Copper transport from Cu(I)-thionein into apo-caeruloplasmin mediated by activated leucocytes

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A study on the transfer of copper from Cu-thionein into apo-caeruloplasmin, using Cu-thionein that was previously oxidised by activated leucocytes, was performed. Cu(I)-thiolate oxidation was conveniently monitored by the progressive decline of the specific Cotton bands between 400 and 300 nm. The characteristic e.p.r. properties and *NN*-dimethyl-*p*-phenylenediamine oxidase activity indicated a successful formation of caeruloplasmin. Taking into account the simultaneous occurrence of leucocytes, apo-caeruloplasmin and Cu-thionein in blood plasma, such an interaction would favour a possible metabolic link between either copper protein.

INTRODUCTION

The molecular mechanism of copper metabolism is unknown. It was proposed that the ubiquitously present metallothioneins may act as storage and/or transport proteins for copper, but no enzymic mechanism is known by which copper may be inserted into the active centres of copper proteins.

Metallothionein levels in liver and kidneys are influenced by factors that include copper and zinc administration, stress and hormonal status. Ten to twenty times higher copper concentrations are found in the liver of foetal and neonatal animals as compared with that in adults (Ettinger, 1984). Although metallothionein has generally been regarded as an intracellular protein, radioimmunoassay procedures revealed low levels in the plasma (0.3 nM) (Mehra & Bremner, 1983). In the plasma of 4-day-old rats, 400-fold higher (0.12 μ M) metallothionein concentrations were found, which declined rapidly to normal after the decrease in the level of metallothionein in the liver (Mehra & Bremner, 1984).

During systemic inflammation, elevated serum copper levels are observed. Essentially all of this copper is bound to caeruloplasmin (Gutteridge *et al.*, 1985).

It was attractive to propose that these increased extracellular copper levels originate from catabolized hepatic Cu-thionein. Several possibilities leading to the release of the firmly sequestered copper of metallothionein have been discussed (Weser *et al.*, 1986). There is a direct Cu(I) transfer from yeast Cu-thionein into some other copper proteins (Morpurgo *et al.*, 1983; Hartmann *et al.*, 1983). Furthermore, liberation of copper occurs after prior oxidation of the copper-thiolate centres. Model reactions for the oxidative cleavage demonstrated that H_2O_2 -generating enzymes were able to oxidize the copper(1)-thiolate chromophores of Cu-thionein (Hartmann *et al.*, 1984). A similar redox reaction is assumed to be mediated by activated leucocytes. These cells are known to release excited oxygen species including superoxide radical ($O_2^{\cdot-}$), H_2O_2 and hydroxyl radical ($OH^{\cdot}$) (Deuschle & Weser, 1985). A rise in the total number of leucocytes is observed during systemic

inflammation and rheumatoid arthritis. This phenomenon may be correlated with the marked increase in the serum copper concentration.

The glycoprotein caeruloplasmin was shown to be an important extracellular antioxidant *in vivo* (Gutteridge, 1985). It can inhibit both lipid peroxidation and the Fenton reaction by catalysing the oxidation of Fe^{2+} to Fe^{3+} . Furthermore, caeruloplasmin, even when heat-denatured, is able to scavenge the active oxygen intermediates $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$, probably in stoichiometric amounts. Also, inflammatory processes may be affected by the binding of this serum protein to specific receptors on leucocyte membranes (Kataoka & Tavasoli, 1985). The inhibition of leucocyte-induced degradation of hyaluronic acid by caeruloplasmin (Hartmann *et al.*, 1985) confirms the pronounced ability of caeruloplasmin to control the activity of leucocytes during systemic inflammation. In addition, approx. 10% of the serum caeruloplasmin is present as the apoprotein (Holtzman & Gaumnitz, 1970), which helps to scavenge unspecifically bound copper.

