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How Bacteria Handle Copper

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1 Introduction: Copper and Life

2 The State of Cytoplasmic Copper

3 Copper Homeostasis in *Escherichia coli*
3.1 CopA Copper ATPase
3.2 CueR Copper-Responsive Regulator
3.3 CueO Periplasmic Multi-Copper Oxidase
3.4 Cus Copper Efflux System
3.5 Copper Biosensors
3.6 Copper Regulons

4 Plasmid-Borne Copper Resistance

5 Copper Homeostasis in *Enterococcus hirae*
5.1 Intracellular Routing of Copper by CopZ
5.2 Proteolytic Degradation of CopZ
5.3 Regulation of the *cop* Operon
5.4 CopZ-Interacting Protein Gls24
5.5 CopA Copper ATPase
5.6 CopB Copper ATPase

6 Chalkophores

7 Conclusion and Outlook

References

Abstract Copper in biological systems presents a formidable problem: it is essential for life, yet highly reactive and a potential source of cell damage. Tight control of copper is thus a cellular necessity. To meet this challenge, cells have evolved pumps for transmembranous transport, chaperones for intracellular routing, oxidases and reductases to change the oxidation state of copper, and regulators to control gene expression in response to copper. These systems are complemented by specific mechanisms for the insertion of copper into enzymes. Copper homeostasis has evolved early in evolution and some components have been conserved from bacteria to humans. This has allowed researchers to apply knowledge across phyla and even involving human copper homeostatic diseases to elucidate the fundamental mechanism of cellular copper homeostasis. After an introduction to the properties of copper and its role in biological systems, some of the best studied bacterial systems for copper homeostasis will be discussed.

1
Introduction: Copper and Life

Copper has been known since prehistoric times. Metallic copper was available in the Middle East around 3500 B.C. It was obtained by reduction of its ores with charcoal. The discovery, some 500 years later, that the addition of tin to copper produced a much harder metal, established the Bronze Age. Copper has continued to this day to be an important metal to human kind. The abundance of copper in the earth's crust amounts to 68 ppm. It occurs mainly as the sulfide, oxide, or carbonate. Its major ores are copper pyrite (chalcopyrite, $CuFeS_2$), copper glance (chalcocite, Cu_2S), cuprite (Cu_2O), and malachite ($Cu_2CO_3(OH)_2$) (Tylecote 1992).

In the primordial, anaerobic word, copper was in the Cu(I) state in the form of water-insoluble sulphides. The ensuing oxygen evolution by microorganisms, a process which started less than 3×10^9 years ago, was a dramatic event for most living organisms. It could be considered to be an early, irreversible pollution of the earth, to which most living organisms adapted by acquiring an oxidative metabolism. While enzymes involved in anaerobic metabolism were designed to operate in the lower portion of the redox spectrum, the arrival of dioxygen created the need for a new redox active metal that could attain higher redox potentials. The oxidation of insoluble Cu(I) led to soluble and thus more bioavailable Cu(II), which was ideally suited to exploit the oxidizing power of dioxygen (Crichton and Pierre 2001). Copper is thus a modern bioelement (Kaim and Rall 1996). Concomitant with the arrival of oxygen, multi-cellular organisms developed.

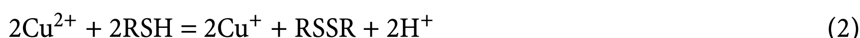
Today, over 30 types of copper-containing proteins are known, prominent examples being lysyl oxidase (involved in the crosslinking of collagen) tyrosinase (required for melanin synthesis) dopamine β-hydroxylase of the catecholamine pathway, cytochrome *c* oxidase, the terminal electron acceptor of the respiratory chain, and superoxide dismutase, required for defense against oxidative damage. Another class of copper proteins, such as plastocyanins or azurines, act as electron carriers. In redox enzymes, copper serves as an electron acceptor/donor by alternating between the redox states Cu(I) and Cu(II) (Karlin 1993). Depending on the type of coordination of the copper to the protein, the redox potential can vary over the range + 200 to + 800 mV.

The redox properties of copper can, on the other hand, also cause cellular damage. A number or mechanisms have been suggested. Reactive hydroxyl radicals can be generated in a Fenton-type reaction:

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

The extremely reactive hydroxyl radical can participate in a number of reactions detrimental to cellular molecules, such as the oxidation of proteins and lipids (Yoshida et al. 1993). Copper can also lead to depletion of sulfhydryls,

such as in cystein or glutathione, in a cycle between reactions 2 and 3:



$$2Cu^{2+} + 2RSH = 2Cu^{+} + RSSR + 2H^{+} \quad (2)$$

$$2Cu^{+} + 2H^{+} + O_2 = 2Cu^{2+} + H_2O_2 \; . \quad (3)$$

The hydrogen peroxide thus generated can participate in reaction (1) and lead to further generation of toxic hydroxyl radicals. It is not clear to what extent reactions 1–3 cause copper toxicity. Cells try to keep H_2O_2 at very low levels and reaction (1) may not be the chief toxic mechanism, although this has been frequently claimed. Alternative routes of copper toxicity include the occupation of zinc or other metal sites in proteins by competition, or unspecific binding to proteins, lipids, and nucleic acids.

The toxic effect of copper is utilized in agriculture for the control of bacterial and fungal diseases (Cha and Cooksey 1991). This has in fact lead to the first thorough investigation of bacterial copper resistance, which is described in a later section.

The same property that makes copper a valuable biometal, namely the redox cycling between copper(I) and copper(II), also complicates experimental work. Copper(I) is the preferred form of copper for handling by the cell. Transporters like copper ATPases or eukaryotic CtrI-type transporters appear to transport copper(I) and copper chaperones bind copper(I) for delivery to cuproenzymes (Finney and O'Halloran 2003). While free copper(II) ions are stable in neutral, aqueous solutions exposed to the atmosphere, free copper(I) ions can only be maintained at very acidic pH or in complexed form. Highly efficient chelators of copper(I) in vitro are *o*-phenanthroline, bathophenanthroline sulfonate or 8-hydroxyquinoline (reported formation constants for copper-phenanthroline complexes are 21, irrespective of the ligands on the phenanthroline dipyrimidine ring system (Bell et al. 1991)). Copper(I) complexes which are stable in air can be formed with acetonitrile, CN^{-}, Tris-buffer and other complexing agents (McPhail and Goodman 1984). Phenanthrolines complex Cu(I) (and Fe(II)) so strongly that they effectively raise the redox potential to a point at which any reducing equivalent can support the reduction of the higher valency metal ion. This reaction can be counteracted by high concentrations (20 mM) of citrate or lactate, which preferentially bind the oxidized form of copper. Another difficulty is the interaction of copper(I) or (II) with all biological molecules, buffer substances, standard reducing agents etc. For example if 2 mM copper is added to complex bacterial growth media, the concentration of free, bioavailable copper remains unknown. This makes it nearly impossible to compare experimental findings by different research groups in terms of copper concentration.

The use of copper(I) rather than copper(II) by copper transporters and chaperones may have thermodynamic reasons. The lower valency states of transition metals are more exchange labile, e.g., the water exchange rate for Fe(II) is $3 \times 10^{6}\ s^{-1}$ compared to $3 \times 10^{3}\ s^{-1}$ for Fe(III). Since most copper

in extracellular fluid or bacterial media is normally complexed to organic molecules, reduction of complexed Cu(II) would strongly facilitate the displacement of the ligand to which the metal ion is bound in the medium, making the metal more bioavailable.

