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CHAPTER 12

Transmembrane α Helices

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I. INTRINSIC MEMBRANE PROTEINS

Intrinsic membrane proteins span their membranes as α helices or as β sheets, driven by the requirement to maximize the number of hydrogen bonds. The cost of transferring a peptide bond from water into a nonpolar environment is about 25 kJ mol^{-1} when not hydrogen-bonded, but only about 2.4 kJ mol^{-1} when hydrogen-bonded (Roseman, 1988). Breaking the hydrogen bonds in a 20-residue α helix in a nonpolar environment would cost about 400 kJ mol^{-1}. This high cost can be avoided if the membrane-spanning region of a membrane protein is organized as an α helix or as a β sheet, because in these structures the peptide backbone can form hydrogen bonds internally.

Formation of β sheets is seen in bacterial outer membrane proteins, which span the membrane as β barrels. Proteins in other membranes span the lipid bilayer as right-handed α helices. An ideal α helix has 3.6 residues per turn and a translation per residue of 1.50 Å. With a thickness for the hydrocarbon core of a lipid

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bilayer of 30 Å, about 20 residues will be required to span the core of the bilayer. The residues in the transmembrane region will be predominantly hydrophobic. However, for membrane proteins such as transporters and ion channels the transmembrane α helices will also have to contain polar groups; the transmembrane α helices will then be amphipathic rather than just hydrophobic. An extreme case could be a transmembrane α helix totally surrounded by other α helices in the center of a helical bundle: Such a helix would not need to be hydrophobic at all, because it is not in direct contact with the lipid bilayer. However, although small in number, the available high-resolution structures for membrane proteins show no evidence for the presence of such purely hydrophilic transmembrane α helices. It may be that the process of insertion of membrane proteins into the membrane during biogenesis requires all the transmembrane α helices to be relatively hydrophobic.

Analysis of the compositions of a large number of membrane proteins predicted to contain single transmembrane α helices has shown that the amino acid composition of a transmembrane α helix is distinctly different from that of hydrophobic α helices in water-soluble proteins. As expected, hydrophobic residues make up the bulk of the residues, the most common being Leu (Landolt-Marticorena *et al.*, 1993; Wallin *et al.*, 1997). Amino acids essentially excluded are the basic (Arg and Lys) and acidic (Asp and Glu) amino acids and their amide counterparts (Asn and Gln). Transmembrane α helices are, however, relatively rich in bulky residues, such as Ile, Val, and Thr, which, in water-soluble proteins, are classed as membrane destabilizers (their bulky side chains interfere sterically with the carbonyl oxygen in the preceding turn of the α helix and thus destabilize the helical conformation). Thus, factors such as residue volume and packing, which are important in determining helix stability in water-soluble proteins, are not so important for transmembrane α helices, at least for membrane proteins containing single transmembrane α helices; effects of large residue volume will, in the membrane, be balanced by the favorable hydrophobic interactions of a large side chain with the fatty acyl chains. In water-soluble proteins, the conformationally flexible Gly residue is also classed as a helix breaker, because it is an intrinsically flexible residue with the potential to adopt most of the dihedral angles available in a Ramachandran plot. The observation that Gly is quite common in transmembrane α helices suggests that its potential flexibility is constrained in the bilayer environment. Because Gly possesses the smallest of all the side chains, it may play a role in mediating helix–helix interactions and packing in the membrane. The polar amino acids most commonly found within transmembrane α helices are Cys, Thr, and Ser. These residues can be stabilized within a hydrophobic environment by hydrogen bonding between their polar side chains and the peptide backbone at positions $i - 3$ and $i - 4$ (Eilers *et al.*, 2000).

Figure 1 shows the positional preferences of the residues in the transmembrane α helices of type I membrane proteins with a single transmembrane α helix oriented

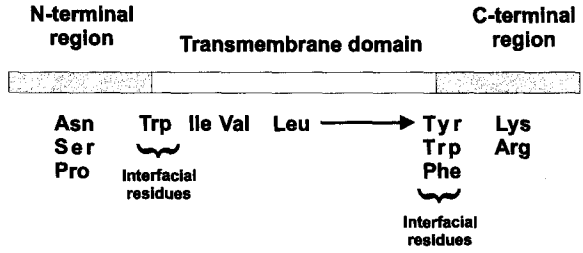

FIGURE 1 Positional preferences for amino acids in the transmembrane domains of human type I membrane proteins with single transmembrane α helices. Modified from Landolt-Marticorena *et al.* (1993).

with its C-terminus on the cytoplasmic side of the membrane (Landolt-Marticoreno *et al.*, 1993). The amino-terminal end of the transmembrane domain contains an Ile-rich region followed by a Val-enriched region. The carboxyl-terminal half of the transmembrane α helix is Leu-rich. Ala is found randomly distributed throughout the transmembrane domain. Aromatic residues are found located preferentially in the boundary regions, with Trp at either end of the transmembrane domain, but with Tyr and Phe only at the carboxyl-terminal boundary. Unlike the other aromatic amino acids, Phe, is also found in the hydrophobic segment as well as in the boundary region.

The polar regions flanking the transmembrane domain are enriched in Arg and Lys on the C-terminal side; Asn, Ser, and Pro are enriched in the N-terminal flanking region. The presence of a positively charged C-terminus (cytoplasmic) could play a role in the process of insertion into the membrane, according to the inside positive rule of von Heijne (1996). The presence of particular residues at the N- and C-terminal ends of the helices could also be important in meeting the requirement to "cap" the ends of the α helices; the initial four −NH and final four −C=O groups of an α helix have no hydrogen-bonding partners provided by the peptide backbone of the α helix itself, and so suitable hydrogen-bonding partners have to be provided in some other way. One way is to extend the helix by three or four residues at each end with polar residues containing suitable hydrogen-bonding partners such as Pro and Asn. Alternatively, if the hydrophobic, nonpolar residues in the transmembrane α helix extend into the headgroup region, hydrogen bonds could form with suitable groups in the glycerol backbone and headgroup regions of the lipid bilayer. Either way, if about 20 residues are required to span the hydrophobic core of the bilayer, the total helix length could be up to 28 residues. The result is that there is a degree of indeterminacy in where the ends of transmembrane α helices should be drawn; the precise ends of transmembrane α helices are often not known.

The observed preference for Trp and Tyr residues for the ends of transmembrane α helices agrees with measurements of the binding of small peptides at the

lipid–water interface, which show that aromatic residues have a preference for the interface (Wimley and White, 1996). Further, a number of small tryptophan analogues have been shown to bind in the glycerol backbone and lipid headgroup region of the bilayer, stabilized partly by location of the aromatic ring in the electrostatically complex environment provided by this region of the bilayer, and partly by exclusion of the flat, rigid ring system from the hydrocarbon core of the bilayer for entropic reasons (Yau *et al.*, 1998). Thus, although it is agreed that aromatic residues at the ends of transmembrane α helices probably act as "floats" at the interface serving to fix the helix within the lipid bilayer, it is unclear whether the aromatic rings are located in the hydrocarbon or the headgroup region of the bilayer. This uncertainty is also apparent in the crystal structures of a number of membrane proteins. For example, the Trp residues in the bacterial potassium channel KcsA (Doyle *et al.*, 1998) are found clustered at the ends of the transmembrane α helices, forming clear bands on the two sides of the membrane, as shown in Fig. 2. However, the Tyr residues clearly form a band on the periplasmic side of the membrane above the band formed by the Trp residues. Similarly, in the bacterial photosynthetic reaction center (Rees *et al.*, 1994) the majority of the Trp residues are found near the periplasmic side of the protein near the ends of the transmembrane α helices, as shown in Fig. 3. However, the band of Trp residues is more diffuse than in KcsA, and some Trp residues are likely to be located in the hydrocarbon core and some in the headgroup region. The average number of

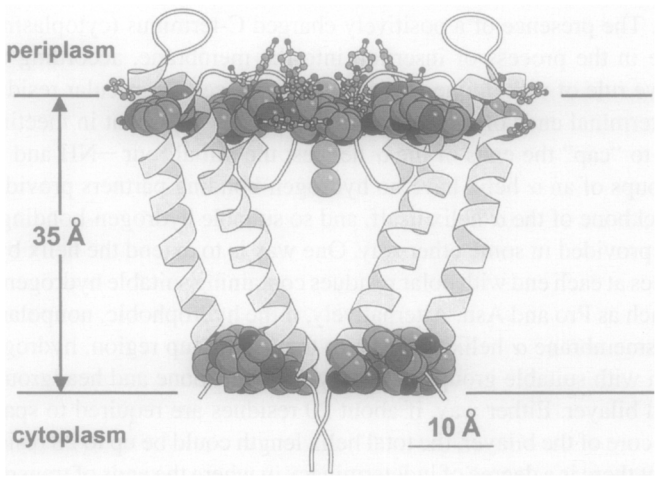

FIGURE 2 The crystal structure of the potassium channel KcsA. A cross section with just two of the four identical subunits is shown. Trp residues are shown in space-fill representation and Tyr residues are shown in ball-and-stick representation. Two potassium ions in space-fill representation are shown moving through the channel. The separation between the two planes representing the outer edges of the Trp residues is 35 Å. (Protein Data Bank [PDB] file 1bl8.)

