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Published in:
FEMS Microbiology Reviews

DOI:
10.1111/j.1574-6976.1993.tb00024.x

Publication date:
1993

FEMS Microbiology Reviews 12 (1993) 293–314

Published by Elsevier

FEMSRE 00335

Bacterial solute transport proteins in their lipid environment

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(Received 5 May 1993; accepted 23 June 1993)

Abstract: The cytoplasmic membrane of bacteria is a selective barrier that restricts entry and exit of solutes. Transport of solutes across this membrane is catalyzed by specific membrane proteins. Integral membrane proteins usually require specific lipids for optimal activity and are inhibited by other lipid species. Their activities are also sensitive to the lipid bilayer dynamics and physico-chemical state. Bacteria can adapt to changes in the environments (respective temperature, hydrostatic pressure, and pH) by altering the lipid composition of the membrane. Homeoviscous adaptation results in the maintenance of the liquid-crystalline phase through alterations in the degree of acyl chain saturation and branching, acyl chain length and the sterol content of the membrane. Homeophasic adaptation prevents the formation of non-bilayer phases, which would disrupt membrane organization and increase permeability. A balance is maintained between the lamellar phase, preferring lipids, and those that adopt a non-bilayer organization. As a result, the membrane proteins are optimally active under physiological conditions. The molecular basis of lipid–protein interactions is still obscure. Annular lipids stabilize integral membrane proteins. Stabilization occurs through electrostatic and possibly other interactions between the lipid headgroups and the charged amino acid residues close to the phospholipid–water interface, and hydrophobic interactions between the fatty acyl chains and the membrane-spanning segments. Reconstitution techniques allow manipulation of the lipid composition of the membrane in a way that is difficult to achieve in vivo. The physical characteristics of membrane lipids that affect protein-mediated transport functions have been studied in liposomal systems that separate an inner and outer compartment. The activity of most transport proteins is modulated by the bulk physical characteristics of the lipid bilayer, while specific lipid requirements appear rare.

Key words: Membrane; Transport; Protein-lipid interactions

Introduction

Membranes play a central role in both the structural organization and function of all prokaryotic and eukaryotic cells. Membranes control the essential movement of solutes (ions and nutrients) in or out of the cell. Passive permeability through a lipid bilayer is slow for most solutes and mostly protein-mediated transport processes allow the entry and exit of solutes into or out of the cell. When the integrity of the membrane is lost, the cells are freely permeable to all solutes, finally leading to their death [1].

Most cell membranes consist of a lipid bilayer. The structure of the membrane depends largely

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on hydrophobic effects (which also control protein structures). The repulsion of the lipid hydrocarbon chains by the water structure drives the chains into an environment free from water. The amphipathic nature of the polar membrane lipids directly defines the bilayer structure. It provides a hydrophobic environment in the middle of the bilayer for hydrocarbon chains, with the lipid polar groups sticking into the aqueous phase (Fig. 1). Dynamically, the lipid bilayer is highly anisotropic; the interior of a bilayer is mostly ordered, and only a small region in the middle is liquid-like. The conformation of the lipid hydrocarbon chains (also the conformation of the lipid headgroups) in the bilayer is well described [1]. A striking feature is the enormous diversity of the membrane lipids. Archaea contain membrane lipids that differ completely from conventional lipids found in eubacteria. These lipids are characterized by ether linkages instead of ester linkages and contain biphytanyl chains instead of fatty acyl chains (Figs. 1 and 2). Lipids of extreme thermophiles are essentially composed of tetraethers with two polar heads linked by two C_{40}

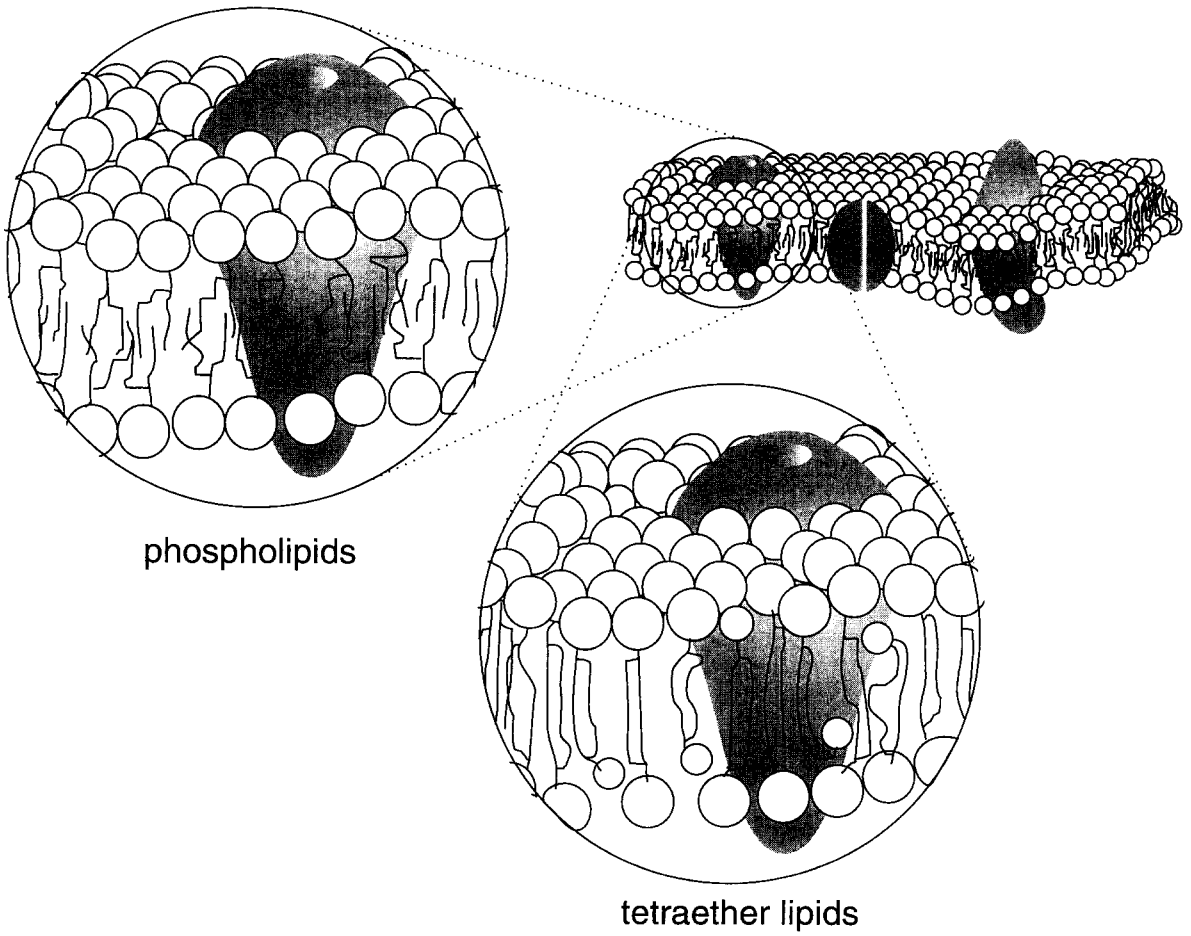

Fig. 1. Sketch of the structure of a biological membrane. Integral membrane proteins are embedded in the liquid-crystalline matrix of the lipid bilayer. Enlargements show a section of a membrane composed of phospholipids (bilayer) or membrane-spanning tetraether lipids (monolayer).

phytanyl chains. This results in a monomolecular arrangement of the membrane instead of a bilayer (Fig. 1) [2].

Biological membranes

The lipid composition of most cell membranes is complex and metabolically tightly regulated, suggesting that lipids play a role in membrane protein activity. Usually biological membranes are in the liquid-crystalline phase, but other phases can also be found, depending on a variety of factors such as temperature, fatty acid composition, pH and the presence of divalent cations. An important parameter in the physico-chemical behaviour of the membrane is the temperature, T_c, at which the transition between gel to liquid-crystalline states takes place. Below T_c the lipids are packed into an ordered semi-crystalline lattice (lamellar gel phase, or L_β) whereas above T_c they are more disordered and fluid (the lamellar liquid crystalline phase or L_α) [3] (Fig. 3).

