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Copper chaperone cycling and degradation in the regulation of the *cop* operon of *Enterococcus hirae*

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Abstract

Extensive insight into copper homeostasis has recently emerged. The Gram-positive bacterium *Enterococcus hirae* has been a paradigm for many aspects of the process. The *cop* operon of *E. hirae* consists of four genes that encode a repressor, CopY, a copper chaperone, CopZ, and two CPx-type copper ATPases, CopA and CopB. CopA and CopB accomplish copper uptake and export, respectively, and the expression of the *cop* operon is regulated by copper via the CopY repressor and the CopZ chaperone. The functions of the four Cop proteins have been extensively studied *in vivo* as well as *in vitro* and a detailed understanding of the regulation of the *cop* operon by copper has emerged.

Introduction

Copper serves as a cofactor for many enzymes due to its biologically suitable redox potential. However, copper excess can lead to toxic effects. Cellular copper levels must therefore be tightly regulated in all organisms. Copper homeostasis in *Enterococcus hirae* has been extensively studied and reviewed (Solioz 2001; Lu & Solioz 2002; Solioz & Stoyanov 2002, 2003; Wimmer *et al.* 2002; Lu *et al.* 2003). It is currently the best understood prokaryotic copper homeostatic system. It consists of four genes *copY, copZ, copA* and *copB*, arranged in the *cop* operon. The gene products of *copA* and *copB* are copper transporting ATPases, *copY* encodes a copper responsive repressor, and *copZ* codes for a chaperone which serves in intracellular copper routing (Odermatt *et al.* 1993; Odermatt & Solioz 1995). The *cop* operon allows growth of *E. hirae* in copper-limiting conditions as well as in up to 8 mM copper. According to the current model, CopA takes up copper when it is limiting, CopB extrudes excess copper, CopY regulates expression of the *cop* operon and CopZ transfers copper intracellularly, e.g. to the CopY repressor (Odermatt *et al.* 1994; Wunderli-Ye & Solioz 1999).

Evolution of copper ATPases

CopA and CopB belong to the P1 subclass of the P-type ATPases (Lutsenko & Kaplan 1995). Because of the conspicuous feature of the intramembranous CPC or CPH motif (sometimes also SPC) of heavy metal ATPases, it has also been suggested to call them CPx-type ATPases (Solioz & Vulpe 1996). The CPx motif is located in the middle of the predicted membrane helix 6 in the most conserved core structure of the enzyme and apparently constitutes part of the ion channel through the membrane. Copper ATPases have been surprisingly conserved from bacteria to humans. Recently, the oldest known microfossils have been discovered in 3.2 billion year old deep-sea volcanic rock (Rasmussen 2000). This suggests that the first living organisms on earth evolved around hydrothermal vents. The hot, acidic

seawater at these vents releases heavy metal ions (Zierenberg *et al*. 2000) and resistance to these metal ions would have been an evolutionary priority for early life. The first heavy metal ATPases may thus have served in the detoxification of the cellular cytoplasm. Early evolution of heavy metal ATPases is supported by the high conservation of these enzymes across phyla and the divergences of heavy metal and on non-heavy metal ATPases before the appearance of eukaryots (Figure 1). Also, copper was probably not an essential trace element for early, anaerobic life forms. Cuproenzymes function almost exclusively in reactions involving oxygen (Figure 2), which only became available by a process which started approximately 10^9 years ago. Copper would thus be a modern bioelement (Crichton & Pierre 2001).

