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Editorial

A new look at Cardiolipin

In 1947 cardiolipin (CL) became the first-characterized phospholipid [1]. Mary Pangborn named it cardiolipin because she had isolated it from beef heart. Although first to be characterized it was the last of the major phospholipids to be stereochemically described [2] and synthesized, in 1966 [3]. In 1972 its biosynthesis in *Escherichia coli* and mitochondria were established [4,5]. That was the era when knowledge of lipid structures and metabolism were emerging largely due to the efforts of Laurens van Deenen in Utrecht. van Deenen was also the Editor of *Biochimica et Biophysica Acta* and nurtured it through the period of its greatest growth.

In 1998, Hoch published the most recent comprehensive review of CL in all its known roles [6]. There are several recent specialized reviews on cardiolipin [7–9]. However, the Hoch review represents a break in the cardiolipin story because in 1996 it was recognized that the defective gene in Barth's syndrome was the taffazin gene [10]. A flurry of research on cardiolipin resulted from that discovery. There had not previously been a disease related to CL. Barth's syndrome (BTHS) [11] is a sex-linked recessive disorder, clinically characterized by the classical symptoms cardio-myopathy, neutropenia and delayed growth with a lethal effect on small boys. The product of the taffazin gene modifies the structure of CL by transacylation, the exchange of fatty acids. That was also unexpected since there had rarely been a reason to think that phospholipid chains were specific to a particular phospholipid.

Textbooks display CL's structure and then stop there, as neither its function nor its conformation had been proposed. I list here the most important facts known about it for decades:

CL occurs primarily in membranes that also contain the F_0F_1 -ATPase. In eukaryotes it is the only phospholipid that is synthesized in the mitochondrion, where it remains until the cell dies. All other phospholipids are synthesized in the endoplasmic reticulum. CL has high binding affinity for each of the membrane proteins that are involved in the synthesis of ATP in the mitochondrion. This includes cytochrome oxidase [12,13], the ATP/ADP exchange protein [14], the F_0F_1 ATP synthase [15], the orthophosphate transporter [16], and its association with the cytochrome bc_1 complex [17]. Most prokaryotes contain the F_0F_1 ATP synthase and CL in their plasma membrane constitutively. Some prokaryotes only contain the F_0F_1 ATP synthase and CL during oxidative phosphorylation and yet anaerobically lack both. These prokaryotes include the photosynthetic bacteria and archaea as described by Corcelli [18]. This evidence suggests CL plays an important role in the bioenergetics of the cell, most likely in ATP synthesis itself. Failure of ATP synthesis appears as a possible explanation of the genetic disease associated with CL in BTHS [19]. Also related to bioenergetics, cardiolipin plays a central role in the destruction of the mitochondrion as the energy center of the cell during apoptosis [20].

Recent research has identified other specialty roles for cardiolipin metabolism, cell biology and physiology. Each of these has been

covered here. Osmoregulation in *E. coli* [21], fatty acid transport in human kidney cells [22], mitochondrial carriers [23,24], mitochondrial kinases [25], the organization of mitochondrial and prokaryote membranes [26,27] and bilayers [28], and its biosynthesis [29]. These subjects are all covered here.

1. Recent Insights into cardiolipin's structure

CL has a high pK_2 (>7.0) based on a titration (Fig. 1). This has changed our understanding of its conformation in bilayers [30,31]. In 1960, Pangborn sent a sample of her original phospholipid preparation to A. V. Few at Cambridge who measured its pK 's by microelectrophoresis [32]. It displayed the expected low pK_1 (1.04), but as the titration continued Few found a high peak of unknown origin which he declared was an artifact since it had not occurred in any of his other membrane samples. This rise stimulated a renewed search for a second pK by Kates et al. [30]. The titration curve they obtained (Fig. 1) was similar to that observed by Few. The pK_2 turned out to be >8.0 as measured by the consumption of 2 equivalents of base. To test the conformation in Fig. 2 Kates synthesized dCL, which lacks the free OH on the connecting glycerol. The dCL titration (Fig. 1) showed that the measured high (>8.0) pK_2 is indeed due to the presence of the free hydroxyl. These experiments required that the headgroup is stabilized by two high-energy H-bonds of the phosphates on that free hydroxyl group (Fig. 2). In order to create the bicyclic headgroup conformation, one phosphate must pick up a proton from solution. The headgroup thus has a single electron delocalized between the 2 phosphates.

