Specific protein–lipid interactions in membrane proteins

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Abstract
Many membrane proteins selectively bind defined lipid species. This specificity has an impact on correct insertion, folding, structural integrity and full functionality of the protein. How are these different tasks achieved? Recent advances in structural research of membrane proteins provide new information about specific protein–lipid interactions. Tightly bound lipids in membrane protein structures are described and general principles of the binding interactions are deduced. Lipid binding is stabilized by multiple non-covalent interactions from protein residues to lipid head groups and hydrophobic tails. Distinct lipid-binding motifs have been identified for lipids with defined head groups in membrane protein structures. The stabilizing interactions differ between the electropositive and electronegative membrane sides. 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 for functional roles are pointed out.

Introduction
Biological membranes are essential for life. They provide specialized permeability barriers for cells and cell organelles. The interplay of membrane proteins and lipids facilitates basic processes of respiration, photosynthesis, protein and solute transport, signal transduction and motility. This broad range of functions in the cellular metabolism is covered by a large number of diverse membrane-embedded proteins, which account for the coding capacity of 20–30% of the genes in a typical organism [1]. The lipid bilayer provides in general the matrix for the membrane proteins, but a tight protein–lipid interaction is required to maintain the diffusion barrier and to keep it electrochemically sealed. The mobile lipid molecules can adhere to the membrane protein surface and flexibly adjust to conformational changes and structural rearrangements. Protein–lipid interactions are specific; numerous biochemical and biophysical studies have demonstrated that defined lipid species confer structural stability, control insertion and folding processes, are involved in assembly or oligomerization of multisubunit complexes or supercomplexes or directly affect the function of membrane proteins [2,3]. The increasing number of membrane protein structures [4,5], which in part contain tightly bound lipids [3,6,7], pave the way to analyse the role of specific lipid-binding sites for the structure and function of membrane proteins.

Key words: cardiolipin, lipid, lipid–protein interaction, membrane protein, phospholipid, X-ray structure.

Abbreviations used: CL, cardiolipin; DOPC, dioleoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; LHC II, light-harvesting complex II; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; RC, reaction centre.

Protein–lipid complexes revealed by X-ray structure analysis
Membrane protein structures account for less than 1% of the known high-resolution protein structures [8]. Structure determination of these amphipathic proteins is still a demanding task, with the main obstacles residing in the overexpression and the choice of the appropriate detergent for purification and crystallization. To date, approx. 100 different X-ray structures have been determined (http://www.mpic-frankfurt.mpg.de/michel/public/memprotstruct.html), 18 of them contain tightly bound lipids with assigned head group providing new insight into specific protein–lipid interactions. Lipids are identified in experimental difference electron-density maps by typical ‘hairpin’-shaped or elongated features adhering to the transmembrane domains and mostly orientated perpendicular to the membrane plane (Figure 1). The majority of lipids identified in membrane protein structures are endogenous [7]. They are reproducibly co-purified with the protein despite the use of detergent in extraction and purification, i.e. membrane proteins are isolated as protein–lipid–detergent complexes. Lipid content and composition are important parameters, which should be controlled during membrane protein preparation to optimize activity, stability and even crystallization properties. For instance, lowering the exposure to the detergent during purification of the yeast cytchrome bc1 complex increased the protein activity by 30% and two additional lipids have been resolved in the respective X-ray structure [7,10]. Procedures suitable for routine qualitative and quantitative lipid analysis have recently been devised [11,12]. In some cases, supplementation of protein preparations with lipids has been essential for crystallization; e.g. addition of the foreign lipid DOPC (dioleoylphosphatidylcholine) facilitated crystallization of...
Figure 1 | In the X-ray structure of the yeast cytochrome bc₁ complex, the interhelical PI is wrapped around the transmembrane helix of the Rieske protein (RIP1)

The integral protein lipid stabilizes the association of this catalytic subunit with the core of the complex including cytochrome b (COB) [16]. The Fo-Fc map (green, contoured at 3σ) calculated prior to insertion of the lipid into the model illustrates the ‘hairpin’-loop typical for bound phospholipids. Stabilization of the phosphodiester group is accomplished via an ion pair with Lys272 of cytochrome c₁ (CYT1), and Ser73 of RIP1 is present as a polar ligand. The 2Fo-Fc electron density map after refinement of the lipid is contoured at 1σ (white).

