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Antioxidant Action of Mobile Electron Carriers of the Respiratory Chain

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1. Introduction

1.1 Evolutionary aspects

Both oxidative photophosphorylation and oxidative phosphorylation are dependent on electron transport chains sharing similarities that are suggestive of evolution of a chemolithotrophy-based common ancestor (conversion hypothesis). Therefore, an early form of electron transport chain with oxidative phosphorylation that is known as prerespitation was able of donating electrons to terminal acceptors available in the primitive reducing biosphere. In the evolutionary pathway this apparatus was supplemented by a photocatalyst capable of a redox reaction. Therefore, oxygenic photosynthesis was a late event during evolution that was preceded by anoxygenic photosynthesis. The development of the manganese complex able to promote water oxidation was a key event in developing oxygenic photosynthesis (Xiong & Bauer, 2002; Bennown, 1982; Castresanal et al., 1994).

The development of oxygenic photosynthesis was one of the most important events in the biological evolution because it changed the redox balance on Earth and created conditions for the biological evolution to more complex life forms. Molecular data showing cytochrome oxidase in the common ancestor of Archaea and Bacteria and an existing cytochrome oxidase in nitrogen-fixing bacteria living in an environment where the level of oxygen was very low are indicia that aerobic metabolism could be present in an ancient organism, prior to the appearance of eubacterial oxygenic photosynthetic organisms. Although the hypothesis that aerobic metabolism arose several times in evolution after oxygenic photosynthesis is not sustained by the above mentioned data, the widespread use of molecular oxygen as final acceptor of electrons resulting from the oxidation of biological fuels was an evolutionary acquisition subsequent to the oxygen photosynthesis. The use of molecular oxygen as final acceptor of electrons removed from biological fuels resulted in a significant improvement of energy yield, a crucial event for the rise of complex heterotrophic organisms. According to the endosymbiotic theory, the respiratory chain present in prokaryotes was transferred to eukaryotes and resulted in cells bearing mitochondria. At the present step of the biological evolution, the aerobic oxidation of biological fuels occurs in the respiratory chain apparatus of the cell membrane of

prokaryotes and in the inner mitochondrial membrane of eukaryotes (Xiong & Bauer, 2002; Bennown, 1982; Castresanal et al., 1994).

Figure 1 illustrates the more recent view of the evolution pathway of electron chain transport correlated to the arising of more complex living organisms.

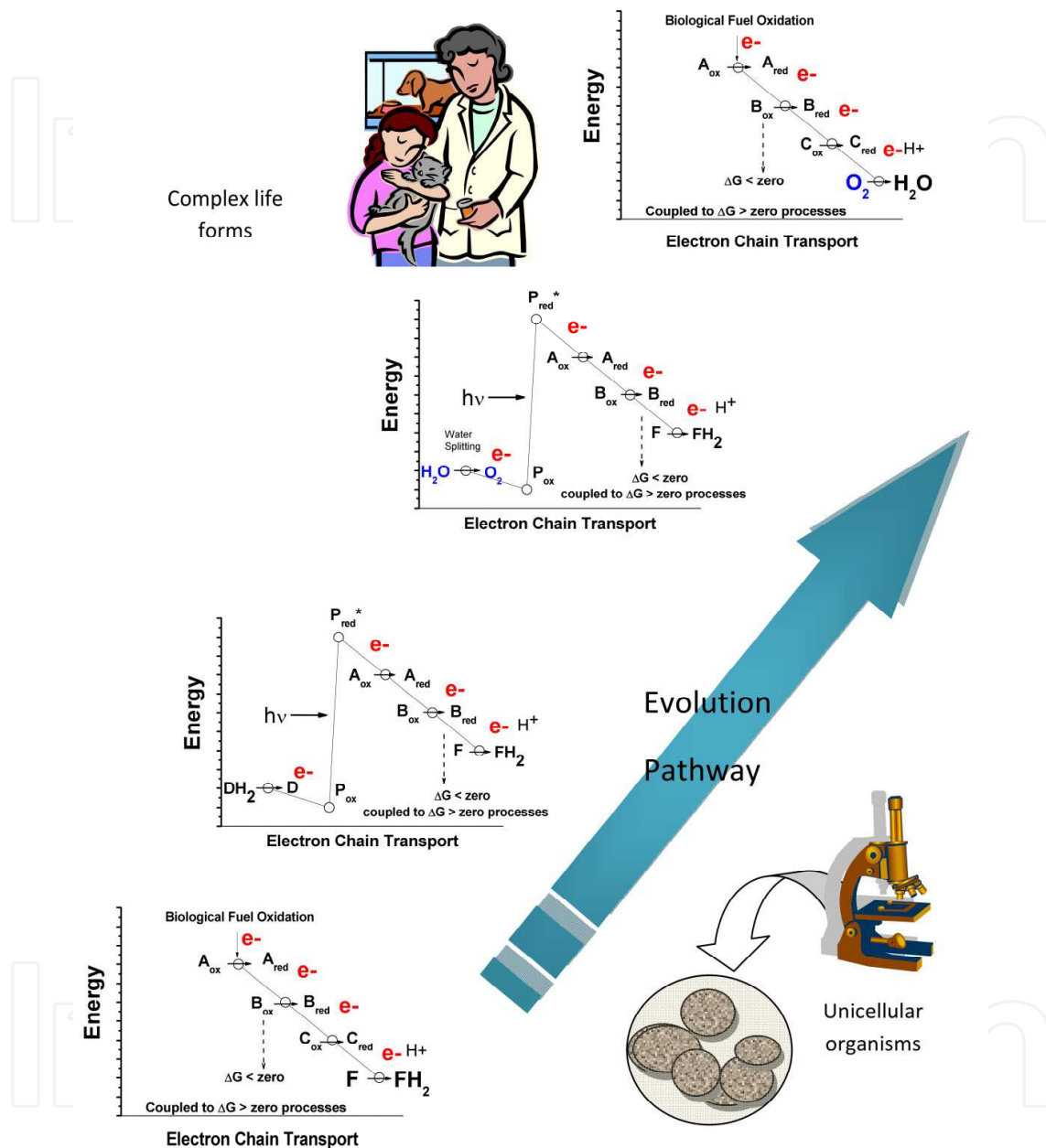


Fig. 1. Evolution pathway of electron transport chain that created conditions for the arising of more complex life forms. A, B and C are representative intermediates in a generic electron transport chain. F represents an electron final acceptor that made feasible the electron chain transport in the primitive reductive atmosphere. P represents a photocatalyst pigment responsible for light harvesting in non-oxygenic and oxygenic photophosphorylation. D represents the electron donor in the non-oxygenic photophosphorylation. The oxygenic photophosphorylation was not represented as Z scheme for clarity. The energy scale is arbitrary. ATP synthesis (phosphorylation) are among the $\Delta G > \text{zero}$ processes coupled to electron transport chains.

1.2 Respiratory chain

The oxidation of biological fuels such as glucose, lipids and amino acids proceeds by the electron transfer to coenzymes NAD^+ and FAD . These metabolic pathways such as glycolysis, citric acid cycle and β -oxidation of fatty acids are totally dependent on the continuous recycling of NADH and FADH_2 coenzymes to the oxidized forms. In aerobic organisms, the recycling of NADH and FADH_2 was done by the electron transfer to respiratory protein complexes I and II, respectively. In the following, electrons are transported through a sequence of redox centers, most of them composed by heme proteins that are known as respiratory cytochromes (Hatefi, 1985; Nantes & Mugnol, 2008). Similarly to the mechanism operating in the photosynthetic apparatus, spontaneous electron transfer through respiratory chain complexes is coupled to proton ejection from the matrix to the intermembrane space resulting in the proton motive force (Eq. 1).

$$\Delta p = \Delta \psi - 60\Delta pH \quad (1)$$

Δp supports ATP synthesis and other energy requiring processes in mitochondria such as ion transport and transhydrogenation. ATP synthesis is done by the enzyme ATP synthase that encloses a membrane extrinsic F_1 and a transmembrane F_0 subunits (Solaini et al, 2002; Zanzami et al, 2007).

