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Abstract

Multicopper oxidases are widely studied enzymes catalyzing the oxygen reduction reaction. Among this family, one class belongs to the copper efflux oxidases (CueOs). They are far less studied in bioelectrochemistry mainly because of the low potential at which they reduce O_2. However, the presence of a specific domain rich in methionine residues covering the first copper electron acceptor induces fundamental issues regarding the electron transfer pathway. In addition, as they are involved in copper homeostasis, the understanding of their catalytic mechanism may have important consequences in therapeutic applications. We present here the last findings reported on CueOs based on electrochemical tools. We focus on the proposed roles of the methionine rich domain on the electron transfer process. Especially, copper binding to this domain and consequences on the interfacial electron transfer process appears to be two fundamental aspects to discuss.

Keywords: CueO; electrochemistry; cuprous oxidase; multi copper oxidase; catalysis; electron transfer; copper homeostasis

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Introduction

Copper is an essential micronutrient in living systems, acting as a cofactor of enzymes involved in many biological processes from photosynthesis and electron transfer (ET) to oxidative stress protection [1,2]. Copper in excess is however toxic for the cell, the different routes for toxicity being dependent on many factors such as oxygen availability, copper redox state, concentration, localization and complexation in the cell. Actually, like iron, copper is known to produce Reactive Oxygen Species (ROS) via Fenton chemistry *in vitro*, potentially occurring *in vivo* in Gram-negative bacteria and inducing copper toxicity in aerobic conditions [3]. On the other hand, copper toxicity for cells is at the origin of attractive strategies to develop novel antibacterial solutions in therapeutic approaches or in agriculture [4]. Among the different systems involved in copper tolerance in bacteria, the copper efflux oxidase (CueO) couples *in vivo* the oxidation of toxic Cu^{+} to the reduction of O_2 into water [1,3,5].

CueOs belong to the multicopper oxidase (MCO) family which is widely distributed in all kingdoms of life and many environments. MCOs transform a variety of substrates including phenols, and share the presence of 4 copper centers. Substrate oxidation occurs at CuT1, and electrons flow intramolecularly to the trinuclear copper cluster (TNC, consisting of one CuT2 and a dinuclear CuT3) where oxygen is reduced into water [6,7]. MCOs are arranged in 3 cupredoxin-like β-barrel domains, the domain 3 hosting CuT1, while TNC lies at the interface between domains 1 and 3. *E. coli* CueO, the most widely studied CueO-enzyme, contains an additional domain covering the CuT1 and rich in Met residues. This particular feature, although with different structural properties [8], seems to be shared by other CueO-like MCOs. It would induce a low phenol oxidase activity but offers an ET pathway for cuprous oxidase activity.

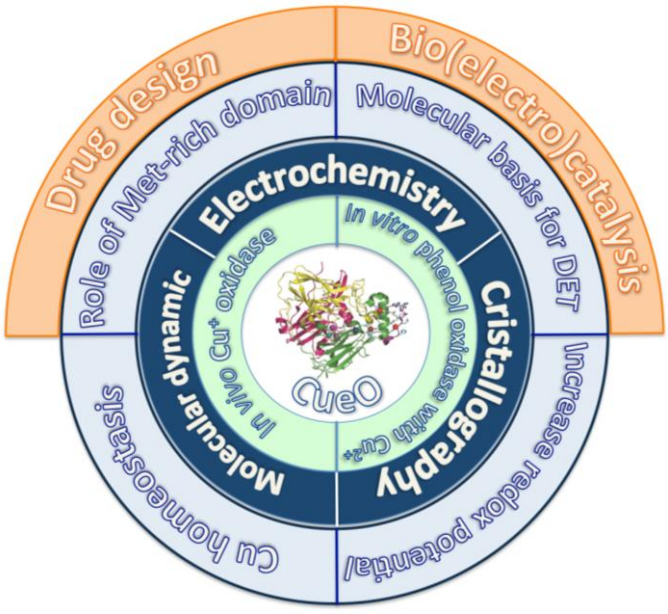

Figure 1. CueO-like enzyme main properties and challenges

Although electrochemists have widely used MCOs during the last years for their efficiency to reduce oxygen with low overpotentials and have defined some parameters that control their efficient wiring on electrodes [9-11], CueOs were much less studied. Indeed, the coordination sphere of CuT1 induces a much lower potential than in other MCO such as fungal laccases (LAC), making CueO less attractive for applications. On the other hand, bacterial MCOs are more stable than fungal LACs, especially at neutral pH and in the presence of classical inhibitors such as halides [12]. Furthermore, they are readily produced and purified in bacterial expression systems.

