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The Chromophore Retinal in Bacteriorhodopsin does not Change its Attachment Site, Lysine 216, During Proton Translocation and Light-Dark Adaptation

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Abstract. In order to determine the attachment site(s) of the chromophore retinal in bacteriorhodopsin and to examine the possible occurrence of light-induced changes in its binding site, all accessible lysine residues in bacteriorhodopsin were modified by reductive methylation with formaldehyde and $NaCNBH_3$. Upon transformation of the ε-amino group of the lysyl residues to the ε-N-dimethyl derivatives, these amino acids lose their potential ability to form a Schiff base linkage with retinal. The influence of this modification on the structure and function of bacteriorhodopsin was determined by applying a variety of techniques to modified or native purple membrane sheets which were either in aqueous suspensions, or attached to or incorporated into planar lipid bilayers and lipid vesicles, respectively. No differences were observed between native and methylated bacteriorhodopsin in the kinetics of laser-flash-induced photocurrents and transmission changes, or in the rate and extent of light-induced pH-changes. The overall rate, the activation energy, and the extent of light-dark adaptation were not severely affected by the modification. Furthermore, to identify the attachment site of the chromophore retinal, premethylated bacterioopsin was treated with trinitrobenzenesulphonate. The results show that lysine 216 is the sole attachment site of retinal in bacteriorhodopsin and that a change in this attachment site does not occur during the photocycle, proton translocation or light-dark adaptation. The ε-amino groups of the lysine residues do not seem to play a crucial role in the coordinated pathway of proton translocation across bacteriorhodopsin.

Key words: Bacteriorhodopsin – Chemical modification – Proton translocation – Purple membrane – Retinal

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Abbreviations used: BR, Bacteriorhodopsin; PM, purple membrane sheets; TNP, trinitrophenyl; TNBS, trinitrobenzenesulphonate

Introduction

Recent investigations indicate that the chromophore retinal of the light-energized proton pump bacteriorhodopsin (BR) of *Halobacterium halobium* is either exclusively linked as a Schiff's base to the ε-amino group of the lysine residue 216 (Bayley et al. 1981; Lemke and Oesterhelt 1981; Mullen et al. 1981) or to both Lys-216 and Lys-40 (Katre et al. 1981; Ovchinnikov et al. 1980) of the protein moiety. Since the actual linkage site was found to depend on the light conditions during reduction as well as on the isomeric state of bacteriorhodopsin, it was speculated that retinal might migrate and change its Schiff base linkage site during the photocycle (Ovchinnikov et al. 1980) and/or during light-dark adaptation (Katre et al. 1981). Obviously, knowledge of the attachment site(s) of the chromophore and of any light-induced alteration of it, is of crucial importance for the understanding of bacteriorhodopsin's structure and function.

In order to get a conclusive answer to this question we have performed reductive methylation of the accessible lysine residues in bacteriorhodopsin with formaldehyde and $NaCNBH_3$. After dimethylation the modified lysine residues cannot form a Schiff base linkage with the chromophore retinal. The structure and function of modified and native bacteriorhodopsin were compared by applying various techniques. Furthermore premethylated bacterioopsin was treated with trinitrobenzenesulphonate to identify the attachment site of the chromophore retinal.

The results obtained show unambiguously that lysine 216 is the sole attachment site of retinal in bacteriorhodopsin and that any change in this attachment site does not occur during the photocycle, proton translocation, or light-dark adaptation.

Materials and Methods

Purple membranes from *Halobacterium halobium* were prepared essentially as described by Oesterhelt and Stoeckenius (1974) and Bauer et al. (1976). Circular dichroism spectra were measured with a Cary 61 spectropolarimeter modified with an 18-kHz modulator. Absorption spectra and the kinetics of the absorption changes accompanying the so-called light-dark adaptation reaction of bacteriorhodopsin were recorded in the scattered transmission accessory of a Cary 118 spectrophotometer. The fast transmission changes during the photoreaction cycle of bacteriorhodopsin were monitored by means of a conventional single-beam flash photometer equipped with a xenon flash lamp (Strobotac GR 1538-A, GenRad, Inc.; flash duration at $\frac{1}{3}$ peak intensity: 3 μs) as the actinic light source. A cut-off filter (Schott OG 515) was used to limit the wavelength range of the originally white excitation light. Light-induced pH-changes in the external medium generated by bacteriorhodopsin-lipid vesicles were measured and calibrated as described by Dencher and Heyn (1979). Purple membranes containing native or modified bacteriorhodopsin were attached to planar bilayer membranes according to Fahr et al. (1981). Illumination by a 10-ns laser pulse

(575 nm) induced electric currents across these planar sandwich structures. The measured photocurrents were digitized by a Biomation 8100 transient recorder and averaged in a Tracor TN 1710. The experimental set-up allowed an analysis of the photocurrent time course in the range between 1 μs and 5 ms.

Reconstitution Procedure

Bacteriorhodopsin was incorporated into phospholipid vesicles by an octylglycoside dilution method according to Racker et al. (1979). Purified soybean phospholipids (asolectin, 27 mM) were sonicated under argon atmosphere in a bath-type sonicator to clarity (about 30–40 min at 10° C). These vesicles (1.1 mg asolectin) were mixed with 30–100 μg of native or methylated bacteriorhodopsin in a final volume of 400 μl containing 75 mM KCl, 1 mM Hepes (pH 7.4), and 1.25% octyl-β-D-glucoside (Calbiochem). Two minutes prior to the addition of the lipid vesicles, bacteriorhodopsin (purple membrane) was mixed with the detergent solution and sonicated for 20 s. The bacteriorhodopsin-asolectin-octylglycoside mixture was incubated in the dark at 20° C. After the given time periods (4–2,800 min after mixing of all constituents) 40-μl samples were taken, diluted with 1 ml 150 mM KCl, pH 6.0, and assayed.