Figure 2 | Yeast cytochrome bc₁ complex with tightly bound phospholipids viewed parallel to the membrane plane with the intermembrane space at the top and the matrix at the bottom

The binding sites of lipid molecules clearly define the vertical positioning of the complex in the membrane. The majority of the identified lipids are from the matrix leaflet of the bilayer. Lipids are shown in space-fill representation and are coloured yellow and for CL cyan [7,10,16]. Phosphor atoms are displayed in red. Cofactors and the Q₁ site occupant ubiquinone are shown as black ball-and-stick models. Helices are depicted as cylinders and coloured according to subunits: cytochrome b (brown), cytochrome c₁ (dark grey), Rieske (green), Qcr6p (cyan), Qcr7p (mid-grey), Qcr8p (white), Qcr9p (magenta), Cor1p (blue) and Qcr2p (purple). Red lines depict the position of the phosphodiester groups of the opposed pair of annular lipids, which are 36 Å apart.

d the cyanobacterial cytochrome b₆f complex [13]. Binding of DOPC at the quinone exchange cavity may stabilize its structure and reduce structural heterogeneity that is detrimental for crystallization.

General principles of lipid binding to membrane proteins

In a recent analysis of all available membrane protein–lipid structures, common features of lipid-binding sites were deduced [7]. Three types of binding modes have been defined for lipid interactions with membrane proteins. (i) A shell of ‘annular lipids’ bound to the protein surface resembles the bilayer structure. (ii) ‘Non-annular surface lipids’ are immersed in cavities and clefts of the protein surface. They are frequently observed for multisubunit complexes and multimeric assemblies and are typically present at contact sites between adjacent subunits or monomers. (iii) ‘Integral protein lipids’ reside within a membrane protein or a membrane protein complex. They often occur in unusual positions, e.g. with the head group below the membrane plane and/or the acyl chains non-perpendicular to it.

The number of lipid molecules surrounding the membrane-spanning domain has been deduced for several proteins from EPR spin-labelling studies [14]. However, most membrane protein structures contain only a few lipid molecules of the first annular shell, apparently a result of extensive purification in the presence of detergents. The partial occupancy clearly indicates different lipid-binding affinities. An EPR spin label analysis revealed a motion-restricted first shell of lipids with an effective rotational correlation time of approx. 10⁻⁸ s [15]. However, binding affinities of individual structurally resolved lipids have not yet been determined.

The annular lipids mediate between the membrane protein and the bulk lipid bilayer. They position the protein vertically in the membrane while providing tight and dynamic integration. In the yeast cytochrome bc₁ complex, phospholipids of the matrix and the intermembrane leaflet [10,16] allow determination of position and thickness of the bilayer associated with the complex (Figure 2). The phosphodiester groups of two oppositely oriented annular lipids, cardiolipin (CL) and phosphatidyl ethanolamine, are 36 Å (1 Å = 0.1 nm) apart. This is in good agreement with the respective distance of 38 Å for pure PC (phosphatidylcholine) bilayers with C₁₈₁ acyl chains [17]. A nearly complete annular shell with 18 tightly bound lipid chains was described for the X-ray
structure of the trimeric bacteriorhodopsin [18]. The most complete example of an annular shell is described for the structure of the membrane rotor ring of the V-type Na\(^{+}\)-ATPase from Enterococcus hirae [19]. Whereas no externally bound lipids are observed, ten molecules of DPPG (dipalmitoyl-phosphatidylglycerol) and ten molecules of DPG (dipalmitoyl-glycerol) cover both bilayer sides of the internal hydrophobic ring surface. Remarkably, a second and third ring of elongated density features, which have been interpreted as acyl chains, is layered on the DPPG shell. This illustrates the notion that central pores in protein complexes or multimeric assemblies are usually plugged with phospholipids. These internal lipid patches are discontinuous from the lipid bulk phase and may have unique compositions and physicochemical characteristics. In the 2.9 Å X-ray structure of bacteriorhodopsin, the central compartment binds three molecules of a haloarchaeal glycolipid [20]. The central lipid patch is shifted by 5 Å towards the membrane centre, this is described as local membrane thinning.

