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Ultraviolet and Visible Absorption Spectra of the Purple Membrane Protein and the Photocycle Intermediates†

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ABSTRACT: The visible and ultraviolet absorption spectra of purple membrane as well as the K, L, and M intermediates of the purple membrane photocycle are reported. The long wavelength absorbance by K (λ_{max} = 628 nm) and L (λ_{max} = 547 nm) strongly suggests that the Schiff base bond between retinal and the purple membrane protein is protonated while the position of the 412-nm band of M suggests that the Schiff base of this intermediate is unprotonated. The extinction of purple membrane at 280 nm is estimated to be ca. 75 000 L cm^{-1} mol^{-1} of which ca. 51 000 can be attributed to individual amino acids (in a hydrophilic environment), ca. 8000 due to retinal's absorption at 280 nm, and ca. 16 000 due to environmental (largely hydrophobic) effects on the aromatic amino acid transitions. There are very large decreases in the near-ultraviolet spectra on conversion to the photocycle intermediates L and M and on bleaching in hydroxylamine; their difference spectra have the shape of the protein itself. Decreasing the polarity of the membrane environment results in a decrease in the extinction changes. These results suggest that ca. 60% of the tryptophans and tyrosines in the protein move from a hydrophobic (interior) environment to a hydrophilic (exterior) environment either on bleaching or on conversion of purple membrane to L or M. Additional evidence of conformational changes during the photocycle includes the inability of hydroxylamine to bleach purple membrane except during the photocycle and the fact that when L or M is irradiated at −196 °C, a temperature at which protein conformational changes probably do not take place, they cannot be converted back to purple membrane until warmed to −100 °C. The conformational changes seen on photoconversion to L and M may well be involved in the mechanism of proton transport across the purple membrane which occurs on illumination.

The purple-colored membrane of *Halobacterium halobium* contains retinal bound to a single protein species by a Schiff base bond (Oesterhelt & Stoeckenius, 1971). When a photon is absorbed by a light-adapted purple membrane protein, a photochemical reaction cycle through a set of intermediates is initiated ending with the pigment returning to its original form (Lozier et al., 1975). The first three intermediates in the cycle are called K, L, and M. Accompanying this cycle is the pumping of protons across the purple membrane (Oesterhelt & Stoeckenius, 1973). Although the actual mechanism of the transfer of protons across the membrane is not known, reversible conformational changes in the protein during the purple membrane photocycle (possibly induced by retinal isomerization) may be involved.

In this study, new evidence of protein conformation changes in purple membrane on bleaching (chromophore loss) is discussed. This evidence includes an ultraviolet difference spectrum indicating large changes in extinction and resembling the shape of the protein absorption spectrum itself. A decrease in the difference spectrum extinction results when the polarity of the membrane environment is reduced indicating that buried aromatic amino acids are more exposed to the external polar media on bleaching. In addition, accurate visible absorption spectra of the purple membrane photocycle intermediates K, L, and M have been determined. The near-ultraviolet absorption and difference spectra of purple membrane and the K, L, and M intermediates have also been measured and indicate significant protein conformational change on conversion of purple membrane to L and M but not to K. Thus, it may be that the purple membrane photocycle effects the transfer of protons across the cell membrane by means of conformational changes in the purple membrane protein.

Materials and Methods

Cultures of *Halobacterium halobium* R_1 were grown and the purple membrane purified according to the procedures of Becher & Cassim (1975). The samples were buffered at pH 7.0 with a 0.02 M potassium phosphate buffer. Purple membrane samples were also placed in a buffered solution of 25% (w/w) NaCl with 1% Ammonyx LO detergent (65% dodecyldimethylamine oxide and 35% tetradecyldimethylamine oxide, Onyx Chemical Co.) to decrease light-scattering (Figure 1, curve 2).

A Cary 118 spectrophotometer was used to record all absorption spectra and difference spectra. Optically matched 0.2-cm pathlength quartz cells were used to record all absorption and difference spectra; these short pathlength cells were fixed within 2 cm of the photomultiplier tube in order to reduce light scattering artifacts by increasing the percentage of scattered light entering the photomultiplier.

The retinal chromophore of the purple membrane was removed by irradiating samples which were suspended in 0.5 M hydroxylamine titrated to pH 7.0 with 1 M NaOH immediately before sample irradiation. The suspensions were placed

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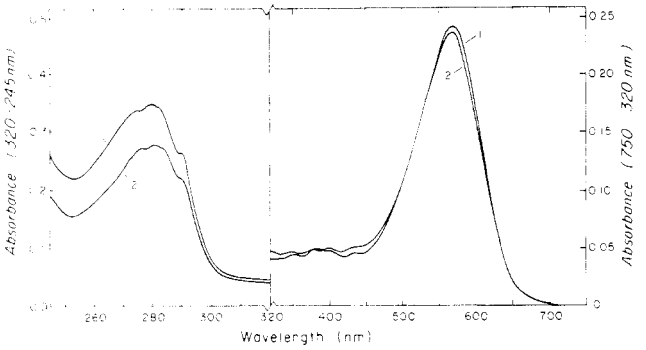

FIGURE 1: Absorption spectra of light-adapted, sonicated purple membrane in 0.02 M phosphate buffer, pH 7.0 (curve 1), with 1% Ammonyx LO and 25% NaCl (w/w) (curve 2). Cell pathlength, 0.2 cm.

in a square 4.5-cm glass bottle, stirred constantly, and bleached by intense irradiation (slide projectors with 500-W lamps) on two sides filtered through 5.5 cm of circulating tap water, 3.2 cm of 2.2% $CuSO_4$ solution, and a Corning glass filter 3-68. The light intensity on the suspensions was ca. 200 mW/cm^2. The suspensions completely bleached to form retinaloxime in approximately 1 h. The samples were then repeatedly washed to remove hydroxylamine. Retinaloxime, which remains bound to the bleached membrane, was then removed as previously described (Tokunaga & Ebrey, 1978).

