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Review

The nature of the rate-limiting step of blue multicopper oxidases: Homogeneous studies *versus* heterogeneous

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Abstract

Multicopper oxidases (MCOs) catalyzed two half reactions (linked by an intramolecular electron transfer) through a Ping-Pong mechanism: the substrate oxidation followed by the O_2 reduction. MCOs have been characterized in details in solution or immobilized on electrode surfaces. The nature of the rate-limiting steps, which is controversial in the literature, is discussed in this mini review for both cases. Deciphering such rate-limiting steps is of particular importance to efficiently use MCOs in any applications requiring the reduction of O_2 to water.

Keywords: Multicopper oxidase; Rate-limiting step; Homogeneous and heterogeneous studies; Electrochemistry

1. Introduction

Blue multicopper oxidases (MCOs) are ubiquitous and several structures have been resolved for ascorbate oxidase, ceruloplasmin, fungal laccases or bilirubin oxidase [1]. MCOs catalyze two half reactions linked by an intramolecular electron transfer (IET) step:

(i) The substrate oxidation which is specific to each MCOs:
(**Eq. 1**: 4 substrate $\rightarrow$ 4 product + $4e^-$ + $4H^+$) followed by,

(ii) A common reduction of O_2 to water, without producing any toxic oxygen intermediates.
(**Eq. 2**: $O_2 + 4e^- + 4H^+ \rightarrow 2\ H_2O$).

MCOs have been characterized in details [2]. Briefly, the catalytic center is composed of four copper atoms: one T1, one T2 and two T3 which composed the trinuclear center (TNC). They are classified according to their optical and magnetic properties. The T1 has characteristic spectrum in EPR and the blue color of the MCOs under oxidized form is due to the intense absorption band around 600 nm due to the binding T1-S(Cys). The oxidation of substrates occurs on the T1 site whose redox potential varies in function of the axial residue of the pseudo-bipyramid trigonal formed by amino acid residues around the T1. The T2 has also a characteristic feature in EPR. The pair of T3 is silent in EPR, because there are coupled by a strong antiferromagnetic exchange interaction, and has a characteristic absorption band at 330 nm. The T1 accepts the electron from the substrates which are then shuttled to the TNC through ~13 Å of Cys−His residues [3]. The reduction of O_2 into H_2O occurs at the TNC.

MCOs present a great interest for any applications requiring the reduction of O_2 to water, among which enzymatic biofuel cells that may power future implantable medical devices [4,5]. The nature of the rate-limiting step of the global reaction is controversial in the literature either for the enzymatic studies in solution or for the electrochemical studies with immobilized MCOs on electrodes surfaces. For both cases it could be associated to any steps shown in Schemes 1

