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Review

Lipids in membrane protein structures

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Abstract

This review describes the recent knowledge about tightly bound lipids in membrane protein structures and deduces general principles of the binding interactions. Bound lipids are grouped in annular, nonannular, and integral protein lipids. The importance of lipid binding for vertical positioning and tight integration of proteins in the membrane, for assembly and stabilization of oligomeric and multisubunit complexes, for supercomplexes, as well as their functional roles are pointed out. Lipid binding is stabilized by multiple noncovalent interactions from protein residues to lipid head groups and hydrophobic tails. Based on analysis of lipids with refined head groups in membrane protein structures, distinct motifs were identified for stabilizing interactions between the phosphodiester moieties and side chains of amino acid residues. Differences between binding at the electropositive and electronegative membrane side, as well as a preferential binding to the latter, are observed. A first attempt to identify lipid head group specific binding motifs is made. A newly identified cardiolipin binding site in the yeast cytochrome bc_1 complex is described. Assignment of unsaturated lipid chains and evolutionary aspects of lipid binding are discussed.

Keywords: Lipid; Membrane protein; Lipid–protein interaction; X-ray structure; Phospholipid; Cardiolipin

Contents

Abbreviations: BC1, cytochrome bc_1 complex; B6F, cytochrome b_6f complex; COX, cytochrome *c* oxidase; RC, photosynthetic reaction center; PSI, Photosystem I; BR, bacteriorhodopsin; AC, ADP/ATP carrier; FDH, formate dehydrogenase N; SDH, succinate dehydrogenase; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PI, phosphatidyl inositol; PG, phosphatidyl glycerol; CL, cardiolipin; DG, phosphatidylglycerophospholipid; SL, sulfolipid; PA, phosphatidic acid; PS, phosphatidylserine; EPR, electron paramagnetic resonance; IMM, inner mitochondrial membrane; IMS, intermembrane space; UM, undecyl maltopyranose

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1. Introduction

Biological membranes are essential for life. They provide specialized permeability barriers for cells and cell organelles, in which the interplay of lipids and membrane proteins facilitates basic processes of respiration, photosynthesis, protein and solute transport, signal transduction, and motility. The lipid bilayer hinders the diffusion of ions, a prerequisite for the generation of electrochemical potentials utilized for synthesis of ATP or active transport. The proportion of putative membrane proteins predicted from sequenced genomes is between 20% and 35% [1]. The large number of membrane-embedded proteins covers a broad range of functions in the cellular metabolism. A tight interaction of these diverse molecules with the phospholipid bilayer is required to maintain the diffusion barrier and keep it electrochemically sealed. This is especially important, as many membrane proteins undergo conformational changes that take place in or affect the transmembrane regions and may be essential for catalytic activity or required for regulatory purposes, as observed for the mechanosensitive channel MscL [2], the bacterial multidrug transporter EmrE [3], bacteriorhodopsin [4], the ATP synthase [5], the cytochrome bc_1 complex [6], and the sodium/proton antiporter NhaA [7]. The interactions between membrane proteins and the lipid bilayer have to allow for structural rearrangements while keeping the sealed nature of the membrane. The mobile lipid molecules are excellent candidates for maintaining this sealing function as they can adhere to the surface of integral membrane proteins and flexibly adjust to a changing environment.

Different aspects of membrane protein–lipid interactions have to be considered. First, the lipid bilayer provides the matrix in which membrane proteins are partially or fully embedded. Within the fluid bilayer, partitioning of the protein complement is enhanced by specific interactions with lipids. Steroid-like lipids may form conglomerates associated with specific membrane proteins and stabilize a microenvironment (patches), also termed lipid rafts, which have a proposed role in signal transduction, membrane transport, and protein sorting (see Refs. [8–12]). Second, it has been shown by spin-label EPR that a first shell of motional restricted *annular* lipids surrounds the membrane domain of proteins (see review in Ref. [13]). Evidently, the binding stoichiometry is related to size and structure of the intramembranous domain of the protein. Here, not only defined stoichiometries have been described, but also the selectivity of different membrane proteins for certain phospholipids [14]. Lipid specificity has been demonstrated as well in numerous biochemical studies, which showed that certain phospholipids are essential for the activity of several membrane proteins [15]. In general, harsh delipidation of membrane proteins results in loss of activity. However, the reasons for specific lipid requirements are in most cases not clear.

In recent years, an increasing number of membrane protein structures have been determined, the majority obtained by X-ray crystallography (see reviews in Refs. [16,17]). Interestingly, some of these integral membrane proteins contain tightly bound lipids, which have been included in the model and refined with the protein (see reviews in Refs. [18–20]). In most cases, these structurally resolved lipids are endogenous and co-purified with the membrane proteins, which are subsequently crystallized as protein–lipid complexes. These binding sites provoke discussion [21] and stimulate research to elucidate their possible functions by for instance a structure-based mutagenesis approach [22]. A wealth of biochemical and biophysical studies have demonstrated the importance of protein–lipid interactions for the assembly, stability, and function of membrane proteins [20], but careful analysis of protein–lipid interactions is required to understand the significance of specific lipid-binding sites for the structure and function of membrane proteins.

In this review, we describe recent information about membrane protein structures with tightly bound lipids. We deduce common features of phospholipid-binding sites, discuss structural and functional roles of specific protein–lipid interactions, and point out evolutionary aspects.

2. Importance of lipids for structural and functional integrity of membrane proteins

Several studies have shown specific lipid requirements for structural integrity and proper function of membrane proteins. Effects and types of protein–lipid interactions are diverse. They may be necessary for stability of the membrane proteins, have a chaperonin-like function in insertion and folding, guide assembly, or be directly

involved in the molecular mechanism, i.e., enzymatic activity or transport processes across the membrane.

Frequently, membrane protein activity decreases upon delipidation [15]. Phospholipids are, for example, essential for the function of the cytochrome bc_1 complex, a membrane protein complex of the respiratory chain, which couples electron transfer between ubiquinol and cytochrome *c* to the translocation of protons across the lipid bilayer. Increased delipidation of this enzyme leads to a gradual decrease in activity up to complete loss of function and destabilization of this multisubunit complex [23,24]. The lipid bilayer exerts lateral pressure that affects structural integrity of membrane proteins. The observed destabilization upon detergent solubilization may, in general, decrease the lateral pressure and increase conformational freedom of the protein. The impact of lateral pressure was discussed for the light-driven proton pump, bacteriorhodopsin. Archaeal membrane lipids are distinct in that they have a branched alkyl ether structure, which provides stability over a wide range of pH and temperature important for adaptation to the extreme environments in which these organisms live. Replacement of the phytanyl tails with alkyl tails resulted in structural perturbation of bacteriorhodopsin indicated by a pronounced blue shift, supporting the importance of these specific lipids for integrity and function of the protein [25].

Often, the activity of membrane proteins is affected by defined lipid species. This has been shown for the unique dianionic phospholipid cardiolipin (bis-phosphatidylglycerol) and the cytochrome bc_1 complex. Biophysical combined with biochemical studies pointed out a firm association of cardiolipin to the complex [14,24]. Digestion of tightly bound phospholipids inactivates the cytochrome bc_1 complex, without disturbing its spectral properties, and reactivation depends on addition of cardiolipin [26]. These results suggest that cardiolipin may carry out a specific function in cytochrome bc_1 complex activity, and the molecular basis for these observed effects is currently under investigation. Two cardiolipin molecules have been identified in the structure of the yeast cytochrome bc_1 complex ([22] and this study). One cardiolipin is bound close to the site of ubiquinone reduction and is proposed to be of importance for the stability of the catalytic site and to be involved in proton uptake [22]. Another example of lipids affecting protein activity is provided by the requirement for anionic lipids in protein translocation, which was initially demonstrated in vitro with cell extracts of mutants defective in phosphatidylglycerol synthesis [27]. The activity of reconstituted bacterial protein translocase, SecYEG, requires the presence of anionic phospholipids. Optimal preprotein translocation is obtained with a composition of anionic and nonbilayer lipids at concentrations that correspond to those found in the natural membrane [28].

