MECHANISM AND ROLE OF DIVALENT CATION BINDING OF BACTERIORHODOPSIN

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ABSTRACT Several observations have already suggested that the carboxyl groups are involved in the association of divalent cations with bacteriorhodopsin (Chang et al., 1985). Here we show that at least part of the protons released from deionized purple membrane ('blue membrane') samples when salt is added are from carboxyl groups. We find that the apparent *pK* of magnesium binding to purple membrane in the presence of 0.5 mM buffer is 5.85. We suggest this is the *pK* of the carboxyl groups shifted from their usual *pK* because of the proton concentrating effect of the large negative surface potential of the purple membrane. Divalent cations may interact with negatively charged sites on the surface of purple membrane through the surface potential and/or through binding either by individual ligands or by conformation-dependent chelation. We find that divalent cations can be released from purple membrane by raising the temperature. Moreover, purple membrane binds only about half as many divalent cations after bleaching. Neither of these operations is expected to decrease the surface potential and thus these experiments suggest that some specific conformation in purple membrane is essential for the binding of a substantial fraction of the divalent cations. Divalent cations in purple membrane can be replaced by monovalent, (Na^+ and K^+), or trivalent, (La^{+++}) cations. Flash photolysis measurements show that the amplitude of the photointermediate, O, is affected by the replacement of the divalent cations by other ions, especially by La^{+++}. The kinetics of the M photointermediate and light-induced H^+ uptake are not affected by Na^+ and K^+, but they are drastically lengthened by La^{+++} substitution, especially at alkaline pHs. We suggest that the surface charge density and thus the surface potential is controlled by divalent cation binding. Removal of the cations (to make deionized blue membrane) or replacement of them (e.g. La^{+++}-purple membrane) changes the surface potential and hence the proton concentration near the membrane surface. An increase in local proton concentration could cause the protonation of critical carboxyl groups, for example the counter-ion to the protonated Schiff's base, causing the red shift associated with the formation of both deionized and acid blue membrane. Similar explanations based on regulation of the surface proton concentration can explain many other effects associated with the association of different cations with bacteriorhodopsin.

INTRODUCTION

Bacteriorhodopsin (bR) is the only protein in the purple membrane of *Halobacterium halobium*. When light is absorbed by its retinal chromophore, it undergoes a photocycle during which protons are translocated across the cell membrane. This leads to a proton gradient that is used by the bacteria for the synthesis of ATP (see reviews in Stoeckenius et al., 1979; Ebrey, 1982).

Purple membrane binds ~4 moles of Ca^{++} and Mg^{++} per mole of bR at ~pH 6 (Chang et al., 1985). Ca^{++} and Mg^{++} are bound quite tightly, but they can be partially or completely removed by treatment with high concentrations of NaCl or KCl, low pH, the divalent cation chelator EDTA, or by reacting the membrane carboxyl groups with 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTSA). After Ca^{++} and Mg^{++} are removed from their binding sites, bacteriorhodopsin loses its purple color and turns blue (λ_{max} ~603 nm, the "blue membrane") (Chang et al., 1985). Blue membrane has a different set of photochemical transformations than purple membrane, indicating that Ca^{++} and Mg^{++} may play an important role in controlling the photochemistry and proton pumping of bacteriorhodopsin. The low pH and ANTSA methods of preparing blue membrane suggest that carboxyl groups are involved in the binding of Ca^{++} and Mg^{++} to purple membrane.

Divalent cations may interact with negatively charged sites on the surface of purple membrane through the surface potential (absorbed in the Gouy-Chapman diffuse double layer, see review in McLaughlin, 1977) and they may bind to the membrane either in association with random negative groups or via a specific conformation of coordination ligands (chelation) like those found in the binding of Ca^{++} to calmodulin (see review in Klee and Vanaman, 1982). Purple membrane contains an excess of negatively charged groups including carboxyls and lipid phosphate groups and thus will be expected to have a significant surface potential. For binding, the oxygen atoms are probably the most important component of the divalent cation binding sites, and these may be from carboxyls, as well as carbonyl or alcohol groups of the protein or phosphate or sulfate groups from acidic lipids.

Because of the surface potential, the surface concentration of cations (including protons) will be related to the bulk concentration by $C_{surface} = C_{bulk} \exp(-Z_i F\Psi_o/RT)$ where Ψ_o is the surface potential, Z_i is the valence of the ith ion, and the other symbols have their usual meanings.

METHODS AND MATERIALS

Preparation of Purple Membrane and Various Types of Blue Membranes

Purple membrane was prepared from *Halobacterium halobium* strain S-9 cells according to the method of Becher and Cassim (1975), except that the DNAase treatment was omitted. EDTA-treated, papain/EDTA-treated, and ANTSA-treated blue membranes were prepared as described previously (Chang, et al., 1985).

Preparation of Bleached Membrane

Bleached membrane was prepared from purple membrane by the method of Tokunaga and Ebrey (1978), except the pH was adjusted to 9.5 and 0.1 M NaCl was added. Residual retinaloxime in the bleached membrane was removed by repetitive washing with hexane. After evaporating the hexane, the bleached membrane was suspended in distilled water.

Modification of Purple Membrane

$MgCl_2$-treated purple membrane was prepared by first incubating purple membrane sheets for 20 min with 5 mM $MgCl_2$ plus 0.5 mM of the pH buffer used. The buffers used were citrate, pH 5.0–5.5; Pipes, 5.8–6.0; Mops, pH 6.2–7.0; Tris, pH 7.3–9.5. The membrane suspensions were washed (20 K, 35 min) six times with the same buffer, and then resuspended in the buffer.

