

The *Enterococcus hirae* copper chaperone CopZ delivers copper(I) to the CopY repressor

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Abstract Expression of the *cop* operon which effects copper homeostasis in *Enterococcus hirae* is controlled by the copper responsive repressor CopY. Purified Zn(II)CopY binds to a synthetic *cop* promoter fragment in vitro. Here we show that the 8 kDa protein CopZ acts as a copper chaperone by specifically delivering copper(I) to Zn(II)CopY and releasing CopY from the DNA. As shown by gel filtration and luminescence spectroscopy, two copper(I) are thereby quantitatively transferred from Cu(I)CopZ to Zn(II)CopY, with displacement of the zinc(II) and transfer of copper from a non-luminescent, exposed, binding site in CopZ to a luminescent, solvent shielded, binding site in CopY.

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1. Introduction

Copper, nickel, cobalt and iron are essential to all organisms because of their use as catalytic and structural elements in enzymes and other molecules. Due to their potential to form radicals and their propensity to bind to biological macromolecules, the availability of these heavy metals in the cell must be carefully controlled. We are only now beginning to understand heavy metal homeostatic mechanisms. Aside from uptake and secretion pathways, a critical part of heavy metal ion homeostasis appears to be the intracellular transport of the metal ion from its point of entry into the cell to its intended recipient molecule. Several proteins involved in such intracellular routing have been identified and indirectly shown to transfer metal ions. Co-factor insertion by these, so called, metal chaperones has been implicated for the biosynthesis of enzymes containing copper, nickel, cobalt and iron [1–6].

The CopZ protein of *Enterococcus hirae* appears to function as a copper chaperone. CopZ belongs to a family of highly conserved chaperones that have been suggested to transfer copper to copper-transporting ATPases in humans and yeast (HAH1, Atx1) [7,8], and mercury (MerP) to a mercury uptake system in bacteria [9]. CopZ-like motifs are also found singly or in multiple copies in the N-termini of the CPx-type ATPases, like the human Menkes and Wilson ATPases, and mercuric reductases (Fig. 1) [10]. In addition to the conservation of the primary sequence (Fig. 1) and the similarity of the predicted secondary structures recent NMR analysis has

shown that these CopZ-like motifs have almost identical global folds but distinct subtle differences around their metal-binding sites [11]. CopZ is one of four genes encoded by the *cop* operon, which controls copper homeostasis in *E. hirae*. Two other *cop* genes encode CPx-type copper ATPases that act as import (CopA) and export (CopB) pumps for Cu(I), and the fourth gene codes for a copper responsive repressor, CopY, that controls the expression of all four *cop* genes [12–14]. CopY is a homo-dimer with an apparent molecular weight of 53 kDa that binds to two distinct 28 base pair sequences in the promoter region of the *cop* operon [15].

Here we use the in vitro interaction of CopY with the promoter region to demonstrate that Cu(I)CopZ can donate copper to CopY, thereby releasing CopY from the DNA. This system defines a minimal set of components required for chaperone-mediated metal transfer. The participating proteins were overexpressed in *Escherichia coli* and could readily be purified, providing for facile mutational analysis. This makes the system ideally suited to investigate, in more detail, the mechanistic aspects of metal chaperone function.

2. Materials and methods

2.1. Protein purifications and cupro-protein preparation

CopY was purified as described [15], with an additional hydroxyapatite column (CHT-II, Bio-Rad), eluted with 0.05–1 M potassium phosphate gradient pH 7.8. Zn(II)CopY eluted at 300 mM potassium phosphate. The eluted Zn(II)CopY was concentrated and buffer exchanged into 20 mM potassium phosphate pH 7.8 in a Centricon-10. CopZ was overexpressed in *E. coli* from the PCR amplified gene cloned into pQE6 (Qiagen) under the control of a *taq* promoter. Cells were induced and extracted as described [15]. Extracts were made 10 mM in dithiothreitol and chromatographed on S-Sepharose, eluted with a 0–300 mM potassium sulfate gradient in 50 mM Tris-sulfate, pH 7.4, 10 mM dithiothreitol. Final purification was achieved by gel filtration on a TSK G3000 column (TosoHaas) in 50 mM (NH₄)₂CO₃, pH 7.4. MNKr2 (residues M¹⁶⁵-K²⁴⁶), the second chaperone-like domain in the human Menkes protein, was overexpressed and purified from *E. coli* as described [16]. The purity of the three proteins was assessed by SDS-PAGE and by quantitative amino acid analysis using a Beckman 6300 analyzer after 24 h in vacuo acid hydrolysis at 110°C in 6 M HCl with 0.01% phenol. The amino acid analysis was also used to determine the protein concentrations. Copper and zinc concentrations were determined by flame atomic absorption spectroscopy on a Varian A-875 instrument. Apo-CopZ and apo-MNKr2 were prepared by acidifying the proteins to pH 2 followed by gel filtration on a Sephadex G-25 column equilibrated with 25 mM HCl. The apo-proteins were lyophilized, resuspended in 100 mM Tris-Cl, pH 8.2, and reduced with 150 mM dithiothreitol in the presence of 10 mM EDTA prior to exchanging the buffer to 25 mM HCl on a Sephadex G-25 column.

