Vol. 114, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 29, 1983 Pages 872-881

EVIDENCE FOR A CARBOXYL GROUP IN THE VICINITY OF THE RETINAL CHROMOPHORE OF BACTERIORHODOPSIN

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Received June 23, 1983

Carboxyl groups of bacteriorhodopsin in purple membranes were activated using a hydrophobic reagent and then covalently labeled with a pH-sensitive reporter group, nitrotyrosine methyl ester. The membrane-bound reporter group had different spectral properties, and a pK 3 units higher than in solution. In purple membranes, an isosbestic point between the 428nm absorption peak of nitrotyrosine methyl ester, and the bacteriorhodopsin 570nm chromophore seen in alkaline titration, indicated interactions between the reporter group and retinal. Modification of white membranes (bacterioopsin from R_1mW strain) revealed similar, unusual spectral and ionization properties. Thus, the hydrophobic environment, not retinal interactions *per se*, are responsible for the ionization behavior of the reporter group. These results indicate that a carboxyl group is near the retinal chromophore of bacteriorhodopsin.

Purple membranes from *Halobacterium halobium* contain bacteriorhodopsin, which functions as a light-driven proton pump. The retinal chromophore of bacteriorhodopsin is bound to the ε-amino group of lysine 216 by a Schiff base linkage (1-2). Light absorption by the chromophore initiates a photochemical reaction cycle which involves configurational changes of retinal and conformational changes of the protein (3). The formation and decay of the M_{412} intermediate are linked to changes in the protonation state of the Schiff base linkage and the release and uptake of protons from the membrane (4-5). Although the complete primary sequence of the 26,000 MW protein has been determined (6), and a high degree of secondary and tertiary structural information is available (7), the structure and amino acid composition of the retinal binding site is relatively unknown. This information is a prerequisite for the understanding of the photochemical reaction cycle and the proton translocation process.

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0006-291X/83 $1.50

Studies of chromophore equilibria in bacteriorhodopsin (8) have led to a model in which the ground state absorption spectrum of the retinylidene-lysine Schiff base is modulated by its protonation state and the interaction with an anionic group. Protonation changes of the protein that occur during reconstitution provide further support for the role of an anionic protein group, such as a carboxyl residue, in chromophore structure (9). In this study chemical modification procedures were employed to attach a pH-sensitive reporter group, nitrotyrosine methyl ester, to a carboxyl group(s). The spectral and ionization properties of this reporter group in bacteriorhodopsin were used to monitor the protein environment in the vicinity of the retinal binding site.

MATERIALS AND METHODS

Purple and White Membranes. Purple membranes from Halobacterium halobium strain S-9 were isolated as in (10) and bacteriorhodopsin protein calculated from 570nμ absorbance measured using $\varepsilon_{570} = 63{,}000\ M^{-1}cm^{-1}$. White membranes from H. halobium, strain R1mW (courtesy Y. Mukahata, Osaka University, Japan) were isolated as described (11). White membranes containing bacterioopsin were reconstituted to bacteriorhodopsin by addition of stoichiometric amounts of all-trans retinal.

Chemical Modification. Carboxyl residues in bacteriorhodopsin (purple membranes) or bacterioopsin (white membranes) were covalently linked to nitrotyrosine methyl ester by using EEDQ[1] as coupling agent (Fig. 1). Nitrotyrosine methyl ester (courtesy D. Koshland, University of California, Berkeley) solutions were in 100% methanol. Membranes suspended in 0.10 M MES buffer, pH 6.0, at 1 mg/ml bacteriorhodopsin were incubated with nitrotyrosine methyl ester for 5 min, then the reaction was initiated by addition of EEDQ for one hour at 25°C. The reaction was stopped by dilution with ice cold 0.1 NaCl, 0.01 M Hepes at pH 7), then centrifuged at 100,000 x g for 30 min to pellet the membranes. Modified samples were repeatedly washed by the above procedure and UV-visible spectra were taken of supernatants to determine complete removal of unreacted nitrotyrosine methyl ester and quinoline products.

