Mapping lipid and detergent molecules at the surface of membrane proteins

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Abstract
Electron-density maps for the crystal structures of membrane proteins often show features suggesting binding of lipids and/or detergent molecules on the hydrophobic surface, but usually it is difficult to identify the bound molecules. In our studies, heavy-atom-labelled phospholipids and detergents have been used to unequivocally identify these binding sites at the surfaces of test membrane proteins, the reaction centres from Rhodobacter sphaeroides and Blastochloris viridis. The generality of this method is discussed in the present article.

Introduction
Our previous site-directed mutagenesis study on the relationship between the structure and function of the Rhodobacter sphaeroides RC (reaction centre) determined the X-ray crystal structure of many such mutants [1]. Careful analysis of these structures revealed two features in the non-protein electron density at the surface of this membrane protein complex. First, there was a tightly bound region of density that could be successfully fitted with the lipid cardiolipin (diphosphatidylglycerol) [2]. Secondly, there were many regions of undefined electron density, some of which were found to lie parallel to each other and could be fitted with either bound detergent molecules or parts of bound phospholipids. While thinking about how to differentiate between these two possibilities, one of us (R.J.C.) remembered some work by Tony Lee that had used brominated phospholipids produced by the addition of bromine atoms across unsaturated double bonds in the fatty acid tails [3]. The element bromine contains considerably more electrons than the other elements that constitute proteins and lipids and will therefore scatter X-rays more strongly, i.e. bromine is a ‘heavy atom’. It was reasoned that, if bromine was added to either phospholipids or detergents, then these atoms should be easily visible in the resulting electron density maps. In this way, it should be possible to distinguish between phospholipids and detergents and be able, hopefully, to characterize their binding sites at the surface of membrane proteins.

Localization of phospholipid-binding sites on the surface of RCs
Figure 1(A) shows the non-protein electron density at a region of the Rb. sphaeroides RC surface and the typical appearance of undefined tube-like density can be clearly seen [4]. The Rb. sphaeroides RC pigment–protein complex was solubilized, purified and crystallized in the presence of the detergent LDAO (N,N-dimethyldecylamine-N-oxide). In Figure 1(A), individual tubes of density have each been fitted with one molecule of LDAO; however, they could equally have been fitted with the fatty acyl chains of a phospholipid. In such a case, it is assumed that the headgroup portion of the phosphatidyl molecule is disordered and so is not visible in the electron-density map. Figure 1(B) shows exactly the same region on the surface of the protein (similar, although not identical, view) when the Rb. sphaeroides RC was co-crystallized in the presence of dibromo-PC (phosphatidylcholine) [4]. Instead of the two adjacent LDAO molecules, as in Figure 1(A), there is one molecule of dibromo-PC [4]. It is important at this point to emphasize a key point about such X-ray crystal structures. These structures are solved assuming that each RC in the crystal is structurally identical. For the protein portion of the RC, this is probably a safe assumption. However, for the non-protein electron density at the surface of the RC, this is undoubtedly not a good assumption. Rather, some putative lipid-binding sites on one RC will be occupied by lipids and some by detergents and, moreover, this will be different on the surface of different RCs in the same crystal. Therefore, when trying to identify putative lipid-binding sites on the surface of membrane proteins, it must be borne in mind that often only partial regions of any given lipid or detergent molecule are sufficiently well ordered to be visible in the electron-density map and also that the final structure at any site will be an average of the different molecules present.

Key words: annular lipid, detergent, heavy atom, phospholipid, photosynthetic bacterium, X-ray crystallography.
Abbreviations used: LDAO, N,N-dimethyldecylamine-N-oxide; PC, phosphatidylcholine; RC, reaction centre.

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Figure 1 | Rb. sphaeroides RC surface features within a groove of the transmembrane domain

(A) LDAO molecules (green) fitted into surface 2F0 − F1 electron density shown at the 0.5σ level at Site A for the 1.95 Å (1 Å = 0.1 nm) structure of the HC(266M) RC mutant from Rb. sphaeroides [5]. The whole displayed area is within the transmembrane region of the RC; ubiquinone UQa molecule is coloured yellow. (B) Site A with bound brominated phospholipid in the groove to the right of (green) helix H of the RC from Rb. sphaeroides co-crystallized with the dibromo-PC [4]. The brominated PC is represented as van der Waals spheres with carbon atoms in yellow, oxygen atoms in red, and bromine and phosphorus atoms in orange. Ubiquinone molecule UQa is drawn as white stick-style models. The RC protein surfaces (including associated pigments) are shown in blue for chain L, green for chain H and pink for chain M. The molecule of cardiolipin is represented in the same colour scheme as dibromo-PC is visible on the left-hand side of (green) helix H. Reprinted with permission from [4]. © 2007 American Chemical Society.