Specific protein–lipid interactions might be important for correct insertion, folding, and topology of membrane proteins. The topology of membrane proteins is determined by positively charged residues in loops connecting the transmembrane helices. Loops enriched in positive charges are not translocated across the membrane, the 'positive-inside' rule [29]. Accordingly, the orientation of the *Escherichia coli* leader peptidase Lep can be altered by the addition or removal of positively charged residues. Interestingly, the orientation of variants is influenced by the anionic phospholipid content of the membranes, suggesting that interactions between negatively charged phospholipids and positively charged amino acid residues guide membrane insertion and topology [30]. Furthermore, it has been shown that phosphatidylethanolamine (PE) is required for the in vivo function of the secondary transporter LacY (lactose permease) of *E. coli*, an integral membrane protein with 12 transmembrane helices [31]. Analysis of function and assembly of LacY (lactose permease) in PE-deficient mutants revealed that PE is not only required for function, but also for proper folding of LacY [32,33]. Lipid-dependent recovery of normal conformation and activity could be restored by postassembly synthesis of PE, thus confirming initial studies in which PE was described as nonprotein molecular chaperone for the folding of LacY (see Refs. [34,35]).

3. Membrane protein structures with bound lipids

The emerging X-ray structures of membrane proteins with tightly bound lipids provide new insight into protein–lipid interactions. Specific requirements and associated difficulties in purifying and crystallizing these amphipathic proteins explains why the total number of membrane protein structures is still low compared to soluble proteins, for which several thousand structures are known (>20,000). To date, 52 different X-ray structures of helical, polytopic membrane proteins derived from the inner membranes of bacteria and mitochondria, as well as a few from eukaryotic membranes, have been determined, of which 23 are unrelated (for a regularly updated list, see http://www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html).

Lipids are identified in experimental difference electron-density maps by typical "hairpin"-shaped or elongated features, adhering to the transmembrane domains and most often perpendicular to the membrane plane. An interesting example is provided by bacteriorhodopsin, for which protein–lipid interactions have recently been reviewed in detail (see Ref. [36]). Bacteriorhodopsin is the main constituent of the purple membrane of Haloarchaea, which is arranged as a native two-dimensional crystal lattice in which it is organized as a trimer [37,38]. Major lipid components of the purple membrane

are archeol (2,3-di-*O*-phytanyl-*sn*-glycerol) derivatives [39]. The specific structural features of phytanyl chains aid in their discrimination from acyl chains of exogenous lipids supplemented during crystallization. Several bacteriorhodopsin structures have been obtained by X-ray and electron diffraction, seven of which hold refined lipids. The crystals for structure determination were acquired by various approaches, yielding either a two- or three-dimensional lattice. The latter were either prepared by conventional means, i.e., in the detergent-solubilized state, or with bicellar and cubic lipidic phase crystallization. The first description of tightly bound lipids was in a 3.5-Å bacteriorhodopsin structure obtained by electron crystallography [40]. NMR studies also showed tight association between halo-archaeal lipids and bacteriorhodopsin in the solubilized monomeric state [41]. Tightness and specificity of lipid binding to bacteriorhodopsin is indicated by the fact that only native lipids are retained during purification and observed in the structures regardless of different experimental approaches and crystallization conditions.

The majority of identified lipids in the diverse membrane protein structures are endogenous and reproducibly co-purified with the protein despite detergent extraction/purification; that is, membrane proteins are isolated as protein–lipid complexes. Comparison of the available membrane protein–lipid structures permits the recognition of common features in lipid-binding modes, which will be outlined below.

3.1. General principles of lipid binding to membrane proteins

Three types of binding modes can be distinguished for lipid interactions with membrane proteins. First, there is an *annular shell* of lipids bound to the protein surface, which resembles the bilayer structure and mediates between protein and bilayer. Second, lipid molecules are immersed in cavities and clefts of the protein surface, frequently observed for multisubunit complexes and multimeric assemblies. These *nonannular surface lipids* are typically present at subunit or oligomeric interfaces. Third, a few examples represent lipids, which reside within a membrane protein or a membrane protein complex and are in unusual positions, e.g., with the head group below the membrane plane and/or non-perpendicular to the bilayer. These *integral protein lipids* could play a role in folding and assembly of membrane proteins.

3.1.1. Lipids of the annular shell

The number of lipid molecules surrounding the membrane spanning domain has been deduced for several proteins from EPR spin labeling studies, which quantify the association of motional restricted lipids with membrane proteins [13]. If the structure of a membrane protein is available, the number of lipids can be calculated from the surface area of the membrane spanning part. Interestingly, a nearly complete annular shell was described for the trimeric bacteriorhodopsin, which has seven transmembrane helices per monomer. Based on the structure, 24 lipids were calculated to enclose the membrane-spanning region of the trimer and six additional molecules to fill the central pore [40] (see also Ref. [36]). A bilayer of up to 18 tightly bound lipid chains was identified in X-ray structures [42,43]. Luecke et al. calculated that 80% of the trimer surface is covered by the described lipids.

However, most membrane protein structures contain only a few lipid molecules, which belong to the first annular shell. This may be the result of exhaustive purification procedures in the presence of detergents necessary to obtain homogeneous and highly purified material for 3D-crystallization. The purification procedure can be optimized by careful adjustment of the protein-to-lipid ratio, to detect additional lipids without compromising uniformity of the preparation. A recent example is provided by the yeast cytochrome bc_1 complex, where minimizing exposure to detergent resulted in a more active preparation with additional phospholipids resolved in the structure [44].

The presence of just a few structurally resolved representatives of the annular shell reflects the different binding affinities. EPR spin-label analysis revealed a motional restricted first shell of lipids with an effective rotational correlation time of $\sim 10^{-8}$ s [45]. Lipid exchange rates, however, vary considerably in the range of 10^4–10^8 s^{-1}, depending on the nature of the lipid–protein interaction, as well as on experimental conditions and applied analytical techniques (see Refs. [13,21]). Endogenous lipids retained during detergent extraction and purification procedures, which are reproducibly detected in membrane protein structures, can be judged as tightly bound. However, exact measurements for the binding affinity of individual structurally resolved lipids are not available.

The annular lipids mediate between the membrane protein and the bulk lipid bilayer and are presumably important for vertical positioning of the protein in the bilayer. The yeast cytochrome bc_1 complex with bound lipids is shown in Fig. 1A and B. Phospholipids of the matrix leaflet [22] and a newly identified phospholipid in the intermembrane leaflet after less delipidation of the complex [44] allow determination of position and thickness of the bilayer associated with the complex. The distance between the phosphodiester groups of two oppositely oriented molecules, cardiolipin and phosphatidyl ethanolamine, is 36 Å (see Fig. 2A). This is in good agreement with the experimentally determined thickness of pure phosphatidylcholine bilayers with 18:1 acyl chains, where the phosphodiester groups are 38 Å apart and the hydrophobic core is 27-Å thick [46]. Annular lipids cover the roughness

