14 Akanuma, S. *et al.* (1998) Serial increase in the thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by experimental evolution. *Protein Sci.* 7, 698–705
15 Merz, A. *et al.* (2000) Improving the catalytic activity of a thermophilic enzyme at low temperatures. *Biochemistry* 39, 880–889
16 Giver, L. *et al.* (1998) Directed evolution of a thermostable esterase. *Proc. Natl. Acad. Sci. U. S. A.* 95, 12809–12813
17 Gershenson, A. *et al.* (2000) Tryptophan phosphorescence study of enzyme flexibility and unfolding in laboratory-evolved thermostable esterases. *Biochemistry* 39, 4658–4665
18 Zhao, H.M. and Arnold, F.H. (1999) Directed evolution converts subtilisin E into a functional equivalent of thermitase. *Protein Eng.* 12, 47–53
19 Wintrode, P.L. *et al.* (2000) Cold adaptation of a mesophilic subtilisin-like protease by laboratory evolution. *J. Biol. Chem.* 275, 31635–31640
20 Taguchi, S. *et al.* (1999) A cold-adapted protease engineered by experimental evolution system. *J. Biochem. (Tokyo)* 126, 689–693
21 Lebbink, H. *et al.* (2000) Improving low-temperature catalysis in the hyperthermostable *Pyrococcus furiosus* β-glucosidase CalB by directed evolution. *Biochemistry* 39, 3656–3665
22 Wati, M.R. *et al.* (1997) Gene from tropical *Bacillus sphaericus* encoding a protease closely related to subtilisins from Antarctic Bacilli. *Biochem. Biophys. Acta* 1352, 56–62
23 Spiller, B. *et al.* (1999) A structural view of evolutionary divergence. *Proc. Natl. Acad. Sci. U. S. A.* 96, 12305–12310
24 Colombo, G. and Merz, K.M. (1999) Stability and activity of mesophilic subtilisin E and its thermophilic homolog: insights from molecular dynamics simulations. *J. Am. Chem. Soc.* 121, 6895–6903
25 Jaenicke, R. (1981) Enzymes under extreme physical conditions. *Ann. Rev. Biophys. Bioeng.* 10, 1–67
26 Wrba, A. *et al.* (1990) Extremely thermostable D-glyceraldehyde-3-phosphate dehydrogense from the eubacterium thermotoga-maritima. *Biochemistry* 29, 7584–7592
27 Zavodszky, P. *et al.* (1998) Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. *Proc. Natl. Acad. Sci. U. S. A.* 95, 7406–7411
28 Lopez-Camacho, C. *et al.* (1996) Amino-acid substitutions enhancing the thermostability of Bacillus polymyxa β-glucosidase A. *Biochem. J.* 314, 833–838
29 Akanuma, S. *et al.* (1999) Further improvements of the thermal stability of a partially stabilized *Bacillus subtilis* 3-isopropylmalate dehydrogenase variant by random and site-directed mutagenesis. *Eur. J. Biochem.* 260, 499–504
30 Van den Burg, B. *et al.* (1998) Engineering an enzyme to resist boiling. *Proc. Natl. Acad. Sci. U. S. A.* 95, 2056–2060
31 Williams, J.C. *et al.* (1999) Structural and mutagenesis studies of leishmania triosphosphate isomerase: a point mutation can convert a mesophilic enzyme into a superstable enzyme without losing catalytic power. *Protein Eng.* 12, 243–250
32 Merz, A. *et al.* (1999) The hyperthermostable indoleglycerol phosphate synthase from *Thermotoga maritima* is destabilized by mutational disruption of two solvent-exposed salt bridges. *J. Mol. Biol.* 288, 753–763
33 McGlynn, S.P. (1969) *Molecular Spectroscopy of the Triplet State*, Prentice-Hall
34 Lower, S.K. and El-Sayed, M.A. (1966) The triplet state and molecular electronic processes in organic molecules. *Chem. Rev.* 66, 199–241

Probing the interface between membrane proteins and membrane lipids by X-ray crystallography

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Biological membranes are composed of a complex mixture of lipids and proteins, and the membrane lipids support several key biophysical functions, in addition to their obvious structural role. Recent results from X-ray crystallography are shedding new light on the precise molecular details of the protein–lipid interface.

Biological membranes are not just inert physical barriers, they are metabolically active and support a wide range of key biochemical processes. These include respiration and photosynthesis, solute transport, motility, cell–cell recognition, signal transduction and protein transport. Those proteins that form an integral part of biological membranes inhabit a complex environment. Over part of their surface they are exposed to the aqueous phase on one or both sides of the membrane, where they interact with water, small hydrophilic ions and molecules, and water-soluble proteins. The remainder of their surface is exposed to the membrane, either the mainly hydrophilic 'interface' region, which forms a layer approximately 15 Å thick on either side of the membrane, or the ~30 Å thick hydrophobic interior of the membrane.

It is widely thought that specific protein–lipid interactions are important for the structural and functional integrity of many key integral membrane proteins from prokaryotic and eukaryotic membranes[1–3]. Specific examples include the plasma membrane Ca^{2+} pump (Ca^{2+}-ATPase)[4], rhodopsin[5], cytochrome *c* oxidase[6,7] and the ADP/ATP carrier from mitochondria[8]. A good match between the hydrophobic protein surface and the surrounding lipids is thought to be important for the stable integration of integral membrane proteins into the lipid bilayer. Interestingly, the introduction of a mismatch through an alteration in the thickness of the hydrophobic core of the membrane, by incorporation of longer or shorter lipids, might provide a mechanism for sorting proteins between different types of membrane in complex membrane systems[9].

