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Electrochemistry of CueO-like multicopper oxidases involved in copper homeostasis

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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 O₂. 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.

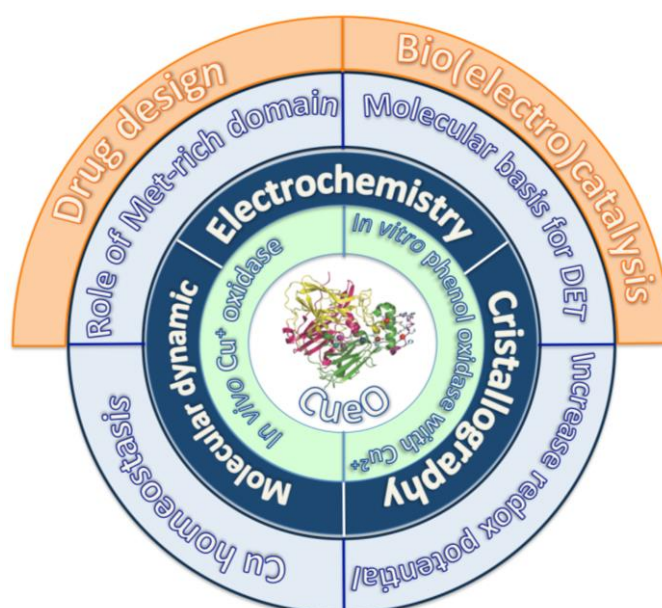


Figure 1. CueO-like enzyme main properties and challenges

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 O₂ 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 Cu²⁺ exogenous addition.

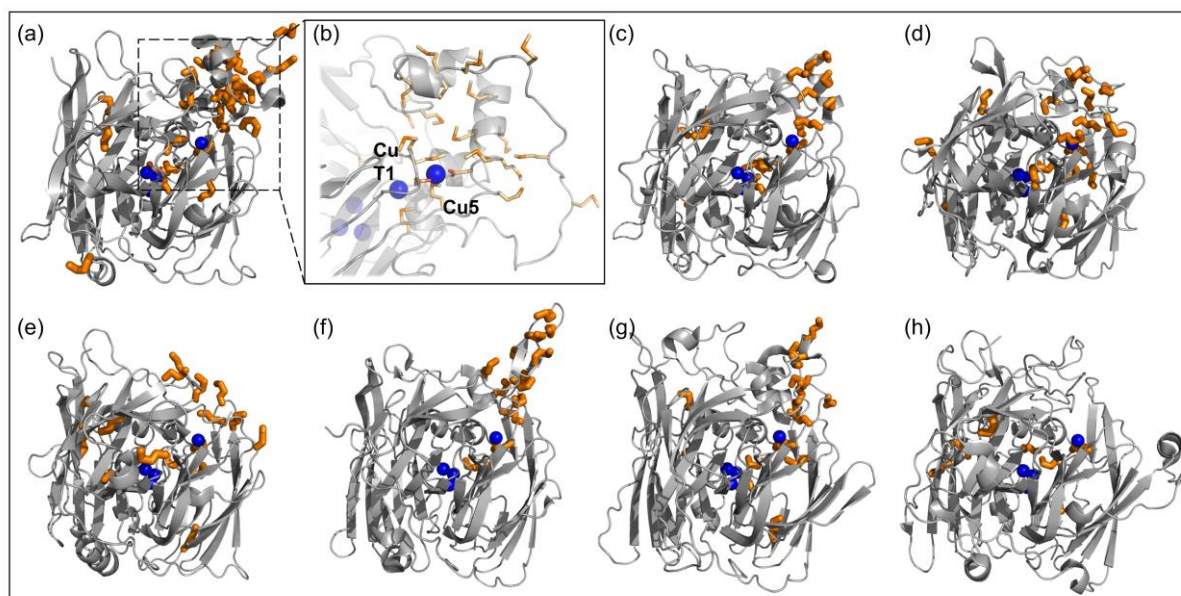


Figure 2. Structural features of CueO-like enzymes (a-g) compared to a non-CueO enzyme (h). (a-b) *E.coli* CueO (PDB 3OD3). The additional domain comprises Met and Asp ligands for a 5th copper atom (Cu5), a helical region with 6 Met that covers the CuT1 site and Cu5, and a loosely constrained loop lying 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 5th 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 $[\text{Cu}(\text{MeCN})_4]^+$ and $[\text{Cu}(\text{bicinchoninate})_2]^{3-}$ 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 $\mu\text{A}/\text{cm}^2$ 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's 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 O₂ reduction.

