Reconstitution of Ceruloplasmin by the Cu(I)-Glutathione Complex

EVIDENCE FOR A ROLE OF Mg^{2+} AND ATP*

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The copper-glutathione complex (Cu(I)-GSH) efficiently acted in vitro as the source of Cu(I) in the reconstitution of apoceruloplasmin. Copper was found to reconstitute the various sites in a multistep process, with metal entry into the protein in a first phase, and a second step involving conformational changes of the protein leading to the recovery of the native structural and functional properties. This latter phase was found to be strongly facilitated by Mg^{2+} or Ca^{2+} and by ATP. Both Mg^{2+} and ATP had to be present for optimal reconstitution.

These results may shed some light on the mechanisms governing the biosynthesis of ceruloplasmin in vivo. Cu(I)-GSH was the only complex able to reconstitute ceruloplasmin at neutral pH. Glutathione may thus function to shuttle the metal from the membrane copper pump, as the Wilson disease ATPase, and ceruloplasmin in the secretory compartments of the cell. The finding that ceruloplasmin acquires the native conformation after metal entry through a complex pathway triggered by Mg^{2+} and ATP suggests that they may act as physiological modulators of this process in vivo.

Ceruloplasmin (CP), an α₂-glycoprotein found in the plasma of all vertebrates, is synthesized mainly in the liver as an apoprotein and secreted into plasma as a holoprotein associated to 5–6 tightly bound copper atoms. The role of the prosthetic metal in the physiologic activity of CP is unclear. The peculiar spectroscopic and functional properties of the copper atoms are typical of multicopper blue oxidases and suggest that CP acts as an enzyme (Rydén, 1984). The metal ions of blue oxidases are divided into three spectroscopically distinguishable types of centers which are referred to as Type 1, or "blue" copper, with an intense optical absorbance around 600 nm and a very small "blue" copper, with an intense optical absorbance around 600 nm and a very small
Copper associated to glutathione as CullGSH may be directly transferred to different copper-dependent proteins like apo-CuZn-superoxide dismutase (Ciriolo et al., 1990) and apohemocyanin (Brouwer and Brouwer-Hoexum, 1992), as well as to thioneins (Freedman et al., 1989; Da Costa Ferreira et al., 1993) and to phytochelatins (Mehra and Mulchandani, 1995).

In this study, we have addressed the problem of the processes governing copper reincorporation into apoCP. A complex pathway involving, after metal entry, conformational changes of the protein stimulated by effectors like divalent cations and ATP has been devised.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**All reagents were of analytical grade and were used without further purification. Mops, bovine serum albumin, thiourea, NADPH, and molecular mass standards were from Sigma. Sepharose 4B and Sephadex G-25 prepacked columns (PD20) were obtained from Pharmacia Biotech (Uppsala, Sweden). Chloroethylamine was from Carlo Erba Farmitalia (Milan, Italy). Reduced glutathione (GSH) and glutathione reductase were obtained from Boehringer (Mannheim, Germany), and CuCl from Aldrich (Steinheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

The CullGSH was aerobically prepared shortly before use as described previously (Ciriolo et al., 1990). A GSH: copper stoichiometric ratio of 3:1 was used. The Cullthione complex was prepared according to Minder and Stocker (1936). All solutions were prepared with water pretreated with Chelex 100 resin, to remove traces of metal ions. The absence of any oxidized copper in the complexes was assessed by EPR.

**Proteins—**Sheep and human CPs were purified as described previously (Calabrese et al., 1989; Musci et al., 1993). ApoCP was prepared by a multistep dialysis at 4 °C under anaerobic conditions, achieved by extensive degassing of the dialysis buffers, and maintained throughout by continuous bubbling with ultrapure nitrogen gas. The various dialysis buffers were exchanged by flask-to-flask transfer under nitrogen pressure. The holoprotein was dialyzed against 100 mM sodium acetate buffer at pH 5.9, containing 10 mM ascorbate. The protein was fully reduced within 1 h, as monitored by complete decolorization of the sample. KCN, 50 mM, was then added and the dialysis continued for 5 h (pH = 9.5, after KCN addition). Removal of the copper-cyanide complex and of excess cyanide from the mixture was achieved by a 5-h dialysis versus 100 mM acetate buffer at pH 5.9 containing 1 mM cysteine, followed by an overnight dialysis versus 50 mM Mops buffer at pH 7.0, containing 150 mM KC1. The residual copper content of the apoprotein was less than 2%. No detectable EPR or optical spectra and no oxidase activity were found.