K^+ or Na^+-purple membrane was prepared by adding ultrapure KCl or NaCl to a blue membrane sample. $LaCl_3$-treated purple membrane was prepared after purple membrane sheets were incorporated into 7.5% polyacrylamide gels (Mowery et al., 1979). The gels were immersed in distilled water for three days with frequent changes then incubated under nitrogen in 5mM $LaCl_3$ buffered at various pHs for one day to allow the displacement of Ca^{++} and Mg^{++} by La^{+++} (Chang et al., 1985). The pH buffers (50 μM) used in the kinetic measurements were: Tris, pH 7.75–8.75; Mops, 7.0–7.5; Pipes, 6.0–6.75 and Citrate, 5.5–5.75.

Atomic Absorption Spectrophotometry

A Perkin-Elmer Model 305 atomic absorption spectrophotometer equipped with either a Ca^{++} or Mg^{++} cathode tube was used to determine the concentration of these cations.

Flash Photolysis

A single-beam kinetic spectrometer was used to measure the M and O photocycle intermediates on the millisecond time scale (Govindjee et al., 1980). The light-induced absorbance changes were initiated with light of wavelengths above 540 nm obtained by placing a Corning CS 3-67 glass cut-off filter in front of a Xenon flash lamp (<6 μs pulse half-width). The light-induced absorbance changes for the large range of times shown in Fig. 6 were monitored with the flash photolysis system described in Austin et al. (1976). A frequency-doubled 30 ns Q-switched Nd glass laser (530 nm) was used to excite the samples. The photomultiplier signal was digitized continuously from 2 μs to 100 seconds after the laser flash with a logarithmic time-base digitizer.

The release and uptake of protons from purple membrane at pH ~7.8 were monitored by measuring the absorbance changes of the pH indicator, 8-hydroxycoumarin (umbelliferone) (Lozier et al., 1976) using the same type of kinetic spectrophotometer used to measure the photocycle intermediates (Govindjee et al., 1980).

RESULTS

Further Evidence for Carboxyl Participation in Divalent Cation Association

Proton release in the blue-purple transition: We reported briefly in Chang et al. (1985) that when cations are added to blue membrane, protons are released. Similar results have been reported by Kimura et al. (1984). Here we present our results in more detail.

A glass electrode was used to monitor the pH change during the addition of various cations to the blue membrane sample. Fig. 1 shows that the pH of the medium decreases when Mg^{++} or La^{+++} are added to the EDTA-treated, papain/EDTA-treated and ANTSA-treated blue membranes whose initial pH is ~4.6. In the EDTA-treated blue membrane sample, the pH decrease caused by La^{+++} addition is greater than for Mg^{++} addition. After correcting for the sample buffering capacity and membrane concentrations the number of protons released by the addition of 10 μM of Mg^{++} to EDTA-treated blue membrane was greater than for papain/EDTA-treated blue membrane, which in turn is much greater than for ANTSA-treated blue membranes. Slightly more protons are released when La^{+++} is added to the EDTA-treated compared with papain/EDTA-treated blue membranes (data not shown). We have examined blue membrane samples at other pH's and found that when salt is added, protons are always released if the initial bulk pH of the solution is >3.7.

The protons that are released from blue membrane samples upon salt addition could either be from the diffuse double layer or from protonated dissociable groups on the

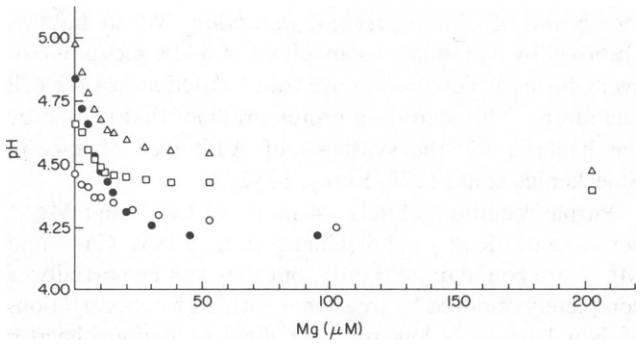

FIGURE 1 The pH change during the blue-purple transition when 2.5, 5, 7.5, 10, 12.5, 15, 20, 27.5, 53, 200, and 1,700 μM of $MgCl_2$ are added to EDTA-treated (△), papain/EDTA-treated (□) or ANTA-treated (○) blue membranes or when 2.5, 5, 7.5, 10, 12.5, 15, 20, 30, 45, and 95 μM of $LaCl_3$ are added to EDTA-treated blue (●) membranes. The absorbance at λ_{max} of EDTA-treated, papain/EDTA-treated, and ANTSA-treated blue membranes are 0.49, 0.52, and 0.58 respectively, temperature 20°C.

membrane, such as the carboxyl groups. The first explanation is probably incorrect because calculations by Masamoto et al. (1980) indicate that the number of protons in the double layer is far too small to account for the magnitude of the pH change that we see. For the second explanation, protons would be released from the dissociable groups if salt addition caused an increase in the degree of ionization. If the membrane is negatively charged and the pH is greater than the pI of the surface, the pH near that surface will be lower than the bulk pH. Addition of salt will decrease the magnitude of the surface potential and thus raise the local pH near the surface of the membrane. Groups (such as carboxyls) that were protonated at the low surface pH will then become ionized as the surface pH increases, leading to the appearance of protons in the bulk medium.