The phase preference of a lipid can be explained by the shape of that lipid molecule (Fig. 3) [4]. Lipids with a large head group compared to the cross-sectional area of the hydrocarbon chains are defined as 'inverted cone' shaped molecules. Such lipids, which include lyso-phospholipids and detergents, pack optimally in micel-

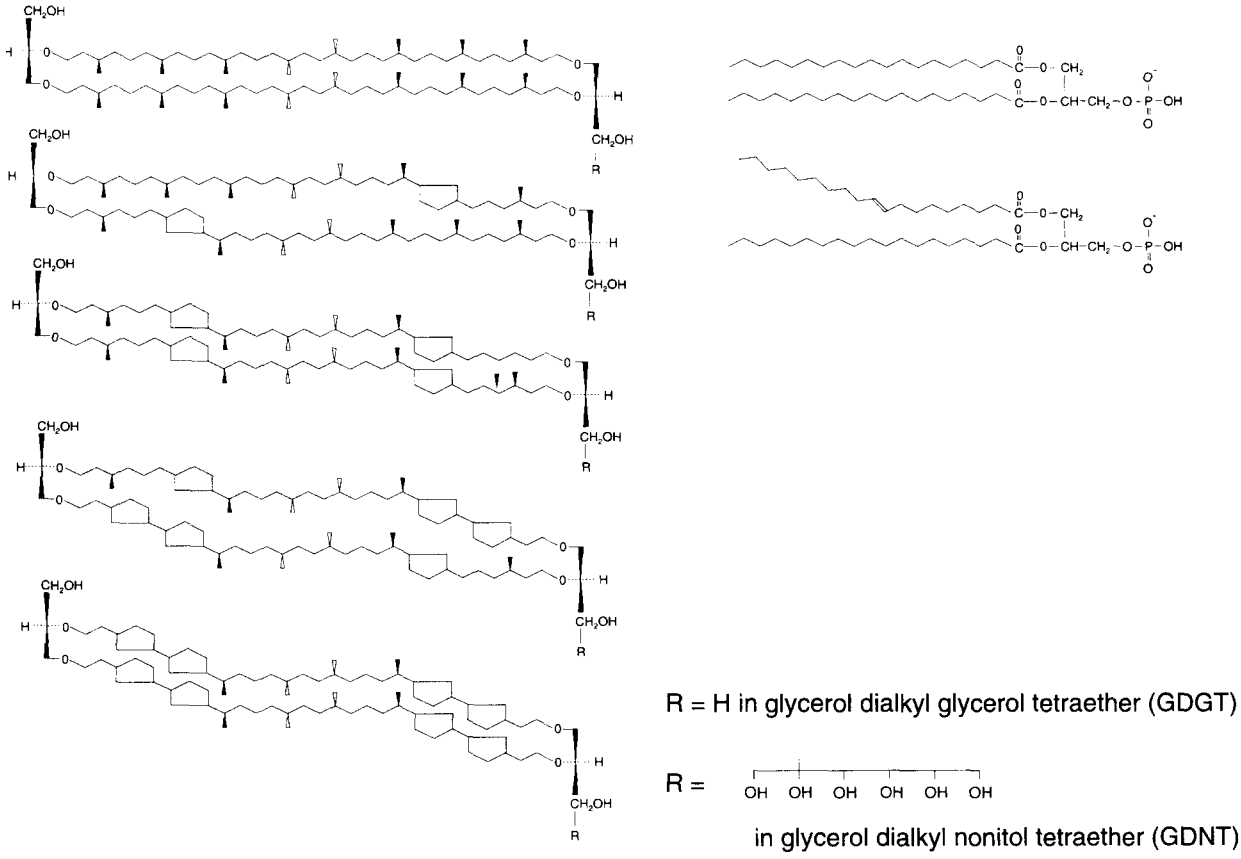

Fig. 2. Backbone structures of tetraether lipids from extreme thermophilic archaea. The lipid molecules contain branched hydrocarbon chains of the phytanyl type containing 0–4 cyclopentane groups per chain, ether-linked to a substituted or unsubstituted glycerol or nonitol group. Also sketched are the backbones of lipid molecules of eubacteria consisting predominantly, though not uniquely, of two linear hydrocarbon chains of variable length and degree of saturation, ester linked to a glycerol group whose third hydroxyl is substituted by a polar residue, in this case a phosphorous moiety.

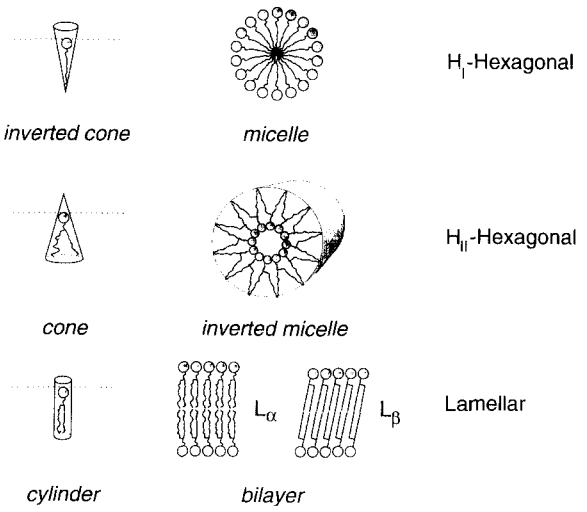

Fig. 3. The molecular shape concept of lipid polymorphism.

lar or H_I type configuration. The opposite case, 'cone'-shaped lipids, includes lipids that prefer inverted structures such as inverted micelles or H_{II} phase. When the headgroup and the acyl chains have comparable cross-sectional areas, the molecules have a cylindrical shape and organize themselves in a bilayer. The shape concept accurately describes the phase properties. The temperature-dependent bilayer to H_{II} transitions can be understood in terms of an increase in hydrocarbon area with increasing temperature. The increased cone shape of the molecule then triggers the transition. Similarly, highly unsaturated phosphatidylethanolamine (PE) species are more cone-shaped and thus have lower transition temperatures. Addition of Ca^{2+} to diphosphatidylglycerol (cardiolipin; CL) liposomes results in Ca^{2+}-binding that reduces the headgroup size because of dehydration, decreases electrostatic repulsion, and (possibly) the distance between the phosphates within one CL molecule. Whereas monogalactosyldiglyceride (MGDG) prefers the H_{II} phase, the additional presence of another glucose in the headgroup to produce digalactosyldiglyceride (DGDG) increases the headgroup size and drives the molecule in a lamellar structure. The bilayer organization of H_{II}-preferring lipids (PE) is stabilized by inclusion of lamellar forming lipids (phosphatidylcholine (PC) or other type I or II lipids), or inclusion of intrinsic membrane proteins. On the other hand, cholesterol stabilizes H_{II} phases [3].

The functional importance of non-bilayer structures is related to the barrier function of the membrane. The bilayer permeability for small solutes usually increases when membrane proteins are incorporated in liposomes composed of one lipid species. On the other hand, incorporation of proteins into mixed lipid systems results in 'sealed' proteoliposomes. The functional roles, suggested for the non-lamellar lipid structures, include: (i) the formation of contact points between membranes; (ii) induction of local high membrane curvature; (iii) induction of packing defects that can be stabilized by membrane polyprenols and hydrophobic proteins; (iv) transmembrane transport of polar molecules; (v) correct integration of proteins into membranes; and (vi) translocation of proteins across the membrane [5].

Membrane proteins

Whereas the primary role of membrane lipids is the formation of a stable bilayer, membrane proteins provide the active components of biological membranes. They are responsible for the transmembrane communication and functional interactions within the plane of the membrane. Membrane proteins may simply be associated with the membrane (extrinsic or peripheral membrane proteins) or may be integrated in the membrane (intrinsic or integral membrane proteins). Peripheral proteins are weakly bound to the membrane surface by electrostatic interactions either with the lipid headgroups or with other proteins. On the other hand, intrinsic membrane proteins are generally bound to the membrane both by electrostatic and hydrophobic interactions. Finally, some membrane proteins are covalently bound to lipids. These are, for instance, glycoproteins with their carbohydrate residues at the outside of the cytoplasmic membrane and binding proteins of Gram-positive bacteria that are attached to the membrane through a fatty anchor [6].

