Biochimica et Biophysica Acta, 547 (1979) 258—269

BBA 47711

REACTION OF THE PURPLE MEMBRANE WITH A CARBODIIMIDE

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(Received November 8th, 1978)

Key words: Purple membrane; Bacteriorhodopsin; Membrane modification; Proton pump; Carbodiimide; (Halobacterium halobium)

Summary

Purple membrane was reacted with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at pH 4.5 and 8.0. At pH 4.5, the reaction yields cross-linked bacteriorhodopsin. The cross-linking is inhibited by pretreatment of the membrane with papain, or by the presence of carbohydrazide or glycine ethyl ester in the reaction mixture. The product of the pH 8.0 reaction is not cross-linked, but it displays altered properties. Two measures of photochemical activity (light-induced change in proton binding ($\Delta\bar{h}$) and decay of photointermediate M) show changes indicative of slowed proton uptake. The $\Delta\bar{h}$ is increased by ethyl dimethylaminopropylcarbodiimide. This increase is unaffected by pretreatment of the membrane with papain, and it is not reversed by NH_2OH. However, the reaction is inhibited by millimolar concentrations of $CaCl_2$. The altered $\Delta\bar{h}$ is not apparent in detergent-solubilized membranes. Ethyl dimethylaminopropylcarbodiimide does not appear to cause a large alteration in the membrane surface charge, as measured by Ca^{2+} binding.

We conclude that (1) at acid pH, ethyl dimethylaminopropylcarbodiimide can be used for cross-linking or for attachment of specific probes to the C-terminal region of bacteriorhodopsin, and hence to the cytoplasmic side of the purple membrane, and (2) at alkaline pH, ethyl dimethylaminopropylcarbodiimide reacts at a different type of site and appears to inhibit the proton pump.

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Abbreviation: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

Introduction

The light-induced proton pump of the purple membrane from extremely halophilic bacteria provides an energy-transducing system for which much structural information is available (for a review, see Ref. 1). The low resolution crystal structure [2] and amino acid sequence analysis [3—5] have already revealed nearly enough information to construct a rough molecular model. The chemical mechanism of proton translocation will soon be elucidated by combining a molecular model with results of chemical and spectroscopic studies.

We have been studying the changes in proton binding ($\Delta\bar{h}$) to purple membranes illuminated with steady light. These changes appear to be related to the proton pump activity [6] and provide unique information about the role of amino acid side chains in the pump mechanism. We found some suggestive titration [6] and spectroscopic [7] evidence for participation of tyrosine in proton transfer reactions during the pump cycle. The following studies were initiated in an attempt to reversibly block tyrosine and measure the effects on proton pump activity. While we apparently did not succeed in modifying tyrosine, we have nevertheless found an interesting and useful reaction of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) with the purple membrane. At low pH, EDC permits highly specific chemical modification of the C-terminal region of bacteriorhodopsin. At alkaline pH the C-terminal region is unaffected, but the proton pump seems to be inhibited through reaction at a different site.

Materials and Methods

Membrane preparation. Purple membranes were isolated from *Halobacterium halobium* S9 or R1 by the procedure of Oesterhelt and Stoeckenius [8]. Stock solutions of approximately 10^{-4} M bacteriorhodopsin in deionized water were stored at 4°C in the dark. Bacteriorhodopsin concentration was measured, after preillumination, on a Beckman Acta Spectrophotometer at 568 nm, using an extinction coefficient of 63 000 M^{-1} [9].

Other materials. EDC was obtained from Calbiochem or Sigma. Papain, ovalbumin, ribonuclease A, and Triton X-100, were from Sigma. Carbohydrazide was obtained from Aldrich. Biorad electrophoresis reagents were used. Omnifluor, scintillation grade Triton X-100 and $^{45}CaCl_2$ were obtained from New England Nuclear. Other reagents and materials used in these experiments were reagent grade and obtained from standard suppliers.

pH measurements. Light-induced changes in proton binding ($\Delta\bar{h}$) were measured as described previously [6] except that the samples were not continuously flushed with nitrogen and were not stirred during the illumination periods. Light-induced pH changes (ΔpH) were measured at various pH. The buffering capacity B of the sample (in units of equivalents per mol of bacteriorhodopsin per pH) was determined from a titration curve. From these two quantities one may calculate, at a particular value of pH, that $\Delta\bar{h} = B\Delta\text{pH}$.