These physico-chemical aspects have led to difficulties in experimentation with copper in biological systems which may have contributed to the delay in studying the homeostasis of this biologically important metal.

2 The State of Cytoplasmic Copper

Since copper is both essential and toxic for cells, they need to tightly control copper availability. To this end, bacteria use several strategies: access of copper to the cell is restricted by extracellular sequestration, relative impermeability of outer and inner bacterial membranes to copper, metallothionein-like copper scavenging proteins in the cytoplasm, and active extrusion of copper from the cell.

The cytoplasmic condition of cells is reducing. Glutathione (GSH) is present in millimolar concentrations and probably acts as the major reducing agent in prokaryotic and eukaryotic cells (McLaggan et al. 1990; Samuni et al. 1981). Other reducing compounds include cysteine or ascorbate. These reduce copper(II) to copper(I) and, in addition, avidly bind to copper. Thus, under normal conditions, cytoplasmic copper is in the reduced Cu^{+}-form and largely complexed by glutathione or other small molecules. Increased levels of GSH were observed in hepatoma cells treated with copper and inhibition of GSH synthesis with buthionine sulfoximine reduced the incorporation of copper into metallothioneins (Freedman and Peisach 1989b). It was also shown in vitro that Cu^{+}-GSH could mediate Cu^{+}-transfer into metal depleted metallothionein or copper-free Cu,Zn-superoxide dismutase (Ascone et al. 1993; Ciriolo et al. 1990; Ferreira et al. 1993). In vivo pulse-chase experiments with radioactive Cu^{+} revealed that Cu^{+} could also be transferred in the reverse direction from metallothionein to GSH and then to Cu,Zn-superoxide dismutase (Freedman and Peisach 1989a). These observations favor the view that GSH plays a key role in eukaryotic copper metabolism. However, inhibition of glutathione synthase in *E. coli* failed to alter the response of cells to copper, so it remains unclear if GSH plays as an important role in copper detoxification by prokaryotes as it does in higher cells.

In eukaryotic cells, resistance to copper toxicity is also influenced by the level of glutathione-peroxidase. An increased level of this enzyme was observed in copper-treated hepatoma cells, allowing efficient accommodation of increased cellular hydrogen peroxide concentrations caused by the oxidation of Cu^{+}-GSH or Cu^{+}-metallothionein (Freedman et al. 1989). To what

extent such a mechanism also operates in bacteria is again not known. In the following section, the best characterized bacterial copper-resistance systems (*Escherichia coli* and *Enterococcus hirae*) will be discussed in detail. Information about copper homeostasis in *Synechocystis* can be found in the chapter by Tottey, Harvie and Robinson.

3
Copper Homeostasis in *Escherichia coli*

In *Escherichia coli* multiple systems evolved for the safe handling of copper (Rensing and Grass 2003). A central component in the copper homeostasis of *E. coli* is the integral inner membrane copper-transporting P-type ATPase CopA, which ensures removal of excess Cu(I) from the cytoplasm. In the periplasmic space, both the multi-copper oxidase CueO and the multicomponent copper efflux system CusCFBA are responsible for the control of copper levels. Additionally, various *E. coli* strains harbor plasmid-encoded genes that increase considerably the copper tolerance, allowing growth in copper-rich environments (Rouch et al. 1985).

3.1
CopA Copper ATPase

The CopA copper-transporting ATPase is the core element of copper homeostasis in *E. coli* both under aerobic and anaerobic conditions (Rensing and Grass 2003) and its expression is regulated by CueR, a transcription activator induced by Cu(I) and Ag(I) (Outten et al. 2000; Petersen and Moller 2000; Stoyanov et al. 2001). The enzyme is an integral inner membrane protein with eight hydropathic transmembrane α-helices and shares common features with other P-type ATPases (Fig. 1). It has conserved ATP binding (GDGIN), phosphorylation (DKTGT), and phosphatase (TGE) domains (Solioz and Vulpe 1996). The cytoplasmic N-terminus harbors two metal-binding CxxC motifs. Interestingly, these motifs are not required for the function (Fan et al. 2001) and apparently do not confer metal-specificity (Rensing and Grass 2003). *E. coli* Δ*copA* that has a disrupted *copA* gene is less resistant to copper than the wild-type, and complementation with the gene on a plasmid restores tolerance (Rensing et al. 2000). Accumulation of ^{64}Cu in everted membrane vesicles derived from a strain overexpressing CopA suggests copper transporting activity. Uptake is inhibited by vanadate, a specific inhibitor of P-type ATPases. DTT, a strong reductant, was required, otherwise no transport could be detected. This suggests that copper is pumped in the form of Cu(I) out of the cell (Rensing et al. 2000). Additionally, it was also shown that CopA is able to transport silver (Stoyanov et al. 2003).

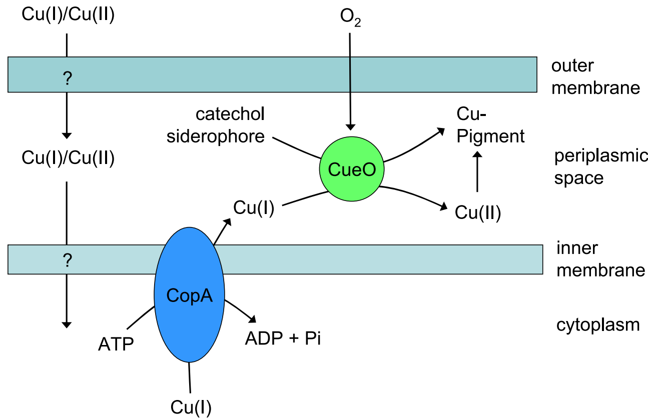


Fig. 1 The CopA ATPase and CueO oxidase of *E. coli*. CopA is an ATP-driven copper pump that expels copper(I) from the cytoplasm into the periplasmic space. There it can get oxidized by the multi-copper oxidase CueO. This enzyme can also oxidize catechol siderophores and the resulting pigments can sequester copper

3.2 CueR Copper-Responsive Regulator

CueR (for copper export regulator) belongs to the MerR-like transcription activators with an N-terminal helix-turn-helix DNA binding region, a C-terminal copper-binding region and a dimerization domain. Expression of *copA* and *cueO* (see below) are both regulated by CueR, but these genes are not organized in an operon. MerR-like regulators activate suboptimal σ^{70}-dependent promoters, in which the spacing between the – 35 and – 10 elements recognized by the σ factor is greater than the optimal 17 ± 1 base pairs. Therefore the conformation of the DNA has to be changed in order to allow access of the RNA polymerase for transcription initiation. The model suggests that there is a structural change of the MerR-like regulator upon binding of the effector molecule and this leads to a distortion of the DNA (Brown et al. 2003).

