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Copper Oxidation State and Mycobacterial Infection

Marc Solioz*

Laboratory of Biochemistry and Molecular Biology, Tomsk State University, Prospect Lenina, Tomsk, Russia

***Corresponding author**: Solioz M, Laboratory of Biochemistry and Molecular Biology, Tomsk State University, Prospect Lenina 36, 634050 Tomsk, Russia, Tel: 923 403 5002; E-mail:marc@solioz-scientific.ch

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Abstract

Copper in aerobic environments prevails as cupric ions, Cu^{2+}, but inside cells, is usually present as cuprous ions, Cu^{+}. Cu^{+} is considerably more toxic to bacteria than Cu^{2+}. Phagosomes employ, among other mechanisms, Cu^{+} to create a hostile environment for engulfed bacteria. Bacteria can reduce or oxidize copper by various mechanisms. How such reactions could affect bacterial survival in phagosomes is discussed, with a focus on *Mycobacteria*.

Keywords: Copper reduction; Multicopper oxidase; Copper oxidation; Mycobacterial infection

Introduction

Copper is an essential trace element for most living organisms due to its role as a cofactor in many enzymes. However, excess copper is toxic to cells. It causes oxidative stress by participating in Fenton-type reactions which lead to the generation of reactive oxygen species, or copper can damage [Fe-S]-cluster-proteins by displacing the iron [1]. Therefore, all cells have elaborated sophisticated systems for copper homeostasis.

Bacteria are particularly vulnerable to copper toxicity, with lethal copper concentrations ranging from µM to low mM, but strongly depending on the experimental setting [2]. Consequently, copper salts are widely used for pest control in agriculture. And as a corollary to this, copper ions participate in the killing of engulfed bacteria by phagosomes. The recent description of non-enzymatic copper reduction by *Lactococcus lactis* bears on several of these issues and moves the oxidation state of copper in the focus of bactericidal mechanisms [3].

Non-enzymatic copper reduction by bacteria

Under aerobic conditions, copper in solution is present as cupric ions, Cu^{2+}. Already forty years ago, it was recognized that in *E. coli* cultures, Cu^{2+} is reduced to Cu^{+} and that the cuprous ions are considerably more toxic to cells than Cu^{2+} [4]. In aerobic cultures, Cu^{+} is steadily re-oxidized by oxygen to the less toxic Cu^{2+}, whereas in anaerobic cultures, Cu^{+} accumulates, creating a much more toxic environment for bacteria. More recently, the copper reducing activity of *E. coli* has been molecularly characterized. It was shown that approximately 70% of this activity was due to menaquinones, 10% to copper reduction by the NADH dehydrogenase, NDH^{-2}, and the remainder due to unknown sources [5-7].

The Gram-positive bacteria *Lactococcus lactis* have also been reported to exhibit extracellular Cu^{2+}-reductase activity [8,9]. It was shown that menaquinones in the bacterial membrane can catalyze copper reduction in a non-enzymatic reaction, presumably by direct interaction of copper with the membrane [3]. Lactic acid bacteria and other fermenting organisms have an excess of reducing equivalents when growing fermentatively and will take advantage of any opportunity to dispose of these. In fermentatively growing *L. lactis*, sub-lethal concentrations of Cu^{2+} ions in the media can apparently serve as electron acceptors [3].

Short-chain menaquinones present in the membrane are reduced by NADH via membrane-bound NADH oxidases, NoxA or NoxB; the reduced menaquinones in turn reduce extracellular Cu^{2+} to Cu^{+} (Figure 1A). Since electrons move from a standard redox potential of -315 mV for the NADH/NAD^{+} couple to one of +150 mV for the Cu^{2+}/Cu^{+} couple, the reaction is thermodynamically very favorable. As expected, a mutant unable to synthesized menaquinones due a deficiency in O-succinylbenzoic acid CoA ligase (MenE) does not reduce extracellular copper and is therefore also less copper sensitive than the wild-type because it does not produce the more toxic Cu^{+} species [3].

The copper reduction by *L. lactis* constitutes a type of metal-based respiration, which is a well-known phenomenon and has been studied extensively in many organisms [10]. In fact, iron respiration was proposed to be one of the earliest forms of respiration that evolved on the Earth [11]. Non-enzymatic copper reduction by *L. lactis* is a 'balancing act' for the bacteria: at copper concentrations which do not inhibit growth, Cu^{2+} reduction can stimulate growth by serving as an electron sink; however, this leads to the accumulation of more toxic Cu^{+}, which could kill the organism.