Reductive Methylation of Bacteriorhodopsin and Bacterioopsin

Reductive methylation of bacteriorhodopsin was performed following the procedure of Jentoft and Dearborn (1979): 30 mg of dark-adapted purple membrane in 10 ml of 0.5 M Na-borate, pH 8.5, were treated in the dark for 16 h with the 10-fold molar excess of cold formaldehyde and the 100-fold molar excess of $NaCNBH_3$ over the free lysine residues in the bacteriorhodopsin molecules. After the reaction, the membranes were washed several times with 0.5 M Na-borate buffer by centrifugation. The whole procedure was repeated five times.

For characterization, methylated membranes were delipidated by dissolving them in 4% dodecylsulfate and subsequent precipitation of the protein with 80% ethanol. The pellet was washed several times with distilled water and lyophilized. The lyophilized methylated protein moiety was hydrolysed with 5.7 N HCl at 105° C for 24 h and subjected to amino acid analysis.

Bacterioopsin of methylated bacteriorhodopsin was obtained by treatment of the modified purple membranes with 2 M solution of hydroxylamine (pH 8.0) in the light. The apomembranes were washed several times with distilled water and suspended in 0.5 M Na-borate buffer (pH 8.5).

TNBS-modification of methylated bacterioopsin was carried out as follows: trinitrobenzenesulphonate (TNBS) (100 μM) was added to 1 μM of methylated bacterioopsin in 3 ml of 0.5 M Na-borate buffer pH 8.5 containing 1% SDS. After 2 h the reaction mixture was dialysed overnight against 3 l of distilled water. The protein was precipitated with 80% ethanol and washed several times to remove SDS. Cyanogen bromide cleavage was performed in 70% formic acid

for 24 h with 100-fold excess of BrCN. BrCN peptides were separated by chromatography on Bio-Gel P-30 columns (1.5 × 100 cm) equilibrated with 80% formic acid. The fraction absorbing at 345 nm was rechromatographed on the Altex Lichrosorb C8 column (0.36 × 25 cm). Peptides up to 100 nM were applied in 80% formic acid (100 µl) and eluted at 1 ml/min over 1 h with a linear gradient of CH_3CN. The initial solvent was 0.1% CF_3COOH in H_2O and the final solvent 0.1% CF_3COOH in 70% CH_3CN.

Amino acid analysis was carried out on a Durrum (D-500) analyser. The phenylthiohydantoin derivative of trinitrophenyl-lysine was identified as lysine after its sequential hydrolysis in 30% ammonia solution at room temperature and then in 5.7 N HCl solution at 140° C (Miyanishi et al. 1982). Automated sequence analysis was performed on an Beckman 890 C sequencer. Polybrene (2 mg) was used as a carrier.

Results

Identification of Retinal's Attachment Site in Bacteriorhodopsin

a) Reductive Methylation of Bacteriorhodopsin. The extent of methylation of bacteriorhodopsin was monitored by amino acid analysis of an aliquot of the purple membrane suspension after each step of the treatment procedure.

Completion of methylation was achieved after the fifth repetition of the methylation procedure. The amount of unmodified lysine residues was estimated by comparing the amount of unmodified lysines in the column eluate with the amount of arginine (7 residues/bacteriorhodopsin) of phenylalanine (13 residues/bacteriorhodopsin). An averaged amount of 1.00 ± 0.05 (mean value and standard deviation of 6 samples) lysines/bacteriorhodopsin remained unmodified (Fig. 1A). No monomethylated ε-amino groups in lysine residues were detected by amino acid analysis.

Amino acid analysis of methylated apomembranes (bacterioopsin) showed no detectable amount of unmodified lysine residues (Fig. 1B).

b) Isolation of Trinitrophenylated (TNP) Peptide from Premethylated and TNBS-Treated Bacterioopsin. Premethylated and TNBS-treated bacterioopsin was cleaved with CNBr. The peptides were fractionated on a Bio-Gel P-30 column, equilibrated with 80% formic acid. Figure 2 shows the elution profile of the resulting peptides. Only one major fraction (see Fig. 2, fraction no. IV) of TNP peptide was obtained. N-Terminal analysis of this fraction demonstrated that it contains two peptides. As could be verified by N-terminal analysis, fractions I and II (see Fig. 2) consisted of aggregated forms of all peptides from the bacterioopsin cleavage procedure, i.e., they also contain the TNP peptide of fraction IV. This explains the observed absorbance at 345 nm of fractions I and II.

The peptide of fraction IV containing the TNP-label was separated from the unlabeled peptide by HPLC (Fig. 3) as described in Materials and Methods. Only band II exhibits the characteristic absorbance of the TNP-label at 345 nm.

