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Interaction of the CopZ Copper Chaperone with the CopA Copper ATPase of *Enterococcus hirae* Assessed by Surface Plasmon Resonance

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Intracellular copper routing in *Enterococcus hirae* can be accomplished by the CopZ metallochaperone. Using surface plasmon resonance analysis, we show here that CopZ interacts with the CopA copper ATPase. The binding affinity of CopZ for CopA was increased in the presence of copper, due to a 15-fold lower dissociation rate constant. Mutating the N-terminal copper binding motif of CopA from CxxC to SxxS abolished this copper-induced effect. Moreover, CopZ failed to show an interaction with an unrelated copper binding protein used as a control. These results show that (i) the CopA copper ATPase specifically interacts with the CopZ chaperone, (ii) this interaction is based on protein–protein interaction, and (iii) surface plasmon resonance is a novel tool for quantitative analysis of metallochaperone–target interactions.

Key Words: **copper homeostasis; *Enterococcus hirae*; metallochaperone; surface plasmon resonance; ATPase; copper homeostasis.**

The ability of copper to cycle between oxidized Cu(II) and reduced Cu(I) is used by cuproenzymes involved in redox reactions, e.g., Cu/Zn superoxide dismutase or cytochrome *c* oxidase. Copper can also be very toxic through the ability to form radicals and cells must protect themselves from copper-induced damage. Intracellular copper should therefore be complexed at all times, putting stringent requirements on the copper homeostatic system. In yeast, it has been estimated that there is less than one free copper ion per cell in the cytoplasm (1).

In the Gram-positive bacterium *Enterococcus hirae,* copper homeostasis has been extensively investigated. The organism possesses two copper ATPases, CopA and CopB, that are localized in the cytoplasmic membrane. Previous studies had indicated that CopA is responsible for copper uptake under copper limiting conditions and CopB for copper export if copper reaches toxic levels (2, 3). The fate of copper that has entered the cell has remained unclear. Cytoplasmic copper could initially be bound to small molecules like glutathione, to copper chaperones, or both. Copper chaperones are specialized proteins which deliver copper to copper utilizing enzymes (4, 5). 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 (6). The homologous eukaryotic chaperones, Atx1 from yeast and HAH1 from humans, have been demonstrated to deliver copper to copper ATPases located in the *trans*-Golgi network (Ccc2 in yeast, Menkes/Wilson ATPase in humans; Refs. 7, 8). These ATPases in turn pump copper into the Golgi network where it is required for the biosynthesis of cuproenzymes, such as the yeast Fet3p iron reductase or human ceruloplasmin (9, 10).

Chaperone–target interactions have been demonstrated using *in vivo* complementation (11), site-directed mutagenesis (12), the yeast two-hybrid system (7, 8, 12), and by *in vitro* binding assays (7, 13). However, none of these methods allows quantitative assessment of the interaction. We here introduce surface plasmon resonance (SPR) analysis to measure the interaction of the purified CopZ chaperone with the purified CopA ATPase. We found that CopZ specifically binds to CopA and that this binding is modulated by copper. The CxxC copper binding motif of CopA was not essential for the interaction, suggesting that docking of CopA to CopZ is governed by protein–protein interaction. This study also demonstrates for the first time the application of SPR analysis to quantitatively assess metallochaperone–target interaction.

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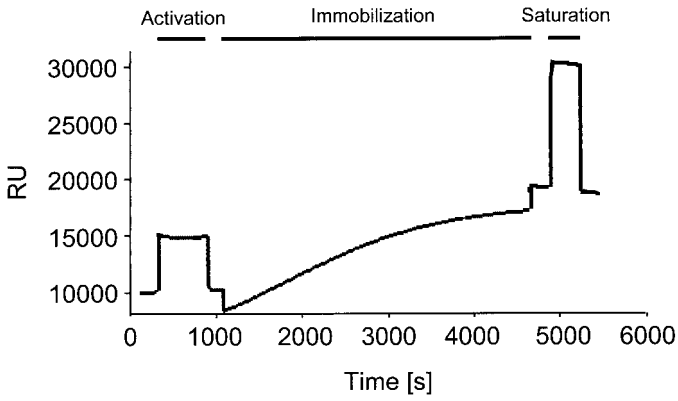

FIG. 1. Chemical coupling of CopA to the sensor chip. Activation and saturation of the BIACORE sensor chip was performed as outlined under Materials and Methods. Binding of CopA for 1 h resulted in 9000 RU, corresponding to 9 ng of CopA bound per square millimeter. Activation, activation of the sensor chip with *N*-hydroxysuccinimide/*N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Immobilization, binding of CopA to the sensor chip; Saturation, quenching of reactive groups with ethanolamine.

