Deprotonation of the Schiff Base of Bacteriorhodopsin Is Obligate in Light-Induced Proton Pumping†

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ABSTRACT: Bacteriorhodopsin (bR) in purple membranes was permethylated with formaldehyde and pyridine–borane with the incorporation of approximately 12 methyl groups. This new pigment, PMbR, absorbed light in the dark-adapted state with a λ_{max} at 558 nm, virtually the same as that of bR. Light adaptation of PMbR produced a λ_{max} of 564 nm with a slightly elevated ϵ. Similar changes occurred with bR. When incorporated into asolectin vesicles, PMbR was able to pump protons in the light with an efficiency similar to that of bR itself. Bleaching of PMbR exposed its active site lysine residue, which was monomethylated to form active site methylated bR (AMbR) after regeneration with *all-trans*-retinal. This blue pigment, which is a cyanopsin rather than a rhodopsin, showed an extraordinary red shift, absorbing light with a λ_{max} of 620 nm in the dark-adapted state. Light adaptation of AMbR resulted in a spectral shift to 616 nm with a decrease in ϵ. This change was completely reversible in the dark. This shift was interpreted to mean that an L-like intermediate was accumulating, as would be expected if deprotonation of the protonated Schiff base could not occur to produce the M intermediate. Furthermore, when incorporated into asolectin vesicles, AMbR proved incapable of pumping protons in the light. It was concluded from these experiments that deprotonation of the Schiff base of bR is obligate for light-induced proton pumping.

Bacteriorhodopsin is a 26-kDa membrane-bound chromoprotein, capable of pumping protons against a gradient for the purpose of generating ATP (Stoeckenius et al., 1979; Henderson, 1977). This process is light dependent and requires the presence of *all-trans*-retinal attached to the protein's active site lysine residue (lysine-226) via a protonated Schiff base (Stockburger et al., 1979; Ovchinnikov, 1982). Absorption of light by the protein results in an all-trans to 13-cis isomerization of the protonated Schiff base chromophore, in a process that is the key to the functioning of the protein (Tsuda et al., 1980). The starting state of the holoprotein is reset by the thermal conversion of the 13-cis to the all-trans form of the chromophore (Pettei et al., 1977). A light-inducible cycle for this process has been delineated, which in some respects is similar to that observed when rhodopsin is bleached (Lozier et al., 1975; Kung et al., 1975). In the case of bR,[1] illumination of its all-trans form produces within picoseconds a bathochromically shifted 13-cis intermediate (K), which thermally decays to produce in a presumably sequential fashion at least four other intermediates, L, M, N, and O, before the cycle is completed. The M intermediate appears to be analogous to metarhodopsin II in that the chromophores in both of them are deprotonated (Doukas et al., 1978).

The deprotonation of the Schiff base of rhodopsin has been shown to be obligate in the formation of activated rhodopsin (R*), which is the conformer(s) of photolyzed rhodopsin capable of catalyzing the exchange of GTP for GDP in the retinal G protein (transducin) (Fung & Stryer, 1980). This was directly shown by monomethylating the active site lysine (lysine-296) of rhodopsin and demonstrating that after photolysis this modified rhodopsin cannot achieve metarhodopsin II nor can it activate the G protein (Longstaff & Rando, 1985; Longstaff et al., 1986). Similar kinds of experiments can be envisaged with bR. Here the initial concern is whether or not an active site monomethylated bR can achieve M and pump protons. M has already been correlated with that (those) conformeric state(s) of illuminated bR capable of moving a proton, and it has been speculated that the deprotonation of the Schiff base at M directly moves the proton to be pumped (Honig et al., 1979; Engelhard et al., 1985). The fact that the stoichiometry of protons moved to quanta absorbed can be greater than one, however, raises questions about this simplest of interpretations (Bogomolni et al., 1980; Govindjee et al., 1980; Rendard & Delmelle, 1980). Nevertheless, it is important to test whether or not deprotonation of the Schiff base is obligate for the proton pumping ability of bR without reference to a particular mechanism and indeed whether an epicycle not involving M can suffice.

In the study reported here, procedures are developed for the dimethylation of the available lysine residues of bR and monomethylation of the active site lysine. The active site monomethylated bO forms stable pigments with both 13-*cis*- and *all-trans*-retinal. The dark-adapted form of the blue pigment (AMbR) shows a λ_{max} at 620 nm, and the light-adapted state shows one at 616 nm. Upon photolysis of AMbR a photochemical change occurs that is consistent with the production of a relatively stable L-like intermediate, and protons are not pumped. It is concluded that deprotonation of the active site Schiff base is critical for the completion of a fruitful proton-pumping cycle.

Experimental Procedures

Materials

Formaldehyde (as *p*-formaldehyde) and pyridine–borane were obtained from Aldrich Chemical Co. [^{14}C]Formaldehyde was from New England Nuclear. 1,4-Piperazinediethanesulfonic acid (PIPES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *o*-phthalaldehyde (OPA),

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[1] Abbreviations: bR, bacteriorhodopsin; bO, bacterioopsin; PMbR, permethylated bacteriorhodopsin; AMbR, active site methylated, permethylated bacteriorhodopsin; OPA, *o*-phthalaldehyde; ME, mercaptoethanol; PIPES, 1,4-piperazinediethanesulfonic acid; Rh, rhodopsin; PMRh, permethylated rhodopsin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide; TLC, thin-layer chromatography.

