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Characterization of the Cation Binding Sites of the Purple Membrane. Electron Spin Resonance and Flash Photolysis Studies†

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Received June 24, 1986; Revised Manuscript Received October 15, 1986

ABSTRACT: The binding of Mn^{2+} and La^{3+} to the blue membrane prepared by deionization of the *Halobacterium halobium* purple membrane has been studied by electron spin resonance (ESR) spectroscopy, visible absorption spectroscopy, and flash photolysis. ESR studies indicated that 10 Mn^{2+} binding sites are present per bacteriorhodopsin monomer. Five high- and medium-affinity sites, normally occupied by Ca^{2+} and Mg^{2+} in the purple membrane, as well as five low-affinity sites were found. Proteolysis and chemical modification experiments indicated that the low-affinity sites are located on the bacteriorhodopsin C-terminal segment, while the high- and medium-affinity sites involve other carboxyl groups of the protein. Competition experiments indicated that La^{3+} binds much more strongly than Mn^{2+} to these sites. Visible absorption spectroscopy and flash photolysis experiments indicated that binding of Mn^{2+} or La^{3+} regenerates both the purple color and formation of the M_{412} intermediate. The effect occurs progressively as cations bind to the high- and medium-affinity sites, bound La^{3+} being more effective than bound Mn^{2+}. In addition, La^{3+} was also shown to inhibit the M_{412} decay but at concentrations higher than those required for binding to divalent cation sites. It is suggested that divalent cations support both the purple color and proton-pumping activity by rendering less negative the surface potential of the purple membrane. This process may promote deprotonation of the counterion of the retinal Schiff base and possibly of other functional groups. On the other hand, it is proposed that the inhibitory effect of La^{3+} is mainly due to binding to a site distinct from those of divalent cations. This latter site may be involved in Schiff base reprotonation.

Bacteriorhodopsin (BR),[1] the retino protein of the *Halobacterium halobium* purple membrane, acts as a light-driven proton pump. Much is known about this protein, with regard to both its structure and its function [for reviews, see Stoeckenius and Bogomolni (1982), Dencher (1983), and Stoeckenius (1985)]. From the functional point of view, absorption of a photon initiates a photocycle during which trans–cis isomerization of the retinal chromophore occurs as well as sequential deprotonation and reprotonation of the retinal–protein Schiff base. Changes in the protonation of several amino acid residues appear to be also involved in the photocycle (Scherrer et al., 1981; Siebert et al., 1982; Hanamoto et al., 1984; Engelhard et al., 1985; Rothschild et al., 1986). How these features relate to the mechanism of active proton transport is the subject of current interest.

Recent studies have indicated that the purple membrane binds about 5 mol of Ca^{2+} and Mg^{2+}/mol of BR and that these divalent cations are essential for the proton transport function (Chang et al., 1985). Indeed, extraction of the cations by various procedures shifts the absorption maximum of light-adapted BR from 568 to 605 nm, yielding the so-called blue membrane (BR_{605}). The pigment thus formed has a severely perturbed photocycle with no deprotonated Schiff base intermediate and does not pump protons (Mowery et al., 1979; Kobayashi et al., 1983; Dupuis et al., 1985; Chang et al., 1985). The purple membrane (BR_{568}) can be regenerated by the addition of mono- or divalent cations with parallel recovery of the proton-pumping activity (Kimura et al., 1984; Chang et al., 1985, 1986; Dupuis et al., 1985). The trivalent cation La^{3+} also restores the purple color but, on the other hand, inhibits proton pumping by perturbing later stages of the photocycle (Drachev et al., 1984; Chang et al., 1986).

Proposals have been made to explain the requirement of cations for the purple color and for the formation of the Schiff base deprotonated M_{412} intermediate (Kimura et al., 1984; Dupuis et al., 1985; Chang et al., 1986). Presently, little is known concerning the nature and properties of the various cation binding sites of the purple membrane as well as their relative involvement in the proton-pumping activity. In this

† This work was supported by the Commissariat à l'Energie Atomique, the Centre National de la Recherche Scientifique (ATP 901 445), and the Comisión Asesora de Investigación Científica y Técnica (0729/85). M.D. was supported by an EMBO fellowship during her stay in Saclay.
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[1] Abbreviations: BR, bacteriorhodopsin; BR_{568}, purple form of bacteriorhodopsin; BR_{605}, blue form of bacteriorhodopsin; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; ESR, electron spin resonance.

0006-2960/87/0426-1179$01.50/0

paper, we present a detailed characterization of divalent and trivalent cation binding to blue and purple membranes using ESR spectroscopy of the paramagnetic cation Mn^{2+}. Besides providing data concerning the number of sites and their affinities, our experiments provide identification of at least part of the binding sites. We also study the relative influence of the various divalent and trivalent cation binding sites upon the BR photocycle monitored by flash photolysis. These data provide some clues to the mechanism by which cations influence the proton-pumping activity.

Materials and Methods

Membrane Preparations. The purple membrane was isolated from *H. halobium* strain S9 as described by Oesterhelt and Stoeckenius (1974). Removal of the C-terminal segment of BR was performed by papain proteolysis of purple membrane according to Liao et al. (1984) and checked by gel electrophoresis. Carboxyl modification of the purple membrane with EDC was performed for 20 h at 0 °C by using the pH 8 method of Renthal et al. (1979). Bleaching of the purple membrane was effected by its illumination in the presence of 0.5 M hydroxylamine, pH 7, for 4 h at 20 °C with a 150-W Intralux lamp equipped with yellow and heat filters. Blue or deionized samples were prepared from purple membrane suspensions by passage through a cation-exchange column (Dowex 50W). After deionization, membranes were resuspended in triple-deionized water. For cation binding studies, membranes were first adjusted to pH 5 with concentrated NaOH, and cations were added to the desired concentration from stock solutions. The pH was then readjusted to 5 or 7. Since partial aggregation of membrane sheets has been suggested (Ovchinnikov et al., 1986; Arrio et al., 1986), we tested whether this could hinder cation binding by studying the effect of sonication. In most cases, no effect was found in either ESR or flash photolysis experiments. Only in the case of samples containing La^{3+} at mole ratios higher than five ions per BR, which showed some precipitation, a brief sonication (i.e., 3 times 30 s using a 3-mm titanium probe) was necessary to obtain the maximum effect of the cation. It was checked separately that sonication by itself did not change the absorption spectrum and the photocycle kinetics of purple and blue membranes.