MATERIALS AND METHODS

Materials. Tris(2-carboxyethyl)phosphine (TCEP) was supplied by Aldrich and dodecylmaltoside by Anawa Trading (Wangen, Switzerland). $Cu(CH_3CN)_4ClO_4$ was prepared as described (14). All other chemicals were from Merck (Darmstadt, Germany) or from Sigma Chemical Corp. (St. Louis, MO) and were of analytical grade.

Protein purifications. CopA was expressed from plasmid pHT1 in *Escherichia coli* and purified as described (15). A CopA double mutant C17S/C20S (CopA–SS) was generated by mutating pHT1 with the QuikChange site-directed mutagenesis kit (Stratagene), using primers 5′-ggaatgacatctgcgaattcttctgctcgaatcgaaaaagaac and 5′-gttcttttttcgattcgagcagaagaattcgcagatgtcattcc. CopZ was purified as previously described (16, 17).

Surface plasmon resonance detection of CopZ interactions. Surface plasmon resonance experiments were performed with a biosensor system (BIACORE) at a sensor temperature of 20°C. Prior to coupling, CopA or CopA–SS were dialyzed against 250 vol of binding buffer (50 mM NaP_i, pH 7.4, 2% acetonitrile, 0.05% dodecylmaltoside) for 14 h at 4°C. For coupling, one volume of a 0.1 mg/ml enzyme solution in assay buffer was mixed with 3 vol of 10 mM Na-formate, pH 3.5 (final pH ≈ 6), and was injected for 1 h at 5 μl/min onto the sensor chip activated by *N*-hydroxysuccinimide/*N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. This was followed by the injection of 1 M ethanolamine, pH 9, for 5 min to quench residual *N*-hydroxysuccinimide groups. The sensor chip was derivatized with 3000 to 9000 response units (RU) of CopA or CopA–SS. Prior to all binding experiments, the chip was conditioned by consecutive injections (injection times indicated in brackets) of solutions containing 1 M NaCl (4 min), 0.1 mM EDTA/0.1 mM bathocuproindisulfonate (2 min), 20 mM TCEP (4 min), and 1 M NaCl (4 min), all in 2% acetonitrile, 0.05% dodecyl maltoside. After each injection, an additional wash of the integrated flow channel was performed to minimize the carryover between injections.

Data evaluation. The data were analyzed using BIAevaluation version 3.0 software (BIACORE). It was assumed that the CopZ–CopA interaction was pseudo-first order. The apparent association (k_a) and dissociation (k_d) rates were derived by fitting of the biosensor curves. Association and dissociation phases were fitted separately. In assessing the fit, the numeric results were always checked for reasonableness and consistency according to the manufacturer's instruction (Software handbook, BIAevaluation, version 3). K_D was calculated from the relation $K_D = k_d/k_a$.

RESULTS

A biosensor assay, which is based on surface plasmon resonance analysis, was established to measure the interaction of CopZ with CopA in real time. The technique can detect the binding of a protein in solution to a protein immobilized ion a sensor chip surface. The mass change when analyte binds to the immobilized ligand causes a change in refractive index at the sensor chip surface and can be measured as a change in the intensity of light reflected from the chip surface. This resonance signal, measured in RU is directly proportional to the change in mass on the sensor chip surface. A response of 1000 RU corresponds to a change in surface protein concentration of approximately 1 ng/mm^2. The working range of the instrument is in the order of 50–20,000 RU (Fig. 1).

In the past, the BIACORE technique was primarily used for relatively stable proteins, such as in antigen-antibody interactions. To apply the method to membrane proteins, we had to apply mild conditions of coupling and regeneration to maintain native protein conformation. This was achieved by working at 20°C throughout all measurements and including 0.05% dodecylmaltoside in all buffers used. Additionally, including 2% acetonitrile allowed binding analyses in the presence of Cu(I), added as copper(I)acetonitrile. We have previously established that neither the structure nor activity of CopA and CopZ are impaired under these conditions (Ref. 17; Wunderli-Ye and Solioz, unpublished observation).

