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Review

Non-covalent binding of membrane lipids to membrane proteins ☆

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ABSTRACT

Polar lipids and membrane proteins are major components of biological membranes, both cell membranes and membranes of enveloped viruses. How these two classes of membrane components interact with each other to influence the function of biological membranes is a fundamental question that has attracted intense interest since the origins of the field of membrane studies. One of the most powerful ideas that driven the field is the likelihood that lipids bind to membrane proteins at specific sites, modulating protein structure and function. However only relatively recently has high resolution structure determination of membrane proteins progressed to the point of providing atomic level structure of lipid binding sites on membrane proteins. Analysis of X-ray diffraction, electron crystallography and NMR data over 100 specific lipid binding sites on membrane proteins. These data demonstrate tight lipid binding of both phospholipids and cholesterol to membrane proteins. Membrane lipids bind to membrane proteins by their headgroups, or by their acyl chains, or binding is mediated by the entire lipid molecule. When headgroups bind, binding is stabilized by polar interactions between lipid headgroups and the protein. When acyl chains bind, van der Waals effects dominate as the acyl chains adopt conformations that complement particular sites on the rough protein surface. No generally applicable motifs for binding have yet emerged. Previously published biochemical and biophysical data link this binding with function. This Article is Part of a Special Issue Entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

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1. Introduction

The membranes of cells (and enveloped viruses) are built largely from membrane proteins and lipids. What effects each membrane component may have on the other, and how they interact to modulate function in, on, and around biological membranes, have tantalized researchers for several decades. It is now possible to synthesize new data from three dimensional structures of membrane proteins with prior data from biophysical experiments to begin to answer the long-standing questions of the nature and extent of lipid–protein interactions in biological membranes, and what roles those interactions may play in biological membrane function.

In the most simple view, biological membranes can be considered to consist of two major classes of molecules: membrane lipids and membrane proteins. Three linked interactions must be considered in such a two component system: lipid–lipid interactions, protein–protein interactions, and lipid–protein interactions. These three sets of interactions are not independent. Perturbations in any one of these linked interactions can be expected to affect the other two. Protein–protein interactions are known from extensive studies with soluble proteins

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to play a central role in modulating and/or enabling function. Lipid–lipid interactions can affect the milieu in which the membrane proteins function (for example, a phase transition of the lipids to a gel state or the incorporation of high levels of cholesterol in the membrane, either of which will inactivate most transmembrane enzymes). Since in a biological membrane one may find dozens of unique membrane proteins and hundreds or thousands of unique membrane lipids, structure and function in the biological membrane are inherently more complex than just outlined. However, such a construct offers a useful structure with which to approach our subject. This review, therefore, will focus on lipid–protein interactions. Lipid–protein interactions, as just noted, can be affected by lipid–lipid interactions and protein–protein interactions. However because the former have been less intensively examined (due to lack of adequate experimental approaches), the focus will be on lipid–protein interactions while remaining cognizant of the other linked interactions.

Lipid–protein interactions will often influence membrane protein function. Lipids may force a protein conformational change to bury the hydrophobic surfaces of the membrane protein within the limited thickness of the hydrophobic interior of the membrane lipid bilayer. This can be particularly dramatic if a substantial hydrophobic mismatch occurs between the width of the hydrophobic surface of the transmembrane domain and the width of the bilayer. Transmembrane proteins express a hydrophobic surface on the transmembrane portion of the protein to coexist with, and match the dimensions of, the hydrophobic interior of the membrane bilayer. If the dimensions of the hydrophobic surface on the membrane protein are similar to the width of the hydrophobic interior of the bilayer, then these two components can find a ready accommodation. If however the protein is in a bilayer that is thinner than the hydrophobic surface on the protein, the protein may alter conformation (such as a tilt [1–3] or a bend [4,5] in the transmembrane portion) to better accommodate the hydrophobic portion of the bilayer, or the bilayer may distort to cover the hydrophobic surface on the transmembrane protein [6,7].

Lipids also can act as effectors that bind and alter membrane protein function. This role is analogous to effectors binding to soluble proteins in solution and inducing a change in protein function. Since the transmembrane proteins are in part within the lipid bilayer and the effective "concentration" of the lipids (solvent) in two dimensions within the bilayer is very high, any binding sites on the protein that can match the properties of one or more membrane lipids can be expected to be occupied by those lipids to some extent. The extent may be dependent upon protein conformation and therefore binding of these lipids can induce changes in membrane protein conformation and consequent changes in membrane protein function.

This latter set of possibilities has engendered considerable hypothesizing and experimenting. The opportunity for specific lipids to bind to particular membrane proteins and affect their function is a compelling concept. Such a concept would begin to provide an explanation for the observation of so many individual lipid species in most biological membranes. Until recently the paucity of atomic level structural information on membrane proteins considerably limited the ability of investigators to address this question directly. Prior to the availability of such detailed structural information, a number of (mostly) indirect experimental approaches were creatively applied that produced considerable support for a role of lipid binding to membrane proteins in regulation of membrane protein function (see for example, [8–12]). Recently considerable new information on atomic structure of transmembrane proteins has become available. Integrating those structural data with the previous biophysical data allows a much more extensive and satisfying exploration of the extent, nature and role of lipid–protein interactions in membranes.

Compared to a decade ago, there is now an abundance of structural data, in particular from X-ray crystallography, on lipid binding to membrane proteins. There are currently over 100 individual examples of a particular lipid binding to a particular membrane protein from X-ray crystal structures of transmembrane proteins (see Table 1). Below, the text will focus on individual classes of lipids, one at a time, that bind to membrane proteins and explore what is known for each of those lipids from X-ray crystallography data. Most earlier reviews on this

Table 1
Number of lipid binding sites in X-ray crystal structures of membrane proteins.

