Nature of forces stabilizing the transmembrane protein bacteriorhodopsin in purple membrane

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ABSTRACT Analysis of the far-ultraviolet solution and the oriented-film circular dichroic (CD) spectra of the purple membrane (PM) has indicated that the α-helical segments of its sole protein bacteriorhodopsin (bR) can undergo a significant tilting from the normal to the membrane plane during light-dependent hydroxylamine-mediated bleaching of the bR. However, this drastic change in tertiary structure is free of any observable secondary structural changes. This phenomenon can provide an excellent means for studying the relative contributions of forces responsible for the stability of this transmembrane protein within the membrane bilayer. Perturbation of the PM by varying degrees of papain digestion (resulting in changes in the bR ranging from only an elimination of the long COOH-terminal tail to the additional eliminations of the short NH_2-terminal tail and a number of linkage amino acids between the helical segments of the bR) and by chemical cross-linking with dimethyl adipimidate (resulting primarily in the formation of intramolecular cross-links) resulted in a significant increase in this bleaching-induced tilting in all cases except the one in which only the COOH-tail was eliminated. The most severe perturbation (2-wk papain digestion) increased the net tilt angle per segment from 24 to 39° with no indication of any secondary structural changes. Although these perturbations drastically reduced the structural stability of the bR to bleaching, they caused virtually no observable changes in the intramolecular structure of the bR or the supramolecular structure of the PM based on analysis of extensive absorption, linear dichroic, and CD spectra. In addition, study of the bleaching rates for the perturbed PM samples indicated that a linear correlation exists between the calculated initial bleaching rates and the net tilt angles.

Considering the forces generally assumed to account for the stability of transmembrane proteins in membranes, (*a*) intersegmental hydrogen bonding and electrostatic interactions, (*b*) electrostatic interactions between hydrophilic polypeptide segments extending outside the bilayer and the many charged lipid heads of the bilayer, and (*c*) hydrophobic interactions, it is clear that the results of the bleaching experiments eliminate all but perhaps the last as contributing significantly to the bR stability in the PM. Furthermore, they provide more compelling evidence than previously available that the bR is capable of undergoing relatively large retinyldiene-controlled tertiary structural changes and that the chromophoric retinal serves as the most important factor in the native bR structural stability. This dynamic view of the bR bears directly on models proposed for bR function, favoring those in which protein structural metastability, rather than rigidity, is an essential factor. The proteinquake or deformation wave model proposed by this laboratory falls into this category.

INTRODUCTION

Two outstanding problems in membrane biophysics are the nature of the forces stabilizing the structures of transmembrane proteins in the membrane bilayers and the involvement of these forces in the active transport functions of these proteins. The popularly held hypothesis is that there are three forces responsible for the in situ transmembrane protein stability: (*a*) intersegmental hydrogen bonding and electrostatic interactions, (*b*) electrostatic interactions between the hydrophilic polypeptide segments of the proteins extending outside the bilayer and the many charged lipid heads of the bilayer, and (*c*) hydrophobic interactions.

The main objective of this study is to explore the relative contributions of these proposed forces to the structural stability of the transmembrane proton-transport protein bacteriorhodopsin (bR) of the purple membrane (PM) of *Halobacterium halobium*. The PM is an ideal model membrane for such studies because an extensive literature is available on its structure and function and that of its sole protein, bR (1, 2). The bR is the best characterized example of a transmembrane protein currently available and serves as an archetype for many others. In addition, there has been considerable success in correlating spectra with structural features of this membrane (3–10).

The bR, which is a chromoprotein with a retinyldiene prosthetic group bound via a protonated Schiff-base linkage to a lysine of the bacteriorhodopsin apoprotein, accounts for 75% of the PM volume with the remainder being lipids. The bR consists of a single 26,866-D poly-

0006-3495/89/10/769/12 $2.00

peptide chain of 248 amino acids. This polypeptide chain is thought to traverse the membrane bilayer seven times, embedding ~80% of the amino acids within the hydrophobic bilayer. The bilayer-spanning segments are generally believed to be α-helical in secondary structure while those located outside are aperiodic. However, the α-helical regions probably exist as a complex mixture of helix types ranging from the α_I to the α_{II} type (11). Although β-sheet structure has also been suggested (12), there is increasing evidence against significant amounts of this structure (11, 13–15). The bR molecules are organized as trimeric clusters arranged in a hexagonal lattice forming a two-dimensional crystal with the polypeptide segments aligned nearly perpendicular to the plane of the membrane. The crystalline lattice of the PM is sufficiently well ordered to promote excitonic interaction among the retinals.

The function of the PM is to transport protons actively against a concentration gradient from the cytoplasmic to the extracellular side of the membrane of the bacterium. This function seems to be intimately correlated with the photocycle of the bR. A question pivotal to the understanding of the molecular mechanisms of this function is the nature and extent of structural changes of the PM during this cycle. Cassim and co-workers have addressed this question by studying the structural changes of the PM during the transition from the light-adapted state, bR_{568}, to the relatively long-lived intermediate state, M_{412}, during the photocycle in which the configuration of the retinal and the protonation of the Schiff-base linkage are altered. In addition, they have studied the structural changes accompanying the light-dependent hydroxylamine-mediated bleaching of the bR which results in the formation of retinaloxime and breaking of the Schiff-base linkage. Changes occurring in both photoprocesses, deduced by PM solution and oriented-film circular dichroic (CD) spectra, were very similar and completely reversible (5, 7, 9). Important changes noted were (*a*) the complete loss of excitonic interaction among the retinals due to the elimination of the native order of the retinals in the PM crystalline lattice, (*b*) the loss of the native structural anisotropism of the PM with respect to the in- and out-of-plane directions, and (*c*) a net tilting of the polypeptide segments away from the normal to the membrane plane as a result of a tertiary structural change of the bR. The only significant difference was that the net tilting was relatively more pronounced in the bleaching process than in the M_{412} formation. Furthermore, Papadopoulos and Cassim (16) showed that the bacterio-opsin polypeptide segments of the native brown apo-membrane are significantly tilted with respect to the membrane normal in contrast to those of the holo-membrane, which is formed from the apo-membrane by the addition of retinal. It is important to note that in all these processes, the nature of the chromophoric retinal is critically involved in the tilting phenomenon. It was concluded from these findings that during these processes the inherently metastable tertiary structure of the bR undergoes transformation from a closed conformation to an open one with the chromophoric retinal functioning as the regulator of this process. This conclusion forms the basis for the proteinquake paradigm of the proton transport function of the PM proposed by this laboratory (7, 9, 16). In addition, the loss of the excitonic interactions is indicative of a significant change in the ordered supramolecular structure of the PM. This apparent metastability in the PM supramolecular structure, indirectly regulated by the retinal, may be essential to the subtle regulation of PM function. However, tilting is also possible without significant tertiary structural changes of the bR but with changes mainly in the out-of-plane orientations of the bR molecules. This type of tilting has been observed during the severe dehydration of the PM which does not directly involve the retinals of the bR molecules (10).