As described above, the respiratory chain comprises proteins assembled as supramolecular complexes; most of them are composed by integral proteins inserted in the inner mitochondrial membrane. This redox system encompasses four complexes: NADH :ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinol:ferricytochrome c oxidoreductase (complex III), ferrocyanochrome c: oxygen oxidoreductase (complex IV) that assembled with ATP synthase constitute the so-called respirasome (Hatefi, 1985; Nantes & Mugnol, 2008; Duchen, 1999; O'Reilly, 2003; Wittig et al, 2006; Fernandez-Vizarra et al, 2009 and Dudkina et al, 2008), (Fig. 2). However, the electron transport among the respiratory complexes is mediated by two mobile electrons carriers: coenzyme Q (CoQ) and cytochrome c.

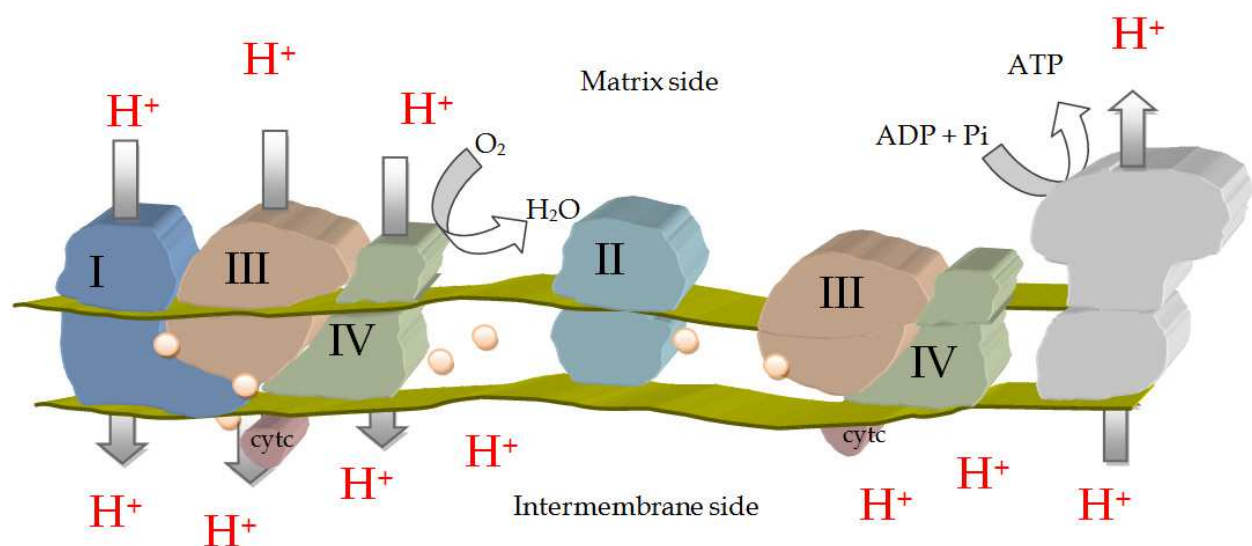


Fig. 2. Pictorial representation of a coupled respiratory chain with respiratory components assembled as respirasomes. CoQ is represented as yellow shadowed spheres.

1.2.1 Fundamental concepts about CoQ

The structure of CoQ was determined by Wolf et al. in 1958. The compound is a 2,3-dimethoxy-5-methylbenzoquinone with the redox active benzoquinone ring connected to a long isoprenoid side chain. According to the isoprenoid chain, five quinones are designated as members of a coenzyme Q group, i.e., CoQ6, CoQ7, CoQ8, CoQ9 and CoQ10 (Fig. 3). Ubiquinol is the product of two-electron reduction of ubiquinone with an ubisemiquinone intermediary form (Fig. 3). The predominant form of ubiquinone in humans presents 10 isoprenoid units in the side chain and it is referred as coenzyme Q₁₀ (CoQ₁₀) or ubiquinone-10. The first studies about coenzyme Q were published in the end 50's with the isolation of a beef heart quinone (Crane et al., 1957) and sequential studies on its redox properties (Moore, 1959; Gale et al, 1963).

The hydrophobicity of this coenzyme results in its partition into the lipid bilayer (Littarru & Tiano, 2007).

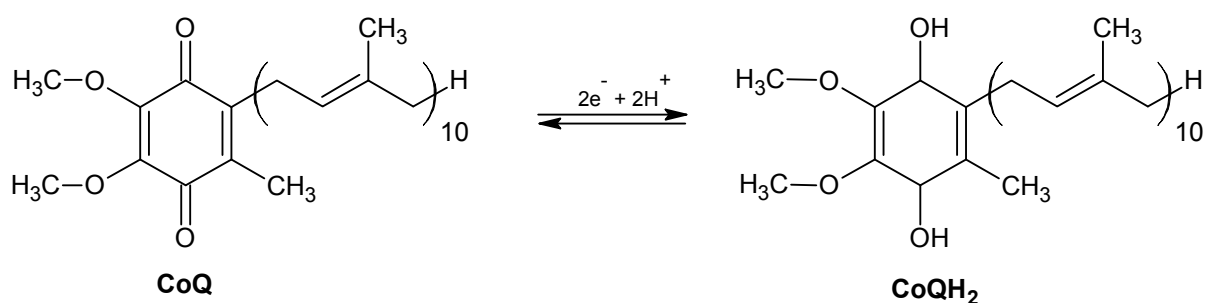


Fig. 3. Chemical structure coenzyme Q in its oxidized (CoQ) and reduced (CoQH₂) forms.

CoQ₁₀ is found in almost all cellular membranes as those of Golgi apparatus and lysosomes. In the inner mitochondrial membrane, CoQ carries electrons from complexes I and II to *bc1* complex but its participation in the respiratory chain involves a redox cycle that also contributes to the generation of the proton motive force. The CoQ redox cycle involves the interaction of the coenzyme with of the *bc1* complex. Several studies are concerned about the mechanism of proton translocation through the cytochrome *bc1* complex related to CoQ cycle and the function of individual subunits of the enzyme in the energy transduction process. Unlike the electron transfer pathway through the *bc1* complex, there is not a consensus on the mechanism that couples the electron transfer to a transmembrane proton electrochemical potential. Two mechanisms of proton translocation by respiratory complexes have been described: the redox loop and the proton pump mechanism. The redox loop mechanism was the mechanism proposed by Mitchell, (1966). This mechanism requires concomitant acceptance of protons from the matrix side followed by proton release at the intermembrane space associated to the redox changes of some respiratory redox centers. The proton pump mechanism requires that the reduction and re-oxidation of protein redox centers would be accompanied by changes in the conformation of proteins with consequent alterations of the pK_a of amino acid side chains and leading to the exposure of these residues alternately at the internal and external side of the membrane (Erecinska, 1982; Trumpower, 1990; Boyer, 1993). Considering exclusively a redox loop mechanism, the CoQ molecules solved inside the membrane lipid fraction are converted to the completely reduced form (CoQH₂) by Complex I or II and the high potential *b562* of Complex III. This process is accompanied by the uptake of two protons from the mitochondrial matrix. The reduction occurs in two steps and consequently semiquinone is generated as intermediate. The

reoxidation of CoQH_2 results from one electron transfer to cytochrome *c*1 via the iron sulfur protein (ISP) and one electron transfer to heme *b*566 that recycles it to heme *b*562 that reinitiates the cycle by transferring one electron to oxidized CoQ. The oxidation of CoQH_2 releases two protons in the intermembrane space. The ratio H^+ /electron transferred to cytochrome *c*1 and consequently to molecular oxygen is 2/1 (Figure 4).