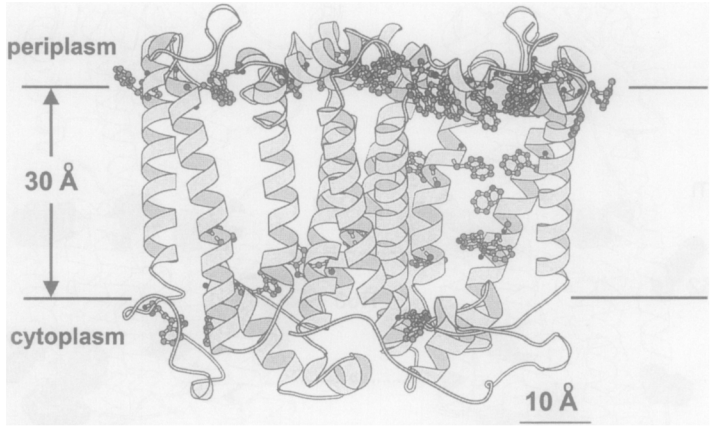

FIGURE 3 The structure of the L and M subunits of the photosynthetic reaction center of *Rhodobacter sphaeroides*. Trp residues are shown in ball-and-stick representation. An approximate location for the hydrophobic core of the bilayer of thickness 30 Å is shown, as defined by the surface covered by detergent. (PDB file 1aij.)

residues in the transmembrane α helices of the bacterial photoreaction center is 26, corresponding to a length of about 39 Å. The stretch of hydrophobic residues in these helices is, however, only about 19 amino acids or about 28.5 Å long (Ermler *et al.,* 1994; Michel and Deisenhofer, 1990). This matches the thickness of the nonpolar region of the complex (about 30 Å) as defined experimentally as the part covered by detergent in the crystal (Roth *et al.,* 1989, 1991). Detergent is seen to cover some of the Trp residues on the periplasmic side of the membrane, but not others (Roth *et al.,* 1991). The distribution of Trp residues on the cytoplasmic side of the complex is much less distinct than on the periplasmic side (Fig. 3). If the hydrocarbon core of the bilayer around the complex does have a thickness of 30 Å, then again the Trp residues on the cytoplasmic side of the membrane will be located in both the hydrocarbon core and the headgroup regions of the bilayer (Fig. 3).

In the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum the situation is more complex, as shown in Fig. 4 (Toyoshima *et al.,* 2000). Many of the transmembrane α helices extend above the membrane surface to form a central stalk linking the transmembrane region to the cytoplasmic head of the protein. As a consequence some of the helices are very long; helix M5, for example, contains 41 residues. A ring of Trp residues can be seen on the cytoplasmic side of the membrane helping to define the location of the membrane surface (Fig. 4). A Lys residue (Lys-262) in transmembrane α helix M3 can be seen pointing up from the hydrophobic core of the bilayer like a snorkel. Because the cost of burying a charged residue in the hydrophobic core of a bilayer is very high (about

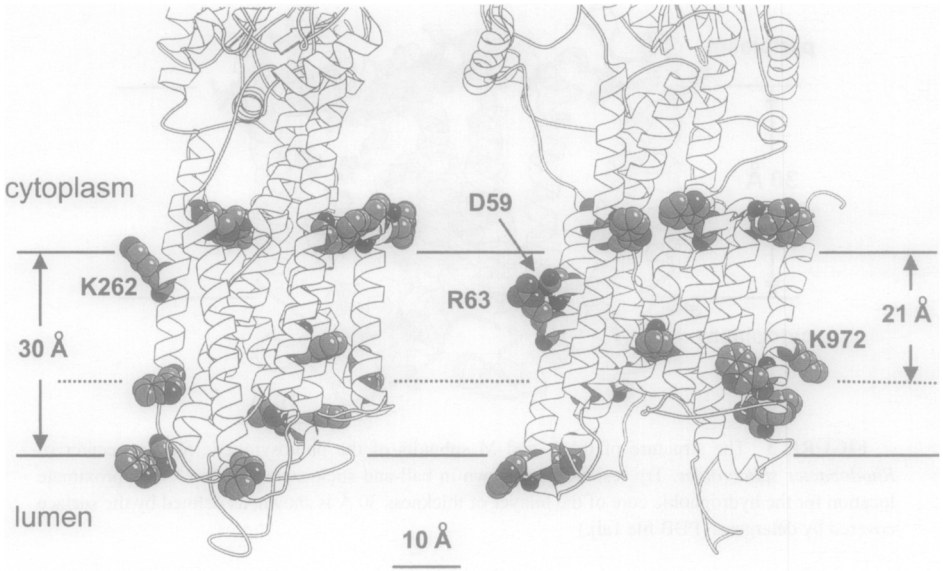

FIGURE 4 The transmembrane region of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum. Two views are shown. The view on the left shows the location of Lys-262 in transmembrane α helix M3 pointing up toward the cytoplasmic membrane surface. The view on the right shows Lys-972 in M10 pointing down toward the luminal membrane surface and Asp-59 stacked against Arg-63 to form an ion pair. A possible location for the cytoplasmic surface and two possible locations for the luminal surface are shown. (PDB file 1eul.)

37 kJ mol^{-1} for a Lys residue; Engelman *et al.*, 1986), it is likely that the amino group on Lys-262 will be located at the interface; the Trp residues in the Ca^{2+}-ATPase will then be located in the headgroup region of the bilayer. The structure of the Ca^{2+}-ATPase is also unusual in that the first transmembrane α helix contains two polar residues, Asp-59 and Arg-63, pointing out into the hydrocarbon core; presumably, stacking of Asp-59 against Arg-63 allows formation of an ion pair.

The distribution of Trp residues on the lumenal face of the Ca^{2+}-ATPase is much more diffuse than on the cytoplasmic side. The hydrophobic thickness of the Ca^{2+}-ATPase could be expected to be about 30 Å, because that is the hydrophobic thickness of a bilayer of di(C18:1)PC,[1] the phospholipid that supports highest activity for the ATPase (East and Lee, 1982). However, as shown in Fig. 4, this definition locates the lumenal loops between transmembrane α helices M5 and M6

[1]Phospholipid designations are PC, PS, and PA for phosphatidylcholine, phosphatidylserine, and phosphatidic acid, respectively. Fatty acyl chains are given in the format *m*:*n*, where *m* is the number of carbon atoms and *n* is the number of double bonds. Thus, for example, dioleoylphosphatidylcholine is di(C18:1)PC.

and between M9 and M10 within the hydrocarbon core. Further, it locates a Lys residue (Lys-972) totally within the hydrocarbon core, which seems unlikely. The hydrophobic thickness of the bilayer would have to be about 21 Å to locate the two interhelical loops and Lys-972 at the lumenal surface (Fig. 4); this is close to the thickness of a bilayer of di(C14:1)PC (22 Å). The crystal structure shown in Fig. 4 corresponds to the Ca^{2+}-bound, E1 conformation of the ATPase. It has been shown that the E1 conformation of the ATPase is favored by di(C14:1)PC, whereas di(C18:1)PC favors the other major conformation of the ATPase, E2 (Starling *et al.*, 1994). Thus, it is possible that conformational changes within the transmembrane region of the Ca^{2+}-ATPase lead to changes in the interhelical loops and thus to changes in the effective hydrophobic thickness of the ATPase (Lee and East, 2001).

II. EFFECTS OF INTRINSIC MEMBRANE PROTEINS ON LIPID BILAYERS

Incorporation of a protein into a lipid bilayer can be expected to have significant effects on the properties of the bilayer. The rough surface presented by a protein to the surrounding lipid bilayer will tend to produce poor packing unless the lipid fatty acyl chains distort to match the surface of the protein. In a liquid crystalline bilayer the lipid fatty acyl chains are disordered, because the chains undergo extensive wobbling fluctuations. The presence of a rigid protein surface would be expected to reduce the extent of these motional fluctuations. However, the chains will have to tilt and become conformationally disordered to maximize contact with the rough surface of the protein. The net result is that the presence of a protein will lead to decreased order for the chains, with a wide range of chain orientations relative to the bilayer normal, but with reduced extent and rate of motion. Because of the reduced motion, lipids adjacent to membrane proteins are often referred to as being motionally restricted.