CueR is also activated by silver and, interestingly, by gold (Stoyanov et al. 2003; Stoyanov and Brown 2003). The basis of the ultrasensitivity of this regulator was further investigated by structural analysis. The sensitivity of CueR to free Cu(I) was determined to be 10^{-21} molar (zeptomolar), which corresponds to less than one copper ion per cell (Rae et al. 1999). The X-ray crystal structure revealed that the copper ion is buried in a solvent-inaccessible site in a loop at the dimer interface and has only two coordinating ligands: the sulfur atoms of the conserved Cys-112 and Cys-120. Mutation of these two residues makes CueR unresponsive to copper, silver and gold, which underscores the importance of these two residues (Stoyanov et al. 2001). The

unusual metal-receptor site restricts the metal to a linear, two-coordinate geometry and uses helix-dipole and hydrogen-bonding interactions to enhance metal binding (Changela et al. 2003) (see also the chapters by Tottey, Harvie and Robinson, and by Helman, Soosanga and Gabriel for a discussions of CueR and MerR-like regulators).

3.3 CueO Periplasmic Multi-Copper Oxidase

CueO is a multi-copper oxidase involved in copper detoxification. Expression of *cueO* (copper efflux oxidase) is regulated by CueR and together with the *copA* constitutes the Cue system. It has been shown that the *cueO* promoter region shares the same features as the *copA* promoter. A copper-dependent increase of β-galactosidase activity of *cueO* promoter-*lacZ* fusions suggested that *cueO* expression is induced by copper (Outten et al. 2000). Disruption of *cueO* rendered cells more sensitive to copper, which supports the assumption that CueO is involved in copper homeostasis (Grass and Rensing 2001). It was also shown that CueO protects alkaline phosphatase from copper-induced damage. The spectroscopic properties indicate that CueO is a multi-copper oxidase. Atomic absorption spectroscopy revealed four copper atoms per molecule.

In vitro assays show comparable properties CueO to related multi-copper oxidases such as Fet3 or laccases. CueO has phenol oxidase and ferroxidase activities. There is also evidence that CueO catalyzes the oxidation of enterobactin in the presence of copper. Enterobactin is a catechol siderophore of *E. coli*. A mixture of copper and enterobactin proved to be very toxic and lead to reduced survival of *E. coli* in the presence of copper. The addition of purified CueO had a positive effect on cell survival (Grass et al. 2004). Oxidized enterobactin can also sequester copper, contributing to the detoxification mechanism. CueO seems to also have cuprous oxidase activity (Singh et al. 2004). The activity is dependent on oxygen, thus CueO works only in aerobic conditions. Therefore, *E. coli* also relies on another copper homeostatic system—the *cusCFBA* operon—which makes cells more resistant to copper under anaerobic conditions (Outten et al. 2001).

The NADH dehydrogenase-2 (NDH-2) of the *Escherichia coli* respiratory chain has been reported to also possess cupric-reductase activity. NDH-2 deficient strains were more sensitive in their growth to high or low copper concentrations than wild-type cells. This suggests that NDH-2 helps to diminish the damaging effects of copper and/or oxidative stress on the respiratory chain and contributes to copper detoxification in *E. coli* (Rodriguez-Montelongo et al. 2006).

3.4 Cus Copper Efflux System

The Cus system of *E. coli* is a chromosomally encoded copper- and silver-resistance system consisting of a CusCBA proton-cation antiporter complex under the control of the CusRS two-component regulatory system (Fig. 2). Initial studies of the Cus (Ylc) system showed that this determinant mediates silver resistance (Franke et al. 2001). The *cusCBA* genes belong to a family of homologous transport complexes involved in the export of metal ions, xenobiotics and drugs (Nies 2003). *cusA* encodes an inner membrane protein belonging to the resistance nodulation cell division family (RND) of proteins. It is assumed that CusA functions as a secondary transporter energized by proton-substrate antiport and is responsible for the substrate specificity. *cusB* codes for a membrane fusion protein (MFP) which serves as an adaptor to link CusA with CusC, which is an outer membrane factor (OMF, Fig. 2) (Rensing and Grass 2003).

CusF is a 10-kDa periplasmic protein. Yeast two hybrid experiments suggested that CusF interacts with CusB and CusC and may function as a periplasmic copper chaperone. Complementation of a *cusF* null mutant with this mutated CusF protein did not restore copper tolerance of the cells to wild-type levels, emphasizing a function of CusF in copper tolerance (Franke et al. 2003). CusF with bound copper is pink and exhibits an absorption maximum at 510 nm. This unusual feature is a consequence of its unusual structure: CusF forms a five-stranded β-barrel, classified as an OB-fold, which is a unique topology for a copper-binding protein. NMR chemical shift mapping experiments suggest that one Cu(I) is bound by conserved residues His-36,

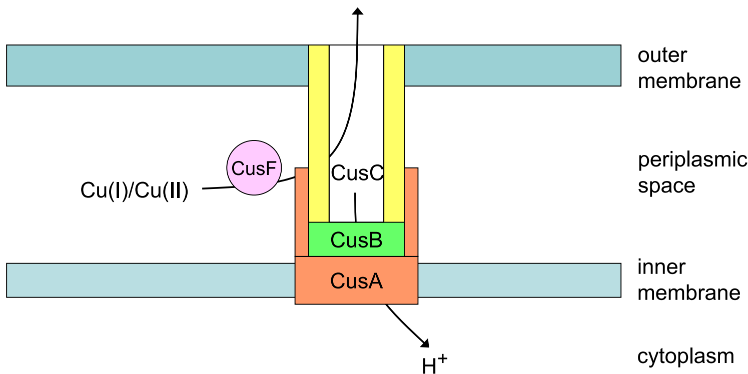


Fig. 2 The Cus system of *E. coli*. The Cus-complex consists of the inner membrane pump CusA, an integral membrane protein with 12 predicted transmembranous helices and two cytoplasmic loop, the periplasmic protein CusB, and the trimeric outer membrane protein CusC, which forms a channel bridging the periplasmic space. Entry of copper probably occurs from the periplasm via the copper chaperone CusF

Met-47, and Met-49 located in β-strands 2 and 3. These residues are clustered at one end of the β-barrel, and their side chains are oriented towards the interior of the barrel (Loftin et al. 2005). CusF thus represents a novel type of periplasmic copper chaperone.

Recently, the structure of OMF TolC, which is related to CusC, has been elucidated. It is a trimer that forms a trans-periplasmic channel embedded in the outer membrane (Koronakis et al. 2000). This suggests a model where CusA, B, and C interact to form a channel spanning the periplasm and connecting the cytoplasm to the extracellular space (Fig. 2). CusCBA belongs to a family of related transport systems that only occur in Gram-negative bacteria and that are involved in the export of metal ions, xenobiotics, and drugs. Such systems transporting various other metals have been characterized in *Pseudomonas*, *Cupriavidus*, *Synechococcus*, *Salmonella* and *E. coli* and are discussed in detail in Chap. 4.

Transcription of *cusCFBA* is regulated by the *cusRS* regulatory system that activates transcription of *cusCFBA* in the presence of copper (Munson et al. 2000). The *cusRS* operon is divergent to the *cusCFBA* operon and encodes a copper-responsive two-component signal transduction system (Franke et al. 2003). CusR is homologous to phosphate receiver response regulators and CusS is homologous to sensor histidine kinases. The closest homologs to CusRS are other two-component copper-regulatory systems: CusR has 61% sequence identity to both PcoR of *E. coli* and CopR of *P. syringae*, whereas CusS has 42% identity to CopS of *E. coli* and 38% identity to PcoS of *P. syringae*. The divergent cusCFB and cusRS operons are activated by the binding of CusR to a unique CusR box between the two promoters.

