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Chapter 2

CYTOCHROME b_5 AS A PLEIOTROPIC METABOLIC MODULATOR IN MAMMALIAN CELLS

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ABSTRACT

Cytochrome b_5 (Cb_5) acts within the cells as a pleiotropic co-factor of multiple enzymes and redox chains that play critical roles for normal function of healthy mammalian organisms. It is maintained in the reduced form largely by the NADH-dependent cytochrome b_5 reductase activity which catalyzes electron transfer to Cb_5 in mammalian cells. The enzyme system cytochrome b_5 reductase/cytochrome b_5 has been shown to be associated with the endoplasmic reticulum, mitochondria and plasma membrane of mammalian cells, where it is involved in multiple metabolic pathways, such as cholesterol biosynthesis, desaturation and elongation of fatty acids, methemoglobin reduction and steroids and xenobiotics metabolism catalyzed by cytochrome P450s. More recently, this system has been shown to be a component of the 'so-called' redox-chain of the plasma membrane present in mammalian cells, where it clusters within

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cholesterol-rich and caveolins-rich lipid rafts-associated sub-microdomains of the plasma membrane. The cytochrome b_5 reductase/cytochrome b_5 is also a relevant system in the redox biology of cell death, as it forms a stable complex with cytochrome *c*, a pro-apoptotic signalling molecules, and it is involved in the modulation of superoxide generation in mammalian cells, playing a major role in the NADH-dependent superoxide anion production of the neuronal plasma membrane accounting for most of the superoxide anion overshot that is observed in an early stage of the neuronal apoptosis. In addition, an expanding family of proteins containing a Cb_5-like domain is also present in mammalian cells. Both, the novel cellular functions described for Cb_5 and for novel proteins containing a Cb_5-like domain point out that Cb_5 can be seen as an intracellular signaling molecule in redox biology and as a potential cellular biomarker of health and disease.

1. INTRODUCTION

Cytochrome *b*'s are hemeproteins characterized by the presence of a heme group not covalently bound to the protein. The heme group of these proteins is a bis(imidazole) coordinated group displaying different physical properties. Mosbauer studies on the heme coordination led to propose a weak binding of a vicinal histidine to the hemin [Medhi, 1990].

Cytochrome *b*'s are part of redox systems as components of electron chains as individual proteins, like cytochrome b_5 (Cb_5), or enzymatic domains, like molybdenum dependent sulfite oxidase. Mammalian cells express several isoforms of Cb_5, which is one of the most studied proteins of the cytochrome *b* family. Cb_5 becomes embedded into membranes after being generated in free polyribosomes rather than in membrane-bound ribosomes, as demonstrated by experiments of Cb_5 incorporation in biological and artificial membranes [Rapoport and Wiedmann, 1985]. *In vivo*, two well defined NAD(P)H-dependent reductases, namely, cytochrome b_5 reductase and cytochrome P450 reductase are the major systems catalyzing the reduction of Cb_5. Cb_5, as a hemeprotein coupled to its reductases, accepts and rapidly transfer electrons to other electron partners [Bond et al., 1990]. Many biological roles of Cb_5 were already known nearly 20 years ago, associated to a large number of functions in cells and tissues, such as lipid biosynthesis through desaturases, plasmalogen and cholesterol biosynthesis, fatty acid elongation, biosynthesis of sialic acid and methameoblogin recycling, reviewed in [Vergeres and Waskell, 1995]. However, novel biological functions of Cb_5 are emerging during last years, pointing out that this protein is also involved in intracellular

signaling pathways whose deregulation play a key role in the apoptotic cell death. In addition, novel proteins containing a Cb_5 –like domain have been found and reported to be involved in the modulation of cellular redox signaling and steroid hormones signal transduction. Despite that the smooth endoplasmic reticulum has a high content of Cb_5, this protein presents a ubiquitous localization within the cells, as it is also found in the soluble cytosolic fraction and also in the cytoplasmic leaflet of organelles like the plasma membrane, endoplasmic reticulum, outer mitochondrial membrane and the nuclear membrane [Oshino, 1978; Rapoport and Wiedmann, 1985].

In this chapter we shall first describe the structure-function relationships and subcellular localization of the different Cb_5 isoforms expressed in mammalian cells. Particular emphasis will be placed in the homology of their primary sequences and conserved structural motifs and amino acids critical for their function as a redox partner, as well as for the regulation of the reduction potential of its heme *b* prosthetic group. In the next section of this chapter, we shall systematically present the enzymes that use Cb_5 as a co-factor, highlighting the functional consequences of the complex formation and the known protein residues identified in specific protein/protein interactions. In this section we have also included a sub-section devoted to the proteins containing a Cb_5 –like domain and the proposed biological roles for the most recently discovered members of this family of proteins. Thereafter, in a different section of this chapter we shall analyze the function of Cb_5 as an electron carrier in redox chains known to be present in mammalian cells and their biochemical and biological functions. As briefly indicated above new roles for Cb_5 in intracellular signaling have been proposed during last years in the field of redox biology. On these grounds, we have devoted the next section to the role of Cb_5 in reactive oxygen species (ROS) signaling and apoptosis. Finally, in the conclusion section we have summarized the major conclusions derived from the points addressed in this chapter, which indicate the potential of Cb_5 as a cellular biomarker for metabolic and age-related diseases.

2. STRUCTURE AND SUBCELLULAR LOCALIZATION

2a. Protein Structure of Cb_5

Mammalian Cb_5 is a globular acidic protein formed by 6 α-helices and 5 β-strands [Mathews et al., 1979], see also the Figure 1, with a molecular

weigth between 16 and 25 kda depending on the species [Velick and Strittmatter, 1956; Spatz and Strittmatter, 1971].

In rat Cb_5 purified from the soluble fraction of liver homogenates the heme group sits in a cavity formed by 4 α-helices and coordinated by His44 and His68. There are two hydrophobic regions recognized in the structure of the protein named core-1 an core-2. The core-1 is formed by the central part of the protein and determines the protein-heme contact. The core-2 is found within the N- and C- terminal segment and has a certain degree of structural independence with respect to these two regions [Knappenberger et al., 2004]. Using trypsin-solubilized bovine liver microsomal Cb_5 (82 residues in length) it has been shown that the Phe40 is an important residue for stabilization of the protein. Mutation of the phenyl residue to tyrosine increase the stability of the protein in its oxidized state while mutation of this residue by Leu or His decrease its thermal stability with a shift in the redox potential [Yao et al.,1997]. Three highly conserved acidic residues, Glu49, Glu53 and Asp65 of membrane Cb_5 from several species (see the Figure 2), and one of the heme propionate are in the same plane in the solvent being implicated in the charge pairing interaction with other redox partners [Schenkman and Jansson, 2003]. NMR experiments of Cb_5 indicated that a conformational change occurs in the α-helices forming the heme-binding pocket when the oxidized and reduced forms are compared, suggesting a high flexibility of this pocket [Fragai et al., 2006].

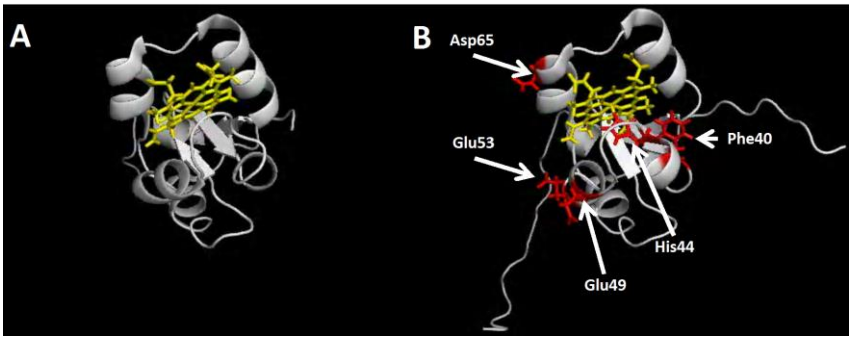

Figure 1. Structure of Cb_5. (A) Soluble fragment of rabbit microsomal Cb_5 (PBD: ID09, [Banci et al., 2000]). (B) Full-length rabbit microsomal Cb_5 (PBD: 2M33, [Ahuja et al., 2013]). Heme group is colored in yellow in panels A and B. Red labeling in panel B, indicates the location of the following side chain residues: Phe40, His44, Glu49, Glu53 and Asp65.

