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**REVIEW**

# Cardiolipin in Energy Transducing Membranes

**E. Mileykovskaya*, M. Zhang, and W. Dowhan**

*Department of Biochemistry and Molecular Biology, University of Texas-Houston, Medical School, Houston, TX 77030, USA; fax: (1-713) 500-0652; E-mail: Eugenia.Mileykovskaya@uth.tmc.edu*

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**Abstract**—Cardiolipin is a phospholipid located exclusively in energy transducing membranes such as the bacterial cytoplasmic membrane and the inner membrane of mitochondria. It plays both a structural and a functional role in many multimeric complexes associated with these membranes. The role of cardiolipin in higher order organization of components of the mitochondrial respiratory chain revealed by a combined molecular genetic and biochemical approach is described.

*Key words*: cardiolipin, supercomplex, yeast mitochondria

The ability of lipids to form subdomains of unique protein and lipid composition provides a mechanism to regulate and compartmentalize enzymes within a biological membrane (for review see [1]). In 1974, V. P. Skulachev and his coworkers suggested that the phospholipid cardiolipin might participate in linking together components of the respiratory chain [2]. This idea was recently experimentally confirmed in our studies and by others [3, 4].

## STRUCTURAL AND FUNCTIONAL PROPERTIES OF CARDIOLIPIN

Cardiolipin (CL), also called diphosphatidylglycerol, was first discovered in beef heart in 1942 [5]. It is located exclusively in the energy-transducing membranes of bacteria and mitochondria. CL differs from all other phospholipids by its dimeric structure, with two headgroups carrying two negative charges and four fatty acyl chains. In animals and higher plants, they almost exclusively contain polyunsaturated fatty acids with 18 carbons (C18:2), while in yeast *S. cerevisiae* they are 16 and 18 monounsaturated chains. Bacterial CLs contain monounsaturated and saturated fatty acids with 14 to 18 carbons [6]. In mammalian and yeast cells, CL fatty acyl chains can be remodeled by phospholipid transacylation, the aberrant process of which is the molecular basis for Barth Syndrome in humans [7]. Despite the apparent symmetry of structure, CL has two chemically distinct phosphatidyl moieties since two chiral centers exist, one in each outer glycerol group, producing diastereomers, such as R/S, S/R, S/S, and R/R. Natural CL is in the R/R configuration. As a result, the two phosphate groups, designated as 1′-phosphate and 3′-phosphate with respect to the central glycerol, have different chemical environments [6].

Many protein complexes involved in oxidative phosphorylation and photophosphorylation contain CL in their quaternary structure and require CL for functional activity (for reviews see [1, 6, 8, 9]). CL is observed in the structure of bacterial reaction centers [10], *Escherichia coli* succinate dehydrogenase [11], $bc_1$ complex in yeast (see below) [12], and *E. coli* formate dehydrogenase-N [13]. Mammalian cytochrome *c* oxidase [14] and ATP/ADP carrier [15] contain tightly bound CL critical for structure and function. CL is the only phospholipid determined in the X-ray crystal structure of formate dehydrogenase-N, the physiological trimer of which is a major component of the *E. coli* nitrate respiration system. CL was identified at the trimer interfaces and is proposed to be essential for the trimer formation [13]. CL dependent dimeric organization of *Rhodopseudomonas viridis* reaction centers was suggested on the basis of spectroscopic and biochemical evidences and predicted by modeling studies [16]. This suggestion is also supported by CL localization at the protein–protein interface in the lipidic cubic phase crystal structure of the *Rhodobacter sphaeroides* reaction center [17].

* To whom correspondence should be addressed.