With the concomitant need of copper homeostasis understanding and rationalization of enzyme-based electrocatalysis, CueO-related studies have been increasingly reported in the last years (Figure 1). The current review discusses how electrochemistry participates to this improved knowledge. We will highlight the diversity of enzymes susceptible to be involved, with their main structural and biochemical properties. We will explore their electrochemical behavior, including the molecular basis that influences the direct electrochemical communication (DET) with the electrode, as well as the interfaces developed for DET. We will especially examine how Met-rich domains may interfere with the electrochemical behavior. We will conclude by fundamental challenges that need to be overcome for understanding and use of CueO based bioelectrocatalytic devices.

Diversity of CueO-like MCOs

In a recent Laccase and Multicopper Oxidase Engineering Database providing a systematic analysis and clustering of known MCO sequences, the bacterial CueO superfamily was shown to regroup almost 5000 proteins [13]. CueO-like proteins have been reported in non-pathogenic and pathogenic bacteria [14-21]. They can be found in a variety of environments, i.e. mesophilic (as *E. coli* CueO), thermophilic (cases of *Thermus thermophilus* laccase (*Tt* LAC), *Aquifex aeolicus* McoA and *Purobaculum aeophilum* McoP) or psychrophilic (case of *Pseudoalteromonas haloplanktis*), or acidophilic bacteria, allowing to tune their properties in terms of activity and stability in diverse conditions [14,17,22,23]. A CueO-like protein has even been identified in the anaerobic sulfate reducing bacterium *Desulfovibrio* sp. A2 raising the question of its role and function in low O_2 availability conditions [24]. As far as this has been

evaluated, they are all involved in copper tolerance, operate as cuprous oxidases and their phenol oxidase activity is most often increased *in vitro* by Cu^{2+} exogeneous addition.

