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Effect of lipid surface charges on the purple-to-blue transition of bacteriorhodopsin

(purple membrane/blue membrane/surface charge density/lipids)

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ABSTRACT **Purple membrane (λ_{max} = 568 nm) can be converted to blue membrane (λ_{max} = 605 nm) by either acid titration or deionization. Partially delipidated purple membrane, containing only 25% of the initial lipid phosphorus, could be converted to a blue form by acid titration but not by deionization. This reversible transition of delipidated membrane did not require the presence of other cations, and the pK of the color change that in native membrane under similar conditions is between 3.0 and 4.0 was shifted to 1.4. We conclude that the purple-to-blue transition is controlled by proton concentration only and that, in native membranes, the cations act only by raising the low surface pH generated by the acidic groups of the lipids. The observation that extraction of lipids from deionized native membrane converts its color from blue to purple further confirms this conclusion. The two states of the membrane probably reflect two preferred conformations of bacteriorhodopsin, which are controlled by protonation changes at the surface of the membrane and differ slightly in the spatial distribution of charges around the chromophore.**

The retinylidene protein bacteriorhodopsin (bR) functions as a light-driven proton pump (1, 2). It is the only protein in the purple membrane (pm) and is arranged in a hexagonal lattice (3, 4). Purple membrane contains a variety of diether lipids, amounting to about 25% by weight, that fill the spaces between bR molecules in the lattice and are all in close contact with the protein (3–6). Most of the lipids are acidic (80%); 70% are phospholipids, mostly the diether analogue of phosphatidylglycerophosphate, and 30% are glycosulfolipids (7, 8).

Acidification or removal of cations from pm suspensions shifts the absorption maximum of bR from 568 to 605 nm (1, 9–15). The chromophores in these acid or deionized blue membranes are indistinguishable in absorption and resonance Raman spectra (16). The color of bR is apparently controlled by the distribution of charges in the chromophore, specifically protonation and a charge pair or dipole near the β-ionone ring that cause a large red shift of the retinal absorbance; a counterion—i.e., a negatively charged amino acid residue near the protonated Schiff base—stabilizes it with a concomitant reduction of the red shift (17–19). Protonation of this counterion has been suggested as the cause for the purple-to-blue transition. The purple color returns upon further acidification and formation of this "acid purple" chromophore has been attributed to restoration of the negative charge by binding of an anion at the site protonated in the purple-to-blue transition (11) or to protonation of a second negatively charged group (12).

The original purple color of the membrane can be restored by addition of salt and alkalinization. Binding of three to five cations to specific sites is generally believed to be required for the color change (13, 14, 20, 21) and the binding sites with the highest affinity may not affect the color change (21). Red shifts of the bR absorption band also occur upon heating (20) or adding anionic detergent to pm suspensions (22). Modification of carboxyl groups by carbodiimides shifts the pK of the color change to lower values (23), whereas acetylation of lysine residues has the opposite effect (24).

It has been suggested that protonation changes near the Schiff base are directly linked to binding of cations to the bR molecule and that carboxyl groups of the protein constitute the binding sites (refs. 14, 20, 21, 25; M. Engelhard, K.-D. Kohl, and B. Hess, personal communication) or that the bound cation itself shifts the absorption of the retinylidene group (26). Here we describe the effect of the negative charges of the pm lipids on the color changes of bR. We show that, in lipid-depleted membranes, the blue-to-purple transition is controlled entirely by the proton concentration and does not require the presence of other cations.

