

Role of proteolysis in copper homeostasis

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Abstract

The *cop* operon of *Enterococcus hirae* controls cytoplasmic copper levels. It encodes two copper ATPases, a repressor, and the CopZ metallo-chaperone. Transcription of these genes is induced by copper. However, at higher copper concentrations, CopZ is degraded by a copper-activated proteolytic activity. This specific proteolysis of CopZ can also be demonstrated *in vitro* with *E. hirae* extracts. Growth of the cells in copper increases the copper-inducible proteolytic activity in extracts. Zymography reveals the presence of a copper-dependent protease in crude cell lysates. Copper-stimulated proteolysis of CopZ appears to play an important role in copper homeostasis by *E. hirae*.

Overview of copper homeostasis in *Enterococcus hirae*

The *E. hirae cop* operon is located on the chromosome and is required for copper homeostasis. It consists of four closely spaced genes, in the order: *copY*, *copZ*, *copA* and *copB*. *copY* encodes a copper-responsive repressor, *copZ* encodes a copper chaperone, and *copA* and *copB* encode a subclass of P-type ATPases (CPx-type) [1a] of 727 and 745 amino acids respectively. Figure 1 depicts our working model of copper circulation in *E. hirae*. An extracellular copper reductase, tentatively called CorA, reduces copper(II) to copper(I) for uptake by CopA. If cytoplasmic copper becomes excessive, it is secreted from the cytoplasm by CopB [1]. While the function of CopB in copper excretion had been shown by direct demonstration of ⁶⁴Cu⁺ as well as ^{m110}Ag⁺ transport, the evidence for CopA being involved in copper uptake is still indirect. It rests on the following three properties of a *copA* knock-out strain: (i) it grows like the wild-type under conditions of normal or elevated copper; (ii) it cannot grow in copper-depleted medium; and (iii) it is more silver-resistant than the wild-type, presumably because CopA can be a route for silver entry into the cell [2].

Key words: *cop* operon, CopZ, *Enterococcus hirae*, metallo-chaperone, oxidative damage.

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Intracellular copper routing: the CopZ copper chaperone

The fate of copper that has entered the cell remains unclear in several regards. However, a major function in intracellular copper routing is taken by the copper chaperones, specialized proteins that deliver copper intracellularly to copper-utilizing enzymes [3,4]. 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* (cf. Figure 1). 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 [5,6]. CopZ has also been shown to interact with CopA, which may be the site of copper loading of CopZ [7], and with a protein, Gls24, of unknown function (Z. H. Lu and M. Solioz, unpublished work).

The solution structure of CopZ has been solved by NMR [8]. It exhibits a $\beta\alpha\beta\beta\alpha\beta$ global structure: two α -helices lying on a four-stranded, antiparallel β -sheet, a structure colloquially called an 'open face sandwich'. A key element of the structure is a CxxC motif, located between the first β -sheet and the first α -helix. It binds copper(I) in a novel, solvent-exposed binding site.

