View the Full Text HTML

Identification of the Peroxy Adduct in Multicopper Oxidases by a Combination of Computational Chemistry and Extended X-ray Absorption Fine-Structure Measurements

Ulf Ryde,*,† Ya-Wen Hsiao,† Lubomír Rulíšek,‡ and Edward I. Solomon§

Department of Theoretical Chemistry, Lund University, Chemical Center, P.O. Box 124, S-221 00 Lund, Sweden, Department of Molecular Modeling, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo náměstí 2, 166 10, Praha 6, Czech Republic, and Department of Chemistry, Stanford University, Stanford, California 94305

Received April 28, 2006; E-mail: ulf.ryde@teokem.lu.se

The multicopper oxidases (MCO) are a group of enzymes that couple the four-electron reduction of molecular oxygen to water with four one-electron oxidations of various substrates.[1] Typical examples are laccase, ascorbate oxidase, ceruloplasmin, and CueO.[2,3] The MCOs contain at least four copper ions: one isolated type 1 Cu near the substrate-binding site and a trinuclear cluster, consisting of a type 2 (T2) Cu ion and a pair of type 3 (T3) Cu ions in a triangular arrangement. Crystal structures of several MCOs are known.[2,3] They show that the T2 Cu is bound to two His ligands and a solvent molecule, whereas the two T3 Cu ions are bound to three His ligands each. Depending on the oxidation state of the Cu ions, there may be a bridging ligand between the two T3 Cu ions, a solvent molecule, or perhaps a derivative of the O_2 substrate.

Extensive kinetic and spectroscopic studies have been performed to deduce the mechanism of the MCOs.[4,5] Four distinct states have been identified in the mechanism: the fully oxidized resting state, a fully reduced state, the peroxy intermediate (PI), and the native intermediate (NI). The PI arises after the binding of O_2 to the reduced state, leading to the two-electron reduction of O_2 to the peroxide level. In the NI, O_2 is reduced by two additional electrons (to the level of two water molecules) and all Cu ions are oxidized.

It is clear that O_2 binds to the trinuclear cluster, but the details of the binding are still unclear. On the basis of spectroscopic evidence, two possible structures have been suggested for the PI: one with O_2^{2-} bridged to the three coppers in the center of the cluster (C_3), and one with HO_2^- bridged to two coppers on the side of the cluster (S_{23}) and with an additional OH^- bridged to a second pair of coppers.[4] In a recent combined quantum mechanical and molecular mechanics (QM/MM) study,[6] based on the crystal structure of CueO (which had the highest resolution, 1.4 Å),[3] these two structures were refined (Figure 1). The results indicated that the C_3 structure was 24−130 kJ/mol more stable than the S_{23} structure, but the multiplicity of the electronic ground state was not in accordance with experimental data (by 6−20 kJ/mol). Both structures fitted the experimental extended X-ray absorption fine structure (EXAFS) data equally well: EXAFS indicates that there is a short Cu−Cu interaction of 3.4 Å in the PI[4] and the best computational structures of the C_3 and S_{23} states had Cu−Cu distances of 3.45−3.57 and 3.42−3.53 Å, respectively.

This illustrates the problem of comparing EXAFS data with structures from other sources: A direct comparison has only a weak discriminatory potential. However, the raw EXAFS spectra contain much more information, including many other Cu−ligand (and Cu−Cu) distances, as well as some angular information, but it is not
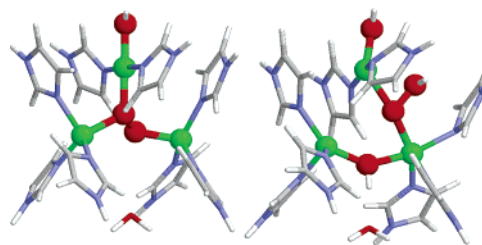
Figure 1. The QM/MM structures[6] of the C_3 (left) and S_{23} (right) states of the PA. Color code: H, white; C, gray; N, blue; O, red; and Cu, green.

easy to use all this information, especially considering possible systematic errors in the methods. Recently, we have developed a technique to combine EXAFS refinements with QM calculations (EXAFS/QM).[7] In essence, it is a standard QM geometry optimization in which we also include numerical forces from a standard EXAFS refinement. Thereby, we obtain a structure that is a compromise between EXAFS and QM. This method has successfully been applied to a number of small inorganic complexes and porphyrins in solution.[7,8] In this paper, we extend this method to QM/MM calculations on a full protein and apply it to the MCOs.

