STRUCTURAL INVOLVEMENT OF CARBOXYL RESIDUES IN THE PHOTOCYCLE OF BACTERIORHODOPSIN

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1. Introduction

The purple membrane is a unique light-energy transducing membrane which is found as part of the plasma membrane of the extreme halophile *Halobacterium halobium*. It contains only a single protein, bacteriorhodopsin (BR), which utilizes ligth energy to translocate protons across the membrane and thereby generate an electrochemical gradient. The retinal chromophore is bound to an ε-amino group of lysine by a Schiff base [1], and absorption of light causes the chromophore to undergo a photochemical cycle. A linear, unidirectional sequence of reactions has been proposed for this cycle, which contains at least 4 intermediates, designed as K_{590}, L_{550}, M_{412} and O_{640} based on the order in which they are believed to appear, and their spectral maxima [2].

Despite the fact that the structure of BR has been well characterized [3], the overall molecular mechanism of proton translocation remains essentially unknown. In this regard, the characterization of the photocycle intermediates is a prerequisite for a detailed understanding of the mechanism involved. Time-resolved resonance Raman spectroscopy has been used to show that the Schiff base linkage is protonated in BR_{570}, unprotonated in M_{412}, and protonated once again in O_{640} [4–6]. Studies of an illumination-independent acid-induced species, which apparently represents stabilization of phototransient O_{640} at low pH, suggest that protonation of a carboxyl-group of an amino acid side chain is involved in the formation of the O_{640} intermediate [7,8].

Several models have been proposed which postulate the involvement of specific amino acid residues in the mechanism of proton translocation. In particular, carboxyl residues have been suggested as:

(i) Interacting directly with the Schiff base nitrogen [8,9];
(ii) Interacting with the β-ionone ring of retinal to form a 'charge stabilized' intermediate [10];
(iii) Participating in a sequence of proton-translocating groups [4,11]; or
(iv) Forming ion-pairs within the membrane with the positively charged groups of arginine and/or lysine [11–13].

Since the primary sequence of BR has been reported [14], it is now possible by chemical modification techniques to gain information on the role of specific amino acid residues in proton translocation. Here we demonstrate that carboxyl modification changes the kinetic parameters but not the pathway of the photocycle in BR. The results show that the modified carboxyl residues are not required for 'O' intermediate formation. However, after carboxyl modification, inhibition of M_{412} decay kinetics is observed and the formation of the blue acid-induced species is prevented. Evidence is presented which indicates that both of these effects are due to intramolecular cross-linking between a carboxyl group and an amino group of lysine. These results indicate that carboxylic acid side chains of aspartic and/or glutamic amino acid residues are important in the photocycle mechanism and therefore in proton translocation by BR.

2. Materials

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC); *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroxyquinoline (EEDQ); glycine methyl ester hydrochloride (GME); ethyl acetimidate hydrochloride (EA); and 2-(*N*-morpholino)ethane sulfonic acid (MES) were obtained from Sigma Chemical Co.

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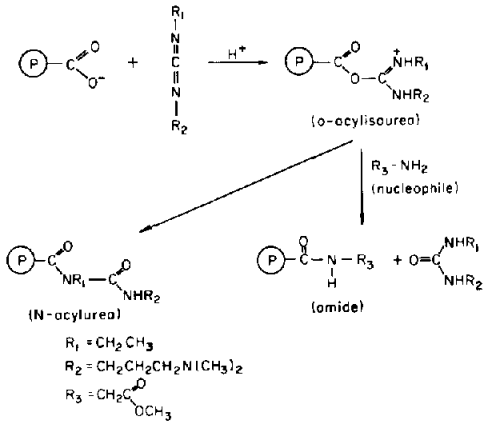
2.1. *Preparation of purple membranes*

Halobacterium halobium strain S-9 was grown according to [15]. Purple membranes were isolated by a modification of the method in [16]. The DNase-treated lysate was centrifuged at 6500 × *g* for 20 min to separate cell debris and the resulting supernatant centrifuged at 42 000 × *g* for 20 min. The purple membrane pellet was resuspended and layered on a step sucrose gradient with increments of 60,52,45, 40,38 and 36% surcrose (w/w). After centrifugation for 16 h at 180 000 × *g*, the purple membrane was separated into a single band of high purity. This preparation was then washed 3 times in distilled water by repeated centrifugation (42 000 ×*g*, 30 min) and stored in 2 M NaCl at 0°C until used.

