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Absorption Spectral Properties of Acetylated Bacteriorhodopsin in Purple Membrane Depending on pH†

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ABSTRACT: The dark-adapted form of bacteriorhodopsin in the purple membrane of *Halobacterium halobium* changes its absorption maximum from 560 to 600 nm if the pH is lowered to about 2 [Oesterhelt, D., & Stoeckenius, W. (1971) *Nature (London), New Biol. 233*, 149; Moore, T. A., Edgerton, M. E., Parr, G., Greenwood, C., & Perham, R. N. (1978) *Biochem. J. 171*, 469; Mowery, P. C., Lozier, R. H., Chae, Q., Tseng, T.-W., Taylor, M., & Stoeckenius, W. (1979) *Biochemistry 18*, 4100; Fischer, U., & Oesterhelt, D. (1979) *Biophys. J. 28*, 211; Muccio, D. D., & Cassim, J. Y. (1979) *J. Mol. Biol. 135*, 595]. We compared the pH dependence of the absorption spectra of acetylated membrane with that of unacetylated native membrane. The completely acetylated membrane showed a midpoint of pH 4.8 for the conversion to the acidic form; that of the native membrane was 3.4. On acetylation, the absorption maximum at neutral pH moved from 560 to 555 nm with about 20% decreases in extinction coefficients as compared with that of the native membrane, whereas the spectrum in acid was not affected. The chloride-dependent blue shift from the acidic form of the acetylated membrane was largely suppressed. The CD spectrum of the acetylated membrane was composed of two bands of an opposite sign with slightly decreased amplitudes. The chromophore of the acetylated membrane was sensitive to hydroxylamine, and the spectrum before bleaching was restored on addition of *all-trans*-retinal to the bleached membrane followed by dark incubation. Blue light irradiation accelerated the conversion to the acidic form in the native membrane but not in the acetylated membrane. Reductive ethylation did not affect the pH dependence of the absorption spectra.

Bacteriorhodopsin (bR)[1] in the purple membrane (PM) of *Halobacterium halobium* carries out a unidirectional shift of protons across the membrane with the aid of light energy absorbed by the retinylidene chromophore (Stoeckenius et al., 1979). These protons are supposed to be conveyed along a series of proton binding groups spanning the membrane as has been postulated for the proton fluxes through the channel of proton-dependent ATP synthetase (Sone et al., 1979). In this respect it would be interesting if chemical modifications of charged residues could affect the spectral properties of the chromophore. Lemke & Oesterhelt (1981) have shown the presence of tyrosine-26 in the vicinity of the chromophore.

Oesterhelt & Stoeckenius (1971) early found the bathochromic shift of the visible absorption spectrum when PM was placed in a weakly acidic medium. Since then, the acidic form of bR has been studied from various aspects: a close examination of its pH dependence (Moore et al., 1978; Mowery et al., 1979; Fischer & Oesterhelt, 1979), its relation to the "O" intermediate in the photocycle of bR (Moore et al., 1978; Fischer & Oesterhelt, 1979; Edgerton et al., 1980), and photoreactions and the analysis of the products (Mowery et al., 1979; Maeda et al., 1980, 1981; Fischer et al., 1981).

Acetylation with acetic anhydride is known to be one of the conventional methods for chemical modification of proteins (Riordan & Vallee, 1972) and is useful, especially in view of its narrow specificity restricted to lysine residues in the case of PM as discussed by Takeuchi et al. (1981). Moreover, acetylation induces pronounced decreases in both light-dependent activities of the proton release from the membrane sheets and of the proton transport across the membrane (Takeuchi et al., 1981). The present studies deal with the effect of acid on the acetylated PM along with its spectral characteristics. The effect of light on the formation of the acidic form of PM is described.