Reconstituted holo and apoCP were chromatographically separated on either DEAE-cellulose or Mono-Q. The mixture loaded on a 25 × 30-cm column of DEAE-cellulose equilibrated with 50 mM phosphate buffer, pH 7.0, was eluted with linear 50–150 mM gradient of the buffer. The holoprotein eluted at ionic strengths corresponding to 85 and 120 mM, while the apo component was recovered at higher ionic strengths. Alternatively, selective elution of the holo- and apo-components was achieved by EPLC (Pharmacia Biotech) on Mono-Q by a linear gradient from 0 mM NaCl at pH 7.5 to 500 mM NaCl at pH 8.5, in 20 mM triethanolamine buffer.

ATP-binding Studies—Solutions containing 38 μM holo or apoCP in 50 mM Mops, 150 mM KC1, 10 mM MgCl2 (pH 7.0) in the presence of different concentrations of (r-32P)ATP (10 μCi/mg) were ultrafiltered in a 0.5-mL Microcon cell (Amicon Co.) with a YM30 membrane. Less than 10% of the initial volume was allowed to filtrate. Retenates and filtrates were assayed for radioactive ATP, and the concentrations of total and free ATP were used to estimate the apparent binding constants.

Miscellaneous—SDS-PAGE was performed with the Bio-Rad Mini Protein II apparatus, with the buffer system of Laemmli (1970), either in reducing or non-reducing conditions (Sato and Gitlin, 1991). PAGE was run according to Davies (1964). Electrophoretic bands were stained for proteins with Coomassie Brilliant Blue R-250, and with diaminodiphenylamine for visualization of the oxidase activity of CP. Protein concentration was determined by the biuret assay (Goo, 1953). Total copper content of holo- or apoCP was estimated either by the biquinoline method (Brumby and Massey, 1967) or by atomic absorption spectroscopy on a Perkin Elmer 2030. CullGSH content was assayed according to Hanna et al. (1988). Oxidase activity versus parahydroxyphenylalanine was assayed as in Calabrese et al. (1989). Total glutathione (GSH+GSSG) content was measured by the 5,5’-dithio-bis(2-nitrobenzoic acid) recycling assay (Anderson, 1985), while GSH and GSSG were separately quantitated by the HPLC method of Reed et al. (1980). Anaerobic experiments were performed with Thunberg cells connected to an optical cuvette or to a quartz EPR tube. Anaerobic conditions were achieved by several cycles of degassing followed by flushing with argon. Optical spectra were recorded on a Perkin-Elmer 330 spectrophotometer equipped with a Hoefr RCB 300 temperature controller. Low-temperature X-band EPR spectra were recorded on a Varian E9 spectrometer interfaced with a Stelar Prometheus Data Acquisition System for analysis and handling of the data. Paramagnetic copper content was calculated with 1.81 mm Cu-EDTA as standard.

**RESULTS**

Samples of CP, both sheep and human, were depleted of copper immediately after isolation from plasma. The reduction of the metal by ascorbate and the rise of pH, up to ~9, which occurred after addition of CN− (see “Experimental Procedures”) were found to be necessary in order to completely and rapidly labilize the metal binding sites. At neutral pH, several days were required to remove most, but not all, of the copper, in agreement with a recent report on rat CP (Terada et al., 1995). Upon metal release, sheep and human CP exhibited a different electrophotometric behavior. The sheep apoprotein migrated with slower mobility than the holo form on PAGE. In these conditions, the apo and holo forms of human CP had the same mobility, while in non-denaturing SDS-PAGE, human holocP ran as a doublet of bands with apparent Mr of 78,000 and 84,000, and the apoprotein invariably ran as a 130-kDa band, as already reported (Sato and Gitlin, 1991).

Reconstitution of Apoceruloplasmin—Reconstitution experiments were carried out at room temperature, unless otherwise stated, on samples of freshly prepared apoCP. No variability in the extent of reconstitution was however observed among samples from the same batch of apoprotein stored for a few hours at 4 °C or for a few days at ~20 °C.