Cb_5 exists in aqueous solution free of detergent as a mixture of monomers and octamers [Konopka and Waskell, 1988]. The monomer form is present at low ionic strength and decreased in a concentration dependent manner with the ionic strength. Binding capacity to vesicles depends on the ionic strength and it has been shown that 30-50 lipid molecules can bind to one molecule of Cb_5 at low ionic strength while up to 250 molecules of Cb_5 have been shown to bind per liposomal vesicle in buffers containing 0.1M salt [Rogers and Strittmatter, 1975; Holloway, 1983]. Studies done with biological and artificial membranes have demonstrated that cholesterol-containing membranes have a lower affinity to Cb_5 [Tajima and Sato, 1979; Enomoto and Sato, 1977]. Stritmatter and co-workers concluded that Cb_5 was stabilized in two different conformations upon binding to membranes, called "loose" and "tight" form [Enoch et al., 1979; Dailey and Strittmatter, 1980].

A water-soluble protein heme domain (wsCyt b_5) from microsomal Cb_5 can be isolated by treatment of the microsomal Cb_5 with trypsin [Strittmatter and Ozols, 1966; Reid and Mauk, 1982]. The properties of this domain and its structure has been studied [Basova et al., 2008]. Proximity to a negatively charged membrane surface destabilizes wsCyt b_5 at neutral pH, and promotes its transition into a more flexible conformational state with molten globule-like properties. It has been observed that wsCyt b_5 can interact with artificial membranes at their surface [Basova et al., 2008]. Its association could be related to the presence of a hydrophobic patch of 320Å^2 adjacent to the anionic residues formed by residues Phe40, Pro45, Leu75 and Phe/Tyr79 (calf), that displays a role in the formation of complexes with redox partners [Mathews et al., 1979]. This hydrophobic patch of the surface of Cb_5 is not part of the hydrophobic core of Cb_5 localized inside the protein and has been associated to redox electron transfer properties to membrane acceptors [Vergeres and Waskell, 1995]. The presence of this hydrophobic patch on the surface of Cb_5 suggests that electrons flow from one side of the protein and could be delivered to membrane associated partners oriented toward this domain.

2b. Cb_5 Homology and Isoforms

Cb_5 keeps a highly conserved region composed by 96 amino acids [Ozols, 1989]. The mammalian form shares an 80% of identity with some conservative substitutions [Schenkman and Jansson, 2003]. Cb_5 from other sources share different percentages of homology with respect to mammals, see the Figures 2 and 3. For example, chicken shares 64%, plants 47%, yeast 26% and bacteria

21% of identical residues. In housefly, there is a form of Cb_5 that shares 44% of the identity with mammal Cb_5 [Guzov et al., 1996]. In rat liver, it has been isolated a type of Cb_5 named outer membrane Cb_5 (OMB5) that has an amino terminal leader sequence that targets the protein to mitochondria. In this type of Cb_5, the heme peptide region has 57% of homology. We will talk about its properties compared to microsomal Cb_5 (MCB5) in a section below in this chapter.

There are two isoforms of Cb_5, named erythrocyte Cb_5 (or soluble isoform) and liver Cb_5 (or membrane anchored), called in that way from the tissues where they were purified and extracted originally. Later it was demonstrated that both proteins are the short and the long isoform from the same protein. The OMB5 isoform contains 146 residues and, therefore, is longer than the MCB5 isoform which has 134 amino acids [Lederer et al., 1983]. The sequence conservation in the central position is very remarkable and includes the heme crevice region [Mathews et al., 1971; Mathews et al., 1972; Mathews et al., 1979]. Both type of proteins contains approximately 100 residues that are exposed to the cytosol, while each protein is anchored to its respective membrane through 20 residues near the protein C-terminus, although the targeting area that attach the protein to the membrane is built by only 10 amino acids [Mitoma and Ito, 1992; De Silvestris et al., 1995; Kuroda et al., 1998]. The first sequence for OMB5 was identified as a human testis isoform, but a nearly identical gene (replacing Lys by Gln95) has been identified in the human, rat and mouse genome [Altschul et al., 1997; Strausberg et al., 2002].

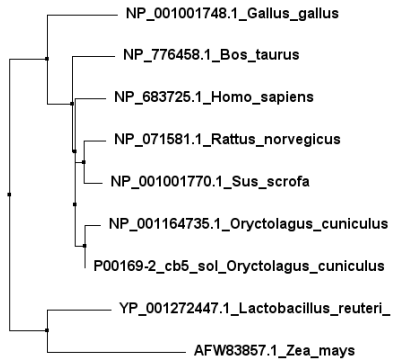

Figure 2. Cladogram-type phylogenetic tree of Cb_5. Shown only the species most used in structural studies of this protein. The phylogenetic tree has been calculated based on neighbour joining using percentage of identity (Waterhouse et al., 2009).

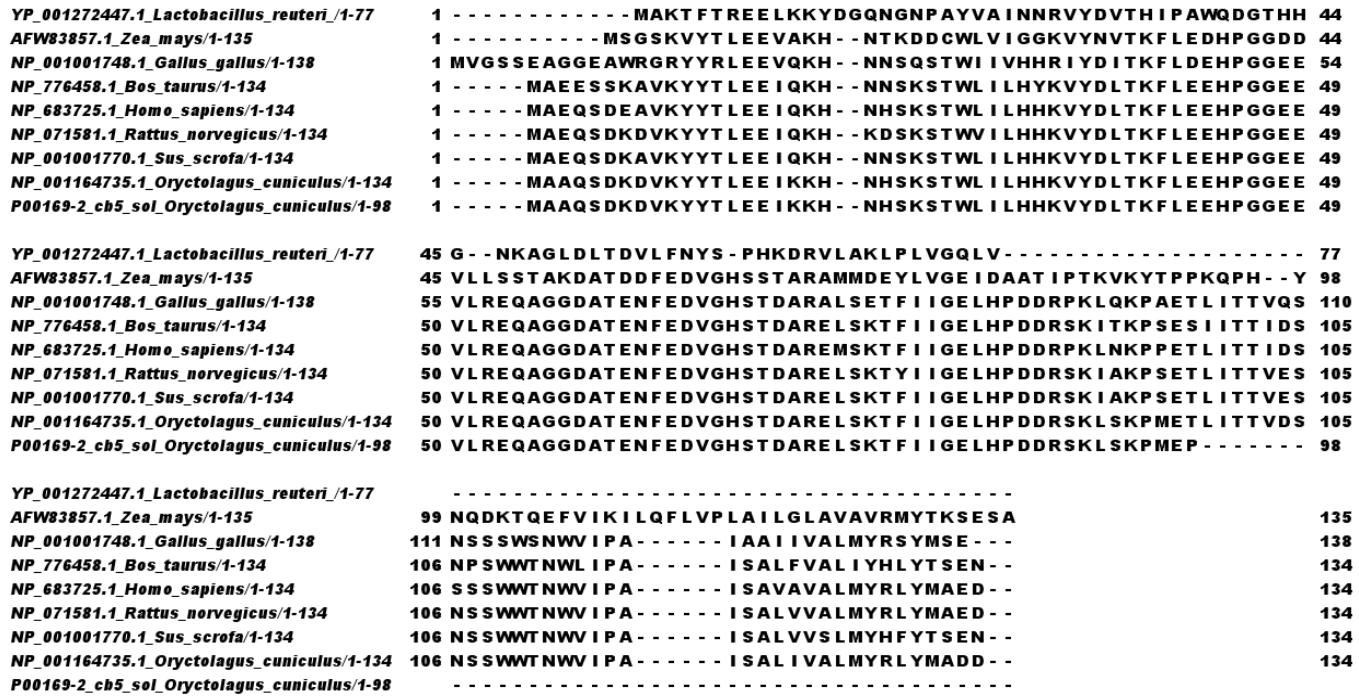

Figure 3. Amino acids sequence alignment of microsomal Cb_5 from the species most often used in structural and functional studies. Also included for comparison the amino acids sequence of the rabbit soluble Cb_5 isoform (98 amino acids), line P00169-2_cb5-sol_*Oryctolagus_cuniculus*.