2.2. *Chemical modifications*

The carbodiimide reaction (fig.1) is initiated by addition of the carboxyl group across one of the double bonds of the diimide system to give an *O*-acylisourea [17]. The activated carboxyl group of this adduct may then react by one of two possible pathways. In the first case, the *O*-acylisourea can rearrange to yield an *N*-acylurea. Alternatively, in the presence of a nucleophile (e.g., an amine), an acyl–nucleophile product will form. it has been shown that the reaction of carboxyl and nucleophile can be driven essentially to completion in the presence of excess carbodiimide and nucleophilic reagent. This procedure has been employed for quantitative estimation of carboxyl groups in proteins [18].

a) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide ; (EDC)



b) N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline ; (EEDQ)

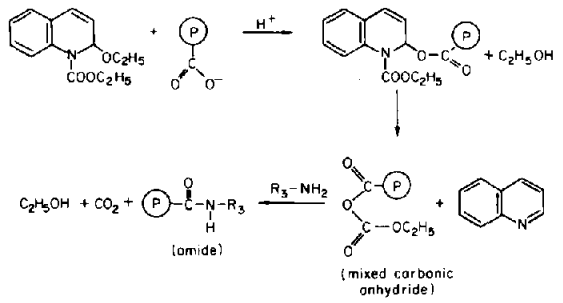


Fig.1. Reaction scheme for carboxyl modification of purple membranes.

2.2.1. Carboxyl residues

(i) EDC: purple membranes were washed and resuspended in 0.10 M MES buffer (pH 4.5) at 1.0 mg protein/ml. Protein was calculated by using ϵ_{568} = 63 000 $mol^{-1} \cdot cm^{-1}$ [19]. EDC was freshly prepared before each experiment and made up in 0.10 M MES (pH 4.5).

(ii) EEDQ: purple membranes were washed and resuspended in 0.10 M MES buffer, pH 6.0 at 1.0 mg protein/ml. EEDQ solutions were prepared immediately before use in 100% methanol. Appropriate controls were run with methanol alone.

(iii) EDC + GME or EEDQ + GME: In reactions employing the nucleophile GME was freshly prepared in 0.10 M MES and adjusted to the appropriate pH with NaOH. Aliquots from this stock solution were taken to give final concentrations from 1–250 mM. GME was incubated with purple membranes for 5 min prior to addition of carboxyl-activating reagent. All reactions were carried out for 1 h in a shaking water bath at 25°C. The reaction was terminated by 10-fold dilution with ice-cold 100 mM NaCl, 1 mM MES, pH 6.5 and immediate centrifugation at 100 000 × *g* for 30 min. Modified samples were washed 3 times by the above procedure.

2.2.2. Lysyl residues

(i) EA: The ϵ-amino groups of lysine are converted to amidine products by monofunctional imidoester treatment [20]. Purple membranes were washed and resuspended in 0.10 M sodium tetraborate (pH 10.0) at 1.0 mg protein/ml. EA was freshly made (100 mM stock solution) in 1.0 M NaOH/0.10 M $Na_2B_4O_7$. The

reaction was carried out for 30 min at 25°C and then terminated by dilution with ice-cold 100 mM NaCl, 1 mM MES (pH 6.5).

(ii) Quantitation: the number of modified lysines was determined by the fluorescamine method as in [21]. In double modification experiments, purple membrane were first modified by the EA procedure, washed, and subsequently modified by one of the above procedures described for carboxyl modification. Final protein concentration in all reactions was 0.50 mg/ml.

2.3. *Flash photolysis kinetics*

Rapid light-induced changes in absorbance were measured in a temperature controlled sample cuvette as in [11] with the following modifications. A Bausch and Lomb monochromator (5 nm half-band width) was placed between the measuring-beam tungsten light source and the sample cuvette and another monochromator after the sample cuvette and prior to the photomultiplier tube. Actinic illumination was provided by a phase-R (model DL-1100) pumped dye laser using rhodamine 575 (0.2 J/flash, 150 ns flash risetime, 1.0–1.5 μs duration). The flash photolysis data was collected with a Biomation 1010 Waveform recorder and repetitive scans (minimum, $n = 20$) accumulated by a Digital PDP-11/34 computer (Digital Equipment Corp.) and stored on Decapack rko5 discs. Subsequent data manipulations (averaging, curve fitting) and analysis were performed by programmed routines and kinetic spectra were printed on a Varian E-80A recorder.