Reaction of purple membrane with EDC. In a typical experiment, a 0.5 ml sample of stock purple membrane was added to 2.5 ml of 0.05 M NaCl and the pH (about 6) was adjusted to 8.0 with about 10 μl of 0.02 N NaOH. The sample vial was tightly capped and placed on ice. Then a 0.4 M EDC solution

was prepared in deionized water. The pH of the EDC solution generally did not need adjustment (pH 7.8). The EDC solution was cooled on ice for about 5 min and 0.5 ml were added to the purple membrane solution to initiate the reaction. The reaction mixture was kept at 0°C in the dark, usually for 1 h. Final concentrations were: bacteriorhodopsin, $1.4 \cdot 10^{-5}$ M; EDC, 0.057 M; NaCl, 0.03 M. After the reaction, the sample was diluted to approximately 10 ml with ice-cold 0.05 M NaCl and centrifuged immediately at 20 000 rev./min in a Beckman JA-20 rotor in a J-21B centrifuge refrigerated to 4°C. After 30 min, the pellet was resuspended in about 10 ml of ice-cold 0.05 M NaCl and centrifuged again. The pellet was washed twice more, using 0.015 M NaCl. The final pellet was resuspended in 2.0 ml of deionized water and either used immediately or stored at 4°C in the dark.

The concentration of the EDC-reacted purple membrane was determined by measuring the absorbance at 568 nm. Because the absorbance spectrum was qualitatively identical to that of unreacted purple membrane, the same molar extinction coefficient was used. Samples reacted for times longer than 1 h were slightly turbid. No correction was made for the additional light scattering.

Samples reacted at pH 4.5 were prepared identically except that the initial pH of both the membrane and the EDC solution was adjusted with about 20 μl of 0.02 M HCl. The product of the low pH reaction was extensively aggregated (except after papain treatment). In order to measure pH changes, the samples were sonicated (three 1 s bursts at 50 W with a Branson Sonifier). Unless otherwise indicated, the EDC-reacted purple membrane preparations discussed below refer to the product of a 1 h reaction.

Electrophoresis. Dodecyl sulfate-polyacrylamide gels were run using 10% acrylamide according to the procedure of Weber and Osborn [10], except that mercaptoethanol was omitted from bacteriorhodopsin samples. Molecular weight markers used were ovalbumin and ribonuclease A. Stained gels were scanned with a Helena Laboratories Quick Scan.

Reaction of EDC-reacted purple membrane with hydroxylamine. A sample of EDC-reacted purple membrane was adjusted to pH 8.0 with Tris buffer to give 2.0 ml of $2 \cdot 10^{-5}$ M bacteriorhodopsin and 0.014 M Tris. A solution of 0.8 M NH_2OH in 0.05 M Tris (1.5 ml) was adjusted to pH 8.0 with 2 M NaOH (0.5 ml). Then 1.2 ml of the NH_2OH/Tris solution was added to the EDC-reacted purple membrane solution under dim light. The reaction mixture was kept in complete darkness at room temperature for 7 h. The membrane was then centrifuged and washed twice with 0.05 M NaCl and twice with 0.015 M NaCl, as described above. Care was taken to avoid exposure of the sample to light until after the second centrifugation.

Reaction of the purple membrane with papain. Papain cleavage was carried out according to the procedure of Arnon [11]. A solution of 0.05 mg/ml papain in deionized water (0.12 ml) was mixed with 0.2 ml of activation solution (0.05 M cysteine, 0.2 M EDTA, adjsted to pH 8.0) and added to a purple membrane pellet (containing about 60 nmol of bacteriorhodopsin) suspended in 1.0 ml of 0.05 M Tris, pH 8.0. The reaction mixture was incubated at 37°C for 30—90 min (depending on the desired extent of cleavage). After the reaction, the sample was diluted with about 10 ml of ice-cold 0.05 M NaCl and

centrifuged and washed as described for the EDC reaction above.