MATERIALS AND METHODS

Purple membrane was purified from *Halobacterium halobium* strain JW-3 as described (27). Membrane suspensions were deionized following the procedure of Kimura (13). To avoid complications due to aggregation, native and delipidated membranes were also incorporated into a 7.5% polyacrylamide gel by mixing the membrane suspension with gel-forming solution and polymerizing the mixture according to standard procedures (12). After embedding, the membranes were washed several times with distilled water followed by Milli-Q water and deionized by overnight incubation with ion-exchange beads. Gel slices (20 × 10 × 3 mm) were soaked in 10 ml of the appropriate solutions for a minimum of 5 hr before recording the effects of pH and salt using a Perkin–Elmer Lambda 4A spectrophotometer. Purple membrane was delipidated by incubating a 10-mg sample overnight at room temperature in 5 ml of 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Calbiochem–Behring) containing 5 mM acetate buffer (pH 5.4). The suspension was pelleted at 45,000 × *g* for 20 min. The delipidation process was repeated three times. Excess CHAPS was then removed by several washes with distilled water. Lipid phosphorus was determined by the molybdate method after ashing (28). Total lipids were extracted according to Kates *et al.* (8) and separated by thin-layer chromatography in a mixture of chloroform/methanol/2-propanol/triethylamine/0.25% aqueous KCl, 30:9:25:18:6 (29). Ca and Mg contents of membrane suspensions were measured by atomic absorption spectroscopy in the presence of La to reduce the effect of phosphorus and to increase sensitivity.

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Abbreviations: bR, bacteriorhodopsin; pm, purple membrane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

To avoid contamination by ions, Milli-Q water and plasticware were used throughout; all chemicals were of analytical grade.

RESULTS

Lipid Extraction and Deionization of Lipid-Depleted Membranes. The visible absorption maximum of pm in 20 mM CHAPS is blue-shifted from 568 nm to 560 ± 1 nm. Integrity of the membranes seems to be retained since they can be sedimented from the suspension under the same conditions as untreated pm. After three extractions, 25% of the initial lipid phosphorus remained in the membrane and thin-layer chromatography of the total lipid showed proportional decrease of all of the main lipid components. The action of CHAPS on pm is similar to that of cholate/deoxycholate (30) in the extent of lipid removal and the absorption shift. In the case of cholate/deoxycholate, it is known that a lattice is still present after lipid removal (6, 30), and the presence of an exciton band in the CD spectra (not shown) indicates that the aggregated state is also preserved after CHAPS delipidation. CHAPS has advantages over cholate/deoxycholate because its use is not restricted to alkaline pH where the protein stability is reduced, and residual detergent will not contribute to the surface charge of the membrane over a wide pH range.

In contrast to native pm, CHAPS-treated membrane, when passed through a cation-exchange column, did not change its color from purple to blue. Also, gel slices with untreated pm became blue after an overnight incubation with beads of cation-exchange resin, with the same absorption maximum (λ_{max}) at 603 nm obtained when pm suspensions were passed through an ion-exchange column. The lipid-depleted membrane gels, however, did not change their color even after several days of incubation and their λ_{max} remained at 561 nm (Fig. 1, curve a).

Acid Titration of Lipid-Depleted Membranes. The lack of a color change in delipidated membranes could be due to an incomplete removal of cations by the ion-exchange resin. Adding increasing amounts of acid to the medium, however, should replace them with protons and the cations should be removed when the incubation medium is changed. Therefore, a delipidated, deionized but still purple gel was passed through a series of HCl solutions of increasing concentration. As shown in Fig. 1 (curve b), the color changes from purple to blue below pH 2. The color shift does not go to completion because the acid purple form begins to appear below pH 1.4 (Fig. 2, curve a), which is expected because its pK in the native membrane is independent of cation concentration (13). If the experiment is then run in the reverse direction (Fig. 2, curve b), the purple color is restored essentially in the same pH range with a small reproducible hysteresis.

These results argue strongly against a role of cations in maintaining the purple color of bR in lipid-depleted membrane and suggest that, in the native pm, they act only through their shielding effect on the high surface charge of the native pm.

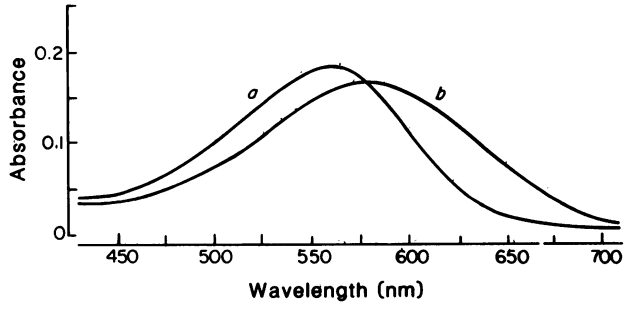

FIG. 1. Absorption spectra of lipid-depleted, deionized membrane (curve a) and its blue form at pH 1.4 (curve b).