Respiration competes with non-enzymatic copper reduction

L. lactis is classified as facultative anaerobe, meaning that it can tolerate oxygen, but cannot respire it. In 1970, Sijpesteijn [12] discovered that in growth media supplemented with heme, *L. lactis* consumes oxygen and grows to much higher cell density. It turned out that *L. lactis* possess functional genes for the two subunits of a terminal cytochrome *bd* oxidase [ubi (mena-) quinol: O2 oxidoreductase], but cannot synthesize heme.

If heme is supplied to cultures, a functional oxidase is synthesized and respiration ensues. In addition, *L. lactis* is endowed with a ferrochelatase, which converts protoporphyrin IX to heme, so aerobic growth is also possible on protoporphyrinogen IX [13]. The ability of

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heme to induce respiration in bacteria classified as "anaerobic" is most likely shared by many firmicutes [14].

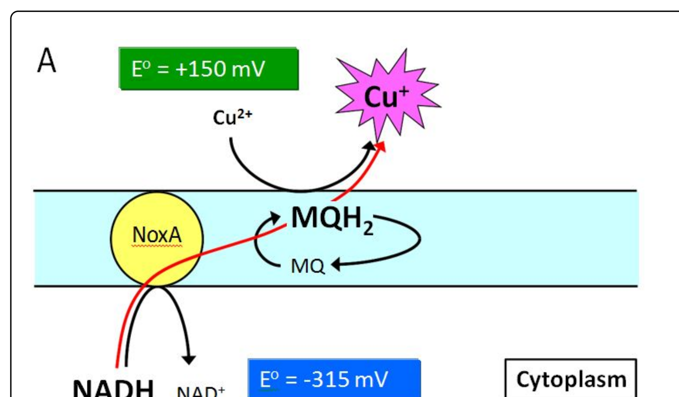

Figure 1A: Electron flow in *L. lactis* under fermentative growth conditions. Under fermentative growth conditions in the presence of Cu^{2+}, the membrane-bound NADH oxidase, NoxA/B, convert oxidized menaquinones (MQ) to their reduced form (MQH_2), with NADH serving as reductant. The reduced menaquinones in turn reduce extracellular Cu^{2+} in a non-enzymatic reaction. The respective standard redox potentials, E°, are given in the figure and the preferred overall electron flow is indicated by the red arrow and that of partial reactions by black arrows.

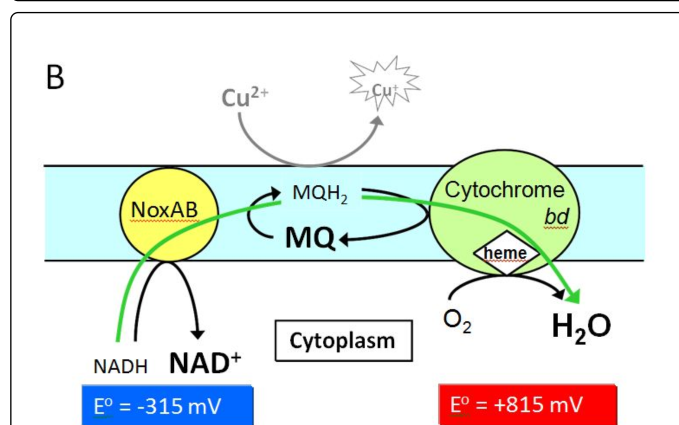

Figure 1B: Electron flow in *L. lactis* under respiratory growth conditions. Under respiring growth conditions in the presence of heme, a terminal cytochrome *bd* oxidase is synthesized. Menaquinones reduced by NADH/NoxA/B are rapidly oxidized by the oxidase, using O_2 as an electron accepter. The respective standard redox potentials, E°, are given in the figure and the preferred overall electron flow is indicated by the green arrow and that of partial reactions by black arrows.s

Surprisingly, when *L. lactis* was supplied with heme, copper reduction was strongly reduced [3]. This could be explained by competition for electrons between the two redox reactions, copper reduction and oxygen reduction. The difference in standard redox potential between O_2/H_2O and $NADH/NADH^+$ is +815 mV - (-315 mV)=+1130 mV, compared to that of the redox couple Cu^{2+}/Cu^+ and $NADH/NAD^+$, which is +150 mV - (-315 mV)=+465 mV (Figure 1B).

Oxygen reduction is thus the thermodynamically favored over copper reduction in respiring cells. Reduction of copper by menaquinones is most likely a fortuitous mechanism. First, copper is usually not present in sufficient concentrations in the natural habitats of *L. lactis* to make a significant contribution to metal respiration. Secondly, it produces toxic Cu^+ ions which can interfere with growth. When growing in milk, *L. lactis* substantially lowers the redox potential of this substrate from +300 mV to -220 mV, presumably as a way of disposing of excess reducing equivalents [15]. This could be of great importance in fermentative processes, but the electron acceptors in milk are unknown and the process remains largely unexplored.