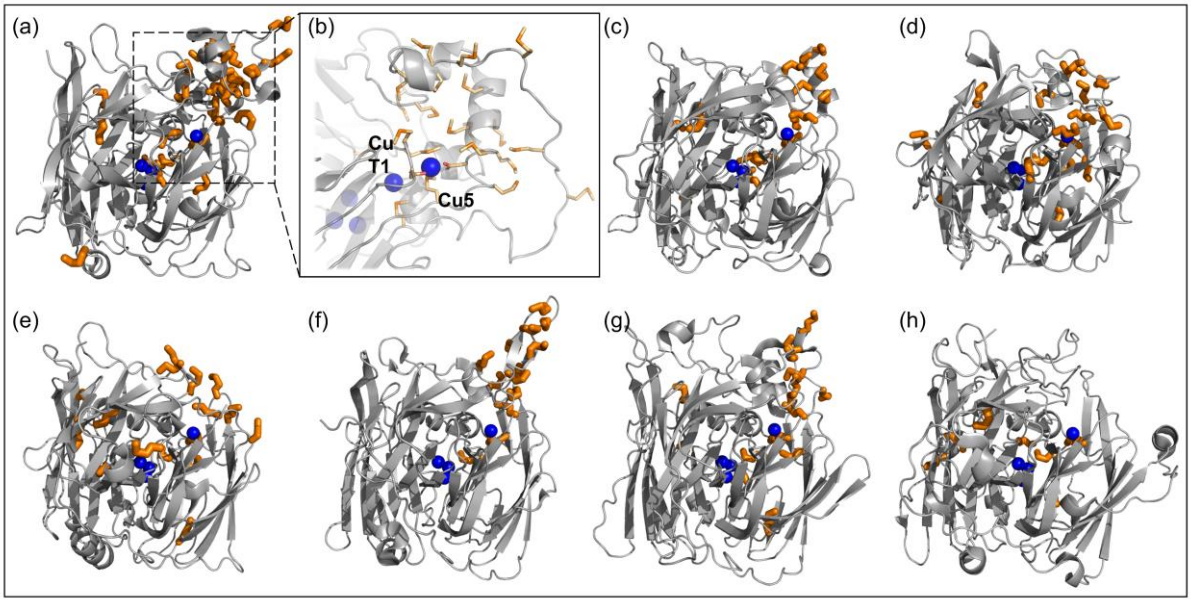

Figure 2. Structural features of CueO-like enzymes (a-g) compared to a non-CueO enzyme (h). (a-b) E.coli CueO (PDB 3OD3). The additional domain comprises Met and Asp ligands for a 5th copper atom (Cu5), a helical region with 6 Met that covers the CuT1 site and Cu5, and a loosely constrained loop lying across the Met-rich helix and carrying 5 Met and 5 His residues [25-29]. (b) Zoom on the Met-rich domain of E.coli CueO (PDB 3OD3) showing an additional copper atom (Cu5) bound close to T1 modelled after another structure (PDB 3NT0). (c) Ochrobactrum sp. CueO (PDB 6EVG). Additional helical domain is covering CuT1 with 12 Met [18]. A part of this domain (A350-A391) is disordered and not resolved in the structure. (d) Purobaculum aerophilum McoP (PDB 3AW5). A large Met-rich loop covers the CuT1 [21]. (e) Aquifex aeolicus McoA (PDB 6SYY). A 29-residues loop is close to the CuT1 comprising 12 Met [30]. A major part of this loop (G327-G354) is disordered and not resolved in the structure. (f) Thermus thermophilus LAC (PDB 2XU9). A Met-rich hairpin is located on the top of the substrate binding pocket near CuT1 which accommodates 6 Met among 16 residues [31,32]. (g) Campylobacter jejuni McoC (PDB 3ZX1). 5 Met residues can be found in a helix over CuT1. (h) Bacillus subtilis CotA (PDB 1W6L) is a non-CueO enzyme given for comparison. Copper atoms are depicted in blue spheres, methionine residues in orange sticks.

All CueOs present an additional domain covering the CuT1 not found in other MCOs. This domain is rich in Met residues, but depending on the organism, it presents a variety of structural organization and a large range of number of Met residues engaged (Figure 2) [15,21,25,29-34]. The consequences of the presence of the Met-rich domain on CueO activity have been mostly discussed in terms of restricted organic substrate accessibility toward CuT1 and additional Cu binding [35]. The first assumption is highlighted by the following results. A thermodynamic study of the CuT1 redox potential of *Tt* LAC, revealed a negative reduction entropy change linked to inaccessibility of CuT1 [34]. Modeling of McoP suggested that CuT1 is less exposed

than in classical MCOs (such as *B. subtilis* CotA), but not so buried as in *E. coli* CueO [33]. Last but not least, in McoA, the loop close to the CuT1 was demonstrated by molecular dynamics simulation and X-ray scattering to control substrate access [30]. As an almost general rule, restricted organic substrate accessibility leads to a 1-2 orders higher Michaelis constant (K_m) for organic substrates such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) than in classical MCOs [15,26,36-40]. Accordingly, *E. coli* CueO mutant lacking the Met-rich region was capable of efficiently oxidizing ABTS [40]. Additional Cu binding is alternatively proposed to offer an ET pathway to the buried CuT1. Hence, the phenol oxidase activity of CueO proteins is greatly enhanced by Cu^{2+} addition in the medium [28]. However, this ET pathway permitted thanks to the Met-rich domain was clarified only in the case of *E. coli* CueO, where in addition to a 5^{th} copper atom (Cu5) found in the insert, at least two other copper atoms were identified bound to the Met-rich helix, with Met residues acting as ligands. Cortes *et al.* proposed a specific role for these additional copper sites for Cu^{+} binding allowing cuprous oxidase activity [41]. They suggested that phenol oxidase activity could only occur upon full occupation of all copper sites, and concluded that this couldn't be the physiological function of CueO *in vivo*, considering the low availability of copper inside the cell. The dual role of the Met-rich domain on the CueO activity was also highlighted in McoA. Shorter loops obtained by increasing truncation of the 29-residues loop close to the CuT1 were shown to induce higher catalytic constant (k_{cat}) for ABTS whereas longer loops increased k_{cat} for Cu^{+} [30]. Cuprous oxidase activity is an important research concept toward the understanding of Cu homeostasis. However, since free Cu^{+} is not stable under air, unnatural Cu^{+} complexes such as $[Cu(MeCN)_4]^{+}$ and $[Cu(bicinchoninate)_2]^{3-}$ are to be used, which do not reflect the *in vivo* environment. Further considerations are required to propose a mechanism fully representative of copper interactions with cells.