Our data provide some evidence that most of the protons initially released by cation addition are probably from protonated carboxyls. Carboxyls are the first groups to be protonated in purple membrane when the pH is reduced below ~6 and thus, are probably the groups to be deprotonated when cation addition lowers the surface potential. Moreover, ANTSA reacts with three to four carboxyl groups per bR (Chang et al., 1985) and each ANTSA introduced replaces one carboxyl with three SO_3^- (The pK of the SO_3^- group is much lower than that of the carboxyl.) Thus in the ANTSA sample, fewer carboxyls are available to release their protons if the surface pH increases due to a decrease in the surface potential.[1] The number of protons released per bacteriorhodopsin when Mg^{++} is initially added is clearly less in the ANTSA-treated blue membrane sample than in the EDTA-treated blue membrane samples, suggesting that the released protons are from the carboxyl groups. Additional evidence comes from the papain/EDTA-treated bR samples. Papain removes four carboxyl groups from the C-terminal tail of bacteriorhodopsin (Renthal et al., 1983). The smaller number of protons released from the papain-treated vs. the native membrane thus also suggests that carboxyl groups are involved in the Ca^{++} and Mg^{++} binding. In summary, these results suggest that some (if not all) carboxyl groups in the Ca^{++} and Mg^{++} binding sites become protonated when Ca^{++} and Mg^{++} are removed from purple membrane and become deprotonated when cations are added back.

pH Dependence of Mg^{++} Binding to Purple Membrane. It is difficult to compare the amount of Ca^{++} or Mg^{++} bound in samples from different purple membrane preparations because the relative amount of these cations bound to bR depends on the growth conditions of the bacteria. However, we previously found that all Ca^{++} could be displaced from the binding sites by incubation with 5 mM Mg^{++} and the apparent dissociation constants of Ca^{++} and Mg^{++} are similar (Chang et al., 1985). Hence we studied divalent cation binding to purple membrane by displacing all Ca^{++} with Mg^{++} at each pH to be tested.

Fig. 2 shows that the binding affinity of Mg^{++} to purple membrane is pH dependent, being much stronger at higher pH. The maximum number of Mg^{++} bound was ~6. The pK of the binding is ~5.85. As discussed below, this pK is that expected for the carboxyl groups of bR, if the membrane surface potential is about −100 mV.

Mechanism of Cation Association

The previous section has strengthened the suggestion that the carboxyl groups of bR are key determinants in divalent cation association. To further investigate the mechanism of divalent cation association (binding vs. Gouy-Chapman adsorption) we found several conditions where cation association by purple membrane decreased, probably without the surface charge density changing.

Cation Release by Heating. If no binding of Ca^{++} and Mg^{++} by specific charged groups in the purple membrane occurs, the interaction of Ca^{++} and Mg^{++} with the purple membrane can be described by simple Gouy-Chapman theory (see review by S. McLaughlin, 1977). For symmetrical salts, Gouy-Chapman theory describes the temperature dependence of the surface potential by:

$$\sigma = 2(RT\, E_r E_0)^{1/2} C_{bulk}^{1/2} \sinh \frac{ZF\Psi_0}{2RT},$$

where σ is the surface charge density, Ψ_0, Z, C_{bulk}, are as previously defined, E_r is the relative permittivity of the solution, and E_0 is the permittivity of a vacuum. Since the permittivity decreases slightly when the temperature

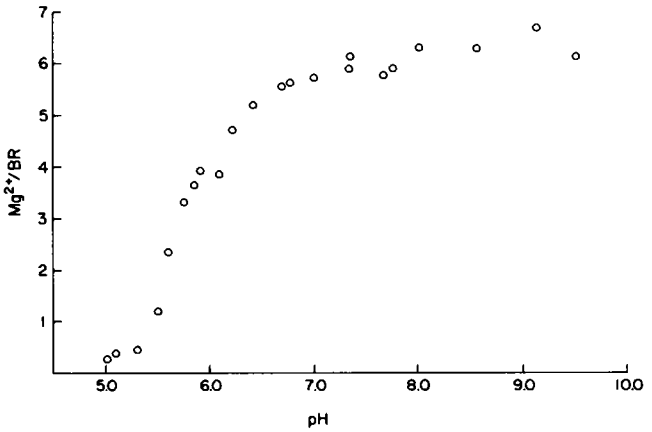

FIGURE 2 pH-dependent binding affinity of Mg^{++} to purple membrane. The buffers (0.5 mM) used are citrate: pH ≤ 5.0–5.3; Mops, pH 5.5–7.0; Tris, pH 7.3–9.5. The concentration of purple membrane used is ~10 μM.

[1]On the other hand, if the protons are released solely from the double layer, then the larger negative charge of the ANTSA membrane should lead to more protons in its double layer and thus more protons will be released when the surface potential is decreased by the cations.

increases, from the above equation the surface potential is predicted to increase slightly when the temperature is raised. Furthermore, since the *pK*'s of carboxyl groups are relatively insensitive to temperature, raising the temperature probably does not change the surface charge density. Therefore, the Gouy-Chapman association of Ca^{++} and Mg^{++} to purple membrane will increase slightly as the temperature increases.