It is clear, therefore, that the reasons for the disorder of the bulk lipids and the disorder of the lipids adjacent to the protein (the boundary or annular lipids) are different; for the bulk phospholipids the disorder is dynamic, whereas for the boundary lipids the disorder is static. An example is provided by the bacteriorhodopsin trimer, whose crystal structure is unusual in showing a few well-defined lipid molecules (Belrhali *et al.*, 1999; Luecke *et al.*, 1999). Figure 5 shows some of the lipids located at the surface of the trimer. The electron densities for the chains are well defined, but the headgroups are disordered, so that the headgroups could not be identified; the lipids were therefore modeled simply as 2,3-di-*O*-phytanyl-*sn*-propane (Belrhali *et al.*, 1999). The considerable static disorder of the chains is clear in Fig. 5, the rotational disorder of the chains being necessary to obtain good van der Waals contacts with the molecularly rough surface of the bacteriorhodopsin trimer. Lipids on the extracellular side of the membrane are better resolved than

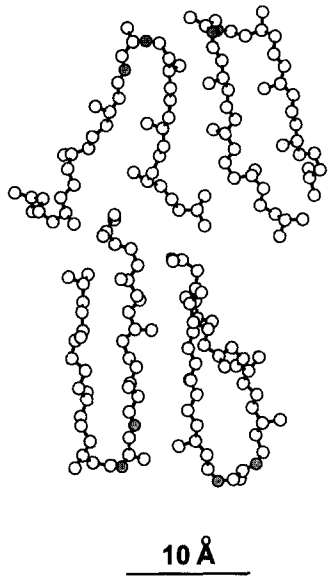

FIGURE 5 Structures of four phospholipid molecules identified in X-ray crystallographic studies of bacteriorhodopsin (Belrhali *et al.*, 1999). The lipids have been modeled as 2,3-di-*O*-phytanyl-*sn*-propane. (PDB file 1qhj.)

those on the cytoplasmic side; the degree of order of the lipids parallels that of the protein, which is also greater on the extracellular side (Grigorieff *et al.*, 1996). The average distance between the glycerol backbone oxygens for phospholipids on the two sides of the membrane was 31.6 Å (Mitsuoka *et al.*, 1999). As expected, this closely matches the hydrophobic length of the transmembrane helices of bacteriorhodopsin; the mean helix length is 23 residues, corresponding to a length of about 35 Å.

The crystal structure also makes clear the very different conformations adopted by the various lipid molecules located on the surface of the trimer. For example, one lipid molecule forms a hydrogen bond from its ether oxygens to a tyrosine —OH group at the end of a transmembrane α helix (Fig. 6; Belrhali *et al.*, 1999; Essen *et al.*, 1998). The result is that the strength of the interactions of individual boundary lipid molecules with the protein will be different.

The disorder of the chains seen in Fig. 6 is consistent with the results of molecular dynamics simulations of the bacteriorhodopsin trimer in a bilayer of diphytanyl phosphatidylglycerophosphate (Edholm *et al.*, 1995). The molecular dynamics simulation agrees with experiment in predicting higher order for both the lipids and the protein on the extracellular side of the membrane; fluctuations in the loops and the ends of helices on the cytoplasmic side of the membrane are greater than on the extracellular side. This is also seen in fluctuations of the lipids, with lipids on

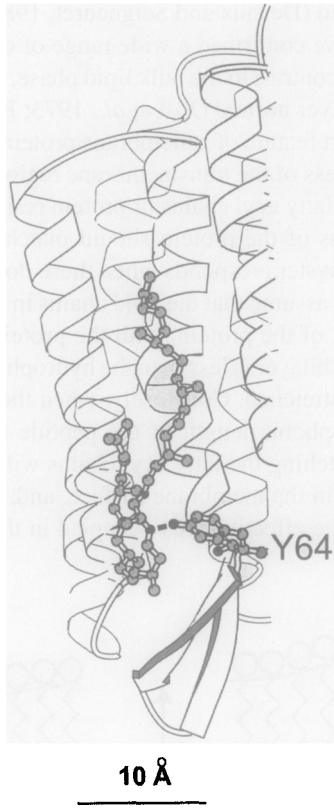

FIGURE 6 Hydrogen bonding in lipid–protein interactions. Tyr-64 at the end of a transmembrane α helix of bacteriorhodopsin is hydrogen-bonded to an ether oxygen of 2,3-di-*O*-phytanyl-*sn*-propane. The hydrogen bond is shown by the dash. (PDB file 1qhj.)

the cytoplasmic side of the membrane fluctuating more strongly than those on the extracellular side (Edholm *et al.*, 1995). The calculated order parameters for the chains are low, mainly due to a static tilt of the chains necessary to allow them to nestle against the rough surface of the protein. The chains in the purple membrane behave more like parts of the protein than parts of a fluid lipid phase, consistent with the idea of boundary lipids (Edholm *et al.*, 1995).

The boundary lipids and the bulk lipids in a membrane can be distinguished experimentally in many systems, because of the static disorder of the boundary lipids and the dynamic disorder of the bulk lipids. Static and dynamic disorder give rise to very different electron spin resonance (ESR) spectra for spin-labeled lipids, and ESR spectra for membrane protein systems usually show two components, one "immobilized," corresponding to boundary lipid, and one relatively mobile,

corresponding to bulk lipid (Devaux and Seigneuret, 1985; Marsh, 1995). Studies with oriented samples have confirmed a wide range of orientational distributions for the boundary lipid, in contrast to the bulk lipid phase, where motion of the lipid long axis is about the bilayer normal (Jost *et al.*, 1973; Pates and Marsh, 1987).

A particularly important feature of a membrane protein as far as the lipid bilayer is concerned is the thickness of the transmembrane region of the protein. The cost of exposing hydrophobic fatty acyl chains or protein residues to water is such that the hydrophobic thickness of the protein should match that of the bilayer. The question then is how the system responds when these do not match. Most models of hydrophobic mismatch assume that the lipid chains in the vicinity of the protein adjust their length to that of the protein, with the protein acting as a rigid body. When the thickness of the bilayer is less than the hydrophobic length of the peptide the lipid chains must be stretched. Conversely, when the thickness of the bilayer is greater than the hydrophobic length of the peptide the lipid chains must be compressed (Fig. 7). Stretching the fatty acyl chains will effectively decrease the surface area they occupy in the membrane surface, and, conversely, compressing the chains will increase the effective area occupied in the surface (Fig. 7). Thus,

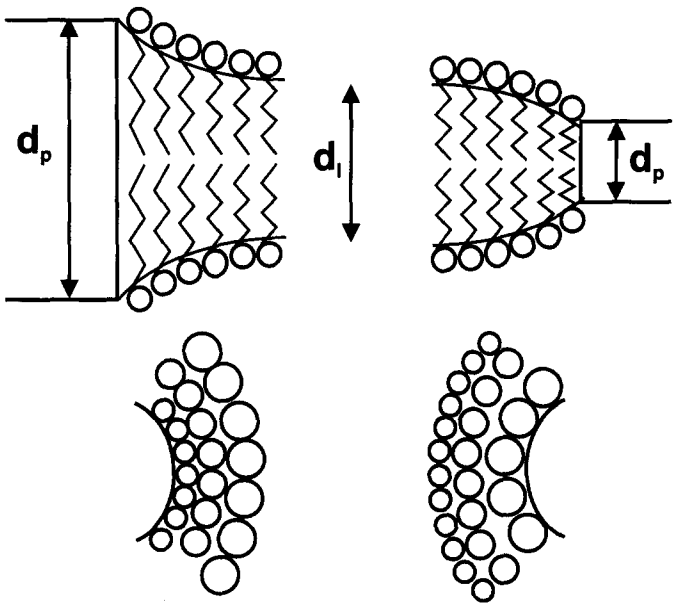

FIGURE 7 The result of a mismatch between the hydrophobic length of a peptide and the hydrophobic thickness of a lipid bilayer. Left: Positive hydrophobic mismatch ($d_P > d_L$). Right: Negative mismatch ($d_P < d_L$). The top shows a "side" view of the chain packing around the peptide and the bottom shows a top view, illustrating the variation in chain cross-sectional area with distance from the peptide. Figure based on Fattal and Ben-Shaul (1993).

changes in fatty acyl chain order are linked to changes in average interfacial areas per lipid molecule.