X-ray crystallography of membrane proteins

As with soluble proteins, much effort has gone into investigating the structure of membrane proteins by

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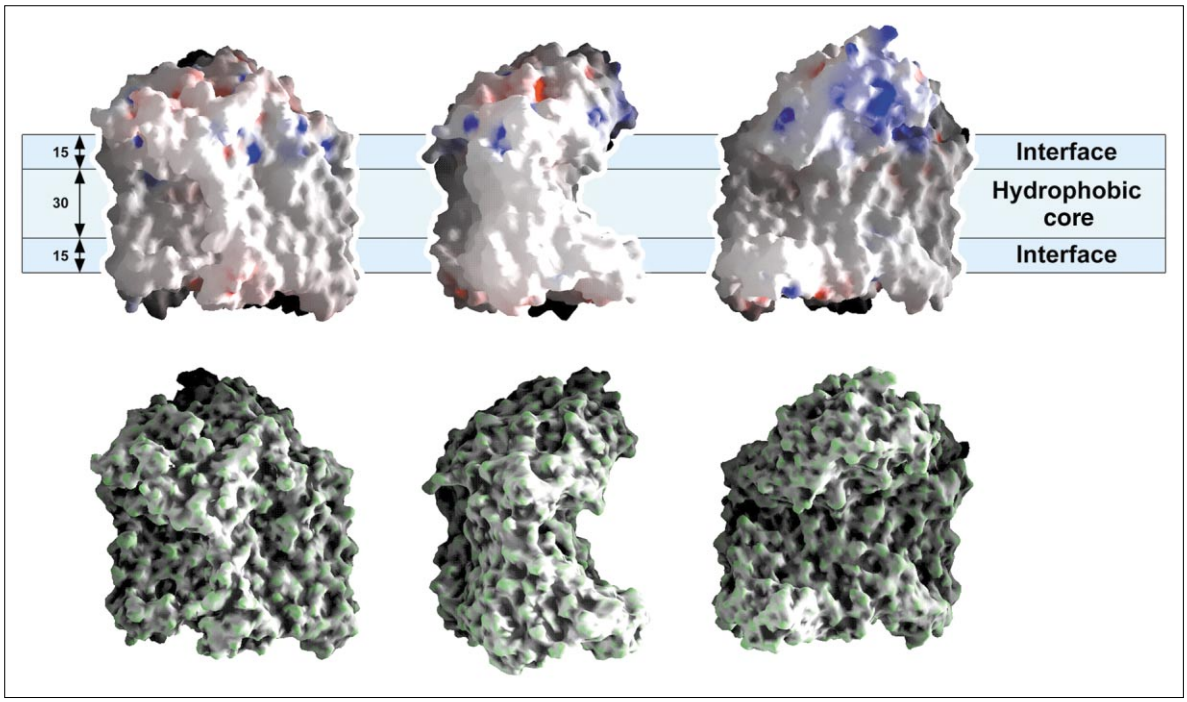

Fig. 1. Views of the reaction centre from the photosynthetic bacterium *Rhodobacter sphaeroides*. In all cases the view is parallel to the plane of the membrane, and the protein is shown as a solid object, rendered with the program GRASP (Ref. 41). In the top row the protein has been coloured according to surface potential (blue, positive; red, negative; grey, neutral). In the bottom row the protein has been coloured according to surface curvature (green, convex; grey , concave). In the top row the approximate position of the membrane is shown. The approximately 30 Å thick core region, corresponding to the hydrophobic acyl chains of the membrane lipids, is distinguished from the approximately 15 Å thick interface regions on either side of the membrane, which correspond to the lipid head groups. The protein surface exposed to the hydrophobic core of the membrane is largely devoid of polar amino acids.

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X-ray crystallography. However, despite this, high resolution structures have been described for only approximately 20 membrane proteins, in stark contrast to the thousands of soluble proteins that have been characterized successfully. X-ray crystallography of membrane proteins is complicated by their amphipathic nature, and the difficulty in obtaining sufficient quantities of highly pure and stable protein for crystallization. However, as knowledge on handling membrane proteins has improved, the rate of production of X-ray structures has increased, particularly during the past three or four years.

Extensive biochemical study has been made of the importance of particular lipids for the activity of a wide range of integral membrane proteins[1–3]. However, it has been difficult to apply techniques such as site-directed mutagenesis to this topic because of a lack of detailed structural information on specific interactions between a given protein and the lipid environment. As the variety of X-ray structures for integral membrane proteins has increased, and the quality of several of these has improved, attention has begun to turn to the question of whether X-ray crystallography can provide information at the molecular level on how these proteins interact with the lipids that surround them in the membrane. For a lipid to be resolved in X-ray data it must be highly ordered and have a high occupancy of its 'binding site' on the protein surface. This implies a highly specific interaction that might have important functional implications.

The protein–lipid interface

What effects do integral membrane proteins have on their lipid environment? The results of spin-label electron paramagnetic resonance (EPR) spectroscopy carried out on a variety of integral membrane proteins indicate that the first shell of lipid that interacts with these proteins is motionally restricted, as a result of the interactions of these lipids with the protein surface[10]. The shell of lipids that coats the protein surface can be distinguished from the lipids in the bulk of the membrane, even though lipids exchange between the two phases at rates of the order of 10^6–10^7 s^{-1}. Therefore, the picture that emerges is one of proteins that are coated with a monolayer of motionally restricted lipid, the so-called annular lipid, which is in exchange with the bulk lipid on the microsecond time-scale[10].