Materials and Methods

PM sheet fragments were prepared by a standard method described by Oesterhelt & Stoeckenius (1974). Acetylation was carried out as described by Takeuchi et al. (1981). PM suspensions (A_{560nm} = ~4) was mixed with an equal volume of saturated solution of sodium acetate. A 10-μL portion of acetic anhydride to every 5 mL of the reaction mixture was added at 10-min intervals with constant stirring at 0 °C. The pH was maintained between 7.5 and 8.0 by adding 1 M NaOH. Acetylation was terminated by diluting with ice-cold water at 80 min unless otherwise specified. The acetylated membrane was precipitated by centrifugation at 34000*g* for 30 min and washed twice with water. Pellet was suspended in water, and the suspensions were dialyzed against 5 mM

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[1] Abbreviations: bR, bacteriorhodopsin; PM, purple membrane; TNBS, 2,4,6-trinitrobenzenesulfonic acid; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; A-PM, acetylated purple membrane; U-PM, unacetylated purple membrane; CTAB, cetyltrimethylammonium bromide.

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phosphate buffer (pH 6.8). Both the native membrane and the acetylated membrane were finally dialyzed against 100 volumes of distilled water by replacing external water 3 times during 48 h before use. All of the procedures were conducted below 4 °C under dim red light.

For spectral measurements, the sample was mixed with 2 volumes of glycerol (Maeda et al., 1980) unless otherwise noted. Absorption spectrum was measured in a Hitachi recording spectrophotometer, Type 124. The pH of the sample was changed by adding small volumes of HCl or NaOH to the unbuffered sample and measured by immersing a Horiba pH electrode, Type 6028-10T, connected to a Radiometer pH meter, Type 26, before spectral measurement. The CD spectrum was measured in a JASCO recording spectropolarimeter, Type J20, at room temperature.

Polyacrylamide gel electrophoresis was carried out on 15% polyacrylamide gels containing 0.1% $NaDodSO_4$ as described by Laemmli (1970). The amount of free amino groups was determined by a 2,4,6-trinitrobenzenesulfonic acid (TNBS) method described by Fields (1972). To 0.45 mL of PM sample in 0.2 M borate buffer (pH 9.5), a solution of 0.05 mL of 10% Emulphogene [poly(oxyethylene-10) tridecyl ether; obtained from Sigma] was added and the protein was denatured by heating at 70 °C for 5 min. The mixture became turbid at 70 °C but returned to clear after cooling. Every four identically prepared samples received 10 μL of 0.1% $NaHSO_3$. Two of them then received 10 μL of 0.1% TNBS solution and the other two served as blanks to subtract the absorption due to retinal. A_{420nm} of each sample was measured after 60-min incubation at 45 °C. Blank samples in the absence of any protein were also measured. The result was presented as an average of these duplicate determinations.

The light-induced pH change of the membrane suspensions was measured as described by Takeuchi et al. (1981). Irradiation with blue light (400–530 nm) or red light (>610 nm) was done at 0 °C as described by Maeda et al. (1981). Retinal isomer composition in PM was analyzed by the method described in the above literature.

Modification of PM with 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC) (purchased from Nakarai, Japan) was carried out under the conditions at pH 8.0 described by Renthal et al. (1979). Ethylation was done by applying the method for reductive methylation described by Jentoft & Dearborn (1979). To PM suspensions (A_{560nm} = ~4) in 0.25 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.5), 0.1 M each of acetaldehyde (a product of Merck; 99%) and sodium cyanoborohydride (recrystallized from a product of Aldrich) was added. After incubation at room temperature for 24 h, the membrane was washed with water by centrifugation. The same reaction was repeated again on this washed membrane. These modified membranes were finally washed with distilled water and dialyzed by the same procedure as described for that of acetylation.

Results

Absorption Properties of Acetylated Purple Membrane. Acetylated purple membrane (A-PM) turned blue during dialysis against distilled water. Its absorption spectrum in 67% glycerol (curve 1 of Figure 1) was very similar to that of the acidic form of unacetylated purple membrane (U-PM) described previously (Maeda et al., 1980). A pH value of the A-PM suspensions in 67% glycerol was 4.2. A decrease in pH to 3.7 did not further influence the spectrum of the A-PM, whereas the absorption spectrum shifted to shorter wavelengths when the pH was increased (Figure 1). The neutral form thus obtained and the acidic form were interconvertible with each other. A deviation from the isosbestic point at acidic pH arose probably from a change of isomer composition at acidic pH as observed with U-PM (Maeda et al., 1980).