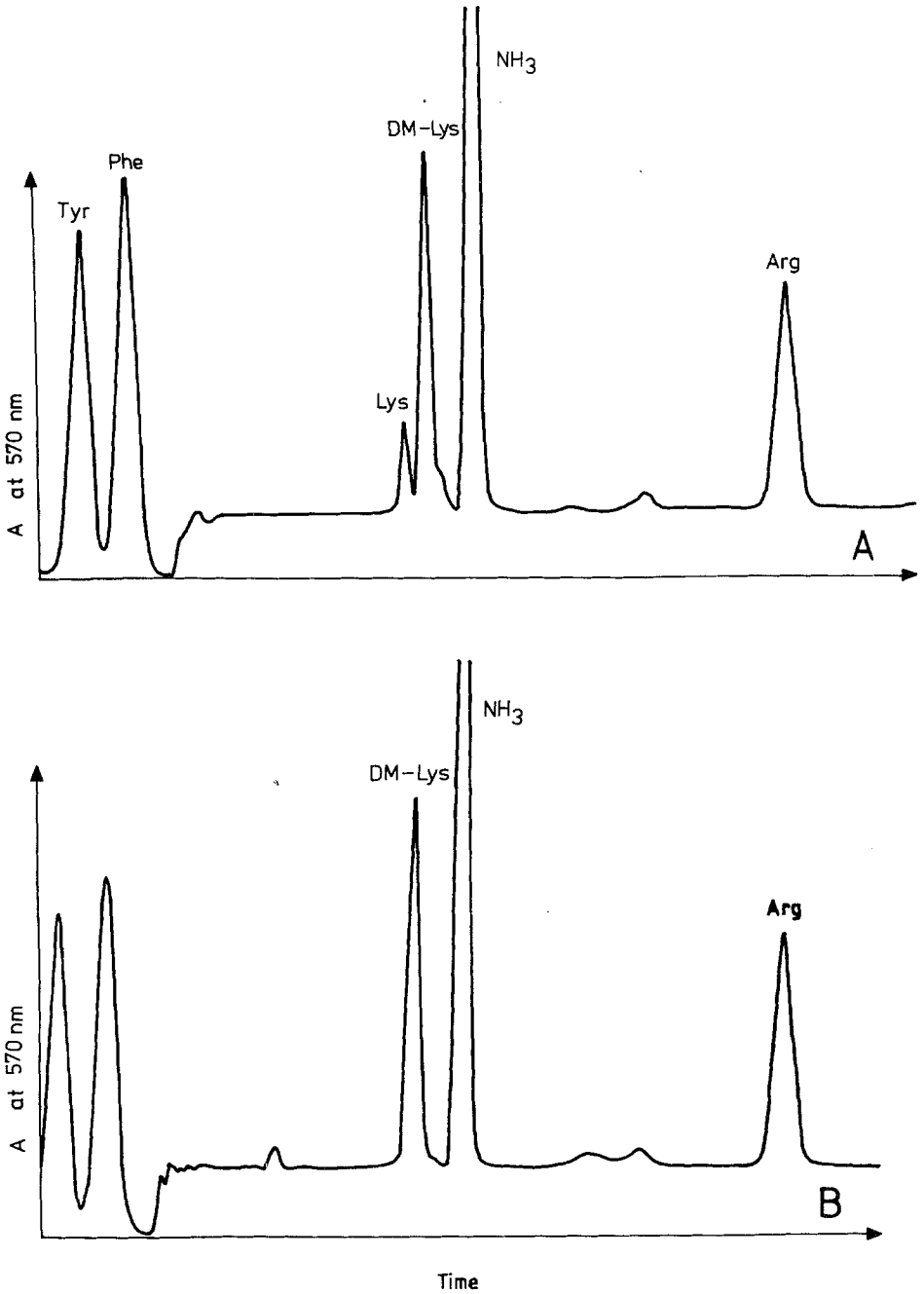


Fig. 1A and B. Amino acid analysis of methylated bacteriorhodopsin (**A**) and methylated bacterioopsin (**B**). Abbreviation: DM-Lys = dimethyllysine. The absorbance A of the column eluate was monitored at 570 nm