Calcium binding. Binding of Ca^{2+} to the purple membrane was measured by centrifugation of purple membrane in varying concentrations of $CaCl_2$. $^{45}Ca^{2+}$ was added prior to centrifugation, and the amount of free Ca^{2+} was estimated by measuring the remaining radioactivity in the supernatant. The concentrations were arranged so that the free Ca^{2+} was between 10% and 90% of the added Ca^{2+}. Samples of 1 ml usually containing 10^{-5} M bacteriorhodopsin and varying amounts of total $CaCl_2$ (from 0 to 10^{-4} M) were placed in 15 ml Corex tubes that had been washed with 0.01 M EDTA. The samples were centrifuged for 45 min in an SS-34 rotor in a Sorvall R2B centrifuge at 20 000 rev./min at 20°C. From the supernatants of each sample, 0.2 ml were withdrawn and placed in a glass vial containing 0.8 ml H_2O. To each vial, 14 ml of scintillation fluid was added (three parts toluene containing 5.33 g Omnifluor/l, and one part Triton X-100). Radioactive disintigrations were counted in a Beckman LS-150 liquid scintillation counter. Each tube contained approximately 0.02 μCi of $^{45}Ca^{2+}$. The free Ca^{2+} was calculated by comparing the counts in the supernatant to a similar sample containing no membrane. Bound Ca^{2+} was calculated by difference from total Ca^{2+} and expressed as $\bar{\nu}$ (mol Ca^{2+} bound/mol of bacteriorhodopsin).

Flash spectroscopy. Flash spectroscopy experiments were done at the Center for Fast Kinetics Research, University of Texas at Austin, using a procedure similar to that of Lozier and coworkers [12,13]. The actinic light source was a 10 mJ Quantaray DCR-1A Nd/YAG laser operated with single pulses of 7 ns width at half-height, doubled to a wavelength of 532 nm. The measuring beam was a 450 W xenon arc lamp with a 100 ms shutter, filtered through a Corning 7-59 filter and an Optics Technology, Inc. above 500 nm cut-off filter. The sample was placed in a 1 X 0.5 cm cell, with the short dimension in the measuring beam. The absorbance changes were measured at 400 nm with a Bausch and Lomb monochromator and a Hamamatsu 928 photomultiplier tube. The photomultiplier tube current was converted to digital form by a Biomation 8100 and stored in PDP 11T34 computer.

The membrane samples were 10^{-5} M in bacteriorhodopsin in deionized water. The pH was measured immediately before and after each experiment. The measurements at alkaline pH were adjusted to the final pH by adding 0.02 N NaOH with a syringe microburet.

Samples were irradiated with ten flashes of actinic light, and the absorbance changes were averaged. Ten measuring beam baselines were averaged for each sample and subtracted from the light-induced absorbance changes.

Results

Reaction of purple membrane with EDC at pH 8.0

Purple membrane was reacted in the dark for various times at pH 8.0, 0°C, with EDC. The light-induced change in proton binding of the product was measured as a function of pH. The results for the 1 h reaction product are shown in Fig. 1. The experimental points fall near the results for purple membrane in 3 M NaCl reported previously [6]. However, in contrast to the effect of high ionic strength on $\Delta\bar{h}$, the EDC effect is not reversed by washing the

membranes with low ionic strength solutions.

An ionic strength effect on $\Delta\bar{h}$ can still be observed in EDC-reacted purple membrane. The increased $\Delta\bar{h}$ of EDC-reacted membrane may be further increased by the addition of 3 M NaCl or 10 mM $CaCl_2$ (data not shown).

Reaction with EDC for longer periods of time produces larger increases of $\Delta\bar{h}$. However, most of the change occurs in the first hour. The value of $\Delta\bar{h}$ at pH 9.2 is shown as a function of time in Fig. 2.

The EDC reaction has no apparent effect on the retinal chromophore. Absorbance spectra before and after EDC treatment are superimposible.

Polyacrylamide gel electrophoresis of EDC-reacted purple membrane in dodecyl sulfate (Fig. 3C) shows that the pH 8.0 reaction product is not an intermolecular cross-link. Only bacteriorhodopsin monomers were observed in the product of the reaction at pH 8.0.

Reaction of EDC-reacted purple membrane with hydroxylamine

Purple membrane that had been reacted with EDC at pH 8.0 was treated with 0.22 M NH_2OH for 7 h in the dark. The membranes were washed free of NH_2OH and then $\Delta\bar{h}$ was measured as a function of pH. As shown in Fig. 4, increase in $\Delta\bar{h}$ induced by EDC is not reversed by NH_2OH. The neutralized NH_2OH solution was about 0.2 M in NaCl, and this ionic strength has been shown to inhibit accessibility of trypsin to a proteolytic cleavage site [4]. Thus, we also reacted EDC-treated membrane with the free base of NH_2OH (0.6 M at