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mercaptoethanol (ME), and ninhydrin were all obtained from Sigma Chemical Co. DMSO was a product of Fisher Scientific Co. HAWP 025 (0.45 μm) filters were from Millipore Corp. Constant-boiling HCl for protein hydrolysis was from Pierce Chemical Co. TLC plates (plastic-backed silica gel 60, without fluorescent indicator, 0.2 mm thick) were a product of E. Merck, West Germany. Scintillation cocktails Filtron-X and Hydrofluor were from National Diagnostics. Asolectin was obtained from Associated Concentrates, Woodside, NY. All other reagents were of the highest grade commercially available.

Methods

Purple Membrane. Purple membrane preparations of 60–130 μM bR obtained from Drs. Thomas Ebrey and Judith Herzfeld were used for further chemical modification. bR was found to be stable (as judged by spectrum) when stored at 4 °C for weeks to months. Protein concentrations were estimated with an $e = 63\,000\ M^{-1}$ at 560 nm for light-adapted bR (and PMbR) after correction for scatter (Oesterhelt et al., 1973).

Permethylation of bR. Permethylation of bR was achieved (forming PMbR) after three rounds of methylation at 22 °C. To an initial 60–80 μM suspension of bR (1 mL) was added water (0.6 mL), 0.5 M HEPES buffer (pH 8) (0.2 mL), and DMSO (0.2 mL). Formaldehyde and pyridine/borane were then added, to give final concentrations of 4 and 20 mM, respectively. After approximately 24 h, the suspension was diluted to 10 mL with water and pelleted at 40000*g* for 30 min. The pellet was resuspended in 1 mL of water, and two successive methylations were carried out as above.

In those experiments where the degree of methylation was determined with varying concentrations of formaldehyde, radiolabeled $[^{14}C]H_2CO$ was used (1 Ci/mol), and two rounds of methylation were performed. Aliquots of labeled bR suspension (20 μL in duplicate) were removed and added to 2 M hydroxylamine (20 μL). Ater 5–10 min at 22 °C, this suspension was filtered with Millipore HAWP filters and washed with 0.1 M hydroxylamine (3 x 5 mL). Filters were dissolved in Filtron X for scintillation counting.

Active Site Methylation of PMbO. PMbR was bleached in the same way as described for bR by Tokunaya and Ebrey (1982), with 2 M hydroxylamine at pH 8–9 at 30–40 °C under intense white light. Excess hydroxylamine was removed by several washings with water, and retinal oxime was extracted by washing with hexane. Part of the dried PMbO was then redissolved in water for regeneration. The rest was resuspended in 10 mM PIPES, pH 6.5, containing 100 mM NaCl, giving a suspension with an $A_{280} = 3$, ready for further active site methylation. This methylation was accomplished over 48 h, by use of two additions of 2 mM formaldehyde and 20 mM pyridine–borane, at 4 °C (the second addition after 24 h with no washing). On completion of this step, excess reagents were removed by washing with water before the remaining, unreacted active site lysine was blocked by use of OPA/ME [as in Longstaff and Rando (1985)]. The suspension was pelleted and washed twice before being resuspended, as above in 10 mM PIPES, pH 6.5, containing 100 mM NaCl. To this suspension was added OPA (10 μL/mL of a solution of 80 mg/mL in methanol) and ME (1 μL/mL of reaction mixture). This reaction was allowed to proceed for a further 48 h by which time no free lysine remained. The absence of lysine was ascertained by following the regeneration with *all-trans*-retinal. No evidence was found for a rapidly (30 min) regenerating peak with a λ_{max} at 555–560 nm—the λ_{max} of dark-adapted, *all-trans*-retinal regenerated product with PMbO. Excess reagents were then removed by pelleting and washing before *all-trans*-retinal (~2-fold excess) was added to regenerate AMbR. Regeneration was found to take at least 24 h at 20 °C. Regeneration was followed, and spectra were recorded on a Perkin-Elmer 552A UV/vis spectrophotometer. When regeneration was complete, excess *all-trans*-retinal could be removed by washing with a 2% solution of defatted albumin (Tokunaga & Ebrey, 1982).

Ratios of dimethyllysine, methyllysine, and lysine produced in AMbR after active site methylation were determined essentially as described by Longstaff and Rando (1985). By use of [^{14}C]formaldehyde, the extent of methylation at the active site was calculated from the moles of $^{14}CH_3$ per mole of bleached, methylated protein, minus the moles of $^{14}CH_3$ per mole of nonbleached, methylated protein (background, <0.2 CH_3/protein). Portions of these two protein samples were then hydrolyzed in 6 M HCl, at 110 °C, in vacuo for 20–24 h. The hydrolysates were then lyophilized and redissolved in a small volume of 10 mM HCl in order to apply 15 000–20 000 dpm to a TLC plate. The plate was developed in MeOH/$CHCl_3$/NH_4OH (8:6:5), dried, sprayed with ninhydrin, and cut into 1-cm strips for counting in Hydrofluor (Longstaff & Rando, 1985). Ratios of dimethyllysine, methyllysine, and lysine were then calculated from the ratio of dimethyl- to methyllysine and from total methyl group incorporation at the active site.