A temporary increase of the absorption at 610 nm was achieved by adding Cu(II)SO4 to apoCP (either sheep or human) at neutral pH in 50 mM Mops, 150 mM KCl buffer in the presence of air (Fig. 1, trace a). The recovery of the blue color took place within the mixing time. However, the band at 610 nm diminished in intensity within a few minutes (Fig. 1, trace b) and eventually converted into a band centered at higher wavelength (Fig. 1, trace c). The phenomenon could be observed at various Cu(II)/CP ratios and a maximum effect (~15% of the expected A610(30 min)) was obtained at a ratio around 10. The derivative was completely inactive versus parahydroxyphenylalanine. Attempts to stop the decay by rapidly chromatographing the apoCP-Cu(II) mixture on G-25 were unsuccessful. Treatment with EDTA followed by G-25 resulted in the complete loss...
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Fig. 2. Time course of the recovery of the absorbance at 610 nm of 1.2 × 10^{-4} M sheep apoCP incubated at 25°C with 7.2 × 10^{-4} M Cu(I)-GSH in 50 mM Mops, 150 mM KCl, containing 5 mM Mg^{2+}.

of the optical features, thus suggesting that Cu^{2+} could not correctly restate in the native copper sites. Cu(I)-thiourea was found to partially restore the spectroscopic properties of apoCP at neutral pH. However, although a spectroscopically stable adduct was obtained, the maximum yield, achieved by anaerobically incubating the protein with the complex for 45 min before reopening to air, was very poor (<15%), and even lower when the incubation was carried out directly in aerobiosis (data not shown).

The complex Cu(I)-GSH, when assayed at neutral pH in different buffers, including phosphate, Mes, and Mops, failed to restore the native spectroscopic properties of CP, which maintained the electrophoretic mobility of the apo form. However, copper stoichiometry, determined after prolonged, i.e. 3 h, incubation of the protein with the complex and gel filtration on G-25, revealed the presence of tightly bound metal atoms, ~6 ions/protein. Of this, about 50% was EPR-detectable, with parameters typical of type 2 copper sites. A chemical assay for reduced copper revealed that the remaining copper atoms were associated to the protein as Cu(I).

In the presence of Mg^{2+}, a divalent cation that binds to CP (Musci et al., 1995), a different result was observed. Addition of Cu(I)-GSH to apoCP (6 Cu(I)/CP molecule) in 50 mM Mops, 150 mM KCl buffer (pH 7) containing 5 mM Mg^{2+} led to a progressive recovery of the absorption at 610 nm, which reached a plateau after 4 h (Fig. 2). Sheep and human apoCP behaved similarly. Within the same species, some variability was observed among different batches of apoCP, with a recovery of the intensity at 610 nm ranging between 50% and 70%. The sheep was slightly more efficient than the human protein. Increasing the Cu(I)/CP ratio did not improve the reconstitution yield. Electrophoretic analyses run, as mentioned before, with non-denaturing SDS-PAGE for the human, and with PAGE for the sheep protein, indicated the presence of only two components in the mixture at the various times of incubation. As shown in the case of sheep CP (Fig. 3A), the intensity of the band with mobility corresponding to the native protein grew up at the expense of the band with R_{p} typical of the apoprotein. Only the component corresponding to the holo form, the intensity of which was consistent with the recovered absorbance at 610 nm, stained positively for oxidase activity (data not shown). The results clearly indicated that a fraction of apoCP never regained the spectroscopic and electrophoretic properties of holoCP, even after several hours of incubation with the Cu(I)-GSH complex.

The presence of Mg^{2+} was found not to improve the reconstitution yield of apoCP by Cu(I)-thiourea or the stability of the protein treated with CuSO_{4}.

The EPR spectrum of the protein reconstituted with Cu(I)-GSH showed, even after gel filtration on G-25, the prevailing presence of resonances with parameters typical of type 2 copper, whose obscured the signals due to type 1 copper (Fig. 4, trace a). Treatment with EDTA (at a final concentration of 50–100 mM), performed at the end of the incubation either by dialysis, or by direct addition of EDTA and passage on G-25, gave a protein with spectroscopic properties similar to those of native CP, although the EPR spectrum (Fig. 4, trace b) showed a content of type 2 copper still slightly higher than that expected.