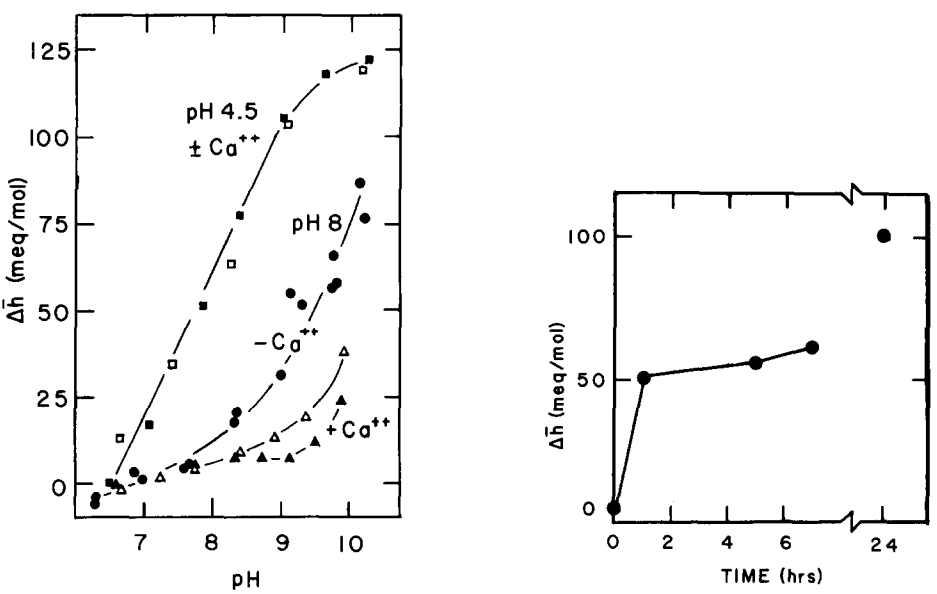

Fig. 1. Effect of pH and Ca^{2+} on steady-state proton release ($\Delta\bar{h}$) from purple membrane reacted for 1 h with EDC. □, purple membrane reacted with EDC at pH 4.5; ■, reaction at pH 4.5 in the presence of 10 mM $CaCl_2$; ●, reaction at pH 8.0; △, reaction at pH 8.0 in the presence of 3 mM $CaCl_2$; ▲, reaction at pH 8.0 in the presence of 10 mM $CaCl_2$. (See text.)

Fig. 2. Increase in steady-state proton release ($\Delta\bar{h}$) from purple membrane reacted for various times with EDC at pH 8.0. Values of $\Delta\bar{h}$ were measured at pH 9.2.

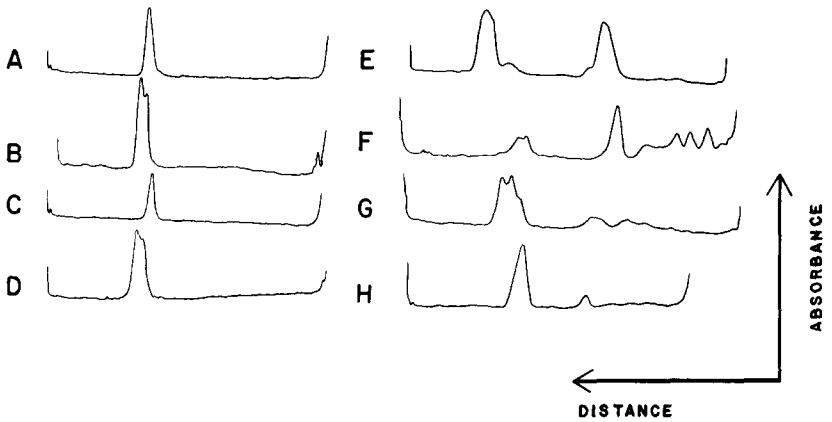

Fig. 3. Polyacrylamide gel electrophoresis patterns of purple membranes. Gels were 10% acrylamide and 0.1% SDS. Electrophoresis proceeded from right (gel top) to left (tracking dye). (A) Purple membrane. (B) Purple membrane reacted with papain for 30 min. (C) Purple membrane reacted with EDC at pH 8.0. (D) Papain-cleaved purple membrane subsequently reacted with EDC at pH 8.0. (E) Standards: RNAase (left) and ovalbumin (right). (F) Purple membrane reacted with EDC at pH 4.5. (G) Papain-cleaved purple membrane subsequently reacted with EDC at pH 4.5. (H) Purple membrane reacted with EDC at pH 4.5 in the presence of 2 M carbohydrazide. Scale: distance/arrow = 7 cm; absorbance: approximately normalized to maximum peaks.

approximately pH 8.5 for 12 h). Again, $\Delta\bar{h}$ before and after NH_2OH treatment of EDC-reacted purple membrane were identical. Thus, the EDC reaction site(s) at pH 8.0 is unlikely to be tyrosine, since an *O*-aryl isourea formed when the phenolic side chain of tyrosine reacts with carbodiimide would be easily removed by hydroxylamine.