The tilting phenomenon provides a means for studying the relative contributions of the forces that may be stabilizing the in situ tertiary structure of bR. In the present study bleaching was used exclusively to achieve tilting because of the advantage that the bleached bR remains stable indefinitely at ambient temperature. The PM was perturbed by varying degrees of papain digestion, which result in changes in the bR ranging from the simple elimination of the long COOH-terminal tail to the additional eliminations of the short NH_2-terminal tail and a number of linkage amino acids between helical segments of the bR (17), and by chemical cross-linking with dimethyl adipimidate (DMA), which results primarily in the formation of intramolecular rather than intermolecular cross-links (18, 19). Analysis of extensive absorption, linear dichroic, and CD spectra of PM solutions and oriented films after modification yielded virtually no indication of change in the intramembrane conformation of the bR or in the supramolecular structure of the PM (see references 3–10, 16, 20 for past studies). However, in all perturbations studied except one, the bleaching-induced net tilt angle per segment, computed to be about 24° in the unperturbed PM, was pronouncedly increased. The most drastic perturbation increased this angle to 39°. In addition, study of the bleaching rates for these variously perturbed states of the PM indicated that a linear correlation exists between the calculated initial bleaching rates and the tilt angles. The only modification found to have no effect was the mild papain digestion, which only eliminates the COOH-terminal tail of bR.

It is concluded that the forces generally believed to stabilize the tertiary structures of transmembrane pro-

teins may play only a minor role in the stability of the bR. It is apparent that the chromophoric retinal serves a much more important role in this capacity. Furthermore, this study provides additional evidence of a much stronger nature than previously available that the bR is structurally metastable and can easily undergo structural transitions from a relatively closed state to an open one. This, in turn, provides further support for the proteinquake paradigm.

MATERIALS AND METHODS

PM preparation

PM was isolated from the S9 strain of *Halobacterium halobium* according to the method of Becher and Cassim (21) with some modifications, notably, the omission of the sucrose gradient step. Centrifugations were done at relatively low speed (22,000 *g*) for 90–120 min and produced preparations uncontaminated by red membrane. It seems likely that a recent discovery of significant red membrane contamination in some PM preparations may be due to the relatively high-speed centrifugation used (100,000 *g*) in these preparations (22).

PM modifications

Cross-linking with dimethyl adipimidate, 10 mM in 0.2 M borate buffer at pH 10 (Pierce Chemical Co., Rockford, IL), was by published methods (18). Bleaching with hydroxylamine HCl, 0.3 M in 20 mM phosphate buffer at pH 6.5 (Sigma Chemical Co., St. Louis, MO) was also by published methods (5). Papain digestion of the PM were achieved by suspended PM at a concentration of 0.4 mg/ml in 20 mM phosphate buffer at pH 7 containing 5 mM cysteine and 1.5 mM EDTA. For cleavage of the COOH-terminal tail alone, papain (Sigma Chemical Co.; type III, 16–40 U/mg protein) was added to give a bR/papain ratio of 200:1, and then the mixture was incubated at 37°C for 2 h. For long-term digestion, the bR/papain ratio was 20:1 and the samples were incubated at 37°C for periods of time from 1 to 6 wk. The samples were washed repeatedly in double distilled demineralized water after digestion. Chymotrypsin cleavage was achieved by adding 1 mg chymotrypsin (Sigma Chemical Co.; type I-S, 40–50 U/mg protein) to each ml of PM at an OD_{568} of 5.0 (2.1 mg protein/ml). The mixture was incubated in the dark for 24 h at 37°C with continual stirring.

Film preparation

The PM was suspended in double-distilled water to an OD_{568} of 0.2 to 0.3 (in the case of the bleached membranes, which lack appreciable absorbance at 568 nm, the solutions were simply made to match the OD_{280} of the native suspensions). The solutions were passed through model GA-1 5-μm filters (Gelman Sciences Inc., Ann Arbor, MI) and degassed. Approximately 0.75 ml of a given solution was then placed on a 25-mm diameter quartz optical flat (Suprasil-S, Precision Cells, Inc., Hicksville, NY) and allowed to dry in a desiccator containing Drierite. Films were incubated for 24 h in a desiccator containing a saturated K_2SO_4 solution (95% relative humidity) before measuring CD spectra to avoid artifacts which can arise in dehydrated films. An average film (OD_{193} of 0.3) had an OD_{568} of ~0.008. Fisher (23) has published spectra for PM bilayers in which he obtained an OD_{568} of 0.0016. Therefore, the films used in this study were probably ~6–12 monolayers thick.

Spectroscopy

Absorption spectra were recorded on a Cary 118C spectrophotometer with a far-UV modification and a scattered transmission accessory (Varian Associates, Inc., Palo Alto, CA). Linear dichroism measurements were made on the 118C using the methods described by Papadopoulos and Cassim (20). CD spectra were recorded on a model J-500A spectropolarimeter (Jasco Inc., Easton, MD). All CD results were subsequently verified on a Cary 60 spectropolarimeter with a 6003 CD attachment (Varian Associates, Inc., Palo Alto, CA). Basic procedures were as previously described (5).

