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Cu(I)-thionein release from copper-loaded yeast cells

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Summary. The release of intact $Cu(I)_8$-thionein from copper-resistant copper-loaded yeast cells, strain X 2180-1Aa, has been shown. This copper(I)-thiolate-rich protein was characterized and compared with the chemical and physicochemical properties of intracellular yeast Cu-thionein. The same molecular mass and stoichiometry of 8 mol copper atoms/mol protein was found. No detectable difference between the Cu-thioneins was seen in luminescence emission, electronic absorption in the ultraviolet region, chiroptical data or amino acid composition. The importance of stable Cu(I)-thiolates in Cu-thionein as a safe vehicle for transporting copper in a non-reactive manner is confirmed.

Key words: $Cu(I)_8$-thionein — Yeast — Extracellular — Circular dichroism — Fluorescence — Electronic absorption

Introduction

The molecular mechanism of copper metabolism is unknown. The essential role of this prominent transition metal requires a finely tuned regulation of cellular copper levels (Weser and Hartmann 1984). Transport of 'free copper ions' must be considered to be rather dangerous as there are many undesired reactions including the following mechanisms.

a) There is virtually no free $Cu(II) \cdot aq$ ion at pH 7. Due to precipitation of $Cu(OH)_2 \cdot aq$, the concentration of hydrated Cu(II) lies beyond the femtomolar region (Bielski and Cabelli 1986).

b) The strong and unspecific binding to biopolymers creates considerable confusion in normal metabolism.

c) Cu(II) displaces, in a competitive manner, many other important metal ions including Mg(II), Cd(II), Mn(II), Zn(II) etc. The respective Cu-complexes are thermodynamically more stable.

d) The Jahn-Teller distorted Cu(II)-coordination compounds, though thermodynamically stable, exchange their axially bound ligands sometimes in less than 0.5 μs (Basolo and Pearson 1967). This kinetic lability can be harmful for many biochemical reactions.

e) Cu(II) acts as an oxidizing agent on —OH, —SH and —CHO moieties.

f) Cu(I) dismutates in aqueous solution to yield Cu_o and Cu(II).

g) Cu(I) can create excited oxygen species according to the Fenton or, as usually known, under the metal-driven Haber-Weiss reaction (Hartmann and Weser 1977; Haber and Weiss 1934). Transiently generated $^{\cdot}OH$ (373 $kJ \times mol^{-1}$) (Gaillard-Cusin and James 1973) may lead to the irreversible destruction of cellular components.

Fortunately, essentially all copper is specifically bound in many different proteins. In the intracellular protein called 'metallothionein' the copper is firmly bound in stable Cu(I)-thiolate clusters. In this form the above uncontrolled reactions of copper will be minimized. In fact, Cu(I)-thionein is increasingly considered to be important for controlling cellular copper metabolism (Weser and Hartmann 1984; Schechinger et al. 1986; Weser et al. 1986; Hartmann et al. 1987). At present, the cleavage of the Cu(I)-thiolate bonding of this ubiquitously present cysteine-rich protein is under intensive investigation. Several catabolic modes have been discussed, including the direct competitive copper extrusion by apo-copper-pro-

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teins. Furthermore, oxidation, proteolysis and alkylation of the Cu-thiolate centres are among the many other possibilities (Weser et al. 1986; Felix and Weser 1988).

Cu-thionein from the yeast *Saccharomyces cerevisiae* is characterized as a 53-residue polypeptide of $M_r = 5655$ containing 12 cysteine residues/molecule. Eight Cu(I)-thiolate centres are known in this protein molecule (Winge et al. 1985). In the presence of chelators specific for Cu(I), 2 mol copper/mol protein were rapidly removed, leaving the remaining 6 mol/mol tenaciously bound. It is concluded that there must be two or more distinct types of coordinated copper (Weser and Hartmann 1988).

Due to the multiplicity of the *CUP1* gene, yeast cells are able to enrich copper from the growth medium very efficiently up to millimolar concentrations. Unlike vertebrate metallothioneins, in which Zn, Cd and Cu are simultaneously bound, copper is exclusively coordinated in the yeast protein. In fact, most of the cellular copper is located in this protein.

One question remains open as to how the accumulated protein is metabolized by the Cu-thionein-loaded cell. Considerable amounts of extracellular copper were observed in the supernatant of washed cells S0 it seemed promising to investigate the nature of released copper-containing compounds. The excretion of intact Cu-thionein seemed attractive as a safe vehicle for transporting copper in a non-reactive manner. The released protein was characterised and compared with the known chemical and physicochemical properties of intracellular yeast Cu-thionein.

