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Bioorganic chemistry of the purple membrane of *Halobacterium halobium*—Chromophore and apoprotein modified bacteriorhodopsins*

ANIL K. SINGH, M. ROY, S. SONAR and M. KAPIL
Department of Chemistry, Indian Institute of Technology, Powai, Bombay 400 076, India

Abstract. Iodophenyl and anthryl retinal analogues have been synthesized. The *trans*-isomers have been isolated and purified by high pressure liquid chromatography. The purified isomers have been further characterized by nuclear magnetic resonance and ultraviolet-visible spectroscopy. Incubation of these retinal analogues with apoprotein (bacterioopsin), isolated from the purple membrane of *Halobacterium halobium* gave new bacteriorhodopsin analogues. These analogues have been investigated for their absorption properties and stability. The iodophenyl analogue has been found to bind to bacterioopsin rapidly. The pigment obtained from this analogue showed a dramatically altered opsin shift of 1343 cm^{-1}. The anthryl analogue based bacteriorhodopsin, however, showed an opsin shift of 3849 cm^{-1}. It has been found that bacteriorhodopsin is quite unrestrictive in the ionone ring site. The apoprotein seems to prefer chromophores that have the ring portion co-planar with the polyene side chain.

The purple membrane has also been modified by treatment with fluorescamine, a surface active reagent specific for amino groups. Reaction under controlled stoichiometric conditions resulted in the formation of a modified pigment. The new pigment showed a band at 390 nm—indicative of fluorescamine reaction with amino group (s) of apoprotein –besides retaining its original absorption band at 560 nm. Analysis of the fluorescamine modified bacteriorhodopsin resulted in the identification of lysine 129 as the modified amino acid residue. Fluorescamine-modified-bacteriorhodopsin suspension did not release protons under photolytic conditions. However, proteoliposomes of fluorescamine-modified-bacteriorhodopsin were found to show proton uptake, though at a reduced rate.

Keywords. Bacteriorhodopsin; purple membrane; bacteriorhodopsin chemical modifications; proton pumping; bacteriorhodopsin analogues.

Introduction

Bacteriorhodopsin (bR) is a 26,000 dalton intrinsic membrane protein that functions as a light-driven proton pump in the purple membrane (PM) of *Halobacterium halobium* (Stoeckenius and Bogomolni, 1982). The discovery (Oesterhelt and Stoeckenius, 1971, 1973) of this pigment as the principal light-utilizing protein of Halobacteria growing under conditions of high salt and light intensity has provoked enormous interest, both in the protein itself and in the Halobacteria. bR has quite unique structural organization. It is arranged into extensive crystalline-like sheets consisting of tens of thousands of molecules tightly packed into hexagonal arrays. The smallest structural unit consists of 3 protein molecules, the trimers being separated from one another by a unimonolayer of tightly bound glycosulpholipid (Blaurock and Stoeckenius, 1971). Complete removal of the endogenous lipids from

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Abbreviations Used: bR, Bacteriorhodopsin; PM, purple membrane; FL, fluorescamine; UV-vis, ultraviolet-visible; NMR, nuclear magnetic resonance; HPLC, high pressure liquid chromatography; bOP, bacterioopsin; CNBr, cyanogen bromide; GPC, gel permeation chromatography; FL-bR, fluorescamine-modified bacteriorhodopsin; OS, opsin shift; LA, light-adapted pigment; DA, dark-adapted pigment.

bR and full recovery of proton translocating activity after reconstitution of the protein with added phospholipids have been accomplished (Huang *et al.,* 1980). The amino acid sequence of bR has been determined (Khorana *et al.,* 1979; Ovchinnikov *et al.,* 1979; Bayley *et al.,* 1981a; Wolber and Stoeckenius, 1984). The single chain of bR is composed of 248 residues, more than 70% of which are hydrophobic in nature. The lysine residue at position 216 is bound to a retinal chromophore *via* a protonated Schiff base (SBH^+) linkage (figure 1) (Lewis *et al.,* 1974; Bagley *et al.,* 1982; Rothschild and Marrero, 1982). Various attempts have been made to understand the general disposition of the protein within the membrane. Based on electron density maps it is believed that the polypeptide spans the membrane in 7 α-helical segments (Henderson and Unwin, 1975; Leifer and Henderson, 1983). In complete contrast, Jap *et al.* (1983), using circular dichroism and infrared spectroscopy, have suggested a model comprising 5 α-helices and 4 strands of *β*-sheet. The position of the chromophore has been refined by neutron diffraction studies using bR reconstituted with deuterated retinal (King *et al.,* 1979; Seiff *et al.,* 1985). Result of these studies place the *β*-ionone ring of the retinal near the centre of the membrane. It has been possible to assign the retinal site to one of two helical rods.