CopA was injected onto the activated sensor chip surface to immobilize the protein to the activated dextran to the extent of 3000 to 9000 RU, corresponding to approximately 3 to 9 ng/mm^2 of covalently linked protein (Fig. 2). The binding of 9000 RU to the sensor chip

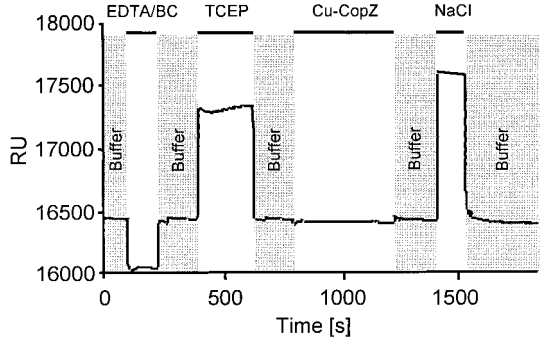

FIG. 2. Overveiw of the washing and incubation steps used in the cycling of the sensor chip. Buffer is "binding buffer." Note the greatly compressed *y*-scale compared to Figs. 3 and 4. See text for other details.

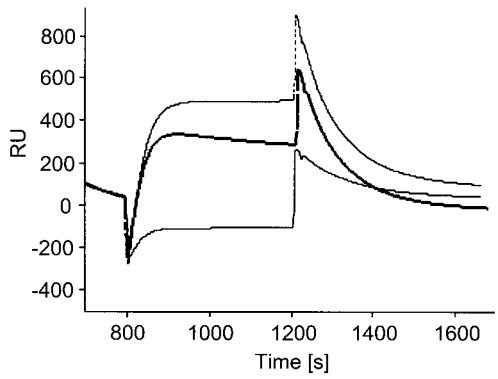

FIG. 3. Association–dissociation of CopZ and wild-type CopA. CopZ at a concentration of 100 μg/ml was interacted with a wild-type CopA sensor chip in the presence of 0 (thick trace), 10 (top trace), and 100 μM (bottom trace) copper(I)acetonitrile, respectively. CopZ injection was started at 800 s and continued until 1200 s, followed by injection of protein-free buffer during the dissociation phase. The rapid drop in signal at 800 s and the rapid rise in signal at 1200 s are artifacts due to a changes in the refractive index of the buffer. The curves were corrected for the baseline obtained without CopZ. Other details of the procedure are described under Materials and Methods.

is in excess of the manufacturer's recommendation, but gave much better signal-to-noise ratios for the binding of the small CopZ. No mass transfer interference was apparent even at the highest chip loading of 9000 RU. The covalent linkage of the proteins to the chip was stable for at least 10 consecutive CopZ injections (no baseline drift).

The regeneration/binding/washing procedure that was employed to analyze CopZ–target interactions is exemplified in Fig. 2. A wash with EDTA and the copper chelator bicinchoninic acid (BC) served to remove bound metal. This was followed by a tris(2-carboxyethyl)phosphine (TCEP) wash. This reagent reduces oxidized thiol groups in proteins without forming adducts or noxious byproducts (18) and was used to reset the conditions for the CxxC metal binding motif of CopA to bind copper. It has been shown for the related metal binding motifs of the ATP7A and ATP7B ATPases of Menkes and Wilson diseases that these sites, when reduced, are readily loaded with copper(I) (19–21).

The association of CopZ with CopA and the dissociation of CopZ from CopA in the absence and presence of copper(I) are shown in Fig. 3. Since copper(I) is virtually insoluble at physiological pH, copper effects were investigated by using the stable copper(I) complex copper(I)acetonitrile (14). It has previously been shown that this complex can donate copper(I) to purified CopZ (17), CopY (Solioz and Dameron, unpublished observation) or the CxxC copper binding motifs of copper ATPases *in vitro* (21). However, it should be noted that due to the use of a copper(I) complex, all copper concentrations must be regarded a relative reference values in regard to free, available Cu^+.

Maximal binding of CopZ to CopA was observed in the presence or substoichiometric amounts of copper(I)-acetonitrile (10 μM copper(I)acetonitrile equals a molar stoichiometry of 0.9 copper(I)acetonitrile/CopZ). Under these conditions, approximately 80% of the theoretical binding of CopZ to CopA was observed. This follows from the fact that the RU are proportional to the mass bound to the sensor chip: the 78-kDa CopA immobilized to 9000 RU on the sensor chip surface could theoretically bind 890 RU of the 7.7-kDa CopZ, anticipating a 1:1 stoichiometry between the two proteins. In the presence of 100 μM copper(I)acetonitrile, the steady-state association of CopZ with CopA was reduced by approximately 75%. In the absence of supplemented copper(I)acetonitrile, a transient binding maximum was observed (loss of RU in the time frame of 900 to 1200 s of the bold curve in Fig. 3). Under these conditions, copper depletion was probably incomplete due to contaminating copper in the running buffer; deionized water contains 50–100 nM residual copper (22). Essentially the same results were obtained using various sensor chips containing from 3000 to 9000 RU of CopA. No binding of CopZ was observed to an empty sensor chip or to a sensor chip with immobilized amyloid precursor protein APP (not shown), a copper binding protein involved in Alzheimer disease (23, 24). This underlines the specificity of the interaction of CopZ with CopA. When CopZ was immobilized on the chip, no interaction with CopA could be observed. This was probably due to steric hindrance from crosslinking of the small CopZ molecule to the chip (see Discussion).