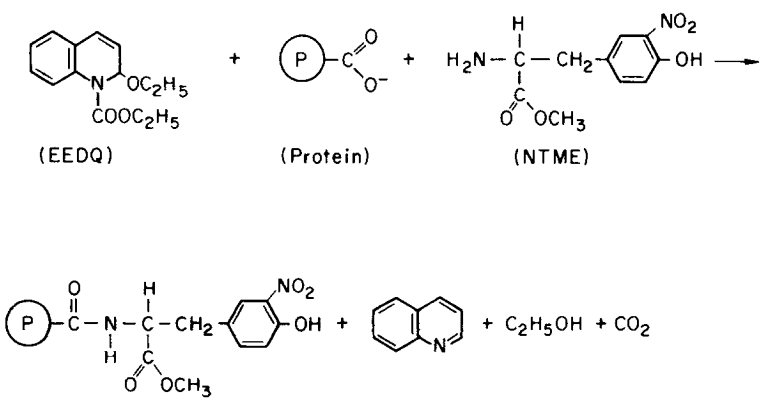

Figure 1. Nitrotyrosine Methyl Ester Labeling Reaction for Carboxyl Residues.

1 EEDQ, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline

The stoichiometry of nitrotyrosime methyl ester covalently bound to bacteriorhodopsin or bacterioopsin was determined spectrophotometrically by a procedure similar to Malan and Edelhoch (12). Modified membranes were centrifuged at 100,000 x g for 30 min and the pellet resuspended in 8 M urea, 1% SDS, 0.01 Hepes at pH 10. The urea-SDS membranes were then heated at 100°C for 10 min to completely denature the protein. Absorption spectra from 300-700 nm were recorded at pH 3 and 10. The absorbance difference between the nitrotyrosyl and nitrotyrosinate chromophores at 436 nm was used to calculate the concentration of nitrotyrosine methyl ester present. The extinction coefficient, (λ_{436}), and pK for the model compound were determined experimentally for nitrotyrosine methyl ester in 8 M urea, 1% SDS, and 0.01 M Hepes; it was found to have a λ_{max} = 436 nm at pH 10, ε_{436} = 5,100 $M^{-1}cm^{-1}$ and pK = 7.6. A value of λ_{436} = 4,800 $M^{-1}cm^{-1}$ was previously determined for nitrotyrosine in 8 M urea, 0.10 M Tris, 0.10 M KCl. The concentration of nitrotyrosine methyl ester determined by the above method was then divided by the known concentration of bacteriorhodopsin or bacterioopsin to obtain a mole ratio of nitrotyrosine methyl ester/bacteriorhodopsin. Control samples examined by the same technique revealed small absorbance changes at 436 nm due to retinal; this small difference was used to correct nitrotyrosine methyl ester absorbance measurements.

M_{412} Photostationary Steady State and Photocycle Kinetics. Purple membranes at protein concentrations of 0.2 mg/ml were used for 412 nm photostationary steady-state determinations (10) and for laser flash photolysis studies of kinetics of formation and decay of M_{412} intermediate (10).

RESULTS

Spectral and ionization properties of nitrotyrosine methyl ester. Spectra of nitrotyrosine methy ester in 0.1 M NaCl shows that in acidic solution, the fully ionized form has an absorbance maximum at 355 nm (ε = 2,980 $M^{-1}cm^{-1}$) and a strong UV peak at 277 nm (Figure 2). Upon alkalinization, the 355 nm peak is shifted to 428 nm (ε = 4,310 $M^{-1}cm^{-1}$) and exhibits an isosbestic point at 381 nm. The pK for the transition was 6.60.