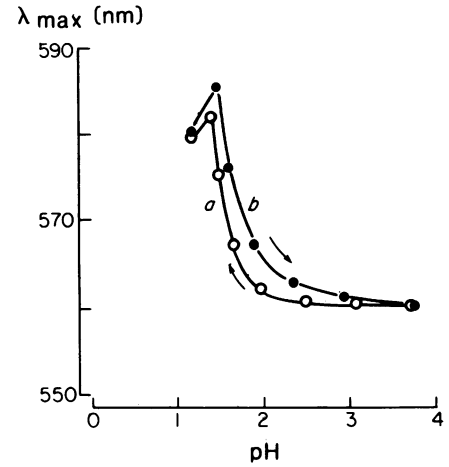

FIG. 2. Titration curve for the absorbance shift of lipid-depleted membrane in the absence of cations. Gel slices containing membrane fragments were deionized and passed through a series of HCl solutions of increasing (curve a) or decreasing (curve b) concentrations. It should be noted that plotting of the absorption value at a fixed wavelength instead of λ_{max} would be a more correct way to express the degree of conversion, but in the case of long-term incubations, especially in acids, bleaching of the chromophore occurs and it is difficult to correct for this.

Salt Dependence of the Purple-to-Blue Transition. If the surface charge density is high, the apparent pK_a of a group on or near the membrane surface may differ substantially from its intrinsic value. We estimate the intrinsic pK of the color change and the surface charge density from the salt dependence of the apparent pK. Fig. 3 shows the titration of

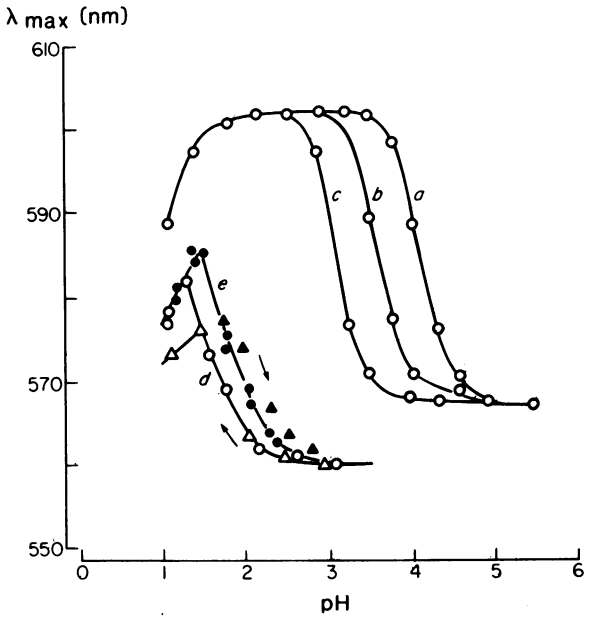

FIG. 3. Titration of pm and lipid-depleted membrane (pm-L) in the presence of $CaCl_2$. Curves a, b, and c show data for pm in the presence of 0.01, 0.1, and 1 mM $CaCl_2$ concentrations, respectively. Curves d and e give the data for pm-L at 0.01, 0.1, 1, and 10 mM $CaCl_2$, which are indistinguishable (○) and only a few data points are shown. The slight shift seen at 100 mM $CaCl_2$ (△) is of questionable significance. The arrows indicate the direction of titration; increasing concentrations (open symbols) were obtained by addition of HCl and decreasing concentrations (filled symbols) were obtained by addition of Milli-Q water.