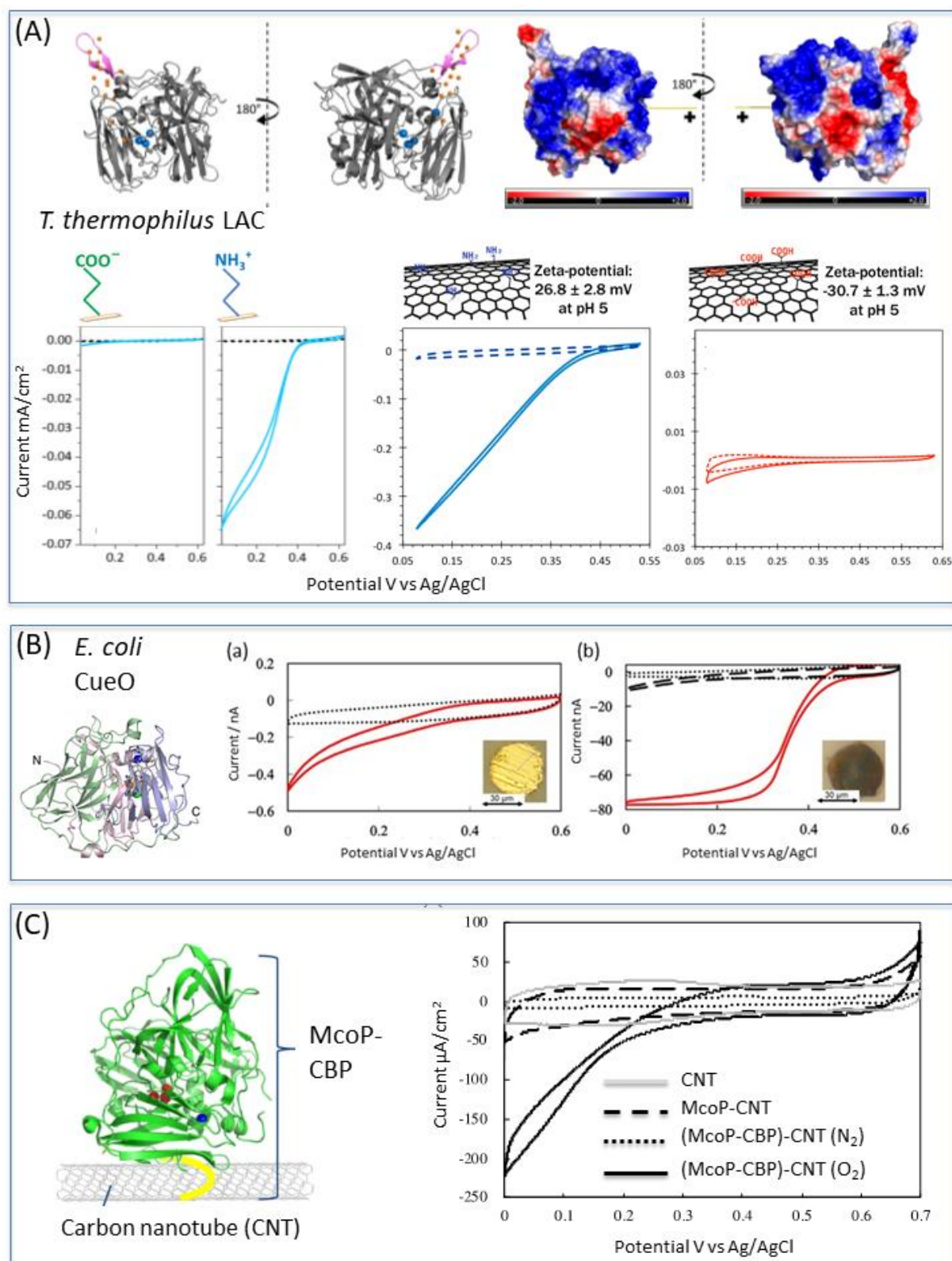


Figure 3. Strategies developed for DET involving CueOs at electrochemical interfaces. (A) Immobilization through electrostatic interactions. First row: due to anisotropy of surface charges and