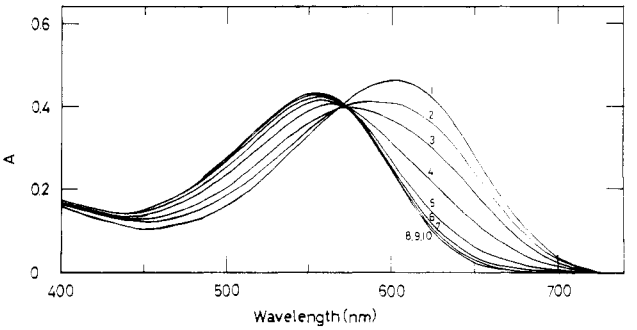

FIGURE 1: Absorption spectra of A-PM at various pH values. The dialyzed A-PM was mixed with 2 volumes of glycerol: curve 1 (pH 4.3). To 2 mL of the sample of curve 1, 2 μL each of 0.025 M NaOH was added successively: curve 2 (pH 4.4) and curve 3 (pH 4.6). Then 1 μL each of 0.1 M NaOH was added successively: curve 4 (pH 5.0), curve 5 (pH 5.5), curve 6 (pH 6.1), and curve 7 (pH 6.5). Curve 8 (pH 7.1), curve 9 (pH 7.4) and curve 10 (pH 7.8), which were obtained by the successive addition of 1 μL of 0.1 M NaOH, coincided completely with each other. The same spectrum as curve 1 was obtained by adding 2 μL of 0.1 M HCl (pH 3.7).

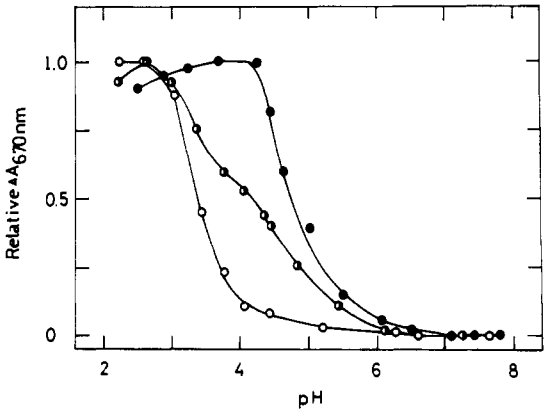

FIGURE 2: pH dependence of the spectral red shift represented by the increase of A_{670nm}. U-PM (○), A-PM (●), and an equal molar mixture of both (◑). ΔA_{670nm} is the difference of A_{670nm} from the value of the neutral pH (less than 5%) divided by the difference of A_{670nm} between the maximum (in acidic pH) and the minimum (in neutral pH). Spectra were measured on the samples prepared by successively adding small volumes of NaOH or HCl as described in the legend of Figure 1 and in the text.

The pH-dependent spectral change of A-PM was compared with that of U-PM by absorbance increases of 670-nm light (Figure 2), which was scarcely absorbed by the neutral form. A midpoint of pH 4.8 for the conversion between the acidic form and the neutral form was greater than that of pH 3.4 observed with U-PM, when experiments were done in 67% glycerol. A curve of A_{670nm} vs. pH of the mixed sample containing both A-PM and U-PM in an equal amount (Figure 2) showed two inflection points at the pH values that corresponded to those of A-PM and U-PM, respectively. The pH of the spectral conversion has been shown to become smaller with the increases of ionic strengths (Fischer & Oesterhelt, 1979). The midpoint for the conversion of A-PM was pH 3.7 in 0.5 M $NaClO_4$, whereas that of U-PM was pH 2.4. The difference between these two values was similar to that in the absence of salt. In the absence of glycerol, the conversion of A-PM also occurred at higher pH than that of U-PM, though progressive aggregation below pH 3 somehow disturbed the determination of the inflection point in the titration curve, especially of that of U-PM.

