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The *Enterococcus hirae* paradigm of copper homeostasis: Copper chaperone turnover, interactions, and transactions

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Abstract

The cop operon is a key element of copper homeostasis in *Enterococcus hirae*. It encodes two copper ATPases, CopA and CopB, the CopY repressor, and the CopZ metallochaperone. The cop operon is induced by copper, which allows uncompromised growth in up to 5 mM ambient copper. Copper uptake appears to be accomplished by the CopA ATPase, a member of the heavy metal CPx-type ATPases and closely related to the human Menkes and Wilson ATPases. The related CopB ATPase extrudes copper when it reaches toxic levels. Intracellular copper routing is accomplished by the CopZ copper chaperone. Using surface plasmon resonance analysis, it was demonstrated that CopZ interacts with the CopA ATPase where it probably becomes copper loaded. CopZ in turn can donate copper to the copper responsive repressor CopY, thereby releasing it from DNA. In high copper, CopZ is proteolyzed. Cell extracts were found to contain a copper activated proteolytic activity that degrades CopZ *in vitro*. This post-translational control of CopZ expression presumably serves to avoid the accumulation of detrimental Cu-CopZ levels.

Copper circulation in *Enterococcus hirae*

Copper is a cofactor in many redox reactions through its ability to cycle between oxidized Cu(II) and reduced Cu(I). Among the over 30 enzymes using copper as a cofactor are Cu/Zn superoxide dismutase, cytochrome c oxidase, lysyl oxidase, tyrosinase, coagulation factors Va and VIII, and dopamine β-hydroxylase, to name just a few. Yet, copper can be very toxic to cells through its ability to form free radicals and cells must protect themselves from such copper-induced damage by tightly controlling the form, complex type, and concentration of cytoplasmic copper. This is accomplished on one hand by keeping intracellular copper complexed at all times. In yeast, it has been estimated that there is less than one free copper ion per cell in the cytoplasm (Rae *et al.* 1999). On the other hand, cellular copper levels are tightly controlled by copper homeostatic machinery that presents itself in ever increasing complexity. Many proteins involved in copper homeostasis have thus far been identified in prokaryotes and eukaryotes (for review see Camakaris *et al.* 1999; Harrison *et al.* 2000; Horn & Tümer 1999; Mercer 2001; Pena *et al.* 1999).

In the Gram-positive bacterium *Enterococcus hirae*, copper homeostasis is understood in some detail and the current model of copper circulation is summarized in Figure 1. 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 (Odermatt *et al.* 1992, 1993). While the function of CopB in copper excretion had been shown by direct demonstration of $^{64}Cu^{+}$ 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* knockout strain: (i) it grows like wild-type under normal or elevated copper conditions, (ii) it cannot grow in

