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Mapping the lipid-exposed surfaces of membrane proteins

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Phospholamban forms a stable complex of five long transmembrane helices. We show that the relative rotational orientation of the helices in the pentameric complex can be distinguished by S–H to S–D exchange of cysteine sulphydryl groups located in the transmembrane segment of the protein and exposed to the lipid environment. Of the three cysteine residues in phospholamban, two residues (Cys 36 and Cys 46) are oriented towards the helix interface and protected from exchange, while the third cysteine (Cys 41) is oriented towards the lipid interface and undergoes exchange with water diffused into the bilayer. Distinguishing the external and internal faces of a membrane protein by sulphydryl exchange provides a general approach for determining the three-dimensional fold of membrane proteins and enhances model building efforts to generate high-resolution structures.

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High-resolution structures of membrane proteins are comparatively rare; their hydrophobic surfaces make crystallization difficult and most are too large for complete NMR studies. On the other hand, establishing the structure of membrane proteins by low-resolution methods combined with computational modelling may prove more tractable for membrane proteins than it has been for their soluble counterparts. This is due to the fact that many membrane proteins exist as bundles of trans-membrane α-helices and only a few key data points are needed to establish how these helices pack relative to one another. Sequence analysis based on hydrophobicity can yield information regarding the length and number of possible trans-membrane helices[1], while short extramembraneous connecting loops or homology to known structures can constrain the relative proximity of helix pairs. The remaining problem is to obtain information on the rotational orientation of the transmembrane helices with respect to one another. One solution to this problem is to selectively label the elements in the complex facing the lipid bilayer (that is, the exterior of the complex). This would enable one not only to assign those helices as being placed at the exterior of the complex, but also to assign their relative orientations with respect to the
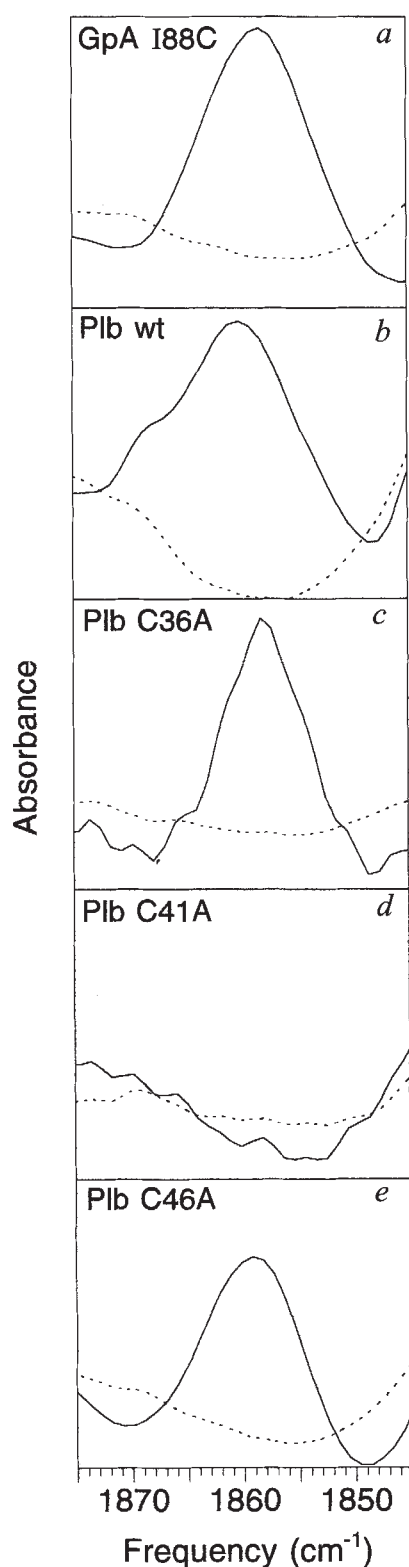
lipid interface. In this paper, we describe a novel approach for mapping the lipid-exposed surfaces of membranes proteins that takes advantage of rapid sulphydryl S–H to S–D exchange of cysteine residues in the protein-lipid interface.

