Cardiolipin binds selectively but transiently to conserved lysine residues in the rotor of metazoan ATP synthases

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The anionic lipid cardiolipin is an essential component of active ATP synthases. In metazoans, their rotors contain a ring of eight c-subunits consisting of inner and outer circles of N- and C-terminal α-helices, respectively. The beginning of the C-terminal α-helix contains a strictly conserved and fully trimethylated lysine residue in the lipid head-group region of the membrane. Larger rings of known structure, from c_8–c_15 in eubacteria and chloroplasts, conserve either a lysine or an arginine residue in the equivalent position. In computer simulations of hydrated membranes containing trimethylated or unmethylated bovine c_8–rings and bacterial c_10– or c_11–rings, the head-groups of cardiolipin molecules became associated selectively with these modified and unmodified lysine residues and with adjacent polar amino acids and with a second conserved lysine on the opposite side of the membrane, whereas phosphatidyl lipids were attracted little to these sites. However, the residence times of cardiolipin molecules with the ring were brief and sufficient for the rotor to turn only a fraction of a degree in the active enzyme. With the demethylated c_8–ring and with c_10– and c_11–rings, the density of bound cardiolipin molecules at this site increased, but residence times were not changed greatly. These highly specific but brief interactions with the rotating c-ring are consistent with functional roles for cardiolipin in stabilizing and lubricating the rotor, and, by interacting with the enzyme at the inlet and exit of the transmembrane proton channel, in participation in proton translocation through the membrane domain of the enzyme.

Cardiolipin and other phospholipids are essential components of active ATP synthases isolated from mitochondria (8–12). It has been suggested that cardiolipin acts to stabilize and lubricate the rotating c-ring (13) or to aid in proton transfer (5), but it is not known where or how cardiolipin binds to the enzyme. Excluding the regulatory protein IF₁, the bovine ATP synthase complex is built from 28 polypeptide chains of 16 varieties (14). About 85% of a mosaic overall structure has been determined to atomic resolution by structural analysis of constituent domains (15–18), and an intact enzyme structure has been described at about 6-Å resolution (19). The enzyme consists of a spherical catalytic F₁-domain in the matrix of the mitochondria, attached to the inner membrane domain by central and peripheral stalks. The central stalk is bound to a ring of c-subunits in the membrane domain, and together they constitute the enzyme’s rotor (13, 20). Each bovine c-subunit is folded into two transmembrane α-helices, and in each rotor-ring, the N- and C-terminal α-helices form concentric inner and outer circles, linked by eight loop regions exposed in the phospholipid head-group region on the matrix side of the inner membrane. Contacts between the loops and the central stalk add to the stability of the rotor. Vertebrate and probably all metazoan ATP synthases have c_9–rings (21). Fungal ATP synthases contain c_10–rings (20), various eubacterial enzymes have rings of c_8, c_11, c_12, c_13, and c_15 (22–28), and the enzyme from spinach chloroplasts has a c_12–ring (29). The turning of the rotor carries energy from the transmembrane proton-motive force generated by oxidative metabolism or photosynthesis to the catalytic domain, to energize the phosphorylation of ADP. The transmembrane path for protons involved in generating rotation is in the interface between the external surface of the c-ring and the membrane subunit a (30), which has not been resolved to high resolution, although a 4-Å X-ray structure of a bacterial enzyme with a c_12–ring (25) and structures of the bovine and an algal enzyme determined by electron cryo-microscopy (19, 31) have revealed its rudiments. In the structures of intact ATP synthases (19, 25, 31), of F₁–c-ring subcomplexes (13, 20), and of c–rings (22–24, 27–29, 32–35), there is no evidence of any bound phospholipid. However, the presence of a fully trimethylated and conserved lysine residue, in the

Significance

ATP, the fuel of life, is produced by a molecular machine consisting of two motors linked by a rotor. One motor generates rotation by consuming energy derived from oxidative metabolism or photosynthesis; the other uses energy transmitted by the rotor to put ATP molecules together from their building blocks ADP and phosphate. The anionic lipid cardiolipin, a component of bacterial membranes and the inner membranes of mitochondria where the machine is found, is an essential component of the enzyme. It interacts specifically, transiently, and repeatedly with the rotor of the machine, possibly lubricating its rotation or participating directly in the generation of rotation from the transmembrane proton motive force.