What sort of surfaces do membrane proteins present to the lipids that surround them in the membrane? In Fig. 1 we show several views of the bacterial reaction centre, an integral membrane protein complex found in the photosynthetic membranes of many species of purple bacteria[11], with the surface of the protein complex coloured according to surface potential (top) or surface curvature (bottom). The structure of the reaction centre from *Rhodobacter sphaeroides* has been determined by X-ray crystallography[12,13]. The transmembrane region of the protein is a tightly packed bundle of 11 transmembrane α-helices, formed by the L-, M- and H-subunits. The helices encase several (bacterio)chlorophyll, ubiquinone and carotenoid cofactors.

Two features of the protein are of particular note. The first is the band of neutral surface potential that girdles the protein (coloured white in Fig. 1), which marks the hydrophobic intra-membrane surface of the protein that is expected to contact the tails of the surrounding lipids (Fig. 1, top row). When the protein is purified, this hydrophobic surface is shielded from the aqueous phase by the detergent micelle, and neutron diffraction experiments have shown the structure of this micelle in crystals of the reaction centre[14,15]. The second point to note is that the protein

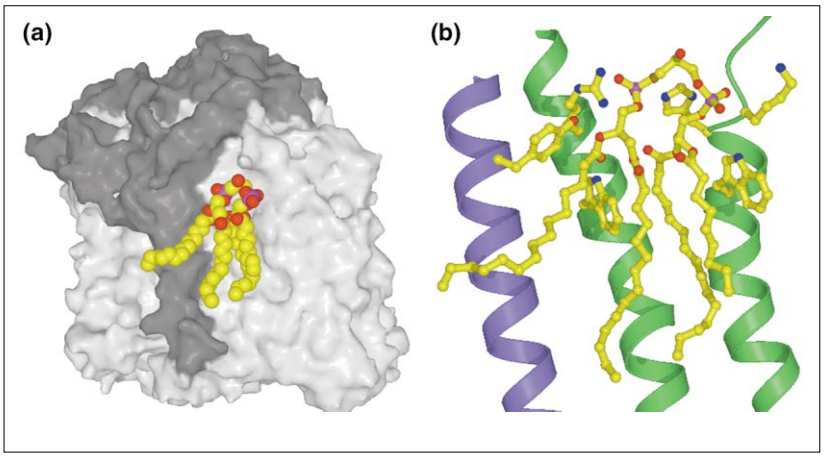

Fig. 2. A molecule of the diacidic lipid cardiolipin associated with the intra-membrane surface of the reaction centre. (a) The protein is shown as a solid object, rendered with the programs SPOCK (Ref. 42) and Raster3D (Ref. 43), with the L and M subunits in white and the H subunit in light grey. The view is parallel to the plane of the membrane, and is the same as that on the left in Fig. 1. The cardiolipin molecule is shown in space-fill format, with carbon atoms in yellow, oxygen atoms in red and phosphorus atoms in pale magenta. (b) Expanded view of the interaction between the cardiolipin (in stick format) and the protein surface. Transmembrane α-helices of the H (purple) and M (green) subunits are shown as ribbons. Residues involved in bonding interactions with the head group of the cardiolipin are shown in stick format. Colour coding is as for (a), with nitrogen atoms in blue.

surface is highly irregular. Focussing again on the hydrophobic intra-membrane region, the surface of the protein features many grooves and channels, some of which are formed by turns of the transmembrane α-helices. The shape of the protein is such that the cytoplasmic domain overhangs part of the transmembrane region (Fig. 1, central views), and the single transmembrane α-helix of the H-subunit is packed against the α-helices of the L- and M-subunits in such a way that a deep cavity is formed between the two. The protein therefore presents a highly irregular surface to the lipids that surround it in the membrane, implying that the adjacent lipids will have to undergo significant distortion from a simple bilayer to fill the space around the protein and thus provide an electrically sealed membrane. This is where nonbilayer lipids play an important role. Estimates from EPR spectroscopy suggest that a protein of the size and shape of the bacterial reaction centre, with 11 transmembrane α-helices, all of which are exposed to the membrane phase, will be surrounded by 30–35 motionally restricted lipids[10].

Insights into the lipid environment of the bacterial reaction centre

In a crystallographic study of an alanine M260 to tryptophan (AM260W) mutant reaction centre from *Rb. sphaeroides*[16], a feature observed in the electron-density map at the intra-membrane surface of the reaction centre was modelled as a molecule of the diacidic lipid diphosphatidyl glycerol, commonly known as cardiolipin (Fig. 2a). This lipid is found as a minor component of many membranes from both prokaryotes and eukaryotes[1]. It has a polar head group composed of three glycerol molecules connected by two phosphodiester linkages, and four acyl tails that can vary in length and in the degree of unsaturation. In *Rb. sphaeroides* the most common acyl chain has 18 carbon atoms and a single unsaturated bond (i.e. 18:1), although both shorter and longer acyl chains are present in minor amounts[17]. The presence of the cardiolipin in crystals of the AM260W reaction centre is not due to this particular mutation, because this lipid has been resolved in X-ray data for other mutant reaction centres (M.R. Jones *et al.*, unpublished). In all cases, the mutations are some distance from the lipid-binding site and do not cause any long-range changes in the structure of the protein. The factors that determine how clearly the cardiolipin molecule is resolved in X-ray data are the subject of ongoing experiments, as is the functional role (if any) of this specific protein–lipid interaction.