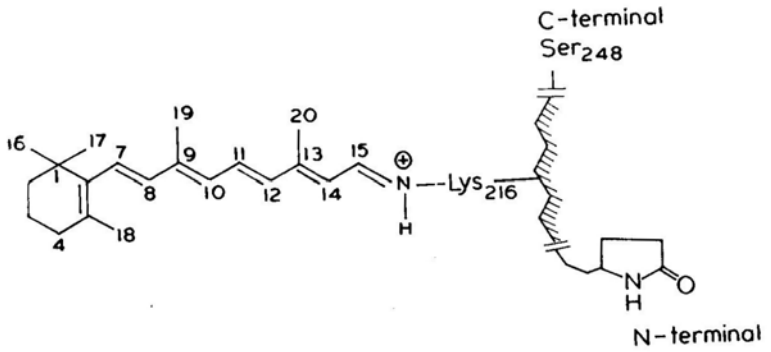


Figure 1. all-*trans*-retinal bound to apoprotein *via* a protonated Schiff base linkage.

There are two forms of bR, the light-adapted (bR^{LA}) absorbing at 570 nm and the dark-adapted (bR^{DA}) absorbing at 560 ran, the chromophores of which are respectively all-*trans*-retinal and a l:l mixture of all-trans- and 13-*cis*-retinal. Both forms undergo a photocycle (figure 2). Absorption of light by bR drives the extrusion of H^+ ions from the cell to generate a proton gradient which can be utilized to fuel active transport and ATP production. Several mechanisms have been proposed, many involving the proton of the Schiff base, for the translocation process but they remain speculative. There has been considerable kinetic and spectrophotometric evidence to suggest that substantial conformational changes occur during the L to M transition (Kuschmitz and Hess, 1982) in which the Schiff base is deprotonated (Bagley *et al.,* 1982). Changes in the environment of tyrosine (deprotonation) and tryptophan residues have been particularly implicated (Hanamoto *et al.,* 1984; Konishi and Packer, 1978), the most attention being directed to Tyr-26 and Tyr-64 whose modification abolished proton transport (Lemke *et al.,* 1982). Crosslinking of lysine residues inhibits the photocycle (Ovchinnikov, 1982). Peptides corresponding

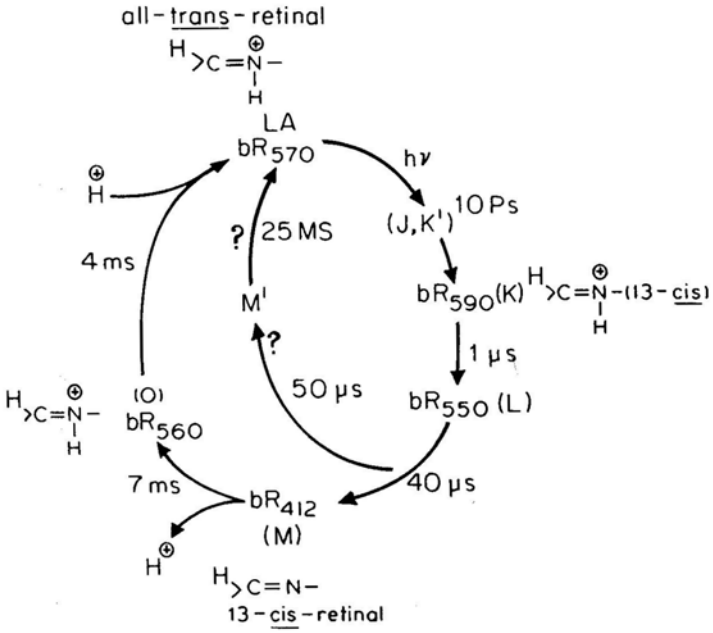
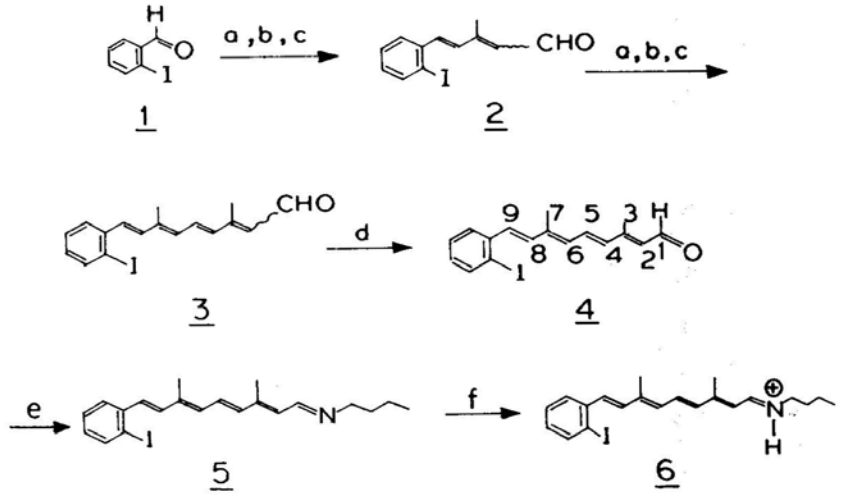


Figure 2. The photocycle of bR (Stoeckenius and Lozier, 1979; Stoeckenius and Bogomolni, 1982). Intermediate designations are shown in brackets; J, K' and M' have not been unambiguously established. The times shown refer to the particular transitions at room temperature. Subscripts denote the absorbance maxima (nm) of the various intermediates.