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Results

Models of c-Rings in Lipid Bilayers. The structures of c-rings at the start and end of the simulations were essentially the same, with RMSDs of less than 0.35 nm between initial and final structures superimposed by backbone beads (Fig. S1). However, c11-rings were the most stable, probably because intersubunit interactions occurred between Lys-50 residues and Asp-52 residues in adjacent c-subunits (23). Sometimes in the c10-ring, the orthogonal residues were sufficiently close to allow the formation of equivalent bonds, whereas the orthogonal residues Lys-43 and Gln-44 in one c-subunit and the other with either Gln-45 or TM-Lys-7 (Fig. 2A). A typical cardiolipin would move into the lipid annulus surrounding the c-ring, binding via its head-group in one of two modes (Fig. 3A). In the outer leaflet of the membrane, cardiolipin 22 of the inner leaflet became bound first to c-subunits s2 and s3, and then to subunits s1 and s2. More cardiolipins were bound simultaneously in the inner leaflet than in the outer leaflet (Fig. 4). The residence times of cardiolipin in the inner and outer leaflets of the membrane were 500 and 220 ns, respectively, and for phosphatidyl lipids in the inner leaflet of the membrane (Fig. 1B).

Interactions of Lipids with the Native c8-Ring. The densities of radial distribution functions of the phosphatidyl moieties of cardiolipin (Fig. 1) were greatest around the side-chains of TM-Lys-43, Gln-44, Gln-45, and Ser-48 in the inner leaflet of the membrane (Fig. 1A). Likewise, the densities of phosphate groups of phosphatidyl lipids were greatest around the same residues, but much lower than those of cardiolipin (Fig. 1A). In the inner leaflet of the membrane, there was a high density of cardiolipin around residue Lys-7, which is at the beginning of the N-terminal α-helix in the vicinity of the lipid head-groups. The densities of phosphatidyl lipid head-groups were much lower (Fig. 1B).

During simulations, several cardiolipins became bound to the native c8-ring and c11-rings and c10-rings of S. cerevisiae and I. tartaricus, respectively, see Figs. S4, S6, and S8.

**Fig. 1.** Interactions between lipids and amino acids in native c8-rings. (A and B) Average radial distribution functions from two independent simulations for lipids in the inner and outer leaflets of the membrane, respectively. Upper and Lower correspond to interactions with cardiolipin and with POPC and POPE, respectively. Their colors correspond to specific amino acids in the bovine c-subunit, as defined in C. (C) Alignment of sequences of c-subunits from cows, S. cerevisiae, and I. tartaricus. Residues interacting with lipids are colored. For the radial distribution curves for the demethylated c8-rings and c10- and c11-rings from S. cerevisiae and I. tartaricus, respectively, see Figs. S4, S6, and S8.

phospholipid head-group region of c-subunits of metazoan ATP synthases, suggested that it could provide a site for cardiolipin to bind selectively, by impeding the binding of the head-group of other phospholipids (21). Therefore, here, we describe coarse-grain molecular dynamics simulations to examine how phospholipids interact with bovine c8-rings, with and without methyl groups, with the c10-Ring in the ATP synthase from Saccharomyces cerevisiae (20) and the c11-rings from the Na+-dependent ATP synthase from Ilyobacter tartaricus (23), where the equivalent lysine residues are conserved but not methylated.
residue (Fig. 2 and Fig. S4), but the binding site and the residence times were unchanged (Figs. 2 and 5 and Fig. S5). The density of cardiolipin phosphates was greatest around residue Lys-43, and significant densities were observed around Ser-48, Gln-44, and Gln-45 (Fig. 2 and Fig. S4A). The densities of cardiolipin phosphates around residues Gln-44, Gln-45, and Ser-48 were similar in trimethylated and demethylated c8-rings, but the density around demethylated Lys-43 was nearly twice that observed around TM-Lys-43, reflecting the weaker Lennard Jones interaction between terminal trimethyllysine side chain bead (modified from the bead type in coarse-grained lysine) and lipid phosphates. Demethylation of Lys-43 had no effect on the interactions of cardiolipin in the outer leaflet with the c8-ring (Fig. 2).