Reaction of papain-cleaved purple membrane with EDC at pH 8.0

Ovchinnikov and coworkers have shown that a 17 amino acid fragment can be removed from the C-terminus of bacteriorhodopsin by brief incubation of purple membrane with papain [3,14]. We find that pretreatment of purple membrane with papain has no effect on the subsequent reaction with EDC at pH 8.0. Fig. 4 shows the light-induced pH changes in papain-cleaved purple membrane, before and after the reaction with EDC at pH 8. The results are indistinguishable from the corresponding experiments with intact purple membrane. The gels in Fig. 3B and D reveal that the papain cleavage of the membrane sample subsequently reacted with EDC was at least 60% complete. Thus, the C-terminal region is unlikely to be the site of EDC sensitivity at pH 8.0.

$\Delta\bar{h}$ of EDC-bacteriorhodopsin monomers in Triton X-100

Purple membranes that had been reacted with EDC at pH 8.0 were dissolved in Triton X-100 under conditions where only bacteriorhodopsin monomers were present [15]. The light-induced pH changes were measured, as shown in Fig. 5. The results indicate no major differences between EDC-reacted and unreacted membrane. Bacteriorhodopsin monomers in Triton X-100 show an increased $\Delta\bar{h}$ which may be interpreted in terms of slowed proton uptake [6]. Yet EDC does not produce any further increase in $\Delta\bar{h}$ above the Triton X-100

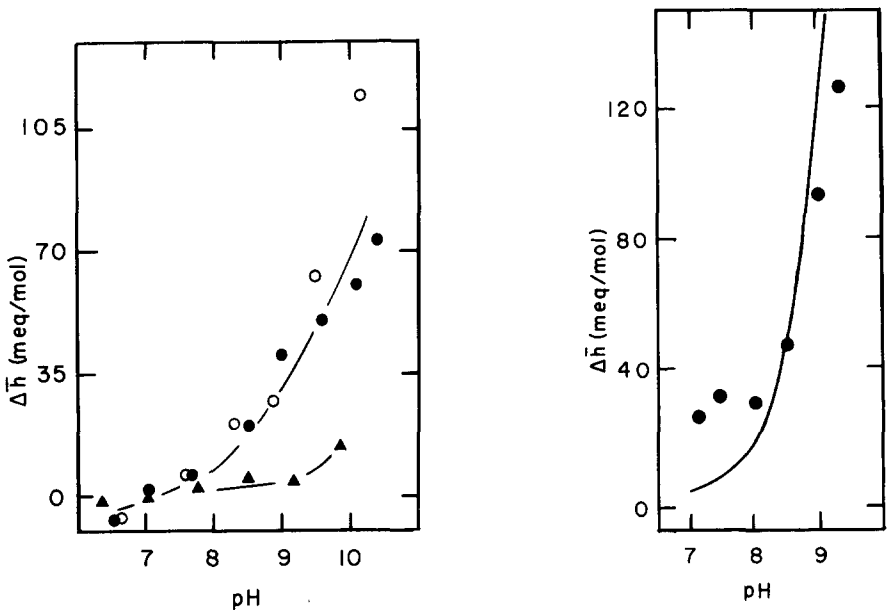

Fig. 4. Effect of NH_2OH and papain on the EDC-induced increase in $\Delta\bar{h}$. ○, EDC-reacted purple membrane (pH 8.0) that was subsequently treated with 0.22 M NH_2OH for 7 h; ●, papain-cleaved purple membrane that was subsequently treated with EDC at pH 8.0; ▲, papain-cleaved purple membranes. Se text for details.

Fig. 5. Effect of detergent solubillization on EDC-reacted purple membrane. Measurement of $\Delta\bar{h}$ after EDC-reacted purple membrane was dissolved in 0.4% Triton X-100 (conditions that produce bacteriorhodopsin monomers). Line is calculated as described previously [6] to fit experimental data for unreacted purple membranes. See text for details.

effect. A possible explanation of this result is that EDC might act on intact membranes to disrupt cooperative interactions between lattice-bound bacteriorhodopsin monomers. Triton X-100 alone would disrupt these interactions, and thus EDC would not be expected to have any additional effect.