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## SUPERCOMPLEXES IN THE MITOCHONDRIAL MEMBRANE

The inner mitochondrial membrane of mammals contains the four major complexes I-IV (NADH dehydrogenase, succinate dehydrogenase, $bc_1$ complex, and cytochrome oxidase, respectively) of the standard respiratory chain and the $F_oF_1$-ATP synthase (complex V). Organization of the complexes of the respiratory chain structurally and functionally as one unit, so called "respirasomes", was originally formulated by Chance [18]. However later, when active redox enzymes were purified and reconstituted into liposomes, his model was gradually changed and finally substituted by the random collision model [19]. In accordance with the later, individual complexes of the respiratory chain are independently imbedded in the lipid bilayer and connected by randomly diffusing small carriers, CoQ and cytochrome *c*. Although this model is included now in all textbooks, active stoichiometric assemblies of individual complexes were demonstrated experimentally by using blue-native electrophoresis (BN-PAGE) after mild solubilization of mitochondria with digitonin [20]. On the basis of these experiments it was recently proposed that the complexes of respiratory chain in mammals are organized as large assemblies constituting a supramolecular network ("respirasome") consisting of two supercomplexes ($I_1III_2IV_4$ and $III_2IV_4$) [20]. A similar organization has been reported for plant mitochondria [21]. In mitochondria of the yeast *Saccharomyces cerevisiae*, which possesses no complex I, but instead two peripheral NADH dehydrogenases (Nde1, external, and Ndi1, internal), only complexes III and IV are assembled into the supercomplexes $III_2IV_1$ and $III_2IV_2$ as revealed by BN-PAGE [22, 23]. In addition, kinetic analysis of yeast mitochondria respiration also supports organization of the yeast respiratory chain into one functional unit [24]. It was suggested that supercomplexes physiologically exist in equilibrium with individual complexes. Thus, electron transfer in the respiratory chain would be through either substrate channeling or random collisions mechanisms depending on metabolic conditions.

## ROLE OF CARDIOLIPIN IN THE SUPERCOMPLEX ORGANIZATION

The yeast model was successfully used to study of the organization of a supercomplex composed of complexes III and IV [22, 23]. Complex III ($bc_1$) in yeast is a homodimer of 10-subunit monomers [25] and Complex IV (cytochrome oxidase) is a homodimer of 12-subunit monomers [26]. A single CL molecule is bound between the cytochromes *b* and $c_1$ of the 10-subunit monomer [12]. CL is positioned in close proximity to the quinone reduction site facing the mitochondrial matrix. This site does not open directly to the matrix solvent, suggesting the requirement for a proton wire for entrance of a proton. CL is positioned at one of two possible proton paths and is proposed to conduct the proton from the bulk solvent to the quinone headgroup [12]. Importantly, mutants in the residues to which CL is bound grew progressively slower with increasing numbers of alterations in the amino acids. It was suggested that CL plays a specific structural and functional role in the cytochrome $bc_1$ complex [12]. There is no structural data available on CL in the yeast complex IV. However, one can expect analogy with the mammalian cytochrome *c* oxidase complex, which contains tightly bound CL essential for structure and function of the complex [14].

A powerful approach to study the lipid requirement for cell processes in general and lipid–protein interaction in particular is genetic manipulation of viable bacterial and yeast mutant strains, in which phospholipid composition can be systematically altered [1]. We developed a "biological reagent", in which the CL content of yeast mitochondria could be regulated *in vivo* [3]. A low-copy "tet-off" plasmid system was employed to exogenously regulate a plasmid copy of the *CRD1* gene (the gene for CL synthase) in a *crd1*Δ background. In this system exogenous addition of the repressor doxycycline to the growth medium showed a dose dependent reduction of CL levels with a parallel increase in the level of phosphatidylglycerol, the immediate precursor of CL. Mutant (*crd1*Δ) cells, which in glucose media did not exhibit any alterations in their phenotype, grew considerably slower and to a lower final density on non-fermentable carbon source than wild type (*CRD1*) cells. Importantly, cells with intermediate CL content displayed growth phenotypes intermediate between wild type and the *crd1*Δ mutant demonstrating the direct dependence of the efficiency of the energetic system on the level of CL [3].

