Minireview

Cardiolipin: a proton trap for oxidative phosphorylation

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Abstract The role of specific lipid structures in biological membranes has been elusive. There are hundreds of them in nature. Why has nature made them? How do they aid in the functioning of membrane proteins? Genetics with its 'knock out' organisms declares that functions persist in the absence of any particular lipid. Nonetheless some lipids, such as cardiolipin (CL), are associated with particular functions in the cell. It may merely expand the variety of culture conditions (pH, temperature, etc.) under which the wild-type organism survives. This article explores a unique role of CL as a proton trap within membranes that conduct oxidative phosphorylation and therefore the synthesis of ATP. CL's pK_2 (above 8.0) provides a role for it as a headgroup proton trap for oxidative phosphorylation. It suggests why CL is found in membranes that pump protons. The high pK_2 also indicates that the headgroup has but one negative charge in the neutral pH range. Data on the binding of CL to all of the oxidative phosphorylation proteins suggest that the CL may aggregate the oxidative phosphorylation proteins into a patch while it restricts pumped protons within its headgroup domain – supplying protons to the ATP synthase with minimal changes in the bulk phase pH. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

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1. Introduction

Biological membranes are rich with a wide variety of the lipid structures. Unlike other constituents of cells, such as amino acids, sugars and nucleic acid bases, their roles as individual molecules are not clear. Some cellular signals are made by enzymatic action on membrane lipids. However, up to one hundred different lipids, not including those lipids that participate in signaling, may be found in the same membrane. Presumably these lipids have specific functions. Occasionally a particular lipid has been identified with a species, with organelles, or with biochemical processes. Cardiolipin (CL) is unique among phospholipids with its four chains. It has been associated with oxidative phosphorylation. A role for it in that process is now emerging.

Remarkable success in the past decade has increased our understanding of the mechanisms of oxidative phosphorylation. Studies on the movements of electrons and of protons through the membrane proteins that conduct this nearly ubiquitous process for making ATP has culminated in obtaining high resolution atomic structures of the key enzymes [1,2]. As the details of the molecular mechanisms of membrane proteins come into focus, our ignorance of the role of lipids, and in particular of CL, stands in stark contrast.

CL, the first phospholipid to be chemically characterized [3,4], has long been associated with the mitochondrion and specifically with the proteins that conduct oxidative phosphorylation. In eukaryotes it is the only lipid that is synthesized in the mitochondrion, where it remains throughout the life of the cell. It presumably has some specific function within the mitochondrion. During the isolation of proteins from the mitochondrion CL has been shown to co-isolate with each of the proteins that participate in oxidative phosphorylation. This includes cytochrome oxidase [5], where it also plays an optimal role in its functioning [6]; in the ATP/ADP exchange protein [7], where its absence decreases the membrane potential [8]; the F_0F_1 ATP synthase [9], where it may also play a role for its optimal functioning [10,11]; the orthophosphate transporter [12]; and the cytochrome bc_1 complex [13]. Two recent reviews are available on CL, its distribution, biochemistry and its association with oxidative phosphorylation [14,15].

No mitochondrial proteins that are not engaged in oxidative phosphorylation co-isolate with CL. Certain proteins not engaged in oxidative phosphorylation in the inner mitochondrial membrane are known to bind phosphatidyl choline (PC). β-Hydroxybutyrate dehydrogenase, a ketone body enzyme, has been shown to depend on PC for its activity [16]. A recent study of a PC photo-labeled analog has been shown to specifically label glycerol-3-phosphate dehydrogenase [17]. In the prokaryote world, CL is identified with eubacteria that conduct oxidative phosphorylation. Such data might suggest that the lipid is required for oxidative phosphorylation, nonetheless there are prokaryote [18] and yeast [19] cardiolipin-less (*cl*-) mutants. These mutants illustrate that oxidative phosphorylation does not *require* CL, nonetheless it appears to play a role that can be replaced by other lipids under the culture conditions used for these cells. It may merely expand the variety of culture conditions (pH, temperature, etc.) under which the wild-type organism survives. We will review the

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Abbreviations: CL, cardiolipin; dCL, 2′-deoxy-cardiolipin; *cl*-, cardiolipin-less; NAO, nonyl acridine orange; PC, phosphatidyl choline