The absorption spectra of purple membrane at several different temperatures are shown in Fig. 3. The appearance of some blue membrane, seen as an increase in absorbance around 630–640 nm, occurs as the temperature is raised from 25 to 70°C. When the temperature is lowered to 25°C, the purple species returns as the blue species disappears (not shown). However, a slight denaturation usually occurs after heating to higher temperatures (e.g., 90°C), as indicated by the absorbance increase at 380 nm. The difference spectra between curve 1 and curves 2, 3, or 4 are identical with the purple membrane/blue membrane difference spectrum, having a λ_{max} at 630 nm (Chang et al., 1985), indicating that the species formed upon heating is identical with the deionized blue species. Since the formation of the blue species is associated with the release of Ca^{++} and Mg^{++} from the membrane, this result indicates that divalent cation association is weaker at higher temperatures, in contrast to the prediction of Gouy-Chapman theory. However, elevated temperatures are expected to disturb the conformation of a protein. The above set of results suggest that a specific conformation of bacteriorhodopsin seems to be necessary for at least that part of divalent cation binding that determines the color of the membrane, and that this conformation is quite sensitive to temperature.

A second kind of observation was made with a sample in which a small amount of Ca^{++} or Mg^{++} was added to EDTA-treated or papain/EDTA-treated blue membrane, shifting the absorption maximum to 590 nm. The λ_{max} of this membrane sample shifts to 600 nm when the temperature is raised from 25°C to 35–40°C and this shift is reversible upon cooling (Fig. 4). This suggests that if only a small fraction of the bacteriorhodopsin in the purple membrane binds cations, the cations can be released more easily than if all the blue membrane has bound enough cations to turn purple. If enough Ca^{++} or Mg^{++} is added to convert all the blue membrane back to purple, then the sample behaves like native bacteriorhodopsin (i.e., it turns blue starting at ~60°C).

A third experiment involves the thermal sensitivity of the various preparations. After blue membrane is formed at high temperatures, it will eventually denature. If we start with pure blue membrane, we find it denatures very rapidly between 65–75°C (data not shown), while purple membrane is stable at these temperatures. When Ca^{++} or Mg^{++} is added to EDTA-treated blue membrane at this temperature, only a small amount of purple membrane is formed, which also shows that at 70°C that part of the membrane involved in determining color binds cations poorly, presumably because the conformation of the binding sites has changed.

Cation Release by Bleaching Purple Membrane

It seems likely that the number and approximate distribution of the charged amino acids in bleached purple membrane is similar to that in purple membrane. First, although bacteriorhodopsin undergoes appreciable tertiary

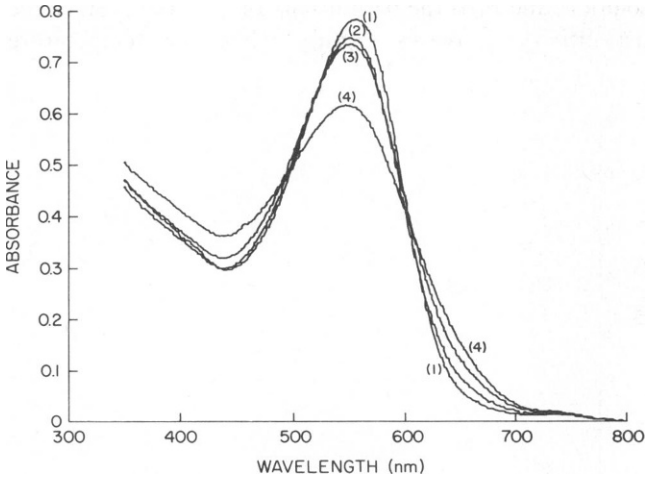

FIGURE 3 Absorption spectra of dark-adapted purple membrane in distilled water at different temperatures. Temperature set with a thermostated cuvette holder; the sample was equilibrated for 30 min at each temperature. (1) 25°C (2) 70°C (3) 80°C (4) 90°C. The absorption maximum at 558 nm decreases and the absorption at around 630 nm increases when temperature is raised, indicating that blue membrane is formed.

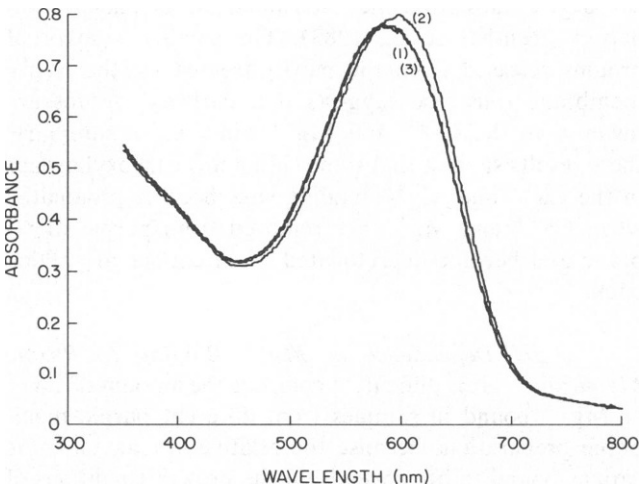

FIGURE 4 Absorption spectra of EDTA-treated blue membrane that has been partially converted to purple membrane by the addition of a small amount of $CaCl_2$, pH = 4.6. (1) 25°C (2) 40°C (3) re-cool to 25°C. The absorption maximum is ~583 nm for (1) and (3), and 600 nm for (2). This indicates that blue membrane is formed at much lower temperatures under these conditions.

structural changes on bleaching, no significant secondary structural change has been detected (Becher and Cassim, 1978). Furthermore, it has been reported that, upon bleaching, purple membrane does not change its surface potential (Ehrenberg and Meiri, 1983; Packer et al., 1984). Thus, if divalent cations associated with the purple membrane only via the electrostatic force of the double layer, bleached purple membrane should bind as many divalent cations as unbleached purple membrane.