Proton-Pumping Assays. Proton-pumping experiments were performed with protein in vesicles made with asolectin, which was partially purified according to the method of Kagawa and Racker (1971). Asolectin (25 g) was stirred for 48 h in dry acetone at 22 °C. The suspension was then filtered, and the remaining solid was dissolved in 100 mL of freshly distilled ether containing 100 mg of butylated hydroxytoluene. The resulting solution was filtered to remove a fine, suspended solid, and the clear solution was evaporated to dryness. A stock solution of asolectin (200 mg/mL) was made up in CH_2Cl_2 and stored at −70 °C under nitrogen. Bacteriorhodopsin and modified bR's were constituted into vesicles according to the method of Racker (1973). Asolectin (20 mg) was dried down in a tube under a stream of N_2 and then washed twice with freshly distilled ether, and traces of solvent were removed in vacuo for 12–16 h. To this was added protein suspension (1 mg in 1 mL of 0.15 M KCl), and the mixture was sonicated for 1–2 min in a bath sonicator to form a cloudy suspension. Vesicles were formed by use of a probe sonicator, giving 1-min bursts of sonication (Branson Sonifier Cell Disruptor) for a total time of 6 min, at 30–40 W, to the protein suspension cooled in a bath of ice–water. The preparation was able to pump protons as soon as the suspension cleared (2–3 min), and optimum pumping was found to occur after ~6 min of sonication. A slight deterioration in proton-pumping activity was observed for periods of sonication longer than 12 min.

Proton-pumping assays were carried out by diluting aliquots of this preparation to 200 μL with 0.15 M KCl. To this was added a solution of 4 M NaCl/0.15 M KCl (0.8 mL), and the pH of the suspension was adjusted to 6.7–6.9 by adding aliquots (2–5 μL) of 5 mM NaOH with stirring in a water bath at 22 °C. The pH of the suspension was monitored on an Orion Research Model 601A pH meter with a Fisher standard microprobe combination electrode in dim light and with intense light provided by a slide projector fitted with an orange Corning glass filter (3-68, transmits light >500 nm). The pH increase was recorded until a new steady state was observed, whereupon the light was switched off and the pH fell toward the dark level.

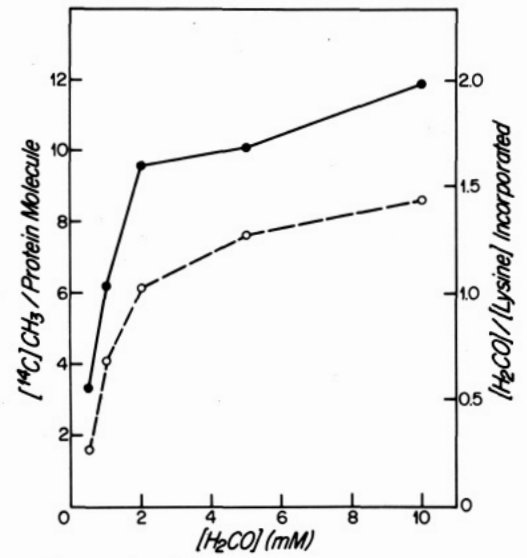

FIGURE 1: Extent of methylation of bR with increasing formaldehyde concentration. One round of methylation was carried out as described under Methods with different concentrations of [^{14}C]formaldehyde in the reaction mixture (○). Each sample was then methylated for a second time under identical conditions (●). Amount of protein was determined spectrophotometrically, and degree of methylation by $^{14}CH_3$ incorporation.

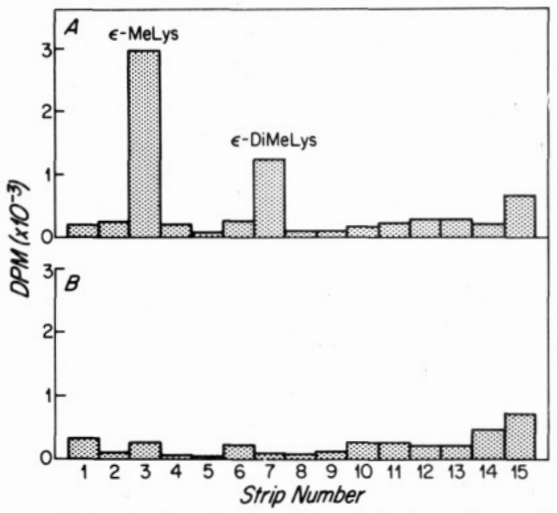

FIGURE 2: Determination of ratios of dimethyllysine and methyllysine at the active site of AMbR. PMbR was bleached and methylated as described under Methods with [^{14}C]formaldehyde and then hydrolyzed, and the ^{14}C-methylated lysines were separated by TLC (A). A control ^{14}C methylation was also performed on nonbleached PMbR, which was hydrolyzed and chromotographed in the same way (B).

