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Substrate and Dioxygen Binding to the Endospore Coat Laccase from *Bacillus subtilis**

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The CotA laccase from the endospore coat of *Bacillus subtilis* has been crystallized in the presence of the non-catalytic co-oxidant 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), and the structure was determined using synchrotron radiation. The binding site for this adduct is well defined and indicates how ABTS, in conjunction with laccases, could act as an oxidative mediator toward non-phenolic moieties. In addition, a dioxygen moiety is clearly defined within the solvent channel oriented toward one of the T3 copper atoms in the trinuclear center.

Laccases (benzenediol oxygen oxidoreductase; EC 1.10.3.2) belong to the multi-copper oxidase family of enzymes that includes 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 Refs. 1 and 2). 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 many key questions still remain. Two of these involve the mechanism of electron transfer from substrates and the mechanism of dioxygen reduction (2, 3). Laccases are the simplest representatives of the family and are therefore used as model systems for investigating structure-function relationships in the multi-copper oxidases. Such fundamental studies are aimed at clarifying the molecular basis for the enzyme redox potential and the specificity toward different substrates.

Most of the known laccases have fungal (*e.g.* white-rot fungi) or plant origins, although a few laccases have recently been identified and isolated in bacteria (4–6). These enzymes have been implicated in many diverse physiological functions such as morphogenesis, pathogenesis, lignin synthesis, and lignolysis (4, 7). Chemically, all these functions are related to the oxidation of a range of aromatic substrates such as polyphenols, methoxy-substituted phenols, diamines, and even some inorganic compounds. The one-electron oxidation of these reducing substrates occurs concomitantly with a four-electron reduction of molecular dioxygen to water. The catalytic centers consist of three structurally and functionally distinct copper centers. T1 copper ("blue copper") is a mononuclear center involved in substrate oxidation, whereas T2 and T3 form a trinuclear center involved in dioxygen reduction to water; for a definition of the copper types, see Ref. 8. The initial substrate reaction products are dioxygen-centered radicals or cation radicals, which usually react further through non-enzymatic routes for the oxidative coupling of monomers or the degradation of polymers.

Because of their high relative nonspecific oxidation capacity, laccases have been found to be useful biocatalysts for diverse biotechnological applications (9). Their biotechnological importance showed a marked increased after the discovery that the oxidizing reaction substrate range could be further extended in the presence of the so-called mediators, small readily oxidizable molecules (10), by a mechanism that remains, as yet, quite unclear. The most extensively investigated laccase mediator is 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS),[1] which allows the oxidation of non-phenolic lignin model compounds and pulp delignification by laccase (10, 11). Several other effective redox mediators have also been reported extensively, and the applications of laccase/mediator systems that target lignocellulosics and other insoluble materials have been described (12). A detailed knowledge of these processes will allow the future design of optimized enzymes by protein engineering techniques and of novel and more efficient mediators by molecular modeling techniques.

Recently, three fungal laccases from *Coprinus cinereus* (13) (Protein Data Bank codes 1A65 and 1HFU), *Trametes versicolor* (14, 15) (Protein Data Bank codes 1KYA and 1GYC), and *Melanocarpus albomyces* (16) (Protein Data Bank code 1GE0) have been structurally characterized, in addition to a bacterial laccase, CotA from *Bacillus subtilis* (17) (Protein Data Bank code 1GSK). The CotA enzyme is thermoactive and thermostable and, thus, an interesting enzyme for industrial applications.

X-ray studies of crystals of the enzyme CotA laccase from *B. subtilis* soaked with the mediator molecule ABTS are described herein. The nature of the binding of ABTS to CotA is described, and the role of ABTS as an oxidative mediator in conjunction with laccases is discussed. In addition, a dioxygen molecule has also been identified in a water channel leading toward the trinuclear copper center, but not bound to it. The implications

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The atomic coordinates and structure factors (code 1UVW) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/)..

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[1] The abbreviations used are: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate; Wat, water molecule.

