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Bacteriorhodopsin: the effect of bilayer thickness on 2D-array formation, and the structural re-alignment of retinal through the photocycle

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Abstract

From our earlier extensive protein–lipid reconstitution studies, the conditions under which bacteriorhodopsin forms organised 2D arrays in large unilamellar vesicles have been established using freeze-fracture electron microscopy. In a background bilayer matrix of phosphatidylcholine ($diC_{14:0}$), the protein can form arrays only when the anionic purple membrane lipid, phosphatidylglycerol phosphate (or the sulphate derivative) is present. Here we have now extended this work to investigate the effect of bilayer thickness on array formation. Phosphatidylcholines with various chain lengths ($diC_{12:0}$, $diC_{14:0}$ and $diC_{16:0}$) and which form bilayers of well defined bilayer thickness, have been used as the matrix into which bacteriorhodopsin, together with minimal levels (c. 4–10 lipids per bacteriorhodopsin) of diphytanyl phosphatidyl-glycerol phosphate, has been reconstituted. Arrays are formed in all complexes and bilayer thickness appears only to alter the type of array formed, either as an orthogonal or as an hexagonal array.

Secondly, we have previously deduced the entire conformation of retinal within the bacteriorhodopsin binding pocket in oriented purple membrane fragments. Using solid state deuterium NMR of the specifically deutero-methylated retinal labelled at each of the methyl positions in the molecule, the C–CD_3 bond vectors of the chromophore have been resolved to $\pm 2°$. The ring conformation is 6-*S-trans*, but the polyene chain is slightly curved when in the protein binding site. Here, we describe studies on the protein in both the ground state and the trapped M_{412}-state of the photocycle, to show that the orientation of the central methyl group (C_{19}) on the polyene chain, which is at $40° \pm 1°$ with respect to the membrane normal, only changes its orientation by approximately 4° upon 13-*cis*-isomerization. Thus, it is the Schiff base end of the chromophore which moves upon light incidence acting as a local switch on the protein in the photocycle, whilst the ring end of the chromophore moves rather less.

Keywords: Bacteriorhodopsin; Electron microscopy; Solid state NMR; Deuterium; Retinal; Membrane protein stucture

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1. Introduction

As a fortuitous consequence of the inherent 2D-array formation of bacteriorhodopsin in the purple membrane, a suggested structure of this integral membrane protein has been determined to 2.7 Å in the membrane plane and 10 Å in the direction of the membrane normal [1]. The factors which are required for 2D-array formation of the protein, a feature which has been pre-requisite to the structural determinations, have now been described [2–4]. It appears that the negative lipid, diphytanyl phosphatidylglyc-