Copper routing and regulation in *E. hirae*

Figure 3 shows an overview of the copper circulation in *E. hirae*. Current evidence indicates that cupric copper, Cu(II), is converted to the cuprous form, Cu(I), at the *E. hirae* cell surface before being imported into the cell by the CopA Cu(I)-ATPase (Wunderli-Ye & Solioz 1998). The reductase has not been molecularly characterized. Subsequent to coppers entry into the cell, it is routed in the cytoplasm by a specific carrier protein, the metallochaperone CopZ. The interaction of CopZ with the CopA ATPase has been characterized by surface plasmon resonance analysis (Multhaup *et al*. 2001). It has been hypothesized that Cu(I) entering the cell via the membrane portion of CopA is transferred to the N-terminal metal binding domain of CopA and subsequently passed forward to CopZ, which then routes the metal to the dimeric Zn(II)CopY (Fig. 3) (Cobine *et al*. 2002). CopY is a Zn containing homodimeric repressor (1 Zn^{2+}/17 kDa monomer) that binds to the promoter region of the *cop* operon, thereby repressing its own synthesis, as well as that of CopA, CopB and CopZ (Strausak & Solioz 1997). Sequence alignment and homology modeling as well as preliminary NMR data (K. R. Poulsen, unpublished) suggest that CopY belongs to the family of winged-helix type repressors (Gajiwala & Burley 2000). Other members of this family are the β-lactamase repressors MecI and BlaI (Melckebeke *et al*. 2003; Garcia-Castellanos *et al*. 2004; Wilke *et al*. 2004). Like most repressors, the CopY repressor is dimeric, with an apparent M_r of close to 50 000 by gel filtration analysis (D. Strausak and M. Solioz, unpublished), suggesting it is in an extended conformation as commonly found in this

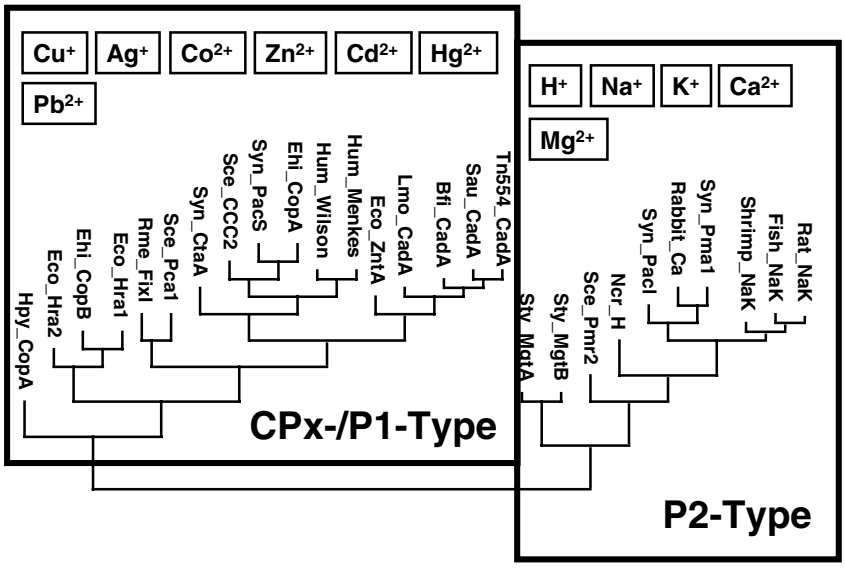

Figure 1. Unrooted phylogenetic tree of P-type ATPases. Divergence was scored for a selected sample of P-type and CPx-type ATPases by the Jukes–Cantor method (Swofford & Olson 1990). Relationships between distant branches are not reliable. The rectangles delineate the subgroups of heavy metal transporting CPx-/P1-type ATPases and the non-heavy metal transporting P2-type ATPases. The ion specificities known for each group are indicated in the two fields.

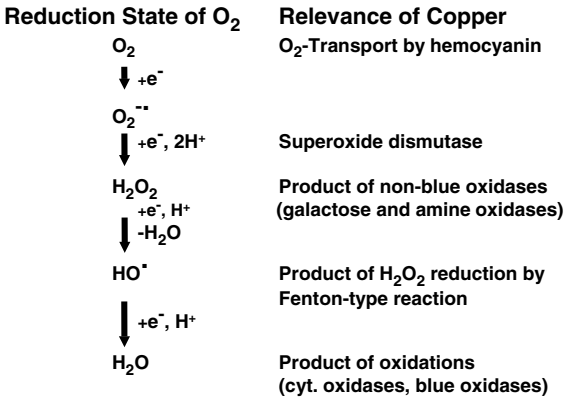

Figure 2. Examples of the involvement of copper at different reduction states of oxygen.

family, whereas it actual molecular weight should be $2 \times 17\,000 = 34\,000$ Da. Footprinting studies also support the proposal that the protein can have an extended conformation and further imply that the protein is a tail-to-tail dimer.