Lipid	PDB ID	Protein	# bound
Cholesterol	3AM6	H^+ pump rhodopsin ARII	2
	2YOO	Turkey β_1-adrenergic receptor	2
	2RH1, 3D4S	Human β_2-adrenergic receptor partial inverse agonist	2
	3PDS	Human β_2-adrenergic receptor agonist	1
	4EIY	Human A_{2A} (bRIL) adenosine receptor	3
	4DKL	Mouse μ-opiod receptor antagonist	2
	4IB4	Human 5-HT_{2B} ERG	1
	4HYT	Porcine Na^+K^+ATPase ouabain phosphorylated	1
	2ZXE	Shark Na^+K^+ATPase FXYD	1
	EMD-1079	Bovine rhodopsin	1
	2BG9	Torpedo nicotinic acetyl choline receptor (model into electron density)	1
DPG	2C3E, 1OKC	Bovine mitochondrial ADP/ATP carrier CATR	3
	1PPG	Bovine mitochondrial cytochrome bc_1, antimycin	1
	1KB9, 1P84	Yeast mitochondrial cytochrome bc_1, inhibitor	1
	2DYR, 1V54	Bovine heart cytochrome c oxidase	2
	4AYT	Human mitochondrial ABC transporter	2
	1NEK	*E. coli* succinate dehydrogenase	1
	1KQF	Formate dehydrogenase N	1
	1OGV, 1QOV	Photosynthetic rxn ctr *R. sphaeroides*	1
	4A2N	*Ma*-ICMT	1
	1M3X	*R. sphaeroides* photosynthetic rxn center	1
	1V54	*B. tau* cytochrome c oxidase	2
PG	1OCC	Bovine heart cytochrome c oxidase	3
	2DYR	Bovine heart cytochrome c oxidase	4
	2AXT	*T. elongatus* photosystem II	1
	1JBO	*S. elongatus* photosystem 1	3
	3LNM	Kv2.1–Kv1.2 chimera potassium channel	1
	2R9R	Kv2.1–Kv1.2 chimera potassium channel	>2
	4DOJ	BetP transporter	1
	4JBW	Maltose transporter	1
	1V54	*B. tau* cytochrome c oxidase	3
PE	1KB9	Yeast mitochondrial cytochrome bc_1, inhibitor	2
	1P84	Yeast mitochondrial cytochrome bc_1, inhibitor	3
	1BCC	Avian cytochrome bc_1	2
	1PPJ	Bovine cytochrome bc_1 inhibitor	2
	1M56	*R. sphaeroides* cytochrome c oxidase	6
	2DYR, 1OCC	Bovine cytochrome c oxidase	3.5
	1QOV	*R. sphaeroides* photosynthetic rxn center	1
	1ZOY, 2FBW	Porcine mitochondrial respiratory complex II	2
	1EYS	*T. tepidum* photosynthetic rxn center	1
	1XIO	Anabaena sensory rhodopsin	>4
	1GZM	Bovine rhodopsin	1
	3W5A	Rabbit Ca pump sarcoplasmic reticulum	3
	3ZUY	Bacterial Na^+ bile acid cotransporter	2
	2ZZ9	Aquaporin	5
	1V54	*B. tau* cytochrome c oxidase	3
	1NEK	*E. coli* cytochrome c oxidase	1
PI	1KB9	Yeast mitochondrial cytochrome bc_1, inhibitor	1
PIP_2	3SYA	Mammalian GIRK2 potassium channel	1
PC	2DYR	Bovine heart cytochrome c oxidase	1
	1P84	Yeast mitochondrial cytochrome bc_1, inhibitor	2
	1KB9	Yeast mitochondrial cytochrome bc_1, inhibitor	1
	1OKC	Bovine mitochondrial ADP/ATP carrier	2
	2Z73	Squid rhodopsin	1
	3AYN	Squid isorhodopsin	1
	3B8E	Porcine renal Na^+K^+ATPase	1
	4AW6	Zinc metalloprotease, ZMPSTE24	1
	3RVY	Voltage gated channel NavRh	1
	4EKW	Voltage gated channel NavRh	4
	2B6O	Aquaporin	Annulus
	2XTV	*E. coli* rhomboid protease GLPG	Annulus
	1QLE	*P. den.* cytochrome c oxidase	2
	1V54	*B. tau* cytochrome c oxidase	1

subject (see for example [7,13–17]) predated much of the X-ray data, and, for the most part, organized their discussion around individual membrane proteins and what lipids bound to them. The recent X-ray data provide for a more detailed examination of lipids binding to membrane proteins than previously possible. Binding of cholesterol and phospholipids, only, to membrane proteins will be discussed here. They account for most of the available data.

As we consider the available data in this review, several themes will emerge from an analysis of the structural information. Some membrane phospholipids and/or cholesterol bind to the transmembrane domain of membrane proteins so tightly that they are not removed by the delipidation treatments used to prepare the samples. These sites often appear in X-ray crystal structures of membrane proteins occupied by a particular membrane lipid. Many if not most of these sites for tightly bound membrane lipids are likely specific for the chemical structure of the particular lipid. Tight binding of these lipids is expected to influence membrane protein structure, and likely function. Biochemical data support the functional role in a number of examples. Most frequently, one to six lipids are bound so tightly to these sites that the detergent isolation techniques do not remove them. These sites may be on the surface of the transmembrane domain. They may be buried within the helical bundle of a transmembrane protein. They may occur between monomers in the crystal structure. In many X-ray structures of transmembrane proteins, detergents are included in the electron density. It is possible that some of these detergent molecules may be occupying sites normally occupied by membrane lipids in the biological membrane.

One limitation should be noted at the beginning of this discussion. The experiments are mostly performed on rigorously delipidated membrane proteins. Usually detergents are used to remove the membrane lipids and other membrane components so that the investigator can work with purified protein and thus hope to crystallize it. The only phospholipids and cholesterol that appear in most of these crystal structures are therefore molecules that are so tightly bound to the membrane protein that extensive detergent treatment does not remove the lipid. This leaves open the likelihood that other lipid binding sites exist that can be depleted of lipid by detergent treatment and therefore are not observed in the crystal structure. Also missing from the X-ray crystal structures will be those lipids whose binding is dynamic such that an ordered lipid in a binding site over the timescale of the X-ray diffraction experiment is not seen.

Other techniques like NMR (nuclear magnetic resonance) and ESR (electron spin resonance of lipid spin labels) allow measurements of lipid–protein interactions in biological membranes, without isolation of the membrane protein, thus reflecting best the biology. These techniques are used as well as in reconstituted membranes in which case during sample preparation membrane lipids are removed and defined lipids are added back. These measurements reveal that a larger number of membrane lipids are interacting with the protein, beyond the one to six described above. Perhaps double that number can be bound at sites on the protein sufficiently strongly to be detected by ^{31}P NMR. With ESR measurements, even more lipids may be identified, enough to completely surround the protein (the lipid annulus). Most of the latter lipids are not tightly bound and exchange rapidly with other lipids in the membrane.

In some cases, data are derived from multiple disciplines, as just noted. The discussion will include only those phospholipids for which atomic level data on binding are available from X-ray crystallography or electron crystallography (or in one case, NMR). For some of the lipids that bind to membrane proteins, other biophysical data are available that precede the X-ray crystallography data and also provide structural evidence for lipid binding. Some of those data will be included in the discussion, data that can now be better understood and that extend our knowledge of lipid–protein interactions in membranes.

2. Cholesterol

Cholesterol is an amphipathic molecule with a polar hydroxyl bonded to four planer fused carbon rings and a short hydrocarbon tail. Cholesterol has a finite but very low solubility in aqueous media so it is usually found incorporated in biological membranes or lipoproteins or bound to proteins, both soluble proteins and membrane proteins.

Cholesterol is an unusually important lipid for mammalian membranes. Most mammals require cholesterol in many of their cell membranes for survival. Sterols closely related in chemical structure (for example epicholesterol) are unable to substitute for this cholesterol requirement. A number of plasma membrane ion pumps are dependent upon cholesterol. Depletion of the plasma membrane of cholesterol renders these ion pumps inactive. That includes the Na^+K^+ATPase, which in many cells is the single most important consumer of ATP in the cell and is responsible for transmembrane ion gradients upon which many cellular functions depend. (Many membrane enzymes lose activity at very high membrane cholesterol content. This is, however, due to another, separate property of cholesterol: at high levels it increases the motional order of the membrane lipids. At high order, transmembrane permeability is reduced and conformational flexibility of the transmembrane protein is inhibited.)

A hypothesis was previously advanced that the cholesterol dependency arose from cholesterol binding to specific sites on certain membrane proteins [18]. The Na^+K^+ATPase provides a case study of this hypothesis, from structural data to stability data to biochemical data [19,20].

