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# Refolding and proton pumping activity of a polyethylene glycol-bacteriorhodopsin water-soluble conjugate

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**Abstract**

Bacteriorhodopsin (BR), from the purple membrane (PM) of *Halobacterium halobium*, was chemically modified with methoxypolyethylene glycol (m-PEG; molecular weight = 5,000 Da) succinimidyl carbonate. The polyethylene glycol-bacteriorhodopsin (m-PEG-SC-BR33) conjugate, containing one polyethylene glycol chain, was water soluble. The secondary structure of the conjugate in water appeared partially denatured, but was shown to contain $\alpha$-helical segments by circular dichroism spectroscopy. The isolated bacteriorhodopsin conjugate, with added retinal, was refolded in a mixed detergent-lipid micelle and had an absorption maximum at 555 nm. The refolded conjugate was transferred into vesicles that pumped protons, upon illumination, as efficiently as did native BR. Modification of the PM with m-PEG did not alter the native structure or inhibit proton pumping, and therefore it is suggested that the glycol polymer is present as a moiety covalently linked to residues unnecessary for proton pumping and proper folding. The site of attachment of m-PEG was determined to be at either Lys 129 or Lys 159, with position Lys 129 the most probable site of attachment. The m-PEG-SC-BR33 could be stepwise refolded to the native conformation by the addition of trifluoroethanol to lower the dielectric constant, simulating the insertion of the BR into the phospholipid bilayer.

**Keywords:** bacteriorhodopsin; chemical modification; circular dichroism; polyethylene glycol

Reaction of methoxypolyethylene glycol reagents, such as 2-methoxypolyethylene glycol-4,6-dichloro-*s*-triazine, with water-soluble proteins yields derivatives that possess decreased antigen activity (Abuchowski et al., 1977) and increased solubility in organic solvents and that retain partial or full biological activity (Takahashi et al., 1984).

It was anticipated that m-PEG modification of bacteriorhodopsin would alter its solvation properties. A water-soluble m-PEG-BR might be a suitable model for the investigation of refolding as the conjugate was inserted into a membrane. It may be possible to simulate folding simply by mixing the water solution with organic solvents of different polarity.

To our knowledge there are no reported attempts to modify membrane proteins to achieve water solubility. The chemical modification of BR, which is a light-driven proton pump in the purple membrane of *Halobacterium halobium*, with methoxypolyethylene glycol succinimidyl carbonate is reported herein. This illustrates that m-PEG is suitable for modifying membrane proteins to yield water-soluble derivatives. The modified water-soluble membrane protein was studied to examine its refolding and its proton pumping activity.

Refolding of BR is, probably, a thermodynamically controlled two-stage process (Popot et al., 1987). The first step proposed was the immersion of $\alpha$-helices into the lipid bilayer, followed by the reassembly of the tertiary structure of seven transmembrane helices. Refolding was reported to take place even if the polypeptide chain was

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*Abbreviations:* BR, bacteriorhodopsin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; MW, molecular weight; m-PEG, methoxypolyethylene glycol (MW = 5,000 Da); m-PEG-BR33, bacteriorhodopsin covalently modified with one chain of methoxypolyethylene glycol, having an apparent MW of 33 kDa; PM, purple membrane of *Halobacterium halobium*; m-PEG-SC, methoxypolyethylene glycol succinimidyl carbonate; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFE, trifluoroethanol; TLCK, 1-chloro-3-tosylamido-7-amino-L-2-heptanon; DMPC, dimyristoyl phosphatidyl choline.

cleaved (Popot et al., 1987), indicating the importance of secondary interactions, as well as the perfect geometrical association of the individual moieties.

A study of the refolding of m-PEG-BR may disclose steric or secondary structural interactions between the parts of the molecule, and possibly the pathway of folding, as well as the molecular structure of the conjugate. It has been shown in this study that m-PEG-BR refolds into a nativelike structure with the capacity for proton pumping.

PEG modification of enzymes also stabilized protein-bound $H_2O$ in organic media (Takahashi et al., 1984). It has also been reported to bind proteins via hydrophobic interactions, and its potential chaperone-like assistance in protein folding has been suggested (Cleland & Rudolf, 1992).

Modification by m-PEG is expected to be highly selective, because PEG is known to be excluded from liposomes (Arnold et al., 1990) and protein surfaces (Timasheff & Arakawa, 1988). m-PEG reagents are anticipated to react only with sites exposed to water at some distance from the surface. On the other hand, sites buried in the bilayer, or located within the membrane surface, are not expected to react. Although there are seven lysines in BR, only Lys 129 and Lys 159 are located within interconnecting loops and are presumably accessible to water (Henderson et al., 1990) and available for reaction with m-PEG.

The stepwise refolding of the conjugate was demonstrated by the stepwise addition of trifluoroethanol to lower the dielectric constant, simulating BR insertion into the bilayer.

## Results

### *Chemical modification of purple membrane*

Bacteriorhodopsin was transformed into two products by the reaction of m-PEG-SC with the purple membrane (Table 1). The primary product was a conjugate with a 33-kDa apparent MW (m-PEG-SC-BR33). Using higher m-PEG-SC ratios to PM, the amount of a 44-kDa conjugate, m-PEG-SC-BR44, increased. Approximately 97% of the BR present in the membrane was modified when the PM was treated twice with m-PEG-SC (Table 1), yielding m-PEG-SC-BR44 as the major product. The addition of m-PEG to the reaction mixture increased the yield (see below). m-PEG-SC-BR33 was isolated by SDS-PAGE electrophoresis, electro-eluted, and purified on a Sephadex LH-60 column with a chloroform-methanol mixture. The solvent was evaporated and the residue dissolved in 1% SDS. SDS was successfully removed from m-PEG-SC-BR33 samples by chromatography on Extractigel columns. The fractions containing protein were slightly turbid. To distinguish between colloidal and solubilized material, the material was centrifuged. Centrifugation at 12,000 × *g* had little effect on the turbidity of the solution; however, centrifugation of the sample at 200,000 × *g* for 45 min (Dencher & Heyn, 1982) removed all the turbidity, and approximately half of the protein remained in solution. The clear supernatant contained m-PEG-SC-BR33, at a protein concentration of 0.34 mg/mL, with as little as 7 μg/mL of SDS. This solution was stable for at least a week at 4 °C, and only a minimal increase of light scattering was observed at 400 nm. The solution could be lyophilized and stored indefinitely at −20 °C.