A number of terms have been suggested to contribute to the total free energy cost of deforming a lipid bilayer around a protein molecule (Fattal and Ben-Shaul, 1993; Nielsen *et al.*, 1998):

1. Loss of conformational entropy of the chains imposed by the presence of the rigid protein wall
2. Bilayer compression/expansion energy due to changes in the membrane thickness
3. Surface energy changes due to changes in the area of the bilayer–water interface
4. Splay energy due to changes in the cross-sectional energy available to the chains along their length, resulting from curvature of the monolayer surface near the protein

A number of models have been proposed to estimate these terms. Fattal and Ben-Shaul (1993) calculated the total lipid–protein interaction free energy as the sum of chain and headgroup terms. For the chains the loss of conformational entropy imposed by the rigid protein wall was positive (unfavorable) even for perfect hydrophobic matching. The other contribution to the chain term arose from the requirement for hydrophobic matching and the consequent stretching or compression of the chain. The term due to the headgroup region was treated as an interfacial free energy, including an attractive term associated with exposure of the hydrocarbon core to the aqueous medium and a repulsive term due to electrostatic and excluded-volume interactions between the headgroups (excluded-volume interactions signify that no two atoms can occupy the same position in space). The resulting profile of energy of interaction as a function of hydrophobic mismatch was fairly symmetrical about the point of zero mismatch. The lipid perturbation energy F (in units of kT per angstrom of protein circumference) calculated by Ben-Shaul (1995) fits to the equation

$$F = 0.37 + 0.005(d_P - d_L)^2, \tag{1}$$

where d_P and d_L are the hydrophobic thicknesses of the protein and lipid bilayer, respectively, and the unperturbed bilayer thickness is 24.5 Å. The hydrophobic thickness of a bilayer of phosphatidylcholine in the liquid crystalline phase is given by

$$d_L = 1.75(n - 1), \tag{2}$$

where n is the number of carbon atoms in the fatty acyl chain (Lewis and Engelman, 1983; Sperotto and Mouritsen, 1993). A simple, but crude calculation gives an idea of the size of the effect that can be expected from hydrophobic mismatch. It is assumed that the protein is very large and so appears flat to a lipid molecule. It is

also assumed that all the lipid perturbation energy is concentrated in the first shell of lipids around the protein. Equation (1) then shows that if a lipid occupies 6 Å of the protein circumference, the lipid–protein interaction energy would change by 3.6 kJ mol^{-1} for a hydrophobic mismatch of 7 Å, corresponding to an increase in fatty acyl chain length of 4 carbons, and by 22.8 kJ mol^{-1} for a hydrophobic mismatch of 17.5 Å, corresponding to an increase in acyl chain length of 10 carbons. Changes in interaction energies of 3.6 and 22.8 kJ mol^{-1} correspond to decreases in the lipid–protein binding constant by factors of 4.3 and 10^4, respectively. If the change in lipid–protein interaction energy were to propagate out from the protein surface to affect more than the first shell of lipids, effects of hydrophobic mismatch would be reduced. For example, if effects were averaged over three shells of lipids, changes in fatty acyl chain lengths by 4 and 10 carbons from that giving optimal interaction would decrease lipid–protein binding constants by factors of 1.6 and 21, respectively.

Energies of the magnitude calculated by Ben-Shaul (1995) are easily sufficient to result in conformational changes on a protein. If a protein conformational change results in a change in the hydrophobic thickness of the protein, the change will result in a deformation of the adjacent bilayer. Because the equilibrium constant describing the equilibrium between two conformational states of a protein is determined by the total free energy difference between the two states, the energetic cost of the membrane deformation will contribute toward the equilibrium constant.

The approach adopted by Nielsen *et al.* (1998) came to rather similar conclusions. The most important energy terms were found to be the splay energy and the compression–expansion term, the splay energy term being most important close to the protein, with the compression–expansion term being more important further from the protein. Even though the bilayer deformation was calculated to extend some 30 Å from the protein, most of the deformation was found concentrated in the component immediately adjacent to the protein.

An alternative model for mismatch is the mattress model of Mouritsen and Bloom (1984, 1993). This again expresses mismatch as two terms. The first is an excess hydrophobic free energy associated with exposing either lipid chains or the protein surface to the aqueous medium. The second is proportional to the contact area between the lipid chains and the hydrophobic surface of the protein. The calculations showed that, for a protein of hydrophobic thickness 20 Å, which matches a bilayer of di(C14:0)PC in the liquid crystalline state, the binding constant for di(C14:0)PC is about a factor of 2.5 greater than for a bilayer of di(C18:0)PC, which will give a bilayer too thick by 7 Å (Sperotto and Mouritsen, 1993). Thus, effects of mismatch calculated in this way are similar to those calculated using the approach of Fattal and Ben-Shaul (1993).

The importance of hydrophobic matching has been confirmed in a number of experimental studies (Dumas *et al.*, 1999; Killian, 1998). For example, although bacteriorhodopsin has relatively little effect on the phase transition temperatures of

di(C14:0)PC or di(C16:0)PC (Alonso *et al.*, 1982), it increases the transition temperature of di(C12:0)PC and decreases that of di(C18:0)PC (Piknova *et al.*, 1993). This is consistent with hydrophobic matching models; because di(C12:0)PC gives a too thin bilayer in the liquid crystalline phase, bacteriorhodopsin favors the gel phase, whereas di(C18:0)PC gives a too thick bilayer in the gel phase, so that bacteriorhodopsin favors the liquid crystalline phase. Hydrophobic matching could also be the explanation for the unexpected observation that, in mixtures of di(C14:0)PC and di(C18:0)PC at temperatures where the mixture contains both gel and liquid crystalline phases, bacteriorhodopsin partitions equally between the two phases (Piknova *et al.*, 1997; Schram and Thompson, 1997). This contrasts with the observed exclusion of bacteriorhodopsin from gel-phase lipid in mixtures containing single species of phospholipid (Alonso *et al.*, 1982; Cherry *et al.*, 1978). It has been suggested that this shows that the requirements of hydrophobic matching are of prime importance; the hydrophobic thickness of bacteriorhodopsin is intermediate between the hydrophobic thickness of di(C14:0)PC in the gel phase and di(C18:0)PC in the liquid crystalline phase, so that bacteriorhodopsin shows little preference between the two. In mixtures of di(C12:0)PC and di(C18:0)PC, where, at low temperatures, two separate gel phases are formed, one enriched in di(C12:0)PC and one enriched in di(C18:0)PC, bacteriorhodopsin partitions very strongly into the di(C12:0)PC-enriched domains; this could be because the hydrophobic thickness of bacteriorhodopsin is better matched to gel-state di(C12:0)PC than to gel-state di(C18:0)PC (Dumas *et al.*, 1997). However, in studies of the Ca^{2+}-ATPase of sarcoplasmic reticulum using either spin-labeled (London and Feigenson, 1981) or brominated phospholipids (East and Lee, 1982), strengths of binding of liquid crystalline-phase phospholipids to the ATPase were found to be independent of fatty acyl chain length. These results are not consistent with the expectations of hydrophobic matching theory and suggest that, in liquid crystalline bilayers, α-helical membrane proteins are not rigid, but, in fact, can distort to match the thickness of the bilayer. Such a distortion could explain why bilayer thickness affects the activity of membrane proteins such as Ca^{2+}-ATPase (Lee, 1998). The structural distortion could take the form of a change in the tilt of the transmembrane α helices with respect to the bilayer normal or could be a change in the packing of the transmembrane α helices.

III. MODEL TRANSMEMBRANE α HELICES

A. Lipid-Peptide Interactions

An alternative approach to these questions, avoiding the complexity of real membrane proteins, is to use simple model transmembrane α helices, which can be synthesized chemically in large quantity. A number of studies have used

peptides of the type Ac-K_2-G-L_n-K_2-A-amide (P_n) consisting of a long sequence of hydrophobic Leu residues capped at both the N- and C-terminal ends with a pair of charged lysine residues. The poly(Leu) region forms a maximally stable α helix, particularly in the hydrophobic environment of the lipid bilayer. The charged Lys caps were chosen both to anchor the ends of the peptides in the lipid headgroup region and to inhibit the aggregation of the peptides in the membrane. The peptide has been shown to adopt the expected α-helical structure in both liquid crystalline- and gel-phase bilayers (Davis *et al.*, 1983; Huschilt *et al.*, 1989; Zhang *et al.*, 1992a). Rates of hydrogen/deuterium exchange for the peptide P_{16} in lipid bilayers suggest that at least 80% of the peptide is in an α-helical conformation in the bilayer, meaning that the whole of the poly(Leu) core must be α-helical (Zhang *et al.*, 1992b). Rates of hydrogen/deuterium exchange were greater at the N- and C-termini of the peptides than in the middle, suggesting some unraveling of the peptide at its ends (Zhang *et al.*, 1992b).

Experiments with the peptides of this type suggest that about 15 lipid molecules are required for complete incorporation of the peptide into a bilayer of the appropriate thickness (Webb *et al.*, 1998). This agrees with estimates from molecular modeling that about 16–18 lipid molecules will be required to form a complete bilayer shell around the peptide. At molar ratios of lipid less than this, non-bilayer phases can be induced, particularly when the hydrophobic length of the peptide is less than the hydrophobic thickness of the bilayer and when the peptide contains interfacial aromatic groups (de Planque *et al.*, 1999; Killian *et al.*, 1996; Morein *et al.*, 1997).

Effects of single transmembrane α helices on lipid bilayers are likely to be less than those of a protein containing a bundle of transmembrane α helices. The cross-sectional area of a single transmembrane α helix is not much greater than that of a phospholipid molecule in the liquid crystalline phase, so that the hydrophobic surface presented to the lipid molecules is rather small. The structure of the lipid bound to bacteriorhodopsin shown in Fig. 6 shows the two chains interacting predominantly with two different transmembrane α helices. This kind of interaction will obviously not be possible with a single transmembrane α helix. Less extensive interactions between lipids and single transmembrane α helices than between lipids and membrane proteins is suggested by ESR experiments. Whereas ESR spectra of spin-labeled lipids in the presence of membrane proteins typically show two-component spectra, as described above, ESR spectra for lipid bilayers containing the peptide L_{24} and for a tryptophan-containing peptide of the type $AW_2(LA)_nW_2A$ are single-component (de Planque *et al.*, 1998; Subczynski *et al.*, 1998). This means either that the lipid fatty acyl chains are not "immobilized" on the peptide surface or that the rate of exchange between bulk and boundary lipid is fast on the ESR time scale (i.e., exchange is faster than 10^7 s^{-1}).