In agreement with the general principles for membrane protein–lipid interactions outlined above, the cardiolipin located on the intramembrane surface of the reaction centre engages in H-bond interactions with polar residues in the membrane interface region (at the cytoplasmic side of the membrane), and hydrophobic interactions with the intramembrane surface of the protein[16]. Bonding interactions involve either direct contacts between the phosphate oxygens of the lipid head-group and basic amino acids or backbone amide groups exposed at the protein surface (Fig. 2b), or indirect contacts with lysine, tryptophan, arginine and tyrosine residues that are mediated by water molecules. Good quality, continuous electron density was obtained for the head-group and upper parts of the acyl tails of the cardiolipin, but their ends were not resolved in the electron density, presumably because they were mobile and therefore disordered[16]. A striking feature was the observation that the acyl tails of the cardiolipin lie along grooves in the α-helices that form the hydrophobic intramembrane surface of the protein. This arrangement is highlighted in Fig. 2b, which illustrates how parts of the acyl tails fit into grooves between turns in the transmembrane α-helices of the H (purple) and M (green) subunits. These van der Waals interactions restrict the motion of the upper parts of the tails of the cardiolipin to a sufficient extent that they can be resolved in the X-ray data.

The lipid environment of bacteriorhodopsin

Bacteriorhodopsin is a light-driven ion pump that is found in the 'purple' membrane of the bacterium *Halobacterium salinarum*[18,19]. The protein is organized in trimers that in turn form a highly ordered 2D hexagonal lattice. Bacteriorhodopsin monomers consist of a bundle of seven transmembrane α-helices that are connected by short inter-helical loops, and enclose a molecule of retinal that is buried in the protein interior, approximately half way across the membrane. Proton pumping by bacteriorhodopsin is linked to photoisomerization of the retinal and conformational changes in the protein, in a series of changes called a photochemical cycle[18,19]. It has been demonstrated that specific lipids can influence the thermal steps in this cycle and, in

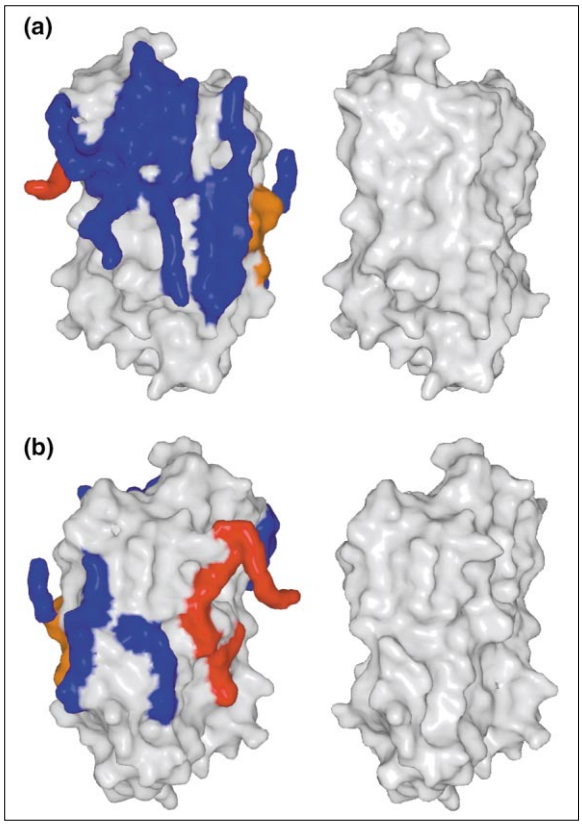

Fig. 3. A monomer of bacteriorhodopsin, with and without the lipids modelled in the X-ray structure of Luecke and co-workers[26] (protein data base entry 1C3W). All views are in the plane of the membrane. The protein is represented as a solid surface (light grey), either with (left) or without (right) the modelled membrane lipids, using the program SPOCK (Ref. 42) and Raster3D (Ref. 43). The top and bottom views show opposite sides of the protein. Lipids located in the central hole around the threefold axis of the bacteriorhodopsin trimer are shown in red, and the molecule assigned as squalene is shown in orange. All other modelled lipids are shown in blue. The views with and without the lipids are shown so that the grooves through which the acyl chains run can be visualized.

particular, that a combination of squalene and phosphatidyl glycerophosphate is required to maintain normal photochemical cycle behaviour[20].