Association and dissociation rate constants were determined by curve fitting using the BIAevaluation software version 3.0 and are summarized in Table 1. For the interaction of CopZ with CopA, the association rate k_a was only slightly affected by 10 and 100 μM copper(I)acetonitrile and maximally exhibited a 2-fold change compared to copper unsupplemented conditions. The dissociation rate k_d was, however, strongly influenced by added copper and was decreased up to 15-fold. This resulted in a 7- and 16-fold increase of the affinity constant K_D in the presence of 100 and 10 μM copper(I)acetonitrile, respectively. Copper could thus modulate the interaction of CopZ with CopA.

CopA possesses an N-terminal metal binding site with the consensus motif CxxC. This motif is found in most copper ATPases and has been shown to specifically bind copper(I) (19–21, 25–27). To test if the CxxC motif of CopA plays a key role in the CopZ–CopA interaction, such as by copper bridging, a CopA mutant which had the cysteine residues of the CxxC motif mutated to serine residues (CopA–SS) was constructed. CopZ still strongly bound to the CopA–SS mutant enzyme, but the interaction was now only insignificantly influenced by added copper; all association and dissociation rate constants resembled those observed for wild-type CopA in the *absence* of added

TABLE 1

Kinetic Parameters of CopZ–CopA and CopZ–CopA–SS Interactions

Interaction	Cu(I) [μM]	k_a [M^{-1} s^{-1}][a]	k_d [s^{-1}][a]	K_D [M]	Figure
CopZ–CopA	0	2.4×10^3	14×10^{-3}	5.8×10^{-6}	3
CopZ–CopA	10	2.8×10^3	1.0×10^{-3}	0.36×10^{-6}	3
CopZ–CopA	100	1.2×10^3	0.95×10^{-3}	0.79×10^{-6}	3
CopZ–CopA–SS	0	2.5×10^3	9.8×10^{-3}	3.9×10^{-6}	4
CopZ–CopA–SS	10	2.3×10^3	8.6×10^{-3}	3.7×10^{-6}	4
CopZ–CopA–SS	100	2.2×10^3	12×10^{-3}	5.5×10^{-6}	4

[a] Association and dissociation rates were determined by curve fitting using BIAevaluation software version 3.0 and corrections were applied as described under Materials and Methods.

copper (Fig. 3, Table 1). Thus, the CxxC motif increases binding of CopZ to CopA in the presence of copper. However, it is not the guiding element for the interaction of CopZ with CopA. Direct protein–protein interaction must occur. Using surface plasmon resonance analysis has allowed us to quantify this interaction for the first time, providing the stage for a detailed analysis of the amino acid residues involved in the CopA–CopZ interaction (Fig. 4).

DISCUSSION

We here introduce SPR analysis as a novel tool to investigate metallochaperone–target interactions and demonstrate that the CopZ copper chaperone strongly and specifically interacts with the CopA copper ATPase of *E. hirae*. The association and dissociation rates measured for CopA–CopZ interactions were 1.2–2.8 $\times$ 10^3 M^{-1} s^{-1} and 0.95–14 $\times$ 10^{-3} s^{-1}. Since only very few cellular interactions have been quantitatively assessed by SPR analysis, a comparison with other such measurements is warranted. For the interaction between the *E. coli* Hsp70 family member, DnaK, and its partner DnaJ, a *k*a of 2.3–3 $\times$ 10^4 M^{-1} s^{-1} and a k_d of 1.6–3 $\times$ 10^{-3} s^{-1} was determined (28). Also using SPR analysis, the *E. coli* σ-sigma factor was found to interact with RNA polymerase with an association rate of 2.1×10^4 M^{-1} s^{-1} and a dissociation rate of 4×10^{-3} s^{-1} (29). It is apparent that these widely different systems exhibit similar rate constants. Future work will have to show if these values represent a general "biological range" for dynamic protein–protein interactions.

