

³¹P NMR Study of the Interaction of Cations with Purple Membrane and of the Purple-Blue Transition†

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ABSTRACT: We have studied by ³¹P NMR both the native purple membrane and the deionized membrane obtained by removal of endogenous cations. The latter membrane was shown to undergo a blue to purple color transition with increasing pH (apparent pK = 5.6). In parallel with this color transition, the ³¹P NMR spectrum obtained at high membrane concentration was dramatically changed from a normal bilayer powder pattern to a seemingly "inverted" line shape. This effect was not observed in native purple membrane and was found to be reversed by addition of cations to the deionized purple membrane. Several data indicated that this inversion of the ³¹P NMR line shape is due to orientation of the membrane fragments perpendicular to the magnetic field. Further studies indicated that both native and deionized purple membranes can undergo such magnetic orientation but that the favorable concentration range is greatly increased for the deionized preparation. This effect is attributed to differences in bacteriorhodopsin conformation and/or membrane surface charge in the two membranes. Binding of divalent cations to the purple membrane was shown to promote an increase of the chemical shift anisotropy of phospholipid phosphate groups as revealed by ³¹P NMR. Accordingly, binding of a trivalent paramagnetic cation promoted strong broadening of the ³¹P NMR spectrum. This suggests a close spatial or structural relationship between phospholipid head groups and cation binding sites in the purple membrane.

The purple membrane of the halophilic bacteria *Halobacterium halobium* appears as a unique striking case with regard to structural organization. It contains a single protein, the light-driven proton pump bacteriorhodopsin (BR),¹ which is organized in a two-dimensional hexagonal lattice of protein trimers [for reviews, see Stoeckenius and Bogomolni (1982) and Dencher (1983)]. Lipids are also present but in remarkably low amounts [lipid to protein ratio 0.3 (w/w)] and have a very unusual composition. Glycolipids and the highly negative phospholipid phosphatidylglycerophosphate (PGP) are the main components, and both contain ether-linked isoprenoid paraffinic chains (Kates et al., 1982).

Nuclear magnetic resonance (NMR) appears to be a suitable technique for studying both structural and functional aspects of the purple membrane. In this regard, the observation of Neugebauer et al. (1977), from neutron diffraction, and of Lewis et al. (1985a), from magnetic birefringence, that purple membrane can orient in strong magnetic fields may be of importance. However, presently no NMR study has investigated this possibility. Most NMR investigations have concentrated on the internal dynamics of BR (Keniry et al., 1984; Lewis et al., 1985b) and on the conformation and environment of its retinal chromophore (Harbison et al., 1983, 1984, 1985). On the other hand, very few NMR data exist concerning the lipids of the purple membrane. Only the study by Ekiel et al. (1981) has investigated the state of phospholipid polar head groups by ³¹P NMR. Lipids are likely to have unusual structural and dynamic properties in the purple membrane and may exert a functional influence. Indeed, several data suggest that lipids may be involved in the inhibitory purple to blue transition of BR induced by low pH or extraction of endogenous divalent cations (Lind et al., 1981;

Lam et al., 1982; Hiraki et al., 1984; Szundi et al., 1987).

In the present study we have used ³¹P NMR to study the effects of cations and pH on the purple membrane, with special emphasis on the purple to blue transition. We show that evidence for magnetic orientation of the purple membrane can be obtained from ³¹P NMR spectra. Cations and pH appear to have a strong influence of this orientational behavior. In addition, the effect of diamagnetic and paramagnetic cations on ³¹P NMR spectra suggests a close structural relationship between the phospholipid head groups and the functionally important cation binding sites of purple membrane.

MATERIALS AND METHODS

Membrane Preparation. Purple membrane was isolated from *Halobacterium halobium* strain S9 according to Oesterhelt and Stoeckenius (1974). Proteolysis of purple membrane by proteinase K was performed during 24 h as described by Dumont et al. (1985). Bleaching of purple membrane in the presence of hydroxylamine was effected as reported in a previous publication (Duñach et al., 1987a). Deionized membrane samples were prepared from the original membrane suspensions by passage through a cation-exchange column (Dowex 50W) and afterward kept in triple-deionized water. Preparation of samples for NMR was usually as follows: membranes (3 mg of protein/mL) in H₂O-D₂O (3:1) were adjusted at the desired salt concentration and pH, spun down, and resuspended, usually at 30 mg of protein/mL although lower concentrations were sometimes used (see Results). The concentrated membranes were then directly transferred into the NMR tube (5-mm internal diameter) and bath sonicated for 10 min for complete equilibration. Sonication was necessary to completely resuspend the highly concentrated mem-

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¹ Abbreviations: BR, bacteriorhodopsin; CSA, chemical shift anisotropy; NMR, nuclear magnetic resonance; PGP, phosphatidylglycerophosphate.