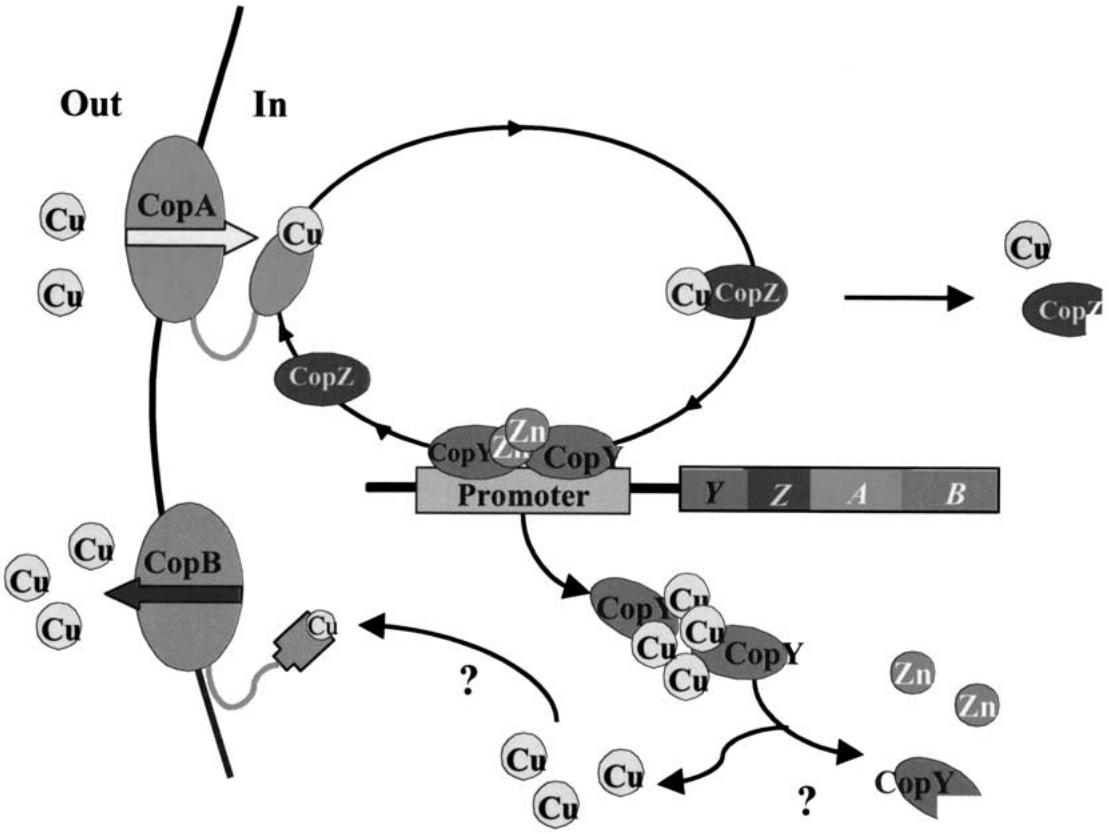

Fig. 1. Copper circulation in *E. hirae*. Model of copper circulation in *E. hirae*. The import and export ATPase pumps, CopA and CopB, regulate the intracellular concentration of copper by pumping metal across the membrane into and out of the cytoplasm. CopZ transfers copper from CopA to the repressor CopY. In CopY, one zinc is replaces by two copper(I) ions, which releases it from the DNA and induces expression of the *cop* operon. Cu-CopY may be degraded, as is excess Cu-CopZ under high copper conditions.

copper-depleted media, and (iii) it is more silver resistant than the wild-type, presumably because CopA can be a route for silver entry into the cell (Odermatt *et al.* 1993).

Intracellular copper routing: the copper chaperones

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, the specialized proteins which deliver copper intracellularly to copper utilizing enzymes (Harrison *et al.* 2000; O'Halloran & Culotta 2000). In *Enterococcus 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, copper displaces its bound zinc and the repressor dissociates from the promoter, allowing expression of the downstream genes (Cobine *et al.* 1999).

The solution structure of CopZ has been solved by NMR. It exhibits a $\beta\alpha\beta\beta\alpha\beta$ global structure: two α-helices laying on a 4-stranded, antiparallel β-sheet, a structure colloquially called an 'open face sandwich'. A key element of the structure is a cysteine-x-x-cysteine motif, located between the firs β-sheet and the first α-helix. It binds copper(I) in a novel, solvent exposed binding site. The same fold identified for CopZ has also been determined for the related eukaryotic copper chaperones, Atx1 from yeast, and Atox1 (formerly HAH1) from humans (Rosenzweig *et al.* 1999; Rosenzweig 2001). CopZ-like structural elements are a widespread feature of proteins involved in metal ion homeostasis and essentially identical struc-

tural building blocks are found singly or in up to six copies as metal binding modules in the N-termini of a number of metalloenzymes. The motif is present in six copies in the N-termini of the human copper ATPases ATP7A and ATP7B which are defective in Menkes and Wilson disease, respectively. One or two CopZ-like elements are also found in bacterial cadmium and copper ATPases, including CopA of *E. hirae*, in mercuric reductases, and in the multi-domain copper chaperones for superoxide dismutase (CCS; Lamb *et al.* 2001; Rosenzweig & O'Halloran 2000).

