

Unravelling copper homeostasis through the study of eukaryotic and prokaryotic model systems

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Introduction

Copper is an essential trace element in biology. In humans, it is required by more than 30 enzymes. Prominent examples are lysyl oxidase, superoxide dismutase, cytochrome oxidase, or dopamine β -hydroxylase. In these and other enzymes, copper functions as a cofactor for the catalysis of redox reactions. On the other hand, excess copper can be toxic by initiating the formation of oxygen reactive radicals which can oxidize proteins, nucleic acids and lipids, leading to cell damage. Thus, the maintenance of balanced copper levels is crucial. Genes involved in copper homeostasis have recently been identified in bacteria, yeast and humans [1–14].

In the Gram-positive bacterium *Enterococcus hirae*, copper homeostasis has been extensively studied. It is accomplished by the *cop* operon, which consists of four genes: *copY*, *copZ*, *copA*, and *copB* (Fig. 1). *CopA* and *CopB* encode P-type ATPases of 727 and 745 amino acids respectively. *CopA* pumps copper into the cells when copper is limiting, while *CopB* is responsible for the extrusion when copper reaches toxic levels [15,16]. The two ATPases are co-regulated in a bi-phasic manner: excess (>0.1 mM) as well as limiting ($<10\mu\text{M}$) copper induces the *cop* operon. This regulation is controlled by *CopY* and *CopZ*, two hydrophilic proteins of 145 and 69 amino acids respectively [2,17]. *CopY* is a copper-responsive repressor of the *cop* operon [18] while *CopZ* functions as a

metal chaperone that routes copper intracellularly.

In humans, mutations in two genes lead to dysregulation of copper. These genes, *ATP7A* and *ATP7B*, encode copper ATPases which share sequence similarity with *CopA* and *CopB* of *E. hirae*. Mutations in *ATP7A* cause Menkes disease, while mutations in *ATP7B* lead to Wilson disease. Menkes disease is a rare X-linked recessive disorder resulting in severe copper deficiency. Affected boys suffer from progressive neurological degeneration, connective-tissue defects, and hypothermia. A hallmark of Menkes disease is the development of *pili torti* or 'kinky hair' some weeks after birth. Therefore Menkes disease is also referred to as kinky hair syndrome. The Menkes ATPase is expressed in several tissues including intestine, heart, and brain, but not in the liver. In the liver, the Wilson ATPase is responsible for copper extrusion. Wilson disease is an autosomal recessive disease leading to an accumulation of copper mainly in the liver, brain, and characteristically in the cornea, leading to Kayser-Fleischer rings. Liver cirrhosis and various neuropsychiatric symptoms dominate the clinical picture.

Since the cloning of the first copper ATPases in *E. hirae* [1], there has been a rapid expansion in the field of heavy metal homeostasis. Mechanistic studies in various model organisms have been put forward. We report here two approaches used to unravel and contribute to the understand-

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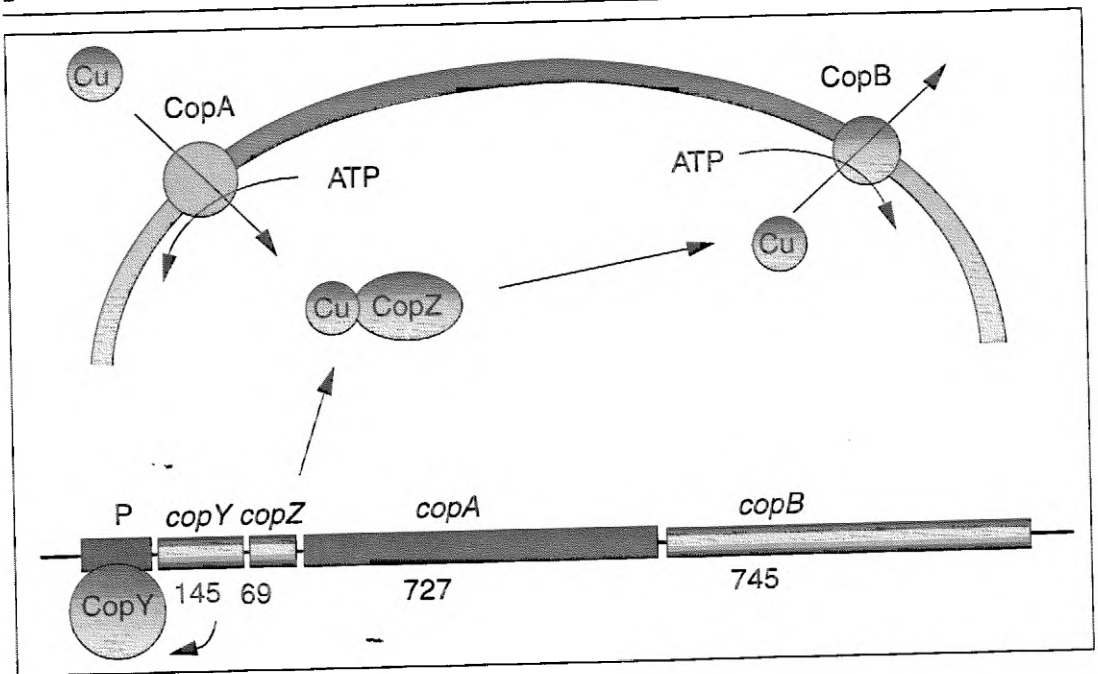