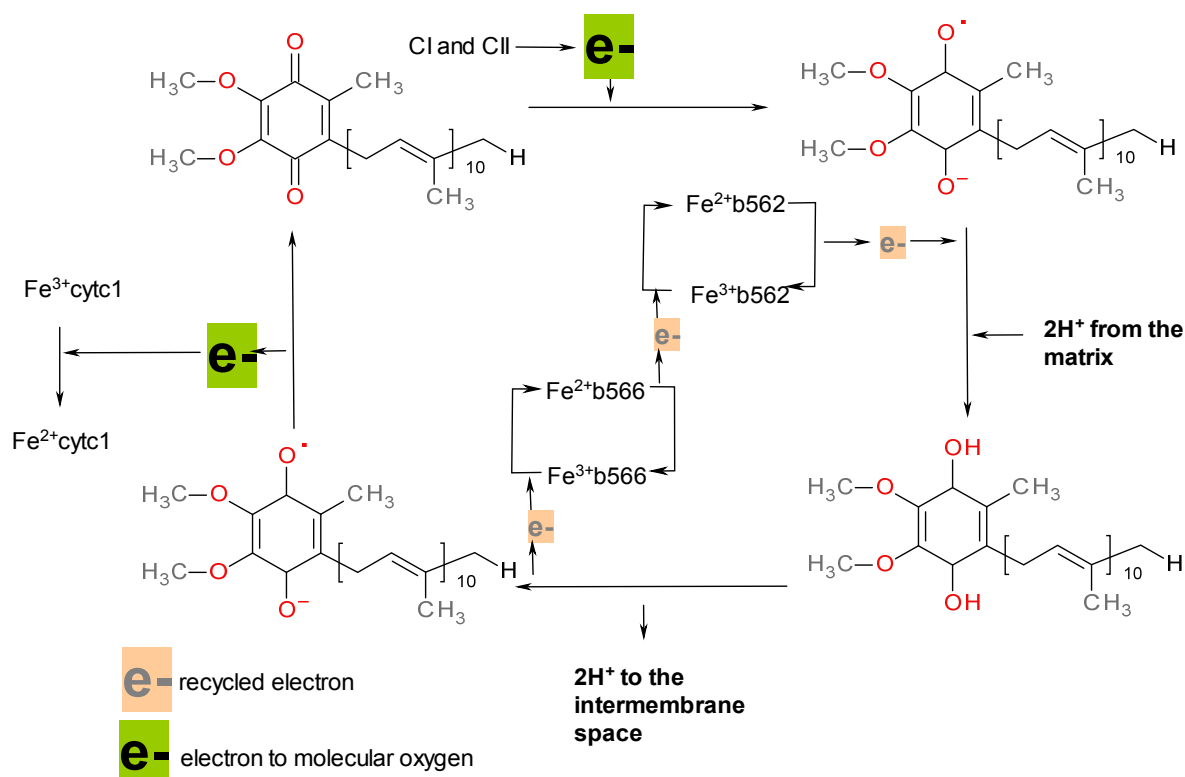


Fig. 4. Coenzyme Q cycle.

Besides the participation in the respiratory chain, literature data has reported, several other important functions of CoQ. The functions include participation in the uncoupling of oxidative phosphorylation and production of heat (Echtay et al., 2001), signaling for gene expression (Doring et al., 2007, Chew et al., 2007) and antioxidant activity (Gomez-Diaz et al., 1997, Papucci et al., 2003). This latter role is the focus of the present chapter and it will be discussed herein.

1.2.2 Fundamental concepts about cytochrome *c*

Respiratory cytochrome *c* is a nuclear-encoded protein located at the external side of the inner mitochondrial membrane. In mammals this protein contains 104 amino acids and a single heme group covalently bound to the protein and with a reduction potential of +260 mV. Unlike the other respiratory cytochromes that assembled in large and membrane bound complexes, cytochrome *c* is a small peripheral protein located at the external side of the inner mitochondrial membrane. Thus, cytochrome *c* is considered a diffusible carrier with pool function in the aqueous phase.

Cytochrome *c* is a basic protein bearing 19 lysine and 5 arginine residues giving a highly positively charged with $\text{pI} = 9.6$ and conferring to this protein a high affinity to acidic phospholipids such as cardiolipin, a lipid component of the inner mitochondrial membrane.

The electrostatic interaction is an important factor for the association of cytochrome *c* with phospholipid membranes and has been focus of several studies. (Rytömaa, 1995, Tuominen, 2002 Zucchi, 2003). The interaction of cytochrome *c* with acidic phospholipids involves both electrostatic and lipid extended interactions, the latter resulting from the insertion of one phospholipid chain in a hydrophobic channel, present in the cytochrome *c* structure, in the region of the heme crevice. Other important aspect about the interaction of cytochrome *c* with the inner mitochondrial membrane is the existence of two membrane binding sites in the cytochrome *c* structure (Kawai et al, 2005, Kawai, 2009).

The biological role of cytochrome is beyond the cell respiration and involves also apoptosis and redox cell balance (Yong-Ling et al, 2008, Huttemann et al, 2011). Cytochrome *c* participates in the mitochondrial electron-transport chain as a mobile electron carrier that shuttles electrons between cytochrome *c*1 of Complex III and cytochrome *c* oxidase. The reduction of molecular oxygen to water catalyzed by cytochrome *c* via complex IV has a $\Delta G^{\circ} = -100$ kJ/mol that is around twice higher as compared to the redox reactions catalyzed by complexes I and III (Hinkle et al., 1991)

Besides the participation in the respiratory chain, cytochrome *c* is a key protein for the intrinsic pathway of apoptosis triggered by some stimulus such as DNA damage, metabolic stress or the presence of unfolded proteins (Yong-Ling et al., 2008; Huttemann et al., 2011). The participation of cytochrome *c* in apoptosis is dependent on its detachment from the inner mitochondrial membrane followed by its translocation through the outer mitochondrial membrane to attain the cytosol. In the cytosol, cytochrome *c* engages the Apoptotic protease-activating factor-1 (APAF1), and composes the apoptosome (Liu et al., 1996; Kluck et al., 1996; Kluck et al., 1997, Yang et al., 1997).