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 O₂ (plain lines) or N₂ (dotted lines). Adapted with permission from [46]; (B) Immobilization of CueO in a matrix with suitable pore size. O₂ reduction catalyzed by E. coli CueO on (a) planar and (b) porous Au microelectrodes. Solid and dotted lines represent CV recorded under O₂ and Ar respectively. In (b) the dashed line corresponds to the control with no enzyme under O₂. The enhancement in catalytic current for O₂ 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 O₂ 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. O₂ 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 O₂ 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 Cu²⁺ 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 Cu²⁺-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 Cu²⁺ activation of CueO-like enzymes in solution and the evidence of a CueO-related electrocatalytic current for O₂ reduction with no requirement of Cu²⁺ 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.

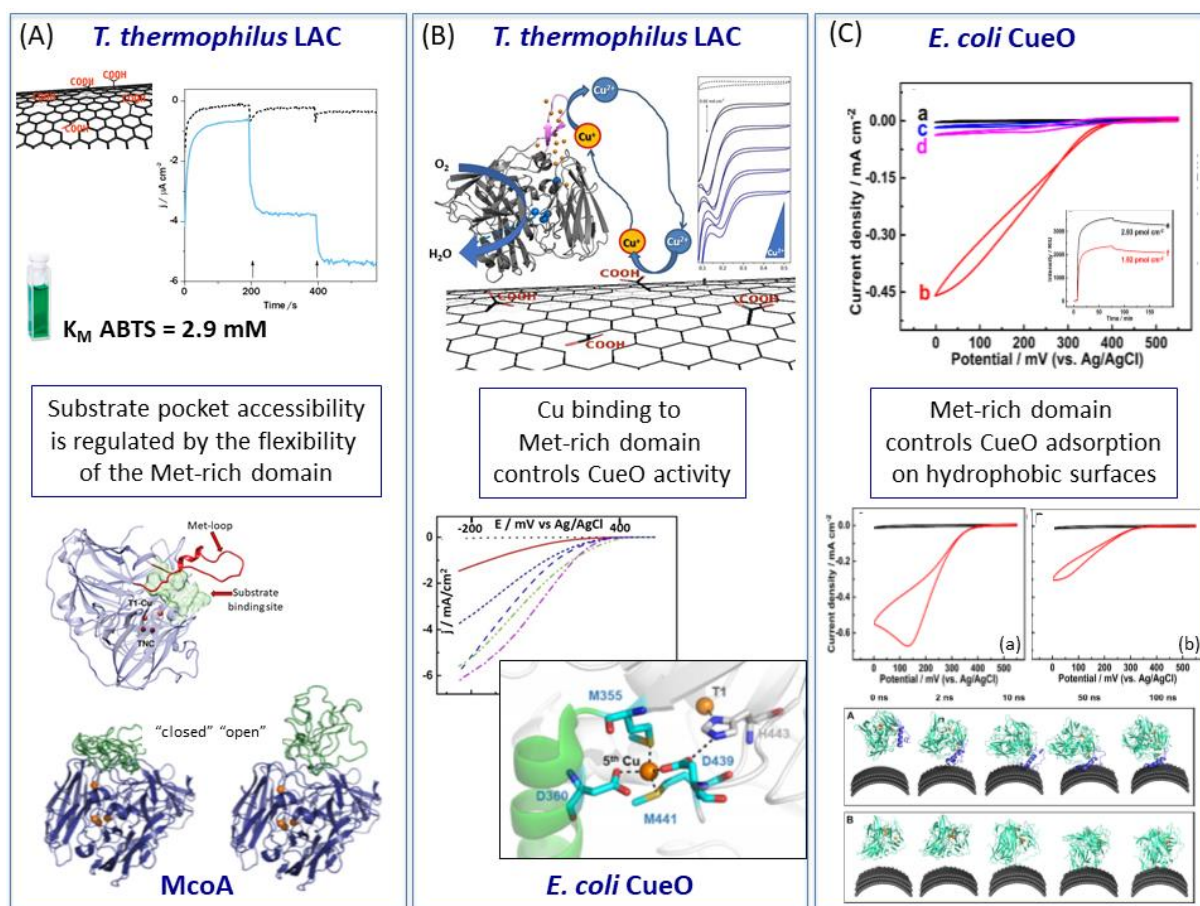


Figure 4. How Met-rich domain affects CueO electrochemistry? (A) Substrate pocket accessibility is regulated by the flexibility of the Met-rich domain. Top schemes: a very low mediated electron transfer process (blue chronoamperometric curve) can be measured after 1 mM ABTS addition when Tt LAC is immobilized 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 through 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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References