Atomic absorption measurements shows that well-washed bleached membrane binds ~1–1.5 Ca^{++} and Mg^{++} per bR. For these experiments the bacteriorhodopsin concentration was determined from sonicated membrane preparations by assuming that the extinction coefficient at 280 nm is 126,000.

Is it possible that Ca^{++} or Mg^{++} is lost because of the manipulations involved in bleaching and that the binding ability of bleached membrane does not change? To remove this ambiguity, bleached membrane was incubated in 5 mM $CaCl_2$ or $MgCl_2$ at pH 5.8 to ensure that all available binding sites of the membrane were full. We found that the total binding ability of $CaCl_2$- or $MgCl_2$-treated samples is similar to unincubated samples (Ca or Mg per bR = 1.7). As another control, when bleached bacteriorhodopsin was regenerated with all-*trans* retinal it regained the ability to bind ~4.3 Mg^{++} per bR at pH = 6.0. The above results—that fewer divalent cations are bound by bleached membrane even though the surface potential does not seem to change—suggest that a specific conformation is essential for a substantial part of the Ca^{++} and Mg^{++} binding to purple membrane. This conformation is altered in the bleached membrane samples.

Effect of Monovalent and Trivalent Cations on the Photocycle and Proton Pumping of bR

K^+-Purple Membrane. The role of Ca^{++} and Mg^{++} in the function of bR can be studied by replacing them in the cation binding sites with monovalent or trivalent cations. EDTA-treated blue membrane is converted into purple membrane when K^+ is added (Chang et al., 1985). The decay kinetics of the M intermediate of this K^+-purple membrane are quite similar to those of native purple membrane in the presence of 150 mM KCl at pH 6.7 (Fig. 5 *a*). However, in the K^+-purple membrane the amplitude of the O intermediate (Fig. 5 *b*) is somewhat smaller than that of native purple membrane. Similar results are seen with Na^+ (data not shown). In contrast, the kinetics of the light-induced proton release and uptake by purple membrane are not affected by the displacement of Ca^{++}/Mg^{++} with K^+ (data not shown).

La^{+++}-purple membrane: Severe aggregation occurs when La^{+++} is added to EDTA-treated blue membrane. To overcome this problem, purple membrane sheets were first incorporated into 7.5% polyacrylamide gels and then

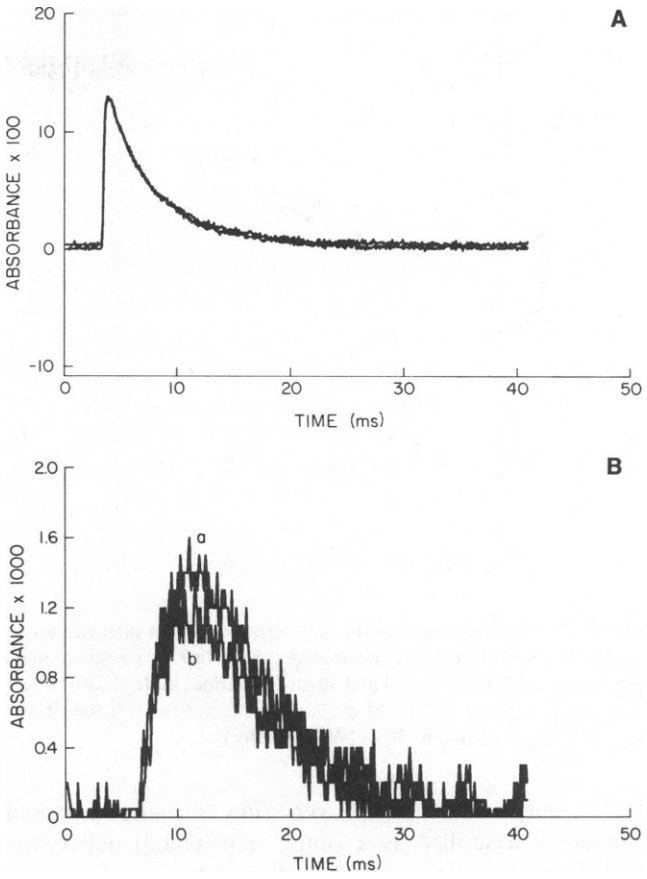

FIGURE 5 (*a*) The M intermediate of native purple membrane and Na^+-purple membrane. The absorbance at λ_{max} is 0.24 for both samples. $\lambda_{actinic} > 540$ nm. (*b*) The O intermediate of (*a*) native purple membrane and (*b*) Na^+-purple membrane; conditions as above.

incubated in La^{+++}/buffer which we have shown displaces all Ca^{++} and Mg^{++} from the binding sites (Chang et al., 1985). The decay time of the M intermediate of La^{+++}-purple membrane is pH dependent and is slower than that of native purple membrane when the pH is higher than 5.5 (Fig. 6). Since the decay of the M intermediate in the

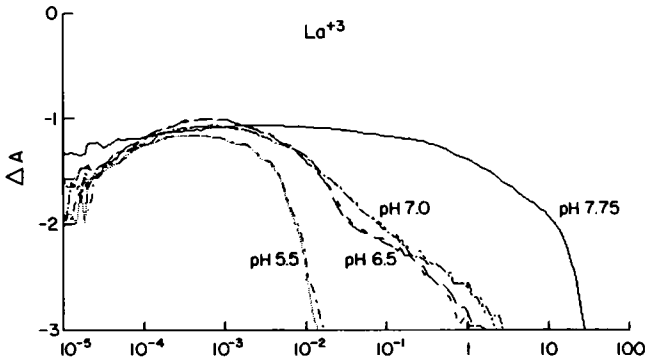

FIGURE 6 pH dependence of the kinetics of the M intermediate of La^{+++}-purple membrane sheets incorporated in 7.5% polyacrylamide gels suspended in 0.15 M KCl and 50 mM buffer. The buffers used are Citrate, 5.5; Pipes, 6.5; Mops, 7.0; Tris, 7.75. $\lambda_{actinic}$ = 530 nm (30 ns Nd glass laser).

