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Decrease of cytochrome c oxidase protein in heart mitochondria of copper-deficient rats

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Copper deficiency has been reported to be associated with decreased cytochrome *c* oxidase activity, which in turn may be responsible for the observed mitochondrial impairment and cardiac failure. We isolated mitochondria from hearts of copper-deficient rats: cytochrome *c* oxidase activity was found to be lower than in copper-adequate mitochondria. The residual activity paralleled copper content of mitochondria and also corresponded with the heme amount associated with cytochrome *aa*3. In fact, lower absorption in the α-band region of cytochrome *aa*3 was found for copper-deficient rat heart mitochondria. Gel electrophoresis of protein extracted from mitochondrial membranes allowed measurements of protein content of the complexes of oxidative phosphorylation, revealing a lower content of complex IV protein in copper-deficient rat heart mitochondria. The alterations caused by copper deficiency appear to be specific for cytochrome *c* oxidase. Changes were not observed for F_0F_1ATP synthase activity, for heme contents of cytochrome *c* and *b,* and for protein contents of complexes I, III and V. The present study demonstrates that the alteration of cytochrome *c* oxidase activity observed in copper deficiency is due to a diminished content of assembled protein and that shortness of copper impairs heme insertion into cytochrome *c* oxidase.

Keywords: copper deficiency, Cytochrome *c* oxidase, heart, mitochondria, rat

Introduction

Copper biochemistry is mainly related to the existence of a number of copper binding enzymes, present both in the intracellular and in the extracellular milieu. These play fundamental roles in metabolic pathways (cytochrome *c* oxidase, lysyl oxidase, dopamine-β-monooxygenase) and in cell defence systems (superoxide dismutase, ceruloplasmin) (Linder & Hazegh-Azam 1996). Thus, copper homeostasis is strictly regulated by complex machinery, controlling its absorption, distribution and excretion. Maintaining copper homeostasis appears to be crucial for human health; alteration of copper metabolism is associated with severe human genetic diseases, characterised by copper deficiency (Menkes syndrome) (Hamer 1993, DiDonato & Sarkar 1997) or copper overload (Wilson disease) (Brewer & Yuzbasiyan-Gurkan 1992, DiDonato & Sarkar 1997). In experimental animal models of copper deficiency, a decrease in the activities of copper enzymes has been reported (Prohaska 1991, Rossi *et al.* 1994). This phenomenon may not only be a consequence of the lack of the metal in the active site; a control exerted by copper at the transcription level might also be envisaged. This occurs in the case of the cytosolic copper enzyme superoxide dismutase (EC 1.15.1.1) which is regulated by copper both at the transcription and at the post-translation level (Carrì *et al.* 1991, Galiazzo *et al.* 1991, Steinkuhler *et al.* 1991, Rossi *et al.* 1994, 1997).

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In *in vivo* studies on copper deficiency it has been pointed out that changes in copper enzyme activities are organ-specific (Paynter *et al.* 1979, Prohaska 1991, Rossi *et al.* 1994). While superoxide dismutase appears to be the target enzyme in the liver, cytochrome *c* oxidase (COX) (EC 1.9.3.1) activity is affected mainly in the cardiac muscle. Impairment of COX may be responsible for the mitochondrial alterations and for the cardiac disorders (especially hypertrophy) associated with copper deficiency in experimental models (Medeiros *et al.* 1993).

COX is the terminal enzyme of the electron transport chain in mitochondria and the site where oxygen is utilised in respiration; it plays a pivotal role in energy supply. Despite the great importance of this enzyme for metabolism, the modalities of regulation of its expression have not yet been elucidated. Their comprehension is made difficult by the genetic origin of the various subunits (up to 13) of this multisubunit enzyme, three of which are codified by the mitochondrial genome, and by the complexity of its active site, composed of three copper ions and two heme groups (Azzi & Muller 1990). The decrease in COX activity concomitant with copper deficiency has been ascribed either to a control on the synthesis of the nuclear- or mitochondrial-encoded subunits (Medeiros *et al.* 1993) or to the failure of assembly of the subunits during scarcity of copper (Keyhani & Keyhani 1975). New insights into the mechanisms by which copper is delivered to COX have recently been provided. In yeast a cysteine-rich cytoplasmic protein (Cox17p) seems to be responsible for copper delivery to mitochondria (Glerum *et al.* 1996a) and overexpression of Sco1p, a mitochondrial inner membrane protein, seems to be essential for inserting copper during assembly of COX (Glerum *et al.* 1996b).