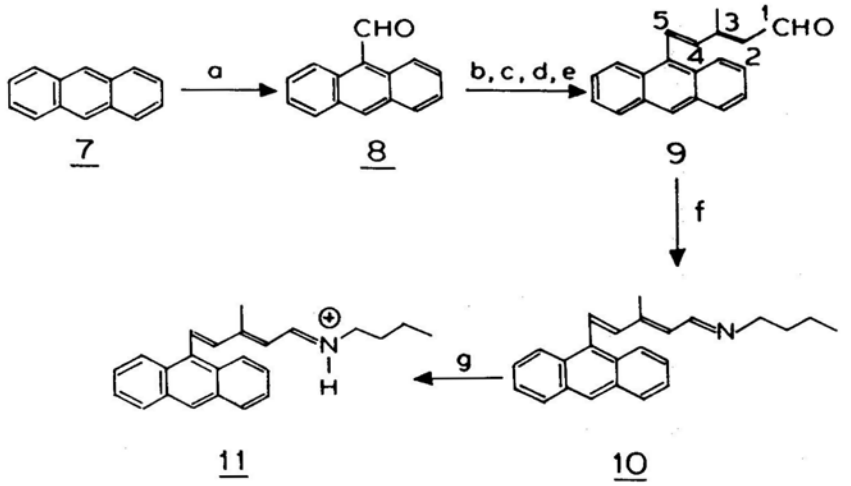
to residues 1-3, 68-72 and 231-248 are not vital for activity (Abdulaev *et al.,* 1978) but arginines (Packer *et al.,* 1979) and carboxylic amino acids (Ovchinnikov *et al.,* 1982) appear to be important. Asp-115, in particular, which reacts with dicyclohexylcarbodiimide on exposure of the protein to light (Renthal *et al.,* 1985), has been suggested to be critical to the proton pumping mechanism.

In order to explain the marked bathochromic shift observed upon binding of retinal to the apoprotein, an experimental and theoretical analysis of the electronic environment of the binding site (Nakanishi *et al.,* 1980) positioned two negative charges (presumed to be carboxylate anions) in close proximity to the Schiff base and the *β*-ionone ring. Suggestions have recently been made (Lugtenburg *et al.,* 1986; Spudich *et al.,* 1986) that there may be a protein-bound positive charge in addition to a negative charges in the vicinity of the *β*-ionone ring. This has opened an interesting new chapter in our understanding of the chromophore-binding site, necessitating further investigation.

Synthetic analogues have been very useful tools for elucidation of the structure and mechanism of function of membrane-bound protein pigments (Mitsner *et al.,* 1986). Similarly, chemical modifications of amino acid residues are expected to provide valuable information regarding the molecular details of the active site as well as clues to the mechanism of proton translocation. The present studies have been undertaken with these viewpoints. Synthetic bRs have been prepared from retinal analogues 4 and 9 (schemes 1 and 2) in order to investigate the effect of stereo-



Scheme 1. Synthesis of iodophenyl analogue of retinal (*4*). a, C-5 Phosphonate, NaH, THF; b, $LiAlH_4$,-EtO; c, MnO_2; d, HPLC; e, *n*-$BuNH_2$; f, dry HCl-MeOH.



Scheme 2. Synthesis of anthryl analogue of retinal (9). a, DMF-$POCl_3$-*o*-dichlorobenzene; b, C-5 Phosphonate; c, $LiAlH_4$-Et_2O; d, MnO_2; e, HPLC; f, *n*-$BuNH_2$; g, dry HCl-MeOH.

electronic perturbations on the active site near the *β*-ionone ring. We have also modified bR with fluorescamine (FL) in order to probe the role of lysine residues in the overall structure and function of PM.

Materials and methods

Studies on retinals were carried out under dim red light and under N_2. The samples were stored at —40°C under N_2 Organic solvents were from Spectrochem, Bombay and were dried before use. Sephadex LH-60 was from Pharmacia Fine Chemical Co., Uppsala, Sweden. Other chemicals used were purchased locally, and were of

either Aldrich, Fluka or Sigma make. Ultraviolet-visible (UV-vis) spectrophotometric measurements were made on a Beckman DU-6 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WP-80 spectrometer using $CDCl_3$ as solvent and tetramethylsilane as internal standard. Ultracentrifugations were done on a Beckman L-8-55 M ultracentrifuge using SW-27 rotors. pH measurements were carried out on a Radiometer make pH meter (PHM-84) equipped with GK 2401C electrodes. High pressure liquid chromatography (HPLC) analyses were performed on a Beckman 110A HPLC instrument (microporacil, 10 μm Si-60, 4·5×250 mm, 1·5 ml/min, 9% ether-hexane, λ_{max} 360 nm, 254 nm). Lyophilizations were performed on Lyophilisers Pvt. Ltd., Bangalore instrument. Sonications were done on a Branson B-12 sonicator.

Retinal analogue synthesis

Ethyl-4-(diethylphosphono)-3-methyl-2-butenoate (C_5 phosphonate) (Mayer and Isler 1971) and activated manganese dioxide (MnO_2) (Fatiadi, 1976) were prepared by following the published procedures. Retinal analogues were synthesized using the Emmons-type chain extension reactions to afford the esters, careful reduction with $LiAlH_4$ to the alcohols, and oxidation to the desired aldehyde with activated MnO_2 (schemes 1 and 2). Pure all-*trans-4* and 9 were obtained after HPLC analysis. Isolated samples were stored at — 40°C under N_2 in dark for further use.

