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Review

How lipids affect the activities of integral membrane proteins

Anthony G. Lee*

Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Southampton SO16 7PX, UK

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Abstract

The activities of integral membrane proteins are often affected by the structures of the lipid molecules that surround them in the membrane. One important parameter is the hydrophobic thickness of the lipid bilayer, defined by the lengths of the lipid fatty acyl chains. Membrane proteins are not rigid entities, and deform to ensure good hydrophobic matching to the surrounding lipid bilayer. The structure of the lipid headgroup region is likely to be important in defining the structures of those parts of a membrane protein that are located in the lipid headgroup region. A number of examples are given where the conformation of the headgroup-embedded region of a membrane protein changes during the reaction cycle of the protein; activities of such proteins might be expected to be particularly sensitive to lipid headgroup structure. Differences in hydrogen bonding potential and hydration between the headgroups of phosphatidycholines and phosphatidylethanolamines could be important factors in determining the effects of these lipids on protein activities, as well as any effects related to the tendency of the phosphatidylethanolamines to form a curved, hexagonal H_{II} phase. Effects of lipid structure on protein aggregation and helix–helix interactions are also discussed, as well as the effects of charged lipids on ion concentrations close to the surface of the bilayer. Interpretations of lipid effects in terms of changes in protein volume, lipid free volume, and curvature frustration are also described. Finally, the role of non-annular, or 'co-factor' lipids, tightly bound to membrane proteins, is described.

Keywords: Lipid–protein interaction; Annular lipid; Hydrophobic mismatch; Membrane structure; Membrane thickness; Lipid headgroup; Non-annular lipid; Integral membrane protein

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Abbreviations: GPS, glycerophosphoserine; GPE, glycerophosphoethanolamine; di(C14:0)PC, dimyristoylphosphatidylcholine; di(C14:1)PC, dimyristoleoylphosphatidylcholine; di(C16:0)PC, dipalmitoylphosphatidylcholine; di(C18:1)PC, dioleoylphosphatidylcholine; di(C22:1)PC, dierucoylphosphatidylcholine; di(C22:6)PC, di(4,7,10,13,16,19 docosahexaenoyl)phosphatidylcholine; di(C12:0)PE, dilaurylphosphatidylethanolamine; (C16:0,C18:1)PE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; di(C18:1)PE, dioleoylphosphatidylethanolamine; di(C18:1)PG, dioleoylphosphatidylglycerol; di(C18:1)PS, dioleoylphosphatidylserine; di(C18:1)PA, dioleoylphosphatidic acid; MscL, mechanosensitive ion channel of large conductance; EcMscL, MscL from *E. coli*

* Tel.: +44 23 8059 4331; fax: +44 23 8059 4459.
E-mail address: agl@soton.ac.uk.

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1. Introduction

Integral membrane proteins operate in an environment made up, in part, by the surrounding lipid bilayer; the composition of the lipid bilayer must therefore be such as to support at least close to optimal functioning for the proteins in the membrane. Effects of lipid structure on membrane protein function can be described in molecular terms, that is, in terms of molecular interactions between the lipid and protein molecules such as hydrophobic effects, hydrogen bonding or charge interactions, or in physical terms, that is, in terms of physical properties of the lipid bilayer such as lipid fluidity, membrane tension, and so on. Although in some cases it is obvious that a description in molecular terms is required, in others it is not obvious whether a molecular or a physical explanation is most appropriate. Where both molecular and physical explanations are available, it is often not clear whether these are actually different explanations or just two different ways of saying the same thing.

An example where a molecular description is clearly the most appropriate is provided by studies of the effect of lipid structure on the binding of annexins to the surface of a lipid bilayer. Binding involves Ca^{2+} ions bridging between the protein and the phospholipid headgroups. Crystal structures have been obtained for rat annexin V in the presence of glycerophosphoserine (GPS) and glycerophosphoethanolamine (GPE) [1]. The phosphoglycerol backbones of GPS and GPE bind in a similar fashion, but with significant differences (Fig. 1). The phosphoryl oxygen coordinates a bound Ca^{2+} ion, capping the Ca^{2+} binding site [1]. In both complexes Ca^{2+} binding leads to extrusion of Trp-185 from the core of the protein; insertion of Trp-185 into the hydrophobic core of the lipid bilayer adds a hydrophobic component to the binding energy. Gly-186 bridges the Ca^{2+} ion to the phospholipid; its carbonyl oxygen coordinates the Ca^{2+} ion and its amide group interacts with the glycerol backbone of the phospholipid analogues. Thr-187 also interacts with the bound Ca^{2+} ion, but, whereas in the GPS complex, its –OH group forms a hydrogen bond with the serine amino group in GPS, in the GPE complex the –OH group of Thr-187 is hydrogen bonded to a water molecule that, in turn, hydrogen bonds to a phosphoryl oxygen. A further difference between the two complexes is that the GPE headgroup extends along the molecular surface in the opposite direction to GPS, in a shallower binding site (Fig. 1). The more extensive interactions observed in the crystal structure with GPS than with GPE suggests that binding to phosphatidylserine will be stronger than to phosphatidylethanolamine. This is

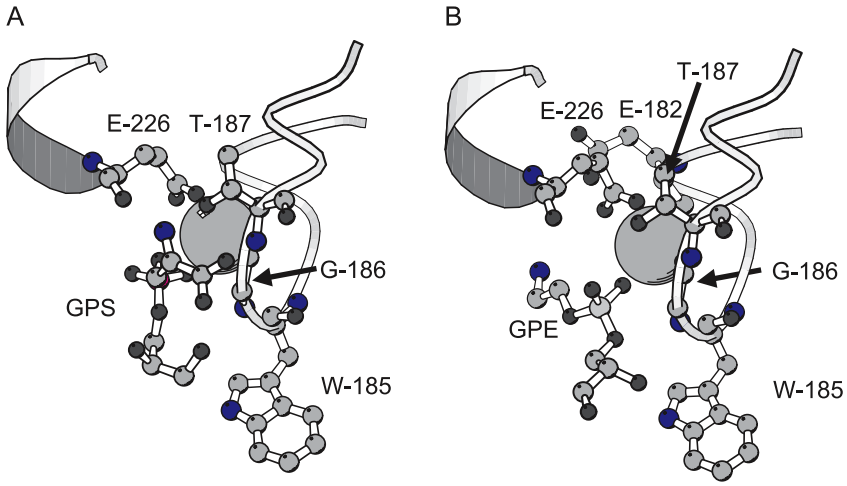

Fig. 1. Binding of GPS and GPE to annexin V. The binding sites for GPS (A) and GPE (B) are shown. The filled sphere is Ca^{2+}. Some of the residues important for binding are shown in ball-and-stick mode (PDB files 1A8A and 1A8B).

indeed what is observed. Although annexin V binds to bilayers of phosphatidylethanolamine [2,3] it binds more strongly to bilayers containing anionic phospholipids, the strength of binding decreasing in the order phosphatidic acid>phosphatidylserine>phosphatidylinositol [2,4–6]. In contrast, annexin V hardly binds to bilayers of phosphatidylcholine or sphingomyelin [2,3]. In this case, therefore, effects of lipid headgroup structure on protein binding can be understood in molecular terms. Although insertion of annexin V into the lipid headgroup region of the bilayer must be affected by physical properties of the lipid headgroup region such as the lateral pressure, an explanation in such terms would miss the heart of the problem. For example, binding to bilayers of phosphatidylethanolamine is stronger than to bilayers of phosphatidylcholine not because of differences in the physical properties of the two bilayers but because the phosphatidylcholine headgroup will be unable to bind well to the headgroup binding site on annexin. However, other proteins that bind in the headgroup region of the bilayer, such as CTP:phosphocholine cytidylyltransferase, show no evidence for specific lipid binding sites on the protein and an explanation for lipid effects on binding in terms of the physical properties of the headgroup region becomes more appropriate (Section 2.8). The aim of this review is to describe the various molecular and physical explanations for the effects of lipids on intrinsic membrane protein function that can be found in the literature and to assess the general applicability of these various explanations.

1.1. Lipids as solvent and lipids as co-factors: annular and non-annular lipid

Whilst the bulk of the lipid molecules in contact with an intrinsic membrane protein act as a 'solvent' for the protein, interacting with the protein relatively non-specifically, some proteins also interact with much greater specificity with a small number of lipid molecules, these lipid molecules often being essential for activity and acting like a traditional co-factor. The solvent lipids have been referred to as boundary lipids or as annular lipids, to denote the fact that they form an annular shell of lipid around the protein [7]. Co-factor lipids, often bound between transmembrane α-helices either within a protein or at protein–protein interfaces in multi-subunit proteins, have then been referred to as non-annular lipids [8]. The lipid molecules in a membrane not in contact with a protein at all are usually referred to as bulk phospholipids; if the effect of a membrane protein on the surrounding lipid molecules is limited to those in direct contact with the protein then the properties of the bulk lipids in a membrane will be the same as those in a simple lipid bilayer.

The rate of exchange of lipid molecules between the annular shell around a membrane protein and the bulk phase is fast, showing that the lipid–protein interaction is a non-sticky one [9–11]. It has sometimes been suggested that annular lipid could only affect the function of a membrane protein if the lifetime of a lipid molecule in the annular shell around the protein were long compared to the turnover number of the protein. However, this is not so, it does not matter which particular lipid molecule is in the annular shell; it is only significant that the lipid molecules that are in the annular shell are in a particular physical state and have a particular effect on the protein. Rapid exchange of the lipids can average the environment sensed by the lipid but will not average the environment sensed by the protein; the environment sensed by the protein (the annular lipid) is the same however fast the lipids exchange. The rate of exchange of non-annular lipid with bulk lipid has not yet been determined, but could be relatively slow given the high specificity of the interaction between non-annular lipid and the protein.

Most of the lipid molecules resolved in high-resolution crystal structures of membrane proteins are likely to be non-annular lipids, their strong binding to the protein leading to immobilization of at least part of the lipid molecule so that they appear in the high-resolution structure [7]. Annular lipids, almost by definition, will normally be too disordered to appear in high-resolution structures. However, annular lipids have been resolved on the surface of crystalline arrays of the bacteriorhodopsin trimer [12], as shown in Fig. 2A. In both X-ray diffraction and electron microscopic studies, the lipid headgroups are disordered but many fatty acyl chains are well resolved, mostly bound in distinct grooves on the surface of the protein [12–15]. Typical of non-annular lipids is the phosphatidylglycerol molecule bound between transmembrane α-helices at monomer–monomer interfaces in the homotetrameric potassium channel KcsA; again the headgroups of the lipid molecules are not resolved, and the lipids have therefore been modelled as diacylglycerol (Fig. 2B) [16]. KcsA requires the presence of phosphatidylglycerol or some other anionic phospholipid for function, and so the phospholipid can be said to act as a co-factor for the protein [17,18].

Binding at non-annular sites will often be headgroup-specific. Fig. 3 shows the binding sites for phosphatidylethanolamine on the photosynthetic reaction centre of *Thermochromatium tepidum* [19] and on yeast cytochrome bc_1 [20]. The very different conformations adopted by the lipid headgroup in the two structures are apparent. In the *T. tepidum* structure the amine group is folded down, allowing the phosphate group to interact with the adjacent Lys and Arg groups. In contrast, in the cytochrome bc_1 structure, the phosphatidylethanolamine headgroup is extended, allowing favourable interaction between the amine group and a neighbouring Glu residue. The ability of other phospholipid molecules to bind to these sites will depend on the size of the lipid headgroup and its charge; size, for example, is likely to prevent binding of phosphatidylcholine to the sites shown in Fig. 3.

Table 1 lists the phospholipid molecules identified to date in high-resolution crystal structures of α-helical membrane

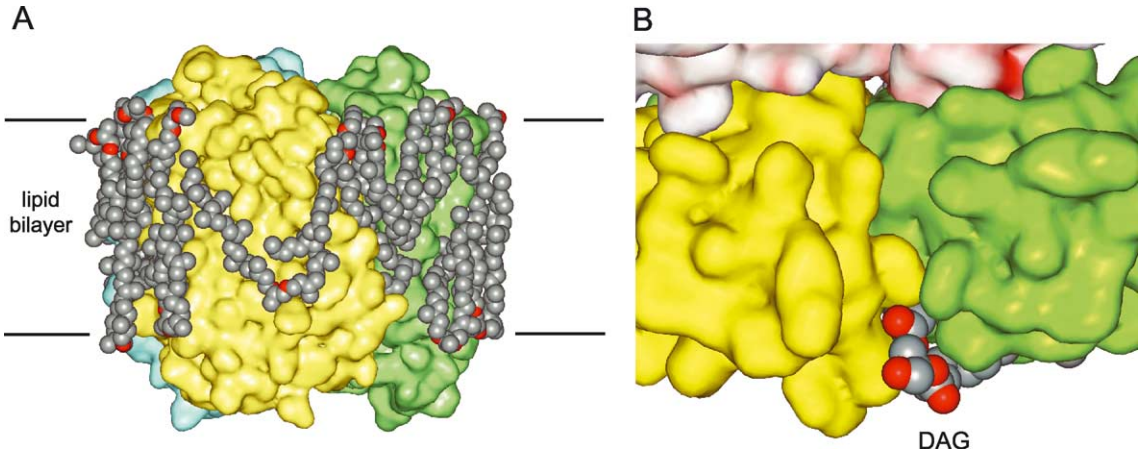

Fig. 2. (A) Lipid molecules bound to the bacteriorhodopsin trimer. The lipid headgroups are not resolved and the lipid molecules have therefore been modelled as 2,3-di-*O*-phytanyl-*sn*-propane (PDB file 1QHJ). (B). A view looking down on the KcsA tetramer, showing a bound lipid molecule DAG, shown in space-fill format, bound between two subunits, shown in yellow and green. The headgroup of the lipid molecule was not resolved, and the lipid has therefore been modelled as a diacylglycerol (PDB file 1K4C).

proteins and gives their classification as annular or non-annular; in the case of bacteriorhodopsin, only one of the several structures available in the PDB database is listed. The table shows that the majority of the lipid molecules resolved in crystal structures of membrane proteins are located between subunits in multimeric membrane proteins. It should perhaps be emphasised that often only partial lipid molecules are identified in electron density maps and there is then the possibility for confusion between, for example, the fatty acyl chains of a phospholipid molecule and the chains of a detergent molecule.