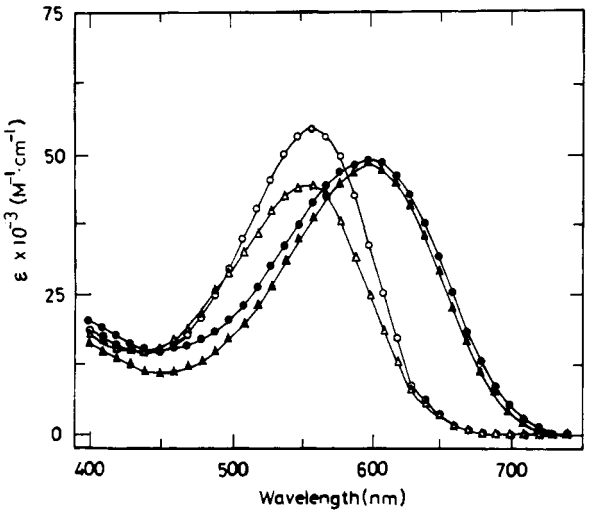

FIGURE 3: Absorption spectra of U-PM a pH 7.3 (○), U-PM at pH 2.6 (●), A-PM at pH 7.4 (△), and A-PM at pH 3.7 (▲). The neutral samples were obtained by adding 6 μL of 0.1 M NaOH to the respective 2-mL samples. The acidic samples were obtained by adding 18 and 2 μL of 0.1 M HCl to 2-mL samples of U-PM and A-PM, respectively. Normalization of the spectra were done by using factors described below. Thus, each sample, adjusted to pH 7.2 by adding 0.1 M NaOH, was divided into several aliquots of 2 mL each. After the spectral measurements the pigments were bleached by the addition of 0.2 mL of 0.1 M CTAB and 0.05 mL of 0.25 M phosphate buffer (pH 9.2), and A_{360nm} was measured after 10 min. Four determinations were done for each pigment. The ratios of the maximum absorbance of the neutral pigments to A_{360nm} of the CTAB-bleached samples were 1.08 ± 0.03 and 0.85 ± 0.02 for U-PM and A-PM, respectively. Molar extinctions of the light-adapted form in neutral pH were calculated from the value of 63 000 M^{-1} cm^{-1} reported by Oesterhelt & Hess (1973) and a ratio of the absorbance maximum of the light-adapted form to that of the dark-adapted form, 1.14.

When the concentration of HCl below pH 2 was increased, the blue shift occurred from the acidic form of A-PM in a manner similar to that from the acidic form of U-PM. The blue shift at strongly acidic pH is known to be anion dependent and is distinct from that at weakly acidic pH (Fischer & Oesterhelt, 1979). At pH 2.3 the acidic form of A-PM underwent the blue shift at a concentration of NaCl of 1 M, 1 order of magnitude higher than that required for the acidic form of U-PM. The blue shift at pH 2.3 was not induced by $NaClO_4$ even at a concentration as high as 4 M.

Figure 1 also showed that the peak of the spectrum of A-PM at acidic pH was slightly higher than that at neutral pH. On the other hand, U-PM lowered the absorbance intensities after conversion to the acidic form (Maeda et al., 1980). The spectra of both A-PM and U-PM were compared with each other after normalization of the maximum extinctions on the basis of the amount of retinylideneamine produced by cetyltrimethylammonium bromide (CTAB) bleaching (Danon & Stoeckenius, (1974) (Figure 3). The maximum wavelength of A-PM at pH 7.4 was 555 nm, being slightly shorter than that of U-PM at the peak. On the other hand, the spectrum of the acidic form of A-PM coincided with the corresponding spectrum of U-PM almost completely. These spectral characteristics were also preserved in the absence of glycerol.

Other Properties of A-PM. The electrophoretic mobility of A-PM was the same as that of U-PM in polyacrylamide gel electrophoresis under the conditions that allowed separation of a slightly smaller fragment deprived of the carboxyl-terminal 17 amino acid residues by limited papain digestion (Ovchinnikov et al., 1979).

The amount of free amino groups was determined by a TNBS method (Fields, 1972) described under Materials and Methods and normalized to the amount of retinylideneamine after treatment with CTAB (see the legend of Figure 3). One bR molecule contains seven lysine residues and no free amino terminus (Khorana et al., 1979; Ovchinnikov et al., 1979), and lipids of PM are devoid of amino groups (Kushwana et al., 1975). Since one of the seven lysine residues was protected from acetylation by retinal, the free amino groups must be decreased to 14% after completion of acetylation. More than 95% of the chromophore was recovered in the A-PM, and the content of the amino groups in the A-PM was 17% to that of U-PM. Therefore, almost all the lysine residues were acetylated exhaustively in the A-PM. A sample withdrawn at 25 min in the course of acetylation containing 34% of free amino groups, or 1.5 mol of free lysine/bR molecule except for the lysine residue bound to retinal. A midpoint for the conversion to the acidic form of this sample was at pH 3.9, indicating that the amino groups reacting relatively fast with acetic anhydride were not responsible for the increase of the pH for the conversion.