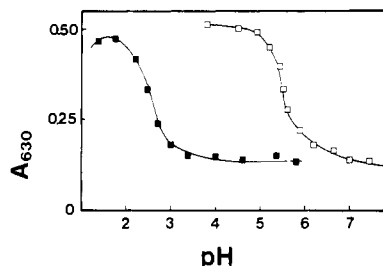


FIGURE 1: pH dependence of the 630-nm absorbance of native purple membrane (■) and of deionized membrane (□) (BR concentration 10 μ M). The difference absorption spectrum of blue membrane (λ_{max} = 605 nm) minus purple membrane (λ_{max} = 558 nm) has its maximum at 630 nm.

brane samples (unsonicated samples only resuspended by repetitive pipetting slowly sedimented in the NMR tube). The pH was routinely checked directly in the NMR tube after the NMR experiments and was always within 0.2 pH unit of the initially adjusted value. For experiments involving paramagnetic broadening, the final sample concentration was 20 mg of protein/mL, and addition of the paramagnetic cation was directly made on this final sample and followed by pH adjustment.

NMR Experiments. ^{31}P NMR measurements were performed on a Bruker MSL-300 spectrometer using broad-band proton decoupling (1.5 W). A $90^\circ(4\ \mu\text{s})-\tau(20\ \mu\text{s})-180^\circ(8\ \mu\text{s})-\tau$ echo sequence was used for signal acquisition. Spectra were obtained by Fourier transformation after exponential multiplication (100-Hz enlargement factor) and with first-order phase correction. The efficiency of proton decoupling was tested on some samples by inserting a decoupling pulse (6.0 W) during the acquisition of the free induction decay, superimposed to the usual continuous decoupling. This resulted in no change in ^{31}P line shapes. Simulation of ^{31}P NMR spectra was performed according to Seelig (1978). For simulation of spectra of magnetically oriented samples, an empirical orientation probability distribution of the following form (unnormalized) was used (Luckhurst, 1974):

$$p(\theta)\ d\theta = \sin\theta \exp\left[\frac{\cos^2(\theta - \theta_0)}{\cos^2\delta}\right] d\theta$$

θ_0 is the most probable orientation of the director axis with the magnetic field and δ the angular width of the distribution.

Freeze-Fracture Experiments. Freeze-fracture electron microscopy was performed according to Gulik-Krzywicki et al. (1986) on some of the samples used for NMR experiments as well as on control unsonicated native purple membrane. Membrane fragment sizes were measured on randomly selected areas of electron micrographs; 150–200 fragments were measured for each sample.

RESULTS

Effect of Cation and pH on the BR Absorption Spectrum. Before describing the effect of pH and cations on ^{31}P NMR spectra of purple membrane, it is important to state clearly how the spectral properties of BR depend on these parameters. Purple membrane (λ_{max} = 558 nm in its dark-adapted form) contains five tightly bound Ca^{2+} and Mg^{2+} ions which can be removed on a cation-exchange column yielding the blue membrane (λ_{max} = 605 nm). Purple membrane can be reformed by binding of a variety of divalent cations (Kimura et al., 1984; Chang et al., 1985; Duñach et al., 1987a). The cation-depleted membrane is thus usually identified as the blue membrane. However, this may be partially misleading. In-

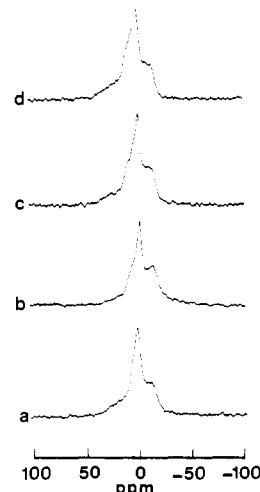


FIGURE 2: ^{31}P NMR spectra of native purple membrane (BR concentration 30 mg/mL) at pH values of (a) 2.5, (b) 5.1, (c) 7.2, and (d) 8.1.

deed, it is well-known that the blue membrane can directly be obtained from the native purple membrane by lowering the pH, with an apparent pK of 2.5 (Mowery et al., 1979; see also Figure 1). Furthermore, as shown in Figure 1, the blue membrane obtained by cation removal can be converted to the purple membrane simply by increasing the pH, with an apparent pK of 5.6.² Thus in the following we will distinguish between native purple membrane, deionized purple membrane, and deionized blue membrane.