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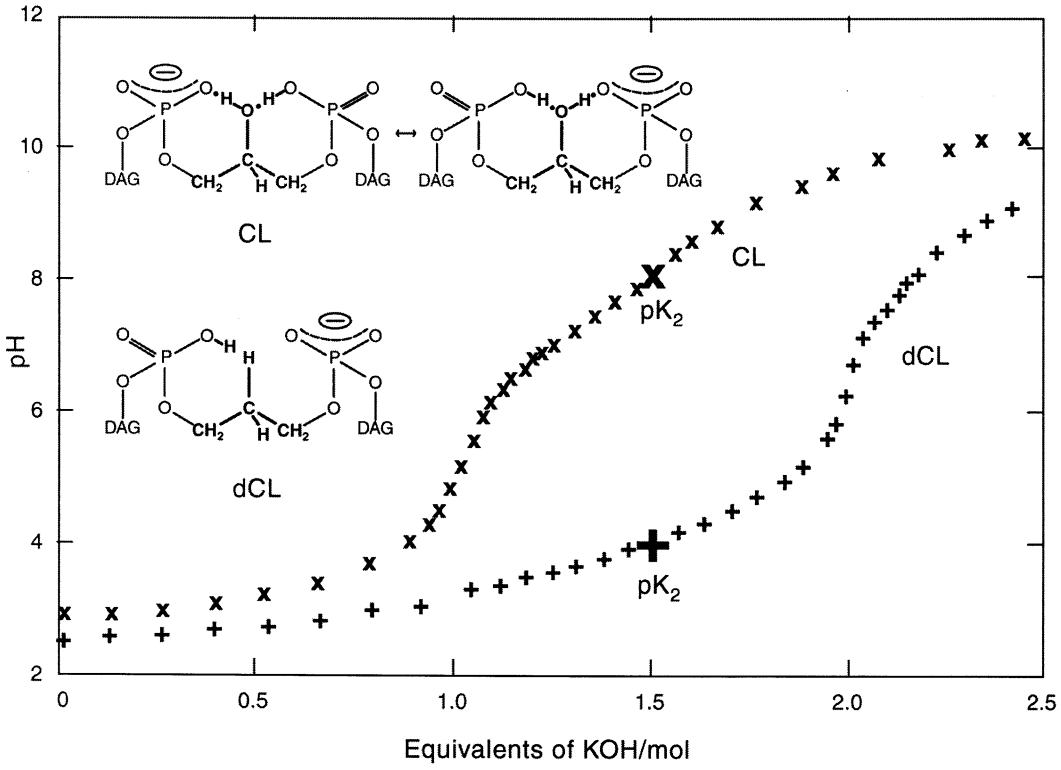

Fig. 1. Titration of CL. CL is titrated with KOH [21] to establish its pK_2. The titration indicates that the pK_2 is above 8.0. The high pK is attributed to the 'acid-anion' structure in the scheme above the titration. The structure, stabilized by the resonance of the single electron between the two phosphates, displays two unexpected features of CL. First, the two phosphates form a small bicyclic structure above the two *sn*-1 chains of the two diacyl moieties below the headgroup. Second, the phospholipid has a single charge per molecule throughout the neutral pH range. Because the pK is a conformational pK, it moves as the titration proceeds [21]. That this unusual pK is due to the hydroxyl on the connecting glycerol is demonstrated by the titration of dCL. Its structure is below the titrations on the right. Taken together these titrations can only be explained by the acid-anion structures of Westheimer and Banfey [22]. The titrations show that the hydrogens in the H-bonds between the phosphate oxygens and the glycerol hydroxyl are vibrating; thus four phosphate oxygens are sharing the single charge.

features of CL that suggest its headgroup is a high capacity buffer that restricts the amount of pumped protons in the bulk phase water.

2. The chemistry of CL

As a glycerophospholipid, CL is unique. The molecule consists of two phosphatidic acids linked by a glycerol. It contains four fatty acid chains per molecule, two phosphates and three glycerols. Until recently no role for the connecting glycerol has been assigned, although it had been shown to form an internal hydrogen bond with a phosphate [20]. The function of the free hydroxyl is to alter the second pK of the headgroup, creating an acid-anion in the headgroup domain [21]. This means that, in contrast to textbooks and the current literature, each CL has only one negative charge per headgroup (Fig. 1).

