Modification of Carboxyl Groups in Bacteriorhodopsin. Chemical Evidence for the Involvement of Aspartic Acid Residues in the Structure and Function of Bacteriorhodopsin

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Covalent modifications of the carboxyl residues of bacteriorhodopsin with α-diazo-*p*-nitroacetophenone (**1**) under different conditions have been performed. The modified proteins have been characterized for their absorption, photochemical and proton pump activities. A partial characterization in terms of modification site has also been carried out. Three carboxyl residues of dark-adapted bacteriorhodopsin undergo reaction with **1** at pH 4.0, and the resulting protein exhibits absorption and proton pump activity similar to that of the native protein. Dark-adapted bacteriorhodopsin does not react with **1** at pH greater than 6.1. More than one carboxyl residues are modified when light-adapted bacteriorhodopsin is treated with **1** at acidic pH of 4.0 and 5.4. However, near physiological pH (7.2) only one carboxyl residue of light-adapted bacteriorhodopsin reacts with **1**. These proteins exhibit absorption bands at 571 nm, fail to show proton translocation, and, upon flash photolysis, exhibit generation of 'M'-like intermediates with $\tau_{1/2}$ of 13.47 ms, and λ_{max} of 400 nm. The modified carboxyl residue is found to be located in the CNBr-9 (residues 72–118) fragment. Reaction of bacteriorhodopsin with **1** at −30 °C under photolytic ($\lambda \geqslant 500$ nm) conditions at pH 7.2 results in the modification of two carboxyl residues, Asp-212 and another one in the CNBr-9 fragment. Such a modified protein exhibits drastically blue-shifted absorption at 400 nm, and does not show any proton translocation or flash photolytic activities. It has been concluded that during the photocycle at least two carboxyl residues exist predominantly in a protonated form. A molecular mechanism for the photocycle is also presented.

Bacteriorhodopsin is the principal intrinsic protein component of the purple membrane of *Halobacterium halobium*.[1] Its unique structural and functional features[2,3] have attracted much attention in recent years, and have led to a feverish multidisciplinary effort directed towards understanding its structural properties, and the mechanism of its function at a molecular level.[4–8] Further, new domains of interest in bacteriorhodopsin and its analogues have come to light because of its great potential in the development of molecular electronic devices and biotechnological systems.[9,10] Bacteriorhodopsin consists of a single polypeptide chain with 248 amino acid residues and a chromophore, retinal.[11,12] The protein molecule is composed of seven closely packed helical rods, spanning the lipid bilayer and oriented nearly perpendicular to the membrane surface. Recently, in a pioneering work, a model for the three dimensional structure of bacteriorhodopsin near atomic resolution has been obtained.[13]

The retinal chromophore in bacteriorhodopsin is bound to the ε-amino group of Lys-216 *via* a protonated Schiff base linkage. There are two forms of bacteriorhodopsin (the light-adapted absorbing at 570 nm and the dark-adapted at 560 nm) the chromophores of which are respectively all-*trans*-retinal and a 1:1 mixture of all-*trans*- and 13-*cis*-retinal. On light absorption bacteriorhodopsin undergoes a photocycle reaction, leading to various intermediates ($BR_{570} \xrightarrow{h\nu} J_{600} \longrightarrow K_{590} \longrightarrow L_{550} \longrightarrow M_{412} \longrightarrow N_{560} \longrightarrow O_{560} \longrightarrow BR_{570}$).[8] Retinylidene chromophore during photoactivation reversibly changes its configuration (all-*trans* ⟶ 13-*cis* ⟶ all-*trans*), as well as its absorption region. One of the intermediates in the photocycle of bacteriorhodopsin is called the M-intermediate with a characteristic blue-shifted absorption maximum at 412 nm. Its formation and lifetime in solution are about 50 μs and 10 ms respectively. The net result of the photocycle is the active transport of protons across the plasma membrane establishing a proton gradient that is used by the cell to drive ATP synthesis. The photocycle and the absorption spectral features have been the object of multiple investigations. The absorption behaviour of bacteriorhodopsin has been suggested to depend largely on the protonation, and configurational and conformational state, of the Schiff base chromophore, and on the nature of electrostatic interactions between the Schiff base chromophore and the side chains of the protein.[14,15] Light-induced proton transfer reaction of bacteriorhodopsin is accomplished mainly through the proton release step of the Schiff base L ⟶ M and the subsequent reprotonation of the Schiff base during M ⟶ N,O. Protonation changes of the amino acid side chains are believed to be involved in the light-induced proton translocation. Chemical modification experiments,[16] and Fourier transform IR spectroscopic studies[17–20] have demonstrated that four aspartic acid residues undergo protonation changes. Site-specific mutagenesis experiments[21–25] have indicated that aspartic acid residues 85, 96, 115 and 212 are important in the structure and function of bacteriorhodopsin. Involvement of aspartic acid residues in protonation–deprotonation processes in the proton translocation has also been suggested in the recent structural model for bacteriorhodopsin.[13] In spite of numerous investigations of bacteriorhodopsin, the properties of bacteriorhodopsin intermediates in the photocycle, and the detailed mechanism of proton translocation through the purple membranes are still under discussion.