CueO electrochemistry : molecular basis for direct enzyme wiring at the electrode

Unlike fungal LACs, electrochemical studies on CueO-like proteins other than *E. coli* CueO are still rare. Most of the works reported to date consider direct ET between the protein and the electrochemical interface with no redox mediators. Various materials were evaluated as electrode support for the enzyme (Figure 3). An early study in Kano's group demonstrated that *E. coli* CueO could generate high current densities more than 10 mA/cm^2 for O_2 reduction when adsorbed on mesoporous carbon support, justifying the interest of CueO as a biocatalyst [42]. As another marked feature, addition of up to 400 mM Cl^- at pH 6.5 did not change the current magnitude, showing the interest of using CueO as O_2 reduction catalyst despite the lower redox

potential when compared to fungal LACs [43]. Unlike homogeneous activity in solution, ET occurred at the electrode even in the absence of the Cu5 and proceeded through CuT1 with an onset potential around 0.4 V vs Ag/AgCl at pH 5. Although the reason for that difference was not elucidated, the mesopore size of the electrode was proposed to favor the direct wiring of the protein [44]. The electrochemical behavior of *Tt* LAC was first investigated by Gray's group after adsorption on ketjen black electrodes functionalized by pyrenebutyric acid [45]. An onset potential of 340 mV vs Ag/AgCl (pH 5) and catalytic current densities in the range of 500 $\mu A/cm^2$ were observed, i.e. tenfold lower than those recorded for *E. coli* CueO on the same electrode material. The knowledge of the electroactive immobilized proteins allowed to calculate an interfacial rate constant of 1 s^{-1}, which implies a large distance between CuT1 and the electrode, further estimated to fall in the range of 24-28 Å.

Attempts to control the orientation of CueO enzymes succeeded to enhance the interfacial ET rate. *Tt* LAC electrochemistry was revisited after immobilization on either SAM-modified gold electrodes [46,47] or CNT-modified graphite electrodes [22,46]. While no catalytic current for O_2 reduction could be observed at negatively charged electrodes, a catalytic process was recorded at amino-terminated SAM electrodes or amino-functionalized CNT-based electrodes. The onset potential reflected a process passing through the CuT1. As surface plasmon resonance and ellipsometry proved the existence of a monolayer of enzyme whatever the electrode, the on-off catalytic current as a function of the electrode charge was proposed to be linked to a specific orientation controlled by electrostatic interaction between the electrode and the environment of the CuT1. Examination of the structure of the enzyme underlines that the CuT1 is buried in the protein shell in a negatively charged environment, which explains the requirement of a positive surface to get a DET orientation of the enzyme. A clear negative charge was also highlighted in the surrounding of *E. coli* CueO CuT1 explaining the benefit of a positively charged electrode for DET orientation [36,43,48].

Alfonta'group studied the electrochemistry of *E. coli* CueO chemically clicked to a glassy carbon electrode through non-canonical amino acids anchorage sites [49]. Different ET efficiency was found according to the anchoring point in the enzyme, although it is difficult to determine whether this is controlled by enzyme orientation or by flexibility of the linker. McoP was immobilized on modified CNTs through either metal affinity bonding with His-tag either positioned at C-ter or N-ter parts of the enzyme [50], or through a CNT-binding peptide introduced at the C-ter of the protein [51,52]. A preferential orientation inducing a shorter

distance between CuT1 and the binding-tag or peptide at the C-ter was proposed to induce higher current densities for O_2 reduction.