Figure 1. Model of copper homeostasis *E. hirae*: CopA, copper(I) import ATPase; CopB, copper export ATPase; P, promoter region; CopY, copper responsive repressor of the *cop*-operon; CopZ, copper chaperone affecting intracellular copper routing.

ing in this field. One is the regulation of copper in *E. hirae* and the other is the functional expression of the Menkes ATPase in frog oocytes.

Copper homeostasis in *E. hirae*

Copper ATPases

E. hirae possesses two copper ATPases, CopA and

CopB. They belong to a new subclass of the P-type ATPases, the CPx-type ATPases. Common to all P-type ATPases are the domains for aspartyl kinase, phosphatase and ATP-binding (Fig. 2). The CPx-type ATPases have, in addition, (1) one or more CxxC heavy metal binding motifs in the N-terminus; (2) a conserved intramembranous CPx motif; (3) a conserved HP locus, and (4) a

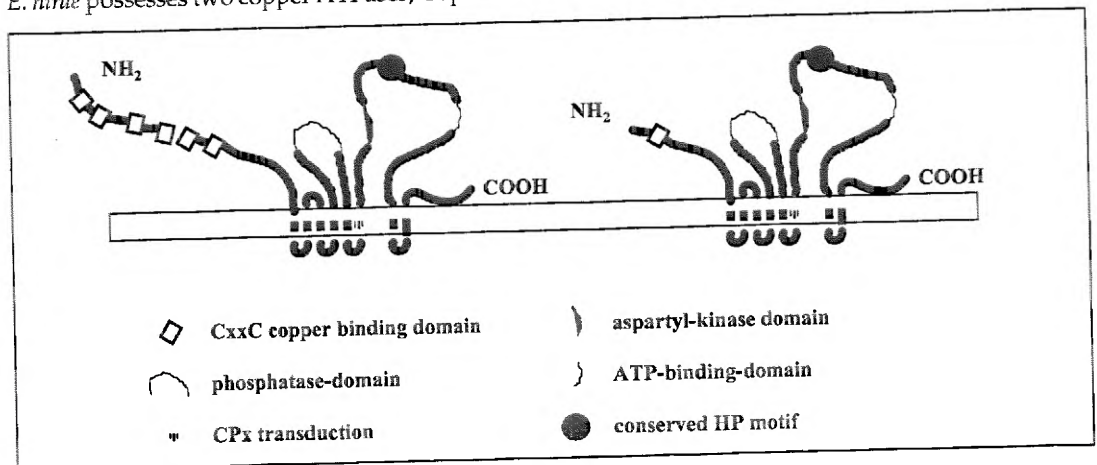


Figure 2. Key features of CPx-type ATPases: Features are depicted on the examples of the Menkes ATPase and the *E. hirae* CopA ATPase. CxxC is the conserved core feature of the heavy metal domains, of which the Menkes ATPase features six and CopA only one.

