A novel copper-binding protein with characteristics of a metallothionein from a clinical isolate of Candida albicans

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It is known that clinical isolates of Candida albicans exhibit a high level of resistance to copper salts, although the molecular basis of this resistance is not clear. To investigate this, a novel copper-binding protein was purified from a clinical isolate of C. albicans. The protein was extracted from yeast cells after an induction period of 10 h in a copper-containing suspension medium. It was further purified by size-exclusion chromatography, ultrafiltration and reverse-phase HPLC. All protein fractions were analysed for their protein and copper contents. The copper/protein ratio increased steadily throughout the purification process; the most highly purified fraction showed a 210-fold increase compared to the whole-cell extract, with a recovery of 0.03%. The molecular mass of the protein was 10000 Da and a reconstitution study using the purified apoprotein suggested that the equivalent extent of Cu(I) binding was approximately 14 mol eq. The amino-terminal segment of the copper-binding protein revealed three Cys-Xaa-Cys motifs, which is typical of a metallothionein (MT), and showed significant homology with mammalian MTs with respect to the positions of the cysteine residues. This is the first report of the isolation of a copper-binding protein from C. albicans.

Keywords: Candida albicans, copper-binding protein, protein purification and characterization, amino-terminal sequence, metallothionein

INTRODUCTION

Copper requirements of micro-organisms are usually satisfied by low external concentrations of the metal (in the order of 1–10 µM), although this is markedly dependent on the copper-complexing capacity of the growth medium. In contrast, copper present at higher levels in its free ionic form (Cu²⁺) can be toxic to microbial cells (Palmiter, 1998; Liu & Thiele, 1997). Copper toxicity is mainly due to its interactions with nucleic acids (Lippert, 1992), to the alteration of enzyme active sites and to the oxidation of membrane components, processes that may be related to the ability of copper to generate toxic hydroxyl free radicals (Simpson et al., 1988). On the other hand, organically complexed copper is relatively nontoxic to micro-organisms (Maret & Valle, 1998). To balance the stimulatory and inhibitory properties of copper, micro-organisms are equipped with a number of homeostatic mechanisms that ensure proper accumulation, distribution and detoxification of the metal. The best understood of these are the metallothioneins (MTs), a family of cysteine-rich polypeptides thought to play a critical role in copper ion storage and detoxification. Fungi invoke a variety of pathways to respond to increased concentrations of metal ions (Cervantes & Gutierrez-Corona, 1994; Fischer & Davie, 1998; Liu & Thiele, 1997; White et al., 1998; Zhu & Thiele, 1996). Among the sequestration compounds that are induced, MTs and (γ-EC)₉G peptides constitute the two most widely used detoxification molecules (Mehra & Winge, 1991; Winge, 1998).

Candida albicans is the most pathogenic and medically important yeast in the genus Candida (Madhani & Fink, 1998). It has been known for some time that clinical isolates of C. albicans and Candida glabrata exhibit high levels of resistance to both copper and cadmium salts, although the molecular basis of this resistance is not known (Malavasic & Cihlar, 1992; Mehra & Winge,
1991). Recent studies with *C. glabrata* have revealed that this yeast employs differing mechanisms to detoxify cadmium and copper salts (Liu & Thiele, 1997). Cadmium salts stimulate the production of (γ-EC)₆G peptides, whereas copper salts induce the synthesis of a family of MTs. Several fungi that are pathogenic in humans, including *C. albicans*, have been screened for the presence of DNA sequences homologous to the *Saccharomyces cerevisiae* MT gene. Southern blot and restriction enzyme analysis showed that one of the *C. albicans* strains examined by Butt & Ecker (1987) contained DNA sequences which hybridize with *S. cerevisiae* MT. However, detailed analysis of the putative MT locus of *C. albicans* has yet to be performed. The cloned MT-like gene could be useful as a selectable marker and for regulated gene expression studies in pathogenic fungi. This paper focuses primarily on the purification of a novel copper-binding protein from a clinical isolate of *C. albicans*. The amino-terminal sequence of the purified protein was determined, and binding of copper was studied.