2.4. *Acid-induced difference spectra*

Acid-induced difference spectra were determined in an Aminco DW-2 spectrophotometer. Purple membranes utilized for acid-induced difference spectra were at 0.20 mg/ml to minimize membrane buffering effects [7]. Changes in pH were measured with a Corning Model 130 pH meter and a Polymark 1885 electrode (Marson Science Inc.). HCl (2.0 N and 0.2 N stock solutions) was added gradually with Hamilton syringes to the sample cuvette. Final added volume was $\leqslant 1\%$ of the sample volume.

2.5. *Other methods*

Polyacrylamide (10%) running gels with 0.1% SDS were prepared with a 5% acrylamide stacking gel according to [22]. Amino acid analysis was performed according to [23] using a Spinco/Beckman 120B amino acid analyzer.

3. Results and discussion

3.1. *Reversal of inhibition of M_{412} decay kinetics by glycine methyl ester*

Reports of chemical modification of carboxyl groups by water soluble carbodiimides have shown an inhibitory effect on the photoreaction cycle of bacteriorhodopsin [11,24]. The mechanism of inhibition was difficult to determine since in native BR intermolecular cross-linking, intramolecular cross-links, and *N*-acylurea products occur. It was therefore of interest to find reaction conditions or other carboxyl reagents which eliminated this multiplicity of reaction products [25]. In this study EEDQ, a hydrophobic, highly specific reagent for activation and modification of carboxyl residues [26] was employed since it had been characterized as behaving like a carbodiimide [27]. Nucleophile promoted amide formation utilizing GME and EEDQ was therefore investigated. Treatment of purple membranes with EEDQ resulted in an inhibition of M_{412} decay kinetics with no effect on M_{412} formation, as found with EDC treatment. Addition of a nucleophile (GME) prior to addition of the carboxyl-activating reagents resulted in partial reversal of the maximal inhibition of M_{412} decay observed after treatment with the carboxyl-activating reagents alone. The effectiveness of reversal was dependent on GME concentration (1–250 mM) during the modification reaction. GME was equally effective when used in conjunction with either EDC or EEDQ reagents (fig.2).

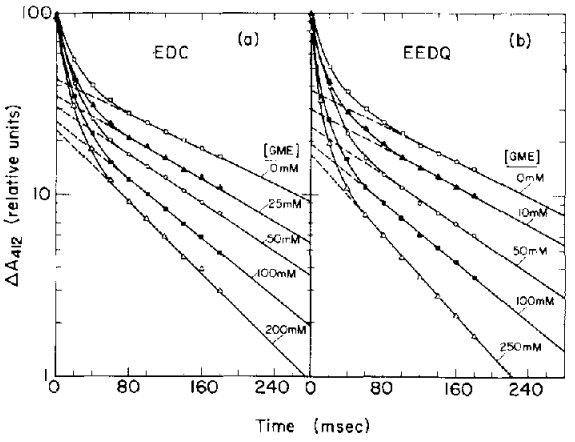


Fig.2. Carboxyl modification in the absence and presence of the nucleophile glycine methyl-ester effect on M_{412} decay.

3.2. *Effect of lysine modification prior to carboxyl-activating reagent treatment*

Purple membranes were first treated with 200 mM ethyl acetimidate and analyzed to determine the % of free amino groups remaining by the fluorescamine method. It was found that 5 out of 7 lysines in BR were modified as shown in [20]. This represents maximal modification since lysine residues 41 and 215 are not reactive [12]. Flash photolysis revealed a minimal effect of lysine modification on M_{412} decay kinetics. Subsequent treatment of lysine-modified BR by EDC (50 mM) showed that inhibition of M_{412} decay was greater than for control or only EA treated, but was substantially less than for EDC treatment alone (fig.3). The kinetics of M_{412} decay in the EA–EDC double modification were virtually identical to those obtained with EDC + 200 mM GME.