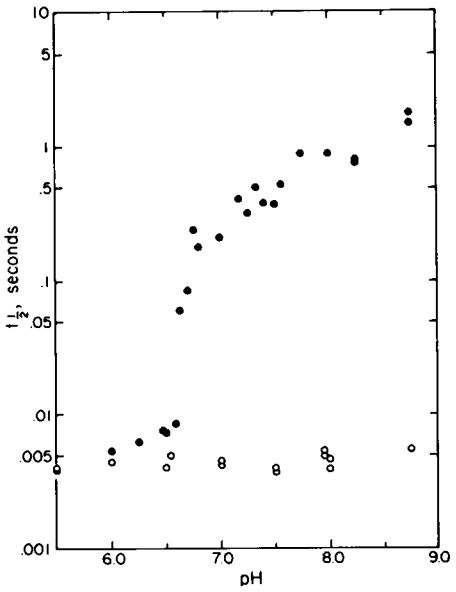

FIGURE 7 pH-dependence of the half-decay time of M intermediate in the La^{+++}-purple membrane sheets incorporated in 7.5% polyacrylamide gels suspended in 0.15 M KCl and 50 mM of various buffers. The buffers used are: Tris, pH 7.75–8.75; Mops, 7.0–7.5; Pipes, 6.0–6.75; and citrate, 5.5–5.75. $\lambda_{actinic}$ = 530 nm (30 ns Nd glass laser).

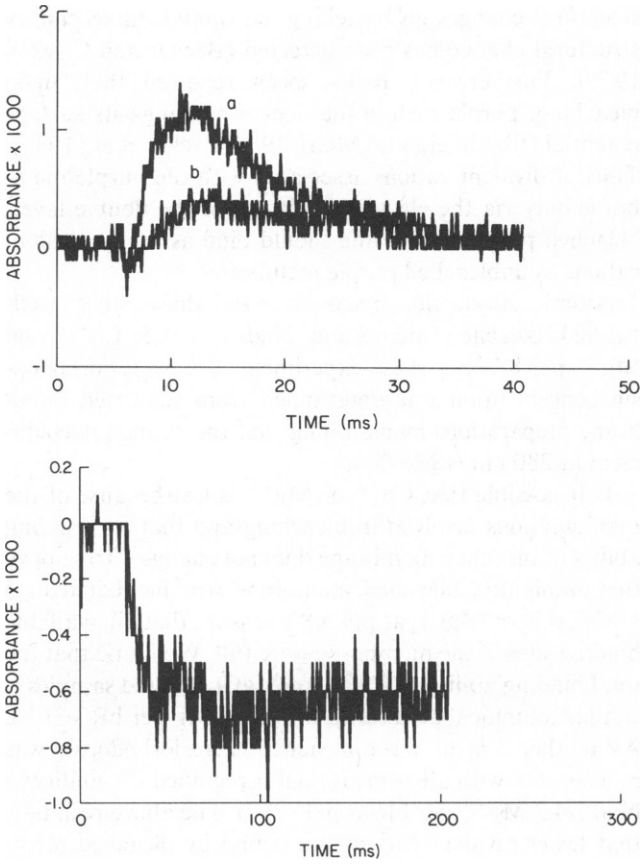

FIGURE 8 (*a*) Flash-induced absorbance change at 660 nm due to O intermediate in purple membrane sheets incorporated in 7.5% polyacrylamide gels suspended in 0.15 M KCl, 50 mM Tris buffer (pH 8.0) with (*b*) and without (*a*) 5 mM $LaCl_3$. $\lambda_{actinic}$ > 540 nm. OD_{570} = 0.22, temperature 20°C. (*b*) Flash-induced absorbance change of the pH-sensitive dye, 7-hydroxycoumarin at 400 nm in purple membrane sheets incorporated in 7.5% polyacrylamide gels. $\lambda_{actinic}$ > 540 nm, 0.15 M KCl 50 μM Tris buffer (pH 7.8). OD_{570} = 0.22. 5mM $LaCl_3$.

La^{+++}-purple membrane is very slow at higher pHs and cannot be described as a simple exponential decay, the half-decay time, $t_{1/2}$, instead of the rate constant, was plotted vs. pH (Fig. 7). The effect of 5 mM La^{+++} on the decay of the M intermediate is drastic between pH 6.5 and 7.5. At pH = 8, the total decrease in half-decay rate is almost 10^3 compared with native membrane.

The amplitude of the O intermediate is reduced by the presence of 5 mM La^{+++} and is almost zero at pH 8.0. The effect of La^{+++} on the amplitude of O intermediate also increases with pH (Fig. 8 *a*).

It has been reported that the M intermediate is closely related to proton release and uptake of bR. Fig. 8 *b* shows the kinetics of proton uptake of La^{+++}-purple membrane in the presence of 50 μM Tris buffer, pH 7.85. Both the release and especially the uptake of protons are greatly slowed down by La^{+++}, suggesting that the decay of the M intermediate is parallel to that of proton re-uptake.