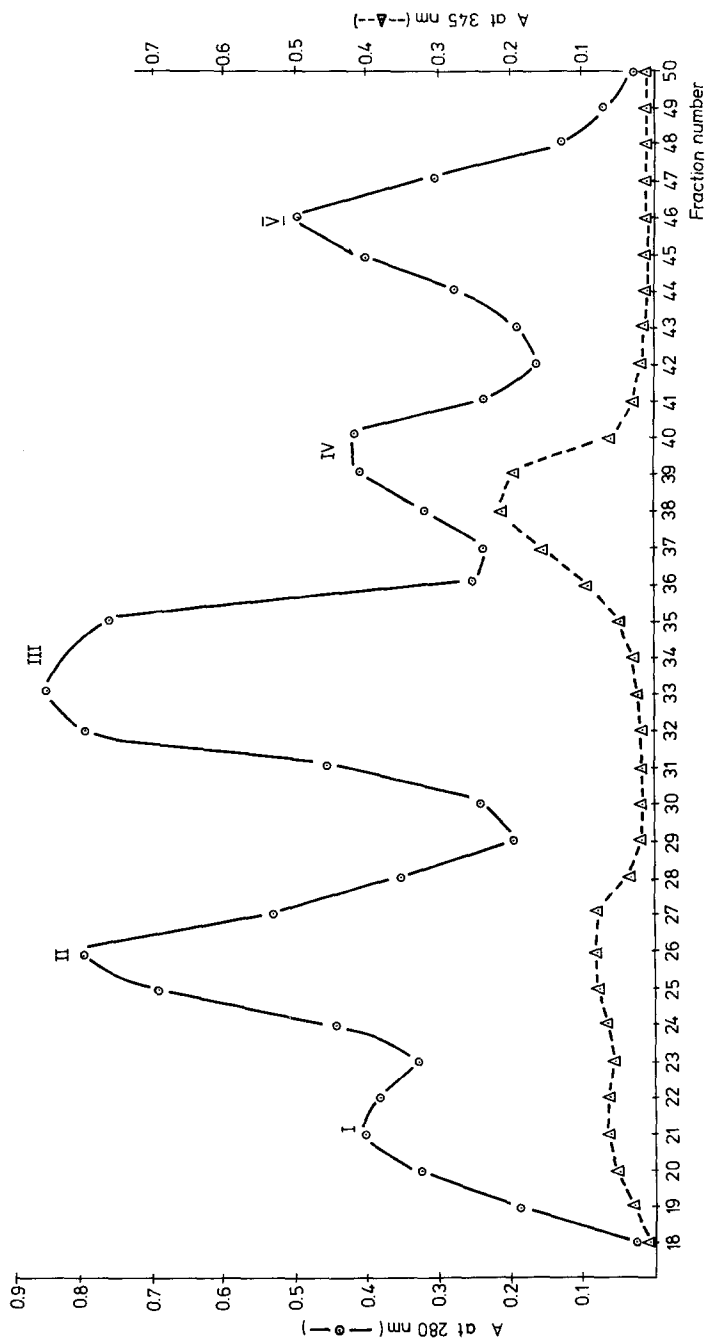


Fig. 2. Separation of CNBr peptides of methylated TNBS-treated bacterioopsin on Bio-Gel P-30; column size (1.5 × 100 cm; flow rate 2.5 ml/min). After cyanobromide-hydrolysis the resulting peptides were lyophilized and diluted in 1 ml of 100% formic and applied to the column. Absorbance A was measured at 280 and 345 nm

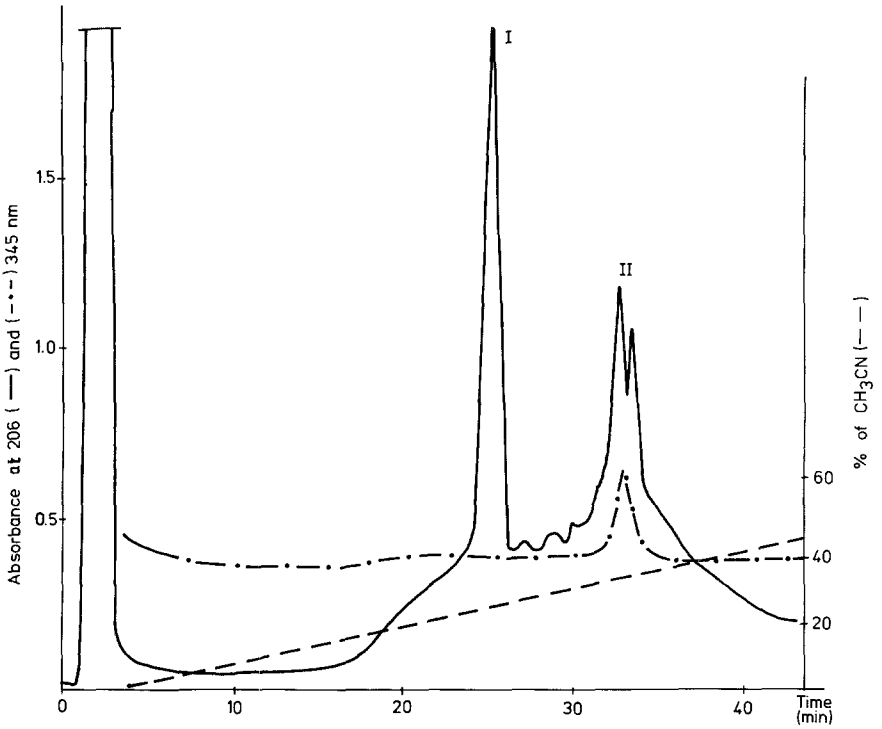


Fig. 3. Separation of fraction IV (see Fig. 2) on the Altex Lichrosorb C8 column (0.36 × 25 cm; flow rate 1 ml/min). Initial solvent was 0.1% trifluoric acid in H_2O; final solvent: 0.1% trifluoric acid in 70% CH_3CN. Detection was performed at 206 and 345 nm

c) Sequence Analysis of the TNP Peptide. The trinitrophenylated peptide (80 nmol) purified as described above was subjected to 10 cycles of automated Edman degradation, which revealed the sequence:

Cycle no.:	1	2	3	4	5	6	7	8	9	10
Sequence:	Val	Leu	Asp	Val	Ser	Ala	–	Val	Gly	Phe

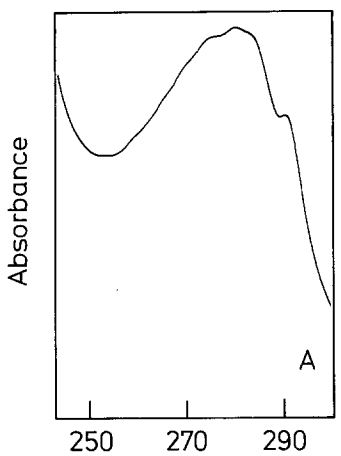
Except for the total absence of phenylthiohydantoin-lysine at cycle 7, the result was in agreement with the sequence data work of Ovchinnikov et al. (1977) and Gerber et al. (1979). The material of the seventh cycle was divided into two parts. One half was hydrolysed with 5.7 N HCl and subjected to amino acid analysis. The other half was sequentially hydrolysed with 30% ammonia at room temperature and 5.7 N HCl at 140° C. Amino acid analysis in that case clearly showed the appearance of a lysine residue at this step.

The results taken together indicate that all but one (i.e., of Lys 216) of the ε-amino groups of bacteriorhodopsin are dimethylated.