RESULTS

Permethylation of bR. The initial objective of the work reported here was to monomethylate the active site lysine of bR. In order to accomplish this, the available non active site lysines of bR first had to be reductively dimethylated to form PMbR before proceeding with the active site modification studies. Purple membrane suspensions containing bR were methylated by procedures similar to those used in the permethylation of rhodopsin (Longstaff & Rando, 1985; Longstaff et al., 1986). The membranes were suspended in buffer, and the non active site lysine residues were reductively dimethylated with formaldehyde–pyridine–borane as described under Methods. Dimethyl sulfoxide was added to improve the extent of methylation with each round of reaction. bR contains a total of seven lysine residues, with the active site lysine being protected from methylation by Schiff base formation with retinal (Ovchinnikov, 1982). The membrane lipids do not contain free amino groups or other methylatable sites (Kates et al., 1982). In Figure 1 is shown methyl group incorporation data for bR at various concentrations of [^{14}C]formaldehyde after a single round (○) and a double round of methylation (●). As can be seen in Figure 1, a high concentration of formaldehyde was required to complete the methylation of the non active site lysines even after two rounds of methylation. The resulting permethylated bR (PMbR) is a stable pigment that proved to be only slightly different from bR itself.

Active Site Methylation of PMbR. As shown above, the non active site lysines can be completely dimethylated under conditions that retain the stability of the holoprotein. Freshly prepared, nonisotopically labeled PMbR was (A) bleached and then labeled with [^{14}C]formaldehyde–pyridine–borane or (B) simply labeled with [^{14}C]formaldehyde–pyridine–borane (Figure 2). The labeled proteins were then digested with HCl, and the ^{14}C-methylated amino acids were separated by thin-layer chromatography (Longstaff & Rando, 1985). Figure 2 indicates where the ε-methyl- and ε,ε-dimethyllysines migrate. The level of ^{14}C methylation at the active site was known for this sample so that from this and the ratios of methylated lysines derived from thin-layer chromatography the proportions of dimethyllysine, methyllysine, and lysine per mole of protein could be calculated. As expected, little or no further methylation of PMbR occurred (Figure 2B). On the other hand, substantial further methylation of PMbO was observed (Figure 2A). The labeling occurred at the ε-amino groups of the remaining active site lysine residue. A substantially higher proportion of monomethylation (61% of total) was observed relative to dimethylation (13% of total). Furthermore, it could be determined (see Methods) that approximately 26% of free, unmethylated lysine remained after this procedure.

Only the monomethylated and unmethylated lysine derivatives are, in principle, capable of forming a pigment with added retinals. In order to remove the 26% of the unmethylated lysine containing bO and to prevent it from interfering with further experiments, the methylated bO's were treated with a mixture of OPA/ME—a reagent previously shown capable of reacting with the ε-amino group of lysine but not its monomethylated counterpart (Longstaff & Rando, 1985; Longstaff et al., 1986). That this reagent is successful here as well was clear from the fact that this treatment led to a modified bO that was incapable of rapidly (10 min) forming a pigment with *all-trans*-retinal absorbing in the 555–560-nm range (unpublished experiments and see below). Nonbleached PMbR was also treated with OPA/ME in an identical manner as a control.

Regeneration and Spectra of AMbO and PMbO. The active site methylated bO (AMbO) previously treated with OPA/ME was incubated with *all-trans*-retinal, and the regeneration rate of the pigment was followed (●) (Figure 3). For comparison's sake, the rate of regeneration with PMbO is also presented (○) (Figure 3). Of interest in Figure 3 is the very slow rate of pigment regeneration observed with the active site methylated material. PMbO was regenerated at approximately the same rate as its unmethylated counterpart, with complete regeneration occurring in ~30 min under these conditions. On the other hand, it took approximately 20 h for the active site methylated pigment to achieve full regeneration. These observations allowed for the determination that the OPA/ME treatment described above was successful. The active site monomethylated derivative produced a pigment with a λ_{max} of 620 nm, whereas PMbO produced a pigment with a λ_{max} of 558, both in the dark (Figure 4). Thus, PMbR is only slightly blue shifted (2 nm) from its unmethylated counterpart,

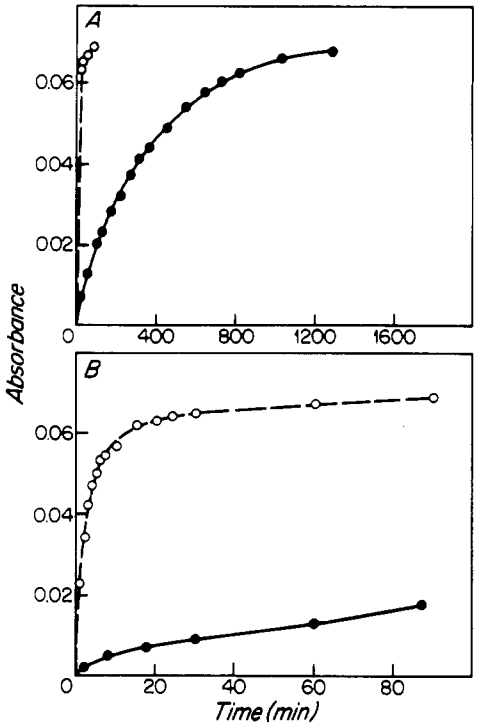

FIGURE 3: Regeneration of PMbO and AMbO with *all-trans*-retinal. Bleached PMbR (○) and AMbR (●) were both regenerated under the same conditions with *all-trans*-retinal at 20 °C as described under Methods. Regeneration was followed by monitoring the increase in λ_{max} for PMbR and by recording spectra over a longer period of time for AMbR. The same data are presented in panels A and B, plotted with different time scales.

