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RAPID REPORT

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Probing the Dioxygen Route in *Melanocarpus albomyces* Laccase with Pressurized Xenon Gas

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Supporting Information

ABSTRACT: Laccases catalyze the oxidation of phenolic substrates and the concominant reduction of dioxygen to water. We used xenon as an oxygen probe in search of routes for the entry of dioxygen into the catalytic center. Two xenon-pressurized crystal structures of recombinant *Melanocarpus albomyces* laccase were determined, showing three hydrophobic Xe-binding sites located in domain C. The analysis of hydrophobic cavities in other laccase structures further suggested the preference of domain C for binding of hydrophobic species such as dioxygen, thus suggesting that the hydrophobic core of domain C could function as a channel through which dioxygen can enter the trinuclear copper center.

The activation of oxygen is one of the key reactions in aerobic life. Enzymes that are directly involved in the activation reaction are oxidases and oxygenases. The mechanism of delivery of oxygen to the active sites, often buried in the core of the protein, is uncertain for many of the oxygen-activating enzymes. Formerly, the general model of how oxygen is delivered to proteins was passive diffusion through the protein matrix.[1] However, more recent studies suggest the existence of specific oxygen entry pathways. Evidence of specific oxygen motions has at least been found for copper amine oxidase, lipooxygenase, cholesterol oxidase, cytochrome *c* oxidase, cyclooxygenase, myoglobin, and the heme−copper respiratory complex.[2−8] These results were obtained using different methods, such as mutagenesis studies, kinetic measurements, molecular dynamic calculations, infrared spectroscopy, and crystallographic studies. On the basis of these studies, it seems that specific hydrophobic paths are used for the flow of oxygen from the protein surface through specific transient binding sites to the active centers where the molecular oxygen is utilized.

Xenon gas can be used to form complexes with proteins under high pressure. These complexes can be used as highly isomorphous heavy-atom derivatives for phasing in X-ray crystallography[9] or detecting hydrophobic cavities in the protein interior.[10] Any site that binds Xe is also a potential and favorable binding site for molecular oxygen[7] because of the similar maximal van der Waals diameter and hydrophobic properties of these two species. First myoglobin[11] and later many other proteins[12] have been reported to bind Xe, predominantly by hydrophobic interactions, via small channels and cavities already existing in the protein structure. More recently, xenon has been used as a specific probe for dioxygen movements in copper amine oxidases.[12,13]

Laccases, benzenediol oxygen oxidoreductases (EC 1.10.3.2), belong to the larger group of blue multicopper oxidases (MCOs). These enzymes oxidize a wide variety of organic substrates, but foremost phenolics. The electrons captured from substrates are used for the reduction of molecular oxygen. The three-domain laccase structure contains three β-barrel cupredoxin-like domains that surround two catalytic copper sites. The T1 mononuclear center (one type 1 copper) is responsible for substrate oxidation and the T2/T3 trinuclear center (TNC, one type 2 copper and two type 3 coppers) for oxygen reduction. We previously observed that a dioxygen molecule binds amidst the three copper atoms in TNC.[14] Suggestions for the mechanism[15] of the redox process have been made, but many details are still being debated. For example, the route by which molecular oxygen reaches the TNC site is unclear. Generally, among laccases, it is thought that a hydrophilic solvent channel leading from the protein surface to the TNC (Figure S1A of the Supporting Information) could be the point of entry for O_2. However, the channel is blocked by the C-terminal end of the amino acid chain in the known ascomycete-type, *Melanocarpus albomyces* (MaL) and *Thielavia arenaria* (TaLcc1), laccase crystal structures.[14,16] Our mutagenesis studies of MaL have not shown any evidence of the conformational change of the C-terminus, but these have clearly shown the critical role of the C-terminus in enzyme activity and stability.[17] Another commonly suggested point of entry to the TNC is a water channel from the protein surface to the T2 copper of the active site, but this channel is again closed in MaL by His98 and a chloride ion coordinated to the T2 copper. In addition, these channels are hydrophilic, making them less likely to accommodate a hydrophobic dioxygen molecule.

We have now resolved two crystal structures of recombinant *M. albomyces* laccase (rMaL) complexed with xenon, which is a known probe for molecular oxygen binding sites. To the best of our knowledge, this is the first time that these kinds of experiments have been used for laccases or other MCOs. rMaL crystals were cryoprotected and placed in a nylon loop inside the pressure cell to form a complex with xenon (10 bar for 5 min). After depressurization, the crystals were flash-frozen in liquid nitrogen. Two X-ray diffraction data sets (Xe_rMaL1 and Xe_rMaL2) were recorded from two different Xe complex crystals of rMaL at resolutions of 1.9 and 2.7 Å (Table S1 of the Supporting Information). The low-resolution data were collected using a wavelength of 1.77 Å for recording the anomalous signal ($f'' = 9.2$ electrons), which was then