Chaperone-target interactions

The function initially demonstrated for CopZ is the transfer of copper from Cu-CopZ to the CopY repressor for regulation (Cobine *et al.* 1999). In contrast, the eukaryotic chaperones Atx1 from yeast and Atox1 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; Larin *et al.* 1999; Pufahl *et al.* 1997)). These *trans*-Golgi copper ATPases in turn pump copper into the Golgi network where it is required for the biosynthesis of cuproenzymes, such as the Fet3p iron reductase in yeast or ceruloplasmin in mammalian cells (Dancis *et al.* 1994; Yuan *et al.* 1995).

How chaperones recognize their targets and how they interact with them at the molecular level has been studied by site directed mutagenesis and structural investigations (Huffman & O'Halloran 2001). The surface residues presented by each partner provide complementarity of the interfaces. Multiple lysine residues clustered on the surfaces of Atox1, Atx1 and CopZ appear to play key roles in protein-protein interactions, albeit the location of these lysine patches is different in CopZ and Atx1/Atox1 (Figure 2; Wimmer *et al.* 1999). In CopZ, the lysine residues 30, 31, 37, and 38 appear to be important for the interaction with the CopY repressor. MNKr2, the second CopZ-like copper binding domain of the human Menkes copper ATPase, cannot donate copper to CopY in spite of its similar predicted structure. However, if four extra lysine residues are introduced into MNKr2 at positions corresponding to the lysine residues in CopZ (cf. Figure 2), the resultant mutant molecule MNKr2K4 becomes competent in donating copper to CopY. This 'gain-of-function' mutation of MNKr2 delineates these four lysine residues as key features in the interaction of the CopZ chaperone with the CopY repressor. Although CopZ and the related chaperones Atx1 and Atox1 have the same protein fold, they differ markedly in the arrangement of critical surface residues. This highlights the varying mode of interaction with different targets by these chaperones.

The CopA ATPase of *E. hirae* has been proposed to be the point of copper entry into the cell. As such, it would be a likely copper loading station for CopZ. The interaction of CopZ with the CopA ATPase was investigated by surface plasmon resonance (SPR) analysis with a Biacore apparatus. The technique can detect the binding of a protein in solution to a protein immobilized on 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. We found that CopZ specifically binds to CopA and that copper modulates this binding. The association of CopZ with CopA and the dissociation of CopZ from CopA in the absence and presence of copper(I) are shown in Figure 3. Maximal binding of CopZ to CopA is observed in the presence of sub-stoichiometric amounts of copper(I) (10 μM copper(I) corresponding to a molar stoichiometry of 0.9 copper(I):1CopZ; copper was added as the stable copper(I)acetonitrile complex; Hemmerich & Sigwart 1963). In the presence of 100 μM copper(I), the steady-state association of CopZ with CopA was reduced to approximately 25%. In the absence of supplemented copper(I), a transient binding maximum is observed due to incomplete copper depletion through contaminating copper in the running buffer. The association rate k_a is 2.4×10^3 $M^{-1}s^{-1}$ without added copper and is only slightly affected copper. The dissociation rate k_d is $14 \times 10^{-3}s^{-1}$ and is strongly influenced by added copper with a decrease of up to 15-fold. This results in a 7 and 16-fold increase of the affinity constant K_D in the presence of 100 and 10 μM copper(I), respectively. It was also shown that mutating the cysteine-x-x-cysteine copper binding motif in the N-terminus of CopA to serine-x-x-serine abolishes the copper induced decrease in the dissociation rate, without significantly affecting the association rate (Multhaup *et al.* 2001). Thus, the CopZ chaperone can interact with the CopA ATPase in a structure and copper dependent manner. Which residues of CopZ and CopA are involved in this interaction remains to be shown. However, the CxxC motif is not the guiding element for this interaction. Figure 4 shows a model of a possible mechanism for CopZ-CopA interaction and metal transfer.