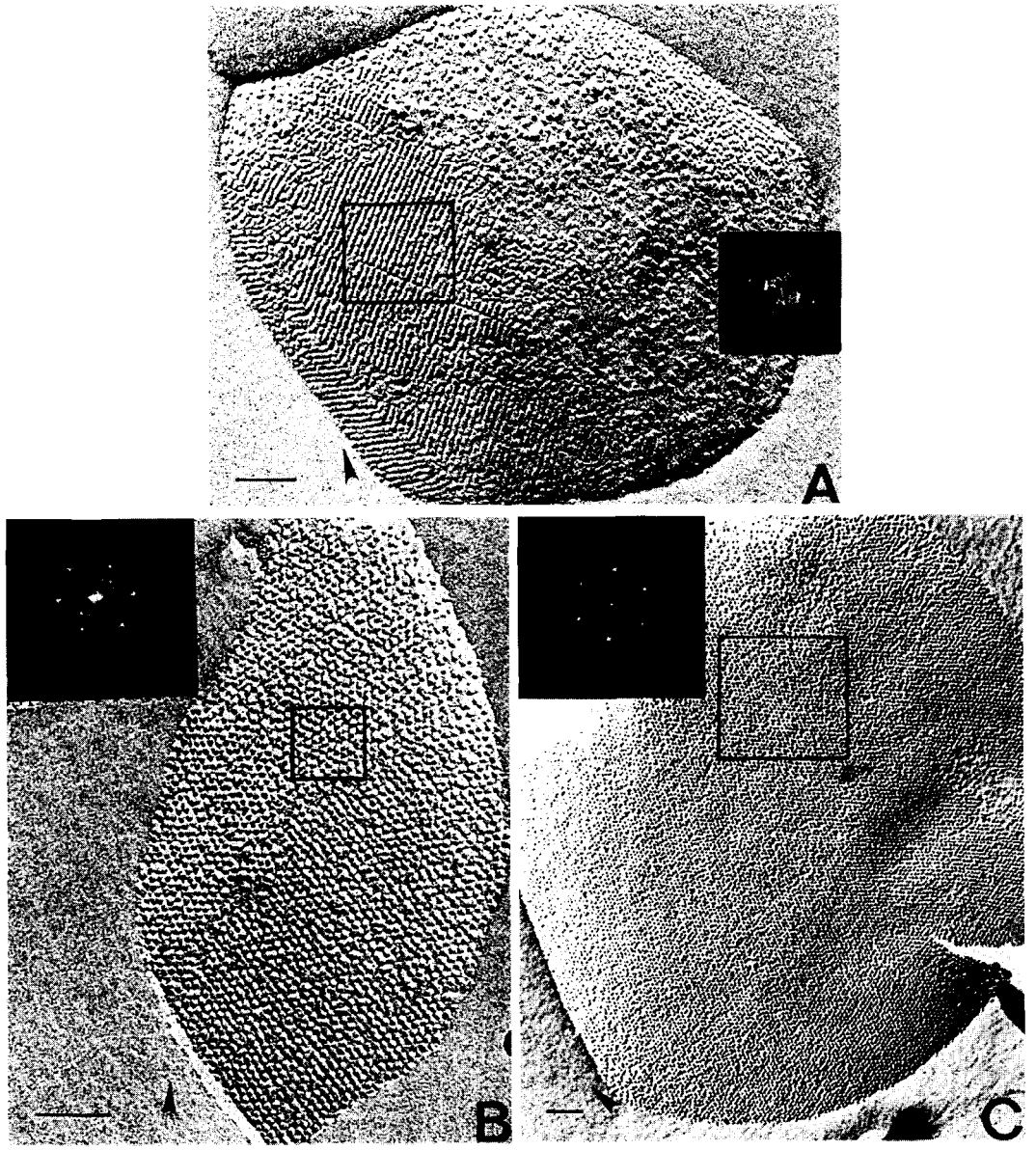

Fig. 1. Freeze-fracture electron micrograph of bacteriorhodopsin–lipid complexes containing phosphatidylcholines of various chain lengths, namely dilauryl ($diC_{12:0}$; $d_b = 2.4$ nm), dimyristoyl ($diC_{14:0}$; $d_b = 2.8$ nm) and dipalmitoyl ($diC_{16:0}$; $d_b = 3.2$ nm). The optical diffractograms from selected areas of the negatives (insets) give evidence for the presence of orthogonal (A) and hexagonal protein arrays (B and C). Shadowing direction is shown by the arrows. Bar 100 nm. From Ref. [16].

erol phosphate, or the sulphated derivative, needs to be an essential component of the membrane, in high (< 2 M) NaCl, for the arrays to form and be stabilised. The mechanism for this array formation for bacteriorhodopsin, or for PS II with galactosyl lipids [5], is not yet clear, neither is it so far reported how lipids interact with any integral or peripheral membrane protein in the molecular detail. Clearly if such information were available and understood, it may be possible to form 2D arrays, and possibly 3D crystals, of a wider range of integral proteins with which to undertake electron diffraction studies to resolve atomic resolution [6,7]. In addition, proteins which form 2D arrays also appear to form 3D crystals [5]. Here we extend this work to study the effect of phosphatidylcholine bilayer thickness on array formation of bacteriorhodopsin.

Although good resolution of the atomic coordinates of the polypeptide chain, and thus the position of most intramembranous residues of bacteriorhodopsin, has been achieved by electron microscopy [1], the detailed conformation of retinal in the protein has not been defined precisely from these studies. In particular, chromophore orientational changes on passing round the photocycle need to be understood in relation to the protein framework. Solid-state deuterium NMR, with which detailed structural information about isotopically labelled groups can be determined on essentially rigid proteins in membranes, does offer this possibility. Thus, in addition to resolving the conformation of the retinal in the dark adapted ground state [8–10], functionally related conformational changes in the retinal have also been detected, as described here. In this present report, we have studied the changes which occur in the prosthetic group in the photoreceptor which has been trapped in one of the stages of the photocycle, the M-state. The approach [8–10] is ab initio, as shown from our earlier work, requiring no further information than the NMR spectral data which reveals unique $C-CD_3$ bond vectors of the protein with respect to the membrane normal.