2. Effects of annular lipids on membrane protein function

2.1. The importance of the lipid headgroup region

The lipid–water interface is not a sharp barrier between a slab of hydrocarbon and a polar region made up of the lipid headgroups and water. Rather, the properties of the interface change gradually over a distance of some 15 Å, the headgroup region having sufficient thickness to accommodate, for example, an α-helix lying parallel to

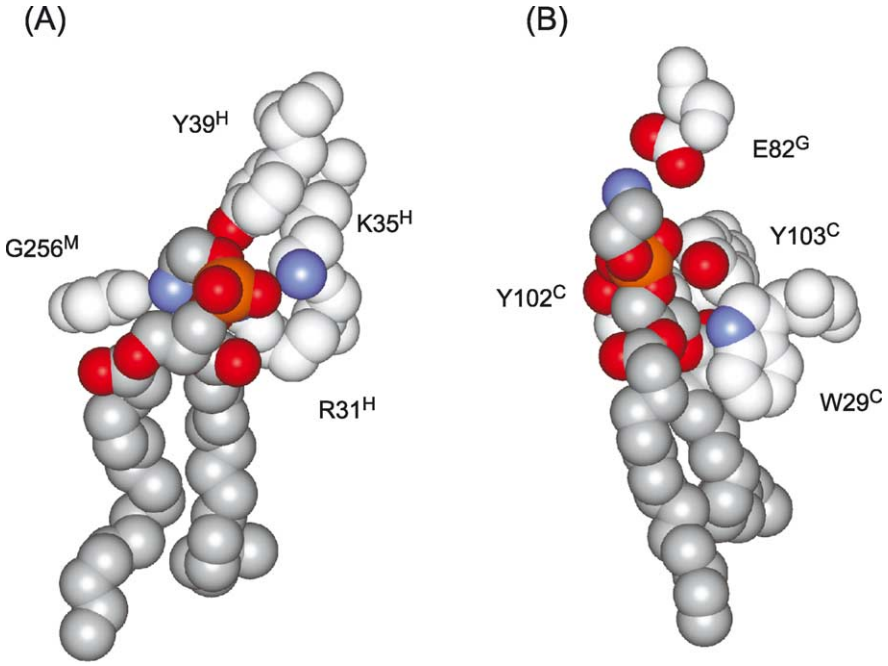

Fig. 3. The structures of bound phosphatidylethanolamine in the photosynthetic reaction centre of *T. tepidum* (A) and yeast cytochrome bc_1 (B), showing residues interacting with the lipid headgroups (PDB files 1EYS and 1KB9).

Table 1
Lipid molecules identified in crystal structures of α-helical membrane proteins

Protein	PDB code	Annular lipids	Non-annular lipids	
			Between helices[a]	Between subunits[a]
Bacteriorhodopsin	1QHJ	6		2
Rhodopsin	1GZM	1		
Bacterial photosynthetic reaction centres				
Rhodobacter sphaeroides	1QOV			1
	1OGV			
	1M3X	1?	1?	1
Thermochromatium tepidum	1EYS			1
Photosystem 1 from *Synechococcus elongates*	1JB0	1	2	1
Light-harvesting complex from spinach	1RWT			2
Cytochrome *c* oxidase from *Paracoccus denitrificans*	1QLE		1	1
Cytochrome bc_1 from *Saccharomyces cerevisiae*	1KB9		1	4
Cytochrome b_6f from *Chlamydomonas reinhardtii*	1Q90			2
Succinate dehydrogenase from *E. coli*	1NEK	1		1
Nitrate reductase	1Q16			1
ADP/ATP carrier from mitochondria	1OKC	7		
Potassium channel KcsA	1K4C			1

[a] Non-annular lipids are classified as either being located between transmembrane α-helices within a monomer or between subunits in a multimeric complex.

the bilayer surface [21]. The complexity of the lipid headgroup region is shown in Fig. 4, which shows a snapshot of a molecular dynamics simulation of a bilayer of 1-palmitoyl-2-oleoyl phosphatidylcholine in the liquid crystalline phase; the degree of disorder and the loose packing in the headgroup region is clear. Packing in the lipid headgroup region will depend on the lipid fatty acyl chain structure as well as on the structure of the lipid headgroup itself. For example, the area occupied in the bilayer surface by a molecule of dipalmitoylphosphatidylcholine [di(C16:0)PC] in the liquid crystalline phase at 50 °C is 64 Å^2 [22], whereas the area occupied by a molecule of dioleoylphosphatidylcholine [di(C18:1)PC] is 72.5 Å^2 [22]; the greater area occupied by di(C18:1)PC follows from effects of the unsaturated oleoyl chains. Similarly, in bilayers of dilaurylphosphatidylethanolamine [di(C12:0)PE] in the liquid crystalline phase the surface area per lipid is 51.2 Å^2 [22] compared to 56 Å^2 for 1-palmitoyl-2-oleoylphosphatidylethanolamine ((C16:0,C18:1)PE) [23]; the greater area occupied by (C16:0,C18:1)PE than di(C12:0)PE can again be attributed to the presence of the bulky *cis* unsaturated chain, and the smaller area occupied by a phosphatidylethanolamine than by an equivalent phosphatidylcholine can be attributed to the smaller headgroup and the greater possibilities for hydrogen bonding.

Differences in the areas occupied by different lipid molecules in the bilayer surface will lead to different patterns of hydrogen bonding and hydration in the headgroup region of the bilayer. Thus the extent of hydration is very different for bilayers of phosphatidylcholine and phosphatidylethanolamine; at full hydration, a bilayer of di(C16:0)PC takes up about 23 molecules of water per molecule of lipid [24], whereas a bilayer of di(C12:0)PE takes up only about 10 molecules of water per molecule of lipid [25]. More details about the nature of hydration is obtained from a molecular dynamics simulation of a bilayer of a phosphatidylethanolamine, which shows a pattern of hydration distinctly different to that for a bilayer of phosphatidylcholine [26,27]; whereas the hydrophobic $-NMe_3^+$ group of phosphatidylcholine induces formation of a clathrate-like hydration shell around the headgroups in order to optimise inter-water hydrogen bonding, direct hydrogen bonds are formed between the $-NH_3^+$ group of phosphatidylethanolamine and the water molecules. Although interlipid hydrogen bonds were observed in the headgroup region of the phosphatidylethanolamine, more hydrogen bonds were formed with water; the hydrogen bonding inter-lipid network was much weaker than that observed in the crystal [26].

The nature of the possible interactions between lipid headgroups and groups in a protein is shown by a molecular dynamics simulation of the tripeptide Ala-Phe-Ala-*O*-*t*-butyl bound to a bilayer of dimyristoylphosphatidylcholine [di(C14:0)PC] [28]. The peptide distribution extended from the headgroup-water interface into the fatty acyl chain region. Both the side chain of Phe and the *t*-butyl group intercalated between the lipids and made van der Waals' contacts with the fatty acyl chains; the N-terminus of the peptide was located close to the lipid phosphate groups. Hydrogen bonding interactions between the N-terminus of the peptide and the non-esterified oxygens of the lipid phosphate groups helped to anchor the peptide at the lipid–water interface. Despite the penetration of the peptide into the hydrocarbon chain region of the bilayer, the presence of the peptide was found not to affect the lipid dynamics or the average structure adopted by the lipid molecules in the simulation. Thus the bilayer was able to accommodate the peptide without significant change in the properties of the lipid molecules. This 'plasticity' of the lipid bilayer environment is not unexpected, given the weak interactions between the lipid molecules [28].

The structure of the lipid headgroup region could affect the structure of a protein penetrating into this region of the bilayer, because of the requirements of the polypeptide backbone and of any polar residues for hydrogen bonding, tending to drive the formation of secondary structures such

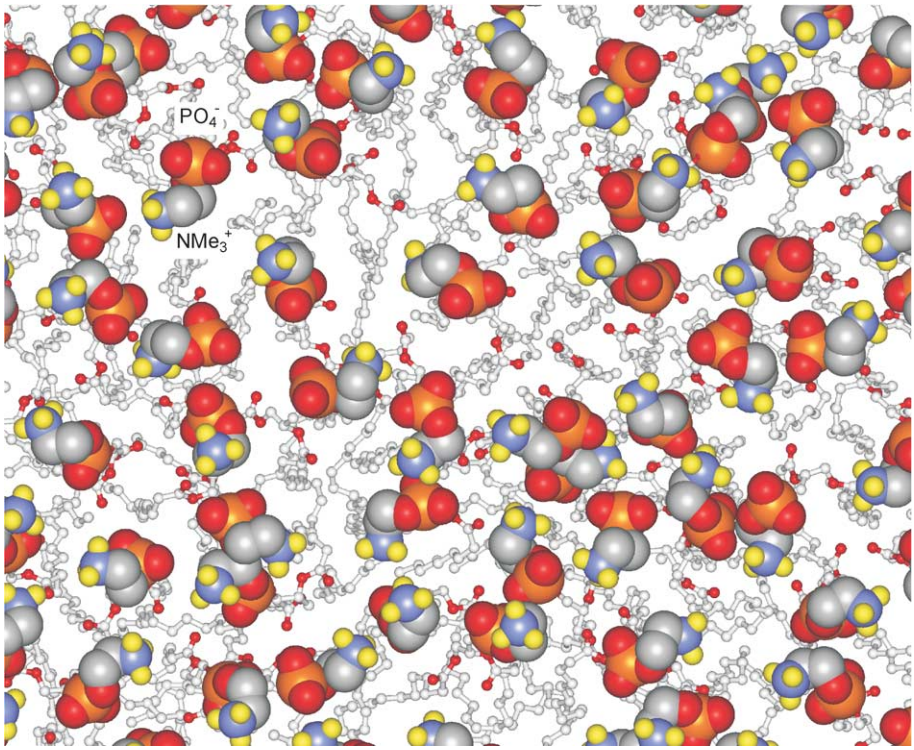

Fig. 4. The headgroup region of a bilayer of 1-palmitoyl-2-oleoyl phosphatidylcholine in a view down onto the bilayer surface. The figure shows a snapshot from a molecular dynamics simulation (Heller, H., Schaefer, M. and Schulten, K.; http://www.umass.edu/microbio/rasmol/bilayers.htm). Fatty acyl chains and the glycerol backbone are shown in ball-and-stick format, with C atoms coloured grey and O atoms coloured red. The phosphocholine groups are shown in space-fill format, with the methyl groups (yellow) reduced in size for clarity. Water molecules are not shown to allow the lipid headgroups to be seen.

as the α-helix and β-sheet. The interface region has been referred to as a catalyst for the formation of secondary structure by peptides [29]. The pattern of hydrogen bonding between peptide, water and lipid will be very different in bilayers of phosphatidylcholine and of phosphatidylethanolamine, potentially leading to different secondary structures for any stretch of peptide located in the lipid headgroup region. The presence of charged lipid headgroups could also have a large effect on the structure of regions of a protein containing charged amino acids and located in the headgroup region.

The structure of the lipid headgroup region could also have a significant effect on the structures adopted by the ends of transmembrane α-helices. The initial four –NH and final four C=O groups of an α-helix have no hydrogen bond partners within the backbone of the α-helix itself. This problem can be overcome by the provision of suitable polar residues at the two ends of the helix and a survey of the ends of α-helical segments of soluble proteins showed that they are often capped in this way [30,31]. However, for a membrane protein, ends of α-helices could also find hydrogen bonding partners in the glycerol backbone and lipid headgroup regions of the bilayer. Changes in the lipid headgroup region could then lead to changes in structure at the ends of the transmembrane α-helices and to consequent changes in the packing of the helices.

A possible example of the importance of the lipid headgroup region for protein function is provided by rhodopsin. The cytoplasmic loop between transmembrane helices C and D, containing a conserved sequence ^{134}E(D)RY, is important in photoactivation [156]. Glu-134 in this loop is located close to the glycerol backbone region of the surrounding lipid bilayer, as shown in Fig. 5 [7]; Glu-134 is unprotonated in unactivated rhodopsin but becomes protonated on formation of metarhodopsin II, the residue then moving into a more hydrophobic environment [156]. Any changes in the headgroup region of the bilayer that affected ionisation of Glu-134 would therefore affect photoactivation of rhodopsin. Also important is the C-terminal region that forms a cationic amphipathic helix lying parallel to the membrane surface (Fig. 5) important for interaction with G proteins [32–34]. A variety of experimental evidence, reviewed in Ref. [33], suggests that this loop changes structure when rhodopsin is activated to the key intermediate metarhodopsin II. Studies with a peptide corresponding to this region showed that the peptide bound in an α-helical conformation to lipid bilayers, but only in the presence of phosphatidylserine; the structure adopted by the loop in rhodopsin could therefore be dependent on lipid structure [33].

