	0.5 (2)	0.5 (2)	0.2 (1)	0.5 (2)

[a] Based on maximum likelihood. [b] rms deviations from standard values. [c] The *G* factor and Ramachandran analysis[37] were determined by PROCHECK.[38]

Results

Overall structure and copper depletion

All the CotA laccase structures reported in this paper show the typical fold of a multi-copper oxidase,[3] There are three domains based on the cupredoxin fold. Domain 3 contains a mononuclear copper centre bound to the protein by a cysteine residue, two histidines and a methionine residue in an approximately tetrahedral configuration, although the methionine is a "soft" ligand and the Cu–SD distance is typically slightly greater than 3.0 Å. The trinuclear copper centre, where the dioxygen reduction takes place, lies in between domains 1 and 3 and is bound by two pairs of histidine residues from each domain. Two solvent channels give clear access to the trinuclear centre[10] as shown in Fig. 1. The first channel is directed towards the two type 3 copper ions on one side of the cluster and the second to the type 2 copper ion on the other side.

For many of the crystals used for data collection in these studies there appears to be a significant depletion of some of the copper centres. This probably results from the methods of purification. In the case of the laccase from *Coprinus cinerus*[7,8] the use of the metal chelator ethylaminediamine tetraacetic acid, EDTA, during the deglycosylation procedure resulted in the complete depletion of the type 2 copper centre. Indeed this centre appears to be the most labile, although the type 3 copper ions also show depletion. In several structures reported previously, a rigorous examination of the isotropic thermal vibration coefficients also suggests that there could be appreciable copper depletion, although this does not always appear to have been taken into account in the structure refinement procedures. Indeed, the apparent ease with which the multi-copper oxidases lose copper may well account for the copper transport function associated with ceruloplasmin (see refs. 3 and 29, and references therein). In more recent purifications of the CotA laccase, strenuous efforts have been made to ensure that copper depletion is minimized by the addition of $CuCl_2$ (see below) until the maximum activity is obtained.

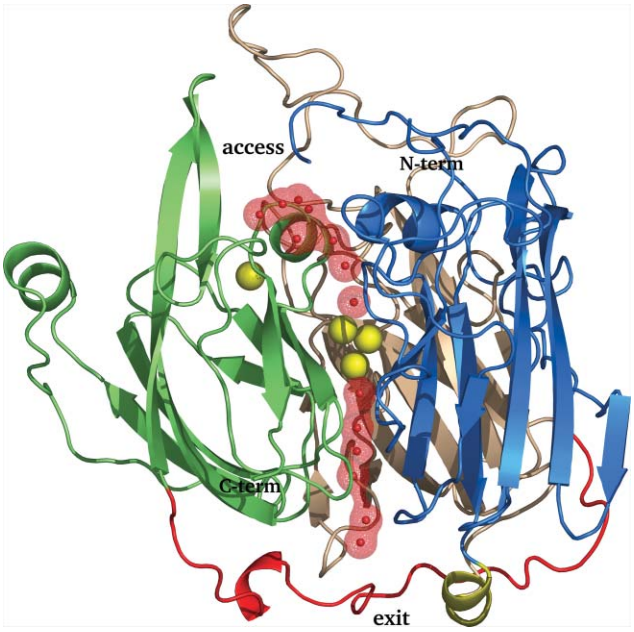

Fig. 1 The channels in the CotA endospore coat laccase from *Bacillus subtilis* giving access to the trinuclear centre for dioxygen (at the top) and egress for the water molecules resulting from the reduction of the dioxygen molecule (at the bottom).