We apply this strategy to determine the rotational orientation of the transmembrane helices in phospholamban (Plb)—a 52-residue integral membrane
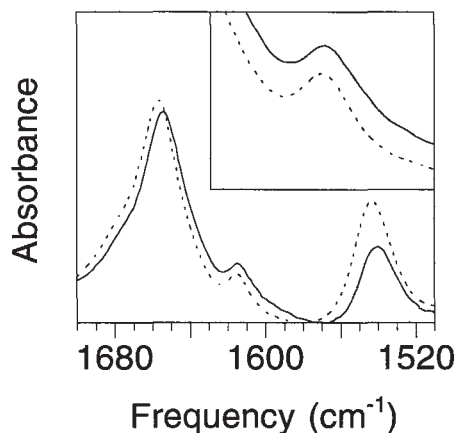
Fig. 1 Amide proton H/D exchange in a helical trans-membrane peptide. Amide I and amide II vibrational modes of the transmembrane domain of phospholamban in H_2O (dotted line) and D_2O (solid line). Inset shows an expansion of the amide I band that has been shifted by ^{13}C labelling. The transmembrane peptides used in this study were synthesized, purified, characterized and reconstituted in a manner identical to that described previously[2]. Lipid to protein ratios ranged from 1:40–1:100 w/w. FTIR spectra were recorded on a Nicolet Magna 550 spectrometer purged with N_2 (Madison, WI) and equipped with a MCT/A detector. 1000 interferograms recorded at a spectral resolution of 4 cm^{-1} were averaged for each sample. Interferograms were processed using 1-point zero filling and Happ–Genzel apodization, followed by automatic base line correction. Samples (400μl) containing 0.4 mg of protein in a buffer of 0.1 mM Na_2PO_4 pH 6.8 were centrifuged for 1 h in an A-95 rotor at 178,000g using an airfuge ultracentrifuge. Pellets were resuspended in 75 μl of either H_2O or D_2O and dried down on germanium IR windows (Grasbey SpeAc, Kent, UK). Spectra were recorded and processed as described previously[2].

GpA I88C — a

Plb wt — b

Plb C36A — c

Plb C41A — d

Plb C46A — e

Absorbance

1870   1860   1850

Frequency (cm⁻¹)

Fig. 2 S–D vibrational modes in H$_2$O (dotted line) or D$_2$O (solid line) of the glycophorin transmembrane peptide with an isoleucine→cysteine substitution at position 88 (a, GpA I88C) and the phospholamban transmembrane peptide with either the wild-type sequence (b, Plb wt), a cysteine→alanine substitution at position 36 (c, Plb C36A), a cysteine→alanine substitution at position 41 (d, Plb C41A) or a cysteine→alanine substitution at position 46 (e, Plb C46A). Exchange is shown by the appearance of an S–D band (solid line).

protein found in cardiac sarcoplasmic reticulum membranes—which is implicated in the regulation of the resident Ca^{2+} pump. The C-terminal half of Plb resides in the bilayer where it forms a pentamer that may serve as an ion-conducting channel. Plb is modelled as a bundle of five long helices[2]. The pentameric complex is held together by noncovalent interactions between residues located in the transmembrane helices[3].

Amide exchange is slow in lipid bilayers

Amide H/D exchange has long been used to assign exposed residues in small soluble proteins (<30,000 M_r)[4], but has limited use for membrane proteins since the amide exchange rates are exceedingly slow within the transmembrane region of an α-helix[5]. This is illustrated in Fig. 1, where the amide I (C=O stretch) and II (N–H deformation) vibrations are shown for the helical transmembrane domain of Plb. Only a fraction of the amide II vibrational band at 1543 cm^{-1} disappears on solvent exchange with D$_2$O. The N–H groups undergoing exchange can be assigned to residues outside of the membrane interior by comparing the frequency shifts observed in the amide I vibration. The amide I at 1657 cm^{-1} normally shifts ~−10 cm^{-1} on amide H/D exchange[5]. In Fig. 1, however, a shift in the amide I frequency of only 2 cm^{-1} is observed, indicating that most of the amide groups are protected. Specific ^{13}C labelling of the peptide backbone at Leu 39 and Leu 42 generates a ^{13}C=O amide I vibration at 1614 cm^{-1} (Fig. 1, inset) and allows a local determina-

tion of exchange in the middle of the transmembrane sequence[6]. The absence of a shift in the 1614 cm^{-1} band upon D$_2$O exchange indicates that the backbone amide groups in the membrane interior are protected from exchange.

S–H exchange in the protein–lipid interface

An alternative approach for mapping the lipid-exposed surface of membrane proteins is by sulphydryl S–H to S–D exchange of transmembrane cysteine residues. Single cysteines provide unique markers for exchange. The S–H vibrational mode is located at 2540–2590 cm^{-1}, in a transparent region of the spectrum, and shifts ~ −700 cm^{-1} on deuteration[7]. We propose that the S–H groups in the lipid interface can undergo rapid H/D exchange. The S–H group is more acidic and more mobile than the backbone N–H, and water is known to rapidly diffuse across membrane bilayers[8] and thus be accessible for the exchange reaction. In contrast, the S–H groups in helix interfaces are expected to be protected from exchange because of their limited access to H$_2$O in a manner analogous to that of the N–H groups buried in the interior of soluble proteins.