TABLE I
Data collection and refinement statistics

Categories	Values
Data collection	
Space group	$P3_121$
Cell Dimensions	
a =	102.278
b =	102.278
c =	136.247
α =	90
β =	90
γ =	120
Number of unique reflections	30797
Resolution range for refinement (Å)	20–2.45 (2.60–2.45)
Completeness (%)	99.9 (100)
$I/\sigma I$ (σ)	6.6 (2)
R_{sym}	8.5 (38.2)
Multiplicity	5
Refinement	
Number of protein atoms	4044
Number of solvent atoms	245
Number of heterogen atoms	51
Final R-factor	10.5 (19.2)
Final free R-factor	13.7 (25.2)
Average B values (Å^2)	35.1
Model r.m.s.[a] deviations from ideality	
Bond lengths (Å)	0.0060
Bond angles (°)	1.400

[a] Root mean square.

of this observation with regard to dioxygen reduction at the trinuclear center are discussed.

EXPERIMENTAL PROCEDURES

Protein Crystallization and Substrate Reaction—Recombinant CotA protein was crystallized as described previously (18). Crystals were grown over a period of 3 to 5 days and then soaked in a crystallization solution containing 0.5 mM of ABTS. These soaking experiments have shown modifications in the color of the resulting mixture that evolved with time, and this was interpreted as an indication of a reaction between ABTS and the enzyme. This enzymatic reaction inside the CotA crystals was followed by observation under a light microscope. The color changed from light blue to dark magenta in a time range of 20 h. No significant changes in crystal color were observed after 20 h of reaction.

Data collection, Model Building, and Refinement—Crystals reacting with the substrates were harvested from the mother liquor at different incubation times, cryo-protected, and frozen for x-ray data collection as described previously (18). Data collection was performed at 100 K using synchrotron radiation at beam line X11, European Molecular Biology Laboratory, Deutsches Elektronen Synchrotron (EMBL-DESY), Hamburg, Germany, with a 165-cm MAR-CCD detector. Data collection and refinement statistics are shown in Table I. Data sets were processed with MOSFLM (19), and scaled with SCALA (20) from the CCP4 program suite (21).

The structure of the CotA-ABTS adduct was elucidated by molecular replacement using MOLREP (22). The starting model was the CotA native structure (Protein Data Bank code 1GSK) (17) from which all the copper and solvent atoms had been removed. One clear solution was evident, giving R-factor and correlation coefficients of 29.9% and 0.801, respectively. An electron density synthesis enabled the location of the four copper atoms in the molecule and showed clear density for the substrate. Refinement was performed using the maximum likelihood functions implemented in REFMAC5 (23). Rounds of conjugate gradient and sparse matrix refinement with bulk solvent modeling according to the Babinet principle (24) were alternated with model building using the O program suite (25) in combination with σ_A-weighted $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ maps (26). After the first round of refinement, solvent molecules were added to the model based on standard geometrical and chemical restraints; two molecules of glycol were also located. The occupancies of the copper atoms in the trinuclear cluster were adjusted so that their isotropic thermal parameters were refined to ~50.0 Å^2; the assignment of full occupancies led to thermal coefficients significantly higher than the local average. The ABTS molecule in the substrate binding pocket was modeled with alternate conformations for the solvent-exposed region. Details of the refinement are shown in Table I.

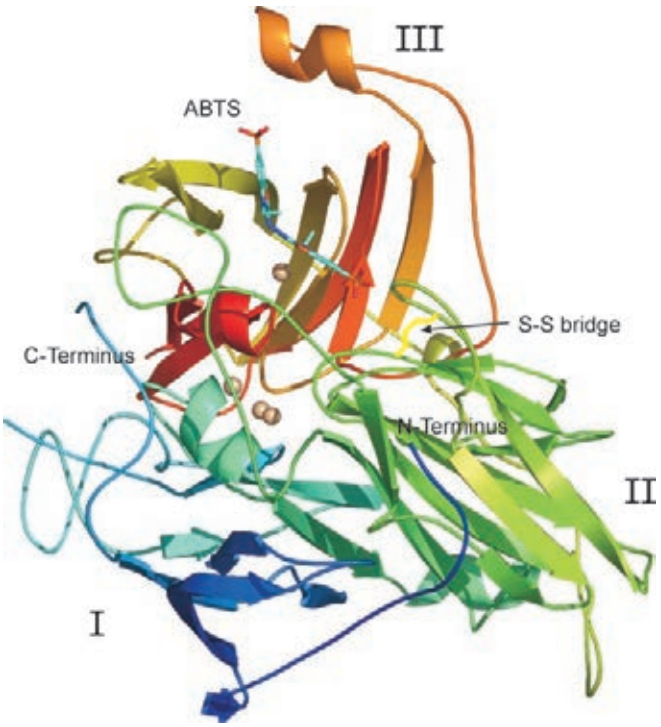

FIG. 1. **Overall CotA adduct structure.** The CotA protein is rainbow colored, and the C- and N termini are marked. The mediator ABTS molecule close to the mononuclear copper center and the nearby disulfide bridge are represented by *blue* and *yellow stick models*, respectively. The copper atoms within the CotA structure are represented as *light brown spheres*. CotA domains are designated by roman numerals according to the nomenclature described previously (17).