**Table 1.** *Product composition of the reaction of purple membrane with methoxypolyethylene glycol succinimidyl carbonate (m-PEG-SC)*

| Reagent | % Conversion | Product composition (%) m-PEG-SC-BR33[a] | m-PEG-SC-BR44[b] | Other |
|---|---|---|---|---|
| m-PEG-SC | 70 | 65 | 30 | 5 |
| m-PEG-SC[c] | 97 | 29 | 47 | 22 |

[a] m-PEG-SC-BR33 (PEG, MW = 5,000 Da). Apparent MW = 33,000 Da on polyacrylamide gels.
[b] m-PEG-SC-BR44 (PEG, MW = 5,000 Da). Apparent MW = 44,000 Da on polyacrylamide gels.
[c] The coupling reaction was repeated twice with the same purple membrane sample.

Samples of the conjugates were analyzed for protein and m-PEG to determine the stoichiometry of the coupling reaction. m-PEG-SC-BR33 was found to contain 1.1 PEG group per 1 molecule of the conjugate, while m-PEG-BR44 contained 2.14. The m-PEG content was determined by NMR and the protein content by absorption spectrophotometry (see Materials and methods).

### *Folding of m-PEG-SC-BR33 in $H_2O$: CD spectroscopy*

The CD spectrum of m-PEG-SC-BR33 in water indicated an α-helical structure (Fig. 1) and was similar to the CD spectrum of PM suspensions in $H_2O$ with lower ellipticity (Fig. 1), as previously reported for native BR in PM (Henderson et al., 1990). Due to the difficulties in the interpretation of the CD spectrum of suspensions of PM (Amons & Schrier, 1981; Gibson & Cassim, 1989), the CD spectra of m-PEG-SC-BR33 and BR were recorded under identical solvent conditions, in 1.25% octylglucoside (Fig. 2), which yielded clear solutions. The $[\theta]_{222}$ value obtained for m-PEG-SC-BR33 was 75% of that measured with BR. The ellipticity value, $[\theta]_{222}$ obtained for m-PEG-SC-BR33, was 51% of that determined for the PM in a suspension of $H_2O$. The addition of unattached m-PEG to the solution of octylglucoside-solubilized BR also reduced the ellipticity. The similarity between the CD curve of m-PEG-SC-BR33 and BR in the presence of unattached PEG is striking, indicating a similar structure

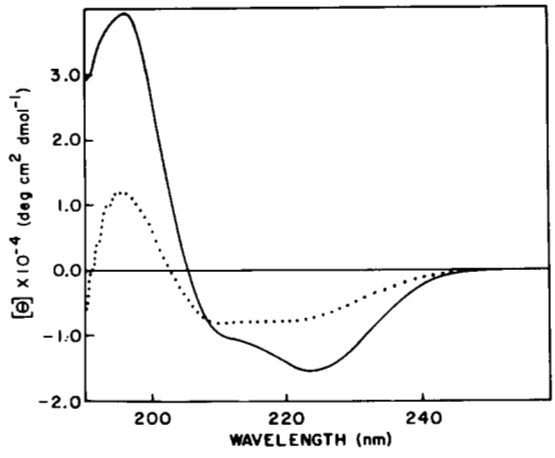


**Fig. 1.** CD spectra of water-soluble m-PEG-SC-BR33 (· · · · ·) and purple membrane (——) in water. Protein concentrations: m-PEG-SC-BR33 = 0.148 mg/mL; PM = 0.487 mg/mL. The pathlength was 0.02 cm. The spectra represent the average of five scans recorded at 20 °C.

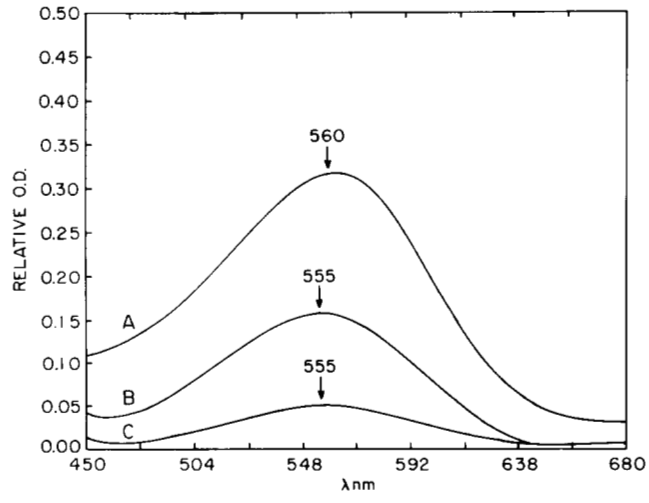


**Fig. 3.** Absorption spectra of purple membrane (**A**) in water at pH 7, 0.132 mg/mL; micellar bacteriorhodopsin (**B**) in the regeneration mixture containing 1% CHAPS, 1% DMPC, 0.2% SDS, 10 mM phosphate buffer, pH 6, 0.077 mg/mL; and micellar solution of m-PEG-SC-BR33 (**C**) in solvent as in B, 0.023 mg/mL. Protein concentrations are different in each spectrum and are shown without adjustment.

and the possibility of PEG–protein interaction. This interaction may be important in solubilizing the conjugate in water.

