Release of copper from yeast copper-thionein after S-alkylation of copper–thiolate clusters

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Our knowledge on the release of copper from Cu-thionein in biological systems is limited. Other than oxidative cleavage or direct transfer, the possibility of an alkylation mechanism seemed attractive. Iodoacetamide and methyl methanesulphonate were successfully employed to alkylate the Cu–thiolate sulphur atom of homogeneous Cu(I)-thionein from yeast. The alkylation caused a weakening of the Cu–S bonding, which led to the release of copper. After equilibrium dialysis a proportion of the released copper was found in the dialysis buffer. When iodoacetamide was used carboxymethylcysteine was detected in the protein hydrolysate. A 10-fold molar excess over cysteine was sufficient for complete alkylation, which could be conveniently monitored by c.d. at 328 and 359 nm. The reaction proceeded under both aerobic and anaerobic conditions. E.p.r. measurements of Cu^{2+} revealed unequivocally the complete cleavage of the Cu–thiolate bonding in less than 5 h. It is possible that this mode of copper release might be of relevance to the molecular transport of this biochemically important transition metal.

INTRODUCTION

Our knowledge on the molecular transport of copper in biochemical systems is very limited (Weser & Hartmann, 1984). The essential role of this biologically important transition metal demands a subtle regulation of cellular copper content. Transport of free Cu^{2+} ions must be discarded, as there would be many undesired reactions that would lead to the irreversible destruction of cellular components.

Cu(I)-thionein has been discussed as a promising candidate in the homeostasis of copper metabolism (Weser & Hartmann, 1984; Schechinger *et al.*, 1986; Weser *et al.*, 1986). Unfortunately, we do not know how the Cu(I)-thiolate bonding is cleaved in the metal-binding centre of this ubiquitous cysteine-rich protein. Several possible ways have been proposed (Weser *et al.*, 1986). There is a controlled Cu(I) channelling 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 leucocytes (Hartmann *et al.*, 1985, 1987; Schechinger *et al.*, 1986), H_2O_2 and enzymes generating activated oxygen species (Hartmann *et al.*, 1984).

A promising field for investigation was the possibility that prior alkylation of the holoprotein would lead to a successful release of copper. We were encouraged by the alkylation studies performed by Bernhard *et al.* (1986) on vertebrate CdZn-thionein and the special situation of the Cu–S bonding in Cu(I)-thionein (Sokolowski *et al.*, 1974; Rupp & Weser, 1975). Alkylation of yeast Cu(I)-thionein was carried out, if not otherwise stated, with iodoacetamide and methyl methanesulphonate. The successful alkylation was monitored by amino acid analysis and by the decline of characteristic Cotton extrema in the c.d. spectrum between 300 and 400 nm. Furthermore, e.p.r. measurements were used to demon-

strate directly the appearance of Cu(II) after the possible destruction of this Cu–thiolate chromophore. The release of a proportion of the copper was monitored by equilibrium dialysis.

MATERIALS AND METHODS

Chemicals

Iodoacetamide was obtained through Serva, Heidelberg, Germany. Methyl methanesulphonate was from Aldrich, Steinheim, Germany. Cu(I)-thionein was isolated from baker's yeast (Weser *et al.*, 1977; Winge *et al.*, 1985; Weser & Hartmann, 1988). All other reagents were of analytical-grade quality.

Alkylation

S-Alkylation of Cu-thionein was carried out at pH 8 for 4 h in 50 mM-Tris/HCl or 50 mM-sodium phosphate buffer. Usually the temperature was 40 °C. The reaction with iodoacetamide was performed in the dark (Carne *et al.*, 1979; Hirs, 1967). Alternatively methyl methanesulphonate was used (Eyem *et al.*, 1976; Segerbäck *et al.*, 1978).

Analytical

Copper was determined quantitatively on a Perkin–Elmer atomic absorption unit equipped with an HGA 76B graphite cuvette. Amino acid analyses were carried out after hydrolysis of 1 mg of protein with 2 ml of 6 M-HCl at 110 °C for 12, 16 and 20 h. The redissolved hydrolysate was separated on CK 10F ion-exchange resin (Mitsubishi) and monitored on a Labotron Liquimat 3 amino acid analyser. Cysteine was oxidized with HClO_4 . C.d. spectra were obtained on a JASCO J 20A spectrophotometer. E.p.r. spectra were obtained on a Varian E-109 spectrometer at 100 K.