Materials and methods

Chemicals. Bathocuproinedisulphonate (disodium 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulphonate) was purchased from Fluka (Buchs), glutathione (GSH) was obtained from Merck (Darmstadt), nitro-blue tetrazolium chloride (NBT) and xanthine were from Serva (Heidelberg) and xanthine oxidase was from Sigma (Heidelberg).

Cu-thionein. The copper-resistant yeast *(Saccharomyces cerevisiae)* strain X 2180-1Aa was kindly donated by Julie Welch (Berkeley, CA) and was grown in a copper-containing medium (1.5 mM $CuSO_4$). Cu-thionein was isolated from ruptured cells as earlier described (Weser et al. 1977; Winge et al. 1985; Weser and Hartmann 1988). Preparation of the excreted Cu-thionein was performed as follows. Yeast (1 g cell paste) was washed twice with phosphate-buffered saline (PBS). The cell pellet was resuspended in 2 ml PBS and shaken for 1 h at 24° C. The cells were separated by centrifugation and the supernatant was chromatographed on a Sephadex G-50 (Pharmacia) column (1.2 × 50 cm), equilibrated with 10 mM $NaHCO_3$/HCl, pH 7 in the presence of 0.1% (by vol.) mercaptoethanol. The Cu-thionein fraction was pooled and lyophilized.

Analytical methods. Copper was analysed on a Perkin-Elmer 3030 atomic absorption spectrometer. Protein concentration was quantified by both the biuret method (Cooper 1981) and N_2 determination with elemental analyses performed on a Perkin-Elmer 240 B elemental analyzer. Electronic absorption was measured on a Hitachi U-2000 spectrophotometer. Amino acid analyses were carried out on a CK 10 F ion-exchange resin (Mitsubishi) and monitored on a Labotron liquimat 3A analyzer after sample hydrolysis using the method given by Moore and Stein (1963). Circular dichroic spectra were run on a Jasco 20A recording spectropolarimeter and fluorescence measurements were performed using a Spex fluorolog 222 double-beam unit.

Results and discussion

The copper-resistant yeast strain X 2180-1Aa was grown for 48 h in a medium containing 1.5 mM $CuSO_4$. After washing five times with 10 vol. water, the copper concentration in the cell was approximately 3 mg/g wet mass. Illumination at 366 nm of this processed yeast produced an orange-red fluorescence which was attributed to intracellular Cu-thionein. This luminescence emission at 610 nm is due to the copper(I)-thiolate chromophore of intact Cu-thionein (Richter and Weser 1988; Beltramini and Lerch 1981) (Fig. 1).

The same phenomenon was observed in the cell-free supernatant when the washed yeast was resuspended in water or buffer. This is indicative for the release of a Cu-thionein-like compound. The time-dependent excretion of the fluorescent product was measured by repeated incubation of 1 g suspended wet cells in 2 ml PBS (Figs 2 and 3). The released-copper concentration in the supernatant of the sedimented cells paralleled both the fluorescence intensity and the amplitude and shape of the characteristic circular dichroic signals of Cu-thionein.

The excretion lasted longer than 96 h, as monitored by the 610-nm emission. However, at this time only about 10% of the initial intracellular copper was extracted. When yeast cells were incubated in the same growth medium omitting any supplementary copper, the copper release was slightly higher. Much to our surprise, the additon of 1 mM reduced glutathione into the buffer or medium caused a 5–6-fold increase in copper concentration, luminescence emission and dichroic amplitude (Fig. 2). It should be emphasized that, even under these conditions, both the fluorescence and the circular dichroism were found to be typical for intact intracellular Cu-thionein. The characteristic chiroptical data can be considered

