Long-Range Effects on the Retinal Chromophore of Bacteriorhodopsin Caused by Surface Carboxyl Group Modification†

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ABSTRACT: Carboxyl groups of bacteriorhodopsin (bR) that are modified by 1-ethyl-3-[3-(trimethylamino)-propyl]carbodiimide (ETC) have been identified. Reaction of deionized purple membrane with a 400-fold molar excess of ETC or [^{14}C]ETC for 1 h at 0 °C incorporates about 3.5 mol of ETC/mol of bR. Proteinase K cleavage of ETC-modified bacterioopsin (bO) produced small ^{14}C-labeled peptides. Amino acid sequence analysis showed three major ETC-modified residues: Glu 234, Asp 38, and Glu 74. Proteolysis of purple membrane with papain removes the ETC site at Glu 234. Treatment of ETC-modified, papain-cleaved purple membrane with hydroxylamine removes half of the remaining ETC label. Subsequent cleavage with chymotrypsin, followed by amino acid sequence analysis, revealed that most of the remaining label was at Glu 74. bR modified by ETC primarily at Glu 74 displays two alterations in the retinal chromophore, located in the membrane interior at a distance more than 2 nm away from the modified carboxyl group. (1) The acid-induced purple-to-blue transition undergoes a shift in apparent p*K* from 3.2 to 2.3. (2) The second-order rate constant for chromophore regeneration from bO and retinal is diminished from 3600 to 1700 M^{-1} s^{-1} in membrane sheets. Most of the shift in the p*K* of the purple-to-blue transition can be explained by the quaternary ammonium ion of ETC attached to Glu 74 overlapping the postulated location of the guanidinium group of Arg 82. The inhibition of regeneration may be attributed to the involvement of the loop connecting helices B and C in a conformational change between bO and bR.

Bacteriorhodopsin (bR),[1] the only protein in the purple membrane from *Halobacterium salinarium* (formerly *Halobacterium halobium*), transduces light energy to a transmembrane hydrogen ion gradient. Amino acid substitution experiments (Khorana, 1988; Soppa et al., 1989; Needleman et al., 1991), along with many spectroscopic studies [reviewed by Mathies et al. (1991)] have provided a plausible molecular mechanism for the light-activated proton pump of bR, involving a sequence of light-triggered proton transfer reactions [for recent reviews see Renthal (1992) and Lanyi (1992)]. The proton release and uptake reactions occur at the membrane surface, but the proton release site has not yet been identified.

Bacteriorhodopsin consists of the protein bacterioopsin (bO) combined with *all-trans*-retinal. Retinal binds within the interior of bR (King, 1979; Leder et al., 1989; Hauss et al., 1990; Henderson et al., 1990) and is inaccessible to the aqueous medium in the dark (Oesterhelt et al., 1974). The absorbance maximum at 570 nm arises primarily from three effects: (1) coupling of retinal to bO as a protonated Schiff base, (2) the orientation of the retinal polyene chain with respect to the β-ionone ring, and (3) specific electronic interactions with protein side chains (Nakanishi et al., 1980; Harbison et al., 1985). Thus, the chromophore absorbance spectrum serves as a sensitive probe of the protein environment near retinal. During the proton pump cycle, bR passes through a number of transient states. These photointermediates are characterized by shifted absorbance maxima. Two states, K and O, are red-shifted, having a blue appearance instead of the native purple color. Consequently, there has been considerable interest in the blue states of bacteriorhodopsin that are observed at low pH or low ionic strength (Fischer & Oesterhelt, 1979; Mowery et al., 1979; Kimura et al., 1984; Chang et al., 1985). Blue membrane is stable in the dark at room temperature, offering the possibility of examining bacteriorhodopsin at equilibrium in a state that may be similar to a proton pump transient.

Blue membrane undergoes a photochemical reaction when exposed to light, but the photocycle lacks the important M intermediate (Varo & Lanyi, 1989) and no protons are pumped (Drachev et al., 1978). Mutagenesis experiments have identified aspartic acid 85 (D85) as an essential residue in the purple-to-blue transition: conversion of D85 to asparagine (D85N) produces bacteriorhodopsin with a permanently blue-shifted chromophore (Mogi et al., 1988; Subramaniam et al., 1990; Otto et al., 1990). When aspartic acid 212 is changed to asparagine (D212N), a pH-dependent chromophore occurs, which resembles D85N above pH 7 (Needleman et al., 1991). Conversion of arginine 82 to glutamine (R82Q) shifts the purple-to-blue transition to neutral pH (Balashov et al., 1993; Brown et al., 1993). The

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[1] Abbreviations: bR, bacteriorhodopsin; bO, bacterioopsin; PM, purple membrane; ETC, 1-ethyl-3-[3-(trimethylamino)propyl]carbodiimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; ETU, 1-acyl-1-ethyl-3-[3-(trimethylamino)propyl]urea; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin.

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three side chains that seem to control the purple-to-blue transition are located near the retinal Schiff base, according to the electron crystallographic molecular model of bacteriorhodopsin (Henderson et al., 1990). These same side chains have also been implicated in formation of the complex counterion to the Schiff base (de Groot et al., 1989, 1990) and also may form a metal coordination site near the Schiff base that appears to control the pigment color (Jonas & Ebrey, 1991).