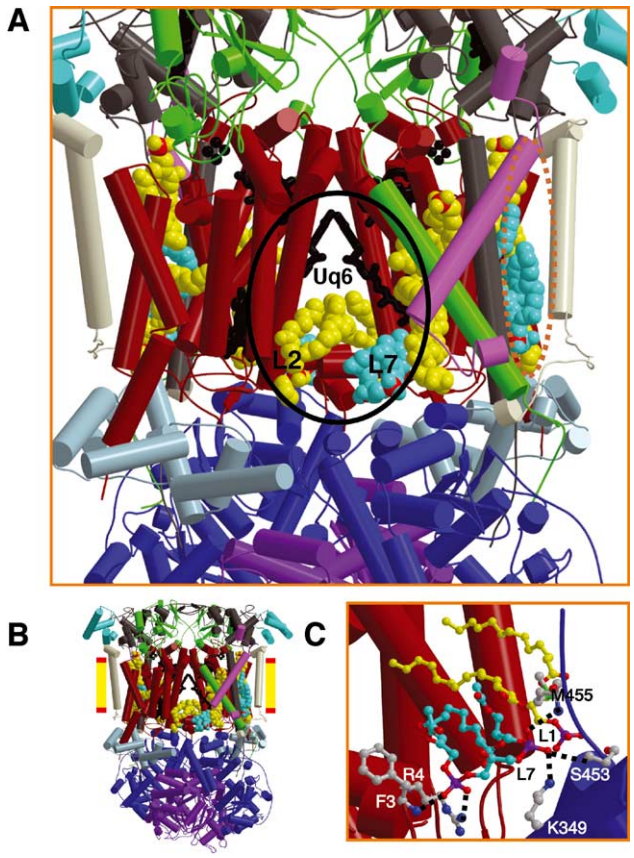

Fig. 1. Yeast cytochrome bc_1 complex with tightly bound phospholipids. (A) The binding sites of lipid molecules to the complex clearly define the vertical positioning of the complex in the membrane. The majority of identified lipids are from the matrix leaflet of the bilayer. Lipids are shown in space-fill representation and colored yellow [(PDB: 1KB9 [22]); (PDB: 1P84 [44])], with the exception of the two cardiolipin molecules, which are shown in cyan [(PDB: 1KB9); L7 (described here)]. Phosphor atoms are displayed in red. Cofactors and Q_i site occupant (ubiquinone, Uq6) are shown as black ball-and-stick models. Helices are depicted as cylinders and colored according to subunits: cytochrome *b* (brown), cytochrome c_1 (dark gray), Rieske (green), Qcr6p (cyan), Qcr7p (midgray), Qcr8p (white), Qcr9p (magenta), Cor1p (blue), and Qcr2p (purple). The two lipid-filled cavities are encircled, at dimer interface (black) and at the side of homodimer (brown, dotted line). (B) View of the homodimeric complex parallel to the membrane plane with the intermembrane space (IMS) at the top and the matrix at the bottom. The inner mitochondrial membrane (IMM) is schematically shown in yellow illustrating the hydrophobic thickness, and red lines depict the position of the polar lipid head group region. (C) The binding site of cardiolipin, L7 (cyan), at the subunit interface of cytochrome *b* (brown) and Cor1p (blue). Hydrogen bonds to direct ligands (light gray) of the phosphodiester moieties are depicted and the neighboring lipid, L1, is shown in yellow. Phosphor, oxygen, and nitrogen atoms are marked in purple, red, and blue, respectively. Interestingly, the main ligands of the polar head group are from subunit termini, i.e., C-terminus of cytochrome *b* and N-terminus of Cor1p. This cardiolipin is, together with L2, positioned at the putative substrate entry point, encircled with black line in A located at the dimer interface. Illustrations were prepared by MolScript [97] and Raster3D [98].

of the protein surface and, importantly, provide a tight integration of the protein into the membrane. Furthermore, these specific protein lipid interactions are likely to be important for correct protein insertion and folding (see Section 2).

3.1.2. Nonannular surface lipids

The surface of multimeric or multisubunit membrane protein complexes exhibits cavities, clefts, or pores. Strikingly, structurally resolved lipids are frequently located at contact sites between adjacent monomers of oligomeric complexes, e.g., for the tetrameric potassium channel and the trimeric bacteriorhodopsin, or at the contact sites between subunits of multisubunit complexes, such as in the cytochrome bc_1 complex. In general, two components for stabilizing contacts of lipids have to be considered—on the one hand, the mainly hydrophilic interactions between the lipid head group and the protein, and on the other hand, the remaining nonpolar but larger area comprising interactions with the hydrophobic tail.

3.1.2.1. Oligomeric assemblies. In oligomeric assemblies, central pores often exist in which internal lipid patches are discontinuous from the lipid bulk phase and may have unique compositions and physicochemical characteristics [47]. In the 2.9-Å X-ray structure of bacteriorhodopsin obtained by heterogenous nucleation with benzamidine, the central compartment binds three molecules of the haloarcheal glycolipid S-TGA-1 (3-HSO_3-Galpβ1-6Manpα1-2Glcpα-1-archaeol). Nanoelectrospray ionization-mass spectrometry (nano-ESI-MS) demonstrated the presence of purple membrane lipids in these crystals. Interestingly, the central lipid patch is shifted by 5 Å toward the membrane center, providing an example of local membrane thinning. The importance of this specific lipid binding is not clear. It has been shown that the presence of native phospholipids, namely phosphatidylglycerophosphate and squalene, are required for a normal photocycle [48]. Increased rates of proton pumping were found when delipidated bacteriorhodopsin was reconstituted in phosphatidylglycerophosphate vesicles in the presence of sulfated triglycosylarchaeol [49].

Filling the crevices between adjacent monomers appears important to provide tight and sealed protein integration into the membrane, as already outlined for annular lipids. In addition, these specific binding sites may be involved in the assembly and/or association of monomers or subunits. For the light-harvesting complex II, it has been shown that the assembly depends on defined phospholipid and glycolipid species [50,51]. In bacteriorhodopsin, a single lipid has been observed at every contact site of adjacent monomers [38,47]. Although the head group was not unequivocally assigned, tight interactions of the phosphate with Lys-40 were proposed based on its important role in purple membrane formation [52]. It was concluded that this specific lipid acts as the "glue" in trimerization of bacteriorhodopsin [36]. Binding of a lipid molecule in the hydrophobic crevice between adjacent monomers has been shown as well for the

homotetrameric potassium channel KcsA [53]. Although the head group could not be clearly assigned, binding of negatively charged lipids to the channel was shown by lipid extraction. In this study, correct refolding of KcsA tetramers in vitro required lipids, but did not appear to be dependent on negatively charged species. However, the biosynthetic assembly of the KcsA tetramer has been shown to be most efficient in the presence of PG [54]. Furthermore, it was confirmed that the potassium channel requires the presence of negatively charged phospholipids for ion conduction [53]. The importance of the lipid-binding site described in the X-ray structure for channel function needs to be investigated.

3.1.2.2. An example for multisubunit assemblies, the yeast cytochrome bc_1 complex. The structure of the homodimeric yeast cytochrome bc_1 complex consists of nine subunits per monomer. The integral membrane domain of the dimer comprises 24 transmembrane helices (TM), which are contributed by the three catalytic subunits—cytochrome *b* (8 TM), cytochrome c_1 (1 TM), and Rieske protein (1 TM)—and the small subunits Qcr8p (1 TM) and Qcr9p (1 TM). Five tightly bound phospholipid molecules, including cardiolipin, were initially identified and refined at 2.3-Å resolution [22]. Subsequently, the protein preparation was compromised for less delipidation by decreasing the duration of an anion exchange chromatographic step by without negatively affecting the crystal quality. Delipidation lowers the enzymatic activity of the cytochrome bc_1 complex [23,24]. Accordingly, the less delipidated protein preparation exhibits a 28% increase in enzyme activity and reveals a new crystallographically resolved phospholipid (L6), which represents the intermembrane leaflet [44]. Inspection of two additional 2.5-Å resolution data sets from independent protein purifications (unpublished data) revealed a second tightly bound cardiolipin molecule in the matrix leaflet of the bilayer (L7; Fig. 1C and Section 3.2.2).

Dynamic properties of an atomic model can be judged by the B-factors, which reflect the disorder for atomic positions. B-factors increase with growing statistical and dynamic disorder, i.e., low occupancy and high mobility, respectively. Table 1 lists all lipid molecules identified in the cytochrome bc_1 complex and marks the interatomic contacts with the respective protein subunits. Ordered binding of the described lipids is manifested by their average B-factors, which are similar to the values obtained for the membrane spanning region of the

