Chapter 2
Copper Toxicity

Abstract Copper is essential for life, yet highly reactive and a potential source of cell damage. Therefore, all cells possess copper homeostatic mechanisms to keep intracellular copper at safe levels. However, under conditions of excess environmental copper, homeostatic system become overloaded and intracellular copper rises to toxic levels. Possible toxic effects of copper span a range of mechanisms and it cannot be known with certainty which mechanism is active to what extent in a particular bacterium of vast and varied bacterial world. For common laboratory species like *Escherichia* or *Bacillus*, the concept has emerged that the main toxic action of copper is the replacement of iron in iron-sulfur cluster proteins, thereby inactivating essential enzyme functions.

Keywords Hydroxyl radical · Hydrogen peroxide · Fenton · Glutathione
Iron-sulfur cluster · Thiol depletion

The mechanism whereby copper is toxic to cells has generally been ascribed to the redox properties of copper, resulting in lethal oxidative damage to cells. However, recent work has put this concept into question and it is currently believed that the main toxic action of copper is the replacement of the iron cofactor in iron-sulfur cluster proteins. Other toxicity mechanism may still be at work to various extents, so to say in the background, depending on environmental and growth conditions. All major possible toxicity mechanisms will be described in this chapter and are summarized in Fig. 2.1. It must be stressed that the toxicity mechanisms discussed in this chapter do not apply to the antimicrobial action of metallic copper surfaces (see Sect. 2.4).

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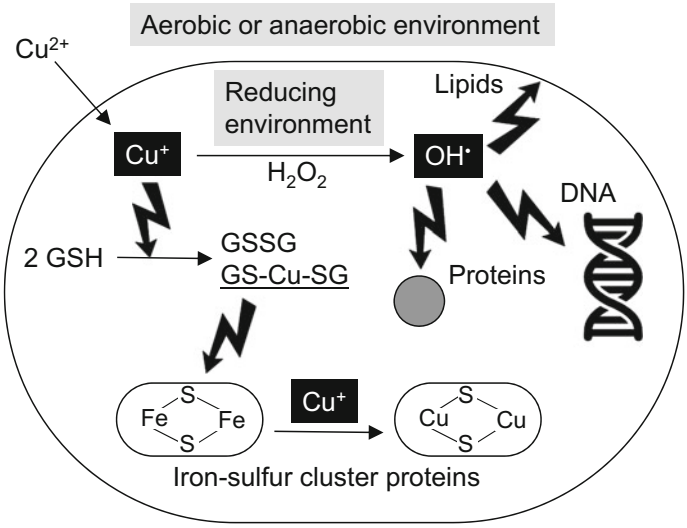

Fig. 2.1 Mechanisms of copper toxicity. Copper enters the bacterial cell by unknown pathways. The reducing condition in the cytoplasm reduces the copper to Cu^+, which can then participate in Fenton-type reactions to produce highly reactive hydroxyl radicals. These can in turn react nonspecifically with lipids, proteins and nucleic acid. Cu^+ can also lead to thiol depletion in the GSH pool, but also in proteins and free amino acids. Under anaerobic conditions, glutathione-copper complexes (GS–Cu–SG) can act as copper-donors for metalloenzymes. The dominant toxicity mechanism most likely is the displacement of iron from iron-sulfur cluster proteins by Cu^+.

2.1 Copper Toxicity Through the Formation of Reactive Oxygen Species

Copper can participate in a number of chemical reactions which lead to the generation of reactive oxygen species (ROS). Reactive hydroxyl radicals, which are extremely reactive in the cellular context, can be generated by a Fenton-type reaction (1):

$$Cu^+ + H_2O_2 = Cu^{2+} + OH^- + OH^{\cdot} \quad (1)$$

ROS production can be amplified by a combination with the Haber–Weiss cycle [(2) and (3)].

$$H_2O_2 + OH^{\cdot} = H_2O + O_2^- + H^+ \quad (2)$$

$$H_2O_2 + O_2^- = O_2 + OH^- + OH^{\cdot} \quad (3)$$

This could provide a particularly rich source of ROS in lactic acid bacteria which can accumulate large amounts of hydrogen peroxide [1, 2]. The rate constant of reaction (3) by itself is negligible, but Cu^{2+} or Fe^{3+} complexes can act as catalysts. Irreversible cell damage by ROS, particularly by the extremely reactive hydroxy

radicals, can come about by a variety of mechanisms, such as inhibition of respiration, lipid peroxidation, or oxidative damage of proteins [3, 4].