When the order of modification was reversed (EDC followed by EA), no difference was observed compared to EDC treatment alone. When EA modified samples were subsequently modified by EDC + GME, instead of EDC alone, the kinetics of M_{412} decay also did not show any additional significant reversal with respect to EA/EDC treatment. Thus, GME was no longer effective in reversing inhibition when lysines were first converted to amidine products by EA. Similarly, EA modification prior to EEDQ treatment also caused partial reversal of M_{412} decay inhibition, although the effect with EDC was more pronounced.

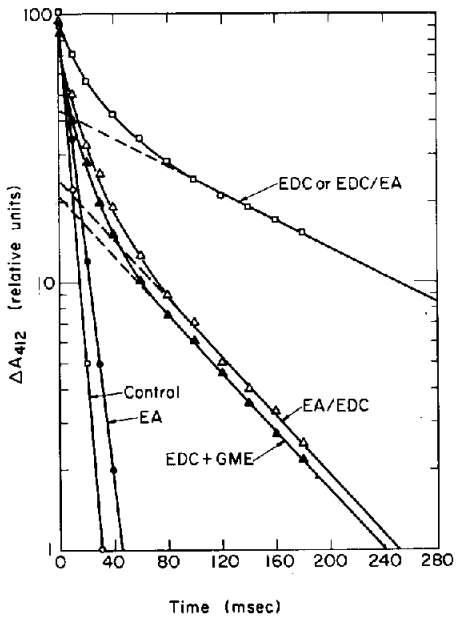


Fig.3. Carboxyl modification in the absence and presence of the nucleophile glycine methyl-ester, and after prior modification of amino groups of lysine with ethyl acetimidate effect on kinetics of M_{412} decay.

Since various concentrations of GME partially prevent the inhibition of M_{412} decay after modification, it suggests that GME competes with an endogenous nucleophilic group on BR. Increased concentrations of GME make it a more effective competitor with respect to the endogenous nucleophile for the activated carboxyl-group. Potential nucleophilic groups on BR that could react with carboxyl groups to form intra- or intermolecular cross-links are the ε-amino groups of lysine and the hydroxyl groups of tyrosine. The possible side reaction with tyrosine does not occur since amino acid analyses showed the same number of tyrosines in control and modified samples [28]. This interpretation is also consistent with other studies utilizing carboxyl-activating reagents and added nucleophiles [27,29]. Thus, it appears that when no added nucleophile is present, an intramolecular cross-link in BR gives maximal inhibition of M_{412} decay kinetics, while glycine methyl-ester amide formation largely prevents cross-linking and yields minimally inhibited kinetics. Lysine–carboxyl double modification experiments further support this idea because lysines that are first modified by EA are unavailable for subsequent reaction with carboxyls activated by EDC or EEDQ and cannot form intramolecular cross-links. The results strongly support the idea of lysine–carboxyl ionic interactions in BR.

EDC treatment results in intermolecular cross-linking as revealed by SDS–polyacrylamide gel electrophoresis, whereas EEDQ treatment does not. Thus, it is clear that the cause of maximal inhibition of M_{412} decay kinetics can only be attributed to intramolecular cross-linking in EEDQ-treated samples. An intramolecular cross-link between a carboxyl and lysine residue in BR may act to constrain conformation changes that take place during the photocycle, which are particularly important for the decay of the M intermediate. It has been shown that intramolecular cross-linking by bifunctional imidoesters resulted in inhibition of M_{412} decay kinetics although the effect was smaller than here [20]. Carbodiimides and EEDQ are 'zero-length' cross-linkers, whereas the bifunctional imidoesters have 8–11 Å spaces between their functional groups. Thus, a cross-link that forms as a result