ESR Spectroscopy. ESR experiments were performed with a Bruker ESP 300 spectrometer operating at X band and equipped with a temperature control system. Spectra were run at 20 °C with a 40-mW microwave power and a 10-G modulation amplitude. Mn^{2+} binding to membranes was monitored from the decrease in intensity of its fourth ESR line. From samples at high protein concentration, it was determined that the ESR amplitude of the bound ion was 3–5% of that of an identical concentration of the free ion. This allowed us to neglect the ESR contribution of the bound ion in composite spectra only in cases where less than 50% of total Mn^{2+} was bound. When more binding occurred, spectral intensities were corrected for this contribution, which was determined from the spectra of the bound ion at high protein concentration. Data were analyzed in the form of Scatchard plots (Scatchard, 1949). When multiple classes of binding sites were present, binding constants and numbers of sites were obtained graphically by first evaluating the slope and abscissa intercept for the higher affinity class, then subtracting the regression line from data points, and finally iterating this procedure until all classes of sites had been processed (Cantor & Schimmel, 1980).

Absorption Spectroscopy. Visible absorption spectra were measured on a Perkin-Elmer 320 double-beam spectrophotometer interfaced with a microcomputer.

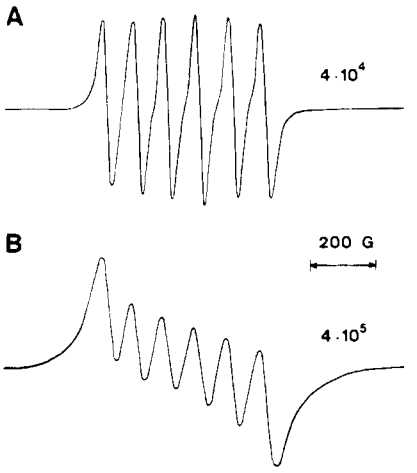

FIGURE 1: ESR spectra of 0.1 mM $MnCl_2$ in aqueous solution in the absence (A) or in the presence (B) of the blue membrane (Mn^{2+} to BR mole ratio 1:1). The spectrometer gain is indicated near each spectrum.

Flash Photolysis. Time-resolved absorption changes were measured with a single-beam absorption spectrometer. The actinic light was provided by two xenon flash lamps equipped with Wratten 12 orange high-pass filters. The measuring beam from a tungsten lamp was passed through a grating monochromator and focused on the thermostated (20 °C) sample quartz cuvette (1-cm path length). The transmitted light was then guided to a photomultiplier (EMI) after being filtered through a band-pass filter to remove scattered light. Signals from the photomultiplier were acquired on a Tracor-Northern signal averager through a differential amplifier. Usually, 10–40 transients were accumulated. Samples were light-adapted before each recording.

Results

ESR Spectra of Mn^{2+} Bound to Blue Membrane. The ESR spectrum of the Mn^{2+} ion in aqueous solution (Figure 1A) shows the well-resolved six-line pattern characteristic of the hexaaquo ion (Reed & Markham, 1984). In the presence of added blue membrane, a decrease of the ESR intensity of Mn^{2+} is observed that depends upon the BR concentration. This is due to binding of Mn^{2+} to the membrane, which decreases its rotational mobility resulting in a broadened ESR powder pattern (Reed & Markham, 1984). Figure 1B shows the Mn^{2+} ESR spectrum at the same concentration in the presence of the blue membrane (Mn^{2+} to BR mole ratio 1:1; note the 10-fold higher gain used). This spectrum represents only membrane-bound Mn^{2+} since a 2-fold dilution only decreases the amplitude proportionally with no change in line shape. The relatively sharp and symmetrical line pattern indicates that Mn^{2+} presents only minimal distortions from cubic symmetry in its membrane binding site(s) (i.e., small zero field splitting) as already found for other Mn^{2+} binding proteins (Villafranca et al., 1976). Similar spectra were obtained at Mn^{2+} to BR mole ratios up to 4, indicating that the corresponding binding sites have comparable symmetry.

ESR Measurements of Mn^{2+} Binding to Blue and Purple Membranes. At higher binding ratios and/or lower protein concentration, the ESR spectrum superimposed spectral features of both free and bound Mn^{2+}. This allowed us to perform Mn^{2+} binding studies on membranes [for an outline of the method, see Materials and Methods and, as an example, Kalbitzer et al. (1978)]. In the case of the blue membrane, we performed such studies at both pH 5 and pH 7.[2] Figure

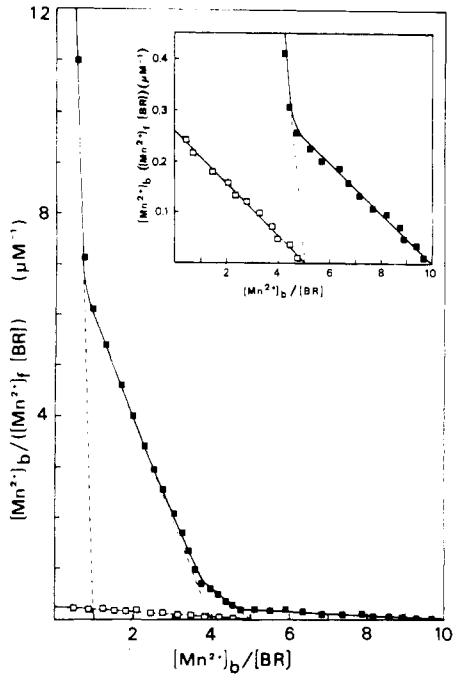

FIGURE 2: Scatchard plots of Mn^{2+} binding to the blue (■) and purple (□) membranes at pH 5 as measured by ESR. BR concentrations ranging from 0.5 μM to 200 μM were used. Inset: expanded plot of the low-affinity region.