Spectroscopic Properties of Lysine-Modified Bacteriorhodopsin

The absorption spectrum and the circular dichroism spectrum of bacteriorhodopsin in the wavelength range between 240 and 700 nm after reductive

methylation of the six accessible lysine residues show no significant differences as compared to the unmodified sample (Fig. 4). The finding that the characteristic peaks and shoulders in the 240–350 nm wavelength region of the absorption and CD spectra (for comparison see Bauer et al. 1976; Heyn et al. 1975; Becher and Cassim 1976) are not altered after methylation excludes any perturbation of the aromatic amino acids by the modification procedure applied. Furthermore, the amplitude and the position of the negative CD band at 318 nm is indicative of unchanged chromophore-protein interactions (Bauer et al. 1976; Becher and Cassim 1976) This conclusion is also supported by the visible absorption spectrum of the modified light-adapted bacteriorhodopsin, which shows an absorption maximum, a band shape and an extinction coefficient as in the unmodified light-adapted bacteriorhodopsin.



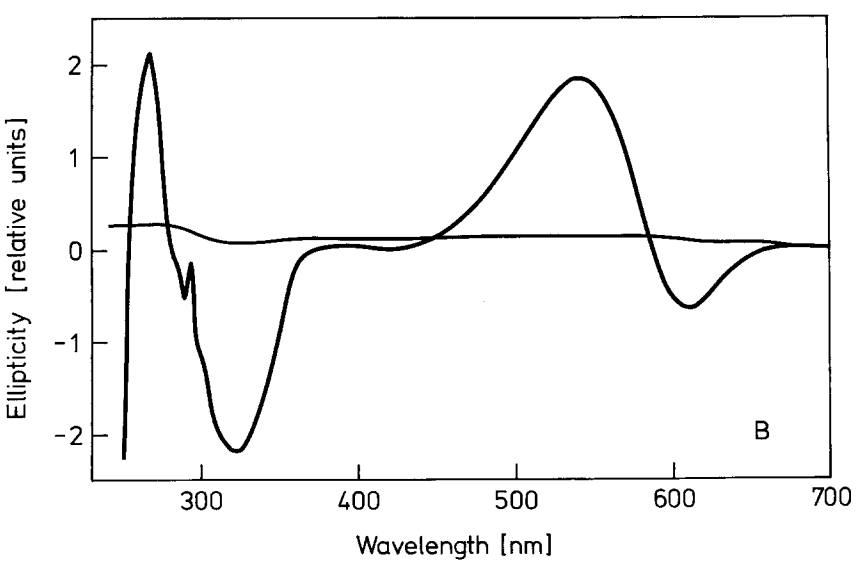


Fig. 4A and B. Near ultraviolet absorption spectrum (**A**) and circular dichroism spectrum (**B**) of methylated bacteriorhodopsin in the purple membrane. The nearly flat baseline in **B** represents the ellipticity of the cuvette with buffer. 20° C

The visible circular dichroism spectrum of the modified purple membrane (Fig. 4B) has the pair of positive and negative exciton bands characteristic of the hexagonally aggregated state of bacteriorhodopsin (Bauer et al. 1976; Heyn et al. 1975; Becher and Ebrey 1976). In contrast to fluorescamine modification of lysine residues in bacteriorhodopsin, which induced mobility of either the chromophore or the entire chromoprotein (Lam and Packer 1981), the treatment applied in the present report does not alter the protein-protein and chromophore-protein interactions typical of the native purple membrane. The reduced intensity of the negative band at 600 nm indicates however that exciton coupling between neighbouring chromophores is somewhat perturbed.

Upon dark adaptation of light-adapted modified bacteriorhodopsin the absorption maximum shifts from 567 nm to 558 nm with a concomitant 12.5% decrease in absorbance (data not shown). The position of the absorption maxima, the wavelength difference of 9 nm, and the difference in absorbance of light- and dark-adapted modified bacteriorhodopsin are very similar to those of unmodified samples. Since these spectral features are an indirect measure of the isomeric state of the chromophore retinal in bacteriorhodopsin, it can be concluded, that as in native bacteriorhodopsin (Oesterhelt et al. 1973; Dencher et al. 1976; Sperling et al. 1977; Ohno et al. 1977; Pettei et al. 1977) light-adapted modified bacteriorhodopsin contains solely all-*trans* retinal whereas dark-adapted modified bacteriorhodopsin represents a 1 : 1 mixture of all-*trans* and 13-*cis* bacteriorhodopsin.

To investigate the kinetics of dark-equilibration from the light-adapted state, the absorption changes at 585 nm were continuously monitored and compared for modified and unmodified samples at different temperatures. In agreement with previous measurements (Oesterhelt et al. 1973; Tokunaga and Ebrey 1978; Dencher et al. 1983) the time-dependent absorption changes of unmodified bacteriorhodopsin followed first-order kinetics. In the case of the modified bacteriorhodopsin, however, the data could not be fitted with a single exponential rate constant at two of the three temperatures investigated, i.e., at 30° C and 40° C. A satisfactory description of the kinetics was obtained by a sum of two exponentials, which differ in lifetime by a factor of 4.4–6.7. The amplitude of the slower component comprises about 75% of the signal. At 20° C the deviation from a single exponential was negligible. If the overall rate for dark equilibration is compared, however, the difference between modified and unmodified bacteriorhodopsin is not large. Dark adaptation of modified bacteriorhodopsin was found to be 1.4–1.8 times faster than of native bacteriorhodopsin (Fig. 5).

From the rate constants of dark-adaptation activation energies of 25.4 kcal/mol and 22.9 kcal/mol for native and modified bacteriorhodopsin, respectively, were calculated (Fig. 5). These activation energies agree fairly well with recently reported values for dark-adaptation of native and reconstituted bacteriorhodpsin in the purple membrane and in lipid vesicles (Tokunaga and Ebrey 1978; Heyn et al. 1981; Keen and Dencher 1976; Dencher et al. 1983).