Apoprotein preparation and its regeneration with synthetic retinals

Master slants of *H. halobium* were kindly supplied by Dr. W. Stoeckenius, Department of Physiology and Cardiovascular Research Institute, University of California, San Francisco, USA. Large-scale cultures were grown under illumination and low oxygen conditions. PM from the bacterial cells were isolated according to standard methods (Oesterhelt and Stoeckenius, 1974). Bacterioopsin (bOP) was prepared by bleaching the PM by irradiating with intense yellow light (Corning filter CS3-67 and 1% $CuSO_4$ solution in H_2O, 3 cm pathlength) in the presence of 0·75 M NH_2OH, pH 7. Bleached membrane protein was purified. The regeneration was achieved by the addition of ethanolic solution of retinal analogues to purified bOP in HEPES buffer, pH 7. The pigment proteins were purified and stored at — 40°C.

Reaction of bR with FL

PM suspension in borate buffer, pH 9, and acetone solution of FL in 1:3 stoichiometry were stirred for 30 s at 4°C. The suspension immediately dialysed. The FL-modified membranes were pelleted by centrifugation at 100,000 *g*. The membranes were lyophilized and then delipidated by dissolving in sodium dodecyl sulphate and keeping overnight at 35°C. The protein (FL-bR) was precipitated by adding ethanol. The pellet was washed several times with ethanol before diluting it with water. Ammonium hydroxide was slowly added and the resulting precipitate was stored at – 40°C.

Chymotryptic digestion of FL-bR

FL-modified bR was taken up in Tris-HCl (50 mM, pH 8) containing 5 mM $CaCl_2$ and

incubated at 37°C with chymotrypsin for 5 h. The membrane was collected by centrifugation (50000 *g,* 45 min) washed and lyophilized, and the fragments C-1 and C-2 were separated on Sephadex LH-60.

Cyanogen bromide cleavage of chymotryptic fragment C-1

The fragment was dissolved in 88% (v/v) formic acid and treated with excess cyanogen bromide (CNBr) (24 h, dark). The fragments were isolated by chromatography on Sephadex LH-60 (88% formic acid-ethanol, 30:70).

Gel permeation chromatography

Gel permeation chromatography (GPC) was carried out on columns (2·5×80 cm) of Sephadex LH-60 equilibrated in 88% formic acid:ethanol (30:70). Lyophilized membranes or fragments were dissolved in 88% formic acid, ethanol was added to an ethanol concentration of 70% by volume, and the solution was used for chromatography. The collected fractions were analyzed for their absorbance at 280 and 390 nm.

Proton translocation measurements

Proton release was measured on bR and fluorescamine-modified –bacteriorhodopsin (FL-bR) suspensions in 4 M aqueous KCl. Proton uptake was determined using proteoliposome preparations. The vesicles were prepared by the sonic method as follows. PM and phosphatidylcholine (freshly isolated from hens' eggs) suspended in 0·2 M KCl were sonicated in a Branson sonicator at 40 W power output in a ice-bath for 3 min. The sonication was repeated 5 times with 2 min intervals. The resulting preparations were used for pH measurements under photolytic condition ($h\nu$, λ_{max}>500 nm, 25°C).

Results and discussion

Retinal analogues

Synthetic analogues have been very useful, tools for the elucidation of the structure and mechanism of function of receptors. In the case of bR and rohodopsin,which are photoreceptors, the interaction between retinal and opsin can be studied by investigating the properties of pigment analogues formed from opsin and retinal analogues With this viewpoint we synthesized the all-*trans*-isomer of iodophenyl analogue (*4*) and anthryl analogue (*9*), which have different stereo-electronic features in the ring portion of the chromophore. In addition, analogue *9* has a side-chain that contains only two ethylenic C = C bonds, in contrast to the natural chromophore which has 4 C = C bonds in the chain. The synthetic strategy for analogues *4* and *9* is outlined in schemes 1 and 2. The *trans*-isomers could be easily separated on normal-phase HPLC and gave satisfactory NMR (table 1) and UV (table 2) data. A typical HPLC trace for the separation of analogue *4* is shown in figure 3.

Table 1. Characteristic NMR[a] signals for *4* and *9*.

Protons	Chemical shift (δ) (multiplicity and J values in Hz)	
	4	*9*
H_1	10·16 (d, $J_{1,2}$, 10)	10·32 (d, $J_{1,2}$, 11·7)
H_2	6·0 (d)	6·18 (d)
H_4	7·60 (d, $J_{4,5}$, 16·5)	6·80 (d, $J_{4,5}$, 16·4)
H_5	7·15 (d, d, $J_{5,6}$, 12)	7·35 (d)
H_6	6·50 (d)	—
H_8	6·46 (d, $J_{8,9}$, 16·5)	—
H_9	7·80 (d)	—
3-CH_3	2·36 (s)	2·68 (s)
7-CH_3	2·16 (s)	—

[a]In $CDCl_3$, TMS, 80 MHz.

Table 2. Absorption maxima of chromophores and pigments.