1. Hyre A, Casanova-Hampton K, Subashchandrabose S: **Copper Homeostatic Mechanisms and Their Role in the Virulence of Escherichia coli and Salmonella enterica**. *EcoSal Plus* 2021:eESP00142020.
2. Andrei A, Ozturk Y, Khalfaoui-Hassani B, Rauch J, Marckmann D, Trasnea PI, Daldal F, Koch HG: **Cu Homeostasis in Bacteria: The Ins and Outs**. *Membranes* 2020, **10**.
- * This review summarizes current knowledge on bacterial Cu homeostasis and describes the strategies that bacteria use for uptake, storage and export of Cu.
3. Giachino A, Waldron KJ: **Copper tolerance in bacteria requires the activation of multiple accessory pathways**. *Molecular Microbiology* 2020, **114**:377-390.
4. Meir A, Lepechkin-Zilbermintz V, Kahremany S, Schwerdtfeger F, Gevorkyan-Airapetov L, Munder A, Viskind O, Gruzman A, Ruthstein S: **Inhibiting the copper efflux system in microbes as a novel approach for developing antibiotics**. *Plos One* 2019, **14**.
5. Yan XX, Yang JL, Wang Q, Lin SS: **Transcriptomic analysis reveals resistance mechanisms of Klebsiella michiganensis to copper toxicity under acidic conditions**. *Ecotoxicology and Environmental Safety* 2021, **211**.
6. Mano N, de Poulpiquet A: **O-2 Reduction in Enzymatic Biofuel Cells**. *Chemical Reviews* 2018, **118**:2392-2468.
7. Sousa AC, Martins LO, Robalo MP: **Laccases: Versatile Biocatalysts for the Synthesis of Heterocyclic Cores**. *Molecules* 2021, **26**.
8. Kaur K, Sharma A, Capalash N, Sharma P: **Multicopper oxidases: Biocatalysts in microbial pathogenesis and stress management**. *Microbiological Research* 2019, **222**:1-13.
9. Xiao XX, Xia HQ, Wu RR, Bai L, Yan L, Magner E, Cosnier S, Lojou E, Zhu ZG, Liu AH: **Tackling the Challenges of Enzymatic (Bio)Fuel Cells**. *Chemical Reviews* 2019, **119**:9509-9558.
10. Adachi T, Kitazumi Y, Shirai O, Kano K: **Direct Electron Transfer-Type Bioelectrocatalysis of Redox Enzymes at Nanostructured Electrodes**. *Catalysts* 2020, **10**.
11. Mazurenko I, Hitaishi VP, Lojou E: **Recent advances in surface chemistry of electrodes to promote direct enzymatic bioelectrocatalysis**. *Current Opinion in Electrochemistry* 2020, **19**:113-121.
12. de Poulpiquet A, Kjaergaard CH, Rouhana J, Mazurenko I, Infossi P, Gounel S, Gadiou R, Giudici-Ortoni MT, Solomon EI, Mano N, et al.: **Mechanism of Chloride Inhibition of Bilirubin Oxidases and Its Dependence on Potential and pH**. *Acs Catalysis* 2017, **7**:3916-3923.
13. Graff M, Buchholz PCF, Le Roes-Hill M, Pleiss J: **Multicopper oxidases: modular structure, sequence space, and evolutionary relationships**. *Proteins-Structure Function and Bioinformatics* 2020, **88**:1329-1339.
14. Roulling F, Godin A, Cipolla A, Collins T, Miyazaki K, Feller G: **Activity-stability relationships revisited in blue oxidases catalyzing electron transfer at extreme temperatures**. *Extremophiles* 2016, **20**:621-629.
15. Kaur K, Sidhu H, Capalash N, Sharma P: **Multicopper oxidase of Acinetobacter baumannii: Assessing its role in metal homeostasis, stress management and virulence**. *Microbial Pathogenesis* 2020, **143**.
16. Kinkar E, Kinkar A, Saleh M: **The multicopper oxidase of Mycobacterium tuberculosis (MmcO) exhibits ferroxidase activity and scavenges reactive oxygen species in activated THP-1 cells**. *International Journal of Medical Microbiology* 2019, **309**.
17. Wen Q, Liu XM, Wang HY, Lin JQ: **A versatile and efficient markerless gene disruption system for Acidithiobacillus thiooxidans: application for characterizing a copper tolerance related multicopper oxidase gene**. *Environmental Microbiology* 2014, **16**:3499-3514.
18. Granja-Travez RS, Wilkinson RC, Persinoti GF, Squina FM, Fulop V, Bugg TDH: **Structural and functional characterisation of multi-copper oxidase CueO from lignin-degrading bacterium Ochrobactrum sp reveal its activity towards lignin model compounds and lignosulfonate**. *Febs Journal* 2018, **285**:1684-1700.