In the present report we show that in the copper-deficient rat model COX activity in heart mitochondria parallels COX protein content. The decrease in activity was also linked to a comparable decrease in heme content of cytochrome *aa*3, while other cytochromes were unaffected. This may indicate that copper deficiency regulates heme insertion into COX.

Materials and methods

Male Wistar rats (Charles River, Calco, Italy), 70 g body weight (about four weeks old) consumed a copper deficient diet (ICN Biomedicals Inc., Costa Mesa, CA, USA) containing 0.28 mg of copper per kg of diet and were provided with double-distilled water. Paired controls were fed the same diet, supplemented with 20 mg of copper as $CuCO_3$ per kg of diet. Both groups had free access to diet and drinking water. After eight weeks, rats were injected intraperitoneally (i. p.) with chloral hydrate and heparin and sacrificed. Hearts were excised after perfusion with saline through the left ventricle.

All procedures were carried out using glassware devoid of metals by treatment with nitric acid followed by washing with double-distilled water that had been equilibrated with Chelex-100 resin (Bio-Rad, Hercules, CA, USA). Chelex-treated water was also used to prepare all solutions.

Mitochondria were prepared essentially as follows: hearts were minced and homogenised in 210 mM mannitol containing 70 mM sucrose, 3 mM Hepes and 1 mM EDTA, at pH 7.4 (1:6, w:v). Homogenates were centrifuged at $700 \times g$ for 10 min, the supernatants collected and centrifuged at $10\,000 \times g$ for 15 min. Pellets were washed in the same buffer, except for omission of EDTA. The last pellet was resuspended at a protein concentration of 1.5–2 mg ml^{-1}.

COX activity in isolated heart mitochondria was measured spectrophotometrically monitoring the oxidation of cytochrome *c* (horse heart, Sigma, St. Louis, MO, USA), which had previously been reduced by treatment with excess ascorbate, followed by passage on Sephadex G25 resin (Pharmacia Biotech AB, Uppsala, Sweden). Assays were performed at 25°C, in a medium containing 30 mM phosphate buffer, pH 7.4, and 0.02 mM ferrocytochrome *c* at 550 nm (Cooperstein & Lazarow 1951). Activity was expressed as units (μmol cytochrome *c* oxidised min^1 mg^{-1} protein, using an extinction coefficient of 27.6 mM^{-1} cm^{-1}.

Difference spectra of COX were recorded, after treatment of isolated mitochondria with 0.5% Triton X-100 and reduction by sodium dithionite, at 25°C by a Perkin Elmer Lambda 9 spectrophotometer. The content of COX was expressed in terms of cytochrome *aa*3 content at 605–630 nm, using an extinction coefficient of 24 mM^{-1} cm^{-1} (Van Gelder 1966).

Protein content was assayed according to Lowry *et al.* (1951).

Measurements of copper levels in the samples were performed, after digestion with 65% nitric acid, by an atomic absorption spectrophotometer (Perkin Elmer 3030), equipped with a graphite furnace.

Electrophoretic analyses were performed essentially as described by Schagger & von Jagow (1991). Briefly: sedimented mitochondria (0.4 mg of total protein) isolated from either copper-adequate or copper-deficient rats were solubilised by addition of 40 μl of 1 M aminocaproic acid, 50 mM Bis–Tris/HCl, pH 7.0, and 7.5 μl of laurylmaltoside (10%) (Boehringer Mannheim, GmbH, Germany). The samples were centrifuged at $100\,000 \times g$ for 30 min at 4°C. The supernatants were supplemented with a suspension of 5% Coomassie blue G250 in 0.5 M aminocaproic acid (2 μl of dye in 40 μl of supernatants) and applied to the native 6→13% polyacrylamide gradient gel (blue native electrophoresis). The complexes of oxidative

phosphorylation were identified by comparison with bovine heart mitochondrial proteins, processed according to Schagger & von Jagow (1991) and, in the cases of complexes IV and V, also by specific activity staining as specified in Zerbetto *et al.* (1997). The activity of complex IV was determined by following the precipitation of oxidised diaminobenzidine and the activity of complex V (F_0F_1ATP synthase activity) by the precipitation of $Pb(NO_3)_2$.

