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The distribution of tetramethylammonium ions on the surface of purple membranes

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Abstract. The two-dimensional distribution of deuterated tetramethylammonium (TMA^+) ions on the surface of purple membranes of *Halobacterium halobium* was determined by neutron diffraction. The measurements were performed on stacks of these membranes with a high concentration of TMA^+ molecules in the water layer between the membranes. A difference Fourier analysis of samples with deuterated and undeuterated ions showed an excess of 8.5 TMA^+ ions per elementary cell in the lipid areas compared to the protein areas. A total number of 90 ions per elementary cell in the intermembrane space was estimated from the preparation procedure. The excess in the lipid domains may result from the higher affinity of TMA^+ ions for the lipid head groups and/or from the fact that the protein (bacteriorhodopsin) protrudes slightly out of the lipid surface.

Key words: Ion distribution, tetramethylammonium, purple membrane, bacteriorhodopsin, neutron diffraction

Introduction

The two-dimensional distribution of ions as seen in projection on the membrane surface can reflect specific electrostatic interactions between the membrane and the ions in the water layer. By changing pH and ion concentrations binding sites of different affinity in the structure could be located. These problems cannot be solved by the conventional X-ray approach of taking the difference structure of a sample with and without ions, because the ions may change the structure itself. Neutron diffraction applied to two specimens containing deuterated and undeuterated ions is the method of choice for this kind of problem (Hentschel et al. 1985). This paper presents our first results on the distribution of the positively charged tetramethylammonium ions (TMA^+) in projection on the surface of the purple membrane of *Halobacterium halobium*. This membrane contains a single protein, bacteriorhodopsin (BR), as a trimer in an extremely well oriented hexagonal lattice (Henderson and Unwin 1975). Because of its crystallinity with well defined protein and lipid areas purple membrane is a good model for the study of ionic interactions in membranes. Also, it was discovered recently that certain bound cations influence the function of BR as a light driven proton pump (Kimura et al. 1984; Chang et al. 1985). In our first experiments the ion distribution was determined for a high TMA^+ concentration (90 TMA^+ ions per elementary cell) in the water layer between the membranes.

Materials and methods

Purple membranes were isolated from *Halobacterium halobium* in the usual way (Bauer et al. 1976). A 15 ml aqueous suspension containing 100 mg of purple membrane was dialysed against a 1 *M* TMA^+ solution for 12 h in order to replace other monovalent cations. The sample was then dialysed for 18 h against distilled water and centrifuged at 140,000 *g* for 45 min. The pellet was redispersed in 2 ml distilled water and divided into two parts. 5 mg of undeuterated TMA-Cl was then added to one sample and 5.5 mg of deuterated TMA-Cl to the other sample. This procedure results in about 90 TMA^+ molecules per elementary cell.

Oriented samples for neutron diffraction were prepared by allowing a purple membrane dispersion

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Abbreviations: BR, bacteriorhodopsin; TMA, Tetramethylammonium

in water to evaporate slowly on five thin quartz slides on areas of 2 × 1 cm for each slide. The drying atmosphere was maintained at 85% relative humidity at room temperature using a saturated KCl solution. When equilibrium was reached each stack of 5 slides was fixed in a holder and mounted on a goniometer with the length axis of the slides in the vertical direction. The slides were aligned perpendicular to the neutron beam inside an aluminium can on diffractometer D16 at the ILL (Jubb et al. 1984). The relative humidity in the can was 85% (controlled using a saturated KCl solution) and the measuring temperature was 23 °C. In-plane diffraction data were collected by scanning both the sample and the two-dimensional multidetector (Jubb et al. 1984).

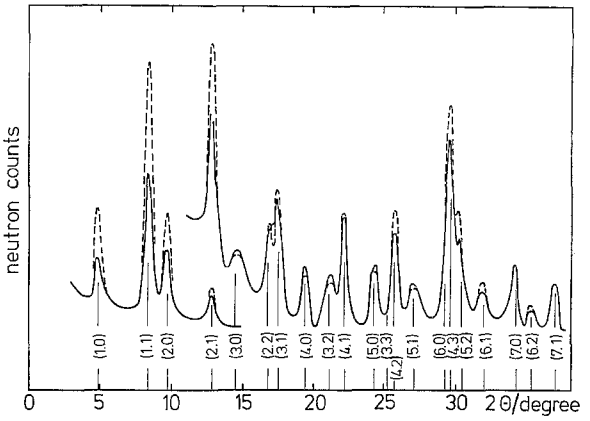

Fig. 1. Neutron counts of purple membranes in H_2O with deuterated (——) and undeuterated (– – –) TMA^+ ions. The data are scaled with respect to each other