**Binding of Lipids to c10- and c11-Rings.** In the c10-rings, cardiolipin phosphates in the inner leaflet of the membrane bound mostly to residue Lys-44 (equivalent to bovine Lys-43) in a single c-subunit.
Residence times for lipids in association with c-rings. They correspond to the duration of binding of a lipid to any part of a c-ring. (A and B) Values for the inner and outer leaflets of the membrane, respectively, averaged over both simulations of each c-ring. The yellow and gray bars correspond to residence times of cardiolipin and POPC/POPE, respectively, in simulations containing cardiolipin, POPC and POPE. Error bars were obtained by bootstrapping.

**Discussion**

**Role of Cardiolipin in Mitochondrial Enzymes.** Bound cardiolipin molecules influence the stabilities and activities of transport proteins and enzyme complexes in the inner membranes of mitochondria and may help to stabilize interactions between respiratory enzyme complexes organized in supercomplexes (36). In some instances, the mode of binding of cardiolipin to proteins has been defined structurally (37). In the ADP/ATP translocase, a cardiolipin is bound between two adjacent transmembrane α-helices, probably providing additional stability (38); in cytochrome c oxidase, a cardiolipin spans between two monomeric complexes, evidently helping to stabilize the dimer (39); and in the cytochrome bc1 complex, a cardiolipin molecule bound in the vicinity of a proton uptake pathway leading from the matrix side of the membrane to the site where quinone reduction takes place, may have a role in proton translocation through the inner membrane (40). In simulations with complexes III and IV (41, 42) and the ADP/ATP translocase, cardiolipin molecules became and remained bound to the same sites occupied by cardiolipin in their crystal structures.

In contrast, the requirement for phospholipids, including cardiolipin, for a fully functional ATP synthase is well established, and it has been proposed that about four cardiolipin molecules are bound on average to each bovine enzyme complex (43). Cardiolipin has been found to be entrapped inside the annulus of the K-ring of a V-type ATPase (44). However, no phospholipids have been detected yet in structures of intact ATP synthases, or in those of F1-c-ring and c-ring subcomplexes, but the fairly harsh conditions for purifying them would be likely to remove any bound lipids. Although the enzyme from the α-proteobacterium, *Paracoccus denitrificans*, has been purified with associated endogenous phospholipids (45), the resolution of the current structure is insufficient to determine whether or not any lipids are bound specifically (25).

**Binding of Cardiolipin to c-Rings.** Cardiolipin, in strong preference to phosphatidyl lipids, became bound to native c-rings at specific sites around TM-Lys-43 on the side of the membrane next to the mitochondrial matrix, and around Lys-7 on the opposite side (Fig. 2). Also, the residence times for cardiolipin around

(Figs. S6 and S7). The head-groups of cardiolipin molecules in the outer leaflet were bound to Lys-8 (Fig. S6 and Fig. 2), which is equivalent to Lys-7 in bovine c-subunit (Fig. 1C). The phosphatidyl lipids were present at a lower density around the c outer-ring than cardiolipin (Fig. S6), and the greatest concentration of their head-groups in the inner leaflet of the membrane was around residues Lys-44, Asp-45 and Thr-46 (Fig. 2 and Fig. S6A), and, in the outer leaflet, around residue Lys-8 (Fig. 2 and Fig. S6B). The residence time of the cardiolipin in the inner leaflet of the membrane (300 ns) was slightly longer than in the outer leaflet (220 ns), whereas the values for the phosphatidyl lipids, 100 ns, were similar in both the inner and outer leaflets (Fig. 5).