Hydroxylamine-bleached and retinaloxime-extracted purple membrane samples (pH 7.0, 0.02 M phosphate buffer) were regenerated by addition of freshly prepared (<24 h) *all-trans*-retinal (retinal 1, Sigma Chemical Co.; retinal 2, gift of Hoffman-Laroche) in ethanol. Typically 10 μL of 5×10^{-3} M retinal solution was used to regenerate 3 mL of bleached membrane with a potential 568-nm absorbance of approximately 1.0 in a 1-cm pathlength. (Test samples of bleached membrane were first regenerated by sequential additions of 1 μL of retinal followed by absorption measurements so that excess retinal was not added to the later samples.) Two bleached-and-extracted membrane samples of equal volume were routinely used. *all-trans*-Retinal in ethanol was added to one sample in the dark and an identical quantity of ethanol alone was added to the reference sample. Both samples were then incubated in the dark at 10 °C for 24 h. The samples were then light-exposed and their absorption and difference spectra recorded (Figure 2). Bleached-and-extracted samples were more than 90% regenerated as indicated by absorption spectra.

The effect of medium polarity on the difference absorption spectra of bleached-extracted and regenerated membrane was studied by suspension of the membrane samples (pH 7.0, 0.02 M phosphate buffer) in glycerol and sucrose as well as in water (Figure 3). Identical volumes and concentrations of bleached-extracted and regenerated membrane were prepared so that final concentrations were 67% or 33% (v/v) glycerol (certified A.C.S. Fisher Scientific Co.) or 50% or 25% (w/w) recrystallized sucrose (Beckman Instruments, Inc.).

In order to study purple membrane conversion to its photointermediates K and L, native purple membrane (membrane which had never been bleached) was suspended in a 2:1 glycerol-water (0.02 M phosphate buffer, pH 7.0) solution to prevent cracks in the sample at low temperatures. For the study of the M intermediate, purple membrane was first suspended in a 25% NaCl solution titrated to pH 10 with 0.1 M NaOH and then mixed with 2 parts of glycerol. Under these conditions, the lifetime of the M intermediate greatly increases allowing accurate spectral measurements (see Becher & Ebrey, 1977).

Low temperature visible and ultraviolet absorption spectra of the K, L, and M intermediates (Figures 4–6) were recorded using either a Pyrex or quartz Dewar flask. For the measurements of the conversion of purple membrane to the K intermediate, purple membrane in a 2:1 glycerol–buffer medium was placed in a stoppered 0.2-cm quartz cell, fully light adapted, and then, in the dark, was lowered into an ethanol bath previously cooled to −100 °C with liquid nitrogen. The cell was then removed from the ethanol bath and, after rapidly blowing off any ethanol remaining on the cell surface with a burst of nitrogen gas, lowered into the Dewar filled with liquid nitrogen. In this manner, the absorption measurements of the sample were not significantly hampered by cracking of the sample on lowering its temperature to −196 °C. Furthermore, this technique prevented a potential artifact, examined in the Results section, which occurs when the sample is directly cooled to −196 °C with liquid nitrogen. light-adapted purple membrane was partially converted to the steady-state mixture of the K intermediate by 500-nm light (interference filter, 12-nm bandwidth) employing the same light source (500-W projector) previously described. The resulting K and purple membrane mixture could be completely converted back to purple membrane with 700-nm light (interference filter, 12-nm band width). All illumination was done with the sample and the Dewar in place in the spectrophotometer by use of a mirror arrangement.

Measurement of the partial conversion of purple membrane to the L intermediate at −100 °C was similarly made. A 0.2-cm quartz cell filled with light-adapted purple membrane in a 2:1 glycerol–buffer medium was lowered, in the dark, into the Dewar flask filled with ethanol cooled to −100 °C. Illumination of the sample was made as described above except that a 640-nm interference filter (12-nm bandwidth) was used in photoconverting the purple membrane to the L intermediate which remains stable at −100 °C. The resulting L and purple membrane photostationary state mixture was partially converted back to purple membrane with 500-nm illumination (interference filter, 12-nm bandwidth). Finally, complete conversion to the M intermediate was made at −40 °C by cooling the ethanol in the Dewar flask to this temperature and by exposure to 600-nm light (interference filter, 12-nm bandwidth). The membrane could be converted back to purple membrane with 420-nm light (interference filter, 12-nm bandwidth). The temperature in the Dewar flask was found to increase less than 5 °C during the time used in measuring either the L or M intermediates.

Results

The Visible Absorption Spectra of Purple Membrane. The visible and near-ultraviolet absorption spectrum of purple membrane in the light-adapted state is shown in Figure 1, curve 1. The visible spectrum is dominated by an intense band at 568 nm with extinction of approximately 63 000 L cm^{-1} mol^{-1} (Oesterhelt & Hess, 1973) having a bandwidth of 3200 cm^{-1}. Curve 2 is the absorption spectrum of purple membrane in 1% Ammonyx LO detergent with 25% (w/w) NaCl. Although the purple membrane pigment in Ammonyx LO without NaCl bleaches, the pigment is stabilized by NaCl and the visible absorption spectrum is essentially unaffected. The effect of the detergent on the protein (near-ultraviolet spectrum) is discussed below.