Both Cu(I)-thionein and caeruloplasmin are present in liver cytosol. Unfortunately, no direct Cu(I) transfer from Cu-thionein into apo-caeruloplasmin was shown. The reactivity of leucocytes with Cu-thionein (Hartmann *et al.*, 1985) and the simultaneous occurrence of both caeruloplasmin and leucocytes in blood plasma prompted us to use apo-caeruloplasmin as a most suitable acceptor protein for copper from oxidised Cu-thionein. Reconstitution of apo-caeruloplasmin employing copper originating from Cu-thionein and in the presence of leucocytes was performed. In addition to the oxidase activity, e.p.r. measurements of the copper in caeruloplasmin were expected to support the genuine formation of the holoprotein. Concomitant degradation of Cu-thionein was monitored by c.d. spectrometry. To minimize uncontrolled reactions of unspecifically bound Cu(II) always present in the vertebrate thionein (Hartmann & Weser, 1985), yeast Cu-thionein was used. This Cu-thionein is a protein homogeneous with respect to Cu(I). It never contains Zn(II) or Cd(II) and is much less susceptible to oxidation. Regardless of its biological

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origin, Cu(I) is exclusively co-ordinated to thiolate sulphur atoms (Bordas *et al.*, 1982; Hartmann & Weser, 1985; Freedman *et al.*, 1986; Abrahams *et al.*, 1986).

MATERIALS AND METHODS

Caeruloplasmin from porcine serum was purified by employing combined anion-exchange and gel-filtration chromatography as described by Rydén & Björk (1976). The apoprotein was obtained by dialysis against 50 mM-KCN in 50 mM-sodium acetate buffer (pH 5.5)/150 mM-NaCl.

The apo-caeruloplasmin contained less than 5% residual copper and could be reconstituted with CuSO_4 /cysteine up to 75% of the theoretical A_{610} value. Oxidase activity was examined by monitoring the oxidation of *NN*-dimethyl-*p*-phenylenediamine (Sigma, Heidelberg, Germany) at 550 nm (Curzon & Vallet, 1960).

Leucocytes were separated as described by Hartmann *et al.* (1985) from fresh citrated porcine blood. Cell viability was assessed by Trypan Blue exclusion. Yeast Cu-thionein was prepared as described by Weser *et al.* (1977) and contained 50 μg of Cu/mg of protein.

Electronic absorption was recorded on a Beckman DU-40 spectrophotometer, c.d. measurements were performed on a JASCO 20A spectropolarimeter and e.p.r. spectra were run on a Varian E-109 spectrometer at 100K. Copper was analysed on a Perkin-Elmer atomic-absorption spectrometer (model 400 S) with a HGA 76B unit. C.d. and e.p.r. measurements were successful in the presence of 10^8 leucocytes in the incubation mixture (1 ml). One-tenth of the leucocyte cells were used in the oxidase-assay experiments. The cells were activated with 10 μl of 2 mM-phorbol myristate acetate (Sigma).

RESULTS AND DISCUSSION

The simultaneous incubation of both Cu-thionein and apo-caeruloplasmin in the presence of activated leucocytes leads to the destruction of copper(I)-thiolate chromophores of Cu-thionein. This could be demonstrated by monitoring the characteristic Cotton bands at 360 and 330 nm. Fortunately, between 400 and 300 nm no chiroptical interference attributable to caeruloplasmin was seen. Upon incubation the Cotton extrema were progressively diminished (Fig. 1). After 4 h the decrease corresponds to a 40% destruction of the Cu(I)-thiolate chromophores.

The formation of caeruloplasmin by copper transfer from Cu-thionein to apo-caeruloplasmin was additionally demonstrated by e.p.r. spectrometry. The characteristic narrow and normal hyperfine structures of Type 1 and Type 2 copper appeared within 6 h (Fig. 2). Reduced copper centres had to be oxidized by H_2O_2 (0.5 mM) before the measurements were made. About 45–50% of the thionein Cu was e.p.r.-detectable in the reconstituted caeruloplasmin. In those samples where leucocytes were omitted, no detectable reconstitution was observed.

To support the structural information obtained by c.d. and e.p.r. spectrometry it was important to examine the functional side of caeruloplasmin reconstitution. The higher sensitivity of an enzymic assay permits physiological concentrations of caeruloplasmin (3 μM to be measured. Furthermore, precipitation of leucocytes by

Fig. 1. C.d. spectra of yeast Cu-thionein after incubation with activated leucocytes and apo-caeruloplasmin

The incubation mixture comprised yeast Cu-thionein (120 μM -Cu), apo-caeruloplasmin (10 μM), 10^8 leucocytes in a buffer composed of 140 mM-NaCl, 10 mM- Na_2HPO_4 , 3 mM- K_2HPO_4 , 2.7 mM-KCl, 1 mM- CaCl_2 , 1 mM- MgSO_4 and 5.5 mM-D-glucose, pH 7.3; the incubation temperature was 22 °C; before the measurements were made, leucocytes were removed by centrifugation.