The $CuCl_2$ soaked structure

The resulting structure is different both from that observed in earlier work[13] and studies involving the ABTS adduct[15] probably reflecting the different methods of purification and crystallisation. Fig. 2(a) shows part of an omit SigmaA weighted $|F_o| - |F_c|$ Fourier synthesis in the vicinity of the trinuclear copper cluster; no dioxygen moiety was included in the structure factor calculations. All three copper ions of the cluster bind to

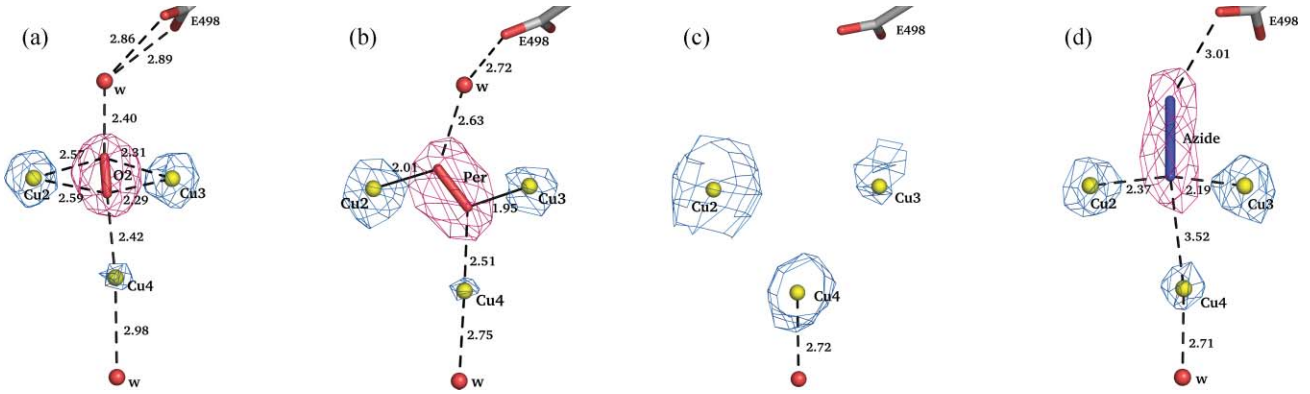

Fig. 2 Sections of electron density syntheses showing details of the trinuclear copper clusters. The numbering of the copper ions is according to the CotA enzyme. In each panel the electron density for the copper ions has been calculated from SigmaA weighted $2|F_o| - |F_c|$ Fourier syntheses. The relative volumes of the electron density peaks reflect the respective occupancies of the copper ions. In panels (a), (b) and (d) the electron density for the adduct is derived from omit Fourier syntheses computed with SigmaA weighted coefficients $|F_o| - |F_c|$; the adduct molecules were not included in the structure factor calculations and five cycles of maximum likelihood refinement were computed using REFMAC[24] prior to Fourier synthesis to minimise phase bias. (a) the $CuCl_2$ soaked structure. A dioxygen molecule is bound into the trinuclear centre. The Cu2 · · · Cu3 separation is 4.72 Å. Contour levels are 10.0 rms for Cu and 8.0 rms for the adduct. (b) the peroxide adduct showing the peroxide bound to the trinuclear centre in a zigzag mode between the two type 3 copper ions. The Cu2 · · · Cu3 separation is 4.66 Å. Contour levels are 6.2 rms for Cu and 6.0 rms for the adduct. (c) the trinuclear copper centre of the fully reduced enzyme in which there are no moieties bridging the type 3 copper ions. The Cu2 · · · Cu3 separation is 5.05 Å. The contour level for the copper ions is 1.7 rms. (d) The binding of the azide inhibitor to the CotA enzyme. The azide binds across the type 3 copper ions so that one terminal nitrogen atom is in an almost identical position to an oxygen atom of the dioxygen moiety observed in the $CuCl_2$ structure. The remaining terminal N atom replaces a water molecule, hydrogen bonded to OE1 and OE2 atoms of Glu498 in the access channel. The Cu2 · · · Cu3 separation is 4.52 Å. Contour levels are 8.0 rms for Cu and 7.0 rms for the azide moiety.