The Ultraviolet Absorption Spectra of Purple Membrane. The near-ultraviolet absorption spectrum (320–245 nm) of light-adapted purple membrane is shown in Figure 1, curve 1. The absorption spectrum includes distinct maxima or shoulders at 290, 280, and 274 nm, which are primarily attributed to the

π-π^* transitions of the amino acids tryptophan and tyrosine. Cystine is not found in purple membrane protein. A previously reported amino acid analysis of purple membrane protein indicated 4 mol of tryptophan and 11 mol of tyrosine per mol of purple membrane protein (Bridgen & Walker, 1976). However, Keefer & Bradshaw (1977) found 7 tryptophans and 10 tyrosines per protein; moreover, their preliminary amino acid sequencing studies indicated the existence of more than four tryptophans. The apparent molar extinction coefficient in water of purple membrane at 280 nm is 93 000 L cm^{-1} mol^{-1} based on a value of 63 000 L cm^{-1} mol^{-1} at 568 nm (Figure 1, curve 1). In 1% Ammonyx LO with 25% NaCl, the apparent extinction is lowered to about 74 000 L cm^{-1} mol^{-1} (Figure 1, curve 2). Based on the Keefer–Bradshaw amino acid composition of the protein, and the extinction coefficients of free tryptophan and tyrosine, at pH 7.1 in water (Mihalyi, 1969), the extinction at 280 nm directly attributable to individual amino acids is no more than 51 000.

The large discrepancy between the extinction values of the membrane in either Ammonyx LO or water and that of the individual amino acids is attributable to several factors: (1) Light scattering in the membrane suspension would be expected to significantly raise the apparent ultraviolet absorbance (discussed below). The smaller extinction observed in Ammonyx LO with NaCl is probably largely due to lower light scattering. (2) Minor transitions of retinal chromophore also contribute to the absorption spectrum of the pigment in the near ultraviolet region. Based on free retinal protonated Schiff base transitions (Erickson & Blatz, 1968), a contribution of 8000 ± 2000 L cm^{-1} mol^{-1} to the extinction of 280 nm is expected. (3) The secondary, tertiary, or quaternary structure of the purple membrane protein may alter the environment of the π-π^* transitions of the aromatic amino acids relative to free amino acids from which the extinction coefficients are estimated. In particular, side chains "buried" within the nonpolar environment of the protein would be expected to have appreciably higher extinctions than those of free amino acids (Donovan, 1969). (4) The structure of the protein may also give rise to interactions between aromatic amino acids including coupling of electronic transitions of equal energy (exciton splitting) and of unequal energy (hyperchromism). Both of these interactions can result in an increase in absorbance relative to free amino acids in this wavelength region. The possibility that dipole coupling between transitions of retinal and the aromatic amino acids might significantly modify extinctions has been excluded (Becher & Cassim, 1977).

Difference Absorption Spectra of Bleached and Regenerated Purple Membrane. Figure 2 shows the near-ultraviolet absorption spectrum of hydroxylamine-bleached and retinaloxime-extracted membrane before (curve 1) and after (curve 2) regeneration by addition of *all-trans*-retinal 1. The difference spectrum is shown for the visible and near-ultraviolet wavelength regions (curve 3). The near-ultraviolet spectrum of regenerated membrane has the same features as the spectrum of native purple membrane including maxima or shoulders at 290, 280, and 274 nm. The near-ultraviolet difference spectrum demonstrates that the extinction at 280 nm of bleached-and-extracted membrane increases by 19 000 L cm^{-1} mol^{-1} on regeneration ($\Delta\epsilon_{280}$ = +19 000), a very large change. The shape of the ultraviolet difference spectrum is very similar to that of the membrane itself, including extrema or shoulders at 290, 280, 270 nm and a minimum at 260 nm. This suggests that the increase in the near-ultraviolet spectrum upon regeneration is largely the result of an enhancement of the absorption bands of the aromatic amino acids at these wavelengths. Although retinal transitions almost certainly contribute 8000 ± 2000 L cm^{-1} mol^{-1} to the extinction in the near-ultraviolet region, these bands would be expected to be relatively unstructured. An exception is the shoulder at 274 nm which can be assigned to the chromophore (see retinal 2 section below). Consequently, both the intensity and shape of the difference spectrum rule out retinal transitions as a significant contributor to the difference spectrum.

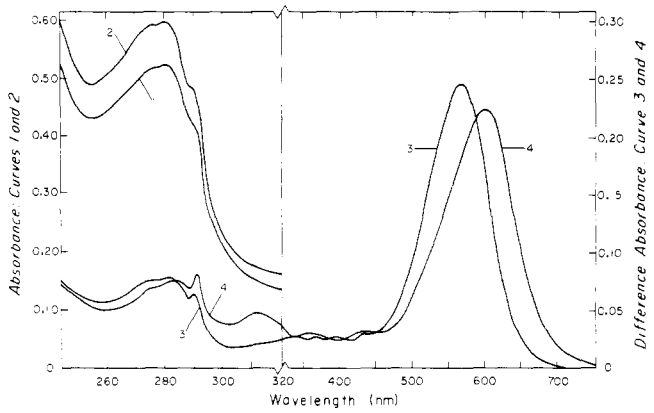

FIGURE 2: Absorption spectra of regeneration of bleached-extracted purple membrane in water (0.02 M phosphate buffer, pH 7.0). (Curve 1) Near-ultraviolet spectrum of bleached-extracted purple membrane. (Curve 2) Near-ultraviolet spectrum of bleached-extracted purple membrane after complete regeneration with *all-trans*-retinal 1. (Curve 3) Visible and near-ultraviolet difference spectra of retinal 1-regenerated purple membrane minus bleached-extracted membrane. (Curve 4) As curve 3, but regenerated with *all-trans*-retinal 2. Cell pathlength, 0.2 cm.

The increased extinction of the absorption bands of the aromatic amino acids upon regeneration of purple membrane almost certainly occurs as a result of conformational changes in the protein. Conformational changes could expose tryptophans and tyrosines in the relatively nonpolar protein environment to the more polar (water) media on bleaching resulting in the loss of the extinction. In addition, conformational changes on bleaching may alter the relative orientation of originally interacting aromatic amino acid transitions resulting in a loss of hyperchromism.