Fig. 2. E.p.r. spectra of caeruloplasmin reconstituted by leucocyte-oxidized Cu-thionein

The incubation mixture comprised yeast Cu-thionein (140 μM -Cu), apo-caeruloplasmin (20 μM) and 10^8 leucocytes; incubation conditions were as in the legend to Fig. 1; instrumental conditions: microwave frequency, 9.24 GHz; microwave power, 20 mW; modulation amplitude, 1 mT; temperature, 100 K.

excessive Cu-thionein concentrations, occasionally observed in the above experiments, was avoided. A time-dependent generation of caeruloplasmin from the apoprotein and oxidized Cu-thionein was seen (Fig. 3).

After 160 min the oxidase activity was elevated 6-fold compared with the original value. In control experiments omitting either Cu-thionein or activated leucocytes the enzymic activity rose to one-third of that found in the complete assay. The incorporated copper is assumed to

Fig. 3. Oxidase activity of caeruloplasmin reconstituted by leucocyte-oxidized Cu-thionein

●, Full incubation [apo-caeruloplasmin (3 μ M), yeast Cu-thionein (50 μ M-Cu) and 10^7 leucocytes]; ■, control, without activation of leucocytes; ▲, control without Cu-thionein; incubation conditions were as in the legend to Fig. 1. One unit is arbitrarily defined as the amount of caeruloplasmin that will cause a ΔA_{550} of 0.01/min (Curzon & Vallet, 1960).

originate from the leucocytes employed or from partially oxidized Cu-thionein. The oxidation of Cu-thionein with H_2O_2 yields Cu(II) (Hartmann *et al.*, 1984). However, according to results with some other copper proteins (Morpurgo *et al.*, 1984; Brüttsch *et al.*, 1984) Cu(I) incorporation into apo-caeruloplasmin is preferred (Morell & Scheinberg, 1958). Even in the absence of additional reducing agents the reconstitution of caeruloplasmin was successful when copper from oxidized Cu-thionein was used. This Cu(II) could be converted into Cu(I) by the $O_2^{\cdot-}$ known to be released from the leucocytes employed.

Alternatively, several transient Cu(I) species in oxidized Cu-thionein are assumed (Weser & Hartmann, 1984), including a Cu(I)-SR state and/or a Cu(I)-mixed-disulphide complex. Thus a direct Cu(I) transfer into apo-caeruloplasmin might be assumed in either case.

In the partially oxidized Cu-thionein occasionally observed during isolation and storage of the protein, this transient copper co-ordination is expected to occur (Hartmann *et al.*, 1986). No e.p.r. signal characteristic for Cu(II) is detectable. However, the molar-ellipticity ($[\theta]_{Cu}$) values are diminished, and this can be attributed to structural changes in the copper-thiolate chromophores. Caeruloplasmin is reduced by this transient form of Cu-thionein, eventually leading to Cu(II) (Hartmann *et al.*, 1979). Although leucocytes are inhibited by caeruloplasmin (Hartmann *et al.*, 1984), copper may be released from leucocyte-treated Cu-thionein via the same mechanism.

The reactivity of leucocytes with Cu-thionein and the concomitant successful formation of caeruloplasmin from the apoprotein present in blood plasma may be considered a basic reaction in copper metabolism. Thus successive loading by copper originating from circulating metallothionein released by the liver seems possible. This may be the reason for the enhanced caeruloplasmin levels in several metabolic conditions, including systemic inflammation and stress.

By way of contrast, the degradation of Cu-thionein in the liver is completely unknown. It is attractive to assume that the formation of caeruloplasmin and other copper proteins proceeds by copper transfer from catabolized Cu-thionein. As proteolysis of Cu-thionein does not appear to be the main route of catabolism (Mehra & Bremner, 1985; Weser *et al.*, 1986), the oxidative cleavage is the more favoured pathway. However, no hepatic Cu-thionein oxidase has so far been detected in many subcellular fractions of liver homogenate.

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