In order to understand the molecular level mechanism of the photocycle of bacteriorhodopsin, it is important to determine the chemical nature of the involved amino acid residues in their microenvironment. The present work is aimed at the chemical modification of carboxyl side chains of the native bacteriorhodopsin. For this purpose we have used a carboxyl-selective reagent, α-diazo-*p*-nitroacetophenone (**1**). The reactivity of **1** towards various bacteriorhodopsin samples has been examined under a variety of conditions, including photochemical

conditions where the protein will be in a dynamic state. The modified proteins have been characterized for their UV–VIS absorption, flash photolytic and proton translocation activities. A partial characterization in terms of the location of modification site has also been done. A molecular mechanism for the photocycle has also been advanced.

Experimental

UV–VIS measurements were made either on Shimadzu UV-260 or on Beckman DU-6 spectrophotometers. Infrared spectra were recorded on Perkin-Elmer-625 IR spectrophotometer. ^1H NMR spectra were taken on 60 MHz Hitachi-600 spectrometer. Amino acids were analysed on Du Pont 8800 gradient high pressure liquid chromatograph. Peptide fractions from GPC column were collected using Gilson fraction collector model TDC-80. Ultracentrifugations were done on a Beckman L-8-55 M ultracentrifuge using rotors type 45 Ti and SW 27. Proton translocations and other pH measurements were done on a pH meter (Radiometer PHM 84) equipped with GK 2410C electrode. Lyophilizations were performed on a Lyophilizer Pvt. Ltd., Bangalore Instrument. Sonications were done on a Branson B-12 sonifier. Flash photolyses were carried out using a Flash Kinetic Spectrophotometer (Model 858, Applied Photophysics, London) equipped with a flash lamp (flash duration 100 μs at 40 J). The absorbance changes were monitored by monochromatized light (20 W, Tungsten-halide lamp, 12 V, bandpass, 5 nm, wavelength range 400–700 nm). Steady-state irradiations were performed by a 250 W Tungsten-halogen lamp in conjunction with a 1% aqueous solution of $CuSO_4$, with a pathlength of 5 cm.

Purple membrane fragments were isolated from the cells of *Halobacterium halobium* according to the literature procedure.[26] The concentrations of bacteriorhodopsin were measured spectrophotometrically using an absorption coefficient of 63 000 $dm^3\ mol^{-1}\ cm^{-1}$ at 560 nm. Light–dark adaptations of the protein samples were done as described earlier.[27] 'Tailless bacteriorhodopsin' was obtained by removing 18 amino acid residues from the C-terminus by papain treatment of bacteriorhodopsin as described in the literature.[28] Light-induced proton translocation measurements were carried out as described earlier.[29] Chymotrypsin was from Sigma. Sephadex LH-60 was from Pharmacia. Other chemicals used were either of Aldrich, Fluka or Merck make. Organic solvents were from Spectrochem, Bombay.