### *Folding of m-PEG-SC-BR33 in micelles*

m-PEG-SC-BR33 was successfully refolded in a mixed CHAPS/DMPC micelle. Both micellar BR and micellar m-PEG-SC-BR33 had an absorption maximum at 555 nm (Fig. 3). The native dark-adapted PM had an absorption maximum at higher wavelength, at 560 nm, as previously reported (Oesterhelt & Stoeckenius, 1974). The extent of the regeneration of the chromophore to that of the native form was approximately 50–75%, as it was for BR (Stern & Khorana, 1989)

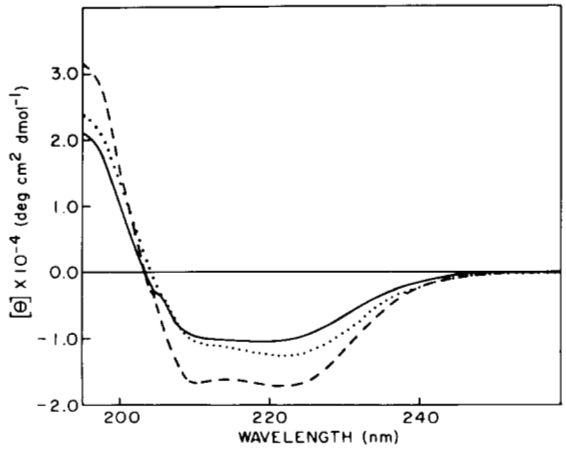


**Fig. 2.** CD spectra of bacteriorhodopsin in octylglucoside (1.25%) (- - - - -), 0.34 mg/mL; bacteriorhodopsin in octylglucoside (1.25%) and 10% PEG (· · · · ·), 0.26 mg/mL; and m-PEG-SC-BR33 in octylglucoside (1.25%) (——), 0.34 mg/mL. Cell pathlength = 0.05 cm; temp. = 20 °C.

### *Folding of m-PEG-SC-BR33 in trifluoroethanol*

By the stepwise addition of TFE to the aqueous solution of m-PEG-SC-BR33, a solvent known to induce secondary structure, the change in secondary structure can be followed by utilizing CD spectroscopy. The resulting ellipticity values, compared to the fully folded BR in 1.25% octylglucoside, is shown in Table 2. A gradual increase in helical content is observed upon increasing the TFE concentration from 0% to 50%. At 50% TFE the $[\theta]_{222}$ value is identical to that of BR in 1.25% octylglucoside (Fig. 4).

### *Proton pumping*

The refolded m-PEG-SC-BR33 from the m-PEG-SC-BR33 stock solution in 1% SDS was transferred to lipid vesicles by detergent dilution, and its proton pumping capacity was determined and compared to a similarly treated BR sample (Fig. 5). The total number of protons pumped after one illumination cycle was 30 ± 4 or 28 ± 3 protons per m-PEG-SC-BR33 or BR molecule, respectively.

The water-soluble m-PEG-SC-BR33 did not regenerate the native chromophore when it was incubated with the mixed DMPC/CHAPS micellar solution unless it had been incubated in a 1% SDS solution prior to the addition of the micellar solution.

**Table 2.** *Circular dichroism [θ] × $10^{-4}$ (deg $cm^2$ $dmol^{-1}$) values of m-PEG-SC-BR33 as a function of trifluoroethanol (TFE) concentration*

| | [θ] | | | | | |
|---|---|---|---|---|---|---|
| | Conc. of TFE in $H_2O$-TFE mixtures (% v/v) | | | | | |
| λ (nm) | 0[a] | 10[b] | 25[c] | 35[d] | 50[e] | BR[f] |
| 222 | −7,780 | −8,800 | −12,800 | −12,700 | −17,080 | −17,030 |
| 212 | −8,030 | −6,390 | −11,270 | −11,260 | −17,650 | −16,310 |
| 196 | 11,860 | 21,230 | 28,440 | 26,460 | 34,640 | 30,330 |

[a] Conc. of m-PEG-SC-BR33 = 0.34 mg/mL; cell pathlength = 0.05 cm; temp. = 20 °C.
[b] Conc. of m-PEG-SC-BR33 = 0.306 mg/mL; cell pathlength = 0.05 cm; temp. = 20 °C.
[c] Conc. of m-PEG-SC-BR33 = 0.255 mg/mL; cell pathlength = 0.05 cm; temp. = 20 °C.
[d] Conc. of m-PEG-SC-BR33 = 0.221 mg/mL; cell pathlength = 0.05 cm; temp. = 20 °C.
[e] Conc. of m-PEG-SC-BR33 = 0.17 mg/mL; cell pathlength = 0.05 cm; temp. = 20 °C.
[f] Bacteriorhodopsin in 1.25% octylglucoside: conc. = 0.487 mg/mL; cell pathlength = 0.02 cm; temp. = 20 °C.

### *Characterization of m-PEG-SC-BR33*

The approximate location of the binding site was determined by two separate enzymatic digestion experiments. Because the attachment of m-PEG to BR increased the apparent MW by 10 kDa, the apparent MWs of fragments of the conjugate having bound m-PEG were expected to differ in a fashion similar to the unmodified fragment. Chymotryptic cleavage of the conjugate (apparent MW 33 kDa) between Phe 71 and Gly 72, by published procedures (Gerber et al., 1979), resulted in a C-1 fragment that was 10 kDa heavier than the C-1 fragment obtained by digestion of the bleached PM. The digestion of the m-PEG-SC-BR44 produced a C-1 fragment that was 19 kDa heavier than the C-1 fragment of PM. This indicated the presence of covalently linked m-PEG in the C-1 fragments of both conjugates (Table 3). All the small fragments (C-2), however, had the same MW, 8 kDa, whether the protein was chemically modified or not (Fig. 6).