Film quality control

To monitor any CD artifacts due to light scattering, samples run on the Jasco spectropolarimeter were measured both on the optical bench (16 cm from the PMT, 11° acceptance angle) and close to the PMT (2 cm, 90° acceptance angle). The films used for this study showed no difference at the two positions, indicating the absence of measurable scattering artifacts. The Cary spectropolarimeter allows very little adjustment of sample-to-PMT distance. The value for this machine was ~2 cm.

Samples were carefully screened for absorption-flattening artifacts caused by excessive sample concentration. Generally, samples with an OD_{193} of greater than 0.5 (films) or 0.3 (solutions) were discarded, as experience has shown that above this concentration artifacts arise. However, many samples which met this criterion were still rejected. Although the ellipticity (θ) at 224 nm is found to have a direct linear relationship to absorbance for OD_{193} <1, the θ at 193 nm is found to decrease relative to that at 224 nm when samples are too concentrated. Solution samples were considered acceptable when they had a $\theta_{193}/\theta_{224}$ ratio of 2.4–2.6. Native PM films were required to have a $\theta_{197}/\theta_{224}$ ratio of 3.5–3.7.

Film quality was also monitored by several indirect methods. A drop of sample to be used for film preparation was placed on carbon-coated formvar grids, dried, shadowed, and examined in an electron microscope to be sure that the membranes were not aggregated and were drying parallel to the film surface. In addition, the linear dichroism of films having an absorption maximum in the visible region was determined. Native PM films were found to have a retinyldiene out-of-plane angle of 21.2 ± 2°. These results are in excellent agreement with those previously published by Heyn et al. (24) who demonstrated that mosaic spread in their film preparations, i.e., the nonparallel alignment of membrane discs, was negligible using neutron diffraction techniques. Finally, thick films made by successive layering and drying of a PM solution showed no evidence of a bilobed excitonic CD band at the visible absorption maximum of the PM. This is indicative of the mosaic spread of the film being negligible, as departure from planarity should give rise to excitonic contributions to the visible CD (5).

It must be emphasized that problems in CD measurements reported by other laboratories such as differential light scattering, absorption flattening, and mosaic spread are in large part due to the fact that the films used were relatively thick or the solutions too concentrated. By taking advantage of the high sensitivity and good signal-to-noise ratio offered by the Jasco spectropolarimeter, it was possible to make extremely thin films which did not present the above mentioned problems.

In situ bleaching

PM films were prepared as described above and placed in specially designed airtight filmholders (Oriel Corp., Stamford, CT). Three drops

of hydroxylamine solution (0.6 M in 40 mM phosphate buffer, pH 6) were placed on each film. Next an o-ring and quartz optical flat were placed in the holder and tightened with a retaining ring. This prevented evaporation and prevented loss of hydroxylamine solution when the films were tilted on edge for spectral measurements. Films were allowed to stand on edge for 20 min before measuring spectra to eliminate artifacts which might be caused by excess solution sheeting across the films. Sometimes films were kept horizontal. Those that were to be bleached were illuminated with light from a Sylvania 500-W DAY projector lamp (GTE Products Corp., Winchester, KY) filtered through a copper sulfate solution and a 500-nm long-pass filter (Corning Glass Works, Corning, NY). Spectra were recorded ~20 min after addition of the hydroxylamine solution and again after bleaching with light or storage in the dark.

Electrophoresis

A modified Laemmli method described by Payne (25) was used for the SDS-polyacrylamide gels. Essential features of the system were a stacking gel containing 5% acrylamide, a separatin gel containing 15% acrylamide, and an electrode buffer (pH 8.3) containing Tris-glycine (25–190 mM) and 0.1% SDS. The slab gel was loaded with samples (10–30 μl) and run for 5 h at 25–30 mA and 21°C. The gels were stained for 1 h with Coomassie Brilliant Blue in methanol-acetic acid (45–9% vol/vol in water) and destained with methanol-acetic acid (5–7.5% vol/vol in water) for several days. Molecular weight standards (SDS-7) were obtained from Sigma Chemical Co.

RESULTS

Papain-treated and cross-linked PM

Fig. 1 is a schematic representation of the bR molecule which has been simplified to emphasize the modified areas. The lysine locations are from Liao and Khorana (26), whereas the papain cleavage sites are based on the

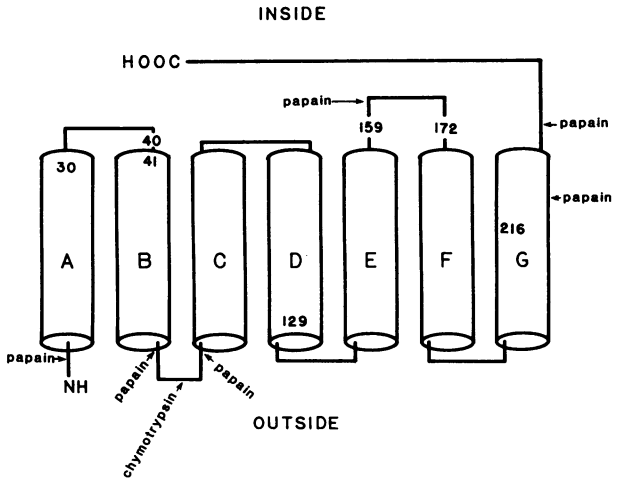

FIGURE 1 A schematic representation of the bR molecule to illustrate the sites of papain cleavage (17) and the location of lysine residues which are indicated by numbers (26).

experiments of Ovchinnikov et al. (17). The latter group found that papain treatment for 2 h at an enzyme-to-bR ratio of 1:200 caused cleavage of the COOH-terminal tail between Gly-231 and Glu-232. They also reported that increasing the ratio to 1:20 and the time to 24 h led to the loss of the three NH_2-terminal amino acids and the linking region between helices B and C, whereas an incubation time of 7 d also caused cleavage between Ser-162 and Met-163.