Recent experimental evidence suggests that the Cus system transports copper from the periplasmic space, rather than from the cytoplasm, to the extracellular environment (Franke et al. 2003). This is supported by the observation that related transporters such as AcrB or MexB can transport substrates that do not cross the cytoplasmic membrane and are thus restricted to the periplasmic space (Zgurskaya and Nikaido 2000). In contrast to the CueO/PcoA-type multi-copper oxidases that require oxygen for function, the CusCBA copper efflux system can work under anaerobic as well as aerobic conditions. It was recently shown that mutation of the Cus system strongly affected copper sensitivity under anaerobic conditions, while both *cueO* and *cusCFBA* had to be mutated to affect copper sensitivity in aerobic conditions (Outten et al. 2001).

A surprising additional function of CusC (IbeB) from *E. coli* K1 was recently described. *E. coli* K1 is the most common Gram-negative organism causing neonatal meningitis. Invasion of brain microvascular endothelial cells is a prerequisite for penetration into the human central nervous system. It was shown that CusC is an important contributing determinant in the process (Huang et al. 1999; Wang and Kim 2002). However, it is currently not apparent how CusC functions in bacterial penetration.

3.5 Copper Biosensors

A reporter plasmid was constructed by inserting the *E. coli* CopA promoter into a plasmid containing the *lux* gene cluster of *Vibrio fischeri* without a promoter (Rogowsky et al. 1987; Stoyanov et al. 2003). *E. coli* cells containing the biosensor exhibited luminescence in response to externally added $CuSO_4$, $AgNO_3$, or $HAuCl_4$. As expected, the biosensor in wild-type cells and in an *E. coli* strain deleted in the CopA copper-exporting ATPase (Δ*copA*) responded differently to metal ions. In a Δ*copA* strain, an eight-fold lower concentration of silver and a 15-fold lower concentration of copper (1 μM), compared to wild-type, was required to elicit maximal luminescence (Stoyanov et al. 2003). At the same time, overall luminescence intensities were enhanced five to seven fold. The more sensitive and more pronounced response of luminescence to copper and silver in the Δ*copA* strain is supposedly due to increased intracellular levels of these ions. This suggests that the homeostatic controls of both copper and silver is impaired in the Δ*copA* strain and that CopA not only effluxes copper ions, but also silver ions. Since the expression of the CopA ATPase is also regulated by silver via the CueR activator (Stoyanov et al. 2001), this appears to represent a true homeostatic control system for silver. Indeed, it could be shown that wild-type cells were more silver-resistant than Δ*copA* cells (Stoyanov et al. 2003). The wild-type still grew at half the maximal growth rate in 0.2 μM Ag^+, which fully inhibited growth of the Δ*copA* strain. The biosensor also responded to gold, with maximal activation observed in 30–40 μM gold. However, there was no significant difference in sensitivity and intensity of the luminescence response between wild-type and Δ*copA* cells. This argues against a participation of the CopA ATPase in gold homeostasis.

A similar biosensor with the *lux* genes under the control of the *E. hirae cop* promoter and carrying the corresponding CopY repressor and CopZ chaperone genes of *E. hirae* was also introduced into an *E. coli* Δ*copA* strain. In this case, half-maximal luminescence was reached at 5 μM extracellular copper (Portmann et al. 2004). It appears that a Δ*copA* strain is completely devoid of a cytoplasmic copper export mechanism. Thus, copper concentrations in the μM range may well represent physiological, cytoplasmic copper concentrations (see also the chapter by Harms for an extended discussion of biosensors).

3.6 Copper Regulons

Our knowledge of copper homeostasis in *E. coli* is still limited, and experimental evidence suggests that it is mainly maintained by controlling the export of excess copper out of the cell. A systematic search for copper-

responsive genes by using the DNA microarray technique was initiated with the aim to better understand the genome-wide response to copper. It was found that 29 genes were specifically and significantly affected by copper. These genes are organized into a hierarchy of the regulation network and form at least four regulons (Yamamoto and Ishihama 2005):

CusR regulon – DNA array experiments confirmed the activation of the *cusCFB* and *cusRS* operons by copper. CusR, the response regulator, binds to the CusR box with the inverted repeat AAAATGACAANNTTGCATTTT. This sequence was only found between the divergent *cusCFBA* and *cusRS* operons. In a CusR-defective mutant, gene expression by copper was absent for only three genes, *cusC*, *cusF*, and *cusB*, which reside in the *cusCFBA* operon. First, this confirms that CusR regulates the transcription of the *cusCFBA* operon, and secondly it shows that no other gene is regulated by this regulator. Similar experiments with DNA microarrays confirmed the strong induction of the *cue* and *cus* systems by copper. Elevated copper concentrations also positively affected the transcription of regulons for superoxide stress response, iron homeostasis, and envelope stress (Kershaw et al. 2005).

CueR regulon – The MerR-like CueR activator binds to the CueR box in the *copA* and *cueO* promoters. An additional 129 CueR box-like sequences in the *E. coli* genome suggest other binding sites for CueR. Among the identified CueR sites, 74 are associated with known genes and the rest are found next to genes with unknown function. The *moa* operon encoding enzymes required for molybdopterin biosynthesis was also shown to be induced by copper. This is not surprising in the light of the recent discovery that molybdenum cofactor synthesis is linked to copper: a copper ion is bound to molybdopterin dithiolate sulfurs as an intermediate in the biosynthetic pathway (Kuper et al. 2004).

CpxR regulon – The CpxR regulon is mainly responsible for the response to cell envelope stress (Raivio and Silhavy 2001; Ruiz et al. 2006). *CpxP* and *spy* are both markedly induced by copper and are under the control of the CpxA-CpxR two-component system. Transcription of 11 genes (*cpxP, spy, JW1832, ybaJ, yccA, ycfS, ydeH, yebE, yecI* and *yqjA*) was also strongly upregulated by copper. In the *cpxAR* null-mutant, these gene products cannot be detected. The promoter from these ten genes all carry the proposed CpxR recognition site GTAAANNNNNGTAAA.

YedW regulon – Microarray experiments revealed that the two-component system *yedVW* is induced considerably by copper. In the CusR-null mutant no activation by copper could be shown, but interestingly, no CusR box-like sequence is located in the promoter region of *yedVW*. YedV is thought to be a sensor kinase and YedW the corresponding response regulator. Recently, it was shown that the YedV kinase transfers phosphate not only to its partner YedW, but also to CusR, and may also be important for the activation of CusR (Yamamoto and Ishihama 2005).

Extracytoplasmic function protein family sigma factor – The sigma factor of the extracytoplasmic function protein family, RpoE, is apparently important for full resistance to Zn(II), Cd(II), and Cu(II). Deletion of *rpoE* leads to a decrease in copper tolerance and promoter gene fusions of copper-resistance genes require lower concentrations of copper for optimum induction in the mutant strain. RpoE is involved in maintaining the integrity of periplasmic and outer membrane proteins (Ruiz et al. 2006). Promoter gene fusion showed that RpoE is induced by metals (Egler et al. 2005).