UV–Vis and EPR studies of recombinant OMB5 suggested that its properties were nearly identical to those of the MCB5 isoform [Rivera et al., 1992], and crystallographic studies showed similar folding patterns for both proteins [Durley and Mathews, 1996; Rodriguez-Maranon et al., 1996]. Human and rat OMB5 has also the same hemin binding properties. Nevertheless calorimetric measurements has shown that the critical unfolding temperature for the rat OMB5 isoform is nearly 20ºC higher than that of the MCB5 isoform, 85.5 and 67.6ºC, respectively, being the midpoints of guanidinium thiocyanate-mediated denaturation 2.04 and 1.52M, respectively [Cowley et al., 2002]. Therefore, the native conformation of the OMB5 isoform seems to be more stable than the native conformation of the MCB5 isoform. Thermal and chemical denaturation properties for human OMB5 (Tm=85.7 and Cm=1.86M) indicates stability properties closer to rat OMB5 than to rat MCB5 isoform.

Interestingly, the redox potential of the rat and human OMB5, -107mV and -40mV, respectively [Rivera et al., 1992; Altuve et al., 2004], differs from that found for MCB5 from rat (~0 to +10mV) [Mauk et al., 1982; Walker et al., 1988; Rodgers and Sligar, 1991; Mauk et al., 1995], bovine liver (~+31mV) [Glenn and Bowden, 1996] and chicken liver (~+40mV) [Bagby et al., 1988]. Human MCB5 isoform have a redox potential approximately 100mV higher than that found for human OMB5 (-40mV). Early electrochemical studies showed that the redox potential of native bovine erythrocyte Cb_5 was varying between values: +0.8 and -26.2mV, and NMR studies using apoCb_5 reconstituted with exogenous hemin allowed to attribute this fact to the two different orientations for the heme in the protein [Walker et al., 1988]. Through the use of modified hemes, it has been shown that both of the orientations are interconvertible and they are the result of heme disorder or rotation of the heme moiety around the α,γ meso axis [La Mar et al., 1981]. The change of heme orientation produces changes in the microenvironment of the heme, placing heme substituents in different microenvironments depending on their interactions with the side chains of the amino acids present in the heme pocket. When the redox potential of Cb_5 is measured by surface modified electrons, the results give more positive values (40-100mV) than when it is measured by potentiometric methods [Rivera et al., 1998]. This difference in redox potentials measured with both type of methods has been related to the neutralization of the surface charge of Cb_5 upon formation of complexes between Cb_5 and the modified electron surface, which leads to exclusion of water from the complex and changes in the dielectric constant of the microenvironment of the heme group and destabilization of the positive

charge of the ferricytochrome respect the ferrous form. These experiments suggest that a shift in the redox potential of Cb_5 should be expected upon formation of complexes of Cb_5 with other protein partners within redox chains, provided that the complex formation elicits a re-orientation of the heme group of Cb_5.

1H NMR experiments have shown that the heme group of MCB5 can be in two interconvertible spatial orientations that differ by a 180 degrees rotation around the α,γ-meso axis of the heme, which are named isoforms A and B of Cb_5 [Keller and Wüthrich, 1980; La Mar et al., 1981; Walker et al., 1988]. When bovine MCB5 is reconstituted, it is observed that the equilibrium between these isoforms is largely shifted towards the A isomer, ratio A:B isoforms of 9:1 [La Mar et al., 1981; Walker et al., 1988]. Other mammalian MCB5s keep favoring the A position in different A:B ratios, for example, 1.6:1 for rat and 20:1 for chicken [Lee et al., 1991]. In this work Lee et al. calculated that in the bovine microsomal protein, orientation A is enthalpically favored by 1.4 kcal/mol, whereas in the rat MCB5**,** orientation B is enthalpically favored by 2.4 kcal/mol. These investigators also pointed out that the steric destabilization of a vinyl substituent at position c in the heme by a hydrophobic cluster of amino acids at positions 23 and 25 is an important factor for heme orientation in Cb_5. For the case of rat OMB5 this A:B ratio is 1:1.2 and for the human form 1:1 [Silchenko et al., 2000].

The large positive shift (+110 mV) observed for rat OMB5 was attributed later to the presence of a multivalent cation site that once is occupied shield the negatively charged protein surface and negatively charged electron surface to facilitate the electron transfer [Glenn and Bowden, 1996]. It has been defined the binding pocket for the multivalent cations being formed by the conserved acidic residues of the rat OMB5 (Glu49, Glu53, Glu61 and Asp60) that correspond to (Glu44, Glu48, Glu56 and Glu65) of human Cb_5. The neutralization of charge as a consequence of the binding of a cation results in water exclusion from the complex inducing a decrease of the dielectric constant in he microenvironment of the heme group that leads to a positive shift of the redox potential [Rivera et al., 1998; Wirtz et al., 2000].

The role of OMB5 has been lately associated to a high reduction of hydroxylamines and imidoximes as a cofactor of molybdenum cofactor sulfurase C-terminal 2 (MOSCO-2), exclusively located at mitochondrial membranes, and cytochrome b_5 reductase [Neve et al., 2012]. Downregulation of MOSCO-2 cause impaired lipid synthesis, and this led these authors to suggest the involvement of these proteins in a new pathway for the synthesis of lipids.

The molecular mechanisms controlling the expression of the *CYB5B* gene encoding for OMB5 or CYB5B protein in the different tissues of mammals, although it has been observed that there is an increase in the levels of this protein in prostate cancer cells treated with luteinizing hormone [Pinski et al., 2011], are a yet unresolved issue despite that cDNA libraries and database analysis has pointed out its expression in many tissues of different mammalian species [Soucy and Luu-The, 2002]. Interestingly, in cancer cells the CYB5B protein has been identified as the 21 KDa protein overexpressed at the cell surface and cytoplasm in Hodgkin lymphoma and aggressive non-Hodgkin lymphomas [Murphy et al., 2010].

3. C*b*$_5$ AS AN ENZYME COFACTOR

C*b*$_5$ plays a pleiotropic role in metabolism acting as cofactor of many enzymes. For the sake of clarity, the role of C*b*$_5$ as enzyme cofactor will be reviewed following the outline indicated next:

(a) Enzymes of lipid metabolism
(b) Methemoglobin reductase activity
(c) Enzymes of xenobiotics detoxification
(d) Proteins containing a C*b*$_5$-like domain

3a. Enzymes of Lipid Metabolism

C*b*$_5$ is used by desaturases to generate an electron-deficiency, to activate oxygen species, and to remove electrons from the saturated hydrocarbon. C*b*$_5$ and its reductase have also been implicated in palmitoyl-CoA elongation in brain microsomes [Takeshita et al., 1985].