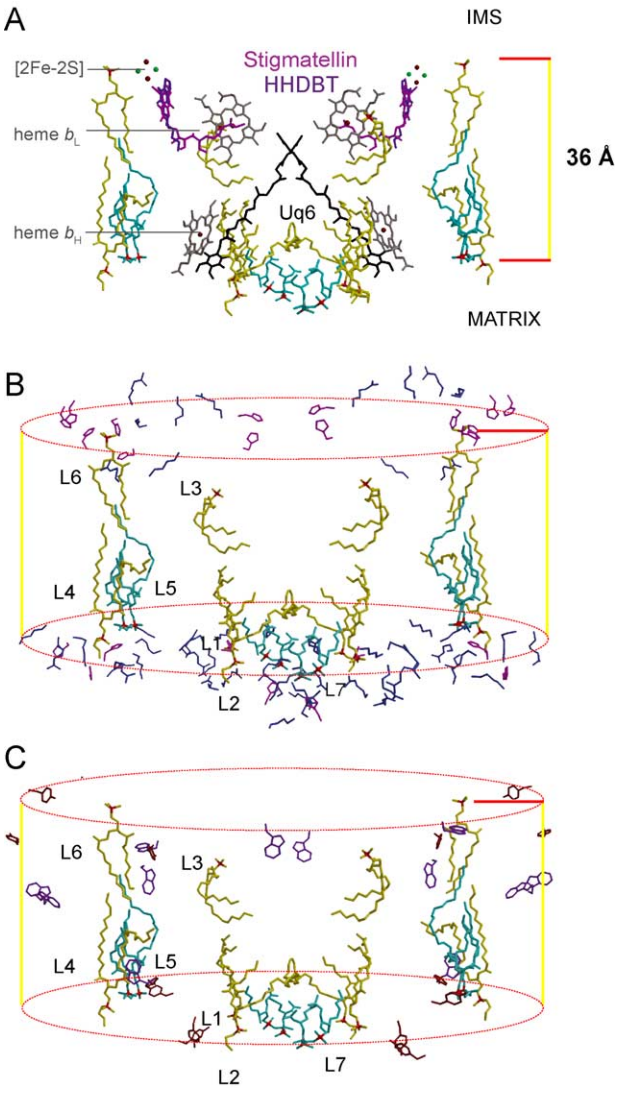

Fig. 2. Characterization of lipid binding sites in the yeast cytochrome bc_1 complex (PDB: 1KB9 and 1P84). (A) Shown in ball-and-stick are the lipids (color coding as in Fig. 1A), the two *b*-type hemes (light gray), Q_i site substrate ubiquinone (Uq6, black), and the superimposed Q_o site inhibitors (HHDBT and stigmatellin). Phosphor, iron, and sulfur atoms are marked in red, brown, and green, respectively. The thickness of the bilayer of 36 Å can be estimated from the distance between the phosphate moieties of matrix leaflet lipid L4 and intermembrane space leaflet L6 (horizontal red and vertical yellow lines). (B) Lipids as well as His (magenta) and Lys/Arg (blue) residues of the complex are depicted. The transmembrane region is sketched as in A, where vertical yellow lines show the bilayer thickness, whereas red ellipsoids mark the interface where the polar lipid head groups form the topmost layer of the membrane. Surface accessible residues (>50-$Å^2$ accessible surface area) were included if positioned 5 Å below or above the marked membrane plane. An asymmetric distribution of the strong basic residues is evident. The applied cutoff criteria restricted the displayed residues to a total number of 41 His/Lys/Arg residues, i.e., 12/14/15, respectively, for each monomer, of which 4/11/10 are positioned on the matrix side. More specifically, the distribution of basic residues was as follows: Cor1p (1/1/4), cytochrome *b* (4/3/4), cytochrome c_1 (3/3/1), Rieske (0/1/1), Qcr7p (2/0/0), Qcr8p (1/2/4), and Qcr9p (1/4/1). (C) Accessible Tyr (brown) and Trp (purple) residues are preferentially located at the protein–lipid interface pointing to their proposed role in positioning the complex by "anchoring" the transmembrane region in the lipid bilayer. The same criteria for residue selection as in B is applied here. This restricted displayed residues to a total number of 14 Tyr/Trp residues (i.e., 8/6 respectively) for each monomer, including subunits: cytochrome *b* (3/3), cytochrome c_1(1/0), Rieske(1/0), Qcr8p (3/2), and Qcr9p (0/1). The program NACCESS was used for surface accessibility calculations [99]. The soluble fragment of Rieske, the hinge protein (Qcr6p), and Cor2p, as well as all lipid species, were omitted from the model used for accessibility calculations. Illustrations were prepared using MolScript [97] and Raster3D [98].

protein (Table 1). In comparison, an average B-factor of 27 Å^2 of the protein model in contrast to 57 Å^2 of the lipid phytanyl chains were observed for the 1.55-Å resolution structure of bacteriorhodopsin [43]. In addition to reproducible B-factor distribution when comparing different data sets, an impressive superimposition of the phospholipid-binding sites in the yeast cytochrome bc_1 complex was observed, including head group specificity, structurally resolved length of acyl chains, and even conserved "kinks", which point to the presence of double bonds (see Section 3.2.3). This reproducibility of lipid binding clearly indicates tight and specific protein–lipid interactions. Interestingly, all structurally resolved lipids were found to interact with at least two subunits, and the lipid L2 interacts with cytochrome *b* of both monomers (see Table 1).

3.1.3. Integral protein lipids

Few lipids have been observed to reside within a membrane protein complex. These integral protein lipids may be of special importance for folding and assembly of membrane proteins. In the yeast cytochrome bc_1 complex, one lipid molecule (L3) was observed in a so-called "interhelical" position [22] (Fig. 3). It is wrapped around the transmembrane helix of the Rieske protein with the ends of the acyl chains reaching into the hydrophobic cleft at the dimer interface extending parallel to the membrane plane. It was assigned as phosphatidylinositol, and its head group is pointing toward the intermembrane space. Remarkably, the position of the phosphate group is 10 Å below the zone of phosphodiester groups of the intermembrane leaflet, exemplified by lipid L6 (Fig. 2). The L3 headgroup is surrounded by transmembrane helices of all catalytic subunits of the complex and subunit Qcr9p. The phosphodiester group of L3 forms a stabilizing ion pair with Lys-272, which is conserved in eukaryotic cytochrome c_1 with the exception of *Neurospora crassa*. Side chains of all catalytic subunits contribute to the stabilization of the headgroup by formation of several hydrogen bonds with the inositol moiety. The acyl chains of L3 fit tightly in a groove between the helices and their position is fixed by hydrophobic interactions with residues of all catalytic subunits. This lipid molecule may stabilize the helix packing between the transmembrane anchor of the Rieske protein and the core of the complex. As it binds close to the pivot point of movement, it could dissipate torsion forces generated by the fast movement of the extrinsic domain of the Rieske protein. The mobility of the latter is essential for the activity of the enzyme [6,55]. Furthermore, it might be important for assembly, as it is located at a point where four out of five membrane spanning subunits of a monomer encounter each other.

There are additional examples of integral protein lipids of suggested functional importance. In the 2.5-Å-resolution structure of the cyanobacterial Photosystem I (PSI), three negatively charged phospholipids and one uncharged galactolipid were observed, all with their head groups bound on the electronegative, i.e., stromal side of PsaA/B [56]. The electron density for the four head groups was well defined and they were assigned as phosphatidylglycerol and monogalactosyldiglycerides, as supported biochemically. Their functional importance was suggested from the observation that two lipids are tightly bound close to the central core of PSI instead of being on the detergent exposed side. Furthermore, one lipid coordinates the antenna chlorophyll *a* (aC-PL1) with its phosphodiester group [56].

In the cytochrome *c* oxidase from *Rhodobacter sphaeroides*, six phospholipid molecules were identified and assigned as phosphatidylethanolamines [57]. Four of

Table 1
Interatomic contacts between ligands and cytochrome bc_1 (BC1) subunits

BC1 subunits			Cor1p	Qcr2p	Rip1p	Cobp	Cyt1p	Qcr6p	Qcr7p	Qcr8p	Qcr9p
B factors (Å^2)			76–79	91–94	60–66	37–40	55–59	78–81	54–56	75–78	72–74
Ligands	B factors (Å)	Chain length									
L1	67–70	5–15; 13	X			X					
L2	64–68	16–18; 7				X/X*					
L3	54–56	14; 12			X	X	X				
L4	62–63	14; 16–18				X			X	X	
L5	74	11; 10; 18; 9				X	X		X		
L6	78–93	9–10; 11				X	X				
L7	86–88	2; 2; 2; 7	X			X					
UM	55	11	X		X						X
UQ_6	77	Isoprenoid (6x)				X					

Abbreviations for BC1 subunits were taken from the YPD database. Style of lipid designation is taken from Ref. [22]: L1–L5), L6 [44] and L7 (described here) were recently identified. The acyl chain lengths show the number of carbon atoms counting from the carboxy-ester bond. The side chains were truncated according to visibility in the experimental electron density and are therefore presented as the range of length as refined in four different data sets. Likewise, the average B factors (Å^2) calculated for subunits and ligands in the analyzed data sets are shown. Chain length gives an indication of the extent of tight binding of acyl chains, but does not necessarily reflect the in vivo nature of the side chains. Asterisk denotes association with the other monomer.

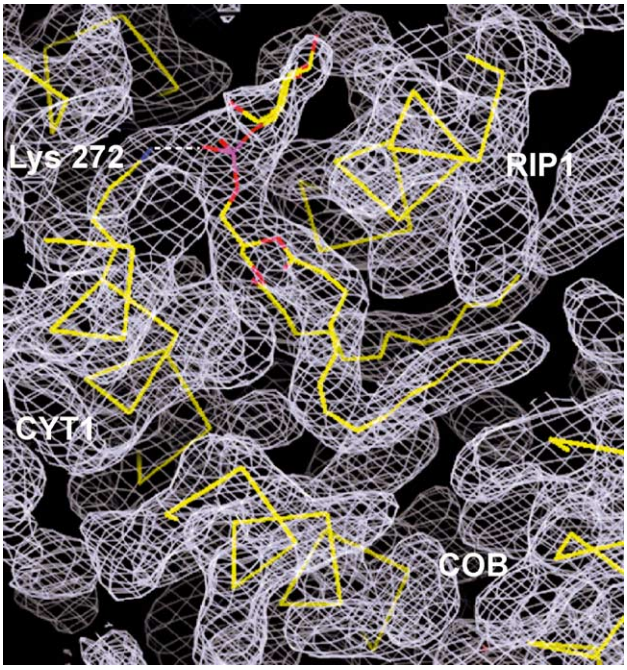

Fig. 3. The interhelical phosphatidyl inositol (L3) is wrapped around the transmembrane helix of the Rieske protein in the cytochrome bc_1 complex (BC1). The integral protein lipid L3 displays an *unusual* position, with the phosphodiester group 10 Å below the membrane plane. Interestingly, side chains of all three catalytic subunits of BC1 contribute to the stabilization of the head group [22]. The 2Fo–Fc electron density map after refinement of the lipid is contoured at 1σ (blue-gray), the ligand of the phosphodiester group Lys-272 (Cyt1p) and L3, are shown in stick presentation, and the protein backbone is drawn as α-carbon trace.

them bind on the interface between subunit IV and subunit I/III. Interestingly, subunit IV has no direct interaction with any other subunit and its position is exclusively stabilized by indirect contacts via the lipid molecules.

