Role of Copper in Mitochondrial Biogenesis Via Interaction With ATP Synthase and Cytochrome *c* Oxidase

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Animals that are copper deficient have cardiac hypertrophy where there is a dramatic increase in mitochondria. Mitochondrial biogenesis is enhanced in this model and there is an upregulation of mitochondrial transcription factor A (mtTFA) and nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2). While the cuproenzyme, cytochrome *c* oxidase (CCO), is an attractive candidate to explain the connection between cardiac hypertrophy in copper deficiency and subsequent mitochondrial biogenesis, studies have revealed that ATP synthase may be impacted by copper depletion. NRF-1 and NRF-2 can bind to some of the subunits of both CCO and ATP synthase to regulate gene expression. Furthermore, oxidative phosphorylation appears to occur unaltered in the copper-deficient state. Copper-deficient mitochondria appear to be less sensitive to the inhibitory effect of oligomycin compared to controls. Decreases in the δ subunit protein and β mRNA transcript have been reported for ATP synthase as a function of copper deficiency. The limited data available suggest that copper, either indirectly or directly, alters ATP synthase function.

KEY WORDS: ATP synthase; copper; cytochrome *c* oxidase; mitochondrial transcriptional factor A; nuclear respiratory factors; mitochondria.

INTRODUCTION

Copper transport across cell membranes via P-type ATPases has generated much recent interest. A major factor for this interest is two human genetic disorders, Menkes and Wilson diseases, that have defective genes encoding peptide portions of ATPases responsible for copper transport. In Menkes disease, the ATP*A* peptide is defective that results in a copper deficiency. In Wilson's disease, there is a defective ATP*B* peptide that causes an accumulation of liver copper but very little copper in other tissues. There are a number of mutations in the genes that encode both of these peptides that have been identified (LaFontaine *et al.*, 1998). Recent data suggest that copper deficiency states may impact a different type of ATPase, referred to as mitochondrial F_1F_0 ATP synthase. This enzyme is critical in proton pumping and ATP formation in mitochondria. While ATP*A* and ATP*B* have known binding sites for copper, it is unknown at this time whether this is the case for F_1F_0 ATP synthase.

A well-recognized role of copper is as a cofactor for the enzyme cytochrome *c* oxidase(CCO) or Complex IV of the electron transport chain, which has a critical role in oxidative phosphorylation. Our group has researched the impact of copper deficiency upon the heart, one hallmark sign of which is cardiac hupertrophy (Medeiros *et al.*, 1991a,b; Medeiros and Wildman, 1997). Scores of studies have reported this observation and a growing body of research implicates that the hypertrophy is due to mitochondria proliferation (Mao *et al.*, 1998; Medeiros *et al.*, 1991a, 1997). In addition, it has been shown that mitochondria respiration in copper deficiency is compromised (Chao *et al.*, 1993; Chen *et al.*, in press; Matz *et al.*, 1995). While CCO is an attractive candidate to explain the biochemical defect leading to impaired electron transport and thus to cardiac hypertrophy, some studies have implicated the oxidative phosphorylation enzyme ATP synthase

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(Complex V) (Chao *et al.*, 1994; Chen *et al.*, in press; Matz *et al.*, 1995; Medeiros *et al.*, 1993). Since the function of CCO and ATP synthase are linked coupling respiration to phoshorylation, it is problematic to separate how a change in one enzyme may impact the other and lead to secondary changes. Mitochondrial ATP synthase is a key factor in regulating energy production in the cell. The energy demands of the cell also have an impact upon mitochondrial biogenesis, which is adjusted in order to meet changing energy demands. Herein we will review how copper deficiency impacts the heart, rich in mitochondria; the pathological aspects of the cardiac tissue; altered structure and function of mitochondria, including mitochondrial biogenesis; and impact upon CCO and ATP synthase.