The atomic coordinates of the CotA-ABTS adduct model and structure factors have been deposited in the Protein Data Bank and assigned the accession code 1UVW. All figures were prepared using PyMol (27).

RESULTS

Overall Structure and Copper Depletion—Crystals of the CotA laccase protein from *B. subtilis* have been soaked with ABTS for periods up to 20 h and then cryo-frozen and used for x-ray studies. Crystal structures have been determined after soaking periods of 1, 6, 8, and 20 h, and in all cases the overall structure appears to be essentially that of the native enzyme (17) (Fig. 1). After soaking periods of 6 h or longer, there is significant copper depletion at the trinuclear site and, in particular, of the T2 copper, Cu4. At the 20-h stage this copper appears to be completely depleted. In this paper, special features observed in the crystal structure of the 1-h soak will be described; the remaining structures will be described elsewhere. The crystal structure of the 1-h soak shows only a relatively low depletion of copper, so that Cu4 appears to be most depleted by ~35%, whereas one of the T3 copper atoms, Cu3, is depleted by only 20%.

The Dioxygen Binding Site—In all of the multi-copper oxidases studied to date, there are water channels that link the trinuclear copper center with the external environment. Dioxygen approaches the trinuclear copper center through these channels, but the precise nature of its subsequent interaction with the center is the subject of debate. Hakulinen *et al.* (16) report the presence of a dioxygen moiety amid the three copper atoms of the trinuclear cluster in the crystal structure of the laccase from *M. albomyces*. In this structure, the distance between the O1 atom of the dioxygen moiety and the T2 copper atom is 2.5 Å, and all the distances between the O1 and O2 atoms of the dioxygen moiety and the T3 copper atoms lie in the range 2.4–2.6 Å. There is no bridging hydroxyl group as ob-

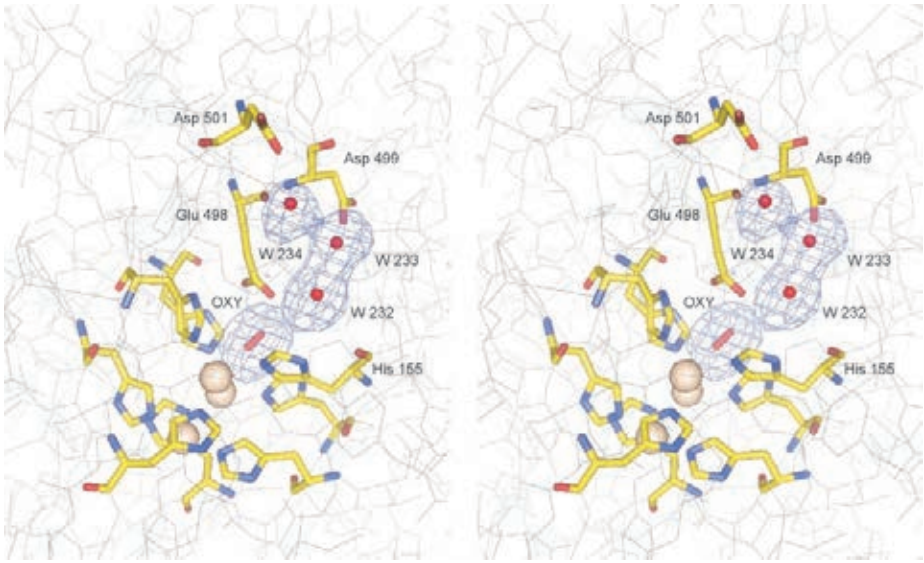

FIG. 2. **Water channel.** A stereo view of a fragment of the water channel by which the dioxygen molecule approaches the trinuclear copper center is shown, together with the side chain residues of Glu-498, Asp-499, and Asp-501 involved in hydrogen bonds with the solvent molecules in the channel. Water (*W*) molecules 233, 234 and 235 are drawn as *red spheres*. The electron density in an omit map contoured at the 4 σ level in the region around the waters in the channel, and the dioxygen molecule is also represented.

served in the fully oxidized structure of ascorbate oxidase (28), and the two T3 copper atoms are separated by 4.8 Å. This value is far closer to the value of 5.1 Å observed in the dithionite-reduced structure of ascorbate oxidize (29) than that of 3.7 Å in the corresponding oxidized structure.