Until these experiments, CL's 2 pK 's, had not been observed because they had not been measured in bilayers. In solution or as a powder, FTIR experiments show CL to have a different conformation in the phosphate region than it has in bilayers (T. H. Haines, H. Deng and R. Callender, unpublished). Thus the conformation shown in Fig. 2 only applies to the lipid in bilayers. Furthermore the titration curve for CL in bilayers is unusual with respect to its pK_2 . A computer simulation [30] did not match a single pK_2 in the range from 6.0 to 8.0. The simulation showed that the pK_2 drifts during the titration presumably due to a changing headgroup conformation during the titration. Significantly it shifts in the direction such that under physiological conditions it maximizes the pool of protons available to the membrane during proton pumping in oxidative phosphorylation.

Note that the conformation of the headgroup in Fig. 2 is remarkably small especially given that CL has four chains. In Fig. 3 (left) the headgroup surface is hydrophobic with the phosphates tucked under the bicyclic ring system. This may explain why pure CL may form Hexagonal II phase complexes rather than bilayers.

This resonance-stabilized bicyclic headgroup may also explain the recent finding that there is an unexpected symmetry in the lengths of CL's 4 fatty acid chains [33–35] in biological membranes. The bicyclic

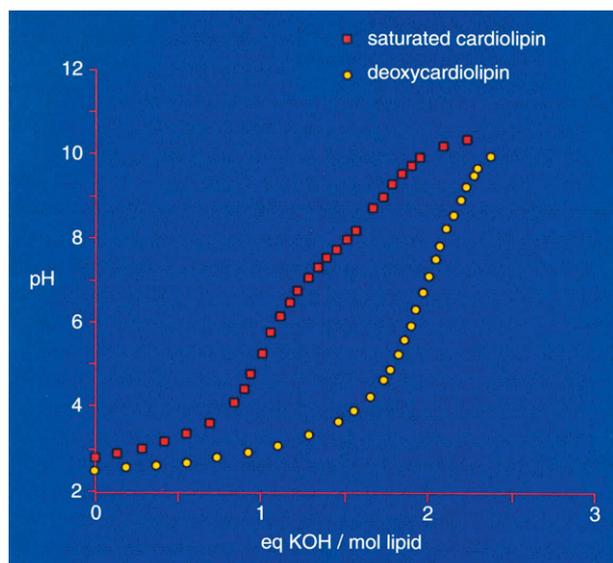


Fig. 1. Titration curve of cardiolipin in bilayers. Saturated beef heart cardiolipin (CL) bilayers potentiometrically titrated (red) along with its analogue missing the $-OH$ (dCL) (yellow). CL displays two pK 's at 4.0 and ~ 8.0 . The dCL displays two pK 's below 4.0. This can only be explained if the headgroup of CL contains two high energy H-bonds formed by the two phosphates with the free hydroxyl of the glycerol.

structure described in Fig. 2 depends upon balanced chain symmetry to stabilize the 2 H-bonds. An imbalance in the length of the pair of chains on the two sides of the headgroup may disrupt the resonance structure in Fig. 2 as shown in an exaggerated form in Fig. 3 (right). Prokaryotes commonly have 16 carbon chains throughout their phospholipid structures but eukaryotes do not. For any prokaryote, four C16 lipid chains will symmetrically stabilize the CL headgroup conformation in Fig. 2. Prokaryotes lack polyunsaturated fatty acid chains. In contrast to the prokaryotes, the polyunsaturation of chains in eukaryotes complicates the "length" estimates of the chains. In the mammalian heart mitochondria, CL's 4 chains are commonly polyunsaturated linoleic acid esters, which solves the length problem. The bicyclic structure allows the application of the high pK_2 to membrane surfaces that contain CL since the ring system would open if the 2 chains on one side of the lipid would be shorter than the pair on the other side as shown in Fig. 3.