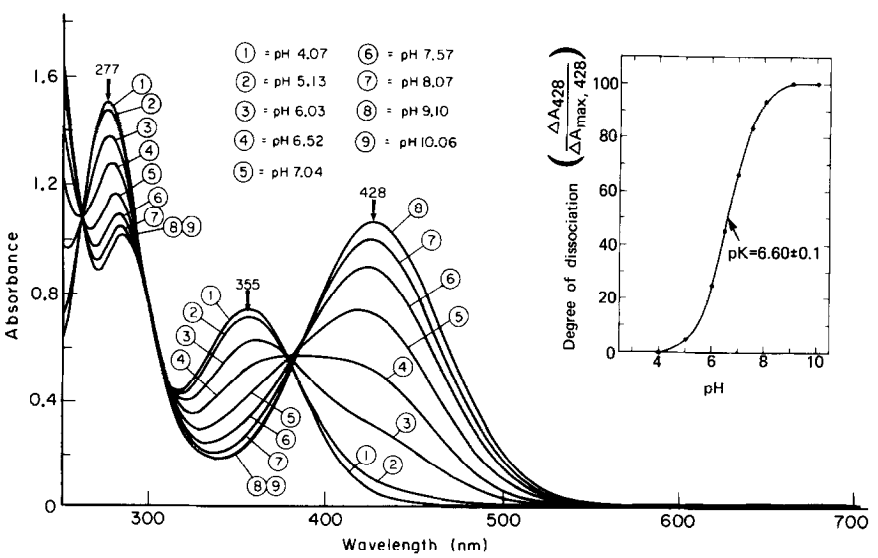

Figure 2. Spectral and Ionization Properties of Nitrotyrosine Methyl Ester. Inset: pK determined from 428 nm absorbance changes.

Nitrotyrosine methyl ester labeling of membranes. The stoichiometry of the labeling reaction was varied by changing the concentration of EEDQ, or nitrotyrosine methyl ester. A combination of short reaction times and low reagent concentrations yielded samples with a stoichiometry of almost 1 nitrotyrosine methyl ester/mole bacteriorhodopsin.

Spectra for modified purple membranes includes the absorption bands of bacteriorhodopsin that arise from retinal-protein interactions, as well as the spectral region of the reporter group. At pH 7.0 modified purple membranes show no alteration in the 570 nm band. Acid titration from pH 7.0 to pH 3.0 results in no changes in the 300-500 nm region of the spectra (data not shown). If the reporter group were in an aqueous environment (as in Figure 2), an absorptionband at 360 nm would have been expected. This was the first indication that the reporter group was in an unusual environment.

Alkaline titration from pH 7.0 to pH 11.0 results in dramatic changes in the reporter group and retinal-protein regions of the spectra. The formation of a new peak at 428 nm characteristic of the nitrotyrosinate ion occurs concomitantly with a large decrease in the extinction coefficient of the 570 nm band and an isosbestic point at 480 nm. Control purple membranes titrated from pH 7.0 to pH 11.0 show only a small decrease in the absorbance maximum at 570 nm and at pH 11.0, 82 percent of the original absorbance remained; no changes in the 300-450 nm spectral region or isosbestic points were observed. Thus, modification of carboxyl groups have a drastic effect on the stability of the retinal binding site indicating a close proximity of a carboxyl residue to retinal. Absorbance changes at 428 nm of modified membranes were used to obtain a pK for the spectral transition associated with the forward titration. The protein-bound reporter had a pK of about 10-11 (Fig.3c), at least 3 pH units higher than the model compound in solution (Fig. 2). The decrease in absorbance at 570nm titrated with an apparent pK of about 10 indicates a close coupling between the appearance of the nitrotyrosinate ion and perturbation of the retinal-protein interaction.