Effects of peptides on chain order in di(C14:0)PC or di(C16:0)PC measured using deuterium nuclear magnetic resonance (NMR) methods are small in both

the liquid crystalline and the gel phases (Davis *et al.*, 1983; de Planque *et al.*, 1998, 1999; Roux *et al.*, 1989). Thus, addition of a peptide $AW_2(LA)_7W_2A$ to bilayers of di(C12:0)PC in the liquid crystalline phase resulted in only a 1.4-Å increase in thickness (de Planque *et al.*, 1998), whereas about an 11-Å increase would be necessary for the bilayer thickness to match the hydrophobic length of the peptide. Similarly, Nezil and Bloom (1992) estimated that the peptide P_{24} increased the thickness of a bilayer of (C16:0,C18:1)PC by just 0.6 Å, despite the hydrophobic mismatch between the peptide and the lipid bilayer being ca. 10 Å. Increases in chain order caused by P_{24} in (C16:0,C18:1)PC in the liquid crystalline phase were detected using ESR, but again the effects were small (Subczynski *et al.*, 1998).

Effects of peptides on chain order will depend on the relative hydrophobic length of the peptide compared to the hydrophobic thickness of the bilayer, with long peptides decreasing order and short peptides increasing order, and such effects have been detected using infrared (IR) spectroscopy, but again effects were small (Zhang *et al.*, 1992a). Thus it appears that lipids will distort slightly to improve the match between the hydrophobic length of the peptide and the hydrophobic thickness of the bilayer, but the extent of these modifications is very limited and much less than required to produce full matching.

A number of studies have been published on the effects of these peptides on the phase transition properties of lipid bilayers. Addition of the peptide P_{16} to bilayers of di(C16:0)PC both broadens the main gel-to-liquid crystalline phase transition and decreases the enthalpy of the transition (Morrow *et al.*, 1985). Similar effects have been seen on incorporation of membrane proteins such as bacteriorhodopsin and Ca^{2+}-ATPase (Alonso *et al.*, 1982; Gomez-Fernandez *et al.*, 1980). The decrease in enthalpy of the transition has often been taken to mean that the lipids adjacent to the protein (the boundary lipids) are very strongly perturbed by the peptide and so are unable to take part in the normal phase transition: They are effectively withdrawn from the transition. However, deuterium NMR spectra of mixtures of P_{24} and di(C16:0)PC above and below the phase transition are typical of liquid crystalline- and gel-phase lipids, respectively, with no evidence for any "special" lipid unable to take part in the phase transition (Huschilt *et al.*, 1985). Similarly, as already described, ESR spectra of spin-labeled lipids show the presence of a single type of lipid in the system, not separate bulk and boundary lipids in slow exchange (Subczynski *et al.*, 1998). Thus the peptides (or proteins) do not remove lipid from the main transition, but, rather, perturb the whole lipid bilayer. The peptide decreases the enthalpy difference between the liquid crystalline and gel phases, whereas the lipids in the bilayer remain recognizably liquid crystalline or gel (Morrow *et al.*, 1985).

Morrow *et al.* (1985) showed that mixtures of lipids and peptides can be modeled in terms of regular solution theory (Lee, 1978). Unfortunately, the number of free parameters in fitting to regular solution theory is high, so that little useful information is obtained from the analysis, apart from showing that the data are

consistent with regular solution theory. Effects of peptides or proteins on phase transition properties have therefore been interpreted qualitatively in terms of a two-component model in which one component is more or less unperturbed bulk lipid and the other is highly perturbed boundary lipid, which undergoes a broad phase transition of low enthalpy; this approach works for peptides of the poly(Leu) type, but, for some reason, does not work with peptides of the $K_2(LA)_nK_2$ type (Zhang *et al.*, 1992a, 1995). Differential scanning calorimetry (DSC) thermograms for mixtures with the poly(Leu) peptides have been fitted to two components, attributed to the phase transitions of peptide-free and boundary lipid, respectively. The phase transition temperature for the peptide-free component is slightly less than that for pure lipid; this is probably due to the normal colligative effects that will follow from mixing the "pure" lipid phase with the boundary lipid, the latter acting as an "impurity." The phase transition temperature for the boundary lipid is higher than the bulk transition temperature for short-chain lipids, but is lower for long-chain lipids (Zhang *et al.*, 1992a). The same observation has been made for membrane proteins (Dumas *et al.*, 1999; Piknova *et al.*, 1993). This is consistent with the idea that short fatty acyl chains have to stretch to match the hydrophobic thickness of the membrane protein, whereas long fatty acyl chains have to compress. However, the experimental changes are much smaller than expected from models of hydrophobic matching.

Further insights into how transmembrane α helices might interact with lipid molecules in a bilayer have come from molecular dynamics simulations. One study was of a transmembrane α helix of 32 alanine residues in a bilayer of di(C14:0)PC in the liquid crystalline state (Shen *et al.*, 1997). The peptide is not an ideal model for a transmembrane α helix, because it lacks charged groups at each end to interact with the polar headgroups of the phospholipids. Nevertheless many features of the simulation are informative. The simulation was started with the peptide as a pure α helix. The central 15 residues (Ala-12–26), which interacted just with the lipid fatty acyl chains, remained as a stable α helix. The N- and C-terminal regions of the α helix, located in the lipid headgroup region, were less stable and fluctuated more, because of transient hydrogen bonding between the peptide bond amide hydrogen and the phosphate or fatty acyl ester oxygen atoms and the water; as a result, the ends of the helices become frayed. The length of the central helical region oscillated slightly about a 22-Å average expected for an α helix, varying between 20 and 23 Å.

The helix was tilted up to 30° with respect to the bilayer normal. Because the helix contains no resides that would make strong contacts in the headgroup region or with water, there is no reason for it not to tilt (Shen *et al.*, 1997). Tilting in fact allows more hydrophobic contact by allowing more of the Ala residues to be located in the core of the bilayer. The presence of the peptide had little effect on the calculated properties of the bilayer. The average bilayer thickness was not significantly changed, although the average order parameter for the CH_2 groups in the chains decreased in the presence of the peptide.

Many different lipid molecules contributed to the immediate surroundings of the peptide (Shen *et al.*, 1997). Even if the fatty acyl chain of a particular lipid was immediately adjacent to the peptide, the headgroup of the lipid could be a substantial distance away. Given the diameter of the helix and the size of the phosphate group, a phosphate immediately adjacent to the helix would be between 8 and 10 Å away from the center of the helix (Shen *et al.*, 1997). On average, five lipid molecules having their chains adjacent to the helix also had their headgroups adjacent, using this definition. However, the headgroup, as given by the position of the phosphorus atom, could be up to 16–18 Å away. Since the average distance between lipid headgroups is 8 Å, this puts these lipids in the "second" shell around the peptide. Other lipids existed between these extremes, suggesting a very diffuse environment around the protein rather than a discrete set of well-ordered shells of lipid. Only rarely did an entire lipid molecule pack tightly around the helix. The shell around the helix contains contributions from a large number of lipid molecules, each contributing a small number of atoms (Shen *et al.*, 1997). Thus there is no evidence for a distinct shell of lipids around the peptide and any perturbation of the lipids extends out just a few Ångstroms.

The lack of a clear shell of lipids around the poly(Ala) peptide contrasts with the boundary lipids observed for membrane proteins and illustrated for bacteriorhodopsin in Figs. 5 and 6. In part this could be an intrinsic feature of single transmembrane α helices, which will not be able to present a large surface area on which fatty acyl chains could be immobilized. The regular structure of a poly(Ala) peptide compared to the rough surface of a typical α-helical peptide might also contribute to the lack of an immobilization shell of lipids. However, a significant factor is also likely to be the lack of polar groups at the ends of the peptide able to interact with the phospholipid headgroups. The importance of polar groups at the ends of the peptides has been shown in a simulation of isolated helices from bacteriorhodopsin (Woolf, 1998). The simulations show that a small number of the lipids surrounding a helix interact with it much more strongly than other lipids, due to a combination of van der Waals (mediated by chains) and charge interactions (mediated by headgroups) (Woolf, 1998).

A molecular dynamics simulation of the peptide L_{16} in a bilayer of di(C14:0)PC in the liquid crystalline phase showed that the peptide tilted with an average angle of 15.3° with respect to the bilayer normal, even though the thickness of the hydrophobic region of the bilayer (22 Å) was a good match to the length of the α helix, 24 Å (Belohorcova *et al.*, 1997). A molecular dynamics simulation has been reported for PF1 coat protein in di(C16:0)PC (Roux and Woolf, 1996). The coat protein contains an amphipathic α helix on the bilayer surface and a hydrophobic transmembrane α helix. Fatty acyl chains next to the transmembrane α helix were slightly more ordered than bulk lipids (Roux and Woolf, 1996). Similarly, in a simulation of the seventh transmembrane α helix of the serotonin 5HT receptor in di(C14:0)PC, lipids in contact with the peptide had slightly higher order parameters than bulk lipids (Duong *et al.*, 1999).