Photon capture by bacteriorhodopsin induces transient absorption changes in the visible and UV wavelength region (Stoeckenius et al. 1979). The

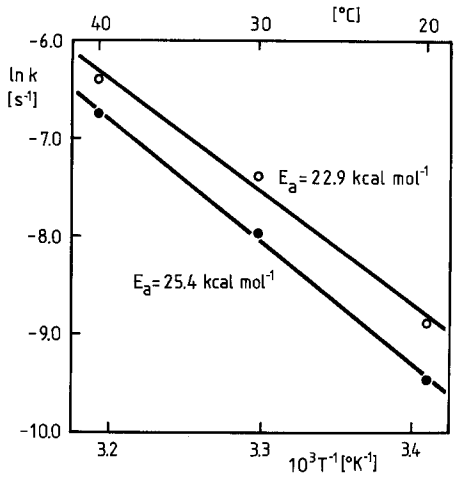


Fig. 5. Arrhenius plot of the rate constant for dark adaptation of methylated (*upper data set,* ○) and native (*lower data set,* ●) bacteriorhodopsin. The purple membranes were suspended in 25 mM phosphate buffer, pH 6.88. Straigth lines are obtained by least square fits. E.: calculated activation energy

photoreaction cycle of all-*trans* bacteriorhodopsin (the bacteriorhodopsin species containing all-*trans* retinal as chromophore) proceeds via a series of distinct spectral intermediates occurring and decaying in the time range of pico- to milliseconds at room temperature (Kayushin et al. 1974; Dencher and Wilms 1975; Dencher et al. 1976; Lozier et al. 1975; Kung et al. 1975; Slifkin and Caplan 1975; Kaufmann et al. 1976; Ippen et al. 1978). Since this photocycle is connected to proton translocation across bacteriorhodopsin in a to-date unknown way, investigation of the effect of specific chemical modifications of both the chromophore and the protein moiety might help to elucidate the underlying molecular mechanism. Flash excitation of the lysine-methylated bacteriorhodopsin generates the same spectral changes as previously reported for purple membrane in living cells and isolated sheets. For a more quantitative comparison the kinetics of the formation and decay of the intensively studied intermediate M-410 were measured.

The time-dependent transmission changes at 407 nm of native and modified bacteriorhodopsin upon excitation by a single light flash shown in Fig. 6 indicate the fast unresolved formation of M-410 and its decay. Since the purple membranes were suspended in low ionic strength buffer the formation and decay kinetics of both processes could be fairly well fitted with a single exponential rate constant (Mäntele et al. 1981; Kuschmitz and Hess 1981; Dencher et al. 1983). The calculated life-times for the formation and decay of M-410 are listed in Table 1 and show no pronounced differences in the kinetics between modified and unmodified bacteriorhodopsin. Therefore the photochemical cycle of bacteriorhodopsin is not influenced by dimethylation of the lysine residues.

Photocurrents Generated by Lysine-Modified Bacteriorhodopsin

During the photocycle protons are translocated across bacteriorhodopsin (Oesterhelt and Hess 1973; Oesterhelt and Stoeckenius 1973). This causes a

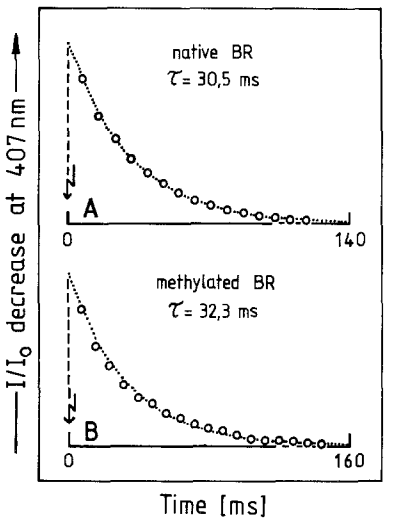


Fig. 6A and B. Flash-induced transmission changes at 407 nm in a suspension of native and modified PM sheets in 25 mM phosphate buffer, pH 6.88 at 7.3° C. The *arrows* depict the exciting flash. Experimental data (O) representing the decay of the intermediate M-410 and calculated fit with one exponential rate constant (. . .) are shown; **A** native bacteriorhodopsin, **B** methylated bacteriorhodopsin

Table 1. Rate of formation and decay of M-410

Sample[a]	Temperature (°C)	τ formation[b] (ms)	τ decay[b] (ms)
Native BR	7.3	0.375 ± 0.016	29.6 ± 0.8
	18.8	–	4.9 ± 0.4
Methylated BR	6.9	0.320 ± 0.013	–
	7.3	–	32.7 ± 1.7
	18.8	–	6.8 ± 0.9

[a] Experiments were carried out in 25 mM phosphate buffer, pH 6.88
[b] Mean and standard deviation of two or three measurements

charge displacement which can be monitored as photocurrents or photovoltages in experimental set-ups in which purple membrane sheets or monomeric bacteriorhodopsin molecules are adsorbed to or incorporated into artificial membranes (Fahr et al. 1981; Bamberg et al. 1979, 1981; Blok et al. 1977; Dancshazy and Karvaly 1976; Drachev et al. 1974; Herrmann and Rayfield 1976; Hwang et al. 1978; Seta et al. 1980; Shieh and Packer 1976). Figure 7 shows laser-flash-induced photocurrents generated by purple membrane sheets containing modified bacteriorhodopsin which had been adsorbed to planar lipid bilayers. The kinetic parameters of these photocurrents are listed in Table 2 and are almost indistinguishable from those of native purple membranes (Fahr et al. 1981). For both samples the photocurrent kinetics can be correlated with the measured transitions in the spectroscopic photocycle.

pH-Changes Generated by Lysine-Modified Bacteriorhodopsin

The photochemical cycle and the photocurrents are accompanied by a vectorial proton transport across bacteriorhodopsin in both modified and unmodified