Structures for bacteriorhodopsin obtained both by electron crystallography[21,22] (at resolutions of 3.0–3.5 Å) and by X-ray crystallography[23–25] (at resolutions between 3.5 Å and 1.9 Å) have included lipids modelled on the basis of full or partial electron density. In a higher resolution (1.55 Å) structure of bacteriorhodopsin[26], Luecke and co-workers observed several long, cylindrical electron density features on the intra-membrane surface of the protein, arranged approximately perpendicular to the presumed plane of the membrane. These were modelled as 18 full or partial lipid acyl chains per monomer, four pairs of which were linked with a glycerol backbone to form diether lipids. Details of the electron density indicated that these chains were attributable to native *H. salinarum* lipids. The acyl chains of the lipids were shown to occupy a large part of the contact surface between monomers in the trimer, in agreement with findings from previous lower-resolution structures[21,23–25]. Other acyl chains were observed to mediate crystal contacts between adjacent trimers in the membrane, again in agreement with earlier findings[21–24]. A noticeable feature of the structure of bacteriorhodopsin is that the central 'hole' around the threefold axis of the trimer is occupied by up to six lipids on the extracellular side of the membrane[21–24,26]; Essen and co-workers have commented that the positions of these lipids indicate that the bilayer in the centre of the trimer is at least 5 Å thinner than that between trimers[23]. In the structure of Luecke and co-workers, one of the lipid acyl chains was buried in the centre of the membrane, where it adopted a distinctive S shape[26]. This chain was modelled as a molecule of squalene, an unsaturated hydrocarbon that is present in the purple membrane in a 1:1 ratio with bacteriorhodopsin, and which is known to be important for maintaining a normal photocycle[21].

Figure 3 shows two opposing views of the surface of one monomer of bacteriorhodopsin, with and without the modelled lipids. These illustrate the extent to which the accessible surface of the protein is coated with the acyl chains of the membrane lipids. Presumably, an additional fraction of the surface is coated by acyl chains that are too disordered to give rise to significant electron density. A final point, highlighted by Luecke and co-workers, is that the lipid chains that could be modelled were closely aligned with grooves on the protein surface, including the molecule assigned as squalene, which resides in an S-shaped cleft formed by valines and leucines[26]. This parallels findings on the binding of the acyl chains of cardiolipin to the bacterial reaction centre, detailed above.

Although density was present that could be attributed to the head group of some of the lipids modelled in the study of Luecke and co-workers, the quality of this density was not sufficient to allow modelling of any of the lipid head groups. However, Essen and co-workers have modelled head groups for two lipids in an X-ray crystal structure for bacteriorhodopsin at 2.9 Å resolution[23]. This structure includes six modelled lipids per bacteriorhodopsin monomer, including three in the central hole of the bacteriorhodopsin trimer, and the head groups of two of these were modelled as sulphated triglycosides that include glucose, mannose and galactose moieties. The model provides insights into possible H-bond and salt bridge interactions between the hydroxyl groups of the glucose and mannose moieties of the triglyceride head group, and the adjacent protein. Points of contact with the protein include the backbone carbonyls of a tyrosine and leucine residue, and the side chain of a threonine. An additional interaction was proposed between a lysine residue and the sulphate of the terminal galactosyl-3-sulphate of the lipid head group. In addition, Essen and co-workers noted that additional interactions between the lipid and protein might be mediated by water molecules that were not crystallographically resolved[23]. Again, this draws an interesting parallel with the study of the bacterial reaction centre[16], where it was proposed that at least four water molecules mediate contacts between the head group of cardiolipin and polar groups on the adjacent protein surface.

Cytochrome *c* oxidase

The crystal structure of bovine cytochrome *c* oxidase has been determined to a resolution of 2.8 Å (Ref. 27). This large integral membrane protein (13 different subunits, giving a total of 1803 amino acids) is responsible for the reduction of molecular oxygen to water during aerobic respiration, with concomitant

proton pumping across the mitochondrial inner membrane. Eight lipids were modelled in this structure, five phosphatidyl ethanolamines and three phosphatidyl glycerols, and both the head groups and acyl tails were successfully modelled. In a higher resolution (2.3 Å) structure of a dimer of this protein[28], a total of 14 lipids have been modelled per monomer – three phosphatidyl ethanolamines, seven phosphatidyl glycerols, one phosphatidyl choline and two cardiolipins. In addition, the crystal structure of cytochrome *c* oxidase from *Paracoccus denitrificans* has been determined, to a resolution of 3.0 Å, and includes two molecules of phosphatidyl choline[29]. Both lipids are embedded in deep grooves in the protein surface, and the phosphate oxygens of the head groups allow interactions with adjacent arginine residues.

The resolution of cardiolipin in the structure of the bovine cytochrome *c* oxidase is particularly intriguing because, whereas in the case of the bacterial reaction centre there is no information on a specific role played by this lipid, it is well documented that cardiolipin cannot be removed from cytochrome *c* oxidase without a loss of enzyme activity[6,7]. One of the cardiolipins modelled in the 2.3 Å structure of cytochrome *c* oxidase is located at the interface of the monomers of the dimer, with the remainder of the phospholipids being located between subunits in the monomer[28]. It is to be hoped that the details of the X-ray crystal structure will provide new insights into why removal of cardiolipin has such an adverse effect on the activity of the enzyme.

β barrel proteins

The ferric hydroxamate uptake receptor (FhuA) from *Escherichia coli* is one of a group of proteins from the outer membrane of Gram negative bacteria that have a membrane-spanning region that consists of a β barrel formed from transmembrane β strands. These proteins interact with lipopolysaccharide (LPS), a complex molecule that forms the outer leaflet of the bacterial outer membrane. Ferguson and co-workers have described the X-ray crystal structure of FhuA with a bound LPS, to a resolution of 2.5 Å (Ref. 30). In general terms, the structure shows many of the features described for α-helical membrane proteins. The acyl chains of the LPS are ordered on the protein surface approximately parallel to the axis of the β barrel, and make numerous van der Waals interactions with surface-exposed hydrophobic residues. The large polar head-group of LPS makes extensive interactions with charged and polar residues near the outer surface of the membrane, principally H-bonds between phosphate or sugar oxygens of the LPS head-group and side-chain amino groups of basic amino acids.