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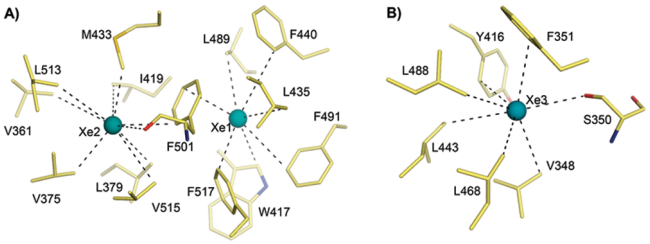

Figure 1. Binding sites for three complexed Xe atoms. The contacts with the nearest atoms of the neighboring residues are marked with dashed lines. The distances are listed in Table S2 of the Supporting Information. (A) Contacts for Xe1 and Xe2. (B) Contacts for Xe3.

used to calculate the anomalous difference maps to confirm Xe binding. Our study revealed three hydrophobic binding sites for Xe (xenons hereafter termed Xe1, Xe2, and Xe3) at the same positions in both molecules in the asymmetric unit that contains two molecules forming a weak dimer.[17] All three sites are located in the same β-barrel structure of domain C. Despite the similar β-barrel structures of domains A and B, we found no traces of Xe binding in those domains. By examining the anomalous electron density map, we again identified three Xe binding sites per protein molecule.

The Xe binding sites in laccases were mainly formed by interactions with aliphatic and aromatic side chains (Figure 1) (Table S2 of the Supporting Information). The binding site for Xe1 was located between two β-sheets in the core of the β-barrel structure of domain C. The Xe1 atom formed hydrophobic contacts with the side chains of seven hydrophobic amino acid residues. Xe1 possessed the strongest electron density, and it was refined with a fixed occupancy of 1.0. The Xe2 atom was also located in the core of the same β-barrel. The distance between Xe1 and Xe2 was only 7.7 Å. The Xe2 atom had contacts with the hydrophobic side chains of eight residues. In addition, Xe2 has a contact with the main chain carbonyl oxygen of Phe501. The Xe2 site occupancy was 0.4, suggesting a lower affinity compared to that of Xe1 (Table S2) possibly because of its hydrophilic interaction with the main chain of Phe501. The Phe501 between the two Xe atoms had contacts with both Xe1 and Xe2. The binding site for Xe3 was different. It was located on a hydrophobic pocket on the protein surface. The pocket is formed between two surface loops and an outermost β-sheet of the barrel in domain C. There are seven contacts with different residues, four with loop residues and three with the residues from the β-sheet; six of the contacts are with the hydrophobic side chains, and only one is with the main chain carbonyl oxygen of Ser350. The Xe3 site was half-occupied, suggesting a binding affinity similar to that of Xe2 (Table S2). When compared to the native structure,[14,18] the xenon binding at sites Xe1 and Xe2 did not cause any major changes to the side chain conformations (Figure S1B of the Supporting Information). However, the binding of xenon at site Xe3 had induced conformational changes to the loop between P347 and P355 (Figure S1C of the Supporting Information).

The entry route for Xe3 is obvious because of the surface-exposed nature of the binding site, but for Xe1 and Xe2, a few possible entry pathways can be suggested. CAVER[19] was used to search for potential paths from the Xe1 and Xe2 sites to the protein surface. It resulted in possible paths from the edge groove of the β-barrel near Leu359, and alternative paths from the surface to the Xe2 site at the opposite edge of the β-barrel near Val408 (outside of the Xe3 site) or near Trp414 (Figure 2). Only a side chain of Phe501, whose conformational change may allow exchange of xenon between sites, separates the Xe1 and Xe2 sites. However,

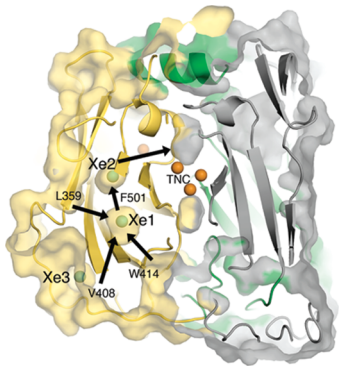

Figure 2. Cartoon with a van der Waals surface (solvent radius of 1.4 Å) representation of the Xe_rMaL1 monomer. Domain A is colored gray, domain B green, and domain C yellow. The observed binding sites for Xe atoms are shown as light blue and copper atoms in orange spheres, and the possible routes for the O_2 to TNC are shown as black arrows.