Compound	λ_{max}(nm)			
	-CHO[a]	SBH[+b]	pigment[c], LA	OS (cm^{-1})
all-*trans*-retinal	380	440	568 (560)[d]	5122
4	373	435	462 (455)[d]	1343
9	386	458	556 (550)[d]	3849

[a]Aldehyde; solvent, ethanol. bSolvent, methanol. cIn 10 mM HEPES buffer, pH 7. [d]pigment, DA.

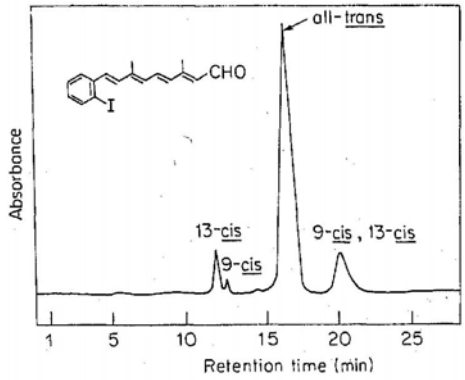


Figure 3. HPLC of synthetic mixture of iodophenyl analogue (4·5 mm × 25 cm micro-poracil, 1·5 ml/min, 9% Et_2 O-HEX 360 nm).

Reconstitution of b R with retinal analogues

When bOP was incubated with iodophenyl analogue (*4*) a new pigment with λ_{max} 462 nm formed rapidly (figure 4). More than 95 % of the binding occurred in the first

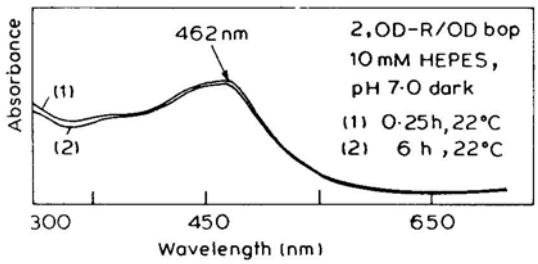


Figure 4. Absorption spectra of bR analogue formed from iodophenyl chromophore (*4*)

minute of incubation. Similarly anthryl analogue *9* gave a new pigment with absorption peak at 556 nm.

Opsin shifts

The opsin shift (OS) is a measure of the influence of the bOP binding site on the absorption spectrum of the chromophore. It is given by the difference between the λ_{max} of the SBH^+ of the chromophore in cm^{-1} and the λ_{max} of the pigment in cm^{-1} We prepared the SBH^+ *6* and *11* corresponding to analogues *4* and , respectively with *n*-butylamine. For the sake of convenience the absorption spectra (figure 5), of SBH^+ were measured in methanol (table 2). Next the model analogues were allowed to bind to bOP, and the absorption spectra of the resulting pigments were obtained The OS (table 2) for bR's with chromophores *4* and *9* were calculated to be 1343 and 3849 cm^{-1}, respectively.

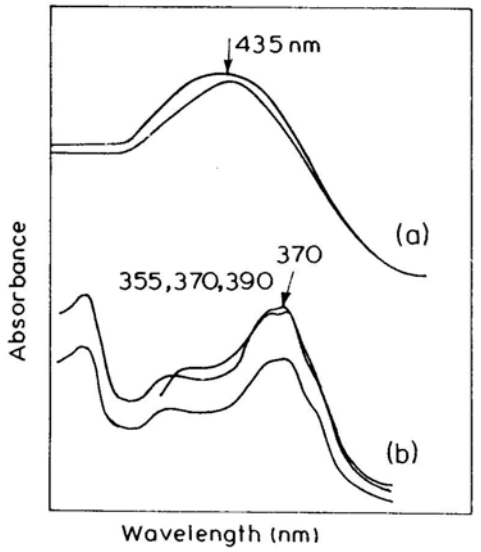


Figure 5. Absorption spectra of (a) protonated Schiff base (*6*) and (b) Schiff base (5)

Dark-light adaptation

Samples of bR analogues in 2 M NaCl with 60% glycerol were cooled to 0°C and photolysed at λ_{max}> 500 nm for light adaptation. For dark adaptation, samples were put in dark overnight at 0°C. The bR-analogues showed dark-light adaptation with absorption maxima for dark-adapted (DA) bR-analogues 5–6 nm less than those for light-adapted (LA) bR analogues (table 2).

Identification of the pigment chromophore

The bound chromophore was extracted from the iodophenyl analogue based-bR with methylene chloride, vortexed for a minute and then centrifuging at 10000 *g* at 4°C. The methylene chloride fraction was analysed by HPLC. The analysis revealed the presence of all-*trans* isomer in the LA form and a mixture of 13-*cis* and all-*trans* isomers in the DA form. This indicated the presence of photocycle in the pigment.

Stability of pigment analogues

The reaction of the pigment analogues with hydroxylamine was measured by adding the reagent (1 mM, pH 7) to the pigment (< 1 mg) and following the absorption spectrum over several hours in the dark. The pigments were found to be unstable in an excess of hydroxylamine.

An assessment of the binding site stability of the analogues was made by measuring of the displacement of the analogue chromophore upon addition of all-*trans*-retinal (0·2 mg/ml in ethanol) to the synthetic pigment. Thus the rates of binding of retinal and iodophenyl analogue of retinal to apomembrane were compared (figure 6). Such an analysis showed that the analogue *4* reacted much more rapidly, the formation of the chromophore being almost complete within the time (1 min) when the first measurement was made. Further, iodophenyl analogue bound to apomembrane was not readily displaced by all-*trans*-retinal. Thus, when retinal was added to bOP that had previously been treated with analogue *4,* the rate of reaction was reduced (figure 6).