Improving the picture of lipid binding by membrane proteins

A common feature of the membrane proteins described above is that they are relatively well-characterized. They constitute mature and robust experimental systems that can be used not only to probe the details of individual protein–lipid interactions, but also to examine the best conditions for the detailed resolution of the full range of interactions between the protein and the first shell of membrane lipids. In the case of lipids that mediate protein–protein contacts within multimeric complexes[21,23–28], which are essentially locked into the protein structure, improvements in the resolution and quality of diffraction data for these proteins will no doubt bring increasingly accurate insights into the precise conformations and interactions of these lipids. Notably, even with relatively high resolution X-ray data, it is often difficult to model accurately the length of an acyl chain, or the number and positions of double bonds. The lipids that are attached to the 'outer surface' of these proteins present a slightly different challenge, as it is easy to envisage that the extent to which this lipid shell is conserved in the protein crystal will depend on the treatments to which the protein is exposed during purification. Furthermore, it is possible that some of the lipids that bind to the surface of purified proteins do so only in place of direct protein–protein contacts that are broken during purification.

A general finding from the crystallographic work carried out thus far is that the extent to which surface features such as bound lipids can be resolved is somewhat variable, even among structures of comparable resolution. This is illustrated by data for the bacterial reaction centre. The report of a cardiolipin attached to the surface of the *Rb. sphaeroides* reaction centre[16] comes nearly 15 years after the first descriptions of the X-ray structure of this protein. The electron density attributed to the cardiolipin is of good quality and is continuous throughout the main part of the molecule, although density is missing for the ends of all four acyl chains. In previous studies, various groups have observed roughly spherical electron density features associated with this region of the protein surface, which have been modelled as ions such as phosphate, sulphate or chloride, and elongated features, which have been modelled as detergent molecules[13,31–35]. This raises an intriguing question: is this molecule of cardiolipin always associated with the crystallized reaction centre? If it is, what factors dictate whether all or only part of the electron density attributable to this lipid is resolved? Obvious candidates include the resolution and completeness of the diffraction data, and data collection conditions such as the length of time that the crystal(s) are exposed to the X-ray beam (reaction centre crystals are prone to radiation damage) and the use of cryo-cooling in some studies. Alternately, if the cardiolipin is not always present, what conditions during the extraction, purification and crystallization of the protein affect the occupancy of this lipid binding site, and what is the lipid replaced by?

Conclusions: the protein–lipid interface at the molecular level

The findings outlined above provide the first detailed pictures of the types of interactions that are found between the highly irregular intramembrane surfaces of

integral membrane proteins and the surrounding lipids. A common observation is that the acyl chains occupy grooves in the protein surface, where they engage in hydrophobic interactions with apolar residues and backbone atoms. These interactions are strong enough to impose sufficient order on the acyl chains of the lipid that they can be resolved in the electron density maps. In some cases the electron density shows the molecular details of how the polar head group of the lipid interacts with the surrounding protein, either directly or indirectly via solvent molecules. Finally, the results on bacteriorhodopsin and cytochrome *c* oxidase highlight the important role that lipids appear to play in mediating interactions between monomers in multimeric proteins, and this might be a widespread phenomenon. In this regard, it is intriguing to note that this role is not restricted to integral membrane proteins. A recent X-ray crystal structure of a dimer of the soluble protein catechol 1,2-dioxygenase revealed the presence of two phospholipids in a 'hydrophobic tunnel' located along the axis of the dimer[36].

To date, we do not have any information on the role of the cardiolipin molecule that is located on the intra-membrane surface of the reaction centre. However, there is some evidence that the membrane-bound form of the *Rb. sphaeroides* reaction centre is a dimer[37,38]. Therefore, it needs to be tested whether the cardiolipin molecule forms part of the contact surface between the monomers in such a structure. However, it is possible that lipids such as this cardiolipin bind to the protein surface only after natural intramembrane protein–protein contacts have been broken during purification. As yet, we have a relatively poor understanding of the extent and nature of direct contacts between proteins that interact within membranes, such as reaction centres and their attendant light harvesting complexes[38]. It remains to be seen whether these direct contact surfaces are extensive or, as illustrated by the structure of the bacteriorhodopsin trimer, the main contacts are mediated by lipid molecules located at the protein–protein interface. The advantage of using lipid molecules to form a significant part of the contact surface between adjacent proteins is that they have a high degree of conformational flexibility, and are usually available in a range of molecular shapes and sizes. Using lipids as a flexible 'jointing material' reduces the need to sculpt interacting protein surfaces in a highly specific, complementary manner. The minimization of direct protein–protein contacts by interposing lipids might be important as a 'lubricant' to ensure sufficient mobility of proteins in membranes.