**METHODS**

**Yeasts strains, medium and culture conditions.** *C. albicans* KULM 83-0300, a clinical isolate, was obtained from the Kitasato University Hospital, Kanagawa, Japan. As a preliminary test of the copper resistance of this isolate, its growth was compared with that of several other yeast strains (*C. albicans* ATCC 10231, *Candida tropicalis* ATCC 750 and *S. cerevisiae* IFO 0565) on YPD agar plates [1% (w/v) yeast extract, 2% (w/v) polypeptone, 2% (w/v) glucose and 2% (w/v) agar] containing various concentrations of CuSO₄. Growth of the tested yeasts was inhibited at concentrations above 30, 15, 10 and 5 mM CuSO₄, respectively. The induction of copper-binding protein was detected in all *Candida* strains by the sodium diethyldithiocarbamate (SDTC) staining method as described below. Thus *C. albicans* KULM 83-0300 was used throughout this study. To prepare the copper-binding protein, yeast cells (5 x 10⁶ cells ml⁻¹) were grown aerobically in YPD liquid medium at 28 °C. CuSO₄ was added to YPD cultures at approximately the midpoint of exponential growth to a final concentration of 5 mM. The cells were harvested after 10 h by centrifugation, washed twice with H₂O and then lyophilized.

**Purification of the copper-binding protein.** Samples (10 g) of freeze-dried yeast cells were physically disrupted with a cold mortar and pestle for 20 min. They were then extracted with 100 ml 20 mM Tris/HCl (pH 7.5) buffer containing: MgCl₂, 2H₂O, 5 mM; KCl, 50 mM; glycerol, 5% (w/v); PMSF, 1 mM; DTT, 3 mM; pepstatin A, 1 µg ml⁻¹; and leupeptin, 0.5–3 µg ml⁻¹. Cell extracts were centrifuged at 28000 g for 30 min to obtain a clarified supernatant. The supernatant was concentrated by lyophilization and suspended in 12 ml N₂-saturated 10 mM Tris/HCl (pH 7.4) containing 0.2% β-mercaptoethanol and then applied to a column of Sephadex G-75 (3 x 40 cm, Pharmacia Biotech) equilibrated in the same buffer. The copper-binding-protein fractions were identified by copper analysis using atomic absorption spectrophotometry (model AA6600G, Shimadzu). The pooled fractions were concentrated by lyophilization. After resuspension in the same buffer, the sample was ultrafiltered (2000 g, 4 °C, 20 h) using an Ultrafree-4 centrifugal filter unit equipped with a high-flux Biomax membrane (nominal molecular-mass cutoff, 30000 Da; Nihon Millipore).

The resulting filtrate containing copper-binding protein was applied to a reverse-phase HPLC μ-Bondapak C₈ column (3.9 x 150 mm; Nihon Waters) connected to a Hewlett Packard HPLC system (HP 1100 series). Adsorbed material was eluted with a linear gradient of 0–10% solution B (60% acetonitrile in solution A) in solution A (10 mM Tris/HCl containing 0.2% β-mercaptoethanol, pH 7.4) at a flow rate of 1 ml min⁻¹. The major copper-containing peaks were pooled, 4 vols ice-cold acetone were added and the samples incubated overnight at −80 °C. The precipitate was collected by centrifugation at 28000 g for 15 min and stored anaerobically at −80 °C with Tris/HCl buffer (pH 7.4). This solution was used as purified copper-binding protein for subsequent studies.

**PAGE.** Nondenaturing PAGE was carried out at pH 8.9 using a 15% acrylamide gel. Protein was measured by the method of Bradford (1976) using bovine serum albumin as a standard. After completion of electrophoresis, the gel was cut into two strips longitudinally. One strip was used for protein staining with Coomassie brilliant blue (CBB) R-250 as follows. The gel was soaked in 10% acetic acid containing 0.25% CBB and 45% methanol for 1 h. It was then destained with 10% acetic acid containing 45% methanol for 1 h and stored in 7% acetic acid containing 5% methanol. The second strip was used for copper staining with SDTC by incubation with 0.2% SDTC solution overnight (Naiki & Yamagata, 1976). During incubation, the brown band corresponding to the copper-binding protein appeared against a transparent background.