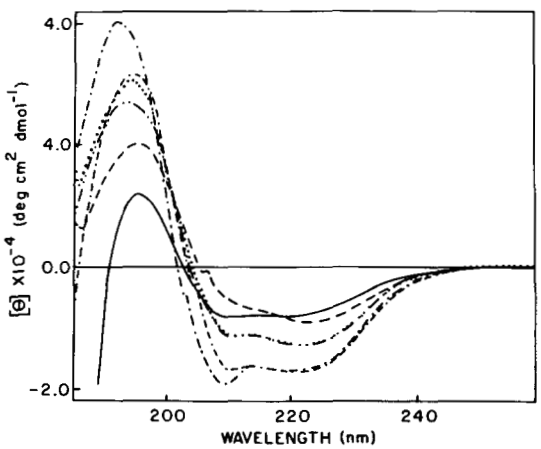


**Fig. 4.** CD spectrum of m-PEG-SC-BR33 as a function of trifluoroethanol concentration and control spectrum of purple membrane in 1.25% octylglucoside in $H_2O$. m-PEG-SC-BR33 in 0% TFE, 100% $H_2O$ (——); 10% TFE (- - - - -); 25% TFE (· · · · ·); 35% TFE (· · - · ·); 50% TFE (· - - -); see Table 3 for conditions. PM in 1.25% aqueous solution (· - - · - -), 0.487 mg/mL; cell pathlength = 0.02 cm; temp. = 20 °C.

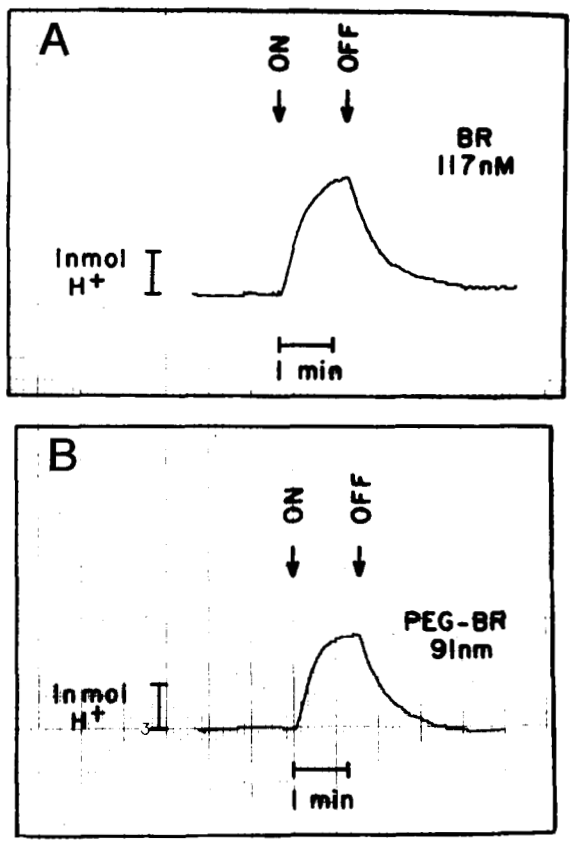


**Fig. 5.** Proton pumping measurements. Typical pH curve upon illumination vs. time for bacteriorhodopsin (**A**) and m-PEG-SC-BR33 (**B**).

**Table 3.** *Apparent molecular weights (Da × $10^{-3}$) of bacteriorhodopsin, m-PEG-bacteriorhodopsin conjugates, and their fragments formed by specific enzymatic digestion,[a] as determined by polyacrylamide gel electrophoresis (Laemmli 1970)*

| | | Fragment | | | |
|---|---|---|---|---|---|
| Sample | Protein | C-1 | C-2 | V-1 | V-2 |
| BR | 23 | 17 | 8 | 16 | 10 |
| m-PEG-SC-BR33 | 33 | 27 | 8 | 25 | 10 |
| m-PEG-SC-BR44 | 42 | 36 | 8 | 34 | 10 |

[a] Digestion was by α-chymotrypsin (C-1 and C-2) or by protease V8 from *S. aureus* (V-1 and V-2).

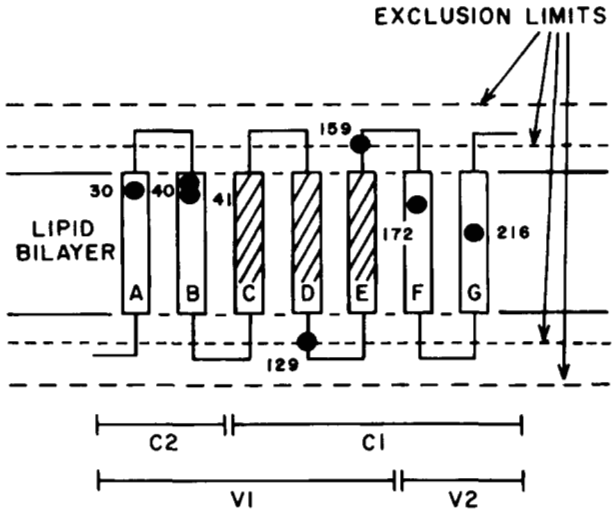


**Fig. 6.** Schematic structure of bacteriorhodopsin. The seven transmembrane helices are denoted A, B, C, D, E, F, and G. The approximate locations of lysine residues are shown by filled circles. The shaded area represents the overlapping sequences of C-1 and V-1 fragments. The broken line indicates the exclusion limits at low PEG concentrations, and the dotted line shows the presumed accessibility of side chains at high PEG concentrations.

Similarly, cleavage of m-PEG-SC–BR33 with *Staphylococcus aureus* protease V8 between Glu 166 and Val 167, by a published procedure (Sigrist et al., 1988), formed a V-1 fragment with a larger MW of 9 kDa, as compared to the V-1 fragment of BR. The small fragment, V-2, was found to be of identical size by SDS-PAGE. This indicated the absence of any m-PEG covalently attached to this part of the conjugate. These results clearly indicate that the binding site was located on the overlapping sequence of C-1 and V-1 (Fig. 6) at either K 129 or K 159.