B. Effects of Hydrophobic Mismatch

The theories described above show that there will be an energetic cost associated with any change in the thickness of a bilayer. This would be reflected in values for the equilibrium constant describing the binding of lipids to the protein. A lipid that can bind to a protein without a change in bilayer thickness would bind more strongly to the protein than one for which binding required a change in bilayer thickness. The strength of interaction between a peptide and a phospholipid in a bilayer can be measured using a fluorescence quenching method (Webb *et al.*, 1998). Peptides used are of the type $KKGL_7WL_9KKA$ (L_{16}) and $KKGL_{10}WL_{12}KKA$ (L_{22}) containing a central Trp residue as a fluorescence reporter group. The peptide is incorporated into bilayers containing the brominated phospholipid dibromostearoylphosphatidylcholine (di(Br_2C18:0)PC); di(Br_2C18:0)PC behaves much like a conventional phospholipid with unsaturated fatty acyl chains, because the bulky bromine atoms have effects on lipid packing similar to those of a *cis* double bond (East and Lee, 1982). Contact between the bromine atoms in the lipid and the Trp residue in the peptide leads to fluorescence quenching. In mixtures of brominated and nonbrominated phospholipids, the degree of quenching of the fluorescence of the tryptophan residue is related to the fraction of the surrounding (boundary) phospholipid molecules that are brominated, and thus to the strength of binding of the nonbrominated lipid to the peptide. An example of the method is shown in Fig. 8. The fluorescence intensity for the peptide L_{16} incorporated into bilayers of di(Br_2C18:0)PC at a molar ratio of lipid to peptide of 100 : 1 is 5% of that in di(C18:1)PC, demonstrating highly efficient quenching of the tryptophan by the bromine-containing fatty acyl chains (Fig. 8). The fluorescence intensity in mixtures of di(Br_2C18:0)PC and di(C18:1)PC decreases with increasing content of di(Br_2C18:0)PC, reflecting the increasing number of boundary lipids that will be di(Br_2C18:0)PC. As shown in Fig. 8, fluorescence quenching curves for L_{16} in mixtures of di(Br_2C18:0)PC and (C14:1)PC show more fluorescence quenching at intermediate mole fractions of di(Br_2C18:0)PC than in mixtures with di(C18:1)PC. This shows that di(C18:1)PC binds more strongly to the peptide than does di(C14:1)PC. The results can be analyzed to give relative lipid-binding constants, as described in Webb *et al.* (1998). These lipid-binding constants for L_{16} and L_{22} are given in Table I.

For L_{22} strongest binding is seen with di(C22:1)PC, for which the relative binding constant is about double that for di(C18:1)PC (Table I). The hydrophobic length of the peptide L_{22} is about 36 Å, calculated for a stretch of 24 hydrophobic residues in total, with a helix translation of 1.5 Å per residue. Thus, strongest binding is seen when the hydrophobic length of the peptide matches the hydrophobic thickness of the bilayer, as expected from theories of hydrophobic mismatch. However, relative binding constants do not continue to decrease with decreasing chain length from di(C18:1)PC to di(C14:1)PC as would have been predicted (Table I). An even

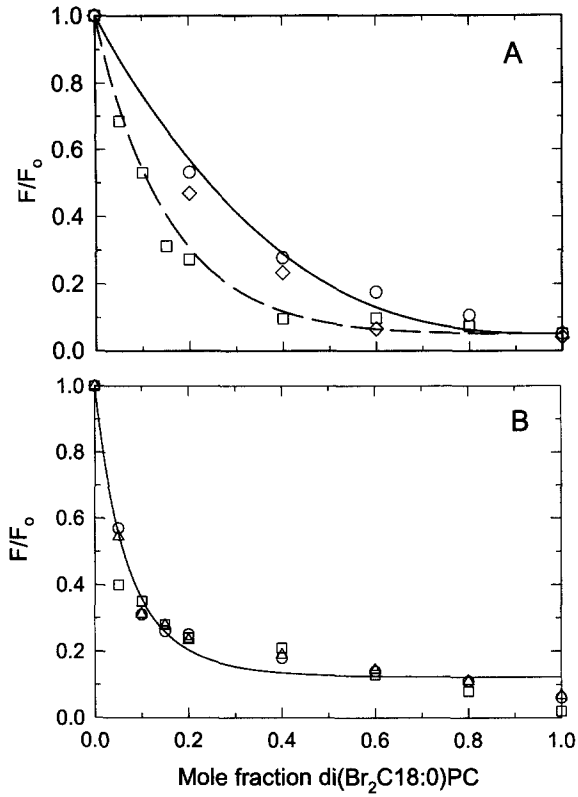

FIGURE 8 Fluorescence quenching method for determining lipid binding constants. (A) Fluorescence intensities for L_{16} in mixtures containing di(Br_2C18:0)PC. The L_{16} was incorporated into a mixture of di(Br_2 C18:0)PC and either di(C18:1)PC (○) or di(C14:1)PC (□), or a mixture of di(C14:1)PC and cholesterol at a 1 : 1 molar ratio (◇), at a molar ratio of peptide : phospholipid of 1 : 100. Fluorescence intensities are expressed as a fraction of that recorded for L_{16} in di(C18:1)PC. (B) Fluorescence intensities in mixtures of di(Br_2C18:0)PC and gel-phase lipid. Fluorescence intensities are shown for L_{16}(○) and Y_2L_{14} (Δ) in mixtures of di(Br_2C18:0)PC and di(C16:0)PC and for L_{16} in mixtures of di(Br_2C18:0)PC and sphingomyelin (□), at 25°C.

larger deviation from theoretical predictions is observed with the short peptide L_{16} (Table I). In this case strongest binding is observed with di(C18:1)PC, with binding decreasing with decreasing chain length to di(C14:1)PC, as expected. However, the peptide was found not to incorporate at all into bilayers of di(C24:1)PC, instead forming aggregates of peptide separate from the bilayer. Ren *et al.* (1999) obtained very similar results, except that under their conditions, unincorporated peptide bound to the surface of the lipid bilayer, with the long axis of the peptide parallel to the surface. Similarly, L_{16} was found to be only partly incorporated into

TABLE I

Relative Phospholipid-Binding Constants for Peptides L_{16} and L_{22}

Lipid	Bilayer thickness[a] (Å)	Relative binding constant[b]	
		L_{16}	L_{22}
di(C14:1)PC	22.8	0.4	0.9
di(C16:1)PC	26.3	0.8	0.7
di(C18:1)PC	29.8	1.0	1.0
di(C20:1)PC	33.3	0.7	1.8
di(C22:1)PC	36.8	—	2.0
di(C24:1)PC	40.3	—	1.5

[a] Bilayer hydrophobic thickness d calculated from $d = 1.75(n - 1)$, where n is the number of carbon atoms in the fatty acyl chain (Sperotto and Mouritsen, 1988).

[b] Estimated hydrophobic length is 27 Å for L_{16} and 36 Å for L_{22}.

bilayers of di(C22:1)PC (Webb *et al.*, 1998). Thus, a short peptide cannot incorporate into a too-thick bilayer. It is suggested that a too-thin bilayer can match to a too-long peptide both by a slight stretching of the lipid and by tilting of the long axis of the helix with respect to the bilayer normal so that its effective length across the bilayer is reduced. However, a too-thick bilayer can only match a too-thin peptide by compression of the lipid, which becomes energetically unfavorable when the difference between the bilayer thickness and the peptide length exceeds about 6 Å (Webb *et al.*, 1998).