Infection raises phagosomal copper

It has long been known that copper deficiency sensitizes mammalian hosts to microbial infections [16] and mechanistic insight into at least one such mechanism is beginning to emerge. The first line of defense against infection is often phagocytic immune cells. Upon activation of these cells, the phagosomes adopts an anti-microbial environment by assuming an acidic pH and elevating reactive oxygen species, nitric oxide, hypochlorite (ClO^-), and proteases [17].

Following infection by *Mycobacteria* and other pathogens, iron and copper also accumulate in the phagosome [18,19]. The mechanism of phagosomal copper accumulation is quite well understood (for review of normal eukaryotic copper homeostasis, see [20]). Ctr1 is the universal copper importer in the plasma membrane of eukaryotic cells. White et al. [21] showed that in cultured macrophage-like RAW264.7 cells, killing of *E. coli* is stimulated by the addition of copper to the culture media, which leads to up regulation of Ctr1 and enhanced transport of copper into the cytoplasm (Figure 2).

There, copper is sequestered by the 7 kDa protein Atox1, which functions as a copper shuttle, or chaperone. Atox1 delivers copper to the copper transporting ATPase, ATP7A, which is located in the trans-Golgi network under basal conditions to deliver copper to secreted cuproenzymes like lysyl oxidase or ceruloplasmin. In activated macrophages, ATP7A traffics from the trans-Golgi network to phagosomes.

Apparently, Ctr1 and ATP7A fulfill cell defense functions by raising cytoplasmic copper levels and transporting copper into phagosome that contain engulfed bacteria. Such a copper-up response may not be limited to macrophage, but occur in a range of cell types in response to internalized bacteria or other factors such as inflammation [20,22].

Mycobacterial defense against copper

Bacteria are well equipped to combat excessive environmental copper and respond by the upregulation of a number of genes which function in the defense against copper. *Mycobacteria*, including the human pathogen *Mycobacterium tuberculosis*, are unique among Gram-positive bacteria in featuring a complex cell wall that contains unusual lipids and thereby forms a kind of outer membrane [23].

This outer membrane functions as a barrier to chemotherapeutic agents and metal ions. A point of weakness are the porins that are required as entry point for essential nutrients, including copper, but may also allow excess copper to get access to the cytoplasmic membrane (Figure 2) [24].

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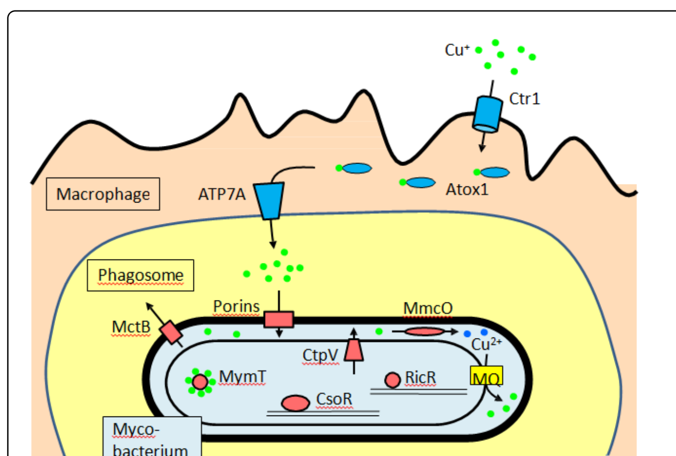

Figure 2: Copper homeostasis in a macrophage with engulfed *M. tuberculosis*.

Cu homeostasis on the host side proceeds by the components indicated in blue: the high-affinity copper transporter of the plasma membrane, Ctr1, is induced by the activation of the macrophage and transports extra copper into the cytoplasm; Atox1, a cytosolic Cu^+ chaperone, transfers the copper to ATP7A, which has trafficked from the trans-Golgi network to the phagosome and pumps copper into the phagosomal lumen. On the pathogen side, *M. tuberculosis* possesses several lines of defense against copper, indicated in red. Copper crosses the cell wall/outer membrane via porins. Excess cytoplasmic copper induces CtpV, a copper export ATPase which is under the control of the CsoR repressor. MctB functions in expelling copper, which has been pumped into the periplasmic space by CtpV, from the cell. Also induced under copper stress are a periplasmic multicopper oxidase, MmcO, which oxidizes Cu^+ to Cu^{2+}, and MymT, a metallothionein which sequesters excess cytoplasmic copper. These proteins are under the control of a second copper-inducible repressor, RicR. Cu^+ is depicted as green dots and Cu^{2+} as blue dots. See text for additional explanations.

