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Copper-release from yeast Cu(I)-thionein by hypothiocyanite ($OSCN^-$)

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In the course of an oxidative burst oxygen free radicals and hypothiocyanite ($OSCN^-$), a transiently abundant derivative of thiocyanate (SCN^-), are formed in the presence of activated polymorphonuclear leukocytes (PMNs). At the same time Cu(I)-thionein is present and the question arose whether or not thiocyanate and its oxidized form may transiently release highly Fenton active copper to improve the efficacy of the above mentioned oxidative burst. Thus, the reaction of yeast Cu-thionein with $OSCN^-$ was examined. Indeed, a release of copper from the Cu(I)-thiolate clusters of the protein was observed *ex vivo*. Both the chiroptic and luminescence emission signals of Cu-thionein essentially levelled off in the presence of a 15-fold molar excess of $OSCN^-$ expressed per equivalent of thionein-copper. The effective copper-releasing activity of this reagent was confirmed by equilibrium dialysis. The demetallized protein could be reconstituted under reductive conditions. SCN^- did not affect the copper-thiolate bonding. It rather acts as a potent metabolic source for the transient copper release from Cu-thionein in the presence of activated PMNs.

Keywords: circular dichroism, copper-release, Cu(I)-thionein, fluorescence, oxidative burst, pseudo halides

Introduction

Our knowledge of the mechanism of biological copper transport on a molecular level is limited. The pronounced reactivity of this prominent transition metal requires finely tuned regulation in biological systems. Transport of 'free copper ions' must be considered to be rather dangerous as there are many undesired reactions that would lead to the irreversible destruction of cellular components (Weser & Hartmann 1984, Felix & Weser 1988). In the course of an oxidative burst the controlled release of copper from Cu-thionein may be considered to be desirable to improve the efficacy of the copper-driven reaction of oxygen free radicals. In this context Cu-thionein has been regarded to be a promising candidate in the control of copper homeostasis (Weser & Hartmann 1984, Schechinger *et al.* 1986, Weser *et al.* 1986). At present there are many different possible functions of this Cu(I)-thiolate protein including the stoichiometric and catalytic reaction with oxygen free radicals (Felix *et al.* 1993) and the control of intracellular redox potentials (Felix *et al.* 1993, Hartmann *et al.* 1984).

We are challenged with the question as to how the Cu(I)-thiolate bonding is formed and/or cleaved in this cysteine-rich protein. To shed some light on the catabolism several possibilities have been proposed (Weser *et al.* 1986). There is a controlled Cu(I) channelling off into the vacant copper-binding sites of apo-Cu-proteins (Morpurgo *et al.* 1983, Hartmann *et al.* 1983). Protein digestion proved successful only after prior destruction of the Cu-thiolate cluster (Weser *et al.* 1986). Numerous studies have been made of possible oxidation mechanisms induced by activated leukocytes (Hartmann *et al.*

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1985, 1987, Schechinger *et al.* 1986), H_2O_2 and enzymes generating activated oxygen species (Hartmann *et al.* 1984, Richter & Weser 1988). Furthermore, *S*-alkylation of the Cu-thiolate clusters led to the release of copper from yeast Cu-thionein (Felix & Weser 1988). Treatment of Cu-thionein *ex vivo* with cyanide is known to break the Cu(I)-thiolate bonding with the consecutive formation of a reconstitutable apo-protein (Narula *et al.* 1991). In biological systems cyanide is immediately converted into thiocyanate (SCN^-). The remaining cyanide concentration is far too low to affect Cu-thionein. SCN^- is found in nearly all body fluids with levels, for example, in blood plasma easily amounting to 120 μM and up to 3 mM in saliva (Thomas & Fishman 1986). Activated polymorphonuclear leukocytes (PMNs) are well able to convert SCN^- into the bactericidal hypothiocyanite ($OSCN^-$) which reacts with thiol groups according to (Thomas 1985) (equations 1 and 2):

$$SCN^- + H_2O_2 \rightarrow OSCN^- + H_2O \quad (1)$$

$$OSCN^- + 2\ R\text{–}SH \rightarrow SCN^- + R\text{-}SS\text{–}R + H_2O \quad (2)$$