Estimating a retinal contribution to the difference in extinction at 280 nm of 8000 L cm^{-1} mol^{-1}, then a large (19 000 − 8000 = 11 000) difference in the extinction may be attributed to conformational change. If the 7 tryptophans and 10 tyrosines were in a totally polar (water) environment, their combined extinction would be ca. 51 000 (7 × 5560 + 10 × 1200 = 50 920) (from Mihalyi, 1969); if they were in a totally nonpolar (protein interior) environment, their extinction would be increased by ca. 18 000 (7 × 1600 + 10 × 700 = 18 200) (from Donovan, 1969) to ca. 69 000. Indeed, our best estimate (see Discussion) is that most of these amino acids are in a nonpolar environment. Therefore, the 11 000 extinction increase on regeneration would require an *average* change of about 60% in the environment of the tryptophans and tyrosines from the polar (water) exterior to the nonpolar interior. Although a loss in hyperchromism might also contribute to the large difference in extinction, we show below that change in polarity of environment is the dominant contributor to the extinction change.

Regeneration of Bleached Membrane with Retinal 2. To identify which ultraviolet transitions might be associated with the chromophore, we compared the difference spectrum for the regeneration of bleached-and-extracted membrane with retinal 1 (discussed above) with that for regeneration with retinal 2 (3-dehydroretinal). The ultraviolet absorption of the two chromophores should be at different wavelengths. The

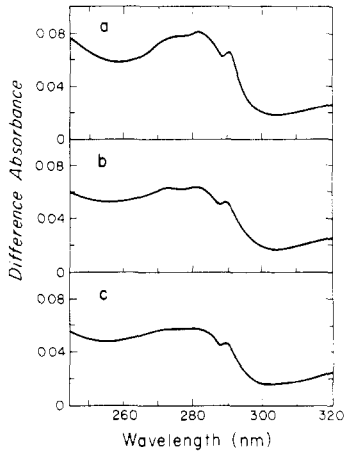

FIGURE 3: Near-ultraviolet difference absorption spectra of retinal 1-regenerated purple membrane minus bleached-extracted membrane (0.02 M phosphate buffer, pH 7.0) in water, sucrose, and glycerol. (a) In water; (b) 50% (w/w) sucrose; (c) 67% (v/v) glycerol. Cell pathlength, 0.2 cm.

retinal 2 regenerated membrane has a blue color [λ_{max} of the light-adapted form is at 603 nm (Tokunaga et al., 1977)] and the near-ultraviolet difference spectrum on regeneration has a large extinction with bands at 291, 284, and 312 nm (Figure 2, curve 4). The 291-nm band in the difference spectrum of blue membrane (associated with the tryptophan transition) is relatively larger than that of purple membrane and the 280-nm band relatively smaller. These results indicate that the regeneration to blue membrane also results in a change in the protein conformation; moreover they strongly suggest that the bands at ca. 274 nm in the retinal 1 spectrum and at ca. 312 nm in the retinal 2 spectrum are transitions of the respective chromophores.

Light-Scattering Artifacts. The possibility that changes in light scattering may induce artifacts in the near-ultraviolet difference spectra of bleached-extracted and regenerated membrane suspensions, and therefore affect the reliability of these conclusions, deserves comment. Light scattering in large particles can be classified as either nonselective or selective scattering. Nonselective scattering occurs without absorption of light and has a relatively smooth, structureless wavelength dependence. Selective scattering occurs with absorption of light and has strong wavelength dependence resulting in a scatter contribution to the recorded absorption spectrum which has the shape of the true absorption spectrum but is shifted by about a half-bandwidth to longer wavelengths (Latimer & Rabinowitch, 1959). Since little or no scattering change occurs on regeneration of purple membrane or between cycle intermediates, scattering effects in the difference spectra are expected to be very small. This assumption was further justified by considering the difference spectra of bleached and regenerated membrane, and of purple membrane and the L or M intermediates (see below). For example, since the near-ultraviolet difference spectrum of bleached and regenerated purple membrane in water (Figure 2, curve 3) has predominantly the shape of the protein absorption spectrum, this demonstrates that changes in nonselective scattering are not a major contributor to the difference spectrum. In addition, since no measureable shift in λ_{max} is indicated by the difference spectrum, then no significant difference due to selective light-scattering exists between bleached and regenerated membrane. Yet another indication that the difference spectra are free from light-scattering artifacts is that the bleached/regenerated difference spectra from highly scattering, unsonicated membrane in water are the same as those of less scattering, sonicated membrane in water.

Effects of Medium Polarity on the Bleached/Regenerated Difference Spectra. One manner in which a change in the protein conformation could account for the near-ultraviolet difference spectrum of bleached and regenerated membrane is by the increased exposure of aromatic amino acid transitions to the more polar water media in bleached membrane. In order to test this hypothesis, the water environment of bleached-and-extracted membrane and of regenerated membrane was replaced by less polar ones (Donovan, 1969). These media included 67% and 33% (v/v) glycerol and 50% and 25% (w/w) sucrose. If, in fact, exposure of aromatic amino acids to a more polar environment were largely responsible for the difference spectrum of Figure 2, then replacement of water with these less polar media would reduce the intensity of the difference spectrum attributable to this protein change.

It should be pointed out here that glycerol or sucrose addition also results in a decrease in light scattering in purple membrane due to more closely matching the index of refraction of the media and the membrane (Ebrey et al., 1977). However, this will not affect the comparison of the *difference* spectra in water, sucrose, and glycerol since both the bleach-extracted and the regenerated membrane undergo similar reduction in light scattering.

Figure 3a is the ultraviolet difference spectrum of retinal 1 regeneration as described in Figure 2. However, in Figures 3b and 3c the membrane samples were placed in 50% (w/w) sucrose and 67% (v/v) glycerol. Although little or no effect is found in the visible difference spectra when these less polar media are used, the shapes of the near-ultraviolet difference spectra are flattened out and intensities significantly decreased. The difference spectra intensities at 280 nm are 19 000 L cm^{-1} mol^{-1} in water, 13 500 L cm^{-1} mol^{-1} in 67% (v/v) glycerol, and 15 500 L cm^{-1} mol^{-1} in 50% (w/w) sucrose. These results are in qualitative agreement with the concept that, in bleached-extracted membrane, tryptophans and tyrosines are more exposed to the medium than in regenerated membrane.