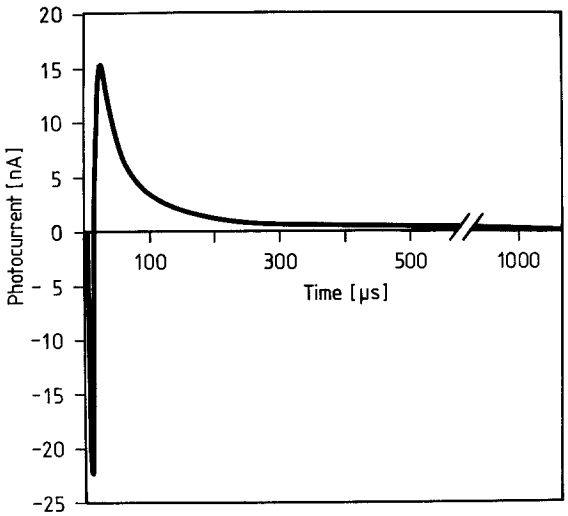


Fig. 7. Laser pulse induced photocurrents of modified purple membrane sheets attached to black lipid membranes. Temperature 25° C; 0.1 M NaCl 5mM *Tris* buffer, pH 7.0; laser pulse 575 nm, 0.1 mJ/cm^2. Black lipid membrane: 2% Diphytanoyllecithine and 0.025% octadecylamine in decane. The graph is an average of 1,500 samples

Table 2. Time constants of the photoelectric currents for methylated and native purple membrane sheets, attached to black lipid membranes

Sample	τ_1 (μs)	τ_2 (μs)	τ_3 (μs)	τ_4 (μs)
Native PM	1.2	17	56	950
Methylated PM	1.2	16	57	930

Temperature 25° C; buffer 0.1 M NaCl, 5 mM *Tris*, pH 7.0; laser pulse $\lambda = 575$ nm, 0.1 mJ/cm^2; 1,500 single sweeps were averaged before computing the time constants

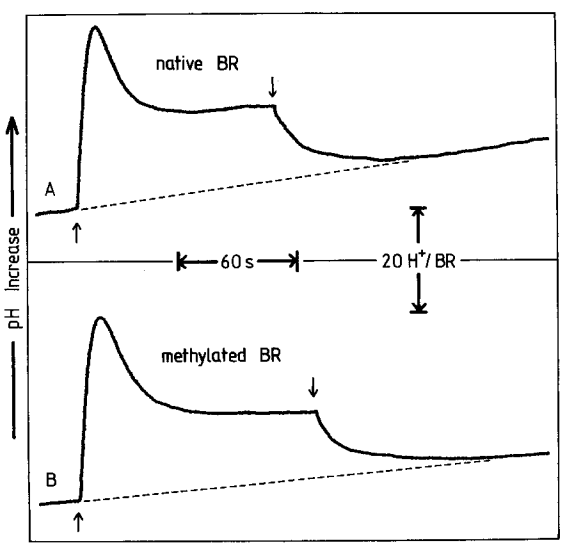


Fig. 8A and B. Light-induced pH-changes of asolectin vesicles reconstituted with native (**A**) and methylated (**B**) bacteriorhodopsin. *Arrows* depict onset and termination of the illumination period. Vesicles were formed by dilution and assayed about 100 min after mixing of all constituents. Molar lipid to BR ratio of about 1,000. 150 mM KCl, pH 6.4; 20.0° C; $9.0 \cdot 10^{-7}$ M valinomycin

samples. This could be demonstrated by monitoring light-induced pH-changes generated by bacteriorhodopsin-asolectin vesicles reconstituted by a octylglucoside-dilution procedure. The observed alkalization of the external medium upon illumination indicates that protons are transported into the vesicles (Fig. 8). Maximal light-energized proton pumping activity upon dilution (see reconstitution procedure) was measured up to about 180 min after mixing the purple membrane with octylglucoside and lipids, but even 2,800 min after incubation half-maximal activity was still present. The time-dependent decline in activity of both reconstituted native and modified bacteriorhodopsin might be due to a less asymmetrical incorporation of the bacteriorhodopsin monomers formed by the action of the detergent (Dencher and Heyn 1978). Under identical experimental conditions neither the kinetics nor the extent of the light-induced pH-changes generated by modified and native bacteriorhodopsin were significantly different. During the steady-state maximal values of 11.3 protons/bacteriorhodopsin are translocated into the vesicles by modified bacteriorhodopsin as compared to 12.4 protons/bacteriorhodopsin by the unmodified bacteriorhodopsin (data not shown). The kinetics and the magnitude of the light-induced pH-changes were altered after abolishing the membrane potential by the addition of the K^+-carrier valinomycin in the presence of KCl. Upon illumination the steady-state value is now reached after passing a larger transient alkalization. Values of 35.4 protons/modified BR and 35.3 protons/native BR for the transient and of 12.9 protons/modified BR and 14.4 protons/native BR for the steady-state were calculated from the data (Fig. 8). Very similar values were obtained from reconstituted bacteriorhodopsin-asolectin vesicles with a molar lipid to BR ratio of 304 instead of 1,000 as used in Fig. 8.

Discussion

Chemical modification of amino acid side chains in bacteriorhodopsin is a promising tool to gain information about their involvement in the vectorial proton transport mechanism of this transmembrane chromoprotein. In the present investigation the ε-amino groups of the lysyl residues of bacteriorhodopsin and bacterioopsin were converted to the ε-N-dimethyl derivatives by reaction with formaldehyde and the reducing agent $NaCNBH_3$. In contrast to other proteins investigated (Jentoft and Dearborn 1979) the α-NH_2 terminus of bacteriorhodopsin remained unmodified, since it is a pyroglutamic acid (Ovchinnikov et al. 1977; Gerber et al. 1979).