Polyunsaturated fatty acids (PUFA) synthesis is catalyzed by the so-called C*b*$_5$ fusion desaturases, since they contain an N-terminal domain orthologous to the microsomal C*b*$_5$ from mammals [Napier et al., 2003]. This is also the case of the moss *Physcomitrella patens* Δ^6-desaturase which is physically associated to its electron donor in the form of an N-terminal extension [Girke et al., 1998], an enzyme that later was found also in in mouse and humans [Cho et al., 1999]. A functional characterization done in *Primula* C*b*$_5$-fusion desaturases revealed that they have strong substrate specificity for α-linolenic acid (ALA; 18:3 n-3). The Δ^6-desaturase enzyme has the ability to work on

both C_{18} and C_{24} substrates (but not on any intermediate chain lengths) [de Antueno et al., 2001; D'Andrea et al., 2002], to catalyze the synthesis of docohexanoic acid [Sprecher et al., 1995]. A Δ^5-desaturase has been reported with a proximal relationship to the human Δ^6-desaturase [Leonard et al., 2000]. By heterologous expression in yeast, one of the ORFs was functionally characterized and identified to be the *Thraustochytrium* orthologue recognized as the Δ^5-desaturase, a member of the Cb_5 fusion desaturases being capable of specifically introducing a Δ^4-double bond into the C_{22} PUFA docosapentaenoic acid (DPA; 22:5 n-3) to yield docohexanoic acid. Other member of this family also include the Δ^8-sphingolipid long chain base (LCB) desaturase from higher plants and Fah1p in yeast and *C. elegans*, an enzyme that catalyzes the hydroxylation of the C_2 position of the very long chain (saturated) fatty acid moiety of sphingolipids [Napier et al., 2003].

The human *FA2H* gene encodes a fatty acid 2-hydroxylase that has been reported to contain a N-terminal Cb_5 domain and four potential transmembrane domains [Alderson et al., 2004]. This enzyme has certain homology with flavoctochrome b_2 and its iron-binding histidine motif is also conserved among membrane-bound desaturases/hydroxylases. On the other hand, C-4-hydroxylation requires an electron-transfer system that includes Cb_5 being degenerative spermatocyte 2 protein (Des2) the enzyme responsible for the hydroxylase activity [Enomoto et al., 2006]. The dihydroceramide:sphinganine C-4-hydroxylase requires the formation of a complex between Des2 and MCB5 (but not the soluble form of Cb_5) via their membrane-spanning domains and the electron transfer from NADH to the substrate via the reduction of MCB5 by cytochrome b_5 reductase [Enomoto et al., 2006].

Cb_5 has also been involved in the conversion of lanosterol to cholesterol and more specifically in the microsomal reaction catalyzed by the 4-methyl sterol oxidase where the C-30 methyl group of lanosterol is oxidized to a steroid -4α-oic acid which can subsequently be decarboxylated. The Cb_5-dependent desaturase called lanosterol *Δ^5desaturase introduces a double bond in lanosterol to produce* 7-dehydrocholesterol [Vergeres and Waskell, 1995]. The biosynthesis of steroid hormones is also modulated by Cb_5, through allosteric modulation of the 3β-hydroxysteroid dehydrogenase/Δ(5)-Δ(4) isomerase (3βHSD) activity resulting in an increase of the affinity of the enzyme toward NAD^+ [Goosen et al., 2013]. A differential modulation by OMB5 and MCB5 has been reported for the 17 α-hydroxylase and 17,20-lyase activities of P450c17 leading to production of 17 α-hydroxypregnenolone (17 α OH-Preg) and dehydroepiandrosterone (DHEA), respectively [Soucy and Luu-The, 2002]. Expression in testis of a cDNA encoding for the outer

mitochondrial membrane Cb_5 (*CYB5B* gene encoding for OMB5) and sharing a 58% of homology with type A Cb_5 gene (*CYB5A* gene encoding for MCB5) has been reported to elicit a stronger increase of the 17,20-lyase activities of P450c17 than that induced by type A Cb_5 [Soucy and Luu-The, 2002]. Later, it was studied the role of the two forms of porcine Cb_5, MCB5 and OMB5, in both the andien-β synthase as well as the 17α-hydroxylase and C17,20 lyase reactions by expression of *CYP17A1*, along with cytochrome P450 reductase, cytochrome b_5 reductase and Cb_5 in HEK-293FT cells [Billen and Squires, 2009]. In this study, the authors reported that an increase in the ratio of MCB5 to *CYP17A1* caused a decrease in 17α-hydroxylase, a transient increase in C17,20 lyase and an increase in andien-β synthase activity, while an increase in the ratio of OMB5 to *CYP17A1* decreased 17α-hydroxylase, but did not affect the andien-*β* synthase activity; however, the C17,20 lyase, was significantly increased. Moreover, immunohystochemical studies have suggested a regionalization of the expression of Cb_5 in the adrenal gland, which has been correlated with the regionalization of the synthesis of 19c steroids as dehydroepiandrosterone in this gland [Mellon, 2005].

In addition to cholesterol biosynthesis, Cb_5 has also been implicated in the formation of plasmalogen [Paltuaf et al., 1974]. Conversion of 1-alkyl-2acyl-*sn*-Gro*P*Etn to 1-alk-1'-enyl-2-acyl-sn-Gro*P*Etn is carried out by the presence the cytochrome b_5 reductase / Cb_5 system in addition to a cyanide-sensitive 1-alkyl desaturase [Snyder et al., 1985]. This result was demonstrated by the use of antibodies against Cb_5 that inhibited the synthesis of ethanolamine plasmalogen. As well as plasmalogen, Cb_5 has been involved in sialic acid metabolism, a metabolite required for the synthesis of gangliosides and other glycoproteins. Cb_5 reduced by cytochrome b_5 reductase forms a complex with the cytidine-5'-monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) hydroxylase, and acts as a cofactor for the CMP-Neu5Ac hydroxylase activity in the presence of oxygen [Gollub and Shaw, 2003; Kohla and Schauer, 2005].

3b. Methemoglobin Reductase Activity

A very important function of Cb_5 has been shown in erythrocytes where methemoglobin (MetHb) can act as the final acceptor of the electron carried out by reduced Cb_5 [Kitao et al., 1974; Kuma et al., 1976].

Cyanosis due to pulmonary and cardiac disease is one of the most common blood disorders. In erythrocytes, MetHb is kept by two systems at levels below 1% of the total hemoglobin [Wang et al., 2000; Kinoshita et al.,

2007]. One of them is the NADH: cytochrome b_5 reductase activity that transfers electrons to Cb_5. This pathway is responsible for more than 95% of the erythrocyte reducing capacity. The second system is formed by a NADPH-dependent flavin reductase pathway (FR) considered as a minor contributor to the reduction of MetHb, because its malfunction do not cause a MetHb reduction-deficient phenotype.

Cytochrome b_5 reductase reduces MetHb and the presence of Cb_5 greatly accelerates this reaction. The proposed mechanism of this reaction can be briefly summarized as follows: (1) NADH binds to the reductase and is transformed into NAD^+, reducing FAD to $FADH_2$; (2) then, the Cb_5-Fe^{3+} is reduced to Cb_5-Fe^{2+} coupled to oxidation of $FADH_2$ back to FAD; and (3) MetHb forms a bimolecular complex with reduced Cb_5 and MetHb is reduced to hemoglobin [Steinberg, 2009].

The complex between Cb_5 and MetHb has been generated by NMR and protein docking approaches [Poulos and Mauk, 1983]. For the α-chain of horse hemoglobin / bovine liver Cb_5 complex, the α-chain residues 56 (Lys), 60 (Lys), and 90 (Lys) interact with the Glu44, Glu43 and Glu60 residues on Cb_5, respectively. Lys61 is implicated in a hydrogen bond between a heme propionate from Cb_5 and a heme propionate from the α-chain. The contacts present in the β-chain/ Cb_5 complex involves hydrogen-bonding between the lysil residues 59, 61, 65, and 95 of the β-chain, and Cb_5 residues Glu48, Glu44, Glu43 and Asp60, respectively. As it happens with the α-chain, a hydrogen bond can be formed between the amino group of the Lys66 bridging the heme propionate from Cb_5 and the α-chain heme propionate [Poulos and Mauk, 1983].