2. Generation of reactive species in mitochondria

The use of molecular oxygen as final acceptor of electrons removed from the biological fuels was an evolutionary acquisition that resulted in a significant improvement of the energy yield, a crucial event for the rise of complex organisms. However, a low percentage of molecular oxygen consumed in mitochondrial respiratory chain is not completely reduced to water generating reactive oxygen species (ROS). Mitochondria clearly represent a primary source of ROS in most aerobic mammalian cells (Turrens et al., 1985). The mitochondrial generation of ROS occurs at electron transport chain as a secondary product of mitochondrial respiratory chain (Murphy, 2009). The primary ROS produced in this process is the superoxide anion ($O_2^{\bullet-}$) resulted from a single electron reduction of molecular oxygen by the electrons leaked from the substrate of respiratory chain. In the mitochondrial matrix a superoxide dismutase (MnSOD) transforms superoxide into a more stable form: hydrogen peroxide (H_2O_2). The rate of H_2O_2 production in isolated mitochondria when in state 4 of respiration is 0.6–1.0 nmol/mg mitochondrial protein/min. (Turrens et al., 1985) but this range was considered over estimated and the superoxide production in normal respiring mitochondria could be around 0.1 nM H_2O_2 /mg mitochondrial protein/min. Besides the components of the respiratory chain, other mitochondrial complexes also can generate superoxide, such as: dihydrolipoamide dehydrogenase-containing FAD-linked pyruvate, α -ketoglutarate dehydrogenase complexes (Starkov et al., 2004), as well as the flavoenzymes α -glycerophosphate dehydrogenase. In the respiratory chain, two sites have been found to be responsible for the vectorial generation of $O_2^{\bullet-}$. Mitochondria respiring with complex I/III substrates release superoxide anion into the matrix while complex II/III

substrates release superoxide anion into the intermembrane space. In complex I the production of $O_2^{\bullet-}$ probably occurs via autooxidation of the reduced flavin mononucleotide (Turrens & Boveris, 1980) and in complex III the partial reduction of molecular oxygen to $O_2^{\bullet-}$ occurs in the Q-cycle via the semiquinone intermediate (Zhang et al., 1998). The vectorial synthesis of superoxide anion indicates that the resultant H_2O_2 formed can act as a mitochondrial second messenger for both nuclear and mitochondrial genomes. This signaling system could be a requirement for appropriate nuclear and mitochondrial gene expression and metabolome modulation. By this point view, the prooxidant formation of the $O_2^{\bullet-}/H_2O_2$ second messenger system is essential for the normal physiological function of the metabolome and the random molecular damage promoted by $O_2^{\bullet-}/H_2O_2$ has been rebutted. However, the physiological function of reactive species is dependent of a fine regulation mechanism warranted by the balance between the generation and decomposition of ROS. Oxidative stress occurs when cells have an imbalance of production and decomposition of ROS that results in damages of biomolecules such as lipids, DNA and proteins. Therefore, H_2O_2 may generate the hydroxyl radical (HO^{\bullet}), the most reactive and damaging oxygen species, through the Fenton reaction catalyzed by transition metals (Halliwell & Gutteridge, 1990, Eq. 2).



Hydroxyl radical can also be generated in the Haber-Weiss reaction with the superoxide radical as shown in Eq. 3 (Valko et al., 2004)



Superoxide ion can also contribute for the generation of hydroxyl radical by recycling Fe^{3+} to Fe^{2+} (Eq. 4)



Mitochondria is also a source of reactive nitrogen species (RNS) derived also from a signaling molecule, the nitric oxide (NO^{\bullet}) (Moncada & Higgs, 1993; Denninger, 1999, Radi et al., 2002). Nitric oxide is generated by the family of nitric oxide synthases (NOS). The NOS family synthesizes NO^{\bullet} using L-arginine as a substrate and NADPH as reducing agent and the reaction is favored by the presence of Ca^{2+} ions and sulfhydryl groups.

Cells of the immune system produce both the superoxide anion and nitric oxide during the oxidative burst triggered by inflammation processes.

Under these conditions, nitric oxide and the superoxide anion may react to produce significant amounts of a highly oxidative molecule, peroxynitrite anion ($ONOO^-$), able to promote DNA fragmentation, lipid oxidation and protein nitrosylation (Valko et al., 2007; Ghafourifar & Cadenas, 2005). Reaction (5) has one of the highest rate constants known for reactions of NO^{\bullet} . Thus, NO^{\bullet} toxicity is linked to its ability to combine with superoxide anion.



To assure the cell redox balance, the evolutionary acquisition of aerobic O_2 -dependent metabolism was accompanied by a highly conserved antioxidant enzymatic apparatus that works in a concerted way to promote decomposition of $O_2^{\bullet-}$ (Cu-Zn/Mn superoxide

dismutase and cytochrome *c*) and H_2O_2 (catalase and glutathione peroxidase) as well as to repair oxidative damage of proteins (thioredoxine, glutaredoxine, thioredoxine reductase and others). In addition to the enzymatic apparatus, low molecular molecules contribute also to the redox cell balance by acting as free radical trapping. CoQ is included among these antioxidant molecules and, unlike cytochrome *c*, its action is not restricted to the mitochondria.

The present chapter is concerned with the state of art of the more recent findings about the antioxidant role played by the two mobile electron carriers present in the mitochondrial respiratory chain of higher organism cells: CoQ and cytochrome *c* and these findings are described herein.

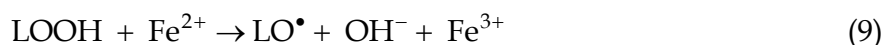
3. Antioxidant properties of CoQ and cytochrome *c*

3.1 CoQ

Antioxidants are molecules able to inhibit the oxidation of other molecules by eliminating free radicals or by decreasing their formation. In biological systems the high effectiveness of antioxidant system is fundamental due to the constant generation of free radicals inside the organism at several sites that potentially may cause oxidative damage and consequently loss of function of protein, lipids and nucleic acids (Halliwell & Gutteridge, 2007). The effectiveness of antioxidants against oxidative damage in biological environments is directly related to their chemical structure.

The role of CoQ₁₀ in biological energy conversions as a redox component of the mitochondrial electron transport chain is well-described. Despite its ability to generate free radicals acting as pro-oxidant, as discussed before, Mellors & Tappel (1966) proposed an antioxidant role for CoQ showing that the reduced and oxidized forms of CoQ were able to prevent heme-catalyzed lipid peroxidation. Up today an increasing number of works has been conducted to understand the mechanisms of CoQ antioxidant action and the *in vivo* situations in which this property is achieved.

Due to the relatively high hydrophobicity, CoQ is partitioned into the lipid bilayers and can play the antioxidant role toward the impairment of lipid oxidation. Impairment of lipid oxidation is biologically important to the maintenance of the membrane integrity and to prevent the oxidation of lipoproteins (Ingold et al, 1993). Lipid oxidation is an oxidative chain reaction that is triggered by the abstraction of a hydrogen atom (H^\bullet) from allylic carbon of an unsaturated chain of a phospholipid (LH) by reactive species such as hydroxyl radical ($^\bullet OH$) resulting in a carbon centered free radical (L^\bullet). After intramolecular rearrangement L^\bullet may react with another LH or with molecular oxygen generating peroxy radicals (LOO^\bullet) that react with another LH resulting in lipid hydroperoxides (LOOH). Such lipid-derived peroxides suffer homolytic cleavage in the presence of metals in Fenton type reactions. The reactions involved in the initiation and propagation of the lipid peroxidation are summarized below.



Molecules that are able to react with these intermediate radical species to form less reactive radicals are considered chain breaking antioxidants, such as vitamin E. One of the mechanisms by which coenzyme Q exerts its antioxidant action inhibiting the lipid oxidation of membranes is by reacting directly with lipid-derived radicals transferring H[•] and generating the ubisemiquinone radical (CoQH[•]) as shown in Eq. 10.