3.2. Specificity of lipid binding

3.2.1. Head group specific binding sites

Frequently, lipid head groups are not clearly resolved, even at high resolution, as shown for the 1.55-Å resolution structure of bacteriorhodopsin [43]. This was interpreted as either low affinity or unspecific binding of the head group, causing noninterpretable electron densities. As more membrane protein structures are determined, the incidences of head group stabilization increase, of which many have been refined and verified by mass spectrometric analysis. Table 2 gives a list of direct ligands of the phosphodiester groups of tightly bound lipids in membrane protein structures with assigned head groups. Although the number of available data is still low, an initial deduction of general principles for stabilization is feasible. In our experience, the position of the phosphodiester group is best defined when considering tightly bound phospholipids, due to strong interactions with the protein via ion pairs and hydrogen bonds. The analysis presented here focuses therefore on interactions between the phosphodiester group and amino acid side chains.

First, the majority of the identified lipids with strongly stabilized head groups is bound on the electronegative side of the membrane (*n* side), i.e., the mitochondrial matrix, the stroma of chloroplasts, and the cytoplasmic side of the plasma membrane; the most evident examples being the mitochondrial cytochrome bc_1 complex [22] and cytochrome *c* oxidase [58] (see Table 2). This is consistent with the asymmetric charge distribution on the membrane protein surface. As described in the 'positive-inside' rule, regions of polytopic membrane proteins facing the negative side of the membrane are generally enriched in arginine and lysine residues, whereas the translocated regions are largely devoid of these residues [29,59]. One can expect that the presence of basic residues allows stronger stabilization of the lipid by formation of ion pairs with the phosphodiester group. It was already pointed out in the description of the first X-ray structure of a membrane protein, the purple bacteria photosynthetic reaction center, that a ring of surface exposed nitrogen atoms from arginine and histidine side chains is located in the transition zone between transmembrane and extrinsic regions at the cytoplasmic side of the membrane [60]. A role of these basic residues in positioning the reaction center perpendicular to the membrane via interactions with the negatively charged phosphate head groups was proposed. In the structure of the yeast cytochrome bc_1 complex, arginine and lysine residues are prevalent at the level of the phosphodiester groups on the matrix side, whereas the ratio is shifted to histidine residues on the side of the intermembrane space (Fig. 2B). Tight stabilization of lipids on the electropositive side of the membrane (*p* side) might occur less frequently due to lack of strong stabilizing interactions. The asymmetric distribution of positively charged residues and the impact of anionic phospholipids in determining membrane protein topology [30] coincides with the distribution of high-affinity lipid-binding sites. This observation supports the importance of tight protein–lipid interactions for integration and folding of membrane proteins.

The following residues are observed as primary ligands of phosphodiester groups on the *n* side (excluding those of phosphatidylcholine) and listed in the order of number of occurrences: Arg>Lys>Tyr>His>Trp, Ser, Asn. Additional stabilizing interactions are provided by Thr and Gln. Although the group is small for binders at the *p* side, one can summarize that Tyr, Thr, Asn, Gln, His, and Arg are present as primary ligands and positive charges are less frequently observed as stabilizing ligands. Stabilization of the phosphodiester group of phosphatidylcholine (PC) appears weaker, with less positively charged ligands in direct vicinity. A combination of His and Ser as a ligand pair is observed (L6) and single interactions with His, Thr, and Ser, are observed. The absence of positively charged ligands at close distance to the PC phosphodiester may be a

Table 2
Direct ligands of the negatively charged lipid head group mostly identified in X-ray structures of membrane proteins

PL	AA	Protein	Comment	Species	PDB	Res. [Å]	Reference
PE	KT	BC1	*n*, annular	*G.gal.*	1BCC	3.2	[6]
PE	**Y**QT	BC1	*n*, same binding site as L4	*G.gal.*	1BCC	3.2	[6]
PE	**Y**NYQK	BC1	*n*, annular, **L4**	*S.cer.*	1KB9	2.3	[22]
PE	**N**	BC1	*n*, dimer interface, **L2**	*S.cer.*	1KB9	2.3	[22]
PE(4)	**R**HS; **R**W; **R**; K	COX	*n*	*R.sph.*	1M56	2.3	[57]
PE(2)	**R**; **K**R	COX	*n*	*B.tau.*	1V54/1V55	1.8/1.9	[58]
PE	**KY**R	RC	*n*, thermophilic bacteria	*T.tep.*	1EYS	2.2	[101]
PE	KK	SDH	*n*	*E.col.*	1NEK	2.6	[73]
PE(2)	**Q**HK; **Y**Q	COX	*p*	*R.sph.*	1M56	2.3	[57]
PE(1)	**T**	COX	*p*	*B.tau.*	1V54/1V55	1.8/1.9	[58]
PC	**S**	BC1	*n*, cavity at dimer interface, **L1**	*S.cer.*	1KB9	2.3	[22]
PC	**R**HE	COX	*n*	*P.den.*	1QLE	3.0	[102]
PC	H	COX	*n*	*B.tau.*	1V54/1V55	1.8/1.9	[58]
PC(3)	T	AC	*n*	*B.tau.*	1OKC	2.2	[74]
PC	**H**S	BC1	*p*	*S.cer.*	1P84	2.5	[44]
PC	**R**D	COX	*p*	*P.den.*	1QLE	3.0	[102]
PI	**K**ST	BC1	*p*, integral protein lipid, **L3**	*S.cer.*	1KB9	2.3	[22]
PG(3)	**R**HS; **RW**	COX	*n*	*B.tau.*	1V54/1V55	1.8/1.9	[58]
PG(3)	**RN**RD; **RS**	PSI	*n*	*S.elo.*	1JBO	2.5	[56]
CL	**K**, **R**H	BC1	*n*, cavity at dimer interface, **L7**	*S.cer.*	–	2.5	here
CL	**KY**K, **Y**HN	BC1	*n*, near Qi site (proton uptake), **L5**	*S.cer.*	1KB9	2.3	[22]
CL	RKYD, **H**KK	COX	*n*	*B.tau.*	1V54/1V55	1.8/1.9	[58]
CL	**HR**, KW	RC	*n*	*R.sph.*	1M3X	2.55	[70]
CL	**H**RN, **WH**	RC	*n*, lipidic cubic phase	*R.sph*	1OGV	2.4	[103]
CL(2)	**S**	AC	*n*	*B.tau.*	1OKC	2.2	[74]
CL	NDY, **HN**	COX	*p*	*B.tau.*	1V54/1V55	1.8/1.9	[58]
CL	**NT**, NS	FDH	*p*, interface of adjacent monomers	*E.col.*	1KQF	1.6	[72]
DG	**K**S	BR	*n*, important for trimer formation	*H.hal.*	1BM1	3.5	[104]
SL	**RK**N	B6F	*n*, sulfolipid, cavity at dimer interface, same position as L1 and L4	*C.rei.*	1Q90	3.1	[78]

Polar interactions of amino acid side chains (AA) with phosphodiester groups are listed. CL has two phosphodiester groups, accordingly the ligands of each group are separated by a comma (,), whereas ligands of distinct lipids are separated by a semicolon (;). Residues within 4.5-Å distance from the phosphodiester group are listed. Primary ligands with interatomic contacts at a distance of less than 3 Å are marked with bold. The abbreviation *n* denotes the negative leaflet of the bilayer: cytoplasm (bacteria), matrix (mitochondria), and stroma (thylakoid). Accordingly, *p* marks the positive side of the membrane, e.g., periplasma (bacteria), intermembrane space (mitochondria), and lumen (thylakoid). All structures were obtained by X-ray crystallography and the PDB code as well as resolution (Res.) of each data set is given in Å.

consequence of the bulky, triple methylated, and positively charged choline head group.