In macrophages, this will induce the *cso* operon, which is under the control of the copper-inducible CsoR repressor [25]. The operon encodes CtpV, which is an enzyme related to known bacterial copper ATPases and pumps cytoplasmic copper to the periplasmic space, and two proteins of unknown function, Rv0970 and Rv0968. MctB is required to transport the periplasmic copper to the cell exterior and is thus functionally, but not structurally, similar to the Cus-system of Gram-negative bacteria [26]. Mutation of the *metB* gene resulted in a dramatically reduced bacterial burden in lungs and lymph nodes in guinea pigs infected with *M. tuberculosis*, supporting the important role of copper in the phagosome [27]. RicR, a second copper-inducible repressor, controls a regulon of six monocistronic gene, namely RicR itself, MymT, a metallothionein which sequesters cytoplasmic copper, MmcO, a multicopper oxidase, and three proteins of unknown function, LpqS, Rv2963, and SocAB [28].

Of special interest is MmcO, a CueO-type multicopper oxidase that has been well characterized in *E. coli* and other bacteria [29]. CueO-type multicopper oxidases are common in Gram-negative bacteria and can oxidize Cu^+, Fe^{2+}, and various polycyclic aromatic hydrocarbons. MmcO has been shown to be required for copper resistance by *M. tuberculosis*, presumably by reducing Cu^+ to less toxic Cu^{2+} [30]. A CueO-type multicopper oxidase may not be of equal importance for all species; for *Brucella melitensis* it was found that the multicopper oxidase did not affect survival in macrophages [31], while mutation of *cueO* in *Salmonella Typhimurium* attenuated survival in liver and spleen, but not macrophages [32]. Clearly, the CueO-type multicopper oxidase contributes to bacterial copper resistance in culture, but it may not be a key factor in all species for survival in phagosomes [33]. This may have multiple underlying reasons. For one, the phagosomal interior may be sufficiently oxidizing for spontaneous copper oxidation to occur. Secondly, the primary role of CueO-like enzymes *in vivo* may not always be the oxidation of copper, but oxidation of iron or organic substrates. Yet, two general observations underline the importance of bacterial copper resistance for survival in macrophages: bacterial mutants which are copper-sensitive in culture are hyper sensitized to macrophage killing and, conversely, chelation of macrophage copper results in increased survival of engulfed organisms [34].

Redox state of copper in phagosomes and Mycobacterial infections

Copper transported into phagosomes by ATP7A is in the reduced Cu^+ form, which is considerably more toxic to bacteria than Cu^{2+} [3]. Reactive oxygen species in the phagosomal compartment could lead to the generation of significant Cu^{2+} by spontaneous oxidation. This Cu^{2+} could, at least in principle, be re-reduced by non-enzymatic copper reduction via menaquinones in the membrane of *Mycobacteria*, restoring the more toxic Cu^+ pool. However, this reaction is unlikely to occur because *Mycobacteria* harbor two terminal oxidases, a cytochrome aa_3 oxidase and a cytochrome *bd* oxidase [35], which would effectively compete with non-enzymatic copper reduction.

But another mechanism may be very significant: compared to wild-type, bacteria deficient in CueO-type multicopper oxidase exhibit much poorer survival in various eukaryotic cell types. This suggests that the oxidation of phagosomal Cu^+ to less toxic Cu^{2+} by multicopper oxidase can partially detoxify the phagosomal lumen and aid the survival of the pathogen. MmcO of *M. tuberculosis* could catalyze such a detoxification reaction. Purified CueO multicopper oxidases of *E. coli* and *Desulfosporosinus* sp. OT were shown be inhibited by Ag+ with an I_{50} of 1 μM ([36] and unpublished observations). This contrasts with the inhibition of bacterial growth, which requires 10- to 1000-fold higher silver concentrations, depending on the species and the media [37]. Low silver ion concentrations could thus potentiate the antibacterial milieu of hagosomes by preventing the oxidation of Cu^+ to less toxic Cu^{2+} by bacterial multicopper oxidases. Silver in various formulations has been used for centuries to treat veneral diseases and other bacterial infections and to dress open wounds and burns [38]. The latter applications continue to be in use up to this day. How silver acts in these applications remains unknown. One possibility is the inhibition of bacterial CueO-type multicopper oxidases by silver, thereby compromising survival in phagosomes. Such a mechanism would certainly deserve further investigation in model systems.

Recent progress in the understanding of both, eukaryotic and prokaryotic copper homeostasis, have greatly advanced our insight into copper's role in phagosomal killing of bacteria. The much higher bactericidal activity of Cu^+ versus Cu^{2+} and thus copper redox reactions appear to play an important role in the process, but addressing this question remains difficult. Although intracellular copper indicators specific for Cu^+ or Cu^{2+} have become available in recent years, their targeting to specific cell compartments is still a

challenge. Clearly, the role of copper redox reactions in phagosomes requires further investigation, but its comprehension may eventually open new avenues to drug development.

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