Besides the inhibition of the lipid oxidation of membranes, CoQ also protects DNA from oxidation induced by H₂O₂ plus metal ions. This process seems to be important especially for mitochondrial DNA oxidation since such damage is not easily repaired (Bentinger, 2010). It was demonstrated in human lymphocytes that incubation with CoQ results in increase of resistance to H₂O₂ damage and less damage by exposure to oxygen (Litarru & Tiano, 2007). Endogenous CoQ also plays an important role in the protection of protein oxidation. The sensitivity of different proteins to oxidative stress varies to a great extent, depending on their structure, composition and localization (Bentinger et al, 2007) but the protective effect of CoQ is probably mediated by a direct scavenger mechanism. Also, the reactive ferrylmyoglobin formed by the reaction of myoglobin with H₂O₂ can use CoQH₂ to be converted to metmyoglobin and oxymyoglobin in a mechanism that allows the heme protein to neutralize peroxides that can be harmful to cells (Mordente et al, 1993; Guo et al, 2002; Litarru & Tiano, 2007).

Although several studies have compared the antioxidant efficiency of vitamin E *versus* CoQ, there is no other biological function described for vitamin E differently of CoQ that participates in the energetic metabolism. This raises some evolutionary questions addressed by Beyer (1994) whether the antioxidant role of CoQ is merely a coincidence of its structure or it was selected on the basis of the advantage to organism against oxidative stress. Furthermore, there is more CoQ than vitamin E in tissues and in mitochondria (Joshi et al, 1963; Mellors & Tappel, 1966; Ingold et al, 1993). Due to its hydrophobicity, the antioxidant efficiency of CoQH₂ is influenced by the polarity of the environment and it is dependent on the accessibility to the free radicals. In aqueous media, ubiquinol is only about 10% as effective as a chain breaking antioxidant like Vitamin E, possibly because the intramolecular hydrogen bonding between the hydroxyl and methoxy groups in CoQ, and also to the electron withdrawing inductive effects of the methoxy groups that stabilize the phenolate relative to the phenoxyl radical (Ingold et al, 1993). These considerations are important to the interpretation of the *in vitro* analysis of the antioxidant activity of CoQ in aqueous systems and apolar environments, such as liposomes and membranes of organelles and cells. In aqueous media, the reactivity of a free radical generated by oxidant agents is modulated by the proton concentration as a function of the pK_a of the radical group. Thus, the reactivity of CoQH[•] radicals generated by the reaction with lipid derived free radicals is lower within the phospholipid bilayer.

By using liposomes as membrane model, Frei et al (1990) showed that CoQH₂, but not its oxidized form CoQ, scavenged free radicals and inhibited lipid peroxidation with similar efficiency than vitamin E. Also, the simultaneous addition of CoQH₂ and vitamin E resulted in oxidation of the quinone sparing vitamin E. In this *in vitro* system, ascorbate or GSH were not able to recycle the oxidized CoQ (Frei et al, 1990). The antioxidant activity of the reduced form CoQH₂ is due to its behavior as a phenolic antioxidant inhibiting not only the lipid

peroxidation but also regenerating vitamin E, preventing DNA and protein oxidation, and reducing ferrylmyoglobin (James et al, 2004; Littarru & Tiano, 2007; Roginsky et al, 2009). Several groups have been concerned to study the mechanisms of the antioxidant action of CoQ. It was proposed that CoQH₂ inhibits lipid peroxidation by decreasing the production of lipid peroxy radicals (LOO[•]) and reducing perferryl radicals. CoQH₂ could eliminate LOO[•] directly acting as a primary scavenger of free radicals (Crane, 2001). Thus, CoQH₂ can exert its antioxidant action inhibiting lipid peroxidation directly by acting as a chain breaking antioxidant and indirectly by recycling vitamin E (James et al, 2004; Cuddihy et al, 2008). It was showed that α-tocopherol recycling in mitochondrial membranes is directly dependent on the CoQ/α-tocopherol molar ratio (Lass & Sohal, 2000) and that such recycle process also occurs in vivo (Lass et al, 1999).



CoQ significantly increases the rate of vitamin E regeneration in membranes a process also observed in low density lipoproteins, presumably by CoQ content present in the blood (Crane, 2001). Alternatively, reduced coenzyme Q could react directly with superoxide and hydroxyl radicals as a free radical scavenger and interfere with the initiation of lipid peroxidation (Beyer, 1990). Differently of others antioxidant compounds, CoQ inhibits both the initiation and propagation of lipid and protein oxidation. (Bentinger et al, 2010). In fact, it is probable that this antioxidant is considerably more efficient than that exhibited by vitamin E (Turunen et al, 2003). The reactivity of CoQ and vitamin E with different radicals, including the reaction rate constants, was reviewed by James et al (2004).

In mitochondria it is proposed that the respiratory chain enzymes and other dehydrogenases are able to recycle CoQ to the reduced form able to protect membranes against oxidation. There are at least three enzymes responsible to maintain CoQ₁₀ in its reduced form: NADH cytochrome b5 reductase, NADH/NADPH oxidoreductase (also called DT-diaphorase) and NADPH coenzyme Q reductase (Turunen et al, 2004). Mitochondrial DT-diaphorase, a two-electron quinone reductase, seems to have a crucial participation in the antioxidant action of CoQ due to its maintenance in the reduced form CoQH₂ (Cadenas, 1995). Differently from NADH and succinate dehydrogenases, which are able to generate the partially reduced coenzyme Q ubisemiquinone, the DT-diaphorase is unique since it can directly reduce CoQ *via* 2 electron transfer without intermediate formation of the semiquinone (Beyer et al, 1996). CoQ can also be reduced by the mitochondrial respiratory chain (Genova et al, 2003; Bentinger et al, 2007). Another mechanism that may contribute to the antioxidant activity of CoQ is the interaction of superoxide dismutase with CoQH₂ and DT diaphorase resulting in inhibition of coenzyme autoxidation (Beyer, 1992). Besides the energetic role of coenzyme Q as mobile electron carrier, the antioxidant activity of CoQH₂ is important to decrease the oxidative modification of mitochondrial CoQ pool associated to the impairment of the electron transport in the respiratory chain observed during the lipid oxidation of mitochondrial membranes (Forsmark-Andrée et al, 1997). It was showed that the antioxidant effects of CoQ in microsomes and mitochondria are also mediated by vitamin E recycling (Kagan et al, 1990). Recently, it was demonstrated that the enzymes: lipoamide dehydrogenase, thioredoxin reductase and glutathione reductase can also reduce CoQ (Olsson et al, 1999; Xia et al, 2001; Xia et al, 2003).

The high antioxidant efficiency of CoQH₂ depends on several factors, including its localization into the membranes, hydrophobicity, the efficiency as scavenger of free radicals and recycling antioxidant cellular systems. Mitochondria are directly implicated with oxidative stress conditions, due to the constant generation of superoxide anions (O₂•⁻) by the respiratory chain, a process which is normally counterbalanced by the antioxidant defense system composed of superoxide dismutase, glutathione peroxidase and reductase, GSH and NAD(P)H. However, in mitochondrial dysfunctions, the excessive formation of O₂•⁻, and consequently of hydrogen peroxide (H₂O₂), leads to the generation of the extremely reactive hydroxyl radical (•OH) by means of the Fenton-Haber-Weiss reaction (Sies, 1997). Stress oxidative is thought to be involved in the etiology of many human diseases (Brookes et al, 2004) but also in cell signaling (Linnane et al, 2007) and endogenous and exogenous antioxidants are crucial to modulate these processes. CoQ and vitamin E addition in cultured cells attenuated ROS production, lipid peroxidation, mitochondrial dysfunction, and cell death induced by amitriptyline (Cordero et al, 2009). In Langendorff preparations of isolated heart, pretreatment with CoQ protected coronary vascular reactivity after ischemia/reperfusion radical scavenger activity (Whitman et al, 1997). CoQ was also able to ameliorate cisplatin-induced acute renal injury in mice (Fouad et al, 2010).