Any attempt to establish quantitative agreement between the experimental results and theory is restricted since the chromophore contribution to the difference spectrum can only be estimated in the near-ultraviolet wavelength region. Nevertheless, based on decreases in the extinction of aromatic amino acids on exposure to glycerol and sucrose (Donovan, 1969) and assuming a retinal contribution to the 280-nm extinction of 8000 L cm^{-1} mol^{-1}, then the difference spectra intensity at 280 nm attributable to the protein effects in water is 11 000 L cm^{-1} mol^{-1}. This protein difference intensity is predicted to drop to 6000 L cm^{-1} mol^{-1} in 67% (v/v) glycerol and to 8500 L cm^{-1} mol^{-1} in 50% (w/w) sucrose. Consequently, the total 280-nm difference spectra intensities are predicted to be 19 000 in water, 14 000 in 67% (v/v) glycerol, and 16 500 in 50% (w/w) sucrose, in good agreement with the values found.

Similar experiments using 33% (v/v) glycerol and 25% (w/w) sucrose resulted in the same flattening of the difference spectrum with approximately half the intensity losses found using 67% (v/v) glycerol and 50% (w/w) sucrose.

Visible Absorption Spectra of the Photocycle Intermediates. In order to study the intermediates in the purple membrane photocycle, light-adapted (λ_{max} = 568 nm) native membrane was suspended in 67% (v/v) glycerol to prevent sample cracking at low temperature and the absorption spectra recorded at various temperatures before and after illumination. The K, L, and M intermediates of the photocycle were studied

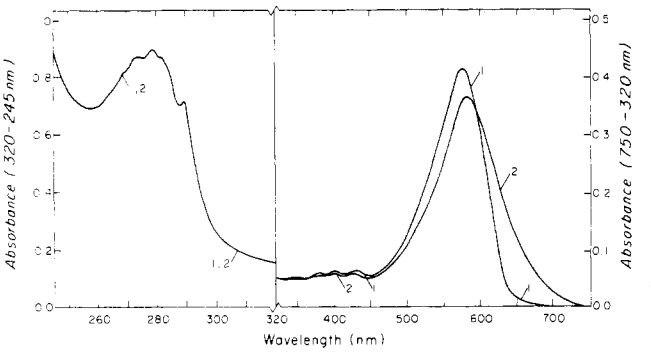

FIGURE 4: Absorption spectra of the conversion of light-adapted purple membrane (curve 1) to 28% K (curve 2) at −196 °C with 500-nm light. In 67% (v/v) glycerol and 0.02 M phosphate buffer, pH 7.0. Cell pathlength, 0.2 cm; baseline subtracted, 750–320 nm.

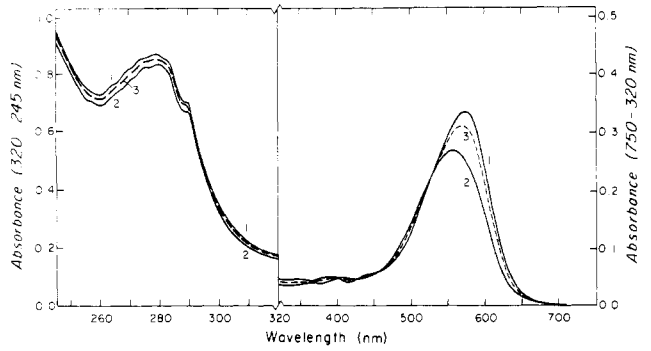

FIGURE 5: Absorption spectra of the conversion of light-adapted purple membrane (curve 1) to 65% L (curve 2) at −100 °C with 640-nm light. After conversion back to 80% PM, 20% L with 500-nm illumination (curve 3). In 67% (v/v) glycerol and 0.02 M phosphate buffer, pH 7.0. Cell pathlength, 0.2 cm; baseline subtracted, 750–320 nm.

at −196 °C, −100 °C, and −40 °C, respectively. The purple membrane is referred to as PM; its absorption maximum is shifted from 568 nm at room temperature to 573 nm at −40 °C, to 574 nm at −100 °C, and to 578 nm at −196 °C.

The visible absorption spectrum of light-adapted purple membrane at −196 °C is shown in Figure 4, curve 1. After correcting for a 7% increase in the absorbance of PM upon cooling due to contraction of the glycerol–water suspension at −196 °C, the absorbance is found to have increased by about 6%. On 500-nm illumination, a steady-state mixture with approximately 28% of the PM converted to K is formed (Hurley et al., 1977), as shown in Figure 4, curve 2. After 700-nm illumination, the spectrum was identical with that of 100% PM (curve 1) indicating complete conversion back to PM. The calculated visible absorption spectrum of 100% K is shown in Figure 7.

The absorption spectrum of purple membrane at −100 °C and its conversion to the L intermediate with 640-nm illumination are shown in Figure 5, curves 1 and 2. In these spectra, approximately 65% of the PM was converted to L. This was determined by first calculating the absorption spectrum of 100% L according to the following procedure. The steady-state mixture of PM and K at −196 °C was formed with 500-nm illumination (as described above) and the K was allowed to decay to L when the sample was warmed to −100 °C. Assuming the resulting absorption spectrum then included 28% L and 72% PM, the absorption spectrum of 100% L was calculated (Figure 7); L has an absorption maximum at 547 nm and an extinction of approximately 51 000 L cm^{-1} mol^{-1}. The photo-steady state of PM and L at −100 °C with 640-nm illumination (Figure 5, curve 2) was calculated to contain 65%

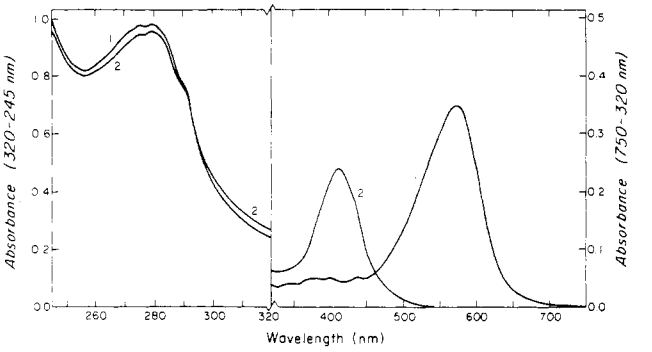

FIGURE 6: Absorption spectra of the conversion of light-adapted purple membrane (curve 1) to 100% M (curve 2) at −40 °C with 600-nm light. In 67% (v/v) glycerol. Cell pathlength, 0.2 cm; baseline subtracted, 750–320 nm. (To increase yield of M, the membrane sample was first suspended in 25% NaCl, pH 10, before glycerol addition.)

