



## Review

# Theoretical studies of the active-site structure, spectroscopic and thermodynamic properties, and reaction mechanism of multicopper oxidases

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## Contents

1. Introduction .....	446
2. Multicopper oxidases: the peculiar spectroscopic and structural features of the trinuclear copper cluster .....	446
2.1. MCO crystal structures: the problem of partially reduced copper ions .....	447
2.2. A brief overview of the spectroscopic observations .....	447
3. Theoretical calculations of the MCO intermediates .....	449
3.1. Structural and energetic characterisation of key intermediates in the MCO reaction cycle by theoretical calculations .....	449
3.2. Ground-state multiplicities and excited states of the MCO intermediates. DFT vs. multi-reference wave function calculations .....	449
3.3. Theoretical calculations of the MCD spectra of trinuclear copper cluster models .....	451
3.4. A comparison with EXAFS spectra: a combined QM/MM/EXAFS procedure .....	452
3.5. A comparison with EPR spectra: g tensors of the MCO intermediates .....	452
4. Dioxygen cleavage by MCOs: the reaction mechanism .....	453
5. Electron-transfer properties of the MCOs .....	454
5.1. Reorganisation energies .....	454
5.2. Reduction potentials .....	455
5.3. The full reaction mechanism .....	456
6. Concluding remarks .....	456
References .....	457

## ARTICLE INFO

## Article history:

Received 20 January 2012

Received in revised form 27 March 2012

Accepted 13 April 2012

Available online 21 April 2012

## Keywords:

Multi-copper oxidases

QM/MM calculations

Multireference calculations

Theoretical spectroscopy

## ABSTRACT

In this article, we review recent theoretical work that has complemented the extensive experimental data available for the multicopper oxidases (MCO) and led to the elucidation of the reaction mechanism of this class of enzymes. The MCOs couple four one-electron oxidations of substrates at the mononuclear type 1 copper (Cu-T1) site with the four-electron reduction of dioxygen at the trinuclear copper cluster (TNC). The TNC consists of three copper ions arranged in a unique triangular fashion. In its oxidised form and in some experimentally observed intermediates (the peroxy and native intermediates), this leads to a magnetic coupling of the unpaired electrons of the three copper ions, resulting in unusual spectroscopic features. By correlating experimental and theoretical data, an unambiguous mapping between the structural, energetic and spectroscopic properties of the various intermediates in the MCO reaction cycle can be established. In terms of theory, these studies involved quantum mechanics (QM; density-functional theory and multi-reference self-consistent field) calculations, combined QM and molecular mechanics (QM/MM) modelling, ranging from standard QM/MM optimisations to the combination of QM/MM optimisation with EXAFS spectroscopy and QM/MM free-energy perturbations to accurately address phenomena such as the Cu-T1 → TNC electron transfer, as well as the reduction potentials and acid constants of all the putative intermediates in the MCO reaction cycle. In summary, we try to demonstrate in this review that the MCOs are not only an attractive playground for theoretical methods, but the results of the calculations (when carefully correlated with and benchmarked against experimental data) can also be used to draw unambiguous conclusions about MCO structure and reactivity.

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## 1. Introduction

The multicopper oxidases (MCOs) form a class of enzymes that play a variety of physiological roles in organisms, having in common the presence of two copper sites that accommodate four copper ions in total. Utilising these specific features, the MCOs couple four one-electron oxidations of a substrate with a four-electron reduction of molecular oxygen to water [1,2]:



This reaction takes place at a trinuclear copper cluster (TNC), whereas the substrate is oxidised at a type 1 copper site (Cu-T1), which is ~13 Å away from the TNC. The two sites are connected via a bifurcated (Cu<sub>TNC</sub>-His)<sub>2</sub>-Cys-Cu<sub>T1</sub> protein chain (where His-Cys-His are three consecutive residues in the protein, of which the two histidines are ligands of two copper ions in the TNC and the Cys is coordinated to the Cu-T1 ion as cysteinate, Fig. 1). This structural arrangement is assumed to provide an efficient electron-transfer (ET) pathway between the two sites, transferring the four electrons that are needed for the dioxygen reduction from the Cu-T1 site to the TNC. Therefore, the oxidation states of four copper ions span the full spectrum between (Cu<sup>2+</sup>)<sub>4</sub> and (Cu<sup>+</sup>)<sub>4</sub>. The MCO family includes enzymes catalysing the oxidation of small organic substrates, such as plant and fungal laccases, ascorbate oxidase, bilirubin oxidase, phenoxazinone synthase, CotA protein, and highly specific enzymatic oxidants of metal ions, such as ceruloplasmin, Fet3p, CueO and MnxG [3].