The plasma concentration of $OSCN^-$ was quantified to be 40–70 μM (Thomas & Fishman 1986). It was of interest to examine whether or not copper can be released from yeast Cu-thionein *ex vivo* in the presence of both SCN^- and $OSCN^-$. The possible breakdown of the oligonuclear Cu(I)-thiolate centres was monitored using circular dichroism (CD) and luminescence emission spectroscopy. Equilibrium dialysis proved to be useful to monitor the possible release of copper during the reaction employing the above mentioned pseudo halides.

Experimental

Cu-thionein from yeast (*Saccharomyces cerevisiae*) was isolated from the copper resistant strain A 2180-1Aa employing repeated gelfiltration (Weser & Hartmann 1991). Thiocyanogen $(SCN)_2$ was prepared by oxidation of lead thiocyanate in the presence of bromine (Aune & Thomas 1977). $Pb(SCN)_2$ (55 mg; 0.17 mmol) was suspended under constant stirring in 10 ml of dried CCl_4 at 0°C; then, 8 μl Br_2 (0.15 mmol) was added in portions of 2 μl each during 15 min. The $(SCN)_2$ concentration was determined spectrophotometrically ($\epsilon_{295} = 140\ M^{-1}\ cm^{-1}$) (Thomas 1985). Hydrolysis of $(SCN)_2$ in alkaline solution resulted in the formation of hypothiocyanite (equation 3) (Thomas 1981). An aliquot of 2 ml of a $(SCN)_2$ solution in CCl_4 was diluted to 50 ml with dry CCl_4 and added dropwise to 0.1 M aqueous NaOH at 0°C.

$$(SCN)_2 + 2\ OH^- \rightarrow OSCN^- + SCN^- + H_2O \quad (3)$$

The concentration of $OSCN^-$ was determined indirectly as $Fe(SCN)_3$ (Aune & Thomas 1977). Luminescence emission was measured on a Perkin-Elmer LS 50 luminescence spectrometer. Excitation was at 300 nm. CD was recorded on a JASCO J720 spectropolarimeter. Due to the lower intensity of the Cotton effects between 400 and 300 nm, the more pronounced bands between 320 and 260 nm were chosen. Fortunately, no reagent and/or solvent-dependent pertubations were seen in this wavelength region. Due to strong interferences, CD spectra below 260 nm were not recorded. Equilibrium dialysis was performed in a polyacrylic two chamber block at 20°C (Felix & Weser 1988). Two sets of 900 μl chambers each were separated by a cellulose membrane (Spectrapor dialysis tubing, M_r cut-off 3500). One compartment contained the mixture of Cu-thionein and the reagent to be examined in 0.1 M Tris-HCl, pH 7.4. In the second chamber only buffer was injected. Copper was quantified on a Perkin-Elmer 3030 atomic absorption spectrometer.

Results and discussion

The reactivity of cyanide with Cu-thionein is long known (Narula *et al.* 1991). The protein can be efficiently demetallized by this reagent. It was of interest to examine whether or not SCN^-, the metabolically converted product of CN^-, may remove copper out of the Cu(I)-thiolate clusters of Cu-thionein. The protein from yeast was chosen as it is well known to be homogeneous with respect to copper. In addition, this metallothionein is substantially more stable against oxidation compared with the mixed Cu,Zn-thionein from vertebrate origin. Thus, yeast Cu-thionein was most appropriate to study in a model-type reaction the reactivity of both SCN^- and $OSCN^-$ on the hexanuclear Cu-thiolate binding centre which was also detected in either α and β domain of rat liver metallothionein (Hartmann *et al.* 1992). The characteristic spectroscopic properties including CD and luminescence emission were used as sensitive tools for monitoring the intactness of the Cu(I)-thiolate centres. Unlike cyanide, titration of Cu-thionein with SCN^- revealed that this pseudo halide did not markedly affect the Cu-thiolate bonding even in the presence of molar ratios amounting to 120 SCN^-/Cu. In either