2. Influence of bilayer thickness on bacteriorhodopsin array formation

In our previous studies, all the bacteriorhodopsin–lipid complexes in which protein arrays are observed by freeze-fracture electron microscopy [2,3,11], have been produced with dimyristoyl ($diC_{14:0}$) acyl chains and added phosphatidylglycerolphosphate or diphytanyl phosphatidylglycerolsulphate [2,3]. Ordered protein arrays can be induced in dimyristoyl phosphatidylcholine bilayers at a range of protein:lipid mole ratios when quenched for electron microscopy from a range of temperatures but *only* when the phosphatidylglycerolphosphate moiety is present [2]. Here, we have now investigated whether matching of the protein hydrophobic surface, which is 3.0–3.2 nm in the direction of the membrane normal, to the bilayer thickness (d_b) is important in array formation.

Bacteriorhodopsin has been reconstituted in bilayers of a range of phosphatidylcholines with varying acyl chain length, including dilauryl ($diC_{12:0}$; d_b = 2.4 nm), dimyristoyl ($diC_{14:0}$; $d_b = 2.8$ nm) and dipalmitoyl ($diC_{16:0}$; $d_b = 3.2$ nm) lipids [14]. In addition, diphytanyl phosphatidylglycerolphosphate at a minimal level (ca. 8–12:45–50:1; phosphatidylglycerolphosphate:phosphatidylcholine:bacteriorhodopsin; mole:mole:mole ratios) has been included in the reconstitution. Arrays of bacteriorhodopsin were observed in all cases, with an hexagonal array being prevalent for all the chain length phosphatidylcholines (Figs. 1B and 1C) except for the shortest chain lipid, dilauryl phosphatidylcholine (Fig. 1A) when an orthogonal array is formed. The bilayer thickness seems, therefore, not to be a significant factor in array formation, although the type of array, whether hexagonal or orthogonal, may be bilayer thickness dependent with an hexagonal array being formed when d_b is close to the protein hydrophobic dimension. Hydrophobic matching of the protein to the bilayer, at least for bacteriorhodopsin, does not seem as important as the lipid–protein interaction between diphytanyl phosphatidylglycerolphosphate (three negative charges) and bacteriorhodopsin in the membrane surface region.

All complexes have been formed by dialysis of lipid-depleted bacteriorhodopsin [12,13] against low salt and are examined either directly or after addition of 4 M NaCl. The arrays of bacteriorhodopsin are induced in the bilayers only after the addition of salt. Even at low salt, it is expected that any charge at the membrane polar–apolar interface would be screened and thus the effects of adding salt is unclear.

3. Changes in the retinal conformation in bacteriorhodopsin during the photocycle resolved by NMR

Conventional high resolution NMR methods are not generally applicable to integral membrane proteins in bilayers. The overall molecular weight of the complex is very high ($\gg 20$ kDa), the molecular reorientation rate with respect to the applied field very slow and the degree of molecular aggregation significant, all properties which give rise to highly anisotropic NMR spectra from which detailed structural information cannot readily be gained. However, nuclear magnetic resonance methods designed specifically to study solids are finding new applications in the resolution of integral proteins whilst in membranes. For deuterium nuclei, the quadrupolar interaction with the electric field gradient of the bond is highly anisotropic and dominates the appearance of the spectrum and analysis of the line-shape provides details about the absolute orientation, as well as the motional averaging, of the labelled group. We have recorded the NMR spectra from the deuterons in a number of selectively deuterated retinals inserted into bacteriorhodopsin to define the conformation of the chromophore when in the binding site, both in the ground state and, to be described here, the M-state of the photocycle.