DISCUSSION

Well-washed purple membrane binds approximately one Ca^{+++} and three to four Mg^{++} ions at the pH of our distilled water (~5.5–6). The divalent cations may interact with negatively charged amino acids and lipids by becoming part of the Gouy-Chapman double layer and/or bind to the membrane perhaps via chelation through oxygen atom coordination. A rather large surface potential should be expected for the C-terminal and perhaps the N-terminal side of bacteriorhodopsin. All of the current models for bacteriorhodopsin structure place 12 negatively charged and nine positively charged amino acids for a net balance of three negative charges (carboxyls) on the C-terminal side of bacteriorhodopsin. In addition, Henderson et al. (1978) find that all of the glycolipids (about five per bacteriorhodopsin, some of which carry one negative charge) are on the N-terminal side of bacteriorhodopsin while all the phospholipids are assumed to be on the C-terminal side. This corresponds to about five phospholipids per bR with each phospholipid carrying two to three negative charges near neutral pH. The net result is that the C-terminal side of bacteriorhodopsin is expected to have ~15–18 negative charges per bR or ~16 charges/1100 Å^2. At low ionic strengths this can give rise to a very large surface potential. From Gouy-Chapman theory (McLaughlin, 1977), in 1 mM monovalent cations and <1 μM divalent cations, the surface potential due to this surface charge density will be about −120 mV. This is two to four times greater than that estimated by Carmeli et al. (1980) and Ehrenberg et al. (1983) with samples having ~1 mM ionic strength. Probably binding of the divalent cations has reduced the effective surface charge of the

membrane; in addition the techniques employed so far may have underestimated the surface potential.

Role of Carboxyls

Several lines of evidence suggest that carboxyl groups in bacteriorhodopsin are involved in the Ca^{++}/Mg^{++} association. (*a*) If the carboxyl groups of bacteriorhodopsin are modified by ANTSA, purple membrane turns blue (Chang et al., 1985) indicating at least some loss of divalent cations, (*b*) When the pH of a membrane suspension is lowered to 2.0, divalent cations are released (Chang et al., 1985) and the purple membrane also turns blue ($pK = 2.9$ to 3.4, Mowery et al., 1979; Renthal and Wallace, 1980; Maeda et al., 1982). (*c*) The number of protons released from blue membrane when cations are added depends on the number of carboxyl groups present. Evidence we have given suggests carboxyls become deprotonated as cations associate with the membrane. (*d*) The binding of Mg^{++} to purple membrane is much stronger at high pH. Presumably the state of protonation of carboxyl groups in the binding site affects the affinity of divalent cations to the purple membrane. The apparent *pK* of this binding is ~5.85, ~1.5–2.0 units higher than expected for carboxyl groups. Since no other known membrane components have a pK higher than that of carboxyls until that of the lipid phosphate group at ca. 6.8 and tyrosine (*pK* 9–11), the *pK* at 5.85 is probably that of the carboxyl groups. The intrinsic *pK* of carboxyl groups, 4.0–4.4, would be expected to be shifted to an apparent *pK* of ~5.8 by the surface potential of about −90 to −120 mV (McLaughlin, 1977). This surface potential is in the range predicted from the calculations noted above. In addition to surface potential, the microenvironment of the carboxyl groups may also modify the apparent *pK*. For instance, if carboxyl groups are in a relatively more hydrophobic region, their *pK* will be higher, since the anionic form is destabilized. (Tanford, 1962; Parsons and Raftery, 1972).

Role of Specific Conformation of the Carboxyls in Binding of Cations

Elemental analyses show that bleached membrane binds only 1 to 1.5 Ca^{++}/Mg^{++} per protein although the number of surface charges in the bleached membrane is similar to that in the native membrane (Ehrenberg and Berezin, 1983). This suggests that some specific conformation in the purple membrane is probably required for at least part (roughly half) of the divalent cation binding to purple membrane. Further evidence for this conclusion comes from ANTSA-treated blue membrane. One molecule of ANTSA carries two more negative charges than the carboxyl it replaces and three to four molecules of ANTSA are bound per mole of bR (Govindjee et al. 1983). Therefore ANTSA treatment introduces six to eight more negative charges per bR and thus will increase the surface potential. However, after treatment, purple membrane loses Ca^{++} and Mg^{++}, suggesting that simple electrostatic interaction between divalent cations and surface charges on the membrane is not the major mechanism for much of the Ca^{++}/Mg^{++} association. Furthermore, when the temperature of purple membrane is raised above 70°C, blue membrane forms, i.e. cations are released, (Fig. 3), whereas the prediction of Gouy-Chapman theory is that binding of cations by surface potential should increase. Additional evidence for conformation dependent binding is that when Ca^{++} is added to the blue membrane suspension at 70°C, the λ_{max} of the absorption spectrum only blue-shifts slightly. However, when the temperature is lowered to 20°C, the λ_{max} shifts closer to that of the purple membrane, indicating that the association of the color determining cations to the membrane is quite sensitive to the temperature changes and thus probably involves a specific conformation of a binding site. Finally, the finding that different cations (such as La^{+++}) (Fig. 7) affect the photocycle of bR differently is contrary to the effect expected if the cations were just lying in the diffuse double layer. Therefore, we conclude that at least part of the Ca^{++} and Mg^{++} are bound to the purple membrane, that some specific conformation is essential for much of this binding, and that this conformation is sensitive to environmental changes.