Effect of pH on the ^{31}P NMR Spectra of Highly Concentrated Native Purple Membrane. Figure 2 shows the ^{31}P NMR spectra of native purple membrane (concentration 30 mg of protein/mL). As already reported by Ekiel et al. (1981), the spectrum of purple membrane superimposes two bilayer powder patterns corresponding to the two phosphate ester groups of PGP, which is the major phospholipid. The broader signal represents the diester phosphorus and the narrower signal the monoester phosphorus. This difference in line shape appears to be attributable to the intrinsically lower rigid tensor CSA of the monoester (Ekiel et al., 1981). A small narrow isotropic component is also present, the intensity of which is variable. This may be due to rapid reorientation of the smaller membrane fragments (see below). Another possibility is that it arises from protein-bound phosphate groups (Kates et al., 1982). Few changes in line shape were observed with decreasing pH except for a progressive reduction of the apparent residual CSA of both phosphate signals. In particular, the transition of the membrane from purple to blue below pH 3 appears to have no dramatic effect on the ^{31}P NMR spectrum.

Effect of pH and Cations on the ^{31}P NMR Spectra of Highly Concentrated Deionized Membrane. A totally different behavior as a function of pH (Figure 3) was obtained for the deionized membrane (protein concentration 30 mg/mL). Below pH 5, i.e., under conditions where this membrane is blue, ^{31}P NMR spectra similar to those of the native purple membrane were obtained, although with a slightly lower apparent residual CSA. However, above pH 5, as the deionized membrane progressively turned purple, a dramatic modification of the ^{31}P NMR line shape occurred with increasing pH. Above pH 6, a totally new spectrum was obtained, the shape

² Note that this effect cannot be due to the presence of Na^+ ions added in the form of NaOH since the final concentration of this ion was an order of magnitude lower than that promoting the blue to purple transition (Chang et al., 1985).

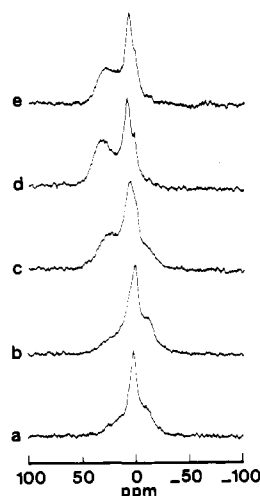


FIGURE 3: ^{31}P NMR spectra of deionized membrane (BR concentration 30 mg/mL) at pH values of (a) 3, (b) 4.5, (c) 5.2, (d) 5.8, and (e) 6.7.

of which has an "inverted" appearance as compared to that obtained at low pH. This inversion evidently occurs for the diester phosphate signal. For the monoester signal, it is sometimes more difficult to observe clearly the inversion, due to the occasional presence of a small spurious isotropic component. However, it can be seen on Figure 3 that the left shoulder of the monoester signal in spectra a and b is not present in spectra c–e, thereby strongly suggesting that inversion also occurs. Spectral simulations also support this view (see below).

The absence of tightly bound cations in the deionized purple membrane appears to be specifically responsible for its inverted ^{31}P NMR spectrum. Indeed, when Ca^{2+} was progressively added to the deionized purple membrane, the pH being kept at about 7, the line shape progressively reversed from the inverted pattern to the normal bilayer pattern (Figure 4). We have previously shown that divalent cations readily bind to the membrane under these conditions (Duñach et al., 1987a). Most of the reversal was obtained at five Ca^{2+} per BR, a ratio which corresponds to complete filling of the high-affinity cation sites of the membrane. A similar reversal was observed upon addition of the trivalent cation La^{3+} and of the monovalent cation Na^+ , the latter requiring however much higher concentrations (>10 mM).

In other experiments, it was found that deionized samples prepared from purple membrane bleached in the presence of hydroxylamine or heavily proteolyzed with proteinase K do not display any inversion in line shape as a function of pH. Thus, this effect appears to be dependent upon the state of BR in the membrane.

Evidence for Magnetic Orientation of Purple Membrane Preparations. A likely explanation for the inverted ^{31}P NMR spectrum of deionized purple membrane is that it is due to orientation of the membrane perpendicular to the magnetic field (see Discussion). Indeed, such orientation has been observed by Neugebauer et al. (1977) with magnetic field strengths significantly lower than those used in our NMR experiments (7.0 T). According to these data, the native purple membrane can orient magnetically only in a narrow concentration range lower than that used in the above experiments. We have thus studied the concentration dependence of the ^{31}P NMR line shapes.