Structurally resolved non-annular surface lipids are frequently located at contact sites between adjacent monomers of oligomeric complexes, e.g., in the homotetrameric potassium channel KcsA [21] and in the trimeric bacteriorhodopsin [20,22], or at the contact sites between subunits of multimeric complexes, like in the yeast cytochrome bc\(_1\) complex [7,10,16]. Interestingly, all structurally resolved lipids in the latter complex interact with at least two subunits of the complex [7]. One may speculate that the binding interactions at subunit interfaces are tighter than for an average annular lipid. Filling the crevices between adjacent monomers appears important to provide tight and sealed protein integration into the membrane. In addition, these specific binding sites may be involved in the assembly and/or association of monomers or subunits. Assembly of the trimeric LHClI (light-harvesting-complex II) depends on defined lipid species including PG (phosphatidylycerol) [23,24]. The molecular explanation is provided by the LHClI X-ray structures. A PG molecule is bound at the monomer interface with one fatty acid chain inserting into the trimer [25,26].

Several integral protein lipids have been observed in membrane protein structures. Their position within the interior of the protein raises the question of whether they are important for folding and assembly. In the yeast cytochrome bc\(_1\) complex, a PI (phosphatidylinositol) molecule is present in a so-called ‘inter-helical’ position [16] (Figure 1). Its head group is 10 Å below the zone of phosphodiester groups of the intermembrane leaflet and the acyl chains are wrapped around the transmembrane helix of the Rieske protein. This lipid molecule may stabilize the helix packing the transmembrane anchor of the Rieske protein and the core of the complex. Another example is cytochrome c oxidase from Rhodobacter sphaeroides. Subunit IV has no direct interaction with any other subunit and its position is exclusively stabilized by indirect contacts via lipid molecules [27].

Endogenous lipids retained during detergent extraction and purification procedures, which are reproducibly detected in membrane protein structures, can be judged as tightly bound. Ordered binding of the described lipids is manifested by their average B-factors, which reflect the statistical and dynamic disorder for atomic positions. An average B-factor of 27 Å\(^2\) of the protein model in contrast with 57 Å\(^2\) of the lipid phytanyl chains was observed for the 1.55 Å resolution structure of bacteriorhodopsin [18]. In the 2.3 Å resolution structure of the yeast cytochrome bc\(_1\) complex [16], the average B-factor of the catalytic subunits cytochrome \(b\), cytochrome \(c\) and Rieske protein is 38, 55 and 63 Å\(^2\) respectively, whereas it varies between 72 and 93 Å\(^2\) for the lipid molecules. In comparison, the average B-factor of the high-affinity (\(K_d < 10^{-10}\) M) inhibitor stigmatellin bound to the catalytic centre P of the complex is 36 Å\(^2\). An interesting side aspect is that lipid binding at centre P overlaps with the binding sites of different inhibitors [7]. Finally, the high reproducibility of structurally resolved lipid-binding sites clearly indicates tight and specific protein–lipid interactions.

### Specific lipid-binding sites

In general, lipid binding is stabilized by specific and mainly polar interactions between the lipid head group and amino acid residues as well as by a larger number of non-polar interactions between the hydrophobic lipid tail and the protein. In a detailed survey of tightly bound lipids in membrane protein structures, general principles for stabilization have been deduced [7]. The study focused on lipids with assigned head groups and on the stabilization of the phosphodiester group, as the latter is best defined in the 16 structures available at that time [7]. Lipid binding in the recently described structures of LHClI [25,26] and of the ATPase-rotor [19] is in accordance with the rules outlined below.