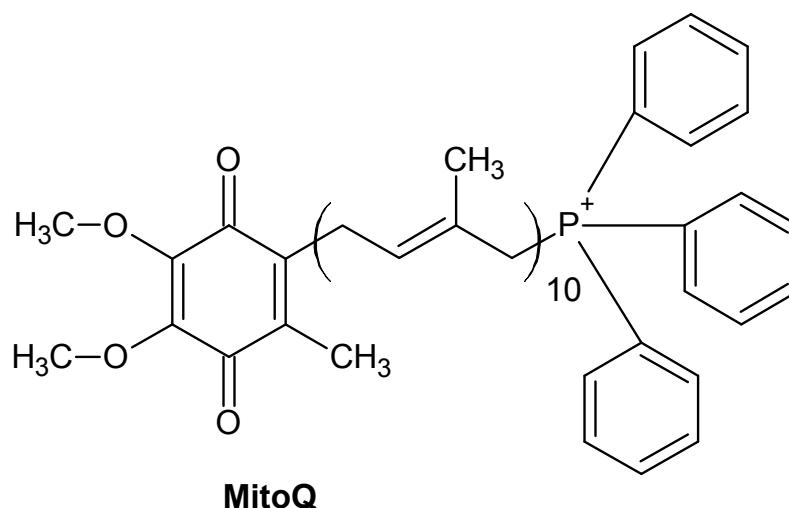


Fig. 5. Chemical structure of triphenylphosphonium-substituted coenzyme Q, MitoQ.

Many *in vitro* and *in vivo* studies showed that CoQ, mainly in its reduced state, may act as an antioxidant protecting membranes from oxidative damage (Beyer, 1990). Besides the antioxidant activity, CoQ participates as cofactor of uncoupling proteins and modulates gene expression associated to cell signaling, metabolism, transport, etc (Linnane et al, 2002). The hydrophobicity of CoQ allows its easy insertion into the mitochondrial inner membrane where it is converted to the reduced form by reductases (Beyer et al, 1996). Although the antioxidant activity of CoQ in biological membranes the relative high hydrophobicity disfavors its use as a therapeutic agent (Kelso et al, 2001). As an alternative, mitochondrial-targeted ubiquinone analogs, including Mito Q, and ubiquinone analogs with a decrease number of carbons in the side chain compared with CoQ10 were developed (Geromel et al, 2002). It was showed that the CoQ analogue decylubiquinone, but not CoQ, decreased ROS production associated to the inhibition of the MPT (mitochondrial permeability transition) and cell death in HL60 cells. Such effect is due to the antioxidant action of decylubiquinone either preventing ROS formation or scavenging ROS generated by cytochrome *bc*1

(Armstrong et al, 2003). MitoQ (Fig. 5) is an orally active antioxidant developed by the pharmaceutical industry to potentially treat several diseases. This compound retains the antioxidant activity of CoQ10 and the triphenylphosphonium cation (TPP⁺) substituent directs this agent to mitochondria (Tauskela, 2007).

In cultured cells MitoQ was able to accumulate into mitochondria and act against oxidative stress (Murphy & Smith, 2007; James et al, 2005). It was also demonstrated a protective effect of MitoQ in a sepsis model by decreasing the oxidative stress and protecting mitochondria against damage as well as by suppressing proinflammatory cytokine release (Lowes et al, 2008). It was proposed that the antioxidant action of MitoQ may be useful in the treatment of diseases associated to the impairment of mitochondrial Complex I (Plecitá-Hlavatá et al, 2009). On the other hand, it was recently showed that MitoQ may be prooxidant and present proapoptotic action due its quinone group that may participates in redox cycling and superoxide production (Doughan & Dikalov, 2007). Thus, the study of the mechanisms of antioxidant action and other effects of CoQ and derivatives must be considered for the development of quinone-based therapeutic strategies.

3.2 Cytochrome c

Similarly to that was described for CoQ, cytochrome c may also contribute to the generation and trapping of prooxidant species. It has been described that besides the participation in the respiratory chain and apoptosis, cytochrome c exhibits also a prooxidant peroxidase activity and an antioxidant superoxide oxidase activity. However, a whole view of the roles played by cytochrome c in cells leads to the conclusion that the respiratory and proapoptotic activities of this protein intrinsically contribute also to the cell redox balance.

It was demonstrate that loss of cytochrome c by mitochondria oxidizing NAD⁺-linked substrates results in respiratory inhibition associated to a significant increase of ROS production (Davey et al., 1998; Gnaiger et al., 1998; Rossingnol et al., 2000) The depletion of cytochrome c results in respiratory inhibition and maintains reduced the electron carriers upstream the hemeprotein with consequent increasing of the NAD(P)H levels. However, the terminal segment of the respiratory chain is more active than the proximal one in such way that only mild respiratory inhibition has been observed in cells undergoing apoptosis accompanied by cytochrome c release and increased production of ROS. Therefore, only almost total cytochrome c depletion could significantly promote respiratory inhibition and enhance of ROS production at complex I (Davey et al., 1998; Gnaiger et al., 1998; Rossingnol et al., 2000; Kushnareva et al., 2002) Considering the role played by cytochrome c in apoptosis, literature data have correlated this event to an increased peroxidase activity of the hemeprotein. In comparison with pentacoordinated heme proteins such as myoglobin and horseradish peroxidase, in the native form, cytochrome c reacts very slowly with peroxides (Radi et al., 1991). However, the peroxidase activity of cytochrome c can be favored in conditions leading to loss of the heme iron sixth coordination position with the sulfur atom of Met80 or the replacement of Met80 by other amino acid lateral chains (Nantes et al., 2000; Rodrigues et al., 2007; Nantes et al., 2001; Zucchi et al., 2003). A condition that can strongly favor the peroxidase activity of cytochrome c is the association with negatively charged membranes (Rytömaa, et al, 1992. Ott, et al., 2002. Mugnol, et al, 2008. Rytömaa & Kinnunen, 1994, Rytömaa, M & Kinnunen, 1995, Kawai, et al, 2005; Kagan, et al., 2005).

According to Kagan et al., (2004) the amount of cardiolipin in the outer side of the inner mitochondrial membrane can be increased in a proapoptotic condition and favor the

peroxidase activity of cytochrome *c*. In this scenario, the peroxidase activity of cytochrome *c* on cardiolipin should be involved in its detachment from the inner mitochondrial membrane to attain the cytosol and trigger apoptosis. In addition, the reaction of cytochrome *c* with lipid-derived carbonyl compounds results in the production of triplet excited species able to generate $O_2(^1\Delta_g)$ by energy transfer to molecular oxygen (Foote, 1968, Nantes et al., 1996; Estevam et al., 2004; Groves, 2006).

Considering the peroxidase activity of cytochrome *c* culminates with its detachment from the inner mitochondrial membrane leading to the death of cells with unbalanced redox processes, the pro-apoptotic activity of cytochrome *c* might be included, if not as antioxidant, but as a protective role of this protein for the whole organism.