Table 1. Numbers of Surface-Excluded Hydrophobic Cavities inside the β-Barrel Domains of Different Laccases[a]

	PDB entry	resolution (Å)	domain A	domain B	domain C
asco-laccases:					
rMaL(Xe)	3QPK	1.70	0 (0)	1 (2)	2 (3)
rMaL	2Q9O	1.30	0 (0)	0 (2)	2 (3)
TaLcc1	3PPS	2.50	0 (0)	1 (3)	2 (3)
basidio-laccases:					
CcL	1HFU	1.68	0 (1)	0 (1)	2 (3)
TvL	1KYA	2.40	0 (0)	1 (1)	1 (3)
RlL	1V10	1.70	1 (2)	2 (4[b])	1 (3)
CmL	2H5U	1.90	1 (1)	1 (1[b])	2 (3)
CzL	2HZH	2.70	1 (1)	1 (1)	1 (3)
TtL	2HRG	1.58	0 (1)	1 (1)	1 (3)
LtL	2QT6	1.50	1 (1)	0 (1)	1 (3)
CgL	2VDZ	1.70	0 (1)	0 (1)	1 (3)
ThL	3FXP	2.05	0 (0)	0 (1)	0 (3)
Tsp.L	3KW7	3.44	0 (2)	1 (2[b])	0 (4)
bacterial:					
CotA	1GSK	1.70	0 (1)	1 (1)	0 (1)
average			**0.3 (0.7)**	**0.7 (1.6)**	**1.1 (2.9)**

[a] The comparison was performed in PyMol[25] using a van der Waals surface model with two different solvent radius settings, 1.40 (default) and 1.20 (in parentheses). [b] Becomes surface-exposed.

CAVER suggests a possible route between the Xe1 and Xe2 sites next to Phe501, but the tunnel is rather narrow.

Upon comparison of different laccase crystal structures in the Protein Data Bank (PDB) and their cavities, it can be estimated that the core of domain A is the most tightly packed and domain C generally contains more hydrophobic cavities than domains A and B (Table 1). The locations of the hydrophobic cavities inside domain C between asco-laccases are equivalent but vary in other fungal laccases. *Trametes versicolor* (PDB entry 1KYA)[20] and *Coriolus zonatus* (PDB entry 2HZH)[21] laccases have a cavity equivalent to the Xe2 site, and *Rigidoporus lignosus* (PDB entry 1V10)[22] and *Coprinus cinereus* (PDB entry 1HFU)[23] laccases have a cavity in the middle of the β-barrel corresponding to the

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position between the Xe1 and Xe2 sites in rMaL (Figure S2 of the Supporting Information). The known asco-laccases, MaL and TaLcc1, have an additional hydrophobic pocket outside the β-barrel in domain C, the Xe3 site. The Xe-induced conformational change to the P347−P355 loop around the Xe3 site clearly opens the pocket, and therefore, it cannot be seen as clearly in the native laccase structures. The pocket does not exist in any basidiomycete fungal laccases or bacterial a CotA laccase. However, some basidiolaccases have a narrow pocket that just reaches inside the β-barrel in domain C but at a different location compared to the Xe3 site of rMaL. The hydrophobic cores of three domains in CotA (PDB entry 1GSK)[24] are all very tightly packed (Table 1), but in addition to solvent channels T2 and T3, there is a deep, partly hydrophobic pocket that reaches to the T3 copper between domains B and C.

The binding sites for the Xe atoms can be used to suggest possible pathways for the entry of dioxygen into the TNC. The Xe1 site is ∼13 Å and Xe2 ∼11 Å from the cavity near the TNC, which is normally occupied by four water molecules (in the T3 space). We could thus speculate that a dioxygen molecule enters the TNC through the hydrophobic core of domain C utilizing Xe1 and/or Xe2 sites. Departure of one water molecule from the cavity in the vicinity of the TNC could make space for dioxygen to enter the TNC. Xe3 is located near the enzyme surface, and there is no obvious route from this xenon to other xenons or to the TNC; the Xe3 site would thus most likely represent one of the hydrophobic pockets on the surface of rMaL.

The recorded data sets provided strong evidence of binding of xenon to domain C of rMaL, and no traces of xenon binding in other domains or generally proposed oxygen entry pathways were found in this study. Because Xe has been reported to bind at sites preferred by dioxygen, we would suggest that rMaL utilizes the hydrophobic cavities of the core of domain C to trap dioxygen molecules from solvent. The analysis of other laccase crystal structures also showed that especially domain C contains more hydrophobic cavities than other domains, further supporting the role of this domain in transferring dioxygen to the trinuclear center. Chemically, it is plausible that hydrophobic dixoygen prefers to bind first to the hydrophobic pockets on the protein surface and then proceed inside to the protein via the hydrophobic route, which is most probably found in the hydrophobic cores of protein domains. In addition to the permanent hydrophobic pore of the β-barrel, diffusion of dioxygen into the TNC requires a set of several transient sites.

■ ASSOCIATED CONTENT

Supporting Information. Experimental and detailed description of Xe binding sites. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The coordinates and structure factors of Xe_rMaL1 have been deposited in the Protein Data Bank as entry 3QPK.