2 shows the titration of Mn^{2+} binding to the blue membrane at pH 5 in the form of a Scatchard plot (Scatchard, 1949). Ten binding sites per BR molecule are found. These correspond to four binding constants that are indicated in Table I (for simplicity, the first to fifth sites will be termed "high- and medium-affinity sites" and the sixth to tenth sites "low-affinity sites"). At pH 7, a similar number of Mn^{2+} binding sites was found, with affinity constants increased 1 order of magnitude as compared to those at pH 5 (Table I).

Binding of Mn^{2+} was also tested on the purple membrane at both pH 5 and pH 7. As indicated in Figure 2 (inset) and Table I, only one class of five sites was found with an association constant corresponding to that of the low-affinity sites found in the blue membrane. This indicates that the five high- and medium-affinity sites evidenced above are those which physiologically bind Ca^{2+} and Mg^{2+} in the purple membrane. No evidence for displacement of the latter ions by Mn^{2+} was found.

Binding of Mn^{2+} to Modified Membranes. Much information concerning the nature of the divalent cation binding sites was obtained from similar Mn^{2+} binding ESR experiments on membranes modified by various treatments. First, the blue membrane lacking the C-terminal cytoplasmic segment of BR was prepared by deionization of papain-treated

Table I: Characteristics of the Mn^{2+} Binding Sites of Blue and Purple Membrane Preparations[a]

	high- and medium-affinity sites		low-affinity sites	
sample	K (μM^{-1})[b]	n[c]	K (μM^{-1})[b]	n[c]
blue membrane, pH 5	26	1		
	2.0	3	0.05	5
	0.60	1		
blue membrane, pH 7	50	1		
	20	3	0.21	5
	5.0	1		
purple membrane, pH 5			0.05	5
purple membrane, pH 7			0.20	5
papain-pretreated blue membrane, pH 5	20	1		
	1.7	3		
	0.51	1		
EDC-pretreated blue membrane, pH 5	26	1		
	2.0	1	0.12	5
	0.22	1		
blue membrane + 1 La^{3+}/BR, pH 5[d]	0.91	3	0.04	5
	0.40	1		
blue membrane + 2 La^{3+}/BR, pH 5[d]	1.0	2	0.04	5
	0.40	1		
blue membrane + 3 La^{3+}/BR, pH 5[d]	0.90	1	0.035	5
	0.30	1		
blue membrane + 4 La^{3+}/BR, pH 5[d]	0.21	1	0.04	5
blue membrane + 5 La^{3+}/BR, pH 5[d]			0.02	5

[a] Determined from Scatchard plots of Mn^{2+} binding after analysis of ESR spectra. [b] Association constant. [c] Number of sites corresponding to the value of K. [d] La^{3+} was added to the sample prior to Mn^{2+} binding study.

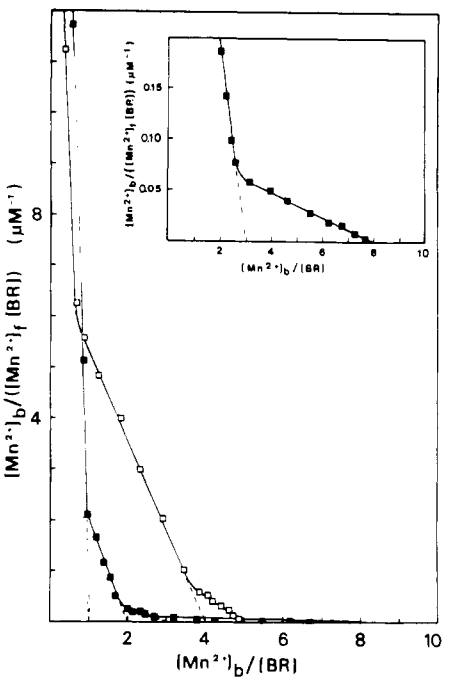

FIGURE 3: Scatchard plots of Mn^{2+} binding to the papain-pretreated (□) and EDC-pretreated (■) blue membranes at pH 5 as measured by ESR. Inset: expanded plot of the low-affinity region for the EDC-pretreated blue membrane.

purple membrane. As shown in Figure 3 and Table I, the five high- and medium-affinity binding sites were still observable in this preparation but with slightly reduced affinities. On the other hand, the five low-affinity binding sites could no longer be detected. This clearly indicates that the low-affinity divalent cation binding sites are located on the C-terminal cytoplasmic segment of BR.

Mn^{2+} binding was also measured on a deionized membrane prepared from purple membrane reacted with the carboxyl-modifying agent EDC (Renthal et al., 1979). As indicated

[2] To obtain the desired pH, NaOH had to be added, due first to the low pH (<4) of the blue membrane eluted from ion-exchange chromatography and second to the fact that Mn^{2+} displaces protons from the membrane. Since monovalent cations can also induce the blue to purple transition [though at concentrations 2 orders of magnitude higher than divalent cations, see Chang et al. (1985)], we tested whether Na^+ could not compete for Mn^{2+} in binding to the blue membrane. Na^+ concentrations of the order of those present in the pH 5 samples did not cause any detectable effect upon Mn^{2+} binding. Na^+ concentrations similar to those of the pH 7 samples slightly decreased Mn^{2+} binding only if Na^+ was added before Mn^{2+}. This indicates that divalent cation–monovalent cation exchange at the binding sites is a slow process. We thus adopted the procedure of adding Mn^{2+} to the sample at pH 5 and then increasing the pH to the desired value. Changes in Mn^{2+} binding elicited by pH variations were immediate, indicating that proton–divalent cation exchange at the binding sites is, on the other hand, a rapid process.