Typical bacterial biosynthesis of CL's headgroup involves an enzyme belonging to the phospholipase D superfamily known as CL synthase (Cls). It catalyzes a reversible phosphatidyl group transfer from one phosphatidylglycerol (PG) molecule to another PG forming CL and glycerol [4]. This enzyme appears to have a conserved N-terminal segment for insertion into the prokaryote membrane [29]. In contrast, in the eukaryote mitochondrion, the Cls is of the CDP-alcohol phosphatidyltransferase superfamily using cytidine diphosphate-diacylglycerol (CDP-DAG) as the donor of the phosphatidyl group which is transferred to a PG to form CL. In a surprising contrast it appears that actinomycetes use the eukaryote biosynthetic system [36]. This paper reviews the recent literature on the eukaryote-prokaryote biosynthetic pathways.

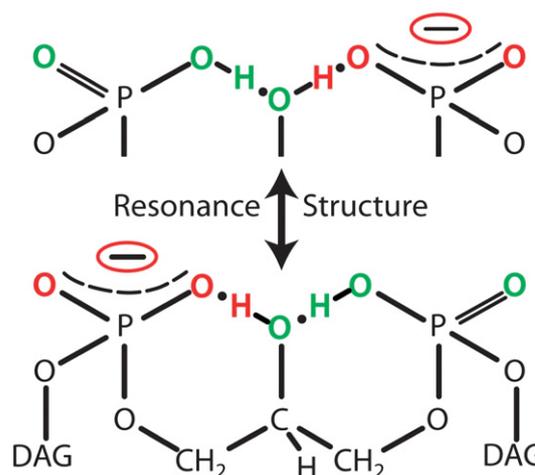
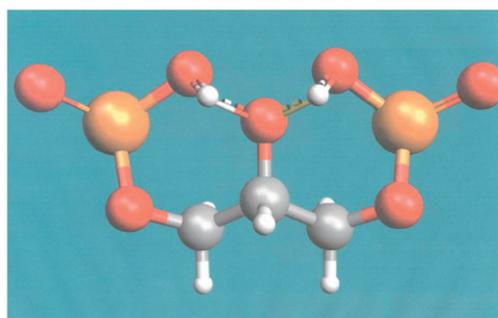
2. Cardiolipin, its organization and role in ATP synthesis

CL is the only phospholipid in eukaryotes that is not synthesized on the cytosolic face of the endoplasmic reticulum. It is synthesized in the mitochondrion and usually remains there until cell death or apoptosis [25,37,38]. Cardiolipin's intimacy with the F_0F_1 ATP synthase together with its high headgroup pK suggests it serves as a proton source for protons used in proton-pumping during ATP synthesis. When the headgroup has a pK in the range of 7 or 8 it is

50% protonated; at 50% protonation little energy is needed to take or deposit protons at each bilayer surface. Protons are rapidly supplied or replaced at the surface by the water. In this unique circumstance there is no ΔpH across the bilayer. The removal of a proton from a bilayer surface means that the energy is immediately converted into $\Delta \Psi$, the membrane potential. Calculations by Junge [39] and by Oster [40] have shown that the proton movement along F_0 during the synthesis of ATP is actually driven by $\Delta \Psi$ and not by ΔpH . This is important, for much of the transport activity in the mitochondrial inner membrane occurs through transporters that are responsive to a membrane potential. CL provides a high concentration of protons in the headgroup surface on both sides, effectively removes the ΔpH as a component of the energy in the $\Delta \mu_{H^+}$.

A second way in which the high pK_2 can facilitate ATP synthesis is by neutralizing negative charge during electron transport on the C-side of the membranes, say from a charged Coenzyme Q. The neutralization of a proton on the C-side of the membrane would create $\Delta \Psi$ energy for proton pumping as computed by Oster [40] and Junge [39]. Therefore simply neutralizing a cardiolipin proton on the C-side of the mitochondrial membrane is equivalent to pumping a proton across the membrane.