the dioxygen moiety: the bond lengths O2–Cu2, O2–Cu3 and O2–Cu4 are 2.59, 2.29 and 2.42 Å, respectively. The O1–Cu2 and O1–Cu3 distances are 2.57 and 2.31 Å, respectively. O1 is 2.40 Å away from a water molecule in the access solvent channel and this water molecule is in turn hydrogen bonded to Glu498. On the opposite side of the cluster to the dioxygen molecule a water molecule is localised 2.98 Å away from the type 2 copper ion, Cu4. This structure is different from those obtained for the "as isolated" state of other multi-copper oxidases with the exception of the lacasse from *M. albomyces*. Usually, the "as isolated" structures of this type of enzyme shows a hydroxyl group sited between the two type 3 copper ions and a hydroxyl group bound to the type 2 copper ion. In the $CuCl_2$ structure, as in the laccase structure from *M. albomyces*,[11] a dioxygen molecule is observed almost symmetrically positioned in between the type 3 coppers ions. However, the laccase structure from *M. albomyces* also shows the access channel blocked by residues from the C-terminus and a chloride anion bound to the type 2 copper ion. In the $CuCl_2$ structure a water molecule is located in the solvent exit channel some 3.0 Å from the type 2 copper ion and, indeed, none of the structures reported in this paper, shows a hydroxyl moiety directly attached to it. There is some evidence of depletion of the type 2 copper site, even with the treatment of the crystals with $CuCl_2$, and its occupancy has been set at 67% in order to give an isotropic thermal vibration coefficient of the same order of magnitude as the other copper ions.

The peroxide adduct

The addition of hydrogen peroxide to the CotA laccase enzyme results in a different structure at the trinuclear copper centre to that observed in the crystals soaked aerobically with $CuCl_2$. A very cautious and careful refinement indicates a model in which a diatomic dioxygen derived species is sited in between the type 3 copper ions, but inclined to the Cu2–Cu3 axis at an angle of 51° as shown in Fig. 2(b). The species was assumed to a peroxide O_2^{2-} anion and in this configuration both type 3 copper ions are bonded to one of the oxygen atoms, so that the distances Cu2–O2 and Cu3–O1 are 2.01 and 1.95 Å, respectively. O1 is some 2.51 Å from the type 2 copper ion whereas O2 is some 2.63 Å from a water molecule that is, in turn, hydrogen bonded to the carboxylate side chain of Glu498. The structure again exhibits copper depletion and the occupancies of Cu2, Cu3 and Cu4 have been set at 75, 70 and 50%, respectively, with a corresponding occupancy for the peroxide moiety of 75%.

This peroxide structure differs from that observed before for ascorbate oxidase.[30] In the latter structure the peroxide is terminally bound to only one of the type 3 copper ions and is oriented along the solvent access channel. Copper depletion is also observed in this structure.

The fully reduced structure

The reduced structure of the CotA laccase is essentially the same as that for ascorbate oxidase,[30] Fig. 2(c). There is no hydroxide moiety bridging the two type 3 copper ions and the distance between them increases to 5.10 Å. There are also increases in the distances between the type 2 to type 3 copper ions so that Cu2 · · · Cu4 and Cu3 · · · Cu4 are separated by 4.20 and 4.22 Å, respectively. Essentially both the type 2 and type 3 copper ions move towards their respective ligands and in the case of the type 3 ions, become three-coordinate, a preferred stereochemistry for Cu(I). However, unlike the reduced form of ascorbate oxidase, there is no hydroxyl moiety attached to the type 2 copper ion, this is replaced by a water molecule at 2.72 Å. The structure of the reduced enzyme exhibits appreciable copper depletion with one of the type 3 copper ions having an occupancy of only 30%

At the resolution of the X-ray data, there are no significant changes on the geometry of the type 1 copper centre upon reduction.

The azide adduct

Fig. 2(d) shows the electron density in an omit SigmaA weighted $|F_o| - |F_c|$ Fourier synthesis in the vicinity of the type 3 copper ions of the trinuclear cluster. The major part of the electron density can be clearly explained by the presence of an azide moiety positioned so that the N1 atom bridges the type 3 copper ions and replacing the O2 atom of the dioxygen moiety in the $CuCl_2$ soaked structure. The N1–Cu2 and N1–Cu3 distances are 2.37 and 2.19 Å, respectively. The other terminal atom of

the azide group, N3, is almost in precisely the same position as a water molecule in the $CuCl_2$ soaked structure and distant by 3.01 and 3.48 Å from the OE1 and OE2 atoms of Glu498. The presence of azide will inhibit the binding of oxygen to the trinuclear cluster and therefore in addition the activity of the enzyme. However, there is some evidence that under the soaking conditions used, although relatively harsh (see below), azide binding to all the molecules in the crystal is not complete and that for a small percentage, probably less than 10%, an oxygenated species remains; no attempt was made to model this species. Thus, in a final difference Fourier synthesis a small positive residual electron density peak appears in a position equivalent to the oxygen moiety.