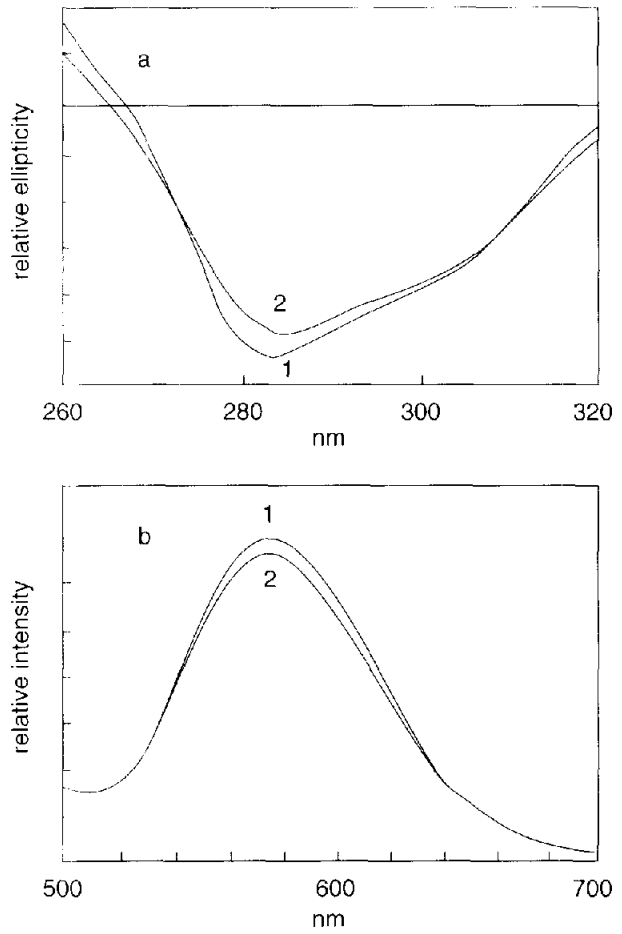

Figure 1. CD (**a**) and luminescence emission (**b**) of yeast Cu-thionein in the presence of potassium thiocyanate. (1) Control, (2) addition of SCN /Cu = 120. The spectra were run 30 min after the addition of SCN^- to the Cu-thionein solution (100 μM Cu).

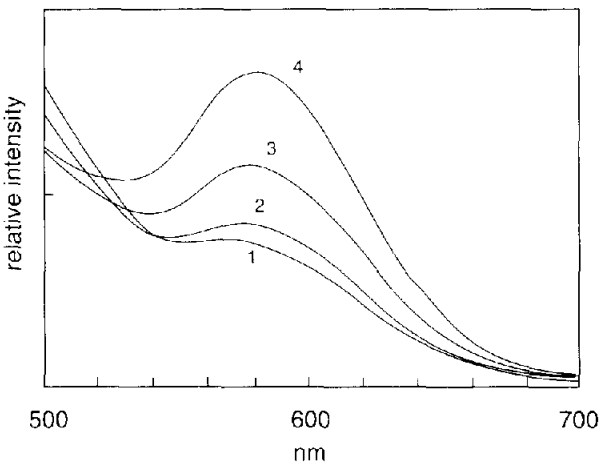

Figure 2. Luminescence emission of yeast Cu-thionein at different molar ratios of OSCN /Cu: (1) Control, (2) 1, (3) 2 and (4) 3. Each titration step was performed by adding 300 μl 130 μM $OSCN^-$ to 2 ml Cu-thionein (20μM Cu in 100 mM Tris–HCl, pH 7.4). Fluorescence spectra were recorded 10 min after each addition of $OSCN^-$.

spectroscopic measurement a negligible decline of Cotton bands or luminescence emission was seen indicating the virtual intactness of the Cu(I)-thiolate centres (Figure 1). At the same time an insignificant copper release was observed during the course of the equilibrium dialysis supporting the above conclusion of a marked stability of the oligonuclear Cu(I)-thiolate clusters.