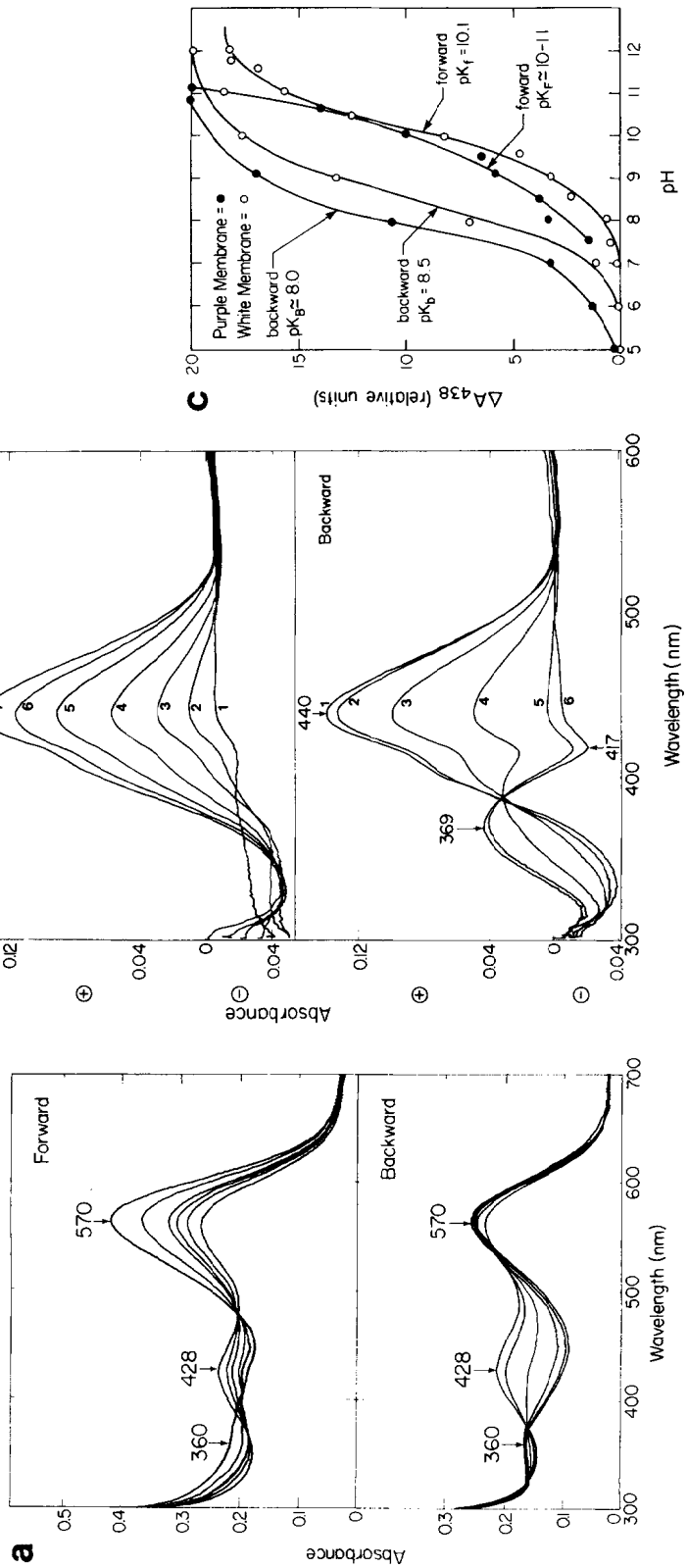

Figure 3. Spectral and Ionization Properties of Nitrotyrosine-Methyl Ester Modified Membranes.
a) Absorption spectra of purple membranes
b) Difference spectra of white membranes
c) pK of reporter group in modified membranes

Backward (acidic) titration of this sample (made after 24 hr under alkaline conditions) reveals a new pattern of spectral changes. The decrease in absorbance at 428 nm is no longer coupled to 570 nm absorbance changes. Instead, a new isosbestic point between a small peak at 360 nm characteristic of the nitrotyrosyl and the 428 nm nitrotyrosinate chromophores appeared (Fig. 3a). The backwards titration also demonstrates a new, substantially lower pK = 8.0 for the reporter group (Fig. 3c).

Circular dichroism spectra (300-700 nm) of modified purple membranes at pH 7.0 (data not shown) show an exciton couplet band with a positive peak at 530 nm and a negative band at 605 nm characteristic of control bacteriorhodopsin (13). In addition, a sharp negative peak at 320 nm is evident. Alkaline titration of modified bacteriorhodopsin resulted in a gradual decrease in the exciton coupling band until about 75 percent of the 530 nm peak remained at pH 11.0.