The binding of azide in the CotA enzyme is different from that reported for ascorbate oxidase[30] and ceruloplasmin.[31] In ascorbate oxidase two azide moieties were found to be terminally bound to only one of the type 3 copper ions, whereas for ceruloplasmin only one of these azide moieties was found to bind under the soaking conditions used. The presence of azide bound to one copper ion will also clearly inhibit enzyme activity, but whether under different soaking conditions an azide moiety binds in a symmetric mode in these two enzymes remains to be seen. In this regard it is interesting to note that in the $CuCl_2$ soaked crystal the dioxygen moiety is slightly closer to one copper ion than the other. Indeed this is precisely the copper ion that binds azide and peroxide in ascorbate oxidase.

In the refinement of the azide soaked structure, setting the occupancies of the copper ions to 100% resulted in a higher value of the isotropic thermal vibration coefficient for the type 2 copper ion of 32.0 $Å^2$ compared with an average value of 24.9 $Å^2$ for the remainder; Cu4, was therefore assigned an occupancy of 80% so that its isotropic thermal vibration coefficient refined to a value close to the average. One unusual feature in the azide structure occurs in the vicinity of the mononuclear copper site. There is evidence that the cysteine normally bound to this copper can adopt two positions. In the second position the sulfur atom moves away from the copper and points directly at the terminal methyl group of the adjacent methionine to form a sulfur–carbon bond. This alternative configuration is present at around the 10% level and presumably results from the relatively harsh soaking conditions of 10 mM concentration of azide over a seven day period. For shorter soaking times the azide moiety still bridges the two type 3 copper ions, but there is little evidence for the formation of the cysteine-methionine bond.

Discussion

In this work four structures are reported,§ corresponding to the CotA laccase in the reduced state, and in the "as isolated" state soaked with $CuCl_2$, with peroxide and with the inhibitor, azide. In addition, a comparison is made between these structures and those of other multi-copper oxidases reported in the literature. Although no significant differences have been observed at the mononuclear copper centre, differences highlighted in the tri-nuclear centre enable a new mechanism for the dioxygen reduction to water to be proposed, as shown in Fig. 3. It should be emphasized that this mechanism is based on known X-ray structures. At some stage in the future it will be important to correlate the details with the plethora of spectroscopic and kinetic data that has been reported. However, the problem of ensuring that spectroscopic and other measurements are synchronised with a given crystal structure are non-trivial. As a consequence, work is ongoing with the group of Professor Ed Solomon (Stanford University, USA) to try to ensure a precise match between a given crystal structure and its respective spectral properties and this work will form the basis of a subsequent publication. However, most of the spectroscopic observations can be reconciled with the proposed mechanism, including the existence of the peroxide intermediate.

The first stage of this putative mechanism involves the movement of molecular dioxygen to the trinuclear copper site. The structures of the ABTS complex of CotA[15] and the peroxide

§ The atomic coordinates and structure factors of these four structure ($CuCl_2$ structure-1w6l; H_2O_2 adduct-1w8e; fully reduced-2bhf; azide adduct-1w6w) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