As shown in Figure 5 (a–c), when the deionized blue membrane was progressively diluted, starting with 30 mg of protein/mL, the ^{31}P NMR spectrum reversed from the inverted pattern to a normal bilayer pattern below 18 mg of

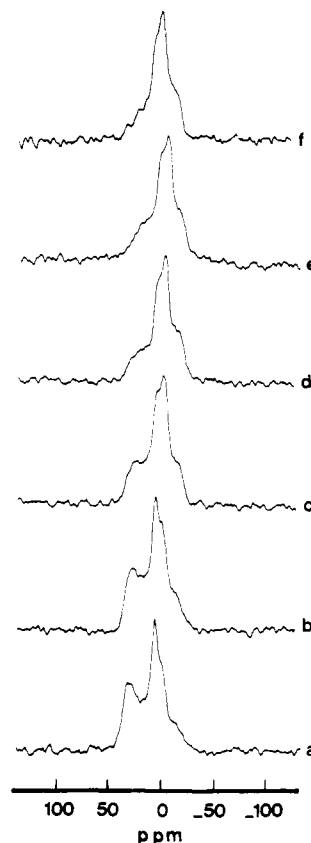


FIGURE 4: ^{31}P NMR spectra of deionized membrane (BR concentration 30 mg/mL) at pH 6.8–7.2 supplemented with (a) 0, (b) 1, (c) 2, (d) 3, (e) 5, and (f) 10 mol of CaCl_2 /mol of BR.

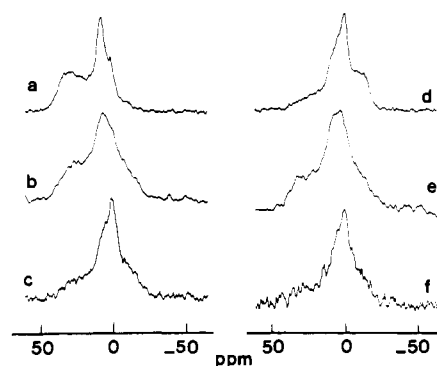


FIGURE 5: ^{31}P NMR spectra of deionized (a–c) and native (d–f) purple membranes at pH 6.8–7.2 resuspended at BR concentrations of (a and d) 30, (b and e) 18, and (c and f) 12 mg/mL.

protein/mL. On the other hand, when the native purple membrane was submitted to similar dilutions [Figure 5 (d–f)], the initially noninverted spectrum at 30 mg of protein/mL changed to an inverted line shape at 18 mg/mL. Further dilution reversed back the spectrum to the normal bilayer pattern. Considering that a similar concentration dependence has been observed for the magnetic orientation of native purple membrane (Neugebauer et al., 1977), this strongly suggests that such an effect is responsible for the inverted ^{31}P NMR spectrum.

Further evidence for this interpretation was provided by an experiment in which deionized purple membrane at 30 mg of protein/mL was included in a Sephadex G-25 gel (extensively washed with EDTA and triple-deionized water to remove all traces of cations). The idea was that if, as proposed by Neugebauer et al. (1977), purple membrane orientation is a macroscopic process requiring cooperative alignment and stacking of many membrane fragments, it would be largely

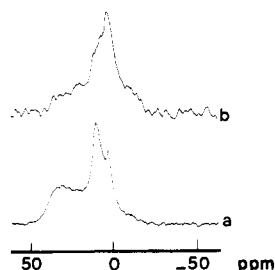


FIGURE 6: ^{31}P NMR spectra of deionized purple membrane (BR concentration 30 mg/mL) at pH 7.1 resuspended in deionized water (a) or embedded in extensively washed Sephadex G-25 gel (b).

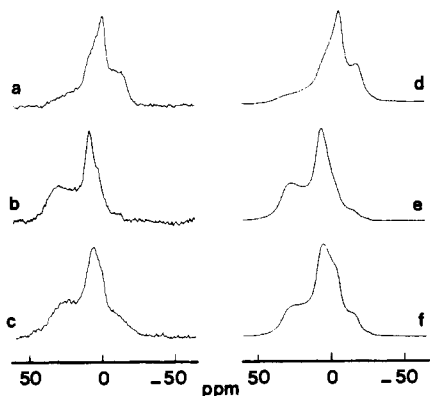


FIGURE 7: Experimental (a–c) and simulated (d–f) ^{31}P NMR spectra of native purple membrane at pH 7.2 (a and d) and deionized membranes at pH 6.7 (b and e) and 5.2 (c and f) (BR concentration 30 mg/mL). Isotropic chemical shift, chemical shift anisotropy, and isotropic and anisotropic line widths for the diester and monoester phosphate (ratio 1:1) were respectively (d) 1.0, 57.2, 3.4, 1.8, 0.2, 15.5, 2.5, and 1.25 ppm, (e) 1.9, 50.1, 3.0, 1.7, 1.8, 14.3, 2.4, and 1.1 ppm, and (f) 1.8, 46.8, 2.8, 1.6, 1.9, 13.7, 2.2, and 1.0 ppm. In (e) and (f) an orientation-dependent distribution with an average orientation of the director axis with the magnetic field of 0° and orientational widths of 30° and 49° , respectively, was used.