19. Wu TL, Wang SH, Wang Z, Peng XW, Lu YL, Wu QM: **A multicopper oxidase contributes to the copper tolerance of *Brucella melitensis* 16M.** *Fems Microbiology Letters* 2015, **362**.
20. Zulfiqar S, Shehzad K, Tahir S, Al-Ghanim KA, Shakoori AR: **Dual Role of Multicopper Oxidase of *Klebsiella pneumoniae* as a Copper Homeostatic Element and a Novel Alkaline Laccase with Potential Application in Green Chemistry.** *Pakistan Journal of Zoology* 2019, **51**:107-115.
21. Sakuraba H, Koga K, Yoneda K, Kashima Y, Ohshima T: **Structure of a multicopper oxidase from the hyperthermophilic archaeon *Pyrobaculum aerophilum*.** *Acta Crystallographica Section F-Structural Biology Communications* 2011, **67**:753-757.
22. Clement R, Wang X, Biaso F, Ilbert M, Mazurenko I, Lojou E: **Mutations in the coordination spheres of T1 Cu affect Cu²⁺-activation of the laccase from *Thermus thermophilus*.** *Biochimie* 2021.
*Study of mutations in the coordination spheres of CuT1 provides evidence that the Met-rich domain in *T. thermophilus* LAC is not the only actor in Cu²⁺-activation of CueO.
23. Miyazaki K: **A hyperthermophilic laccase from *Thermus thermophilus* HB27.** *Extremophiles* 2005, **9**:415-425.
24. Mancini S, Kumar R, Mishra V, Solioz M: **Desulfovibrio DA2_CueO is a novel multicopper oxidase with cuprous, ferrous and phenol oxidase activity.** *Microbiology-Sgm* 2017, **163**:1229-1236.
25. Singh SK, Roberts SA, McDevitt SF, Weichsel A, Wildner GF, Grass GB, Rensing C, Montfort WR: **Crystal Structures of Multicopper Oxidase CueO Bound to Copper(I) and Silver(I) FUNCTIONAL ROLE OF A METHIONINE-RICH SEQUENCE.** *Journal of Biological Chemistry* 2011, **286**:37849-37857.
26. Wang HQ, Liu XQ, Zhao JT, Yue QX, Yan YH, Gao ZQ, Dong YH, Zhang ZY, Fan YL, Tian J, et al.: **Crystal structures of multicopper oxidase CueO G304K mutant: structural basis of the increased laccase activity.** *Scientific Reports* 2018, **8**.
27. Akter M, Inoue C, Komori H, Matsuda N, Sakurai T, Kataoka K, Higuchi Y, Shibata N: **Biochemical, spectroscopic and X-ray structural analysis of deuterated multicopper oxidase CueO prepared from a new expression construct for neutron crystallography.** *Acta Crystallographica Section F-Structural Biology Communications* 2016, **72**:788-794.
28. Decembrino D, Girhard M, Urlacher VB: **Use of Copper as a Trigger for the in Vivo Activity of *E. coli* Laccase CueO: A Simple Tool for Biosynthetic Purposes.** *Chembiochem* 2021, **22**:1470-1479.
29. Cui H, Zhang L, Söder D, Tang X, Davari MD, Schwaneberg U: **Rapid and Oriented Immobilization of Laccases on Electrodes via a Methionine-Rich Peptide.** *ACS Catalysis* 2021, **11**:2445-2453.
**This work combines electrochemistry, SPR and molecular dynamic simulations to demonstrate the efficient adsorption through the Met-rich domain of *E. coli* CueO WT and various mutants on hydrophobic carbon materials.
30. Borges PT, Brissos V, Hernandez G, Masgrau L, Lucas MF, Monza E, Frazao C, Cordeiro TN, Martins LO: **Methionine-Rich Loop of Multicopper Oxidase McoA Follows Open-to-Close Transitions with a Role in Enzyme Catalysis.** *Acs Catalysis* 2020, **10**:7162-7176.
*A combination of molecular dynamic simulations and small-angle X-ray scattering is used to probe the open-to-close transitions of Met-loop in *Aquifex aeolicus* McoA.
31. Liu HP, Zhu YY, Yang XR, Lin Y: **Four second-sphere residues of *Thermus thermophilus* SG0.5JP17-16 laccase tune the catalysis by hydrogen-bonding networks.** *Applied Microbiology and Biotechnology* 2018, **102**:4049-4061.
32. Serrano-Posada H, Centeno-Leija S, Rojas-Trejo SP, Rodriguez-Almazan C, Stojanoff V, Rudino-Pinera E: **X-ray-induced catalytic active-site reduction of a multicopper oxidase: structural insights into the proton-relay mechanism and O-2-reduction states.** *Acta Crystallographica Section D-Structural Biology* 2015, **71**:2396-2411.
33. Fernandes AT, Damas JM, Todorovic S, Huber R, Baratto MC, Pogni R, Soares CM, Martins LO: **The multicopper oxidase from the archaeon *Pyrobaculum aerophilum* shows nitrous oxide reductase activity.** *Febs Journal* 2010, **277**:3176-3189.