The term acid-anion was introduced by Westheimer in 1956 [22]. He offered an explanation for the two remarkably divergent pKs of fumarate ($pK_1 = 3.4$; $pK_2 = 4.2$), with its *trans* double bond, and maleate ($pK_1 = 2.6$; $pK_2 = 6.8$), with its *cis* double bond. He suggested that maleate trapped a proton between its carboxyls, not permitted for fumarate. The trapped proton allows the single electron to resonate among the four maleate carboxyl oxygens (for a review see [23]). Thus the acid-anion concept explains the remarkably high pK_2 of maleate. It can also be used to explain why, at neutral pH range, CL has a single negative charge on the headgroup instead of the standard textbook assumption that it has two charges, one for each phosphate. The mono-protonated species is an acid and also a singly charged anion.

Models of CL showed [21] that its phosphates would form a tight bicyclic structure H-bonding to the hydroxyl on the connecting glycerol if it trapped a proton forming an acid-anion (Fig. 1). Using vesicles of chain-saturated CL, a titration yielded a pK_1 below 4.0, as is expected for a phospholipid [21]. However, its pK_2 is above 8.0 (Fig. 1), as is suggested by the formation of an acid-anion. To confirm this interpretation, the pK of the molecule lacking the hydroxyl (dCL) was measured. Both pKs are below 4.0 (Fig. 1), similar to analogs such as phosphatidic acid or phosphatidylglycerol [24].

The CL pK_2 (> 8.0) has two important implications for the role of CL in membranes. It implies a compact structure for the headgroup in which both phosphate groups are above the two central (*sn*-1) chains of the four-chained phospholipid. This tight conformation explained [25] how the fluorescence spectrum of nonyl acridine orange (NAO) can be used to identify CL in bilayers and biological membranes that contain it (Fig. 2). NAO, with its flat fluorescent domain, anchors itself to the bilayer with its nonyl group. CL's small headgroup combined with the fact that it has four chains permits stacking of its aromatic domain. These two features of the CL structure permit fluorescence microscopy visualization of CL domains in live mitochondria [26,27], vesicles [28] and in live

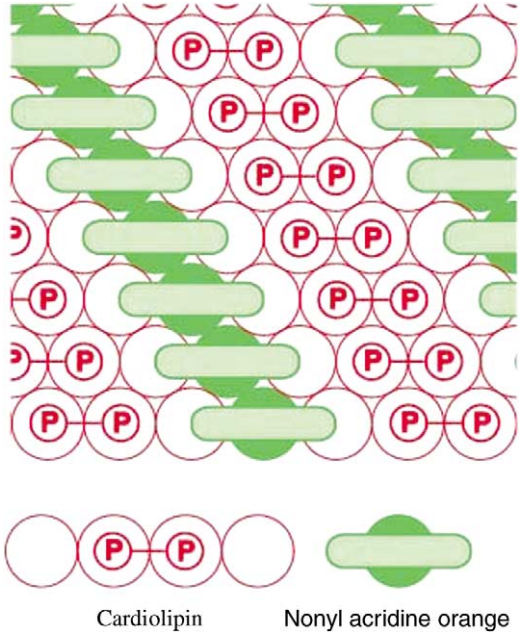

Fig. 2. A top view of a monolayer containing CL and NAO. NAO has been successfully used in fluorescence microscopy to localize CL in mitochondria and prokaryotes. The chains of both the CL (four chains; open red circles) and the NAO (one chain; filled green circles) are in a hexagonal array. The small, singly charged headgroup of the CL is paired between its two phosphates. This allows space for the NAO molecules to stack between the CL strings. Thus NAO fluoresces at CL surfaces because CL contains four chains and a small headgroup. The interaction between adjacent CL headgroups in the strings suggests that there might be intermolecular acid-anion interactions in addition to the bicyclic CL acid-anion.

Escherichia coli [29], where it forms patches in the live *E. coli*. Studies comparing the spectral fluorescence of pure NAO to that in the presence of CL on membranes indicate that the CL molecules and the NAO molecules are arranged in rows [25] (Fig. 2). This linear organization of NAO in CL bilayers may not correspond to CL in the absence of NAO, although living cells stained with NAO are fully active [26–29].