precluded within the small void volume between gel beads. Indeed, as shown in Figure 6, for the gel-embedded deionized purple membrane, the ^{31}P NMR line shape is almost completely reversed to a normal bilayer powder pattern.

Spectral Simulation of ^{31}P NMR Line Shapes. We have used spectral simulation to obtain quantitative spectral parameters of purple membrane phospholipid phosphates as well as angular distribution parameters associated with oriented membrane spectra (Figure 7). The normal bilayer ^{31}P NMR spectra could be adequately simulated by use of an isotropic distribution of axially symmetric chemical shift tensors with orientation-dependent line widths. For inverted ^{31}P NMR spectra, a nonrandom angular distribution of the director axes with a finite angular width was introduced. Completely inverted spectra could be best simulated with an average membrane orientation perpendicular to the magnetic field (i.e., membrane normal director axis oriented parallel to the magnetic field) and an angular distribution width of $25\text{--}30^\circ$. Intermediately inverted spectra could be accounted for by increasing the angular width.

The residual CSA values of the diester phosphate obtained from spectral simulations are plotted in Figure 8 as a function of pH for native purple membrane, deionized purple membrane, and proteinase K proteolyzed deionized membrane. The CSA of the monoester appears to behave in the same manner (not shown). For all three preparations, the CSA increases with pH. A strong decrease of the CSA is associated with heavy proteolysis of BR with proteinase K. On the other hand, an increase of the CSA appears to be promoted by the presence

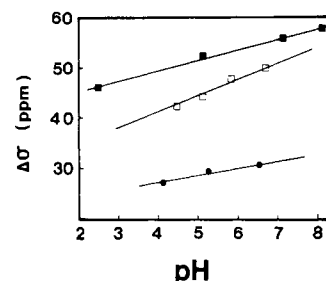


FIGURE 8: pH dependence of the CSA of the diester phosphate of PGP obtained from spectral simulations for native purple membrane (■), deionized membrane (□), and proteinase K treated, deionized membrane (●).

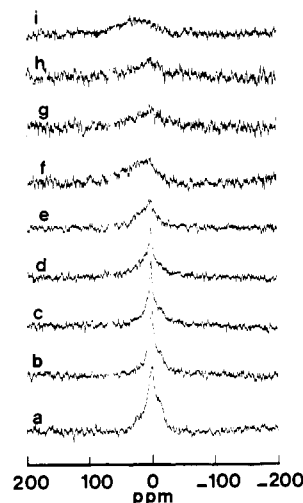


FIGURE 9: ^{31}P NMR spectra of deionized blue membranes (BR concentration 20 mg/mL) at pH 5.0–5.2 supplemented with (a) 0, (b) 0.1, (c) 0.5, (d) 1, (e) 2, (f) 3, (g) 4, (h) 5, and (i) 10 mol of $\text{Nd}(\text{NO}_3)_3$ /mol of BR.

of endogenous divalent cations in the native purple membrane. This suggests that bound cations influence the conformation and/or motion of phospholipid head groups.

Paramagnetic Cation-Induced Broadening of ^{31}P NMR Spectra. To further characterize the structural relationship between the cation binding sites of purple membrane and the phospholipid head groups, we have studied the effect of the paramagnetic cation Nd^{3+} on the ^{31}P NMR spectra. We have chosen conditions where membrane orientation does not occur in order to avoid changes in line shape due to this effect, namely, the deionized blue membrane at pH 5. Our previous data indicated very strong binding of trivalent cations to this preparation (Duñach et al., 1987a). Figure 9 shows that binding of Nd^{3+} promoted a very efficient broadening of the ^{31}P NMR spectrum. Most of the effect was obtained upon binding of five Nd^{3+} per BR, that is, saturation of the high-affinity cation sites of the membrane. Interestingly enough, broadening appears to occur more efficiently for the diester than for the monoester. It must be noted that precipitation of the membrane by the trivalent cation only occurs above this ratio (Duñach et al., 1987a).