After papain digestion and washing with double-distilled demineralized water, the PM suspensions became blue, with an absorption maximum at ~605 nm. This effect has been widely described in recent years and is apparently due to a loss of calcium and magnesium ions after loss of the COOH-terminal tail (27, 28). The purple color was regenerated on addition of small quantities of mono- or divalent cations. In this study all references to papain-treated PM will denote the purple form unless otherwise stated. Absorption studies in which papain-treated PM was suspended in a water-glycerol (1:2) mixture at −70°C showed that all papain-treated samples were still capable of forming the M_{412} photointermediate when excited by light of wavelength >500 nm, as long as sufficient cations were present. After removal of the papain, the samples of PM which had been digested for 6 wk actually became partially autobleached after several days in the dark at 4°C. Absorption spectra of these samples showed, in addition to the 605-nm peak, a second peak at ~380 nm characteristic of free retinal. All further experiments were therefore done with 1–2 wk digested samples which did not show any degradation with storage. It is interesting to note, however, that samples of PM that were first cross-linked with DMA and then digested for 6 wk did not show this tendency to autobleach. PM treated with DMA alone showed no visible change from the native form, but proved to be more susceptible to deionization after repeated washes.

Linear dichroism

Linear dichroism studies detected little, if any, difference in retinal orientation. The polyene chains were calculated to have angles of 21.2, 21.5, and 20.6° from the membrane plane for native, cross-linked, and long-term papain-treated membranes, respectively. The oxime form of the retinals in bleached membranes appear to have an average orientation of 38.8° from the membrane plane.

CD of native and perturbed samples

CD studies in the visible region from 700–300 nm demonstrated no significant differences between the native, papain-treated, and cross-linked samples. The curve for

the papain-treated sample was characteristic of the dark adapted form of PM. Papain-treated PM was found to dark adapt much faster than native PM, therefore making measurement of the CD for light adapted papain-treated PM impractical. In the near-UV region from 300–250 nm, the modified samples had spectra identical to the native samples, indicating that there were no significant changes in the local environments of the aromatic amino acids after cross-linking or papain digestion.

The far-UV CD spectra for native, cross-linked, and papain-treated PM solutions were essentially similar, indicating that no significant changes in secondary structure had occurred after modification. The papain-treated samples had greater amplitudes for the positive band centered at ~193 nm. Long-term cleavage of PM with papain results in the loss of various interhelical polypeptide segments, as well as the COOH- and NH_2-terminal tails. These can be expected to give spectrum of an aperiodic protein which is basically a single negative band at ~196 nm (29). The loss of these segments should, therefore, result in an increase in the positive band at 193 nm. To check this hypothesis, published values from CD curves for proteins with aperiodic and α-helical secondary structures (30) were used to calculate the change in the CD spectrum which would result if a protein which was 80% α-helix and 20% aperiodic were to have the aperiodic moiety removed. The match between the resulting curve (not shown) and the papain-treated CD curve was excellent.

Far-UV CD studies of oriented films are quite informative because of their extreme sensitivity to changes in the tertiary structure of α-helical proteins. In solution, such proteins appear to demonstrate three rotatory bands in the region from 250 to 190 nm (29). A positive band at ~193 nm and a negative one at ~206 nm are due to the three allowed excitonic amide π-π^* (NV_1) transitions, resulting in moments polarized perpendicular and parallel to the helix axes, respectively. Polarization of the amide n-π^* transition responsible for the negative band at ~225 nm of α-helical protein is not well established, but this band seems to show little change with orientation in the case of the bR in PM (11). These three bands are apparent in solution CD spectra (see, for example, Fig. 2 *C*). In the case of the PM films, however, alignment of the membrane patches parallel to the quartz plate results in the protein helical segments being oriented parallel to the measuring light beam. Therefore, only transitions perpendicular to the helical axes can occur. This analysis is confirmed by the film spectrum of native PM (Fig. 2 *A*), which clearly lacks the parallel polarized band which is centered in the PM spectrum at ~207 nm (11).

Film CD spectra of the modified samples showed no change except that the papain-treated membranes consistently gave a greater peak at 197 nm (compare Fig. 2, *A* and *B*). As with the solution spectra, this is thought to be due to the loss of aperiodic conformation. The absence of a band at ~207 nm for the cross-linked samples indicates that the DMA molecules are inserted without perturbing the orientation of the helical segments. Its absence for the papain-treated samples shows that the COOH-terminal tail, as well as various interhelical connecting segments, are not required to maintain the helices in their native orientation. To summarize, CD spectra from 700 to 182 nm demonstrate that the chemical modifications have virtually no effect on the intramembrane protein structure of the bR or the supramolecular structure of the PM.

CD of bleached PM

Similar studies were next conducted on the above samples after bleaching with hydroxylamine. Previous studies with bleached PM (5) and with PM in the M_{412} photointermediate state (7) have shown the appearance of contributions from a negative band centered at ~207 nm in the film CD spectra. This has been interpreted in both cases as being due to the tilting of one or more helical segments of the protein, thus allowing the components of the transition dipole moments polarized parallel to the helical axes to interact with the incident light. The connection between this tilting and the pumping of protons across the membrane is unclear, but the possibility exists that the tilting of helical segments may be either the cause or the result of transient channel formation. It is worth noting here that vesicles containing bleached membranes have been shown to be leaky to protons in contrast to vesicles containing only native membranes (31, 32). In addition, this phenomenon can be reversed by reconstituting the bleached membranes with retinal.