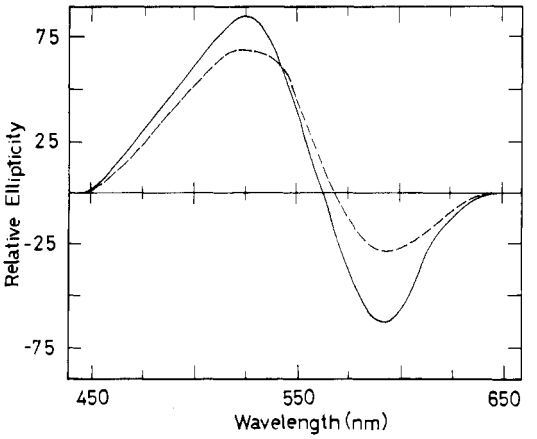

FIGURE 4: CD spectra of U-PM (—) and A-PM (- - -) in 5 mM phosphate buffer (pH 6.8) at 20 °C. The concentration of the samples was 2.3 × 10^{-5} M, which was determined as described in the legend of Figure 3.

Both the CD spectra of A-PM and U-PM are shown in Figure 4. The spectrum of U-PM over a range of 450–650 nm coincided well with that presented by Becher & Cassim (1976). The spectrum of A-PM showed smaller negative ellipticities and intersected at a longer wavelength with the base line than U-PM did but still retained a spectral shape consisting of two bands with an opposite sign.

A-PM bleached slowly in 0.67 M hydroxylamine (pH 7.0) even in the dark with a half-time of about 200 min at 20 °C. The maximum wavelength was kept constant at 555 nm during this process. After incubation for 18 h at 20 °C, the hydroxylamine was removed by dialysis against 5 mM phosphate buffer (pH 6.8) at 4 °C. A pigment generated with *all-trans*-retinal showed the same maximum wavelength as that of native A-PM. These results also indicated the absence of any influence of acetyltyrosine, which would remain in the A-PM, on the spectrum, since hydroxylamine was known to break *O*-acetyl bonding (Balls & Wood, 1956).

The content of 13-*cis*-retinal in the dark-adapted form of A-PM was 48 ± 0%, being less than that of U-PM, 56 ± 1%. When A-PM in 5 mM phosphate buffer (pH 6.8) was irradiated with yellow light (>400 nm) at 3 °C, the spectrum moved by only 5 nm to longer wavelengths and 13-*cis*-retinal of 31 ± 1% remained. Since complete conversion to the all-trans form was not attained even at 3 °C by irradiation, all the spectral measurements were done throughout with the dark-adapted samples.

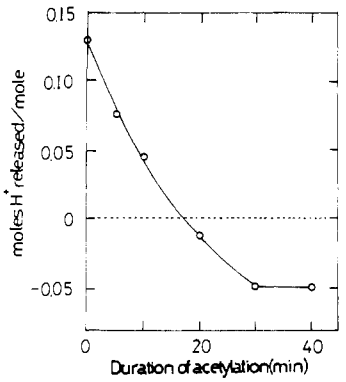

FIGURE 5: Changes of the light-induced proton release and uptake in the course of acetylation reaction. Aliquots were taken at desired times, and washed and dialyzed membranes were suspended in unbuffered 0.2 M KCl (pH 5.5). The amount of proton released from or bound to the membrane sheets was determined at 2 °C by irradiating with >490-nm light.