A recent X-ray crystal structure of the phosphorylated pig kidney Na^+K^+ATPase with ouabain bound reveals a cholesterol binding site on the transmembrane portion of the protein between the γ and α subunits [21] (Fig. 1). This site is close to a suggested phospholipid binding site (see below). Together binding of these two lipids to these sites on

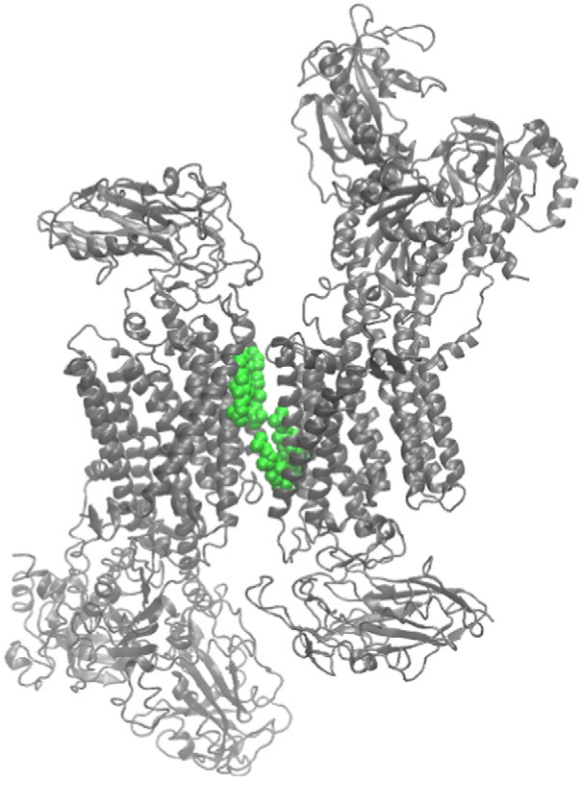

Fig. 1. X-ray crystal structure of the porcine Na^+K^+ATPase (PDB ID: 4HYT) in gray. Cholesterol molecules in the crystal are shown in green.

the Na^+K^+ATPase stabilizes activity of the enzyme. The cholesterol binding site is specific for cholesterol; ergosterol is not as effective as cholesterol in stabilization of enzyme activity. An earlier study examined the effects of membrane cholesterol content in a biological membrane on Na^+K^+ATPase activity. Increasing cholesterol from low concentrations in the membrane promoted increasing enzyme activity up to the native membrane cholesterol level (extrapolating to zero cholesterol content suggested that in the absence of cholesterol the enzyme was inactive). Other sterols were unable to duplicate these activating effects of cholesterol [22]. All these data can be better understood in the light of the new structural information. As the early biochemical data suggested, the Na^+K^+ATPase has a cholesterol binding site which is specific for the cholesterol molecule. It is reasonable to speculate based on these studies that occupancy of this cholesterol binding site by cholesterol promotes and stabilizes activity of this important plasma membrane enzyme. Therefore, cholesterol does bind to at least one transmembrane protein at a site specific for cholesterol, altering the properties of the protein. Such results begin to describe a specific and critical role for one membrane lipid in membrane function through lipid–protein interactions.

There are data that cholesterol binds to other transmembrane proteins as well. The transmembrane domain of the amyloid precursor protein has a cholesterol binding site. In this case, the three dimensional structure was determined from nuclear magnetic resonance (NMR) data. Titrations with cholesterol not only identified which amino acid residues formed the binding site but also revealed some information about the affinity of the binding site for cholesterol [23].

The class of G-protein coupled receptors (GPCR) shows cholesterol binding to the receptor in the recent set of X-ray crystal structures of this family of membrane receptors. In each case, one to two cholesterol molecules are found in binding sites on the transmembrane portion of the receptor.

Human β_2 adrenergic receptor has been successfully crystallized and its atomic resolution structure determined. Cholesterol is found bound at the interface between two monomers of the receptor [24]. Cholesterol is known to affect the function of this receptor, but whether that occurs by promoting association of two receptor monomers as the crystal structure suggests, or perhaps keeps the monomers apart, is not known.

In a more recent crystallographic analysis [25], two binding sites for cholesterol are found, at sites separate from the protein–protein interface described above. These latter have been suggested to be candidate sites for known effects of cholesterol on the receptor function, including effects on ligand binding and thermal stability. The cholesterol binding sites in the structure of the receptor are in the transmembrane region, next to helices I, II, III, and IV, on one face of the transmembrane domain. The suggestion is made that binding of cholesterol increases the packing density of the transmembrane helical bundle, thus increasing the thermal stability of the molecule.

Other GPCR also have cholesterol (or analog) binding sites. Turkey β_1 adrenergic receptor shows a binding site for cholesterol hemisuccinate [26].

The crystal structure of the proton pumping rhodopsin, ARII, shows two cholesterol binding sites, bounded by helices I and VII of one protein, and helix I of an adjacent protein in the crystal [27].

The structure of the human A_{2A} adenosine receptor contains three cholesterol binding sites. While two are at crystal contacts, one cholesterol is not. Cholesterol binding at the latter site is suggested to stabilize the transmembrane helical bundle, much like the case of the human β adrenergic receptor, and possibly orient a key residue in the ligand binding pocket of the receptor [28].

One cholesterol binding site is seen in the μ-opiod receptor [29]. The binding site is bounded by two transmembrane helices from one receptor molecule and one from another, much like the ARII receptor. Biochemical evidence shows a sensitivity of the receptor function to cholesterol in the membrane, and furthermore shows that the site of binding can discriminate sterol structure [30].

The 5-HT_{2B}ERG receptor also has a binding site for one cholesterol [31].

Finally, no discussion of GPCR and cholesterol would be complete without mention of bovine rhodopsin. Rhodopsin function in membranes is inhibited by the presence of high levels of membrane cholesterol [32]. This GPCR exhibits a binding site for cholesterol in the electron crystallography data [33]. The site is next to helix VI, close to a phospholipid binding site. Fluorescence experiments with a fluorescent derivative of cholesterol are consistent with a binding site on the transmembrane domain of the protein specific for the cholesterol structure, near a tryptophan (helix VI includes a tryptophan) [34]. Molecular calculations also provide support for cholesterol binding to the transmembrane domain of rhodopsin [35]. Cholesterol influence on rhodopsin behavior is well studied [32,36,37].

In summary, X-ray crystallographic data shows that cholesterol binds to a number of membrane proteins at specific sites. This binding provides a mechanism for cholesterol to influence transmembrane protein conformation and thus transmembrane protein function. These observations are consistent with the previous suggestion that the specific effects of cholesterol on biological membrane function are mediated by binding to membrane proteins. Enough data are now available to form the basis for targeted investigations to detail the molecular mechanisms by which cholesterol effects its influence through binding to the protein structure (these effects of cholesterol through binding to membrane proteins are separate and independent from the effects on membrane proteins due to the ordering by cholesterol of the lipid bilayer in which the membrane proteins are found).