Copper can also lead to depletion of glutathione (GSH), which is a major protective substance against heavy metal toxicity (see Sect. 2.3). This could occur in a cycle between reactions (4) and (5):

$$2Cu^{+} + 2H^{+} + O_2 = 2Cu^{2+} + H_2O_2 \quad (4)$$

$$2Cu^{2+} + 2GSH = 2Cu^{+} + GSSG + 2H^{+} \quad (5)$$

These combined reactions catalyze redox cycling of copper at the expense of GSH and O_2 to produce GSSG, the oxidized, dimeric form of GSH. Other cellular thiols could be depleted by the a similar mechanism. Hydrogen peroxide generated by reaction (4) could in turn participate in reactions (1)–(3) and amplify toxic hydroxyl radical production. While reasonable on paper, it is not clear if these reactions really occur in the cytoplasm under copper stress. The free copper concentration is probably far too low to catalyze these reactions.

The concept of cellular damage by copper *via* the production of ROS, thiol depletion, and oxidative damage of proteins, lipids, and DNA appears logical and has for years been claimed to be the toxicity mechanism of copper, but has never thoroughly been proven. Clearly, detrimental ROS production and oxidative damage can lead to cell death under certain stress conditions. For example, Woodmansee et al. [5] showed that nitric oxide (NO) accelerated the rate at which H_2O_2 killed *Escherichia coli* cells, apparently by greatly enhancing DNA damage through Fenton chemistry. Since NO damages the iron-sulfur clusters of dehydratases, the released iron could catalyze the Fenton reaction. However, NO also blocks respiration, which makes cells more susceptible to oxidative damage, making the mechanism of cell death unclear.

In the analysis of copper toxicity in *E. coli*, it was observed that the majority of H_2O_2-oxidizable copper is located in the periplasm and copper-mediated hydroxyl radical formation mainly occurs in this compartment, away from the DNA [6]. Copper-loading of cells actually increased their resistance to killing by H_2O_2 by eliminating iron-mediated oxidative killing and reducing the rate of DNA damage. These observations do not explain how copper suppresses iron-mediated damage but it is clear that copper does not catalyze significant oxidative DNA damage *in vivo*; therefore, copper toxicity must occur by a different mechanism. This challenges the oxidative-damage copper toxicity concept and newer work strongly supports a mechanism whereby the main toxic action of copper is the displacement of iron by copper in essential iron-cluster enzymes, thereby rendering key enzymes inactive.

2.2 Copper Toxicity by Iron-Sulfur Cluster Damage

First, it was demonstrated that the intracellular free concentration of copper, the Fenton reagent in the oxidative damage concept, is in the zeptomolar (10^{-21} M) range [7]. Intracellular copper, which appears to always be in the Cu^+ form, binds to sulfhydryl-bearing proteins, amino acids, and small molecules like glutathione (GSH). This, in combination with the copper-homeostatic machinery which pumps copper out of the cell, keeps the intracellular concentration of water-coordinated and thus reactive copper vanishingly small. Secondly, it was convincingly shown for *Escherichia coli* that the primary cause of copper toxicity is not the generation of hydroxyl radicals, but the displacement of iron by copper from the iron-sulfur clusters ([4Fe–4S] clusters) of important enzymes [8]. This is a thermodynamically favorable reaction because Cu^+ is a softer Pearson acid than Fe^{2+} and can thus effectively compete with iron for the cysteine ligands of iron-sulfur clusters (see Chap. 1). Primary targets of copper-iron exchange were dihydroxy-acid dehydratase of branched-chain amino acid synthesis, isopropylmalate isomerase involved in leucine biosynthesis, and fumarase A. The toxicity mechanism did not appear to be influenced by aerobic or anaerobic growth conditions.