Visible-CD solution spectra for the bleached forms of native, cross-linked, and papain-treated PM were identical. They consisted of a single positive band at ~365 nm which is the absorption maximum for retinaloxime. The loss of the characteristic biphasic exciton band is due to the elimination of the native order of the retinals in the PM crystalline lattice so that their transitions become decoupled (5). The near-UV CD spectra for the bleached membranes were also alike. They all demonstrated a similar large change from the unbleached spectra. Such a change has been previously interpreted as indicative of alterations of the local environments of the aromatic amino acids in a manner that results in the loss of the native structural anisotropism of the PM with respect to the in- and out-of-plane directions (5). However, the similarity among the bleached samples suggests that modification does not further enhance these changes. Far-UV solution spectra showed no change from the

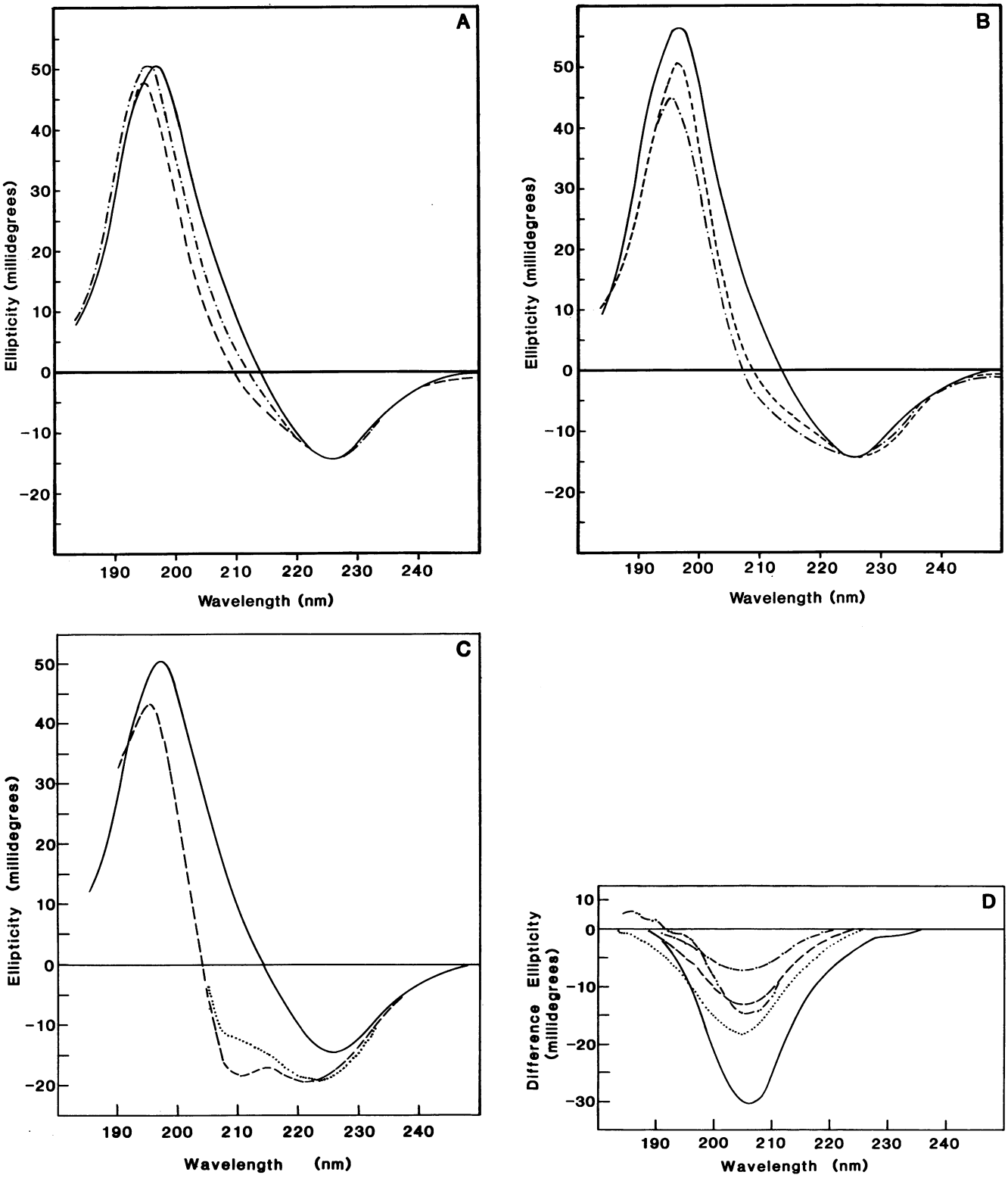

FIGURE 2 Far-UV CD film spectra of PM. All spectra are normalized to an OD_{193} of 1.0. (*A*) Native (——), bleached (—·—), and cross-linked then bleached (---). (*B*) Papain-treated (——), papain-treated (1 wk) then bleached (---), and papain-treated (2 wk) then bleached (—·—). (*C*) Native (——), and ethanol-treated with addition of a drop of ethanol (---). A PM solution spectrum (···), normalized to match the ethanol-treated film at 222 nm, is also included for comparison purposes. (*D*) Difference spectra to demonstrate the magnitude of the bands at ~207 nm of variously perturbed PM samples in *A*, *B*, and *C* after bleaching: native (—·—), cross-linked (---), papain-treated (1 wk) (—··—), and papain-treated (2 wk) (···). Film difference spectrum for ethanol-treated PM (——), which provides a 90° tilt standard for calculating tilt angles of variously perturbed PM samples. (See text for details.)

unbleached samples, indicating that no detectable change in secondary structure had occurred. However, important changes were evident in the far-UV film spectra. Whereas modification of the PM did not change the cross-over point at ~214 nm before bleaching, modification followed by bleaching resulted in a shift of the cross-over points to shorter wavelengths (Fig 2, *A* and *B*). Difference spectra for bleached and unbleached films of the various preparations showed increasingly large negative bands at ~207 nm for native, cross-linked, and long-term papain-treated samples after bleaching (Fig. 2 *D*). Samples treated with papain for only 2 h behaved like native PM, indicating that removal of the COOH-terminal tail alone has no effect. As discussed earlier, the appearance of a band at 207 nm in the protein's film spectrum can only be explained as being caused by the orientation of one or more helical segments tilting at an angle in respect to the incident light.