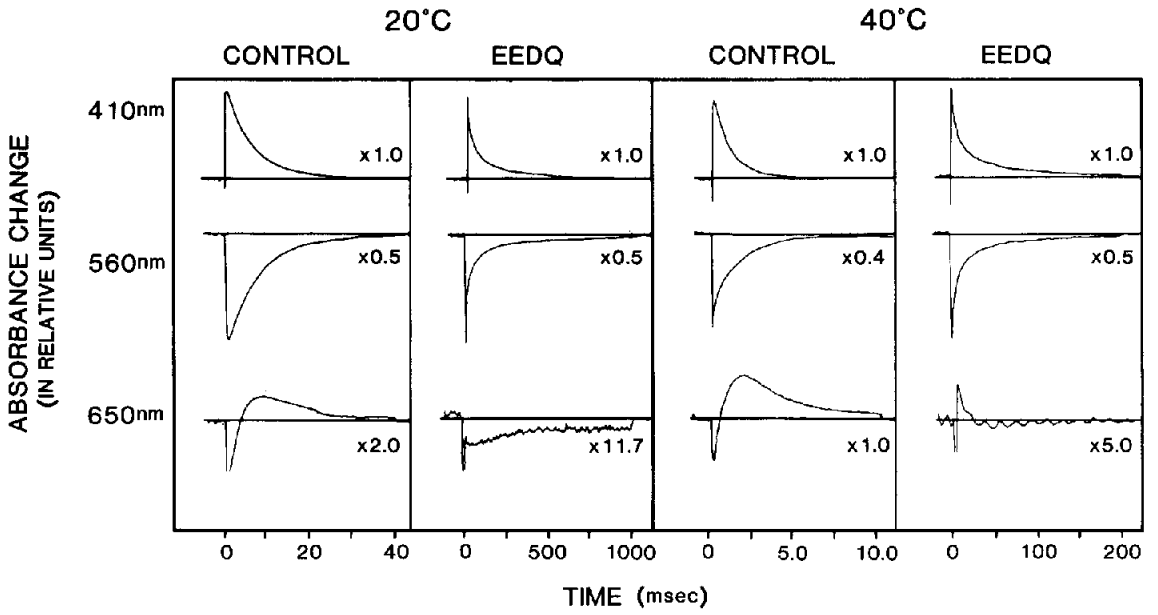


Fig.4. Effect of carboxyl modification on the kinetics of BR, M and O.

of carboxyl-activating reagent treatment must occur between a lysine and carboxyl residue that are in very close proximity to each other and probably interacting ionically.

3.3. *Apparent absence of photointermediate O_{640} and kinetics of reformation of BR_{570}*

At 650 nm control purple membranes (pH 6.5, 20°C) showed the typical pattern for the O_{640} intermediate of an initial negative absorbance change which is quickly reversed and yields a positive peak followed by a slow decay (fig.4). The magnitude of the initial negative absorbance is apparently a contribution from the large negative change in absorbance that occurs at 570 nm. This was visualized by computer subtraction of the flash photolysis signal at 560 nm from the signal obtained at 650 nm. The magnitude of the subtracted signal is based on the relative absorbance at the two wavelengths. The corrected flash photolysis kinetics are true kinetics for the 'O' intermediate (not shown).

Carboxyl-modified samples exhibited an entirely different pattern at 650 nm from controls. In both EEDQ and EEDQ + GME modified preparations analyzed at 20°C, an initial negative transient absorbance response appeared, but it was followed by a gradual increase in absorbance towards the baseline and no further. Kinetic analysis of these traces at longer time priods confirmed that positive absorbance at 650 nm did not develop. Increasing the temperature to 40°C, which normally substantially favors O_{640} intermediate formation [7], revealed a positive absorbance at 650 nm in the modified samples. This positive absorbance appeared in the same time domain as the signal from control samples at 40°C indicating the kinetics of the O_{640} intermediate are substantially unaltered.

The apparent kinetics of the increase in absorbance at 650 nm were similar to both the second phase of M_{412} decay and reformation of BR_{570} for all carboxyl-modified samples. The results from EDC and EDC + GME modified samples also revealed the absence of positive absorbance at 650 nm and similar kinetic parameters for M_{412} decay and BR_{570} reformation. The kinetics measured at 650 nm in the modified samples at 20°C thus represent changes of the BR_{570} species whose broad absorption band extends out to 700 nm. Typically, the absorbance at 640 nm represents 10% of the absorbance at 570 nm peak value. Flash photolysis kinetic traces of modified samples at 650 nm (20°C and 40°C) are therefore the composite of the fast O_{640} intermediate changes superimposed upon slow changes of the regeneration of BR_{570}. This result is consistent with a scheme where

$M \overset{k_1}{\rightarrow} O \overset{k_2}{\rightarrow}$ BR and $k_2 >> k_1$ at 20°C but $k_2 \simeq k_1$ at 40°C as is the case in control samples at 20°C.