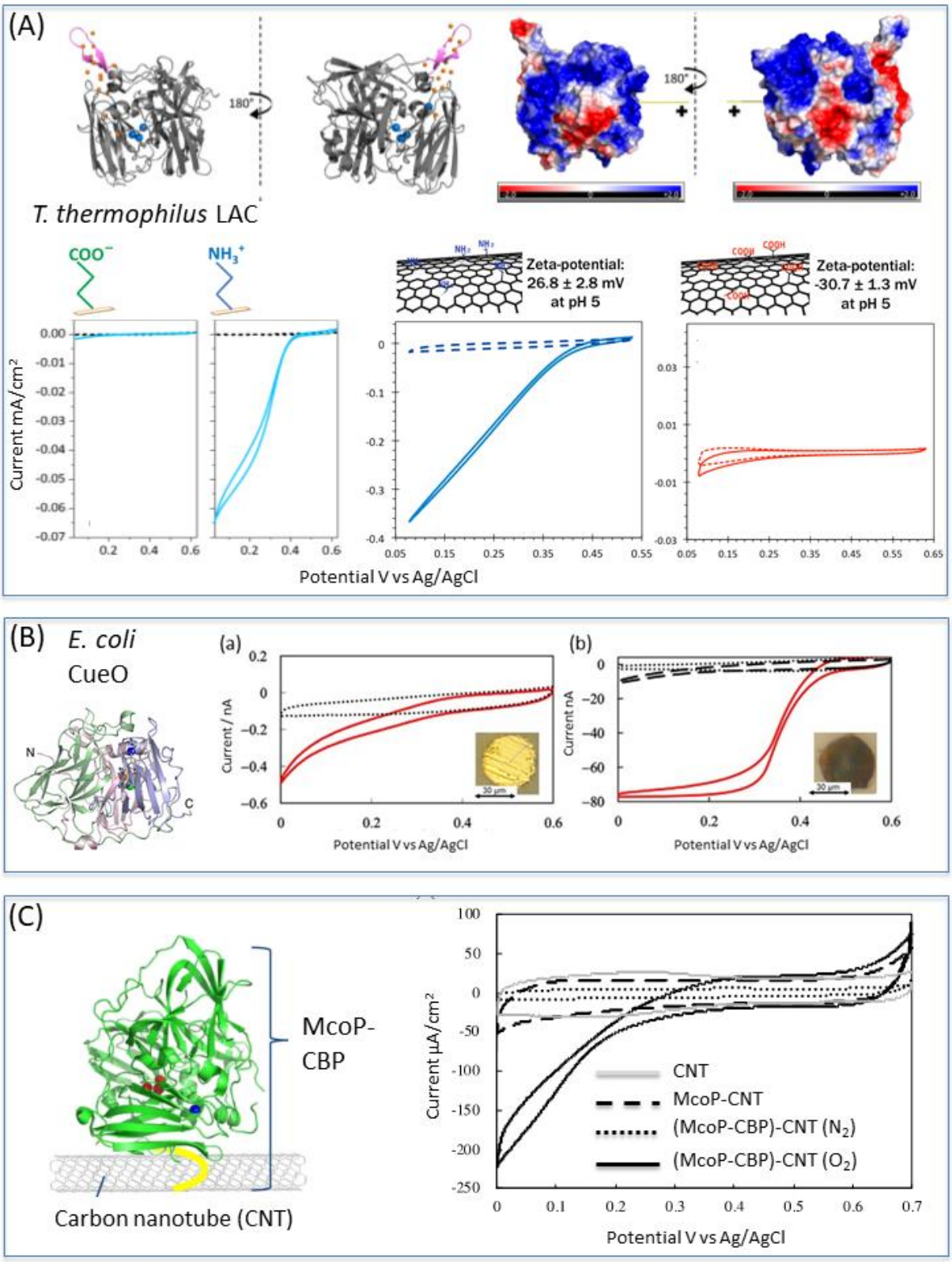

Figure 3. Strategies developed for DET involving CueOs at electrochemical interfaces. (A) Immobilization through electrostatic interactions. First row: due to anisotropy of surface charges and

the presence of a negative patch in the environment of CuT1 (colored in red), the CueO-like enzyme Tt LAC presents a dipole moment of more than 800 Debye pointing positive opposite to the CuT1 (represented in yellow line). The consequence is the control of the enzyme orientation through electrostatic interactions (second row). DET can occur only on positively charged electrodes, either on amino-terminated self-assembled monolayers on gold or on amino functionalized carbon nanotubes. Cyclic voltamograms are run at 5 mV/s, under O_2 (plain lines) or N_2 (dotted lines). Adapted with permission from [46]; (B) Immobilization of CueO in a matrix with suitable pore size. O_2 reduction catalyzed by E. coli CueO on (a) planar and (b) porous Au microelectrodes. Solid and dotted lines represent CV recorded under O_2 and Ar respectively. In (b) the dashed line corresponds to the control with no enzyme under O_2. The enhancement in catalytic current for O_2 reduction suggests an enhanced enzyme loading as well as an enhanced proportion of enzyme correctly oriented. Adapted with permission from [44]; (C) Immobilization through CueO engineering. McoP was genetically engineered near CuT1 with a peptide able to bind to carbon nanotubes (CBP). DET on CNTs was only observed via this engineered CueO. Adapted with permission from [51].