Different aggregation states of the complexes of oxidative phosphorylation may occur upon treatment of the samples for electrophoretic separation. Both in control and in copper-deficient samples complex III is in a dimeric form, while complexes I and V are in the monomeric state, as previously reported (Schagger & von Jagow, 1991 Reinheckel *et al.* 1995). On the contrary, complex IV exists both in dimeric and monomeric forms in samples from copper-adequate rats (Schagger & von Jagow 1991, Reinheckel *et al.* 1995), while in samples from copper-deficient rats the monomeric form is prominent. The higher ratio detergent/complex IV protein content occurring in the samples from copper-deficient rats is responsible for this phenomenon. In fact, by adding to mitochondria of copper-adequate rats higher amounts of detergent just before loading the sample onto the gel, the prominent form of complex IV becomes the monomer, as in the copper-deficient samples. However this does not affect results of complex IV activity on gels. Complex II appears only as a very weak band.

To quantify the intensity of the bands corresponding to mitochondrial complexes, the gels were scanned in an Ultrascan XL Laser Densitometer (Pharmacia). Complex V was used as internal standard, as it was the most abundant band and was not altered by the treatment of the rats. The content of complex IV protein and its activity was then determined by calculating the ratio between the area of complex IV (monomer plus dimer) and the area of complex V.

Data were expressed as means ± standard deviations. Statistical analysis was performed by the Student's t test for unpaired data; P values < 0.05 were considered significant.

Results and discussion

Copper deficiency in rats leads to impairment of COX activity mainly in the heart, although this organ is less affected than liver in terms of decreased copper content (Rossi *et al.* 1994). Consequently, we hypothesised this copper enzyme to be the target of copper deficiency in the muscular tissue, particularly rich in mitochondria, whilst superoxide dismutase mainly decreased in the liver. The depletion of copper content in heart was found to be 40% of copper-adequate values. This reflected a decrease in COX activity accounting for about 60% (Rossi *et al.* 1994).

In the present study, measurements of copper content in mitochondria isolated from copper-deficient rat hearts revealed the same trend: indeed, a greater depletion of copper (70%) occurred (Figure 1A). The steeper depletion of copper content observed in isolated mitochondria with respect to homogenates of copper-deficient rat hearts may be due to the fact that copper content is differently distributed in the various cell compartments (Evans 1973).

Changes in COX activity in the mitochondria from copper-deficient rat hearts were evaluated by two independent methodological approaches. These were: (1) spectrophotometric analysis (data reported in Figure 1B), which detects the activity of the enzyme in bulk; and (2) activity staining of the bands obtained by gel electrophoresis, which detects the activity of the complex IV (corresponding to COX) (Figure 1C) extracted from the mitochondrial membrane. Both methods revealed a lower COX activity in mitochondria from copper-deficient rat heart, accounting for 40% (spectrophotometric measurements) and 25–20% (densitometric analysis of diaminobenzidine stained bands) of the values of copper-adequate rats.

Metals represent a class of important transcription modulators that regulate gene expression in different ways, either by activation or repression of gene transcription (Gralla *et al.* 1991, Thiele 1992). In the last years, several studies from this laboratory have suggested that a copper-containing enzyme, superoxide dismutase, can be regulated in a versatile way by copper. In the yeast *Saccharomyces cerevisiae*, the synthesis of Cu, Zn

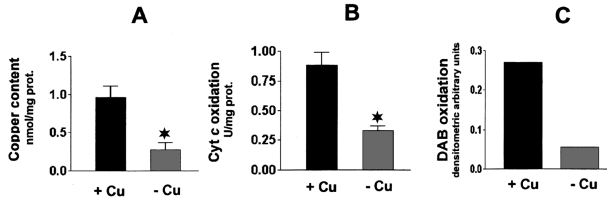

Figure 1. Copper content and cytochrome *c* oxidase activity of mitochondria isolated from hearts of copper-deficient (-Cu) or copper-adequate rats (+Cu). Panel A: copper content, measured by atomic absorption spectroscopy. $n = 11$; $^*P < 0.001$. Panel B: cytochrome *c* oxidase activity was measured spectrophotometrically, monitoring the oxidation of reduced cytochrome *c* at 550 nm. $n = 11$; $^*P < 0.001$. Panel C: cytochrome *c* oxidase (complex IV) activity was measured by densitometric analysis of activity stained gels. Results are from one experiment representative of five.