Estimation of the degree of tilting

In an effort to estimate the extent of tilting represented by these 207-nm bands, experiments were performed in which the far-UV CD spectra of PM films were measured both before and after addition of a drop of ethanol or acetone (Fig. 2 *C*). These solvents disrupt the crystalline structure of the PM by solubilizing the lipids, therefore causing the helical segments to lose their parallel orientation with respect to the membrane normal (7, 10, 11). For the purpose of this estimation, the helices were assumed to be oriented at random after addition of solvent. This assumption appears to be valid, because the ethanol-treated film spectrum is very similar to the native solution when the two spectra are normalized at 222 nm (Fig. 2 *C*). The similarity also demonstrates that ethanol treatment had no lasting effect on the protein secondary structure. In addition, IR linear dichroism studies of the solvent-treated films indicated that the average segmental tilt-angle was 53 ± 2°, which is in excellent agreement with the theoretical expected value of 54.7° for the completely randomized helix. A detailed description of this procedure was previously given (11). The difference spectrum for ethanol-treated films was found to be a single Gaussian band centered at ~207 nm (Fig. 2 *C*). A random orientation can be represented as one-third of the helical segments being oriented parallel to the incident light and two-thirds being perpendicular to it due to the symmetry of the helix (33). Because transitions parallel to the direction of the incident light cannot occur, this band represents two-thirds of the ellipticity which would be observed if all segments were to tilt 90°. Multiplying the magnitude of this 207-nm band by 1.5 therefore provides a 90° tilt standard with which to compare the various bleached films. These films should show 207-nm bands in which the magnitudes differ from the corrected ethanol difference spectrum by a factor of $\sin^2 \alpha$, where α is the angle between the helical segments and the membrane normal which is parallel to the direction of the incident light, because $\sin^2 \alpha$ must vary linearly with the magnitudes of the 207-nm band according to the formalism of the exciton theory of α-helical polypeptides (33). Recently, this linearity has been demonstrated in the spectra of the oriented α-helical 20-mer peptide alamethicin in phospholipid multibilayer liquid crystals for α values varying from 0 to 45° (34). Based on this analysis, an average α of 24, 32, 35, and 39° can be estimated for the native, cross-linked, 1- and 2-wk papain-treated bleached films, respectively, assuming that all seven helical segments of the protein tilt. If fewer than seven segments are assumed to tilt, then it is clear that the ones which do tilt must move through greater angles than those calculated above. A minimum of two segments would have to tilt to produce the 207-nm band that occurs in the case of bleached, native films; a minimum of three segments would have to tilt in the other cases.

In situ bleaching

At this point it is worth mentioning that an alternate explanation to the tilting hypothesis to account for the presence of the 207-nm bands may be mosaic spread due to the very remote possibility that bleached membranes may not lie as flat as native ones when dried in a film. To check this possibility, PM films were also bleached in situ by exposing them to hydroxylamine and intense light (data not shown). Membranes bleached in this manner showed the appearance of a band at ~207 nm, similar to the ones found for solution-bleached films. However, its magnitude suggested an average helix tilt of 19° rather than 24°. Films exposed to hydroxylamine but kept in the dark showed no change. From these results, it is concluded first that hydroxylamine alone does not alter the flatness of the PM discs, and secondly, that bleaching must involve microscopic changes within the membrane discs which do not appreciably alter their macroscopic shape. The close packed nature of the films would not allow major three-dimensional changes without a large input of energy to overcome the electrostatic interactions between membranes. It is a well-known fact that once the membranes have aggregated in solution they can only be separated by extensive sonication.

DISCUSSION

It is important to note that the tilting observed in films bleached in situ, as estimated from the magnitude of the resultant 207-nm band, was less than in ones made from

PM bleached in solution (19° vs. 24°). Therefore, it would appear that the many interactions present in a multilayered film may constrain the movement of helical segments after bleaching. This observation has important implications for the interpretation of results of experiments involving the formation of the M_{412} intermediate in films. Estimates of degree of tilting involved in the bR_{568} to M_{412} transition in films, using such techniques as far-UV CD and FTIR of films, may be low due to constraints on movement of the helices (7, 35–37).

The greater average tilting, after bleaching, of both cross-linked and papain-treated films are most likely due to different phenomena. In the case of the cross-linked films, it could be due either to a greater tilting of the segments that normally tilt on bleaching of the native PM, or to a "domino effect" in which segments normally unaffected by bleaching of the native PM are pushed or pulled to a tilted position by neighboring helices to which they are cross-linked. It is possible that the covalent bonding of DMA to the lysine residues can result in a reduction of the conformational stability of the PM without altering the native conformation of the PM. This could result in a greater helical motion during bleaching. On the other hand, the "push-pull" mechanism may be more reasonable depending on the location of the lysine residues. Cross-linking with the DMA would largely serve to couple the cytoplasmic ends of various helical segments. This arrangement would permit the transfer of movement of one helical segment to its neighbors without immobilizing them with numerious cross-links, as is presumable the case with glutaraldehyde.

Although the locations of various amino acids within the bR molecule have not yet been conclusively established, model A of Agard and Stroud (38) appears to be a promising conformation for bR on the basis of work done by a number of laboratories (19, 39–42). It is reproduced in Fig. 3. Using the method of mapping radii of interaction for lysines plus DMA (19) and taking into account current models for the locations of lysines relative to the membrane surface (26), intramolecular cross-links are possible between Lys-30 (helix A) and -40 or -41 (helix B), Lys-159 (helix E) and -172 (helix F), and Lys-172 and -40. Additionally, Lys-159 could bind to Lys-30 or -40 of an adajacent molecule of the trimer, and Lys-172 and -30 could bind to their counterparts on adjacent trimers.

A protease experiment was performed in an effort to reduce these possibilities. Chymotrypsin is known to cleave bR between Phe-71 and Gly-72 into two polypeptide fragments (17), C-1 (amino acids 72–248) and C-2 (1–71). If cross-links existed between Lys-172 and Lys-40, then the cleavage with chymotrypsin would not break the molecule into two fragments. This is easily monitored using SDS polyacrylamide gel electrophoresis. No bands

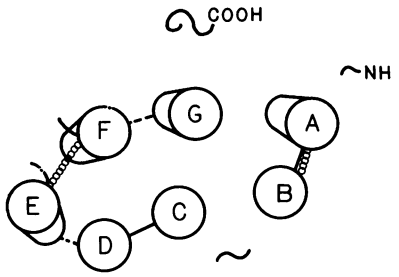

FIGURE 3 The most likely arrangement of the helical segments of bR after a drawing by Agard and Stroud (38). The molecule is viewed from the cytoplasmic side. Solid lines between helices represent linking regions on the cytoplasmic side whereas dotted lines are linking regions on the extracellular side. Chains represent likely cross-linking sites whereas gaps represent sites of proteolysis.