The absorption and CD spectrum of the modified bacteriorhodopsin (Fig. 4) did not show any alterations in the UV and visible range indicating the absence of severe perturbations in both the protein structure and the protein-chromophore interactions. The exciton coupling between adjacent bacteriorhodopsin molecules was slightly affected in modified purple membrane as expressed by the value of 2.3 for the amplitude ratio of the positive and negative exciton band (Fig. 4B) as compared to reported values of 1.4–1.8 for native purple membrane (Bauer et al. 1976; Becher and Ebrey 1976; Heyn et al. 1975). The preservation of the natural features might be due to the fact that the methyl

groups added to the lysyl residues are relatively small compared to other protein-modifying reagents and that the tertiary amines formed retain the charge of the parent amines with only small differences in pK values (Jentoft and Dearborn 1979).

Whereas in bacterioopsin all seven lysines could be modified, 1.00 lysine remained unmodified in bacteriorhodopsin due to blockage by the chromophore (Fig. 1). To identify the specific lysine residues forming the Schiff base linkage with retinal, the chromophore of completely methylated bacteriorhodopsin was removed and the bacterioopsin subsequently modified with TNBS. Since the TNP group was found to be predominantly at lysine 216, this amino acid seems to be the sole attachment site for the chromophore. This result is in accordance with recent experiments of other groups applying different strategies (Bayley et al. 1981; Lemke and Oesterhelt 1981; Mullen et al. 1981).

Although in our investigation we modified bacteriorhodopsin in the dark-adapted state which is composed of a 50 : 50 mixture of all-*trans* bacteriorhodopsin and 13-*cis* bacteriorhodopsin (Dencher et al. 1976; Sperling et al. 1977; Ohno et al. 1977; Pettei et al. 1977) only one predominant attachment site was identified. Therefore both retinal isomers occupy the same Schiff base linkage site, i.e., the lysyl residue 216. This is in contrast to the suggestion by Katre et al. (1981) that the 13-*cis* retinal might be attached to lysine-216 and the all-*trans* isomer to lysine 40/41.

Upon transformation of the ε-amino group of the lysyl residues of bacteriorhodopsin to the ε-N-dimethyl derivatives these amino acids lose the potential ability to form a Schiff base linkage with the chromophore retinal. Therefore this modified bacteriorhodopsin sample is suited to test the hypothesis that retinal might migrate and alter its attachment site during the photocycle and/or during light-dark adaptation (Katre et al. 1981; Ovchinnikov et al. 1980). Different features were studied. The lysine-modified bacteriorhodopsin was found to undergo a photochemical cyle not significantly different from the one of the native photopigment. The kinetics of the formation and decay of the intermediate M-410 investigated are not influenced by blocking the lysines (Fig. 6, Table 1). Also the kinetics of flash-induced transient photocurrents generated by modified bacteriorhodopsin molecules in purple membrane sheets adsorbed to planar lipid bilayers are not altered (Fig. 7, Table 2). The modified bacteriorhodopsin is still able to transport protons across the membrane upon illumination as shown with reconstituted bacteriorhodopsin-asolectin vesicles. Both the rate of the light-induced pH-changes and the maximal value of protons translocated per bacteriorhodopsin (up to 35 H^+/BR) do not differ in the modified and unmodified samples (Fig. 8).

The only difference observed between both samples is the kinetics of dark adaptation. Whereas the absorption changes of unmodified bacteriorhodopsin are always single exponential, the corresponding ones of the modified bacteriorhodopsin represent a superposition of two exponentials at two of the three temperature investigated. However, since on the one hand the overall rates are not drastically different and the calculated activation energies are similar (Fig. 5) and on the other hand the isomeric composition of the light- and dark-adapted bacteriorhodopsin is the same in both samples, it can be concluded

that the molecular mechanism of light-dark adaptation is not affected by the lysine modification.

All the results described show unambiguously that bacteriorhodopsin with blocked methylated lysine side chains is as active as the native molecule. A transient change of retinal's Schiff base linkage site from lysine 216 to another lysine residue as an essential step during both the light-induced proton transport and chromophore isomerization during light-dark adaptation is therefore excluded by our studies, which do not exclude the possibility, however, that the Schiff base linkage might be transiently broken during these reactions. Furthermore, the results demonstrate that the ε-amino groups of the lysines do not seem to participate in the coordinated pathway of proton translocation across bacteriorhodopsin. This observation is in agreement with previous experiments in which either bulky groups were introduced onto most of the lysine residues or their charge was changed (Packer et al. 1981; Lemke et al. 1982).

Since the completion of this work two papers have been published, which confirm our results. Resonance Raman spectroscopy on the recombined chymotryptic fragments C-1 and C-2 of ($\varepsilon - {}^{15}N$) lysine-labeled and unlabeled bacteriorhodopsin showed that in both the light-adapted bacteriorhodopsin and the M-410 intermediate the chromophore is attached to the large C-1 fragment (amino acids 72–248) (Rothschild et al. 1982). Dimethylation of the ε-amino groups in Lys-30, 40, and 41 of C-2 fragment (amino acids 1–71) by the procedure of Jentoft and Dearborn (1979) did not influence the regeneration of the bacteriorhodopsin chromophore or proton translocation (Huang et al. 1982). These results rule out Lys-41 as a functional site of attachment of retinal in bacteriorhodopsin and provide additional strong evidence that the chromophore remains attached as a Schiff's base to Lys-216 during the entire photocycle.

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