4 Plasmid-Borne Copper Resistance

A plasmid-borne copper-resistance system, identified in *Pseudomonas syringae* pathovar tomato, was one of the first bacterial copper-resistance systems that was studied in detail. The system was discovered in copper-resistant bacteria isolated from tomato cultures in Southern California that had been sprayed with copper sulfate for disease control (Mellano and Cooksey 1988b). The copper resistance of these bacteria is conferred by the *copABCDRS* operon on plasmid pPT23D. It is homologous to the Pco system on plasmid pRJ1004 of *E. coli*. It was isolated from a strain from the gut flora of pigs fed a copper-enriched diet to promote growth (Tetaz and Luke 1983). Gene clusters similar to *pcoABCDRS* (and to *cop* of *P. syringae*) operons can be identified in the genomes of many bacteria. Often only homologues of PcoA and PcoB are encoded on a genome, suggesting that CopCD is an accessory system required for maximal resistance. The following discussion of the function of the *pco* system of *E. coli* in most parts also applies to the *P. syringae copABCDRS* system and related operons of other bacteria.

The *pco* gene cluster of the pPT23D plasmid of *E. coli* contains, in addition to the *pcoABCDRS* operon present in many organisms, also the *pcoE* gene, which is transcribed from its own promoter (Brown et al. 1995; Cooksey 1994; Rouch and Brown 1997). Radioactive ^{64}Cu transport measurements with whole cells showed that resistant cells containing the *pco* determinants exhibited decreased copper accumulation during logarithmic growth. It was suggested that an energy-dependent copper efflux mechanism is associated with the *pco* copper-resistance system of plasmid pRJ1004 (Brown et al. 1995). However, the observed decrease in copper accumulation could also have been due to diminished uptake. Thus, the molecular mechanism of copper resistance by the *pco* and the related *P. syringae cop* systems is still not fully understood (see also below).

Copper resistance directed by the *E. coli pco* (and *P. syringae cop*) operons have been shown to be copper inducible (Mellano and Cooksey 1988a; Rouch et al. 1985). A two-component regulatory system, PcoRS, induces transcription of the *pcoABCDRS* operon, where PcoR acts as a transcriptional activator

that binds to DNA (Mills et al. 1994). PcoS appears to be a periplasmic histidine kinase that senses copper. The *pcoE* gene is transcribed from a separate promoter which is also under the control of PcoRS (Rouch and Brown 1997). Both promoters are also induced by copper in the absence of the plasmid-borne *pcoRS* two-component regulatory system due to the presence of a second, related two-component regulatory system on the chromosome, named cusRS, which is also required for the copper-dependent expression of the cusCFBA periplasmic efflux system (Franke et al. 2003; Munson et al. 2000).

A different type of regulation was identified for the *pco*-like, plasmid-borne copper-resistance system of *Xanthomonas axonopodis* pv. vesicatoria. Instead of the CopRS two-component regulatory system, a novel protein, CopL, was found to regulate copper-inducible expression of the downstream multi-copper oxidase CopA in *X. axonopodis*. CopL encodes a histidine and cysteine-rich 130-kDa protein. Inactivation of *copL* caused a loss of transcriptional activation by copper, which could not be complemented by the *E. coli cusRS* or the *P. syringae pcoRS* systems. This suggests that CopL is a novel transcriptional activator and shows an interesting divergence in the mechanisms of regulation of the copper resistance by Pco-like systems.

Recently, progress has been made in understanding the function of some of the components of *cop* and *pco* proteins involved in conferring copper resistance (Fig. 3). PcoA of *E. coli* is a 66-kDa protein and appears to be a multi-copper oxidase similar to CueO of *E. coli* and CopA of *P. syringae*.

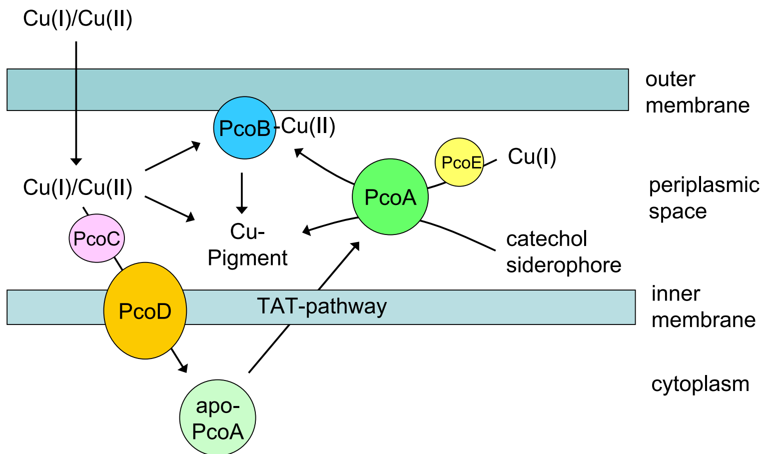


Fig. 3 The Pco/Cop systems of *E. coli* and *P. syringae*. Copper enters the periplasmic space by an unknown pathway. In the periplasm, PcoC shuttles copper to PcoD, which could provide the necessary copper for the PcoA multi-copper oxidase, but the copper may not necessarily transit through the cytoplasm in the process. Periplasmic copper can be detoxified by binding to oxidized catechol siderophores, generated by the PcoA oxidase, or to the outer membrane protein PcoB. PcoE shuttles copper Cu(I) to PcoA for oxidation to less toxic Cu(II)

PcoA exhibits oxidase activity (Huffman et al. 2002) and may, in analogy to CueO, function in copper detoxification by oxidizing Cu(I) to less toxic Cu(II) and/or by oxidizing catechol siderophores, such as enterobactin, which can then sequester copper (Fig. 1). Indeed, the formation of brown colonies by *E. coli* harboring the Pco-plasmid pRJ1004 and grown in the presence of $CuSO_4$ has been reported more than two decades ago (Tetaz and Luke 1983). Interestingly, *P. syringae* harboring the *copABCDRS*-system turn bright blue when grown in the presence of copper. The similarity of the function of PcoA and CueO is underlined by the observation that PcoA could functionally complement *E. coli cueO* deletion strain (Rensing and Grass 2003). Also similar to CueO, PcoA possesses a arginine-arginine motif in its leader sequence and is probably exported through the cytoplasmic membrane by the TAT-pathway.

PcoB is a 33-kDa protein, predicted to be localized in the outer membrane. PcoA and PcoB together confer higher copper resistance than PcoA alone, indicating that they act in concert, but the exact function of PcoB remains elusive. Its most feasible function at the current state of knowledge is that of a copper sequestration protein to buffer periplasmic copper excess.

PcoC is a 12-kDa periplasmic protein (Huffman et al. 2002; Lee et al. 2002). Recent structural studies showed that PcoC can bind copper(I) as well as copper(II) concomitantly (Zhang et al. 2006). A closely related protein from *Bacillus subtilis*, YcnJ, appears to be a fusion of two proteins, one homologous to PcoC and one homologous to PcoD. This observation and the biochemical properties of PcoC from *P. syringae* suggests that PcoC serves as a copper chaperone, delivering copper to PcoD. A PcoC homologue, YobA, is encoded by the *E. coli* chromosome but its function is unknown.