The relative amounts of the two major intermediates of the rhodopsin photocycle, metarhodopsin I (MI) and metarhodopsin II (MII) depend on lipid structure and on

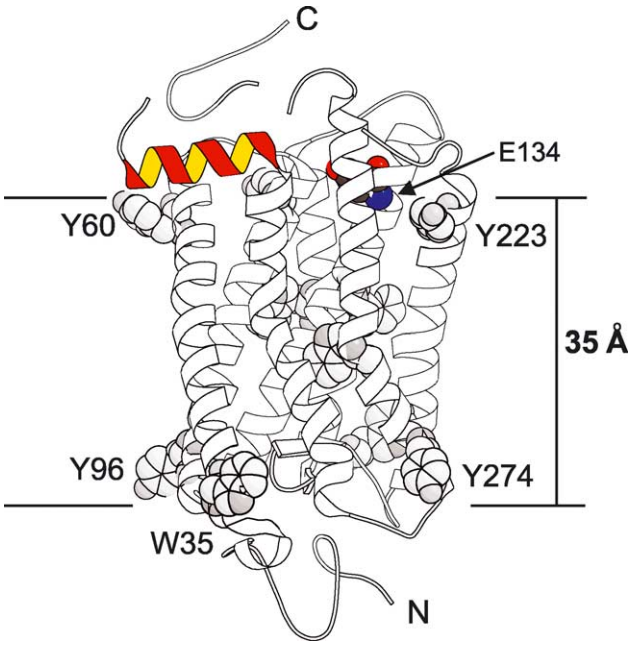

Fig. 5. The structure of rhodopsin. The hydrophobic thickness of rhodopsin is defined largely by the Tyr and Trp residues shown in space-fill representation. The amphipathic helix from Asn-310 to Cys-322 is shown coloured, as is Glu-134. (PDB file 1F88).

pH [35,36]. Small amounts of MII are formed when rhodopsin is reconstituted with egg phosphatidylcholine, but the amounts are much less than those formed in the native membrane. Increasing the chain length and unsaturation of the phosphatidylcholine to di(4,7,10,13,16,19-docosahexaenoyl)phosphatidylcholine [di(C22:6)PC] results in a very significant increase in the amount of MII formed [37]. Incorporation of phosphatidylethanolamines into the bilayer also results in an increase in the level of MII. MI/MII ratios equal to those in the native membrane are seen in mixtures of di(C18:1)PC and dioleoylphosphatidylethanolamine [di(C18:1)PE] when the di(C18:1)PE content is increased from the value of about 40% characteristic of the native membrane, to about 75% [35,36,38]. However, when rhodopsin is reconstituted into mixtures of phosphatidylcholine and phosphatidylethanolamine containing C22:6 chains, high levels of MII are achieved at phosphatidylethanolamine levels comparable to those in the native membrane [36]. Although these effects are usually interpreted in terms of the preference of phosphatidylethanolamines for a curved, hexagonal H_{II} phase (see Section 2.8), it is possible that effects of lipid headgroups on the ionisation of Glu-134 in the CD loop [156] or on the structure of the C-terminal amphipathic helix are important. For example, phospholipid composition has significant effects on the interaction between MII and the G protein transducin, the association constant between MII and transducin being higher in bilayers of di(C18:0,C22:6)PC than in bilayers of (C18:0,C18:1)PC [39] and protonation of Glu-134 increases interaction with transducin [156] and, as already described, the C-terminal amphipathic helix in rhodopsin is involved in interaction with transducin. Another possible example where the differences in hydrogen bonding potential between phosphatidylethanolamine and phosphatidylcholine could lead to conformational changes in a protein is provided by the mechanosensitive channel of large conductance (MscL) discussed in Section 2.10.

High-resolution structures of the Ca^{2+}-ATPase of sarcoplasmic reticulum in the Ca^{2+}-bound and thapsigargin-bound states, believed to correspond to the E1 and E2 conformations of the Ca^{2+}-ATPase, respectively, are significantly different in parts of the protein that are likely to be located in the lipid headgroup region of the bilayer (Fig. 6) [40–42]. Of the 10 transmembrane α-helices in the Ca^{2+}-ATPse, helix M1 is unusual in containing a number of charged residues and helix M1 undergoes a large change in structure during the transformation from E2 to E1 [40]. In the E2 state, the region N-terminal of Asp-59 forms an amphipathic helix located in the lipid headgroup region, with hydrophobic residues on one side and Glu-58 and Asp-59 on the other. In the Ca^{2+}-bound, E1 form Glu-51 and Glu-55 are now located at the top of the first transmembrane α-helix with Glu-58 facing in towards one of the Ca^{2+} binding sites. Arg-63, which snorkels up to the lipid–water interface in the E2 conformation, forms an ion pair with Asp-59 in the Ca^{2+}-bound, E1 form, exposed to the lipid bilayer. Mutation of Asp-59 results in large changes in the rate of dissociation of Ca^{2+} from the Ca^{2+}-bound ATPase [158]. Changes in the lipid headgroup region might well, therefore, be expected to have significant effects on the activity of the Ca^{2+}-ATPase. In agreement with this expectation, the activity of the Ca^{2+}-ATPase is lower in bilayers of di(C18:1)PE than in bilayers of di(C18:1)PC, but only at temperatures where pure di(C18:1)PE would adopt a hexagonal H_{II} phase [43]. The activity of the Ca^{2+}-ATPase is also low in bilayers of dioleoylphosphatidylserine [di(C18:1)PS] or dioleoylphosphatidic acid [di(C18:1)PA] [44]. Similarly, di(C18:1)PS, di(C18:1)PA and cardiolipin all support lower activities for the diacylglycerol kinase of *E. coli* than that supported by di(C18:1)PC [45]. Interestingly, activity for the diacylglycerol kinase is higher in bilayers of dioleoylphosphatidylglycerol [di(C18:1)PG] than in bilayers of the other anionic phospholipids, which could be significant since phosphatidylglycerol is the major anionic phospholipid in the *E. coli* cell membrane [45].

Finally, the lipid headgroup region could affect the activity of a membrane protein by changing the concentrations of charged molecules or ions close to the surface of the membrane. Incorporation of a negatively charged lipid into a membrane will increase the negative charge on the surface of the membrane and thus increase the concentration of positively charged molecules or ions close to the surface of the membrane and, correspondingly, decrease the concentration of negatively charged molecules or ions close to the surface. The simplest description of these charge effects is given by Gouy–Chapman theory [46,47]. By

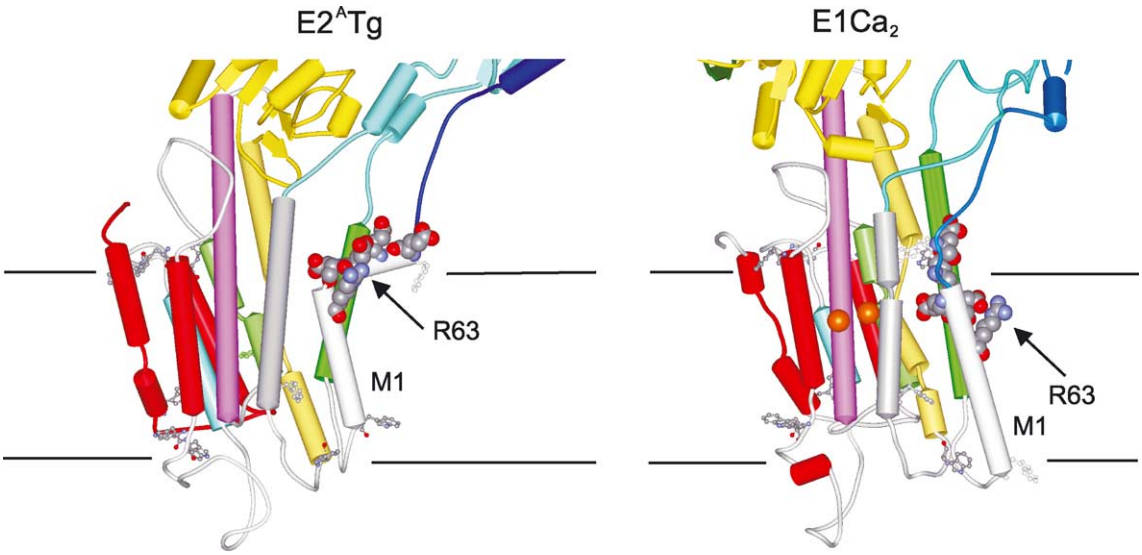

Fig. 6. The transmembrane region of the Ca^{2+}-ATPase in its Ca^{2+}-free, thapsigargin-bound ($E2^{A}Tg$) and Ca^{2+}-bound ($E1Ca_2$) conformations. Particularly large changes are seen in the first transmembrane α-helix, M1, shown in light grey. Charged residues in M1 are shown in space-fill representation. The two bound Ca^{2+} ions in the $E1Ca_2$ structure are shown in orange. Trp residues that help to define the probable location of the hydrophobic core of the lipid bilayer are shown in ball-and-stick representation, and a possible location for the hydrophobic core of the lipid bilayer is shown by the horizontal lines (PDB files 1EUL and 1IWO).

changing local concentrations of charged species, incorporation of negatively charged lipids into a membrane will change the apparent affinities of membrane proteins for charged substrates. Interpretation of these effects may, however, need to take into account the effects of membrane charge on the local concentration of H^+. For example, the presence of negatively charged phospholipids in a membrane might be expected to increase the apparent affinity for Ca^{2+} of a membrane protein that binds Ca^{2+}. However, the presence of negatively charged phospholipids will also increase the concentration of H^+ close to the membrane and, since Ca^{2+} ions often bind to proteins in competition with binding of two H^+ ions, the effects of membrane charge on local H^+ concentrations could cancel out the effects on local Ca^{2+} ion concentrations.

Effects of charge on local H^+ concentrations will also, of course, be important for any process that depends on protonation. For example, the MI/MII equilibrium for rhodopsin is pH-dependent and the presence of phosphatidylserine in the membrane has a large effect on this equilibrium, changing the apparent p*K* describing the equilibrium through an effect on the H^+ concentration at the surface [48]. The presence of anionic lipid can also, by increasing the local H^+ concentration, increase protonation of acidic residues in a stretch of peptide located close to a membrane surface and so increase the effective hydrophobicity of the peptide and increase its penetration into the bilayer [49].

2.2. Hydrophobic thickness

An obvious and important property of a lipid bilayer is the thickness of the hydrophobic core of the bilayer, generally taken, for glycerophospholipids, to correspond to the separation between the glycerol backbone regions on the two sides of the bilayer. The hydrophobic thickness of the lipid bilayer is expected to match well the hydrophobic thickness of any protein embedded in the bilayer, because of the high cost of exposing either fatty acyl chains or hydrophobic amino acids to water. Any mismatch between the hydrophobic thicknesses of the lipid bilayer and the protein would be expected to lead to distortion of the lipid bilayer, or the protein, or both, to minimize the mismatch. In cases of extreme hydrophobic mismatch, it is possible that a membrane protein will be excluded from the lipid bilayer, as has been seen with simple model transmembrane α-helices [50]. Extreme mismatch could also lead to the formation of non-bilayer phases by the lipids, particularly at low molar ratios of lipid to protein [51,52].

Most models of hydrophobic mismatch assume that fatty acyl chains in the vicinity of a membrane protein adjust their length to match the hydrophobic thickness of the protein, the protein acting as a rigid body [53–55]. When the hydrophobic thickness of the bilayer is less than that of the protein, the lipid chains will stretch to provide a thicker bilayer. Conversely, when the hydrophobic thickness of the bilayer is greater than that of the protein, the lipid chains will compress to provide a thinner bilayer (Fig. 7). Fattal and Ben-Shaul [53] have estimated the deformation energy required to change the thickness of a lipid bilayer in this way. If it is assumed that all the lipid perturbation energy is concentrated in the annular shell of lipids around the protein, the equations of Fattal and Ben-Shaul [53] can be used to calculate the binding constant for a lipid that has to distort to bind to the protein, relative to that of a lipid that

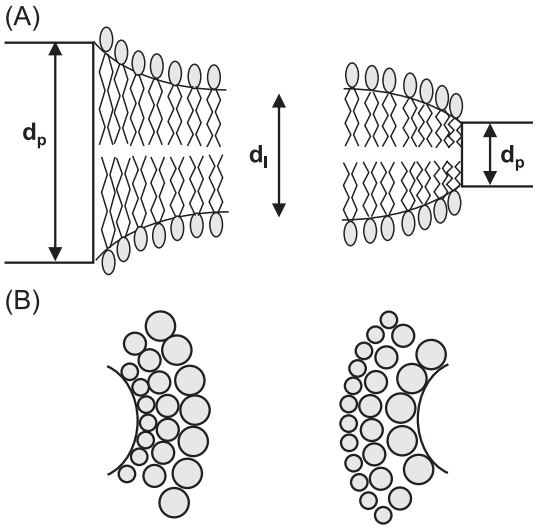

Fig. 7. Hydrophobic mismatch. The diagram shows how a lipid bilayer could distort around a membrane protein whose hydrophobic thickness is greater than that of the lipid bilayer (left; $d_p > d_l$) or less than that of the lipid bilayer (right; $d_p < d_l$). Panel (A) shows a side view of the membrane and panel (B) shows a view down onto the surface of the membrane. When the hydrophobic thickness of the protein is greater than the hydrophobic thickness of the bilayer, the lipid chains must be stretched so that the surface area occupied by a lipid molecule will be less in the vicinity of the protein than for bulk lipid. Conversely, to match a protein with a thin transmembrane region, the fatty acyl chains of neighbouring lipids will be compressed and will therefore occupy a greater surface area.

can bind without distortion [56,57]. For the β-barrel protein, OmpF, the observed variation in lipid binding constant over the chain length range C12 to C20 is close to that calculated using the theory of Fattal and Ben-Shaul [53], suggesting that β-barrel proteins are rigid in the sense that they distort only slightly in mismatched bilayers [56]. However, for α-helical membrane proteins, the theory predicts a much steeper dependence of relative binding constant on chain length than observed experimentally for the proteins KcsA, MscL and Ca^{2+}-ATPase [57–59]. For example, Fig. 8 compares the observed binding constants for MscL with the theoretical values [57]. The relatively small changes in the measured lipid binding constant with changing fatty acyl chain length suggests that the fatty acyl chains do not stretch or compress by as much as would be required to provide full hydrophobic matching. Thus, for example, the measured binding constant for di(C12:0)PC is that expected if the chains stretch by only ca. 40% of the amount required to provide full matching to the hydrophobic thickness of MscL. Interestingly, this estimate is very close to that made on the basis of molecular dynamics simulations of MscL in a bilayer with C12 chains [60]. As discussed elsewhere [7,50,61], binding constants for simple transmembrane α-helices also vary much less with changing fatty acyl chain length than expected if the lipid bilayer were to distort around the helix to provide hydrophobic matching. Indeed, this has now been confirmed by direct measurements of lipid bilayer thickness where no increase in thickness was detected on incorporation of a transmembrane α-helix into a thin bilayer [62].

