Copper(I) transfer into metallothionein mediated by glutathione

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Rabbit liver metallothionein depleted of Cd(II) and Zn(II) was fully reconstituted using a Cu(I)–GSH complex under strictly anaerobic conditions. Anaerobic fluorescence titration, using an emission band at 625 nm which is diagnostic of the correct insertion of Cu(I) into the thiolate clusters of metallothionein, showed that the fluorescence maximum was obtained on addition of as many Cu(I) equivalents as the available Cu(I)-binding sites in the protein (i.e. 12). Binding was nearly complete within 1 min, and Cu(I)–GSH was much more efficient than Cu(I)–thiourea or Cu(I)–acetonitrile in metallothionein reconstitution. In air, full reconstitution was obtained with stoichiometric copper only

when an excess of GSH was present in the reaction mixture. Cu(I)–GSH was also able to displace Zn(II) and Cd(II) from natural metallized thionein. It is concluded that: (a) Cu(I)–GSH is a potential physiological Cu(I) carrier, not only for Cu²⁺/Zn²⁺ superoxide dismutase [Ciriolo, Desideri, Paci and Rotilio (1990) *J. Biol. Chem.* **265**, 11030–11034] but also for metallothionein; (b) in the case of metallothionein, physiological concentrations of GSH protect the protein from autoxidation in air and facilitate Cu(I)–thiolate exchange; (c) the natural metal composition of metallothionein may be related to metal bioavailability rather than to evolutionary changes in protein structure.

INTRODUCTION

Metallothioneins (MTs) are small (6–7 kDa) thiol-rich proteins, which are able to bind potentially toxic metal ions (Webb, 1987; Waalkes and Goering, 1990). In native mammalian MTs, 20 cysteine residues bind seven bivalent metal ions, such as Cd(II) and Zn(II) (arranged in two tetrahedral tetrathiolate clusters, Kagi and Schaffer, 1988), and 12 Cu(I) ions [six metal ions per cluster, trigonally co-ordinated (Nielson et al., 1985)].

It has been proposed that these proteins may also control both storage and transport of essential metals such as Cu(I) and Zn(II) (Li et al., 1980; Hartmann et al., 1987). This function is particularly important in the case of Cu(I), which, besides being an essential cofactor of many enzymes, is extremely reactive in redox reactions when not properly chelated, giving rise, in the presence of oxygen, to the superoxide radical, hydrogen peroxide, and the hydroxyl radical. Such a role seems to be validated by changes in MT synthesis as a function of the intracellular Cu(I) level, in either normal lymphoblastoid cells or cells of patients with Menkes' disease, a rare hereditary disorder of Cu(I) transport (Sone et al., 1987; McArdle et al., 1989, 1990; Palida and Ettinger, 1991).

However, the mechanisms of Cu(I) transport inside the cell and the possible involvement of other molecules are still largely unknown.

It has long been known (Hopkins, 1929; Osterberg et al., 1979) that GSH has a strong affinity for Cu(I), forming stable complexes with it. In fact, inhibition of incorporation of Cu(I) into MT was obtained by depleting hepatoma cells of GSH (Freedman et al., 1989). Furthermore, the Cu(I)–GSH complex was shown to act very efficiently in the donation of Cu(I) to Cu²⁺/Zn²⁺ superoxide dismutase, another major copper depository in eukaryotic cells (Ciriolo et al., 1990).

These results suggest that, in addition to its remarkable function in intracellular anti-oxidant defence (Winterbourn and Mundy, 1990), GSH might also co-operate in the metabolism of Cu(I). To corroborate this hypothesis further, it seemed worth

while to investigate the incorporation of Cu(I) into mammalian apo-MT from the Cu(I)–GSH complex. The results obtained showed that insertion of copper into apo-MT was distinctly more efficient with Cu(I)–GSH than with other Cu(I) complexes of routine use in the reconstitution of Cu(I)-MT and other Cu(I)-containing proteins (Rupp and Weser, 1974; Morpurgo et al., 1984; Brutsch et al., 1984; Byrd et al., 1988; Schechinger et al., 1988). In addition, the Cu(I)–GSH complex was also shown to be able to displace Zn(II) and Cd(II) in the naturally occurring Zn(II)-containing MT and to transfer Cu(I) to metal-depleted MT even in air.