PcoD is a 34-kDa cytoplasmic membrane protein with eight predicted transmembranous helices. A homologue of PcoD (YebZ) with unknown function can be predicted from the *E. coli* genome sequence.

PcoE, which is transcribed from its own promoter, is a small periplasmic protein that is required for full copper resistance. It is related to SilE, which is a silver binding protein of the plasmid-borne *sil* silver-resistance system (Gupta et al. 1999). PcoE can bind copper and expression of PcoE by itself leads to copper accumulation in the periplasm. Likely, PcoE functions as a periplasmic copper chaperone (Rensing and Grass 2003).

5 Copper Homeostasis in *Enterococcus hirae*

Starting with the discovery of the two copper ATPases in *E. hirae* in 1992, copper homeostasis has been systematically elaborated in this Gram-positive bacterium. It possesses a *cop* operon that is required for copper homeostasis and which consists of the four genes *copY*, *copZ*, *copA*, and *copB*. *copY* en-

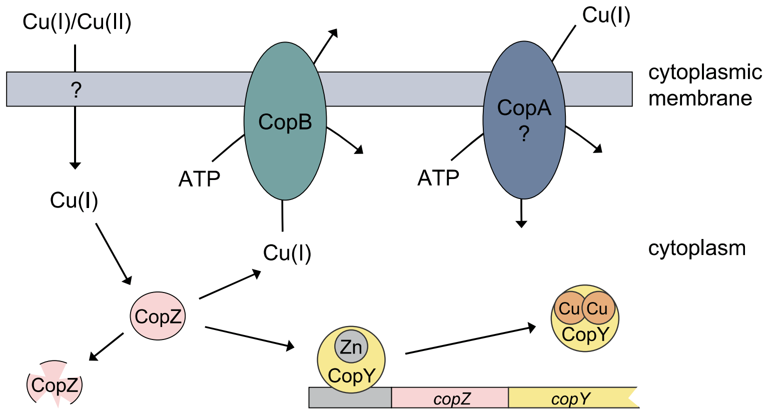


Fig. 4 Copper homeostasis in *E. hirae*. Under high copper conditions, copper enters cells by unknown pathways. Inside the cell, CopZ picks up copper(I) and can deliver it to CopB for expulsion and to the CopY repressor to induce transcription of the *cop* operon. CopY in its zinc form binds to the promoter and represses transcription. Two copper(I) can replace the zinc(II) in CopY, thus abolishing DNA binding and inducing transcription. If intracellular copper is excessive, CopZ is degraded by a copper-activated protease. CopA is a putative copper uptake ATPase under copper-limiting conditions

codes a copper-responsive repressor, *copZ* a copper chaperone, and *copA* and *copB* code for CPx-type copper ATPases. Figure 4 depicts the current working model of copper circulation in *E. hirae*.

copA and *copB* were the first genes encoding copper ATPases to be described (Odermatt et al. 1992). The function of CopB in copper excretion had been shown by direct demonstration of $^{64}Cu^{+}$ as well as $^{m110}Ag^{+}$ transport (Solioz and Odermatt 1995). The evidence for CopA being involved in copper uptake is still indirect. It rests on the following four properties of a *copA* knock-out strain: (i) it grows like wild-type under normal or elevated copper conditions, (ii) it is more silver resistant than the wild-type, presumably because CopA can be a route for silver entry into the cell, (iii) it is not deficient in silver extrusion from the cytoplasm, and (iv) cytoplasmic copper accumulation in high copper is similar to that of wild-type (Odermatt et al. 1993, 1994). However, it cannot be ruled out at the present time that CopA has another function.

5.1 Intracellular Routing of Copper by CopZ

Intracellular routing of copper is accomplished by copper chaperones, specialized proteins which deliver copper intracellularly to copper-utilizing enzymes (Harrison et al. 2000; O'Halloran and Culotta 2000). In *E. hirae*, the 69-amino-acid protein CopZ has been shown to function as a chaperone and

to specifically deliver copper to the CopY repressor. In its zinc form, the CopY repressor binds to the *cop* promoter and represses transcription of the four *cop* genes, *copY*, *copZ*, *copA*, and *copB*. When CopZ donates copper to CopY, its bound zinc is displaced by copper and the repressor dissociates from the promoter, allowing expression of the downstream genes (Cobine et al. 1999, 2002a; Lu et al. 2003; Magnani and Solioz 2005) (see also below).

The solution structure of CopZ has been solved by NMR (Wimmer et al. 1999). It exhibits a $\beta\alpha\beta\beta\alpha\beta$ global structure: two α-helices laying on a four-stranded, antiparallel β-sheet, a structure colloquially called an "open-face sandwich". CopZ belongs to a conserved family of copper chaperones, which have been identified in yeast, plants, and higher cells (Huffman and O'Halloran 2001). The same structural element is also found in the N-terminal metal binding domain of heavy metal transporting ATPases in one to six copies, but the function in these enzymes remains unclear (Arnesano et al. 2002). A key element of CopZ and related copper chaperones and metal binding domains is the CxxC metal-binding motif located between the first β-sheet and the first α-helix. It binds copper(I) or silver(I) in a solvent-exposed binding site. The solution structure of CopZ allowed to make predictions about surface residues involved in the interaction with other proteins.

Using surface plasmon resonance analysis (Biacore apparatus), it was shown that CopZ also interacts with the CopA copper ATPase (Multhaup et al. 2001). The binding of CopZ to CopA was modulated by copper. Maximal binding of CopZ to CopA was observed in the presence of roughly stoichiometric amounts of copper(I) (10 μM) corresponding to a molar stoichiometry of 0.9 copper(I) per CopZ. The association rate k_a was $2.4 \times 10^3\ M^{-1}\ s^{-1}$ without added copper and was only slightly affected by copper. The dissociation rate k_d was $14 \times 10^{-3}\ s^{-1}$ and was strongly influenced by copper, with a decrease of up to 15-fold. This results in a 16-fold increase of the affinity constant K_D in the presence of 10 μM copper(I). It was also shown that mutating the CxxC copper-binding motif in the N-terminus of CopA to SxxS abolished the copper-induced decrease in the dissociation rate without significantly affecting the association rate. Thus, the CopZ chaperone can interact with the CopA ATPase in a copper-dependent manner. Preliminary studies showed that CopZ also interacts with the CopB copper ATPase.

5.2 Proteolytic Degradation of CopZ

Under high copper conditions, proteolysis of CopZ was observed. Levels of *copZ* mRNA increased with increasing ambient copper levels and reached a 1000-fold induction at 0.25 mM copper, as assessed by real-time quantitative PCR. However, CopZ protein increased only up to 0.5 mM copper and declined at higher copper concentrations, to become nearly undetectable at 3 mM copper (Lu and Solioz 2001). It was concluded that CopZ overexpres-

sion is toxic to cells, because growth of a strain overexpressing CopZ from a plasmid ceased to grow in 1.5 mM copper and was more sensitive to oxidative stress induced by to H_2O_2 or paraquat (Lu and Solioz 2001).