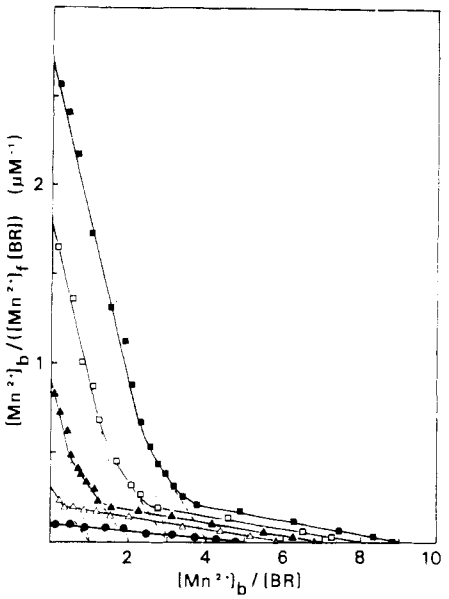

FIGURE 4: Scatchard plot of Mn^{2+} binding to the La^{3+}-pretreated blue membrane at pH 5 as measured by ESR. $LaCl_3$ to BR mole ratio: (■) 1, (□) 2, (▲) 3, (△) 4, and (●) 5.

(Figure 3 and Table I), two of the high- and medium-affinity Mn^{2+} binding sites were no longer detectable in this preparation. This suggested that these sites had been destroyed by the EDC treatment. However, a less likely explanation was that, after carboxyl modification, part of the endogenous divalent cations of the purple membrane becomes no longer removable by deionization (the visible absorption maximum of the modified membrane was shifted only to 576 nm after deionization, instead of 605 nm for the native membrane). To test this possibility, the EDC treatment was repeated on a blue membrane preparation to which Mn^{2+} had been added in order to occupy the five high- and medium-affinity sites (allowing it to turn purple, see below). After deionization, this preparation contained no residual Mn^{2+} as judged from ESR, indicating that removal of endogenous cations efficiently occurs. It also displayed Mn^{2+} binding characteristics similar to those depicted above. Thus, it can be concluded that BR carboxyl groups are structurally involved in at least some of the high- and medium-affinity divalent cation sites of purple membrane. These carboxyl groups seem to be distinct from those of the C-terminal segment.

Finally, Mn^{2+} binding to bleached and deionized membrane was found to be very weak by ESR (data not shown). Thus, it appears that high- or medium-affinity divalent cation sites no longer exist in bleached membranes.

ESR Measurement of La^{3+} Binding to Blue Membrane. The trivalent cation La^{3+} is known to regenerate the purple membrane from the blue membrane (Kimura et al., 1984; Chang et al., 1985) but inhibits the proton-pumping function (Drachev et al., 1984; Seigneuret & Rigaud, 1986). To study La^{3+} binding, we performed Mn^{2+} ESR binding experiments with blue membrane samples pretreated with various concentrations of La^{3+}. As shown in Figure 4 for each La^{3+} added per BR up to five, one Mn^{2+} binding site was found to disappear. Additionally, the affiniti s of the remaining binding sites for Mn^{2+} were found to decrease with increasing La^{3+} present (Table I). La^{3+} concentrations higher than five ions per BR resulted in residual affinities for Mn^{2+} that were too low to be measured. Similar results were obtained with the papain-pretreated blue membrane.

These experiments indicate that La^{3+} is a strong competitive inhibitor of Mn^{2+} binding to the blue membrane. Namely,

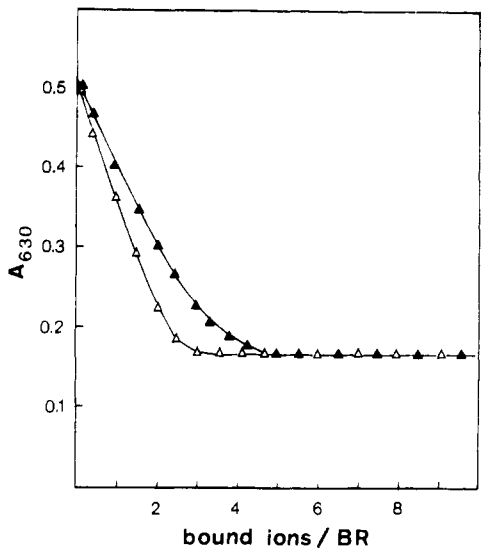

FIGURE 5: Dependence of the 630-nm absorbance of the blue membrane upon the amount of bound Mn^{2+} (▲) and La^{3+} (△) per BR (mol/mol) at pH 5. Bound cation was inferred from ESR experiments performed at the same BR concentration (10 μM).

each added La^{3+} occupies one Mn^{2+} binding site and cannot be displaced by Mn^{2+}. On the other hand, it was found that La^{3+} could displace membrane-bound Mn^{2+} from its binding sites (not shown).

Effect of Mn^{2+} and La^{3+} upon the BR Visible Absorption Spectrum. As already found by other authors (Kimura et al., 1984; Chang et al., 1985), both Mn^{2+} and La^{3+} made the blue membrane turn purple. Absorption spectra of dark-adapted membranes showed that this occurred as a progressive transformation of BR_{605} into BR_{558} (i.e., the dark-adapted form of BR_{568}) as ions were added (not shown). The presence of a single isosbestic point at 578 nm indicated that only these two species are involved. The range of concentration at which Mn^{2+} elicited the purple color was similar to that found with Ca^{2+} and Mg^{2+}. On the other hand, lesser concentrations of La^{3+} were necessary. This may be due to differences in binding affinity and/or to differences in efficiency of the bound ion. To distinguish between these two effects, the absorbance at 630 nm (where the $BR_{605} - BR_{558}$ difference spectrum shows a maximum) is plotted in Figure 5 as a function of the amount of *bound* Mn^{2+} and La^{3+}. In the case of Mn^{2+}, the number of bound ions was determined from the ESR spectrum of the sample. In the case of La^{3+}, the amount of bound ion is equal to the total ion present as judged from Mn^{2+}–La^{3+} competition ESR experiments. As can be seen, binding of five Mn^{2+} per BR is necessary to achieve the complete BR_{605} to BR_{558} transformation. This corresponds to complete filling of the high- and medium-affinity sites. On the other hand, La^{3+} appears to be more efficient, even on the basis of bound ions. Namely, binding of about three ions per BR is sufficient to completely regenerate BR_{558}.