In the course of an oxidative burst oxygen free radicals are formed concomitantly with a substantial rise of Cu(I)-thionein secretion into the medium (Hartmann *et al.* 1989). Likewise, pseudo halides other than CN^- and SCN^- must be considered. Of particular interest was the formation of $OSCN^-$. This potent bactericidal and oxidizing agent is generated from SCN^- in the presence of activated leukocytes or peroxidases and H_2O_2 (Thomas 1985). In the event of an oxidative burst, unfortunately, the contribution of the attack of $OSCN^-$ on Cu-thionein cannot be distinguished from the overall reaction. The Cu(I)-thiolate clusters would be oxidatively destroyed in an uncontrolled manner even in the absence of thiocyanate (Hartmann *et al.* 1985, 1987, Schechinger *et al.* 1986, Richter & Weser 1988). To avoid this uncontrolled reaction chemically generated OSCN from $(SCN)_2$ was employed (Thomas 1981). A marked reactivity was observed. Equimolar $OSCN^-$ expressed per equivalent of thionein-copper diminished the intensity of the luminescence emission by 30%. In the presence of a 3-fold molar excess the signal height declined to half of its initial magnitude (Figure 2). This suggested that the oligonuclear Cu(I)-thiolate chromophores were cleaved.

The prior separate preparation of $OSCN^-$ from $(SCN)_2$ yielded only diluted solutions (130 μM) of this transiently formed species which proved to be unsuitable for the titration experiments. The concentration of this short-lived $OSCN^-$ was substantially improved by adding $(SCN)_2$ directly to aqueous Cu-thionein solutions. During the hydrolysis (equation 2) the initial $OSCN^-$ concentrations rose to 15 700 μM. Fortunately, the accompanying SCN^- did not significantly interfere with the Cu(I)-thiolate chromophores (see also Figure 1). In the presence

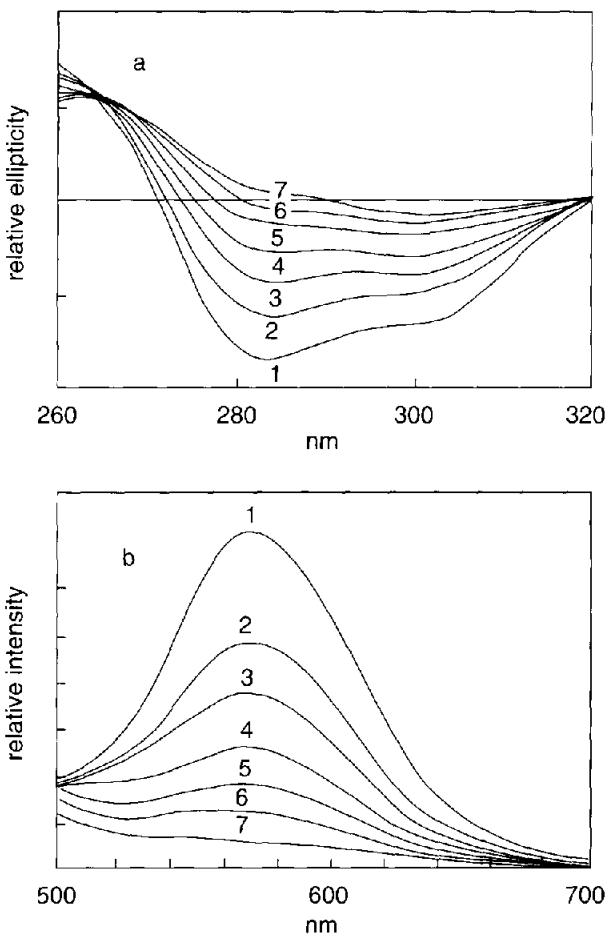

Figure 3. CD (**a**) and luminescence emission (**b**) of yeast Cu-thionein at different molar ratios of $(SCN)_2$/Cu: (1) control, (2) 3, (3) 6, (4) 9, (5) 12, (6) 15, and (7) 18. Cu-thionein (2 ml; 100 μM Cu) was titrated with $(SCN)_2$ (15.7 mM) at 20°C (1 molar equivalent =15 μl). Both CD and fluorescence spectra were run 10 min after each titration step.