To summarize, lipid phosphodiester groups (excluding PC) are often stabilized by two or more residues, with motifs frequently combining a positively charged and a polar ligand, for instance KT, KW, KY, RS, RW, RY, RN, HS, HW, and HY. Furthermore, a specific binding pattern for the diacidic cardiolipin is suggested. Tight binding interactions with three residues are observed: KKY, RKY, and HRN. XXY, where X is a positively charged and Y a polar residue, can be suggested as a preliminary cardiolipin binding motif. It is important to point out that the suggested motifs are nonlinear, even ligands from different subunits may contribute; therefore, structure-based searches are required to identify lipid-binding sites. It should be noted that in addition to the above-described amino acid side chain interactions, backbone nitrogen or oxygen atoms are also observed to contribute to stabilization of the phosphodiester group, although not pointed out in Table 2.

Aromatic residues are frequently involved in lipid stabilization. Tyrosine residues are typically present as polar ligands of phosphodiester groups, either as main stabilizing ligands or in combination with a positively charged ligand. In the yeast cytochrome bc_1 complex, they are observed with their side chains at the level of the phosphodiester groups at both sides of the membrane domain surface (Fig. 2C). Within the transmembrane region, tryptophan residues are preferentially located in the hydrophobic/hydrophilic transition zone with their indole ring oriented toward the center [60]. Fig. 2C depicts solvent-accessible tryptophan residues in the yeast cytochrome bc_1 complex. Two main types of phospholipid-stabilizing interactions via tryptophan exist. Hydrogen

bonds are frequently observed between the indole nitrogen atom and the phosphodiester group. In addition, a lamellar orientation of the side chain permits stabilization of the acyl chain position often contributing with large surface contact area (see Fig. 4, and Section 3.2.3).

In addition to interactions with the phosphodiester groups, the head group moieties are stabilized by multiple interactions with the protein. A detailed description of interactions between lipid molecules and protein residues has been given, e.g., for the yeast cytochrome bc_1 complex [22]. An attempt to identify head group specific binding motifs would be premature, as the number of occurrences in the database is still too low and in some cases head group assignment is pointed out as tentative. Exact assignment and refinement of tightly bound lipids is crucial for future studies to improve our knowledge about lipid-specific binding sites.

3.2.2. Cardiolipin

Cardiolipin is an anionic phospholipid that consists of two phosphatidyl residues linked by a glycerol moiety [61]. In eukaryotes, it is found almost exclusively in mitochondria or plastids, and is also present in energy-transducing membranes of prokaryotes [62–64]. A large number of mitochondrial proteins that reside in the inner membrane interact with cardiolipin [63–65]. The presence of this phospholipid molecule is, e.g., essential for full activity of the ADP–ATP carrier [66] and of cytochrome *c* oxidase [67,68]. However, it is not clear how cardiolipin contributes to full catalytic activity of these enzymes. The importance of this dianionic phospholipid may result from its unique large and charged headgroup, requiring a specific and tightly interacting binding site, which may stabilize a protein domain in a clamp-like manner.

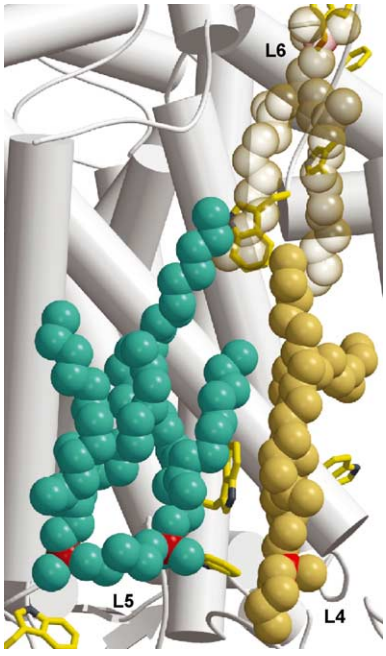

Fig. 4. Lamellar-oriented tryptophan residue structures the protein surface for lipid binding. Phospholipids (in space-fill presentation L4, L5, and L6) line each side of the cytochrome bc_1 complex (PDB 1P84) and mark the transmembrane region as viewed from the membrane plane. Transmembrane helices are shown as white cylinders and Trp residues visible in this orientation are painted yellow with the nitrogen depicted in blue. Notably, Trp residues line up with the polar nitrogen pointing to the phosphodiester groups and the indole ring is positioned vertically between the acyl side chains, demonstrating the specific role of Trp in aligning and orienting the lipids on the protein surface.

Cardiolipin binding was first reported for the photosynthetic reaction center of the purple bacteria *R. sphaeroides* [18] and has consequently been described in different structures of the wild-type and variant protein [69,70]. The quality of the electron density is variable; this was discussed as substoichiometric binding of the lipid to the detergent-purified reaction center [71]. Cardiolipin is bound by direct interaction of the polar head group with the side chains of Arg-267, His-145, and the backbone amide of Lys-144, all from subunit M. Additionally, the head group is stabilized by water-mediated hydrogen bonds.

One cardiolipin molecule has been described in the structure of cytochrome bc_1 complex [22]. The observed hydrogen bonding pattern at the Q_i site suggests a direct involvement of this cardiolipin in proton uptake at the ubiquinone reduction site, the Cl/K pathway. When examining the electron density maps of the yeast cytochrome bc_1 complex structures, which were obtained from a less delipidated and concomitantly more active protein preparation [44], a second cardiolipin molecule (L7) was identified. The dianionic head group of L7 was unambiguously identified in all Fo–Fc difference density maps calculated from different data sets of crystals from the modified protein preparation. The molecule was included in the model and its position refined. L7 adheres to the protein surface in the hydrophobic cleft close to the homodimer interface, the head group facing the matrix side (Fig. 1C). It is stabilized via several polar and nonpolar interactions with residues of the C-terminal domain of the core 1 protein subunit (Cor1p) and the N-terminal region of cytochrome *b* (Cobp) from the same monomer. More specifically, Lys-349^{Cor1p} is the primary ligand of phosphodiester group A (P_A) and phosphodiester group B (P_B) is stabilized by polar interaction with Arg-4^{Cobp}. Also, backbone nitrogen atoms of Phe-3^{Cobp} and Arg-4^{Cobp} are within hydrogen bond distance to P_B-O12 and the backbone nitrogen atom of Met-455^{Cor1p} is within hydrogen bond distance to P_A-O4′. Additionally, several nonpolar interactions contribute to the binding of the head group. L7 binds at the interface of different subunits; it might therefore be important for structural integrity of the complex. Interestingly, the main ligands of the head group of L7 are key players in a tightly interwoven hydrogen bond network, which also includes structural water molecules, at the core 1 protein and cytochrome *b* interface.

The acyl chains of L7 are largely unresolved pointing to conformational flexibility of these side chains extending into the lipid bilayer, rather than being fixed by tight

interactions on the protein surface. The observed mobility of the acyl chains suggests that they contribute to the lipid layer. This may suggest that this lipid participates in forming a feasible environment to promote substrate diffusion from the membrane to the active sites and/or substrate exchange between sites of quinone/quinol catalysis within the complex. Interestingly, several acyl chains terminate in the cavity leading to the active sites (encircled in Fig. 1A). Structural and/or functional relevance of the L7 binding site will be explored by site-directed mutagenesis.

Tightly bound cardiolipin has also been observed at the interface of the homodimeric bovine cytochrome *c* oxidase [58] and at the interface of adjacent monomers in the trimeric formate dehydrogenase N [72]. In addition, cardiolipin binding sites have been reported for the succinate dehydrogenase from *E. coli* [73] and the ADP/ATP carrier [74].

Apparently, cardiolipin binds preferentially at monomer interfaces of oligomeric assemblies and at subunit interfaces of multisubunit complexes. In addition, its importance for formation of supercomplexes (see Section 6) has been suggested. Functional implications of cardiolipin binding need to be addressed in more detail.

3.2.3. Binding of lipid chains

A common feature of the structurally resolved lipids is that the fatty acyl or phytanyl side chains are snuggled in shallow grooves and clefts, the position of the chains stabilized by multiple van der Waals contacts [22,43]. The extent to which the acyl chains can be exactly traced varies considerably, and chains should be truncated according to visibility in experimental electron density, before inclusion in the model for refinement. Attraction from the protein frequently forces the hydrophobic tails into distinct often highly curved shapes, which deviate considerably from the sprawled though mobile conformation expected in a lipid bilayer [75,76]. These patterns of acyl chain stabilization are amazingly reproducible, as observed for the cytochrome bc_1 complex when comparing the electron density maps of four data sets obtained from different membrane and protein preparations. Phospholipid positions are exactly superimposed and even the resolved length of the tails is very similar. Tight binding of these lipids is indicated by the distribution of average B factors for the lipids, which are only slightly higher than those for the associated protein subunits (Table 1).