COPPER DEFICIENCY AND CARDIAC HYPERTROPHY

In rodents and swine, feeding a diet deficient in copper to weanling animals, leads to cardiac hypertrophy within 4–5 weeks (Jalili *et al.*, 1996; Wildman *et al.*, 1994, 1996). Older animals are less susceptible to this condition when on a copper-deficient diet, presumably because they have sufficient stores of liver copper (Davidson *et al.*, 1992). Many of the copper-deficient animals develop anemia and at one time the anemia resulting from copper deficiency was thought to produce the cardiac hypertrophy. This concept has since been discredited and it is now known that the development of cardiac hypertrophy precedes the development of anemia in copper deficiency (Medeiros *et al.*, 1991a). Anemia alone leads to hypertrophy in which the shape of the heart is eccentric and the ventricular lumen volume is greatly enhanced, the ventricular walls are thin and overall circumference of the heart increased (Medeiros and Beard, 1998). In copper deficiency with associated anemia, the type of hypertrophy is concentric in shape, where the ventricular walls are significantly thickened and the ventricular lumen decreased in volume. Normally this type of hypertrophy is observed in hypertension (pressure overload) but copper-deficient rats often exhibit low blood pressure (Medeiros *et al.*, 1984; Wu *et al.*, 1984). Evaluation of the myocardium in copper deficiency reveals that the heart enlargement is largely due to increased volume density of mitochondria, leading to the concentric pattern (Mao *et al.*, 1998).

Electron microscopy studies demonstrated that copper-deficient myocardium also had increased deposits of lipids and glycogen granules (Davidson *et al.*, 1992; Medeiros *et al.*, 1991a). More significant, in addition to mitochondrial proliferation, there was a change in the pathology of the mitochondria. Mitochondria from copper-deficient animals are fragmented, have vacuoles, and the cristae have lost their usual parallel arrays. These pathologies have led us to study biochemical changes in the heart, particularly the mitochondria.

BIOCHEMICAL AND MOLECULAR ASPECTS

While electron microscopy studies reported changes in some myofibrillar components, the major myofibrillar proteins did not appear to be affected by copper deficiency using gel electrophoresis (McCormick *et al.*, 1989). However, our lab discovered that among the nonmyofibrillar portion of hearts from copper-deficient rats, there were low molecular weight peptides that appeared to be diminished or absent, most notably peptides in the 23 and 16-K range (McCormick *et al.*, 1989). Potential candidate peptides considered were those that comprise CCO since it is a known cuproenzyme. Western blot analysis of the nonmyofibrillar proteins revealed that in the copper-deficient models, all of the nuclear encoded peptides of CCO were markedly diminished and the higher molecular weight mitochondrial encoded subunits were unaffected. The CCO complex consists of 13 peptide subunits, the largest three contain copper and the catalytic site for electron transfer and are mitochondrially encoded. The nuclear encoded subunits are thought to be regulatory, but the real function is obscure as the enzyme can still function without the nuclear encoded subunits present. It was apparent that the 23-K subunit that was diminished in the gels was Subunit IV of CCO and confirmed by Western blot analysis (Medeiros *et al.*, 1993). However, the 16-K subunit did not correspond to any of the other known molecular weights of the nuclear encoded subunits of CCO. Peptide sequencing of the 16 K found 80% homology to the bovine δ subunit of ATP synthase (Medeiros *et al.*, 1993). These findings were the first suggestion that copper deficiency could impact an enzyme other than CCO in oxidative phosphorylation. Questions arose as to whether there were other changes in ATP synthase as a function of copper deficiency; were the changes secondary to cardiac hypertrophy and/or mitochondrial pathology and proliferation; or were the changes in response to an alteration in CCO function. To get a better idea of the various scenarios, what follows is a review of ATP synthase structure and function, mitochondrial respiration and nucleotide pools in copper deficiency; and molecular signals that regulate mitochondrial proliferation and may be impacted by changes in ATP synthase.