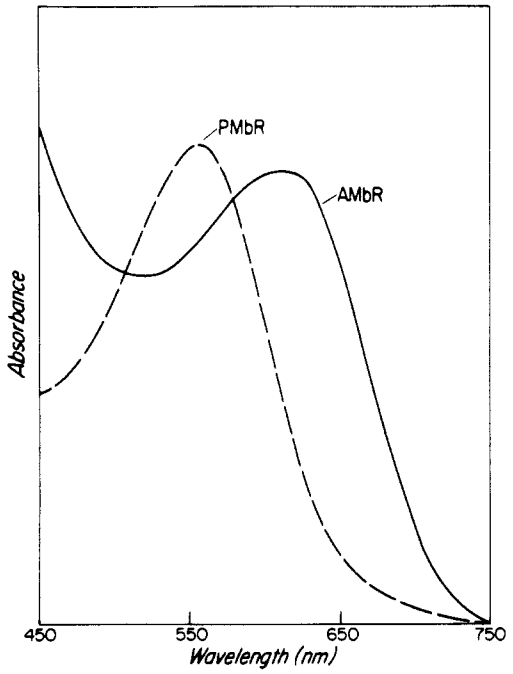

FIGURE 4: Partial UV/vis spectra of PMbR and AMbR. Shown here are overlaid, dark-adapted spectra of PMbR and AMbR (regenerated with *all-trans*-retinal), normalized to give similar peak heights. The spectrum of PMbR is only very slightly affected by permethylation (~2 nm blue shifted), whereas active site methylation and regeneration produce a blue pigment with a dark-adapted broad λ_{max} centered around 620 nm.

but the active site methylated derivative is radically red shifted. This can be visually observed, because this pigment is blue and hence is of the cyanopsin family.

Light and Dark Adaptation of PMbR and AMbR. In panels A–C of Figure 5 are shown the light- and dark-adapted

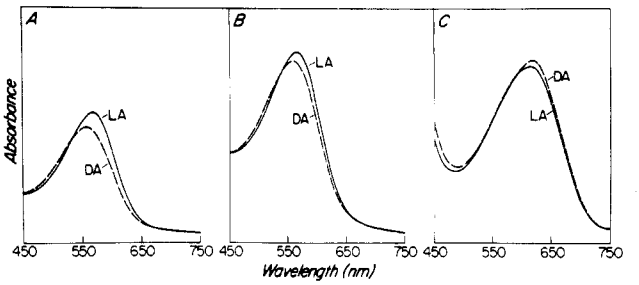

FIGURE 5: Partial light-adapted/dark-adapted spectra for PMbR and AMbR. Unmodified bR (panel A) and PMbR (panel B) behaved similarly in light/dark adaptation cycles, so that the light-adapted pigment was slightly red shifted and had a slightly higher extinction coefficient than the dark-adapted pigments. AMbR (panel C) demonstrated the opposite behavior such that, in light, the pigment changed to one with a lower wavelength λ_{max} (~4 nm) and a slightly lower extinction coefficient (0.96 of the dark-adapted species). In all cases, dark adaptation was for 20–24 h at 4 °C, and light was provided by a slide projector with an orange Corning glass filter (3-68) as described under Methods for proton-pumping experiments.

spectra for bR (A), PMbR (B), and AMbR (C) respectively. bR undergoes a small (10 nm) bathochromic shift along with a 10% increase in ϵ (Figure 5A)—a result consistent with previously reported studies (Oesterhelt et al., 1973). A similar shift is observed with PMbR with respect to both the λ_{max} and ϵ (Figure 5B). It is noteworthy that the opposite kinds of shifts are observed with AMbR (Figure 5C). Here a small hypsochromic shift (4 nm) occurs, accompanied by a small (4%) decrease in ϵ. This shift would result from the accumulation of an intermediate prior to the completion of the cycle. The effects reported in Figure 5C are reversible in the dark within 30 min.

Proton-Pumping Abilities of Modified Pigments. Asolectin-based vesicles containing bR, PMbR, and AMbR were prepared by published procedures as described under Methods. Light-dependent pH changes were measured, according to procedures previously described (Racker, 1973). In part A of Figure 6 are presented the data for bR. A light-dependent increase in pH was observed that was rapidly reversed when the light was turned off. In Figure 6B similar experiments are shown for PMbR that had previously been treated with OPA/ME. The rate at which the pH changes were manifest was slower than that with bR, but the magnitude of the changes was unaltered. In Figure 6C is shown a sample of PMbR that was bleached and incubated at 4 °C for 96 h before regeneration with *all-trans*-retinal, in parallel with a sample undergoing active site methylation and OPA treatment. In Figure 6C, the pH changes measured were somewhat less than those observed in panel B, perhaps due to the occurrence of some denaturation during the bleaching regeneration cycle. Finally, and most noteworthy, AMbR showed no ability to move protons photochemically (Figure 6D). Figure 7 shows the proton-pumping abilities of bR (■), PMbR (as in Figure 6B) (○), and AMbR (▲) to a new steady state as a function of protein concentration. By this measure, AMbR again shows no ability to pump protons at any concentration tested. PMbR is probably a little less efficient than unmodified bR by this assay, but the observed differences are clearly minor.