Copper transfer from the chaperone to the repressor

The copper transfer from Cu(I)CopZ to the CopZ repressor has been shown by a variety of techniques. By gel filtration analysis, it could be shown that in a mixture of Cu(I)CopZ and Zn(II)CopY, copper is quantitatively transferred to CopY and its zinc is released (Cobine *et al.* 1999). A copper-thiolate in a solvent-excluded environment displays luminescence. While Cu(I)-CopZ is not luminescent, $Cu(I)_2$CopY exhibits a strong luminescence. This shows that copper is transferred from a solvent exposed binding site on CopZ to a solvent excluded pocket in CopY. This copper transfer requires specific protein–protein interaction. The second metal binding domain of the Menkes copper ATPase (MNKr2) could not transfer copper to CopY, although it has a structure similar to CopZ. However, CopZ has one very basic surface formed by four lysine residues and the N-terminus, which is not present in MNKr2. If four lysine residues were inserted in similar positions into MNKr2, a gain-of-function mutant which could donate copper to CopY could be generated. This suggests that a basic surface on the chaperone is required for the interaction of the chaperone with the repressor. For the interaction of the related yeast Atx1 chaperone, it could similarly be shown that a basic surface on Atx1 interacts with an acidic surface on its target protein, the copper ATPase CCC2 (Rosenzweig 2001).

Repressor-DNA interaction: the cop-box

Expression of the *cop* operon is low in standard growth media but can be induced up to 50-fold by exposing the cells to extracellular copper.

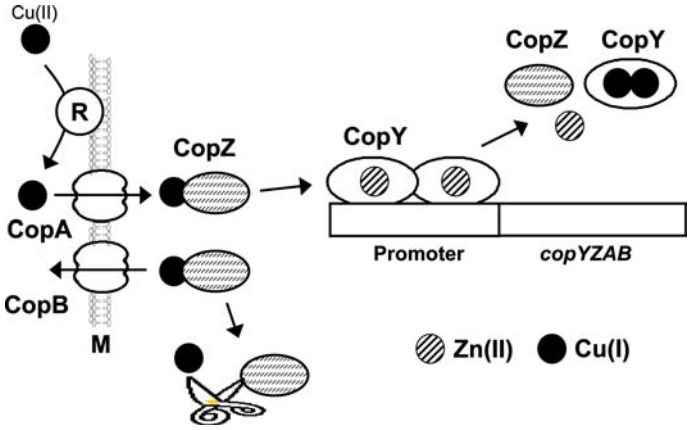

Figure 3. Model of copper circulation in *E. hirae*. A reductase (R) at the cytoplasmic membrane (M) reduces Cu(II) to Cu(I), which can be taken up by CopA. Copper is then transferred to the CopZ chaperone, which in turn donates it to the CopY repressor. Two Cu(I) per CopY monomer are transferred to the repressor, thereby releasing the bound zinc and releasing CopY from the promoter. This allows transcription of the four *cop* genes to proceed. Under high copper conditions, excess CopZ is degraded by a copper-activated protease.

The CopY repressor binds to the consensus binding site TACANNTGTA, which was termed the 'cop-box' (Portmann *et al*. 2004) and suppresses transcription if no excess copper prevails in the cytoplasm. Mutation of the cop-box by site-directed mutagenesis abolished the interaction with the repressor. Cop-boxes are also found in related bacteria such as *Streptococcus mutans, Lactococcus lactis, or Lactobacillus sakei*. All these organisms also contain CopY homologues which appear to regulate related copper homeostatic systems. The interaction of the *E. hirae* CopY repressor and those of the related bacteria mentioned above with the promoter were assessed in kinetic terms by surface plasmon resonance analysis. All homologous repressors exhibited essentially identical interaction kinetics with the *E. hirae* promoter, corroborating the universal nature of the cop-box. For the interaction of *E. hirae* CopY with its native promoter, association and dissociation constants for all the anticipated partial reactions could be derived (Figure 4).