34. Shin J, Gray HB, Winkler JR: **Stability/activity tradeoffs in Thermusthermophilus HB27 laccase.** *Journal of Biological Inorganic Chemistry* 2020, **25**:233-238.
35. Kataoka K, Komori H, Ueki Y, Konno Y, Kamitaka Y, Kurose S, Tsujimura S, Higuchi Y, Kano K, Seo D, et al.: **Structure and function of the engineered multicopper oxidase CueO from Escherichia coli - Deletion of the methionine-rich helical region covering the substrate-binding site.** *Journal of Molecular Biology* 2007, **373**:141-152.
36. Hiraishi T, Tachibana K, Asakura N, Abe H, Maeda M: **Enhanced expression of a recombinant multicopper oxidase, CueO, from Escherichia coli and its laccase activity towards aromatic substrates.** *Polymer Degradation and Stability* 2019, **164**:1-8.
37. Kumari A, Kishor N, Guptasarma P: **Characterization of a mildly alkalophilic and thermostable recombinant Thermus thermophilus laccase with applications in decolourization of dyes.** *Biotechnology Letters* 2018, **40**:285-295.
38. Ezike TC, Udeh JO, Joshua PE, Ezugwu AL, Isiwu CV, Eze SOO, Chilaka FC: **Substrate specificity of a new laccase from Trametes polyzona WRF03.** *Heliyon* 2021, **7**.
39. Sadeghian I, Rezaie Z, Rahmatabadi SS, Hemmati S: **Biochemical insights into a novel thermo/organo tolerant bilirubin oxidase from Thermosediminibacter oceani and its application in dye decolorization.** *Process Biochemistry* 2020, **88**:38-50.
40. Kataoka K, Sakurai T: **Role of Hydrogen Bond Connecting Ligands for Substrate and Type I Copper in Copper(I) Oxidase CueO.** *Chemistry Letters* 2013, **42**:1102-1104.
41. Cortes L, Wedd AG, Xiao Z: **The functional roles of the three copper sites associated with the methionine-rich insert in the multicopper oxidase CueO from E. coli.** *Metallomics* 2015, **7**:776-785.
42. Tsujimura S, Miura Y, Kano K: **CueO-immobilized porous carbon electrode exhibiting improved performance of electrochemical reduction of dioxygen to water.** *Electrochimica Acta* 2008, **53**:5716-5720.
43. Chumillas S, Maestro B, Feliu JM, Climent V: **Comprehensive Study of the Enzymatic Catalysis of the Electrochemical Oxygen Reduction Reaction (ORR) by Immobilized Copper Efflux Oxidase (CueO) From Escherichia coli.** *Frontiers in Chemistry* 2018, **6**.
44. Miyata M, Kitazumi Y, Shirai O, Kataoka K, Kano K: **Diffusion-limited biosensing of dissolved oxygen by direct electron transfer-type bioelectrocatalysis of multi-copper oxidases immobilized on porous gold microelectrodes.** *Journal of Electroanalytical Chemistry* 2020, **860**.