Effect of Mn^{2+} upon M_{412} Amplitude and Decay. The effect of Mn^{2+} upon the BR photocycle was measured by flash photolysis at 420 nm in the millisecond time scale. The native purple membrane shows a pronounced absorption transient in this region corresponding to formation and decay of the unprotonated Schiff base M_{412} intermediate (Lozier et al., 1975).

In agreement with other reports (Mowery et al., 1979; Kobayashi et al., 1983; Chang et al., 1985), the blue membrane at pH 5 disclosed a very small flash-induced response at 420 nm. Addition of Mn^{2+}, even at ratios of less than one ion per BR promoted the appearance of a transient response of

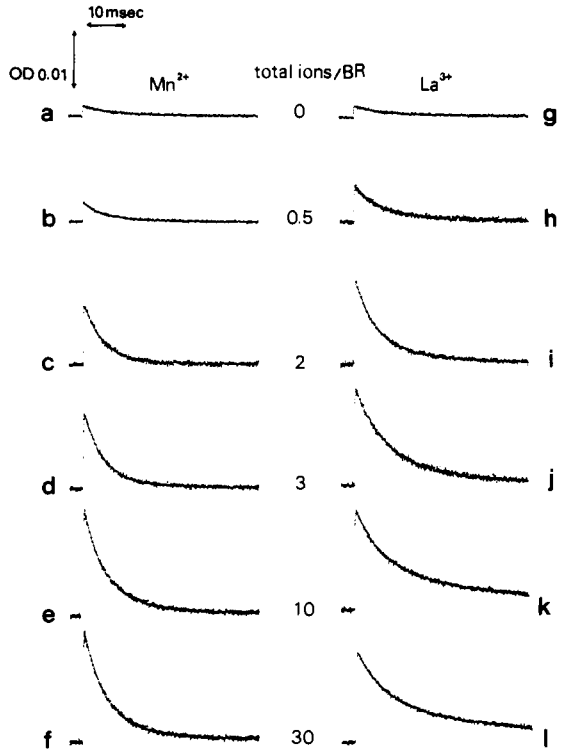

FIGURE 6: Effect of Mn^{2+} (a–f) and La^{3+} (g–l) upon the flash-induced 420-nm transient absorbance change of blue membrane (BR concentration, 10 μM) at pH 5. The total cation to BR mole ratios are indicated near each transient. The bound cation to BR mole ratios (inferred from ESR experiments) are (a) 0, (b) 0.5, (c) 1.91, (d) 2.7, (e) 5.4, (f) 10, (g) 0, (h) 0.5, (i) 2, (j) 3, and (k and l) unknown.

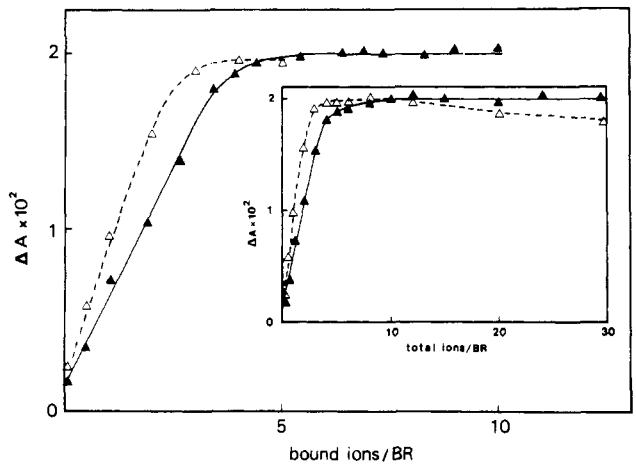

FIGURE 7: Dependence of the amplitude of the flash-induced 420-nm transient absorbance change of the blue membrane upon the amount of bound Mn^{2+} (▲, full line) and La^{3+} (△, dotted line) per BR (mol/mol) at pH 5. Bound cation was inferred from ESR experiments performed at the same BR concentration (10 μM). Inset: same plot as a function of the total amount of cation added per BR (mol/mol).

growing amplitude (Figure 6a–f). At all Mn^{2+} concentrations tested, the spectral maximum of this transient was determined to be in the 400–420-nm region (not shown). Thus, in all cases the species involved is identical with the M_{412} intermediate of native purple membrane. In Figure 7, the amplitude of the 420-nm response is plotted as a function of *bound* Mn^{2+} per BR determined by ESR (the plot vs. total Mn^{2+} is shown in the inset). The maximum regeneration of the M_{412} transient is obtained when five Mn^{2+} are bound per BR. The half-time for M_{412} decay did not depend significantly upon the Mn^{2+} concentration (see Figure 8). At ratios of five bound Mn^{2+} per BR, the M_{412} transient of regenerated purple membrane was similar to that of the native one with respect to both