Proteolysis of CopZ could be demonstrated in vitro. When cytosolic extracts were mixed with purified CopZ, it was rapidly degraded. Cu(I)CopZ was more rapidly degraded than *apo*-CopZ, in line with the notion that the exposed copper on CopZ can be toxic. The serine protease inhibitors *p*-phenylmethylsulfonyl fluoride and *p*-aminobenzamidine inhibited the degradation of CopZ, while N-α-tosyl-L-lysine chloromethyl ketone and N-tosyl-L-phenylalanine chloromethyl ketone, which are also serine protease inhibitors, were without effect. The metallo-proteinase inhibitor *o*-phenanthroline also did not inhibit CopZ degradation. It was thus concluded that the protease-degrading CopZ is a serine-type protease. On zymograms, the CopZ degrading activity was tentatively identified as a protein of 58 kDa. This protein displayed the expected properties, namely activation by copper and inhibition by *p*-aminobenzamidine (Magnani and Solioz 2005).

5.3 Regulation of the *cop* Operon

CopY is a copper-responsive repressor that binds as a homodimer to two distinct regions in the *cop* promoter (Strausak and Solioz 1997). It contains one zinc per monomer, which plays an important structural and functional role. CopY has a modular structure with a DNA-binding and a copper-binding domain. The N-terminal part shows approximately 30% sequence identity to bacterial transcription repressors of β-lactamase genes such as MecI of *Staphylococcus aureus*, PenI of *Bacillus licheniformis*, or BlaI from an *S. aureus* transposon (Himeno et al. 1986; Suzuki et al. 1993; Wittman and Wong 1988). These are members of the family of "winged-helix" proteins, which form a winged-helix structure in the N-terminal DNA-binding domain. Winged-helix proteins belong to the superfamily of helix-turn-helix DNA-binding proteins (Gajiwala and Burley 2000; Portmann et al. 2006).

The C-terminal domain contains four cysteine residues in the arrangement Cys-x-Cys-x_4-Cys-x-Cys. The same consensus motif is also present in other copper-responsive transcriptional regulators such as MacI, a transcription factor for the Ctr1 and Ctr3 copper transporters of *Saccharomyces cerevisiae*, or ACE1 and AMT1, which regulate transcription of metallothionein (Dameron et al. 1993; Zhou and Thiele 1991).

When transcription of the *cop* operon is activated, Cu(I)CopZ transfers two copper ions per Zn(II)CopY monomer, displacing the single Zn(II) (Cobine et al. 2002b). This appears to lead to conformational changes and reduced affinity to the operator. The copper transfer reaction is rapid, for there was no stable complex detected by gel filtration (Cobine et al. 1999). Two copper ions are required to remove the zinc(II) ion bound per CopY monomer

and CopY has a higher affinity for copper than CopZ. Lysine residues 30, 31, 37, and 38 of CopZ appeared to be important for the interaction with the CopY repressor. MNKr2, the second CopZ-like copper-binding domain of the human Menkes copper ATPase, cannot donate copper to CopY in spite of its similar predicted structure. However, if four extra lysine residues are introduced into MNKr2 at positions corresponding to the lysine residues in CopZ, the resultant mutant molecule MNKr2K4 becomes competent in donating copper to CopY (Cobine et al. 2002a; Magnani and Solioz 2005). This "gain-of-function" mutation of MNKr2 delineates lysine residues as key features in the interaction of the CopZ chaperone with the CopY repressor and supports the concept that protein–protein interaction governs copper transfer from the chaperone to the repressor. Finally, the kinetic parameters of *apo*-CopZ and Cu(I)CopZ interaction with Zn(II)CopY were determined by surface plasmon resonance. The affinity for Cu(I)CopZ was 440-fold higher than that for *apo*-CopZ (Portmann et al. 2004).

There seems to be no cooperativity of CopY binding to the two different binding sites in the operator region, but the half-association concentration of CopY differs at the two sites: 5 nM for the upstream site and 2 nM for the downstream site. A common conserved motif consisting of an inverted repeat of the sequence TACAnnTGTA, the "cop-box", appears to be the binding site for CopY-like copper-responsive repressors. The *E. hirae* CopY has the same binding activity to the operator region of the *Lactococcus lactis* and *Streptococcus mutans cop* operons as to its endemic promoter. The equilibrium dissociation constant for Zn(II)CopY–DNA interaction in absence of copper was 1.7×10^{-10} and the dissociation rate of Zn(II)CopY was increased sevenfold in the presence of copper, showing that copper reduces the affinity of CopY to the repressor site (Portmann et al. 2004).

Previously, it was reported that mutations of ACA to TCA at the positions A-61 and A-30 strongly reduces the affinity of CopY to the operator sequence highlighting the importance of these bases for the interaction of CopY with this operator region. However, only the double mutant completely abolished the interaction of CopY with the operator region and lead to hyperinduction of the *cop* operon by copper stimulation. When only one of the inverted repeats was mutated, the interaction of CopY still was allowed with the other site and the expression was not affected. The *cop* operon seems to be induced not only by higher copper concentrations, but also by low copper. The mechanism of this regulation is not known and seems to involve other components (Strausak and Solioz 1997; Wunderli-Ye and Solioz 1999).

5.4 CopZ-Interacting Protein Gls24

Using the yeast two-hybrid system with truncated CopZ as a bait, Gls24 was identified (Stoyanov and Solioz, unpublished). Gls24 belongs to the family of

Gls24-like stress response regulators (Giard et al. 2000), which may also have a role in pathogenicity (Teng et al. 2005). Gls24 was overexpressed and purified and was shown by surface plasmon resonance analysis to interact with CopZ in vitro. Cu(I)CopZ exhibited a high affinity for Gls24, with a K_D of 7.5×10^{-6} M. This affinity is five-fold higher than the affinity of Cu(I)CopZ for the CopY repressor or the CopA copper ATPase, which are both around 3.5×10^{-5} M. This suggests that the interaction between CopZ and Gls24 is biologically relevant. The expression of Gls24 was induced by glucose starvation and exposure of cells to copper. By NMR and circular dichroism, the protein appeared to contain unfolded domains.

Because of its interaction with Cu(I)CopZ, one could envision that ehGls24 functions as a protective cap. Copper bound to CopZ and other metallochaperones is in a solvent-exposed position at one end of the molecule. This copper could conceivably still exert toxic effects. A "cap" protein could protect the exposed copper in CopZ and prevent it from interacting in transit. While attractive, this concept remains hypothetical and requires experimental verification.

5.5 CopA Copper ATPase

For the in vitro study of CopA, an expression and purification scheme was developed. CopA was expressed in *E. coli* with a cleavable 6xHis tag. Membranes were extracted with dodecyl maltoside and CopA purified by Ni-affinity chromatography. The enzyme was better than 95% pure after a single chromatographic step, appeared fully active, and exhibited essentially the same properties with or without the His-tag (Wunderli-Ye and Solioz 2001).