Size Distribution of Purple Membrane Fragments. Since magnetic orientation of purple membrane fragments is dependent upon their size (Neugebauer et al., 1977), it was important to determine the latter, especially considering that the preparation of NMR samples involved a slight sonication step (see Materials and Methods). Figure 10A shows the fragment size distribution of unsonicated native purple membrane obtained by freeze-fracture electron microscopy. This distribution (average size $0.47\ \mu\text{m}$) is in reasonable agreement

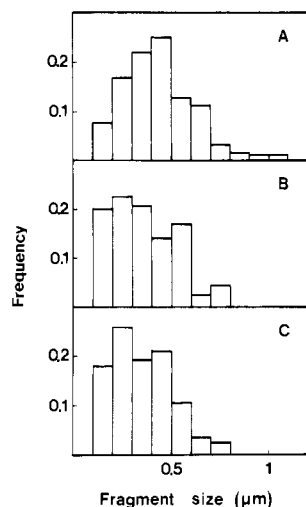


FIGURE 10: Size distributions obtained by freeze-fracture electron microscopy for (A) unsonicated native purple membrane, pH 7.1, (B) sonicated native purple membrane, pH 7.0, and (C) sonicated deionized purple membrane, pH 6.8. The preparation of samples B and C was identical with that used for NMR experiments.

with previous data (Lewis et al., 1985a). Figure 10B, shows the size distributions obtained for NMR samples (i.e., sonicated preparations) of native and deionized purple membranes. In both case, more of the small fragments ($<0.3 \mu\text{m}$) and less of the large fragments ($>0.6 \mu\text{m}$) are present than in the unsonicated material, presumably as a result of sonication. On the other hand, the size distributions of the two sonicated preparations appear similar (average size 0.36 and $0.35 \mu\text{m}$, respectively, for native and deionized membranes). The freeze-fracture data also indicated that in none of the above preparations was any aggregation present.

DISCUSSION

The ^{31}P NMR results presented in this study were mainly obtained on two types of BR preparations: the cation-containing native membrane and the cation-depleted deionized membrane. Both preparations can be made either blue or purple depending on pH and have an apparent pK for this color transition of 2.5 and 5.6, respectively. These features can be accounted for in the framework of our previous model in which the purple to blue transition of BR is mainly due to protonation of a protein group. Binding of cations only has the effect of decreasing the apparent pK of this group by reducing the negative surface potential at the cytoplasmic side of the purple membrane (Duñach et al., 1987a, 1988).

Our NMR data show that both the native and the deionized purple membrane can orient perpendicular to a magnetic field. Other explanations of the occurrence of an inverted ^{31}P NMR line shape are unlikely. Formation of a hexagonal lipid phase would be associated with a much lower value of the apparent CSA (Cullis & De Kruijff, 1979) and is furthermore incompatible with the structural organization of the purple membrane. Conformational change of the lipid head group leading to inversion of the axes of the chemical shift tensor is also theoretically a possible explanation (Thayer & Kohler, 1981). However, in our case, as can be directly observed on spectra, such an effect would have to be accompanied by a large isotropic shift, at least in the case of the diester signal (the maximum of the diester signal shifts about 35 ppm upon inversion; see, for example, Figure 3). Indeed, our attempts to simulate the inverted line shape in this framework (not shown) indicate that not only a negative CSA value but also an isotropic σ value of about 15 ppm for the diester (as com-

pared to 1 ppm in the noninverted case) are necessary for a proper simulation to be obtained. This latter value appears unrealistic since the highest ^{31}P isotropic shifts of phosphate signals are of the order of 3–4 ppm (Gorenstein, 1984). Additionally, it is not obvious to conceive how dilution of the membrane or inclusion in gel could affect a lipid conformational change. On the other hand, orientation of the membrane perpendicular to the magnetic field is expected to lead to an increase in the spectral intensity in the high-ppm region ($\theta = 0^\circ$) and to a decrease in the low-ppm region ($\theta = 90^\circ$) and can thus produce an apparently inverted line shape. Spectral simulations appear to confirm this view and further indicate that the highest orientation available is associated with a mosaic spread of about 25° . The effect of dilution and inclusion of the membrane in Sephadex gel is also in keeping with this hypothesis.