lipid-depleted membranes in the presence of 0.01–100 mM $CaCl_2$ concentrations and compares them to the pH titration curves for native pm in the presence of 0.01, 0.1, and 1 mM $CaCl_2$. Though the apparent pK of the pm color change decreases with increasing $[Ca^{2+}]$ in accordance with the Gouy–Chapman formalism, the pK of the lipid-depleted membrane remains at pH 1.4 over a much larger salt concentration range. Only at 100 mM $CaCl_2$ is the titration curve shifted to slightly higher pH, but only to an extent which may not be significant. Apparently, the lipid-depleted membrane has zero or a small net positive surface charge in the pH range where the color change takes place. This is expected, because the surface carboxyls should be protonated over most of this pH range and the number of protonated bases on the protein molecule should be sufficient to compensate for the two or three negative charges of the remaining lipids. The observed pK of the group(s) controlling the purple-to-blue transition in lipid-depleted membrane must therefore be close to the intrinsic one, assuming that the color change is linked to the titration of one or more specific groups and that delipidation does not affect the charge distribution on the protein itself. We will argue in the *Discussion* that the color change does not involve protonation of a group in the chromophore but that protonation occurs somewhere else and affects the chromophore indirectly.

Blue-to-Purple Transition Induced by Lipid Extraction. The acid titration experiment suggests that removal of negative surface charges by extraction of lipids from deionized blue membranes should suffice to induce the blue-to-purple transition without addition of cations. After incubation of blue membrane with deionized CHAPS solutions overnight at room temperature, the absorbance maximum shifted from 603 nm to 561 nm, which corresponds to a complete transition from blue to purple and has been achieved without adding any cations. The absorbance at 630 nm as a function of CHAPS concentration is shown in Fig. 4. The transition is incomplete if the detergent concentration is below the critical micelle concentration (≈8 mM), which indicates that the color change is indeed due to extraction of lipids. This conclusion is further supported by the time course of the spectral change. The major part of the absorbance change required >10 min in 20 mM CHAPS and even longer times at concentrations much below the critical micelle concentration. Binding of a detergent molecule or a cation to a specific site would be a much faster process, and cations do indeed restore the purple color in less than a second (13, 31).

To rule out stronger binding of cations, and their retention in delipidated samples, the membrane preparations were checked by atomic absorption spectroscopy. Deionized blue preparations contained 0.1 Ca and 0.4 Mg atom per molecule of bR, and the total Ca plus Mg content was further reduced to 0.1 per bR by the lipid extraction, which returned their color to purple.

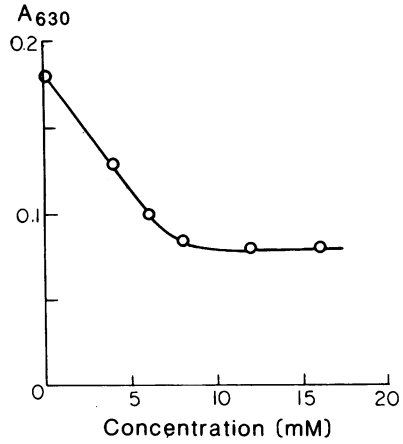

FIG. 4. Absorbance changes at λ = 630 nm of deionized blue membrane suspensions after incubation overnight in deionized CHAPS solutions of different concentrations at pH ≈ 5.

DISCUSSION

Though the different published techniques to produce blue and reconstituted purple membrane all entail the dissociation and rebinding of other cations, we have shown here that in delipidated and deionized membranes, changes in proton concentration alone are sufficient. The small absorbance change observed upon delipidation, which is similar to that seen with other detergents or lipid replacement, where the function of bR is apparently not substantially impaired (e.g., refs. 30, 32, 33), argues that the structure of the molecule is not altered significantly by delipidation. This is borne out by structural studies on partially delipidated membranes (6). We therefore assume that we are observing the same transition seen in native pm.