Another important role for cardiolipin in energy metabolism is to anchor two very large kinases, mitochondrial creatine kinase (MtCK) and nucleoside diphosphate kinase (NDPK-D) [25]. The two kinases bind to CL on the inner and probably the outer mitochondrial



CARDIOLIPIN HEADROUP IN BILAYERS

Fig. 2. The resonance-stabilized structure of CL headgroup when it is in a bilayer. The two phosphates of the headgroup can maintain this structure only when one of the headgroup phosphates picks up a proton from water to H-bond with the pair of electrons on the free $-OH$. Under these circumstances the single electron is dispersed across the headgroup. In a bilayer at neutral pH, the headgroup has a single charge and the pK of the headgroup is above that. (Appreciation is expressed to Dr. Michael Schlame for contributing of the upper figure.)

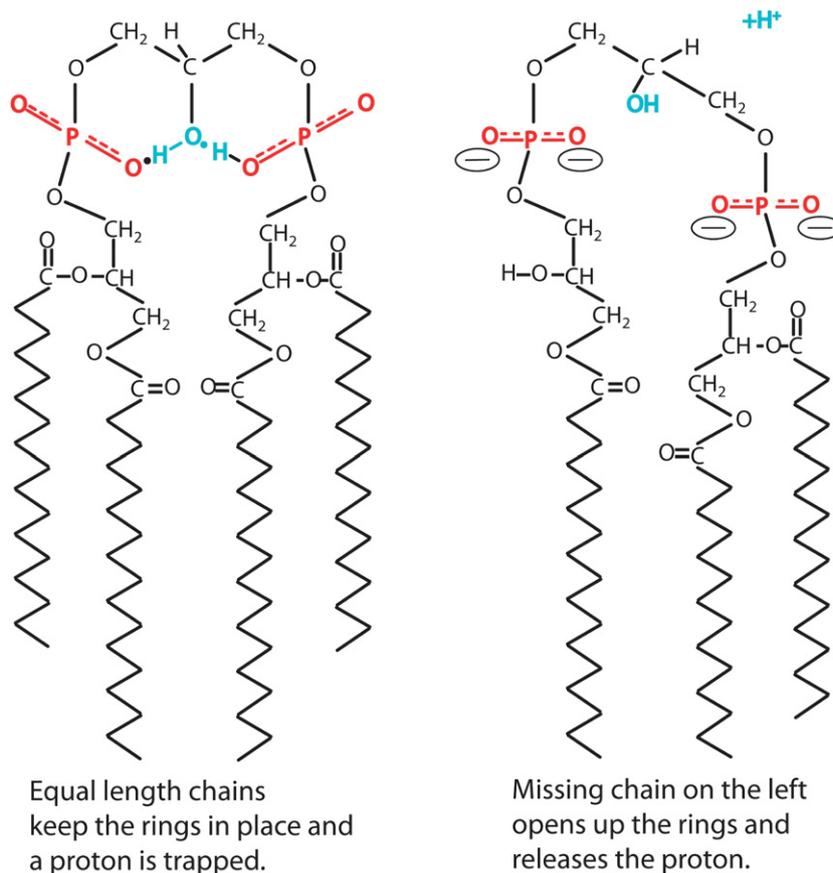


Fig. 3. Two structures of CL demonstrate the disruptive effect of a disparity in the “length” of the fatty acid chains of the lipid on the headgroup’s resonance structure. The four chains on the left maintain the headgroup resonance. Lyso-cardiolipin (lysoCL) on the right cannot support the balance required to maintain the resonance. Its pK_2 is below 4.0, as is that of dCL which lacks the central OH-group that stabilizes the bicyclic headgroup stability (R. F. Epand, R. M. Epand and T. H. Haines unpublished). If the high pK_2 of CL is important for the synthesis of ATP [31] then the cleavage of a chain from CL to form lysoCL would hinder ATP synthesis in vivo.

membranes at the contact points between the membranes. They appear to provide channels for substrates, such as creatine phosphate, between the matrix and the cytosol. The kinases share important functional/structural analogies and roles in the bioenergetics of mitochondria. Creatine kinase (MtCK) and by analogy dinucleotide kinase NDPK-D are both oligomeric complexes. A quantitative analysis of the properties of MtCK, in comparison with the X-ray structure, shows the attachments with CL [41]. These proteins have many isozymes, presumably characteristic of specific tissues. In muscle and brain, for example, creatine kinase provides a reserve supply of high energy phosphate (creatine phosphate) during initial signal periods when the ATP is rapidly consumed. The cell needs an instant source of ATP when glycolysis begins. Creatine phosphate is used to phosphorylate the ADP rapidly until glycolysis establishes the appropriate ATP concentration (2 to 7 seconds).