DISCUSSION

Both rhodopsin and bR are holoproteins containing vitamin A aldehydes covalently linked to active site lysine residues via Schiff base linkages (Ovchinnikov, 1982). It is generally accepted that these Schiff bases are protonated and that this protonation is important to the normal functioning of these proteins. However, deprotonation of the Schiff bases is also

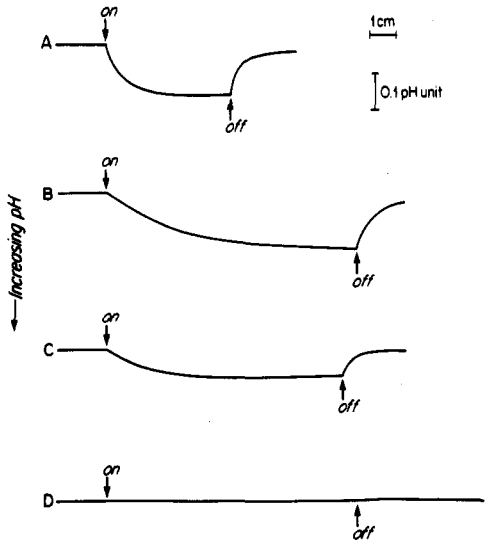

FIGURE 6: Traces of pH changes produced by vesicles of bR and modified bR. Unmodified bR in vesicles made with asolectin was shown to actively take up protons when light was shone under the conditions described under Methods (A). When the light was off, the pH returned to normal. Also shown (B) is a preparation of nonbleached PMbR made as described under Methods and also treated with OPA/ME at 4 °C along with the bleached PMbR during the preparation of AMbR. A similar pH change was observed although the rate of proton pumping was roughly half that observed for PM. A sample of bleached/regenerated PMbR is also shown (C) in the same proton-pumping assay, and exhibits the slower rate of proton pumping. The pH change observed for this preparation was also less than that for panels A and B. AMbR cannot transfer protons across the vesicle surface with light energy (D). AM vesicle suspensions were matched to have the same absorbance at their λ_{max} values (equivalent to ~75 μg/mL of regenerated protein, assuming the same extinction coefficient). The traces were recorded at a speed of 0.2 mm/s in the light and 2 mm/min in the dark.

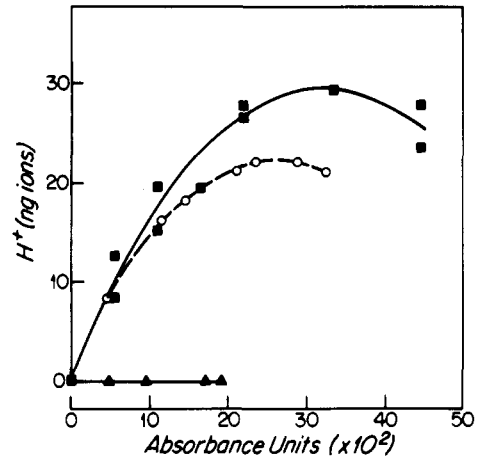

FIGURE 7: Proton pumping by bR and modified bR at different protein concentrations. bR (■) and PMbR (○) showed a similar relationship between protein concentration and amount of protons pumped with increasing regenerated protein concentration (expressed as absorbance at the λ_{max}), although PMbR is a little less efficient. AMbR (▲) was found to be completely inactive in this proton-pumping assay at all the concentrations tested.

important. For example, metarhodopsin II, which is the spectroscopic signature of photochemically activated rhodopsin, appears to contain a deprotonated Schiff base (Doukas et al., 1978). Activated rhodopsin is that conformeric form of the holoprotein capable of catalyzing the exchange of GTP for GDP in the retinal G protein (transducin) (Fung & Stryer, 1980). It has been suggested that deprotonation of the Schiff base of bR is critical in photochemically activated proton pumping as well as in rhodopsin function (Honig et al., 1979; Engelhard et al., 1985). In the simplest case, the Schiff base proton is the one actually pumped. However, the fact that the stoichiometry of proton pumping per cycle can be greater than 1 might lead one to argue against adopting this interpretation in an unmodified form (Bogomolni et al., 1980; Govindjee et al., 1980; Rendard & Delmelle, 1980). Nevertheless, it appears that the Schiff base does deprotonate during cycling, because at least one of the intermediates occurring in the cycle (M_{412}) contains an unprotonated Schiff base as determined spectroscopically (Doukas et al., 1978; Engelhard et al., 1985).

In previous studies, we had investigated the behavior of active site methylated rhodopsin (Longstaff & Rando, 1985; Longstaff et al., 1986). It was found that this pigment, which absorbs at 523 nm (bovine rhodopsin absorbs at 498 nm), showed unusual bleaching behavior. Immediately upon the absorption of light, the spectrum of the modified rhodopsin shifted to ~485 nm, whereupon it slowly hydrolyzed over many hours to afford *all-trans*-retinal and the modified opsin. No evidence for the formation of either metarhodopsin II or metarhodopsin III was found, and the slowly decaying 485-nm intermediate was ascribed to a stabilized metarhodopsin I like intermediate. The G protein was not activated in the presence of the photolyzed active site methylated rhodopsin (Longstaff et al., 1986), thus showing that Schiff base deprotonation at the active site is necessary to achieve the protein conformation corresponding to activated rhodopsin detected at the membrane surface by the G protein.