The resolution of lipid binding at the level of detail outlined in this article provides the first opportunities to design mutagenesis experiments that can probe the importance of specific protein–lipid contacts for the structural and functional integrity of membrane proteins. Mutagenesis has been used to identify residues responsible for interactions between soluble proteins and anionic phospholipids (Refs 39,40), but these studies have not been guided by structural information on the actual protein–lipid contact. Using the new crystallographic information outlined above, it will be interesting to examine the effects of disrupting the specific interaction between squalene and bacteriorhodopsin, or the binding of specific cardiolipin molecules to cytochrome oxidase. In the case of the reaction centre, the obvious next step will be to remove the histidine and arginine residues that bond to the head-group of the cardiolipin, and to examine the consequences for assembly, stability and function of the reaction centre. Studies to this end are underway. It might be that removal of these bonding interactions will prevent the binding of cardiolipin at this position on the surface of the reaction centre. However, this is far from certain, because a clear message that comes out of the work summarized in this article is the strength and importance of hydrophobic interactions between the intra-membrane surface of the protein and the acyl chains of the adjacent lipids. The detailed structural information now becoming available will provide opportunities for examining the consequences of disrupting these hydrophobic interactions in a highly controlled manner. Finally, it will be interesting to see whether the extent and characteristics of the boundary layer of lipids can be better defined through crystallographic studies employing lipids that have been labelled with heavy atoms.

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References

1 Ioannou, P.V. and Golding B.T. (1979) Cardiolipins: their chemistry and biochemistry. *Prog. Lipid Res.* 17, 279–318
2 Hoch, F.L. (1992) Cardiolipins and biomembrane function. *Biochim. Biophys. Acta* 1113, 71–133
3 Dowhan, W. (1997) Molecular basis for membrane phospholipid diversity: Why are there so many lipids? *Annu. Rev. Biochem.* 66, 199–232
4 Lee, A.G. (1998) How lipids interact with an intrinsic membrane protein: the case of the calcium pump. *Biochim. Biophys. Acta*, 1376, 381–390
5 Epand, R.M. (1998) Lipid polymorphism and protein–lipid interactions. *Biochim. Biophys. Acta*, 1376, 353–368
6 Awasthi, Y.C. *et al.* (1971) Tightly bound cardiolipin in cytochrome oxidase. *Biochim. Biophys. Acta* 226, 42–52
7 Robinson, N.C. (1982) Specificity and binding-affinity of phospholipids to the high-affinity cardiolipin sites of beef-heart cytochrome-*c* oxidase. *Biochemistry* 21, 184–188
8 Hoffman, B. *et al.* (1994) The reconstituted ADP/ATP carrier activity has an absolute requirement for cardiolipin as shown in cysteine mutants. *J. Biol. Chem.* 269, 1940–1944
9 Munro, S. (1998) Localization of proteins to the Golgi apparatus. *Trends Cell Biol.* 8, 11–15
10 Marsh, D. and Horvath, L.I. (1998) Structure, dynamics and composition of the lipid-protein interface. Perspectives from spin-labelling. *Biochim. Biophys. Acta* 1376, 267–296
11 Hoff, A.J. and Deisenhofer, J. (1997) Photophysics of photosynthesis. Structure and spectroscopy of reaction centers of purple bacteria. *Phys. Rep.* 287, 1–247
12 Allen, J.P. *et al.* (1987) Structure of the reaction center from *Rhodobacter sphaeroides* R-26 – the cofactors. *Proc. Natl. Acad. Sci. U. S. A.* 84, 5730–5734
13 Ermler, U. *et al.* (1994) Structure of the photosynthetic reaction-center from *Rhodobacter sphaeroides* at 2.65-angstrom resolution – cofactors and protein-cofactor interactions. *Structure* 2, 925–936
14 Roth, M. *et al.* (1989) Detergent structure in crystals of a bacterial photosynthetic reaction center. *Nature* 340, 659–662
15 Roth, M. *et al.* (1991) Structure of the detergent phase and protein-detergent interactions in crystals of the wild-type (strain-Y) *Rhodobacter sphaeroides* photochemical-reaction center. *Biochemistry* 30, 9403–9413
16 McAuley, K.E. *et al.* (2000) Structural details of an interaction between cardiolipin and an integral