Even though any distortion of the lipid bilayer around a membrane protein to provide hydrophobic matching appears to be rather small, the efficiency of hydrophobic matching between lipid bilayer and protein appears to be high. For example, experiments with the potassium channel KcsA have shown that the Trp residues at the ends of the transmembrane α-helices maintain their interfacial position when the fatty acyl chain lengths of the surrounding phospholipids are varied between C10 and C24 [59], suggesting highly efficient matching of the hydrophobic thickness of the protein to that of the lipid bilayer. This conclusion is in apparent contradiction with molecular dynamic simulations of MscL in thin bilayers which suggest that distortions on the protein in thin bilayers are very small, so that hydrophobic matching between MscL and a lipid bilayer with C12 chains is only ca. 50% complete [60]. However, it is possible that this is a consequence of the relatively short time scale of the simulation [60].

If distortion of the lipid bilayer does not provide full hydrophobic matching with the protein then the protein itself must distort to match the hydrophobic thickness of the lipid bilayer. Possible distortions of the membrane protein include changes in the tilt of the transmembrane α-helices and changes in the packing of the transmembrane α-helices. Distortion of the α-helical structure of the central core of a transmembrane α-helix is also possible in principle, but studies with model transmembrane α-helices suggest that this is rather unlikely in practice [63]. However, one form of distortion away from an ideal α-helical structure that might be possible is rotation of side chains about the Cα–Cβ bond linking the side chain to the polypeptide backbone; for a residue at the end of a helix such a rotation would change

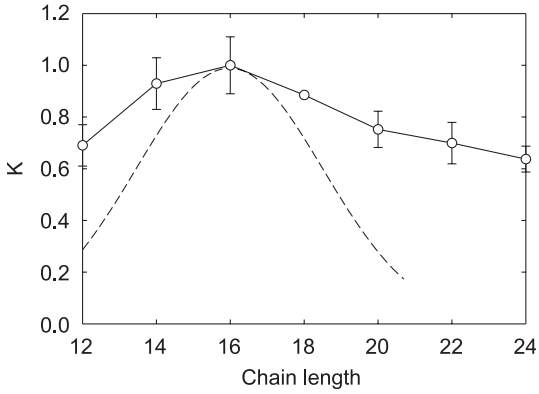

Fig. 8. The dependence of lipid binding constants on chain length for the mechanosensitive channel MscL. The chain length dependencies of the binding constants for phosphatidylcholines relative to di(C18:1)PC are plotted for MscL (○; solid line), scaled to a value of 1 for di(C16:1)PC. The dotted line shows the theoretical dependence of lipid binding constant on chain length calculated from the data of Fattal and Ben-Shaul [53] as described in Powl et al. [57], shifted along the chain length axis to match the experimental optimum binding at di(C16:1)PC.

the effective length of the helix. For example, rotation of a Tyr residue to lie roughly parallel to the long axis of a helix would extend the length of the helix by about 3 Å and rotation of the larger Trp residue would have an even larger effect.

Effects of hydrophobic matching on protein structure will be highly cooperative [7,44]. Although, as described above, lipid binding constants change little with changing fatty acyl chain length, small differences in the free energy of binding of a fraction of a kJ mol^{-1} for any one phospholipid molecule (which would not result in any detectable change in lipid binding constant) will become significant when summed over the large number of lipid molecules making contact with the protein in the membrane.

The strongest evidence that membrane proteins do distort significantly in response to hydrophobic mismatch comes from the observed changes in activity for a variety of membrane proteins with changes in fatty acyl chain length [58,64–70]. Many of the observed profiles of activity against membrane thickness are similar, with highest activity in phospholipids with a chain length of about C18, matching the average chain length of most biological membranes, with lipids with shorter or longer chains supporting lower activities. Fig. 9 shows the chain length dependencies of the activities of the Ca^{2+}-ATPase from sarcoplasmic reticulum and *E. coli* diacylglycerol kinase [7,67]. Despite their similar profiles, the reasons for the low activities of the two proteins in short and long chain lipids are, of course, very different. Low activities for the Ca^{2+}-ATPase follow from changes in the rates of phosphorylation and dephosphorylation, from changes in the rates of various conformation changes, and from changes in the stoichiometry of Ca^{2+} binding [44,65,71,72]; low activities for diacylglycerol kinase follow from changes in the affinity for substrate and changes in the maximal rate [67]. Clearly, there will be no universal relationship between hydrophobic mismatch and effects on enzyme activity; the effect of mismatch on activity will be unique for each particular membrane protein, depending on the change in structure resulting from the mismatch and on the effect that that particular change in structure has on activity.

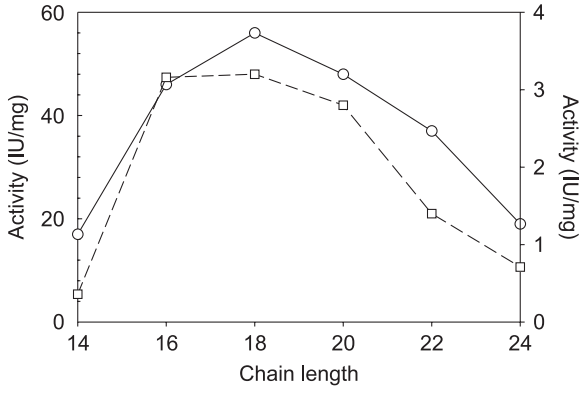

Fig. 9. The effect of fatty acyl chain length on enzyme activity in bilayers of phosphatidylcholine in the liquid crystalline phase. Ca^{2+}-ATPase (□; right-hand axis) or diacylglycerol kinase (○; left-hand axis) were reconstituted into phosphatidylcholines containing monounsaturated fatty acyl chains of the given chain lengths. ATPase activities were determined at 25 °C. For diacylglycerol kinase the substrate was dihexanoylglycerol [7,67].

The chain length dependence of the activity of the plasma membrane Na^+,K^+-ATPase is slightly different to that of the Ca^{2+}-ATPase shown in Fig. 9, with an optimum chain length of C22 in the absence of cholesterol but C18 in the presence of cholesterol [66]. This difference in chain length optimum could reflect the fact that the Na^+,K^+-ATPase is located in the plasma membrane where the presence of cholesterol will make the membrane thicker than other membranes in the cell. However, since the activity of the Na^+,K^+-ATPase in di(C18:1)PC in the presence of cholesterol is very considerably greater than that in di(C22:1)PC in the absence of cholesterol, cholesterol must have effects on the Na^+,K^+-ATPase additional to any effects following from the changes in bilayer thickness [66]. These additional effects could follow from binding to non-annular sites, as discussed in Section 3.

If a membrane protein can adopt more than one conformation, each with a different hydrophobic thickness, then a change in the thickness of the surrounding lipid bilayer would be expected to shift the equilibrium between the various conformations towards the one that best matches the hydrophobic thickness of the bilayer. For example, Ca^{2+}-ATPase is thought to exist in one of two major conformational states, denoted E1 and E2 [73]. Because the E1 conformation of the ATPase appears to be favoured in the short-chain lipid dimyristoleoylphosphatidylcholine [di(C14:1)PC] it was suggested that the hydrophobic thickness of the E1 conformation of the ATPase could be less than that of the E2 conformation [44]. However, crystal structures of the Ca^{2+}-ATPase in its Ca^{2+}-bound form (E1 conformation) and bound to the inhibitor thapsigargin (E2 conformation) provide no evidence for a major change in hydrophobic thickness between the two conformations [40], so that better hydrophobic matching for the E1 conformation in di(C14:1)PC is unlikely to be the explanation for the shift towards E1 in di(C14:1)PC. Similarly, the relative amounts of the two major intermediates of the rhodopsin photocycle MI and MII also depend on bilayer thickness [36]. The ratio of MII/MI increases slightly with increasing chain length in phosphatidylcholine bilayers over the chain length range C14 to C18 [36], as expected if the hydrophobic thickness of the MII intermediate were greater than that of the MI intermediate. However, although activation of rhodopsin involves movement of transmembrane α-helices, the extent of the motion is probably quite small [34] arguing against any major change in hydrophobic thickness for rhodopsin on formation of MII.

The possibility that hydrophobic mismatch results in protein aggregation is discussed in the following section.

2.3. Effects of lipid structure on protein aggregation and helix–helix interactions

A membrane protein could reduce the extent of unfavourable interactions with the surrounding lipid bilayer by aggregating to reduce the lipid-exposed surface area of the protein, but the extent to which this is possible will depend on the shape of the protein. If the extramembranous domains of the protein are small, protein aggregation will lead to contact between the transmembrane domains of the proteins, leading to displacement of lipid from the surface of the transmembrane domains. However, if the extramembranous domains of the protein are large, contact between the extramembranous domains would prevent the transmembrane domains from coming into contact [7]. A protein for which contact between transmembrane domains seems possible is bacteriorhodopsin, which has a cylindrical shape. Bacteriorhodopsin is largely monomeric in phosphatidylcholines in the liquid crystalline phase when the chain lengths are between C12 and C22 in length, but aggregates in bilayers of di(C10:0)PC or di(C24:1)PC [74]; it is not known whether or not this aggregation affects the function of bacteriorhodopsin.

The idea that protein aggregation could be the explanation for the effects of hydrophobic mismatch on protein function has been tested for the Ca^{2+}-ATPase [75]. Low activities were observed for the Ca^{2+}-ATPase in bilayers of short or long-chain phospholipids when the ATPase was reconstituted into sealed vesicles containing isolated, single ATPase molecules, where aggregation is not possible, so that, in this case at least, aggregation could not be responsible for the low activities observed [75]. Further, the large cytoplasmic domain on the Ca^{2+}-ATPase would prevent any extensive interaction between transmembrane domains of adjacent protein molecules.

Effects of lipid structure on the aggregation of transmembrane α-helices have been studied and interpreted in terms of the energetics of lipid–lipid interactions compared to lipid–helix and helix–helix interactions. The free energy of association of two transmembrane α-helices in a lipid bilayer, ΔG_a, can be written as

$$\Delta G_a = \Delta G_{HH} + n/2\Delta G_{LL} - n\Delta G_{HL} \quad (1)$$

where ΔG_{HH}, ΔG_{LL}, and ΔG_{HL} are the free energies of helix–helix, lipid–lipid and helix–lipid interactions, respectively, and it is assumed that formation of a helix–helix pair displaces n lipids from around the two helices [21,76]. Dimerisation of the helices could be driven by a favourable value for ΔG_{HH}, arising, for example, from salt bridge or hydrogen bonding interactions between the two helices. Good packing at the helix–helix interface with strong van der Waals' interactions could also contribute to a favourable value for ΔG_{HH}. Weak interactions between the polar headgroups of the lipids and the helices and poor packing between the lipid fatty acyl chains and the rough surface of the transmembrane α-helices would also drive dimerisation since ΔG_{HL} would then be unfavourable compared to ΔG_{HH} and ΔG_{LL}. Any decrease in motional freedom for the lipid fatty acyl chains due to the presence of the relatively rigid transmembrane α-helices will lead to a decrease in chain entropy, also leading to an unfavourable ΔG_{HL}.

The free energy of dimer formation by a pair of transmembrane α-helices in a lipid bilayer has been determined by measuring the quenching of the fluorescence of a Trp-containing helix by a dibromotyrosine-containing helix [77]. The free energy of dimerisation was found to increase with increasing fatty acyl chain length in bilayers of phosphatidylcholine, but to depend rather little on the length of the helix. In di(C18:1)PC the free energy of dimerisation was 8.4 kJ mol^{-1} [77]. As described by White and Wimley [21] this can be compared to the free energy cost of creating a void equivalent to a methyl group in the hydrophobic core of a soluble protein, which is about 6.7 kJ mol^{-1}. The free energy change favouring helix dimer formation in di(C18:1)PC is therefore that expected if helix–helix packing were more efficient than helix–lipid packing by an amount equivalent to the volume of about one methyl group. A comparison can also be made with the entropy change corresponding to disordering of the lipid fatty acyl chains at the gel to liquid crystalline phase transition [78], which corresponds to a free energy change of ca. 2.9 kJ mol^{-1} per carbon atom. The increase in free energy for dimer formation with increasing fatty acyl chain length is about 0.5 kJ per mole per carbon atom [77]. Thus a relatively small increase in chain order caused by the presence of the peptide could make a significant contribution to the free energy for oligomerisation of transmembrane α-helices. A chain-length dependence of the energy of helix–helix packing could be part of the explanation for the chain-length dependence of the activities of some membrane proteins discussed above; changes in the energies of helix–helix interactions as a result of changing phospholipid chain length could have significant effects on the packing of the transmembrane α-helices in a multi-helix protein and so affect activity. It has also been suggested that environmental effects on helix–helix interactions could mean that the packing of transmembrane α-helices observed in crystal structures of detergent solubilized proteins is slightly looser than the packing that would occur in a lipid bilayer [79].

2.4. Effects of the gel to liquid crystalline phase transition

The transition from the liquid crystalline to the gel phase results in a very marked change in the physical properties of a lipid bilayer, which might be expected to have significant effects on the activities of membrane proteins. Some membrane proteins such as the Ca^{2+}-ATPase do indeed show low activities in gel phase lipid, as described in Ref. [7]. The low activity observed for the Ca^{2+}-ATPase in gel phase lipid is not due to any aggregation of the Ca^{2+}-ATPase since low activities are seen for the Ca^{2+}-ATPase recon-

stituted at high dilution into sealed vesicles where the number of ATPase molecules per vesicle is close to one, so that aggregation is not possible [75]. Thus the effects of gel phase lipid follow directly from effects of the gel phase on the conformational state of the Ca^{2+}-ATPase [7,80].