CueO electrochemistry : how to enhance CuT1 redox potential

In CueOs, CuT1 presents a four-coordinate trigonal pyramid with copper ion ligated by two His, one Cys and one Met. Generally speaking, a copper site with a strong axial ligand bond will present a low formal potential owing to stabilization of the oxidized state [7]. The Met axial ligand of CuT1 in CueOs, compared to Leu or Phe found in most MCOs from fungi, induces a redox potential 200-300 mV lower than recorded in fungal LACs. Redox potentials for CuT1 in CueO-like enzymes were reported to be 200-350 mV vs Ag/AgCl [34,53]. Many works reported various mutations in the CuT1 coordination spheres with the aim of increasing the operational voltage of CueOs [13,54-56]. Site directed mutagenesis was realized in *Tt* LAC and McoP by substitution of the Met CuT1 axial ligand to Leu or Phe. Direct electrochemistry experiments underlined a positive shift between 60 and 100 mV of the onset potential for O_2 reduction [22,57,58]. Poor stability of the mutants however precluded an enhancement of the activity. Random mutation and site-saturation mutagenesis libraries were generated on *E. coli* CueO. O_2 bioelectroreduction was measured through an electrochemical platform based on CueO containing crude extracts dispatched in 96-well microtiter plates and adsorbed on a CNT-modified electrode [59]. 1500 clones were tested, among which 4.8% exhibited increased redox potentials. A double mutant allowed a decrease of 140 mV in the overpotential. The amino acids involved (D439 and L502) are located in the CuT1 second coordination sphere and form hydrogen bonds with the coordinated ligands explaining the enhanced potential. The so-built

electrode was stable for 1 week. The same electrochemical platform was used to evaluate site mutation of the ligands of Cu5 in *E. coli* CueO [60]. 11 variants were identified showing enhanced electroactivity for O_2 reduction. In particular mutation of the residue D439 involved in the bonding with Cu5 showed 4-fold catalytic current enhancement because of a synergetic effect between CuT1 redox potential positive shift and distance between Cu5 and CuT1 decrease. Search for CuT1 enhanced redox potential also induced side effects on the activity. A directed evolution strategy allowed to isolate a McoP mutant exhibiting an enhanced activity both in solution or at the electrode, although the CuT1 redox potential was only slightly enhanced [61]. The amino acid F290, not involved in CuT1 coordination, was shown to be essential. From a structural point of view, this mutation was found to increase the loop flexibility adjacent to the CuT1. A marked feature in the case of *Tt* LAC M455L mutant is the inhibition of the activity upon Cu^{2+} addition, suggesting that the Met-rich domain would not be the only actor in copper activation of CueO-like proteins [22]. Future research must definitely be directed towards the consideration of both thermodynamics and kinetics to understand the mechanisms under Cu^{2+}-related activation/inhibition of the CueO activity.

CueO electrochemistry : role of the Met-rich domain

Additional Cu atoms binding to the Met-rich domain in CueOs and consecutive ET pathway to CuT1 is expected to play a crucial role in the interfacial ET. Nevertheless, as clearly highlighted in the previously described electrochemical studies, the potential influence of this Met-domain and even of Cu5 presence is rarely considered. The apparent discrepancy between Cu^{2+} activation of CueO-like enzymes in solution and the evidence of a CueO-related electrocatalytic current for O_2 reduction with no requirement of Cu^{2+} is also largely neglected.

Different roles of the Met-rich domain in the interfacial ET can be discussed (Figure 4). It could be first expected that the Met-rich domain would block the electrical wiring to the electrochemical interface. However, the heterogeneous ET at electrodes does not proceed through the same mechanism as in solution. Actually, ET can proceed at the electrochemical interface provided that the distance to CuT1 is short enough, with no restriction due to steric hindrance as in the case of organic substrates. Adachi *et al.* reported the electrochemical behavior of *E. coli* CueO variants lacking parts of the Met-rich helix on differently charged porous carbon electrodes [48]. DET was obtained no matter the mutation, and the heterogeneous ET rate was unchanged strongly suggesting that electrons were transferred from

the electrode to the CuT1 not passing through the Met-rich helix. The same situation was found with *Tt* LAC either WT or hairpin-deletant immobilized on CNT bearing different charges [46].

However, it was shown that addition of Cu^{2+} in the electrolyte induces a new catalytic process at a lower potential than expected for catalysis passing through the CuT1. This new process illustrates a second role for the Met-rich domain in the interfacial ET induced by additional copper binding to the Met-rich domain. It was tentatively attributed to the cuprous oxidase activity of the *Tt* LAC immobilized on CNTs [22,46]. Potential role of occupancy of Cu5 in *E. coli* CueO-based bioelectrocatalysis was reported in one other work, where the increase in the catalytic current was linked to a specific orientation of the enzyme through the Met-rich domain (see next section) [60]. No cuprous oxidase activity could be however identified. Clearly, this topic is still in its infancy and further investigation are required to fully understand the involved mechanisms. The fact that the deletion of the Met-rich domain in *Tt* LAC did not suppress the Cu-induced electrocatalytic process, and that Met residues in the coordination sphere of CuT1 were also involved in the Cu^{2+}-activation process further support this conclusion [22,46].