membrane protein. *Proc. Natl. Acad. Sci. U. S. A.* 96, 14706–14711
17 Russell, N.J. and Harwood, J.L. (1979) Changes in the acyl lipid composition of photosynthetic bacteria grown under photosynthetic and non-photosynthetic conditions. *Biochem. J.* 181, 339–345
18 Lanyi, J.K. (1997) Mechanism of ion transport across membranes – bacteriorhodopsin as a prototype for proton pumps. *J. Biol. Chem.* 272, 31209–31212
19 Oesterhelt, D. (1998) The structure and mechanism of the family of retinal proteins from halophilic archaea. *Curr. Opin. Struct. Biol.* 8, 489–500
20 Joshi, M.K. *et al.* (1998) Importance of specific native lipids in controlling the photocycle of bacteriorhodopsin. *Biochemistry* 37, 14463–14470
21 Grigorieff, N. *et al.* (1996) Electron-crystallographic refinement of the structure of bacteriorhodopsin. *J. Mol. Biol.* 259, 393–421
22 Mitsuoka, K. *et al.* (1999) The structure of bacteriorhodopsin at 3.0 angstrom resolution based on electron crystallography: implication of the charge distribution. *J. Mol. Biol.* 286, 861–882
23 Essen, L-O. *et al.* (1998) Lipid patches in membrane protein oligomers: crystal structure of the bacteriorhodopsin-lipid complex. *Proc. Natl. Acad. Sci. U. S. A.* 95, 11673–11678
24 Belrhali, H. *et al.* (1999) Protein, lipid and water organization in bacteriorhodopsin crystals: a molecular view of the purple membrane at 1.9 angstrom resolution. *Structure* 7, 909–917
25 Sato, H. *et al.* (1999) Specific lipid–protein interactions in a novel honeycomb lattice structure of bacteriorhodopsin. *Acta Crystallogr. D*, 55, 1251–1256
26 Luecke, H. *et al.* (1999) Structure of bacteriorhodopsin at 1.55 angstrom resolution. *J. Mol. Biol.* 291, 899–911
27 Tsukihara, T. *et al.* (1996) The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 angstrom. *Science* 272, 1136–1144
28 Mizushima, T. *et al.* (1999) Structure of phospholipids in a membrane protein complex, bovine heart cytochrome *c* oxidase. *Acta Crystallogr.* A55 (Suppl.) Abstr. P06.04.069
29 Harrenga, A. and Michel, H. (1999) The cytochrome *c* oxidase from *Paracoccus denitrificans* does not change the metal center ligation upon reduction. *J. Biol. Chem.* 274, 33296–33299
30 Ferguson, A.D. *et al.* (2000) A conserved structural motif for lipopolysaccharide recognition by procaryotic and eucaryotic proteins. *Structure* 8, 585–592
31 Deisenhofer, J. *et al.* (1995) Crystallographic refinement at 2.3-angstrom resolution and refined model of the photosynthetic reaction-center from *Rhodopseudomonas viridis*. *J. Mol. Biol.* 246, 429–457
32 Lancaster, C.R.D. and Michel, H. (1997) The coupling of light-induced electron transfer and proton uptake as derived from crystal structures of reaction centres from *Rhodopseudomonas viridis* modified at the binding site of the secondary quinone, Q(B). *Structure* 5, 1339–1359
33 Stowell, M.H.B. *et al.* (1997) Light-induced structural changes in photosynthetic reaction center: implications for mechanism of electron-proton transfer. *Science* 276, 812–816
34 McAuley-Hecht, K.E. *et al.* (1998) Structural studies of wild-type and mutant reaction centers from an antenna-deficient strain of Rhodobacter sphaeroides: monitoring the optical properties of the complex from bacterial cell to crystal. *Biochemistry* 37, 4740–4750
35 Axelrod, H.L. *et al.* (2000) Determination of the binding sites of the proton transfer inhibitors Cd^{2+} and Zn^{2+} in bacterial reaction centers. *Proc. Natl. Acad. Sci. U. S. A.* 97, 1542–1547
36 Vetting, M.W. and Ohlendorf, D.H. (2000) The 1.8 angstrom crystal structure of catechol 1,2-dioxygenase reveals a novel hydrophobic helical zipper as a subunit linker. *Structure* 8, 429–440
37 Francia, F. *et al.* (1999) The reaction center-LH1 antenna complex of *Rhodobacter sphaeroides* contains one PufX molecule which is involved in dimerization of this complex. *Biochemistry* 38, 6834–6845
38 Loach, P.A. (2000) Supramolecular complexes in photosynthetic bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 97, 5016–5018
39 Mehdi, H. *et al.* (2000) A hydrophobic sequence at position 313-316 (Leu-Ala-Phe-Trp) in the fifth domain of apolipoprotein H (beta(2)-glycoprotein I) is crucial for cardiolipin binding. *Eur. J. Biochem.* 267, 1770–1776
40 Makise, M. *et al.* (2000) Identification of amino acids involved in the functional interaction between DnaA protein and acidic phospholipids. *J. Biol. Chem.* 275, 4513–4518
41 Nicholls, A. *et al.* (1991) Protein folding and association – insights from the interfacial and thermodynamic properties of hydrocarbons. *Protein Struct. Funct. Genet.* 11, 281–296
42 Christopher, J.A. (1998) *SPOCK: The Structural Properties Observation and Calculation Kit*, The Center for Macromolecular Design, Texas A&M University
43 Merritt, E.A. and Murphy, M.E.P. (1994) Raster3D Version 2.0: a program for photorealistic molecular graphics. *Acta Crystalogr. D*, 50, 869–873

A mitochondrial perspective on cell death

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The role of mitochondria as crucial participants in cell death programs is well established, yet the mechanisms responsible for the release of mitochondrial activators and the role of BCL2 family proteins in this process remain controversial. Here, we point out the limitations of current approaches used to monitor the physiological responses of mitochondria during cell death, the implications arising from modern views of mitochondrial structure, and briefly assess two proposed mechanisms for the release of mitochondrial proteins during apoptosis.

Mitochondria are essential for cellular energy metabolism and Ca^{2+} homeostasis[1]. It is therefore not surprising that mitochondrial dysfunction can cause cell death through ATP depletion and Ca^{2+} dysregulation[2]. However, the picture is more complex owing to the role played in cell death by proteins released from mitochondria such as cytochrome *c* (Ref. 3), apoptosis-inducing factor (Ref. 4) and smac–diablo (Refs 5,6), a process that can be modulated by regulated targeting of BCL2 family members to the outer mitochondrial membrane[7] (OMM). In general terms, antiapoptotic members of the BCL2 family tend to inhibit the release of mitochondrial proteins, whereas proapoptotic members (such as BID, BAX and BAK; see Fig. 1)[7] favor release. The mechanism(s) for cytochrome *c* release, as well as the mechanistic link(s) with BCL2 proteins have not been solved[8]. It is reasonable to ask whether the controversies, in particular about the role of the permeability transition (PT) and voltage-dependent anion channel (VDAC), arise because of differences in the experimental models of cell death or because the interpretation of the results is not as unequivocal as it appears, or both. These questions

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