In this study, we were interested in preparing active site methylated bR in order to begin to study its photochemical and functional behavior. The major question addressed here is whether or not Schiff base deprotonation is obligate in photochemically induced proton pumping. In order to prepare active site methylated bR and to quantitate the reaction, all available peripheral lysines first had to be reductively dimethylated. This was performed in membrane sheets, with formaldehyde and pyridine–borane—reagents previously used in the successful permethylation of rhodopsin (Longstaff & Rando, 1985). It was found that the dimethylation process is highly dependent on the formaldehyde concentration (Figure 1). As long as extreme conditions were avoided, i.e., pH >9 or DMSO concentrations >20% (v/v), bleaching of the protein did not occur, and a methylated protein capable of regeneration was obtained. Under more forcing conditions, i.e., pH >9, DMSO or ethanol concentrations >20% (v/v), or temperatures >30 °C, more denaturation was observed. With milder conditions, but with higher formaldehyde concentrations (>10 mM), or with multiple rounds of methylation at lower concentrations, protein denaturation during the reaction was reduced, but the methylated product was incapable of regeneration after bleaching and chromophore extraction (data not shown). The reasons for this are not known, but in this respect bR is more sensitive to this type of chemical modification than was rhodopsin. Permethylated bR proved to be stable and to have a λ_{max} virtually the same as that of bR (558 nm vs. 560 nm in the dark-adapted state) (Figure 4). Furthermore, the PMbR also underwent reversible light–dark adaptation similar to bR (Figure 5) and, most importantly, pumped protons with a similar efficiency to that of bR itself after incorporation into asolectin-based vesicles (Figures 6 and 7). The only difference noticed was that the rate of reaching a given steady-state pH change was slower than that for unmodified bR by itself (Figure 6). Methylation of the non active site lysine residues of bR with formaldehyde–sodium cyanoborohydride was also reported to produce a PMbR that was functionally similar to

bR itself (Abdulaev et al., 1984).

The fact that PMbR behaved similarly to bR allowed us to proceed with the active site methylation studies. Bleaching of PMbR and further methylation at the active site lysine with formaldehyde–pyridine–borane led to a mixture of active site lysine dimethylated, monomethylated, and unmethylated entities (Figure 2). As compared to the active site methylation of opsin, a relatively larger amount of the monomethyl derivative was found compared to its dimethyl counterparts ($\sim$ 4/1 vs. 1/1) (Longstaff & Rando, 1985). The unmethylated lysine containing AMbO could be quantitatively removed by treatment with OPA/ME (Longstaff & Rando, 1985), leaving the active site lysine monomethylated and dimethylated derivatives. Since the latter modified protein cannot engage in Schiff base formation with retinal, it was possible to proceed with studies on the monomethylated material. Several striking differences were observed between the behavior of PMbO and the behavior of AMbO. First of all, PMbO underwent regeneration with *all-trans*-retinal at about the same rate as bO itself, whereas AMbO was markedly slower (Figure 3). Complete regeneration with AMbO was only achieved after 24 h vs. $\sim$30 min for PMbO. Second, the regenerated AMbR, which is blue and should more correctly be called a cyanopsin, shows a λ_{max} of 620 nm whereas PMbR absorbs 2 nm blue shifted from bR (Figure 4). The striking bathochromic shift (60 nm) of AMbR is shared, albeit to a lesser degree, by active site methylated rhodopsin, which absorbs 25 nm red shifted compared to Rh or PMRh (Longstaff et al., 1986). These shifts are not due to differences in the λ_{max} values of charged retinal Schiff bases formed with secondary and primary amines. λ_{max} values of Schiff bases formed between *all-trans*-retinal and either *N*-methylbutylamine or *N*-butylamine (when the resultant Schiff base is protonated) are identical (Longstaff et al., 1986). The etiology of this effect must be looked for elsewhere and perhaps is related to small changes in the relative positioning of the charged Schiff base and its negatively charged counterion at the active site of bR. The shift in the λ_{max} values of protonated Schiff bases are important in the functioning of the visual pigments and bR, and possible mechanisms by which opsin or bO could effect these shifts have been written on extensively by others (Honig et al., 1979; Nakanishi et al., 1980; Motto et al., 1980).

A further observed difference between PMbR and AMbR relates to the light–dark adaptation cycles of the two pigments. Dark-adapted PMbR, which presumably contains a mixture of chromophores in the all-trans and 13-cis configurations, light adapts similarly to bR itself; that is, a small red shift is observed along with a small increase in ϵ (Figure 5). This shift presumably occurs because all of the chromophore is photoisomerized to the trans configuration during light absorption. On the other hand, photolyzed AMbR behaved distinctly differently (Figure 5). A small blue shift is observed along with a small decrease in ϵ during light adaptation (Figure 5). It is assumed that dark-adapted AMBr contains a mixture of all-trans and 13-cis chromophores. A probable interpretation of the observed light-adaptation results is that an all-trans–13-cis isomerization has occurred to produce the intermediate L, which cannot proceed to M_{412} because deprotonation of the Schiff base is critical for this conversion. Strikingly similar spectral charges are observed with bR at low temperatures (−74 °C), where the L $\rightarrow$ M transition is blocked (Tsuda et al., 1980). The 550-nm intermediate L, with lower ϵ than light-adapted bR, accumulates under these conditions. It should be noted that the light adaptation observed with AMbR is completely reversible. After 30 min in the dark the spectrum of the dark-adapted AMbR was obtained again. Future experiments will test the hypothesis of an accumulating L-type intermediate with flash photolysis techniques and low-temperature chromophore extraction to analyze the isomeric state of the retinal at various stages of the photocycle. If indeed an L-like intermediate is accumulating in the light because the L $\rightarrow$ M conversion is blocked, then it is interesting that nevertheless the dark-adapted state can be thermally achieved. This would of necessity mean that there must be a thermal isomerization pathway from L to the dark-adapted state that does not involve any intermediates past M.

