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Lipid interactions with bacterial channels:

fluorescence studies

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Abstract

Interactions between a membrane protein and the lipid molecules that surround it in the membrane are important in determining the structure and function of the protein. These interactions can be pictured at the molecular level using fluorescence spectroscopy, making use of the ability to introduce tryptophan residues into regions of interest in bacterial membrane proteins.

Fluorescence quenching methods have been developed to study lipid binding separately on the two sides of the membrane. Lipid binding to the surface of the mechanosensitive channel MscL is heterogeneous, with a hot-spot for binding anionic lipid on the cytoplasmic side, associated with a cluster of three positively charged residues. The environmental sensitivity of tryptophan fluorescence emission has been used to identify the residues at the ends of the hydrophobic core of the second transmembrane α -helix in MscL. The efficiency of hydrophobic matching between MscL and the surrounding lipid bilayer is high. Fluorescence quenching methods can also be used to study binding of lipids to non-annular sites such as those between monomers in the homotetrameric potassium channel KcsA.

Keywords

bacterial channel; fluorescence; KcsA; lipid-protein interaction; mechanosensitive channel; potassium channel

The biological membrane as a system

A first step to understanding how the biological membrane works as a system is to study, separately, the structures and properties of the lipid and protein components of the membrane, and much progress has been made to this end. A next step is to understand how the lipid and protein components interact in the membrane. This is considerably more difficult. X-ray crystal structures of integral membrane proteins generally include few, if any, lipid molecules, and those lipid molecules that are observed are generally located at protein-protein interfaces in oligomeric structures and so are not typical of the bulk of the lipid molecules interacting with a membrane protein [1]. Other methods have, therefore, to be developed to study lipid-protein interactions.

Some functions of a biological membrane will depend on the bulk physical properties of the membrane such as elasticity, internal pressure, elastic strain etc. For example, the organelles in an eukaryotic cell are linked by a constant flow of membrane vesicles, budding off from one organelle and fusing with another. Although the process of vesicle budding involves a complex set of protein interactions, it is also likely to depend on properties of the lipid bilayer such as ease of bending [2]. In contrast, it is important that membrane proteins be

insulated from these bulk properties of the membrane; for the function of any one membrane protein to be independent of what the other proteins in the membrane are doing, it is important for the function of a membrane protein to depend only on its immediate environment. We need, therefore, to focus on how membrane proteins interact with the lipid molecules that surround them in a membrane and develop a molecular-level picture of these interactions.

Lipid-protein interactions are of fundamental importance since they determine, in part, the structures of integral membrane proteins [3] and, therefore, their activities [4]. Some membrane proteins show a requirement for small amounts of a particular lipid or class of lipid for function, the lipid acting like a cofactor. For example, the potassium channel KcsA only opens if the membrane contains a small amount (10 mol%) of an anionic lipid and the crystal structure of KcsA shows an anionic lipid molecule bound at the protein-protein interfaces (non-annular sites) in the homotetrameric structure [5]. The functions of membrane proteins are also affected by the large number of lipid molecules interacting with the hydrophobic, membrane-penetrant surface of the protein, the so-called boundary or annular lipids. This is most readily demonstrated in reconstituted systems where the membrane protein of interest is reconstituted into lipid bilayers of defined composition; changing the lipid fatty acyl chains and changing the lipid head groups lead to large changes in activity for all the membrane proteins studied in this way [4]. There is considerable debate in the literature about how these effects on activity come about. It has been suggested that they could follow indirectly from changes in bulk physical properties of the membrane such as lipid-free volume, internal pressure, elastic strain etc. (for a discussion, see [4]). However, it is also possible that the observed effects follow directly from differences in the molecular interactions between proteins and the various classes of lipid molecules. For example, the hydrogen-bonding potential of the $-\text{NH}_3^+$ group of PE (phosphatidylethanolamine) is very different from that of the corresponding $-\text{NMe}_3^+$ group in PC (phosphatidylcholine) and it could be differences of this type that explain the different effects that these lipids have on the functions of some membrane proteins. The debate has been hindered by the lack of methods to probe lipid-protein interactions at the molecular level. Since X-ray crystallography cannot give us a complete picture of how a membrane protein interacts with the lipid molecules that surround it in a membrane, it is necessary to develop other techniques to provide the required information.

Studying lipid binding using fluorescence quenching

The first spectroscopic technique used to study lipid-protein interactions was ESR, using spin-labelled lipids [6]. This technique can be used to determine an average binding constant for lipids over all the sites on the transmembrane surface of a membrane protein but cannot detect any heterogeneity in binding. We introduced a more convenient fluorescence quenching method for looking at lipid-protein interactions, making use of the ability of bromine-containing phospholipids to quench the fluorescence of tryptophan residues in a membrane protein [7]. When used with a membrane protein containing many tryptophan residues, the fluorescence quenching method, like ESR, can only give an average of the lipid binding constants at all the annular sites. So, for example, fluorescence quenching methods were used to show that SERCA1 (sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase 1), which contains 13 tryptophan residues, had an average binding constant for PEs of approx. one-half of that for PCs [7], but we do not know if this means that PEs bind less well than PCs at all sites or whether PEs and PCs bind equally well at some sites, with PEs binding much less strongly than PCs at other sites. However, an advantage of the fluorescence quenching method is that, for a membrane protein containing a single tryptophan residue, the fluorescence method gives lipid-binding constants just for the lipid-binding sites close to the tryptophan residue [8].