α-Diazo-*p*-nitroacetophenone (**1**) was prepared[30] by the reaction of *p*-nitrobenzoyl chloride with diazomethane in dry diethyl ether at 0 °C in the dark. It gave satisfactory physicochemical analyses, m.p. 114–117 °C (lit.,[31] m.p. 114 °C); λ_{max}(EtOH)/nm 248, 310 (ε 31 328 $dm^3\ mol^{-1}\ cm^{-1}$); ν_{max}(KBr)/cm^{-1} 2100; δ_H($CDCl_3$, TMS) 6.12 (s, 1 H, α-C*H*-N_2).

Freshly prepared solutions (10 mmol dm^{-3}) of **1** in absolute ethanol were used in all the reactions. Cu^{2+} ion treatment of bacteriorhodopsin samples was done by adding appropriate amounts of an aqueous solution of $CuCl_2$ (0.01 mol dm^{-3}). Lower pH values of the reaction samples were attained by addition of 6.25 mmol dm^{-3} perchlorate ions.

Ester groups in the modified proteins (approx. BR, 5×10^{-5} mol dm^{-3}, 1 cm^3) were determined by conversion of the ester to hydroxamic acid residue using hydroxylamine at pH 7.0, followed by estimation of the hydroxamate as described in the literature.[32] The procedure showed reasonable sensitivity and the absorbance for 0.1 μmol of monoesterified sample was about 0.35.

Reactions of Dark- and Light-adapted Bacteriorhodopsin with **1**.—In a typical procedure, aqueous suspensions of bacteriorhodopsin samples (approx. 4.8×10^{-5}–10^{-6} mol dm^{-3} BR) (Table 1) in distilled water at the desired pH were first treated with a 5–10 fold molar excess of Cu^{2+} ions. A freshly prepared ethanolic solution of **1**, in approx. 35-fold mole excess over bacteriorhodopsin, was introduced and the mixture was gently stirred at temperatures as indicated in Table 1. The esterified protein samples were isolated by subjecting the reaction mixture first to dialysis against distilled water, and then to centrifugation (*ca.* 15 000 rpm, 45 Ti, 45 min, 4 °C) followed by lyophilization.

Reaction of Bacteriorhodopsin with **1** *at* −30 °C *under Photolytic Conditions.*—Aqueous dark-adapted bacteriorhodopsin and 'tailless bacteriorhodopsin' suspensions in 67% glycerol were light adapted at 0 °C. The temperature of the reaction vessel was then lowered to −30 °C by circulation of ethylene glycol–water (1:1) through a cryostat. Light-adapted protein (5×10^{-5} mol dm^{-3}) samples were then treated with $CuCl_2$ (0.01 mol dm^{-3}) solution (protein to Cu^{2+} ratio, 1:35) and photolysed at $\lambda > 500$ nm (250 W, Tungsten-halogen lamp, 1% aqueous $CuSO_4$, 5 cm). Further, 16 aliquots (6.0 mm^3 each) of ethanolic **1** were added after every 30 min over a period of 8 h during photolysis. The reaction mixture was gently stirred while irradiation continued. The reaction was carried out at pH 4.0 and 7.2 and the modified proteins were isolated by the usual procedure.

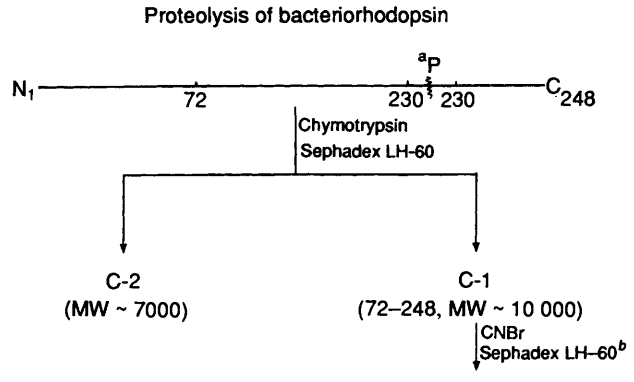
Proteolysis of Bacteriorhodopsin and Peptide Analysis of Modified Proteins.—In order to determine the modification sites, the modified proteins were subjected to proteolytic studies which were identical to the proteolytic methods described for native bacteriorhodopsin (Scheme 1).[29,33,34] The proteolytic behaviour of modified proteins was similar to the proteolytic behaviour of native bacteriorhodopsin. Chymotrypsin cleaves bacteriorhodopsin into two fragments C-1 (residues 72–248) and C-2 (residues 1–71). These chymotryptic fragments can further be cleaved to smaller peptides by cyanogen bromide. The cyanogen bromide fragments of chymotryptic peptides C-2 and C-1 can be separated on Sephadex LH-20 and LH-60 columns respectively. Further the amino acid sequences of the cyanogen bromide peptides are known.