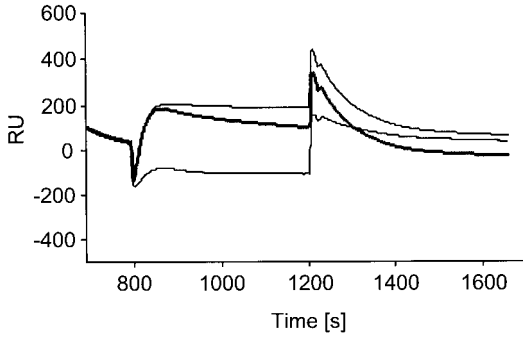

FIG. 4. Association–dissociation of CopZ and mutant CopA–SS. CopZ at a concentration of 100 μg/ml was interacted with a CopA–SS sensor chip in the presence of 0 (thick trace), 10 (top trace), and 100 μM (bottom trace) copper(I)acetonitrile. Other details are as in the experiment of Fig. 3.

By site-directed mutagenesis it was shown that the CxxC motif of CopA is not an essential element for the interaction with CopZ. CopA–SS with the CxxC motif mutated to SxxS still exhibited a high affinity for CopZ. Thus, the interaction is not governed by copper bridging of the two CxxC motifs in CopA and CopZ, and other protein domains must be involved in protein–protein interaction. However, the CxxC motif of CopA modulated the interaction with CopZ in response to copper. With wild-type CopA, the addition of copper(I) decreased the dissociation rate k_d by 15-fold (cf. Table 1). Mutation of the CxxC motif of CopA to SxxS eliminated this modulating activity of copper, presumably by preventing copper binding to this site.

In the reverse experiment, immobilized CopZ did not display CopA binding activity. Since CopZ is a protein of only 69 amino acids, it is likely that immobilization to the sensor chip resulted in steric hindrance for CopA binding. The basic amino acid residues of CopZ, which are the reactive groups for immobilization, are all cultered on one side of CopZ (17). These residues are also most likely required for docking to CopA. This finds support from studies of HAH1 and Atx1, the human and yeast homologue of CopZ, where basic amino acids have been shown to be critical for the interaction with the respective copper ATPases (12, 30, 31).

According to our working hypothesis, copper enters the cell via CopA under copper limiting conditions. The interaction of CopA with CopZ suggests that the chaperone can subsequently serve to route copper to other sites. Evidence for copper uptake by CopA is derived from the observation that *copA* knock-out strains do not exhibit a change in copper resistance, but are unable to grow under copper starvation. Furthermore, *copA* knock-out strains show increased resistance to

silver compared to wild type (32), presumably because CopA can be a route for silver entry into the cell. Other related copper ATPases have also been implicated in copper uptake. Disruption of the gene for CtpA of *Listeria monocytogenes*, which has 44% sequence identity with CopA, leads to reduced growth in copper limiting media and to reduced virulence (33, 34). A CopA homologue was also identified in *Staphylococcus aureus* as a gene induced during infection and therefore involved in virulence (35). Likely, the product of this gene serves in copper accretion by the cell. Since there is essentially no free copper in eukaryotic cells (1), pathogenic bacteria must be furnished with highly efficient copper uptake mechanisms to supply the cell with essential copper. CopA of *E. hirae* and homologous enzymes may have evolved in response to this need. However, additional experiments are required to further support this hypothesis.

Irrespective of the function of CopA, its interaction with CopZ demonstrates that these two proteins "communicate" with each other. The interactions of CopZ homologues, such as yeast Atx1 or human HAH1, with their respective targets, Ccc2 and Menkes/Wilson copper ATPase, respectively, has been semiquantitatively assessed with the yeast two-hybrid system (7, 8, 12). In this *in vivo* method, proper folding of the chimeric proteins is not assured and cannot be tested easily. In the BIACORE method used here, purified, native proteins were employed as starting material. The covalent binding of the proteins to the sensor chip is not believed to disturb their conformation. In fact, immobilization of proteins to solid supports is a standard method to enhance the stability of enzymes for medical and biotechnological applications (36–38). The validity of our approach is underlined by the specific binding of CopZ to CopA with reasonable kinetic parameters, and the lack of binding of CopZ to the unrelated copper binding protein APP. Functional competence of CopA bound to the sensor chip is also supported by the extent of CopZ binding, which amounted of 80% of the theoretical binding capacity, assuming a CopZ:CopA stoichiometry of one.

Taken together, these results show that the *E. hirae* CopZ copper chaperone specifically interacts with the CopA copper ATPase and thus reveal a new link in intracellular copper routing in *E. hirae*. The present study also demonstrates the utility of SPR analysis as a novel method to quantitatively assess metallochaperone-target interactions.

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