With c11-rings, cardiolipin phosphates were bound around residue Lys-50 (equivalent to bovine Lys-43) for long periods, with appreciable additional density around residues Gly-51 and Ser-55 (Fig. 2 and Fig. S8). There was also significant density for cardiolipin phosphates about 1 nm from Asp-52, corresponding to the second coordination sphere. The region of the c11-ring in contact with the outer leaflet of the membrane has no lysine residues in the head-group region with which cardiolipin might interact. However, there was some density of cardiolipin and phosphatidyl lipids around residues Tyr-80 and Asn-82 (Fig. S8). The residence time for cardiolipin in the inner and outer leaflets of the membrane were ca. 600 and 300 ns, the highest values observed in the simulations (Fig. 5). Moreover, at any instant, several cardiolipins were bound to the c11-ring (Fig. S9).
these sites and elsewhere in the lipid annulus next to the c-ring were longer than those for phosphatidyl lipids (Fig. 5). However, there was a greater tendency for cardiolipin molecules to be bound in the vicinity of TM-Lys-43 than of Lys-7. At any instant, three or four cardiolipins were bound to c-rings in the inner leaflet, similar to estimates of the number of cardiolipin molecules bound per ATP synthase complex (43). Demethylation of Lys-43 increased the density of the head-groups of cardiolipin molecules, but had little effect on their residence times. Increase in the ring size to c10 also had little effect, but increasing the ring to c11 increased both the density of cardiolipin molecules and their residence times (Figs. 3 and 4). Again, cardiolipin became bound in two modes via equivalent amino acids in the head-group regions of the lipid bilayer (Figs. 1C, 2, and 3), and it tended to diffuse around the rings (Fig. 4 and Figs. S3, S5, S7, and S9).

These simulations show that the regions of the c-subunit in contact with the head-group regions of the inner membranes of mitochondria have evolved to attract cardiolipin molecules preferentially over phosphatidyl lipids. Lysine residues, or arginine residues in some bacteria, and their positive charges are intrinsic components of many of these binding sites, although the positive charge is not an absolute requirement (Fig. S10). Irrespective of the size of the ring, and the methylation status of Lys-43 in the c8-rings, the site in the inner leaflet is more attractive than the site in the outer leaflet, and given the asymmetric distribution of cardiolipin molecules in the inner membranes of mitochondria, the site around Lys-43 is more significant than the site around Lys-7. However, in the c8-rings, the selectivity in the inner leaflet is not, as proposed (21), endowed by the complete trimethylation of Lys-43. Rather, trimethylation has little effect on the attraction of cardiolipin. The complete trimethylation of Lys-43 of the c-subunit of ATP synthase has been found uniquely throughout all classes of metazoaans that have been examined, and it probably persists throughout the approximately two million species in this kingdom of life. It is an intrinsic feature of ATP synthases with c8-rings, which according to current models experience the greatest rotational torque of all c-rings, during catalysis. However, the exact role of the modification remains obscure.

**Role of Cardiolipin Molecules in ATP Synthase.** The sites where cardiolipins are bound permanently to complexes III and IV and ADP/ATP translocate are characterized by having positively charged and other polar residues in the region of the lipid head-group, in a groove in their surfaces where the head-groups and acyl chains can nestle. The sites of interaction of cardiolipin with c-rings also have positively charged and polar residues in the lipid head-group region, but in contrast, the convex surface of the c-ring provides no associated grooves where the phosphate groups can associate stably. Thus, cardiolipin molecules interact repeatedly, but transiently, with the c-ring via two belts of continuous binding sites formed by the repeating pattern of polar and basic residues in each c-subunit, one on the inner leaflet exposed part of the protein, and the other on the outer one, and then they diffuse away without being affected by rotation. In an active ATP synthase rotating at 100 Hz, one rotation of the c-ring takes 10 ms, and so during the ~500 ns of interaction with a cardiolipin, the ring turns about 0.02°. At any given instant, there are approximately three to four cardiolipins interacting with the c-ring; the cumulative effect of these repetitive, but brief, specific interactions could contribute to the stability of the ring, and may also lubricate its passage through the lipid bilayer. In the integral ATP synthase complex, the c-ring and the a subunit are in intimate contact (25), leaving about 70% of the external surface of the c-ring exposed to the bilayer and available for these transient interactions. It is unlikely that any cardiolipin molecules can bind, even transiently, in this tight interface region.