Transmembrane segments of integral membrane proteins consist predominantly of hy-

drophobic amino acids that make these domains compatible with the hydrophobic interior of the lipid bilayer. At the end of these hydrophobic transmembrane regions, a high incidence of charged amino acids is often found. These transmembrane segments, spanning the hydrophobic region of the phospholipid bilayer, may adopt the α-helical conformation. The α-helices will be organized into a specific three-dimensional structure that depends partly on the interaction energy of matching of the hydrophilic and hydrophobic portions of the protein with those of its lipid environment. Folding is further driven by the self-association of small hydrophilic regions of the different α-helices. As a result, the transmembrane proteins are firmly locked in their position in the lipid bilayer by the hydrophobic effect; the charged regions cannot enter the hydrophobic interior of the membrane and the hydrophilic portions of the protein are in contact with water [7].

The nucleotide and amino acid sequences of many bacterial membrane proteins are known, including facilitated diffusion transport proteins, proton- and/or sodium-dependent sugar and amino acid symporters, anion exchange proteins, and ATP-coupled ion pumps (for Reviews see [8–10]). Secondary structure models of these proteins have been mathematically derived from the primary structure. In all these models, multiple membrane-spanning α-helices are packed so that hydrophilic pores are formed which are lined by the polar faces of several membrane-spanning helices. These α-helices contain at least 20–22 amino acids, i.e. the minimum number of amino acid residues that can span a lipid bilayer in α-helical conformation. The α-helices are largely or entirely composed of hydrophobic residues, or the α-helices are amphipathic with non-polar faces interacting with the hydrophobic fatty acyl chains [8]. The axes of the transmembrane α-helices are thought to be roughly parallel to the lipid acyl chains. In some proteins, transmembrane regions of β-strands parallel to the lipid chains have been shown to be present. Turns or bends within the membrane have been suggested, although this is thought to be energetically unfavourable [11]. Short α-helices or β-sheets could facilitate the conformation changes involved in the translocation cycle of transport proteins [8].

A high resolution of the molecular structure by diffraction methods requires three-dimensional crystals, which are difficult to obtain from integral membrane proteins [12]. There is no high resolution structural information on transport proteins. The structure has been elucidated of two integral membrane proteins that reside in the cytoplasmic membrane. Bacteriorhodopsin from *Halobacterium halobium* spontaneously forms two-dimensional crystals in its natural purple membrane. The three-dimensional structure has been solved at 3.5 Å resolution with high-resolution cryomicroscopy. The structure is largely similar to previously proposed arrangements. The protein is composed of seven rods roughly arranged as a cylinder with a radius of 15 Å. The helices are perpendicular to the membrane surface. Seven rods are α-helices and are oriented with their hydrophobic phases pointing toward the lipid phase and their hydrophilic phases inwards to the active site. Each α-helical segment is about 45 Å long, which is in agreement with X-ray data on the total thickness of the purple membrane (about 49 Å). The segments connecting the loops are extramembranous and non-helical and contain the charged and polar amino acid residues. A model of the protein has been presented, in which the amino acid residues are shown that form the retinal binding site and the proton pathway [13].

The structure of the photosynthetic reaction center of *Rhodopseudomonas viridis* has been solved at 3 Å resolution. Crystallization of this protein was most likely eased by the large fraction of the protein complex that sticks out of the lipid bilayer. Reaction centers consist of four subunits, i.e. H, M, L, and cytochrome *c*. The H and M subunits each contain five α-helical transmembrane segments, while the H subunit contains one membrane-spanning-helical segment. Each segment is about 40 Å long, sufficient to traverse the membrane. The amino acids within these stretches are largely non-polar. The transmembrane helices (A, B, C, D, E) within each subunit run antiparallel, but helices C and E run parallel. The helices are tilted less than 25° away

from the bilayer normal, but helix D (in L and M) has a tilt of 38°. Helices vary in length from 24 to 30 residues. The portions of the L and M subunits connecting the transmembrane segments lie mainly flat on each side of the membrane and form the contacts for the two hydrophilic subunits. The polar ends of the transmembrane helices are more negatively charged at the periplasmic side than at the cytoplasmic side of the membrane. This is energetically favourable, since the membrane potential is negative at the cytoplasmic side [14].

Indirect proof for hydrophobic interactions between the α-helices and the membrane lipids is given by the localization of detergent in *R. viridis* reaction center crystals. Most of the detergent is disordered and associated with the reaction center in a ring-shaped region, covering the 11 α-helices over a length of about 30 Å along their most hydrophobic surfaces, perpendicular to the plane of the membrane [15]. Very similar hydrophobic interactions have been observed in *Rhodobacter sphaeroides* reaction centres crystals in the presence of another detergent. These results point to those regions of the reaction center protein that, in situ, are most likely to be involved in hydrophobic interactions with lipids (or hydrophobic proteins) [16].

Lipid–protein interactions

Membrane proteins require a lipid bilayer to maintain activity due to the extensive hydrophobic regions. Separation of the membrane proteins from their native environment often results in the loss of activity, which can be restored by re-addition of lipids [17]. Certain integral membrane proteins require specific lipids for optimal activity and are inhibited by other lipid species. The activity of integral membrane proteins is usually sensitive to the lipid composition, the lipid bilayer dynamics and its physico-chemical state [18].

Bulk lipid

The lipid environment is defined as the bulk lipids in which the proteins are embedded. The bulk lipids determine the physico-chemical state of the membrane, often called the 'membrane fluidity' and quantitative expressed as the inverse of viscosity (η). Fluidity is a somewhat ambiguous term as it combines the effects of both lipid dynamics and acyl chain order, while often properties of membrane lipids other than fluidity change discontinously at for instance the main-phase transition [19]. Dynamic processes include lateral and rotational diffusion of the lipid molecule, and also rotation around single carbon–carbon bonds. Acyl chain order or lipid packing refers to the average orientation of each carbon along the chain [20]. In model and biological membranes, the term fluidity is usually used in a qualitative sense. Generally, fluidity is measured by observing the motion of spin-labelled probes or fluorescent probes incorporated in the bilayer. These probes are small molecules, comparable to the size of the phospholipids in the membrane. The measurements are sensitive to both rates of motion and any constraints to that motion. Therefore, fluidity gives information about both dynamics and molecular order. It has proven to be a useful parameter, especially in characterizing alterations in the physical state of the membrane due to changes in for instance temperature, pressure, cholesterol content, and phospholipid composition [21].

Membrane phase

Intrinsic membrane proteins function in the liquid-crystalline phase of the membrane. Most membrane proteins become inactive or at least show a dramatic loss of activity during the phase transition from the liquid-crystalline phase to the gel phase. Induction of the phase transition, by lowering the temperature, will lead to a squeezing out of proteins by lateral diffusion ending up in patches of high protein and low lipid content. In the patches, lipids with the lowest transition temperature may segregate, forming microdomains that remain in a fluid state [20,22].

The influence of the physical state of the lipids on the activity of different membrane proteins has been shown [23–27]. The $(Na^+\text{-}Mg^{2+})$-ATPase from *Acholeplasma laidlawii* B senses the lipid-phase transition, as indicated by abrupt

changes in enzyme activity at a lower temperature than suggested calorimetrically. Still, some activity remains even at temperatures well below the cooperative lipid-chain melting transition. It has been suggested that the enzyme partitions preferentially in the liquid-crystalline phase at temperatures at which gel and liquid-crystalline lipid coexist [24].

The internal dynamics of the lactose permease of *Escherichia coli* have been found to be affected by the physical state of dimyristoyl-phosphatidylcholine (DMPC) bilayers. Internal dynamics were determined by measuring the fluorescence anisotropy decay of tryptophanyl residues and covalently bound pyrene labels. Tryptophanyl residues and cysteinyl residues bearing a pyrene label were located on membrane-spanning helices. The slow relaxation processes (50 ns) of the fluorophores were assigned to orientation fluctuations of the polypeptide backbone. Below the lipid-phase transition the transport rate was drastically decreased, while binding of lactose was unaltered. Also, the slow orientation fluctuations of the polypeptide backbone decreased. It was argued that the slow orientation fluctuations of the membrane-spanning helices lead to conformation changes of the protein, in which the binding site is exposed alternatively at both sides of the membrane. Binding or release of the substrate and the transport step will lead to subsequent conformation changes. These data suggest that the physical state of the membrane directly affects the conformation freedom of the transport protein that is necessary for the transport step [28].

Membrane fluidity

Generally, fluidity as measured by spin probes and fluorescent probes is strongly determined by the lipid packing in the bilayer. The tightness of acyl chain packing increases with increasing length of the acyl chain and decreasing *cis*-unsaturation, which resulted in the membrane becoming more ordered. All perturbations that decrease the area per lipid molecule, such as increased hydrostatic pressure, lower temperature, or the addition of cholesterol to phospholipids in the liquid crystalline state, result in a decrease of fluidity [29].