Flash photolysis conducted on samples at pH 3.0 showed that the overall shape of the control response was similar to that obtained at neutral pH, although the magnitude of the response at 650 nm is increased and the decay process is greatly slowed. However, EDC-modified samples photolyzed at pH 3.0 showed no positive absorbance at 650 nm, but a complex negative absorbance response.

3.4. *Effects of carboxyl-modification on the formation of the acid-induced species*

Acid titration of purple membranes is known to induce a red shift in the absorption spectrum, leading to formation of an acid species absorbing maximally at 600–610 nm. This effect is reversible since the original spectrum can be regenerated by addition of base [7]. Difference spectra of control purple membranes at pH 7.0 (25°C) and samples at various acid pH-values revealed a substantial decrease in absorbance at λ = 550 and a concomitant absorbance increase of a broad band centered at λ = 640 nm (fig.5A). A plot of ΔA_{640} *vs* pH revealed that the 640 nm peak had a $pK \simeq 3.5$ (inset), in agreement with [30]. At pH <3.0

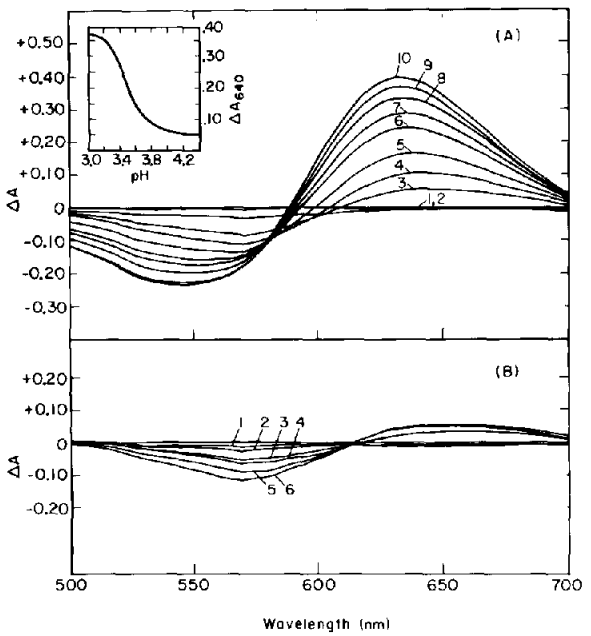


Fig.5. Inhibition of the formation of the blue acid-induced species of BR following carboxyl-modification of purple membranes.

some membrane aggregation was observed, although reversal of aggregation by addition of NaOH was still effective. Acid titration of carboxyl-modified purple membranes showed substantial differences in their spectral behavior relative to controls. Samples modified by EDC, EDC + GME, EEDQ, and EEDQ + GME treatment consistently did not show the formation of spectral species absorbing at 600–610 nm at acid pH up to pH 3.0. However, the modified preparations revealed a decrease in 570 nm absorbance with increasing acidity. Carboxyl-modified samples appeared purple at pH 3.0 while control purple membranes appeared blue. Difference spectra of EDC modified BR (fig.5B) showed a negative peak with λ_{max} = 570 nm and a very small broad positive band centered at λ = 650 nm.

An acid-induced species may represent a stabilization of the 'O' intermediate [7,8]. It is clear that the O_{640} intermediate is capable of forming in samples in which the acid-induced species is not. This indicates but does not prove that a carboxyl residue which is necessary for O_{640} formation is not modified by our procedures and that intramolecular cross-linking is responsible for preventing formation of the acid-induced species. Formation of the acid species is the result of the titration of 1 or 2 groups and not due to the 'random' titration of lipid or protein [7,8]. It is not known which residue is involved but it must be a residue which can move into contact with the retinal chromophore in order to create a large spectral shift [9,10]. This residue may share properties of the Schiff base that render it inaccessible or unreactive under the mild conditions employed in this study for modification.

The formation and location of ion pairs buried within the purple membrane are essential elements distinguishing several current models of bacteriorhodopsin structure [13,31]. The future determination of the location of lysine–carboxyl cross-links may allow a basis for precise assignment of the location of α-helical rods within the 3-dimensional structure. Furthermore, a method now exists for coupling of a wide variety of nucleophilic agents (radioactive, chromophoric, fluorescent energy transfer acceptors) to carboxyl-groups that can be utilized to map BR structure and probe its proton pumping function.

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