Possible effects of aromatic residues at the ends of transmembrane α helices have been studied using peptides $K_2GFL_6WL_8FK_2A$ (F_2L_{14}) and $K_2GYL_6WL_8YK_2A$ (Y_2L_{14}), in which one Leu residue at each end of the poly(Leu) stretch is replaced by either a Phe or a Tyr (Mall *et al.*, 2000). In contrast to the results with L_{16}, peptide F_2L_{14} incorporated fully into bilayers of di(C24:1)PC, and Y_2L_{14} partitioned partially into di(C24:1)PC. The fluorescence quenching method was again used to obtain binding constants for phosphatidylcholines to the peptides, measured relative to the binding constant for di(C18:1)PC (Table II). The effective hydrophobic length of the peptide Y_2L_{14} might be expected to be somewhat greater than that of L_{16}; if the peptide is modeled as an α helix with the two Tyr residues oriented to be roughly parallel to the long axis of the α helix, the distance between the two Tyr—OH groups is ca. 33 Å, about 6 Å greater than the hydrophobic length of L_{16}. The hydrophobic length of Y_2L_{14} calculated in this way matches the hydrophobic thickness of a bilayer of di(C20:1)PC, whereas the relative lipid-binding constants increase from di(C14:1)PC to di(C22:1)PC (Table II). Similarly, relative binding constants for F_2L_{14} increase with increasing chain length from di(C14:1)PC to di(C24:1)PC. The results with Y_2L_{14} and F_2L_{14} show that introduction of aromatic

TABLE II

Relative Lipid-Binding Constants for Peptides Containing Aromatic Groups

Lipid	d^a (Å)	Relative binding constant		
		Y_2L_{14}	F_2L_{14}	Y_2L_{20}
di(C14:1)PC	22.8	0.46	0.50	0.47
di(C16:1)PC	26.1	0.74	0.83	0.45
di(C18:1)PC	29.8	1	1	1
di(C20:1)PC	33.3	1.02	1.16	1.22
di(C22:1)PC	36.8	1.37	1.62	0.96
di(C24:1)PC	40.3	—	2.33	1.19

[a]Hydrophobic thickness of the bilayer calculated from $d = 1.75(n - 1)$, where n is the number of carbon atoms in the fatty acyl chain (Sperotto and Mouritsen, 1988).

residues at the two ends of the hydrophobic sequence increases the ability of the short peptides to partition into thick lipid bilayers. The observation that the highest relative binding constant is obtained with bilayers considerably thicker than the calculated hydrophobic length of the peptides suggests that the presence of aromatic residues at the ends of the helices could lead to marked thinning of the bilayer around the peptides (Mall *et al.*, 2000).

The chain length dependence of lipid binding to Y_2L_{20} is much less marked than for the shorter peptides; the relative binding constant increases from di(C14:1)PC to di(C18:1)PC, but then hardly changes with increasing chain length between di(C18:1)PC and di(C24:)PC (Table II). This contrasts with L_{22}, which shows markedly stronger interaction with di(C22:1)PC than with phospholipids with shorter or longer chains. This again suggests that the introduction of the two Tyr residues leads to an increase in the thickness of the bilayer with which optimal interaction of the peptide is observed.

C. *Interactions with Anionic Phospholipids*

Interactions between transmembrane α helices and the phospholipid headgroups also have to be considered. Using the fluorescence quenching method, it was shown that a small number of anionic phospholipid molecules (possibly just one) bound strongly to the peptide L_{16}, the remaining molecules binding with an affinity equal to that of phosphatidylcholine. The binding constant for the strongly bound phosphatidic acid molecule relative to phosphatidylcholine in a medium of low ionic strength was 8.6 (in mole fraction units), corresponding to a difference in unitary

binding energies of -5.3 kJ mol^{-1} (Mall *et al.*, 1998). At pH 7.2, phosphatidic acid bears a single negative charge (Cevc, 1990). The binding constant for phosphatidic acid changed little with ionic strength, suggesting that the interaction with the positively charged peptide did not follow simply from a high positive potential in the vicinity of the positively charged Lys residues on the peptide, increasing the local concentration of anionic phospholipid. The energy of interaction between two ions U is given by

$$U = z_1 z_2 e^2 / 4\pi\varepsilon_o\varepsilon_r r, \tag{3}$$

where z_1 and z_2 are the charges on the two ions ε_o is the permittivity of a vacuum, ε_r is the relative permittivity (dielectric constant) of the medium, and r is the distance between the two ions. Assuming a dielectric constant of 78.5 (water), we find that an energy of interaction of 5.3 kJ mol^{-1} corresponds to a distance of separation between two monovalent ions of 3.3 Å. This therefore suggests that strong interaction requires the anionic headgroup of phosphatidic acid to be in close contact with one of the Lys residues on the peptide. Once this strong interaction with a single phosphatidic acid molecule has been made, other phosphatidic acid molecules will then interact with L_{16} relatively nonspecifically, with a binding constant relative to phosphatidylcholine close to 1.

The relative binding constants for phosphatidylserine were less than for phosphatidic acid and are more sensitive to ionic strength (Mall *et al.*, 1998). For phosphatidylserine, the presence of the positively charged ammonium group as well as the negatively charged carboxyl group in the headgroup region may reduce interaction with the positively charged peptide. In contrast to L_{16}, the binding constants for anionic phospholipids to L_{22} are very similar to those for zwitterionic phospholipids, with a relative binding constant close to 1. It could be that tilting of L_{22} in the bilayer, necessary to match the hydrophobic length of L_{22} to the hydrophobic thickness of a bilayer of di(C18:1)PC, locates the Lys residues on the peptide too far from the lipid headgroup region to allow a strong interaction between the anionic phospholipid and the peptide.

Both phosphatidylserine and phosphatidic acid bind more strongly to the peptides Y_2L_{14} and Y_2L_{20} than does phosphatidylcholine, the effect of anionic phospholipid decreasing slightly with increasing ionic strength. However, in this case the experiments are consistent with a model in which the binding constants for all the anionic phospholipid molecules binding to the peptide are increased slightly (Mall *et al.*, 2000). This suggests that the presence of the Tyr residues prevents close association of the anionic phospholipid group with the cationic Lys residues.

These results suggest that the effects of charge on the interactions between phospholipids and transmembrane α helices will often be rather small and will be strongly dependent on the detailed structure of the peptide and its orientation in the membrane. This picture is consistent with the results of the molecular

dynamics simulations of individual α helices of bacteriorhodopsin in bilayers of di(C14:0)PC, which showed that a small proportion of the lipid molecules interacted with the α helices much more strongly than the others, and that these strong interactions were dominated by electrostatic terms rather than van der Waals terms (Woolf, 1998).

In general, binding constants for phospholipids to membrane proteins also show relatively little selectivity for anionic phospholipids. For example, binding constants for phosphatidic acid and phosphatidylserine relative to phosphatidylcholine are close to 1 for the Ca^{2+}-ATPase (Dalton *et al.*, 1998), and binding constants for phosphatidic acid and phosphatidylserine for the (Na^+–K^+)-ATPase are about twice those for phosphatidylcholine (Esmann and Marsh, 1985). However, there is evidence for the presence of a small number of "special" anionic phospholipids binding to some membrane proteins, acting as "cofactors." An example is provided by cytochrome *c* oxidase, whose crystal structure shows the presence of a lipid molecule bound between the transmembrane α helices (Iwata *et al.*, 1995). Interaction between an anionic phospholipid and a binding site on a membrane protein would be specific if strong binding requires close interaction between the anionic headgroup and a positively charged residue on the protein, as suggested by the results presented here.

D. *Effects of Phospholipid Phase*

The phase of the phospholipid is important in determining interactions with transmembrane α helices. As shown in Fig. 8, fluorescence quenching is much more marked in mixtures of di(Br_2C18:0)PC and di(C16:0)PC at temperatures where both liquid crystalline and gel phases are present than in mixtures of di(Br_2C18:0)PC and di(C18:1)PC (Mall *et al.*, 2000). Thus, L_{16} is excluded from regions of lipid in the gel phase and accumulates in regions in the liquid crystalline phase. The binding constants of L_{16} and L_{22} for di(C16:0)PC in the gel phase relative to di(C18:1)PC in the liquid crystalline phase are ca. 0.15 (Mall *et al.*, 2000). This is consistent with the expectation that van der Waals contacts between an all-*trans* fatty acyl chain and the molecularly rough surface of a peptide will be poor; one way that this poor packing can be overcome is by exclusion of the peptide from the gel phase. Quenching plots for Y_2L_{14} are very similar to those of L_{16} (Fig. 8), showing that the presence of bulky aromatic residues does not have any significant effect on the selectivity for liquid crystalline- over gel-phase lipid (Mall *et al.*, 2000). Further, since Y_2L_{14} shows a preference for longer chain phospholipids than L_{16}, Y_2L_{14} might have been expected to show a greater preference for gel-phase lipid than L_{16}, because phospholipid in the gel phase gives a thicker bilayer than the corresponding lipid in the liquid crystalline phase. Because Y_2L_{14} and L_{16} show equal preferences for liquid crystalline- over gel-phase lipid,

any effects of hydrophobic matching between the peptide and the bilayer must be small compared to effects of lipid phase on the interaction energies between the peptides and the lipid (Mall *et al.*, 2000). Preferential partitioning of proteins from domains in the gel phase into domains of liquid crystalline lipid has been demonstrated for a variety of membrane proteins, including bacteriorhodopsin (Cherry *et al.*, 1978) and Ca^{2+}-ATPase (East and Lee, 1982; Kleeman and McConnell, 1976).

Effects of sphingomyelin at 25°C are very similar to the effects of gel-phase di(C16:0)PC (Fig. 8; Mall *et al.*, 2000). Mixtures of bovine brain sphingomyelin and di(C18:1)PC are in a two-phase region at 25°C, with gel-phase domains enriched in sphingomyelin (Untracht and Shipley, 1977). Thus, partitioning of the peptides between gel- and liquid crystalline-phase lipid shows little dependence on the structure of the phospholipid. It has been suggested that plasma membranes of mammalian cells contain domains or "rafts" enriched in sphingomyelin and that particular enzymes, particularly those associated with cell signaling, are concentrated within the rafts (Simons and Ikonen, 1997). The results presented here suggest that membrane proteins containing transmembrane α helices will tend to be excluded from these rafts, and it may therefore be significant that many of the signaling proteins suggested to be contained within the rafts are anchored to the membrane by glycosylphosphatidylinositol anchors (Harder and Simons, 1997).