Table 1

Phenotypic thermoadaptations of fatty acyl/alkyl composition to modulate membrane fluidity

Organisms	Change in fatty acyl/alkyl composition
Bacteria	Unsaturation
	Chain length
	Branching
	Cyclization
Archaea	Cyclization

The physiological relevance of fluidity is evident from the adaptations of various organisms to environmental stress. A variety of organisms can alter the phospholipid composition of their cell membrane in response to environmental changes to maintain the bulk of their phospholipids in the liquid crystalline state [30]. This is most clearly observed with thermal stress. It has been suggested that the exact viscosity of the phospholipid component of the membrane is important for optimal functioning of the membrane. The concept of 'homeoviscous adaptation' postulates that organisms alter the lipid composition to maintain a constant membrane viscosity at different temperatures [31]. While eukaryotes modulate membrane fluidity by varying the phospholipid/cholesterol ratio, the temperature-dependent change in fatty acid composition of bacteria is very complex (Table 1) (for review, see [32]). The reason for this complexity of fatty acid changes may be to provide refined protein–lipid interactions by creating lipid domains with different fatty acid acyl chain composition. Bacteria and archaea do not contain cholesterol, but equivalents of sterols, hopanoids and related compounds are widely found. These compounds can alter membrane fluidity in model membranes but have not yet been identified as part of any thermal-regulating mechanism in vivo [33,34].

An example of thermal adaptation is presented by the fatty acid alterations of *E. coli* grown at low temperatures. The predominant fatty acids in *E. coli* are palmitoyl ($C_{16:0}$), palmitoleoyl ($C_{16:1}$), and *cis*-vaccenoyl ($C_{18:1}$). At low temperatures the proportion of unsaturated fatty acids increased, more *cis*-vacceneic acid is incor-

porated in the bilayer, whereas the amount of palmitoleic acid remains constant. These global changes maintain the averaged fluidity of the membrane and allow the cell to survive at temperatures that would otherwise be lethal. These changes are realized by alterations in the activity of one of the two enzymes that catalyze the elongation of fatty acids. At low temperatures the enzyme 3-ketoacyl-ACP synthase II is more active in the conversion of palmitoleic acid, resulting in an increase of the pool size of unsaturated fatty acids to be incorporated into the phospholipids (for review, see [35]). Thermophilic bacilli such as *Bacillus stearothermophilus* change their fatty acid composition so that the upper end of the phase transition from liquid-crystalline to gel phase is always below the growth temperature. However, the exact level of fluidity is not kept constant throughout the growth temperature range [32].

Pressure can also induce changes in fatty acid composition of the membrane as an adaptation for the organism to survive [36]. At high pressures, the proportion of unsaturated fatty acids of the barophilic marine bacterium (NPT3) was found to increase. The packing properties of the unsaturated fatty acids maintain the membrane in the liquid-crystalline state (for review, see [37]).

Several examples have been reported in which a clear linear correlation between enzyme activity and membrane fluidity was found [38–40]. The dependency of enzyme activity on membrane fluidity is largely correlated with the lipid packing density. Changes in this density account for most of the thermally induced changes in fluidity.

The rate constants of the valinomycin mediated Rb^+ flux through membranes composed of different monoglycerides varied with the hydrocarbon chain composition. An increase in chain length from C_{16} to C_{22} or a decrease in the number of C = C double bonds from four to one resulted in a decrease of the rate constants of translocation. The association and dissociation constants, however, remained the same. These effects on translocation rate constants were attributed to the decreased order and smaller microviscosity of membranes [40].

In many studies cholesterol is used as a modulator of membrane fluidity [27,41–43]. Studies on the effect of composition changes on the bulk lipid structure have shown that acyl chain order increases with the cholesterol content above the phase transition temperature and decreases below this temperature [20]. The activity of the human erythrocyte hexose transporter reconstituted in dipalmitoylphosphatidylcholine (DPPC) liposomes was shown to decrease by the presence of cholesterol. However, at temperatures below the transition temperature of DPPC (30°C), cholesterol restores transport activity consistent with its ability to increase fluidity below the transition temperature [27].

Also the activity of the branched-chain amino acid transport system of *Lactococcus lactis* subsp. *cremoris* (formerly named *Streptococcus cremoris* [44]) was studied in a model system in which the cholesterol content was varied [45]. The introduction of cholesterol had a more pronounced effect on protonmotive force (Δp) driven leucine transport than on leucine counterflow, suggesting that the membrane fluidity acts primarily on H^+-coupled leucine translocation [45]. The results are consistent with a model that assumes that the rate of carrier-mediated solute transport across the membrane is proportional to the (rotational and/or lateral) diffusion constant of the protein in the membrane. This diffusion constant is inversely proportional to microviscosity [46]. The effect of membrane fluidity on H^+-coupled leucine transport has also been shown in membranes in which the fluidity was varied by the introduction of monolayer lipids, extracted from an extreme thermophilic archaea *Sulfolobus acidocaldarius* [47].

Lipid shape

As an alternative to homeoviscous adaptation, McElhaney [48] has suggested that it is more important to maintain the liquid-crystalline phase than some absolute value of membrane fluidity. Therefore it is envisioned that the temperature-dependent changes in lipid composition constitute a mechanism to prevent the formation of non-bilayer phases, which would disrupt membrane organization and increase permeability. This 'homeophasic theory' may explain why so many bacterial thermophiles have such large con-

tents of branched-chain fatty acids. These fatty acids form less well-ordered gel phases and do not give rise to phase separation of proteins at temperatures below the liquid-crystalline to gel phase transition temperature [32].

Evidence for regulation of the lipid composition based on an apparent balance of molecular shapes instead of 'fluidity' has been presented for *Acholeplasma laidlawii*. The organism is a mycoplasm species that lacks a cell wall. Exogenous fatty acids are readily incorporated into the plasma membrane. The major lipid components are MGDG and DGDG. The phase properties of *A. laidlawii* membranes are determined primarily by the MGDG/DGDG ratio. The lipid composition has been examined in organisms grown with particular fatty acids at particular temperatures or in the presence of membrane perturbants such as alcohols and detergents. When the perturbation resulted in an increased content of cone shaped lipids, the MGDG/DGDG ratio was decreased and vice versa. The results are interpreted in terms of lipid packing parameters as they influence the phase properties of the membrane lipid mixture (for review, see [49]).

In *E. coli*, PE accounts for 70–80% of the total glycerophospholipids. PE readily adopts an inverted hexagonal phase (H_{II}) conformation. The function of PE has been investigated by constructing an inactivated allele of the gene encoding the phosphatidylserine synthase (*pss*) that catalyses the committed step to the synthesis of PE [50]. Growth of this mutant stopped when the PE content had dropped to 30%, whereas a further decrease in the PE content to 0.007% was observed when cells are grown in the presence of divalent metal ions at millimolar concentration. The efficiency by which these ions suppressed the growth phenotype of the mutant strain decreased in the following order: $Ca^{2+} > Mg^{2+} > Sr^{2+}$. The remainder of the phospholipid was primarily phosphatidylglycerol (PG) and CL. It has been suggested that suppression of the growth phenotype of this *pss* mutant by Mg^{2+} is due to the ability of CL to substitute for the H_{II} phase-forming ability of PE in the presence of Mg^{2+} [50,51]. The results suggest that PE fulfils a structural role in *E. coli*, and may imply a need for 'homeophasic' regulation of the lipid composition.

Some proteins have been shown to strongly influence the shape of the phospholipids and to be able to stabilize lamellar or hexagonal forms of the lipid. The hydrophobic polypeptide gramicidin A converts dioleoylphosphatidylcholine (DOPC) from a bilayer to a hexagonal H_{II} form when the lipid/protein molar ratio is 10:1. Interaction of the lipid to gramicidin is followed by dehydration of the lipid that stabilizes the non-bilayer form [52]. In contrast, cytochrome *c* oxidase stabilizes the lamellar bilayer structure and prevents salt-induced transition to inverted hexagonal phase. The protein is incorporated only in the lamellar phase [53]. Such an influence on lipid polymorphism has also been observed for other integral membrane proteins (e.g. rhodopsin [54] and glycophorin [55]). These lipid–protein interactions may contribute to the stability of the bilayer.