Inhibition of EDC-induced increase in $\Delta\bar{h}$ by Ca^{2+}

The reaction of purple membrane with EDC at pH 8.0 is inhibited by the presence of 3 mM or 10 mM $CaCl_2$. Purple membrane was reacted with EDC in the presence of $CaCl_2$ and then washed free of Ca^{2+}. The subsequent measurement of light-induced pH changes shows (Fig. 1) that there has been only a small effect of EDC on $\Delta\bar{h}$. By itself, Ca^{2+} causes an increase in $\Delta\bar{h}$. The presence of 10 mM $CaCl_2$ has a similar effect to that previously reported for 3 M NaCl [6]. Thus, both Ca^{2+} and EDC treatment have similar effects on light-induced pH changes, and the presence of Ca^{2+} prevents EDC from reacting with the purple membrane at pH 8.0. Since this suggests a competitive inhibition, we studied the binding of Ca^{2+} to the purple membrane. The data for intact membranes follows the type of non-linear Scatchard plot expected for binding of a cation to a membrane with a high negative surface change density [16].

Both purple membrane and EDC-treated purple membrane showed one or

two apparent binding sites/mol of bacteriorhodopsin that were too tight to be accurately measured by the centrifugation technique. Essentially all of the $^{45}Ca^{2+}$ was bound at low stoichiometries. Weaker binding was also found, with an apparent dissociation constant of $4 \cdot 10^{-5}$ M and a stoichiometry of ten sites/mol of bacteriorhodopsin. As can be seen in Fig. 6, the weaker binding was identical for both purple membrane and EDC-treated purple membrane. Presumably the phosphate and carboxyl groups are the sites of Ca^{2+} binding. Although Ca^{2+} and EDC both increase $\Delta\bar{h}$, and Ca^{2+} inhibits the EDC reaction, EDC does not appear to affect Ca^{2+} binding. Two possible explanations are: (1) since EDC is positively charged below about pH 9, the EDC reaction is driven in part by the negative surface charge of the purple membrane. In the presence of Ca^{2+}, the surface charge is reduced and the reaction is slowed. (2) A small number of Ca^{2+} binding sites are blocked by EDC, which could not be detected by the binding assay.

Effect of EDC on the photoreaction cycle

The product of the EDC reaction at pH 8.0 displays altered photoreaction cycle kinetics. Light-induced absorbance changes of membranes irradiated by ns pulses of 532 nm light were monitored at 400 nm. At this wavelength for intact membranes, there is a transient increase in absorbance with a half-time of about 50 μs and a single exponential decay with a half-time of about 3 ms (room temperature, 22°C; solvent, deionized water; pH 6.4) (Fig. 7A). By contrast, the EDC-reacted purple membrane under indentical conditions shows a strongly biphasic decay (Fig. 7B). The rise-time of the photointermediate is unaffected. The decay may be fitted with two exponentials, one with a half-time of 2 ms and the other of 6 ms. The longer half-time increases to 10 ms at pH 9, under conditions where unmodified purple membrane still shows a single exponential of 2 ms half-time. This result, along with the steady-state experiments described above, suggests that EDC inhibits the proton pump.

Reaction of EDC with purple membrane at pH 4.5

Reaction of purple membrane with EDC at pH 4.5 produces a highly aggregated product which must be sonicated to disperse it into a suspension. Steady-state light-induced pH changes were found to be even larger than those found for purple membrane reacted with EDC at pH 8.0 (Fig. 1). The value of $\Delta\bar{h}$ at pH 9.2 for membranes reacted at pH 8.0 was about 52 mequiv./mol, while for membranes reacted at pH 4.5 it was 102 mequiv./mol. Furthermore, the reaction at pH 4.5 is unaffected by the presence of Ca^{2+} during the reaction. The product of the pH 4.5 reaction was subjected to polyacrylamide gel electrophoresis in dodecyl sulfate. In contrast to the pH 8.0 reaction product, it appeared to contain polymerized bacteriorhodopsin, with dimers, trimers, and higher polymers (Fig. 3F). This cross-linking reaction requires the presence of an amino group to form an amide with an EDC-activated carboxyl group. The small amount of unpolymerized bacteriorhodopsin in the product shows an interesting higher molecular weight form, so that the monomer bacteriorhodopsin migrates as a doublet. This might be the result of cross-linking to lipid, or intramolecular cross-linking. The doublet is a major product of the EDC reaction at pH 5.5 (not shown).