E. Effects of Cholesterol

The presence of cholesterol has a marked effect on incorporation of the peptides into phospholipid bilayers (Webb *et al.*, 1998). Incorporation of cholesterol at a 1 : 1 molar ratio to phospholipid leads to a general reduction in incorporation of the peptides L_{16} and L_{22}, but superimposed on this effect is a chain length effect. In the presence of cholesterol, the binding constant of P_{16} for di(C14:1)PC relative to di(C18 : 1)PC increased from 0.4 to about 1, as expected if the presence of cholesterol increases the effective chain length of the C14 chain so that it more nearly matches the hydrophobic length of the peptide (Fig. 8). Consistent with this interpretation, the presence of high molar ratios of cholesterol prevented the incorporation of P_{16} into bilayers of di(C18:1)PC. Nezil and Bloom (1992) showed that incorporation of cholesterol at 33 mol% increases bilayer thickness by about 4 Å.

Studies with brominated analogues of cholesterol showed that cholesterol binds to the peptides with a binding constant only a factor of about two less strongly than di(C18:1)PC (Mall *et al.*, 1998). This is rather surprising, given the relatively rigid structure of the steroid ring of cholesterol and the molecularly rough surface of the peptide. In other studies, it has been shown that cholesterol binds relatively

weakly at the lipid–protein interface of the ATPase (Simmonds *et al.*, 1982, 1984); comparison with the peptide studies reported here suggests that weak binding of cholesterol to the ATPase involves interactions in the lipid headgroup region rather than interactions between the sterol ring and the hydrophobic transmembrane α helices.

IV. BIOLOGICAL CONSEQUENCES OF HYDROPHOBIC MISMATCH

The requirement to match the hydrophobic thickness of a membrane protein to that of the surrounding lipid bilayer could be important in a number of ways. Targeting of proteins to their correct final destinations in a cell is essential in maintaining cell integrity. In the bulk flow model, the vast majority of proteins synthesized in the endoplasmic reticulum (ER) are believed to leave the ER by default and flow along the exocytic pathway until they reach the plasma membrane (Nilsson and Warren, 1994). Some proteins, however, have to be retained at particular points along the exocytic pathway. Compartmental localization could be achieved in one of two ways. The first involves a retention signal in the protein, which, at the appropriate point in the exocytic pathway, prevents forward movement of the protein by denying it access to budding transport vesicles of the onward pathway. The second involves a retrieval signal, leading to recapture of the protein after it has left the compartment in which it resides. The classical retrieval signal is the KDEL sequence found in many ER-resident proteins; the situation appears to be different for Golgi-resident proteins, where membrane-spanning domains act as retention signals (Nilsson and Warren, 1994).

Despite the extensive flux of proteins through the Golgi, the Golgi maintains its own distinctive population of resident proteins. Furthermore, the distribution of enzymes within the Golgi is organized according to function, so that, for example, the distributions of glycosyltransferases and glycosidases, although overlapping, are distinct (Colley, 1997; Roth, 1987). Many of the proteins in the Golgi membrane are predicted to contain a single transmembrane α helix, oriented with the N- and C-termini on the inner and outer faces of the membrane, respectively. The Golgi retention signal in such proteins has been shown to involve the membrane spanning domain (Munro, 1991, 1995; Nilsson *et al.*, 1991; Swift and Machamer, 1991). However, the membrane-spanning domains show no sequence homology, and it has not been possible to identify any particular motif leading to retention (Bretscher and Munro, 1993; Colley *et al.*, 1992; Munro, 1991). Thus, sialyltransferase remains localized in the Golgi even when its 17-amino-acid transmembrane domain is replaced by 17 Leu residues (Munro, 1991). However, a longer stretch of 23 Leu residues did not provide an efficient retention signal (Munro, 1991). Similarly, a 4-residue insertion into the transmembrane domain of galactosyltransferase reduced its retention in the Golgi (Masibay *et al.*, 1993). The reverse

effect has been shown with the influenza virus neuraminidase, which shifted from the plasma membrane to the Golgi and ER when the number of residues in the transmembrane domain was reduced (Sivasubramanian and Nayak, 1987).

The lack of a clear retention motif, together with the inability to saturate the mechanism for Golgi retention by overexpression, suggests that retention is not a receptor-mediated event (Nilsson and Warren, 1994). One possible model is then retention by preferential interaction with membranes of optimal thickness (Nilsson and Warren, 1994). Both Bretscher and Munro (1993) and Masibay *et al.* (1993) showed that transmembrane domains of Golgi proteins are shorter (average 15 residues) than transmembrane domains of plasma membrane proteins (average 20 residues). It has therefore been suggested that if the Golgi membrane is thinner than the plasma membrane, membrane proteins with short transmembrane domains will interact "more strongly" with the lipid bilayer of the Golgi than with that of the plasma membrane, leading to retention in the Golgi (Bretscher and Munro, 1993; Masibay *et al.*, 1993). The studies with model peptides described above show that a protein containing a transmembrane α helix with a hydrophobic length greater than the hydrophobic thickness of the Golgi membrane will be able to move out of the Golgi into the plasma membrane. However, a protein whose transmembrane α helix has a hydrophobic length less than the hydrophobic thickness of a particular membrane will not be able to enter that membrane, and such a protein would then be retained in the Golgi (Webb *et al.*, 1998).

Studies of targeting of proteins in yeast are also consistent with a lipid-based model (Rayner and Pelham, 1997). The length of the transmembrane domain is important in targeting with long helices (24 residues), ensuring transport to the plasma membrane. However, for proteins with shorter transmembrane domains, the relative hydrophobicity of the transmembrane domain has been suggested to be important as well as its length, this determining targeting to the Golgi and the vacuole (Rayner and Pelham, 1997).

Retention of some membrane proteins in the ER could also depend on the length of the transmembrane domain of the protein. An important class of ER membrane proteins are those with an N-terminal catalytic domain exposed to the cytoplasm and a C-terminal membrane anchor. Such proteins are inserted into the ER membrane post-translationally by a signal-recognition-particle-independent pathway. No ER retrieval signals have been identified in these proteins. Instead, it has been observed that the hydrophobic domain is rather short. For example, cytochrome b_5, a protein of this type, has a transmembrane domain containing just 17 hydrophobic amino acid residues (Pedrazzini *et al.*, 1996). If the length of the hydrophobic stretch is increased to 22 residues, the protein is transported out of the ER along the secretory pathway (Pedrazzini *et al.*, 1996). It could therefore be that matching of the thickness of the lipid bilayer and the transmembrane length of the protein is important in retention in ER, as was suggested for the Golgi complex. Although the length of the transmembrane domain appears to be the most important

factor, the structure of the C-terminal, lumenal, region has also been shown to contribute to retention (Honsho *et al.*, 1998). Experiments with another C-terminal-anchored protein, the ubiquitin-conjugating enzyme UBC6 from yeast, suggest that the thickness requirements of the ER and Golgi membranes may be different, explaining targeting between these two organelles (Yang *et al.*, 1997). Whereas UBC6 containing the wild-type 17-residue transmembrane domain targets to the ER, increasing the length of the transmembrane domain to 21 residues results in movement to the Golgi, and increasing the length further to 26 residues allows movement to the plasma membrane.

These experiments show that the length of the transmembrane α helix is often an important factor in targeting, although it is likely to be only one of a number of important factors. The lengths of the transmembrane α helices are also likely to be important in the proper function of membrane proteins containing multiple transmembrane α helices. An example already described is that of the Ca^{2+}-ATPase, which shows highest ATPase activity in di(C18:1)PC and lower activities in bilayers of phospholipids with longer or shorter fatty acyl chains (Lee, 1998). Changes in the ATPase underlying these changes in ATPase activity are complex (Lee, 1998), but all must be mediated by the transmembrane α helices, because these are the parts of the ATPase that can "sense" the change in bilayer thickness. In the case of the Ca^{2+}-ATPase it seems that, as described above, the two major conformational states of the ATPase (E1 and E2) have different preferences for bilayer thickness, the E1 conformation favoring thin bilayers and the E2 conformation favoring thick bilayers (Lee, 1998). Changing the bilayer thickness could change the tilt of the transmembrane α helices in the Ca^{2+}-ATPase, it could change the packing of the helices, and, possibly, it could lead to changes in the structures of the loops connecting the helices, changing the effective lengths of the helices. All these changes could be linked to changes in the phosphorylation domain of the Ca^{2+}-ATPase, located well above the surface of the membrane.

If, as seems likely, the various membranes in a cell have different thicknesses because of their different lipid compositions, the structure of each membrane protein will have evolved to match the thickness of the membrane in which it resides.

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