2.2. Copper titrations

One mole equivalent of Cu(I), stabilized as [Cu(I)Cl₂][−], was titrated

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CopZ	mkqefsvk	GMSCNHC	varieeavgr	isgvkkvkq	lkkekavvkf	deanvqatei	cqainelgyq	aevi
Atx1	maeikhyqfnv	VMTCSGC	sgavnkvltk	lepdvskidi	slekqlvdvy	ttlpydfile	kikktgkevr	sgkq
CCH	maqtvvklv	GMSCQGC	vgavnrvlgk	megvesfdid	ikeqkvtkvg	nvepeavfqt	vsktgkktssy	wpveaeae
Hah1	mpkhefsv	DMTCGGC	aeavsrvinl	lggvkydidl	pnkkvciese	hsmatlal1	kktgktvsyl	gle
MNKR2	gevvlnkve	GMTCHSC	tstiekgikg	lqgvqrikvs	ldnqeativy	qphlisveem	kkqieamgfp	afvkk
MNKR4	getvinid	GMTCHSC	vqsiegvsk	kpgvksirvs	lansngtvey	dplltspetl	rgaiedmgfd	atlsqtnep
MerP	atqvtvlavp	GMTCAAC	pitvkkalsk	vegsvkdvg	fekreavvtf	ddtkasvqkl	takatadagyp	ssvkkq
CadHMA	mnvyrvq	GFTCANC	agkfeknvkk	ipgvqdakvn	fgaskidvvg	nasveeleka	gafenlkvsp	eklanqti

Fig. 1. Sequence alignment of metallochaperones and other proteins with chaperone-like motifs. The consensus motifs are in bold and the asterisks indicate the conserved cysteine residues involved in metal binding. CopZ, copper chaperone of *E. hirae*; Atx1, chaperone donating copper to the CCC2 Cu-ATPase in yeast; CCH is a chaperone of *Arabidopsis thaliana* [3]. HAH1, human homologue of CopZ associated with copper delivery to Menkes/Wilson ATPases; MNKR2 and MNKR4, two of the six CopZ-like motifs in the N-terminus of the human Menkes ATPase; MerP, mercury chaperone from *Shigella flexneri*; CadHMA, heavy metal associated domain of the cadmium ATPase from *Staphylococcus aureus* [21].

into apo-CopZ and -MNKR2 under anaerobic conditions as described [17]. Reconstitutions were performed at 25 nmol/ml in degassed 50 mM potassium phosphate, pH 7.8, 100 mM NaCl for both MNKR2 and CopZ.

2.3. Band shift assays

Band shift assays were performed as described [15]. The procedure was modified to use a double stranded 36-mer oligonucleotide (5' CCC CGG ATC CAA TTT TCG ATT ACA GTT GGA ATC TAT TAT CGA AGT 3', sense strand), representing one CopY-binding site of the *cop* promoter.

2.4. Luminescence and gel filtration analysis

Zn(II)CopY samples were incubated with freshly reconstituted Cu(I)CopZ and Cu(I)MNKR2 in 100 mM potassium phosphate, pH 7.8, 100 mM NaCl under anaerobic conditions. Luminescence spectra were recorded using a Perkin-Elmer LS 50B luminescence spectrometer at 23°C with an excitation wavelength of 295 nm and a 350 nm band pass filter in sealed screw-topped anaerobic fluorescence cuvettes (Spectrocel). For the gel filtration analysis, 5 mM citrate was added prior to chromatography on a Superdex 30 (Pharmacia) equilibrated in 50 mM potassium phosphate, pH 7.8, 100 mM NaCl, 2 mM di-thiothreitol.

3. Results

3.1. Band shift analysis of copper(I) transfer from CopZ to CopY

The efficient interaction of purified CopY with a synthetic, radiolabeled *cop* promoter fragment in vitro is demonstrated in the band shift experiment shown in Fig. 2. Purified CopY

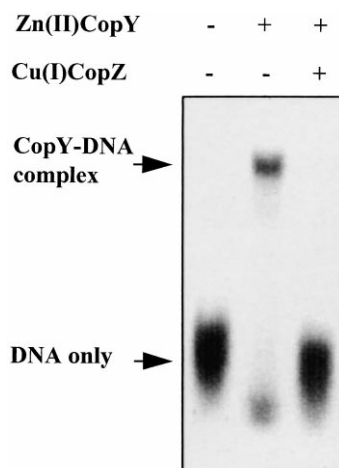


Fig. 2. Metalloregulation of CopY analyzed by band shift assays. Native CopY bound to and retarded ³²P-labeled oligonucleotide promoter fragment. Cu(I), provided as Cu(I)CopZ, abolished binding of CopY to the promoter.