MATERIALS AND METHODS

Reagents and assay

The Cu(I)–GSH complex was prepared shortly before use as previously described (Ciriolo et al., 1990), by adding CuSO₄ dissolved in water to solutions of GSH in 0.1 M phosphate buffer, pH 7.0, either under anaerobic conditions or in air, in a stoichiometric ratio of 3 GSH:1 Cu. The Cu(I)–(thiourea)₃Cl complex was prepared as described by Brutsch et al. (1984) by dissolving CuCl (Aldrich) in an aqueous solution of thiourea (Sigma) under N₂. Cu(I)–(acetonitrile)₄ClO₄ was prepared in 2 M HClO₄ and aq. 2 M acetonitrile (Hemmerich and Sigwart, 1963).

GSH and glutathione reductase were obtained from Boehringer (Mannheim), and CuSO₄ was from E. Merck (Darmstadt). Other chemicals were purchased from different commercial sources, and were of the highest available purity. All solutions were prepared with water pretreated with Chelex 100 resin (Bio-Rad, Richmond, CA, U.S.A.) to remove traces of metal ions.

Protein concentration was determined by using the absorbance of the apo-MT at 220 nm ($\epsilon = 48\,200\text{ M}^{-1}\cdot\text{cm}^{-1}$) (Buhler and Kagi, 1979) and/or by the method of Lowry et al. (1951), with BSA as standard. U.v. and visible spectra were recorded on a Perkin–Elmer Lambda 9 spectrophotometer.

Abbreviations used: MT, metallothionein; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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Cu, Zn and Cd concentrations were determined by atomic absorption spectrometry, on a Perkin–Elmer 3030 spectrometer fitted with an HGA-400 graphite furnace.

Thiol groups in the different samples were determined by the modified Ellman's method (Suzuki et al., 1990), using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma) in imidazole buffer, at pH 7.0.

Total glutathione ($\text{GSH} + \frac{1}{2}\text{GSSG}$) was determined by a DTNB/ $\frac{1}{2}$ glutathione reductase recycling assay (Anderson, 1985), and it was expressed as GSH equivalents.

Preparation and characterization of starting protein materials

MT I, from rabbit liver, was obtained from Sigma as Cd(II)/Zn(II)-MT. The protein was essentially pure as shown by SDS/PAGE analysis (Laemmli, 1970). Its metal analyses gave an average value 0.10 g-atom of Cu(I), 5.4 g-atoms of Cd(II) and 1.7 g-atoms of Zn(II) per mol of protein. The apoprotein was prepared by gel filtration at low pH, as described elsewhere (Good and Vasak, 1986). The commercial Cd(II)/Zn(II)-containing protein was dissolved in 0.01 M HCl and passed down a Sephadex G-25 (Pharmacia) gel column equilibrated with 0.01 M HCl, to remove the metal ions. The protein fractions, monitored by absorbance spectra, were subsequently lyophilized in a Speed-Vac Savant concentrator, or kept in acidic solution under liquid N_2 . The correct quality of apo-MT samples was assessed by assaying their metal contents, the SH/protein ratio and metal-binding capacity. Cu(I), Zn(II) and Cd(II) were undetectable in the prepared apo-MT. An average value of 18 ± 3 mol-equiv. of SH groups/mol of protein was determined, and this value was periodically checked before the reconstitution experiments. In order to test the number of available metal-binding sites, titration of a 0.025 mM solution of apo-MT in 0.05 M acetic acid was performed with a 5 mM AgNO_3 solution. The absorbance at 250 nm was monitored as a function of the addition of increasing equivalents of AgNO_3 (Nielson et al., 1985). A stoichiometry of 12 g-atoms of Ag(I)/mol of protein was obtained (Zelazowski et al., 1989). Further determination of the Ag(I) content, after purification of the reconstituted Ag(I)-MT by gel-filtration chromatography, gave 12.0 ± 0.8 g-atoms/mol of protein.