Equilibrium dialysis

The equilibrium-dialysis unit was composed of two tightly fitting polyacrylic blocks (240 mm × 60 mm ×

Fig. 1. Ion-exchange chromatogram of carboxymethylated yeast Cu-thionein after 16 h hydrolysis

A 25 μ l portion of a 1.4 mM hydrolysate was passed through a column (180 mm $\times$ 4 mm) containing polystyrene cation-exchange resin (CK 10F; Mitsubishi) and monitored on a Labotron Liquimat 3 amino acid analyser.

15 mm) each containing five cylindrical chambers (15 mm $\times$ 5.7 mm) of 1000 μ l volume. The two sets of chambers were separated by a cellulose membrane (Spectrapor dialysis tubing, M_r cut-off 3500; Spectrum Medical Industries, Los Angeles, CA, U.S.A.) Only 900 μ l was injected perpendicularly into each cylindrical cell through channels 1.5 mm wide. A second channel allowed the displacement of air. A remaining air bubble served as an internal stirrer. In the rear cylindrical chambers only buffer was injected. The front chambers contained the alkylation mixture and Cu-thionein. The apparatus was rotated at approx. 20 rev./min. Equilibrium was reached in less than 50 min at 22 $^{\circ}$ C. In general dialysis was continued up to 90 min.

RESULTS AND DISCUSSION

Cu-thionein from yeast was selected as it is well known to be homogeneous with respect to copper and contains no zinc or cadmium (Weser & Hartmann, 1984). It was expected that the native protein would be successfully alkylated because the charge at the sulphur site was close to the charge of sulphur found in the amino acid cysteine. According to X-ray photoelectron spectrometric measurements the binding energy value of the unresolved S_{2p} ($\frac{1}{2}$, $\frac{3}{2}$) level of protonated cysteine sulphur lies at 163 eV (Sokolowski *et al.*, 1974; Rupp & Weser, 1975, 1976). The sulphur in Cu(I)-thionein has a binding energy value of 162 eV, and the S_{2p} value of the thiolate sulphur atom of Zn(II)-thionein appears at 161 eV.

Fig. 2. Release of copper from Cu-thionein during alkylation: dependence on the concentration of iodoacetamide ($\blacktriangle$) and methyl methanesulphonate ($\blacksquare$)

Alkylating reagents were added in 0-, 1-, 2-, 5-, 10-, 20-fold molar excess over each cysteine residue of Cu-thionein (35 μ M). The reactions were carried out for 4 h at 40 $^{\circ}$ C in 50 mM-Tris/HCl buffer, pH 7.5, in 1 ml polypropylene cups under constant agitation and in the presence of N_2 . Portions (900 μ l) were placed in a rotating (20 rev./min) microdialysis unit (see the Materials and methods section) and dialysed for 90 min at 22 $^{\circ}$ C. With iodoacetamide both alkylation and dialysis were performed in the dark to minimize uncontrolled I_2 -dependent oxidative deterioration. The initial Cu-thionein concentration was as above.

Fig. 3. pH-dependence of released copper from Cu-thionein alkylated with methyl methanesulphonate ($\blacksquare$) and iodoacetamide ($\blacktriangle$)

A 10-fold excess of alkylation reagents were used. For further conditions see the legend to Fig. 2. The initial Cu-thionein copper concentration was 178 μ M in either case.

Zn(II)-thionein has been shown to be readily alkylated (Bernhard *et al.*, 1986). Thus the more covalently co-ordinated sulphur atom of Cu(I)-thionein should also react with iodoacetamide. Indeed, in the elution diagram of the chromatographed hydrolysate of Cu(I)-thionein carboxymethylcysteine was clearly detectable (Fig. 1). This reaction also proceeded under anaerobic conditions.

In the alkylation process stretching of the Cu-S bonding should eventually lead to the cleavage of this

Fig. 4. C.d. of Cu-thionein previously incubated with iodoacetamide

Reaction conditions were as follows: yeast Cu-thionein, 0.5 mg/ml; iodoacetamide, 10-fold molar excess over each cysteine residue. Alkylation was performed in 50 mM-Tris/HCl buffer, pH 8.5, at 37 °C. The spectra were recorded at 2 h intervals. The inset shows the progressive decline of the $[\theta]_{\text{Cu},328}$ value in the presence of iodoacetamide. For alkylation conditions see the legend to Fig. 2.