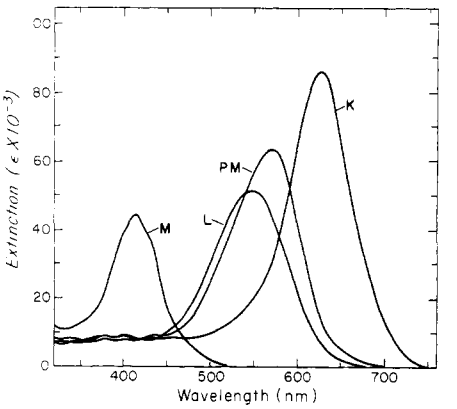

FIGURE 7: Absorption spectra of the intermediates of the purple membrane photocycle. The spectra of the intermediates after 100% conversion were calculated for K and L from Figures 4 and 5 (see text) and were directly recorded for M and purple membrane (PM). The absorption maxima were: K, 628 nm (−196 °C); L, 547 nm (−100 °C); M, 412 nm (−40 °C); PM, 568 nm (23 °C).

L. This photo-steady-state mixture could be converted back to 80% PM and 20% L with 500-nm illumination at −100 °C (Figure 5, curve 3). Complete conversion back to PM was prevented by the significant overlap of PM and L absorption.

PM could be 100% converted to the M intermediate at −40 °C with 600-nm illumination using the conditions described in Methods and Materials and in Becher & Ebrey (1977) (Figure 6). The M intermediate could be converted back to PM with 420-nm illumination at −40 °C.

Near-Ultraviolet Absorption Spectra of the Photocycle Intermediates. The near-ultraviolet absorption spectra of light-adapted purple membrane before (curve 1) and after (curve 2) 28% conversion to K, 65% conversion to L, and 100% conversion to M are shown in Figures 4, 5, and 6. In addition, the near-ultraviolet difference spectra are shown (Figure 8). Since all these spectra were recorded with the membrane in 67% glycerol, only about 60% of any potential decrease in the near-ultraviolet absorption spectra attributable to exposure of the tyrosines or tryptophans to the more polar (water) environment is seen.

The near-ultraviolet spectra of light-adapted purple membrane in 67% glycerol at −196 °C are shown in Figure 4, curve 1. The bands are considerably more intense than those recorded at room temperature due to an increase in non-selective light scatter, unavoidable with low temperature spectroscopy. Nevertheless, the −196 °C spectrum shows considerably

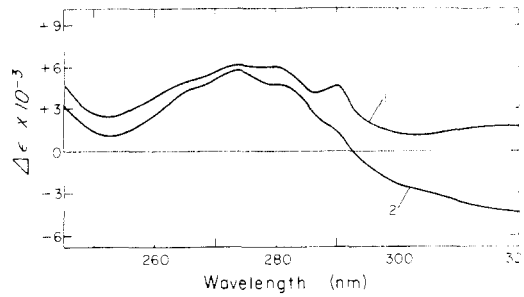

FIGURE 8: The ultraviolet difference spectra of purple membrane to L conversion at −100 °C (curve 1) and purple membrane to M conversion at −40 °C (curve 2). (From Figures 5 and 6.)

greater band resolution than that of purple membrane recorded at 23 °C (Figure 1). The 290-nm tryptophan shoulder becomes quite distinct and the maxima at 279 and 273 nm become more prominent. A small blue shift (~1 nm) is also observed. The 500-nm illumination results in a photo-steady state containing 28% K (Figure 4, curve 2). No significant change in the near-ultraviolet absorption spectrum is found ($\Delta\epsilon \leq 1000$). In these experiments we estimate that a near-ultraviolet extinction change of 1000 could not be detected on conversion to 28% K at −196 °C; this translates to a smallest possible detectable change in extinction of about 3000 on complete conversion to K.

In both the visible and ultraviolet absorption studies of PM to K conversion, special care was taken in the preparation of the sample. As described in the Methods and Materials section, the membrane sample in 67% glycerol was first cooled to −100 °C before plunging into liquid nitrogen. When, instead, the sample at room temperature was cooled directly to −196 °C, on 500-nm illumination the extinction at 280 nm was found to decrease by about 4000 L cm^{-1} mol^{-1} with lesser decreases throughout the near-ultraviolet wavelength region (spectra not shown). However, on conversion back to 100% PM with 700-nm light (and with any further conversions to K or PM) the near-ultraviolet spectra remained unchanged. In addition, in the visible wavelength region after conversion back to 100% PM the maximum at 578 nm did not return exactly to the intensity of the original PM but was about 2000 L cm^{-1} mol^{-1} less. Since these effects were not seen when the sample was precooled before dropping to −196 °C, we believe the irreversible extinction changes seen in the samples cooled directly to −196 °C were artifacts of the rapid cooling process.

In conclusion, no measureable change in the near-ultraviolet spectrum directly resulting from conversion of PM to K is found indicating (1) no protein conformation change at −196 °C (as expected) and (2) little change in absorbance due to iosmerization of the chromophore.