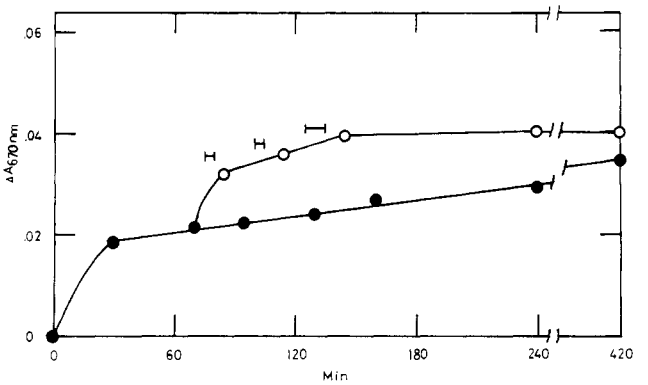

FIGURE 6: Course of the conversion of U-PM in 2×10^{-4} M HCl to the acidic form at 0 °C. U-PM ($A_{560nm} = 0.16$) in 2×10^{-4} M HCl was divided into two parts at 70 min. The one part was kept in the dark (●), while the other was irradiated with blue light (○) for the durations indicated by horizontal bars flanked by vertical bars. The absorption spectrum was measured at indicated time at 3 °C, and the data were expressed by the increase of A_{670nm}.

Acetylation has been known to result in decreases in the light-dependent activity to release protons from the membrane sheets (Takeuchi et al., 1981). Several aliquots were withdrawn from the reaction mixture at intervals during the course of the acetylation and then subjected to centrifugation and dialysis as described under Materials and Methods. The membrane was then suspended in 0.2 M KCl and the pH was adjusted to 5.5. The amount of protons released from the membrane sheets was measured (Figure 5). The light-dependent proton-releasing activity was decreased by 40% even with the sample at 10 min, while the pH dependence for the conversion to the acidic form was unchanged. The sample at 20 min, when the acetylation was still not complete as described above, lost activity almost completely. Light-dependent proton uptake activity emerged during further acetylation as reported previously (Takeuchi et al., 1981).

Effect of Blue Light, Modification with EDC, and Ethylation. U-PM was mixed with HCl to a final concentration of 2×10^{-4} M (pH 3.6) and kept at 0 °C. A small extent of a rapid conversion to the acidic form occurred, followed by a slower process, as stated in the previous paper (Maeda et al., 1980) on U-PM in 8×10^{-4} M HCl. The sample was divided into two parts at 70 min after addition of HCl. One was irradiated with blue light (400–530 nm) for short durations as depicted by horizontal bars in Figure 6, while the other was kept in the dark. Figure 6 expressed the shifts of the spectrum to longer wavelengths as the increases of A_{670nm}. The sample irradiated with blue light converted more rapidly to the acidic form than that kept in the dark. No further shift was observed beyond a level after complete conversion. A rapid conversion induced by light was also observed with U-PM in 6.5×10^{-4} M HCl at either 0 or 20 °C but neither with U-PM in 2×10^{-3} M HCl, where the conversion was rapid nor with A-PM at any pH values examined.

By treatment with a water-soluble carbodiimide reagent, EDC, the pH for the conversion was lowered to below pH 2. The conversion was apparently partial, probably owing to the competition with the acid-induced blue shift. Reaction of PM with acetaldehyde in the presence of sodium cyanoborohydride under the conditions described under Materials and Methods led to an almost complete ethylation (15% of free amino groups). It should be noted that much higher concentrations of reagents were required for a completion of the reaction than those used for soluble proteins by Jentoft & Dearborn (1979). In such a modified PM sample, the pK value for the conversion was kept at the same low pH as that of U-PM.

Discussion

Spectral measurements of the acidic form of PM must be conducted by special devices in order to prevent it from aggregation in acid. Mowery et al. (1979) incorporated PM into polyacrylamide gels; Muccio & Cassim (1979) measured spectra repeatedly with intermittent stops for mixing. We have used PM suspensions in 67% glycerol for the purpose of spectroscopy over a range of subzero temperature (Maeda et al., 1981). Increases in light scattering due to aggregation in acid was also avoidable in this way (Maeda et al., 1980). Glycerol has been shown to affect kinetic parameters of the long-lived intermediates in the photocycles of bR (Beece et al., 1981). Our pK value (the apparent pH of the midpoint for the conversion between the acidic form and the neutral form) of 3.4 obtained with U-PM in the absence of added salt was decreased by about 1 pH unit in 0.5 M $NaClO_4$. These values and a tendency to decrease the pK values with the increase of salt concentrations were nearly consistent with those obtained by previous authors (Fischer & Oesterhelt, 1979; Mowery et al., 1979; Muccio & Cassim, 1979). A molar amount of protons of 1.7 required for the conversion to the acidic form from U-PM, which was estimated from the data of Figure 3, was identical with the value obtained by Mowery et al. (1979). In 67% glycerol the pK value of the A-PM was 4.8, being markedly larger than that of 3.4 of the U-PM. Such a large pK shift due to the acetylation was also found in a high ionic strength medium or in the absence of glycerol.