CopZ	: -----MKQEF SVKGMSCNHC VARIEEAVGRISG-----	: 28
ATX1	: -----MAEIKHYQFNVV-MTCSGGSGAVNKVLT KLEPD	: 32
HAH1	: -----MPKHEFSVD-MTCGGCAEAVSRV LNKLG--	: 27
CCS	: MASDSGNQGT LCTLEFAVQ-MTCQSCVDAVRKSLQGVAG-	: 38
LYS7	: -----MTTNDTYEATYAIP-MHCENCVNDIKACLKNVPG-	: 33
CopZ	: VKKVKVQLKKEKAVVKFDEANVQATEICQAINELGYQAEV	: 68
ATX1	: VSKIDISLEKQLVDVYTTLPYDFILEKIKKTGKEVR-SGK	: 71
HAH1	: GVKYDIDL PNKKVCI ESEHSMDTLLATLKKTKGTVSYLGL	: 67
CCS	: VQDVEVHLEDQMLVHHTLPSQEVQALLEGTCRQAVLKGM	: 78
LYS7	: INSLNFDIEQQIMSVESVAPSTIINTLRNCCKDAIIRGA	: 73

Figure 3 Amino acid sequence alignment of copper chaperones: Identical amino acids are shaded and the conserved motif CxxC is boxed. ATX1, yeast chaperone donating copper to the CCC2 Cu-ATPase; HAH1, human homologue of ATX1; CCS and LYS7, the respective Cop human and yeast copper chaperones donating copper to superoxide dismutase.

membrane topology very different from that of non-heavy metal ATPases [19,20].

The evidence for the role of CopA in copper uptake is still indirect. Disruption of the *copA* gene did not significantly affect cell growth. However, these mutant cells ceased to grow in media where copper was complexed with 8-hydroxyquinoline or *o*-phenanthroline, suggesting a role for CopA in importing copper under limiting conditions. This is further supported by the observation that cells disrupted in the *copA* gene are more resistant to silver(I) than the wild-type. Thus, the CopA ATPase appears to provide a route for Ag⁺ (and Cu⁺) into the cell [17].

For the CopB ATPase, copper transport could directly be demonstrated. In inside-out membrane vesicles of *E. hirae*, CopB catalyzed ATP-dependent accumulation of Cu(I) and Ag(I). This would correspond to copper extrusion in whole cells [16]. In addition, *copB* disrupted cells were intolerant to copper, while *copA* null mutants were not affected [17].

Regulation

CopY regulates the *cop* operon by acting as a copper-inducible repressor. The consensus copper binding domain of CopY, CxCx₄₋₅CxC, is also found in the yeast copper responsive transcriptional activators, ACE1 and MAC1 [21,22]. Null-mutants in *copY* strongly overexpressed CopA and CopB [2]. Two CopY binding sites on the promoter, each approximately 30 basepairs in length, were mapped by DNaseI footprinting and found to encompass an inverted repeat. The transcriptional start site mapped between the two repressor binding sites in the center of this inverted

repeat. Binding of CopY to the promoter was also demonstrated *in vitro* by band shift experiments. The metal ions Cu⁺, Ag⁺ and Cd²⁺ that induced the *cop* operon *in vivo* also released CopY from the DNA *in vitro*, suggesting metal induced release of CopY from the promoter as the induction mechanism [18].

Intracellular copper routing

In recent years, a new class of copper homeostatic proteins, known as the chaperones, has attracted considerable attention. These intracellular proteins bind copper at the point of entry and transfer the ions to specific sites. Thereby, they protect cells from the potentially toxic effects of reactive heavy metal ions. CopZ, an 8 kDa protein, appears to act as a copper chaperone in *E. hirae*. It shares a conserved copper binding motif of consensus CxxC with a number of known heavy metal binding proteins and chaperones (Fig. 3). The overall sequence similarity between these proteins is 46–55%. Strong indirect evidence indicates that the yeast homologue of CopZ, ATX1, catalyzes copper transfer to the CCC2 copper ATPase, localized in the *trans*-Golgi network [23]. HAH1 is the human homologue of ATX1 and probably functions similarly in the delivery of copper to the Menkes and Wilson copper ATPases [24]. Similarly, the human CCS protein and its yeast homologue LYS7 appear to catalyze Cu(I) incorporation into Cu-Zn superoxide dismutase [25].