(or possibly without any molecules present) at that site in the different RCs in the single crystal. There are at least two factors that determine whether a site on the surface of the membrane protein is filled with lipid or detergent or remains empty. The first is the relative ‘strength’ of the detergent itself. Of course, not all proteins are stable in all detergents, rather some, notably overexpressed mammalian membrane proteins, are only stable in very mild alkyl sugar detergents such as DDM (dodecyl maltoside), whereas more robust membrane proteins, for example some bacterial photosynthetic complexes, can be solubilized, purified and crystallized in much more robust detergents such as LDAO. Thus a delicate balance must be achieved with the detergent so that it disrupts completely the lipid–lipid interactions in the membrane, but does not perturb the interactions within a protein that keep it stable. However, some of the annular lipids may have stronger interactions with the surface of the protein than with the detergent micelle and so will remain in their sites.

The second factor that determines the occupancy of sites on the membrane surface is the conditions chosen for solubilization of the membranes. Generally, a higher concentration of detergent or a higher temperature for solubilization will lead to harsher conditions and the removal of more tightly bound lipids. Again, a balance must be achieved between solubilization conditions that are too harsh and risk denaturing or inactivating the protein in question and those that are too mild, resulting in an insufficiently solubilized protein preparation that contains too many lipids. Such a sample may be too inhomogeneous to be able to form crystals. The concept of binding sites on the surface of the protein with various degrees of affinity for lipids and detergents has consequences for the common procedure of exchanging one detergent for another before crystallization. If the detergent used for solubilization has a high affinity for sites on the protein surface, then it may not be completely removed during detergent exchange, even if the procedure is performed with great diligence.

The occupancy of the site shown in Figure 1(B), designated Site A, with dibromo-PC is approximately 25% and so illustrates the need for heavy-atom tagging. Without the increased X-ray scattering from the bromine atoms and corresponding bulges on the electron-density maps, it would not have been possible to definitively assign the density to dibromo-PC. A second site, designated Site B, on the other side of the RC was also found with the dibromo-PC bound (Figure 2).

In order to try to investigate the specificity of lipid-binding sites, such as Site A and Site B, for other phospholipids, the co-crystallization experiment was repeated using phospholipids with different headgroups, such as PG (phosphatidylglycerol), PE (phosphatidylethanolamine) and PS (phosphatidylserine), and with two different positions of the bromine atoms on the fatty acid tails (6,7- or 9,10-dibromination) [4]. All of these lipids were found to bind Site B, whereas their binding in Site A was non-existent or very limited. The occupancy of the brominated phospholipid...
in Site B was again low (∼12–25%), but it was clear that different sites show different degrees of specificity. In this case, Site A would only accept 6,7-dibromo-PC, whereas Site B showed a lower degree of specificity. Comparison of these two sites suggests that Site A has a limited length of the groove on the RC surface, including specific locations of the hydrogen-bond donor groups, and is therefore very specific in the lipid it accepts, whereas Site B is located in the flexible region of the RC (in the extended groove near the site of the distal ubiquinone, UQB) and can accept a variety of lipids.

**Binding of heavy-atom derivatives of detergents on the surface of RC from *Rb. sphaeroides* and *Blastochloris viridis***

Br-LDAO is not commercially available and so, in order to carry out the converse experiment, i.e. labelling the detergent rather than the phospholipid, Br-LDAO was synthesized. Crystals of the RC were soaked in Br-LDAO and bromine atoms were looked for in the crystal structure. Figure 3 shows a site on the RC from *Rb. sphaeroides* where a molecule of Br-LDAO is clearly visible in the electron-density map (structural data to be published). This detergent-binding site corresponds to Site A described above, where it was shown that dibromo-PC could bind. This result illustrates the usefulness of heavy-atom-tagged detergents in combination with X-ray crystallography to locate detergent-binding sites on the surface of membrane proteins. Experiments have also recently been started to identify, compare and contrast the binding sites on the surface of the *Blc. viridis* RC with those identified previously on the surface of the *Rb. sphaeroides* RC. An example is given in Figure 4 of initial electron density from a *Blc. viridis* RC and Br-LDAO co-crystallization experiment, fitted with an LDAO molecule usually found at this site. Features of this electron density clearly indicate dibromination in the middle of the aliphatic chain (structural data to be published).