MITOCHONDRIAL ATP SYNTHASE

Mitochondrial ATP synthase is the terminal enzyme in oxidative phosphorylation and produces the energy,
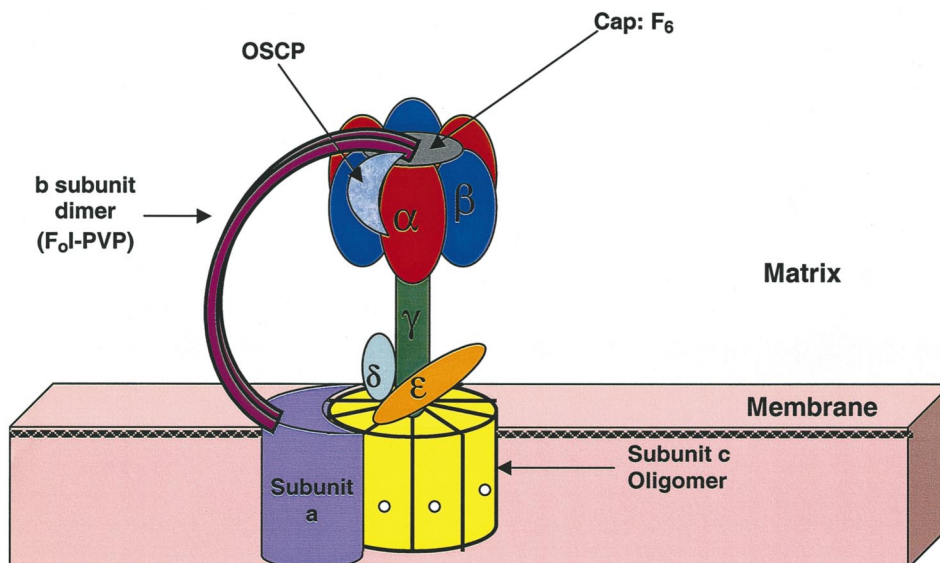
Fig. 1. Diagram of our current understanding of the structure of mitochondrial ATP synthase. The F_1 subunit is comprised of the α, β, δ, ε, and γ subunits. All of the other subunits are assigned to the F_0 subunit. See text for details. (Adapted from Papa *et al.*, 2000; Pedersen *et al.*, 2000a,b.)

ATP for the cell. It consists of two major units, F_1 in the matrix, which binds ADP/ATP and catalyzes ATP synthesis and hydrolysis, and F_0, which spans the inner mitochondrial membrane and directs protons to the F_1 moiety. As a result of this composition this enzyme is often referred to as F_1F_0 ATP synthase. Figure 1 shows the proposed structure of ATP synthase (Papa *et al.*, 2000; Pedersen *et al.*, 2000a,b). F_1 is made up of the three α subunits, three β subunits, and one subunit each of γ, δ, and ε all of which are nuclear encoded. F_0 also contains multiple subunits including an oligomycin sensitivity conferring protein (OSCP), 10–12 c subunits involved in gating of the proton channel (Saraste, 1999), an a subunit that facilitates transfer of protons from the inter membrane space to the c subunits, two b (FoI-PVP) subunits that are thought to function as stators between F_1 and F_0 and two F_6 subunits that also mediate sensitivity to oligomycin. Also present in F_0 are d, e, f, and g subunits that are found in close association with the OSCP subunit (Belongrundov *et al.*, 1996).

F_1F_0 ATPase is able to catalyze both ATP synthesis and hydrolysis. The switch between these two activities is dependent on the proton gradient between the matrix and the inter membrane space. ATPase can use the energy from ATP hydrolysis to pump H^+ into the intermembrane space or if a large enough proton gradient exists, use the proton flow through the complex (from inter membrane space to matrix) to drive ATP synthesis.