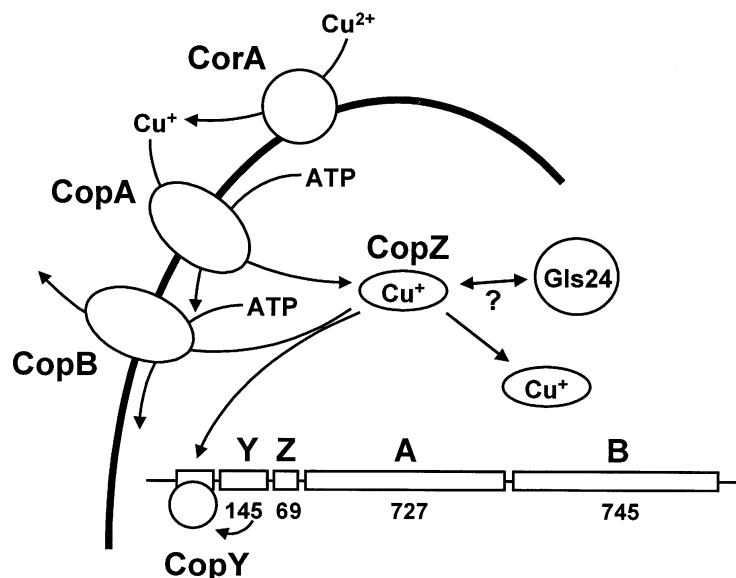
Proteolytic degradation of CopZ

It was observed that CopZ of *E. hirae* is more labile under high copper stress. Levels of *copZ* mRNA increase 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 expression increased only up to 0.5 mM copper and declined at higher copper concentrations, as shown by Western blotting of crude cell lysates [9]. It was concluded that CopZ overexpression is toxic to cells, based on the following observations. (i) Growth of a strain overexpressing CopZ from a plasmid was inhibited by ambient copper in excess of 0.1 mM, and ceased in the presence of 1.5 mM copper, while growth of wild-type *E. hirae* was not

Figure 1

Schematic drawing of the *cop* operon and model of copper homeostasis in *E. hirae*

CorA is an extracellular copper reductase that generates copper(I) for uptake by CopA. Inside the cell, CopZ picks up copper(I) from CopA to deliver it safely to the CopY repressor, which regulates expression of the *cop* operon. If intracellular copper is excessive, CopZ may deliver copper to CopB for secretion, and excess CopZ is degraded. CopZ also interacts with Gls24, a putative stress response protein of unknown function.



markedly affected by this copper concentration. (ii) CopZ overexpression made *E. hirae* more sensitive to oxidative stress, as shown by its increased sensitivity to H_2O_2 and paraquat [9].

Proteolysis of CopZ could also be demonstrated *in vitro*. When cytosolic extracts of *E. hirae* were mixed with purified CopZ, the protein was rapidly degraded. Interestingly, apo-CopZ was significantly more resistant to degradation than Cu-CopZ. The more rapid degradation of Cu-CopZ is in line with its proposed toxicity. Copper bound to CopZ is solvent exposed [5], and this copper can probably participate in Fenton-type reactions, leading to the generation of reactive hydroxyl radicals and cell damage.

The protease responsible for CopZ degradation was not induced by exposure of cells to copper, and was present in cells in which protein synthesis had been inhibited for 1 h with chloramphenicol. Rather, the proteolytic activity was constitutive, but was stimulated by copper(I) or by Ag(I), a potential Cu(I) mimetic. The serine protease inhibitors PMSF and *p*-aminobenzamidine inhibited the degradation of CopZ, while tosyl-lysylchloromethane and tosylphenylalanyl-

chloromethane, 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 that degrades CopZ is a serine-type protease [10]. 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.

Discussion

Proteolysis provides cells with an additional means of modulating protein availability in response to alterations in cellular physiology or external stress. However, these processes have received considerably less attention than transcriptional or translational regulation, and our understanding of them is only fragmentary (see [11] for a review). Other examples of metal-induced proteolytic regulation come from recent studies with the yeast zinc and copper transporters. The yeast zinc transporter, ZRT1, is a plasma membrane protein that is required for high-affinity zinc uptake. Its expres-