If the structure of bR in the lipid-depleted membrane is not significantly changed, the main difference to native pm is a large decrease in negative surface charge density and this can explain the observed phenomena at least qualitatively. We note that the simple Gouy–Chapman formalism to calculate the surface pH may not be applied to pm in the absence of salt because dissociation of surface groups will not be complete and a more complex treatment will be required; however, a rough estimate should suffice for our purposes. In the pH range where the purple-to-blue transition occurs, the net surface charge will be dominated by the acidic groups of the lipids and in the absence of other cations it has to be balanced by protons. Ten sulfonyl and phosphate groups per bR in a 100-Å-thick water layer is equivalent to a 60 mM solution of a strong acid. Even if we consider only the phospholipids, this means that the pH at the surface is below 2.0. Cations added to the suspension will concentrate near the surface and displace protons. Vice versa, acidification will displace other cations from the surface and further decrease the surface pH, even though it is substantially below the medium pH. In a titration experiment, a high binding constant will be measured even in the case of unspecific electrostatic binding and cannot be distinguished from the stronger chemical binding to the phosphate groups, for example. If more than one cation per bR is required to increase the surface pH sufficiently for the blue-to-purple transition to occur, several apparent binding sites with decreasing affinity will be observed. The possibility, that surface charges are involved in the color change, has been acknowledged in several publications (e.g., refs. 12, 13, 20, 26, 34), and our results show that their effect on the surface pH suffices to cause the transition.

Quantitatively, the pK of 1.4 observed for the transition in lipid-depleted membrane agrees reasonably well with the pK found in native membranes when the surface charge was screened by a cationic polymer (34), and the curves for the dependence of pK on the Ca^{2+} and Na^+ concentrations (13), when extrapolated, intersect at pH 1.5. The physical meaning of pK 1.4 and its connection with the mechanism of color change is not clear. It could reflect titration of specific phospholipids retained in the delipidated pm since the pK of glycerophosphate is around 1.4 or it could be a group on the protein shifted far from its normal pK. Most published data on the purple-to-blue transition (but not most conclusions) are consistent with the concept given here. Two exceptions are the observations that monomeric, lipid-depleted bR in micelles of lauryl sucrose show the same pK of ≈3.1 as washed, native membrane (35) and that removal of the C-terminal amino acids by papain shifts the pK for the transition to somewhat higher pH (13). In the former case, the extent of delipidation was not documented. In the case of

papain-treated bR, which removes four carboxyl groups from the C terminus, the positive charges of two remaining arginine residues may now prevail and be accessible.

That changes in the screening of surface charges are sufficient to cause the purple-to-blue transition does not mean that specific protein binding sites for cations do not exist, and the extent to which they affect the surface charge may also affect the purple-to-blue transition. Differences in the affinity constants for different cations (ref. 21; K.-D. Kohl, M. Engelhard, and B. Hess, personal communication) and effects of lanthanide ions on the photoreaction cycle kinetics and proton-pumping function of pm (14, 36) are arguments for the existence of such sites. However, the data available also indicate that the effects on the function of bR can be separated from the effects on the color transition. The triphenyltin cation, which strongly affects the bR photocycle, also shows an apparent higher binding affinity in titration of the blue-to-purple transition than equivalent single metal cations (M. K. Mathew, D. B. Bivin, S. L. Helgerson, and W.S., unpublished data), and defined binding sites for Pb on the protein have also been found in diffraction studies (25).

The purple-to-blue transition may or may not be caused by protonation of one or a few specific groups in the protein. In either case, the effect on the chromophore is probably indirect and not due to titration of a counterion to the retinal Schiff base. If the latter were the case, formation of the acid purple form at still lower pH would imply either protonation of a second group near the ring (12) or, in view of the anion effect on acid purple formation, substitution of Cl^- for the first protonated counterion (11). Neither explanation is compatible with the restoration of the resonance Raman spectrum, to an extent that it is essentially the same as at neutral pH (16) and placement of a chloride ion in the exact place of the native counterion, presumably a carboxylate group, is difficult to rationalize. We prefer the view that the purple-to-blue transition is a transition between two preferred conformational states of the protein, which depend mainly on solvent and solute interactions at its surfaces and which differ only in the arrangement of the charges around the chromophore and not in their number. Titration through the blue-to-acid purple transition in this view would restore more or less accurately the native arrangement, depending on the anion species, which necessarily is present in molar concentrations.

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