Two recent studies by ^{31}P or ^2H NMR have obtained evidence for the orientation of pure lipid membranes of particular composition in a magnetic field (Seelig et al., 1984; Speyer et al., 1987). However, this appears to be the first NMR report of the magnetic orientation of a biological membrane. Pure lipid membranes orient parallel to the magnetic field and give rise to "single-crystal-like" sharp ^{31}P NMR spectra. In the case of purple membrane, the orientation is perpendicular to the magnetic field. It may be remarked that, for an identical mosaic spread, a perpendicular orientation is expected to display less sharp spectra than a parallel orientation. This is inherent in the axially symmetric angular dependence of the chemical shift. It is thus difficult to compare the extent of orientation of purple membrane with those of pure lipid systems since no estimation of the mosaic spread was made in the above-mentioned reports.

Our observation by ^{31}P NMR of orientation of the native membrane only in a narrow concentration range is qualitatively in agreement with previous results obtained by neutron diffraction (Neugebauer et al., 1977). The significant difference in the concentration at which maximal orientation is obtained in our case (18 mg of protein/mL) and in the neutron study (12 mg/mL) is probably related to the different size distribution of membrane fragments. Indeed, Neugebauer et al. (1977) used unsonicated purple membrane while we used slightly sonicated samples which, according to our freeze-fracture data, have a smaller average fragment size. In the same instance, Lewis et al. (1985a) could detect magnetic birefringence with very low concentrations of unsonicated purple membrane at field strengths comparable to ours. Such birefringence is possibly mainly contributed by magnetic orientation of the large membrane fragments which may not occur or which may be undetectable in our case.

In addition, our ^{31}P NMR data clearly show that native and deionized purple membranes have a different orientational behavior in the magnetic field. Namely, the deionized purple membrane is able to orient magnetically in a wider concentration range extending to much higher concentrations (30 mg/mL). Freeze-fracture controls rule out the possibility that these differences are related to a nonidentical size distribution of membrane fragments in the two preparations.

For the native purple membrane, the occurrence of concentration-dependent magnetic orientation appears to be due to the combination of three effects (Neugebauer et al., 1977): (1) diamagnetic anisotropy of membrane fragments due to the high content of transmembrane protein α -helices perpendicular to the membrane plane; (2) interactions between membrane fragments allowing cooperative macroscopic alignment above a critical concentration; (3) viscosity of the suspension which

precludes orientation at high membrane concentrations. Thus, the higher capacity of the deionized purple membrane for magnetic orientation may be attributed to the more favorable nature of one or several of these three properties. A higher diamagnetic susceptibility of the membrane fragments can arise from a different conformation of BR involving a more perpendicular orientation of those α -helices known to be tilted in the native membrane (Henderson & Unwin, 1975) or re-orientation of tryptophan residues (Neugebauer et al., 1977). Indeed, conformational changes of BR have been shown to occur upon deionization of the membrane (Kimura et al., 1984; Duñach et al., 1987b). Alternatively, the more negative surface charge associated with the absence of cations in the deionized purple membrane may imply more cooperative interactions between membrane fragments (repulsive antistacking interactions or permanent dipole lateral interactions), allowing a better alignment in the magnetic field. This higher negative charge could also lower the viscosity at high membrane concentration. In any case, it appears that the ability to orient magnetically at high concentration is an intrinsic property of the deionized purple membrane. Indeed, this capacity is generated as a function of pH in parallel with formation of this pigment³ and is abolished in parallel with binding of cations. This suggests that the structural properties of the membrane are not univocally related to its color nor to the binding of cations.

Analysis of the ³¹P NMR spectra has also allowed us to determine the CSA of phospholipid phosphate groups in the membrane. All CSA values increase with pH, an effect which may be attributed to the progressive deprotonation of lipid head groups as already observed for other acidic phospholipids (Browning & Seelig, 1980). In the native purple membrane, the high CSA values appear to be the result of two effects. The stronger effect is that of the transmembrane protein BR. Indeed, an important lowering of the CSA is obtained after proteinase K treatment of the membrane. This enzyme is known to heavily proteolyze BR, even removing parts of the transmembrane regions (Dumont et al., 1985). Thus, it seems that the wobbling motion of the PGP polar head group is strongly hindered in amplitude in the purple membrane due to the presence of native BR. A similar conclusion was drawn by Ekiel et al. (1981). Such a restricting effect is consistent with the report that proteins are separated by a single layer of lipids in the purple membrane (Glaeser et al., 1985). In addition, another factor increasing the CSA of native purple membrane appears to be the presence of tightly bound divalent cations since deionization reduces the CSA. This suggests either a direct involvement of PGP head groups in some of the cation binding sites or a close proximity between the head groups and the sites.