The 10–12 c subunits form a ring at the base of the γ subunit and the pumping of protons by the a subunit to the c ring and is thought to result in the turning of both the c ring and the entire F_1 complex. This rotation is believed to provide the energy for the synthesis of ATP by the F_1 complex. Tsunoda *et al.* (2001) showed that the ε subunit can exist in two conformations in the F_1 complex. When the ε subunit is perpendicular to the inner membrane, ATP hydrolysis is inhibited, proton pumping is inhibited, but ATP synthesis is not. On the other hand, when the ε subunit is parallel to the inner membrane, ATP hydrolysis is activated and proton pumping functions normally, but there is no effect on ATP synthesis. This data indicates that the ε subunit may be important in the regulation of switching between ATP synthesis and ATP hydrolysis.

ATP synthase is also regulated by a natural protein inhibitor that minimizes futile ATP hydrolysis in de-energized mitochondria. This inhibitor (IF_1) is found in 1:1 stoicheometry with the enzyme and has been shown to bind to one of the β subunits (Klein *et al.*, 1980) and inhibit ATP hydrolysis. IF_1 may function by affecting the flow of protons though F_0 (Guerrieri *et al.*, 1987) and thereby modify the kinetics of ATP synthase such that the rate of ATP hydrolysis is diminished (Gomez-Puyou *et al.*, 1983). There is some evidence to suggest that Cu and/or Zn may prevent the inactivation of ATP synthase by IF_1 (Bronnikov *et al.*, 1990).

Oligomycin is also a specific inhibitor of ATP synthase. It binds to the membrane portion of the F_0 complex of the enzyme and inhibits proton translocation (Hopp *et al.*, 1986). Sensitivity of ATP synthase to oligomycin

appears to be mediated not only by the OSCP but also by the F_6 subunit and the F_0I-PVP(b) subunits (Guerrieri *et al.*, 1991; Papa *et al.*, 1999; Zanotti *et al.*, 1988). OSCP is found in close association with the α and β subunits of F_1, with which it crosslinks (Belogrudov *et al.*, 1995), and is nearby some F_0 components of the stalk (Archinard *et al.*, 1986).

MITOCHONDRIAL BIOGENESIS

Mitochondria have a high degree of genetic and metabolic autonomy. Mammalian mitochondrial DNA is a small double standard circular molecule located in the mitochondrial matrix. The mtDNA is strictly maternally inherited, does not recombine, and has a high incidence of spontaneous mutations (Marin-Garcia and Goldenthal, 1997). Thirteen respiratory chain polypeptides, 2 ribosomal RNAs, and 22 transfer RNAs required for translation are encoded in mtDNA. However, the majority of respiratory subunits and all of the proteins required for expression, maintenance, and replication of mitochondrial DNA are nuclear encoded (Gugneja *et al.*, 1995). Consequently, normal mitochondrial respiratory function and mitochondrial biogenesis are under the dual control of mitochondrial and nuclear genomes.

Mitochondrial transcription factor A (mtTFA) is the major transcription factor governing mitochondrial DNA replication and transcription during mitochondrial biogenesis (Vibasius and Scarpulla, 1994). Low levels of mtTFA transcript and protein are associated with overall decreased mitochondrial gene transcription in HeLa cells (Virbasius and Scarpulla, 1994). On the other hand, expression of human mtTFA in *Saccharomyces cerevisiae* devoid of mtTFA, restores mitochondrial DNA transcription and function (Parisi *et al.*, 1993). Functional human mtTFA is a 25-kDa protein, which specifically recognizes and binds mtDNA sequence from 12 to 39 bp upstream of the transcription initiation site in the light-strand and heavy-strand promoters (Dairaghi *et al.*, 1995; Montoya *et al.*, 1997). This binding is an absolute requirement for transcriptional activation of the two promoters located in the displacement-loop region of human mtTFA. Transcriptional activation initiates the synthesis of mitochondrial RNAs by mitochondrial RNA polymerase (Dairaghi *et al.*, 1995). Thus, mtTFA is also involved in the replication and maintenance of mtDNA by facilitating the synthesis of heavy strand replication primers (Dairaghi *et al.*, 1995; Gopalakrishnan and Scarpulla, 1995).