The functional importance of non-bilayer forming lipids is evident from studies in which the Ca^{2+}-ATPase of sarcoplasmic reticulum was reconstituted. The Ca^{2+}/ATP coupling ratio is stabilized by cone-shaped lipid molecules as dioleoyl-phosphatidylethanolamine (DOPE) and MGDG. In DOPE and MGDG, high initial rates of ATP-dependent Ca^{2+} uptake are found. Methylation from DOPE to DOPC or glycosylation from MGDG to DGDG resulted in a progressive decrease of both ATP-dependent Ca^{2+}-uptake and coupling ratios. Methylation increases the size of the polar head and reduces the cone shape of MGDG and PE. These lipids tend to adopt cylindrical shapes that in turn increases the H_{II} transition temperature. It has been suggested that the Ca^{2+}-to-ATP coupling by the Ca^{2+}-ATPase is controlled by a specific arrangement of cone-shaped lipids at the lipid–protein interface [56].

Membrane thickness

Another aspect of lipid–protein interactions is the matching between the length of the hydrophobic part of the protein and lipid hydrophobic thickness. This aspect has been discussed theoretically by Mouritsen and Bloom [7]. Their

'mattress model' of lipid protein interactions in membranes is a thermodynamic model describing the phase diagram of mixtures of lipid bilayers and amphiphilic proteins or polypeptides. Variables are the thickness of the hydrophobic region of the lipid bilayer and the length of the hydrophobic region of the proteins. The model considers the elastic properties of the lipid bilayer and the proteins and identifies the perturbations caused by mismatches between lipid and protein hydrophobic thicknesses. Many proteins that mediate membrane-associated biological activities span the entire thickness of the membrane. The three-dimensional structure of integral membrane proteins is pictured as stabilized by the matching of pairs of oppositely charged or polar residues in different α-helices within the hydrophobic depths of the membrane. By varying the number of hydrophobic residues of a transmembrane protein and/or acyl chain lengths of the phospholipid molecules, the mismatch between their hydrophobic regions can be varied. Variations in the mismatch can cause irregularities in the lateral distribution of membrane proteins and modify the temperature range of coexistence of lipid phases [7].

Since the Gibbs free energy ($\Delta G^{\circ\prime}$) of the interaction of hydrophobic residues to water is high, it is unlikely that any mismatch between the thickness of the hydrophobic portions of membrane proteins and the lipid bilayer will result in significant exposure of these regions to water. Changes in conformations of either the protein or the lipid can be expected to minimize any mis-

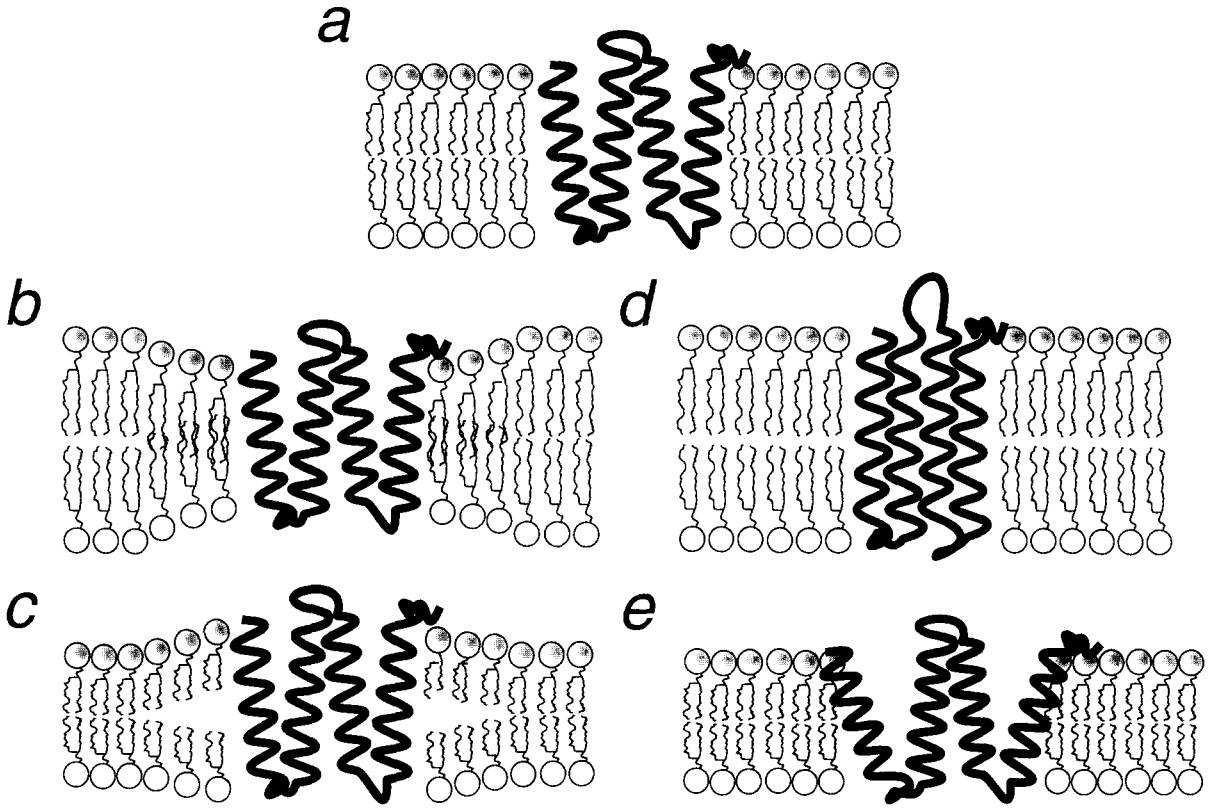

Fig. 4. Scheme showing the possible consequences of mismatching between the hydrophobic thickness of the membrane and an integral membrane protein in a model system composed of defined, synthetic phospholipids. When a perfect match (**a**) cannot be realized due to the length of the acyl chains, matching is achieved through interdigitation of the phospholipid acyl chain (**b**), packing defects at the lipid/protein interface (**c**) or by conformational change in the membrane protein (**d** and **e**).

match in thickness. There must be a mechanism by which the lipids or the membrane-spanning segments of such proteins (or both) adjust under circumstances where the 'inherent thickness' of a given bilayer does not match that of the membrane-spanning domain of the protein. In principle, this could involve increases or decreases in the conformation disorder of the lipids when the lipid-crystalline bilayer is 'thinner' or 'thicker', respectively, than the inherent length of the membrane-spanning domains of the protein. There could also be adjustments to the protein ranging from net tilting of the protein relative to the bilayer normal to some distortions of the hydrophobic α-helices that presumably comprise the membrane-spanning domains. Since free energies of distortion of proteins can be expected to be large, it is more likely that the bilayer distorts to match the hydrophobic thickness of membrane proteins [57]. Lewis and Engelman [58] have suggested that only small energy changes are involved in thickening or thinning a bilayer. By definition, there must be limits to which such 'adjustments' can occur and the function of proteins can be maintained. Adjustments may occur easier in a thicker lipid bilayer [24]. In a heterogeneous lipid mixture, it is also possible that the lipids rearrange so that lipids with the correct length could interact with the protein. To minimize deformation strain in the bilayer, elastic deformations will cause specific lipids to bind to particular proteins by shape or size matching and not due to specific chemical interactions (Fig. 4) [21].

The hydrophobic thickness of the membrane can be obtained most accurately from continuous X-ray scattering that measures the distances between the phosphate groups across the bilayer. By subtracting the thickness of the polar headgroup region known from neutron-diffraction studies of specifically deuterated samples, the hydrophobic thickness is obtained. The hydrophobic thickness is a linear function of the acyl chain length and is about 30% larger in the gel phase. It is now anticipated that integral membrane proteins are approximately matched by the fluid bilayer thickness of their natural membranes to function optimally [59]. By manipulating the hydrophobic thickness either by changing the lipid acyl chain length or by incorporating alkanes it is invariably found that the activity of different membrane proteins is influenced by the degree of matching and is optimal at a defined thickness [24,26,57,60–63] (Fig. 5).