Each of the three methyl groups on the retinal polyene chain, and the methyl groups of the cyclohexene ring (Fig. 2) have been deuterated ($-CD_3$) to give a retinal analogue with non-perturbing isotopic substitutions. When inserted into bacteriorhodopsin in oriented purple membranes from a *H. salinarium* deficient in retinal synthesis (strain JW5), the deuterium NMR spectra are characteristic of quickly rotating methyl groups (with T_1s of the order of 60 ms at $-60°$ C) [9]. The spectra recorded with their normal parallel to the spectrometer magnetic field direction, consist of pairs of spectral lines (Fig. 3), with separations (quadrupole splittings, $\Delta\nu_Q$) which are directly related to the angle (Θ) of the C–CD_3 bond vector with the membrane normal [17]. Although the magnitude of the quadrupole splitting can be accurately determined, its sign can only be determined by examining the spectral shape as the sample is tilted in the applied field and comparing with computer simulated line shapes [9]. The large

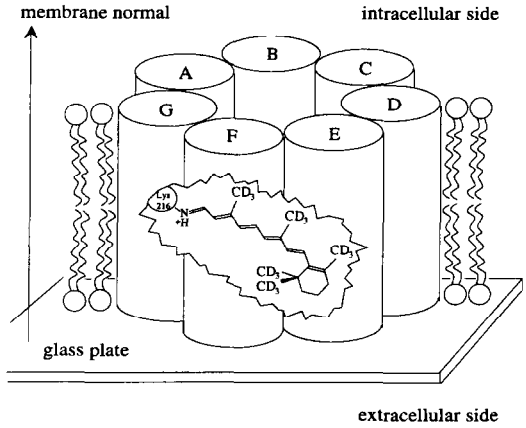

Fig. 2. Structure of bacteriorhodopsin showing the individually deuterium labelled sites deuterated for the deuterium NMR studies. Oriented purple membrane samples were prepared on small glass plates and measured with their normals parallel to the spectrometer magnetic field direction.

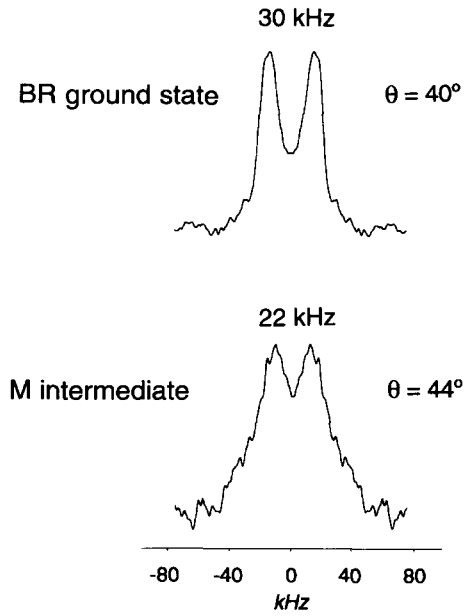

Fig. 3. Experimental deuterium NMR spectra of bacteriorhodopsin containing retinal with a selectively labelled deuteromethyl group at carbon C_{19} (central methyl group in the polyene chain, Fig. 2), showing the dark-adapted state (upper) and M_{412}-intermediate trapped in guanidinium hydrochloride at $-60°$ C (lower). The quadrupole splitting decreases notably upon isomerization and some loss in the quality of the microscopic alignment is observed which leads to line-broadening.