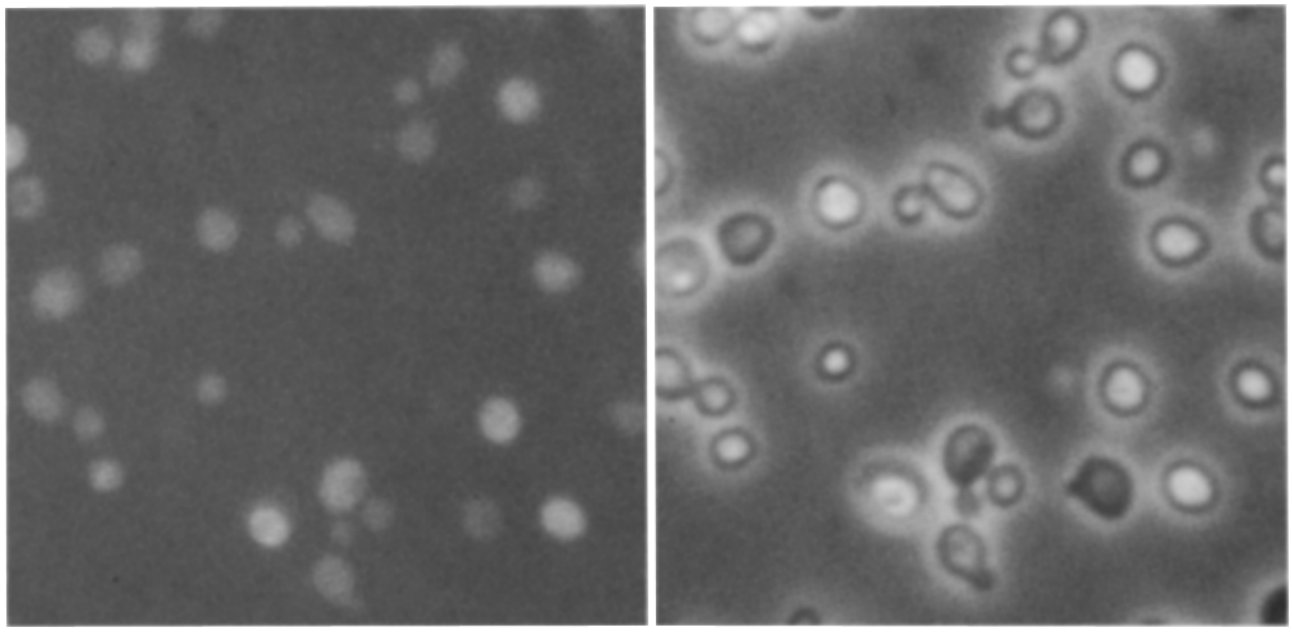

Fig. 1a, b. Microscopic fluorescence photograph of copper-loaded yeast cells. An identical area of cells was viewed throughout **a** fluorescence filter and **b** optics (magnification ×800)

the most specific proof indicating the existence of yeast Cu-thionein.

Characterization of the copper-binding compound was carried out by gel-filtration of the cell-free supernatant on Sephadex G-50. There was a similar elution pattern as obtained in the case of yeast cell homogenates with the intriguing exception that high-M_r compounds were essentially absent. About 70% of the eluted copper was associated with the Cu-thionein fraction. The remaining portion was analysed in the $M_r = 1000$ region. In order to compare the separated Cu-thionein with the intracellular species isolated earlier, a series of spectroscopic measurements was carried out. Identical ultraviolet electron absorption, luminescence emission and circular dichroism were obtained (Figs 4 and 5).

Quantification of protein and copper revealed a stoichiometry of 7.5 ± 0.3 mol copper/mol protein which is close to both the value of the intracellular Cu-thionein and the theoretical amount

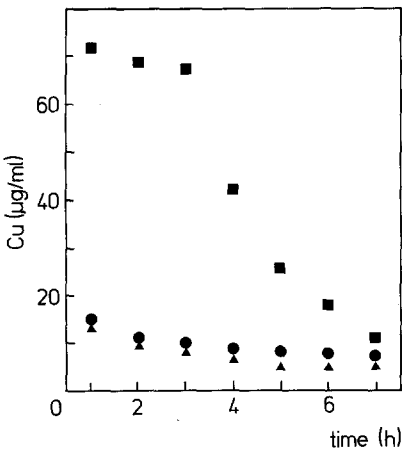

Fig. 2. Time-dependent copper release from copper-loaded yeast in the presence of different incubation media. (▲) PBS; (●) copper-free culture medium; (■) copper-free culture medium +1 mM reduced glutathione. Copper excretion was measured in the supernatant of 1 g wet yeast. Cells were repeatedly suspended in 2 ml incubation medium after shaking for 1 h at 24° C

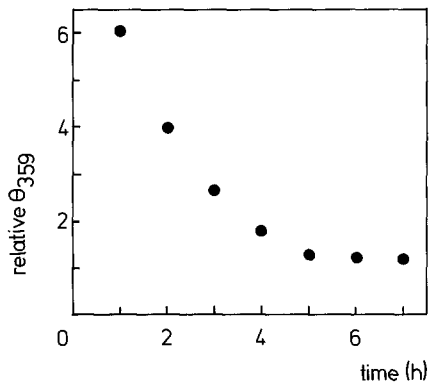

Fig. 3. Time-dependent copper release from copper-loaded yeast. Dichroic amplitude at 359 nm of excreted yeast Cu-thionein after repeated incubation of cells in PBS. The supernatants were directly used for the measurements. Incubation conditions were the same as in Fig. 2