**Molecular mass of the copper-binding protein.** The molecular mass of the purified copper-binding protein was measured by HPLC gel filtration on a Shodex Protein KW-802.5 column (8 x 300 mm, Showa Denko) at a flow rate of 0.3 ml min⁻¹ with 50 mM Tris/HCl containing 0.2 M NaCl (pH 7.5) as an eluent. The molecular mass of the copper-binding protein was measured by comparison with the following calibration standard proteins from the LMW gel filtration calibration kit (Pharmacia Biotech): bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (137 kDa); and insulin chain B (4 kDa, Sigma).

**Preparation of apoprotein and amino-terminal sequence analysis.** The removal of copper ions from the copper-binding protein was accomplished by boiling the protein with a solution of 10 mM EDTA in 7 M guanidinium hydrochloride containing 0.5 M Tris (pH 8.5) for 5 min (Mehra et al., 1988). The mixture containing apoprotein was filtered with a 0.22 µm pore-size membrane filter. The resulting filtrate was fractionated by HPLC on a Shodex Protein KW-802.5 column. Adsorbed material was eluted with 0.02% trifluoroacetic acid. The protein peak was localized by measuring absorbance at 214 nm. The copper-free protein peak was pooled and concentrated by lyophilization. The amino-terminal sequence of the copper-binding protein was determined by automated Edman degradation of carboxymethylated apoprotein (Winge et al., 1985) using a Shimazu Protein Sequencer PPSQ-10 system (Shimadzu). Cysteine was determined by Edman degradation of S-pyridylethylated cysteine residues.

**Reconstitution of the apoprotein with Cu(I).** Copper reconstitution was carried out by the addition of increasing molar equivalents of Cu(I) to the apoprotein. A solution of Cu(I) prepared by the method of Mehra et al. (1988) was added to apoprotein (4 nmol) dissolved in 0.02 M HCl to achieve 1–20 mol eq. copper (mol protein)⁻¹. The samples were neutralized with 200 µl 0.2 M dibasic potassium phosphate and diluted to a final volume of 500 µl with water. The absorbance and luminescence spectra of the reconstituted...
Copper-binding protein from *Candida albicans*

Samples were measured using UV spectrophotometry (model UV-1200, Shimadzu) and spectrofluorometry (model FP-777, Japan Spectroscopic), respectively.

**Proton displacement of Cu(I) from the copper-binding protein.** Samples of the native protein (4 nmol) were adjusted to the desired pH by adding 500 µl 0.2 M potassium phosphate preadjusted to that pH. The absorbance spectra of these samples were then recorded. In this experiment, the base line absorbance changed in response to pH change. A base line correction was performed.

**RESULTS**

**Induction and isolation of a copper-binding protein from *C. albicans***

*C. albicans* KULM 83-0300 is capable of growth in YPD medium containing high concentrations of copper salts. Inhibition of growth by 50% requires approximately 15 mM CuSO$_4$. We sought to determine if metal-sequestering macromolecules contributed to this apparent copper resistance. A summary of the purification procedure for the copper-binding protein from *C. albicans* KULM 83-0300 is presented in Table 1. Since copper-binding proteins are readily oxidized (Berger *et al*., 1997), it was necessary to minimize the duration of the purification procedure. In the present work, all purification steps were carried out on two consecutive days with purging using nitrogen. The samples of purified copper-binding protein for subsequent studies were prepared just prior to use. However, the results shown in Table 1 suggested that the copper-binding protein accounts for less than 0.1% of the protein in cell extracts. Fractionation of the clarified cell extract prepared from cells grown in copper-supplemented medium by Sephadex G-75 gel filtration yielded a single copper-containing component, making up 42% of total starting protein. After further fractionation of the eluent by ultrafiltration, the copper-containing fraction was chromatographed using a reverse-phase HPLC µ-Bondapak C$_{18}$ column to obtain a pure sample of the copper-binding component. Assuming no copper release from protein during purification, the copper-binding component was purified 210-fold relative to whole-cell extract. Release of free copper ions could occur as a consequence of oxidation of the copper-binding protein. This final fraction contained 72% of total copper and 0.03% of total protein. Only one copper-containing component was detected during the purification steps.