In order to extend this concept, another set of detergents were synthesized incorporating mercury atoms as the heavy atoms rather than bromine. This change was undertaken because it has been suggested that carbon–bromine bonds are susceptible to radiation damage. This damage could...
**Figure 5 | Binding of Hg-LDAO molecules at the surface of RC from *Rb. sphaeroides***

The $2F_o - F_c$ electron density at 1.0σ level (blue) and $F_o - F_c$ electron density at 3.0σ level (green) as found in the initial maps calculated for the 3.25 Å (1 Å = 0.1 nm) structure of RC co-crystallized with Hg-LDAO (model in yellow). Also shown (in magenta) is the superimposed 1.95 Å structure of the HC(266M) RC mutant from *Rb. sphaeroides* [5] with the LDAO molecule passing through the high blue/green unmodelled density. (A) Electron density visible in Site B for a putative mercury atom of the Hg-LDAO molecule in a position to co-ordinate the carbonyl oxygen of the valine residue at position 220 of the L subunit (L220-Val). (B) Electron density for a putative mercury atom of the Hg-LDAO molecule residing in the wide groove of which Site A is part. This density coincides with the LDAO molecule of the HC(266M) RC mutant, shown in Figure 1(A) as the most horizontally oriented of the modelled (green) LDAO molecules. The mercury atom in this position is co-ordinating carbonyl oxygen of the alanine residue at position 25 of the H subunit (H25-Ala).

possibly contribute to the low occupancy of the bound brominated lipids as the occupancy of the aliphatic atoms in the fatty acid tails was higher than occupancy of the bromine atoms. Figure 5 shows the result of a preliminary experiment where RCs were co-crystallized with Hg-LDAO. Again the enhanced X-ray scattering of the heavy atom, in this case mercury, is clearly seen in the initial electron-density maps, although the density for the aliphatic atoms of LDAO are not visible next to the very high mercury-density area (structural data to be published). Further work on these mercury-tagged detergents is continuing.

**Investigating an idea of phasing of X-ray diffraction data for membrane proteins through the use of heavy-atom derivatives of lipids and detergents**

In the study described, the usefulness of the heavy-atom detergent derivatives mentioned was tested for phasing of the X-ray diffraction data. The aim was to establish whether these detergent derivatives could be used as ‘easy/natural’ heavy-atom compounds for phasing of diffraction data for membrane protein crystals using anomalous dispersion methods. Membrane proteins require detergents for solubilization and stability, so replacement of these detergents by heavy-atom-containing derivatives would be a much simpler and straightforward method to introduce the heavy atom into the crystal than the toxic and hazardous heavy-atom compound soaks required at present. Additionally, the RC electron-density features suggest that detergent molecules bind in the grooves of the membrane protein’s hydrophobic surface (Figure 1A). However, the limited degree of occupancy compounded by possible effects resulting from X-ray damage to the carbon–bromine bond mean that the anomalous signal produced by these heavy atoms is not yet sufficient to allow successful phasing. Further efforts are being undertaken to (i) increase the level of occupancy, and (ii) modify the data-collection strategy in order to reduce the experimental errors, which should increase the chances of successful phasing.

**Discussion**

The idea behind the study described was inspired by previous work carried out by Tony Lee. It is a great pleasure therefore to dedicate this short contribution to him on the occasion of his retirement. This study has shown the usefulness of heavy-atom-tagged phospholipids and detergents in combination with X-ray crystallography to explore surface binding sites for these molecules around membrane proteins. Of course, the molecules in these binding sites must be sufficiently well ordered to be visualized in the electron-density maps. Often, only portions of these molecules can be seen in the crystal structures and then identification becomes very difficult. The heavy-atom tagging, when successful, allows unequivocal identification even when only part of the molecule can be seen. These molecules are a very useful addition to a crystallographer’s toolkit.

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