## Discussion

BR, the membrane protein from *H. halobium*, has been successfully conjugated with m-PEG (MW = 5,000 Da). Methoxy-polyethylene glycol succinimidyl carbonate and other m-PEG reagents are known to couple to the nucleophilic side chains of proteins, mainly the $\epsilon$-amino group of the lysine side chains. There are seven lysines in BR, and several lie buried within the membrane (Henderson et al., 1990). According to the Henderson model (Henderson et al., 1990; Kinemage 1), Lys 216 and Lys 172 are buried deep in the membrane bilayer, and Lys 30, Lys 40, and Lys 41 are located near the end of membrane-spanning helices, either in the lipid phase or on the surface of the membrane. Lys 129 and Lys 159 are part of interconnecting loops and are very likely exposed to water. Lys 41 and Lys 216 of PM are known to be accessible to hydrophobic reagents, such as phenylisothiocyanate (Sigrist et al., 1984) or the latter to retinol, suggesting a location within the hydrophobic lipid bilayer. In the native PM, these sites are not accessible to m-PEG reagents, which cannot penetrate the hydrophobic bilayer. Lys 30, Lys 40, and Lys 172 do not react with the hydrophobic reagent phenylisothiocyanate (Sigrist et al., 1984). Although relatively high ratios of reagent to protein are necessary to achieve significant modification of BR, the majority of the protein molecules in the native lipid bilayer reacted (Table 1). The reaction produced two major products, m-PEG-SC–BR33 and m-PEG-SC–BR44. The amount of m-PEG-SC–BR44 formed in the reaction increases with increasing reagent-to-protein ratios. The stoichiometry of the coupled products was found to be one m-PEG molecule per one protein molecule in m-PEG-SC–BR33, and two m-PEG groups per one protein in m-PEG-SC–BR44. It appears that m-PEG-SC–BR44 is formed by further reaction of m-PEG-SC–BR33 with m-PEG-SC.

Reaction conditions were varied to investigate the optimum conditions for obtaining a maximum yield of the conjugate. Increasing the ratio of m-PEG-SC to PM resulted in an increase in yield. However, the maximum yield was obtained by having the chemically inert m-PEG present. At a concentration of 25% (w/w) of m-PEG, a ninefold increase in yield was obtained when the reactive reagent, m-PEG-SC, was held at a concentration of 1% (w/w). This can be related to the relationship between the exclusion phenomena and chemical reactivity of the m-PEG-SC. Because a change in the reagent concentration also changes the exclusion limit, due to the m-PEG moiety of m-PEG-SC, the addition of m-PEG was found to maximize the yield. PEG is known to be excluded from protein surfaces (Arakawa & Timasheff, 1985). Anomalous values of the $\zeta$-potential of liposomes measured in the presence of PEG have been reported and interpreted as resulting from the physical exclusion of PEG from the solvation shell of the liposomes (Arnold et al., 1990). It is also known that the association of water with lipid bilayers creates a large repulsive force that opposes the close approach to membranes (LeNeveu et al., 1977; McIntosh et al., 1987). The amount of work required to remove these water molecules, which is necessary for the close approach and reaction of m-PEG reagents, is significant. Increasing PEG concentrations reduces the chemical potential of water in the bulk phase by forming hydrogen bonds with water molecules (Tilcock & Fisher, 1982), which, in turn, decreases the work necessary to remove water from the solvation shell of the membrane. As a consequence, the approach of an m-PEG reagent molecule to the membrane surface is expected to be hindered, and only coupling of sites located beyond the exclusion shell is to be expected.

The hypothesis of the exclusion mechanism during the modification reaction of BR emerges from several observations. The product composition did not change significantly when the protein concentration was increased sevenfold. Had the reaction been kinetically controlled, a significant effect on the conversion and selectivity would

have been expected. The size of the exclusion zone is concentration dependent (Arakawa & Timasheff, 1985). For example, an increase of the total concentration of PEG from 10% to 40% (w/w) reduced the exclusion volume to less than half. The thickness of the zone of exclusion decreased from 7.4 Å to less than 4.3 Å. For PEG, MW 5,000 Da, the exclusion shell can be estimated to be 16–17 Å at very low PEG concentrations, based on the correlation of the radius of gyration and the exclusion zone thickness determined experimentally (Arakawa & Timasheff, 1985). Coupling sites outside this zone, which is expected to diminish at higher m-PEG concentrations, can be expected to react. The exclusion mechanism in the reaction of PM with another m-PEG reagent (2-*O*-methoxypolyethylene glycol-4,6-dichlorotriazine) was investigated by adding chemically inert m-PEG to the reaction mixture. The presence of m-PEG in the reaction mixture is expected to reduce the amount of tightly bound water in the solvation shell of the membrane, and, therefore, the accessibility and chemical reactivity of the water-exposed coupling sites are expected to be enhanced (Fig. 6). This was observed to occur. It can be concluded that (1) the approach of the polymeric reactant is physically hindered in dilute solutions; (2) the reaction proceeds by the exclusion mechanism, which is the cause of the high selectivity; (3) only Lys 129 and Lys 159 are exposed to the water phase at an appropriate distance from the membrane surface and hence to the reagent; and (4) Lys 30, Lys 40, Lys 41, and Lys 172 are buried in the bilayer and are therefore inaccessible to the macromolecular m-PEG reagents. Thus, m-PEG reagents are suitable for the investigation of the topology of lysine residues in membrane proteins, being able to distinguish between lysines in different locations and reacting only with the water-exposed sites, implying that nonreactive sites are located within the membrane surface or in the bilayer.