| Table 1. Summary of the purification of the copper-binding protein from *C. albicans*** |
|----------------------------------|-------|-------|--------|-------|-------|
| Step                            | Total protein (mg) | Total Cu (µg) | Cu/protein (µg mg$^{-1}$) | Recovery (%) | Purification (-fold) |
| Cell extract                    | 231   | 4708  | 20     | 100   | 100   | 1     |
| Sephadex G-75                   | 9.8   | 2036  | 208    | 43.2  | 4.2   | 10    |
| Ultrafiltration                 | 3.2   | 1135  | 355    | 24.1  | 1.4   | 18    |
| Reverse-phase HPLC             | 0.08  | 337   | 4212   | 7.2   | 0.03  | 210   |

Native-PAGE analysis of the purified component indicated the presence of a single band when stained with CBB and SDTC (Fig. 1). The molecular mass of the purified molecule was determined by HPLC gel filtration to be approximately 10000 Da.

**Preparation of apoprotein and amino-terminal sequence analysis**

To ascertain the nature of this copper-binding protein, further fractionation of the apoprotein prepared by guanidinium hydrochloride treatment was carried out using HPLC on a KW-802.5 column. The removal of copper ions from the protein is commonly accomplished by the proton displacement method (Nielsen *et al*., 1985). This procedure was not effective with our protein, as it was found to precipitate upon acidification. Instead, copper removal was accomplished by boiling in a solution of 0.1 M EDTA in 6 M guanidinium hydro-
chloride containing 0.1 M Tris (pH 8.6), using techniques described by Mehra et al. (1988). Fractions encompassing the copper-free protein peak were pooled and the sequence of the amino-terminal 24 residues was determined by Edman degradation (Fig. 2). The amino-terminal 24 residues of the polypeptide contained three repeating sequences of Cys-Xaa-Cys (Xaa refers to an unspecified residue), the Cys-Xaa-Cys motif being typical of MT. Furthermore, the first four cysteine residues of the copper-binding protein occupied exactly the same positions as the first four cysteine residues in the amino-terminal sequence region of MTs characterized in other organisms (Fig. 2). In addition, serine residue 5 and lysine residue 19 were invariant with three vertebrate MTs. Note the complete lack of aromatic amino acids in the copper-binding protein from C. albicans as well as in all the MTs sequenced so far (Lerch, 1980; Mehra et al., 1988, 1989). Because of these common structural features, we categorize the C. albicans copper-binding protein as a member of the MT superfamily.

The stoichiometry of copper binding determined by reconstitution of the apoprotein with Cu(I)

The copper-binding protein exhibited electronic transition in the near-UV similar to the typical charge-transfer transitions seen in copper-binding MTs (Fig. 3a). The copper-binding protein was luminescent with an uncorrected emission maximum of 530 nm (Fig. 3b) when excited with UV light (from 300 to 370 nm). The luminescence is characteristic of Cu(I)-thiolate coordination in an environment shielded from solvent interactions (Mehra et al., 1988). Cu(I)-thiolate electronic transitions are salient features of copper-binding MTs.

It is the presence of repeating Cys-Xaa-Cys sequences in vertebrate MTs that makes these proteins effective metal-binding molecules (Lerch, 1980). Because the C. albicans copper-binding protein described here contained three Cys-Xaa-Cys sequences within its amino-terminal 24 residues, it could be expected to show metal-binding characteristics similar to those of vertebrate MTs. The stoichiometry of metal binding was determined by reconstitution of the apoprotein with Cu(I). Titration of the apoprotein with increasing mole equivalents of Cu(I) resulted in increased absorbance at 250 nm until 14 mol eq. copper had been added (Fig. 4). No further increase in absorbance occurred when up to 20 mol eq. of Cu(I) were added. The equivalence point of titration of the apoprotein with Cu(I) may be defined as the minimum number of mole equivalents of Cu(I) required to disrupt the metal-thiolate clusters. Proton displacement studies have shown that the pH at which 50% of bound Cu(I) ions dissociate from rat and probably other mammalian MTs is 2.7 (Nielson et al., 1985). The pH of half-dissociation of Cu(I) bonding to S. cerevisiae MT and C. glabrata MT-II is 0.3 and 0.8, respectively (Byrd et al., 1988; Mehra et al., 1989). In this study, the C. albicans copper-binding protein lost 50% of Cu(I), as determined by loss of absorption at 250 nm, at pH 1.6 (Fig. 5). Since the absorbance in the near-UV is dominated by sulfur → metal charge transfer transitions (Nielson et al., 1985), the pH-dependent loss of absorbance is a reflection of the dissociation of Cu(I).