For technical reasons, we have used the EXAFS data from the peroxy adduct (PA), which is the complex of H_2O_2 with the fully oxidized resting state of the protein.[1,9] Spectroscopic and computational studies have shown that the structures of the PI and the PA are very similar, although the T2 Cu is reduced in the former, but oxidized in the latter.[6,9]

To start with, we optimized the two putative structures of PA in a vacuum with QM[10] and made a standard EXAFS fit, in which only E_0 (the absorption edge energy) was free to vary.[11] The results of these calculations are collected in Table 1 and show that the C_3 model fits the EXAFS data better than the S_{23} model (χ^2_{red} is 76, compared to 113). If three Cu−ligand distances are also fitted (QM + EXAFS fit), the difference between the two structures is reduced (the shortest Cu−Cu distance is 3.41−3.42 Å in both structures), but the C_3 structure is still better ($\chi^2_{red} = 72$ and 77). Similar results are obtained if a different number of distances are fitted, but when the number is increased, we soon obtained distances that would give unphysical structures (e.g., strongly distorted His imidazole rings).

The EXAFS/QM method[7] avoids this problem: It always ensures that the system is close to a realistic QM structure and also gives a measure of the deviation from the ideal QM structure in energy terms, ΔE_{QM1}. Our previous experience indicates that realistic structures should have $\Delta E_{QM1} < \sim 10$ kJ/mol.[7,8] Thus, we use the QM calculations in the same way as MM is used in almost all NMR

† Lund University.
‡ Academy of Sciences of the Czech Republic.
§ Stanford University.

Table 1. Results of the Combined QM and EXAFS Calculations[a]

calculation	model	E_0	χ^2_{red}	ΔE_{QM1} kJ/mol	Cu–Cu distances (Å) 2–3	2–3'	3–3'
QM	C$_3$	5.5	76		3.41	3.80	4.63
	S$_{23}$	3.4	113		3.34	3.81	3.75
QM + EXAFS fit	C$_3$	4.2	72		3.41	3.80	4.63
	S$_{23}$	3.8	77		3.42	3.78	3.72
EXAFS/QM	C$_3$	4.8	48	3.6	3.44	3.71	4.60
	S$_{23}$	3.5	50	10.9	3.42	3.85	3.71
QM/MM	C$_3$	2.7	218		3.52	4.04	4.49
	S$_{23}$	6.3	134		3.72	4.03	3.72
QM/MM + EXAFS fit	C$_3$	3.6	83		3.42	4.04	4.49
	S$_{23}$	3.3	86		3.85	3.95	3.86
EXAFS/QM/MM	C$_3$	3.5	38	9.9	3.41	4.02	4.44
	S$_{23}$	4.5	74	5.0	3.70	4.00	3.77

[a] E_0, χ^2_{red}, ΔE_{QM1}, and the distances between the three Cu ions are given for the two PA models, using six different methods: An EXAFS fit (of only E_0) directly to the QM or QM/MM structure, a standard EXAFS fit of three distances (in addition to E_0), starting from the QM or QM/MM structures, and the combined EXAFS/QM and EXAFS/QM/MM methods.
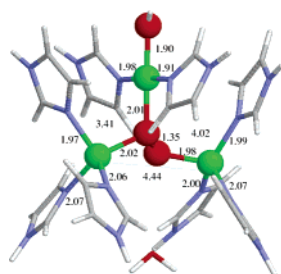
Figure 2. The final EXAFS/QM/MM structure of the C$_3$ state of the PA in CueO, with Cu–ligand distances in Å. Color code: H, white; C, gray; N, blue; O, red; and Cu, green.

and crystallographic refinements to supplement the experimental data and ensure that a realistic structure is obtained.

The EXAFS/QM results in Table 1 show that the two structures give a similar Cu–Cu distance (3.42–3.44 Å). They are both strongly improved by the optimization, but the C$_3$ structure is still best ($\chi^2_{red} = 48$ and 50). The ΔE_{QM1} energies were 4 and 11 kJ/mol, respectively.

Next, we made a similar investigation of the QM/MM structures obtained in our previous study.[6] Interestingly, the original structures fitted the EXAFS data quite poorly, and the C$_3$ structure gave worse results than the S$_{23}$ structure ($\chi^2_{red} = 218$ and 134), although the shortest Cu–Cu distance, 3.52 Å, is closer to 3.4 Å than that in the S$_{23}$ structure, 3.72 Å. This illustrates the problem of comparing only distances in QM calculations with those obtained in EXAFS fits. However, a standard EXAFS fit of three distances (QM/MM + EXAFS fit) made the C$_3$ structure better than the S$_{23}$ structure again.

Of course, the ideal procedure would be to optimize the copper site inside the protein with both QM and EXAFS data. We have therefore implemented such an EXAFS/QM/MM procedure by combining our EXAFS/QM and QM/MM software.[7,12] The results of this approach (last two rows in Table 1) show that the C$_3$ structure is strongly improved, much more than the S$_{23}$ structure ($\chi^2_{red} = 38$ and 74). The resulting structure (Figure 2) has a Cu–Cu distance of 3.41 Å, that is, close to what was found in all the other structures which included EXAFS data in the fits. However, the other two Cu–Cu distances are quite far from what was obtained in the EXAFS/QM structure (4.02 and 4.44 Å compared to 3.71 and 4.60 Å). This shows that both the EXAFS and the QM energies are insensitive to these distances. The ΔE_{QM1} energy is 10 kJ/mol.