The study of the MCOs has a long history. However, judged by the number of crystallographic studies and spectroscopic observations, the experimental research has greatly intensified during the last two decades. One of the most remarkable achievements that can be attributed to a careful interpretation of the spectroscopic data is the postulation of the TNC itself (i.e. three copper ions bound at a distance of 3–5 Å) [4]. In the early 1990s, this hypothesis was confirmed by the crystal structures of ascorbate oxidase [5], showing a TNC, consisting of a pair of type 3 copper ions (Cu-T3), coordinated to three histidine groups each and in the oxidised state bridged by an oxo or hydroxide ion, and one type 2 copper ion (Cu-T2), coordinated by two histidine residues and a water molecule or hydroxide ion (Fig. 1). Most of these achievements are well documented in many extensive and excellent reviews [2,6–11] on the MCO structure, reactivity and spectroscopy written over the years, which also

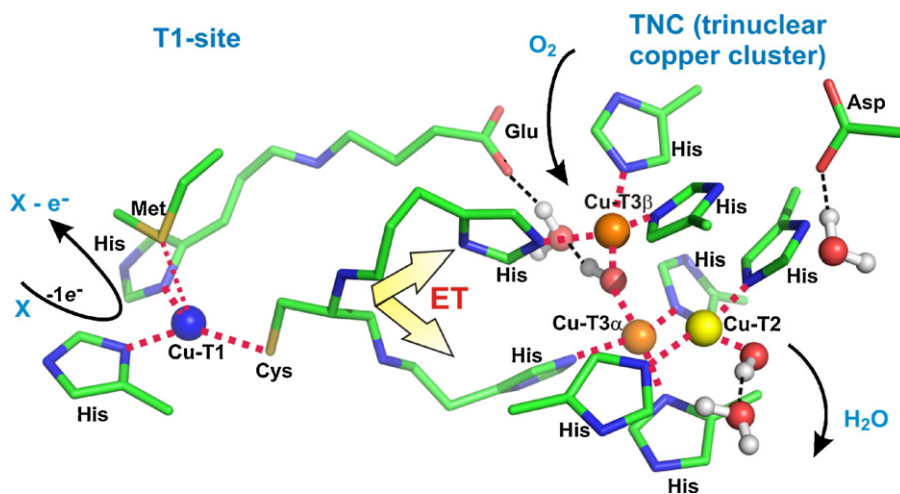
provide a historical record of the evolution of our understanding of their complex molecular architecture.

The aim of this review is to show that the MCOs and smaller inorganic models of the TNC present a highly challenging playground for computational chemistry and demonstrate how recent theoretical contributions have assisted in our understanding of the details of the MCO reaction mechanism. It must be mentioned that this task would not have been possible without the large amount of spectroscopic data and crystal structures of MCOs available.

What makes the MCOs so interesting for a computational bioinorganic chemist? We can conceive at least three reasons. The first is the inherent (in vacuo) instability of many plausible intermediates in the catalytic cycle of MCOs (i.e. the geometrical arrangements of the TNC) as a consequence of the four distinct redox states of the three copper ions (i.e. (Cu<sup>2+</sup>)<sub>3</sub>, (Cu<sup>+</sup>)(Cu<sup>2+</sup>)<sub>2</sub>, (Cu<sup>2+</sup>)(Cu<sup>+</sup>)<sub>2</sub> and (Cu<sup>+</sup>)<sub>3</sub>) coupled with the various accessible protonation states of the copper ligands originating from water or dioxygen (e.g. oxo, hydroxo and peroxo species) [13]. Therefore, the TNC site (the Cu ions with their first-sphere ligands) possesses a high positive charge (+3 or +4 according to the suggested reaction mechanism), which is partly compensated for by two carboxylate residues in the second coordination sphere of the TNC, which are conserved throughout the MCO family (Fig. 1) [14]. The second reason is the complicated electronic structure of the TNC. In the putative structure of the so-called native intermediate, NI, the TNC contains three unpaired spins at the vertices of a triangle, all coupled via an O<sup>2-</sup> ion in the centre. This leads to so-called spin-frustration, which means that the exchange coupling between the three pairs of Cu<sup>2+</sup> ions cannot be satisfied. In this oxidation state, there are two doublet states and one quartet state close in energy (within a few hundred cm<sup>-1</sup>) [15]. The third reason is the unique opportunity to couple the theoretical calculations directly to experimental data and provide their theoretical interpretation [16].

## 2. Multicopper oxidases: the peculiar spectroscopic and structural features of the trinuclear copper cluster

In this section, we shortly review two important sources of experimental information that have been crucial for our understanding of the structure and function of the MCOs: X-ray crystallographic data and observations spectroscopic (absorption, circular dichroism – CD, magnetic circular dichroism – MCD,



**Fig. 1.** The general architecture of the trinuclear copper cluster site (the site of the four-electron  $\text{O}_2 \rightarrow \text{H}_2\text{O}$  reduction) and of the Cu-T1 site (the site of one-electron oxidations of organic substrates or metal ions). The figure shows a QM/MM model of the resting oxidised state in the CueO enzyme (PDB code 1KV7) [12]. The likely electron-transfer pathway via the bifurcated Cys-(His)<sub>2</sub> chain is depicted together with an alternative His-X-Glu connection between the Cu-T1 and Cu-T23 sites. The coordination bonds are depicted by red dotted lines (with a thinner line used for the coordination of the Met residue in Cu-T1 site, which is not present in all MCOs). Three solvent molecules with positions obtained by QM/MM modelling are displayed as well, which can be part of a water exit channel. Black dashed lines are used for hydrogen bonds.

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