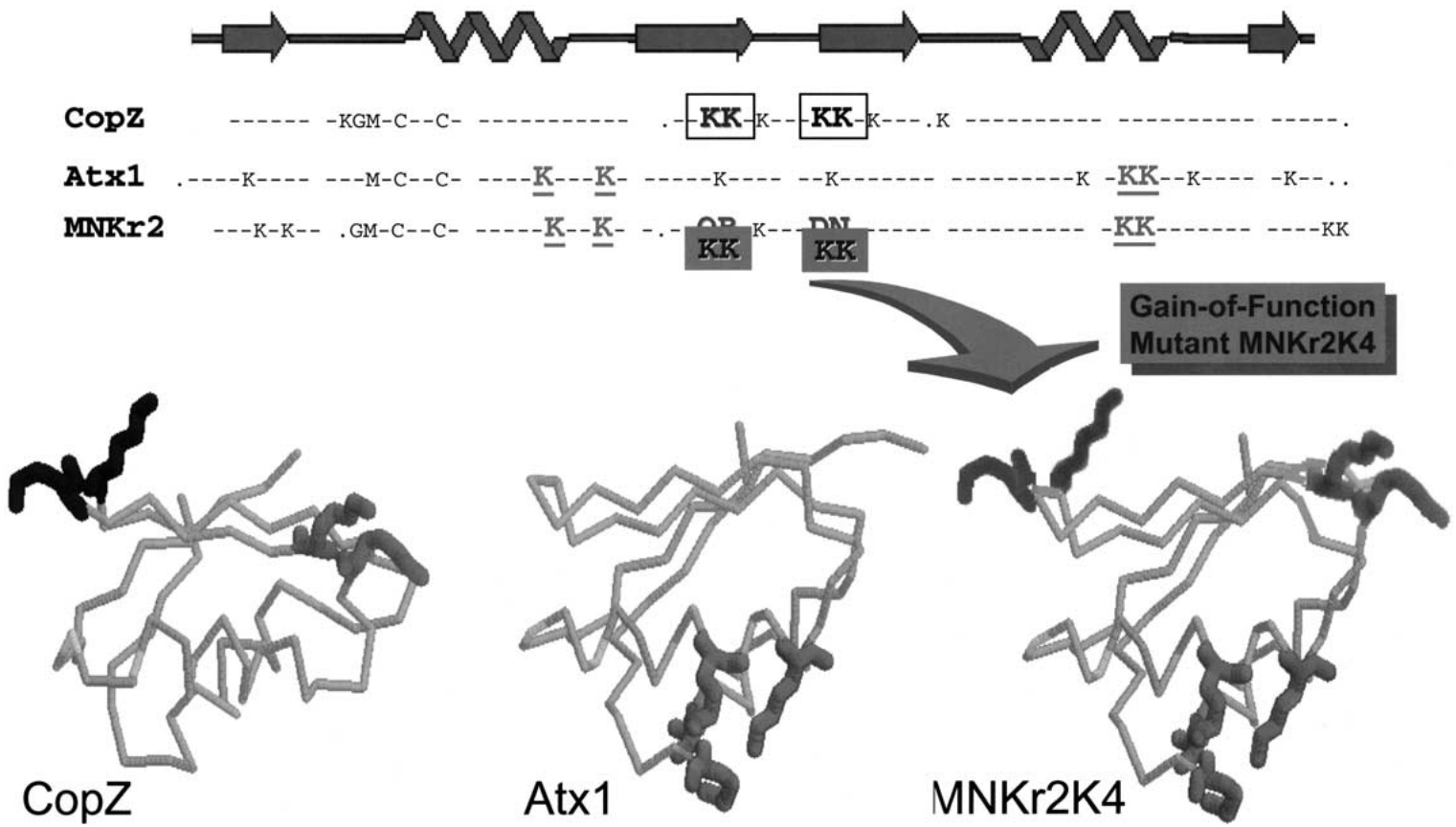

Fig. 2. Critical lysine residues of CopZ, Atx1, and MNKr2K4. The lysine residues critical for CopZ function are boxed and are on the upper side of the CopZ fold shown. These residues are not present in Atx1 or MNKr2. The residues critical for Atx1 chaperone function are enlarged and underlined, as are the corresponding lysine residues of unmodified MNKr2. In the representations of the Atx1 and MNKr2K4 folds, these lysines are located in the lower half. The four lysine residues that have been introduced into MNKr2 to obtain the gain-of-function mutant molecule MNKr2K4 are shown below the sequence alignment. The lysine mutations introduced into MNKr2K4 are Q30K, R31K, D37K, and N38K. The structure of MNKr2K4 with the four extra lysine residues was modeled.

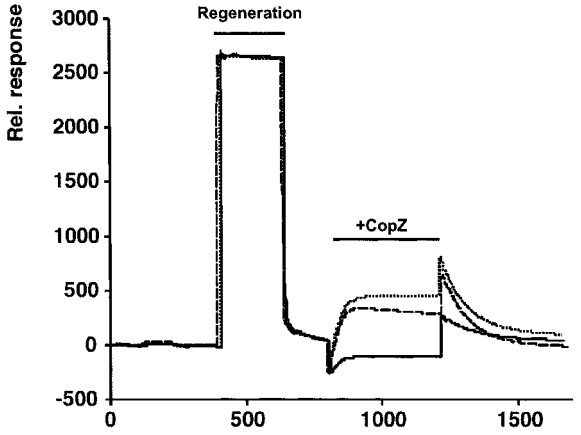

Fig. 3. Association-dissociation of CopZ and wild-type CopA. CopA-CopZ interaction was measured by surface plasmon resonance analysis with the Biacore apparatus. Following regeneration of a CopA loaded chip with high salt and a reducing agent, CopZ at a concentration of 100 μg/ml was interacted in the 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.

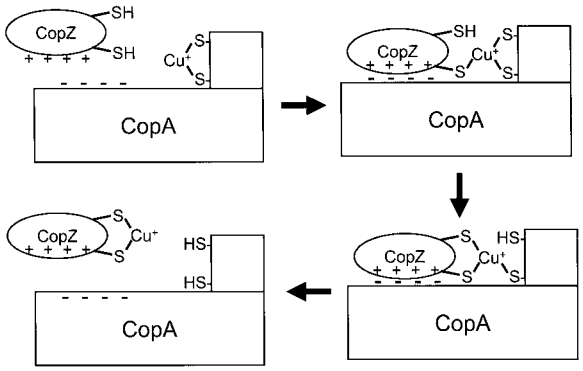

Fig. 4. Model of CopA-CopZ interaction and copper transfer. The CopZ chaperone docks on the CopA ATPase by protein-protein interaction involving positively charged surface lysine residues on CopZ and negatively charged amino acids on CopA. Copper is then transferred from CopA to CopZ by sequential ligand transfer. Finally, CopZ dissociates from CopA. This step could be facilitated by conformational changes of CopA associated with the pumping cycle.

Proteolytic degradation of CopZ

Proteolysis provides cells with an additional means to modulate 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 Gottesman & Maurizi 1992; Gottesman 1996 for review). In *Saccharomyces cerevisiae* copper induced degradation of the copper transporter Ctr1p has been described (Ooi *et al.* 1996). In this organism, the transcription factor Mac1, which is involved in high affinity copper uptake, is also degraded under high copper concentrations (Zhu *et al.* 1998). In the green algae *Chlamydomonas reinhardtii, apo*-plastocyanin, a copper binding protein involved in photosynthetic electron transport, is rapidly degraded under copper deficient conditions. The degradation liberates the copper for use in essential cuproenzymes (Li & Merchant 1995). However, the proteases involved in any of these processes have not been identified.