Another possible function for cardiolipin in the ATP synthase, discussed before, is that its negative charges might participate directly in proton translocation through the membrane by trapping a proton in a resonance structure, leaving it with one negative charge overall (5). However, the re-estimated values of the pKa values of cardiolipin are consistent with each cardiolipin carrying two negative charges at physiological pH values, negating the proton-trap model. Nonetheless, proton transport along the membrane surface between proton pumps and ATP synthase has recently been shown to enhance ATP production by ATP synthase (46), and the negative charges of cardiolipin could still be intrinsic components of the transmembrane proton pathway. In the current structural models of the bacterial and bovine ATP synthases (19, 25, 31), the a subunit contains a bundle of four α-helices tilted at about 30° to the plane of the membrane. This bundle appears to provide two polar half channels. In mitochondria, one of them would lead from the intermembrane space, carrying protons to the essential glutamate residue in the middle of the C-terminal α-helix of a c-subunit in the a-c-ring interface. It has been proposed that once neutralized, this carboxyl residue moves by Brownian motion in a counter clockwise direction generating the first element of a rotary cycle. More negatively charged glutamates are brought successively to the proton half channel and neutralized by protons, providing further incremental rotary steps. Eventually, after being rotated through the lipid bilayer the neutralized glutamates are brought successively into a second polar half-channel in the subunit c-ring interface, where they reionise, regenerating the negatively charged carboxylate, and releasing the proton into the half-channel leading to the matrix of the mitochondrion (30). It is conceivable that the negative charges of cardiolipin molecules bound around TM-Lys-43 near to the exit of this second half-channel could be accepting protons from the ATP synthase and then be dispensing them to respiratory complexes. Such a mechanism would avoid the local accumulation of positive charges around the proton exit half channel. Similarly, in the outer leaflet of the inner mitochondrial membrane they could be bringing protons to the inlet channel by associating briefly in the vicinity of Lys-7. Although this mechanism can apply to both bacterial and mitochondrial ATP synthases, it cannot apply to the chloroplast enzyme, as chloroplast membranes are devoid of cardiolipin, however, they do contain the anionic lipid, sulfoquinovosyl diacylglyceride, which may substitute functionally for cardiolipin (47).

**Limitations and Future Directions for Simulation Work.** The current simulations, with a length of 2 × 4 μs, are short in comparison with simulations performed by Arnarez et al. of cytochrome bc1 and cytochrome c oxidase with cardiolipin (41, 42). However, the length of simulations here is justified as the symmetry of the c-ring means that the sampling is enhanced and the cardiolipin exchange at the c-ring is fast. The coarse-grained approach has the advantage of accessing timescales appropriate to observe protein–lipid interactions, with enough molecular detail to elicit meaningful results. However, secondary structure is fixed during simulations, thus preventing the observation of detailed conformational changes at protein–lipid and protein–protein interfaces. All-atom simulations of the system would provide an excellent complement to the results presented here.

**Materials and Methods**

**Coarse-Grained Models of Proteins and Lipids.** Coordinates of c-rings were taken from the bovine F1-Clp (13), the S. cerevisiae F1-Clp (20), and the I. tartaricus c11 complexes (23) (Protein Data Bank ID codes 2XND, 2X0K and 2WGM, respectively). Each structure was converted to a coarse-grained model with MARTINI scripts (48). Parameters for POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine) and POPC (1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylcholine) were taken from MARTINI (49). In those for cardiolipin (50-52), each phosphate bead had a negative charge as previously (52), and in recognition of its reassessed charged state (6). Each cardiolipin model had four 18:1 acyl groups.

**Coarse-Grained Simulations.** The simulations and the analysis of trajectories are described in SI Materials and Methods.
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