Fig. 5. C.d. of yeast Cu-thionein in the presence of methyl methanesulphonate

The inset shows the dependence of $[\theta]_{\text{Cu},328}$ on the reaction time. For further details see the legends to Fig. 2 and 4.

bond. Equilibrium dialysis was chosen to remove considerable proportions of this loosely bound copper. Under the given conditions a 10-fold molar excess of iodoacetamide over cysteine residues of Cu(I)-thionein yielded maximal copper release (Fig. 2). In the presence of methyl methanesulphonate the concentration of dialysed copper was somewhat lower. Maximum values were again obtained at a 10-fold molar ratio. Chromatographic separation of methylcysteine, unfortunately, was unsuccessful, owing to overlapping with glutamate and proline elution peaks.

The pH-dependence of the respective alkylation

Fig. 6. Temperature- and time-dependence on the alkylation of yeast Cu-thionein monitored by c.d. spectroscopy at 328 nm

No difference in the alkylation reagents was noticed. ●, 22 °C; □, 37 °C; ▲, 40 °C. Cu-thionein (88 μM) was incubated with a 10-fold molar excess of either iodoacetamide or methyl methanesulphonate over each cysteine residue.

reactions was compared with either compound in a 10-fold molar excess. Methyl methanesulphonate showed maximal reactivity at pH 7 with a progressive decline to alkaline pH. By contrast, iodoacetamide showed a maximum at pH 8.5 (Fig. 3).

There was no detectable difference in the rate of copper release when the alkylation experiments were performed in phosphate or Tris buffer solutions. In the course of the equilibrium dialysis a considerable proportion of released thiolate copper was expected to be trapped by non-specific chelation to the many other peptide bondings of the *S*-alkylated protein. An approach to detect the decomposition of the Cu-thiolate clusters, both directly and comprehensively, was the recording of the characteristic Cotton extrema in the c.d. spectrum between 300 and 400 nm. In this wavelength region no perturbations originating from the alkylation reagents were noticed. After the addition of iodoacetamide a progressive decline of the Cotton bands was seen (Fig. 4).

Methyl methanesulphonate reacted in the same way, leading to the complete disappearance of the 328 and 359 nm Cotton extrema (Fig. 5). The linear mode of decay should be emphasized. There was no detectable sign of labile intermediates and/or co-operativity (see insets of Figs. 4 and 5). This is consistent with the pseudo-first-order kinetics observed in the oxidative destruction of Cu-thionein (Richter & Weser, 1988). As in the equilibrium-dialysis experiments, there was a temperature-dependence. At 37 °C and above the alkylation was complete after 8 h (Fig. 6).

Direct evidence of non-specifically bound Cu(II) after cleavage of the Cu-thiolate centres was obtained by e.p.r. measurements. Cu(I) co-ordinated to thiolate sulphur exerts no e.p.r. signal at all, this being attributable to its completely filled $3d^{10}$ orbital. Thus only negligible Cu(II) signals are seen at zero time. Only 5 h was required to demonstrate the complete conversion into

Fig. 7. Time-dependent e.p.r. measurements of yeast Cu-thionein after alkylation with iodoacetamide (a) and methyl methanesulphonate (b)

Alkylations were performed in 50 mM-Tris/HCl buffer, pH 8.5 (a), and pH 7.5 (b), at 40 °C in the presence of 42 mM-iodoacetamide or -methyl methanesulphonate respectively. The concentration of Cu-thionein copper was 0.67 mM. For the complete oxidation of copper 10 mM- H_2O_2 was added. Recording conditions: temperature, 100 K; microwave frequency, 9.24 GHz; microwave power, 20 mW; gain, 10×10^3 for alkylation with iodoacetamide and 5×10^2 for alkylation with methyl methanesulphonate.

e.p.r.-detectable Cu(II) (Fig. 7). Excess H_2O_2 did not increase the magnitude of the e.p.r. signals, confirming the quantitative cleavage of the Cu-S bonding. The displaced fully e.p.r.-detectable $3d^9$ copper exerts the signals of non-specifically co-ordinated Cu(II) of the biuret type in both series of e.p.r. measurements.

Possible biological consequences are that, unlike the Cu(I)-thiolate centres, this Cu(II) is far more loosely bound and can easily be chelated by many apo-Cu-proteins, e.g. apo- Cu_2Zn_2 -superoxide dismutase (Hartmann *et al.*, 1987) or apo-caeruloplasmin (Schechinger *et al.*, 1986). Of course, during equilibrium dialysis only limited copper concentrations are detectable in the dialysis buffer (see Figs. 2 and 3). The major proportion remains co-ordinated to the many peptide bondings of the alkylated protein.