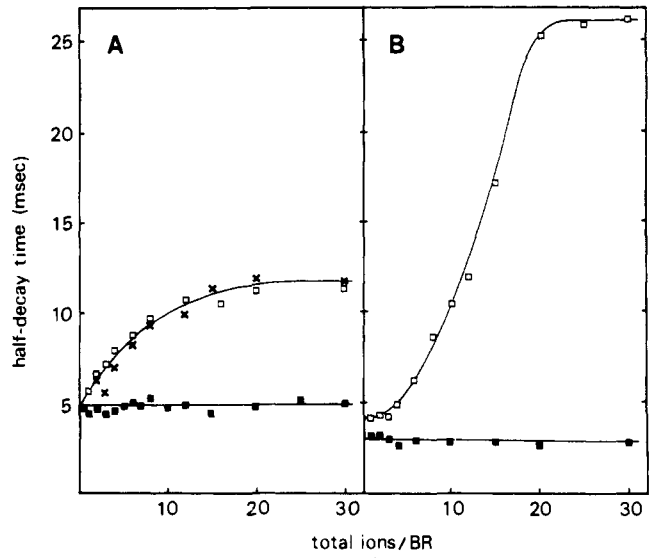

FIGURE 8: Dependence of the half-decay time of the flash-induced 420-nm transient absorbance change upon the total amount of cation added per BR (mol/mol) at pH 5 (A) and pH 7 (B) for (■) blue membrane plus Mn^{2+}, (□) blue membrane plus La^{3+}, and (×) papain-pretreated blue membrane plus La^{3+} (BR concentration, 10 μM).

amplitude and lifetime. Similar results were obtained with papain-pretreated blue membrane.

Thus, these experiments indicate that the activating effect of divalent cations upon M_{412} yield is related to their binding to the five high- and medium-affinity sites of purple membranes. Each bound divalent cation appears to be able to restore part of the M_{412}.

Effect of La^{3+} upon M_{412} Amplitude and Decay. The effect of La^{3+} upon the photocycle was more complex than that of Mn^{2+}. Binding of La^{3+} to blue membrane also promoted a strong increase in the amplitude of the M_{412} absorbance transient (Figure 6g–l). Bound La^{3+} was more effective than bound Mn^{2+} in that the maximum amplitude was obtained after binding of only about three ions per BR (Figure 7).

In addition, La^{3+} had another effect upon the M_{412} transient. Namely, addition of the trivalent cation promoted a progressive decrease of the M_{412} decay rate (Figure 6). The decay was no longer monoexponential with an increasing slow phase. In Figure 8, the apparent half-time for the decay is plotted as a function of the total amount of La^{3+} per BR added to blue membrane. As can be seen, La^{3+} appears to promote a much stronger decrease of the M_{412} decay rate at pH 7 than at pH 5. At both pH, the maximum effect is observed at about 20 La^{3+} per BR.[3] Since this concentration is much higher than that required to fill the five high- and medium-affinity cation sites, we investigated whether the inhibitory effect of La^{3+} upon M_{412} decay was related to its binding to the low-affinity C-terminal segment sites. However, as shown in Figure 8A, addition of La^{3+} to papain-pretreated blue membrane pro-

[3] Addition of more than five La^{3+} per BR elicited progressive precipitation of the membrane. A similar effect has been observed by Drachev et al. (1984). The precipitate could be cleared out by sonication and, up to 10 La^{3+} per BR, reappeared only some minutes later, allowing flash photolysis experiments to be performed on clear samples. However, it must be stressed that once the precipitate had reappeared, no significant changes in the absorption transient occurred, provided that sedimentation was avoided. Only at higher La^{3+} concentrations did the amplitude of the transient slightly decrease as a result of precipitation (see Figure 7, inset). It is unlikely that the La^{3+}-induced decrease of the M_{412} decay rate is related to precipitation since it can already be observed at La^{3+} to BR ratios at which precipitation does not occur. Furthermore, a similar inhibitory effect of La^{3+} has been observed for the purple membrane embedded in gels (Drachev et al., 1984; Chang et al., 1985, 1986).

moted a decrease of the M_{412} decay rate that was identical with that found for the unproteolyzed preparation.

Finally, it was tested whether high concentrations of divalent and monovalent cations could have a similar inhibitory effect upon M_{412} decay. Mn^{2+} at concentrations up to 10^4 ions per BR and Na^+ at concentrations up to 10^5 ions per BR (that is, respectively 100 mM and 1 M in our experimental conditions) had no decreasing effect upon the rate of M_{412} decay but rather a small accelerating effect (not shown).

Discussion

The first result of this study is that Mn^{2+} not only can substitute the Ca^{2+} and Mg^{2+} divalent cations physiologically bound to the purple membrane but also yields functionally active BR. Indeed, Mn^{2+} can restore the purple color in a concentration range similar to that found for Ca^{2+} and Mg^{2+}. The Mn^{2+}-substituted purple membrane discloses a M_{412} intermediate upon illumination that is of comparable yield and lifetime to that of the native one. Futhermore, we have found that reconstituted liposomes (Rigaud et al., 1983) prepared from Mn^{2+}-substituted purple membranes have light-induced proton uptake activities similar to those observed when native membranes are used (unpublished results). All these features indicate that Mn^{2+} can be used as a functional probe of divalent cation binding to BR-containing membranes.

In this regard, our ESR studies of Mn^{2+} binding clearly indicate that 10 divalent cation sites per BR molecule are present on blue membrane. Among these, the five high- and medium-affinity sites can be unambiguously identified as those that physiologically bind Ca^{2+} and Mg^{2+} in purple membrane and support both the purple color and the proton-pumping activity. Indeed, binding of Mn^{2+} to these five sites (starting from the blue membrane) allows the complete recovery of the BR_{568} species and of the M_{412} intermediate. Furthermore, on the native purple membrane, Mn^{2+} can no longer bind to these sites, suggesting that these are already occupied by other cations. The inability of Mn^{2+} to displace the endogenous Ca^{2+} and Mg^{2+} is striking, considering that all these divalent cations restore the purple color at similar concentrations. This may indicate that exchange of divalent cations at the binding sites is a slow process.

Apart from these high- and medium-affinity functional sites, Mn^{2+} binding ESR experiments allowed us to evidence five low-affinity sites that are accessible on both blue and purple membranes. Binding of Mn^{2+} to the latter sites appears to have no effect upon either the BR absorption spectrum or the M_{412} intermediate.