An alternative role for the Met-rich domain of CueOs, which is closely related, is linked to the flexibility of the Met-rich domain with an even enhanced flexibility upon Cu binding, as demonstrated in CueO enzymes [26,62-65]. However, this flexibility hasn't been yet demonstrated to influence the electrochemical behavior.

Finally, the Met-rich domain may serve to anchor the protein in a favorable orientation for DET. In agreement, Climent *et al.* explained the DET process they observed with *E. coli* CueO on bare gold by the formation of sulfur-gold bonds that place CuT1 at a short distance of the electrode [43]. The role of the Met-rich domain in *E. coli* CueO was recently rationalized by considering the adsorption and electrocatalytic properties of WT CueO and two mutants, one lacking the Met-rich domain and the other one in which Met in the helix was mutated to Ser residues [29]. Only WT CueO was electroactive towards O_2 reduction on gold electrode, although both WT and mutants were proved by SPR measurements to be adsorbed. The involvement of the Met-rich domain for DET orientation was thus proposed. Upon adsorption on hydrophobic carbon materials, the ABTS-mediated catalytic current was largely enhanced with the helix mutant compared to the WT, but the direct electrocatalytic current was greatly decreased. Such results were explained based on an adsorption taking place through the Met-rich helix and controlled by hydrophobic interactions.