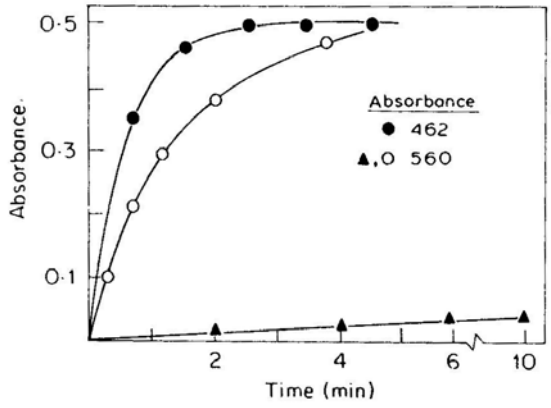


Figure 6. Timecourse of binding of iodophenyl analogue (●) and retinal (○, ▲) to bOP. (○), Retinal binding to bOP; (▲), retinal binding to iodophenyl analogue-bR.

New bR-analogues

Previous studies (Mitsner *et al.,* 1986) have shown that numerous retinal analogues form pigments with bOP. The present investigations show that pigments can be formed with aldehyde side-chain containing even two ethylenic trans double bonds. Another finding of this work is that the iodophenyl analogue of retinal binds to the bOP more rapidly than retinal. This lends some support to the postulate that retinal binds in a sterically strained planar form (Schreckenbach *et al.,* 1977; Bayley *et al.,* 1981b) rather than in a conformation that is twisted about the 6–7 bond.

The reduced OS of iodophenyl analogue-bR (1343 cm^{-1}) in comparison to that of anthryl analogue-bR (3849 cm^{-1}) is because of a less planar conformation in the former. On the other hand the difference may simply be due to slightly different orientations of the two chromophores within the binding site and hence different influences from the external point charges (EPC) residing near the ring (figure 7).

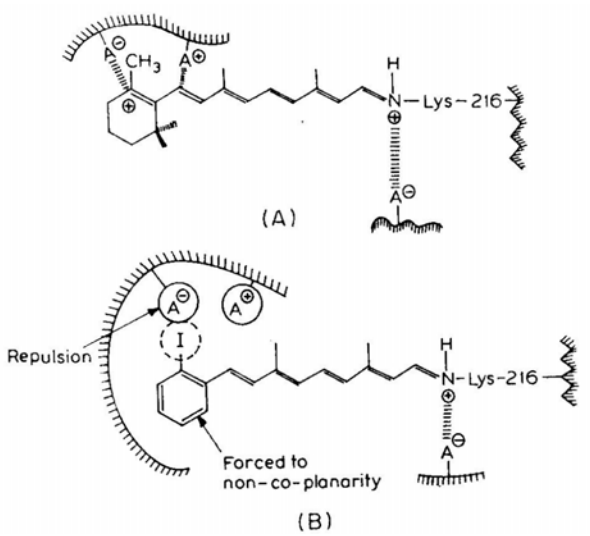


Figure 7.A. Model for bR binding site where 6-s-*trans* chromophore interacts with a pair of charges on bOP in the vicinity of *β*-ionone ring. **B.** Model for iodophenyl retinal analogue-bR, where due to soft, polarizable iodo group interaction with dipolar charge pair on bOP makes the phenyl group orientation non-co-planar.

According to the EPC model, electronic and/or steric perturbations at the ring binding site are expected to strongly affect the absorption maxima of bR-analogues. Therefore, the drastic deviations of the OS in the case of iodophenyl analogue of retinal lends support to the EP C model (Nakanishi *et al.,* 1980).

Though both chromophores *4* and 9 have an aromatic group with electronic features different from those of the natural chromophore. The phenyl chromophore exhibited rather small OS compared to the anthryl analogue, the anthryl chromophore may be considered as having some of the required double bonds in the tricyclic aromatic ring system; this imparts to the chromophore a more planar disposition compared to the phenyl analogue. There is apparantly a less planar conformational orientation in the iodophenyl analogue.

Electronegative groups on the ring have been found to destabilize the excited state of the chromophore. Thus, the 5-trifluoro-methylretinal based bR-analogue has been found to show an OS of only 2400 cm^{-1} (Rao *et al.,* 1986). The rather soft and polarizable iodo group on analogue *4* is expected to undergo electrostatic interactions with the opsin-bound pair of positive and negative charges in the vicinity of the ionone ring. The orientation of the ring will thus be governed by the resultant force of these interactions. The negative iodo group would like to keep away from the negative charge on the opsin. In addition, the migrating positive charge on the polyene in chromophore *4* would be repelled by the positive charge of the opsin (figure 7). The iodophenyl chromophore is influenced by these opsin-bound charges to adopt a non-co-planar conformation, leading to a reduced OS. Thus, in one way this model chromophore validates the very recent proposition that there is a protein-bound positive charge in addition to a negative charge in the vicinity of the *β*-ionone ring (Derguini *et al.,* 1986; Lugtenburg *et al.,* 1986; Spudich *et al.,* 1986).