The conversion to the acidic form has been suggested to result from the protonation of a small number of anionic residues (Fischer & Oesterhelt, 1979; Mowery et al., 1979). The pH for the conversion to the acidic form is known to be lowered with the increase in the ionic strengths (Fischer & Oesterhelt, 1979) or by the addition of a cationic polyelectrolyte, diethylaminoethyldextran (Bakker-Grunwald & Hess, 1981). A modification with a water-soluble reagent, EDC, of carboxyl groups also inhibited the conversion. Thus, the decreases of pH for the conversion could result from the suppression in an effective charge density on the surface of the membrane.

On the contrary, the acetylation shifted the pH for the conversion to higher pH. This could be explained in terms of a decrease in positive charge in the membrane due to the acetylation of lysine residues by assuming the loss of the positive charge from the lysine as a primary cause of the effect of the acetylation. An increase in the pK has also been shown

in the presence of anionic molecules with hydrophobicity (Fischer & Oesterhelt, 1979; Drachev et al., 1981; though without detailed accounts) or with bR incorporated into the vesicles composed of acidic lipids derived from *H. halobium* (Lind et al., 1981). The acetylation increased the p*K* value for the conversion by about 1 pH unit, independently of the ionic strength. Thus, the lysine residues responsible for this process might be shielded from the effect of salt in the aqueous environment.

Acetylation did not affect the spectrum of the acidic form at all but somehow suppressed the anion-dependent blue shift from the acidic form. This is in agreement with the idea that the anion-dependent blue shift is resulted from anion binding by positive charges (Fischer & Oesterhelt, 1979).

The increase of the 13-*cis*-retinal content generally accompanies with the blue shift of the spectrum, either in native membrane (Oesterhelt & Schuhmann, 1974; Maeda et al., 1977) or in detergent-treated membrane (Casadio et al., 1980). The content of 13-*cis*-retinal in the dark-adapted form of A-PM, whose maximum was at 555 nm, was less than that of U-PM: the direction of the spectral shift was the reverse of that expected from isomer composition. The treatment of PM with detergent shifts the spectrum to the blue (Dencher & Heyn, 1978; Casadio et al., 1980). But, this is not entirely due to a structural distortion of the membrane (Casadio et al., 1980). The blue shift of the spectrum has also been shown to occur at alkaline pH (Muccio & Cassim, 1979; Lemke & Oesterhelt, 1981). Moreover, the CD spectrum of A-PM resembled that of U-PM at alkaline pH measured by Muccio & Cassim (1979). An increased sensitivity of A-PM to hydroxylamine might be an indication of some structural distortion of the membrane. Acetylated apomembrane, however, still was able to regenerate the spectrum of A-PM on addition of *all-trans*-retinal.

These results may leave a possibility that the acetylation induces some structural distortion within a framework of the membrane and eases the conversion to the acidic form. The observed distortion, however, could also be a secondary effect resulting from a loss of positive charges. Reductive alkylation of lysine residues can introduce alkyl groups in place of acetyl groups, while keeping a net positive charge. An almost complete ethylation of PM did not result in the p*K* shift. This supported a view that the effect of the acetylation is primarily due to the loss of the positive charges.

In view of a notion that the acidic form resembled with the "O" intermediate in the photocycle of bR (Moore et al., 1978; Fischer & Oesterhelt, 1979), it is interesting to point out that irradiation with blue light accelerated the conversion to the acidic form. This could be the result of some perturbation of the anionic residues during the photocycle.

The decrease of light-induced proton release from the membrane sheets, one of the consequences from the acetylation, was observable in an early step of the modification, indicating that the other lysine residues are responsible for the photoactivity described by Takeuchi et al. (1981). Apart from these, A-PM is useful to prepare the acidic form of PM at weakly acidic pH.

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