Spectroscopic investigation of the modified PM yielded results consistent with retention of the native protein structure. m-PEG-SC-BR33, solubilized by SDS, readily refolded when mixed with a DMPC/CHAPS micelle solution. Because both BR and m-PEG-SC-BR33 had an absorption maximum at 555 nm (Fig. 3), which had been previously reported for BR (Huang et al., 1981; Subramaniam et al., 1990), the protein structure of micellar m-PEG-SC-BR33 must be similar, if not identical, to that of the micellar BR. The wavelength of the absorption maximum is somewhat different from that of the dark-adapted PM (Fig. 3). The visible spectrum of two dark-adapted modified PM samples, containing approximately 40% and 60% modified protein, was recorded (Fig. 3). The light-adapted form, generated by illumination through a yellow filter for 5 min, had an absorption maximum at 568 nm, identical to that of the PM (Becher & Cassim, 1976). Also, only a slight difference in the tyrosine fluorescence maximum at 315 nm for PM and at 309 nm for m-PEG-SC-BR33 was observed (not shown).

The refolding proceeds spontaneously as the SDS environment is replaced by DMPC/CHAPS. It is known that, in its SDS-solubilized form, much of the helical structure of BR is still present (Huang et al., 1981). m-PEG, on the other hand, is readily soluble in water but insoluble in hexane, which is similar in polarity to the interior of an SDS micelle. On these premises, it is reasonable to assume that the apolar helices, in a disordered array, occupy the interior of the SDS micelle, whereas the m-PEG, attached to a water-exposed loop, is located outside of the micelle in the water phase. By changing the lipid environment, the detergent–helix interactions are weakened and helix–helix interactions become dominant, and a nativelike structure is restored in the hydrophobic interior of the micelle. m-PEG, assumed to be located on the outside of the micelle, is thus expected not to interfere with folding. The structural and functional integrity of the conjugate was verified by its capacity for proton pumping. The conformational changes of m-PEG-SC-BR33 required for proton pumping are not altered by the presence of m-PEG (Fig. 5).

The CD spectrum of BR (in 1.25% octylglucoside) in the presence of unattached m-PEG is similar to that of m-PEG-SC-BR33 (Fig. 2). Thus, a similar interaction between attached or unattached PEG and BR is proposed. This interaction reduces the α-helix content of the protein, resulting in similar conformations. This interaction may be important in solubilizing the conjugate in water.

The modified PM was investigated by CD spectroscopy. The visible CD spectra characteristic of the secondary and tertiary structures are shown in Figure 7. The far-UV CD spectra of the native and modified PM were nearly identical, with a minimum at 224 nm, with $[\theta]_{222} = 1.65 \times 10^4$ deg cm$^2$ dmol$^{-1}$, and similar to that previously reported for PM (Gibson & Cassim, 1989). The visible CD spectra of two modified PM samples, 40% and 60% modified, were compared to that of the native mem-

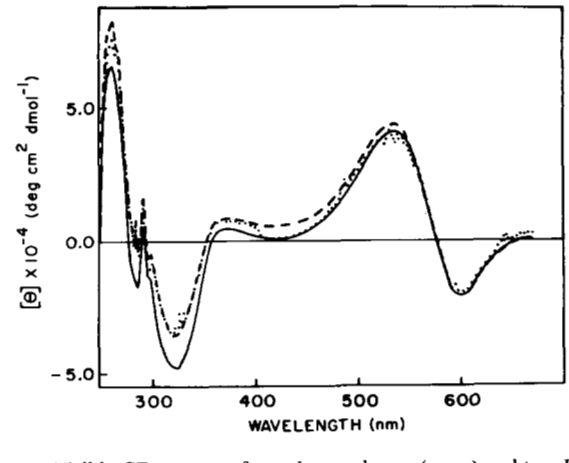


**Fig. 7.** Visible CD spectra of purple membrane (——) and two PEG-modified purple membrane samples. Degrees of modification were 40% (- - - -) and 90% (· · · · ·).

brane (Fig. 7). All samples had the same characteristic CD spectra, with a negative peak at 317 nm and a double peak consisting of a positive and negative trough centered around 560 nm, with a crossover at 577 nm. The intensity of the 317-nm peak was of smaller magnitude than that of the native membranes, and the ratios of the positive and negative troughs were slightly different, the ratio being 1.9 for the native membrane, 1.6 for the 40% modified PM, and 1.3 for the 90% modified PM.

Because m-PEG-SC-BR33 readily refolds into a native structure capable of proton pumping, this derivative may be suitable to investigate refolding induced by changing solvent polarity.

Future studies will attempt to determine the order of the formation of the seven helices in BR. The aromatic amino acids are unevenly distributed and are found as follows: two Trp, one Tyr, and one Phe in helix A; two Tyr and two Phe in helix B; two Trp, one Tyr, and one Phe in helix C; no aromatics in helix D; two Trp, three Tyr, and two Phe in helix E; two Trp and one Tyr in helix F; and two Phe in helix G. Advantage will be taken of this distribution to detect conformational changes in the environment of these residues. m-PEG modifications of BR, as well as the presence of unattached m-PEG, in the solution of octylglucoside-solubilized BR, reduce the $[\theta]_{222}$ to about 75% of the original value (Table 2). Because the CD spectrum of BR in the presence of unattached m-PEG and octylglucoside is similar to that of m-PEG-SC-BR33, an interaction between the m-PEG and the protein of the conjugate is likely. In contrast, the CD spectrum of the PM in the presence of the same amount of m-PEG, as found in the 40% modified sample, indicated a relatively small change of secondary structure. The membrane structure protects the protein from interactions with PEG by the exclusion mechanism (Arakawa & Timasheff, 1985). The structure of the protein in the octylglucoside micelle may expose some apolar parts of the protein capable of binding PEG just as well as the hydrophobic regions, as was found for the molten globule state of bovine carbonic anhydrase (Cleland & Rudolf, 1992). This interaction is likely to be accompanied by the minor partial unfolding of the helices present in BR, as indicated by the slightly altered CD spectrum (Fig. 2).