It was shown that CopZ could bind copper(I) or silver(I). Preliminary NMR structural data indicated that CopZ has a $\beta\alpha\beta\beta\alpha\beta$ fold (Fig. 4, R. Wimmer, M. Solioz, and K. Wüthrich, unpub-

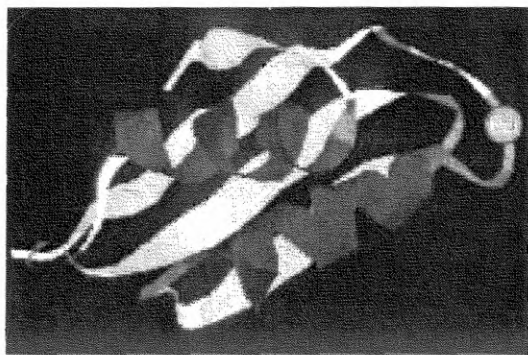


Figure 4 Model of the three-dimensional structure of CopZ. The structure was modelled after the known structure of MerP, using SWISS-MODEL [30].

lished). This structure resembles those found for the bacterial mercury chaperone MerP and the fourth copper binding domain of the Menkes ATPase [26,27]. Striking features of the CopZ structure are a distinct acidic and an alkaline surface patch, respectively. They may be important for specific protein-protein interactions of CopZ with copper donating and copper accepting sites and thus the transfer of copper between proteins. The most convincing evidence, thus far, of CopZ functioning as a chaperone comes from the demonstration of direct transfer of copper from Cu(I)CopZ to the repressor CopY *in vitro*. This interaction appeared to be highly specific: the CopZ-like fourth metal binding domain of the Menkes ATPase failed to donate copper to CopY (Cobine et al, submitted).

However, CopZ does not appear to be essential for the cells. Mutants disrupted in the *copZ* gene did not exhibit increased sensitivity to copper. To the contrary, when CopZ was overexpressed in a $\Delta copZ$ mutant, the cells became copper sensitive (Fig. 5). This suggests that excessive Cu(I)CopZ is toxic, possibly because copper bound to CopZ is relatively exposed and can still engage in free-radical forming reactions. Interestingly, the yeast homologue of CopZ, ATX1, did not induce copper sensitivity in *E. hirae*, possibly due to lower expression level or a lack of specific interaction with copper donating and/or accepting proteins.

Expression of ATP7A in *Xenopus laevis* oocytes

The Menkes disease gene encodes a copper ATPase that bears surprising similarity to CopA of *E. hirae* (43% sequence identity). The predicted protein product of 1500 amino acids clearly belongs to the subclass of CPx-type ATPases. It

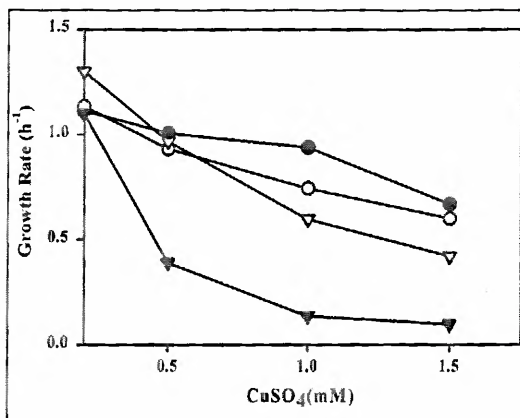


Figure 5 Effect of CopZ expression on the copper resistance of *E. hirae*: Cells were grown in media containing increasing concentrations of CuSO₄ and growth monitored by following the optical density at 546 nm. Growth rate was calculated as 1/doubling time. ●, *E. hirae* wild-type; ○, $\Delta copZ$ mutant; △, $\Delta copZ$ mutant complemented with a plasmid bearing the yeast *Atx1* gene; ▼, $\Delta copZ$ mutant complemented with a plasmid bearing the *copZ* gene.

has eight predicted transmembrane helices and six copper binding domains, instead of one as in CopA of *E. hirae* (cf. Fig. 2). Copper transport by the Menkes ATPase has recently been demonstrated in membrane vesicles from Chinese hamster ovary cells expressing the enzyme approximately 50-fold [28]. However, this system is not very versatile and we endeavored to develop an oocyte system for the functional expression of human wild-type and mutant forms of the Menkes ATPase.