3. Diphosphatidylglycerol (cardiolipin)

Diphosphatidylglycerol (DPG), or cardiolipin, is a highly negatively charged phospholipid that is a major component of, for example, the inner mitochondrial membrane. The chemical structure of DPG is more complex than the other lipids that will be discussed here. It has 4 acyl chains esterified to one headgroup that contains two phosphates that confer the negative charge. While in biology one normally thinks of this phospholipid in membrane bilayers, in pure form in the presence of some cations, DPG is unstable in the bilayer structure and instead forms non-bilayer structures including the Hexagonal II phase [38]. In the inner mitochondrial membrane, DPG is present at about 20% of the lipids [39] and the membrane is in the form of a bilayer.

In the inner mitochondrial membrane, DPG plays critical roles in structure and in function. Recent X-ray crystallographic analysis has revealed DPG binding sites on several transmembrane proteins in the inner mitochondrial membrane.

The ADP/ATP carrier protein is one transmembrane protein that binds DPG. The ADP/ATP carrier is responsible for transport of ADP into the mitochondria for oxidative phosphorylation to ATP which is then transported out of the mitochondria for use as a cellular fuel. Three DPG molecules are found bound to the bovine ADP/ATP carrier in the crystal structure [40] (Fig. 2). The phosphates are coordinated to backbone nitrogens. ^{31}P NMR experiments on bovine ADP/ATP carrier also demonstrated the tight binding of DPG to the ADP/ATP carrier protein [41]. (^{31}P NMR is a non-perturbing means to monitor the behavior of the phosphorus in the polar headgroups of phospholipids.) Six DPG were found to be bound. The techniques used for preparation of the sample may have been less perturbing than the detergent extraction used for the crystallography, perhaps explaining the higher number of DPG molecules bound. Biochemical experiments suggest possible roles for DPG in stability of the carrier but apparently more experimentation is required to rigorously establish the nature of the biological role [42,43].

DPG has also been found bound to the yeast cytochrome bc_1 complex from the inner mitochondrial membrane. This enzyme catalyzes electron transfer from ubiquinol to cytochrome c. One DPG is bound and appears to play a role in the biological function [44]. One of its

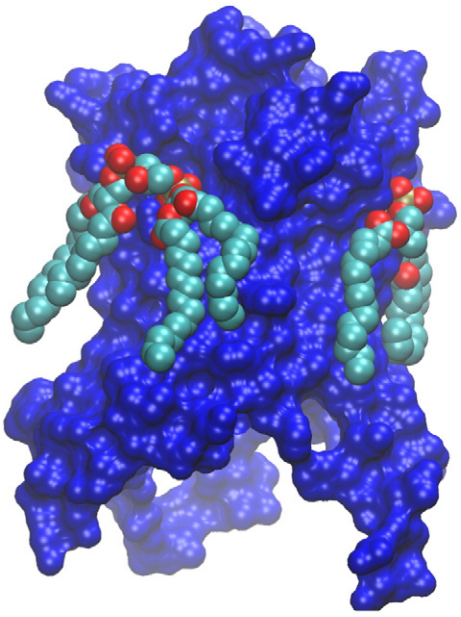

Fig. 2. X-ray crystal structure of bovine ADP/ATP carrier (PDB ID: 2C3E) in blue, showing DPG molecules bound to the protein.

phosphates is coordinated in part with two lysines and an arginine, and the other phosphate in part through a water molecule to a lysine. The side chain of a tryptophan is in close proximity to the acyl chains of the DPG. These interactions help stabilize the binding of the amphipathic DPG to the protein. Recent molecular calculations on cytochrome bc_1 in a lipid environment are in agreement with the X-ray crystal structure in finding the same DPG binding site and, in addition, suggest 5 other binding sites for DPG on the protein [45].

Cytochrome c oxidase, found in the inner mitochondrial membrane, catalyzes the final electron transfer to water of the mitochondrial electron transport chain. DPG binds to cytochrome c oxidase and its removal leads to loss of activity which can be restored by addition of DPG [46]. In an X-ray structural analysis of bovine heart cytochrome c oxidase, two DPG are found bound, both in the transmembrane domain [47]. One DPG bridges two monomers to stabilize a dimer. The other DPG binds near subunit III. The acyl chains of the DPG adopt particular conformations in their binding sites, accommodating to the rough surface of the protein. The phosphates are stabilized by interactions with hydrogen bond donors from the protein.

ABC10 is an ATP-binding cassette (ABC) transporter in the inner mitochondrial membrane. X-ray structural analysis shows that DPG bound to the transmembrane domain of the protein [48]. Interestingly, some of the acyl chains of the DPG are intercalated among the transmembrane helices of the protein.

Formate dehydrogenase-N (Fdn-N) is a component of *Escherichia coli* nitrate respiration. In the crystal structure, one DPG is bound to a site on the transmembrane subunit [49]. The site is at the trimer interface. The headgroup of the DPG is hydrogen bonded to serine, asparagine on one subunit and threonine on another.

Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate coupled to the reduction of ubiquinone. In the X-ray crystal structure, DPG is found bound to the transmembrane domain of this membrane protein [50]. DPG is also found bound to the transmembrane domain of isoprenylcysteine carboxyl methyltransferase [51].

The photosynthetic reaction center, *Rhodobacter sphaeroides*, contains a binding site for one DPG. The X-ray crystal structure shows that a DPG molecule is bound to the transmembrane domain. The negatively charged headgroup of the DPG is stabilized by interactions with a positively charged arginine, lysine, and histidine [52]. These residues appear to be conserved across species [53]. The acyl chains contact two subunits, contributing to stabilization of the protein structure, essentially providing a means of binding the subunits together by the phospholipid.

Experimental evidence shows that DPG binds to a number of membrane proteins, particularly in those membranes involved in electron transport. By binding, the DPG is able in some cases to facilitate association of one protein subunit to another (by binding one more of its acyl chains to each of the subunits), thus stabilizing the structure of a complex of multiple subunits. In fact, DPG may be critical in the formation of supercomplexes in mitochondrial membranes [54,55] even while the specific roles in function remain to be clarified. Perhaps as a consequence, the fatty acyl chain composition of the DPG is well defined in these membranes.

In other cases, DPG with its multiply-charged headgroup can directly play a role in membrane protein function. The headgroup can form resonance structures that can absorb and release a proton. This concept gains credence as a possible role for DPG in the observation that in some cases the DPG headgroup is found near a catalytic site in the structure of the protein.

4. Phosphatidylglycerol

Phosphatidylglycerol (PG) is typical of many phospholipids in that it contains two acyl chains esterified to a glycerol, which in turn is bonded to a headgroup structure that contains net charges. PG contains one phosphate in its headgroup and no other groups with compensating positive charges (the remaining structure is a glycerol). PG therefore has a net negative charge and thus introduces negative charge into a membrane surface and to the lipid–protein interface.

In one of the transmembrane protein structures already introduced, cytochrome c oxidase, 4 PG are found bound in the crystal structure [47]. The headgroup of PG is not well resolved in this structure so it is difficult to determine the contacts between the phospholipid headgroup and the residues of the protein. However, the electron density describing the acyl chains of the PG binding to the protein is defined. The PG acyl chains adopt particular conformations on the protein hydrophobic transmembrane surface. Those conformations are driven by the acyl chains conforming to the rough (on the atomic scale) protein surface sculptured by the size and shapes of the various hydrophobic amino acid side chains. It is interesting that these chains appear to bind to sites with some conservation of structure. The acyl chains of the PG adopt a nearly superimposable conformation on the surface of this protein in structures of cytochrome c oxidase from different species. In an interesting variant, PG in some sites is bound with their acyl chains within the transmembrane bundle of alpha helices. That is, the acyl chains find their way into sites among the transmembrane helices, not just to sites on the surface of the transmembrane bundle of helices of the protein (Fig. 3). Such partially buried sites likely affect the stability of the transmembrane domain of the protein. Finally, some lipids are bound at the interface between subunits in the X-ray crystal structure [56].