Comparable results were obtained in samples incubated for 3 h with Cu(I)-GSH in the absence or in the presence of air. The sample incubated in anaerobiosis recovered the blue color within 10 min after admission of air into the optical cuvette, a time consistent with reoxidation of copper at the native sites (Calabrese et al., 1989). Substitution of Ca^{2+} for Mg^{2+} did not vary the extent of the recovery of the 610 nm absorption, which proceeded, however, with noticeably slower kinetics.

In order to evaluate the copper stoichiometry of the holo fraction, this was separated from the mixture, at the end of the incubation, by ion-exchange chromatography on DE52 or, alternatively, on Mono-Q by FPLC (see “Experimental Procedures”). With both methods, and for both sheep and human CP, only two peaks were resolved. The peak eluting at lower ionic strength contained a protein with a copper content of ~5 and ~6 copper ions/CP for sheep and human CP, respectively, and with spectroscopic properties (Fig. 5) and catalytic parameters, K_{m} and V_{max}, indistinguishable from those of the respective native CP. The electrophoretic behavior was also that of the corresponding native protein (Fig. 3, panel B, lanes 1 and 3;
DE52.

CP after reconstitution with Cu(I)-GSH and separation on G-25. All steps were performed both in the presence (closed circles) and in the absence (open circles) of Mg²⁺. See text for details.

Fig. 6. Change of the optical absorbance at 610 nm of sheep apoCP incubated with Cu(I)-GSH for 15 min and then chromatographed on G-25. All steps were performed both in the presence (closed circles) and in the absence (open circles) of Mg²⁺. See text for details.

Fig. 5. Optical (panels A and C) and EPR (panels B and D) spectra of sheep (panels A and B) and human (panels C and D) CP after reconstitution with Cu(I)-GSH and separation on DE52. Dotted curves are the native holoproteins shown for comparison.

panel C, lanes 1 and 2). The other peak was due to a protein that, although with the electrophoretic mobility typical of apoCP (Fig. 3, panel B, lane 2; panel C, lanes 3 and 4) and lacking any oxidase activity (Fig. 3B, lane 4), nevertheless contained ~2 copper ions/molecule. These copper ions had no optical features and were in the oxidized state, with an EPR spectrum typical of type 2 copper ions. Quantitative measurements carried out by HPLC techniques (Reed et al., 1980) revealed that no glutathione, either reduced or oxidized or as a mixed disulfide, had remained associated to the fraction of holoCP or to the protein that had not recovered the spectroscopic properties. Therefore, an irreversible modification of apoCP by glutathione could not be invoked as the cause of the only partial reconstitution of the protein.

Mechanism of Copper Incorporation into ApoCP—To better analyze the mechanism of Cu(I)-GSH-mediated copper transfer into apoCP, and the role of Mg²⁺, sheep apoCP, in 50 mM Mops, 150 mM KCl (pH 7), was incubated for 15 min with stoichiometric Cu(I)-GSH and then quickly (i.e. ~ 3 min) separated on G-25 to remove the unrecombined copper complex. Both steps, incubation and gel filtration, were performed either in the absence or in the presence of 5 mM Mg²⁺, yielding a total of four samples. In the first 15-min interval, only the samples incubated in the presence of Mg²⁺ had, as expected, an appreciable recovery of the blue color (Fig. 6, a and b versus c and d). Afterward, the samples chromatographed in the presence of Mg²⁺ recovered the absorbance at 610 nm independent of whether the cation was initially present (Fig. 6, a' and c'), while the samples separated in the absence of Mg²⁺ (Fig. 6, b' and d') maintained the absorbance at 610 nm that had reached at the end of the 15-min incubation period. Addition of Mg²⁺ to these latter samples caused the development of the blue color (Fig. 6, b' and d'), with kinetics comparable to those observed for samples a' and c'. These results indicated that copper entry into the protein was not affected by Mg²⁺, which was apparently required only for the recovery of the absorbance at 610 nm. This was clearly seen in the sample represented by curve in Fig. 6 that, incubated and separated in the absence of the cation, nevertheless promptly recovered the blue color after addition of Mg²⁺. In the continual presence of Mg²⁺, the rate of recovery of the blue color became faster after G-25 (Fig. 6, a versus a'). Since GSH can reduce the blue sites of CP even in the presence of oxygen,² it is likely that some GSH kept part of reconstituting CP in the reduced state before the chromatography. Alternatively, the slower kinetics of the first phase could be due to the presence of a Cu(I)-GSH-CP ternary complex. Quantitative measurements of total glutathione content (Anderson, 1985) revealed however that no glutathione was present in the sample chromatographed on G-25, suggesting that a ternary complex, if formed upon addition of Cu(I)-GSH, would dissociate on G-25. Altogether these results indicated that the reconstitution of CP proceeded through two distinct phases, the first, Mg²⁺-dependent, involving copper entry into the protein, the second, stimulated by the cation, leading to recovery of the spectroscopic properties. This recovery was not due to a reoxidation phenomenon, since it took place with comparable kinetics also when oxygen was removed from the sample immediately after G-25, provided that Mg²⁺ was present during the anaerobic incubation. Thus, the regain of the spectroscopic properties seemed as if it was due to a cation-mediated conformational rearrangement of the protein leading to the correct geometries of the copper sites.