Reconstitution and purification of Cu(I)-MT prepared with Cu(I)-GSH

Apo-MT was neutralized at pH 7.0 with phosphate buffer (0.1 M), under N_2 or Ar, in a Thunberg apparatus. Appropriate samples of a deaerated solution of Cu(I)-GSH complex were then added with a gas-tight syringe to the reaction mixture. The reconstitution process was monitored by measuring fluorescence at 610 nm (with excitation at 366 nm) which has been assigned to the Cu(I)-thiolate complex typical of Cu(I) co-ordination in yeast and vertebrate Cu(I)-thioneins (Krauter et al., 1989). Fluorescence measurements were made on a Jovin Yvon Spectrofluor JY3, using a Thunberg apparatus sealed to a fluorescence cuvette, under N_2 . Samples of the solutions containing Cu(I)-GSH or other Cu(I) complexes were added to the apo-MT solution, and the absorbance band with a maximum at 625 nm (excitation at 336 nm) was recorded. The minimum time for such a measurement was approx. 1 min. Spectra were usually recorded a second time after 10 min: in all cases, only slight increases (less than 10%) were observed at equilibrium with respect to the value at 1 min.

The extent of reconstitution was determined after isolation of the Cu(I)-MT. After 2 h of incubation at 25 °C, the reconstitution

incubation mixture was chromatographed on a Sephadex G-25 column (40 cm $\times$ 1 cm; 0.3 ml/min) equilibrated with 0.05 M phosphate buffer, at pH 7.0, and made anaerobic by purging with N_2 . In some experiments, before chromatography, 0.1% (v/v) 2-mercaptoethanol was added. The isolated Cu(I)-containing fractions were then analysed for homogeneity and metal content.

RESULTS

Evaluation of correct Cu(I)-thiolate binding by fluorescence

Titration of apo-MT with the Cu(I)-GSH complex, under N_2 , produced a typical increase in the intensity of the fluorescence band at 625 nm, which is indicative of incorporation of Cu(I) at the correct binding site (Krauter et al., 1989; Li et al., 1991). Under the same experimental conditions, GSH and the Cu(I)-GSH complex displayed fluorescence signals at the same wavelength that were much less intense (1% and 5% respectively). Furthermore, anaerobic titration of the protein revealed a maximum in the fluorescence curve near 12 Cu(I) equivalents which accounts for the saturation of both metal clusters (Figure 1). The rate of Cu(I) incorporation into the thionein from the Cu(I)-GSH complex was relatively fast with respect to the time limits of the fluorescence determination. In fact, the characteristic orange-red fluorescence of Cu(I)-MT was detectable at near maximum intensity within 1 min of the addition of the Cu(I)-GSH complex.

The Cu(I)-GSH complex was much more efficient in the process of MT reconstitution than other complexes of standard use for reconstitution of Cu(I)-containing proteins, such as Cu(I)-(thiourea)₃Cl and Cu(I)-(acetonitrile)₄ClO₄ (Figure 1). Moreover, in the case of acetonitrile, the corresponding Cu(I) complex was not stable under our experimental conditions (pH 7.0), and partial oxidation of the metal was observed even under N_2 , with quenching of the Cu(I)-MT fluorescence (Figure 1).

Isolation and characterization of reconstituted Cu(I)-MT

MT anaerobically reconstituted with 12 mol-equiv. of Cu(I)-GSH complex was chromatographed by gel filtration after

Figure 1 Fluorescence of reconstituted copper-thionein with different Cu(I) complexes

Titration of apo-MT (26 μM) with Cu(I)-GSH complex (○), Cu(I)-(thiourea)₃Cl (●) and Cu(I)-(acetonitrile)₄ClO₄ (△) monitored by the fluorescence band with maximum intensity at 625 nm. Excitation was at 336 nm with filter at 389 nm. Measurements were made at 25 °C, under anaerobic conditions, in 0.1 M phosphate buffer, pH 7.0.

Figure 2 Gel-filtration chromatography of Cu(I)-MT in 0.1 M phosphate buffer, pH 7.0, at 25 °C, under anaerobic conditions

The reaction mixture (1 ml) was applied to the column after 2 h incubation of apo-MT with Cu(I)-GSH. Initial concentrations: 56.5 μ M apo-MT; 680 μ M Cu(I)-GSH complex in Cu(I). For experimental procedures, see the Materials and methods section. ●, MT; ■, Cu(I); ○, GSH; ×, SH.