corresponding to the intact protein were found after cleavage of cross-linked PM with chymotrypsin for periods of 24–72 h at 37°C. The most prominent band had an apparent molecular weight of ~19,200 D, which is close to that expected for the C-1 fragment. Furthermore, a fluorescamine assay analysis has indicated that between four and five lysines in a given bR molecule react with DMA (18). In addition, most reactions lead to intramolecular cross-links, as evidenced by the small percentage (<10%) of dimers and trimers formed. Therefore, it appears that the predominant links formed are between Lys-172 and -159 and Lys-30 and -40 or -41 (see Fig. 3). It should be noted that there are 12 amino acids between Lys-159 and Lys-172, a large enough number to allow their respective helical segments to move somewhat independently of each other in the absence of some other binding force. In view of this, cross-linking of Lys-159 and Lys-172 (helices E and F) seems the most likely cause for the increase in tilt after bleaching of cross-linked PM. It is interesting to note that in a recent electron diffraction map, helix F appears to be either tilted or distorted (43). Distortion is possible because this helix contains a proline at position 186. However, it is also possible that the relatively severe experimental conditions under which the electron diffraction maps are made may have caused this helix to tilt (10, 43–46). These experimental conditions may also explain why the diffraction methodology has failed to detect any tertiary structural changes of the bR during the bR_{568}-to-M_{412} transformation contrary to the findings of the far-UV CD methodology (7, 44).

The effect of long-term papain digestion is opposite to that of cross-linking in that it decouples the ends of the helical segments at several points, specifically, between helices B and C and between helices E and F. Therefore, the bleaching results for papain-treated PM are probably due to the tilting segments being less constrained by adjoining helices and thus being able to tilt farther. The

fact that the two-hour papain-treated PM in which the COOH-terminal tail of the bR is eliminated showed no change in tilt on bleaching indicates that this cannot significantly contribute to the structural stability of the molecule. However, with proteins such as visual rhodopsin, in which ~50% of the mass of the protein is located outside the lipid bilayer rather than ~20% as in the bR, this may not be the case (47). The role of the COOH-terminal tail in the structure and function of bR is still the subject of debate. Whereas Govindjee et al. (27) find differences in proton pumping in certain vesicles after papain cleavage of the COOH-terminal tail, studies by others (26, 48) suggest that the tail does not affect bR function except in that its absence allows back-to-back aggregation of membrane sheets. This could lead to liposomes that pump in both directions and thus explain the results of Govindjee et al. (27).

The near-UV CD results for the bleached samples can now be reexamined in light of the far-UV film results. The fact that there is no difference between the samples suggests that there is no further change in the local environments of the aromatic amino acids. In view of the tilting hypothesis, this implies that the exposure of the interior of the molecule to the aqueous environment is an all-or-none phenomenon. This view is supported by experiments in which ethanol was added dropwise to a PM solution until the suspension changed from purple to yellow, indicating that the molecules had opened sufficiently for the retinal-apoprotein interactions to vanish. After several washes to remove the ethanol, the CD spectrum in the near-UV region was identical to those for the bleached solutions.

Both cross-linked and long-term papain-treated samples had faster initial bleaching rates than native purple membrane (Fig. 4), whereas samples treated with papain for only 2 h showed no change in rate (not shown). In light of the results with the CD film studies, these different rates can be interpreted to mean that a greater degree of tilting in the M_{412} state results in a longer period during which the Schiff base linkage is susceptible to cleavage by hydroxylamine. It is well known that hydroxylamine can only bleach PM in the presence of intense green light. In addition, the bleaching rate can be greatly increased even in the dark by attaching hydrophobic benzene derivatives to hydroxylamine (49). These facts imply that the Schiff base exists in a hydrophobic environment in bR_{568} but becomes exposed to the aqueous phase during the photocycle. Apparently, a most likely mechanism by which this may be achieved is the tilting of certain helical segments. At any rate, it is significant that there is a direct correlation between the estimated degree of tilting and the bleaching rate.

The shapes of the curves in Fig. 4 raise some interesting possibilities concerning the interactions that occur during

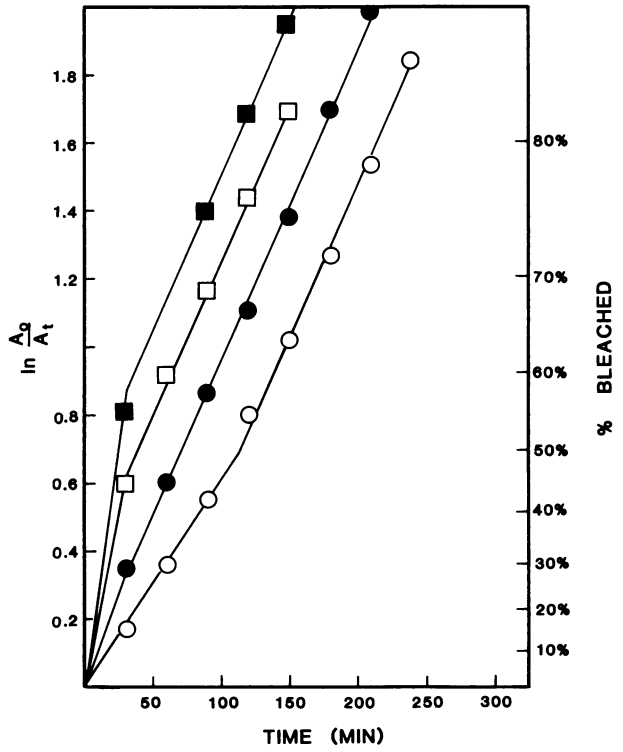

FIGURE 4 The time dependence of bleaching of PM samples. The left vertical coordinate indicates ln (A_0/A_t) where A_t is the absorbance at time t, whereas the right one indicates percent bleached on time. (○) Unmodified, ● cross-linked, □ 1 wk papain-treated, and (■) 2 wk papain-treated samples. The slopes give the rate constants for the bleaching reactions.