To sustain this hypothesis, the behavior of the copper sites during the process was analyzed in deeper detail. Taking advantage of the ability of EDTA to remove both Mg²⁺ and copper loosely bound to the protein (cf. Fig. 4), aliquots of the sample a' (Fig. 6) were treated with EDTA at different times after the gel filtration on G-25 performed in the presence of Mg²⁺. The optical and the EPR spectra were measured after an additional passage on G-25 equilibrated with Mops/KCl buffer to remove free and EDTA-complexed metal ions. The optical spectra showed that the absorbance at 330 nm was totally recovered in the sample treated with EDTA 1 min after G-25, at variance with the absorbance at 610 nm, suggesting that the copper atoms of the trinuclear cluster were already oxidized at this stage. The EPR spectra, shown in Fig. 7 (panel A) for the samples treated 1 and 150 min after G-25, had a completely different lineshape, mostly due to type 2 copper at shorter times and to type 1 copper at longer times. Fig. 8 (upper panel) graphically reports the content of paramagnetic, type 1, and type 2 copper atoms of the samples quenched with EDTA at different times. Type 1 copper was evaluated from the absorbance at 610 nm, while type 2 copper was obtained by subtracting the contribution of type 1 copper to the paramagnetic copper content of the samples. The amount of paramagnetic copper remained essentially stable, suggesting that a modification of type 2 centers into type 1 copper sites was at the base of the regain of the spectroscopic properties. PAGE analysis of the aliquots revealed again the presence of the two bands, the oxidase-inactive one, with mobility corresponding to that of apoCP, prevailing at shorter times, and the oxidase-active one.

² L. Calabrese and G. Musci, unpublished results.
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Fig. 7. Panel A, X-band EPR spectra of sheep apoCP incubated with Cu(I)-GSH for 15 min, chromatographed on G-25, and then incubated for 1 min (spectrum a) and 150 min (spectrum b) before treatment with EDTA. All steps were in 50 mM Mops, 150 mM KCl, pH 7.0, containing 5 mM Mg²⁺. Panel B, X-band EPR spectra of sheep apoCP incubated with Cu(I)-GSH for 1 min, chromatographed on G-25, and then incubated for 1 min (spectrum a) and 150 min (spectrum b) before treatment with EDTA. All steps were in 50 mM Mops, 150 mM KCl, pH 7.0, containing 5 mM Mg²⁺ and 10 mM ATP. Experimental settings are described in Fig. 4.

Fig. 8. Content of paramagnetic (open circles), type 1 (closed squares), and type 2 copper (open squares) of sheep apoCP incubated with Cu(I)-GSH for 15 min, chromatographed on G-25, and then treated with EDTA at different times. All steps were in 50 mM Mops, 150 mM KCl, pH 7.0, containing 5 mM Mg²⁺. Upper panel, in the absence of ATP; lower panel, in the presence of 10 mM ATP.

Fig. 9. Elution profiles on Mono-Q of the samples depicted in Fig. 8 (upper panel), treated with EDTA 6 min (a), 22 min (b), and 47 min (c) after G-25. The peaks of the genuine holoCP and apoCP are shown by the dotted and the dotted broken lines, respectively.