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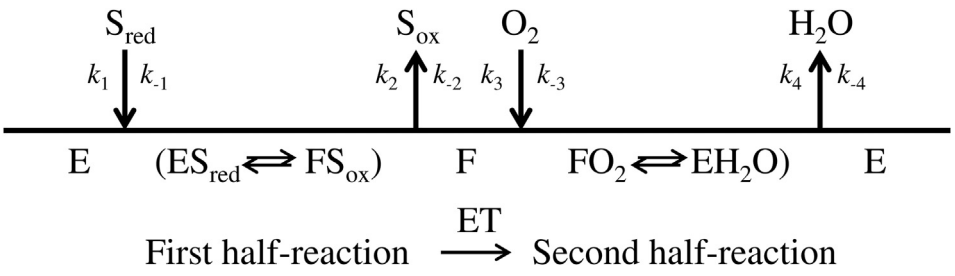
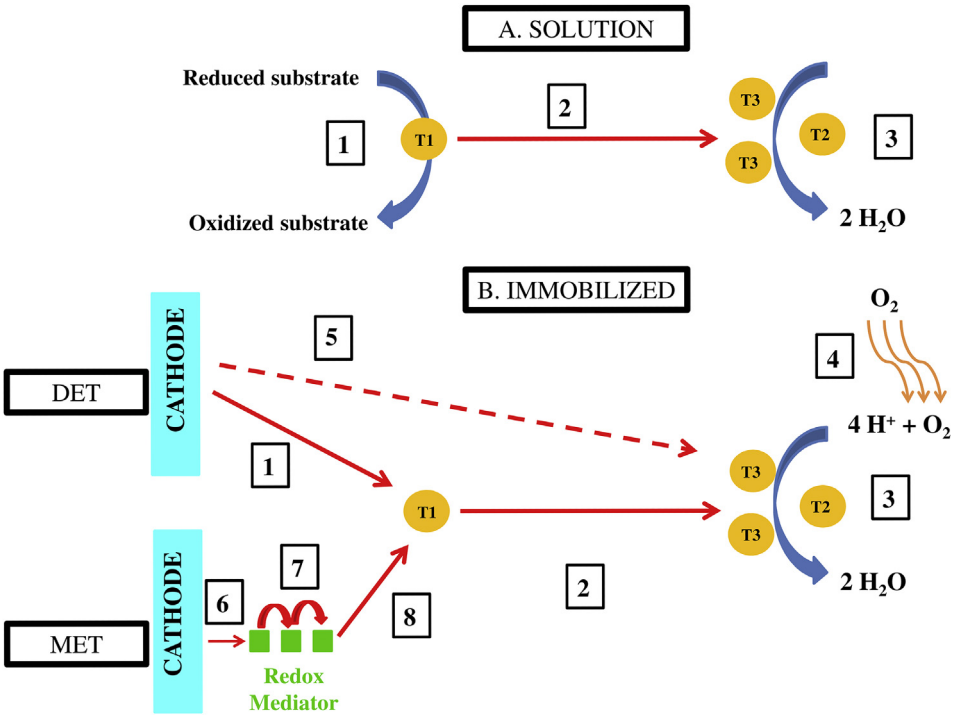


Scheme 1. General reaction and individual steps of the Ping-Pong Bi Bi system using the Cleland notation. E and F are two different stable enzyme forms. Each substrate addition (S_{red}, O_2) is followed by a product release (S_{ox}, H_2O).



Scheme 2. Different steps for electron transfer for enzymes in solution (A) and immobilized (B) with Direct electron transfer **(DET)** or Mediated electron transfer **(MET)** techniques.

and 2. In addition, in solution it can also be due to any isomerization step or conformational rearrangement [6,7].

In this mini review, we summarized the different arguments through examples of the assignment of the rate-limiting step of MCOs. We will distinguish two types of experiments: in homogenous solution where occur classical redox reactions and immobilized on electrodes surfaces where the enzymes are artificially reduced.

2. Enzymatic studies in solution

To determine the rate-limiting step of the global reaction, the classical method is to compare the k_{cat} determined at steady-state with the individual rate constant of each molecular step. Steady-state kinetic studies showed that MCOs followed BI BI Ping-Pong [6] type mechanism as represented with the Cleland notation (Scheme 1).

Scheme 2A represents the well admitted different steps for electron transfer (ET) for MCOs in solution. Substrate oxidation occurs at the T1 site (**step 1**). The reduction rate constant of the T1 is very efficient and fast as shown by the bleaching of the T1 followed at 600 nm after addition of a reductant [8]. The difference of reduction rate constants observed between different MCOs depends on the rate of intermolecular ET between the substrate and the T1. It also depends on the accessibility of the T1 site of each MCOs [8]. Steady-state kinetic studies on laccases with several substrates have permitted to highlight a linear relationship between the $\log(k_{cat}/K_m)$ and the redox potential difference between the T1 site and the substrate [9]. From those experiments, the rate-limiting step is attributed to the substrate oxidation [2,3,9].

As said by Page and coworkers [10] and according to the Marcus theory [11] the tunneling rates remain higher than catalytic rates (k_{cat}) at typical physiological ΔG (0 to −0.1 eV). The IET (**step 2**) rate constant (*k*IET) from the T1 to TNC has been studied by pulse radiolysis or laser flash photolysis experiments [12–14]. In laccase from *Rhus vernicifera*, *k*IET is >*k*cat so IET is not the rate-limiting step [15].