As observed previously (17), CotA has a clearly defined channel directed toward the trinuclear copper cluster. Aspartate and glutamate residues act as channel markers as the channel penetrates the protein structure (Fig. 2); Asp-501 lies at the surface, followed by Asp-499 and Glu-498 as the channel progresses toward the trinuclear center. In the CotA structure reported herein, three water molecules occupy the first section of this channel (Fig. 2). Wat235 is hydrogen bonded to Asp-501 near the surface (OD1 . . . O_w = 3.20 Å) and to the carboxyl group of Glu-498 (O . . . O_w = 2.65 Å) and is also in close proximity to Wat234 with an O_w . . . O_w separation of 3.42 Å. Wat234 is, in turn, hydrogen bonded to Asp-499 (OD1 . . . O_w = 2.86), the amino group of Ala-158 (N . . . O_w = 2.83 Å), and Wat233 (O_w . . . O_w = 2.70 Å). Wat233 forms hydrogen bonds with Glu-498 (OE2 . . . O_w = 2.84 Å), Ser-160 (OG1 . . . O_w = 2.99 Å), and His-155 (ND1 . . . O_w = 3.31 Å), one of the ligands to Cu3. The channel then proceeds downwards to the site of the dioxygen molecule, which is ~5.15 Å from Wat233 and directly above a T3 copper atom, Cu2 (Fig. 3). One atom of the dioxygen molecule, O1, appears to be held by Glu-498 in a bifurcated manner (OE1 . . . O1 = 2.70 Å; OE2 . . . O1 = 2.92 Å), whereas the second oxygen atom, O2, is 3.25 and 3.30 Å from the T3 copper atoms Cu2 and Cu3, respectively, and therefore not directly bound to them. It is probable that this dioxygen molecule has been trapped in a holding position prior to binding to one or more of the copper atoms in the trinuclear center. Under the soaking conditions used, the enzyme has probably been placed in a dormant state awaiting further substrate oxidation activity. The reception of further electrons will then cause the dioxygen to bind to its active site at the trinuclear copper center. In an omit electron density synthesis whereby the copper atoms and their ligands and all the channel moieties are omitted from the phase calculations, there is continuous electron density at the 7 root mean square level between the dioxygen molecule and Cu2, but not between it and Cu3. The implication is that the dioxygen molecule will most probably bind to Cu2 in a manner almost identical to the binding of azide and peroxide to the T3 copper, Cu3 (equivalent to Cu2 in CotA), in the ascorbate oxidase structure and that this is the first stage of binding of dioxygen to the trinuclear center.

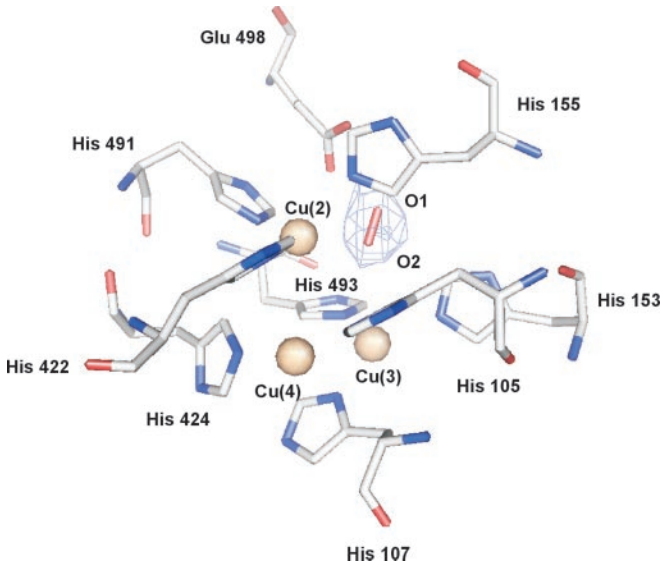

FIG. 3. **Trinuclear copper center and dioxygen molecule.** The figure shows the overall structure of the trinuclear copper center, including the coordinating histidine residues and the refined dioxygen molecule. The electron density in an omit map contoured at the 4 σ level is shown around the dioxygen molecule. The Glu-498 side chain, which binds to one of the oxygen atoms of the refined dioxygen molecule, is also represented.