Closely similar results are obtained with other density-functional methods (cf. Table S2 in the Supporting Information).

In conclusion, we have implemented a combination of EXAFS refinement and QM/MM calculations, which allows for the refinement of an EXAFS structure inside a protein, keeping the structure chemically reasonable. By this method, we show that the C$_3$ structure in Figure 2 fits the experimental EXAFS data better than the alternative S$_{23}$ structure. Thus, this investigation provides an independent confirmation of our previous energetic results[6] that O$_2$ binds in an all-bridged mode to the center of the trinuclear cluster in the MCOs. This is also in accordance with a recent QM investigation of other spectroscopic properties of the two tentative models of the PI in the MCOs.[13]

Supporting Information Available: A detailed description of the EXAFS/QM/MM interface; a more detailed account of the QM calculations and EXAFS fits; calibration of the EXAFS/QM/MM method on the oxidized state of the MCOs; influence of the QM method on the results; the final C$_3$ EXAFS/QM/MM structure of PA in CueO; a comparison of the experimental and calculated EXAFS for the C$_3$ and S$_{23}$ structures in both k and R' space; and a description of the considered EXAFS paths. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. *Chem. Rev.* **1996**, *96*, 2563−2605.
(2) Messerschmidt, A. In *Handbook of Metalloproteins*; Messerschmidt, A., Huber, R., Wieghardt, K., Poulos, T., Eds.; Wiley: New York, 2001; pp 1345−1357, 1359−1368, 1369−1379.
(3) Roberts, S. A.; Weichsel, A.; Grass, G.; Thakali, K.; Hazzard, J. T.; Tollin, G.; Rensing, C.; Montfort, W. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2766−2771.
(4) Shin, W.; Sundaram, U. M.; Cole, J. L.; Zhang, H. H.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **1996**, *118*, 3202−3215.
(5) Palmer, A. E.; Lee, S.-K.; Solomon, E. I. *J. Am. Chem. Soc.* **2001**, *123*, 6591−6599. Palmer, A. E.; Quintanar, L.; Severance, S.; Wang, T.-P.; Kosman, D. J.; Solomon, E. I. *Biochemistry* **2002**, *41*, 6438−6448. Lee, S.-K.; George, S. D.; Antholine, W. E.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **2002**, *124*, 6180−6193. Torres, J.; Svistunenko, D.; Karlsson, B.; Cooper, C. E.; Wilson, M. T. *J. Am. Chem. Soc.* **2002**, *124*, 963−967. Solomon, E. I.; Chen, P. I.; Metz, M.; Lee, S.-K.; Palmer, A. E. *Angew. Chem., Int. Ed.* **2001**, *40*, 4570−4590.
(6) Rulíšek, L.; Solomon, E. I.; Ryde, U. *Inorg. Chem.* **2005**, *44*, 5612−5628.
(7) Hsiao, Y.-W.; Tao, Y.; Shokes, J. E.; Scott, R. A.; Ryde, U. *Phys. Rev. B* **2006**, *74*, 214101.
(8) Hsiao, Y.-W.; Ryde, U. *Inorg. Chim. Acta* **2005**, *359*, 1081−1092.
(9) Sundaram, U. M.; Zhang, H. H.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **1997**, *119*, 12525−12540.
(10) QM calculations were performed with the Turbomole software (Schäfer, A.; Horn, H.; Ahlrichs, R. *J. Chem. Phys.* **1992**, *97*, 2571−2577) using the Becke-Perdew86 (BP86) density functional, the DZP basis set (Schäfer, A.; Huber, C.; Ahlrichs, R. *J. Chem. Phys.* **1994**, *100*, 5829−5835) on Cu and 6-31G* on the other atoms.
(11) EXAFS fits were performed with the IFEFFIT software (Newville, M. *J. Synchrotron Rad.* **2001**, *8*, 322−324), using theoretical scattering amplitudes and phase shifts calculated with the FEFF program [Ankudinov, A.; Bouldin, C.; Rehr, J. J.; Sims, J.; Hung, H. *Phys. Rev. B* **2002**, *65*, 104107 (up to four scattering legs and a maximum effective path length of 10 Å)]. Debye−Waller factors were calculated by FEFF with the equation-of-motion method using force constants obtained from a frequency calculation on vacuum-optimized structures of the C$_3$ and S$_{23}$ models of the PA. The fitting range was the same as in the original EXAFS investigation,[9] $k = 3.5−12.5$ Å^{-1} and R' = 0.8−4.0 Å.
(12) Ryde, U. *J. Comput.-Aided Mol. Des.* **1996**, *10*, 153−164. Ryde, U.; Olsson, M. H. M. *Int. J. Quantum Chem.* **2001**, *81*, 335−347.
(13) Chalupsky, J.; Rulíšek, L.; Neese, F.; Solomon, E. I.; Ryde, U. *Inorg. Chem.* **2006**, *45*, 11051−11059.

JA062954G