It was of interest to determine whether the unusual spectral and ionization behavior of the nitrotyrosine methyl ester residue bound to bacteriorhodopsin was caused by retinal-nitrotyrosine methyl ester interactions. For these studies white membranes which have bacterioopsin were used. Visible spectra of white membranes prior to retinal reconstitution are featureless except for a small peak at 414 nm which is due to small amounts of a contaminating respiratory pigment (11). Difference spectra of alkaline and acidic titrations of modified white membranes (Fig 3b) showed that formation of the nitrotyrosinate ion had a band at 438 nm with a pK = 10.1 (Fig.3c). A small decrease in absorbance at 320 nm accompanied this change, but the transition did not show a clear isosbestic point. However, the backward titration demonstrated a clear isosbestic point at 385 nm as a result of conversion of the 438 nm nitrotyrosinate ion to a 369 nm absorbance peak characteristic of the nitrotyrosyl chromophore. As in the case of modified purple membranes, the backward titration of modified white membrane exhibited a decreased pK = 8.5 compared to the forward titration.

pH dependence of M_{412} steady-state and kinetics. The activity of bacteriorhodopsin as a function of pH in the region of the pK of protein bound reporter group should be a sensitive measure of nitrotyrosine methyl ester effects on the

photocycle. Alkaline titrations of control and nitrotyrosine-modified bacteriorhodopsin were carried out to quantitate the steady-state level of the M_{412} intermediate (Fig. 4). The pH dependence of M_{412} levels was compared by normalizing the nitrotyrosine methyl ester values to the control value at pH 7.0 to correct for initial differences in protein concentration and steady-state levels. Modified membranes showed an elevated level of the M_{412} intermediate in the alkaline pH range with the largest difference in activity at pH 10-11 (Fig. 4, Inset). This suggests that the reporter in bacteriorhodopsin which has a pK of 10-11, is responsible for the increased levels of M_{412} in the steady state.

Laser flash photolysis showed the same kinetics for formation of M_{412} in control and modified purple membranes. At pH 7.0 the decay kinetics for modified preparations were slightly inhibited. Control samples exhibited a monophasic decay with a $t_{1/2}$ = 6 msec at 20°C while modified preparations showed slightly biphasic kinetics, with $t_{1/2}$ 9.3 msec. This indicates that the protonated nitro-

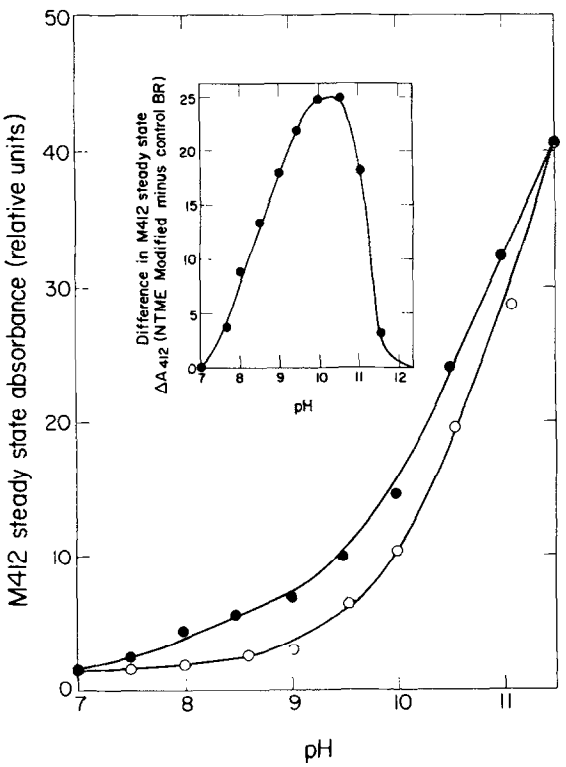

Figure 4. M_{412} Steady-State of Nitrotyrosine-Methyl Ester Modified Purple Membranes. Inset: difference between control and modified samples.