Induction of the *cop* operon by excess copper thus proceeds by donation of two Cu(I) to Zn(II)CopY. The resultant $Cu(I)_2$CopY has an increased dissociation rate for the cop-box and an immeasurable association rate. CopY leaves the cop-box and allows transcription to proceed. *In vitro*, it proved impossible to return copper-loaded CopY to the zinc form and it appear likely that $Cu(I)_2$CopY is degraded rather than recycled.

Degradation of CopZ

Under high copper conditions, the *cop* operon is induced and the level of the polycistronic *cop* mRNA increases up to 10^3 fold (Lu & Solioz 2001). However, the expression of the CopZ chaperone does not increase concomitantly. CopZ expression is highest at 0.75 mM media copper and declines at higher copper concentrations (Figure 5a). It was argued that high Cu(I)CopZ concentrations could be toxic because the exposed copper could participate in Fenton-type reactions, thereby generating toxic radicals. Indeed, it was found that cells in which overexpression of CopZ was forced from a plasmid, became sensitive to oxidative stress by

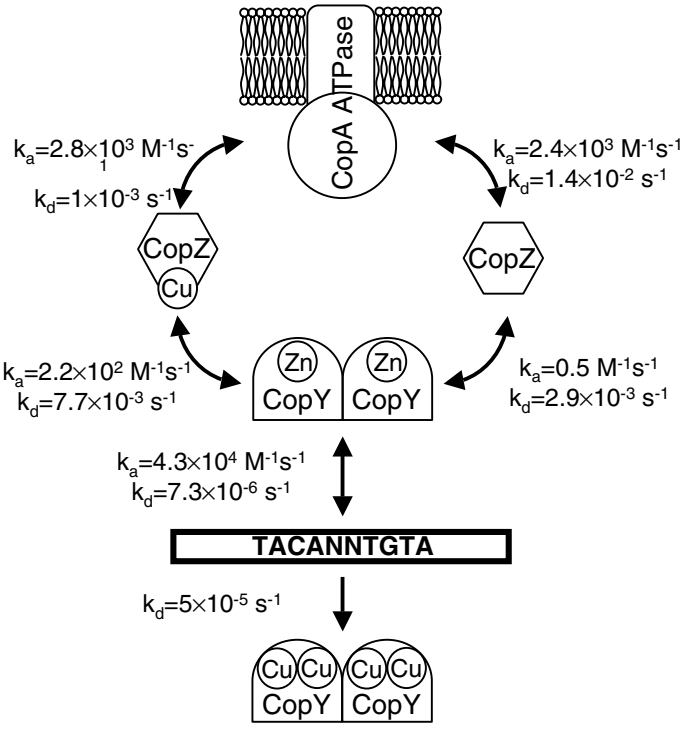

Figure 4. Overview of the interaction kinetics between elements of copper homeostasis and regulation in *E. hirae*. The kinetic values for the respective interactions are indicated. Values for the interaction of CopZ and Cu(I)CopZ with CopA were taken from Refs. Multhaup *et al*. (1996), Strausak *et al*. (1993), all other values are from Ref. Portman *et al*. (2004).

paraquat or hydrogenperoxide. They also became markedly copper sensitive (Figure 5b). Degradation of CopZ or Cu(I)CopZ was found to be catalyzed by a copper induced protease, which could be detected in extracts of cells grown under high copper conditions (Figure 6). A major proteolytic activity was detected at an M^r of around 58,000. This proteolytic activity was specific to CopZ as it did not react on gels containing bovine serum albumin or chicken egg lysozyme. *In vitro* degradation experiments with purified CopZ showed that the degradation of Cu(I)CopZ was faster than that of apo-CopZ, with half-times of degradation of 7 and 22 min respectively.