The majority of the lipids with strongly stabilized head groups are 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 are the mitochondrial cytochrome bc\(_1\) complex [16] and the cytochrome c oxidase [28]. This preferential location is consistent with the ‘positive-inside’ rule, meaning the protein surface on the negative membrane side is enriched with positively charged residues [29]. Lipids on the electropositive side of the membrane (\(p\) side) may lack strong stabilizing interactions. The asymmetric distribution of positively charged residues and the impact of anionic phospholipids in determining membrane protein topology [30] coincide with the distribution of high-affinity lipid-binding sites. This observation supports the importance of tight protein–lipid interactions for insertion and folding of membrane proteins.

The majority of the phospholipids are stabilized by two (or more) polar interactions between amino acid residues and the phosphodiester group, typically with motifs combining a positively charged and a polar ligand, as exemplified in Figure 1 and listed below (using single-letter amino acid codes).

\[ KT, KW, KY, RS, RW, RY, RN, HS, HW, HY \]

At the \(n\) side of the membrane, the following residues (listed
according to the number of occurrences) act as primary ligands of the phosphodiester groups.

Arg > Lys > Tyr > His > Trp, Ser, Asn

Additional stabilizing interactions are provided by Thr and Gln. Based on the small number of binders at the p side, one can summarize that positive charges are less frequently observed and that the following residues are present as primary and as stabilizing ligands.

Tyr, Thr, Asn, Gln, His, Arg

The stabilization of the phosphodiester group of PC is an exception: a combination of His and Ser as ligand pair or single interactions with His, Thr and Ser occur. The absence of positively charged ligands at close distance to the PC phosphodiester may be a consequence of the bulky, triple methylated and positively charged choline head group. CL consists of two phospha tidyl residues linked by a glycerol moiety [31]. This diacidic lipid has a very specific binding pattern. Tight binding interactions with three residues have been described for the phosphodiester groups: e.g. KKY, RKY and HRN. The following CL binding motif has been suggested [7]: XYZ, where X and Y are positively charged residues and Z is a polar residue.

Most of the described binding motifs are non-linear and may even consist of ligands from different subunits. Therefore structure-based searches are required to identify lipid-binding sites. In addition to the above-described amino acid side-chain interactions, backbone nitrogen or oxygen atoms contribute to stabilization of the phosphodiester group.

Aromatic residues are frequently involved in lipid stabilization. Tyrosine residues are typically present as polar ligands of phosphodiester groups, either as primary stabilizing ligand or in combination with a positively charged ligand. Within the transmembrane region, tryptophan residues are preferentially located in the hydrophobic–hydrophilic transition zone with their indole ring oriented towards the centre [32]. 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.

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 bc₁ complex [16]. In some cases, lipid head groups are not clearly resolved even at high resolution, e.g. in the 1.55 Å resolution structure of bacteriorhodopsin [18]. This was interpreted as either low affinity or unspecific binding of the head group. Exact assignment and refinement of tightly bound lipids are crucial to improve our knowledge about lipid-specific binding sites in the future.

### Binding of lipid tails

A common feature of the structurally resolved lipids is that the fatty acyl or phytanyl chains are snuggled in shallow grooves and clefts, the position of the chains stabilized by multiple van der Waals contacts [16,18]. Attraction from the protein frequently forces the hydrophobic tails into distinct often highly curved shapes (Figures 1 and 2), which deviate considerably from the sprawled though mobile conformation expected in a lipid bilayer [33]. These patterns of acyl chain stabilization are remarkably reproducible as observed for different X-ray structures of the cytochrome bc₁ complex [7]. However, the ends of the acyl chains are largely unresolved, indicating their higher conformational flexibility. Surface accessible tryptophan residues have been proposed to fulfill a functional role as interfacial anchoring residues [34]. A ring of lamellar-oriented surface-exposed tryptophan residues may provide an interlocking tooth system for the first shell of annular lipids [7].

The acyl chains of bound lipids are usually refined as fully saturated. However, some examples show extreme bending angles, so-called ‘kinks’, which can only be explained by the presence of one or more double bonds [7]. Higher-resolution structures may allow in the future the correct assignment of unsaturated lipid species, as recently shown for phosphatidylethanolamine bound to the orphan nuclear receptor steroidogenic factor-1 [35].