Results and discussion

Figure 1 shows the intensity data for the samples with undeuterated and with deuterated TMA^+ ions in H_2O. There are pronounced changes in the lower order reflections between the two samples. These reflections are decreased in intensity for the samples with deuterated TMA^+, indicating that the contrast between lipid and protein areas is lower than in the sample with undeuterated TMA^+. This supports the hypothesis that the number of TMA^+ molecules per $Å^2$ in projection on the membrane surface is higher in the lipid than in the protein areas. The diffraction patterns are very similar to those of oriented purple membranes in H_2O and D_2O at 100% relative humidity (Zaccai and Gilmore 1979). The difference Fourier map showed more D_2O per $Å^2$ in the lipid areas and therefore the contrast between protein and lipid domains was decreased.

In order to follow these observations further data reduction was done as described earlier (Zaccai and Gilmore 1979; Jubb et al. 1984). The neutron counts in a reflection were integrated and corrected by the Lorentz factor $(h^2+h\,k+k^2)^{1/2}$. Intensities with the same value in $(h^2+k\,h+k^2)$ are superimposed on the same powder ring. They were divided according to the intensity ratios measured by electron diffraction on single sheets. The square roots of these intensities (Table 1) were combined with phases from electron microscopy (Henderson and Unwin 1975). Figure 2a shows the scattering length density map calculated from structure factors for the sample with undeuterated TMA^+ in H_2O.

In order to prove that the observed changes in the low order reflections result from the increased number of TMA^+ molecules in the lipid areas, the density in the lipid regions was reduced by a constant number for the deuterated sample until the

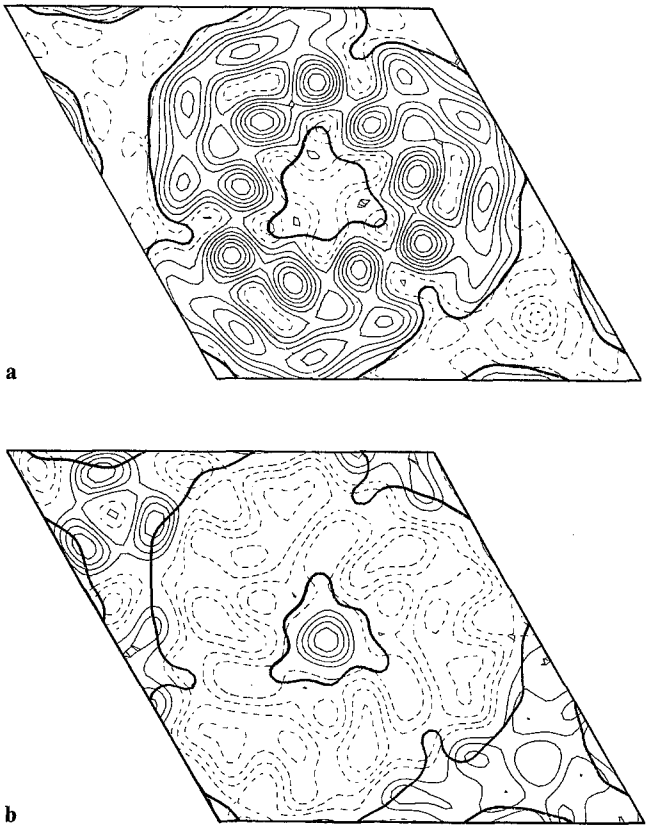

Fig. 2. a Neutron scattering density maps of purple membrane in H_2O with undeuterated TMA^+ ions calculated from the structure factors in Table 1. Contour levels corresponding to negative scattering length densities are plotted by *broken lines*. The lipid areas separated by the *strong line* from the protein parts were used in the fit procedure described in the text. **b** Difference Fourier map according to the structure factors in Table 1 showing the distribution of TMA^+ ions. *Full lines* indicate density levels above average (excess of TMA^+ ions), and *broken lines* indicate below average density