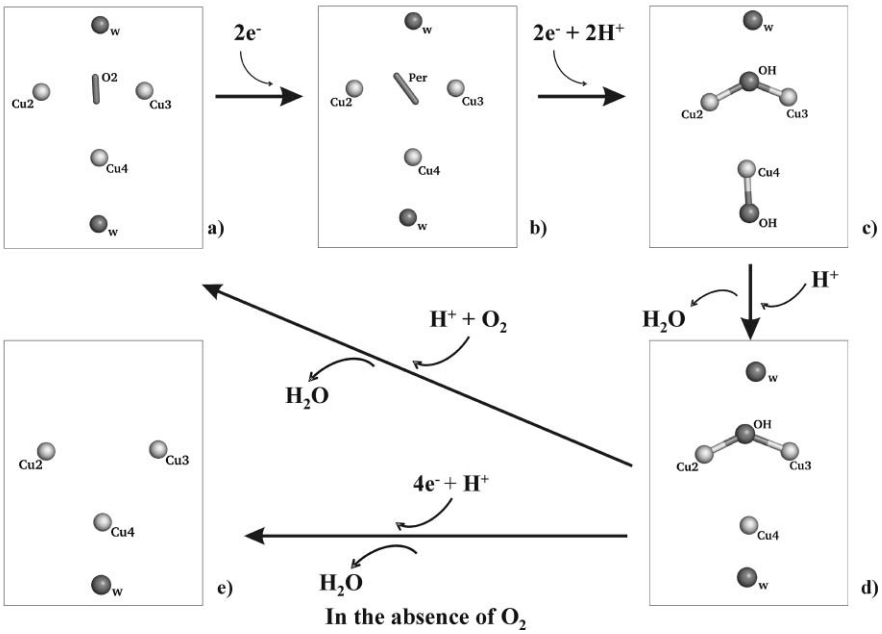

Fig. 3 Schematic view of structures representing stages in the mechanism of dioxygen reduction. (a) The "resting" state for CotA with a dioxygen molecule bound almost symmetrically between the type 3 copper ions and interacting with the type 2 copper. (b) The peroxide intermediate for the CotA enzyme. (c) The "resting" state of the ascorbate oxidase and ceruloplasmin enzymes with one hydroxyl group bridging the type 3 copper ions and a second attached to the type 2 copper ion at the entrance to the egress channel. (d) The molecule with one bridging hydroxyl group as observed in the enzyme from *Trametes versicolor*. (e) The enzyme in its fully reduced state. In neither (a) nor (b) is there an hydroxyl group attached to the type 2 copper ion.

adduct of ascorbate oxidase[30] clearly show that access to the trinuclear copper cluster by dioxygen (or a dioxygen derived species) is through a well defined channel, normally occupied with solvent molecules. For CotA the channel involves the side chains of residues Glu498, Asp499 and Asp501. In the peroxide complex of ascorbate oxidase, the peroxide is bound to one of the type 3 copper ions in a terminal manner and the distance between the two type 3 copper ions is 4.8 Å. However the studies presented herein on CotA and for the laccase from *M. albomyces*[11] suggest that in the case of dioxygen, the molecule will progress to an almost symmetrical binding site within the trinuclear cluster, Fig. 3(a), whereby all the copper oxygen distances are in the range 2.2–2.6 Å. In this structure all the copper ions will be in the oxidised +2 state; the blue colour of the enzyme is certainly consistent with the type 1 copper being oxidized. Whether there is an intermediate stage with the dioxygen bound terminally to only one of the type 3 copper ions remains unknown.

The next stage of the putative mechanism involves the formation of a peroxide intermediate, Fig. 3(b). Two electrons are transferred to dioxygen and the diatomic O_2^{2-} moiety becomes inclined to the line between Cu2 and Cu3 so that the closest Cu–O distances are 1.95 Å (Cu3) and 2.01 Å (Cu2). The pathway of electron transfer in CotA involves substrate molecules donating electrons to the type 1 copper ion through His497 and then the transmission of these electrons through Cys492 and the adjacent histidines, His491 and His493 to Cu3 and Cu2 respectively. Each of the type 3 copper ions can then transfer one electron to an oxygen atom of the bound dioxygen. After participating in the transfer process, the type 3 copper ions will be left in the +2 state.

