Electrostatic sensor for identifying interactions between peptides and bacterial membranes

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Abstract

The use of the membrane probe fluorescein phosphatidylethanolamine (FPE) to investigate membrane binding is well established. However, until now, its use has been restricted to studies involving peptides and eukaryotic membranes. This useful tool has been developed to interrogate peptide:prokaryotic membrane interactions by introducing novel methodology to incorporate FPE into the membranes of UV killed, whole bacterial cells. The electrostatic potential of the membrane in the immediate vicinity of the probe affects the protonation state of the xanthene ring system in the fluorescein head group, which is held close to the membrane surface. When altered, e.g. by peptide binding and insertion, a change in fluorescence results, which can be measured spectrophotometrically. Applicability of this technique to bacterial surface interactions was confirmed by production of a binding curve for both a synthetic peptide and a 37 kDa protein. Future investigations are anticipated to utilize this technology to characterize interactions of other toxins plus antimicrobial peptides such as lactoferricin and defensins with their target membranes.

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1. Background

Three electrostatic potentials are associated with a biological membrane; the transmembrane potential (∆ψ), the dipole potential (ψD) and the surface potential (ψS). The transmembrane potential arises from a charge gradient across the membrane, the dipole potential originates from molecular dipoles on the membrane lipids, and the surface potential represents the potential difference between the membrane surface and the bulk aqueous phase. The surface potential results from the net electric charges at the membrane surface, and changes in ψS caused by a binding event can be measured using the membrane probe fluorescein phosphatidylethanolamine (FPE).

FPE has been well characterised for use in membrane binding studies (Wall et al., 1995a) and incorporates into lipid bilayers via the phosphatidylethanolamine segment maintaining the fluorescein moiety close on the surface (Fig. 1A). The electrostatic potential in the immediate vicinity of the probe affects the protonation state of the xanthene ring system in the fluorescein head-group, and when altered causes a change in fluorescence which can be measured spectrophotometrically.

Addition of positive charge, for example, reduces the size of the membrane’s negative electrostatic potential, resulting in an increase of the apparent pK (pKapp) of FPE, which in turn causes deprotonation of the fluorescein xanthene ring and an increase in fluorescence (Wall et al., 1995a) (Fig. 1B). Conversely, addition of a negative charge results in a fluorescence decrease, and a non-charged molecule binding to the labelled membrane will have no effect. Only charges present on, or close to the surface of the membrane affect the electrostatic potential, and hence the protonation state of FPE. Therefore, when a peptide inserts into a labelled bilayer any charges along the inserted section are effectively hidden from the probe, and consequently may result in a change in fluorescence (Fig. 1B). Unincorporated probe in the surrounding media of labelled membranes is eliminated before addition of test samples by centrifugation and resuspension of cells, or in the case of liposomes separation through a size
Fig. 1. Characteristics of FPE. (A) Structure of fluorescein phosphatidylethanolamine (FPE) alongside representation of FPE in a phospholipid bilayer. (B) Schematic representation of a positively charged peptide binding to FPE-labelled membranes followed by insertion of charged residues.

exclusion column. As the response of FPE is determined by the protonation state of fluorescein, charges in the surrounding medium, in the form of salt or pH changes for example, will also affect fluorescence. Because of this, the medium used is essentially Tris buffer at a pH (pH 7.4) where incorporated FPE fluorescence is between the minimum and maximum levels, thereby allowing an increase or decrease upon addition of test samples. Where whole cells are used, sucrose is added to the medium to eliminate osmotic effects, and in all cases the medium containing the test protein is the same as that in which the membrane systems are suspended.

FPE has been extensively used for labelling both artificial liposomes and eukaryotic cells (Asawakarn et al., 2001; O’Toole et al., 2000; Wall et al., 1995b) but not whole bacteria, and has predominantly been used to measure the interaction of chemically synthesised peptides. To investigate interactions between large proteins and bacterial cells, existing labelling procedures were modified.

2. Example application

Two Gram-negative bacteria, Escherichia coli and Helicobacter pylori, were used to develop this methodology. Both have two membranes, however provided the integrity of the outer membrane is maintained, exogenously added FPE would only be incorporated here since access to the inner membrane would be denied. E. coli was chosen as it is the most extensively studied bacterium, whereas the choice of the gastric pathogen, H. pylori was based on its impact on human health. H. pylori infects over 50% of the world population, and infection by H. pylori is a major risk factor in the development of a number of diseases, including peptic ulcers, gastric lymphoma and gastric adenocarcinoma (Ernst and Gold, 2000).

Amongst the many virulence factors of H. pylori (CagA, urease, outer membrane proteins, adhesins, mucinase), one of the most extensively studied is the vacuolating toxin VacA (Cover, 1996). VacA is a member of the family of
autotransporter proteins (Cover, 1996; Telford et al., 1994), and once released from the bacterium it attacks eukaryotic cells (Cover and Blaser, 1992; Galmiche et al., 2000; Kimura et al., 1999; Kuck et al., 2001; Molinari et al., 1997, 1998a; Papini et al., 1998; Pelicic et al., 1999). Autotransporter proteins are characterised by three functional domains. An N-terminal targeting domain delivers the autotransporter to the inner membrane and is cleaved during translocation to the periplasm. This translocation may be achieved via the SRP/Sec pathway (Henderson et al., 1998; Sijbrandi et al., 2003). The C-terminal domain is proposed to insert into the outer membrane forming a \( \beta \)-barrel structure through which the mature protein (or passenger domain) is translocated. Cleavage of this C-terminal domain, which may be mediated by the passenger protein or surface proteases (Henderson et al., 1998, 2000) then releases the mature protein (Fig. 2). VacA shows homology to this family both by possession of an extended N-terminal signal peptide and by the presence of a C-terminal domain, which shows homology to other autotransporters and is cleaved during secretion (Cover, 1996; Schmitt and Haas, 1994). Once secreted, the \( \sim 90 \) kDa passenger domain of VacA is processed further, yielding subunits of \( \sim 58 \) and \( \sim 37 \) kDa. These subunits form a flower-like rosette structure (Lupetti et al., 1996) proposed to combine to form dodecamers (Cover et al., 1997). In acidic conditions, these oligomers dissociate into monomers, exposing hydrophobic regions and allowing efficient insertion into target membranes (Molinari et al., 1998b).