The results presented in this paper show that Cu(I)-GSH is effective in transferring the metal to ceruloplasmin. A protein with spectroscopic, enzymatic, and physicochemical properties...
**Table I**

### Effect of Mg<sup>2+</sup> and of ATP on the yield and kinetics of reconstitution of sheep CP

| Sample       | Yield<sup>a</sup> | t<sub>1/2</sub><sup>b</sup> |
|--------------|-------------------|-----------------|
| Apo          | 3.4               | -                |
| ApoMg<sup>2+</sup> | 36.0            | 30              |
| ApoATP       | 21.0              | 35              |
| ApoMg<sup>2+</sup>ATP | 48.0            | 15              |
| ApoGTP       | 4.0               | ND<sup>c</sup>  |
| ApoADP       | 2.5               | ND<sup>c</sup>  |

<sup>a</sup> Evaluated by the absorbance at 610 nm.

<sup>b</sup> Time for half-maximal reconstitution.

<sup>c</sup> ND, not determined.

indistinguishable from those of the native protein has in fact been obtained with good yields by incubating apoCP with stoichiometric amounts of the complex. These results are at variance with those obtained with a different Cu(I) complex, namely Cu(I)-thiourea, or with a source of oxidized copper. The Cu(I)-thiourea complex has been used in the past to reconstitute apoCP, due to its structural analogy with the metal binding site of metallothioneins (Schechinger et al., 1988). The experimental conditions were however quite different, especially as the pH value (−6) was concerned, and the reconstituted protein, although regaining the oxidase activity, showed a fairly high amount of EPR-detectable type 2 copper. When assayed at neutral pH, the Cu(I)-thiourea complex has turned out to be rather inefficient in transferring copper to apoCP, independent of the presence of other effectors. On the other hand, apoCP could regain the absorbance at 610 nm when incubated with CuSO<sub>4</sub>, but the derivative turned out to be highly unstable, possibly because of incorrect or missing filling of non-blue sites by Cu(I).

The Cu(I)-GSH adduct appears the most suitable source of copper so far investigated to re-establish the native structural and functional properties of CP at neutral pH. Two different aspects should however be pointed out: (i) there is a stringent requirement for the presence of effectors like Mg<sup>2+</sup> and ATP, as will be better discussed later on; (ii) even under these conditions, reconstitution yields never reach 100%, leaving a fraction of apoCP only partially saturated with copper and incapable to recover the correct metal stoichiometry and spectroscopic properties. This latter phenomenon can be reasonably explained on the basis of some denaturation induced by the extreme conditions necessary for copper removal, in particular by the high pH value attained during dialysis of the protein versus cyanide.

The data obtained with Cu(I)-GSH allow the outlining of a mechanism for copper incorporation into apoCP. GSH is able to transfer copper to the protein, but cannot promote the recovery of the spectroscopic properties unless a divalent cation like Mg<sup>2+</sup> and/or ATP is present. ApoCP reconstitutes in two distinct steps. In a first phase, the metal binds to the protein moiety and reoxidizes, possibly at the right sites, but with incorrect geometries, reflected in spectroscopic properties different from those of native CP. The protein recovers the correct optical and EPR features in a second phase. During this phase, which strictly depends on the presence of a divalent cation (Mg<sup>2+</sup> or Ca<sup>2+</sup>) or ATP, the chromophores of CP behave quite differently. The 330 nm absorption band readily regains its native shape and intensity, suggesting that the trinuclear cluster promptly recovers its structure. The blue absorption and the native EPR lineshape of the type 1 copper sites, on the other hand, are recovered slowly, in a process not involving a redox phenomenon, as it occurs also in the absence of oxygen. Therefore, it is likely that the role of Mg<sup>2+</sup> and of the nucleotide is to induce some conformational rearrangements which affect the protein organization. Such a structural change is in fact not confined to the ligands of the blue sites, as it also produces the abrupt change, in an all-or-none fashion, of the electrophoretic and chromatographic behavior of the protein.