The general methodologies involved in delipidation, chymotryptic and cyanogen bromide fragmentations, and separation of peptide fragments were adapted from the literature.[29,33,34] Peptides were identified by monitoring the absorbance of effluent at 280 nm. Location of the *p*-nitrophenyl residue in the peptides was done by measuring the effluent absorbance at 310 nm. The eluents corresponding to desired peptides were collected under a N_2 stream and were subjected in some cases to *o*-iodosobenzoic acid treatment. The identity of the peptides was established by analysing their amino acid composition by HPLC.[35]

Isolation of Ester-labelled Peptides from Light-adapted Bacteriorhodopsin Modified at pH 7.2, 0 °*C.*—The modified protein was subjected to chymotrypsin treatment and the C-1 fragment bearing the *p*-nitrophenyl group was separated from the C-2 fragment by Sephadex LH-60 chromatography. The C-1 peptide (residues 72–248) was subjected to cyanogen bromide cleavage when five fragments, CNBr-6 (residues 210–248), CNBr-7 (residues 119–145), CNBr-8 (residues 164–209), CNBr-9 (residues 72–118) and CNBr-10 (residues 146–163) were obtained. The eluents corresponding to CNBr-fragments 6, 8 and 9 were pooled together, and dried under a stream of nitrogen and the concentrate was suspended in 40 mm^3 of 88% formic acid. To this solution was added 100 mm^3 of *o*-iodosobenzoic acid solution (100 μg cm^{-3} in 4 mol dm^{-3} guanidine hydrochloride dissolved in 80% acetic acid). The reaction was allowed to proceed at 25 °C in the dark for 4 h.

Table 1 Results of the reactions between α-diazo-*p*-nitroacetophenone (**1**) and bacteriorhodopsin

Sample	pH	*T*/°C	No. of groups modified	λ_{max} of modified protein/nm	Modification site
Dark-adapted bacteriorhodopsin	4.0	12	3	560	—
	6.1	12	0	560	—
Light-adapted bacteriorhodopsin	4.0	0	4	571	—
	5.4	0	2–3	571	—
	7.2	0	1	571	CNBr-9
Papain-treated bacteriorhodopsin, dark-adapted	4.0	12	2	560	—
	6.1	12	0	560	—
Papain-treated bacteriorhodopsin, light-adapted	4.0	0	3	571	—
	5.4	0	1	571	—
	7.2	0	1	571	CNBr-9
Light-adapted bacteriorhodopsin, under photolysis	4.0	−30	4	440	—
	7.2	−30	2	440	CNBr-9 and Asp-212
Light-adapted, papain-treated bacteriorhodopsin under photolysis	7.2	−30	2	440	CNBr-9 and Asp-212



CNBr fragment	No. of amino acids	Range	Asp/Glu residues
6	39	210–248	Asp-212, 242 Glu-232, 234, 237
7	27	119–145	—
8	46	164–209	Glu-166, 194, 204
9	47	72–118	Asp-85,96,102,104,115 Glu-74, 105
10	18	146–163	Glu-161

Scheme 1 *a* Papain cleavage. *b* Elution rate: CNBr-8, CNBr-9 > CNBr-6 > CNBr-7 > CNBr-10

The peptides were separated on the precalibrated Sephadex LH-60 column when CNBr-8 fragment was displaced giving the CNBr-6 and CNBr-9 fragments. Absorbance monitored at 310 nm indicated the CNBr-8 fragment to be devoid of *p*-nitrophenyl residue.