The comparison of the complexes formed by Cb_5 with human MetHb reductase and with rat and corn nitrite reductase shows that there is a large main chain shift caused by a single-residue replacement from proline to threonine. A model of the complex between Cb_5 and the human reductase has been built and compared to the heme-containing domain of the nitrate reductase molecule. The interaction between cytochrome b_5 reductase and Cb_5 differs from that described for the nitrate reductase because of differences in the amino acid sequences [Bando et al., 2004]. Modeling analysis of cytochrome b_5 reductase has found 5 mutations key in the formation of a stable complex b_5-cytochrome b_5 reductase : Pro64Leu, Leu72Pro, Val105Met and Pro144Leu [Bando et al., 2004]. The type of electron transfer between Cb_5 and MetHb is the same by formation of an intracomplex electron transfer that it is characterized to be transient, with a low affinity complex formation [Xiong et al., 2009]. In these complexes water plays a double role in the

electron-transfer system, decreasing the tunneling barrier and inducing protein interface remodeling that screens the repulsion between the negatively charged propionates of the two hemes [Keinan et al., 2012].

3c. Enzymes of Xenobiotics Detoxification

Cb_5 acts as a cofactor in NADPH dependent cytochrome P450 monoxygenations, reviewed in [Schenkman and Jansson, 2003]. Antibodies against Cb_5 has been reported to produce an 80% blockade of the metabolism of benzo-α-pyrene and ethoxycoumarin, and to strongly inhibit laurate hydroxylation and the microsomal metabolism of testosterone and methoxyflurane. Interestingly, the metabolism of benzphetamine, ethylmorphine, aniline oxidation, and aminopyrine demethylation was not affected, the last three compounds when NADPH was used as a substrate [Schenkman and Jansson, 2003]. Furthermore, an increase of cytochrome P450-mediated metabolism of some xenobiotics in the presence of Cb_5 has been observed, but this was not extensive to all xenobiotics and metabolites that are putative substrates of the large family of cytochrome P450s, as Cb_5 did not stimulate the metabolic rate of all of them. An increase of the cytochrome P450 activities in the presence of Cb_5 has been shown for oxidation of 7-ethoxycoumarin and p-nitrophenatole by CYP2B4 and CYP1A2; benzphetamine demethylation, testosterone hydroxylation, aminopyrine demethylation, p-nitroanisole demethylation by CYP2C11; benzphetamine demethylation, 7-ethoxycoumarin demethylation, acetanilide hydroxylation, testosterone hydroxylation by CYP2B4. For the case of CYP1A2 and the metabolism of prostaglandin E1, E2 and A1, Cb_5 is a mandatory cofactor that enhances the activity [Schenkman and Jansson, 2003]. The steroidogenic cytochrome P450s CYP17A1is also activated by the presence of Cb_5 where it has been observed a buried CYP17A1 active site composed by residues Arg-347, Arg-358, or Arg-449 [Estrada et al., 2013]. Interestingly, apoCb_5 has also been shown to enhance the activities of CYP3A4, CYP2A6, CYP2C19, and CYP17A1 but not that of CYP2E1 or CYP2D6 [Peng and Auchus, 2013]. In addition, it has been noticed that some controversial data reported in the bibliography regarding modulation of cytochrome P450s by Cb_5 can be attributed to the order of addition of substrates and the presence of lipids [Peng and Auchus, 2013]. The potentiation by Cb_5 of the metabolism through cytochrome P450s catalyzed reactions could reflect a more efficient coupling of the system in the presence of Cb_5, which decreases the rate of collateral

reactions releasing non-productive superoxide anion and its dismutation metabolite hydrogen peroxide and results in an increasing rate of product formation [Schenkman and Jansson, 2003]. It has been demonstrated that Cb_5 promotes that the electron transfer reaction becomes faster than the competing reaction of superoxide anion diffusion out from the catalytic center, see for example [Gorsky et al., 1984; Schenkman and Jansson, 2003]. Indeed, formation of hydrogen peroxide has been used to monitor uncoupling of the cytochrome P450 system [Gorsky et al., 1984; Jansson and Schenkman, 1987; Locuson et al., 2007].

Recently, it has been identified a system implicated in N- hydroxylation of prodrugs through the molybdenum enzyme mitochondrial Amidoxime Reducing Component (mARC), cytochrome b_5 reductase and Cb_5 [Froriep et al., 2013]. mARC is formed by two isoforms mARC-1 and -2 (also known as MOSCO1 and MOSCO2). These proteins form a novel system that consists in three separate proteins where each mARC reduces N-hydroxylated compounds through the electrons provided by the cytochrome b_5 reductase/Cb_5 redox system. The reconstituted system produces the reduction of *N*-hydroxy-sulfonamides to sulfonamides like it happens for some prodrugs, e.g. the reduction of N-hydroxy-valdecoxib to valdecoxib, an inhibitor of COX2 activity [Havemeyer et al., 2010].

3d. Proteins Containing a Cb_5-like Domain

A family of proteins of diverse biological functions contains Cb_5-like domains, i.e. with the hemeprotein domain covalently bound to other redox protein domains [Lederer, 1994]. This is the case of multiple enzymes including lactate dehydrogenase [Guiard et al., 1974], sulfite oxidase [Ito, 1971] and nitrate reductase [Le and Lederer, 1983]. The aminoacid homology between Cb_5 and the heme binding region of bakers' yeast cytochrome b_2 [L-(+)-lactate dehydrogenase, EC 1.1.2.3] was shown by [Guiard et al., 1974]. This domain has a strong similarity with the sequence of microsomal Cb_5. Some enzymes involved in lipid metabolism like some "front-end desaturases" or also called Cb_5 fusion desaturases and the fatty acid 2-hydroxylase have also been defined as proteins containing a Cb_5 domains, as indicated above in section 3a of this chapter. Many of these proteins are directed to subcellular membranes through a specific signal-sequence tail that targets and anchors them to mitochondrial or endoplasmic reticulum membranes. The structure of the heme binding domain of the baker's yeast flavocytochrome b_2 or of lactate

dehydrogenase, remains similar to the one described for Cb_5, consisting in two cores on each side of a β-sheet (heme crevice). For both proteins heme is bis-His coordinated. The working mechanism of this type of proteins reported for sulfite oxidase can be used to illustrate the cooperation between redox centers in these proteins. Indeed, they can be seen as tight and efficient built-in redox chains. Briefly, after substrate oxidation two electrons are transferred from sulfite to the molybdenum center that are subsequently transferred to the Cb_5 domain in a two-step reaction and finally accepted by cytochrome *c* (Cyt *c*) as the final electron acceptor [Rudolph et al., 2003]. The molybdenum cofactor domains of nitrate reductase and sulfite oxidase are homologous [Lederer, 1994]. All members of the cytochrome b_5 family, exhibit a Pro-Gly-Gly motif (residues 41-43 in HSO b_5) at the top of the cleft that promotes the heme exposition to the solvent. The presence of a proline induces a twist in the peptide chain that separates it away from the heme group, producing the exposure of the propionate group due to the presence of two consecutive glycine residues [Rudolph et al., 2003]. This structure has been implicated in protein-protein interactions [Durham et al., 1995] and regulation of the redox potential of Cb_5 [Rivera et al., 1998]. The comparative analysis of a high-resolution structure of human sulfite oxidase cytochrome b_5 domain (HSO b_5) and of Cb_5 has revealed the physical-chemical features that account for the heme redox potential differences found between these proteins.

In addition to the role of Cb_5 as an electron acceptor in the molibdenopterin dependent sulfite oxidase, Cb_5 acts as an acceptor of flavoproteins like cytochrome b_5 reductase, nitrate reductase, cytochrome P450 reductase and flavocytochrome b_2. The final acceptor of the electrons taken up by Cb_5 reduction is Cyt *c* for the case of flavocytochrome b_2 and sulfite oxidase systems, and in nitrate reductase this final acceptor is Mo-pterin cofactor. In microsomal systems, cytochrome P450 [Henderson et al., 2013] and acyl-CoA desaturases [Oshino and Omura, 1973; Montgomery, 1976] are the electron acceptors from Cb_5.