Photosystem II is involved in photosynthesis. Photosystem II catalyzes the light-driven oxidation of water. In contrast to PG binding to cytochrome c oxidase, PG binding to photosystem II is characterized by well-defined headgroups bound to the protein, indicating high affinity binding mediated by PG headgroup structure. In support of this, the binding site for the headgroup contains arginine, threonine, and asparagine, residues that are conserved. These residues offer a positively charged region on the protein compatible with the negatively charged phosphate in the PG headgroup [57].

PG also binds to photosystem I which catalyzes another part of the photosynthesis reaction. PG binding to photosystem I is well defined in the density map. Three PG molecules are bound. The headgroups are in some cases near the chlorophylls. Some of the PG acyl chains are bound between protein subunits in the transmembrane domain of the protein [58,59].

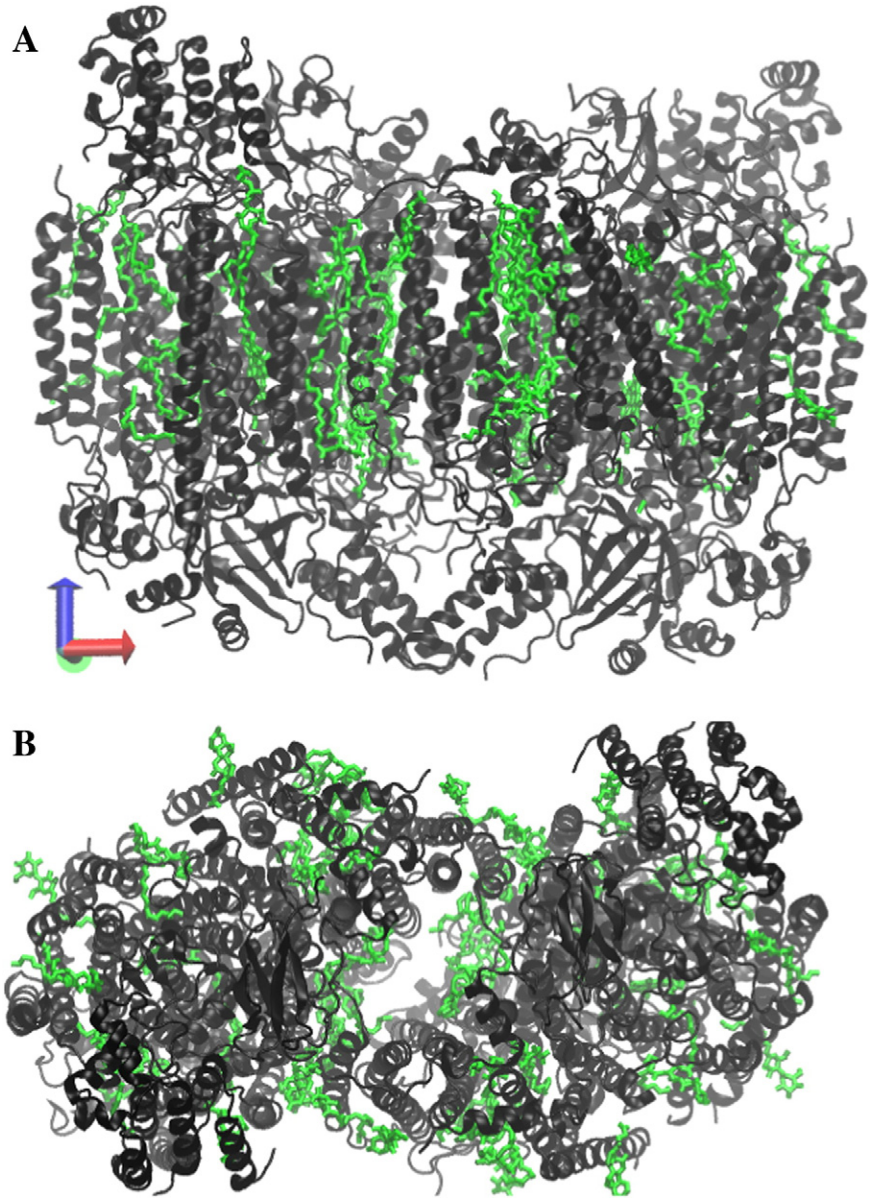

Fig. 3. X-ray crystal structure of bovine heart cytochrome c oxidase (PDB ID: 2DYR) in gray, showing phospholipids in green. A. Side view showing phospholipids in a configuration approximating a bilayer. B. Top view showing some phospholipids partly intertwined with the helices of the transmembrane domain of the protein.

There is X-ray crystallographic evidence that PG binds to some transmembrane channel proteins. Channel proteins provide a means for small molecules or ions to transverse a membrane that the lipid bilayer would otherwise render impermeable. The X-ray crystal structure of the voltage dependent potassium channel provides an interesting example [60,61]. Possibly because the protein was crystallized from mixed micelles of PG and phosphatidylethanolamine, a number of phospholipids are found in the structure. All the electron density of the well-defined phospholipid sites have been modeled with PG (though whether PE might occupy some sites probably cannot be ruled out). Overall the phospholipids organize to approximate a bilayer, with molecules of PG occupying sites on both sides of the putative bilayer structure. However closer inspection reveals that considerable variation in PG location is observed. This is likely due to specific interactions between amino acid side chains on the channel and headgroup structures in the phospholipid. One feature in particular is revealed by this crystal structure: there are likely more sites for interactions between membrane proteins and membrane lipids than the relatively rare ones observed on severely delipidated (by detergent treatment) proteins already discussed in this review. The latter sites may just be lower affinity sites that are more readily displaced by detergents used in purification.

PG also binds to the betaine transporter, BetP. The binding site is between protein subunits with one acyl chain contacting one subunit and the other acyl chain contacting the other subunit [62].

One further example of PG binding to a membrane transporter protein is the maltose transporter. PG is bound at a site between two transmembrane helices of one subunit with the headgroup at the putative membrane–aqueous interface. The binding site does not appear to be between subunits [63].

PG binding to transmembrane proteins shows much of the range of interactions exhibited by DPG binding to transmembrane proteins. In some cases the PG headgroup is the best defined part of the phospholipid, suggesting a fairly specific and tight interaction between the PG headgroup and the site on the protein. These headgroup-specific sites in some cases contain hydrogen bond donors and positive charges that may stabilize the interaction with the negatively charged PG headgroup.

In other cases, the acyl chains of the PG are best defined in the crystal structures, suggesting strong interactions between these portions of the lipid and the surface of the protein. In some cases the acyl chains of one PG contact more than one subunit and may stabilize the overall structure of a multisubunit protein.