This pathway of autotransport predicts that mature VacA would be found in the culture supernatant, however, we observed that approximately 20% was associated with the bacterial cells that produce it (unpublished data this laboratory). To determine whether this interaction occurs post, or co-secretion, we investigated the nature of VacA:prokaryote membrane association and its in vivo relevance.

Immunofluorescent microscopy revealed that cell-surface VacA forms tight foci on the surface of \( H. pylori \) cells (Fig. 3). This association of mature VacA with the bacterial cell surface can occur post-secretion, since it occurs when culture supernatants containing VacA are incubated with non-toxin producing \( H. pylori \) cells (strain 8823/A130, Cover et al., 1994). Furthermore, VacA is able to adsorb to the surface of \( E. coli \) cells (unpublished data, this laboratory). Although the tightly bound adsorbed VacA is accessible to trypsin digestion (unpublished data, this laboratory), pre-treatment of bacterial cells with trypsin did not preclude VacA interaction, indicating that the interaction was unlikely to be protein mediated. Furthermore, the direct interaction of VacA with LPS is doubtful since VacA successfully

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**Fig. 2.** Schematic representation of a generalised autotransporter secretion mechanism. The precursor polypeptide is targeted to the SRP-Sec machinery by the N-terminal signal peptide (shown in red); and translocated into the periplasm where the signal peptide is cleaved. The C-terminal domain (shown in blue) inserts into the outer membrane forming a \( \beta \)-barrel structure through which the passenger domain (shown in black) is translocated. The C-terminal domain is then cleaved, releasing the extracellular mature protein. IM: bacterial inner membrane; OM: bacterial outer membrane.
Adhesion of other molecules with the membrane. Further analysis with live bacteria in conjunction with electron and fluorescent microscopy to verify membrane integrity and FPE localisation remains crucial.

However, with these caveats, FPE was employed successfully to label both *E. coli* and *H. pylori* cells. Measurement of the fluorescence of *H. pylori* strain 8823/A130 in the presence and absence of salt over a range of pHs confirmed that the bacterial-incorporated probe was responsive to alterations in electrostatic potential (Wall et al., 1995a,b; Cladera and O’Shea, 2001). Moreover, increased fluorescence was evident upon addition of charged ions (Ca$^{2+}$) and peptides (poly-L-lysine) at pH 7.4. Since sufficient quantities of purified mature VacA were not available to investigate the interaction of mature toxin with labelled bacterial cells, the VacA p37 subunit was overproduced in *E. coli* with a C-terminal His-tag to aid purification (Lettley and Atherton, unpublished data). At pH 7.4, the purified recombinant p37 bound to FPE-labelled *H. pylori* cells in a manner suggesting co-operative binding of at least two ligand molecules with a $K_d$ of $\sim 1.7$ nM in a single step with a rate constant of approximately 1.4. The binding constants were determined by plotting the cumulative amplitudes of the FPE fluorescence signals against the protein concentration and fitting to standard binding models (Wall et al., 1995a) according to the following equations:

$$y = \left(\frac{ax}{b + x}\right) + c$$  \hspace{1cm} (1)

$$y = \left(\frac{ax^c}{b^c + x^c}\right) + c$$  \hspace{1cm} (2)

where $a$ is the maximum amplitude, $b$ the binding affinity $K_d$, $x$ the concentration of peptide/protein, and $c$ the Hill coefficient for co-operativity. To ascertain if a specific receptor was responsible for the binding of p37 to the bacteria, the recombinant p37 was added to FPE-labelled artificial vesicles composed of 85% phosphatidylcholine and 15% phosphatidylethanolamine. This composition was chosen as it mimics the eukaryotic cell membranes used in previous studies which indicated non-specific binding (Moll et al., 1995). The p37 bound with much lower affinity to these artificial lipid bilayers than to the bacterial cells, implying the presence of additional factors in the bacterial outer membrane capable of promoting p37 binding.

### 3. Summary

A method of fluorescently labelling bacterial cell membranes has been developed which relies on the modulation of the fluorescence of FPE-labelled bacterial cells. Although requiring further optimisation, this approach is capable of monitoring the insertion of charged molecules and should be applicable to analysis of membrane active agents such as antimicrobial peptides. As an example, the interaction of VacA with bacterial cell surfaces was assessed. VacA adsorption...
to bacterial cell surfaces requires a bacterial factor distinct from LPS or trypsin-accessible surface proteins, and may be mediated via the p37 subunit since recombinant p37 was capable of inserting into FPE-labelled bacterial cells more efficiently than into artificial lipid bilayers.

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References