The NAD(P)H cytochrome b_5 oxidoreductase (NCB5OR) is another example where a flavohemeprotein contains a cytochrome b_5-like domain [Zhu et al., 1999]. NCB5OR has been found in vertebrates and invertebrates and is expressed in a variety of cells and tissues. Because of its weak NAD(P)H oxidase activity, e.g. it has a very low k_{cat} value of ~0.05 s^{-1} and K_M ~12 mm(O_2) for this activity, and based on the experimental evidences showing that cytochrome *b*-type NAD(P)H oxidase could regulate the gene expression through oxidative modification of hypoxia-inducible factor 1, it has

been proposed that NCB5OR can act as an oxygen sensor within the cells [Zhu et al., 2004].

A novel family of proteins containing a cytochrome b_5-like domain is composed by the progesterone receptor membrane component 1 and 2 (PGRMC1 and PGRMC2), neudesin, and neuferricin, all containing a cytochrome b_5-like heme/steroid-binding domain and belong to the membrane-associated progesterone receptor (MAPR) family of proteins [Kimura et al., 2012].The putative membrane receptors for progesterone PGRMC1 and PGRMC2 are transmembrane receptors [Thomas, 2008; Thomas et al., 2009] for progesterone that differs from the nuclear receptors with genomic effects [Peluso, 2007; Kimura et al., 2013]. The activity of PGRMC1 and PGRMC2 is mediated by activation of mitogen-activated protein kinase (MAPK) signaling and intracellular Ca^{2+} increases [Kimura et al., 2012]. It has been shown that PGRMC1 binds and activates cytochrome P450 proteins [Min et al., 2005; Hughes et al., 2007] linking this protein to modulation of drug, hormones and lipid metabolism [Han et al., 2012]. Neudesin (neuron-derived neurotrophic factor; NENF) is a ~18KDa protein that was identified as a neurotrophic factor involved in neuronal differentiation and survival being its neurotrophic activity exerted via the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) [Kimura et al., 2013]. Neudesin is highly expressed in the central nervous system and could be involved in the rapid non-genomic actions of progesterone due to similarities to PGRMC [Kimura et al., 2013]. The structure of human neudesin has been obtained by NMR. It is formed by a structure containing 4 α-helices and 6 β-strands (β1-α1-β2-β3-α2-β4-α3-α4-β5-β6), being the heme-binding pocket predicted to be formed between α2 and α3 [Han et al., 2012]. The putative heme-binding domains is located from residues 45–143, in which Tyr-81 and Tyr-87 form the predicted binding site for the heme iron. It has been suggested a role of neudesin as a protein with ability to form a complex with progesterone suppressing the translocation of progesterone to the nuclear receptor in addition to a possible role as a progesterone receptor at the cell surface of the cells [Kimura et al., 2013]. As well as neudesin, another extracellular heme binding protein of ~18KDa with cytochrome b_5-like heme/steroid-binding domain has been recently identified and called neuferricin [Kimura et al., 2010]. Indeed, cytochrome b_5 domain 2 containing protein (CYB5D2 or neuferricin) belongs to the family of membrane-associated progesterone receptors (MAPRs) [Xie et al., 2011]. The expression of the mRNA has been mainly observed in the brain since the embryo stage and increases during development. In primary cultures of mouse neurons, it has been observed that recombinant mouse neuferricin

promote neurogenesis providing new insights about the function of heme-binding proteins as extracellular signal transmitters. In addition, neuferricin has been shown to improve HeLa cells survival after etoposide (ETOP)-mediated cytotoxicity [Xie et al., 2011]. Cytochrome b_5 domain-containing protein 1(Cyb5d1) (~18-26KDa**)** is a protein belonging to the PGRMC1 family with unknown function. There is not information about the function of this protein although there are some studies showing its role a as a survival gene reducing the malignant potency of breast cancer cells [Grade et al., 2006; Grade et al., 2007; Yasrebi et al., 2009].

4. CB_5 AS AN ELECTRON CARRIER IN REDOX CHAINS

Acidic residues of Cb_5 have been proposed as mediators for the complex formation with multiple protein partners in redox reactions catalyzed by Cb_5. The three-dimensional structure of the Cb_5/Cyt *c* complex has been resolved at atomic level. Glu44, Glu48, Asp60 and the heme propionate group of bovine Cb_5 have been shown to play an important role for the complex stability [Rodgers et al., 1988]. In soluble Cb_5 all residues are mobile except Glu43 that forms an intramolecular salt bridge with Arg47 constraining the mobility of both residues [Mathews and Czerwinski, 1985]. By direct mutagenesis, it was observed that modification of hydrophobic residues didn't have any effect on the binding between Cyt *c* and Cb_5 although the substitution of residues Phe35, Pro40, Val45, Phe58, and Val61, which provide the thermodynamic driving forces for hydrophobic-mediated interactions in this complex, resulted in changes in the efficiency of the electron transfer reaction [Ren et al., 2004].

The role of acidic residues surrounding the heme group of Cb_5 has been shown also to be critical for its redox coupling to other final electron acceptors. This is the case of proteins like MetHb [Poulos and Mauk, 1983], metmyoglobin [Livingston et al., 1985], cytochrome P450cam (CYP101) [Stayton et al., 1989] and cytochrome b_5 reductase [Meyer et al., 1995]. The residues of Cb_5 involved in complex formation with MetHb have been discussed in the previous section of this chapter. Strittmatter characterized the crosslinking of the complex formed by bovine cytochrome b_5 reductase and bovine Cb_5 and showed also the importance of Glu43, Glu44, Glu48, Glu56, Asp60 and the heme propionate for interfacial interactions [Strittmatter et al., 1990]. The interaction of human Cb_5 with human cytochrome b_5 reductase implicates charge-pair interactions between Lys-41, Lys-125, Lys-162, and Lys-163 of the enzyme, and Glu-47, Glu-48, Glu-52, Glu-60, Asp-64 (group

A), and heme propionate of Cb_5 [Shirabe et al., 1998]. In addition, flash photolysis studies with cytochrome b_5 reductase demonstrated that in the presence of NAD^+ there is an increase in the rate constants of formation and stabilization of the Cb_5/cytochrome b_5 reductase complex resulting in a complex more stable against dissociation at high ionic strength [Meyer et al., 1995]. Experiments done with cytochrome b_5 reductase mutants of Thr66, a critical residue for the one-electron transfer reaction catalyzed by this enzyme, showed that the ternary complex formed with NADH and NAD^+ is a major intermediate in the turnover and that the release of NAD^+ from this complex is the rate limiting step [Kimura et al., 2003].

Cb_5 is also part of the complex involved in aquabalamin reduction. This activity was described early in the presence of NADH or NADPH by Watanabe [Watanabe et al., 1992a; Watanabe et al., 1992b], who showed that partially purified Cb_5/cytochrome b_5 reductase and cytochrome c/ cytochrome P-450 reductase catalyzed aquabalamin reduction. These observations suggested that both enzyme systems could reduce cob(III)alamin as a secondary substrate, being responsible for inner mitochondrial membrane cob(III)alamin reductase activity that participate in the biosynthesis of adenosylcobalamin. Microsomal cytochrome P450 reductase was later shown to efficiently activate methionine synthase but only in the presence of soluble Cb_5 [Chen and Banerjee, 1998], catalyzing the formation of methylcobalamin (MS; 5-methylthetrahydrofolate (Me-H4folate): L-homocysteine S-methyltransferase, EC 2.1.1.13) with simultaneous production of methionine and tetrahydrofolate from homocysteine and 5-methylthetrahydrofolate, respectively [Banerjee and Matthews, 1990]. The authors suggested that either NADPH or NADH can serve as the electron donor for activation of porcine methionine synthase and that one of the two components in the electron transfer pathway is a microsomal component. This suggested that either cytochrome P450 reductase or cytochrome b_5 reductase were the redox partner of porcine methionine synthase [Banerjee and Matthews, 1990]. More recently, a second soluble human dual flavoprotein oxidoreductase NR1 homologous to methionine synthase reductase has been reported [Paine et al., 2000]. This homologous protein was fully activated in the presence of soluble Cb_5 to levels comparable to those seen for methionine synthase reductase [Olteanu and Banerjee, 2003].