However, the protective antioxidant activity of cytochrome *c* can also be exerted in a preventive rather than a destructive way. The elimination of superoxide ion by SOD generates hydrogen peroxide. As discussed before, hydrogen peroxide is a signaling molecule but its accumulation in cells should be prevented to avoid undesirable reaction with transition metal ions and the consequent generation of hydroxyl radical. Hydrogen peroxide can react with Fe^{III} respiratory cytochrome *c* and convert it to high valence species (oxoferryl forms) that are highly prooxidant species. The high valence species of cytochrome *c* can attack lipids and trigger a radical propagation leading to oxidative damages of mitochondrial membranes. Therefore, the cellular antioxidant apparatus includes catalase and GPx (glutathione peroxidase) that are responsible for hydrogen peroxide reduction. Fe^{III} cytochrome *c* competes with SOD for one electron reduction by superoxide ion. The reduction of cytochrome *c* by superoxide ion is more efficient than SOD to prevent oxidative stress because, by this way, the electron is devolved to the respiratory chain, does not generate hydrogen peroxide and further prevents the generation of high valence species of the heme protein. In an apparent paradox but consistent with the competition with cytochrome *c*, over expression of SOD1 has been related to an increase of the oxidative stress (Goldsteins et al., 2008). Cytochrome *c* can efficiently act as a true antioxidant by scavenging $O_2^{\cdot-}$ without producing secondary and potentially harmful ROS (Pereversev et al., 2003).

Also, the reduction of cyt *c* heme iron by $O_2^{\cdot-}$ impairs peroxidase activity on hydrogen peroxide and the consequent generation of radical and excited prooxidant species. As discussed before, even the conditions favoring the peroxidase activity of cytochrome *c* should not be considered exclusively harmful and damaging events since they culminate with detachment of cytochrome *c* from the inner mitochondria membrane to participate in the apoptosis in cytosol. It is important to note that the participation of cytochrome *c* in oxidative and nitrosative stress can also promote damages in the heme protein (Estevam et al., 2004; Rodrigues et al., 2007), including impairment of the proapoptotic activity (Suto et al., 2005). However, the association of cytochrome *c* with unsaturated lipid bilayers is shown to prevent these oxidative damages and preserve the apoptotic activity (Estevam et al., 2004; Rodrigues et al., 2007).

The contribution of cytochrome *c* for hydrogen peroxide elimination is probably not restricted to the peroxidase mechanism and superoxide ion trapping. It has been proposed the reduction of hydrogen peroxide by Fe^{II} cytochrome *c* in a mechanism named as electron-leak pathway.

At this point it is important to consider the role played by testicular cytochrome *c*. Reactive oxygen species generated in the respiratory chain are responsible for damages in biomolecules such as DNA, lipids and proteins of sperm that culminate with loss of cell

viability and infertility. Sperm are particularly susceptible to the undesirable effects of ROS because their high content of polyunsaturated fatty acids present in the plasma membrane and a low concentration of ROS scavenging enzymes in the cytoplasm (Jones et al, 1979, Huttemann et al., 2011; Sharma et al, 1999; Liu et al., 2006). Mammalian germ cells express two types of cytochrome c during their development: the somatic cytochrome c and a testis specific cytochrome c that shares 86,5% identity with the somatic counterpart. During meiosis, the expression of somatic cytochrome c declines and testis cytochrome c becomes the predominant form in sperm. Liu et al, reports that testis cytochrome c is three fold more efficient than the somatic one in the catalysis of H₂O₂ reduction and is also more resistant to be degraded by the side products of this reaction. In line with the proposal that apoptosis is also an antioxidant protective mechanism, testis cytochrome c exhibited higher apoptotic activity in the well established apoptosis measurement system using *Xenopus* egg extract.

Therefore, testis cytochrome c can protect sperm from the damages caused by H₂O₂ as well as promote the elimination of sperm whose DNA was damaged. Taken together the electron-leak pathway and apoptosis, probably related to a peroxidase activity are the contribution of testis cytochrome c for the biological integrity of sperm produced by mammalian cells.

Therefore, a delicate balance controls both antioxidant and prooxidant activities of cytochrome c with repercussions on both bioenergetics and cell death. In this regard, it is noteworthy that cytochrome c import to mitochondria, synthesis and activities underlying life and death fates for cells are regulated by signaling mechanisms and involves thiol redox balance, allosteric regulation and chemical modifications including nitration and phosphorylation. Cytochrome c is a nuclear-coded protein that is imported by mitochondria as apoprotein and, in the intermembrane space, is converted to the holoprotein by the covalent ligation of the heme group to cystein residues 14 and 17, a process catalyzed by the enzyme heme lyase (Dumont et al, 1991). The addition of the heme group confers redox properties for cytochrome c and enables it to participate, as terminal oxidant agent, in the thiol redox cascade involved in the import and assembly of TIMs (transporters of the inner mitochondrial membrane) (Chacinska et al, 2004, Riemer et al., 2011; Allen et al., 2005). The participation of cytochrome in the respiratory chain as electron carrier is also controlled by allosteric and covalent modification mechanisms. ATP has been characterized as a downregulator of the electron-transfer activity of cytochrome c. The mechanism may involve changes of both charge and structure of cytochrome c and is consistent with the adjustment of respiratory chain activity to the energy demand of cell signaled by the ATP/ADP ratio. Recent findings have strongly shown that the well-known mechanism of protein phosphorylation operates also in the control of proteins responsible for the oxidative phosphorylation. The technique of cytochrome c isolation in the presence of nonspecific phosphatase inhibitors enabled the identification of tissue-specific sites of cytochrome c and evidenced the activities of this protein is under the control of this specific cell signaling mechanism mainly operating in higher organisms (Huttemann et al., 2011). Previously, it was demonstrated that redox reaction of cytochrome c with a model aldehyde, diphenylacetaldehyde, is under the control of the protonation of two tyrosine residues (Rinaldi et al, 2004). More recent findings established the phosphorylation of cytochrome c tyrosine residues is involved in the control of the transmembrane potential that in health conditions should not attain the maximal to avoid increase of ROS generation (Yu et al., 2008; Zhao et al., 2010). The consequences of cytochrome c nitration in biological systems

have been investigated and demonstrated that a small structural change promoted by nitration of tyrosine 74 does not preclude ligation of cytochrome c to Apaf-1 but this cytochrome c form became unable to activate caspases (Garcia-Heredia et al., 2010).

Similarly to CoQ, the more recent findings about cytochrome c biological functions operating under the control of cell signaling mechanisms led to investigations concerning the therapeutical use of cytochrome c properties by administration of exogenous proteins as well as by controlling its phosphorylation in pathological conditions (Huttemann et al, 2011; Piel et al., 2007, 2008).

Fig. 6 summarizes the antioxidant activity of cytochrome c.