Table 1. The moduli of structure factors F_D and F_H of purple membrane in H_2O with deuterated and undeuterated TMA^+ ions, combined with phases from the electron microscopy work of Henderson and Unwin (1975). The structure factors are scaled with respect to each other as described in the text

h, k	F_D	F_H	α/degree
10	48	66	342
11	114	142	162
20	84	99	190
21	27	32	210
12	57	66	312
30	29	18	96
22	48	47	118
31	61	63	188
13	18	18	120
40	51	44	282
32	45	30	5
23	30	20	342
41	56	61	318
14	43	47	308
50	57	56	345
33	< 15	< 15	345
42	23	26	103
24	66	74	240
51	15	13	28
15	52	46	268
60	< 15	< 15	329
43	91	99	125
34	57	62	162
52	64	59	130
25	26	24	272

structural amplitudes approached those of the undeuterated sample times a constant factor independent of h, k. This factor is the scaling factor between F_H and F_D, the amplitudes for the samples with undeuterated and deuterated TMA^+. If the ratio $S(h,k) = F_H(h,k)/F_D(h,k)$ is not constant within the experimental errors for all reflections the assumption of an homogeneous excess of TMA^+ in the lipid part is invalid. As a test the weighted mean square deviation is used, which tends to zero if the assumption is true,

$$r_s = \sum_{h,k} w_{hk} (S(h,k) - \bar{S})^2 / \sum_{h,k} w_{hk} \Rightarrow \text{Zero},$$

where

$$\bar{S} = \sum_{h,k} w_{hk} S(h,k) / \sum_{h,k} w_{hk}$$

is the weighted averaged scaling factor. As weighting factors, w_{hk} we used the reciprocal relative experimental errors

$$w_{hk} = F/\Delta F .$$

Subtracting a constant density of (0.008 ± 0.001) units from the lipid areas in the map for the sample with deuterated TMA^+ a minimum r_s-value of 0.0055 was obtained which is close to zero, taking into account the experimental error of each order. The corresponding scaling factor was determined to be $\bar{S} = (0.908 \pm 0.023)$.

Using this scaling factor Fig. 2b shows the difference Fourier map, which clearly demonstrates that most of the unevenly distributed TMA^+ is in the lipid domains.

How many extra TMA^+ molecules are spread in the lipid part? The answer is given from an absolute scaling of the map. Considering the fact that in the sample with the undeuterated TMA^+ the scattering length density in the lipid region is approximately zero and calculating the average density in the protein domain from the known amino acid sequence (Ovchinikov et al. 1979), an estimate of an excess of (8.5 ± 1.0) TMA^+ ions in the lipid part can be made.

We next examine whether or not the TMA^+ molecules distributed in the lipid areas are too strong a label for the application of the difference Fourier approach. As shown in an earlier paper (Plöhn and Büldt 1986) we analyzed this question by a model calculation.

A rough model of the purple membrane was constructed and each density point assigned with atomic scattering factors for neutrons and electrons to simulate neutron and electron diffraction respectively. The model for the neutron diffraction was then modified by distributing the scattering power of 8.5 TMA^+ molecules homogeneously in the lipid region. From the intensities of these models the difference Fourier map was calculated using the same approximations as in the experimental case. The distribution of the 8.5 TMA^+ molecules appeared in the difference Fourier map and it was interesting to note, that the map did not show half of the TMA^+ molecules as expected for a single heavy atom site (compare Blundell and Johnson 1976) but did show nearly all of the 8 TMA^+ molecules. This fact was also observed with the D_2O/H_2O exchange experiments (Zaccai and Gilmore 1979) and can be understood in terms of contrast variation arguments for the lower order reflections. These reflections are mainly due to the difference in scattering between lipid and protein areas of the unit cell, where each area can be considered as homogeneous at this resolution. The deuterated TMA^+ associating preferentially with one of these areas would lead to a change in the scattering density difference between them (contrast variation). This would result in a change in the amplitude of these reflections which are proportional to contrast, and would not alter their phases.

What is the interpretation of our result? Do lipid head groups in the purple membrane attract more TMA^+ ions than the protein areas? We think that

this is not necessarily true. Part of the effect might simply be caused by the slight protrusion of bacteriorhodopsin out of the lipid surface. This may lead to the small increase of TMA^+ molecules in the lipid part. However, the other effect of greater affinity of TMA^+ ions for lipid head groups cannot be excluded. Here further experiments with lower TMA^+ concentrations are necessary. In addition the pH and the concentration of other ions should be varied in order to study their influence on TMA^+ binding. By this method positions in the protein with different binding constant for TMA^+ could be detected. It should be noted that recent experiments gave evidence for the fact that cations at certain binding sites affect the photo-chemical-cycle of bacteriorhodopsin (Kimura et al. 1984; Chang et al. 1985).

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