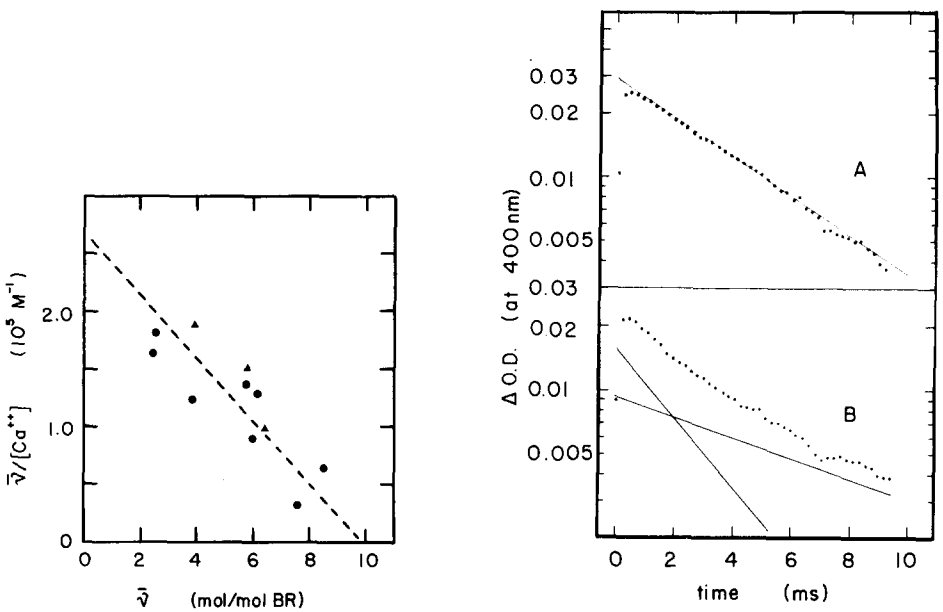

Fig. 6. Ca^{2+} binding to purple membrane (•) and to purple membrane reacted with EDC at pH 8.0 (▲). Each 1 ml sample of purple membrane contained 10^{-5} M bacteriorhodopsin, varying amounts of $CaCl_2$, and about 0.02 μCi of $^{45}Ca^{2+}$. Samples were centrifuged and the supernatant radioactivity counted as described in the text. pH 6.3—6.6.

Fig. 7. Kinetics of decay of light-induced absorbance changes at 400 nm. (A) Purple membrane; pH 6.4. (B) Purple membrane reacted with EDC at pH 8.0; pH 6.5. Samples were resuspeded in deionized water. Temperature = 22°C. Data are the average of ten laser flashes. A baseline averaging the measuring beam ten times was subtracted from the data.

Inhibition of the pH 4.5 EDC cross-linking reaction by papain or by added nucleophiles

Papain-cleaved purple membrane was reacted with EDC at pH 4.5. The product of the reaction, in contrast to the product from intact membranes, shows almost no polymerized bacteriorhodopsin by gel electrophoresis (Fig. 3G). The product is almost entirely monomer. It still has an increased $\Delta\bar{h}$, but it is similar to the $\Delta\bar{h}$ of the pH 8.0 EDC reaction product. Under the conditions used for papain cleavage (short incubation time) the only alteration of the purple membrane is the removal of 17 amino acids from the C-terminus. This peptide is known to contain five carboxyl groups: two asparates, two glutamates, and the terminal carboxyl group [3,4]. Thus, the cross-linking reaction must be between bacteriorhodopsin's C-terminal tail and an amino group elsewhere in the molecule.

If an external nucleophile is added to the reaction mixture, it would be expected to compete with the membrane-bound amino group and prevent polymerization. This is in fact the result observed when glycine ethyl ester or carbohydrazide is added to the reaction mixture (Fig. 3H).

Discussion

We have reacted purple membrane with EDC at pH 8.0 and find an increased light-induced change in proton binding ($\Delta\bar{h}$) (Fig. 1) and a slowing of the photoreaction cycle (Fig. 7). Both observations are consistent with an inhibitory effect of EDC on the uptake side of the proton pump.