The suggested model of the water-soluble conformation of m-PEG-SC-BR33 consists of a large random coil of polyethylene glycol that associates with hydrophobic segment of the $\alpha$-helices of BR. The minor rearrangement of the apolar groups of BR induces a slight conformational change, which may include the unfolding of some of the $\alpha$-helical structure, as indicated by the altered CD spectrum (Fig. 2). This transformation allows the more hydrophilic side chains to be exposed to the water associated with the outside of the associated polymer-protein structure. These interactions between m-PEG and BR are likely to facilitate the water solubility of the conjugate. It is known that BR readily refolds in mixed lipid-detergent micelles to a nativelike structure (Stern & Khorana, 1989). In its SDS-solubilized form, much of the helical structure of BR is still present (Huang et al., 1981). By changing the lipid environment from SDS to the DMPC/CHAPS/SDS micelles, the detergent-helix interactions are weakened, helix-helix interactions become dominant, and a nativelike structure is restored in the hydrophobic interior of the micelle. The refolding of m-PEG-SC-BR33 dissolved in 1% SDS also proceeds spontaneously as the SDS environment is replaced by DMPC/CHAPS. Because both refolded BR and m-PEG-SC-BR33 had an absorption maximum at 555 nm (Fig. 3), as previously reported for micellar BR (Huang et al., 1981; Stern & Khorana, 1989), the protein structure of micellar m-PEG-SC-BR33 must be similar to, if not identical with, that of the micellar BR.

To determine the approximate location of the binding sites, the BR and m-PEG-SC-BR33 were cleaved, separately, by two enzymes, $\alpha$-chymotrypsin and proteinase V8. It has been previously shown that $\alpha$-chymotrypsin cleaves the polypeptide chain between F71 and G72 (Gerber et al., 1979). This produces a smaller fragment (C-2), which contains the A and B helices of BR, and a residual larger fragment (C-1), which contains helices C, D, E, F, and G (Fig. 6) (Liao et al., 1983). Protease V8, on the other hand, cleaves off a fragment (V-2) between E166 and V167, containing helices F and G, leaving the residual large fragment (V-1) intact (helices A, B, C, D, and E). In both cases, the V-1 and C-1 fragments of m-PEG-SC-BR33 had MWs approximately 8.5–9.5 kDa higher than the corresponding fragments of BR (27.5 kDa vs. 18 for C-1 and 25 vs. 16.5 for V-1). This is evidence of the binding of PEG to both large fragments (C-1 and V-1). As a consequence, the binding sites must be located in the overlapping sections, including helices C, D, E, and their interconnecting loops.

In addition, the small fragments formed by protease V8, of BR and m-PEG-SC-BR33, had the same apparent MW (10.5 kDa), indicating the absence of any PEG bound to this part of the molecule (V-2).

Assuming that lysines are the most likely sites for coupling, the attachment of two m-PEG molecules to the only available two lysines on helices C, D, and E and their interconnecting loops, namely K129 and K159, may be proposed.

Previous studies on labeling PM with biotin *N*-hydroxy succinimide ester (Henderson et al., 1978) or fluorescein (5(6)-carboxyfluorescein succinimidyl ester) (Heberle & Dencher, 1992) indicated coupling at Lys 129. Thus, the K129 position is the most probable site of attachment.

## *Conclusions*

1. Bacteriorhodopsin, a membrane protein, has been modified by reaction with methoxypolyethylene gly-

col succinimidyl carbonate to yield a water-soluble m-PEG-SC-BR33 conjugate.
2. m-PEG-SC-BR33 can be refolded in lipid vesicles to restore the native structure, which is capable of pumping protons at a rate similar to that of native purple membrane.
3. The attached m-PEG polymer chain is proposed to be covalently linked to a sequence not involved in proton pumping, namely on one of the loops between helices C, D, and E on Lys 129 or 159, with Lys 129 the most probable site.
4. Stepwise refolding of m-PEG-SC-BR33 can be achieved by stepwise addition of trifluoroethanol to yield the native conformation.

## Materials and methods

### *Materials*

*Halobacterium halobium* was grown and the PM isolated as described by Oesterhelt and Stoeckenius (1974). m-PEG (MW = 5,000 Da) was obtained from Fluka; triethylamine, *N*-hydroxy-succinimide, TFE, and DMPC were purchased from Aldrich. CHAPS was obtained from Pierce, and phosgene came from Matheson. The all-trans retinal was an Eastman Kodak product. m-PEG-SC was synthesized as previously described by Zalipsky et al. (1991). Both TLCK-treated $\alpha$-chymotrypsin and protease V8 from *S. aureus* were purchased from Sigma. The Extractigel column was a Pierce product. Dilinoleic phosphatidic acid, dioleoyl phosphatidyl choline, and dimyristoyl phosphatidyl choline were obtained from Avanti Polar Lipids, Inc.

### *Coupling of PM with m-PEG-SC to yield MeO-($CH_2$-$CH_2$-O)$_x$-CO-NH-BR (m-PEG-SC-BR)*

m-PEG-SC (10 $\mu$mol) was dissolved in 190 $\mu$L 0.1 M sodium tetraborate buffer solution at pH 9.3 with vigorous stirring. To this solution was added 55 $\mu$L PM suspension containing 270 $\mu$g (10 nmol) of protein. After stirring the reaction mixture for 1 h at room temperature, the modified membrane was separated by centrifugation (20,000 $\times$ *g*, 60 min) and washed three times with water. Finally, the sediment was resuspended in 55 $\mu$L water.