The OS of bR-analogue obtained from the planar anthryl chromophore *9* supports the recent hypothesis (van der Steen *et al.,* 1986) that retinal binds to the apoprotein in its planar 6-s-*trans* conformation and not the 6-s-*cis* conformation.

Thus, in conclusion it can be said that the ring binding site in bOP is quite unrestrictive as even the highly modified anthryl arid iodophenyl chromophores could be accomodated. Alterations to the side chain do not seem to prevent pigment formation. However, the apoprotein prefers a chromophore which has a ring site co-planar with the side polyene chain.

Modification of bR by FL

Chemical modifications of amino acid side chains can provide significant information regarding structural and functional features of the binding site. Various amino acid residues have been implicated in maintaining the structure and photo-biological functions of PM. bR pumps protons most effectively in the pH range 4 to 10. Possible groups involved in H^+ translocation by bR are the *ε*-amino groups of lysine, which have a pK of 10·5. Indeed schemes hypothesizing involvement of lysine in trans-membrane proton movement driven by pK shifts linked to Schiff base deprotonation have been postulated (Stoeckenius and Lozier, 1979). It has also been postulated that lysine groups play a structural role in maintaining a protein conformation that is essential for activity. Accordingly, it was of interest to evaluate the role of lysine residues by modifying them. We modified lysine residues of PM using FL, which is a surface-labelling and amino group-selective reagent. Treatment of reconstituted PM with FL has been shown to modify one lysine residue on the cytoplasmic side of the membrane (Tu *et al.,* 1981).

Controlled treatment of PM suspension with FL in acetone at pH 9 resulted in a new pigment with absorption peaks at 560 and 390 nm (figure 8). Use of excess reagent and prolonged treatment gives a pigment with absorption at 500 and 390 nm; the 560 nm peak disappears, and denaturation of pigment occurs. The peak at 390 nm is characteristic for pigment formed by reaction of FL with $-NH_2$ groups of bR. The new pigment showed an emission peak at 472 nm. FL as well as its hydrolysis products are non-fluorescent. Therefore, the observed fluorescence is because of a fluorophore, *12* (scheme 3) which is formed only.as.a result of the reaction between FL and free *ε*-amino groups of lysine residues of bR. Under the

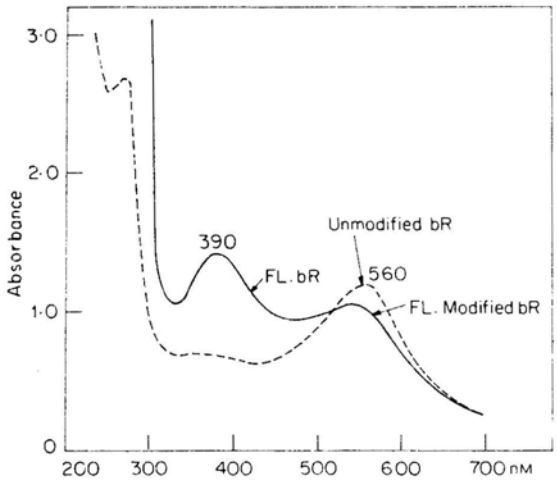
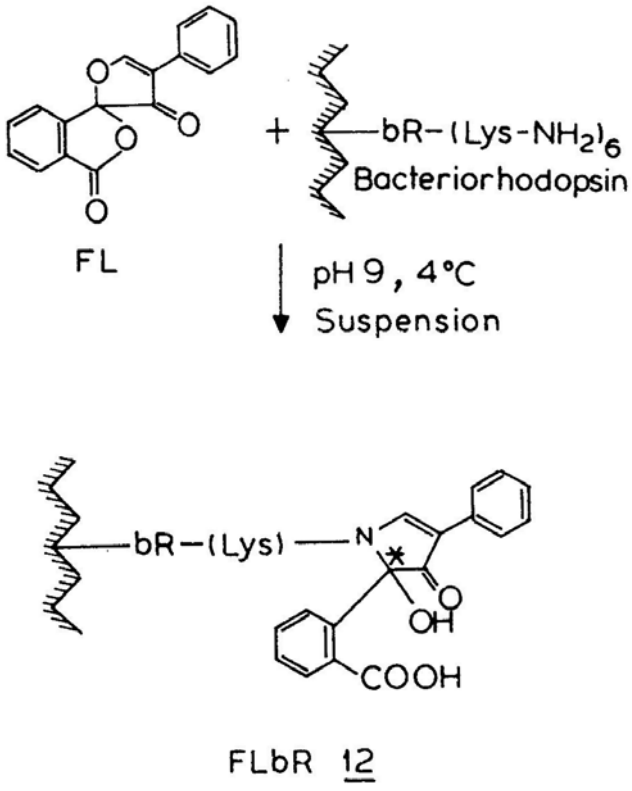


Figure 8. Absorption spectra of bR and FL-bR in suspension.



Scheme 3. Reaction of FL with bR.

present conditions modification of lysine residues on extracellular as well as cytoplasmic side of the membrane are expected.