superoxide dismutase is regulated at the gene level by copper via the transcription factor ACE 1, which folds in an active form only in the presence of copper. It can then bind to the promoter of the superoxide dismutase gene, thus activating the transcription process (Carrì *et al.* 1991, Galiazzo *et al.* 1991). Regulation at the post-translation level also occurs, since copper, which is present at the active site of the enzyme, can activate a pre-existing apoform of the enzyme. In higher eukaryotes, such as the copper-deficient rat (Rossi *et al.* 1994, 1997) or the cultured cells K562 (Steinkuhler *et al.* 1991), post-translation regulation is also operative.

COX is composed of several subunits, whereby the largest three are encoded by the mitochondrial genome and the remaining ones are nuclear encoded (Azzi & Muller 1990). The active site of COX is localised on the mitochondrial encoded subunits, which hold three copper ions and two heme groups. It has already been reported that copper is needed to assemble the nuclear encoded subunits to form mitochondrial complex IV, but this was found in lower eukaryotes, such as the yeast *Candida utilis* (Keyhani & Keyhani 1975) and *S. cerevisiae* (Glerum *et al.* 1996a,b). The COX17 gene of *S. cerevisiae* has been shown to encode for a copper-binding cytoplasmic protein (Cox17p), which targets copper to mitochondria (Glerum *et al.* 1996a). Copper is then transferred to Sco1p, a mitochondrial copper carrier, which translocates the metal to the mitochondrial matrix and donates it to subunits 1 and 2 of COX (Glerum *et al.* 1996b). Cox17p and Sco1p are essential for assembly of COX, since their mutants fail in forming the mature complex.

Fewer details are known about the regulation of the synthesis of COX by copper in higher eukaryotes. The mechanisms of copper delivery to mitochondria might be similar to those described for yeast: recently, a cDNA encoding for the human homologue of COX17 has been isolated (Amaravadi *et al.* 1997). It has been suggested that mitochondrial encoded peptides, those holding the active site, are not altered in copper-deficient rat hearts, while the nuclear encoded peptides seem to be decreased (Medeiros *et al.* 1993, 1997). Northern hybridisation studies demonstrate that mRNA transcripts for subunits 2 and 4, which correspond to mitochondrial and nuclear encoded subunits, do not differ between copper-deficient and copper-adequate rats (Medeiros 1997). These results suggest that copper might act on the subunits post-transcriptionally.

In the present study, an evaluation of COX assembly condition has been performed by means of specific electrophoretic analysis. Figure 2 shows the gel electrophoretic pattern (A) and the relative densitometric profile (B) obtained following extraction of the complexes of the respiratory chain from mitochondrial membranes by treatment with detergents. The densitometric quantification of Coomassie blue stained gels obtained from four copper-deficient and five copper-adequate rats revealed that the complex IV content was $75 \pm 7\%$ lower in copper-deficient than in copper-adequate rats. These results parallel the decrease of COX activity shown in Figure 1B and C and in Figure 2C. From the same analysis we were also able to establish whether possible changes in COX protein are specific and not resulting from a general impairment of mitochondrial membrane apparatus, consequent to copper deprivation. Indeed, both complex III and complex V of the respiratory chain could also be identified on the same gel (Figure 2A and B); their contents appeared to be unchanged between copper-deficient and copper-adequate rat heart mitochondria. The lower activity of complex IV in copper-deficient samples appears to be specific, since the activity of complex V, corresponding to F_0F_1ATP synthase was unaffected (Figure 2C).