5. Phosphatidylethanolamine

The phosphatidylethanolamine (PE) headgroup is a zwitterion, with a phosphate (negative charge) and a free amine (positive charge). PE shares with DPG the ability to form pure lipid structures different from the phospholipid bilayer. Specifically, many forms of PE readily adopt the Hexagonal II structure [64]. While PE carries two charges, in a mixed phospholipid bilayer (with another phospholipid component that stabilizes the bilayer structure) the PE headgroup is strongly hydrogen bonded intermolecularly to an immediate neighbor phospholipid. This forms an ion pair between the positively charged amine of one phospholipid and the negatively charged phosphate of a neighbor phospholipid. This close favorable interaction greatly diminishes the polar character of the headgroup and the corresponding membrane surface, and facilitates the formation of non-bilayer phases as well as interactions with membrane proteins.

X-ray crystallography reveals two (or three) binding sites for PE on cytochrome bc_1. Interactions between the protein and the PE are manifest for both the headgroup and the acyl chains. For one of the headgroups, the amine of the PE is hydrogen bonded to the carboxyl of a glutamic acid side chain. The phosphate is coordinated to two tyrosine hydroxyls and to an ordered water that is turn hydrogen bonded to a tryptophan. The acyl chains make close contacts to a number of aromatic amino acids [44,65–67].

PE also binds to cytochrome c oxidase [47,56]. In one X-ray crystal structure of *R. sphaeroides* cytochrome oxidase, six molecules of PE are found in defined binding sites [68]. Two of the PE are bound in a cleft created by transmembrane helices of the protein. The other four PE are bound between subunit IV and subunits I and III. In these sites, PE appears to play a role in the association between subunit IV with the rest of the complex. Binding of PE headgroups to another cytochrome c oxidase had been previously observed through ^{31}P NMR experiments, in which 3–8 PE molecules were found bound, similar to the observation in the above X-ray crystal structure [69].

Two PE molecules were found bound to the mitochondrial respiratory complex II in the X-ray crystal structure [70,71]. Both of these binding sites extend the acyl chains along the axis of the transmembrane domain of the protein. The acyl chains of the PE have a more defined interaction with the protein than the phospholipid headgroup, in contrast to cytochrome c oxidase described above.

One PE molecule is found bound in the X-ray crystal structure of *Thermochromatium tepidum* photosynthetic reaction center [72]. The binding site is on the transmembrane domain of the protein. In this case the headgroup of the PE is in a distinct binding site created in part by an arginine and a lysine (which can favorably interact with the PE phosphate). The acyl chains bind in a cleft between the transmembrane helix of the H subunit and the transmembrane helices of the L and M subunits of the protein.

Anabaena sensory rhodopsin in its X-ray crystal structure binds a number of PE molecules [73]. Some sites appear to be at the interface between proteins in the crystal and some sites are on the surface of the transmembrane domain interacting with only the monomer. Specific interactions between residues and the phospholipid are not reported. The headgroups are not well defined in the electron density while the acyl chains are (Fig. 4).

Bovine rhodopsin is the photosensitive pigment in rod cells responsible for black and white at low light levels. Rhodopsin functions in a membrane rich in PE. It is not surprising then to find, even for a transmembrane protein that has been extensively delipidated, a PE molecule bound in the X-ray crystal structure (though the identification of this lipid molecule as PE is somewhat ambiguous). The binding site for the acyl chains, which are well defined, is between helices 6 and 7. The headgroup is not visualized in the crystal structure [74].

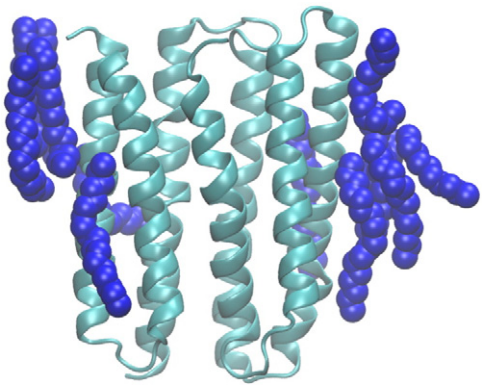

Fig. 4. X-ray crystal structure of *Anabaena* sensory rhodopsin (PDB ID: 1XIO) in cyan. Bound PE acyl chains appear in blue. The PE headgroups are not well defined. The identity of the phospholipid is made by chemical analysis since the unique structure of the PE headgroup cannot be seen in the electron density.

Bovine rhodopsin of the retinal rod outer segment disk is a highly studied system, and several independent biophysical techniques have been employed to study the lipid–protein interactions in this system. The disk membrane is relatively simple in composition in that 90% of the protein in the membrane is rhodopsin. Rhodopsin therefore is a good subject to explore what techniques other than X-ray crystallography can tell us about lipid–protein interactions in this system. ^{31}P NMR measurements (which reflect primarily the behavior of the phospholipid headgroups) are among the few biophysics approaches that allow study in the intact rod outer segment disk membranes. These experiments revealed a subcomponent of the membrane phospholipid that is bound to the protein (at least in the headgroup region) while the majority of the phospholipid is in a bilayer structure [75–77]. While the composition of this bound component could not be determined at the time, it likely contains PE and PC, the two major phospholipids of the disk membrane. About 20 phospholipids were found bound per rhodopsin. Electron spin resonance measurements with lipid spin labels also sense two different lipid environments, one relatively immobilized and the other a lipid bilayer structure [78]. ^{2}H NMR measurements of specifically labeled acyl chains of phosphatidylcholine in reconstituted rhodopsin–bilayer system show only one component [79]. However, the solid echo used in those experiments decays to an insignificant intensity when lipids are bound to membrane proteins with typical correlation times for rotational diffusion. Therefore the latter technique could not be expected to reveal a strongly bound lipid component. Solid state NMR measurements of saturation transfer between protein and lipid suggest a close approach between phospholipid components and rhodopsin in reconstituted membranes with a preference for PE in the vicinity of the protein [80]. Finally chemical labeling experiments to determine accessibility of phospholipid headgroups clearly identify two phospholipid components in the native disk membrane, one protected by the protein component [81].

The calcium pump from rabbit sarcoplasmic reticulum transports calcium against a concentration gradient into the lumen of the sarcoplasmic reticulum in the muscle. This calcium pump crystallizes with three PE molecules (per monomer) in the structure [82]. In this case the headgroups of the PE are well defined while the acyl chains of the PE are not, suggesting specific interactions between the headgroup and the pump protein (Fig. 5). This is emphasized by the observation of PE bound in each of the sites without contributions from other phospholipids of the sarcoplasmic reticulum membrane. PE is bound to the pump at the interfacial region of the protein surface between

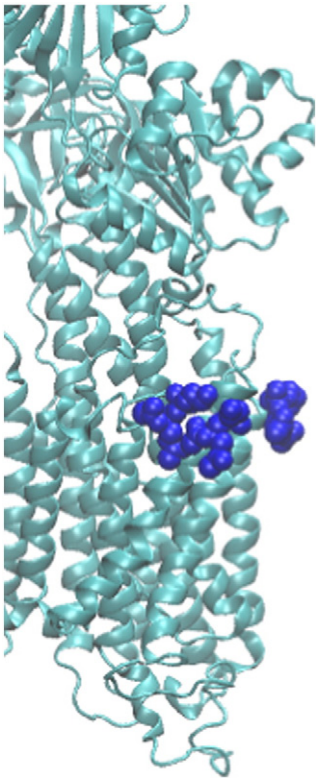

Fig. 5. X-ray crystal structure of the rabbit (fast twitch) sarcoplasmic reticulum Ca^{2+} ATPase (PDB ID: 3W5A) in cyan. Bound PE headgroups appear in blue. The acyl chains of the PE are not well defined in this structure.

the transmembrane domain and the large extramembraneous domain of the protein.