The Copper Sites—The T2–T3 copper distances, 4.07 Å for Cu2–Cu4 and 3.83 Å for Cu3–Cu4 in the CotA adduct, are significantly less than those recorded in the native enzyme, 4.64 Å and 4.67 Å, respectively, and lie intermediate between the oxidized and reduced forms of ascorbate oxidase. The copper ligand distances for the T1, T2, and T3 copper types are comparable with those observed in the native CotA structure, except in the case of the His-155 ligand to Cu3, which moves in such a way as to accommodate the dioxygen molecule (see Fig. 3) from a standard distance of 2.05 Å in native CotA to 2.48 Å in the adduct structure. The trinuclear center in the CotA adduct structure loses the bridging hydroxide moiety, and the distance between the two T3 copper atoms shows a small increase from 4.28 Å in the native structure (17) to 4.56 Å in the adduct structure (Fig. 3).

The Adduct Binding Site—The crystal structure of the 1-h soak of the CotA laccase with ABTS clearly shows that the ABTS binds in a large pocket mainly formed by apolar residues

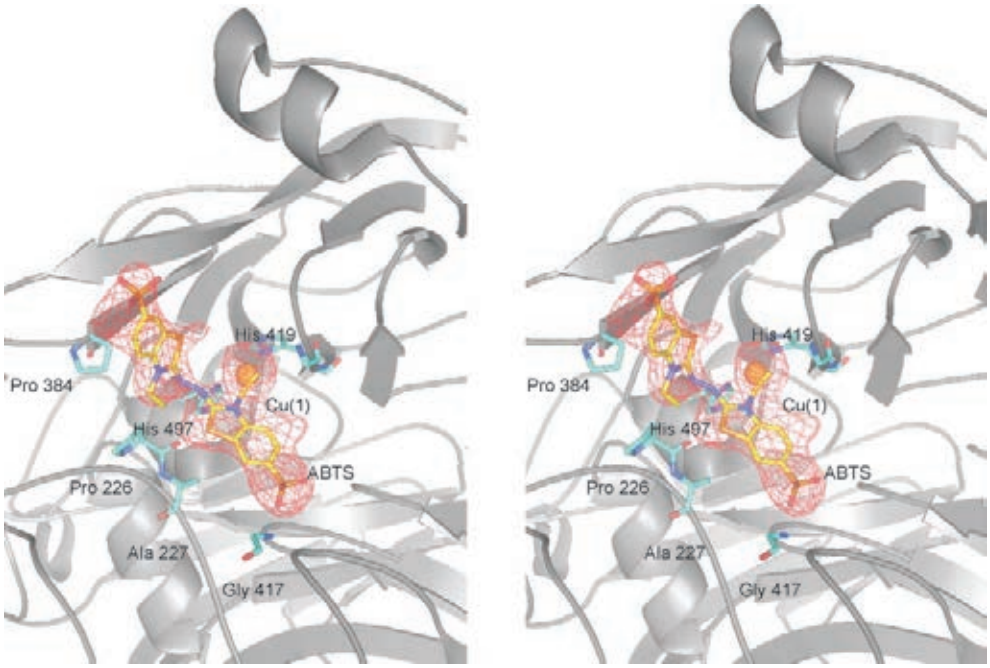

FIG. 4. **ABTS mediator in the binding site.** A stereo view of the region of the CotA molecule close to the mononuclear copper center shows the position of the ABTS molecule. The electron density in an omit map contoured at the 4 σ level in the region around the substrate is also represented. Residues in close proximity to the substrate are represented as *blue stick models*. The half of the substrate exposed to the solvent, close to Arg-416, has been modeled with two alternative configurations as described in the text.