Isolation of Ester-labelled Peptide from Light-adapted Tailless Bacteriorhodopsin Modified at pH 7.2, 0 °*C*.—The modified protein was subjected to the usual chymotrypsin and cyanogen bromide treatments, and Sephadex LH-60 chromatography. CNBr-8 and -9 fragments showing the presence of the *p*-nitrophenyl moiety were collected. *o*-Iodosobenzoic acid treatment followed by Sephadex LH-60 chromatography resulted in the isolation of separated peptide fragments. The CNBr-9 fragment contained the ester group. Its identity was further established by examining the amino acid composition. The peptide was completely hydrolysed in 6 mol dm^{-3} HCl at 110 °C for 22 h. 3-Aminobutyric acid was used as internal standard. The hydrolysates were converted to dansyl derivatives and the dansyl derivatives were separated by reverse phase HPLC [Ultrasphere, ODS-C_{18}, 250 × 4.6 mm, solvents: (A) acetonitrile–0.02 mol dm^{-3} phosphate buffer, pH 6.2, 60:40; (B) acetonitrile–0.02 mol dm^{-3} phosphate buffer, pH 6.2, 5:95; gradient: 3 linear segments from 7% A to 45% A in 30 min, from 45% A to 60% A in 1 min, from 60% A to 70% A in 7 min; flow rate 1.5 cm^3 min^{-1}).

Isolation of Ester-labelled Peptides from Bacteriorhodopsin and Tailless Bacteriorhodopsin Modified under Photolytic Conditions at pH 7.2, −30 °*C*.—The procedure adapted was identical to the one described earlier. The ester functions were present in coelution peaks corresponding to CNBr-fragments 6, 8 and 9. To determine the modification site, tailless bacteriorhodopsin was modified under photolytic conditions and the modified protein was subjected to the chymotrypsin and cyanogen bromide cleavage as usual. In this way the CNBr-6 fragment could be isolated separately by Sephadex LH-60 chromatography. The identity of the CNBr-6 fragment obtained from modified tailless bacteriorhodopsin was confirmed by examining the amino acid composition on HPLC.

Results and Discussion

The results of the reactions of bacteriorhodopsin with **1** are summarized in Table 1. Dark-adapted bacteriorhodopsin underwent modifications of three of its carboxyl groups at pH 4.0. There was no effect of this modification on the absorption properties. However the modified protein exhibited diminished proton translocation. It showed the generation of an 'M'-like intermediate with $\tau_{1/2}$ of 13.1 ms. Dark-adapted bacteriorhodopsin, however, did not react with **1** at pH higher than 6.1, indicating the absence of unionized accessible carboxyl groups in the protein under these conditions.

The reaction of light-adapted bacteriorhodopsin with **1** was examined at three pH values. All the three modified proteins showed similar absorption behaviour with λ_{max} at 571 nm (Fig. 1). None of the modified proteins showed any proton pumping activity. At pH 7.2, the reaction of light-adapted bacteriorhodopsin was more specific, with modification of only one carboxyl group. Flash irradiation of this modified protein showed formation of a blue-shifted (λ_{max} 410 nm) species with $\tau_{1/2}$ of 13.47 ms, which is similar to the M intermediate of native bacteriorhodopsin. Papain-treated bacteriorhodopsin,[28] which is known to be devoid of 18 amino acid residues at the