The calcium pump has also been the subject of study by ^{31}P NMR of the phospholipid headgroup phosphates [83]. These studies revealed a component of the phospholipid bound to the pump protein. While it was not possible to determine the phospholipid composition of the bound component at that time, the number of bound phospholipids ranged from 12 phospholipids per pump protein in light sarcoplasmic reticulum to twice that number in heavy sarcoplasmic reticulum. Direct observation of two component ^{31}P NMR powder patterns, corresponding to phospholipid headgroups in two different environments, has been observed in the sarcoplasmic reticulum where the calcium pump is the major protein component, and in reconstituted membranes containing the pump [84]. ^{13}C NMR experiments with the ^{13}C label in the phosphatidylcholine headgroup of the pump reconstituted in defined phospholipid membranes also revealed two components of similar magnitude to the ^{31}P NMR measurements [85]. Likewise electron spin resonance measurements with lipid spin labels show evidence of two lipid components of similar relative magnitude [86,87]. As in the case of rhodopsin, ^{2}H NMR experiments on reconstituted membranes containing the calcium pump show one component [88]. As noted above, this is likely the result of rapid decay of the solid echo in those experiments. Another factor is the ability of an acyl chain to move off and back on the protein when the lipid headgroup is bound to the protein [89]. Finally other techniques also suggest the presence of two lipid components in membranes containing the calcium pump. Differential scanning calorimetry experiments of reconstituted membranes show two lipid components in the presence of the protein [90]. Phospholipase digestion experiments also are consistent with the presence of two lipid components [86].

The X-ray crystal structure of a bacterial sodium bile acid cotransporter shows two PE molecules bound per monomer [91]. Both are bound to the transmembrane helical domain. One is located between monomers at a contact point and the other is located at a site away from the contact point. Both the headgroup and the acyl chains are defined in the crystal structure.

Aquaporin is responsible for rapid movement of water across a membrane. Five PE molecules are found in a crystal structure of aquaporin [92]. The PE molecules are bound to the exterior surface of a tetramer, with the acyl chains lying alongside the transmembrane helices of the protein [93].

PE exhibits many of the same patterns of binding to membrane proteins as exhibited by DPG and PG. In some cases, PE appears most strongly bound to the membrane protein through the headgroup of the phospholipid. The phosphate in these cases is stabilized in the binding site by interactions with lysine and arginine side chains, or with hydroxyls from tyrosine side chains. In other cases, PE is bound to the membrane protein by strong interaction with acyl chains of the phospholipid. The PE acyl chains may interact with more than one subunit, thereby possibly stabilizing the quaternary structure of the membrane protein.

6. Phosphatidylinositol

Phosphatidylinositol (PI) has the inositol in its headgroup and the inositol can be phosphorylated on the sugar hydroxyls. The various phosphorylation states of PI play multiple roles in biology. PI has been found bound to two transmembrane proteins in X-ray crystal structures. In the structure of yeast cytochrome bc_1 one molecule of PI is bound to the transmembrane domain, in contact with 4 of 5 subunits with transmembrane domains. The inositol is hydrogen bonded to residues on the protein and the phosphate of the PI forms an ion pair with a conserved lysine. This PI is suggested to contribute to the stability of the protein [44].

One phosphatidylinositol bisphosphate (PIP_2) is found bound to each subunit of the protein in one of the X-ray crystal structures of the mammalian GIRK2 potassium channel. PIP_2 binds with its acyl chains in contact with the transmembrane helices of the protein and its headgroup at the interface between the transmembrane domain and the extramembraneous domain. The phosphate of the headgroup is stabilized in the binding site by interactions with more than one lysine on the protein. This PIP_2 enables a conformational change that appears to be important in the transport function of the protein [94].

One other example of PI binding to a transmembrane protein is human erythrocyte glycophorin, a transmembrane glycoprotein of the erythrocyte plasma membrane. At least 3 PI molecules from the human erythrocyte bind so tightly and specifically to glycophorin that when this protein is isolated by detergent treatment, the PI molecules remain bound. While there is no crystal structure of this protein (the protein is heavily glycosylated inhibiting crystallization), ^{31}P NMR studies have been published. Among other experiments, glycophorin was reconstituted into a lipid bilayer of diglactosyldiglyceride (DGDG). Since DGDG is a bilayer-forming lipid without phosphate, the only phospholipid visible in the ^{31}P NMR experiments is the PI. The ^{31}P powder pattern showed an immobilized lipid with the only degree of motional freedom that of rotational diffusion of glycophorin to which the PI was bound [95]. This is the only example of a ^{31}P NMR experiment that solely reflects the behavior of phospholipids bound to a membrane protein. This PI is thought to influence binding of glycophorin with other proteins associated with the plasma membrane of the human erythrocyte.

The examples of PI binding to membrane proteins so far available show the PI headgroup predominating in the lipid–protein interaction. The phosphate of the headgroup is stabilized in the binding site by interactions with the positively charged amino acid side chain of lysine.

7. Phosphatidylcholine

Phosphatidylcholine (PC) is the last phospholipid for which there are multiple examples of binding to membrane proteins from X-ray crystallography. PC is a strong bilayer-forming lipid and the most

common phospholipid in mammalian membranes. The headgroup consists of a quaternary amine (positive charge) and a phosphate (negative charge).

One PC is found bound to yeast cytochrome bc_1 [44]. The acyl chain of this PC is bound alongside a transmembrane helix of one of the subunits, cytochrome b. The phosphate of the headgroup is stabilized in the binding site by interacting with a nearby histidine from the cytochrome b. In a subsequent study of a less severely delipidated enzyme, an additional PC binding site was found in the position expected for a normal lipid bilayer around the protein. This PC binds next to helices from both cytochrome b and cytochrome c_1. The phosphate is stabilized by interaction with a histidine from cytochrome c_1 [65].

In cytochrome c oxidase, PC is bound in a location compatible with a lipid bilayer around the protein. The acyl chains are bound in a groove on the surface of the protein. The choline headgroup is in an extended conformation pointing to what would be the aqueous phase from the membrane [47].

The X-ray crystal structure of the ADP/ATP carrier protein has two PC binding sites, one on each side of the protein. In each case, the PC headgroup is located at the interface between the transmembrane domain and the extracellular portion of the protein. In one site, the phosphate of the headgroup is near an arginine. The acyl chains of the PC lie alongside one of the transmembrane helices of the protein. The other site is in a position consistent with the opposite leaflet of the lipid bilayer with the headgroup at the interface between the hydrophobic transmembrane domain and the hydrophilic regions [96].

In squid rhodopsin, one PC is found bound. Its headgroup is bound underneath helix VIII, a helix that is roughly parallel to the putative membrane surface plane. Its acyl chains lie along helices I and VII in the transmembrane domain [97].