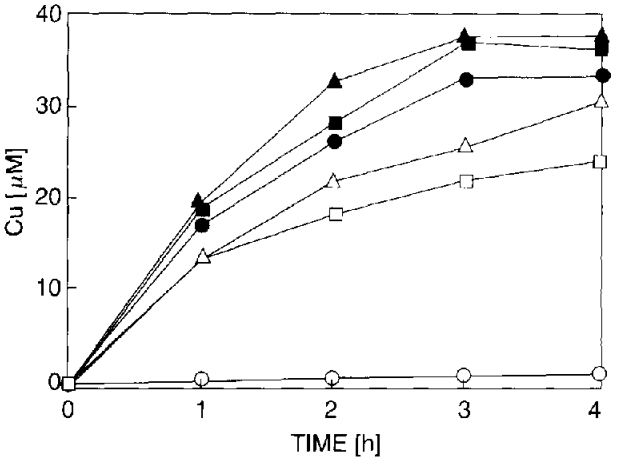

Figure 4. Release of copper from yeast Cu-thionein at different molar ratios of $(SCN)_2$/Cu: (○) control, (□) 5, (△)10, (●) 15, (■) 20 and (▲) 25. The initial thionein-copper concentration was 75 μM in the respective compartment containing the reaction mixtures. Values were taken in 1 h intervals from either buffer compartment. All measurements were carried out in triplicate. The reproducibility was better than ±5%.

of a 15-fold molar excess of $OSCN^-$ both the luminescence emission and chiroptical signal intensities were nearly completely levelled off (Figure 3).

Equilibrium dialysis was additionally performed to support the former conclusion of a copper release from Cu-thionein in the presence of $OSCN^-$. After the addition of a 20-fold molar excess of $(SCN)_2$, equilibrium was reached at 35–38 μM Cu in either compartment of the dialysis apparatus after 4 h, which indicates successful copper depletion under the given experimental conditions (Figure 4). The buffer compartment remained completely protein-free during the course of the experiment.

It was of interest whether or not the protein moiety was affected during the $OSCN^-$-treatment. SDS–PAGE revealed that no protein damage at all was observed up to a molar excess of 5 over thionein-copper. At that stoichiometric ratio about 60% of the copper was released. At higher $OSCN^-$/thionein-copper ratios some polymerized metallothionein species were noticed due to the formation of inter- and/or intramolecular disulphide bridges according to equation (1). The released copper remained electron paramagnetic resonant non-detectable, attributable to the concomitant formation of Cu(I)SCN from the richly abundant SCN^-. Much to our surprise some 95% of the Cu(I)-thiolate centres of the previously demetallized protein could be restored after the addition of 0.1% (v/v) 2-mercaptoethanol as monitored by both CD and luminescence emission. In contrast to biogenic glutathione 2-mercaptoethanol was chosen as the reducing agent to avoid the perturbing fluorescence of Cu(I)glutathione (Steinkühler *et al.* 1991). It should be pointed out that 2-mercaptoethanol without Cu-thionein and in the presence of the same concentration of copper salt exhibited no Cotton effect and luminescence emission at all under these conditions.

In conclusion, $OSCN^-$ represents an efficient and reactive metabolite for the transient release of Cu from copper-thionein. The reversibility of this process is both highly advantageous and economic as the protein moiety may be used repeatedly as a

copper shuttle. The most appropriate candidate under *in vivo* conditions for disulphide reduction certainly will be glutathione. Although the present findings are based on *ex vivo* investigations, they convincingly confirm earlier proposals that metallothionein is an excellent system for both copper storage and transport under physiological conditions. At the same time it can be considered to be a most powerful site-directed Fenton reagent in biological defence mechanisms, e.g. the scavenging of intruding pathogenic microorganisms.

Acknowledgement

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References

Aune TM, Thomas EL. 1977 Accumulation of hypothiocyanite ion during peroxidase-catalyzed oxidation of thiocyanate ion. *Eur J Biochem* **80**, 209–214.

Felix K, Weser U. 1988 Release of copper from yeast copper-thionein after *S*-alkylation of copper-thiolate clusters. *Biochem J* **252**, 577–581.