Similar damage to iron-sulfur clusters was also demonstrated in the Gram-positive bacterium *Bacillus subtilis*. It was found that Cu^+ damaged the iron-sulfur cluster of SufU, which serves as the major scaffold for iron-sulfur cluster assembly and transfer to target proteins [9]. No significant induction of the PerR regulon, involved in oxidative stress defense, was observed under these conditions, indicating that oxidative stress did not play a major role. In *E. coli* grown under anaerobic conditions and amino acid limitation, but without exogenously added copper, endogenously liberated copper ions were found to damage the iron-cluster enzyme fumarate reductase and iron-cluster biogenesis [10]. These growth conditions also led to the induction of the alternative Suf system for iron cluster biogenesis and the CusCFBA copper transporter which pumps copper out of the periplasmic space (see Chap. 4). In *Rubrivivax gelatinosus* defective in the cytoplasmic copper exporter, CopA, and grown under microaerobic or anaerobic conditions in the presence of copper, a substantial decrease of cytochrome *c* oxidase and the photosystem was observed [11]. This led to reduced cytochrome oxidase and photosystem biogenesis, but also to coproporphyrin III extrusion from cells.

All these studies are in support of the newly emerged concept that copper toxicity is primarily due the poisoning of iron-sulfur cluster proteins by copper rather than oxidative damage. This raises questions about the applicability of the well-established in vitro Fenton redox processes of aqueous copper to the physiological regime. The concept of iron cofactor-displacement is further supported by the toxicity mechanisms of other heavy metal ions. Cd^{2+}, Ag^+, Zn^+, and Hg^{2+} have in common with copper that they have a soft Pearson character (high thiophilicity [12]), and thus also efficiently compete iron out of iron-sulfur clusters [13]. Cd^{2+}, Ag^+, and Zn^+ are not redox active and can thus not catalyze Fenton-type reactions. In line with this copper toxicity concepts, Park et al. showed that intracellular hydroxyl radical levels are not

significantly changed by the addition of Cu^{2+} to *E. coli* [14]. Rather, the biocidal action of Cu^{2+} is attributable to the cytotoxicity of cellularly generated Cu^{+}, which does not appear to be associated with oxidative damage by Cu(I)-driven ROS. Cu^{+} is considerably more toxic to cells than Cu^{2+} due to its higher thiophilicity and thus higher avidity for sulfhydryl residues of proteins, but also the higher permeability of the cytoplasmic membrane for Cu^{+} than Cu^{2+} [15].

The concept of metal toxicity by replacement of iron in iron-sulfur clusters is further supported by the finding that cobalt stress also affects the function of iron-sulfur cluster proteins. Exposure of *E. coli* to cobalt resulted in the inactivation of the three iron-sulfur cluster enzymes tRNA methylthiotransferase, aconitase, and ferrichrome reductase. However, cobalt did not directly displace the iron from these protein. Rather, cobalt affected the iron-sulfur cluster assembly machinery *via* the scaffold proteins SufA and IscU, in which the iron-sulfur clusters are more labile [16]. Co^{2+}, being an intermediately-soft Pearson acid like Fe^{2+}, can successfully compete with iron for thiolate binding sites, particularly if it is present in excess.

In a transcriptomics study on Cd^{2+} toxicity in *E. coli*, the following genes/functions were observed to be upregulated: disulfide bond repair, oxidative damage repair, cysteine and iron-sulfur cluster biosynthesis, proteins with iron-sulfur clusters, iron storage proteins, and cadmium resistance proteins; general energy conservation pathways and iron uptake were down-regulated [17]. These findings are in line with the concept that Cd^{2+}, like Cu^{+}, effectively competes with iron for sulfur ligands. Released iron would then result in down-regulation of iron uptake and upregulation of iron storage proteins.

The concept of iron displacement from iron-sulfur cluster proteins does not preclude intracellular ROS generation. Indeed, displacement of iron from iron–sulfur clusters leads to increased cytoplasmic Fe^{2+}, which can catalyze Fenton chemistry. Copper ions induce the *soxRS* regulatory system of *E. coli* under aerobic conditions, indicating the generation of ROS, and this SOS response system can apparently cope with the resulting oxidative stress; hypersensitivity to copper is only observed in mutants deficient in superoxide dismutases or repair enzymes for oxidative DNA damage [18].

Possible alternative routes of copper toxicity include the occupation of zinc or other metal sites in proteins, and unspecific binding to proteins, lipids, and nucleic acids. Given the diversity of the bacterial community, there will be many variations to the scheme of copper toxicity, but the frequently stated copper-induced oxidative damage concept currently falls short of explaining bacterial copper toxicity. For an exhaustive discussion of metal ion toxicity in general, see Ref. [19].