The asymmetric distribution of surface-accessible tryptophan residues has been pointed out (see Section 3.2.1) and is obvious in the structure of yeast cytochrome bc_1 complex (Fig. 2C). A biochemical study of interfacial tryptophan residues has shown that they do not determine the topology of membrane proteins, but they have been proposed to fulfil a functional role as interfacial anchoring residues [77]. In membrane protein structures, tryptophan residues not only contribute to polar stabilization of the phosphodiester group via the indole nitrogen atom, but also lamellar orientation of the indole ring between adjacent phospholipid chains is observed as shown in Fig. 4. A ring of lamellar oriented surface exposed tryptophan residues may provide an interlocking tooth system for the first shell of annular lipids.

The acyl chains of lipid are usually refined as fully saturated, although it is often evident from the density that one or more double bonds would be energetically more feasible to produce the extreme bending angles that are frequently observed. For instance, the acyl chain of L4 in the yeast cytochrome bc_1 complex was reproducibly found to bend at a close to 90° angle to extend into a narrow and highly hydrophobic dent coated by cytochrome *b* residues, including side chains of Phe-94, Leu-101, Ile-330, and Phe-333 (Fig. 5A). An interesting aspect is that the fully resolved side chain invariably takes this position, despite no obvious sterical reason hindering it from extending into the bilayer. The observed 90° curvature of the lipid chain points to the presence of two consecutive double bonds. These unsaturated lipid species have been observed by nano-ESI-MS analysis of crystals of this complex (Lange and Hunte, in preparation).

The tight and specific interactions between lipids and a highly structured protein surface mediate between protein and bilayer and support a sealed integration of the protein in the bilayer. Lipids have also been discussed as lubricants, which ensure mobility of proteins in membranes and minimize unwanted protein–protein contacts [19]. They may enhance protein–protein recognition and promote supercomplex formation (see Section 6). The lipid coating of irregular surfaces of internal cavities may facilitate uptake of hydrophobic substrates as suggested for the cytochrome bc_1 complex [22] and the cytochrome b_6f complex [78,79].

3.2.4. Binding of lipids to porin-type proteins

The outer membrane of gram-negative bacteria has a specific lipid composition containing the characteristic lipopolysaccharides (LPS). Structures of several members of the outer membrane porin-type β-barrel proteins have been determined by X-ray crystallography and NMR [80,81]. Protein–LPS interactions have been studied for the ferrichrome–iron receptor FhuA from *E. coli* [82]. In the X-ray structure, one LPS molecule was bound to the receptor and modeled as the most abundant *E. coli* K-12 LPS chemotype. The complex LPS molecule contains lipid A with phosphorylated glucosamine disaccharides to which six fatty acid chains are attached. Similar as described for the α-helical membrane proteins, two rings of aromatic residues at the protein surface indicate the transition zone between the apolar core of the protein and the area facing the polar head groups [80,82]. The LPS molecule bound to FhuA is located with the glucosamine moieties slightly above the ring of aromatic residues and five of the six fatty acid chains are positioned close to the barrel surface. Their orientation parallel to the barrel axis corresponds to the position expected for the external LPS monolayer. The

binding of the negatively charged phosphate moieties of LPS to FhuA is stabilized by interactions with 11 charged or polar residues, mainly arginines and lysines [83]. A subset of four positively charged residues was identified as a conserved LPS-binding site in a structure-based search. Using this conserved motif, a putative LPS binding site in the structure of the outer membrane protease OmpT was identified [84]. Furthermore, lipid interactions with β-barrel proteins are reported by NMR studies. Specific stabilization of dihexanoyl-phosphatidylcholine (DHPC) head group and acyl chains have been described for the porin OmpX embedded in a DHPC micelle [85], which mimics interactions with the phospholipid bilayer. Specific stabilization of DHPC hydrophobic tails with Val, Ile, Leu, as well as amide groups of the backbone, were described. The strength of intermolecular interactions was shown to be largest for residues centrally located on the barrel surface and decreased toward the edges.

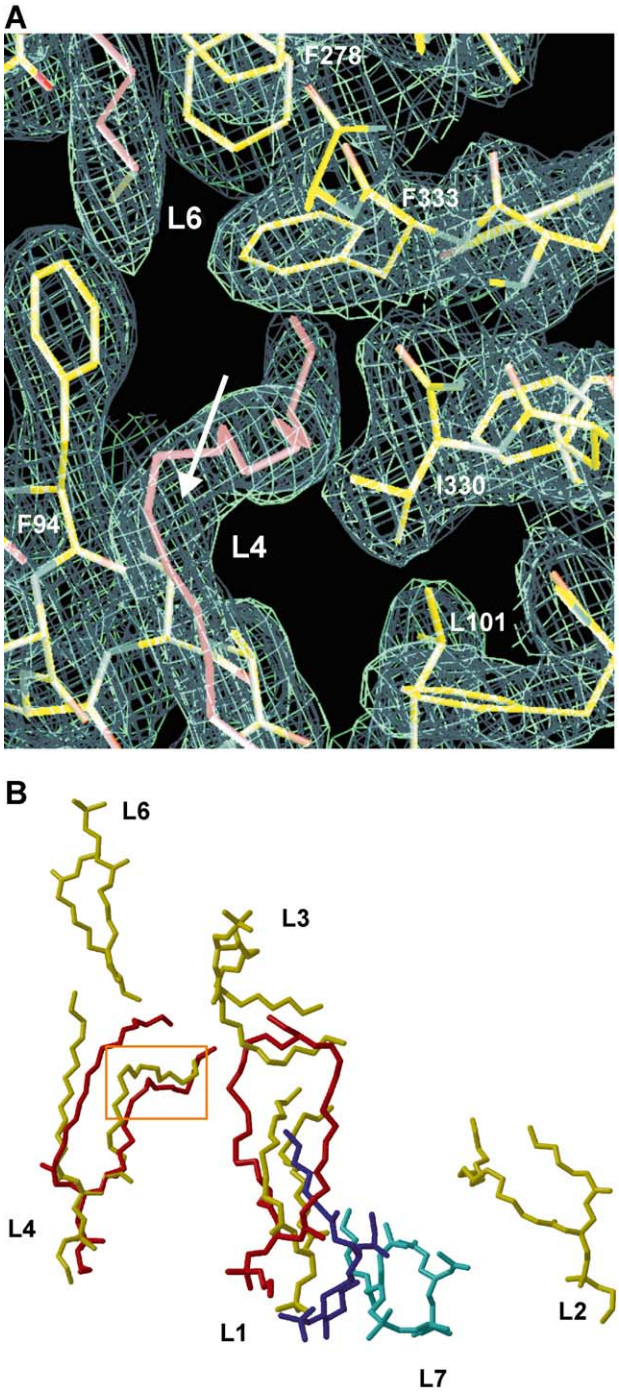

Fig. 5. "Kinked" acyl chains and conserved binding sites. (A) The kinked side chain of L4 was reproducibly identified and refined to its full length. It is bent and inserts into a narrow and highly hydrophobic dent of cytochrome *b* residues. The pronounced kink in the electron density for the lipid chain prohibits a suitable refinement of a saturated acyl chain. The presence of a double bond is suggested for the kink position, as indicated by the white arrow. The side chain of the IMS leaflet lipid L6 extends into the opening of the cleft where the oppositely oriented matrix leaflet L4 is fixed. (B) Lipid positions observed in the cytochrome bc_1 complex from yeast (yellow/cyan) and chicken (red) and that of the photosynthetic b_6f complex (blue) are remarkably similar in distinct regions of the proteins. Notably, in a lipid-filled cavity on the side of the homodimer (see brown dotted line Fig. 1A), the PE modeled in the chicken complex (red) displays a perfect "match" to the yeast L4 upon superimposition of the structures. Even the "kinked" side chains point to a remarkably conserved feature (orange rectangle). A second cavity with high lipid affinity is present at the dimer interface and is discussed as substrate entry point. The isoprenoid chain of the natural yeast substrate Uq6 extends into this cavity (see Fig. 1A). Interestingly, in the photosynthetic b_6f complex from alga, the binding of an endogenous sulfolipid (blue) coincides with the binding site of L7 and L1. The structures were aligned using the LSQ option in program O [100].