Other evidence of the spatial relationship between the PGP phosphates and the cation binding sites is the observation that binding of the paramagnetic cation Nd³⁺ to these sites has a strong broadening effect on the ³¹P resonances of the membrane. It must be stressed that our previous results indicated that the affinity constant of trivalent cations toward the sites is higher than 1 μ M⁻¹ (Duñach et al., 1987a). Thus at the concentrations used in the NMR experiments, the unbound cation concentration is negligibly small and cannot account for the broadening effect. Indeed, although Nd³⁺ usually acts

as a shift reagent in membranes (Bergelson, 1978), it promotes a broadening in the present case, suggesting a long lifetime of the cation-membrane complex.

Data by other groups (Chang et al., 1985, 1986) and ourselves (Duñach et al., 1987a,b) indicate that BR carboxyl groups are involved in the high-affinity cation binding sites of purple membrane. However, a contribution of phospholipid head groups to the sites is suggested by the influence of the lipid environment on the purple-blue transition (Lind et al., 1981; Lam et al., 1982; Hiraki et al., 1984) and by the recent observation that cations do not affect this transition in partially delipidated purple membrane (Szundi & Stoeckenius, 1987). Our NMR data are consistent with this proposition, though we are aware that these do not constitute a definitive proof. In any case, since phospholipids are known to be asymmetrically located in the cytoplasmic leaflet of the purple membrane (Henderson et al., 1978; Renthal & Chua, 1984), the Nd³⁺ broadening data presented here support the idea that the cation binding sites are located at the cytoplasmic surface as previously suggested (Chang et al., 1986; Duñach et al., 1987a).

CONCLUSIONS

This study confirms that purple membrane can orient in strong magnetic fields and furthermore demonstrates that this property is affected by binding of cations to the functionally important sites. These sites may involve polar head groups of phospholipids at the cytoplasmic surface. The magnetic orientation data also imply that purple membranes obtained in the presence and absence of cations have different structural properties. A functional comparison of these two membranes may thus be important.

Additionally, the possibility to magnetically orient purple membranes at relatively high concentration may prove particularly useful in future NMR studies.

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³ We have also found (unpublished results) that, after papain cleavage of the C-terminal segment of BR, the apparent pKs for the blue to purple transition and for magnetic orientation at 30 mg/mL of the deionized membrane are both shifted to a higher value (6.8). This further suggests a strong correlation between these two processes.

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Assessment of the Number of Nucleotide Binding Sites on Chloroplast Coupling Factor 1 by the Continuous Variation Method[†]

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ABSTRACT: The method of continuous variation (Job plot analysis) and difference absorbance spectroscopy were used to investigate the binding of 2'(3')-(trinitrophenyl)-ADP and -ATP to chloroplast coupling factor 1 (CF₁). Experiments performed at a low total concentration (30 μM) of nucleotide and enzyme binding sites (assuming three or four binding sites per CF₁) could be interpreted in terms of approximately three nucleotide binding sites per CF₁. At higher total concentrations (100 and 400 μM), the number of apparent binding sites increased to almost four. Computer-generated Job plots, using a protein-ligand complex formation scheme of *n* independent, nonequivalent binding sites, gave good fits to the experimental data at all concentrations when four binding sites were modeled. The dissociation constant of the fourth site was estimated to be ~20 μM. Additional nucleotide binding sites were not directly observed by this method and, if they exist, have very weak binding affinities (dissociation constants > ~1 mM).

The soluble, extrinsic portion of the ATP synthetase isolated from spinach chloroplasts, chloroplast coupling factor 1 (CF₁),¹ is comprised of five types of polypeptides (α, β, γ, δ, and ε). The two largest polypeptides, α and β, have been associated with nucleotide binding (Kambouris & Hammes, 1985; Admon & Hammes, 1987). The stoichiometry of the polypeptide chains comprising CF₁, α₃β₃γδε (Moroney et al., 1983), is

identical with that of F₁ sectors isolated from proton ATPases of bacteria and mitochondria [cf. Senior and Wise (1983)]. Recent studies with *Escherichia coli* and mitochondrial F₁ provide strong evidence for the existence of six distinct nucleotide binding sites (Wise et al., 1983; Kironde & Cross, 1986). Ligand binding studies with rat liver mitochondrial

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¹ Abbreviations: CF₁ and ECF₁, F₁-ATPases isolated from spinach chloroplasts and *Escherichia coli*, respectively; EDTA, ethylenediaminetetraacetic acid; TNP-AMP, -ADP, or -ATP, 2'(3')-(trinitrophenyl)adenosine mono-, di-, or triphosphate, respectively; Tris, tris(hydroxymethyl)aminomethane.