Our study also provides some clues to the structural nature of these cation binding sites and their location on the membrane. Many potential candidates as chemical groups involved in cation binding exist on the purple membrane. The fact that the affinities for Mn^{2+} increase with pH indicates that the sites originate at least in part from protonable negative groups. Experiments with papain-treated membranes indicate clearly that the five low-affinity sites are located on the C-terminal cytoplasmic segment of BR. Indeed, the latter is known to contain five negatively charged carboxyl residues (Ovchinnikov, 1982). The absence of functional involvement of these low-affinity sites is consistent with the idea that the C-terminal moiety of BR has no influence upon activity (Liao & Khorana, 1984; Ovchinnikov et al., 1986).

Concerning the five high- and medium-affinity functionally important sites, the observation that their number is reduced after EDC treatment clearly shows that carboxyl groups are directly involved. These carboxyls are distinct from those of the BR C-terminal segment (since all five sites are preserved after papain treatment) and may constitute binding sites together with noncharged protein groups, as found in soluble calcium-binding proteins. This does not preclude the possibility that lipids may also contribute to some of the sites. We have recently observed that purple and blue membranes have markedly different ^{31}P-NMR spectra, suggesting the involvement of lipid phosphate groups in cation binding (M. Roux, M. Seigneuret, and M. Duñach, unpublished data). Indeed, binding of Mn^{2+} to negative lipids has been shown to be very strong (Bergelson, 1978). In any case, the high- and medium-affinity binding appears to be critically dependent upon the protein conformation and the lipid–protein arrangement of the membrane, since it is completely abolished by bleaching. This treatment has been reported to promote modifications of the BR tertiary structure and of the lattice structure of purple membrane (Becher & Cassim, 1977; Hiraki et al., 1978).

An important point is to know on which side of the purple membrane the high- and medium-affinity sites are located. As already discussed by Chang et al. (1986), a localization on the cytoplasmic surface is more likely since most of the negatively charged groups that may provide strong binding for cations (i.e., protein carboxyls and phospholipid phosphates) are located on this surface. Moreover, we have shown here that removal of the BR C-terminal cytoplasmic segment has the effect of slightly but significantly decreasing the affinity of these sites for Mn^{2+}. This is in agreement with the finding of Kimura et al. (1984) that higher concentrations of divalent cations are necessary to restore the purple color for the papain-treated membrane than for the native one. Such data argue in favor of a cytoplasmic surface localization of the binding sites. The highly charged C-terminal peptide may induce a more negative charge density in the vicinity of this surface and thereby increase the affinity of cation binding to the sites.

This last point serves to illustrate the more general fact that the affinity of a site may depend not only upon its specific structure but also upon the charge density created by neighboring charged groups. In this instance, the differences in binding constants evidenced here for the various high- and medium-affinity sites (up to a factor of 50) may at least in part come from the fact that binding of each divalent cation to the membrane locally renders its surface potential less negative, thus decreasing the affinity of further binding. Consistently with this idea, prior binding of La^{3+}, which carries a higher positive charge, decreases even more the affinity of Mn^{2+} binding to the remaining sites. Mn^{2+} affinity constants in the absence and presence of La^{3+} suggest that binding to the first site influences the second to fourth sites, which on the other hand have no mutual influence. The fifth site is influenced by binding to one or several of the four first sites. This suggests that all sites are located on the same side of the membrane, since it would appear unlikely that two sites electrically interact if located on opposite sides. However, the interactions between sites could also be conformationally mediated and thus be transmembranous. ESR studies involving magnetic interactions between different paramagnetic cations could help to strictly define the relations between sites.

It has previously been reported that the endogenous divalent cations associated with the purple membrane are necessary for the proton-pumping function of BR since the deionized blue membrane does not display a light-induced M_{412} deprotonated intermediate (Chang et al., 1985). Our study confirms this view and, furthermore, indicates that all five bound divalent cations are functionally important. Indeed, binding of Mn^{2+}

to all five high- and medium-affinity sites is necessary to completely restore in parallel both the BR_{568} species and its M_{412} intermediate. There are some striking features concerning the way this regeneration takes place. First, it is only a two-state process since there is no spectroscopic or functional evidence for any intermediate state between BR_{605} and BR_{568}. Second, it is a continuous and monotonous process in that each amount of bound divalent cation, even at less than one per BR, restores part of the functional BR_{568} with its M_{412} intermediate. Thus, all sites are functionally equivalent and independent and need not be all occupied for functional BR_{568} to occur.

Such features are compelling in the evaluation of the mechanism by which divalent cations activate BR. Any mechanism by which binding of divalent cations changes the protein conformation from the inactive BR_{605} state to the BR_{568} state through conformational-dependent chelation is difficult to conciliate with our results. Indeed, considering that regeneration begins at less than one bound cation per BR but completes only at five bound cations per BR and that only two species are involved, such a mechanism would require that cations bind five to one BR molecules before binding to one another. This can only be achieved through either strong positive cooperativity between sites within one BR molecule or strong negative cooperativity between sites among different BR molecules, neither of which is observed in our Mn^{2+}-binding data (as mentioned above, a slight negative cooperativity may occur but is very limited since the second to fourth sites have an identical affinity).