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■ REFERENCES

(1) Calhoun, D. B., Vanderkooi, J. M., Woodrow, G. V., III, and Englander, S. W. (1983) *Biochemistry 22*, 1526–1532.
(2) Smith, M. A., Knowles, P. F., McPherson, M. J., and Pearson, A. R. (2010) *Faraday Discuss. 148*, 1–14.
(3) Saam, J., Ivanov, I., Walther, M., Holzhütter, H.-G., and Kuhn., H. (2007) *Proc. Natl. Acad. Sci. U.S.A. 104*, 13319–13324.
(4) Lario, P. I., Sampson, N., and Vrielink, A. (2003) *J. Mol. Biol. 326*, 1635–1650.
(5) Koutsoupakis, K., Stavrakis, S., Soulimane, T., and Varotsis, C. (2003) *J. Biol. Chem. 278*, 1562–1532.
(6) Furse, K. E., Pratt, D. A., Schneider, C., Brash, A. R., Porter, N. A., and Lybrand, T. P. (2006) *Biochemistry 45*, 3206–3218.
(7) Scott, E. E., Gibson, Q. H., and Olson, J. S. (2001) *J. Biol. Chem. 276*, 5177–5188.
(8) Solomonsson, L., Lee, A., Gennis, R. B., and Brzezinski, P. (2004) *Proc. Natl. Acad. Sci. U.S.A. 101*, 11617–11621.
(9) Vitali, J., Robbins, A. H., Almo, S. C., and Tilton, R. F. (1991) *Appl. Crystallogr. 24*, 931–935.
(10) Prangé, T., Schiltz, M., Pernot, L., Colloc'h, N., Longhi, S., Bourguet, W., and Fourme, R. (1998) *Proteins: Struct., Funct., Genet. 30*, 61–73.
(11) Schoenborn, B. P. (1965) *Nature 208*, 760–762.
(12) Duff, A. P., Trambaiolo, D. M., Cohen, A. E., Ellis, P. J., Juda, G. A., Shepard, E. M., Langley, D. B., Dooley, D. M., Freeman, H. C., and Guss, J. M. (2004) *J. Mol. Biol. 344*, 599–607.
(13) Johnson, B. J., Cohen, J., Welford, R. W., Pearson, A. R., Schulten, K., Klinman, J. P., and Wilmot, C. M. (2007) *J. Biol. Chem. 282*, 17767–17776.
(14) Hakulinen, N., Kiiskinen, L.-L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A., and Rouvinen, J. (2002) *Nat. Struct. Biol. 9*, 601–605.
(15) Solomon, E. I., Augustine, A. J., and Yoon, J. (2008) *Dalton Trans. 30*, 3921–3932.
(16) Kallio, J. P., Gasparetti, C., Andberg, M., Boer, H., Koivula, A., Kruus, K., Rouvinen, J., and Hakulinen, N. (2011) *FEBS J.*, in press.
(17) Andberg, M., Hakulinen, N., Auer, S., Saloheimo, M., Koivula, A., Rouvinen, J., and Kruus, K. (2009) *FEBS J. 276*, 6285–6300.
(18) Hakulinen, N., Andberg, M., Kallio, J., Kruus, K., Koivula, A., and Rouvinen, J. (2008) *J. Struct. Biol. 162*, 29–39.
(19) Medek, P., Beneš, P., and Sochor, J. (2007) *J. WSCG 15*, 107–114.
(20) Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C., and Mougin, C. (2002) *Biochemistry 41*, 7325–7333.
(21) Lyashenko, A. V., Zhukova, Y., Zhukhlistova, N., Zaitsev, V., Stepanova, E., Kachalova, G., Koroleva, O., Voelter, W., Betzel, C., Tishkov, V., Bento, I., Gabdulkhakov, A., Morgunova, E., Lindley, P., and Mikhailov, A. (2006) *Crystallogr. Rep. 51*, 817–823.
(22) Garavaglia, S., Cambria, M. T., Miglio, M., Ragusa, S., Iacobazzi, V., Palmieri, F., D'Ambrosio, C., Scaloni, A., and Rizzi, M. (2004) *J. Mol. Biol. 342*, 1519–1531.
(23) Ducros, V., Brzozowski, A. M., Wilson, K. S., Brown, S. H., Ostergaard, P., Schneider, P., Yaver, D. S., Pedersen, A. H., and Davies, G. J. (1998) *Nat. Struct. Biol. 5*, 310–316.
(24) Enguita, F. J., Marçal, D., Martins, L. O., Grenha, R., Henriques, A. O., Lindley, P. F., and Carrondo, M. A. (2004) *J. Biol. Chem. 279*, 23472–23476.
(25) *The PyMOL Molecular Graphics System*, version 1.3, Schrödinger, LLC.