contains one mole equivalent of zinc, as determined by mass spectroscopy and atomic absorption spectrophotometry; this bound zinc is essential for the interaction of CopY with DNA (data not shown). Incubation of Cu(I)CopZ with Zn(II)-CopY-DNA caused the protein-DNA complex to dissociate. Complete dissociation of CopY from the promoter required two equivalents of Cu(I). The Cu(I)-mediated dissociation of the CopY-DNA complex is presumably brought about by a structural change in CopY upon Cu(I) binding. Due to the high affinity of Cu(I) for thiolate ligands, it most likely binds to the -Cys-x-Cys-x-x-x-Cys-x-Cys- motif in CopY. Although CopY has little homology to other known proteins, the spacing of the cysteines in this putative metal-binding site is similar to that of the copper responsive transcription factor, ACE1. The Cu(I)-binding site in ACE1 shields the copper(I) from solvent interactions, which results in the formation of a unique Cu(I)-thiolate fluorescent center that emits at approximately 600 nm [17,18]. It was, therefore, of interest to investigate the luminescence properties of Cu(I)CopY.

3.2. Detection of solvent shielded copper thiolates in Cu(I)CopY by luminescence spectroscopy

Titration of Zn(II)CopY with two mole equivalents of Cu(I)CopZ resulted in the formation of a highly luminescent species with an emission maximum at 600 nm, characteristic of Cu(I)-thiolates in a solvent shielded environment (Fig. 3). Neither Cu(I)CopZ nor native Zn(II)CopY were luminescent. These findings suggest that Cu(I) was transferred from a solvent exposed non-luminescent environment in Cu(I)CopZ to a

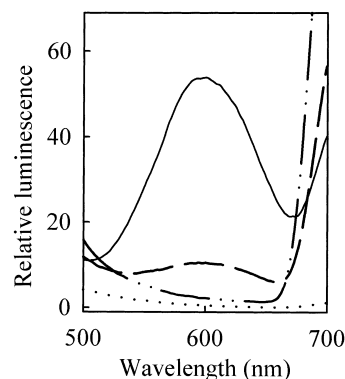


Fig. 3. Copper(I) transfer from the copper chaperone CopZ to CopY, as monitored by the formation of a Cu(I) lumophore in CopY. Emission spectra of native Zn(II)CopY (dot-dashed line), Cu(I)CopZ (dotted line), CopY pre-incubated with Cu(I)CopZ (solid line), and CopY pre-incubated with Cu(I)MNKR2 (dashed line).

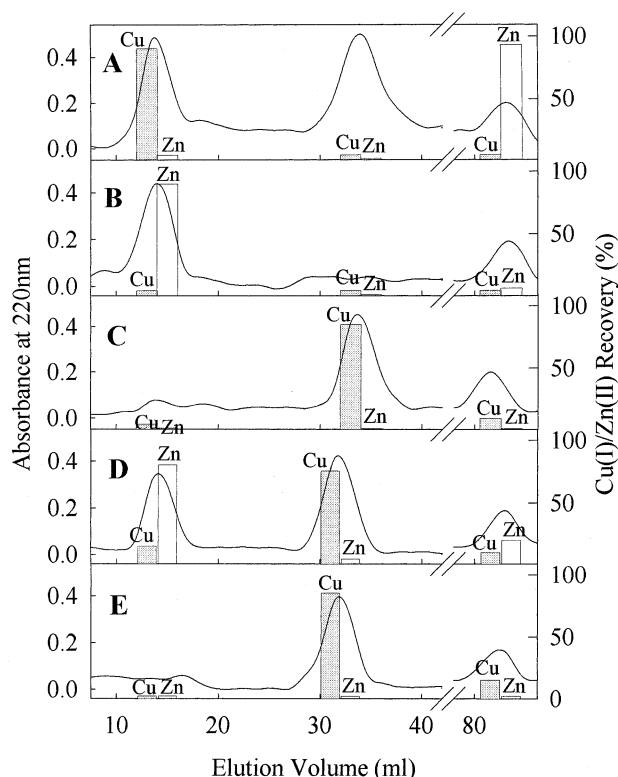


Fig. 4. Transfer of Cu(I) to CopY assessed by gel filtration. The solid lines show the protein elution profiles (absorbance at 220 nm), and the bars represent the percent of copper or zinc in the corresponding peaks. A: Zn(II)CopY pre-incubated with Cu(I)CopZ; B: Zn(II)CopY; C: Cu(I)CopZ; D: Zn(II)CopY pre-incubated with Cu(I)MNKr2; E: Cu(I)MNKr2.