Two other lines of evidence suggest that arrays similar to that displayed in Fig. 2 might occur in living cells. First, the unusual titration curve in Fig. 1, when it is compared to computer-simulated pKs [21], shows that CL's pK_2 is not a simple pK but that the pK_2 shifts as the titration proceeds. This does not happen with dCL, which displays theoretical titration curves for both its pKs [21]. Thus the CL titration curve implies that the headgroup is interacting with adjacent CL molecules on the membrane surface in some manner consistent with the array found in CL doped with NAO (Fig. 2). This also suggests why the four chains are useful to its function. They provoke such association between the small headgroups of adjacent CL molecules. There is no evidence that this array occurs in the absence of NAO. Thus, the important bioenergetic function of trapping protons by CL may be to simply supply a high buffering capacity to the membrane–water interface. In this way CL bridges the gap between proton source and proton consumer with highly mobile CL monomers or oligomers. Here mobile CLs would laterally shuttle protons from oxidative phosphorylation complexes to the ATP synthase.

Typically, membrane proteins do not have highly specific binding sites for a particular lipid. Nonetheless all of the oxidative phosphorylation proteins and many proteins in prokaryote photosynthesis bind to CL. If there are headgroup arrays within patches of CL, however, there is an advantage for all of the oxidative phosphorylation proteins to bind this lipid. In that way the CLs can be used for direct access to pumped protons. Anionic lipids have been shown to have a high buffering capacity at physiological pH in membranes [30]. The phospholipid headgroups are in contact with each other and buffering the protons in much the same way as the acid-anion/extended-resonance structure stabilizes the CL conformation. Thus the contact between the adjacent CL headgroups is appropriate for the formation of additional acid-anions *between* the CL molecules, making the phospholipid singularly appropriate for buffering protons in the headgroup domain. The high pK_2 indicates that it will do so through wide ranges of pH including bulk phase water above pH 7.0. This buffering is within its headgroup region and not in the bulk water phase.

The pumping of a proton across the bilayer, from one headgroup domain to the other (CL is on both sides of the bilayer), is thus not an issue of the ΔpH but rather of the $\Delta\Psi$. The proton is essentially moved as a charge, so that the energy of the movement is vested in the $\Delta\Psi$, or membrane potential, rather than the ΔpH. This is assumed to be the driving force for the movement of protons through F_0 of the ATP synthase [31,32].

This ability of CL to diminish the ΔpH and enhance its $\Delta\Psi$ may explain the marked increase in CL found in prokaryotes exposed to uncouplers [33], which are well established to specifically reduce a ΔpH. If protons are pumped across the membrane directly into a headgroup buffer and consumed directly by the ATP synthase from that headgroup domain, then the uncoupler is less effective in diminishing ATP synthesis by altering the ΔpH. In this study [33] of the effect of pentachlorophenol, a well-known uncoupler of oxidative phosphorylation on microbes that are resistant to it, the phospholipid composition was shifted to a greater proportion of CL. There are two important implications to this interpretation of these data. First, it suggests that sorting out the ΔpH and the $\Delta\Psi$ experimentally may be more complex than is generally assumed. Second, it illustrates that the role of lipids in selective processes may not be an absolute requirement but rather that it expands the living conditions under which the organism survives.

3. CL and the oxidative phosphorylation proteins

Evidence from the laboratories of crystallographers supports a special relationship between CL and the oxidative phosphorylation complexes. In addition to the yeast cytochrome bc_1 complex [34], a unique binding site for CL has been identified in several prokaryote proton-pumping proteins associated with the photoreaction centers of prokaryotes [35,36]. These proteins, that are equivalent to the cytochrome bc_1 complex in other prokaryotes and in mitochondria, are also engaged in ATP synthesis through a proton gradient. In the identified CL-binding sites in these proteins, the two phosphates of the CL are separated, one bound to a lysine. Close examination of the data [34] suggests the binding site for CL is in the 'exit position' for the proton to the C-side of the