The investigation of nuclear control of mitochondrial gene expression has lead to the discovery of several other important transcription factors. Nuclear Respiratory Factor-1 (NRF-1) is one of the candidates that may coordinate nuclear encoded respiratory chain expression with mitochondrial gene transcription and replication. Functional NFR-1 binding activity resides in a single polypeptide of 68 kDa (Chau *et al.*, 1992). NRF-1 recognition sites have been found in many genes encoding respiratory functional subunits, such as rat CCO subunit VI*c* and the bovine ATP synthase γ subunit. A subunit of human mitochondrial RNA processing endonuclease (MRP RNA) and human mtTFA genes also contain NRF-1 binding sites (Virbasius *et al.*, 1993). Therefore, NRF-1 may activate mitochondrial gene expression by upregulating mtTFA.

Another nuclear gene product, NRF-2, has also been implicated in the coordination between nuclear and mitochondrial gene expression. Purified NRF-2 contains 5 subunits. The 56-kDa subunit of NRF-2 can bind DNA with a GGAA/T motif (Gugneja *et al.*, 1995). Although the majority of genes encoding proteins in respiratory functions have a NRF-1 recognition site, some genes, such as CCO subunit IV and ATP synthase β subunit, lack a NRF-1 recognition site but contain a NRF-2 recognition site indicationg that these respiratory chain genes may be differentially regulated (Villena *et al.*, 1998). In some genes, both NRF-1 and NRF-2 recognition sites have been identified, such as mtTFA, although the promoter is much less dependent on NRF-2 compred to NRF-1 (Gugneja *et al.*, 1995; Vibasius and Scarpulla, 1994). In any case, it is apparent that NRF-1 and NRF-2 may convey nuclear regulatory events to the mitochondria via mtTFA, and coordinate the gene expression between the nuclear and mitochondrial genomes. A summary of all these events and how they may affect mitochondrial biogenesis is given in Fig. 2.