Conclusion

The study of the copper homeostasis in *E. hirae* has yielded important insight into the molecular mechanism of copper regulation and a number of new concepts have emerged. It has allowed to extensively characterize the function of the CopZ chaperone in delivering Cu(I) to the CopY repressor. This is a novel induction mechanism whereby the inducer is delivered to the repressor by a protein. Surface plasmon resonance analysis of the repressor-DNA interaction has been used to identify the novel cop-box as the consensus binding sequence and as a widespread DNA motif in copper regulation by Gram-positive bacteria. A complete set of kinetic constants could be derived for the CopA–CopZ–CopY–DNA interaction chain. Finally, the copper dependent degradation of CopZ is a novel mechanism in copper homeostatic regulation.

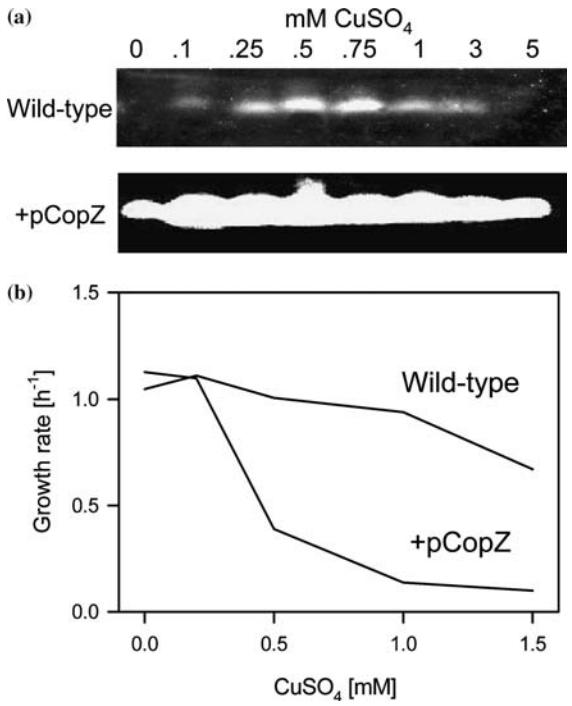

Figure 5. Inhibitory effect of CopZ overexpression. (a) Western blot showing CopZ levels of wild-type cells or cells containing a CopZ overexpressing plasmid (+pCopZ) grown at different copper concentrations. (b) Growth rates of wild-type and CopZ overexpressing (+pCopZ) cells as a function of the media copper concentration.

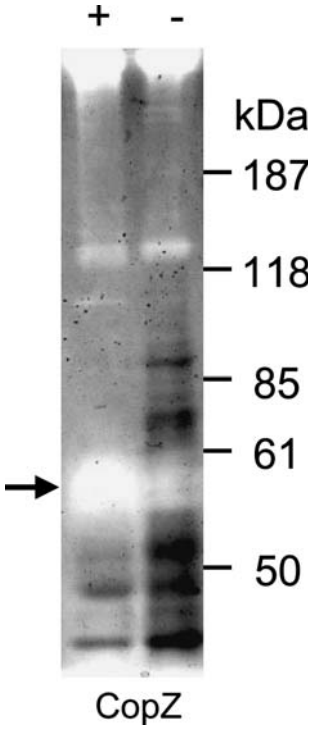

Figure 6. Zymogram of CopZ proteolytic activity. The presence of a copper dependent proteases was detected by zymography. Extracts (50 μg/lane) from cells induced with 3 mM copper (+) or from uninduced cells (–) were resolved on a 7.5% SDS polyacrylamide gel, renatured and allowed to reacts with the CopZ embedded in the gel. The vertical scale shows the migration of standards of the corresponding molecular weights in kDa and the arrow the major CopZ-proteolytic activity.

Acknowledgements

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