Figure 3 Gel-filtration chromatography of Cu(I)-MT, in 0.1 M phosphate buffer, pH 7.0, at 25 °C, under anaerobic conditions

The reaction mixture (1 ml) was applied to the column after 2 h incubation of apo-MT with 12 mol-equiv. of either Cu(I)-(thiourea)₂Cl (●, ■) or Cu(I)-(acetonitrile)₄ClO₄ (○, ×). Initial concentrations: 50 μ M apo-MT. For experimental procedures, see the Materials and methods section. ●, MT; ■, Cu(I); ○, MT; ×, Cu(I).

2 h of incubation at 25 °C under strictly anaerobic conditions. A typical elution profile is shown in Figure 2. Two major peaks were observed at 220 nm and these were Cu(I)-MT and GSH as demonstrated by u.v. spectra for Cu(I)-MT and by a DTNB/glutathione reductase assay for GSH. Most of the added metal was eluted with the MT fractions, and 90% recovery of protein was achieved. A 100% recovery of GSH was also obtained, and it was eluted practically free of metal. No apparent GSH binding to the thionein was observed. Under these conditions 11.8 ± 1.2 Cu(I) atoms bound to MT were determined. The metal content was unaffected if the sample was pretreated with 0.1% 2-mercaptoethanol before chromatography, and 19 ± 2 SH groups were still titrated after acidification, under anaerobic conditions, of the Cu(I)-MT.

Figure 4 Gel-filtration chromatography of Cu(I)-MT in 0.1 M phosphate buffer, pH 7.0, at 25 °C, under aerobic conditions in the presence of excess GSH

The reaction mixture (1 ml) was applied to the column after 2 h incubation in air of apo-MT with Cu(I)-GSH in the presence of hundredfold excess of GSH. Initial concentrations: 100 μ M apo-MT; 1200 μ M, Cu(I)-GSH complex in Cu(I). For experimental procedures, see the Materials and methods section. ●, MT; ■, Cu(I); ○, GSH; ×, SH.

Similar experiments performed in air showed pronounced oxidation of the protein. In fact, the fractions containing the protein, after acidification, showed an absorbance at 220 nm that was higher than the one observed with the starting material. The value of Cu(I) incorporation obtained by using 12 mol-equiv. of the complex was 6.8 ± 0.6 g-atoms per mol of protein. Less than 10% of the glutathione was eluted in the reduced form, and only 5 ± 3 SH groups were determined after protein acidification.

An altered migration of the protein sample in gel chromatography was observed under N₂ when reconstitution was performed with either thiourea or acetonitrile, indicating either oxidation of the protein or insertion of the metal ions in sites different from the native one (Figure 3). In these experiments, additional evidence for partial oxidation of the protein was the lower value of the SH/protein ratios (5.5 ± 1.0 and 2.5 ± 0.6 respectively) with respect to GSH. Furthermore, under these conditions 5.8 ± 0.8 and 3.5 ± 0.5 Cu(I) atoms bound to MT were determined.

When the reconstitution experiments were performed in the presence of excess GSH under aerobic conditions, different behaviour was observed. Figure 4 shows the elution profile obtained in air and in the presence of a one hundredfold excess of GSH. In this case a 95% recovery of MT was achieved and the metal/protein ratio was 11.2 ± 0.6 using 12 mol-equiv. of Cu(I), comparable with the value obtained in the absence of air. Glutathione was eluted as both the oxidized (fractions 20–24) and the reduced (fractions 26–31) form, with the latter one being approx. 43% of the added glutathione. These results clearly indicate that GSH acts as an efficient Cu(I) donor to thionein, and is able to protect the protein from oxidation in air. This may be considered an obvious result in view of similar effects obtained with other thiols, but it should be kept in mind that GSH is the only thiol that is present at high concentration under physiological conditions.