LINKAGES BETWEEN COPPER DEFICIENCY AND ATP SYNTHASE, CYTOCHROME *c* OXIDASE AND MITCHONDRIAL BIOGENESIS

Rusinko and Prohaska (1985) reported that despite a marked decreased in cyotchrome *c* oxidase activity in the copper-deficient state, ATP levels did not appear to be altered and similar energy charges between copper-adequate and copper-deficient rats' hearts were reported. A subsequent study by Chao *et al.* (1993) reported similar findings regarding ATP levels and energy charge. In the latter study, in addition to ATP levels, nucleotide pools were evaluated. There was significantly greater NAD levels and the sum of nucleotides was greater in hearts from copper-deficient rats. These increases could be due to the increased mitochondrial volume observed in copper-deficient rats. Phosphocreatine levels were also significantly higher in copper-deficient rats, which could explain
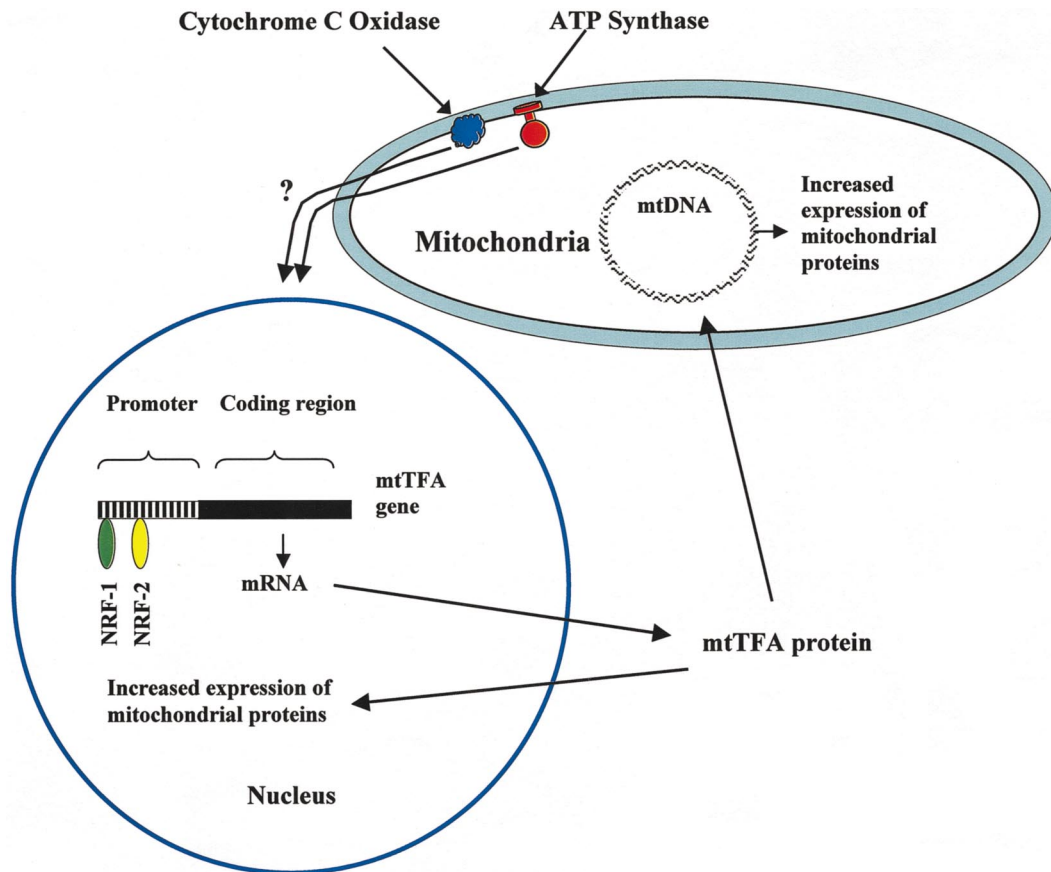
Fig. 2. Diagram of nuclear control of mitochondrial biogenesis via mtTFA. NRF-1 and NRF-2 can bind to the promoter regions of mtTFA as well as to promoters of some of the subunits comprising cytochrome *c* oxidase and ATP synthase. mtTFA can bind to promoters of nuclear genes that encode mitochondrial proteins as well as bind to mtDNA to increase DNA replication as well as to enhance expression of mitochondrial proteins. Defects in activity or function of either cytochrome *c* oxidase or ATP synthase, and/or ATP levels may feedback to the nucleus to upregulate mtTFA, NRF-1, and NRF-2. See text for details.

how ATP levels were maintained. Cardiac mitochondrial function was also evaluated by Chao *et al.* (1993). State 3 respiration and respiratory control ratios, an indication of coupling, were significantly depressed in copper-deficient hearts compared to controls. ADP:O levels, however, did not differ by group. Later we (Chao *et al.*, 1994) reported that the F_1F_0 synthase δ subunit protein level was decresed in hearts from copper-deficient rats. These two reports were the first to suggest that ATP synthase may be involved in the pathologies associated with copper deficiency. A later study by Matz *et al.* (1995) reported decreases in both State 3 and State 4 respiration in mitochondria from copper-deficient rats, but no significant difference in respiratory control ratios or ADP:O, using a variety of substrates to verify their results. Furthermore, they reported that in cardiac mitochondria from copper-deficient rats, oligomycin, which inhibits ATP synthase activity, had less

inhibitory effect compared to controls. There was no difference in the uncoupling ability of 2,4-dinitrophenol between copper-deficient rats and controls. This suggested that while CCO activity may be dramatically decreased by copper deficiency, electron transport appeared to unaffected and that perhaps the alteration could be due to changes in ATP synthase. ATPase activity did not differ by dietary copper treatments, nor did 2,4-dinitrophenol addition alter ATPase activity. On the other hand, oligomycin, either in the presence or absence of 2,4-dinitrophenol, did result in greater ATPase activity in the copper-deficient group, which again suggested an incomplete inhibition of ATP synthase by oligomycin. These authors suggested that the function of the oligomycin-sensitive subunits in ATP synthase are altered in mitochondria from copper-deficient rats. New data from our lab support the findings of Matz *et al.* (1995) that oligomycin had less of an effect