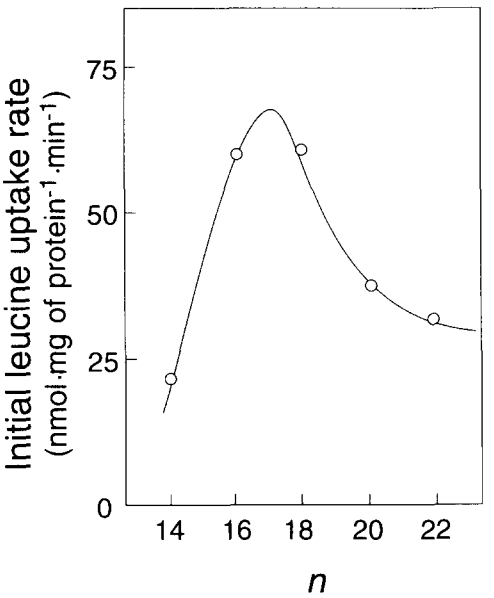

Fig. 5. Initial rates of counterflow uptake of leucine in hybrid membranes obtained by fusion of *Lactococcus lactis* membrane vesicles with liposomes composed of di(18:1)PE/di(n:1)PC. n is the number of carbon atoms in the acyl chain was varied between 14 and 22. From [62], with permission.

Membrane thickness can also be estimated by deuterium nuclear magnetic resonance (^{2}H-NMR) measurements, since a linear relation exists between the average orientation order parameter of the acyl chains and the membrane thickness. This is only true for the liquid-crystalline phase, because in the gel phase the chains are tilted with respect to the bilayer normal. It has been suggested that cholesterol may regulate membrane function indirectly by changing the hydrophobic thicknesses of the membrane. There is a strong relation between orientation order of the lipids and microviscosity, but this is not so for cholesterol. The introduction of cholesterol into phospholipid bilayer membranes increases the orientation order (the associated decrease in the average membrane surface area per polar head is

often called 'condensation'), but does not appreciably increase the microviscosity. An increase in orientational order of the lipid acyl chains by the addition of cholesterol, as measured with fluorescent probes, is correlated with an increase in membrane thickness [64,65].

'Annular lipid'

The lipid annulus is the lipid in a special environment of the membrane protein that is sufficient to cover the surface of the protein. The surface will be heterogeneous, both due to the molecular 'roughness' of the amino acid residues making up the hydrophobic α-helices and the presence of charged amino acid residues close to the phospholipid water interface. Annular lipids, dissolving the protein, will constitute a monolayer, fitting the hydrophobic and hydrophilic regions. It is believed that the lipid annulus stabilizes a functional conformation of the membrane protein [1]. The bulk (mobile) lipid and annular (immobile) lipid are separately recognized with electron spin resonance (ESR) of spin-labelled membrane components. The timescale of spin label ESR is such that the population of lipids interacting directly with the membrane proteins can be resolved from the bulk lipids, even if the lipid diffusion rate would be as in a fluid bilayer. The selectivity of the proteins toward particular lipid species is measured with low concentration of lipid probe in a matrix of 'reference' phospholipid (or phospholipid mixture). The relative association constant of the probe with the protein versus the reference lipid and the rates of lipid exchange between the probe and the bulk lipid at the binding sites can be estimated. This latter rate at the lipid interface is determined by the first order rate constant for exchange of the protein-interacting lipid component. Off-rates are generally in the region of $10^7\ s^{-1}$, which is of the same order but significantly slower than the free diffusion rates in lipid bilayers. In this way it has been possible to identify the boundary layer of annulus lipids, or first shell, surrounding integral membrane proteins [11,18].

A fixed number of lipid binding sites per protein is found independent of the lipid/protein ratio. Only at low lipid/protein ratios the stoichiometry may decrease, and often non-specific aggregation of protein is observed. The number of annulus lipids that have restricted mobility and do not exchange readily with the bulk lipids, varies from approximately 60 for the (Na^+-K^+)-ATPase dimer to 22 for the rhodopsin monomer. The Ca^{2+}-ATPase was found to be exceptional since no selectivity for lipids was found [18]. Another interesting exception is the M13 phage coat protein, for which a stronger lipid selectivity and a slower interfacial lipid exchange rate was found for the β-sheet conformation compared to the α-sheet conformation of the protein. Differences in lipid selectivity are attributed directly to the two different conformations. Amino acid residues, which are responsible for the selectivity of negatively charged lipids, will be more exposed in the extended nature of the β-structure than in the compact α-structure. The retarded exchange rate is explained by entrapment of lipids within aggregates, which are only formed when the protein has a β-sheet conformation. Additionally, it is proposed that the β-sheet conformation results in a more extended shape of the lipid acyl chains at the protein interface and therefore a reduction of the acyl chain flexibility and exchange rate. This increased lipid–protein interaction with the β-sheet conformation may be directly relevant for outer membrane proteins in *E. coli* and other Gram-negative bacteria [66].

A particular preference for interacting with proteins is observed for negatively charged lipids. PC exhibits mostly the lowest selectivity, although the selectivity of PE is sometimes even lower. CL, phosphatic acid (PA) or free fatty acids are found to have the highest selectivity, depending on the particular protein [18]. For thylakoid membranes DGDG appears to take the role played by PC in non-plant systems while phosphatidylglycerol (PG) displays a marked selectivity in this system [67]. The relative association constant (K_r) for CL with cytochrome *c* oxidase is 5–6-fold that of PC [68]. Studies on the association of spin-labelled CL with cytochrome *c* oxidase showed that the binding sites for CL are not highly specific. CL is an efficient activator for cytochrome *c* oxidase. Activation is based on the increased surface

charge conferred by CL at the boundary layer of the enzyme, resulting in an increase of the surface concentration of both cytochrome *c* and protons. Secondly, an enhanced interconversion between different conformations of the enzyme was observed in the presence of CL [69].

Lipid selectivity is generally considered to arise in part from polar interactions between charged lipid headgroups and amino acids of the protein hydrophilic surface. Lipid selectivity depends on ionic strength and on the protonation state of the lipid [18]. A clear demonstration of these polar interactions is provided by the differential selectivity of two myelin proteolipids, PLP and DM20, for acidic phospholipids. DM20 is derived from PLP by the deletion of a 35-amino acid hydrophilic segment, which is considered to form an extramembranous loop. PLP induces the formation of domains, enriched in charged lipids from mixtures of neutral and charged lipids. Whereas PLP is associated with motional restricted lipids, DM20 does not show this selectivity. The five positively charged residues (3 arginine and 2 lysine residues) in the hydrophilic loop of PLP are possible candidates for preferential associations with acidic phospholipids [70,71].

There are indications that the interactions between the charged headgroups of the lipids and the hydrophilic surface of the protein are not purely electrostatic. A decrease of the surface charges by high ionic strength does not entirely suppress the lipid selectivity. Covalent modifications indicated that lysine residues are involved in the lipid selectivity of cytochrome *c* oxidase [72], and the subunits bearing some of the lysine groups have been identified [73].

Hydrophobic interactions between the lipid acyl chains and the membrane-spanning region of the protein occur at the protein surface. The strong binding of CL, compared to other phospholipids, to both $(Na^+–K^+)$- and $(Ca^{2+}–Mg^{2+})$-ATPase has at least in part been attributed to the four fatty acyl chains of CL compared to the two fatty acyl chains of the other phospholipids [74].

Reconstituted systems revealed that annulus lipids influence the protein activity. Extraction of proteins by non-ionic detergents in the presence of large amounts of phospholipids leads to an exchange of the lipids associated with the protein in the membrane and the phospholipids in the detergent [75]. This is evident from studies on the leucine transport carrier of *L. lactis*. Although leucine transport activity depends on aminophospholipids (PE and phosphatidylserine (PS)) or glycolipids [76], activity is only recovered in proteoliposomes when cytoplasmic membrane vesicles are solubilized in the presence of acidic phospholipids [77]. Many of the mitochondrial carriers, such as the ADP/ATP and phosphate carrier, require CL during solubilization and purification [78]. The eukaryotic $(Na^+–Ca^{2+})$-exchanger of cardiac sarcolemmal vesicles only functions when it is reconstituted into liposomes containing PS, CL or PA, whereas phosphatidylinositol (PI) and PG provide a poor environment for $Na^+–Ca^{2+}$ exchange. PS, PG and PA can apparently interact with the protein in a different manner than PG and PI. Both PS and PA have an anionic charge at the hydrophilic terminus of the molecule. PG, likewise, has relatively well-exposed anionic sites. On the other hand, the negative charge on both PG and PI is shielded by a terminal neutral moiety that may prevent interaction with the protein [79].