Cb_5 as an electron carrier is also involved in fatty acid synthesis through modulation of microsomal cytochrome P450 activities. Reduced Cb_5 can act as an electron donor to reduce the cytochrome P450 ferrous-dioxygen complex, promoting the formation of ferryl-oxo cytochrome P450 [Bonfils et al., 1981;

Pompon and Coon, 1984]. This reductive process was competitive with decomposition of the protein complex through superoxide anion release and dismutation to hydrogen peroxide. The increase in the formation of the ferryl-oxo complex was dependent on Cb_5 and limited to 30%. This effect was observed during steroids oxidation by this system [Perret and Pompon, 1998]. Experiments done with some cytochrome P450s ($P450_{LM2}$ or $P450_{RLM5}$) in the presence of Cb_5, showed that there was a shift of the heme group to high spin in parallel to the increase of the affinity for substrates of the cytochrome P450s, pointing out that binding of Cb_5 altered the conformation of cytochrome P450 [Bonfils et al., 1981, Tamburini et al., 1985; Tamburini and Schenkman, 1987; Perret and Pompon, 1998]. Later, it was reported that Cb_5 inhibits electron transfer from the NADPH: cytochrome P450 reductase to the CYP2B4 isoform [Zhang et al., 2008]. This opposite functional modulation of different cytochrome P450 isoforms by Cb_5 has been proposed to correlate with the presence of two different types of Cb_5 orientations in the multimeric structures of the mixed oxidase system formed between cytochrome P450 reductase and different cytochrome P450 isoforms, reported more recently [Sulc et al., 2012].

Cb_5 can also act as an electron carrier of the 'so-called' redox-chain of the plasma membrane present in plant and mammalian cells, because cytochrome b_5 reductase has been shown to be a component of this redox chain [Villalba et al., 1995; May, 1999; Kim et al., 2002]. Moreover, the cytochrome b_5 reductase pool associated with plasma membranes is clustered within cholesterol-rich and caveolins-rich lipid rafts-associated sub-microdomains of the plasma membrane [Chatenay-Rivauday et al., 2004; Samhan-Arias et al., 2009; Marques-da-Silva et al., 2010]. Thus, the cytochrome b_5 reductase/ Cb_5 complex is a component of the 'so-called' endoplasmic reticulum-plasma membrane junction, which is also known to be associated with lipid rafts of the plasma membrane [Pani et al., 2008; Cahalan, 2009]. Interestingly, the soluble form of Cb_5 has been found to stimulate NADH-dependent cytochrome b_5 reductase activities associated with synaptic plasma membranes [Samhan-Arias et al., 2009], and increase of Cb_5 expression levels are associated with an increase in the production of superoxide anion and clustering of cytochrome b_5 reductase at the plasma membrane at the early steps of the neuronal apoptosis [Samhan-Arias et al., 2012]. Noteworthy, in the type II form of recessive congenital methemoglobinemia due to NADH-dependent Cb_5R deficiency, cyanosis is associated with severe mental retardation and neurologic impairment, and the enzymatic defect is systemic, involving both soluble and membrane-bound isoforms [Leroux et al., 1975;

Vieira et al., 1995]. On the other hand, as noted above in this chapter (section 3a) Cb_5 is a key element in cholesterol synthesis from lanosterol, being the reduced form of the heme protein essential in this metabolic step [Fukushima et al. 1981], and caveolins play a relevant role in cholesterol traffic within the cells [Yamauchi et al., 2007]. Indeed, cytochrome b_5 reductase is inhibited by antibodies directed against the amino terminal domain of caveolins [Samhan-Arias et al., 2009]. Caveolins binding site to cytochrome b_5 reductase is close to the NADH binding site of this flavoprotein and this suggests a possible regulatory role of the enzyme activities of cytochrome b_5 reductase by its interaction with caveolins [Samhan-Arias et al., 2012].

5. CB_5 IN ROS SIGNALING AND APOPTOSIS

Cb_5 as all *b* type cytochrome can be considered as anionic peroxidases [Kiel, 1995], although cytochrome *b*s in general show very weak peroxidase activity due to self-bleaching. The reason of this weak peroxidase activity could be generation of an abortive compound II yielding hydroxyl radical that could destroy the heme [DeFilippi et al., 1979; Xu et al., 1993].

Autoxidation of cytochrome *b* can lead to the generation of superoxide anion and hydrogen peroxide, and later the hydroxyl radical, although only three types of cytochrome *b*-containing proteins have been proposed as terminal oxidases, namely, erythrocytic green hemeprotein, NADPH oxidase and nitric oxide synthase [Kiel, 1995].

The hypothesis of a role for Cb_5 in apoptosis was postulated by Davydov based on the experimental evidences pointing out that Cyt *c* and Cb_5 can form a complex [Davydov, 2001]. In this review, it was hypothesized that after induction of the apoptotic stimulus and release of Cyt *c* from mitochondria, Cyt *c* would interact with Cb_5 forming a complex that blocks the interaction of Cyt *c* with other proteins like Apaf or caspase-9. Because of the high affinity for the interaction between both cytochromes, dissociation constant of the Cb_5/Cyt *c* complex ~10^{-7}M [McLean and Sligar, 1995; Sun et al., 1999], Cb_5 seems to be one of the major targets for Cyt *c* after being released from mitochondria.

In addition, the presence of cytosolic Cyt *c* should also be expected to affect microsomal monoxygenase activities due to competition of cytochrome P450 for association with Cb_5. As indicated above, the interaction of cytochrome P450 with Cb_5 increases the coupling between electron transfer and microsomal monoxygenase activities. Therefore, the presence of Cyt *c*

would hamper this interaction and therefore produce a ROS burst associated to electron leakiness through the microsomal cytochrome P450 monoxygenase systems. The level of microsomal monoxygenase activity and their role in electron transfer are normally underestimated, although microsomal monoxygenases components (including cytochrome P450s, Cb_5 and their reductases) account for at least half of the electron transfer proteins in liver cells [Archakov, 1975; Venditti et al., 1998]. Moreover, the later discovery of activation of a inner peroxidase activity of Cyt *c* during apoptosis due complex formation with anionic lipids like cardiolipin, phosphatidylserine and also phosphatidylinositol [Jiang et al., 2004; Kagan et al., 2005; Kapralov et al., 2007], should further potentiate further the pro-oxidant effect of Cyt *c* on the microsomal oxygenase activity. The redox status and the presence of cellular lipophilic antioxidants like tocopherol are key to stop this reaction [Samhan-Arias et al., 2011]. Additionally, it was early described that at the outer surface of the mitochondria there is an NADH oxidase activity catalyzed by cytochrome b_5 reductase and Cb_5 that could work coupled to the inner mitochondrial membrane cytochrome *c* oxidase activity using intermembrane Cyt *c* as an electron shuttle [Nicholls et al., 1969; Bodrova et al., 1998], being this activity increased after addition of exogenous Cyt *c* to the assay when purified mitochondrias are used. The authors also suggested that Cyt *c* itself could be responsible for the oxidation of NADH when Cyt *c* is released from mitochondria rather than solely modulate the NADH:cytochrome *c* reductase activity. A role of Cyt *c* in detoxifying endogenous hydrogen peroxide by the presence of NADH has also been proposed recently, producing an increase in the formation of superoxide radical [Velayutham et al., 2011]. In addition, it has also been reported the stimulation by Cyt *c* of NADH consumption catalyzed by cytochrome b_5 reductase [Bobba et al., 1999], which was proposed to support the hypothesis that a moderate release of Cyt *c* could contribute to maintaining adequate levels of cellular ATP via direct reduction of Cyt *c* by cytochrome b_5 reductase through an electron shuttle with cytochrome *c* oxidase [Skulachev, 1998].