(Fig. 4) and previously identified in the structure of the native protein (17). The ABTS can be readily built into the electron density as a shallow U-shaped molecule with the bottom of the U in close proximity to His-497. This contrasts with the almost linear structure of ABTS found in the crystalline form (30) and is consistent with the ABTS being bound in a radical and/or cationic state. At a resolution of 2.4 Å it is difficult to precisely define the details of the adduct binding, but a plausible model involves one in which one-half of the adduct is partially buried in the CotA structure and the other half is relatively exposed to the external environment. In the partially buried half, the sulfonate moiety approaches the disulfide bridge between cysteines Cys-229 and Cys-322, (Cys-322 SG − O14 = 3.22 Å), and one of the oxygen sulfonate atoms forms a hydrogen bond with the Gly-323 (O13 − Gly-323 n = 3.06 Å). With respect to the exposed half, there appears to be some evidence of disorder, and two conformations have been modeled. In one conformation, the thiazoline ring is *trans* to the other half of the molecule as reported in the crystal structure (30), and in the other a rotation about the central N-N bond of the bis-azino moiety places it in a *cis* conformation. In either case, the sulfonate group is ~5–6 Å distant from Arg-416. In this model, the thiazoline ring of the partially buried half of the ABTS molecule is roughly perpendicular to the aromatic ring of His-497, one of the T1 copper ligands, with a closest approach between C2 and the NE2 atom of His-497 of 3.32 Å. This geometry would favor the transfer of an electron from the adduct to the T1 copper center through His-497; subsequently the electron would be transferred to the trinuclear cluster through Cys-492, the copper ligand that confers the blue color to CotA.

DISCUSSION

There are two key features observed in the present structure: (i) the presence of an dioxygen molecule in the solvent channel pointing toward the T3 copper atom with the highest occupancy, Cu2; and (ii) the adduct itself bound at the site of substrate oxidation. These structural differences, when compared with the enzyme native structure, reflect the enzymatic activity of CotA in the crystal toward ABTS (5). Neither of these features has been observed in previous structural studies on the multi-copper oxidases or in the other crystals of CotA with longer ABTS soaking periods.

In the crystal structure of the *M. albomyces* enzyme, the C terminus residues block access to the channel through which it is believed the dioxygen molecule approaches the trinuclear site. This may account for the stability of the dioxygen adduct, but it also raises the question of whether this structure represents the active state of the enzyme. The authors have even suggested that a conformational change has to occur to allow the entry of the dioxygen molecule. They also noted that the absorption spectra of the enzyme in the range 300–700 nm are similar to those obtained from the laccase from *Rhus vernicifera* (31–33) and state that this supports the structure of a dioxygen moiety bound to all three copper atoms in the trinuclear center. These studies contrast with those undertaken on ascorbate oxidase (29), whereby peroxide (an dioxygen substitute) and azide (an inhibitor) were found to bind only to one of the T3 copper atoms with a concomitant loss of the bridging hydroxide moiety and an increase in the separation of the two T3 copper atoms from 3.7 to 4.8 and 5.1 Å for peroxide and azide, respectively. In the case of azide, two azide moieties were found to bind to the same T3 copper atom in the trinuclear cluster in ascorbate oxidase. The addition of azide to ceruloplasmin produced only one binding position under the soaking conditions used, but, again, this involved only one T3 copper atom, equivalent to that used by ascorbate oxidase (34). Thus, the precise nature of dioxygen binding to the trinuclear center in the multi-copper oxidases appears to remain undefined and, indeed, may be different for different members of this enzyme family. It is possible that, after the binding of the dioxygen molecule to one of the T3 copper atoms in CotA, a subsequent stage involves binding to the trinuclear center as observed in the laccase from *M. albomyces.*

As yet, the precise nature of substrate binding to the multi-copper oxidases is not well defined, and many of the binding