The near-ultraviolet absorption spectra before and after the 65% conversion by 640-nm light of PM to L at −100 °C is shown in Figure 5, curves 1 and 2. No shifting of the bands at 275, 280, and 290 nm is apparent on conversion to 65% L but the extinction at 280 nm decreases by 6000 ± 1000 L cm^{-1} mol^{-1}. This change in the ultraviolet absorption spectrum is reversible to the extent that L can be converted back to PM with 500-nm light (curve 3).

The PM to L difference spectrum (Figure 8, curve 1), like that found on bleaching purple membrane in hydroxylamine, has a shape which is similar to that of the purple membrane protein itself including maxima or shoulders at approximately 290, 280, and 270 nm. As previously argued for bleaching, this strongly suggests that a change in protein conformation occurs on conversion of PM to L. A conformational change in the protein could lead to decreased absorbance at 280 nm by exposure of the aromatic amino acids in the relatively nonpolar protein interior to the more polar (water) media. In addition, a conformational change could alter the relative orientation of originally interacting aromatic amino acid transitions resulting in a loss of hyperchromism. Isomerization of the retinal chromophore between PM and 65% L may contribute to the difference spectrum, although such a contribution is unlikely to be more than 2000 L cm^{-1} mol^{-1}.

Near-ultraviolet absorption spectra showing the complete conversion of PM to M at −40 °C with 600-nm light are shown in Figure 6, curves 1 and 2. The samples have increased light scattering due to the use of NaCl. However, the light scattering is nonselective and does not contribute to the difference spectrum (see previous discussions of scattering). A large decrease in the near-ultraviolet absorption (5000 ± 1000 L cm^{-1} mol^{-1} at 270 nm) occurs on conversion of PM to M and is nearly completely reversible with 420-nm illumination. The difference spectrum of PM to M conversion is shown in Figure 8, curve 2. Much of the potential extinction loss in the aromatic amino acid bands is offset by the tail of the 412 nm (retinal chromophore) band of the M intermediate which is estimated to contribute about 2000 L cm^{-1} mol^{-1} to the extinction of M at 290 nm but less than 1000 L cm^{-1} mol^{-1} at 270 nm. Based on previous arguments and the intensity and shape of the difference spectrum below 290 nm, a change in the conformation of the protein in going from PM to M is indicated.

Consequently, the near-ultraviolet absorption spectra of purple membrane on conversion to the L and M intermediates strongly suggest that a change in the conformation of the protein occurs on conversion to these intermediates. Most of this change has occurred by L, with little additional net change in going on to M. If the extinction losses of 5000 to 6000 L cm^{-1} mol^{-1} at 270 and 280 nm in 67% glycerol media on conversion to M and L are attributed to a change in environmental polarity alone, this would require an average change of at least 50% in the environment of the 7 tryptophans and 10 tyrosines in the protein from the nonpolar interior to the more polar (67% glycerol–33% water) exterior (see Donovan, 1969). Although loss of contributions from hyperchromism would cause this approximation to be too high, this would also be indicative of a protein conformational change.

We have also found that purple membrane that is suspended in a solution of 25% NaCl and 1% Ammonyx LO (pH 7.0) also converts to ca. 100% M at −20 °C with 600-nm light (not shown). The trimer structure of the membrane protein remains intact as demonstrated by a comparison of its visible circular dichroic spectrum to that of native purple membrane (Becher & Ebrey, 1976). The near-ultraviolet absorption spectrum was found to decrease by 5000 L cm^{-1} mol^{-1} at 270 nm on 100% conversion to M and to result in a difference spectrum similar to that found on conversion to M in the 25% NaCl–pH 10 medium. These spectral results are significant because they corroborate the previous M conversion experiments in a situation at neutral pH and where there is drastically reduced light scattering.

Discussion

Visible Absorption. The 568-nm band in purple membrane is due to a π–π^* transition of the retinal chromophore. Other transitions to higher excited states of the chromophore are responsible for the minor absorption bands from 450 to 320 nm. In addition, a comparison of the retinal 2 and retinal 1 difference spectra indicates that the chromophore also has minor transitions in the 250–320-nm region.

The main absorption bands of the first two intermediates

in the purple membrane photocycle, K and L, also absorb in the red. The K absorption spectrum (Figure 7, see also Hurley et al., 1977) has a maximum at 628 nm, in reasonable agreement but somewhat further to the red than had been previously been reported (Lozier & Niederberger, 1977; Goldschmidt et al., 1976). We find that the absorption maximum for the L intermediate is at 547 nm (Figure 7), again close to the value given by Lozier & Niederberger (1977), although their L spectrum also includes a band at ca. 410 nm—probably due to the presence of the M intermediate. It can readily be shown that such long wavelength absorption maxima are possible for a retinal-based chromophore only if the π electrons are delocalized along the chain by protonation of the Schiff base (Honig et al., 1976; Honig & Ebrey, in preparation). Thus, the red absorption maxima of both the K and the L intermediates strongly suggest that, as in the original pigment, the retinal Schiff base is protonated. The absorption maximum of the M intermediate at 412 nm, however, suggests that it is an unprotonated Schiff base. This has also been shown by resonance Raman studies comparing the vibrational positions of unprotonated Schiff base model compounds to the corresponding lines in M (Lewis et al., 1974; Aton et al., 1977).

Ultraviolet Absorption. As discussed in the text, many factors—especially light-scattering—make it difficult to know the true extinction coefficient of the purple membrane at 280 nm. Our best estimate is that, based on an extinction coefficient of 63 000 L cm^{-1} mol^{-1} for the 568-nm band, the extinction of the 280 nm band is about 75 000. The sum of the extinctions of the individual aromatic acids in water is 51 000. Of the difference, 24 000, we estimate that ca. 8000 is due to the retinal chromophore's absorption at 280 nm and ca. 16 000 arises from environmental effects on the aromatic amino acid transitions and secondarily to hyperchromism of these interactions. Since ca. 18 000 L cm^{-1} mol^{-1} is the maximum possible contribution from environmental effects, this suggests that most of the tyrosines and tryptophans are in a nonpolar environment (protein interior).