Cu(I)-GSH has been employed here for the first time with CP. This tripeptide is the most abundant non-protein thiol in mammalian cells, and has been shown to be able to chelate and detoxify metals soon after they enter the cell (Fukino et al., 1986; Andrews et al., 1987; Singhal et al., 1987; Kang and Enger, 1988). GSH can form very stable complexes with Cu(I), and the Cu(I)-GSH complex has been implicated in the incorporation of Cu(I) into metallothionein (Freedman et al., 1989) and phytochelatins (Mehra and Mulchandani, 1995), as well as in copper donation to both intra- and extracellular proteins like Cu,Zn-superoxide dismutase (Ciriolo et al., 1999) and hemocyanin (Brouwer and Brouwer-Hoexum, 1992). Glutathione plays a crucial role in the ER, where the GSH/GSSG couple constitutes the principal redox buffer and has been implicated in the correct folding of nascent proteins (Hwang et al., 1992). Therefore, a role for glutathione in the metal traffic control within this compartment, as in the cytosol, is not unlike.

It is now generally accepted that CP incorporates copper early during biosynthesis of the polypeptide chain, although the exact subcellular localization of the process is not clear, i.e. whether it takes place in the ER or in the Golgi (Sato and Gitlin, 1991; Terada et al., 1995). Our finding that Cu(I)-GSH reconstitutes apoCP at neutral pH is consistent with the ER being the site of copper incorporation (Mellman et al., 1986). The genetic studies carried out on patients with Wilson disease, a metabolic disorders of copper, have allowed to identify an ATPase as the copper pump involved in copper incorporation into nascent apoCP (Yamaguchi et al., 1992; Bull et al., 1993; Tanzi et al., 1993). However, also in this case the subcellular localization remains to be clarified. It can not be excluded that, in vivo, copper incorporation into CP is directly mediated by this membrane-bound ATPase (Bull et al., 1993; Tanzi et al., 1993). This mechanism would imply that the pump can specifically interact with the many different copper-dependent proteins to be processed within the ER, including secretory proteins other than CP (extracellular superoxide dismutase and lysyl oxidase) or membrane proteins like yeast Fet3p (Yuan et al., 1995). It is easier to figure out that a soluble molecule shuttles the metal from the pump to the target. Glutathione is, in this respect, a likely candidate, since, as stated above, its presence in the ER is well documented (Hwang et al., 1992; Young et al., 1993).

A role for divalent cations and for ATP in the ER is well established, and the effects exerted by these molecules in vitro on apoCP may therefore have a physiological relevance. All these species exert a common effect, they are able to stimulate recombined CP to establish the proper spatial relationships at the blue copper sites. Both Ca<sup>2+</sup> and Mg<sup>2+</sup> have been recently demonstrated to bind to CP, with affinities in the millimolar range (Musci et al., 1995). Calcium is actively stored in the ER, where it can reach millimolar levels being bound to specific ER proteins (Sambrook, 1990). However, Ca<sup>2+</sup> showed to be less efficient with apoCP than Mg<sup>2+</sup>, which is at millimolar concentrations in the ER (Gunther, 1990). ATP is required by different ER systems including chaperones (Hendrick and Hartl, 1993), and it is involved in a number of crucial phenomena, including translocation of proteins to the cis-Golgi (Beckers et al., 1987, 1990). It also binds to CP, with a slightly higher affinity for the apo form. However, our data do not allow to unequivocally assess that the Mg<sup>2+</sup>-ATP complex is the active species, although the observation that the maximum effect was observed with both Mg<sup>2+</sup> and ATP, with respect to Mg<sup>2+</sup> or ATP alone, strongly suggests a role for the complex in assisting...
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Reconstitution of Ceruloplasmin by the Cu(I)-Glutathione Complex: EVIDENCE FOR A ROLE OF Mg AND ATP
Giovanni Musci, Stefania Di Marco, Gian Carlo Bellenchi and Lilia Calabrese

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Giovanni Musci, Stefania Di Marco, Gian Carlo Bellenchi,
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Pages 1976-1977, Figs. 6 and 7: The quality of the reproduction of these figures was inadequate. An improved version is shown below:

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Cloning of antizyme inhibitor, a highly homologous
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Yasuko Murakami, Tamotsu Ichiba, Senya Matsufuji, and
Shin-inchi Hayashi

Page 3340: A data base accession number was omitted from this paper. The data appear under the accession number D50734.

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