Functional experiments were performed on AMbR incorporated into asolectin-based vesicles to determine if proton pumping could occur in the light. Although PMbR was clearly capable of light-induced proton pumping, AMbR was clearly not (Figures 6 and 7). This result would be compatible with the idea that M cannot be achieved in this pigment and that M is requisite in the proton-pumping cycle. In any case, the fact that proton pumping cannot occur with AMbR is taken to mean that deprotonation of the Schiff base in bR is obligate in proton pumping. How deprotonation of the Schiff base leads to proton translocation is the subject of future study.

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Stoichiometry of the Sodium–Calcium Exchanger in Nerve Terminals†

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ABSTRACT: The stoichiometry of the Na^+–Ca^{2+} exchanger from synaptic plasma membranes was studied in both native and reconstituted preparations. In kinetic experiments performed with the native preparation, initial rates of Na^+ gradient-dependent Ca^{2+} influx were compared to Ca^{2+}-dependent Na^+ efflux. These experiments showed that 4.82 Na^+ ions are exchanged for each Ca^{2+} ion. A thermodynamic approach in which equilibrium measurements were made with the reconstituted preparation resulted in a similar (4.76) stoichiometry. The effects of membrane potential generated by valinomycin-induced K^+ fluxes could be demonstrated in the reconstituted preparation. In addition, the direct contribution of the Na^+–Ca^{2+} exchanger to the membrane potential across the reconstituted vesicle membrane could be demonstrated by using the lipophilic cation tetraphenylphosphonium.

The sodium–calcium exchanger (or antiporter) is one of the major calcium-transporting molecules found in most, if not all, excitable and secretory cells (Reuter & Seitz, 1968; Baker et al., 1969; Kaczorowski et al., 1984). It transports calcium across the membrane in exchange for sodium ions. The amount and direction of calcium flux depend on the direction and magnitude of sodium and calcium gradients and the membrane potential (Mullins, 1977). From experiments performed in the squid giant axon (Mullins, 1977; Blaustein & Russell, 1975), the heart (Mullins, 1975; Reeves & Sutko, 1980; Ledvora & Hegyvary, 1983), and synaptosomes (Blaustein & Ector, 1976), it is known that the sodium–calcium antiporter is electrogenic. The number of sodium ions that are reported to be exchanged for each calcium ion varies from three to six (or even more) (Mullins, 1977; Reeves & Sutko, 1980; Ledvora & Hegyvary, 1983; Blaustein & Ector, 1976; Horackova & Vassort, 1979). This difference is not trivial at all, since it determines the range of membrane potential at which the exchanger will be active, the direction of its activity, and its capability to regulate cytoplasmic free Ca^{2+} ion concentration (Mullins, 1981a). The variability in the stoichiometry of the Na^+–Ca^{2+} exchanger may be due to genuine differences among different biological systems, or, alternatively, it also might reflect the fact that in most preparations studied, including membrane vesicles derived from native sarcolemma (Pitts, 1979; Reeves & Hale, 1984), many other unrelated ionic permeabilities were present; these permeabilities may be responsible for some of the fluxes attributed to the sodium–calcium exchanger or may affect the exchanger indirectly by acting on the membrane potential and thus on the driving force acting on sodium–calcium antiporter.

In this work, we have measured the stoichiometry of Na^+–Ca^{2+} antiport both in a native synaptic plasma membrane (SPM)[1] vesicle preparation and in a functionally reconstituted preparation of the synaptic plasma membrane sodium–calcium exchanger. The reconstituted preparation eliminates most of the unrelated ionic permeabilities for two reasons. First, during reconstitution, a great excess of lipids is provided; in the native vesicles, the protein:phospholipid ratio is 1:1.4, while in the reconstituted vesicles this ratio is 1:50. Thus, the probability of having the exchanger and another channel (or transporter) in the same vesicle is greatly reduced. Second, the reconstituted vesicles have a smaller diameter (550 Å) compared to the native vesicles (850 Å). The surface area decreases with the square of the diameter, to 42%. Therefore, most of the proteins that were in the same native vesicles before reconstitution are probably separated and localized in different vesicles after the reconstitution. Thus, the combination of higher phospholipid content, which also imposes a hydrophobic barrier, and the fact that different permeabilities reside in different veiscles leads to the expectation that measurements of sodium–calcium antiport activity will have a better "signal to noise" ratio and offer a biochemical preparation which might yield more meaningful measurements from the physiological viewpoint.

Experimental Procedures

Preparation of synaptic plasma membranes (SPM) was done essentially as described (Rahamimoff & Spanier, 1979; Erdreich & Rahamimoff, 1983), except that a three-step ficoll gradient (2%, 8%, and 12%) instead of a five-step one was used. Reconstitution was performed as described in our previously published procedure (Barzilai et al., 1984) with minor modifications. The method consists of solubilization of SPM in 2% cholate in the presence of a 50-fold excess (by

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[1] Abbreviations: SPM, synaptic plasma membrane; TPP^+, tetraphenylphosphonium; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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