In addition to the contribution of chelation to binding, the surface potential will greatly increase the apparent association constant of divalent cations to the membrane. If one takes the charge density we estimated above, 16 charges/1100 Å^2, then under the conditions that we form blue membrane, washing in distilled water (ionic strength <1 μM), the surface potential should be about −180 to −210 mV, which would increase the intrinsic binding of Ca^{++} and Mg^{++} to any groups on the surface by a factor of ~10^7. That is $K_{apparent} = 10^{-7} K_{intrinsic}$ when $\Psi_o = -240$ mV.

Consequences of Cation Removal on the Formation of the Blue Membrane

The results presented above indicate that a large fraction of the divalent cations associated with purple membrane at low ionic strengths are not just sitting in the double layer but actually have some affinity for negatively charged groups on the surface of the purple membrane. One interesting consequence of these bound divalent (and trivalent) cations is that they can have strong effects on the surface charge density of the purple membrane and hence on the surface potential. Monovalent cations have a much smaller effect on surface charge density. Through this effect on the surface potential, divalent cations can thus greatly decrease the local proton concentration near the membrane, leading to the deprotonation of groups that would not be deprotonated if the surface charge density was not decreased by their binding. Moreover, the initial surface charge density of native purple membrane can be

greatly increased as cryptic negative membrane sites are exposed when divalent cations are removed. This would lead to an increase in the surface potential and thus a lowering of the surface pH. Indeed this protonation will eventually be self limiting because as negatively charged groups are protonated, this will lead to a decrease in the surface charge density and, eventually, the surface potential.

It seems reasonable to suppose that the blue membrane formed from purple membrane by lowering the bulk pH is due to the protonation of some color regulating group, and that this low pH blue membrane is quite similar to the blue membrane formed by deionization. In acid blue membrane the group protonated is probably the counter ion of the protonated Schiff base as proposed by Honig (1978) and others (Fischer and Oesterhelt, 1979; Warshel and Ottolenghi, 1980). The main evidence for this is that the protonation of the counter-ion would give a bathochromic shift of the chromophore similar to that seen when blue membrane is formed from purple membrane. For deionized blue membrane, the increased surface potential proposed above due to removal of the divalent cations would lead to the protonation of the same color regulating anionic group by an increase in the local proton concentration. This would explain how both acid and deionized blue membranes have the same absorption and Raman spectra, (Kimura et al., 1984; Chang et al., 1985; Smith et al., 1985; Pande et al., 1985). One difference between the two types of blue membranes would be that in the deionized purple membrane, the local proton concentration may be increased on just one side of the membrane, perhaps explaining the difference in aggregation properties of the two types of blue membranes.

Thus the above proposal for the origin of the color of the blue membrane only indirectly couples the cations to the blue color. That is, the binding of divalent cations and the color of the purple/blue membrane are coupled only through the ability of the cations to control the protonation of the Schiff base counter-ion. Many of the observations that we have made here and elsewhere, as well as observations by others on both the deionized and acid blue membranes, can, indeed, be explained by the simple hypothesis outlined above.

Effect of Cations on Photochemistry and Proton Pumping

As noted above, when Ca^{++} and Mg^{++} are removed from their binding sites on the purple membrane, bacteriorhodopsin becomes blue, indicating that cations must interact with the chromophore directly or indirectly. We have found that removing the cations also drastically changes the photochemistry of bacteriorhodopsin (Chang et al., 1985 and this paper), suggesting Ca^{++} and Mg^{++} may play an important role in the function of bacteriorhodopsin. The effects of Ca^{++} and Mg^{++} were studied here by replacing them in purple membrane samples with monovalent and trivalent cations. Flash photolysis measurements showed that the amplitude of the O intermediate is greatly reduced when Ca^{++} and Mg^{++} are replaced by La^{+++}. The kinetics of the M intermediate are also significantly affected by La^{+++}. Therefore, the presence of Ca^{++} and Mg^{++} is necessary for bacteriorhodopsin to function properly.

Although the kinetics of light-induced proton release and uptake by bacteriorhodopsin are not affected by monovalent cations such as Na^{+} or K^{+}, they are significantly affected by La^{+++}, especially proton uptake. Because of limitations of our equipment, we could not measure light-induced pH changes at very long times after the flash; however, our data show that the kinetics of the light-induced pH change (Fig. 8 *b*) seems to be parallel those of the M intermediate (Fig. 6).

The qualitative explanation of the effects of cations on the photocycle can also be given in terms of cation binding modifying the surface charge density, and thus the surface potential, and local proton concentration. Thus to the first approximation, at the usual cation or buffer concentrations used, the surface potential will be quite small and the local proton concentrations will be approximately the same in the presence of either sodium or calcium. In contrast, the trivalent cation lanthanum displaces Ca^{++} and Mg^{++} from the membrane and so probably binds to the same negative membrane sites; La^{+++} binding would lead to a significant decrease in the surface charge density and it is indeed quite possible that the surface now becomes positively charged with La^{+++} bound to it. More particularly, it is interesting to speculate that there is a divalent cation binding site close to where protons are taken up by the membrane after the photocycle is initiated, leading to an approximately neutral complex when the ligands bind a divalent cation. When a trivalent cation is in this site, this would place a net positive charge in the region where a positively charged proton has to be taken up by the photocycling bacteriorhodopsin. This positive charge would be expected to lead to a much slower rate at which protons are taken up and thus a slower rate of decay for the M intermediate of La-purple membrane. The change in surface charge due to La^{+++} binding may also explain the decrease in the amount of the O intermediate in La-purple membrane. It is well known that the concentration of O decreases as the pH is raised. The binding of La^{+++} to bacteriorhodopsin will result in a significant increase in the local pH, and thus explaining the decrease in the amount of O.

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