sion is induced by zinc-limited conditions. When cells are exposed to high levels of extracellular zinc, not only is ZRT1 synthesis turned off, but ZRT1 protein in the plasma membrane is rapidly endocytosed and subsequently degraded in the vacuole [12]. Similarly, copper regulates the synthesis of the yeast high-affinity copper uptake protein Ctr1 at the transcriptional level. Exposure of cells to 10 μ M copper is sufficient to repress transcription and to trigger proteolytic degradation of Ctr1. Unlike ZRT1, degradation of Ctr1 occurs at the plasma membrane by an as yet unidentified protease [13]. The yeast transcription factor Mac1, which is involved in high-affinity copper uptake, is also degraded under conditions of high copper concentrations [14]. Conversely, in the green alga *Chlamydomonas reinhardtii*, apoplastocyanin, a copper binding protein that catalyses electron transfer during photosynthesis, is degraded rapidly under copper-deficient conditions. This presumably serves to ensure that copper is available to indispensable cuproenzymes [15]. Interestingly, this work suggested the involvement of a copper-activated protease in the process, similar to our concept for CopZ degradation. Although no copper-stimulated protease has so far been described, the fact that trypsin can be engineered such that it can be strongly activated by copper [16] suggests that Nature could adopt a similar strategy for copper-induced degradation. The above examples and our studies highlight the important role of controlled proteolysis as an additional safeguarding mechanism in heavy metal homeostasis, and provide a lead towards the identification of the proteolytic process involved.

The proteolytic degradation of CopZ in wild-type cells grown in high copper concentrations not only implies that the chaperone is dispensable under these conditions, but also suggests that it becomes toxic to the cell. The chief cause, or at least a contributing factor to this, could be the observed rise in cytoplasmic copper in cells grown at elevated ambient copper [17]. Increased intracellular copper could result in excessive Cu–CopZ concentrations. Elevated Cu–CopZ levels, in turn, could compromise the specificity of the intracellular copper delivery process, which would lead to the transfer of copper to unqualified sites. Alternatively, the exposed copper in Cu–CopZ [8] could participate in radical formation. In support of the latter mechanism, we observed that over-expression of CopZ led to increased sensitivity of cells to copper, hydrogen peroxide and paraquat, a generator of superoxide anions. In this light, the

three-times faster degradation of Cu–CopZ compared with apo–CopZ that we observed in our *in vitro* assay would be biologically meaningful: toxic Cu–CopZ has to be removed as a priority.

The copper-induced degradation of CopZ raises a number of exciting questions. First, by what mechanism is the CopZ-degrading protease activated? Does activation take place by the binding of copper to a special binding site of the protease, or does it proceed more indirectly via activation of a proenzyme? Secondly, how does the preferred degradation of Cu–CopZ over that of apo–CopZ come about? Is there a modification of Cu–CopZ or apo–CopZ to signal preferred degradation of Cu–CopZ to the pathway, or is the preference for Cu–CopZ an intrinsic property of this degradative activity?

Conclusion

The finding that high ambient copper levels trigger the degradation of the CopZ copper chaperone of *E. hirae* illustrates a novel control mechanism for an intracellular metal routing protein. Given the high degree of conservation of the copper homeostatic machinery from bacteria to humans, proteolysis is likely to play a role in copper homeostasis in all cell types.

This work was supported by grant 32-56716.99 from the Swiss National Foundation, and a grant from the International Copper Association.

References

- 1 Solioz, M. and Odermatt, A. (1995) *J. Biol. Chem.* **270**, 9217–9221
- 1a Vulpe, C. and Solioz, M. (1996) *Trends Biochem. Sci.* **21**, 237–241
- 2 Odermatt, A., Suter, H., Krapf, R. and Solioz, M. (1993) *J. Biol. Chem.* **268**, 12775–12779
- 3 Harrison, M. D., Jones, C. E., Solioz, M. and Dameron, C. T. (2000) *Trends Biochem. Sci.* **25**, 29–32
- 4 O'Halloran, T. V. and Culotta, V. C. (2000) *J. Biol. Chem.* **275**, 25057–25060
- 5 Cobine, P., Wickramasinghe, W. A., Harrison, M. D., Weber, T., Solioz, M. and Dameron, C. T. (1999) *FEBS Lett.* **445**, 27–30
- 6 Cobine, P. A., George, G. N., Jones, C. E., Wickramasinghe, W. A., Solioz, M. and Dameron, C. T. (2002) *Biochemistry* **41**, 5822–5829
- 7 Multhaup, G., Strausak, D., Bissig, K.-D. and Solioz, M. (2001) *Biochem. Biophys. Res. Commun.* **288**, 172–177
- 8 Wimmer, R., Hermann, T., Solioz, M. and Wüthrich, K. (1999) *J. Biol. Chem.* **274**, 22597–22603
- 9 Lu, Z. H. and Solioz, M. (2001) *J. Biol. Chem.* **276**, 47822–47827