4. Probing importance of specific lipid-binding sites by site-directed mutagenesis

Increased knowledge about the molecular interactions between membrane proteins and specific lipids resulted in a number of hypotheses for their general and specific roles for structural and functional integrity of membrane proteins in vivo. These models need to be challenged by complementary techniques. Some evidence can be obtained from null mutants, which are devoid of certain phospholipids (see Sections 2 and 6). Furthermore, to pinpoint effects to an individual lipid modification of the ligands by site-directed mutagenesis is informative [22,86].

The first analysis was shown for the yeast cytochrome bc_1 complex [22]. Primary ligands of the phosphodiester group of the interhelical lipid L3 (Lys-272 of cytochrome c_1) and of cardiolipin (K288, K289, K296 of cytochrome c_1) were exchanged. The mutation K272A did not affect growth rate or properties of the enzyme while in the membrane. However, the enzyme activity of the purified variant K272A was only ~1% compared to the complex from the wild-type strain. Immunochemical analysis showed nearly complete loss of the Rieske protein during purification. Apparently, disruption of the ion pair between Lys-272 (Fig. 3) and the phosphodiester of L3

does not disturb the assembly of the complex, but is sufficient to destabilize binding of the interhelical phospholipid. This supports the assumption that the interhelical phospholipid is important for a stable association of the Rieske protein with the entire complex. Double and triple replacements of cardiolipin lysine ligands by leucine resulted in drastically reduced amounts of cytochrome b and cytochrome c_1 for the mutants K289L/K296L and K288L/K289L/K296L. The turnover number of the triple mutant was reduced to 38%. In contrast, the mutants K288L/K289L and K288L/K296L showed little effect. However, all mutations affect stability of the multisubunit complex, thus impeding structural analysis of the variants. The cardiolipin-binding site seems to be important for the structural integrity of the complex. Work on more stable variants is in progress and should permit analysis of the role of cardiolipin in the proposed proton uptake pathway (Cl/K pathway).

Recently, the importance of a previously identified interaction between a molecule of the diacidic lipid cardiolipin and the purple bacterial reaction center (see Section 3.2.2) was addressed by disrupting the main bonds to residues by site-directed mutagenesis [86]. Two highly conserved basic residues of the M-subunit of the reaction center, which provide the main bonds to the cardiolipin headgroup—His-145 and Arg-267—were changed to phenylalanine and leucine, respectively. Both mutations did not affect the photosynthetic growth rate or the functional properties of the reaction center. The X-ray structure of the variant R267L was not altered compared to WT. Differential scanning calorimetry showed that in detergent micelles, the leucine variant exhibits an approximately 5 °C decrease in melting temperature, suggesting a role of the interaction in thermal stability. The authors suggest that one function of this specific cardiolipin binding site is the stabilization of the interaction between adjacent membrane-spanning α-helices in a region where there are no direct protein–protein interactions.

5. Conserved lipid-binding sites

Growing evidence for important structural and functional roles of specific binding sites raises the question, whether conserved lipid-binding motifs exist. The observation that the lipid-binding sites identified in PSI are related by a pseudo-C2 axis pointed to an evolutionary conserved feature [87]. Residues stabilizing cardiolipin in the photosynthetic reaction centers are highly conserved among species of photosynthetic bacteria [88]. Also, ligands of phospholipids in the yeast cytochrome bc_1 complex are highly conserved [22,44], including those of the newly identified cardiolipin. Interestingly, lipid-binding sites are conserved among the cytochrome bc_1 complex from yeast and chicken, as well as lipid-filled cavities coincide with those identified in the photosynthetic cytochrome b_6f complexes (see Fig. 5B). The position of a phosphatidyl ethanolamine (L4) superimposes with a phosphatidylethanolamine described in the structure of the enzyme from chicken. Even the “kinked” side chain appears as a conserved feature (Fig. 5A). A second cavity with high lipid affinity is present at the dimer interface. This intermonomer cavity is coated with lipids and is discussed as a substrate entry point with quinol/quinone exchanged between the catalytic sites and/or with the membrane pool. Interestingly, in this lipophilic cavity, the binding of an endogenous sulfolipid (SL) was shown in the structure of the photosynthetic cytochrome b_6f complex from alga [78], which coincides with the binding site of two phospholipids identified in the yeast cytochrome bc_1 complex, including the cardiolipin (L7) first described here (see Section 3.2.2) (see Fig. 5B). A conserved feature when comparing L7 and SL binding mode is that the headgroups of both lipids are clearly resolved, whereas the acyl chains appear highly mobile and are therefore truncated in the structures. The importance of this lipid-enriched cavity for structural integrity of the homodimer is illustrated in the structure determination of the homologous cyanobacterial enzyme [79]. The stability of this preparation was enhanced by addition of exogenous lipids [89], two of which were retained and refined in this cavity. Structural instability of the cytochrome b_6f complex preparation from alga was overcome by introduction of an affinity tag for rapid purification; thus, a total of three endogenous lipids were tightly bound and resolved in this structure [78]. Interestingly, two of these endogenous lipids were assigned at the lipophilic side cavity of the homodimer, where the binding site for the intermembrane space lipid (L6) was described in the yeast enzyme [44]. These lipids exhibit well-resolved acyl chains, similar to the lipids binding in this cavity in yeast (see Fig. 4: L4, L5, and L6).

6. Lipids important for association of supermolecular complexes

Experimental evidence suggest that the multisubunit complexes of the respiratory chain in both prokaryotes and eukaryotes are assembled into supercomplexes or “respirasomes” [90]. Supercomplex formation is thought to improve the efficiency of oxidative phosphorylation by substrate channeling. Initially, cardiolipin was thought to be essential for the formation of supercomplexes from cytochrome bc_1 complex and cytochrome c oxidase [91,92]. In wild-type yeast, these two complexes are associated into a supercomplex, but 90% of individual complexes were observed in crd1Δ strain, which cannot synthesize cardiolipin [92]. Further analysis led to the proposal that cardiolipin stabilizes supercomplexes, as well as the individual complexes, but is not essential for their formation in the inner mitochondrial membrane [93]. In this context, a putative

interaction site of cytochrome *c* oxidase (COX) and cytochrome bc_1 complex has been proposed, which involves a depression in the latter complex flanked by helices of cytochrome *b* and cytochrome c_1 and coated by a tightly bound cardiolipin molecule and two additional lipids identified in the structure. Importance of this cardiolipin binding site for supercomplex formation is currently under investigation by site-directed mutagenesis (Wenz, Schagger, Hunte, in preparation).

Supermolecular assemblies have been described for photosynthetic complexes [94–96]. In this context, an observation of Fyfe et al. is noteworthy. When destabilizing the cardiolipin binding by site-directed mutagenesis, purification of the arginine to leucine variant (R267L) required one anion exchange column step less to obtain highly pure protein. These results were interpreted by the authors as destabilization of protein–protein interactions. Possible functional significance is under investigation [86].

7. Conclusions

Tightly bound lipids mediate between the bilayer and the membrane immersed domain of the protein. They have to adjust for the shape, surface, and mobility of the membrane protein, and allow integration into the bilayer without compromising the electrical seal. Binding constants for tightly bound phospholipids are not known. However, the observed tight and specific interactions with membrane proteins reflects their importance for the structural and functional integrity of the protein. Specific interactions are likely to direct insertion of integral membrane proteins into the bilayer, thereby determining topology and guiding assembly.

The described lipid-binding sites reflect general principles in stabilizing interactions between membrane proteins and phospholipids. The strongest and most frequently found interaction is between the negatively charged phosphodiester moiety and a positive countercharge. Depending on the individual headgroup moiety, several hydrogen bonds and/or ion–pair interactions stabilize head group binding, whereas hydrophobic lipid side chains fit tightly into hydrophobic grooves at the protein surface and are stabilized by multiple nonpolar, van der Waals interactions with amino acid residues. Remarkably, many of the ligands to lipid head groups are conserved, pointing to an important role of this stabilizing interaction. Also, conservation of lipid-enriched binding sites was found to cross species boundaries. In the case of yeast vs. the chicken cytochrome bc_1 complex and even when compared to the distantly related cytochrome b_6f complex, coinciding lipid-binding sites were observed by X-ray structural analysis. The controversial question whether the conserved lipid-binding sites described here have a functional/structural role awaits further analysis, especially through structure-based-mutagenesis approach, as initiated for the cytochrome bc_1 complex and the bacterial reaction center.

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