tyrosine methyl ester species introduces only a slight perturbation to the overall photocycling mechanism. At pH 10.0 where the M_{412} steady-state levels showed the greatest difference between control and modified samples, as expected control samples showed slower M_{412} decay kinetics ($t_{1/2}$ = 55 msec) which were strongly biphasic. The difference between the decay kinetics for the modified samples at pH 7.0 and 10.0 was even greater, $t_{1/2}$ = 125 msec for the second, slow phase. The ratio of the half time for M_{412} decay in the control sample at pH 10 versus pH 7 is 9.17, while the same ratio for the nitrotyrosine-modified bacteriorhodopsin is 13.4. The relative inhibition of M_{412} decay kinetics between the modified and control sample is 1.47 times. Since the M_{412} steady state level is determined by the ratio of the M_{412} decay to formation kinetics, these changes should be reflected in the steady state level. Indeed, comparison of the values at pH 10 (Fig. 4) shows that the increase is very close to a factor of 1.5.

DISCUSSION

Nitrotyrosine methyl ester appears to be a sensitive probe to monitor changes in bacteriorhodopsin's microenvironment near the retinal binding site. The results suggest that this reporter group is bound to a carboxyl group which resides in a hydrophobic membrane domain in the native state.

Implications of chemical modification for the microenvironment of the retinal binding site. The unusual absorption spectra of the reporter group in modified purple membranes that is evident at neutral pH is correlated with the unusually high pK of 10-11. These spectral and ionization properties may be due to either an electrostatic interaction with a nearby group or a change in the polarity of the environment. Examples of nitrotyrosine pK changes, as well as theory (14), demonstrate that a change to a more hydrophobic environment will increase the pK of nitrotyrosine residues.

Exposure to alkaline pH results in a localized configurational change that transfers the reporter group from a buried hydrophobic membrane domain into a more aqueous environment near the membrane surface. Thus, the backwards (acidic) titration demonstrated an irreversible hystersis that produces a lower pK near

8.0, closer to that of the model compound in solution. Numerous examples of irreversible configurational changes as evidenced by titration curves of water-soluble globular proteins have been reviewed (15). As a result of the configurational change, reporter group retinal interactions are lost. CD spectra of modified membranes are unchanged. Since the excitonic interactions among retinal chromophores have been correlated with a trimeric organization of bacteriorhodopsin in the membrane (16), no significant change has occurred in this property. Also, the presence of the reporter group results in a minimal perturbation of the protein and chromophore structure, since other chemical modification procedures which affect the chromophore result in the loss of this feature.

To determine if nitrotyrosine methyl ester interactions with retinal "per se" were responsible for either the unusual spectral or ionization behavior, we examined these properties in modified white membranes (lacking retinal). The alkaline titration of nitrotyrosine-modified white membrane also showed unusual spectral and pK properties for the reporter group, nearly identical to that found in purple membranes. The acidic titration after alkaline conditions also demonstrated an irreversible hysteresis that shifted the reporter group to a lower pK and caused a return to spectral properties similar to that of the model compound. The extreme similarity between reporter group properties in purple and white membranes indicates that its spectral and ionization properties must be due to the protein microenvironment in the vicinity of the reporter group.

Location of a carboxyl residue at the retinal binding site. Given the current lack of knowledge concerning the three-dimensional structure and functional group composition of the "active site" of bacteriorhodopsin, it is not possible to accurately specify the distance and geometry of the reporter group with respect to retinal in the binding site. Nevertheless, the location of the modified carboxyl can be suggested by reference to current models of bacteriorhodopsin secondary and tertiary structure (7,17,18). Of seven possible buried carboxyl residues, several residues are situated within approximately 10 Å of the surface of the membrane; Glu-9, Asp-96, Glu-194, Glu-204, Asp 212. Huang et al., (18) used a retinal photoaffinity label to identify Glu-194 on helix F as a

residue adjacent to the β-ionone ring of a retinal derivative. The modified carboxyl group in the chromophore environment could likely be Glu-194 since it is within the required distance for interaction, but does not perturb the chromophore structure.

ACKNOWLEDGEMENTS

Research was supported by grants from the Office of Biological Energy Research, Division of Basic Energy Sciences, Department of Energy . J.M.H. was a recipient of a National Research Service Award from the United States Public Health Service.

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