DISCUSSION

This is the first report of the isolation of a copper-binding protein from C. albicans. It is known that clinical isolates of C. albicans and C. glabrata exhibit elevated resistance to copper salts (Butt & Ecker, 1987). These species have been analysed for the induction of MT-like proteins in response to copper, cadmium, zinc and gold (Butt et al., 1984). Neither fungus produced a low-molecular-mass cysteine-rich protein in response to these metals. C. albicans strain 792 produced a protein similar to the MT found in S. cerevisiae in response to copper, and in C. glabrata strain 62 a unique, approximately 4500 Da protein was induced by copper (Mehra et al., 1988, 1989, 1990). In this study, C. albicans
Copper-binding protein from *Candida albicans*

KULM83-0300 cells cultured in the presence of 5 mM CuSO₄ synthesized a copper-binding protein. Only one copper-containing component was detected during the purification steps. The purified molecule had a polypeptide molecular mass of 10000 Da, as determined by HPLC gel filtration.

MTs have been described in most vertebrate and invertebrate species as low-molecular-mass proteins with high cysteine content (approx. 30%), a lack of aromatic residues and the presence of 7–12 heavy metal atoms per molecule (Kagi, 1993). Vertebrate MTs constitute a family of highly conserved proteins and the positions of the cysteine residues involved in metal binding are invariant (Hamer, 1986; Kagi & Kojima, 1988). Isometallothioneins from the invertebrate *Scylla serrata* show significant homology to each other as well as to vertebrate MTs (Hamer, 1986; Kagi & Kojima, 1988). In contrast, the two MT genes in the invertebrate *Drosophila* and *C. glabrata* encode proteins which show little sequence homology to each other (Lastowski-Perry et al., 1985; Mehra et al., 1989). In this study, the *C. albicans* copper-binding protein exhibited the typical MT sequence motif, Cys-Xaa-Cys. The role played by these sequence motifs in the formation of metal clusters in MTs is well recognized (Hamer, 1986; Kagi & Kojima, 1988). Thus the *C. albicans* copper-binding protein is structurally analogous to other well-characterized MTs.

In many cells, MT appears to exist with varying ratios of bound copper. Our reconstitution study suggested that...
the equivalent extent of Cu(I) binding of the purified C. albicans apoprotein was approximately 14 mol eq. Concentrations of copper in excess of 14 ions per molecule did not alter the absorption properties of the molecule. These results suggested that binding was specific. It is stressed that the value of 14 mol eq. is not rigorous since the UV absorption method provides only an approximation. Substantiation of these results could be obtained using luminescence (Mehra et al., 1989). Mammalian MT usually binds seven zinc ions, but it also can contain copper, cadmium and traces of other metals. Although binding stoichiometries and coordination geometry have not been clearly established for metal ions, Cu-MT is one form of the protein that deviates from the usual coordination of seven tetrahedrally bound metal ions per polypeptide (Boulanger et al., 1983; Nielsen & Winge, 1983). Nielsen et al. (1985) found that 11 or 12 copper ions were bound to MT. The S. cerevisiae CR55 MT-like protein (Culotta et al., 1994; Jensen et al., 1996) and C. glabrata MT (I and II) (Mehra et al., 1989) bind in excess of 10 copper ions per molecule. It is important to understand the coordination properties and structure of Cu-MT because the protein may function in cellular processes involving copper (Aschner, 1996; Jasani & Schmid, 1997). Further studies to elucidate the gene sequence and the cluster structure of the Cu-protein are in progress.

REFERENCES


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