In order to investigate the ability of a single sulphydryl group to exchange in a lipid interface, we synthesized a peptide corresponding to the transmembrane domain (residues 71–99) of human glycophorin A with a cysteine substituted for isoleucine 88 (GpA I88C). The transmembrane domain of glycophorin A provides a good model system for these studies as its structure has been investigated by several different approaches[9–14] which establish that the transmembrane domain is helical and dimerizes in membranes with residue 88 oriented towards the lipid acyl chains. As observed with the transmembrane domain of phospholamban, the amide protons in the transmembrane domain of glycophorin A are protected from exchange (data not shown). In contrast, the appearance of an S–D vibrational band at 1858 cm^{-1} (Fig. 2a) clearly demonstrates that the cysteine S–H at position 88 exchanges with D$_2$O.

**Establishing rotational orientation of Plb helices**

Based on these studies, we measured the S–H to S–D exchange of the three naturally occurring cysteine residues in the transmembrane domain of Plb. Comparisons were made between four different transmembrane peptides (residues 25–52): a peptide with the wild-type sequence (Plb WT) and three peptides each containing a substitution of an alanine residue for a wild-type cysteine (Plb C36A, Plb C41A and Plb C46A). These substitutions do not affect pentamerization of Plb at ambient temperatures[15], and all of the peptides exhibit nearly identical FTIR spectra and amide exchange properties indicating that the alanine

substitutions do not disrupt the structure of the complex (data not shown).

The S–H vibrations of the three cysteine residues in PLB WT overlap and result in a single band at 2560 cm^{-1} (data not shown). The S–H frequency is sensitive to the nature and strength of hydrogen-bonding[7]; the frequency of the observed S–H band in phospholamban argues that all of the S–H groups are hydrogen bonded. To determine whether or not the S–H groups can exchange, it is only necessary to observe the S–D region of the spectrum around 1860 cm^{-1}. In the absence of exchange, this region exhibits no protein or solvent bands. Comparison of the S–D vibrations (Fig. 2b–e) shows that exchange occurs in Plb WT as well as Plb C36A and Plb C46A. The intensity of the S–D peak corresponds roughly to that expected for exchange at a single site. Only Plb C41A lacks an S–D band (Fig. 2d). These results indicate that the sulphydryls at positions 36 and 46 are protected from H/D exchange. Only Cys 41, present in Plb WT, Plb C36A and Plb C46A, is able to exchange. These results argue that Cys 41 is oriented towards the lipid-exposed interface and that Cys 36 and Cys 46 are packed in the interior of the complex.

The spacing of the three cysteine residues around the helix axis (i, i+5 and i+10) in the transmembrane sequence ensures that at least one cysteine sulphydryl is buried in a helix interface and at least one sulphydryl is oriented towards the surrounding lipids. The observed pattern of protection corresponds well with the locations of the cysteine residues in a model of the phospholamban pentameric complex based on mutagenesis[3] and molecular modelling[16]. Using saturation mutagenesis, it was found that several positions in the transmembrane sequence are able to accommodate conservative substitutions without disrupting the pen-