pockets appear to be broad and able to accommodate a number of different substrates. For ceruloplasmin it has been found that the major binding site for metal cations and biogenic amines is close to the T1 copper in domain 6, the closest T1 copper atom to the trinuclear center. On the other hand, aromatic diamines, including *p*-phenylenediamine, which is often used in assays for oxidase activity, bind in an entirely different place at the base of domain 4 (34). These binding studies with ceruloplasmin were restricted to a resolution of 3 Å, or worse, and precise details of substrate binding are therefore not available. In the case of the laccases, only the arylamine complex of the laccase from *T. versicolor* has, to date, been reported (14). The arylamine, 2,5-xylidine, is used as a laccase inducer in the fungus culture. The binding of ABTS in CotA differs from that of 2,5-xylidine, presumably because of its quite different overall size and shape. In the most abundant isozyme present in the crystal structure of the laccase from *T. versicolor*, the aromatic ring system of the 2,5-xylidine is again almost perpendicular to His-458 (equivalent to His-497 in CotA). However, it is also almost perpendicular to the thiazoline ring of the ABTS and is pointing in the opposite direction. The amino group of the 2,5-xylidine is 2.61 Å from the NE2 atom of His-458 and 3.20 Å from the OD2 atom of Asp-206 (equivalent to Thr-260 in CotA). It should also be noted in the laccase from *T. versicolor* that Phe-463 replaces the weak ligand Met-502 in CotA.

Bourbonnais and Pace (10) have shown that the laccase substrate range can be extended to include non-phenolic lignin subunits in the presence of a non-catalytic mediator such as ABTS. The involvement of a laccase-generated ABTS radical cation was suggested, although this radical cation in the absence of laccase was ineffective for non-phenolic oxidations (10). The ABTS has two oxidation states, namely the cation radical, $ABTS^{\cdot+}$, and the dication, $ABTS^{2+}$, and these two oxidized forms are relatively stable and electrochemically reversible (35). The dication species has been proposed as responsible for the oxidation of the non-phenolic lignin model compounds and for the role of the enzyme confined to the production of this dication (35). If laccases in solution adopt a similar mode of binding of ABTS as is shown by CotA in the crystalline form, then this may provide an explanation regarding the ability of ABTS and other mediators (36) to act as laccase co-oxidants in the reactions with phenolic and non-phenolic molecules. Thus, ABTS will initially bind to the laccase as the radical cation and then, with the transfer of a further electron, as the dication, which has a low solubility compared with the radical cation. Subsequent reactions can then occur through at least two routes. First, non-phenolic moieties can react directly with the exposed part of the bound ABTS dication without having to penetrate deeply into the binding pocket; second, the bound ABTS dication can react readily with other ABTS molecules in solution. The latter will involve a co-proportionation reaction of ABTS to $ABTS^{\cdot+}$, as has been observed at high concentrations of ABTS (35); the electrons acquired by such a process are transferred to the T1 copper site of the laccase. The $ABTS^{\cdot+}$ in solution can then participate in oxidative reactions with phenolic compounds. The mechanism suggested above provides a useful model for the oxidation of non-phenolic moieties by the ABTS-coupled laccase systems.

The present study shows that the cryo-freezing of crystals of the CotA laccase from *B. subtilis* soaked with ABTS for 1 h induces the ABTS to form a stable complex with the laccase. Soaking times longer than this appear to cause dissociation of the ABTS and substantial loss of copper from the trinuclear center. The enzyme has probably been trapped in a dormant form with a dioxygen molecule in the solvent channel awaiting binding to one of the T3 copper atoms. When the enzyme receives further electrons, the dioxygen will move closer to the copper and be reduced to two molecules of water. Clearly, further studies are required to define the precise mechanisms of both dioxygen reduction and the role of oxidative mediators.