upon mitochondrial respiration in the copper-deficient rats compared to controls (Chen *et al.*, in press). Chen *et al.* (in press) reported that State 3 oxygen consumption rates were significantly lower in copper-deficient mitochondria and the inhibitory effect to oligomycin was less than in controls. Since mitochondria from copper-deficient hearts often exhibit pathology, Chen *et al.* (in press) also checked oxygen consumption in the presence of NADH. If the mitochondria becomes leaky or permeable to substrates, such as NADH, oxygen consumption is dramatically increased. However, this was not the case, in fact oxygen consumption was lower in mitochondria from hearts of copper-deficient rats. These results suggested that the mitochondrial membrane was intact. The decrease in mitochondria respiration may also be reflective of the observation by Chen *et al.* (in press) that mitochondrial membrane potential was significantly decreased in copper deficiency. Mitochondria membrane potential is critical for facilitating the import of nuclear encoded and cytoplasmically synthesized proteins into the mitochondria.

The linkages of some of the subunits of CCO and ATP synthase with NRF-1 and NRF-2, prompted our group to evaluate expession of certain transcripts of ATP synthase subunits and potential changes in mtTFA, NRF-1, and NRF-2. Foremost, we found that mtTFA protein in hearts from copper-deficient rats was markedly increased compared to controls (Mao *et al.*, 2000). A subsequent study revealed that NRF-1 and NRF-2 were also upregulated (Mao and Medeiros, 2001). In addition we found increased expression of ATP synthase Subunit *c* in hearts from copper-deficient rats (Mao *et al.*, 2000), a key component in the proton pumping portion of ATP synthase. This subunit forms a ring structure in the F_0 sector, which functions as a proton channel in the mitochondrial inner membrane structure (Saraste, 1999). Molecular signals stimulating mitochondrial biogenesis may simultaneously upregulate expression of F_1F_0 ATP synthase Subunit *c*. This is supported by a previous finding in our laboratory that Subunit *e* mRNA of the F_0 sector was increased in the hearts of copper-deficient rats compared with controls.

Our group also reported that in hearts of copper-deficient rats, the ATP synthase β subunit mRNA was markedly depressed. The β subunit is the catalytic site for the ATPase reaction (Pedersen and Amzel, 1993). The subunit δ, which also decreased in copper-deficient rat hearts, is important for the structural stability of the F_1 sector (Giraud and Velours, 1997; Pan *et al.*, 1998). The lower mRNA levels for the β subunit may be the result of adaptation to the decrease peptide of the δ subunit in hearts from copper-deficient rats. Another possible explanation for the change in the β mRNA may be a potential influence that thyroid hormone has upon the mammalian

β subunit gene promoter (Martin *et al.*, 1996), which can increase expression of the transcript. Decreased thyroid hormone levels have been reported in copper-deficient rats (Lukaski *et al.*, 1995) and may indirectly decrease the β subunit gene expression. Also, a decrease in the activity of CCO activity may provide a feed-forward mechanism to reduce β subunit gene expression and conserve ATP.

SUMMARY

Indirect evidence suggests that copper may be an important part in the function of ATP synthase. This evidence stems from observations in animals made copper-deficient from a dietary means and subsequent studies on intact mitochondria and changes in peptide and message levels of ATP synthase. Decreased respiration and decreased sensitivity to oligomycin across several studies in copper deficiency implicates a potential role for copper in regard to ATP synthase. Impaired ATP synthase function may be responsible for the observed mitochondrial biogenesis in hearts from copper-deficient animals and the transcription factors that appear upregulated as a result. The precise binding site of oligomycin to ATP synthase could help unravel the greater resistance to oligomycin inhibition in copper deficiency.

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