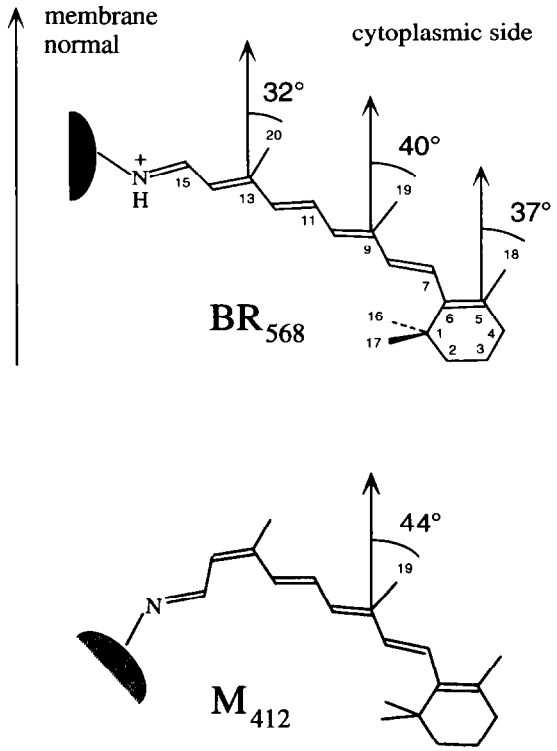

Fig. 4. Orientation and conformation of retinal in bacteriorhodopsin in the BR_{568} ground state and in the M_{412} intermediate of the photocycle, constructed from the C–CD_3 bond vectors that were determined by deuterium NMR [8,10]. The angle between the C_{19} methyl group and the membrane normal was evaluated from the quadrupole splittings of the spectra shown in Fig. 3 and this angle increases from $40 \pm 1°$ in the ground state to approximately $44 \pm 2°$ in the M-state [11].

anisotropic range in the spectra enable a high degree of precision in the measurement of the bond vector (Θ) to be realised, to $\pm 2°$. Each NMR spectrum recorded and analysed is an independent piece of data and the analysis relies on no other information making the method ab initio and very precise in this kind of system.

In previous work we have shown that in the ground state, the retinal orientation and conformation has thus been fully described by combining the measured bond vectors and model building (Fig. 4). The angles were obtained for the methyl groups C_{18} ($37° \pm 1°$), C_{19} ($40° \pm 1°$), and C_{20} ($32° \pm 1°$) along the polyene chain [10], and the two geminal methyls on the ring were found to lie close to the horizontal of the membrane [8]. The roughly parallel orientations of C_{18} and C_{19} confirm that retinal must have a 6-*S-trans* conformation when bound to bacteriorhodopsin. However, since the three methyl groups along the polyene chain are not entirely parallel to one another, the chromophore backbone must possess a certain degree of curvature and possibly some out-of-plane twist, although this cannot be resolved by these present measurements.

Here, we have repeated the kind of experiments performed earlier, but on bacteriorhodopsin which has been trapped in the M-state of the photocycle in the presence of guanidinium hydrochloride at $-60°$ C [11]. The retinal in this case was deuterated in the C_{19} position (Fig. 2) since this would be a good reporter of any orientational or conformational changes in the bulk of the chromophore backbone. The spectra recorded for the ground state and M-state (Fig. 3) have notably different quadrupole splittings and some loss of signal-to-noise. Also, the quality of the alignment in the sample is lower, something which can be quantified and compensated for in the spectral simulations [9]. Thus, comparison of the angle of the C_{19} methyl group of retinal in the ground state ($40° \pm 1°$) [10] with that of the M-intermediate ($44° \pm 2°$) [11], shows that the local tilt of the retinal increases by only approximately 4° upon 13-*cis*-isomerization [11]. This implies that the bulk part of the chromophore remains relatively firmly held in the binding pocket, while it is the Schiff base end of retinal at the covalent protein–chromophore linkage that moves.

4. Conclusions

The current studies either of bacteriorhodopsin array formation or of the retinal conformation in bacteriorhodopsin, are in good agreement with earlier investigations. Electron diffraction studies of reconstituted bacteriorhodopsin in dimyristoyl phosphatidylcholine bilayers were performed on bacteriorhodopsin which had not been totally delipidated [15]. Any residual endogenous lipid, which does remain during the solubilization process of bacteriorhodopsin when no column chromatography of the protein is included in the preparative method [12], will produce arrays of bacteriorhodopsin, as shown

[16]. Although the precise nature of the lipid-induced array formation of bacteriorhodopsin is not described in molecular detail, it is clear that such effects may have important implications for the crystallisation and stabilisation of a range of other integral membrane proteins; this has yet to be demonstrated in the light of the two currently demonstrated cases where such interactions have been shown to be important [5,6].

The retinal chromophore conformation has been resolved rather precisely and the results agree with other spectroscopic [18] and optical data [19], as well as neutron scattering data [20]. The deuterium NMR work described here gives details about the retinal chain and ring, as well as the small orientational changes which occur in the chromophore through the photocycle. Isomerization of the retinal could couple to the protein backbone in the region of the Schiff base, causing it to move as a local switch during the photocycle.

Acknowledgements

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