A role of Cb_5 in the attenuation of lipid radical cycles as well as in the scavenging of lipid radical reactions in biological membranes has been proposed [Dmitriev, 2007]. It has been shown that Cb_5 can act as electron donor for reduction of lipid peroxyl radicals (LOO·) via protein-lipid interactions [Dmitriev, 1995; Dmitriev, 1998], resulting in the chemical reaction:

$$Cb_5\,(\text{reduced}) + LOO^{\cdot} \rightarrow Cb_5\,(\text{oxidized}) + LOO^{-}$$

Therefore a key role of cytochrome b_5 reductase in protection against lipid peroxidation in cells has been suggested [Dmitriev, 1998; Dmitriev, 2007]. This protective role can be important in pathophysiological phenomena like aging in liver, where it has been shown a drop of Cb_5 [Plewka et al., 1998]. A *in vivo* form of NCb5OR and Cyb_5r4 (b_5+b_5 reductase) have been shown more recently to protect against endoplasmic reticulum stress-induced lipotoxicity [Zhang et al., 2010]. These findings support the previously suggested role of the cytochrome b_5 reductase/Cb_5 system against lipid peroxidation. On these grounds, it has been suggested that inhibitors of cytochrome b_5 reductase may abolish the cooperation between outer and inner membranes and, therefore, stimulate mitochondrial lipid peroxidation [Dmitriev, 2007]. Therefore, several conditions and treatments that modulate Cb_5 levels could collaterally modulate this function in cells. For example, an increase in Cb_5 levels has been shown during hypothyroidism and after treatment of patients or animals with propylthiouracil, carbon tetrachloride, p-nitroanisole, malotilate and griseofulvin, whereas ethanol consumption in hamsters produce a decrease in Cb_5 levels [Schenkman and Jansson, 2003].

As noted in section 4 of this chapter, part of the neuronal cytochrome b_5 reductase pool is associated with lipid rafts of the plasma membrane, and its deregulation is largely responsible for the early superoxide anion overshot that precedes the activation of caspases in cerebellar granule neurons apoptosis [Valencia and Moran, 2001]. Moreover, the increase of superoxide anion production by cytochrome b_5 reductase in this neuronal model of apoptosis correlated with the increase of the expression level of soluble Cb_5 [Samhan-Arias et al., 2012], which stimulates the cytochrome b_5 reductase associated with synaptic membranes [Samhan-Arias et al., 2009]. Noteworthy, the redox properties of brain Cb_5 have been reported to be different from those of Cb_5 isoforms found in other tissues [Yoshida et al., 1984]. The measurements of redox potential done in this work highlighted the presence of two pools of microsomal brain Cb_5, one with a redox potential near 50 mV and another with a redox potential of ca. -30mV. As brain tissue is a complex tissue, containing many types of neurons, microglia and a large network of capillar-size blood microvessels, the expression of different Cb_5 isoforms in neurons deserved to be demonstrated. The presence of the cDNAs encoding for a soluble and for a membrane-bound isoform of Cb_5 in isolated neuronal and glial cultures was shown in [Yoo, 1999], and this has been recently confirmed by our group using primary cultures of cerebellar granule neurons from rat tissue [Samhan-Arias et al., 2012]. Indeed in this publication, we found that the ratio between the expression of soluble and membrane isoforms of Cb_5 changed during the

early stages of the apoptosis of these neurons, i.e. before Cyt *c* release form mitochondria and loss of mitochondrial membrane potential and well before the activation of caspases. On these grounds, we have proposed a function of soluble Cb_5 associated with deregulation or uncoupling of the plasma membrane-bound cytochrome b_5 reductase, which shows a marked clustering within plasma membrane lipid rafts in primary cultures of mature cerebellar granule neurons [Samhan-Arias et al., 2009; Samhan-Arias et al., 2012].

The molecular mechanisms that control the expression levels of Cb_5 remain to be established. Cb_5 gene has two promoters and 1 silencer segment of the 5'-terminal region of the gene [Li et al., 1995]. The gene is formed by six exons, including a non-functional exon 4 and a span of about 28 kb. Since this is a housekeeping gene in the 5' region a TATA box is absent, but two CAAT boxes and several G:C-rich SpI motifs are present [Cristiano et al., 1993]. The expression of Cb_5 gene has been reported to be under regulation of steroid hormones. Transcription of the gene is regulated by Sp3, GATA-6, and Steroidogenic Factor 1 in human adrenal NCI-H295A cells [Huang et al., 2005]. In cells were silencing of the methylase DNMT3b has been performed using siRNA, it has been observed an increase of apoptosis, decreased growth and migration in parallel to an increase in the expression of Cb_5, caspase-7 and the kinase CDKN3, suggesting that the promoter of Cb_5 gene could be under control of methylation/demethylation [Yaqinuddin et al., 2008]. In addition, the increase of the Cb_5 gene expression is a common feature between this apoptotic process and the apoptosis of cerebellar granule neurons (see above).

CONCLUSION

Cb_5 acts within the cells as a pleiotropic co-factor of multiple enzymes and redox chains that play critical roles for normal function of healthy mammalian organisms, and thus it is a potential biomarker of health and disease. It is maintained in the reduced form largely by the NADH-dependent cytochrome b_5 reductase activity which catalyzes electron transfer to Cb_5 in mammalian cells. In mammalian cells Cb_5 shows a ubiquitous distribution, being present in the cytoplasm and also associated with the components of the endoplasmic reticulum, mitochondria and plasma membrane of mammalian cells. Subcellular compartmentation of Cb_5 also modulates its redox properties, as the redox potential of the heme group of Cb_5 is largely dependent of the spatial conformation of this group within the protein structure

and also of the dielectric constant of the microenvironment around the heme group.

The implication of Cb_5 in the modulation of multiple metabolic pathways, such as cholesterol biosynthesis, desaturation and elongation of fatty acids, MetHb reduction and cytochrome P450-dependent reactions of steroids and xenobiotics metabolism, points out a key role of Cb_5 in the maintenance of healthy intracellular metabolic homeostasis in mammals. More recently, Cb_5 has been suggested to be involved in the recycling of lipid peroxyl radicals and also to modulate the methionine synthase reductase activity. In addition, the family of proteins containing a Cb_5-like domain in mammals is expanding, highlighting the relevance of Cb_5 in biological evolution and opening new perspectives on modulation of cellular signaling pathways by b_5-type hemeproteins.

As a co-factor of cytochrome b_5 reductase, Cb_5 has been shown to act as an electron carrier in mitochondria and in the plasma membrane coupled to cytosolic NADH consumption. In addition, as a redox co-factor of cytochrome P450s/cytochrome P450 reductase systems and of cytochrome b_5 reductase Cb_5 is also implicated in the modulation of the production of superoxide anion in mammalian cells. Since deregulation of superoxide anion production can elicit a cellular oxidative stress leading to activation of caspase-9 by Cyt *c*, the finding that Cb_5 binds Cyt *c* with high affinity suggested a role for Cb_5 in apoptosis. Indeed, in a well established model for neuronal apoptosis the time course of the increase of superoxide anion and of the increase of the content of soluble Cb_5 are correlated events during the early stage of apoptosis [Samhan-Arias et al., 2012]. Interestingly, several proteins containing a Cb_5-like domain in mammals are NAD(P)H oxidoreductases. Therefore, Cb_5 can also be seen as an intracellular signaling molecule in the field of redox biology, aging and related diseases.

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