*This work reports the performance of *E. coli* CueO immobilized on porous gold electrode for O₂ biosensing.

45. Agbo P, Heath JR, Gray HB: **Modeling Dioxygen Reduction at Multicopper Oxidase Cathodes.** *Journal of the American Chemical Society* 2014, **136**:13882-13887.

46. Hitaishi VP, Clement R, Quattrocchi L, Parent P, Duche D, Zuily L, Ilbert M, Lojou E, Mazurenko I: **Interplay between Orientation at Electrodes and Copper Activation of Thermus thermophilus Laccase for O₂ Reduction.** *Journal of the American Chemical Society* 2020, **142**:1394-1405.

**Based on the structural examination of *T. thermophilus* LAC, this work put forward the molecular basis for efficient DET between SAM-modified golds and carbon nanotube-modified electrode. It demonstrates for the first time how cuprous oxidase activity of CueO can be measured by electrochemistry.

47. Hitaishi VP, Mazurenko I, Vengasseril Murali A, de Poulpiquet A, Coustillier G, Delaporte P, Lojou E: **Nanosecond Laser-Fabricated Monolayer of Gold Nanoparticles on ITO for Bioelectrocatalysis.** *Frontiers in Chemistry* 2020, **8**.

48. Adachi T, Kitazumi Y, Shirai O, Kawano T, Kataoka K, Kano K: **Effects of Elimination of a Helix Regions on Direct Electron Transfer-type Bioelectrocatalytic Properties of Copper Efflux Oxidase.** *Electrochemistry* 2020, **88**:185-189.

**This paper investigates the direct electron transfer between positively or negatively charged mesoporous electrode and CueO WT and variants with different truncated helix. It shows that DET

proceeds at positive electrode through the negative CuT1 environment no matter of the existence of the MET-domain.