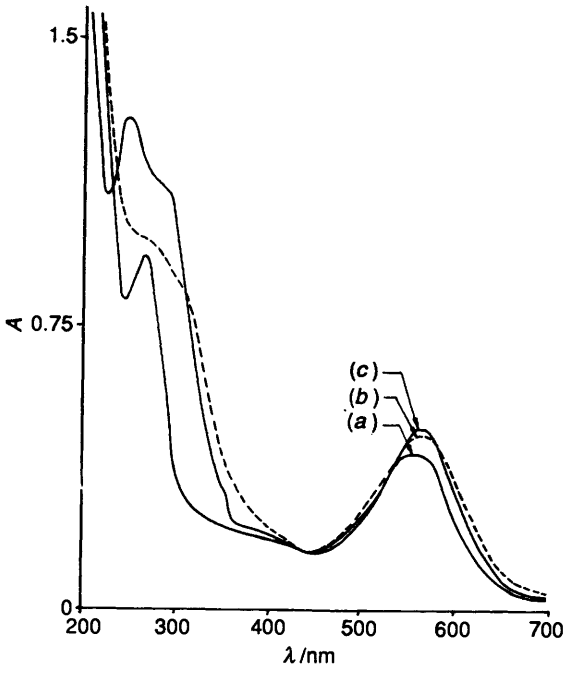


Fig. 1 Absorption spectra of (*a*) dark-adapted bacteriorhodopsin, (*b*) light-adapted bacteriorhodopsin modified with **1** at pH 7.2, and (*c*) light-adapted tailless bacteriorhodopsin modified with **1** at pH 7.2

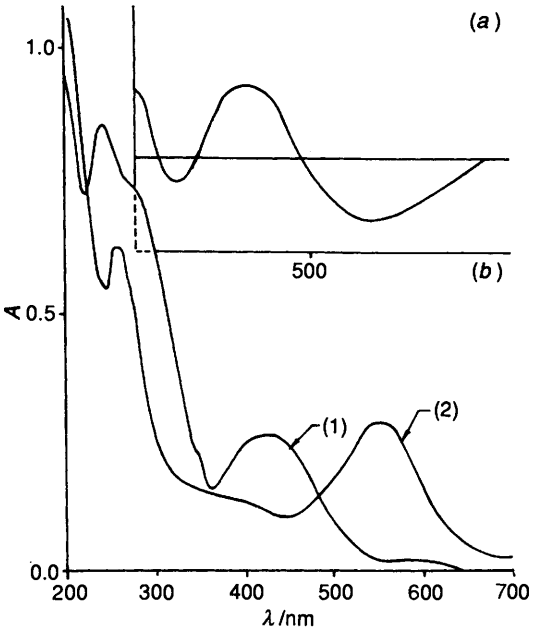


Fig. 2 (*a*) Difference absorption spectrum of bacteriorhodopsin modified with **1** under photolysis at −30 °C, and light-adapted bacteriorhodopsin. (*b*) Absorption spectra of, (1) bacteriorhodopsin modified with **1** under photolysis at −30 °C and (2) dark-adapted bacteriorhodopsin

C-terminus, also underwent modification of one of its carboxyl groups when treated with **1** at pH 7.2. This modified tailless protein showed the characteristic red-shifted band at 571 nm. It did not show any proton pumping activity, but showed the formation of an 'M'-like intermediate with absorption at 410 nm and $\tau_{1/2}$ of 13.7 ms. Thus, while modification of a carboxyl group of tailless bacteriorhodopsin does not significantly influence the absorption behaviour, it drastically affects the proton pumping activity. Hence, the inability of the modified tailless protein to pump protons is due to modification of one of its carboxyl groups. These results also show that glutamic acid residues 232, 234 and 237 and aspartic acid residue 242 are not involved in the reaction of bacteriorhodopsin with **1** at pH 7.2.

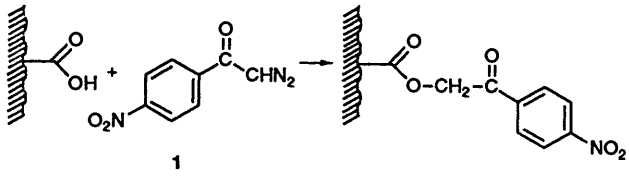
The reactions of light-adapted bacteriorhodopsin and papain-treated bacteriorhodopsin with **1** (pH 7.2) at −30 °C under continuous illumination resulted in the disappearance of the purple complex band with concomitant formation of a new band at 440 nm (Fig. 2). The modified proteins did not exhibit any proton translocation. The hydroxamate method indicated that two acidic residues underwent esterification.