The magnitude of $\Delta\bar{h}$ may be considered a measure of the difference between the rates of proton release and uptake of the proton pump [6] plus the alteration of proton binding that is due to light-induced conformational changes. Lozier and coworkers found that proton release follows the formation of photoreaction cycle intermediate M, and that proton uptake follows the decay of M (at pH 7.8) [13]. While the causal relationship between M and proton pumping remains to be established, it is clear that M represents a deprotonated retinal Schiff base [17], and thus it is central to the molecular mechanism of the pump. Therefore, the formation and decay of M may be taken as a crude measure of pump activity. The increase in $\Delta\bar{h}$ of EDC-reacted purple membrane, by itself, could mean either an increase in the proton release rate or a decrease in the proton uptake rate. But the slowed decay of M along with the increase in $\Delta\bar{h}$ suggest a slowing of the proton uptake side of the pump. This result could be obtained by one of the following mechanisms: (1) a direct modification by EDC of one of the side chains involved in proton translocation; (2) an effect of EDC on a site that undergoes a conformational change during the pump cycle, of (3) an effect of EDC on the aggregation state of the membranes. Evidence against mechanism 1 is that EDC-modified membrane, when dissociated in Triton X-100, does not seem any different from unmodified membranes in Triton X-100 (Fig. 5). Purple membranes in high ionic strength solutions tend to aggregate, and this aggregated membrane displays both an increase in $\Delta\bar{h}$ [6] and a biphasic decay of photoreaction cycle intermediate M [18]. The parallelism between high ionic strength and EDC-treated membranes with regard to these two properties indicates that the EDC reaction product may be aggregated, and that the aggregation is responsible for the apparent alteration of proton pump activity. However, the aggregation of purple membrane in high ionic strength has been explained by a decreased surface charge effect [19]. Since we have evidence against a significant change in the surface charge (Fig. 6) either the EDC-treated membrane is not aggregated, or aggregation has occurred by a highly specific electrostatic interaction involving only a few side chains/bacteriorhodopsin molecule.

The most likely explanation of our observations of increased $\Delta\bar{h}$ and biphasic decay of M is a chemical modification of a site (or sites) which undergoes a conformational change during the pump cycle. This same site must also be affected by (or involved in) membrane aggregation.

Chemical modification of proteins by reaction with carbodiimides may lead to a variety of products. In the presence of a suitable nucleophile, carboxyl groups may be converted to amides or esters. The *O*-acyl isourea initially formed in the reaction may also rearrange to form an *N*-acylurea [20]. Furthermore, carbodiimides may react with tryosine side chains to form *O*-arylisoureas [21]. The product of the pH 8.0 EDC reaction has not been conclusively identified. We have shown it is not likely to be an intermolecular cross-link,

since only monomers are detected by gel electrophoresis (Fig. 3C). We also have evidence against an *O*-aryl isourea product, since the altered $\Delta\bar{h}$ is not reversed by hydroxylamine (Fig. 4). Thus, the pH 8.0 reaction product is most likely either an intramolecular cross-link or an *N*-acyl urea. Both would have the effect of eliminating negatively charged sites. We do not yet know the stoichiometry of the reaction. However, because the Ca^{2+} binding seems to be unaffected by the EDC reaction (Fig. 6), it is improbable that EDC drastically alters the charge of the membrane surface. Therefore, we think the stoichiometry is relatively low.

We have also found that EDC at pH 4.5 promotes extensive cross-linking of the purple membrane from the C-terminal tail to an unidentified nucleophilic side chain (Fig. 3F and G). Previously reported cross-linking experiments with purple membrane [22,23] have used imidoesters to couple amino groups. The products of these reactions have not yet been identified. It will be interesting to see if the group to which we have cross-linked the C-terminal tail is the same amino group cross-linked by imidoesters.

The low pH EDC reaction will be quite useful for attaching new functional groups specifically to the C-terminal region [24], which is known to be on the cytoplasmic side of the membrane [4].

Acknowledgments

We thank Florence Tilley, Don Oster and Margaret Tse for technical assistance and Dr. W.H. Woodruff for the use of his laser. Flash spectroscopy was performed at the Center for Fast Kinetics Research (CFKR) University of Texas at Austin, where Dr. Mike Rodgers and Dave Foyt generously offered their assistance and advice. CFKR is supported by NIH grant RR-00886 from the Biotechnology Branch of the Division of Research Resources, and by the University of Texas at Austin. This research was initiated with a grant from Research Corporation. We are grateful to the Robert A. Welch Foundation (grant AX-736) and the NIH (grant GM 25483) for supporting parts of this work. G.H. is a Robert A. Welch Foundation Predoctoral Fellow.

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