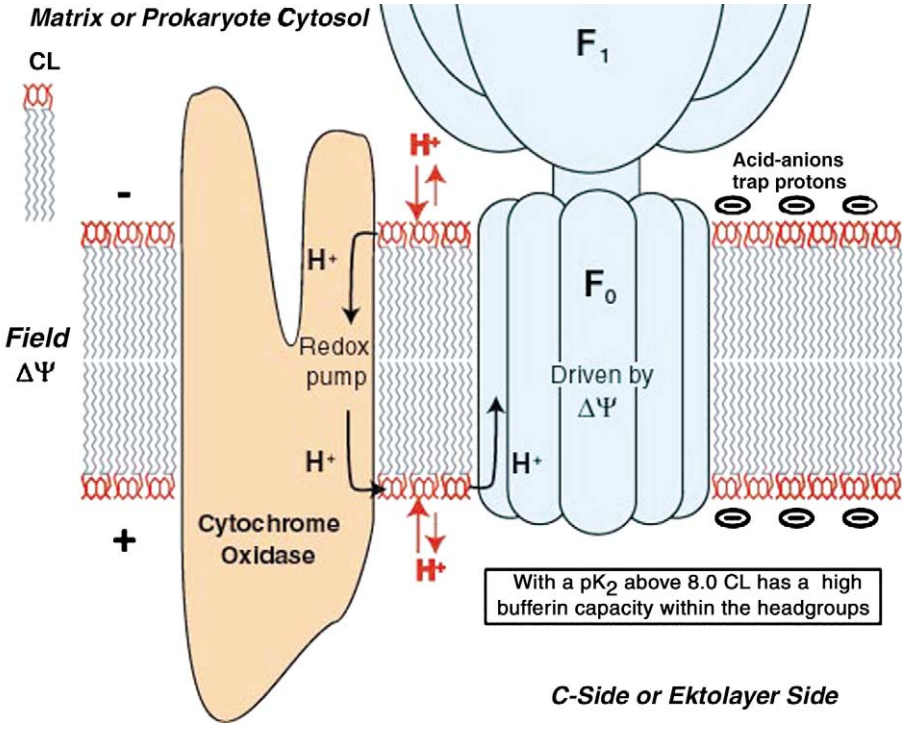

Fig. 3. A cartoon of the unique role of CL as a proton trap for protons in the headgroup domain during oxidative phosphorylation. Its pK (>8.0) is above that of water. It absorbs protons from the bulk phase at both high and low pH. All seven proteins of the oxidative phosphorylation system have high affinity binding sites for CL, suggesting a patch of the lipid may be formed in their presence. A redox-pumped proton need not leave the CL headgroup domain to be delivered to F_0. The model implies that it is not the proton, nor the ΔpH, but a single charge, driven by $\Delta\Psi$, that is used for ATP synthesis.

inner mitochondrial membrane. All this is consistent with an array of CL molecules buffering the protons in the headgroup domain.

Why do *all* of the oxidative phosphorylation proteins bind to CL? Skulachev and coworkers [37] have suggested that CL can be used to bind one membrane protein to another and this may be essential for oxidative phosphorylation. Or perhaps can these proteins be assembled into patches or rafts? A patch of CL containing such proteins can be functionally useful if protons are trapped in the headgroup domain (Fig. 3). Evidence for this was obtained in *E. coli* [29], where investigators found identifiable domains of fluorescence using NAO. Such domains were not found in the *cl*-mutants. Supercomplexes of the oxidative phosphorylation proteins are functional in yeast and mammalian systems [38]. It will be useful to seek CL in these supercomplexes.

4. Protons at the bilayer surface

Fig. 3 is a cartoon that shows how CL acts as a proton trap in the headgroup domain. It provides a source of protons as charges for F_0 regardless of the bulk phase pH. Here CL provides a direct and intimate proton sink for both the protein donor and the protein acceptor domains during oxidative phosphorylation. It was suggested early that protons in proton-pumping membranes are entrapped in the headgroups of lipids in proton-pumping membranes [23]. A consideration of the relationship between protons in the headgroup domain and those in the bulk water phase was addressed by Junge and McLaughlin [39]. The kinetics of the diffusion of protons along membrane surfaces was tested on monolayers of synthetic lipids [40–42], on 'bilayers' of *Sulfolobus*, an archaebacterium with lipids that cross the bilayer [43], and on purple membranes [44,45]. These studies focused on the speed with which such lateral conduction occurs compared to that of bulk water. The measured rates of lateral proton migration are 20× faster [41], equal to [45], or 100× slower [44] than that of proton diffusion in bulk water. However, conduction speed is clearly irrelevant to proton-pumping systems since the measured rates of lateral conduction by the lipids are orders of magnitude greater [40,43,44] than the utilization of the energetic protons.

Fig. 3 illustrates how CL is a sink for protons. It acts as both a reservoir for protons-to-be-pumped and a reservoir for pumped protons consumed in energy transduction. CL allows the proteins engaged in oxidative phosphorylation to use proton-pumping to generate a $\Delta\Psi$ while it diminishes the bulk-phase ΔpH across the membrane. Thus the $\Delta\Psi$ alone may provide the ΔG for the synthesis of ATP, although proton pumps create it.

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