Treatment of modified proteins with aqueous NaOH (pH 9.1) caused the 440 nm band of these proteins to disappear with the regeneration of 560 nm band. The hydroxamate test on the regenerated protein indicated the absence of ester group.

Proteolytic studies on light-adapted bacteriorhodopsin modified at pH 7.2 resulted in the isolation of CNBr fragments 6 and 9. To locate the site of modification, tailless bacteriorhodopsin was modified and the modified protein was subjected to proteolysis and peptide analyses. The modified carboxyl group was found to be present in the CNBr-9 fragment with amino acid residues 72–118, containing five aspartic and two glutamic acid residues.

Similarly, proteins modified under photolytic conditions were also analysed. The CNBr-6 fragment from modified tailless bacteriorhodopsin, with only the N_1–C_{230} sequence, was found to contain the ester label. The analysis of the primary structure of the CNBr-6 fragment from tailless bacteriorhodopsin shows the presence of only one (Asp-212) residue with a carboxyl side chain. Hence it is deduced that of the two carboxyl groups modified under photolytic conditions, one is Asp-212. The other one was found to be present in CNBr-8 and 9.

As diazo compounds[32] preferentially react with unionized carboxyl groups (–CO_2H), it is believed that the –CO_2H group in bacteriorhodopsin gets converted to an ester function bearing a *p*-nitrophenyl label. Bacteriorhodopsin contains ten Glu and nine Asp residues. All these residues can react with **1** (Scheme 2) provided their acidic side-chain exists as –CO_2H.



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Scheme 2

The covalent modifications of the carboxyl residues in dark-adapted bacteriorhodopsin do not show any significant effect on the colour or the photochemical proton pump activity of bacteriorhodopsin, although at low pH (4.0) as many as three carboxyl groups underwent modification. The reaction of light-adapted bacteriorhodopsin near physiological pH (7.2) is more specific. It leads to the modification of one of the seven carboxyl residues (Asp-85, -96, -102, -104, -115 and Glu-74, -105) of peptide CNBr-9 containing amino acid residues 72–118 between the C and D helices. The modified carboxyl group is unlikely to be the one involved in electrostatic interaction with the retinal chromophore, as no significant absorption changes are observed. However in view of the loss of proton pump activity, it is inferred that the modified carboxyl group plays a crucial role in the photocycle. The loss of the characteristic 570 nm band and proton translocation during protein modification under photolytic conditions at −30 °C is noteworthy. The modified protein with a blue-shifted absorption (440 nm) failed to generate photocycle intermediate 'M' when flash photolyses were carried out. Site determination experiments indicate that of the two modified carboxyl groups one is Asp-212 (G helix) and the second carboxyl group could be one out of the seven

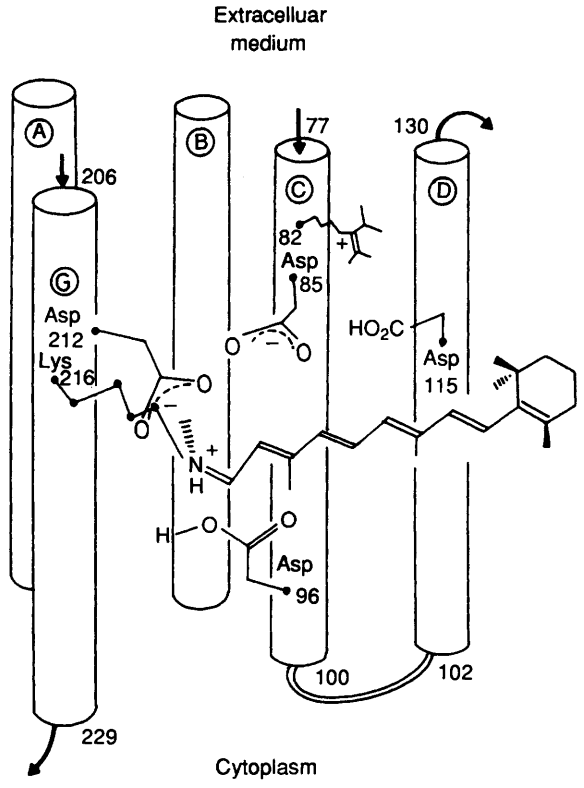

Fig. 3 Disposition of aspartic residues at the chromophoric site in bacteriorhodopsin

carboxyl groups present in the C and D helices (Asp-85, -96, -102, -104, -115 and Glu-74, -105).