For some membrane proteins, it is necessary to take into account the increase in bilayer thickness that occurs on transition into the gel phase; if the bilayer thickness in the liquid crystalline phase is less than optimum for the protein then the increase in bilayer thickness that occurs on transition into the gel phase will tend to lead to an increase in activity. For example, the activity of diacylglycerol kinase in di(C14:0)PC is comparable to that in di(C14:1)PC at all temperatures even though the activity in gel phase di(C16:0)PC is less than that in di(C16:1)PC at the same temperatures [45]. Similarly, the Na^+,K^+-ATPase has a very low activity in di(C18:0)PC in the gel phase but its activity in di(C14:0)PC is greater than that in di(C14:1)PC at all temperatures [66].

2.5. Effects of membrane viscosity

Proteins, in carrying out their functions, undergo changes in shape. Any molecule or part of a molecule moving in a liquid environment experiences some frictional drag, opposing the movement. In the simplest model, frictional forces can be pictured as arising from attractive interactions between molecules of the liquid; in order to push a solid object through the liquid, it is necessary to move some of the molecules of the liquid with respect to others. The resistance to motion through the liquid is expressed in terms of the viscosity η or its inverse, fluidity. In a lipid bilayer, the resistance to motion will come predominantly from the lipid fatty acyl chains. Molecular dynamics simulation suggests an effective viscosity of about 1 cP (1 P=0.1 Pa s) for chain motion in a liquid crystalline bilayer, a value comparable to that for a simple alkane [81,82].

The balance between the frictional forces and the mechanical restoring forces acting on a group will determine the type of motion adopted by the group. Underdamped motion in which a group vibrates about its equilibrium position occurs when frictional forces are low. High frictional forces give rise to overdamped motion, where a group returns, after displacement, to its equilibrium position without vibrating. Vibrations of covalent bonds will be underdamped because of the large restoring forces together with the small frictional effects that follow from the small size of the structural unit. Large-scale motions in a protein, such as hinge-bending motions, will be overdamped because of the large surface displacements involved and the consequent strong frictional effects of the solvent.

Over short time periods ($<10^{-12}$ s) small amplitude motions in a protein are similar to motions in a simple liquid. Each group is temporarily trapped, rattling in a cage made up of other neighbouring groups in the protein and, at the surface of the protein, of molecules of the surrounding solvent [83]. Frequent collisions of the cage atoms with the encaged group rapidly randomise its motion. Such collisions are the microscopic basis of the frictional forces that limit the rate of movement of the group. It is therefore important to understand the relative importance of the solvent and of neighbouring groups on the protein in making up the cage. In a study of the rotation of Trp residues in small proteins it was concluded that, at low temperatures, small fast motions of Trp residues were dominated by the frictional resistance of the solvent [84]. However, as the temperature was increased, the amplitude of motion increased, until the amplitude was such that further increases became limited by the local peptide environment. When motion is limited by the local peptide environment, the peptide can be considered to be effectively unsolvated. The small fast motions of Trp residues appeared to be strongly dependent on viscosity only for solvent viscosities of the order of several poises. At viscosities of the order of 1 cP, the amplitude of the local motions was largely determined by neighbouring groups on the peptide, which thus made up the effective ‘solvent cage’ [84]. Molecular dynamics simulations give a similar picture. Brooks and Karplus [85,86] concluded that for a solvent to have a significant effect on the motion of a particular group in a protein, not only must the group be in direct contact with the solvent (i.e., be exposed on the surface of the protein) but also the rate of motion of the group must be comparable to the rate of motion of the solvent (they must be dynamically coupled). Thus small amplitude, high-frequency motions are independent of solvent, whether or not the group is on the surface of the protein, because their frequency is too high to couple with solvent motion.

These expectations largely agree with experimental data on the model ion channel gramicidin. Gramicidin dimerises in a lipid bilayer to form a channel with a $\beta^{6.3}$ helix structure, spanning the bilayer with all its side chains in contact with the lipid bilayer [87]. In an informative experiment, the fluorescence polarization of dansyl-labelled gramicidin was compared, as a function of temperature, with that of a fluorescently labelled fatty acid [88]. The results suggest that motion of the dansyl group in dansyl-labelled gramicidin is extensively coupled to motion of the peptide in the liquid crystalline phase; the lipid environment is, therefore, of relatively low effective viscosity, allowing underdamped motion of the dansyl group and considerable interaction with other groups in the peptide. Extensive coupling between the dansyl group and the peptide was detected even in gel phase lipid; membrane viscosity seems to play little part in the control of side chain dynamics. This is consistent with the results of molecular dynamics simulations that suggest that the frequency of side-chain conformational transitions for the Val and Leu residues in gramicidin are the same in the presence or absence of a surrounding lipid bilayer [89]. Similar conclusions were drawn from NMR studies of the rates of motion of particular side chains in gramicidin. Thus of the valine residues in gramicidin, Val-1 and Val-7

were free to undergo restricted motion in gel phase bilayers and large amplitude motions in liquid crystalline phase bilayers. In contrast, Val-6 and Val-8 are immobile, confined to a single conformational state [90]. In the channel structure Val-6 makes van der Waals' contact with Trp-13 and Val-8 makes van der Waals' contact with both Trp-9 and Trp-15; packing of the Val residues next to the Trp residues could be the explanation for their restricted motion. Of the Trp residues, Trp-9 and Trp-15 have the smallest amplitude of motion, suggesting that these two rings could be stacked against each other [91]. Thus there is no gradient of motion for the side chains of the peptide going from the bilayer surface to the bilayer centre to correspond to the well-defined motional gradient in the lipid fatty acyl chains. A dynamic phase boundary has been said to exist between the peptide and the lipid [90].

A description of any effects of solvent viscosity on the rates of the conformational changes in a protein requires an extension of conventional transition state theory, since conventional transition state theory does not include effects of viscosity. In transition state theory, a particular molecule going from A to B crosses the barrier only once before being trapped for a long time in state B (Fig. 10); at some later time, the molecule might revert from state B back to state A, but this would then be a completely unconnected event. The rate of the transition from A to B (k_{AB}) depends on the height of the energy barrier between A and B (ΔH^*_{AB} in Fig. 10), the rate decreasing with an increase in the size of the energy barrier [92,93]. In those cases where the viscosity of the surrounding solvent has no significant on the rate of the transition, traditional friction-independent transition state theory describes the system very well. However, for those systems where the viscosity of the surrounding solvent does affect the rate of the transition, some way has to be found to incorporate effects of friction into the theory. One approach is that of Kramers [94] who introduced frictional effects by assuming that when the system is poised at the transition state and going from A to B, it will not necessarily get there, because the direction of motion may change to go back to A. Thus, even when the molecule has sufficient energy to overcome the barrier, it will not do so in one attempt. The frictional forces exerted by the environment will cause the molecule to undergo Brownian motion to-and-fro over the barrier. As described by Frauenfelder and Wolynes [95], in a typical reaction path, the reactants do not go directly from one side of the energy barrier to the other, but rather may cross the transition region many times, tottering back and forth before reacting. To illustrate the viscosity dependence of the activation energy, the potential energy diagram for the reaction can be redrawn as in Fig. 10, where E_η is the extra barrier imposed by viscosity; this formulation takes account of both the internal rearrangement of the molecules necessary to form the transition state and the need to move solvent molecules to allow the required rearrangement of the reacting molecules.

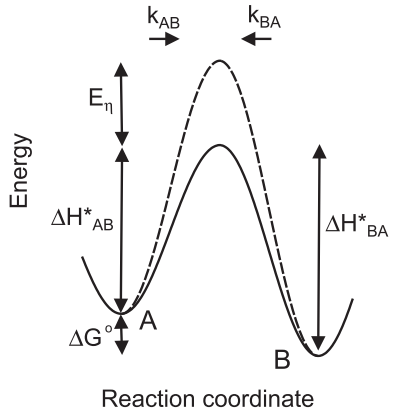

Fig. 10. The effect of an increase in viscosity on the free energy barrier over which a system has to go from A to B. The intrinsic energy barriers for the reaction in the forward and backward directions are ΔH_{AB}* and ΔH_{BA}*, respectively. The free energy difference between A and B is given by ΔG^o. The pathway between A and B in the absence of any viscosity effects is shown by the solid line. The effect of viscosity is to add an extra component E_η to the energy barrier, reducing rates in both the forward and backward direction, with no effect on ΔG^o, as shown by the broken line.

An important point that needs to be made about viscosity is that viscosity cannot affect any equilibrium property of a system [92,93]. This follows from the time-independence of thermodynamic quantities. Increasing the viscosity of the medium will increase the activation energy in both the forward and the back directions, decreasing the forward and the back rates equally (Fig. 10). Since the equilibrium constant is equal to the ratio of the forward and backward rate constants

$$K_{equil} = k_{AB}/k_{BA} \tag{2}$$

this means that the equilibrium constant is unaffected by viscosity. Since ΔG^o is related to the equilibrium constant by

$$\Delta G^o = -RT \ln K_{equil} \tag{3}$$

ΔG^o is also unaffected by viscosity and, indeed, as shown in Fig. 10, changing viscosity does not change the relative energies of A and B. This provides a simple way to distinguish between dynamic (viscosity) and static (equilibrium) effects of a solvent. Static effects of a solvent occur when solvent interacts preferentially with either the initial or final state changing the energy of state A relative to that of B and so changing the equilibrium constant, whereas dynamic effects leave the equilibrium constant unchanged.

Experimental studies of the effects of membrane viscosity on the activities of membrane proteins are difficult because membrane viscosity can only be changed at a constant temperature by changing the composition of the membrane and these changes in composition could directly affect the properties of the proteins in the membrane through changes in lipid–protein interaction.

Any direct, static effects of lipid composition need to be sorted out before any possible dynamic influences of lipid on rates can be clearly revealed. At one time, it was common to expect that changes in membrane viscosity would have significant effects on the activities of membrane proteins. One form of this idea was 'homeoviscous adaptation', the idea that the exact viscosity of the lipid component of the membrane was held constant for optimal functioning of the membrane, organisms altering the lipid compositions of their membranes to maintain a constant membrane viscosity [96]. Whilst it is undoubtedly true that many organisms change the lipids in their membranes in response to a change in temperature in such a way as to reduce the effects of the temperature change on the viscosity of their membranes, it would not be true, in general, to say that this actually maintains a constant viscosity in the membrane [97].

Studies of the effects of phospholipid composition on the activities of many membrane proteins in reconstituted systems suggests that changes in viscosity are unlikely to be a significant factor in determining activity. For example, changing the phospholipid composition around the Ca^{2+}-ATPase changes the equilibrium constant between the E1 and E2 conformations of the protein and changes the stoichiometry of Ca^{2+} binding to the protein [44], and changing the phospholipid composition around rhodopsin changes the pK value for the ionisation controlling MI/MII ratio [36]. These changes, being changes in equilibrium properties, cannot follow from changes in viscosity.

2.6. Effects of changes in membrane protein volume

A possible response to an unfavourable interaction between a membrane protein and the surrounding lipid bilayer (poor solvation of the protein by the lipid) would be for the protein to reduce the diameter of its transmembrane domain and so minimize its lipid-exposed surface area. Effects of lipid solvation on the diameters of transmembrane domains have been interpreted in terms of the surface tension at the lipid–protein interface [68]. Effects of surface tension were first described for liquid–air interfaces [98], but the same arguments apply in principle for any interface, including the lipid–protein interface. Surface tension arises at an interface because of the tendency of molecules in a liquid to stick together. When a molecule is on the surface, it experiences a force which tends to pull it back into the bulk phase, because it has more neighbouring liquid molecules in the bulk phase than it does at the interface. The force is attractive, and is referred to as a tension, the surface tension. Increasing the area of the surface involves doing work against the cohesive forces in the liquid, that is, against the surface tension. A tension is a negative pressure, and so an alternative description of surface tension is in terms of a surface pressure. To relate these effects to membrane proteins, we can consider a solid cylinder (the protein) embedded in a liquid (the lipid bilayer). The work done, dW, in forming a surface of area A is proportional to the area of the surface formed so that

$$\mathrm{d}W = \gamma A \tag{4}$$

where γ is the surface tension. For a cylinder of radius r and height h, the total surface free energy will be $2\pi rh\gamma$. If the radius of the cylinder were to decrease by δr, the surface free energy would decrease by $-2\pi h\gamma\delta r$. The tendency of the cylinder to lower its surface free energy by shrinking is counterbalanced by an excess pressure inside the cylinder as compared to outside. If the radius of the cylinder decreases by δr, then the volume will decrease by $2\pi rh\delta r$. The increase in free energy $P\Delta V$ of the cylinder is therefore $\Delta P(2\pi rh\delta r)$ where ΔP is the change in pressure. At equilibrium, the two changes in free energy must add to zero, so that

$$2\pi h\gamma\delta r = \Delta P 2\pi rh\delta r \tag{5}$$

and so

$$\Delta P = \gamma/r \tag{6}$$

The interface between a membrane protein and the lipid bilayer can be characterised in terms of the interfacial surface tension γ_{PL} [68]. The pressure within the protein, P_P, is then given by

$$P_P = P_L + \gamma_{PL}/r \tag{7}$$

where P_L is the pressure within the lipid bilayer. The small radius of curvature of a membrane protein means that quite small interfacial tensions produce very large effective pressures on protein molecules. Thus for a protein of radius 20 Å, a surface tension of 10 dyn/cm, typical of organic liquid interfaces, will produce a pressure of 50 atm. Poor solvation of a protein will lead to a high surface tension γ_{PL} and thus to a high internal protein pressure, shifting the equilibrium in favour of the conformation of the protein with smallest volume. If the two conformations differ in radius by δr, then the free energy difference ΔG^o between the two conformations becomes

$$\Delta G^o = \Delta G^{o\prime} + 2N_o\pi h\gamma_{PL}\delta r \tag{8}$$

where $\Delta G^{o\prime}$ is the free energy difference when γ_{PL} is 0. For example, a change in radius of 2 Å for a protein of height h=50 Å, with a surface tension γ_{PL} of 10 dyn/cm, would correspond to a change in free energy of 38 kJ mol^{-1}, sufficient to bring about a significant change in the conformation of a membrane protein. A change in radius of 2 Å would correspond to an increase in circumference equivalent to about four lipid molecules in the bilayer around the protein. It is not yet possible to say whether or not changes in radius of this magnitude are likely. Attwood and Gutfreund [99] estimated an increase in volume on formation of the MII state of rhodopsin of 179 $Å^3$ and Klink et al. [100] estimated a volume change of no more than 200 $Å^3$ between the various intermediates of bacteriorhodopsin. These measured changes in volume will include contribu-

tions from changes in solvation, but even if all the measured changes in volume were due to changes in volume of the protein they would corresponding to an increase in radius for the protein of only ca. 0.03 Å. A change in radius of this magnitude would be too small to lead to any significant effects of solvation on the rhodopsin MII to MI transition. Similarly, comparing the crystal structures of the Ca^{2+}-ATPase in the E1 and E2 conformations suggests that any difference between the circumference of the transmembrane domain of the protein in the two conformations is very small [40].