Another explanation that fits more satisfactorily with our data is that the equilibrium between the inactive BR_{605} and the active BR_{568} states is controlled by the local membrane surface potential. The more negative surface potentials would favor BR_{605} and the less negative surface potentials BR_{568}. The cytoplasmic surface of the membrane may bear 10–20 excess negative charges per BR (Renthal & Cha, 1984; Chang et al., 1986). Thus, binding of up to five divalent cations is likely to drastically modify its surface potential. Under the framework of this model, the change toward less negative surface potential promoted by each bound cation shifts the BR_{605}–BR_{568} equilibrium to a certain amount toward BR_{568}. The model can account for the two-state continuous nature of the regeneration process (this without implying cooperative cation binding). Moreover, we have clearly shown that lesser amounts of bound La^{3+} than of bound Mn^{2+} are necessary to completely restore the BR_{568} species and the M_{412}. This does indicate that it is the total positive charge bound to the membrane (up to about 10 elementary charges), rather than the number of bound cations, that determines the amount of BR_{568} present. This bears out for a local surface potential controlled process. Of course, this interpretation does not preclude the possibility that cation chelation by the protein modifies locally its conformation. In fact, circular dichroism (Kimura et al., 1984), thermal denaturation (Chang et al., 1986), and proteolysis (M. Duñach, M. Seigneuret, J.-L. Rigaud, and E. Padros, unpublished results) data suggest that such conformational changes are taking place. However, as explained above, it is difficult to conceive that these changes are directly involved in the BR activation process, except possibly for the first site (indeed, we cannot exclude the possibility that the cation bound to this site exerts its influence through both conformational and surface potential effects). It is also possible that the change in surface potential associated with cation binding modifies the protein conformation.

A surface potential controlled purple–blue transition is also suggested by other data. It accounts for the fact that monovalent cations can restore the purple form at millimolar concentrations (Kimura et al., 1984; Chang et al., 1985). Monovalent cations can decrease the negative surface potential through a diffuse double-layer charge-screening effect (McLaughlin, 1977). More generally, it has been found that treatments that increase the negative charge of the membrane favor the blue form (Maeda et al., 1982; Padros et al., 1984) while those that decrease this negative charge favor the purple form (Oesterhelt & Stoeckenius, 1971; Bakker-Grunwald & Hess, 1981). That divalent cations might act through a surface potential effect has also been suggested by other authors (Kimura et al., 1984; Chang et al., 1986).

How can the surface potential control the color and the activity of BR? The early suggestion concerning the mechanism of induction of the blue membrane by acid was that protonation of the counterion of the retinal Schiff base would occur, thus changing the electronic environment of the chromophore (Mowery et al., 1979; Fisher & Oesterhelt, 1979). Such an hypothesis can easily be integrated into a surface potential model. Indeed, the more negative surface potential promoted by dissociation of divalent cations is likely to increase the apparent pK of the Schiff base counterion (by increasing the interfacial proton concentration and by destabilizing the deprotonated form) and may thus elicit its protonation. Reassociation of cations would have the reverse effect. In this instance, the suggestion that cations bind to the cytoplasmic surface is consistent with reports indicating that the retinal Schiff base is located closer to this surface (Ovchinnikov, 1982; Huang et al., 1982).

A presently unanswered question is why the BR_{605} species does not pump protons. A relevant suggestion by Dupuis et al. (1985) is that cations may provide a positive charge necessary to promote deprotonation of the Schiff base and of a tyrosine at the level of M_{412} formation. Alternatively, recent work (Siebert et al., 1982; Engelhart et al., 1985) suggests that the Schiff base proton is donated to a nearby carboxyl group. As invoked above in the case of the Schiff base counterion, it is possible that divalent cations stabilize the deprotonated form of this carboxyl group, allowing it to function as a proton acceptor (note that this carboxyl group could itself be the Schiff base counterion). Another explanation is that M_{412} formation involves electrogenic conformational changes of the protein that are influenced by the surface potential. However, recent data seem to indicate that very few conformational changes occur at this level of the photocycle (Nabedryk & Breton, 1986).

The trivalent cation La^{3+} has been shown to inhibit proton pumping by BR (Drachev et al., 1984; Seigneuret & Rigaud, 1986). With purple membrane embedded in gels and treated with high concentrations of La^{3+}, it was found that the M_{412} decay was considerably slowed down (Drachev et al., 1984; Chang et al., 1985, 1986). Since La^{3+} also displaces divalent cations from the purple membrane, it was suggested that the inhibitory effect was due to La^{3+} binding to sites normally occupied by Ca^{2+} and Mg^{2+}. Our experiments with La^{3+} were performed with blue membrane samples not embedded in gel (despite minor problems due to precipitation). This allowed us to use low La^{3+} concentrations and to discriminate between the different effects of the trivalent cation. ESR data indicate that La^{3+} does bind to the high- and medium-affinity sites much more strongly than divalent cations. Visible absorption and flash photolysis data show that this is accompanied by restoration of BR_{568} and M_{412} formation. Moreover, we confirm that La^{3+} also has another effect, namely, a decrease of the rate of M_{412} decay. However, it is clearly shown here

that the La^{3+} concentration necessary to yield the maximum inhibitory effect ($\sim$20 La^{3+} per BR) is much higher than that necessary for binding to the high- and medium-affinity sites (five La^{3+} per BR). We thus have to conclude that the inhibitory effect of La^{3+} is, at least in part, due to binding of the trivalent cation to one (or several) specific low-affinity site(s). This later class of site is distinct from the low-affinity C-terminal segment sites (since papain has no effect upon the inhibition) and is likely to be also located at the cytoplasmic surface on the basis of previous experiments (Drachev et al., 1984; Seigneuret & Rigaud, 1986).

The inhibitory effect appears to be specific for La^{3+} since mono- or divalent cations are ineffective in this regard. The La^{3+}-induced decrease of the M_{412} decay rate being stronger at higher pH, it is possible that the trivalent cation interferes with the process of Schiff base reprotonation (which seems to be associated with M_{412} decay). For example, since it is suggested that this reprotonation occurs by proton donation from a tyrosine (Scherrer et al., 1981), La^{3+} may stabilize the deprotonated tyrosinate either by direct binding or through a distance surface potential effect.

ACKNOWLEDGMENTS

We are indebted to Dr. H. Conjeaud with whom preliminary flash photolysis experiments were carried out. We thank Drs. P. Setif, A. W. Rutherford, and J. Farineau for providing us with many experimental facilities for this work. We acknowledge helpful discussions with Drs. A. Sanson and M. Sabés.

Registry No. Manganese, 7439-96-5; lanthanum, 7439-91-0; hydrogen ion, 12408-02-5.

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