tamer[3]. These positions fall on one face of a helical wheel diagram of the phospholamban monomer (Fig. 3) that includes position 41. In the pentameric model of phospholamban, only Cys 41 is exposed to
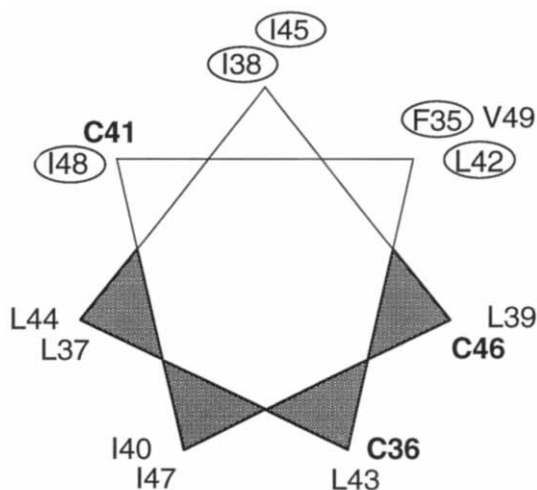
Fig. 3. Helical wheel diagram of the human phospholamban transmembrane domain. The amino-acid positions that were able to accommodate conservative substitutions without disrupting the pentamer were circled[4]. These positions are thought to be facing the surrounding lipids. The helix faces that are thought to interact are shaded. Conservative substitutions of amino acids in these positions may disrupt the pentameric complex[4]. The three cysteine residues are shown in bold.
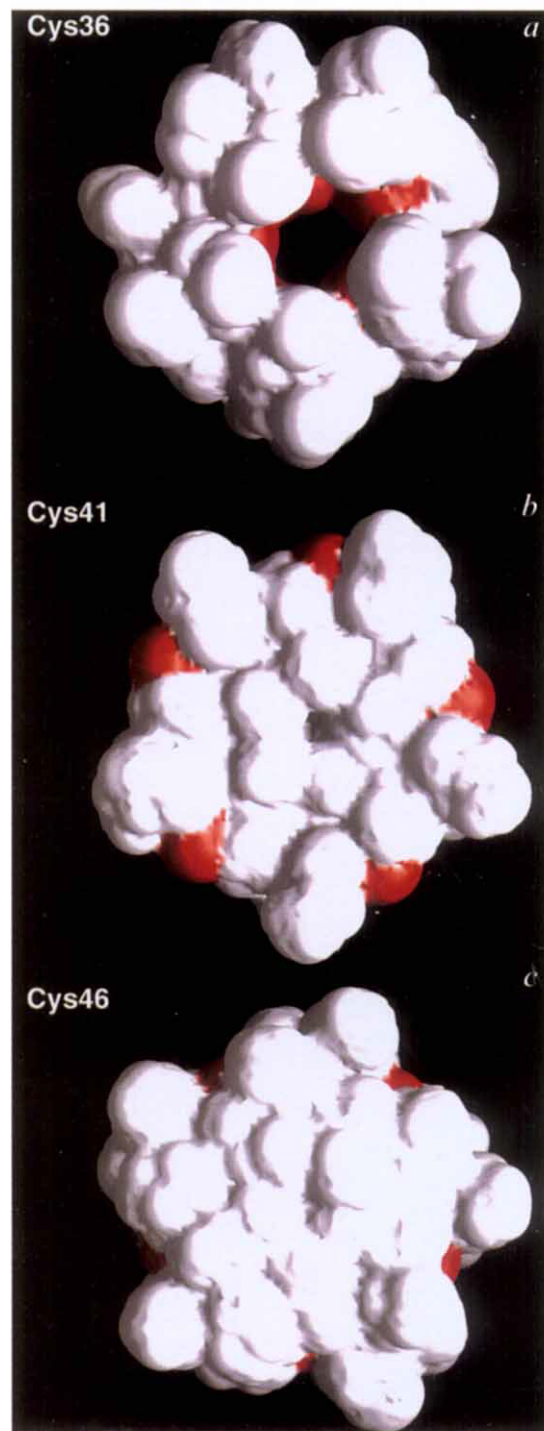
Fig. 4. Model of the phospholamban pentameric complex showing the solvent accessibility of the transmembrane sulphydryl groups. The surfaces correspond to five residue cross-sections of the complex containing either a, Cys 36 (residues 34–38), b, Cys 41 (residues 39–43) or c, Cys 46 (residues 44–48). The cysteine side chains are shown in red. The figure was made using the program GRASP[20].

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the lipid interface. The other two cysteine residues are packed in the interior of the complex. This is illustrated in Fig. 4 which shows the solvent-exposed surfaces of the cysteine side chains labelled in red. Interestingly, although Cys 36 is oriented towards the channel in the interior of the protein, it is protected from exchange. This may reflect the specific orientation and mobility of the Cys 36 S–H or the nature of water bound in the pore. Moreover, protection was observed with or without Ca^{++} in the sample buffer arguing that the structure of the complex does not change in the presence of divalent cations.

General application to membrane proteins
The results presented here illustrate a new method for determining the lipid-exposed surfaces of membrane proteins based on sulphydryl exchange. The small cysteine side chain, being slightly larger than an alanine methyl, should not disrupt the three-dimensional fold of membrane proteins. This method may be regarded as a hybrid between cysteine scanning mutagenesis[17–19] and amide H/D exchange. Our method removes the need for large chemical labels present in cysteine scanning mutagenesis while making use of isotope-detected accessibility.

Using this approach, we are able to directly establish the rotational orientation of the helices in the pentameric complex of phospholamban. The exchange data support the model of the complex derived independently from mutagenesis studies and molecular modelling, and demonstrate that the complex is stable, in contrast to many channel-forming peptides that only transiently associate in membranes. These studies illustrate a general approach for determining the three-dimensional fold of membrane proteins and promise to greatly enhance model-building efforts to generate high-resolution structures by supplying key structural constraints.

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