2.7. Effects of lipid free volume

Litman and Mitchell [35] related effects of lipid structure on the MI/MII ratio in rhodopsin to the free volume present in the lipid bilayer. Voids or pockets of 'free volume' are present within a bilayer because of the low order of the fatty acyl chains and their low packing density. The free volume V_f (per mole) present in a bilayer can be defined as:

$$V_f = V_T - V_{VDW} \quad (9)$$

where V_T is the measured volume and V_{VDW} is the volume calculated from the van der Waals' dimensions of the lipid. The amount of free volume in a liquid can be very significant. For example, in liquid hexadecane at 20 °C, the free volume makes up 42% of the total volume (see Ref. [101]). Similarly, a comparison of the volumes occupied by chain methylene and terminal methyl groups in a bilayer of di(C16:0)PC in the liquid crystalline phase (28.7 and 54.6 $Å^3$, respectively) with the van der Waals' volumes for these groups (21.7 and 32.9 $Å^3$, respectively) emphasises the extent of the free volume present in a lipid bilayer [102]. As a measure of the free volume present in a lipid bilayer, Straume and Litman [103] introduced a parameter f_V referred to as the fractional volume, derived from the dynamic fluorescence anisotropy properties of the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Increasing disorder in the lipid bilayer, leading to an increased free volume, results in an increased range of motion for the molecules of DPH in the bilayer and so to an increase in the parameter f_V; f_V gives a measure of the cohesiveness of the phospholipids in the bilayer. Mitchell et al. [104] observed a correlation between the MI/MII ratio in egg phosphatidylcholine/cholesterol systems and f_V, which held whether the system was modified by addition of cholesterol or by changing temperature. However, when a wider range of phospholipids was studied, the correlation broke down, although for each particular phospholipid, a correlation was observed between f_V and the ratio MI/MII in that phospholipid [37]. The molecular basis for the observed correlation was suggested to be an increase in molecular volume for the protein at the MI to MII transition, the increase in volume being favoured by a bilayer with a large free volume [35]. The model is therefore related to that described in Section 2.6 and depends on there being a significant change in volume between different conformational states of the protein.

2.8. Effects of interfacial curvature and elastic strain

Most, if not all, biological membranes contain lipids that, in isolation, prefer to adopt a curved, hexagonal H_{II} phase rather than the normal, planar, bilayer phase [105,106] and it has been suggested that bacteria control the lipid compositions of their membranes to maintain a constant proportion of lipids favouring the hexagonal H_{II} phase (for a recent review, see Ref. [107]). The effects of lipids favouring the hexagonal H_{II} phase have been tested on some membrane proteins. For the Ca^{2+}-ATPase and for *E. coli* diacylglycerol kinase, the presence of phosphatidylethanolamine, a lipid favouring the hexagonal H_{II} phase, leads to decreased activity [43,45]. However, for rhodopsin, the presence of a lipid favouring the hexagonal H_{II} phase is required for proper function [36]. The highest level of the MII intermediate is seen when rhodopsin is reconstituted into mixtures of phosphatidylcholine and phosphatidylethanolamine containing C22:6 chains [108] but MI/MII ratios equal to those in the native membrane are seen in mixtures of di(C18:1)PC and di(C18:1)PE if the di(C18:1)PE content is increased from the value of about 40% characteristic of the native membrane, to about 75% [38,108]. The presence of a lipid such as a phosphatidylethanolamine will lead to significant changes in the lipid headgroup region, as described in Section 2.1, which could be the explanation for the observed effects. However, an alternative explanation for the effects of these lipids has been developed, based on the fact that, in a membrane, phosphatidylethanolamine will be forced to adopt a bilayer structure rather than its preferred non-bilayer structure, as will now be described.

Mixtures of two lipids, one preferring the bilayer phase and one the hexagonal H_{II} phase, adopt a bilayer phase if the mixture contains more than about 20 mol% of the bilayer-preferring lipid [109]. The presence of an intrinsic membrane protein also has a strong tendency to force a bilayer phase onto phospholipids preferring the hexagonal H_{II} phase. For example, the presence of cytochrome *c* oxidase causes cardiolipin to adopt a bilayer structure under conditions (presence of Ca^{2+}) where normally it would adopt a hexagonal H_{II} phase [110]. The effect of glycophorin on di(C18:1)PE is also to force it into a bilayer structure [111]. A major protein of the thylakoid membrane, the light harvesting complex (LHC-II) of Photosystem II, also called the chlorophyll *a*/*b* protein, forces the hexagonal H_{II}-favouring lipid of the thylakoid membrane, monogalactosyldiacylglycerol (MGDG), to adopt a bilayer structure [112]. It is therefore assumed that the lipid molecules surrounding an intrinsic membrane protein in a membrane will all be in the bilayer phase, even when the lipid molecules would, in isolation, adopt a hexagonal H_{II} phase. The fact that phospholipids preferring to adopt a curved structure are forced to adopt a planar structure has been said

to result in a state of 'curvature frustration' for these lipids and it has been suggested that this could be important for the proper function of the membrane [113].

The tendency of phospholipids such as phosphatidylethanolamines to adopt a curved structure can be understood in terms of the forces present in a lipid bilayer [113–115]. The stability of a lipid bilayer is determined largely by the balance between the hydrophobic interactions, which tend to decrease the interfacial area between the lipids and water, and the inter- and intramolecular interactions, including headgroup hydration, which give rise to a net repulsion, tending to increase the surface area of the bilayer [116]. Thus a cohesive hydrophobic tension at the polar–apolar interface is balanced by a repulsive two-dimensional lateral pressure due to repulsion of chains and headgroups. The lateral stresses responsible have been illustrated by Seddon [114] as in Fig. 11. At about the position of the glycerol backbone region, just below the lipid headgroups, an attractive force F_γ arises from the unfavourable contact of the hydrocarbon chains with water (the hydrophobic effect). Tight packing in this region ensures the minimum exposure of the hydrocarbon interior of the membrane to water, leading to a negative lateral pressure (a positive membrane tension), tending to contract the bilayer. A positive lateral pressure F_h arises in the headgroup region because of steric, hydrational and electrostatic effects; these will normally be repulsive but may contain attractive contributions from, for example, hydrogen bonding interactions. Similarly, in the hydrocarbon interior of the membrane, attractive van der Waals' interactions between the chains will be opposed by the repulsive interactions due to the thermal motions of the chains, the net effect being a positive lateral pressure F_c tending to expand the membrane.

For a lipid monolayer to stay flat, the pressures illustrated in Fig. 11 must be in balance across the monolayer. If the lateral pressure in the chain region becomes greater than that between the headgroups, the monolayer will curl towards the aqueous region (Fig. 11). This is defined as a negative curvature (the direction of monolayer curvature is defined from the point of view of an observer in the fatty acyl chain region of the bilayer looking out towards the lipid headgroup region; a concave curvature for the monolayer is defined as being positive and a convex curvature is defined as being negative, as shown in Fig. 11). Conversely, if the lateral pressure between the headgroups becomes greater than that between the chains, the monolayer will curl towards the chain regions, a positive curvature (Fig. 11). The tendency to curl becomes frustrated in a lipid bilayer. In a symmetrical bilayer (with identical conditions on each side) the two monolayers will both want to curve in the same way (either positive or negative) and so will counteract each other; the two monolayers cannot both curve in the same direction since this would create free volume in the interior of the bilayer. Thus the bilayer has to remain flat, in a state of physical frustration. Confining a monolayer with a nonzero spontaneous curvature to a planar form results in an elastic free energy stored in the bilayer—it is analogous to bending a curved rubber into a planar sheet. If the stresses in the bilayer become too great, the bilayer structure will become unstable and a non-bilayer phase will form.

Attard et al. [117] have estimated the stored curvature elastic energy for di(C18:1)PE in a flat monolayer to be ca.

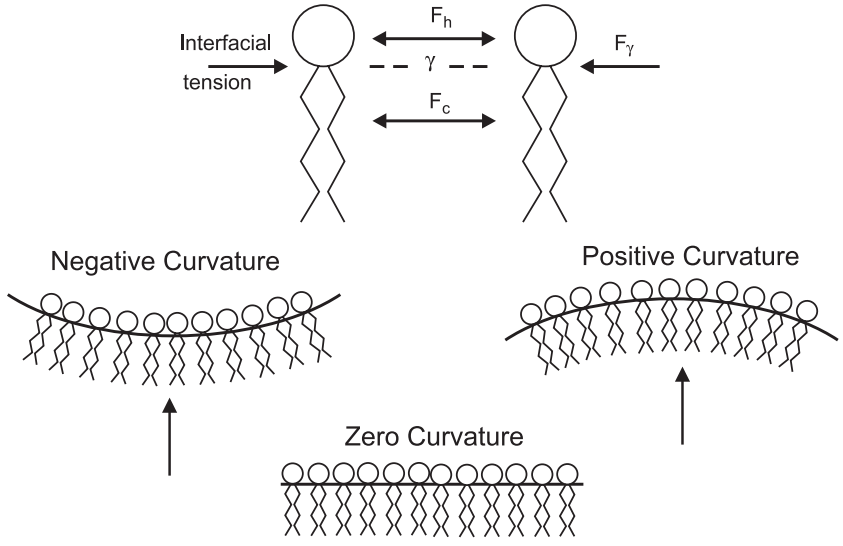

Fig. 11. Spontaneous curvature depends on interactions in a lipid bilayer. The distribution of lateral pressures and tensions across a lipid monolayer is shown at the top of the figure. The repulsive lateral pressure F_c in the chain region is due to thermally activated bond rotational motion. The interfacial tension γ, tending to minimize the interfacial area, arises from the hydrophobic effect (unfavourable hydrocarbon-water contacts). Finally, the lateral pressure F_h in the headgroup region arises from steric, hydrational and electrostatic effects; it is normally repulsive, but may contain attractive contributions from, for example, hydrogen bonding interactions (after Ref. [114]). Below, the tendency for spontaneous curvature of a lipid monolayer arising from an imbalance in the distribution of lateral forces across the monolayer is shown. The arrows show the direction of observation used in the definition of negative and positive curvature.

2.5 kJ mol^{-1}. Insertion of a protein into the lipid headgroup region could release some of this stored curvature elastic energy. For example, the fatty acyl chains of phospholipid molecules adjacent to a helix located in the headgroup region of the bilayer will have to splay out to fill the space below the helix (Fig. 12) and a phospholipid such as di(C18:1)PE with a large chain area but small headgroup area is ideally suited to do this; insertion of a protein into the headgroup region of a bilayer containing di(C18:12)PE will release some of the stored curvature elastic energy. Attard et al. [117] have suggested that release of stored curvature elastic energy contributes significantly to the binding of the extrinsic membrane protein CTP:phosphocholine cytidylyltransferase to bilayers containing phospholipids favouring the hexagonal H_{II} phase. Nevertheless, the chemical structure of the headgroup region could also be important since the membrane binding domain of CTP:phosphocholine cytidylyltransferase adopts a mixed structure in water and only adopts its final α-helical structure after insertion into the headgroup region [118].

Brown [36,38] has suggested a mechanism by which stored curvature elastic energy could be linked to a mismatch between the hydrophobic thicknesses of a membrane protein and the surrounding lipid bilayer. As described in Section 2.2, one possible response of a system when the hydrophobic thickness of a protein is greater than that of the surrounding lipid bilayer is for the fatty acyl chains of the lipids around the protein to stretch, to provide hydrophobic matching (Fig. 7). The lipids around the protein will show negative curvature. For phospholipids that favour a planar bilayer structure this will be unfavourable but formation of a membrane with negative curvature will be favourable for phospholipids such as phosphatidylethanolamine. Thus if a membrane protein can adopt two conformational states in the first of which its hydrophobic thickness matches that of the planar bilayer but in the second of which its hydrophobic thickness is greater than that of the planar bilayer, then the presence of phosphatidylethanolamine will favour the thicker form (Fig. 13). An effect of this type could explain why phosphatidylethanolamine favours the MII conformation of rhodopsin, if the hydrophobic thickness of MII is greater than that of MI [36,38]. As described in Section 2.2, the fact that the ratio of MII to MI increases slightly with increasing chain length in phosphatidylcholine bilayers over the chain length range C14 to C18 [36] would be consistent with a greater hydrophobic thickness for MII than MI although the changes in helix packing during the MI to MII transition seem unlikely to result in any major change in hydrophobic thickness. Salamon et al. [157] have estimated from surface plasmon resonance experiments that the average thickness of the rhodopsin membrane increases by ca. 4 Å on photoactivation.

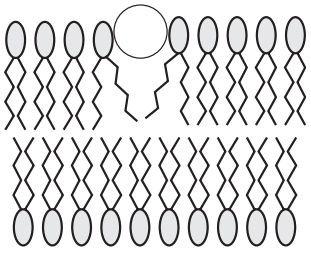

Fig. 12. Binding of an α-helix in the headgroup region of a lipid bilayer can create free volume in the fatty acyl chain region below the helix, and the fatty acyl chains of neighbouring phospholipids will need to distort to fill this free volume. Adapted from Attard et al. [117].