Lack of both CL and phosphatidylglycerol results in more severe phenotypes. Mutants with no phosphatidylglycerol (*pgs1*Δ mutants) or CL do not grow on non-fermentable carbon sources since they fail to synthesize subunits of complex IV (Cox1p, Cox2p, Cox3p, Cox4p) and the Cobp subunit of complex III [27]. CL lacking strains (*crd1*Δ) at 30°C have the normal steady state levels and the same ability to synthesize these subunits as wild type cells [28].

In agreement with previous results [22, 23], digitonin extracts of mitochondria isolated from *CRD1* cells subjected to BN-PAGE showed exclusively a $III_2IV_2$ supercomplex. On the other hand, extracts of mitochondria from *crd1*Δ cells showed 90% of the individual homodimers of complexes III and IV not organized into a supercomplex. Remarkably, digitonin extracts from cells with intermediate levels of CL contained a mixture of individual homodimers of complexes III and IV and $III_2IV_2$-supercomplex in a proportion of about 30 : 70 demonstrating that the formation of supercomplex was dependent on CL in a dose-response manner [3]. We sug-

gested that CL is required for high affinity association of complex III and complex IV for each other and cannot be effectively substituted by phosphatidylglycerol [3].

Although BN-PAGE analysis was developed to study native assembly of mitochondrial complexes, the possible dissociation of loosely bound subunits caused by the dye in the absence of CL was suggested. Colorless native (CN)-PAGE provides a safer way to identify membrane protein complexes since there is no introduction of anionic dye during the process [29]. Therefore, this more gentle technique was employed. In comparative study [4], no significant amount of the supercomplex was detected in mitochondria lacking CL (but with an increased level of phosphatidylglycerol) using BN-PAGE, but the supercomplex was almost exclusively found in the mutant lacking CL when CN-PAGE was employed. It was suggested that anionic dye induced dissociation of the loosely bound complexes III and IV in the absence of CL, and that the supercomplex is still exists in the mutant mitochondria but that CL is essential for its stabilization. On the other hand, since no detergent was introduced during gel separation, and highly hydrophobic membrane complexes migrate through the native gel by their own intrinsic charges, the possibility of aggregation between the complexes was not excluded. However, no supercomplex in mitochondria lacking CL was resolved using CN-PAGE analysis when levels of digitonin lower than that used to solubilize mitochondria were added in the gel [30].

These results strongly indicate that CL plays a central role in the association between complexes III and IV. A combination of a structural approach with kinetic studies further supported this conclusion [30]. Under physiological conditions, a linear relationship between respiratory activity and inhibitor antimycin concentration for wild type mitochondria was observed consistent with a single functional unit model of the electron transport chain of *S. cerevisiae* [24, 30]. However, we found that CL-lacking mitochondria displayed a hyperbolic relationship indicating that cytochrome *c* interacts with complexes III and IV as a freely diffusible carrier and the functional III–IV-supercomplex does not exist in the mutant [30]. The presence of phosphatidylglycerol may be sufficient to support activity and a weak association of the homodimers, but it is not efficient in formation of stable supercomplex.

What special properties provide CL the ability to facilitate a specific interaction between complexes III and IV? X-Ray crystal structures of several multimeric membrane protein complexes strongly support a role for lipids as a deformable rather than a rigid interface between subunits of these larger complexes [31]. X-Ray crystallography has been used to examine the structural details of an interaction between CL and the photoreaction center, where CL was for the first time resolved [10]. X-Ray diffraction data showed that binding of the lipid to the protein involved a combination of ionic interactions between the protein and the lipid headgroup and van der Waals interactions between the lipid tails and the electroneutral intramembrane surface of the protein. Essential in this interaction is the ability of phospholipid to fill cavities and grooves between hydrophobic interfaces of the protein located within the membrane bilayer while providing specific ionic bridges at the water–hydrophobic interface. Deformability of this "cushion" between subunits would allow greater movement at the interface. CL, with unsaturated fatty acids, is especially suited for this role with four twisted hydrocarbon domains and a negatively charged hydrophilic domain [13, 31].