Brominated phospholipids are easily prepared by the addition of bromine across the double bond in phospholipids containing *cis*-monounsaturated fatty acyl chains [7]. Phospholipids containing brominated fatty acyl chains behave much like conventional phospholipids with unsaturated fatty acyl chains because the bulky bromine atoms have effects on lipid packing that are similar to those of a *cis* double bond [7]. The mechanism of quenching of a tryptophan residue by bromine is not certain and could be by a collisional mechanism or by Forster energy transfer since dibrominated molecules show significant absorption at the wavelengths of tryptophan emission [9]. However, irrespective of the mechanism, quenching of tryptophan fluorescence by dibrominated quenchers fits an equation with a sixth-power dependence on the distance of separation between the tryptophan residue and the quencher, with a value for R_0 (the distance at which quenching is 50% efficient) of approx. 8 Å ($1 \text{ Å} = 10^{-10} \text{ m}$), comparable with the diameter of a lipid molecule [9]. Only a brominated lipid molecule bound in the immediate vicinity of a tryptophan residue will therefore cause quenching. Furthermore, the fluorescence lifetime for tryptophan is considerably less than the time for two lipid molecules to exchange position in the bilayer so that the quenching method gives an essentially time-frozen picture of the membrane: the extent of fluorescence quenching in a mixture of a brominated lipid and a non-brominated lipid will therefore depend on the probability that any of the lipid-binding sites close to the tryptophan residue is occupied by a brominated lipid molecule [10]. Because mixing of lipids in the liquid crystalline state is close to random [11], the observed level of fluorescence quenching will depend simply on the mole fraction of the brominated lipid in the mixture and the binding constant of the sites on the protein for the brominated lipid [10].

The lipid annulus is heterogeneous: hot-spots for binding anionic lipid

A suitable protein for study by the fluorescence quenching method is the mechanosensitive channel MscL from *Mycobacterium tuberculosis*. This homopentameric protein has the dual advantage that its structure has been determined [12] and that it contains no tryptophan residues so that tryptophan residues can be introduced into chosen regions of the protein to report on lipid binding in that region. For example, a tryptophan residue introduced at position 69 reports on lipid binding on the periplasmic side of the protein, whereas a tryptophan residue introduced at position 87 reports on lipid binding on the cytoplasmic side of the protein (Figure 1) [8]. We found that di(C_{18:1})PC (dioleoylphosphatidylcholine) and di(C_{18:1})PE (dioleoylphosphatidylethanolamine) bound with equal affinity, on both sides of the protein [8]. In contrast, although anionic lipids bound with equal affinity for di(C_{18:1})PC on the periplasmic side of the protein, anionic lipid bound with higher affinity than di(C_{18:1})PC at a single site per monomer on the cytoplasmic side (Figure 2) [8]. This preference for anionic lipid is associated with a cluster of positively charged residues, Arg⁹⁸, Lys⁹⁹ and Lys¹⁰⁰, on the cytoplasmic side of the protein, at a location suitable for interaction with the lipid head group [8]. Mutation of one of these positively charged residues to an uncharged residue leads to a decrease in affinity for anionic lipid [8]. The results of removing all three positively charged residues is more complex since it leads to a major conformational change on the protein, probably from a closed form to an open form of the channel [8]. The positively charged cluster on any one subunit of MscL interacts with acidic residues on the two neighbouring subunits, the charge interactions acting as a molecular Velcro, holding the ends of the transmembrane α -helices together, in a closed channel. The channel opens on increasing the tension in the membrane, which involves tilting the transmembrane α -helices [13], increasing the separation between the ends of the helices, which can only be achieved by disrupting the interactions between the positively and negatively charged residues in neighbouring subunits; removing the positive charges allows the channel to transform into an open state in the absence of tension in the membrane.

How a membrane protein sits in the surrounding lipid bilayer

Because crystal structures of membrane proteins do not include a full complement of lipid molecules, the location of the lipid bilayer around a membrane protein is not usually obvious from the crystal structure [1]. Complications arise from the fact that transmembrane α -helices often extend beyond the likely position of the lipid bilayer, the fact that transmembrane α -helices usually have to span the lipid head-group region as well as the hydrophobic core of the bilayer and the fact that the ends of transmembrane α -helices may not be well defined because of the need to provide suitable hydrogen-bonding partners for residues at the ends of the helices.