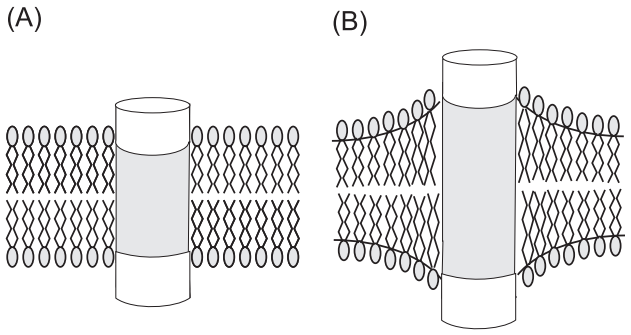

Fig. 13. Coupling of the spontaneous curvature of a lipid bilayer to conformational changes in a membrane protein. In panel (A) the hydrophobic thickness of the protein matches that of the lipid bilayer. In panel (B) the hydrophobic thickness of the protein is greater than that of the lipid bilayer, leading to stretching of the lipids in the vicinity of the protein, this being favoured by non-bilayer forming lipids with negative spontaneous curvature. Adapted from Botelho et al. [38].

Curvature frustration has also been suggested to be important in channel formation by gramicidin [119]. In a too-thick bilayer, dimer formation by gramicidin will only occur if the lipid bilayer can thin around the dimer, resulting in positive curvature for the two monolayers making up the bilayer. Any change in the lipid structure favouring a negative monolayer equilibrium curvature (increasing the propensity to form the hexagonal H_{II} phase) will make dimer formation less favourable. Lundbaek et al. [119] have shown just such an effect in membranes containing phosphatidylserine; addition of Ca^{2+}, or any other change that reduces electrostatic repulsion between the lipid headgroups, decreases channel formation, as expected since these changes will lead to negative monolayer curvature. Effects of lysophospholipids on dimer formation by gramicidin are also consistent with effects mediated by curvature frustration, the larger the headgroup the larger being the effect [120].

2.9. Effects of lateral pressure profile

As described in the previous section, lateral pressures in the headgroup and chain regions of a lipid bilayer must balance to give a stable planar structure. However, the distribution of lateral pressures within the chain region is not uniform [115]. The lateral pressure in a bilayer with simple saturated chains shows a maximum in the centre of the

bilayer and at a position roughly half way up the fatty acyl chains [115]. It has been suggested that this profile of lateral pressures will be very sensitive to changes in the structures of the fatty acyl chains, the presence of *cis* double bonds, for example, having a large effect on the pressure profile [121]. If the shape of the transmembrane region of a membrane protein were to change significantly during a conformational change, for example, changing in width at one depth in the bilayer more than at another, then the conformational change would be affected by the lateral pressure profile within the bilayer. Anything that changed the lateral pressure profile would then affect the conformational equilibrium of the protein; the lateral pressure profile would be an important determinant of protein function [122]. There appears to be little experimental data concerning this possibility. However, experiments in which the Ca^{2+}-ATPase was reconstituted into a series of phosphatidylcholines containing fatty acyl chains of the same length but containing different numbers of *cis* double bonds showed no dependence of activity on chain structure, as long as the chain length remained constant [123], in disagreement with a role for the lateral pressure profile, at least for the Ca^{2+}-ATPase. Further, most changes in structure in the transmembrane domains of membrane proteins appear at present to be rather small and global, resulting from rotation, tilting, and translation of transmembrane α-helices. If localised changes in structure in the transmembrane domains of membrane proteins turn out to be rare, as seems likely, then changes in lateral pressure profile are unlikely to be important for membrane protein function.

2.10. Effects of membrane tension: mechanosensitive ion channels and osmoregulated transporters

One of the most fundamental of homeostatic processes in prokaryotic organisms is regulation of the balance between the internal and external osmotic forces across the cytoplasmic membrane. Exposure of bacterial cells to a medium of low osmotic pressure leads to an influx of water into the cell, to cell expansion, and to an increase in tension within the cell membrane that would result in cell lysis unless the osmotic gradient is reduced in some way. For this reason bacteria (Gram-negative, Gram-positive, and Archaea) contain mechanosensitive channels that, when activated by stretching of the membrane, allow a rapid and non-selective flux of solute out of the cell, thus preventing lysis of the cell. The most studied of the mechanosensitive channels is MscL, the mechanosensitive channel of large conductance [124]. The trigger for opening MscL is a change in the physical properties of the surrounding lipid bilayer since an increase in membrane tension applied by negative pressure in a patch clamp pipette opens MscL in simple reconstituted membrane systems containing MscL as the only protein [124]; the lipid bilayer is an essential part of the gating mechanism of the channel. The opening of other ion channels, such as the two-pore domain K^+ channels TREK and TRAAK is also induced by increased tension in the membrane [125].

Because lipid bilayers have a very low compressibility, the volume of the bilayer will not change significantly when the pressure across the bilayer is increased; an osmotic downshift will therefore lead to an increase in membrane area and a decrease in membrane thickness [124]. The relative area expansion of the bilayer is related linearly to the membrane tension t

$$t = K_A \Delta A / A_0 \tag{10}$$

where ΔA is the increase in surface area, A_0 is the original area and K_A is the area expansion modulus [124]. Lipid bilayers have a relatively high resistance to area expansion because separation of the lipid headgroups exposes the hydrophobic core to water; typical values of K_A are between 10^2 and 10^3 mN/m, depending on the lipid composition. The lytic tension range for a lipid bilayer is ca. 3–30 mN/m so that a bilayer can be expanded by only about 2–4% before rupture. The result is that, at near lytic tensions, the area of the bilayer will increase by ca. 4%, and the bilayer thickness will decrease by almost as much. For a membrane that is 30 Å thick, a 2–4% change in thickness would represent a decrease in thickness of ca. 1 Å. The membrane tension required to open MscL is close to this lytic tension [124].

The free energy of the MscL channel is a linear function of membrane tension t

$$\Delta G = t\Delta A - \Delta G^o \tag{11}$$

where ΔG^o is the difference in free energy between the closed and open forms in the absence of an externally applied membrane tension and ΔA is the difference in membrane area occupied by open and closed channels at a given membrane tension; $t\Delta A$ is the work required to keep the channel open. The mechanosensitivity of the channel is then determined by ΔA; the larger the change in area the more sensitive will be the channel to changes in membrane tension. It has been estimated that the change in cross-sectional area of the MscL channel on opening (ΔA) is 6.5 nm^2 [126,127].

A high-resolution structure is available for MscL from *Mycobacterium tuberculosis* in the closed state [128]. MscL is a homopentamer, each subunit containing two transmembrane α-helices, TM1 and TM2, and a cytoplasmic helix. The five TM1 helices form an inner helical bundle with the five TM2 helices forming a peripheral skirt that contacts the lipid molecules. A large loop between TM1 and TM2 extends into the central pore and may contribute to the properties of the channel. TM1 and TM2 are tilted with respect to the membrane, coming together at their C-terminal ends to block the channel. Opening the channel probably involves an iris-like expansion of the pore with a tilting of the TM1 helices, and several models have been presented for this process [129–131].

Maurer and Dougherty [132] found that loss of function mutants of MscL (mutants that could not be gated or that

required more tension than the wild-type channel to gate) are concentrated in regions of the protein near the headgroups of the lipid bilayer on both surfaces of the membrane. This suggests that interactions involving the lipid headgroup region of the bilayer could be responsible for tension sensing in MscL. A molecular dynamics simulation of MscL from *T. tuberculosis* in bilayers of (C16:0,C18:1)PE showed a large number of hydrogen bonds between the lipid molecules and MscL, about half involving the NH_3^+ group of the phosphatidylethanolamine headgroup [133]. Other lipid headgroups such as phosphatidylcholine and phosphatidylglycerol do not have a hydrogen bond donating group analogous to the NH_3^+ of phosphatidylethanolamine and so show a very different pattern of hydrogen bonding. A molecular dynamics simulation of MscL in bilayers of (C16:0,C18:1)PC suggests that the loss of hydrogen bonding observed on replacement of the phosphatidylethanolamine headgroup by the phosphatidylcholine headgroup is compensated for by a conformational change in the C-terminal region of the protein, bringing the C-terminal region closer to the membrane, leading to stronger interactions with the membrane [60]. This compensating conformation change could be the reason why binding constants of MscL for di(C18:1)PE and di(C18:1)PC are very similar [57]. It is also interesting that the gating tension for MscL from *T. tuberculosis* expressed in *E. coli* spheroplasts is higher than that for the MscL from *E. coli* itself [124]. This could be related to the fact that the cytoplasmic membrane of *T. tuberculosis* is rich in cardiolipin and phosphatidylinositol-mannosides [134], whereas that of *E. coli* is rich in phosphatidylethanolamine.

MscL from *E. coli* (EcMscL) has been reconstituted into a series of phosphatidylcholines of different chain lengths [135]. In di(C16:1)PC, EcMscL opened with significantly lower activation threshold than in di(C18:1)PC, whereas in di(C20:1)PC the threshold was higher, so that thin bilayers favour channel opening and thick bilayers stabilize the closed form [135]. This would be expected if the transmembrane α-helices in the open state were more tilted towards the plane of the membrane than in the closed form. The results of spin-labelling of Cys residues in TM1 in the narrowest section of the permeation pathway are consistent with changes in conformation for MscL with changing bilayer thickness, but these conformational changes appear not to correspond to a change to the fully open state; it was suggested that thin bilayers could stabilize one or more of the several closed states that lead to the fully open state rather than stabilizing the actual fully opened state [135].

Interestingly, the response to hyperosmotic stress is very different to that just described for a hypoosmotic stress. Bacteria counteract hyperosmotic stress by accumulating compatible solutes by uptake and/or synthesis, including amino acids, polyols, and quaternary ammonium compounds such as glycine betaine and carnitine. The osmoregulated transporter for quaternary compounds (OpuA) in *L. lactis* is the main system that protects the organism against hyperosmotic stress. It was first proposed that changes in transmembrane osmotic gradient were transmitted to the OpuA system via distortions in the membrane bilayer [136]. However, it is now known that high ionic strength at the cytoplasmic face of OpuA activates the transporter under iso-osmotic conditions, leading to the suggestion that charge interactions could be important in activation [137]. In reconstituted systems, it was found that anionic lipid is essential for function of OpuA [137,138]. In mixtures with di(C18:1)PC, ca. 25 mol% di(C18:1)PS or di(C18:1)PG gave the lowest activation threshold; the fact that di(C18:1)PS and di(C18:1)PG had the same effect suggests that specific interactions with OpuA are not important. Replacing di(C18:1)PC with di(C18:1)PE in mixtures with di(C18:1)PG led to increased maximal activity but had no effect on the threshold for activation. Changing the chain length of the phosphatidylcholine in mixtures with di(C18:1)PG did not change the threshold, although maximum activity was found to be chain length dependent, with a maximum at C18. Similarly, replacing a *cis* double bond with a *trans* double bond did not affect the threshold [137]. These results argue against the bulk properties of the bilayer being important for activation. Rather, it is suggested that changes in osmotic strength, by changing the volume of the reconstituted vesicles, lead to changes in the ionic strength in the lumen of the vesicles, which, in turn, alters anionic lipid–protein interactions and leads to activation [137]. BetP, a glycine betaine transporter of similar function to OpuA but unrelated in structure, also responds in the same way to the intracellular concentration of K^+ [139]. Thus the mechanism of activation of these proteins is very different to that of MscL.

3. Effects of non-annular lipids on membrane protein function

Some membrane proteins only show activity when in the presence of particular classes of lipid, these ‘special’ lipids often co-purifying with the protein and being resolved in high-resolution structures of the protein. Lipids of this type have been referred to as non-annular lipids, to distinguish them from the annular lipids that interact with the bulk hydrophobic surface of a membrane protein. Most non-annular lipids are bound between transmembrane α-helices, often between subunits in multi-subunit proteins (Table 1) as shown in Fig. 2A for the phosphatidylglycerol bound to KcsA, for which the fatty acyl chains are resolved although the headgroup is not. However, tightly bound lipid is not only found between transmembrane α-helices. For example, a resolved phosphatidylglycerol molecule is seen bound on the surface of the transmembrane domain of the heterotrimeric nitrate reductase A (Fig. 14) [140]. Only the first seven and four carbons, respectively, of the two fatty acyl chains are resolved, but the lipid headgroup is well resolved.

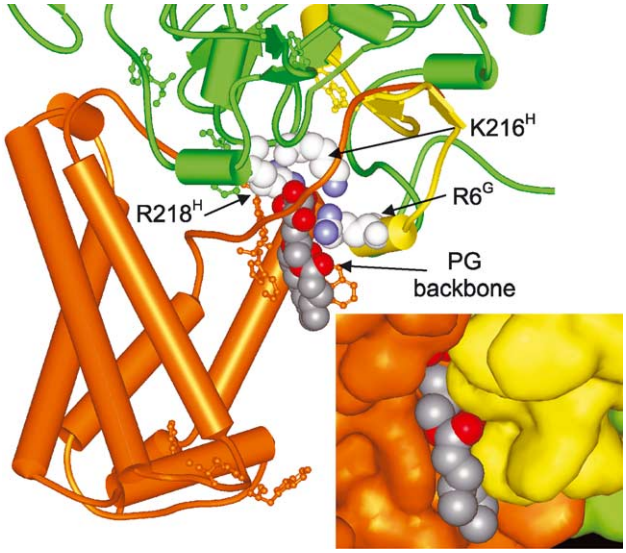

Fig. 14. The heterotrimer of nitrate reductase A. The subunits NarG, NarH and NarI are coloured yellow, green, and brown, respectively. Trp residues are shown in ball-and-stick representation and the bound phosphatidylglycerol (PG) molecule is shown in space-fill representation; the glycerol backbone of PG is marked. Also shown in space-fill format are Arg-6 in NarG and Lys-216 and Arg-218 in NaH that contribute to the binding of the PG molecule. The insert shows how PG binds between the surfaces of the three monomers making up the heterotrimeric structure (PDB file 1Q16).