Conformational Changes upon Bleaching. We have studied difference absorption changes in the ultraviolet under two general conditions: (a) bleached and extracted vs. regenerated pigment; and (b) the intermediates K, L, and M vs. the native pigment. Bleaching of purple membrane appears to involve protein conformational change as indicated by absorption and circular dichroic spectra (Becher & Cassim, 1977) and the evidence reported here. This evidence includes the near-ultraviolet difference spectrum of bleached and regenerated membrane showing large extinction changes in the ultraviolet which cannot be assigned to changes in the chromophore absorption. Moreover, the shape of the difference spectrum is quite similar to that of the protein itself, suggesting that regeneration has increased the extinction of many of the aromatic residues. Decreasing the polarity of the membrane environment results in a decrease in the magnitude of the difference spectrum extinction, further indicating that the difference spectrum is largely the result of increased exposure to water of a number of aromatic amino acid residues upon bleaching (see Donovan, 1969). We estimate that the magnitude of these changes requires an *equivalent* of about 60% of the tryptophans and tyrosines of each purple membrane molecule moving from a hydrophobic to a hydrophilic environment upon bleaching. Although this might result from direct exposure of tryptophans and tyrosines to water when the chromophore is removed, this would require most of the 7 tryptophans and 10 tyrosines in the protein to line the chromophore binding site—an unlikely localization of these amino acids. Moreover, similar ultraviolet absorption changes are seen on bleaching the pigment even if the retinaloxime, which is thought to remain in the binding site, is not extracted (Becher & Cassim, 1977). Perhaps related to the evidence of protein change is the observation by Henderson (cited in Bauer et al., 1976) that there is a disordering of the crystalline structure of the membrane upon bleaching; this is presumably due to a change in the packing of the pigment molecules in the membrane lattice.

Conformational Changes in the Photocycle. Our study also provides similar evidence of protein conformational changes in the intermediates of the purple membrane photocycle. The ultraviolet absorption changes indicate an *equivalent* of at least 50% of the aromatic amino acids changing environmental polarity on conversion of PM to L or M. Evidence of protein conformational changes in the photocycle of the purple membrane protein comes from several different observations. The most obvious is simply the existence of several different intermediates in the photocycle (Lozier et al., 1975) all having different absorption spectra and temperature-dependent rates of transformation. A second observation is that several agents such as NH_2OH and $NaBH_4$ react only or much more readily when the purple membrane is cycling through its photointermediates. (Recently, we have found that if hydroxylamine is mixed with a sample photoconverted to M at 6 °C, the chromophore bleaches to retinaloxime within a few seconds.) This suggests a greater reactivity of the hydrophobic reagents with the chromophore during one or more of its stages in the photocycle. Another kind of evidence is the temperature dependence on the photoreversibility. We have found (Hurley et al., 1978) that, even though the L and M intermediates can return to PM at −100 °C upon absorption of a photon, these photoreversals cannot take place at −196 °C. This is readily understandable if protein conformational changes occur in going from irradiated L or M back to PM but cannot occur at liquid nitrogen temperatures.

In contrast to the conformational changes we have seen here, Becher & Cassim (1977) found no evidence of any change in the pigments helical structure on bleaching. One simple explanation of both the large changes in the environmental polarity of many of the aromatic amino acids with a lack of change in the helical structure of the protein is the following: Henderson & Unwin (1975) showed that the purple membrane protein consists of seven roughly parallel helical segments perpendicular to the membrane. Such packing would probably place the aromatic amino acids in a hydrophobic environment. Then if the photocycle leads to a *moving apart* of the closely packed helices, this would result in a large increase in the environmental polarity of the aromatic amino acids, all with no significant change in the amount of helical structure of the protein.

Exactly how photon absorption and the resulting purple membrane photocycle result in the transport of protons across the cell membrane is unknown. One appealing possibility is that the Schiff base in PM, which is protonated, becomes deprotonated (M) with proton release on one side of the membrane and subsequently becomes reprotonated from the other side. This would result in the net transport of one proton across the membrane. However, a difficulty with this scheme is that preliminary measurements of the quantum efficiency of pumping protons across the cell membrane yield a value of ca. 0.6 (Bogomolni, 1977) about twice the value of the photocycle quantum efficiency (Becher & Ebrey, 1977; Goldschmidt et al., 1977). Thus, it may be that two protons are required to be transported per photocycle. Nevertheless, in either case, the protein conformational changes during the photocycle that are discussed above are probably involved in the actual mechanism of proton transport.

Photochemical Considerations. A property of both pigments which is important in establishing photoisomerization (Rosenfeld et al., 1977) is photoreversibility—the ability of light absorbed by one of the intermediates to start a sequence which leads to the reformation of the pigment. There is one rather surprising difference between the photoreversibility behavior of the intermediates of rhodopsin compared with those of the purple membrane protein. Cooling metarhodopsin I to −65 °C prevents photoreversal of this intermediate, presumably because the protein cannot refold at this temperature, although isomerization of retinal occurs (Hubbard et al., 1959). Moreover, examination of the ultraviolet protein absorbance indicates there are no detectable changes in protein conformation on rhodopsin to lumirhodopsin conversion at −100 °C (Ebrey & Honig, 1972; Ebrey & Yoshizawa, 1973). In contrast to both of these observations, we have shown here that (a) the L intermediate of the purple membrane can photoreverse to PM at −100 °C and the M intermediate can photoreverse to PM at −40 °C and (b) large changes in the ultraviolet absorbance, indicating protein conformation changes, are seen upon the formation of the L intermediate at −100 °C and the M intermediate at −40 °C. A rather surprising conclusion is indicated—that the purple membrane protein despite its hexagonal lattice structure has considerably more conformational flexibility at low temperature than rhodopsin, at least rhodopsin in a detergent micelle.

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