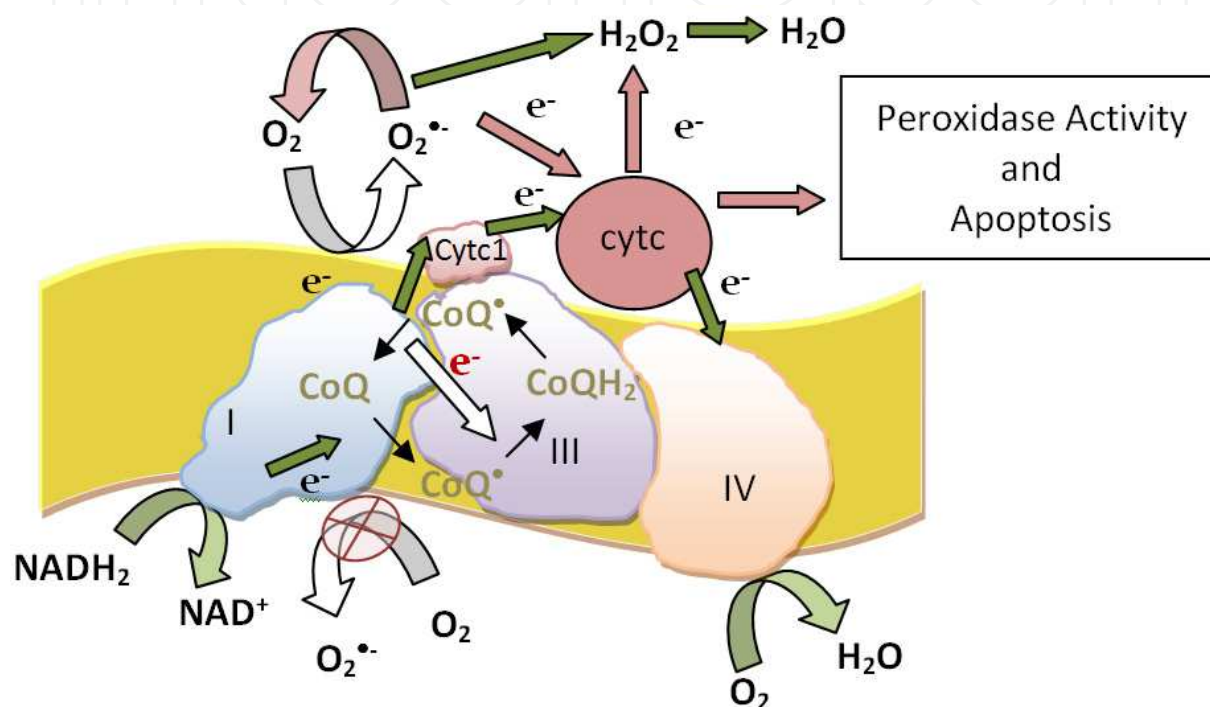


Fig. 6. Antioxidant activity of cytochrome c. In the respiratory chain, cytochrome c affects the generation of O₂^{•-} and H₂O₂ by making the electron transfer of the respiratory chain more fluent (green arrows). The heme protein also eliminates the generated O₂^{•-} and H₂O₂ through a cytochrome c mediated electron-leak pathway (red arrows). Further, the peroxidase activity of cytochrome c associated to apoptosis is also a protective mechanism for the whole organism as in the case of testis cytochrome c.

4. Conclusion

The evolutionary acquisition of the O₂-dependent aerobic metabolism resulted in a highly more efficient use of the energetic fuels and a cell signaling mechanism based on reactive species. Concerning the ROS, the primary species produced in mitochondria is O₂^{•-} from which both the signaling molecule, H₂O₂, and the highly deleterious derivative, hydroxyl radical are generated. Therefore, a very efficient antioxidant apparatus was also evolved to assure cell redox balance and repair of oxidative damages. The antioxidant apparatus encompasses enzymes able to decompose reactive species (SOD, catalase) and repair oxidative damages (thioredoxin, glutaredoxine) and free radical trapping (ascorbic acid,

tocopherol, lipoic acid). More recently, cytochrome c was included in the category of antioxidant enzymatic apparatus due to its capacity to oxidize superoxide ion and devolve the electron to the respiratory chain as well as by the capacity to reduce hydrogen peroxide. The electron transport in the respiratory chain can also be considered an antioxidant activity of cytochrome c because it contributes for the fluency of electron transport. The antioxidant activity of CoQ is based on the direct and indirect trapping of free radicals and it is not restricted to mitochondria but exerted in the whole cellular and extra-cellular media. The beneficial antioxidant activity of CoQ has been studied with the aim to develop an antioxidant therapy by the use of CoQ analogous and derivatives. Figure 7 summarizes the antioxidant activity of mobile electron carriers of the respiratory chain.

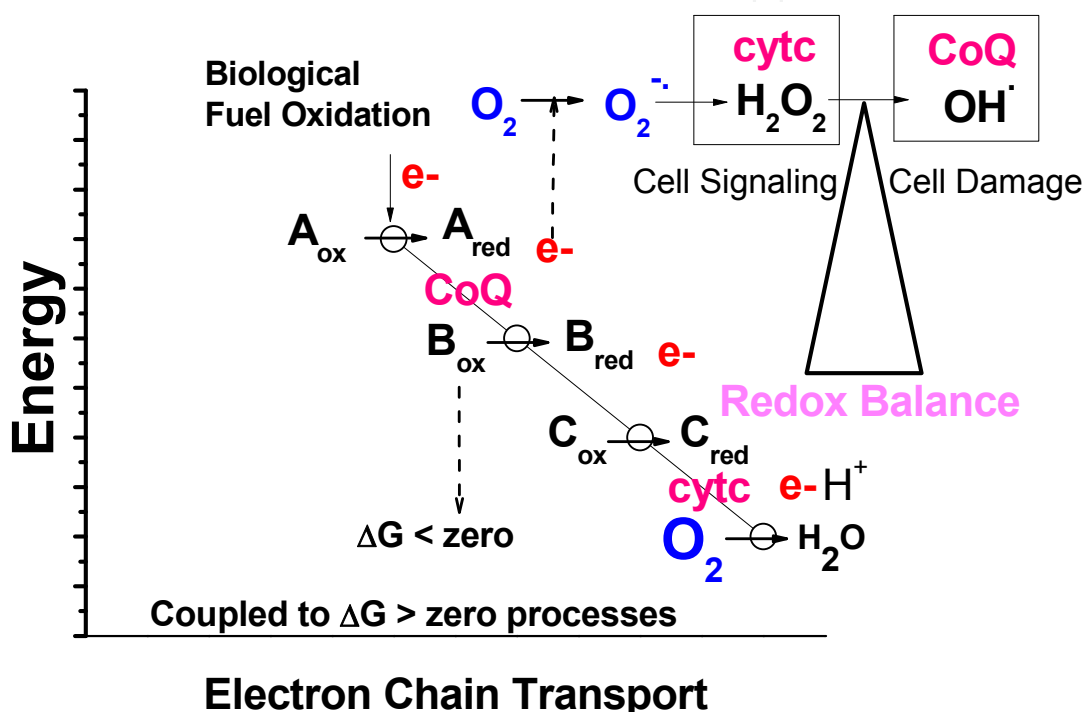


Fig. 7. Antioxidant activity of mobile electron carriers of the respiratory chain. The aerobic oxidation of biological fuels by using molecular oxygen as final acceptor of electrons in an electron transport chain allowed an efficient mechanism of withdrawing energy from biological fuels concomitant with the generation of reactive species for cell signaling but also able to promote cell damage. The redox cell balance is achieved by prevented the accumulation of reactive species without prejudicing the signaling function. Cytochrome c contributes to the maintenance of the adequate levels of hydrogen peroxide in cells by means of fluency of electron transport in the respiratory chain, oxidation of superoxide ion and reduction of hydrogen peroxide and CoQ by means of direct and indirect trapping of free radicals.

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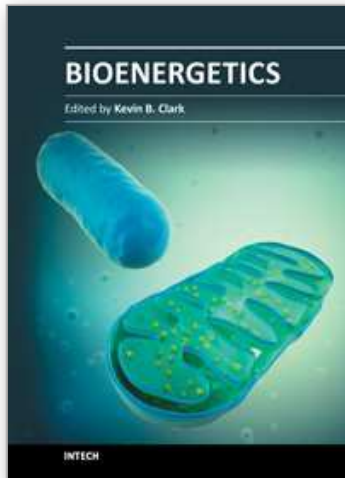
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