The headgroup is bound in a pocket formed by all three subunits with a number of positively charged residues contributing to the binding site (Fig. 14). With major contributions to the binding site coming from two subunits that are not transmembranous, the headgroup binding site resembles that on an extrinsic membrane protein binding to the surface of a membrane, such as that on annexin V shown in Fig. 1. Although the effect of phosphatidylglycerol on the function of nitrate reductase A appears not to have been defined, it is likely that it plays a major structural role, holding the heterotrimeric structure together.

Properties of non-annular lipids have been reviewed at length elsewhere [7] and so will only be touched on briefly here. A classic example of a non-annular lipid is provided by the requirement for cardiolipin by a number of proteins involved in bioenergetics, including NADH dehydrogenase, cytochrome bc_1, ATP synthase, cytochrome oxidase, and the ADP/ATP carrier [141,142]. Bovine heart cytochrome oxidase copurifies with a small number of tightly bound cardiolipin molecules whose removal leads to loss of activity [143,144]. The reason for the importance of cardiolipin is unknown. Cytochrome oxidase prepared from dogfish contains no cardiolipin but shows normal activity, arguing against an absolute requirement for cardiolipin for cytochrome oxidase activity [145]. Similarly, cardiolipin-deficient strains of *Saccharomyces cerevisiae* are viable under fermenting and non-fermenting conditions, except at high temperatures, showing that cardiolipin is not essential for oxidative phosphorylation [159]. In *S. cerevisiae* the presence of cardiolipin was found to stabilize supercomplexes between complexes III and IV of oxidative phosphorylation, although it was not essential for their formation [159]. The tightly bound cardiolipin molecule found in the purple bacterial reaction centre [160] also appears to play a role in protein stability [161]. Thus after mutation of an Arg residue involved in interaction with the bound cardiolipin molecule the crystallized reaction centre no longer contained a cardiolipin molecule; this had no effect on the structure of the reaction centre or on its function, but did reduces its thermal stability [161].

In cytochrome bc_1 it has been suggested that the cardiolipin molecule resolved in the crystal structure could be important for the stability of the protein and could also be part of a proton 'wire' conducting protons from the aqueous phase to the site of quinone reduction since it is located close to the site of quinone reduction [20]. Activity of the mitochondrial ADP/ATP carrier shows an absolute dependence on the presence of cardiolipin [146]. The high-resolution structure of the ADP/ATP carrier shows three bound cardiolipin molecules per monomer, and four phosphatidylcholine molecules (Fig. 15) [147]. Unusually, these lipid molecules appear to occupy annular sites on the protein, being bound to the surface of the protein molecule rather than being buried between transmembrane α-helices (Table 1). The binding sites for cardiolipin do not show the large numbers of positively charged residues that might have been expected for an anionic lipid binding site of high affinity; the binding site for the cardiolipin molecule illustrated in Fig. 15, for example, contains only one positively charged residue, Arg-151. The bound cardiolipin molecules are, however, contained within distinct grooves

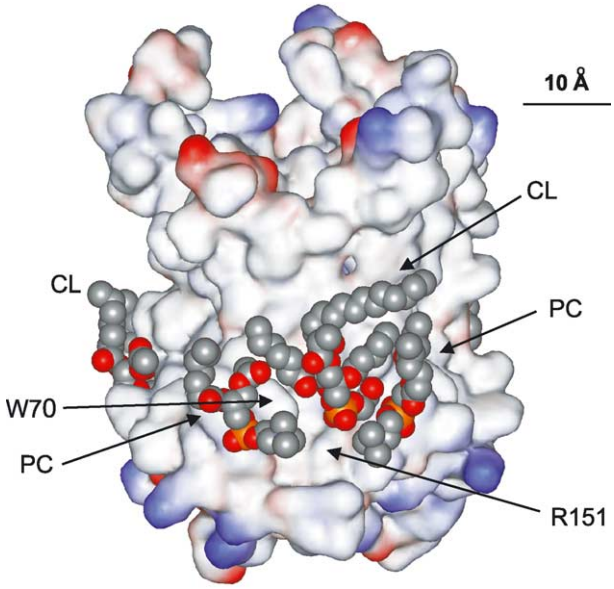

Fig. 15. The surface of the mitochondrial ADP/ATP carrier. Shown are the binding sites for two cardiolipin (CL) and two phosphatidylcholine (PC) molecules. The binding site for the cardiolipin molecule shown in the centre is defined by Arg-151 and Trp-70. The surface is colour-coded by electrostatic potential (red, negative; blue, positive; grey, neutral) (PDB file 1OKC).

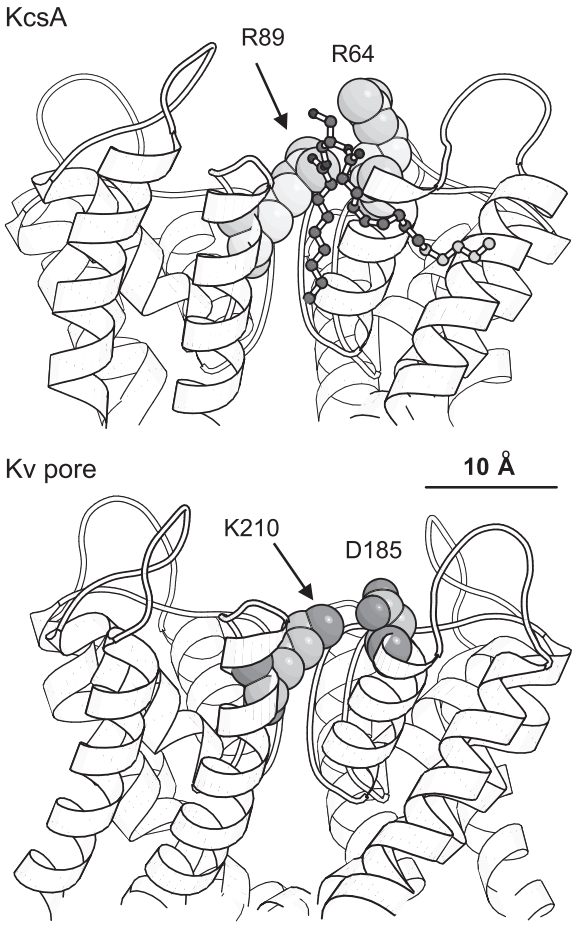

Fig. 16. Comparison of the monomer–monomer interfaces in the potassium channel KcsA from *Streptomyces lividans* and in the pore region of the voltage-gated potassium channel Kv from *A. pernix*. The headgroup of the phosphatidylglycerol molecule tightly bound to KcsA is not resolved and so is modelled as a diacylglycerol, shown in ball-and-stick representation. The phosphatidylglycerol molecule in KcsA is bound between Arg-64 and Arg-89 in adjacent monomers, shown in space-fill representation. The corresponding residues in the voltage-gated potassium channel (Kv) are Asp-185 and Lys-210, also shown in space-fill representation (PDB files 1K4C and 1ORQ).

on the surface, often containing prominent Trp residues, such as Trp-70 shown in Fig. 15. Although the ADP/ATP carrier crystallises as a monomer, the native structure is a dimer and so it is possible that some of the resolved lipid molecules will be located at protein–protein interfaces in the dimeric structure.

Non-annular lipid molecules located at protein–protein interfaces could help to ensure good packing at the interface. For example, the tightly bound molecule of phosphatidylglycerol in KcsA is located at the monomer–monomer interface in the homotetrameric structure (Fig. 2B), between two Arg residues, one coming from each of the monomers at the interface (Fig. 16). When KcsA is reconstituted into lipid vesicles, it shows a requirement for anionic phospholipid for function, with no specificity for which anionic lipid, which could, for example, be phosphatidylglycerol, phosphatidylserine, or cardiolipin [18]. It is possible that the role of the anionic phospholipid molecule is to reduce electrostatic repulsion between the two Arg residues. It is interesting that the equivalent residues in the voltage-gated potassium channel from the thermophilic bacterium *Aeropyrum pernix* [148] are Lys and Asp (Fig. 16) forming a salt bridge at the interface; it is not known whether or not the voltage-gated potassium channel shows a functional requirement for anionic phospholipid.

Non-annular lipid molecules might have functional effects on membrane proteins by affecting the movement of transmembrane α-helices in the protein. Such a possibility is suggested by studies of the effects of the hydrophobic inhibitor thapsigargin on the function of the Ca^{2+}-ATPase [40]. Thapsigargin binds to the Ca^{2+}-free form of the ATPase, in a cleft between transmembrane α-helices M3, M5, and M7 with the polar part of the thapsigargin molecule located between Phe-256 and Ile-829, with the key –OH groups in thapsigargin [149] hydrogen bonding to Glu-255 (Fig. 17). In the Ca^{2+}-bound form of the ATPase, the space between helices M3 and M7 is much less than in the thapsigargin-bound form, and so thapsigargin acts as an inhibitor by keeping helices M3 and M7 apart, preventing the Ca^{2+}-ATPase from adopting the conformation in which it can bind Ca^{2+} and so be active. Thapsigargin therefore acts as a simple wedge, preventing the relative movement of helices necessary for function. This could be a general mechanism for the function of non-annular lipids. For example, binding of cholesterol and phospholipids to the Ca^{2+}-ATPase was interpreted in terms of binding of cholesterol to non-annular sites on the Ca^{2+}-ATPase from which phospholipids were excluded, the effect of binding to such sites depending on the chain lengths of the phospholipids present in the system [7,8]. It is possible that the non-

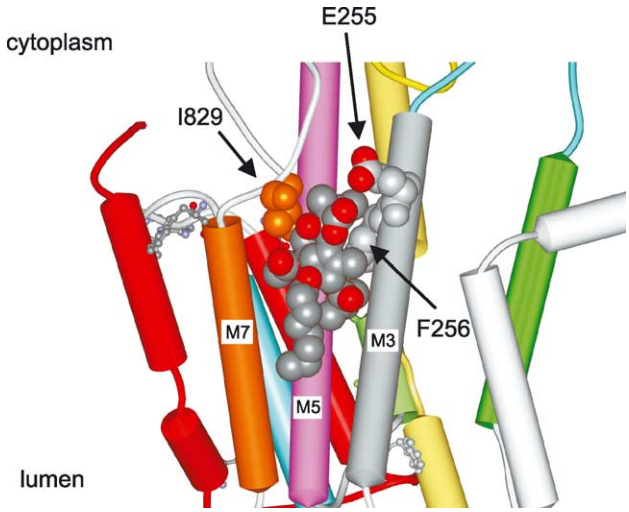

Fig. 17. The binding site for thapsigargin on Ca^{2+}-ATPase. The bound thapsigargin is shown in space-fill representation, binding between Phe-256 and Ile-829 and hydrogen bonding with Glu-255. Transmembrane α-helices M3, M5, and M7 are coloured grey, lilac and brown, respectively (PDB file 1IWO).

annular binding sites for cholesterol are also located between transmembrane α-helices. The presence of cholesterol has significant effects on the activities of a number of other membrane proteins. Thus the nicotinic acetylcholine receptor (AChR) requires the presence of cholesterol for function [150,151] and, on the basis of competition studies, Jones and McNamee [152] suggested the presence of non-annular sites on AChR to which cholesterol can bind, but from which phospholipids are excluded. The binding sites for cholesterol showed little selectivity and many other hydrophobic molecules such as α-tocopherol, coenzyme Q_{10} and vitamins D_3 and K_1 were able to substitute for cholesterol [153,154]. The presence of cholesterol has also been shown to result in increased activity for the Na^+,K^+-ATPase [66], as described in Section 2.2.

Not all members of a particular class of membrane protein necessarily show the same requirements for non-annular phospholipids. For example, phosphatidylglycerol is enriched in the photosynthetic membranes of purple bacteria such as *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* where it has been suggested that it could interact preferentially with the light-harvesting complex 2 (LH2) antenna pigment–protein complex, but it is not present in the LH2 complex of *Rhodopseudomonas acidophilia*, showing that it cannot play an essential role in the photosynthetic membrane [155,160]. This is what would be expected if phosphatidylglycerol were playing a role in protein packing; differences in the amino acid sequences of various members of a family would lead to different requirements for optimal packing.

4. Conclusions

The availability of a range of crystal structures for intrinsic membrane proteins means that we can now start to explain, at the molecular level, how lipids interact with membrane proteins to affect their function. With the exception of mechanosensitive channels, it seems likely that gross changes in the transmembrane regions of membrane proteins do not occur when membrane proteins carry out their functions. We need therefore to understand how rather subtle changes in the transmembrane regions of membrane proteins are affected by interaction with the surrounding lipid molecules; changes of this type are probably best understood at the molecular level. Membrane proteins, particularly those made up of bundles of transmembrane α-helices, are not rigid entities around which the lipid bilayer distorts to provide the strongest interactions. Rather, both the lipid and the protein molecules will distort to provide the best interaction, with the result that protein function will be affected by the structure of the surrounding lipid bilayer. Some membrane proteins also have a specific requirement for a small number of tightly bound lipids, referred to as non-annular lipids, acting as co-factors for the protein, and often being required to ensure good packing at protein–protein interfaces in multi-subunit membrane proteins.

An important feature of a lipid bilayer is the thickness of the hydrophobic core of the bilayer, defined by the lengths of the fatty acyl chains in the component lipids. The conformation adopted by a membrane protein in a lipid bilayer will be such that the hydrophobic thickness of the protein will be close to the hydrophobic thickness of the surrounding lipid bilayer. A change in the hydrophobic thickness of the lipid bilayer away from its normal value would therefore result in significant changes in the conformation of the membrane protein, away from that required to ensure maximum activity, resulting in large decreases in activity for the protein. Lipid headgroup structure will also have important effects on membrane protein function. The structures adopted by the parts of a membrane protein that are located in the lipid headgroup region will be determined, in part, by hydrogen bonding to the headgroups, which will be very different, for example, for phospholipids such as phosphatidylcholine and phosphatidylethanolamine. Effects of phospholipid headgroup structure on membrane protein function could, therefore, depend on specific interactions between the lipid headgroups and the protein.

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