solvent shielded Cu(I)-thiolate environment in CopY. The data support the suggestion, from the gel mobility shift assay, that Cu(I) is transferred from CopZ to CopY. To test if the transfer of copper from CopZ to CopY requires specific protein-protein interaction, we expressed and purified the second CopZ-like metal-binding domain (MNKr2) of the Menkes ATPase and loaded it with Cu(I). MNKr2 is a structural analog of CopZ and is believed to have a Cu(I) transfer role in the Menkes ATPase [11]. Incubation of Cu(I)MNKr2 with Zn(II)CopY, however, does not lead to copper specific luminescence, indicating that a specific protein-protein interaction must occur for the transfer of copper(I) from CopZ to CopY.

3.3. Quantification of copper and zinc transfer

Analytical gel filtration experiments were conducted to quantify Cu(I) transfer from Cu(I)CopZ to CopY and to detect any stable, tertiary Cu(I)CopZ-CopY or Cu(I)MNKr2-CopY complexes. As shown in Fig. 4, Cu(I) was quantitatively transferred from Cu(I)CopZ to CopY, displacing the bound Zn(II) ion. The displaced Zn(II) ion migrated as a low molecular weight citrate complex in the salt fractions. Cu(I)MNKr2 was unable to transfer Cu(I) to CopY effectively, as previously observed in the luminescence analysis. Cu(I)CopZ and Cu(I)MNKr2 by themselves did not lose bound copper under the experimental conditions. Thus, Cu(I)-CopZ, but not the structural analog Cu(I)MNKr2, transfers copper(I) to CopY.

4. Discussion

This study shows for the first time the direct transfer of stoichiometric amounts of metal from a copper chaperone, CopZ, to a recipient/target protein, Zn(II)CopY. Previous studies on the unrelated copper chaperones LYS7 and CCS showed that they are required for the synthesis of copper/zinc superoxide dismutase (SOD1) in *Saccharomyces cerevisiae* and humans, respectively. However, the evidence for copper transfer between these proteins was indirect and rested on knock-out and substitution assays [2]. Based on similar evidence, a yeast homologue of CopZ, Atx1, has been nominated as a copper chaperone for the CCC2 copper ATPase [1]. The direct transfer of the metal has not been demonstrated.

In *E. hirae*, previous studies have shown that CopY regulates expression of the *cop* operon by binding to the promoter in the absence of copper; in the presence of copper the protein releases the promoter [15]. As shown here, CopY binds to DNA as a Zn(II)CopY complex. Cu(I)CopZ can donate copper to CopY, thereby displacing the bound Zn(II) and releasing CopY from the DNA. The displacement of Zn(II) by copper suggests that the metal ions bind to the same thiolate-binding site within CopY. The luminescence of the Cu(I)-CopY complex implies that Cu(I) is sequestered in an environment where it is protected from solvent. It is plausible that the Cu(I) ions are being sequestered in a Cu(I)-thiolate cluster as found in the Cu(I)-regulated transcription factor ACE1 and the metallothioneins [17,18]. The inability of the displaced Zn(II) to bind to CopZ indicates that the metal-binding site in CopZ is specific for Cu(I).

The transfer of Cu(I) from the -Cys-x-x-Cys- metal-binding site of CopZ is probably driven by the higher affinity of the more cysteine-rich -Cys-x-Cys-x-x-x-Cys-x-Cys- metal-binding site in CopY. The failure of the structural analog MNKr2 to deliver its copper to CopY suggests that the presence of the conserved metal-binding site and global fold is not sufficient to effect copper transfer. Rather, the mechanism probably involves the specific docking of the chaperone to the recipient protein, with subsequent transfer of the metal ion. A two step mechanism of this type would help protect the cell from the toxic effects of copper ions by preventing their non-specific release to inappropriate sites. No stable complex formation between CopY and CopZ or MNKr2 was detected by gel filtration, suggesting that the transfer reaction is rapid.

Presumably, the binding of Cu(I) to CopY introduces or stabilizes a tertiary fold that is distinct from that of the Zn(II) form. Since CopY requires Zn(II) for DNA binding, the key component of the process that releases CopY from the DNA maybe the displacement of the Zn(II). The formation of distinct Cu(I) and Zn(II) species of the same protein has also been characterized in the ACE1 system [19,20]. Importantly, the simple in vitro system described here defines all the components required for copper chaperoning, namely, Cu(I)-CopZ, Zn(II)CopY, and *cop* promoter. Mutational analysis of CopZ and CopY will enable the residues involved in the docking and/or transfer of copper to be identified and will allow us to work toward a detailed mechanism of metal transfer by a chaperone.

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