In our experimental model lower COX activity in mitochondria of hearts of copper-deficient rats was accompanied by lower heme content of cytochrome *aa*3, as demonstrated by the diminished absorption of the alpha-band at 605 nm (Figure 3). Quantitative measurements showed a decrease of about 70% (1.14 ± 0.05 μmol vs. 0.38 ± 0.02 μmol per mitochondria protein, $n = 5$, $P < 0.001$). These results are in line with the values obtained for copper content of the mitochondrial preparations. Difference spectra also showed that cytochromes *c* and *b*

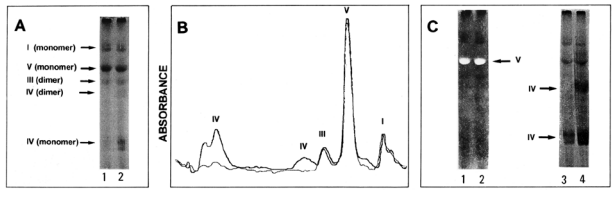

Figure 2. Coomassie blue and activity stained gel electrophoresis of the complexes of oxidative phosphorylation from copper-deficient or copper-adequate rat heart mitochondria. Panel A: Coomassie blue stained gel of samples prepared from copper-deficient (lane 1) and copper-adequate (lane 2) rats. The protein load is 20 μg. Panel B: densitometric traces of lane 1 (thin line) and lane 2 (bold line), showing complexes I, IV and V. Panel C: ATPase activity (lanes 1, 2) and complex IV activity (lanes 3, 4) stained gels of copper-adequate (lanes 2, 4) and copper-deficient (lanes 1, 3) rats. The protein load is 20 μg.

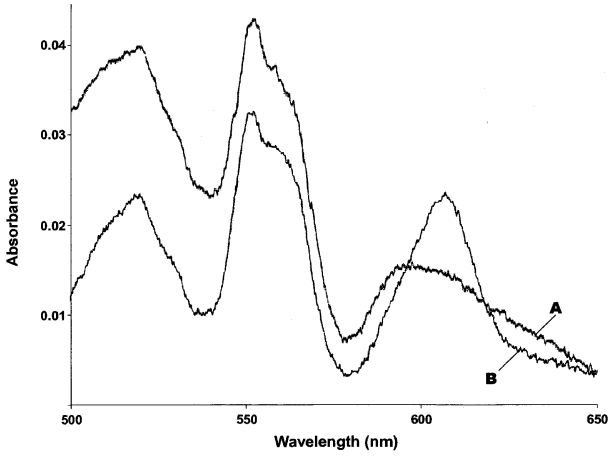

Figure 3. Difference spectra of cytochromes of mitochondria isolated from copper-deficient and copper-adequate rat hearts. A: dithionite reduced minus oxidised difference spectrum of mitochondria from the heart of one copper-deficient rat; B: dithionite reduced minus oxidised difference spectrum of mitochondria from the heart of one copper-adequate rat. Difference spectra of mitochondria from six copper-deficient and six copper-adequate rat hearts were compared, giving similar results to those showed.

contents were unmodified: therefore copper deficiency selectively affected the heme content of cytochrome *aa*3.

Metabolisms of copper and iron are strictly linked. In yeast, a multicopper oxidase, Fet3p, mediates high affinity iron uptake (Askwith *et al.* 1994, Yuan *et al*. 1995). Consequently, mutations in the FET3 gene or shortness of copper impair iron uptake, as a secondary effect. Fet3p is homologous to the human multicopper oxidase ceruloplasmin. Copper deficiency has been associated with iron deficiency because of lack of ceruloplasmin activity (Askwith *et al.* 1994, Rossi *et al.* 1994). This has been suggested to result in impaired heme production and anemia (Linder & Hazegh-Azam 1996). However, our results show that other hemoproteins, namely cytochromes *b* and *c*, are unaffected by copper deficiency. Thus, iron availability might selectively affect COX assembly.

In conclusion, our data demonstrate that the deficiency of COX activity in the heart mitochondria of copper-deprived rats is due to a lower content of assembled COX protein. This is in line with the decrease of immunoreactive COX found in cerebellum, spinal cord and other regions of the central nervous system in Menkes disease (Sparaco *et al.* 1993) and in related animal models (Seki *et al.* 1989) with altered metabolism of copper. At which step during COX maturation (transcription of the mitochondrial or nuclear encoded subunits, import of subunits into mitochondria, altered assembly into the membrane or rate of degradation) copper exerts its control is presently under investigation in our laboratory.

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