- 10 Lu, Z. H., Dameron, C. T. and Solioz, M. (2002) *Biometals*, in the press
- 11 Gottesman, S. (1996) *Annu. Rev. Genet.* **30**, 465–506
- 12 Gitan, R. S., Luo, H., Rodgers, J., Broderius, M. and Eide, D. (1998) *J. Biol. Chem.* **273**, 28617–28624
- 13 Ooi, C. E., Rabinovich, E., Dancis, A., Bonifacino, J. S. and Klausner, R. D. (1996) *EMBO J.* **15**, 3515–3523
- 14 Zhu, Z., Labbe, S., Pena, M. M. and Thiele, D. J. (1998) *J. Biol. Chem.* **273**, 1277–1280
- 15 Li, H. H. and Merchant, S. (1995) *J. Biol. Chem.* **270**, 23504–23510
- 16 Willett, W. S., Brinen, L. S., Fletterick, R. J. and Craik, C. S. (1996) *Biochemistry* **35**, 5992–5998
- 17 Odermatt, A., Krapf, R. and Solioz, M. (1994) *Biochem. Biophys. Res. Commun.* **202**, 44–48

Received 10 March 2002

Metal Transport

Microbial siderophore-mediated transport

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Abstract

Microbial iron chelates, called siderophores, are synthesized by bacteria and fungi in response to low iron availability in the environment. The present review summarizes structural details of siderophore ligands with respect to their transport properties. This presentation is largely centred on the occurrence and function of siderophores in the various bacterial and fungal genera.

Introduction

Siderophores are defined as low-molecular-mass microbial compounds with a very high affinity for iron. Their function is to mediate iron uptake by microbial cells. For a more comprehensive review of the structures and functions of siderophores the reader is directed to reviews on iron transport [1] and microbial transport systems [2]. Although most siderophores are water soluble and are excreted into the environment, there are some siderophores that are not excreted at all, such as the mycobactins, synthesized by mycobacteria, that are located within the cell envelope [3,4]. This is in contrast to the carboxymycobactins and exochelins that represent the real extracellular siderophores of the mycobacteria. Fungal siderophores may also be divided into extracellular and intracellular siderophores, as found in spores and mycelia of *Neurospora* and *Aspergillus* [5].

Also, extremely lipophilic siderophores have been found in marine bacteria that do not readily diffuse into the surrounding medium, but which form vesicles [6]. Thus the environmental distribution of siderophores may vary to some extent. However, their general iron-transport function is obvious and has been documented by radioactive labelling experiments in a variety of microbial organisms. Although a number of transport mechanisms are based on non-destructive shuttle systems, some of the ligands may be degraded by esterases after iron delivery to the cells. In general, most siderophore transport systems are highly specific for certain siderophores, although some broad-range siderophore-recognition systems have been described. Several novel siderophore-transport systems have recently been proposed based on ligand-exchange mechanisms [7,8].

Functions of siderophores

Although their main function is to acquire iron from insoluble hydroxides or from iron adsorbed to solid surfaces, siderophores can also extract iron from various other soluble and insoluble iron compounds, such as ferric citrate, ferric phosphate, Fe-transferrin, ferritin or iron bound to sugars, plant flavone pigments and glycosides or even from artificial chelators like EDTA and nitrilotriacetate by Fe(III)/ligand-exchange reactions. Thus, even if siderophores are not directly involved in iron solubilization, they are required as carriers mediating exchange between extracellular iron stores and membrane-located siderophore-transport systems.

Key words: biosynthesis, ecology, stereochemistry.

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