In *E. hirae*, proteolysis of CopZ under high copper conditions was observed. CopZ is encoded by a polycistronic message also encoding CopA, CopB, and CopY and these four genes are thus co-induced by copper. Levels of 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 increases only up to 0.5 mM copper and declines at higher copper concentrations, to become nearly undetectable at 3 mM copper (Lu & Solioz 2001). 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 is inhibited by ambient copper in excess of 0.1 mM and ceases to grow in 1.5 mM copper, while growth of wild-type *E. hirae* is not markedly affected by this copper concentration, and (ii) CopZ overexpression makes *E. hirae* more sensitive to oxidative stress, evident by increased sensitivity to H_2O_2 and paraquat.

Proteolysis of CopZ could also be demonstrated *in vitro*. When cytosolic extracts are mixed with purified CopZ, it is rapidly degraded (Lu & Solioz 2001). Interestingly, *apo*-CopZ is 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 (Cobine *et al.* 1999) and this copper can most likely participate in Fenton-type reactions, leading to the generation of reactive hydroxyl radicals and cell damage.

The protease responsible for CopZ degradation is not induced by exposure of cells to copper and is also present in cells in which protein synthesis has been inhibited for one hour with chloramphenicol. Rather, the proteolytic activity appears to be constitutive, but stimulated by copper(I) or Ag(I), a potential Cu(I) mimetic. The serine protease inhibitors *p*-phenylmethylsulfonyl fluoride (PMSF) and *p*-aminobenzamidine inhibit

the degradation of CopZ, while N-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which are also serine protease inhibitors, are without effect. The metallo-proteinase inhibitor *o*-phenanthroline does also not inhibit CopZ degradation. It was thus concluded that the protease degrading CopZ is a serine type protease (Lu & Solioz 2001). On zymograms, the CopZ degrading activity was tentatively identified as a protein of 58 kDa. This protein displays the expected properties, namely activation by copper and inhibition by *p*-aminobenzamidine.

Taken together, these results show the specific degradation of the CopZ copper chaperone under high copper conditions. This step adds another level of control to the copper homeostatic system of *E. hirae* (cf. Figure 1).

Conclusions

The *cop* operon of *E. hirae*, encoding the four genes CopY, CopZ, CopA and CopB, is regulated at the transcriptional level by the copper responsive repressor, CopY. It is released from the promoter by increased copper levels, thereby allowing transcription to proceed. For this process, Cu-CopZ donates copper to the CopY repressor, which leads to the loss of the zinc(II) from CopY and its ability to bind to DNA. In addition to this transcriptional control, the intracellular concentration of CopZ is also regulated at the post-translational level by proteolytic degradation of CopZ under high copper conditions. The latter implies that CopZ is dispensable under high copper conditions. Conceivably, excess Cu-CopZ becomes toxic to cells as such or interferes with another detoxification process that sets in under high copper conditions. A corollary to CopZ degradation is that CopZ is dispensable for the secretion of excess copper, which proceeds *via* the CopB copper ATPase. Conceivably, copper complexed to glutathione or other biomolecules is the substrate for CopB. The proteolytic degradation of CopZ is a novel mechanism in copper homeostasis. Given the high evolutionary conservation of some components of copper homeostatic systems, similar mechanism are likely to operate in other cell types as well. The interaction of CopZ with the putative copper import ATPase CopA, revealed by surface plasmon resonance analysis suggests that CopZ picks up copper from CopA under copper limiting conditions. Surface plasmon resonance analysis is a novel, powerful tool for the analysis of protein-protein interactions in copper homeostasis.

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