GENERAL DISCUSSION

Clearly, these alkylation studies are no more than a model. However, enzyme-catalysed protein modifications, including hydroxylation, phosphorylation, acylation and methylation, occur quite frequently in biological systems (Paik & Kim, 1971; Alberts *et al.*, 1983). For example, suitable enzymes could be methyltrans-

ferases known to use S-adenosylmethionine as the methyl donor. Methyltransferases are located in exactly the same tissues where metallothionein is also present. Unlike the oxidative cleavage (Schechinger *et al.*, 1986), copper release from Cu-thionein after alkylation is successful under anaerobic conditions. This pathway would conveniently furnish with copper many vital apo-Cu-proteins that are present in these cellular compartments known to be deficient in oxidizing components. Further digestion of the remaining alkylated protein will proceed in exactly the same manner as for non-alkylated proteins (Katznelson & Kulka, 1983). The present alkylation of the holoprotein adds another possibility to the facilitation of proteolysis of the normally resistant metal-thiolate clusters of metallothioneins. Protein digestion was previously only known after oxidative cleavage of the copper-binding centres (Winge & Miklossy, 1982; Bremner & Mehra, 1983; Mehra & Bremner, 1985; Weser *et al.*, 1986). However, we are still far away from discovering the detailed molecular mechanism of copper transport.

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REFERENCES

- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J. D. (1983) *Molecular Biology of the Cell*, pp. 759–763, Garland, New York
- Bernhard, W. R., Vařák, M. & Kägi, J. H. R. (1986) *Biochemistry* **25**, 1975–1980
- Bremner, I. & Mehra, R. K. (1983) *Chem. Scr.* **21**, 117–121
- Carne, T., Tipping, E. & Ketterer, B. (1979) *Biochem. J.* **177**, 433–439
- Eyem, J., Sjö Dahl, J. & Sjöquist, J. (1976) *Anal. Biochem.* **74**, 359–368
- Hartmann, H.-J., Morpurgo, L., Desideri, A., Rotilio, G. & Weser, U. (1983) *FEBS Lett.* **152**, 94–96
- Hartmann, H.-J., Gärtner, A. & Weser, U. (1984) *Hoppe-Seyler's Z. Physiol. Chem.* **365**, 1355–1359
- Hartmann, H.-J., Gärtner, A. & Weser, U. (1985) *Clin. Chim. Acta* **152**, 95–103
- Hartmann, H.-J., Schechinger, T. & Weser, U. (1987) *Inorg. Chim. Acta* **136**, 107–110
- Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197–203
- Katznelson, R. & Kulka, R. G. (1983) *J. Biol. Chem.* **258**, 9597–9600
- Mehra, R. K. & Bremner, I. (1985) *Biochem. J.* **227**, 903–908
- Morpurgo, L., Hartmann, H.-J., Desideri, A., Weser, U. & Rotilio, G. (1983) *Biochem. J.* **211**, 515–517
- Paik, W. K. & Kim, S. (1971) *Science* **174**, 114–119
- Richter, A. & Weser, U. (1988) *Inorg. Chim. Acta* **151**, 145–148
- Rupp, H. & Weser, U. (1975) *Bioinorg. Chem.* **5**, 21–32
- Rupp, H. & Weser, U. (1976) *Biochim. Biophys. Acta* **446**, 151–165
- Schechinger, T., Hartmann, H.-J. & Weser, U. (1986) *Biochem. J.* **240**, 281–283

- Segerbäck, D., Calleman, C. J., Ehrenberg, L., Löfroth, G. & Osterman-Golkar, S. (1978) *Mutat. Res.* **49**, 71–82
- Sokolowski, G., Pilz, W. & Weser, U. (1974) *FEBS Lett.* **48**, 222–225
- Weser, U. & Hartmann, H.-J. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., ed.), pp. 151–174, CRC Press, Boca Raton
- Weser, U. & Hartmann, H.-J. (1988) *Biochim. Biophys. Acta* **953**, 1–5
- Weser, U., Hartmann, H.-J., Fretzdorff, A. & Strobel, G. (1977) *Biochim. Biophys. Acta* **493**, 465–477
- Weser, U., Mutter, W. & Hartmann, H.-J. (1986) *FEBS Lett.* **197**, 258–262
- Winge, D. R. & Miklossy, K.-A. (1982) *J. Biol. Chem.* **257**, 3471–3476
- Winge, D. R., Nielson, K. B., Gray, W. R. & Hamer, D. H. (1985) *J. Biol. Chem.* **260**, 14464–14470

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