Oocytes of *Xenopus laevis* allow the heterologous expression and functional testing of membrane proteins. Previously, several membrane pumps and transport activity measures have been expressed in this system [29]. To monitor expression levels and to track the cellular location of the Menkes ATPase in oocytes, we fused the C-terminus of the ATPase with the green fluorescent protein (GFP). This chimera was transcribed *in vitro* from a T3 promoter with T3 polymerase, 5' capped and polyadenylated. Oocytes injected with this mRNA expressed the Menkes-GFP fusion protein, as determined by Western blotting (Fig. 6). The native form of the Menkes ATPase was similarly expressed. Confocal fluorescent microscopy allowed us to localize the Menkes-GFP fusion protein to the plasma membrane. This system should thus be ideally suited to directly measure copper transport by the Menkes ATPase. CopA of *E. hirae* has been shown

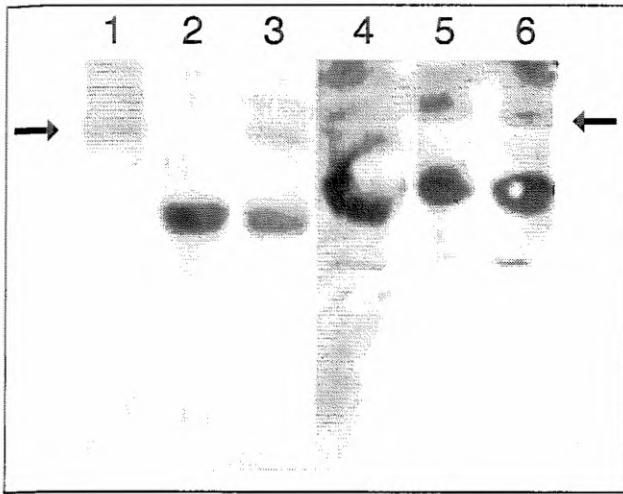


Figure 6. Menkes ATPase expression in oocytes: Oocytes were injected with water or Menkes-GFP mRNA and expression analyzed by Western blotting with an anti-Menkes antibody. Lane 1, Chinese hamster ovary cells overexpressing Menkes ATPase; lanes 2 and 4, water injected controls; lanes 3 and 6, oocytes injected with Menkes mRNA; lane 5, oocytes injected with Menkes-GFP mRNA. The arrowhead corresponds to the 190 kDa Menkes ATPase. The prominent band of lower molecular weight is due to a nonspecific interaction of the antibody with an endogenous oocyte protein.

to transport not only copper(I) but also silver(I) [16]. The measurement of silver transport therefore represents an alternative to investigate the function of the Menkes ATPase. In our oocyte system, transport can be measured in both direction by either incubating eggs with copper or silver isotopes and measuring uptake, or by injecting eggs with the radioisotope and assessing efflux.

Conclusion

Heavy metal homeostasis is a relatively unexplored field, though, in the last few years there has been a huge effort to understand the regulation of trace metals in living organisms. We have come to realize that copper homeostasis is an active process. Several copper ATPases and regulatory proteins have been cloned and studied. A relatively complete picture of copper homeostasis has emerged for *E. hirae*. In humans, the inherited Menkes and Wilson disease have now been clearly linked to defects in transmembranous copper transport. Therapeutic treatments of these diseases are not always satisfactory. This has provided a strong impetus to understand the molecular function of the Menkes and Wilson ATPases and, more generally, the basis of mammalian copper homeostasis. Many components of copper homeostatic systems appear to have been conserved from bacteria to man. Study of bacterial, yeast and mammalian systems can thus provide complementing pieces to the puzzle of the complex copper homeostatic systems that operate in living organisms.

Acknowledgement — We thank Thomas Weber for excellent technical assistance and Simona Berardi

for help with the oocyte work. This work was supported by grant 3200-046804 from the Swiss National Foundation.

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