Experiments in this laboratory previously showed that modification of purple membrane with water-soluble carbodiimide (1) alters light-induced steady-state changes in H^+ binding, (2) slows the photocycle of the retinal chromophore, and (3) inhibits the low pH purple-to-blue transition of the chromophore (Renthal et al., 1979; Renthal & Wallace, 1980). We had assumed that the modified sites must be surface carboxyl side chains on the cytoplasmic side of the membrane (the uptake side of the proton pump) because the photocycle and proton binding effects were consistent with inhibition of a rate-limiting pump uptake step. However, we were prompted to reinvestigate this reaction by two recent findings from mutagenesis experiments. First, only interior (rather than surface) carboxyl groups have been identified as essential for proton pumping (Mogi et al., 1988; Khorana, 1988; Butt et al., 1989); and second, the groups that seem to control the purple-to-blue transition—D85, D212, and R82—are all found on the extracellular (proton-release) half of the membrane (Henderson et al., 1990). We have now located the major sites of modification of bacteriorhodopsin with 1-ethyl-3-[3-(tri[^{14}C]methylamino)propyl]carbodiimide (ETC). We can identify the amino acids responsible for the inhibitory effect of water-soluble carbodiimides on the purple-to-blue transition. In addition, we find that this same modification inhibits the rate of chromophore formation.

EXPERIMENTAL PROCEDURES

Membranes. Purple membrane (PM) was prepared from *H. salinarium* (formerly *H. halobium*) S9 by the method of Oesterhelt and Stoeckenius (1974) and stored in 0.025% NaN_3 at 5 °C. Blue membrane was prepared by passing purple membrane through a column of the H^+ form of AG 50W X2 cation-exchange resin (Bio-Rad) and then washing twice with deionized water by centrifugation and resuspension.

Carbodiimides. [^{14}C]ETC was prepared by methylation of EDC free base (prepared by ether extraction of a Na_2CO_3-neutralized aqueous solution of EDC-HCl, Aldrich Chemical Co.) with $^{14}CH_3I$ (Sigma, 43 Ci/mol, or Dupont NEN, 7.5−15 Ci/mol) and a 3-fold excess of distilled CH_3I in dry ether (reaction time 1.5 h or overnight, in darkness, with stirring, under N_2, 23 °C). The precipitated ETC was decanted, rinsed with ether, dissolved in $CHCl_3$, vacuum-evaporated, and recrystallized twice from $CHCl_3$/ethyl acetate. The product was protected from light and stored desiccated at −20 °C. Purity was estimated by UV absorbance of the aniline adduct (Williams et al., 1980) and found to be 95%, equivalent to a nonradioactive standard (Aldrich Chemical Co., recrystallized). The specific activity obtained was in the range of 7×10^{10} to 7×10^{11} cpm/mol.

Reaction of Purple Membrane with Carbodiimide. The reaction was done essentially as previously described (Renthal et al., 1979, 1983, 1987; Renthal & Wallace, 1980), except blue membrane (55 μM bR), titrated with NaOH to pH 7.0−7.2, was used. Reaction conditions were 1 h. 0 °C, in darkness, with a 400:1 ETC:bR molar ratio (24 mM final ETC concentration containing approximately 10^{11} cpm of ^{14}C/mol). Samples were washed by centrifugation and resuspension. For CNBr or proteinase K fragmentation, the modified membranes were lyophilized.

Fragmentation Methods. Prior to CNBr and proteinase K cleavage, bR was delipidated by passage down a column of LH-60 [eluting solvent 70/30 (v/v) ethanol/88% formic acid], followed by vacuum evaporation of fractions containing bO. Cleavage with CNBr was done as previously described (Gerber et al., 1979). Cleavage with proteinase K was done by a procedure similar to that of Gerber et al. (1977). Cleavage procedures for papain (Renthal et al., 1983) and chymotrypsin (Gerber et al., 1979) were described previously. Prior to chymotrypsin cleavage, the purple chromophore was bleached by suspension of the membranes in 0.2 M hydroxylamine, pH 7.0, with 1.5−2.5 h of irradiation from an Osram Xenophot HLX tungsten source (11 V, 7.5 A) in an Ace photochemical reaction vessel (Model 7878). Nitrophenol (0.5 mM in 0.05 M Tris, pH 8) circulated through the reaction vessel as a coolant and blue filter. Small samples (approximately 10 nmol of bR) were irradiated in 1-cm cuvettes with a projector lamp filtered through 1% $CuSO_4$.

Separation Methods. Separation of bO fragments by liquid chromatography was done by methods that were previously described: LH-60 chromatography in formic acid/ethanol (Gerber et al., 1979) or chloroform/methanol (Sigrist et al., 1988) and reverse-phase HPLC separations in 5% formic acid (Gerber et al., 1979) or 10 mM TFA (Engelhard et al., 1989). Analysis of purple membrane lipids was done on silica layers by previously described methods (Renthal et al., 1987). Gel electrophoresis was done on 12% polyacrylamide by the method of Laemmli (1970).