2.3 Glutathione and Copper Toxicity

The small tri-peptide γ-L-glutamyl-L-cysteinylglycine, or glutathione (GSH), is present in the cytoplasm of all eukaryotes. In the prokaryotic world, GSH is absent in most Actinomycetes, which contain mycothiol instead [20]. GSH is present in many

other Gram-positive bacteria and in most Gram-negative ones [21]. While most bacteria synthesize GSH in the cytoplasm, some take it up from the environment [22]. Some bacteria devoid of GSH contain bacillithiol or γ-L-glutamyl-L-cysteine instead [23, 24]. GSH is in equilibrium with its oxidized, dimeric form, GSSG, but GSH is the predominant form inside the cell. In *E. coli*, GSH/GSSG is the main redox couple that helps to maintain the cytoplasmic redox potential in growing cells at about −240 mV [25]. It is reasonable to assume that all microorganism contain a small-molecular weight thiol that can function similar to GSH.

GSH strongly complexes a variety of metal ions and has been shown to have a protective role in heavy metal stress in a number of organisms (see Chap. 3). However, recent work shows that under anaerobic conditions, GSH enhances copper toxicity in *Lactococcus lactis*. To specifically address detoxification independent of ROS formation, Obeid et al. [26] used an *L. lactis* strain which could neither synthesize GSH nor import it from the culture medium. GSH synthesis could be activated in this strain by inducing GSH biosynthetic enzymes from a plasmid [27]. Under anaerobic, fermentative conditions, GSH rendered *L. lactis* more sensitive to copper, particularly during the first phase of exponential growth [26]. It was proposed that GSH binds copper and facilitates its delivery to metal binding sites of enzymes, such as to iron-sulfur clusters. Such a mechanism would further support the hypothesis that metal sites of enzymes are the primary target of copper toxicity, as described in Sect. 2.2. That GSH-metal ion complexes facilitate the metallation of enzymes has been well documented in vitro [28–30]. These findings differ from those obtained under aerobic conditions, where GSH has been shown to exert a protective effect against copper toxicity both, in Gram-positive and Gram-negative organisms (see Chap. 3).

2.4 Copper Toxicity in Contact Killing

Bacteria are killed on dry copper surfaces in minutes to hours, a process referred to as contact killing [31]. The process has received considerable attention in recent years because copper could be used for critical touch-surfaces to curb the spread of infections [32]. There have been several hospital studies and the results look promising, but further work is needed [33–36]: Other applications of copper that appears attractive include the coating of surfaces of implants to avoid periprosthetic infections [37] or the use of copper-impregnated fabrics in health care settings [38].

There is now substantial insight into the mechanism of contact killing and it has become clear that the contact-killing process follows principles different from those in killing or growth arrest of bacteria by copper ions in suspension or in culture. Contact-killing as it is understood today proceeds as follows [31, 39, 40]:

(1) Copper dissolves from the copper surface and mM concentrations accumulate in the limited aqueous space within minutes.
(2) Severe membrane damage occurs and the cytoplasm is flooded with copper ions.

(3) Cytoplasmic content is lost and copper inhibits most metabolic activities.
(4) Genomic and plasmid DNA gets degraded by unknown mechanisms.
(5) No structurally intact bacteria can usually be detected after exposure to metallic copper.

These dramatic changes are in stark contrast to inhibition experiments in culture, where the bacteria are under growth conditions and thus have an energy supply to combat copper entry and repair some cell damage. Also, cells exposed to copper in culture do not appear to structurally disintegrate.

Finally, the meaning of "copper concentration" must be considered. In contact killing, there are usually no copper-binding growth media components around, so the copper liberated from a copper surface will lead to very high 'free' copper concentrations. Copper avidly binds to growth media components, so the 'free' or active copper concentration in *culture* is not anywhere near to the 'added' copper concentration. Estimates from our lab suggest that if 5 mM $CuSO_4$ is added to standard LB growth media, the free copper concentration is <10 μM, and is further lowered with increasing cell density (M. Solioz, unpublished observation). There are two corollaries to this: first, it is virtually impossible to compare copper 'concentrations' between labs and, secondly, it is impossible to compare copper concentrations between contact killing experiments and inhibition experiments in culture.

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