Felix K, Lengfelder E, Hartmann H-J, Weser U. 1993 A pulse radiolytic study on the reaction of hydroxyl and superoxide radicals with yeast Cu(I)-thionein. *Biochim Biophys Acta* **1203**, 104–108.

Hartmann H-J, Morpurgo L, Desideri A, Rotilio G, Weser U. 1983 Reconstitution of stellacyanin as a case of direct Cu(I) transfer between yeast copper thionein and 'blue' copper apoproteins. *FEBS Lett* **152**, 94–96.

Hartmann H-J, Gärtner A, Weser U. 1984 Oxidation of Cu(I)-thionein by enzymically generated H_2O_2. *Hoppe-Seyler's Z Physiol Chem* **365**, 1355–1359.

Hartmann H-J, Gärtner A, Weser U. 1985 Copper dependent control of the enzymic and phagocyte induced degradation of some biopolymers, a possible link to systemic inflammation. *Clin Chim Acta* **152**, 95–103.

Hartmann H-J, Schechinger T, Weser U. 1987 Cu(II) transfer into apo-Cu_2Zn_2-superoxide dismutase from Cu-thionein oxidized by activated leukocytes. *Inorg Chim Acta* **136**, 107–110.

Hartmann H-J, Schechinger T, Weser U. 1989 Copper-thionein in leucocytes. *Biol Met* **2**, 40–44.

Hartmann H-J, Yue-Jin L, Weser U. 1992 Analogous copper(I) coordination in metallothionein from yeast and the separate domains of the mammalian protein. *BioMetals* **5**, 187–191.

Morpurgo L, Hartmann H-J, Desideri A, Weser U, Rotilio G. 1983 Yeast copper-thionein can reconstitute the Japanese-laquer-tree (*Rhus vernicifera*) laccase from the Type 2-copper depleted enzyme via a direct copper (I)-transfer mechanism *Biochem J* **211**, 515–517.

Narula SS, Mehra RK, Winge DR, Armitage IM. 1991 Etablishment of the metal-to-cysteine connectivities in silver-substituted yeast metallothionein. *J Am Chem Soc* **131**, 9354–9358.

Richter A, Weser U. 1988 Kinetics of the H_2O_2 dependent cleavage of Cu-thiolate centres in yeast Cu_8-thionein. *Inorg Chim Acta* **151**, 145–148.

Schechinger T, Hartmann H-J, Weser U. 1986 Copper transport from Cu(I)-thionein into apo-caeruloplasmin mediated by activated leucocytes. *Biochem J* **240**, 281 283.

Steinkühler C, Pedersen JZ, Weser U, Rotilio G. 1991 Oxidative stress induced by a di-Schiff base copper complex is both mediated and modulated by glutathione. *Biochem Pharmacol* **42**, 1821–1827.

Thomas EL. 1981 Lactoperoxidase-catalyzed oxidation of thiocyanate: equilibria between oxidized forms of thiocyanate. *Biochemistry* **20**, 3273–3280.

Thomas EL. 1985 Products of lactoperoxidase-catalyzed oxidation of thiocyanate and halides. In: Pruitt KM, Tenovuo JO, eds. *The Lactoperoxidase-Catalyzed Oxidation of Thiocyanate and Halides*. New York: Marcel Dekker; 31–53.

Thomas EL, Fishman M. 1986 Oxidation of chloride and thiocyanate by isolated leukocytes. *J Biol Chem* **261**, 9694–9702.

Weser U, Hartmann H-J. 1984 Copper-thiolate proteins (metallothioneins). In: Lontie R, ed. *Copper Proteins and Copper Enzymes*, Vol. III Boca Raton, FL: CRC Press; 151–173.

Weser U, Hartmann H-J. 1991 Purification of yeast Cu-thionein. *Methods Enzymol* **205**, 274 278.

Weser U, Mutter W, Hartmann H-J. 1986 The role of Cu(I)-thiolate clusters during the proteolysis of Cu-thionein. *FEBS Lett* **197**, 258 262.