Apparently, carboxyl side chains of aspartic acid residues appear to be important in the structure and mechanism of function of bacteriorhodopsin. The role of Asp-212 in maintaining the purple colour of bacteriorhodopsin is evident from these results. Further, it is found that Asp-212 of light-adapted bacteriorhodopsin reacted with **1** only when the protein was subjected to continuous irradiation at low temperature of −30 °C. It is implied that the side chain of Asp-212, which exists as a carboxylate counter-anion in bacteriorhodopsin, became protonated under the photolytic and low temperature conditions of modification. The protonation state of the carboxyl residues may be altered under photolytic conditions (λ 500 nm) of modification, as the protein can undergo light-induced conformational changes. Alterations in the state of protonation of the carboxyl groups due to the local effect of a modified carboxyl cannot be ruled out, however. Nonetheless, drastic changes in the absorption and light-induced proton pump activities can only occur if important external carboxyl residues in close proximity to the retinylidene chromophore get modified. The current structural model of bacteriorhodopsin,[13] point charge mutation results[24,25] and FTIR studies[36] have suggested the involvement of aspartic acid residues (Asp-212, Asp-96, Asp-85) in the structure and function of bacteriorhodopsin. Thus, it may be said that Asp-212 and Asp-85/96 are among those residues, which play crucial roles in maintaining the structural features and light-induced proton pump activity of bacteriorhodopsin.

A structural model depicting the relative disposition of retinal and aspartate residues important in the structure and function of bacteriorhodopsin is depicted in Fig. 3. Out of seven α-helical rods only five (A–D and G) are shown. It is further believed that in light-adapted bacteriorhodopsin Asp-212 is ionized and acts as counter-anion to the imine nitrogen and Asp-96 is in an unionized form. It is further suggested that three amino acid residues *viz.* Asp-85, 96 and 212 actively participate in the protonation–deprotonation processes occurring during the photocycle of bacteriorhodopsin. Absorption of photons by bacteriorhodopsin leads to the formation of K_{590} in which the 13-*cis* chromophore is believed to be present. The twisting of the chromophore leads to separation of the ion-pair formed between Asp-212 $-CO_2^-$ and the protonated Schiff base nitrogen. The destabilized proton of the Schiff base goes to Asp-212 $-CO_2^-$ leading to the deprotonation of the Schiff base. There may be partial twisting about the C_{14}-C_{15} single bond resulting in a large decrease in the pK of the Schiff base. The deprotonation of the Schiff base renders the C_{14}-C_{15} single bond flexible, and allows back isomerization about this bond. Consequently the pK of the Schiff base is increased leading to its reprotonation by Asp-96. Further, with deprotonation of Asp-212 bacteriorhodopsin-570 is regenerated.

In conclusion, chemical modification of bacteriorhodopsin by the carboxyl-selective reagent α-diazo-*p*-nitroacetophenone (**1**) has shown the following.

(a) It is possible to make a distinction between ionized and unionized carboxyl groups in their microenvironment.

(b) During the photocycle, at least two carboxyl residues exist predominantly in a protonated form.

(c) Asp-212 serves as counter-anion to the retinylidene Schiff base. As it is present in the carboxylate form (CO_2^-), it failed to react with **1** under the conditions of either dark- or light-adaptation. However, as the photocycle progresses and the photoisomerization occurs, Asp-212 $-CO_2^-$ gets protonated causing it to react with **1**.

(d) Light-adaptation of bacteriorhodopsin involves protonation of at least one of the carboxyl residues.

Further selective chemical modifications of bacteriorhodopsin in its functional state and while the protein is photochemically active, may provide additional information, significant for theories of the structure and mechanism of functions of the opsin family of proteins.

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