bleaching and presumably also during the normal photocycle. For both native and papain-treated PM there appears to be a change in the bleaching rate constant when about half of the bR molecules are bleached. Furthermore, cross-linking leads to a fairly constant bleaching rate. The curve for the native membranes suggests that there is a cooperative effect because when about half of the molecules are bleached, the rate constant increases. This can be explained if conformational changes in the bleached molecules cause a change in the forces exerted on the unbleached molecules in such a way that the latter are rendered more susceptible to bleaching. Indeed, it has been demonstrated that noncycling bR molecules undergo transient rotations induced by the initial rotation of a cycling neighbor (50). Furthermore, EPR studies have shown bleached PM lipids to have a more rigid environment than those of native PM, suggesting that the bR monomers either expand on bleaching or rotate in such a way that the trimeric units increase their surface area (51). In addition, fluorescence resonance energy transfer studies suggest a similar phenomenon during the normal PM photocycle (52). Increased suscep-

tibility to bleaching most likely results from an increase in the decay half-life of the M_{412} intermediate. This may in turn be caused by a greater degree of tilting or a decrease in the restoring forces which counteract the tilting. The curves for cross-linked and papain-treated PM can then be explained in this context. The cross-linked samples seem to demonstrate the greater bleaching rate from the beginning. It may be that cross-linking mimics the "crowding" effect of the bleached molecules on the unbleached molecules by tightly coupling certain helical segments. The papain-treated samples, on the other hand, show a faster initial rate. This is presumably caused by the greater freedom of movement of helices in the papain-treated samples, allowing for a more open conformation. Again, a point could be reached when sufficient molecules were bleached to force the remaining, unbleached molecules into a conformation characterized by the rate constant seen in the other two curves.

CONCLUSION

Analysis of the absorption and CD spectra indicates that neither cross-linking with DMA nor proteolysis with papain result in measurable changes in the intramembrane conformation of the bR. Yet these two modifications, which are essentially opposite in nature, do cause important changes in the response of the bR to light. Analysis of the bleached-film CD spectra indicates the helical segments of the modified proteins tilt more during the photocycle than native ones. This interpretation is supported by the present finding that modification also leads to an increased bleaching rate, and by the previous observations that cross-linking with DMA leads to an increase in the decay half-life of M_{412} (18, 19). Therefore, the results of both modifications of the PM is an alteration in the structural stability of the bR, but not the structure of its ground state conformation.

Possible mechanisms for proton transport by PM should be examined in light of these findings. Several models present in literature depend on the precondition of extensive hydrogen bonding between adjacent α-helical segments (53–55). Although such models cannot be entirely discounted based on these findings; nevertheless they now appear very questionable. In addition, these findings provide evidence of a more compelling nature than previously available for the ability of bR to undergo significant tertiary structural changes that may result in the tilting of several helical segments during the photocycle. The helices that tilt most likely are not extensively hydrogen bonded to those which do not tilt because of the energy required to break a large number of hydrogen bonds. The additional helix or helices that tilt after cross-linking must also not be hydrogen bonded to nontilting helices, for the same reason. Therefore, each bR molecule can be divided into three subgroups of helical segments, between which there is apparently no extensive hydrogen bonding. Additional support for this hypothesis is provided by the papain experiments, which show that the interhelix linking segments are important constraints to tilting some helices. If hydrogen bonding were an important stabilizing force, papain cleavage should make little difference in the structural stability of the protein.

On the other hand, it is clear that the retinal serves as the most important factor in stabilizing the bR molecules. The tilting seen here on hydrolysis of the retinal-lysine bond has also been seen to occur in the brown membrane, which lacks retinal due to nicotine-induced blockage of the retinal-synthesizing pathway (16). In addition, there has been evidence that the retinal greatly stabilizes the secondary structure of C-1 + C-2 fragments suspended in DMPC/cholate/SDS (56). Finally, recent work in this laboratory indicates that PM samples in which the retinal-lysine double bond has been reduced to a single bond by sodium borohydride undergo a tilting of helices after photooxidation of the retinal C=C bonds (unpublished observation). From these results one must conclude that the native structure of bR is maintained largely as a result of interactions between the retinal and the apoprotein. It appears that in the PM, the retinal regulates the tertiary rather than secondary structure of the bR. Despite the crystalline nature of the membranes, these tertiary structure changes can be increased by cross-linking or papain digestion, suggesting that the protein tertiary structure is extremely susceptible to alteration by the retinal molecule. Other studies done in this laboratory indicate that the surrounding lipids may also have an important effect on the protein tertiary structure (9). It now appears that the predominant stabilizing forces in PM must be the hydrophobic interactions between the protein and retinal plus the surrounding lipids. Hydrogen bonding, on the other hand, seems to play a lesser role that was previously thought.

It is clear that the results presented here tend to favor models for bR function in which protein structural metastability, rather than rigidity, is an essential factor. Certainly the proteinquake, or deformation wave, model proposed by this laboratory falls into this category. It should be mentioned, however, that transient tilting of the helices could also lead to the formation of temporary proton wires. Indeed, this could be accomplished by sequentially joining short segments of a "wire" as the deformation wave travels through the proton. This would be analogous to a system of locks in a canal and would provide directionality to the proton movement.

Attempts to verify the existence of a proton wire have been unsuccessful so far (57, 58). Indeed, the discovery of the importance of Pro-186 to bR function (58) once again

argues for the importance of a metastable protein tertiary structure. In addition, the proton wire model does not provide a ready explanation for the ability of halorhodopsin to transport chloride ions rather than protons although it is structurally very similar to bR (59, 60). In addition, Rothschild et al. (61) used difference FTIR spectra to conclude that the evidence supported the existence of some common elements in the molecular mechanisms of the active transport by these two proteins. For the present, therefore, evidence would seem to favor the transport of protons through transient channels by deformation waves or proteinquakes resulting from global tertiary structure changes during the bR photocycle. This model could easily be altered to accommodate other substrates transported by other transmembrane proteins.

Received for publication 6 March 1989 and in final form 9 June 1989.

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