Specifically bound lipids

Some proteins have a small number of high-affinity lipid binding sites that are specific for particular not readily exchangeable lipids. The mitochondrial ADP/ATP carrier has a high binding activity for CL that is not determined by the acyl chain composition of the CL, at least not when the protein is reconstituted in saturated PC environment. The high binding affinity appears to be determined mainly by the structure of the headgroup and to involve electrostatic interaction between the negatively charged CL headgroup and positively charged protein surface [80]. Mitochondrial cytochrome c oxidase [68] and β-hydroxybutyrate dehydrogenase [81] have been shown to tightly bind CL and PC, respectively.

The stimulation of the $(Na^+–K^+)$-ATPase in the presence of low levels of cholesterol has been shown to depend on a direct interaction between

an integral membrane protein and a sterol. Stimulation was shown to be structurally specific. Lanosterol is less capable than cholesterol in stimulating the enzyme, while ergosterol is virtually ineffective. This stimulating effect was explained by a direct sterol–protein interaction, mediated by specific sites on the cholesterol-sensitive protein [43].

Modulation of the lipid environment of membrane proteins in vitro

When membrane proteins are moved from their natural environment to an artificial membrane system, the activities have to be preserved, i.e. membrane proteins have to be functionally reconstituted in the model membrane (Fig. 6).

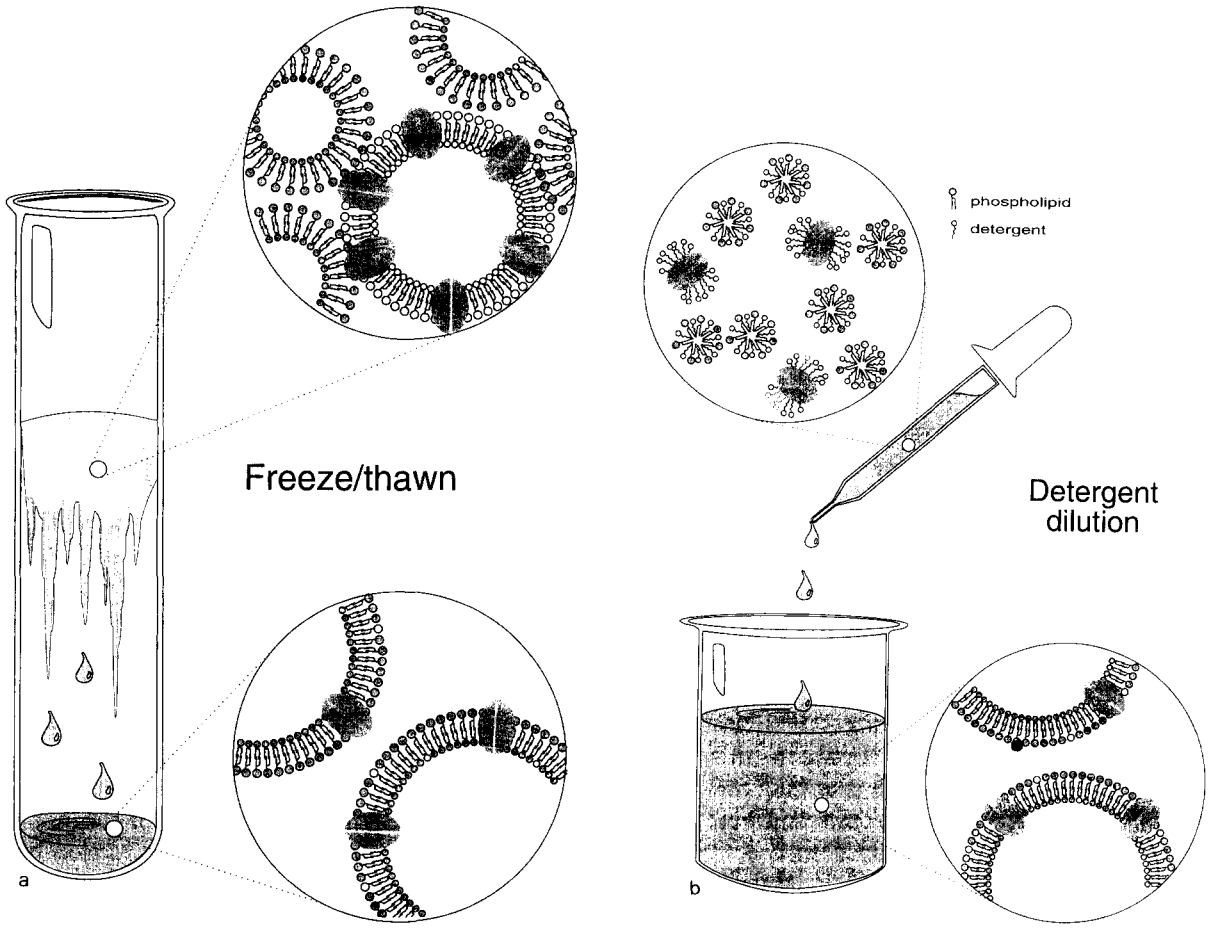

Fig. 6. Sketches showing in vitro techniques to enrich membrane proteins with exogenous lipids. (**A**) Membrane fusion: Membrane vesicles are fused with an excess of liposomes through a freeze-thaw step, and subsequently sonicated or extruded through polycarbonate filters to obtain unilamellar vesicles. (**B**) Detergent solubilization and dilution: Proteins are extracted from the membrane vesicles with detergent in the presence of excess phospholipid. The mixed micellar solution of detergent–phospholipid–protein is diluted into buffer to allow the removal of the detergent and the spontaneous formation of proteoliposomes. Membrane proteins are temporarily delipidated during detergent extraction in contrast to the fusion method where the protein remains embedded in lipid.

Full activities of membrane proteins are expressed only when they are correctly oriented and inserted in the lipid bilayer. In secondary solute transport proteins the efficiency of reconstitution is reflected only by the transport activities. This activity can be monitored by measuring the transport of a solute from one compartment to another [78,82]. Antibodies against the exposed hydrophilic domains [83–85] or binding of the solute to the reconstituted carrier can be used to assess the extent of incorporation. The lipid environment of membrane proteins in vitro can be manipulated with or without the use of detergents.

Membrane fusion

Membranes can be isolated from intact cells, yielding closed cytoplasmic membrane vesicles with the same polarity as intact cells [86,87]. Alternatively, cells can be subjected to low shear forces in a French pressure cell, yielding membrane vesicles with an inside-out orientation [88]. These membrane vesicles have been used to study the role of the protonmotive force (Δp) in bioenergetic processes, particularly with respect to secondary transport [86,89]. Many biological membranes do not contain a H^+-translocating electron transfer system. Therefore, a method was developed to incorporate a Δp-generating system in these membranes vesicles. This method is applicable to cell membranes of eukaryotic cells and fermentative bacteria, in which no accessible proton pump is present. By incorporating proton pumps, like cytochrome *c* oxidase from beef heart or other sources, bacteriorhodopsin, or bacterial reaction centers, a Δp can be generated continuously with energy supply from the outside. The primary proton pump, reconstituted into liposomes, is inserted in the biological membrane by membrane fusion. This is accomplished by freeze/thaw-sonication of a mixture of cell membrane vesicles and the proteoliposomes and results in the formation of tightly sealed hybrid membranes (for review, see [90]). This procedure has been applied successfully to bacterial membranes [91–94], purified submitochondrial membranes ([95]; for review see [94]) and yeast [97] and fungus (D. Hillinga, H. Versantvoort, A.J.M. Driessen, and W.N. Konings, submitted) plasma membranes.

Membrane fusion has also been used to enrich membrane vesicles with exogenously supplied phospholipids (Fig. 6a). In this way bacterial membrane vesicles of *L. lactis* have been fused with liposomes, composed of a defined lipid composition, to investigate systematically the lipid requirement of the leucine transport system [45,47,63,76,98]. Membrane fusion does not always yield membrane structures that are tightly sealed. Mitochondrial inner membranes [99,100] and right-side-out membrane vesicles of *E. coli* (A.J.M. Driessen, unpublished results) loose their energy-conserving properties upon fusion with liposomes. A possible explanation is that contaminating outer membranes containing porins are incorporated in the hybrid membranes upon fusion. Fusion is also the most commonly used technique for incorporating spin-labelled probes in proteoliposomes. Spin labels are first sonicated to form small unilamellar vesicles and subsequently incubated with protein-containing membranes [101].