REFERENCES

1. Messerschmidt, A. (1997) *Multi-copper Oxidases,* World Science Press, Singapore
2. Lindley, P. F. (2001) in *Handbook on Metalloproteins* (Bertini, I., Sigel, A., and Sigel, H., eds) pp. 763–811, Marcel Dekker, Inc., New York
3. Cole, A. P., Root D. E., Mukherjee, P., Solomon, E. I., and Stack, T. D. (1996) *Science* **273,** 1848–1850
4. Gianfreda, L., Xu, F., and Bollag, J.-M. (1999) *Bioremediation J.* **3,** 1–25
5. Martins, L. M., Soares, C. M., Pereira, M. M., Teixeira, M., Costa, T., Jones, G. H., and Henriques, A. O. (2002) *J. Biol. Chem.* **277,** 18849–18859
6. Claus, H. (2003) *Arch. Microbiol.* **179,** 145–150
7. Mayer, A. M., and Staples, R. C. (2002) *Phytochemistry* **60,** 551–565
8. Malmström, B. G. (1982) *Ann. Rev. Biochem.* **51,** 21–59
9. Xu, F. (1999) in *The Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation* (Flickinger, M.-C., and Drew, S. W., eds) pp. 1545–1554, John Wiley & Sons, Inc., New York
10. Bourbonnais, R., and Paice, M. G. (1990) *FEBS Lett.* **267,** 99–102
11. Bourbonnais, R., Paice, M. G., Reid, I. D., Lanthier, P., and Yaguchi, M. (1995) *Appl. Environ. Microbiol.* **61,** 1876–1880
12. Xu, F., Palmer, A. E., Yaver, D. S., Berka, R. M., Gambetta, G. A., Brown, S. H., and Solomon, E. I. (1999) *J. Biol. Chem.* **274,** 12372–12375
13. Ducros, V., Brzozowski, A. M., Wilson, K. S., Brown, S. H., Østergaard, P., Schneider, P., Yaver, D. S., Pedersen, A. H., and Davies, G. J. (1998) *Nat. Struct. Biol.* **5,** 310–316
14. Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C., and Mougin, C. (2002) *Biochemistry* **41,** 7325–7333
15. Piontek, K., Antorini, M., and Choinowski, T. (2002) *J. Biol. Chem.* **277,** 37663–37669
16. Hakulinen, N., Kiiskinen, L.-L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A., and Rouvinen, J. (2002) *Nat. Struct. Biol.* **9,** 601–605
17. Enguita, F. J., Martins, L. M., Henriques, A. O., and Carrondo, M. A. (2003) *J. Biol. Chem.* **278,** 19416–19425
18. Enguita, F. J., Matias, P. M., Martins, L. O., Plácido, D., Henriques, A. O., and Carrondo, M. A. (2002) *Acta Crystallogr. Sec. D* **58,** 1490–1493
19. Leslie, A. (1992) *Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography* **26**
20. Weiss, M. (2001) *J. Appl. Crystallogr.* **34,** 130–135
21. Collaborative Computational Project, Number 4 (1994) *Acta Crystallogr. Sec. D* **50,** 760–763
22. Vagin, A., and Teplyakov, A. (1997) *J. Appl. Crystallogr.* **30,** 1022–1025
23. Murshudov, G. N., Lebedev, A., Vagin, A. A., Wilson, K. S., and Dodson, E. J. (1999) *Acta Crystallogr. Sec. D* **55,** 247–255
24. Tronrud, D. (1996) in *Proceedings of the CCP4 Study Weekend: Macromolecular Refinement* (Dodson, E., Moore, M., Ralph, A., and Bailey, S., eds) pp. 1–10, Daresbury Laboratory, Warrington, UK
25. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. Sect. A* **47,** 110–119
26. Read, R. J. (1986) *Acta Crystallogr. Sect. A* **42,** 140–149
27. DeLano, W. L. (2002) *The PyMOL User's Manual*, DeLano Scientific, San Carlos, CA
28. Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A., and Finazzi-Agro, A. (1992) *J. Mol. Biol.* **224,** 179–205
29. Messerschmidt, A., Luecke, H., and Huber, R. (1993) *J. Mol. Biol.* **230,** 997–1014
30. Mousty, C., Therias, S., Aboab, B., Molinie, P., Queignec, M., Leone, P., Rossignol, C., and Palvadeau, P. (1997) *New J. Chem.* **21,** 1321–1330
31. Huang, H.-W., Zoppellaro, G., and Sakurai, T. (1999) *J. Biol. Chem.* **274,** 32718–32724
32. Lee, S.-K., George, S. D., Antholine, W. E., Hedman, B., Hodgson, K. O., and Solomon, E. I. (2002) *J. Am. Chem. Soc.* **124,** 6180–6193
33. Sundaram, U. M., Zhang, H. H., Hedman, B., Hodgson, K. O., and Solomon, E. I. (1997) *J. Am. Chem. Soc.* **119,** 12525–12540
34. Zaitsev, I., Zaitsev, V., Card, G., Moshkov, K., Bax, B., Ralph, A., and Lindley, P. (1996) *J. Biol. Inorg. Chem.* **1,** 15–23
35. Bourbonnais, R., Leech, D., and Paice, M. G. (1998) *Biochim. Biophys. Acta* **1379,** 381–390
36. Fabbrini, M., Galli, C., and Gentili, P. (2002) *J. Mol. Catal. B Enzym.* **2002,** 231–240