49. Schlesinger O, Pasi M, Dandela R, Meijler MM, Alfonta L: **Electron transfer rate analysis of a site-specifically wired copper oxidase.** *Physical Chemistry Chemical Physics* 2018, **20**:6159-6166.
 50. Takamura E, Nakamura T, Sakamoto H, Satomura T, Sakuraba H, Ohshima T, Suye S: **Effects of multicopper oxidase orientation in multiwalled carbon nanotube biocathodes on direct electron transfer.** *Biotechnology and Applied Biochemistry* 2019, **66**:137-141.
 51. Sakamoto H, Futamura R, Tonooka A, Takamura E, Satomura T, Suye S: **Biocathode design with highly-oriented immobilization of multi-copper oxidase from *Pyrobaculum aerophilum* onto a single-walled carbon nanotube surface via a carbon nanotube-binding peptide.** *Biotechnology Progress* 2021, **37**.
 52. Sakamoto H, Futamura R, Fujiwara I, Meboso T, Li N, Takamura E, Satomura T, Suye S: **Immobilization of multicopper oxidase from *Pyrobaculum aerophilum* onto an electrospun-aligned single-walled carbon nanotube surface via a carbon-nanotube-binding peptide for biocathode.** *Journal of Applied Polymer Science* 2021, **138**.
- *This study aimed to orient *Pyrobaculum aerophilum* McoP on single-walled carbon nanotubes by engineering a CNT-binding peptide at the C-terminus of McoP near CuT1.
53. Mate DM, Alcalde M: **Laccase engineering: From rational design to directed evolution.** *Biotechnology Advances* 2015, **33**:25-40.
 54. Sakurai T, Yamamoto M, Ikeno S, Kataoka K: **Amino acids located in the outer-sphere of the trinuclear copper center in a multicopper oxidase, CueO as the putative electron donor in the four-electron reduction of dioxygen.** *Biochimica Et Biophysica Acta-Proteins and Proteomics* 2017, **1865**:997-1003.
 55. Kurose S, Kataoka K, Shinohara N, Miura Y, Tsutsumi M, Tsujimura S, Kano K, Sakurai T: **Modification of Spectroscopic Properties and Catalytic Activity of *Escherichia coli* CueO by Mutations of Methionine 510, the Axial Ligand to the Type I Cu.** *Bulletin of the Chemical Society of Japan* 2009, **82**:504-508.
 56. Miura Y, Tsujimura S, Kurose S, Kamitaka Y, Kataoka K, Sakurai T, Kano K: **Direct Electrochemistry of CueO and Its Mutants at Residues to and Near Type I Cu for Oxygen-Reducing Biocathode.** *Fuel Cells* 2009, **9**:70-78.
 57. Takamura E, Taki S, Sakamoto H, Satomura T, Sakuraba H, Ohshima T, Suye S: **Site-Directed Mutagenesis of Multicopper Oxidase from Hyperthermophilic Archaea for High-Voltage Biofuel Cells.** *Applied Biochemistry and Biotechnology* 2021, **193**:492-501.
 58. Zhu YY, Zhang Y, Zhan JB, Lin Y, Yang XR: **Axial bonds at the T1 Cu site of Thermophilus SG0.5JP17-16 laccase influence enzymatic properties.** *Febs Open Bio* 2019, **9**:986-995.
 59. Zhang LL, Cui HY, Zou Z, Garakani TM, Novoa-Henriquez C, Jooyeh B, Schwaneberg U: **Directed Evolution of a Bacterial Laccase (CueO) for Enzymatic Biofuel Cells.** *Angewandte Chemie-International Edition* 2019, **58**:4562-4565.
 60. Zhang LL, Cui HY, Dhoke GV, Zou Z, Sauer DF, Davari MD, Schwaneberg U: **Engineering of Laccase CueO for Improved Electron Transfer in Bioelectrocatalysis by Semi-Rational Design.** *Chemistry-a European Journal* 2020, **26**:4974-4979.
- **This paper investigates the role played by Cu5 in *E. coli* CueO using an electrochemical screening platform of a library of engineered mutants obtained by site-directed mutagenesis. Some mutants exhibit a high enhancement of the catalytic current explained by conformational changes that increases the stability and decreases the distance between CuT1 and Cu5.
61. Satomura T, Hirano T, Inagaki K, Horinaga K, Takamura E, Sakamoto H, Ohshida T, Ohshima T, Sakuraba H, Suye S: **Activity enhancement of multicopper oxidase from a hyperthermophile via directed evolution, and its application as the element of a high performance biocathode.** *Journal of Biotechnology* 2021, **325**:234-240.
 62. Meir A, Weike G, Schwerdtteger F, Airapetov LG, Ruthstein S: **Exploring the role of the various methionine residues in the *Escherichia coli* CusB adapter protein.** *Plos One* 2019, **14**.

63. Zhu YY, Zhan JB, Zhang Y, Lin Y, Yang XR: **The K428 residue from *Thermus thermophilus* SG0.5JP17-16 laccase plays the substantial role in substrate binding and oxidation.** *Journal of Biomolecular Structure & Dynamics* 2021, **39**:1312-1320.
64. Zhang Y, Dai ZJ, Zhang SM, Yang XR: **The catalytic properties of *Thermus thermophilus* SG0.5JP17-16 laccase were regulated by the conformational dynamics of pocket loop 6.** *Biochimica Et Biophysica Acta-General Subjects* 2021, **1865**.
65. Miranda-Blancas R, Avelar M, Rodriguez-Arteaga A, Sinicropi A, Rudino-Pinera E: **The beta-hairpin from the *Thermus thermophilus* HB27 laccase works as a pH-dependent switch to regulate laccase activity.** *Journal of Structural Biology* 2021, **213**.
66. Zou Z, Gau E, El-Awaad I, Jakob F, Pich A, Schwaneberg U: **Selective Functionalization of Microgels with Enzymes by Sortagging.** *Bioconjugate Chemistry* 2019, **30**:2859-2869.