CopA was reconstituted into soybean phospholipid vesicles by detergent dialysis. In proteoliposomes, ATPase activity was 300 to 400% of the starting activity. This suggested that the reconstituted ATPase was of high functional integrity and suitable to assess enzyme kinetic parameters. ATPase activity was maximal at pH 6.25, but exhibited a broad pH optimum in the range of pH 5.7 to 6.5. From kinetic analysis, a K_m for ATP of 0.2 mM and a V_{max} of 0.15 μmol/min/mg were derived (Wunderli-Ye and Solioz 2001). Turnover of CopA was slow in comparison to non-heavy metal ATPases, such as eukaryotic Ca^{2+} or Na^+/K^+-ATPases (Heyse et al. 1994; Pickart and Jencks 1984). Slow turnover was also observed for purified CopB of *E. hirae* and may be a general property of copper ATPases (Solioz and Camakaris 1997). CopA was fully active without added copper, presumably through the presence of contaminating copper ions (see below). Vanadate inhibited CopA with an I_{50} of 200 μM. The formation of an acyl phosphate intermediate could also be shown for CopA. The slow turnover of CopA is a major obstacle in the logical extension of this work, namely the measurement of copper transport by CopA in proteoliposomes.

5.6 CopB Copper ATPase

For the overexpression and purification of CopB, an engineered strain of *E. hirae* with a deletion of the CopY repressor was used. The enzyme was solubilized from the membranes with dodecyl maltoside and purified by Ni-affinity chromatography (Bissig et al. 2001b). Since CopB has a very histidine-rich N-terminus, it contains an "endogenous" His-tag and avidly binds to Ni-NTA resins.

The *E. hirae* CopB ATPase confers copper resistance by expelling excess copper and is related to the human ATPases *ATP7A* and *ATP7B* which are defective in Menkes and Wilson disease, respectively. Many mutations in these latter genes have been identified in patients (Gitlin 2003; Mercer 2001). Some mutations occur in highly conserved sequence motifs, which has allowed us to test their effect using the homologous CopB enzyme. The Menkes disease mutation C1000R changing the conserved CPC motif was mimicked in CopB as a C396S mutation. This mutant enzyme was unable to restore copper resistance in a CopB knock-out mutant in vivo. The purified mutant ATPase still formed an acyl phosphate intermediate, but had no detectable ATPase activity. The most frequent Wilson disease mutation H1069Q was introduced into CopB as H480Q. This mutant ATPase also failed to confer copper resistance to a CopB knock-out strain. Purified H480Q CopB formed an acyl phosphate intermediate and retained small, but significant ATPase activity. These results show that S396 and H480 of CopB are key residues for ATPase function and suggest similar roles for S1000 and H1069 of Menkes and Wilson ATPase, respectively (Bissig et al. 2001b). This work opened a new approach to test structure-function relationships in eukaryotic copper ATPases, which have so far not been purified.

An enigma had been the lack of activation of purified CopB by copper. We thus had hypothesized that there is sufficient contaminating copper in the buffers to fully activate the enzyme. Common copper chelators such as bicinchoninic acid or bathocuproine disulfonate did inhibit the ATPase activity, but only at concentrations in the high millimolar range. Tetrathiomolybdate avidly interacts with copper and has recently been employed to reduce excess copper in patients with Wilson disease (Brewer et al. 1994, 1996). It was found that tetrathiomolybdate inhibits CopB with an IC_{50} of 34 nM. Dithiomolybdate and trithiomolybdate, which commonly contaminate tetrathiomolybdate, inhibited the copper ATPases with similar potency. Inhibition could be reversed by copper or silver, suggesting inhibition by substrate binding. These findings for the first time allowed an estimate of the high affinity of CopB for copper and silver (Bissig et al. 2001a). Tetrathiomolybdate is thus a new tool for the study of copper ATPases.

6 Chalkophores

For iron acquisition, so-called siderophores are widely used in nature. They are extracellular iron-chelators that are secreted by cells. Their high complexing power for iron help microorganism acquire the scarce iron via uptake of the siderophore-iron complexes by specialized transport systems. Recently, evidence of analogous molecules for copper transport from methane-oxidizing bacteria has emerged. A fluorescent 1.2-kDa chromopeptide secreted by *Methylosinus trichosporium* OB3b was isolated and structurally analyzed (Kim et al. 2004). The molecule, which was called methanobactin, is composed of a tetrapeptide, a tripeptide, and several unusual moieties, including two 4-thionyl-5-hydroxy-imidazole chromophores that coordinate the copper, a pyrrolidine, and an amino-terminal isopropyl ester group. Copper is coordinated by a dual nitrogen- and sulfur-donating system derived from the thionyl imidazolate moieties. In methanotrophs, copper is required for the biosynthesis of methane monooxygenases and these organisms thus have a high demand for copper. They must therefore have an effective mechanism for acquiring copper from the environment. The secretion of a copper "siderophore" or chalkophore would support growth of the organism in a copper-limiting environment (Choi et al. 2006).

7 Conclusion and Outlook

There has been major progress in our understanding of copper homeostasis (and trace metal ion homeostasis in general) in the last decade. However, major questions still need to be tackled. The first unresolved question is that of the intracellular copper concentration and the experimental difficulty of distinguishing free, bioavailable copper from bound copper that is not available for participation in copper-homeostatic processes. Estimates for the concentration of free, intracellular copper range from 10^{-21} M (= less than one free copper ion per cell) based on thermodynamic calibration of the *E. coli* CueR copper response regulator of *E. coli* (Changela et al. 2003), through 3×10^{-8} M based on half-maximal activation of the purified CopB copper ATPase of *E. hirae* (Bissig et al. 2001a) to 10^{-6} M using the copper-responsive repressors CopY of *E. hirae* and CueR of *E. coli* in *lux*-based biosensors in vivo (Stoyanov et al. 2003). New tools such as the membrane-permeable, copper(I)-selective fluorescent indicator CTAP-1 could aid in defining the state of cytoplasmic copper (Yang et al. 2005).

A second unresolved question is that of the bacterial requirement for cytoplasmic copper. Known cuproenzymes like cytochrome *c* oxidase or superoxide dismutase are embedded in the cytoplasmic membrane and face

the periplasmic space. Copper loading of these enzymes during biosynthesis could occur with periplasmic copper. Currently, the only know system that suggests a need for cytoplasmic copper is the biosynthesis of the molybdenum cofactor (Kuper et al. 2004). Its synthesis involves the insertion of molybdenum into molybdopterin by the Cnx1 G-domain. The identification of copper bound to the molybdopterin dithiolate sulphurs in Cnx1G, coupled with the observation that copper inhibited Cnx1G activity, suggests a link between molybdenum and copper metabolism, which would require cytoplasmic copper (Schwarz and Mendel 2006).

The third unanswered question is that of how copper enters bacterial cells. It is not yet known what the pathways for entry across the outer and inner membranes are. Porins such as OmpC or OmpF have been proposed as entry pathways for copper and silver into the periplasmic space but isogenic OmpF and/or OmpC deficient mutants of *E. coli* did not differ significantly in copper or silver sensitivity (Egler et al. 2005; Li et al. 1997). It is also not clear how copper enters the cytoplasm. In higher cells, copper is taken up actively by CtrI-like transporters in the cytoplasmic membrane (Puig and Thiele 2002). No similar system has so far been discovered in bacteria. In *E. hirae*, CopA has been proposed to serve in copper uptake, but final proof of this hypothesis is lacking. Conceivably, copper enters the cytoplasm as substrate-copper complexes by substrate transporters such as sugar or amino acid transporters. Alternatively, transporters for other metal ions may be leak pathways for copper. Finally, the recent discovery of copper "siderophores", or chalkophores, in methanogenic bacteria opens entirely new avenues of copper entry into bacterial cells.

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