The evidence that these kinases are intimate with the bioenergetics of the mitochondrion itself is clear because they bind to VDAC and the ATP/ADP exchange protein. An extensive review by the Schattner and Epand groups, major researchers in this field, details some unifying pathways for the operation of these proteins in energy metabolism.

Cardiolipin also affects ATP synthesis by organizing proteins and lipids in those membranes that make ATP. An exhaustive and masterful application of physical techniques has helped our understanding of the organization and properties of CL-containing lipid bilayers is provided by Lewis and McElhaney [28]. Their data bring cardiolipin up to the standard of our understanding of the more familiar double-chain lipids. On the other hand bilayers are static by their nature whereas biological membranes are not.

Cardiolipins in membranes, especially in the inner membrane of mitochondria and in bacteria are very dynamic as shown by Mileykovskaya and Dowhan [26]. Here the dynamism relates to the exchanges of the lipid components of the multiprotein complexes involved in diverse cellular functions including cell division, energy metabolism and membrane transport. Examining the cardiolipin deacylation/transacylation enzymes Schlame and Ren [27] propose a dynamic structural homeostasis in the inner membrane of the mitochondrion. It is based on the dynamics of the transfer of the chains of the CL.

The regulation of the biosynthesis and degradation of cardiolipin can be a response of osmotic activity in bacteria as shown in the elegant experiments of Romantsov and Wood [21]. The response of bacteria to osmotic induction of the gene encoding CL synthase which alters the proportion of CL in membranes.

Most known mitochondrial carrier proteins bind CL [23,24,42]. The isolation and purification of these proteins is often facilitated by the addition of CL during their preparation. Since CL may convert the ΔpH to the $\Delta\Psi$, the effect of $\Delta\Psi$ on the transport activity and function might profitably be explored. This applies to all carriers that bind cardiolipin including the transmembrane proteins dicarboxylate [43], tricarboxylate [44], and uncoupling proteins [45]. For the latter, CL also seems to regulate aspects of the uncoupling protein’s activity including nucleotide binding.

Each of the six mitochondrial inner membrane proteins engaged in ATP synthesis via its proton gradient displays high binding affinity for CL. This includes the ATP/ADP exchange protein [42,46], the F_0F_1 ATP synthase [47], the phosphate uptake protein [48–50], cytochrome oxidase [51], the b_c1 complex [52,53], and NADH dehydrogenase

[54,55]. There appears to be no evidence for the binding of CL to succinate dehydrogenase, an enzyme that participates in oxidative phosphorylation but not in the proton-gradient during ATP synthesis.

CL is largely in membranes that contain the F_0F_1 ATP synthase, however the chloroplast thylakoid membrane lacks CL; it contains a different ATP synthase (CF_0CF_1). It does, however, contain a unique lipid, the plant sulfolipid, (sulfoquinovosyl diacylglyceride) that can potentially serve a similar role since it always occurs in the specific chloroplast membrane that contains CF_0CF_1 . In some Gram-positive cocci the sulfolipid replaces cardiolipin and phosphatidyl glycerol [56]. When prokaryote facultative anaerobes are denied oxygen both the F_0F_1 ATP synthase and the CL disappear together.

3. Barth's syndrome

The recognition that Barth's Syndrome (BTHS) [11] is caused by a mutation resulting in the remodeling of CL's chains [57,58] motivated researchers to investigate them. The Schlame group examined CL's chains in natural membranes. They noted that the four chains are symmetrical in length and in some cases like mammalian liver mitochondria, identical [35]. These data indicate that the chains are capable, *in vivo*, of maintaining the headgroup conformation shown in Fig. 2. Were they not symmetrical, the bicyclic resonance-stabilized headgroup shown in Fig. 3 for lyso-CL would collapse.