The m-PEG-SC-BR conjugate was isolated by preparative gel electrophoresis (Huang et al., 1981). Three bands were observed, with apparent MWs of 25 kDa (unreacted BR), 33 kDa (m-PEG-SC-BR33), and 44 kDa (m-PEG-SC-BR44). After separating the bands by slicing the gel, the protein was isolated by electro-elution in a Bio-Rad model 422 electro-eluter and the solution was lyophilized. The product was further purified on a Sephadex LH-60 column (Wüthrich & Sigrist, 1990). The sample was dissolved in a chloroform/methanol 1:1 mixture, which contained 0.1 M sodium acetate. The PEG-protein sample was eluted in the same solvent. After evaporation of the solvent by applying a stream of $N_2$, the residue was treated with water to extract sodium acetate from the conjugate. The protein was pelleted by centrifugation (12,000 $\times$ *g*) and dissolved in 1% SDS. This eluant may be lyophilized and stored at $-20$ °C. This solution may be applied to the Extractigel column to remove the SDS (see below).

### *Removal of SDS from m-PEG-SC-BR33 dissolved in water*

SDS was removed from m-PEG-SC-BR33 (in 1% SDS) samples by loading onto an Extractigel column. The protein eluted with 0.1 M $Na_2PO_4$ buffer, pH 7.0, in the void volume. The slightly turbid solution was centrifuged at 200,000 $\times$ *g* for 45 min (Dencher & Heyn, 1982) to yield a clear aqueous solution. The clear supernatant was analyzed for protein and detergent. At least 99.5% of the detergent was removed. The final aqueous solution contained only 30 molecules of SDS per protein molecule; that is less than the average amount of SDS bound by proteins (about 100–200 molecules for SDS for bacteriorhodopsin [Nelson, 1971]). A concentration of 0.34 mg/mL was obtained. This may be lyophilized for storage at $-20$ °C.

### *Enzymatic digestion of BR and the m-PEG-SC-BR33 conjugate*

The PM and m-PEG-SC-BR33 modified PM were bleached and digested with $\alpha$-chymotrypsin by the method previously described (Gerber et al., 1979) at a protein concentration of 2 mg/mL. The ratio of protein to enzyme was 100:1.

Digestion of BR and the m-PEG-SC-BR33 conjugate by protease V8 was performed as previously described (Sigrist et al., 1988). The protein concentration was 0.4 mg/mL, and the protein:enzyme ratio was 10:1.

### *Refolding and proton pumping of BR and m-PEG-SC-BR33*

m-PEG-SC-BR33 was refolded by adding a stock solution of the conjugate (0.8 mg/mL of protein) in 1% SDS to a solution containing DMPC and CHAPS (1% w/v of each in the final solution) and a final concentration of 0.2% for SDS and 0.15 mg/mL for protein (Stern & Khorana, 1989). All-trans retinal (0.285% solution), dissolved in ethanol (10 mM), was added in a twofold excess. This mixture was incubated for 16 h at room temperature.

Refolded m-PEG-SC-BR33 and BR were incorporated into lipid vesicles containing dilinoleic phosphatidic acid, dioleoylphosphatidyl choline, and dimyristoyl phosphatidyl choline (20:10:10) (Subramaniam et al., 1990) by the detergent dilution method previously described (Stern & Khorana, 1989) for proton pumping assays. Proton

pumping was measured in 1 mL of the vesicle suspension placed in a reaction vessel at 25 °C (Racker & Stoeckenius, 1974). The light of a 500-W slide projector filtered through an orange Corning 3780 yellow filter and a 3965 heat filter was focused on the solution, and the pH change was monitored with a glass electrode (MI 410; Microelectrodes, Inc.) and recorded on a recorder (Fisher Recordall series 5000). The system was calibrated by the addition of 4-$\mu$L aliquots of 0.5 mM hydrochloric acid. Proton pumping activity was calculated from the pH change measured at the steady-state level and expressed as the total number of protons translocated per mole of protein.

### *Other methods*

UV and visible spectra were recorded on a Gilford 2600 spectrophotometer. CD spectra were recorded on a computer-operated Jobin Yvon Auto-Dichrograph Mark V instrument (Prevelige & Fasman, 1987). CD spectra of the m-PEG-SC-BR33 were recorded at a protein concentration of $\approx$0.3 mg/mL in a 2-mm cell under various conditions. Apparent MWs were determined on 15% or 18% polyacrylamide gels by SDS-PAGE (Laemmli, 1970). The Coomassie-stained gels were quantified with an ISCO 1312 gel scanner. The m-PEG content of the conjugate was determined by NMR. The SDS content of the sample was determined spectrophotometrically with *p*-rosanilide (Amons & Schrier, 1981). The concentration of BR was determined using the extinction coefficient of $\epsilon_{280}$ = 66,000 $cm^{-1}$ $M^{-1}$ (Braiman et al., 1987).

### *Stepwise refolding of m-PEG-SC-BR33 in TFE*

m-PEG-SC-BR33, with added retinol, was successfully refolded in a mixed DMPC/CHAPS micelle. The extent of regeneration of the native absorption spectra of PM was approximately 60–75%. Similarly treated BR also yielded a 75% regenerated product. Both micellar BR and micellar m-PEG-SC-BR33 had an adsorption maximum at 555 nm, indicating identical protein structures.

The water-soluble m-PEG-SC-BR33 did not regenerate the chromophore when it was incubated with the mixed DMPC/CHAPS micellar solution. However, the native chromophore absorption was efficiently regenerated if the water-soluble conjugate was first incubated in a solution containing 1% SDS prior to mixing with DMPC/CHAP micellar solution.

A solution of m-PEG-SC-BR33 in water was prepared as described above for the refolding experiments. The samples for CD spectroscopy were prepared by mixing the appropriate volume of the water solution with the necessary amount of TFE. For example, 50 $\mu$L of m-PEG-SC-BR33 solution (0.34 mg/mL) was mixed with 50 $\mu$L of TFE to make a 50% v/v mixture. The spectra were recorded in a 0.05-cm cell, and three scans were averaged for each measurement.

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