The freeze/thaw-sonication method has been successfully applied to incorporate purified membrane proteins into liposomes [102,103]. However, this application is limited due to the risk of denaturation of the membrane proteins. Sonication of the proteins with liposomes can be used to facilitate reconstitution of membrane proteins. Most likely the protein can insert into small vesicles because of the high degree of curvature of these liposomes. Co-incubation of the proteins and preformed vesicles has also been applied successfully for proteins with limited hydrophobic surfaces such as cytochrome b_5 and β-hydroxybutyrate dehydrogenase that do not penetrate across the bilayer. Low concentration of amphiphiles in the protein–phospholipid mixture can facilitate the incorporation of membrane proteins, such as bacteriorhodopsin or cytochrome *c* oxidase, into liposomes. Cholesterol [104], short-chain PCs [105], and fatty acids [106] have been used as amphiphilic catalysts.

Detergent-mediated reconstitution

Treatment of biological membranes with detergents or with organic solvents results in the disintegration of the lipid bilayer. Detergents presumably bind to membrane proteins at the nonpolar binding sites normally in contact with the bilayer interior. This process is called solubilization, while reconstitution refers to the reincorporation of a solubilized membrane protein into a lipid bilayer. Solubilization/reconstitution is often accompanied with a loss of enzymatic activity. The loss of function is the result of denaturation caused by detergent solubilization. This is explained by the amphiphilic properties of the membrane proteins that tend to make them unstable in a solubilized state in both aqueous and organic solvents. In aqueous media, intermolecular self-association of the hydrophobic surfaces of the protein is favoured, leading to the formation of high-molecular mass protein aggregates that usually precipitate. In organic solvents, on the other hand, the polar domains tend to associate, which leads to exposure of the hydrophobic domains to the solvent. Therefore it is important to search for conditions that minimize irreversible denaturation [6].

The stability of membrane proteins in detergent solution is influenced by a number of parameters such as the type of detergent used; the presence of lipids and of osmolytes, like glycerol; the ionic strength of the medium and the type of salt added during solubilization and in some cases by the presence of reducing agents and proteinase inhibitors [82]. Usually a detergent with a high critical micellar concentration and small micelle size is chosen so that it can be removed easily by dialysis, gel filtration or dilution (Fig. 6b). Sodium cholate or octylglucoside are the most common choices. The ratio of detergent to phospholipid and the method and rate of detergent removal are important factors that determine the size distribution of the resulting proteoliposomes [107,108]. Dialysis is a slow process and is usually the method of choice [109]. Occasionally it is advantageous to eliminate the detergent more rapidly, especially if the protein is unstable in the presence of excess detergent. Gel filtration chromatography has been used successfully in effectively separating the proteoliposomes from the detergent [110,111]. Even more rapid is the dilution procedure, in which the protein–lipid–detergent mixture is diluted far below the critical micelle concentration of the detergent. Proteoliposomes are formed spontaneously and can be separated from the detergent by centrifugation [112]. Triton X-100 is commonly used for the purification of membrane enzymes, but it is not an ideal detergent in reconstitution experiments since it is difficult to remove. Polystyrene beads have proven to be useful for removal of this detergent, leaving protein and phospholipid behind although binding of protein to the resin can be a problem [113]. Finally, reverse phase evaporation, in which diethyl ether disperses a phospholipid–protein mixture, has also been applied for reconstitution [114], but most proteins do not tolerate this procedure.

Secondary transport proteins have been reconstituted from solubilized membrane vesicles [77,115–118]. The preparation of membrane vesicles can be eliminated by solubilization of membrane proteins directly from whole cells [118,119]. The proteoliposomes obtained are physically stable, non-leaky and can sustain an electrical potential ($\Delta\psi$). Porins do not co-reconstitute apparently due to their low solubility in non-ionic detergents, the relatively hydrophilic nature of these outer membrane proteins or a failure of the porin monomers to reassemble correctly [119]. For the further purification of solubilized membrane proteins different techniques are used that are often similar to those used for the purification of soluble proteins. These include DEAE cellulose or sepharose, gel filtration chromatography, hydroxylapatite, sucrose density gradients, etc. Furthermore, separation techniques have been developed for intrinsic membrane proteins, such as hydrophobic phase separation [121] and hydrophobic chromatography [122].

Toward a stable membrane system

Liposomes prepared from natural or synthetic phospholipids usually suffer from chemical and

mechanical instability. Stabilization of such systems would offer attractive experimental possibilities. Different approaches to stabilize membranes have been described such as intermolecular cross-linking of lipid headgroup and acyl chains with or without spacers, the use of polymerizable sterol analogues, or fixation of amphiphilic polymers via hydrophobic anchor groups to the lipid bilayer [123]. A major problem with these artificial lipid species is their incompatibility with membrane proteins. Although their use often confers a greater stability to the lipid vesicles, they support little or no activity of integral membrane proteins. Membrane lipids of archaea are receiving a growing interest for use in liposome preparation as they differ considerably from conventional lipids in their chemical structure (Fig. 2). The natural resistance to oxidation and esterases makes these ether lipids suitable for the preparation of liposomes with enhanced stability and storage characteristics. Closed and stable unilamellar liposomes can be formed from a specific lipid fraction obtained from a total lipid extract from *Sulfolobus acidocaldarius* [124]. These monolayer liposomes bear a dramatically reduced H^+- and Cl^- permeability compared to bilayer liposomes prepared from a natural phospholipid mixture extracted from *E. coli*. Low-molecular mass compounds entrapped in the liposomal lumen are retained for periods up to months (M.G.L. Elferink, J. de Wit, A.J.M. Driessen and W.N. Konings, unpublished results). Liposomes prepared from membrane-spanning lipids have been used for the functional reconstitution of integral membrane proteins, such as the cytochrome *c* oxidases of beefheart mitochondria [47,124] and thermophilic bacilli, bacteriorhodopsin from *H. halobium* [125], the quinol oxidase from *S. acidocaldarius* (M. Gleißner, M.G.L. Elferink, J. de Wit, A.J.M. Driessen, W.N. Konings and G. Schäfer, unpublished results) and the precursor protein translocator of *E. coli* (A.J.M. Driessen, unpublished results). The leucine transport system of the mesophilic bacterium *L. lactis* has been introduced into these liposomes by fusion with isolated membrane vesicles [47]. Maximum activity was observed when the monolayer lipids were mixed with bilayer lipids to rigidify the membrane at 25°C. Monolayer lipids can provide a suitable matrix for the reconstitution of membrane proteins. Due to their membrane-spanning nature, monolayer of these lipids as formed on a water–air interface may represent a better model system for studies on protein–lipid interactions than the conventional phospholipids that form only a monolayer of half the width of the membrane. Another potential application of monolayer lipids is their use in black lipid membranes [126].

Concluding remarks

All available data indicate a supportive role of lipids in the stabilization of solute transport proteins in a native and active conformation. However, the picture about the interaction of transport proteins with lipids is far from complete. Many questions remain, e.g. Do lipids also fulfill a catalytic role in solute transport such as proposed in the functioning of the multiple drug resistance protein [127]? The multiple drug resistance protein actively extrudes a large variety of structurally unrelated drugs across the membrane and may expose its substrate-binding site to the hydrophobic interior of the membrane. Little is known about the precise molecular pathway by which solute pass the membrane in a protein-mediated manner. A common view is that the membrane-spanning α-helices of solute transport proteins are arranged as a bundle to form a cylinder-like particle with a hydrophobic surface internalizing a hydrophilic interior [128]. The often polar solutes would pass the membrane through the channel-like interior of the transport protein, and amino acid residues of the inner wall would confer specificity to the transport process and catalyze the energy-coupling reactions. Such a molecular view of a transport reaction only assumes a supportive role of lipids. There is, however, no information on the precise packing of the membrane-spanning α-helices, nor is it known whether lipids have access to the solute binding site. In this respect, the lactose transport protein of *E. coli* exhibits greater affinity for galactosides with increasing lipophilicity [129],

suggesting some interaction between the solute and hydrophobic domains. The latter may consist of lipid acyl chains, apolar side-chains of amino acid residues or both. Structural information at molecular resolution will be needed to resolve mechanistic questions, and to understand interactions between lipids and transport proteins.

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