It has been suggested that the cavity within the membrane imbedded domain of complex III formed by a transmembrane helix of cytochrome $c_1$ and three transmembrane helices of cytochrome *b* can interact with the core components of complex IV, namely Cox1p, Cox2p, and Cox3p, to form a supercomplex [20]. We hypothesized that CL may fill this cavity and provide a flexible amphipathic linkage between the above subunits of complexes III and IV [3]. Molecular modeling of CL in complex III in the putative site of its interaction with complex IV was performed [4]. The primary attractive forces appear to be hydrophobic interactions since detergents dissociate the supercomplex at low ionic strength [22]. This is consistent with an observation that some supercomplex can be detected in cells lacking CL but with elevated phosphatidylglycerol that might facilitate low affinity interactions.

## FROM SUPERCOMPLEXES TO SUPRA-MOLECULAR ARCHITECTURE OF MITOCHONDRIAL MEMBRANES

The important factor for formation of supercomplexes is a high protein density of the mitochondrial crista membrane. Experimental data show that around 94% of Complex III and the ATP synthase are located in crista membranes and only around 6% of these complexes are located in the inner boundary membrane [32]. It was suggested that diffusion of complexes between the crista and inner boundary membrane may be restricted by formation of crista junctions, which serve as a barrier for diffusion between the two membranes [33]. According to Frey et al. [34], the crista junction is a thermodynamically favorable arrangement of the mitochondrial inner membrane, which might mediate compartmentalization of complexes. As a result integral membrane proteins cover up 50% of the total membrane area. The center to center distance between energy-transducing proteins may be well within the range of intermolecular forces which could cause aggregation of complexes [35]. It was suggested that the clustering of supercomplexes would depend on the energetic state of mitochondria since tight relationships exist

between the respiratory function and the structural changes [36].

Interestingly, a change in the respiratory state induced changes in CL distribution in the mitochondrial membrane as shown by experiments with the CL-specific dye Nonyl Acridine Orange (NAO). A measurement of the red shift in the emission spectra of NAO has been used for decades as a tool for CL detection in mitochondria. There are several models explaining specificity of NAO–CL interaction [37-39]. It was reported that the fluorescence of NAO due to interaction with CL is significantly modified by factors that control the spatial arrangement of CL molecules within artificial membranes [39]. It was also demonstrated that in isolated respiring mitochondria NAO fluorescence is related to the respiratory state of mitochondria but does not depend on the membrane potential. The high dependence of NAO emission on mitochondrial energetic state was interpreted as re-localization of the CL in the crista membrane [39, 40].

The suggestion that CL might play an essential role in cristae morphology was made in studies of mitochondria of unfed amoebae (*Chaos carolinensis*). Electron tomography demonstrated that under these conditions mitochondrial cristae were converted from long narrow tubular invaginations into membrane structures of highly ordered cubic crystalline form [41]. It was found that fasting strongly increased accumulation of $H_2O_2$ and reactive oxygen species in the amoeba [42]. The authors suggested that highly ordered internal architecture of mitochondria was a protective response to oxidative stress and CL might play an essential role by changing curvature of the membrane. Another possibility is that CL participates in protein–protein contacts resulting in formation of highly ordered membrane structure. From this standpoint, it is important to mention that CL mediates an important crystal contact between two symmetry related protein molecules in the lipidic cubic phase crystal structure of the reaction center from *Rhodobacter sphaeroides* [15]. It is well established that the content of CL in the membrane becomes a critical factor for mitochondrial function under different stress conditions (low and high temperature, stationary phase of growth, low substrate level, oxidative stress) [6].

Thus, one can suggest a specific and unique role of CL in the organization of ordered membrane structures in response to different metabolic or stress conditions.

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