In several X-ray crystal structures of transmembrane proteins in which PC binding sites are identified, only minimal information is available about PC binding. PC is found bound at one site on the Na^+K^+ATPase in the X-ray crystal structure. The site is near the transmembrane domain of the protein. Only the headgroup is well defined, suggesting binding affinity is strongest at the headgroup of PC [98]. PC is also reported in the X-ray crystal structure of the zinc metalloprotease, ZMPSTE24. The PC binding site is in the groove defined by two transmembrane helices of the protein [99]. In the voltage gated channel, NavRh, a PC molecule is found bound between subunits of this transmembrane protein [100]. In a later crystal structure, 4 PC molecules are found bound, again between subunits of the protein [101].

Two very different examples of PC molecules in X-ray crystal structures of membrane proteins address the problem that most X-ray crystal structures of membrane proteins are obtained from delipidated preparations of the membrane protein to facilitate crystallization. Since much of membrane function depends upon the lipids in the membrane, it is important to derive structural information from membrane proteins in as membrane-like an environment as possible. It was to this end that the ^{31}P NMR studies described above were directed. ^{31}P NMR reports on the behavior of the phospholipid headgroups in a non-perturbing manner. Furthermore, this measurement can be made on intact biological membranes, not just reconstituted systems or delipidated protein preparations. Although much can be learned, the approach has its limitations in that the atomic detail characteristic of X-ray crystallography is absent.

Therefore the next two studies are of interest in that they are X-ray crystal structures yet they retain substantial lipid bilayer in the crystal. Aquaporin is one example (Fig. 6). Structures have been reported from dimyristoyl PC bilayers and mixed PE, PG, and DPG bilayers. In each case, the phospholipids are found in a bilayer configuration around the perimeter of a protein tetramer. The number of phospholipids is sufficient to cover the surface of the transmembrane domain of the protein. In an extended membrane, this structure would suggest a minimum of two layers of lipids between tetramers. Biological membranes typically have 2 to 4 layers of lipids between membrane proteins in the plane of the membrane. No particular motif of binding of the phospholipids to the membrane protein was identified. Acyl chains adopt a variety of conformations (though ordered structures) to accommodate the surface of the transmembrane domain of the protein and the thickness of the hydrophobic region [102].

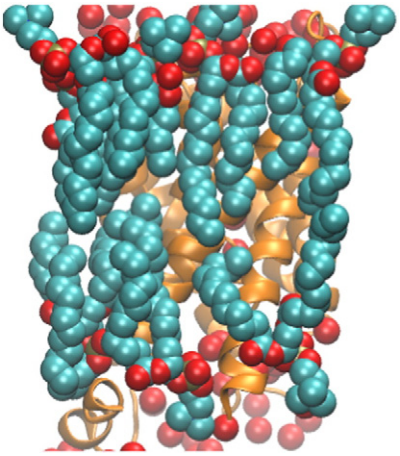

Fig. 6. X-ray crystal structure of lens aquaporin (PDB ID: 2B6O) in orange showing several PC molecules (in cyan) in a configuration approximating a bilayer.

Rhomboid protease has been crystallized from bicelles of PC and the detergent CHAPSO. By crystallizing from an environment that itself approximates a lipid bilayer, it was hoped to obtain a structure for this protein more related to the structure in a membrane. Reminiscent of squid rhodopsin, two PC binding sites are in a pocket underneath two short helices, H1 and H2, that extend parallel to the membrane surface. Three additional PC molecules bind in a groove formed by transmembrane helices TM1 and TM3. A PC binds in the groove between TM1 and TM2. A PC also binds in the groove between TM2 and TM5. While other sites are not as well defined, evidence was found for a total of about 14 PC molecules on the surface of the transmembrane domain of this protein. These phospholipids approximate the locations expected for a lipid bilayer [103].

The crystal structure of these membrane proteins that PC binds to the proteins by interactions with both the headgroup and the acyl chains. In some cases, the former provides the best defined interactions, and in other cases, the latter. In the former, the phosphate is stabilized in the binding site by histidine in one case and arginine in another. In the latter case, examples exist of acyl chains of PC binding to more than one subunit, possibly stabilized quaternary structure of the protein. In other cases, the acyl chains lie alongside the transmembrane helices in a groove on the surface.

8. Summary

With over 100 examples of lipid binding sites in the crystal structures of membrane proteins, some limited motifs emerge for possible lipid–protein interactions in biological membranes involving those lipids that are most tightly bound (i.e., so tightly bound that they are not removed through delipidation by detergents during protein purification). In some cases, binding to the membrane protein appears mediated through the entirety of the lipid structure. Cholesterol binding sites and some phospholipid binding sites provide examples. In other cases, binding appears dominated by either lipid headgroup interactions with the membrane protein or interactions between the acyl chains and the membrane protein.

In the case of mediation of lipid binding through phospholipid headgroups, most often the negatively charged phosphate is stabilized by interactions with positively charged amino acid side chains on the protein. As an alternative in some cases the stabilization is provided by polar ligands from amino acids rather than full opposite charges.

The available data do not support a single motif for binding of the lipid headgroups. Experiments, including the ^{31}P NMR experiments, suggest that these interactions occur even in biological membranes, not just in crystals of delipidated proteins.

When mediation of lipid binding is dominated by acyl chain interactions with the protein, the interactions are stabilized by hydrophobic effects. However, perhaps because of the wide range of amino acid side chains that are hydrophobic, no consensus for a specific motif has yet emerged. Acyl chains of the lipids may be bound on the surface of the transmembrane domain of the protein. They may be bound to more than one subunit and thus contribute to the stability of the quaternary structure. Interestingly in more than one case, the acyl chains of the lipids penetrate the bundle of helices of the transmembrane domain of the protein. Here they must play a structural role, but much remains to be investigated concerning that role.

These X-ray crystal structures do not yet reveal a discernable motif for cholesterol binding to transmembrane proteins, in contrast to the CRAC motif for cholesterol binding to other classes of proteins.

Data are available from many studies demonstrating that specific membrane lipids modulate membrane function through regulation of the activity of membrane bound enzymes. For example, the essential requirement for cholesterol in mammalian cells can be traced to cholesterol interactions with membrane proteins, which as can now be seen in this review, could arise from cholesterol binding to membrane proteins at specific sites. DPG provides an important parallel example from the phospholipids. DPG has been shown to be essential for function of particular mitochondrial enzymes. Those same enzymes have now been found to have specific binding sites for DPG in their structure. Occupation of these binding sites by DPG likely provides the mechanism for DPG modulation of enzyme activity.

From the beginnings of the field of studies of structure and function of biological membranes, investigators have been aware of the coexistence of lipids and proteins in these membranes [104–106]. It has been a basic hypothesis driving the study of membranes that these two classes of compounds must interact with each other in the biological membrane in a way that builds the biological functions manifest from those membranes. As methodologies and technologies have improved over the decades since, data gradually accumulated that support that hypothesis. Now the advances in X-ray crystallography and electron crystallography of transmembrane proteins have given firm substance to the hypothesis. At the same time, many experiments published before the advent of X-ray crystallography of membrane proteins take on greater significance in the light of these more recently available atomic level structures. The field now seems set to explore in detail the structural and mechanistic origins of the modulation of membrane function by interactions between membrane lipids and membrane proteins.

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