This work [35] involved the development of a yeast system that mimicked the human defective tafazzin CL chain array, which included small quantities of lyso-CL. Vaz et al. [59] took advantage of this observation to develop a simple assay for BTHS. Using HPLC-tandem mass spectrometry they were able to identify BTHS in infants by locating lyso-CL in blood spots. The presence of lyso-CL suggests that the defective transacylase of BTHS may both lower the pK_2 of the CL's and also reduce ATP synthesis. Major clinical features of the patients of BTHS are myopathy, (weakness) and often cardiomyopathy, neutropenia and growth delay. All are accountable in cells that lack energy. This combined with the location of CL among proteins involved in ATP synthesis suggests ATP deficiency in a variety of organs. Whatever the role of CL, the manner in which the chains are placed on CL in various systems remains unclear. The combination of chains found on symmetrical CL in eukaryote systems results from both from direct biosynthesis and subsequent remodeling [60]. This requires two or more enzymes which vary from yeast to fruitfly to mouse to human. A recent review describes the manner in which these processes occur in yeast as determined via knock-out genes [60].

Heart mitochondrial monolysocardiolipin acyltransferase is an enzyme that models CL by its choice of fatty acids. The function of this enzyme may be altered by alcohol, resulting in fetal alcohol syndrome [61]. A number of recent studies have unraveled much of the transacylation mechanisms of cardiolipin transacylation [33,34,62,63], comparing them to similar transacylases of inositides [64]. This work has allowed richer insights into the tafazzin gene product that is defective in BTHS [10,35]. Another inference on chain selection during the synthesis of CL in the mitochondrion is the uptake of fatty acids. A major enzyme that regulates the fatty acid uptake is the human fatty acid transport protein 1 (FATP-1) [65].

4. Apoptosis

For a cell in a multicellular organism to commit suicide for the benefit of the whole organism, it must first destroy the energy source of the cell—the mitochondrion [20,66]. If so one would expect a critical target that is unique to mitochondria. Cardiolipin, rarely found elsewhere in the cell, is the target of choice. CL is also present at the contact sites between the outer and inner membranes; this CL is the target of the tBid protein that approaches the mitochondrion with the suicide message, possibly as a result of changes in the intrinsic curvature of the membrane [67]. This first step is followed by the

binding of CL to Bax/Bak, provoking oligomerization and cristae remodeling that begins the destruction of the organelle.

Cardiolipin in mammalian mitochondria typically have 4 polyunsaturated linoleic acid chains which are subject to oxidation. This oxidation leads to "reactive oxygen species" (ROS), which in turn releases cytochrome c (cyt c), an important water-soluble very basic protein in the intermembrane space (IMS) and ultimately to the cytosol.

CL binds to cyt c in a surprising manner; cyt c binds one of CL's chains with the other 3 chains embedded in the membrane. This "lipid anchor" arrangement is made possible by a hydrophobic pocket in the protein [68,69]. This unique binding alters the redox potential of cyt c so that it no longer picks up electrons from the bc_1 complex to reduce cytochrome oxidase. This necessarily cuts off ATP synthesis. The details on the relationship of CL and ROS have not yet become clear. The ROS structure and details of its oxidation state(s) will play a major part of the apoptosis story as it unravels.

5. Coda

Finally, in contrast to commercial *E. coli* CL, commercial beef heart CL does not easily form bilayers. It has four linoleic (polyunsaturated) chains, which spread laterally in a bilayer, thinning the bilayer. Taken together with its very small bilayer headgroup conformation (Fig. 2) this presents as a concave surface. It tends to form hexagonal II (H_{II}) phase rather than a bilayer. For this reason the commercially available *E. coli* CL preparation with its C_{16} chains, with only some mono-unsaturates, forms more stable bilayers. Such stable bilayer properties may actually facilitate CL's usefulness for *E. coli* in the initiation of DNA replication [70].

A student today will find cardiolipin interesting beyond the dull textbook structure of 20 years ago. We have yet to recognize its role in ATP synthesis, why and how its chains have length requirements (no other phospholipids do), whether or not it forms rafts in membranes sequestering membrane proteins, how it is an initiator in DNA synthesis in *E. coli* as Crooke and Kornberg found, how osmotic effects regulate it, and the details of how it works in apoptosis. There is still too much mystery in it.

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