Replacement of bound Zn(II) and Cd(II) by Cu(I)-GSH

Cu(I) is known to be able to displace both Cd(II) and Zn(II) from their binding sites in mammalian MT (Stillman et al., 1986). Under our experimental conditions, a slight excess of Cu(I)-GSH

Table 1 Metal analyses of MT samples purified after addition of Cu(I)-GSH to Cd(II)/Zn(II)-MT or ZnSO₄ to Cu(I)-MTResults are means $\pm$ S.E.M. ($n = 3$).(a) Addition of Cu(I)-GSH [0.33 mM Cu(I)] to Cd(II)/Zn(II)-MT (10.5 μ M) under N₂ in 0.025 M phosphate buffer (pH 7.0)

	[Cu(I)]/[protein]	[Cd(II)]/[protein]	[Zn(II)]/[protein]
Commercial Cd(II)/Zn(II)	0.1 $\pm$ 0.01	5.4 $\pm$ 0.30	1.7 $\pm$ 0.12
Purified MT (fraction 1)	7.0 $\pm$ 0.50	0.3 $\pm$ 0.01	0.1 $\pm$ 0.01
Purified MT (fraction 2)	3.5 $\pm$ 0.28	1.3 $\pm$ 0.08	0.2 $\pm$ 0.01

(b) Addition of ZnSO₄ (1.03 mM) to partially reconstituted Cu(I)-MT (11.6 μ M) under N₂ in 0.10 M phosphate buffer (pH 7.0)

	[Cu(I)]/[protein]	[Zn(II)]/[protein]
Initial Cu(I)-MT	4.5 $\pm$ 0.25	0.2 $\pm$ 0.01
Purified MT	4.4 $\pm$ 0.27	3.0 $\pm$ 0.15

was found to produce the same effect (Table 1a). On the other hand, when a 90-fold excess of ZnSO₄ was added to a partially reconstituted Cu(I)-MT sample, under conditions routinely used for reconstitution of MT with Zn(II) (Winge et al., 1985), metal analyses of the reconstituted protein showed that Zn(II) was able to occupy the binding sites available up to the expected saturation value, but it was not able to replace bound Cu(I) (Table 1b).

DISCUSSION

Cu(I)-MT is the major copper-storage protein in eukaryotic cells, and GSH was previously suggested to be a possible site of Cu(I) residence, before incorporation into MT, during the process of Cu(I) loading of hepatoma cells (Freedman et al., 1989). Here, we have shown that GSH is actually able, and much better than the most efficient non-physiological complexing agents of routine use in Cu(I) biochemistry, to transfer Cu(I) to metal-depleted thionein. This effect was not at all related to reduction by GSH of disulphide bridges that may have been formed during inadequate preparation of apo-MT, since all controls (SH groups, Ag(I)-binding capacity) demonstrated the full integrity of the starting protein material used in the reconstitution experiments. On the other hand, correct Cu(I) incorporation also occurred under aerobic conditions, if GSH excesses comparable with physiological concentrations were used. In this case, at variance with the conclusions from the anaerobic experiments, GSH apparently protects MT from its extreme reactivity to oxidation in air. This effect may point to a more general role for GSH as an actual partner of MT in Cu(I) storage and transfer in cells.

Cu²⁺/Zn²⁺ superoxide dismutase and MT behave in different ways in the process of Cu(I) uptake from Cu(I)-GSH. In fact, a Cu(I)-GSH-protein ternary complex was detected by n.m.r. with the former enzyme (Ciriolo et al., 1990). In the case of MT, the incorporation process was very fast with respect to the time limits of the fluorescence measurements and did not allow any transient species to be observed. Even if the formation of a ternary complex cannot be excluded, the higher rate of incorporation may be due to the occurrence of a simple thiolate exchange process in the case of MT, which cannot take place

with the co-ordination sphere (four histidines) of Cu²⁺/Zn²⁺ superoxide dismutase.

A final comment takes note of the ability of Cu(I)-GSH to remove Cd(II) and Zn(II) from mammalian MT (Table 1), which, at variance with yeast Cu(I)-MT (Winge et al., 1985), is usually isolated as a Cd(II)/Zn(II)-containing protein. This result indicates that displacement of Zn(II) and Cd(II) by Cu(I) in mammalian MT can also occur in the presence of a physiological Cu(I) complex, such as Cu(I)-GSH. The metal replenishment of MT *in vivo* may depend on the concentration of different metal ions available to the cell besides the species-specific structural properties of the protein.

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