### Functional importance of individual lipids

Specific roles of defined lipid-binding sites for membrane protein folding, assembly, structural integrity and function have been suggested. These hypotheses can be challenged by structure-based site-directed mutagenesis [16,36]. Disrupting bonds with the phosphodiester moiety should destabilize the lipid binding.

The first analysis was carried out for the yeast cytochrome bc₁ complex [16]. Primary phosphodiester ligands of the interhelical lipid (Lys²⁷² of cytochrome c₁, Figure 1) and of CL (Lys²⁸⁸, Lys²⁹⁹ and Lys²⁹⁶ of cytochrome c₁) were modified. The K²⁷²A (Lys²⁷² → Ala) mutation does not affect the assembly of the complex, but destabilizes the association of the catalytic Rieske-protein subunit with the complex. Double and triple replacements of the CL ligands by leucine resulted in drastically reduced amounts of cytochrome b and cytochrome c₁ for the mutants K²⁸⁹L/K²⁹⁶L and K²⁸⁸/ K²⁸⁹L/K²⁹⁶L. The catalytic-centre activity of the triple mutant was reduced to 38%. All mutations affect stability of the multisubunit complex, thus the binding site seems to be important for the structural integrity of the complex. This CL molecule close to the catalytic site of quinone reduction has been suggested as a proton entrance for quinone reduction, the Cl/K pathway [16]. Site-directed mutagenesis in combination with Fourier-transform infrared spectroscopy analysis is in progress to address this question.

CL binding to the purple bacterial RC (reaction centre) was analysed by site-directed mutagenesis disrupting the main direct bonds between two highly conserved basic residues.
of the M-subunit to side chains and CL [36]. The mutations H145F and R267L did not affect the photosynthetic growth rate or the functional properties of the RC and no difference was observed in the X-ray structure. However, the CL binding appears to support thermal stability of the RC as shown by differential scanning calorimetry.

Apparently, CL binds preferentially at monomer interfaces of oligomeric assemblies and at subunit interfaces of multimeric complexes [7]. In addition, it stabilizes supercomplexes in the respiratory chain [37]. Its unique large head group combined with a specific and tightly interacting binding site appears to be well suited to stabilize proteins in a clamp-like manner.

More examples of individual lipids with suggested functional importance have been summarized recently [7]. Further experimental evidence is needed to support the diverse models.

Conserved lipid-binding sites

Residues that stabilize lipid binding in membrane proteins are often highly conserved [7]. An interesting example is the cytochrome bc\(_1\) complex. Here, lipid-binding sites are not only conserved among the respiratory chain complexes from yeast and from chicken but the lipid-filled cavities coincide with those identified in the related photosynthetic cytochrome bc\(_6\) complex [7]. The lipid coating of the irregular surfaces of the internal cavities may facilitate the uptake of the lipophilic quinone substrates.

Conclusions

X-ray structures have revealed tight and specific binding interactions of lipids with membrane proteins. Bound lipids mediate between the bilayer and the membrane-immersed domain of the protein maintaining the electrical seal. They may direct the insertion of the protein into the bilayer, thereby determining the topology and guiding assembly; they may be important for the structural and functional integrity of the protein. General features of lipid binding have been deduced. Depending on the individual head-group moieties, 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 non-polar interactions with amino acid residues. Lipid-binding motifs for the stabilization of the phosphodiester group have been identified. They differ for the electropositive and electronegative sides of the membrane and often involve conserved residues. There is initial evidence that lipid species-specific binding motifs exist and a first suggestion has been made for the dianionic CL. Further structures with correctly assigned and refined lipids are essential to improve the database. The motifs should assist identification or prediction of lipid-binding sites and may be useful for protein folding analysis. The controversial question of whether conserved lipid-binding sites have a functional/structural role awaits further analysis, especially through a structure-based-mutagenesis approach, as initiated for the cytochrome bc\(_1\) complex and the bacterial RC.

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