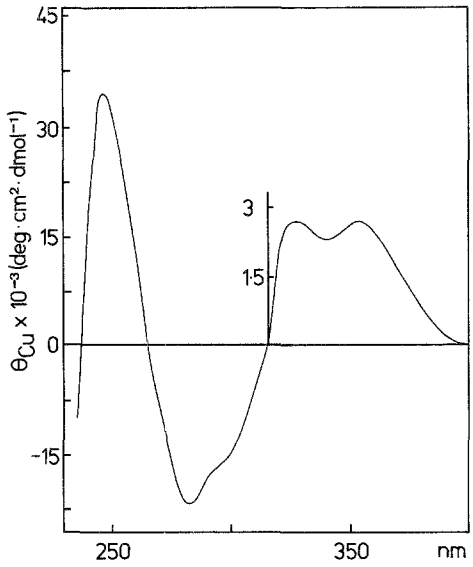

Fig. 4. Circular dichroism of excreted yeast Cu-thionein

of copper (8 mol/mol). At the same time, the amino acid composition was in accordance with earlier work (Weser et al. 1977). In addition, the reactivity with the specific Cu(I)-chelator bathocuproinedisulphonate was examined. Again the same 20–25% Cu(I)-reactive copper was measured without any change in the intensity of the observed Cotton bands (Weser and Hartmann 1988). Identical specific molar ellipticities expressed for copper were obtained (i.e. $\theta_{Cu}^{359} = 1700 \pm 100$ deg · cm² · dmol⁻¹).

In conclusion, we are dealing with an intact Cu-thionein excreted from living yeast cells in considerable concentration. A similar excretion was seen when normal baker's yeast previously treated with copper salts was employed. Proof that the yeast cells were alive was obtained by growing the cells in the above copper-containing

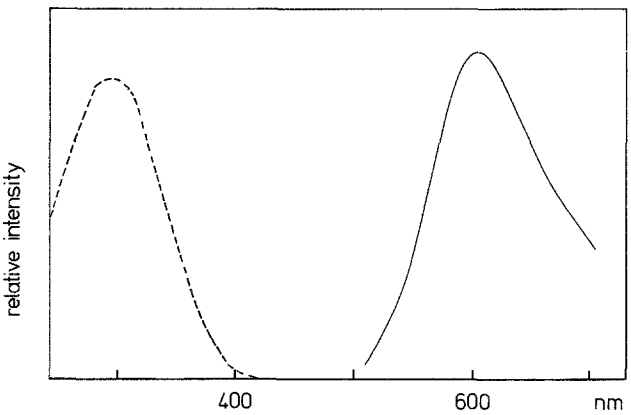

Fig. 5. Corrected excitation and emission spectra of released yeast Cu-thionein

medium for a further 24 h, both before and after 96 h Cu-thionein release. An identical growth rate was found in both cases, as calculated by estimation of the final cell volume. Furthermore, if any cell damage had taken place, characteristic cytosolic enzymes such as Cu_2Zn_2-superoxide dismutase, should have been detected. No superoxide dismutase activity at all was seen in the supernatant using the NBT assay.

The mechanism of the Cu-thionein release is still unknown. Whether there is an active membrane transport or just a simple discharge process by free diffusion needs further investigation. However, due to the reactivity of glutathione which dramatically accelerates the Cu-thionein excretion, it is obvious that an energy-consuming process is involved. An increased intracellular glucose supply may be the cause for this phenomenon. In the presence of NADPH the glycolytic pathway of glucose is favoured which results in an elevated production of ATP. The glutathione-supported NADPH generation from $NADP^+$ is well known (Stryer 1975).

A relationship of the Cu-thionein release from yeast cells and the known production of microbial siderophores is very attractive (Winkelmann and Huschka 1987). This would be the first known case of a membrane-transferable low-molecular-mass copper-containing protein.

Conclusion

Although the intracellular catabolism of Cu-thionein is still unknown, the excretion of intact Cu-thionein is of utmost importance in general copper metabolism. Cu-thionein would be a most appropriate compound to avoid the many uncontrolled reactions of free copper briefly mentioned in the Introduction. In other words, the safest way to transport copper(I) is in the Cu(I)-thiolate form. It would be very interesting to search for similar extracellular thionein-like proteins in vertebrates. Apart from all these biochemical considerations, the suspension of copper-loaded yeast cells in simple buffers woud be a most convenient, rapid and efficient method for the isolation of Cu(I)-thionein.

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