Light-induced reaction of FL-modified bR

Light-induced H^+ uptake was measured using lipid vesicles with FL-bR or bR incorporated in the membrane. Proton uptake was determined to be 3 mol H^+ per mol of FL-bR and 6 mol H^+ per mol of bR. FL-bR, however, did not show light-induced H^+ release, thereby implying that bR cannot transport protons anymore when the light-induced proton release is blocked.

Identification of modified lysine

FL-bR was cleaved with chymotrypsin. Sephadex LH-60 GPC yielded fragments C-l and C-2. Fragment C-l was found to absorb at 390 nm indicating the presence of the FL modified lysine in this fragment. CNBr cleavage of C-l followed by Sephadex separation of the fragments (figure 9) showed that fragment CNBr-7 contained the modified lysine residue (λ_{max} 390 nm).

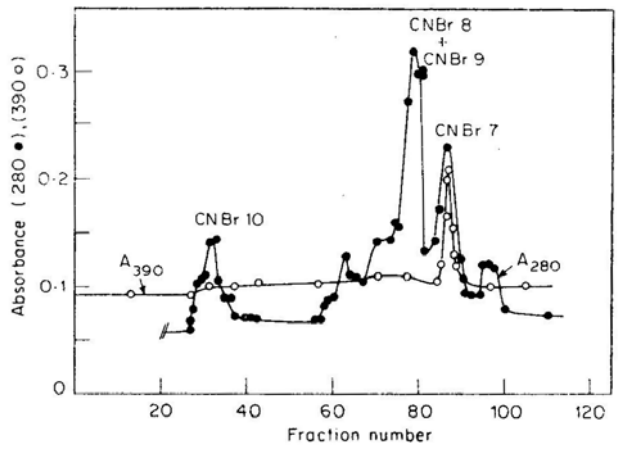


Figure 9. Chromatography of CNBr fragments of chymotryptic fragment C-l of Fl-bR on Sephadex LH-60.

In principle CNBr cleavage of C-l (residues 72–248) is expected to give 5 fragments (CnBr-6-10) (Gerber *et al.,* 1979; Huang *et al.,* 1982) (figure 10). As expected, CNBr-8 and 9 were co-eluted. CNBr-10 was eluted just after the void volume. CNBr-8 and 9 were followed by CNBr-7 in accordance with their sizes (figure 9). Unmodified bR fragments and FL-bR fragments were found to have similar elution pattern under the conditions employed. Thus, elution pattern does not change because of lysine modification by FL. CNBr-7 (residue 119-145) containing a total of 26 amino acids, has only one lysine at position 129. Lysine 129 is situated on the extracellular side in rod D of the polypeptide chain (figure 10).

Thus, modification of lysine 129 while not influencing the electrostatic interactions between the chromophore and apoprotein, definitely seem to influence proton release by bR suspension. This shows that at least one lysine residue is important for bR function. Lysine 40 has been shown (Harris *et al.,* 1979) not to be involved in the

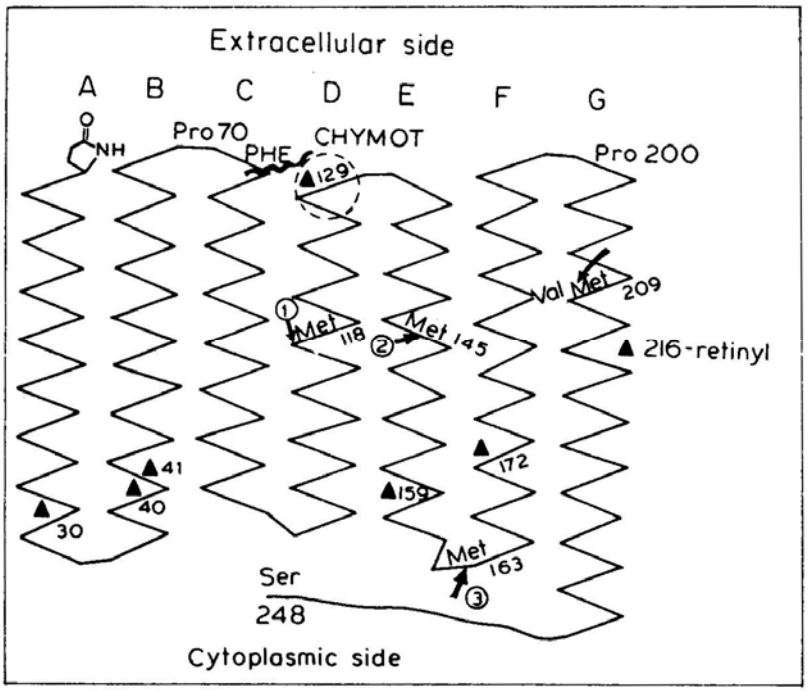


Figure 10. Primary sequence of bR showing lysine residues (▲) and sites of chymotrypsin (~ ~) and CNBr (→) cleavage sites in fragment C-l (72-248)-Fl modified Lys-129 residue is circled. Retinal interacts with lysine-216.

proton pump mechanism. Lysine 216 is involved in retinal binding. The roles of lysine 30, 41 and 159 are yet to be established.

In conclusion, it can be said that chemical modification of bR or its apoprotein is capable of providing useful chemical information on the structural and functional properties of PM of *H. halobium.*

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