One experimental approach to defining where the hydrophobic core of the lipid bilayer is located on a membrane protein surface makes use of the environmental sensitivity of fluorescence emission [14]. The method is particularly sensitive since the glycerol backbone region of the lipid bilayer, which defines the extent of the hydrophobic core of the bilayer, is a region where environmental properties change very rapidly with distance, going from the non-polar fatty acyl chain region to the polar head-group region over a distance of a few Å. Experiments with the potassium channel KcsA have shown that a tryptophan residue located just below the glycerol backbone region of a lipid bilayer emits maximally at 332.6 nm [15]. Figure 3 shows fluorescence emission maxima for tryptophan residues placed at defined positions in the second, lipid-exposed, transmembrane α -helix of MscL. The plot locates the interfacial positions as being Leu⁶⁹ on the periplasmic side and a residue between Val⁹¹ and Tyr⁹⁴ on the cytoplasmic side (Figure 3). The locations of the interfaces on the periplasmic and cytoplasmic sides clearly follow from the presence of an exposed aspartic residue at position 68 on the periplasmic side and from the presence of exposed charged residues at positions 11 and 16 on the cytoplasmic side (Figure 1). An important observation that follows from these studies is that the region of the second transmembrane α -helix that spans the hydrophobic core of the lipid bilayer cannot be determined from consideration of the second helix alone; the charged residues marking the C-terminal end of the hydrophobic core-spanning region of the helix, Arg¹¹ and Asp¹⁶, are located at the N-terminal end of the first transmembrane α -helix.

The hydrophobic thickness of MscL estimated from these experiments is 25 Å (Figure 1), in good agreement with experiments showing that the PC that binds with highest affinity for MscL is that with a fatty acyl chain length of C₁₆, corresponding to a bilayer of thickness 24 Å [9]. Figure 3 also shows that the residues defining the ends of the hydrophobic core of the transmembrane α -helix do not change when the lipid fatty acyl chain length changes from C₁₂ to C₂₄, corresponding to a change in bilayer thickness of approx. 21 Å, showing that hydrophobic matching between the protein and the lipid bilayer is highly efficient [14].

Non-annular lipid-binding sites

X-ray crystallography shows the presence of a number of lipid molecules bound not to the exposed hydrophobic surfaces of membrane proteins but buried between transmembrane α -helices, often at protein-protein interfaces [1]. These sites have been referred to as non-annular sites. Binding at these sites often shows significant structural specificity and is often essential for functioning [4]. Binding at non-annular sites can also be characterized using fluorescence quenching methods; the fluorescence of a tryptophan residue located close to a non-annular lipid-binding site will be quenched by a brominated lipid molecule binding to the non-annular site. In this way, it was shown that binding to the non-annular site between protein subunits in the homotetrameric potassium channel KcsA was specific for anionic lipid [16].

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Abbreviations used

di(C_{18:1})PC	dioleoylphosphatidylcholine
di(C_{18:1})PE	dioleoylphosphatidylethanolamine
PE	phosphatidylethanolamine

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Figure 1. The structure of the mechanosensitive channel of large conductance, MscL
(A) A surface polarity plot for MscL showing the location of the hydrophobic domain defined by fluorescence studies. The positions of Leu⁶⁹ on the periplasmic side of the membrane and of Val⁹¹ and Tyr⁹⁴ on the cytoplasmic side are marked. For clarity, only one set of defining residues is shown. The hydrophobic thickness of the protein, defined by the positions of Asp⁶⁸ and Asp¹⁶ is 25 Å. **(B)** A similar view but showing the relative locations of residues 69 and 87, used to study binding on the periplasmic and cytoplasmic sides of the protein respectively and the location of the positively charged cluster Arg⁹⁸, Lys⁹⁹ and Lys¹⁰⁰ that gives rise to a hot-spot for binding anionic lipid on the cytoplasmic side of the membrane.

Figure 2. Lipid binding on the two sides of MscL

Binding constants for di(C_{18:1})PE (PE), dioleoylphosphatidylserine (PS) and dioleoylphosphatidic acid (PA) are shown relative to that for di(C_{18:1})PC (PC) on the periplasmic (filled bars) and cytoplasmic (hatched bars) sides of the protein.

Figure 3. Defining the hydrophobic thickness of MscL

A plot of the tryptophan fluorescence emission maxima (nm) against the position of the tryptophan residue introduced into the lipid-exposed second transmembrane α -helix of MscL allows the identification of residues defining the ends of the hydrophobic core-spanning region of the helix: ○, di(C_{12:0})PC; □, di(C_{14:1})PC; ●, di(C_{18:1})PC; △, di(C_{24:1})PC. The dotted line at 332.6 nm marks the expected fluorescence emission maximum for a tryptophan residue immediately below the glycerol backbone region of the bilayer. The lower panel shows the locations of the mutated residues in MscL.