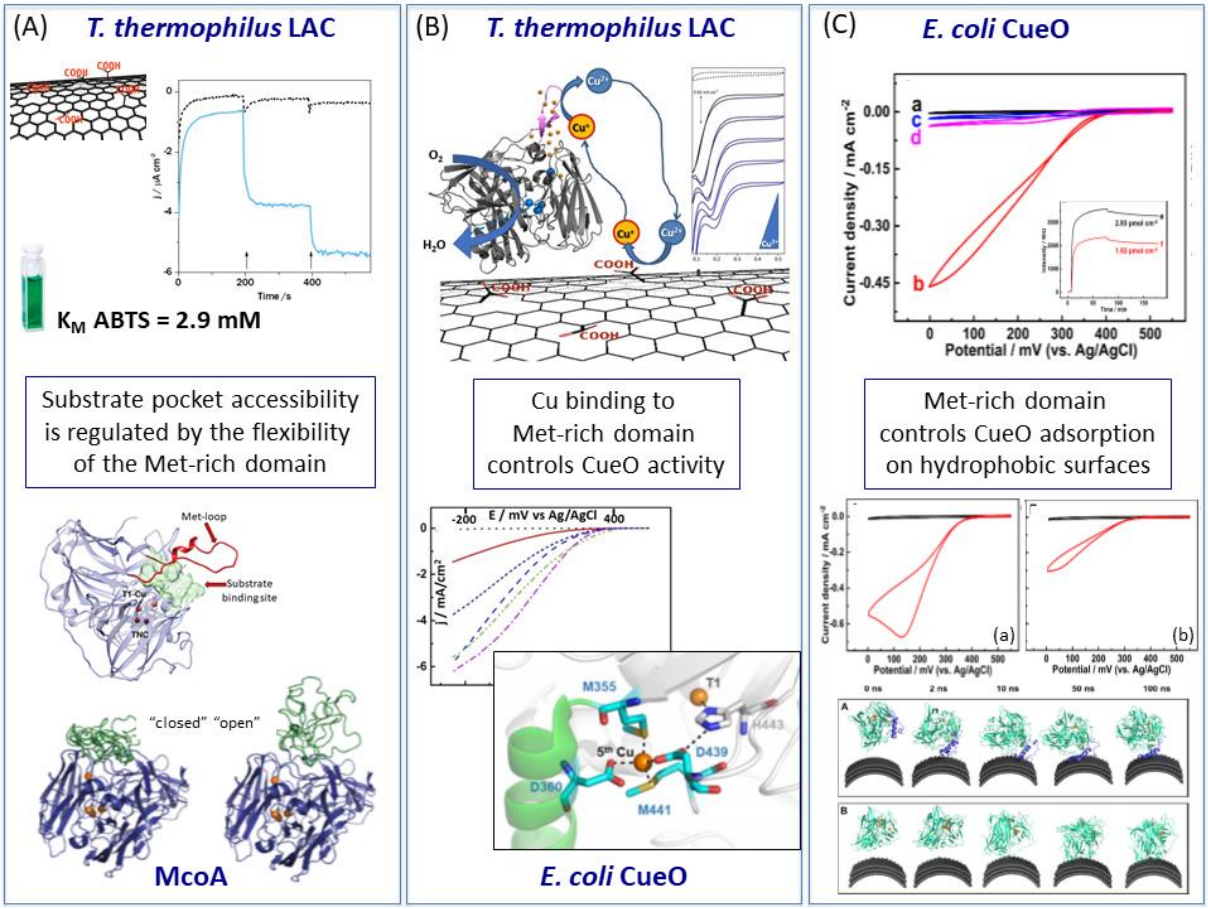

Figure 4. How Met-rich domain affects CueO electrochemistry? (A) Substrate pocket accessibility is regulated by the flexibility of the Met-rich domain. Top schemes: a very low mediated electron transfer process (blue chronoamperometric curve) can be measured after 1 mM ABTS addition when Tt LAC is immobilized on negatively charged CNTs in a non-efficient orientation for DET. Inaccessibility of CuT1 is also reflected by the high value of K_m for ABTS (2.9 mM) obtained by spectrophotometric homogeneous assays. Adapted with permission from [46]. Down schemes: the flexibility of the Met-loop in McoA is demonstrated by molecular dynamic simulation to switch from open-to-close transition, having a regulatory role for CueO activity. Adapted with permission from [30]; (B) Additional Cu binding to the Met-rich domain controls CueO activity. Top schemes: in the presence of Cu^{2+} in the electrolyte, the electrogenerated Cu^{+} binds to the Met-Rich domain of Tt LAC, inducing a new catalytic process at a potential 150 mV lower than the expected O_2 reduction passing through the CuT1. This catalytic process is Cu^{2+} dependent and has been attributed to the cuprous oxidase activity of the enzyme. Adapted with permission from [46]. Down schemes: Mutations of the ligand of Cu5 in E. coli CueO allows to increase catalytic currents and to reduce the overvoltage for O_2 reduction. Results have been attributed to synergetic effects of redox potential change of CuT1, increase in the hydrophobicity of Cu5 and decrease of the distance between Cu5 and CuT1 offering an effective ET pathway. The red CV curve is the WT CueO, while other coloured dotted lines are obtained for M355A, D360K, D439T, M441H. Adapted with permission from [60]; (C) The Met-domain controls CueO adsorption on gold

and carbon nanotube-based electrodes. Top schemes: O_2 reduction only occurs on gold electrode with the WT CueO (b), while the Met-rich domain (c) and Ser-rich domain (d) mutants are ineffective, although adsorbed as proved by SPR measurement (insert). Down schemes: comparative CVs for O_2 reduction by (a) CueO WT and (b) CueO Met-rich mutant suggesting a process controlled by fast enzyme adsorption through the Met-rich domain on hydrophobic carbon nanotubes. Such an assumption is further attested by MD simulation of (A) CueO WT adsorption and (B) Cueo Met-rich mutant. Adapted with permission from [29].

Conclusion and remaining challenges

We have shown in this short review the particularity of CueOs compared to other classical MCOs, linked to the presence of a Met-rich domain covering Cu T1, decreasing the substrate accessibility but allowing new ET pathway by additional Cu binding. Although recent works have highlighted the role of this Met-rich domain, many questions are still open. The first question concerns the requirement of many copper binding sites, with a predominance of Met residues as ligands in CueOs. What is the exact role of these Met residues and do they all have the same role, are all the sites occupied sequentially or at the same time, is this dependent on the environment conditions, i.e. O_2 availability and copper concentration? The other question that arises from the *E. coli* CueO structure is the way and state copper can be transferred to the Met-rich domain. The final question concerns the role of Met residues in the presence of ROS. Electrochemistry should provide clues by the in-situ generation of Cu^+ at the electrode, hence elucidating the affinity and role of Cu^{2+}/Cu^+ binding, by studying the effect of ROS produced for example trough ring-disk electrode experiments, and by filling the gap between *in vivo* copper tolerance of CueO-containing bacteria and *in vitro* CueO activities. But most of all, it will be crucial in future works to consider thermodynamics and kinetics of the ET pathway through CuT1 for understanding the unique properties of CueOs. Such fundamental research will allow other proteins than CueOs also involved in copper homeostasis to be studied [62]. It will enlarge the applicative potential of CueO-like enzymes as biocatalysts for oxidative polymerization of phenols [28,36,66], or for O_2 [44] or copper biosensor development.

Author's contributions

The author's responsibilities were as follows: IM realized bioinformatic for structure analysis. EL prepared the manuscript. All the contributors reviewed the manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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