Two further electrons are then assimilated in a similar manner and these electrons, together with two protons, then give rise to a species in which the peroxide moiety is split into two hydroxyl groups, one of which remains in a bridging position between the type 3 copper ions, Fig. 3(c). The source of the protons is likely to be the acidic residues occupying the walls of the access channel and in particular Glu498. The second hydroxyl moiety migrates to the opposite side of the type 2 copper and forms a Cu–OH bond to give a structure that is equivalent to the "resting" state[32,33] proposed in the mechanism of dioxygen reduction by ascorbate oxidase. At a first inspection, the migration of the hydroxyl group appears difficult to explain from the X-ray structures alone, since the amount of space available is limited and the procedure must involve a movement of the type 2 copper ion, Cu4, and some transient local distortion of the protein geometry. Cu4 is bound to the protein by only two histidine residues, His105 and His422 in CotA, and is known to be more labile than the two type 3 copper ions. A mechanism can be envisaged whereby the Cu4 moves out of the plane defined by the trinuclear cluster using the histidine residues as pivot points, so that it can bind a hydroxyl group and transfer it into a position at the beginning of the outlet solvent channel. In this context it should be remembered that X-ray crystallography gives rise to static structures that serve as excellent templates for further studies. At physiological temperatures the atoms in the molecule are subject to vibrational movements about a mean point, some of which are concerted and others apparently random. The classic example is the case of myoglobin for which a model where the atoms are represented by space-filling spheres according to the respective atomic radii suggests that there is no room for a dioxygen species to penetrate inside and bind to the iron in the heme moiety or indeed to be released from the protein after binding. Yet this access and egress is a fundamental property of myoglobin. However several studies, such as those involving pico-second time-resolved X-ray techniques,[34] have indicated how movements of the atoms can create pathways to permit myoglobin to bind and release oxygen. Similar movements must operate in the case of the migration of hydroxide groups around the type 2 copper ion.

The following stages involve the addition of a proton to the hydroxyl group bound to Cu4 and the release of the first water molecule into the outlet channel. This will leave a structure with only a single hydroxyl moiety bridging the two type 3 copper ions,[9,10] Fig. 3(d). This hydroxyl moiety will then migrate to the far side of Cu4, become protonated and finally released as the second water molecule. The enzyme can then bind a second dioxygen molecule for a re-iteration of the cycle. Alternatively, in the absence of dioxygen the assimilation of four electrons will leave the enzyme in the fully reduced state, Fig. 3(e), ready to bind dioxygen as soon it becomes available.

In this putative mechanism the role of the type 2 copper is therefore two-fold; firstly it helps to anchor the dioxygen molecule to the trinuclear cluster and secondly it temporarily binds the hydroxyl groups arising from the reduction of the dioxygen molecule prior to these moieties being released as water molecules into the outlet channel. The type 3 copper ions serve to transfer electrons to the bound dioxygen so that it can be reduced. The mechanism proposed also indicates why there is a separate electron transfer path between the type 1 copper ion and each of the type 3 copper ions. Removal of the type 2 copper should still enable the enzyme to bind dioxygen in a manner similar to that observed in haemocyanins,[35] but should result in loss of oxidative activity through its inability to reduce dioxygen.

The mechanism described above is consistent with all the X-ray structures so far reported and with a mass spectrometric investigation of the reaction between $^{18}O_2$ and a reduced tree laccase.[36] These experiments, undertaken before any three-dimensional structural information was available, assumed that the access route to the trinuclear copper cluster used by the dioxygen was the same as that used to release the water molecules. They were interpreted as one water molecule being rapidly formed and released, whereas the second remains associated with the type 2 copper ion for a longer period. The three-dimensional structural information now available indicates that different channels are used for access by dioxygen and release of water molecules, but the concept of sequential release of water is consistent with the mechanism proposed.

It is pertinent to ask what is the "resting" state of the enzyme? For ascorbate oxidase[32,33] and ceruloplasmin[31] the "resting" state appears to be one in which one hydroxyl group bridges the type 3 copper ions and a second is attached to the type 2 copper, Fig. 3(c). In earlier work on CotA[13] the enzyme was purified and crystallized in a similar configuration. In the presence of the mediator 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate), however, a different configuration was observed with a dioxygen moiety apparently trapped in the access channel[15] and a water molecule instead of a hydroxyl group at the type 2 copper ion. In the present work and with the method of purification described in the experimental section, the "resting" state appears to be one with a dioxygen moiety bound to the trinuclear cluster and a water molecule interacting with the type 2 copper ion, Fig. 3(a). Presumably the "resting" state of the molecule depends on a number of factors that include the method of purification and crystallisation, the pH, the redox potentials of the copper ions and the availability of dioxygen and a supply of electrons. Clearly the multi-copper oxidase enzymes are able to adopt a number of different stable configurations as indicated in Fig. 3.

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