Amino Acid Analysis and Sequence Analysis. Peptides were cleaved with HCl vapor under Ar for 24 h at 110 °C prior to amino acid analysis on a Beckman 7300 instrument. Sequence analysis was performed on an Applied Biosystems 477B gas-phase sequencer, with samples bound to Immobilon (Millipore) film. HPLC fractions were transferred to Immobilon in a solution of 10 mM TFA in 50% CH_3CN/water (v/v). Peptides in polyacrylamide gels were transferred to Immobilon films electrophoretically (Matsudaira, 1987). Coomassie-stained bands were excised for sequencing.

Spectrophotometric Titrations. Purple membrane samples were polymerized in polyacrylamide gels (5% acrylamide, 3 × 18 × 0.2 cm) by methods similar to those of Fisher and Oesterhelt (1979), Mowery et al. (1979), and Renthal et al. (1990). After polymerization, the gels were soaked in 3 changes of deionized water. Prior to spectroscopy, the gels were cut into 1- × 3-cm pieces and equilibrated for 30 min with buffer. Buffers were as follows: pH 7.1−5.9, 20 mM sodium phosphate; pH 5.6−3.6, 20 mM acetic acid/sodium acetate; pH 3.2−2.0, 20 mM sodium sulfate titrated with sulfuric acid; below pH 2, sulfuric acid.

For papain-cleaved purple membrane, the proteolysis was done prior to gel formation. For ETC-modified purple membrane, the membrane samples were deionized with the H^+ form of AG 50W X2 cation-exchange resin (Bio-Rad) prior to gel formation and then treated as follows. The purple membrane gel was cut into 3-cm squares and reacted with

25 mM ETC on ice in the dark for 1 h. The gels were then washed successively for 10 min with ice-cold 50 mM NaCl, with 15 mM NaCl twice, and with deionized water twice. For NH_2OH-cleaved membrane, the ETC and NH_2OH reactions were done prior to polymerization of the gel.

Spectra were measured on an Aviv/Cary 14 spectrophotometer. The fraction of bacteriorhodopsin in the blue form, f_B, was calculated from

$$A_\lambda/A_I = f_P(\epsilon_{\lambda,P}/\epsilon_I) + f_B(\epsilon_{\lambda,B}/\epsilon_I) \quad (1)$$

where A is absorbance, ϵ is molar extinction coefficient, subscript λ refers to the measuring wavelength, subscript I refers to the isosbestic point (587 nm), subscript P refers to the purple form, measured at pH 5, and B refers to the blue form, measured at pH 2. Two different values of λ were used, 550 and 640 nm, and the values of f_B obtained were averaged.

Chromophore Regeneration. Regeneration of hydroxylamine-bleached purple membrane was done in 0.1 M sodium phosphate buffer, pH 7.0, at a concentration of approximately 5 μM bO. *All-trans*-retinal or retinol was added from an ethanol stock solution (2.6 mM) to a final concentration of 6.5 μM. The refolding and regeneration of bR in detergent micelles was similar to previously described methods (Liao & Khorana, 1984; Braiman et al., 1987): 0.4 mL of bO (about 10 μM) in dodecyl sulfate plus 0.15 mL lipid solution (32.5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and 29.5 mM dimyristoylphosphatidylcholine in 50 mM phosphate buffer, pH 6.0) plus 2 μL of *all-trans*-retinal (2.6 mM in ethanol). Absorbance changes at 550 nm (detergent samples), 565 nm (membrane samples with retinal), or 356 nm (membrane samples with retinol) were monitored as a function of time with an Aviv/Cary 14 spectrophotometer.

The kinetics were fit to a second-order rate equation:

$$d[bR]/dt = k(C_T - [bR])(A - [bR]) \quad (2)$$

where C_T = total protein concentration (bO plus bR), A = total retinal concentration (free retinal plus bR), [bR] = concentration of regenerated bR at time t, and k = the second-order rate constant. Integration of eq 2 gives

$$[bR] = \frac{AC_T(\exp[kt(A - C_T)] - 1)}{A\exp[kt(A - C_T)] - C_T} \quad (3)$$

The sum of the squares of the differences between measured and calculated values of [bR] was minimized by varying k.

RESULTS

Stoichiometry of ETC Modification of Purple Membrane. Deionized purple membrane was modified with [^{14}C]ETC and the lipids were separated from bO by LH-60 chromatography. Bacterioopsin traveled in the void volume and typically contained about 90% of the applied radioactivity. The remaining radioactivity eluted with the included volume. Analysis of lipids in the modified membrane by thin-layer chromatography showed no change in the polar lipid distribution and essentially no radioactivity migrating as a distinct modified component. Thus, the counts in the included volume probably represent labile or noncovalently bound ETC. Reaction stoichiometry was estimated on individual fractions. The average of peak fractions was 3.5 $\pm$ 0.1 mol of ETC/mol of bO.

Fragmentation of [^{14}C]ETC-bO. ETC-bO was cleaved with CNBr and the fragments were separated by reverse-phase HPLC with a water/ethanol gradient. A comparison of the elution profiles (data not shown) of ETC-modified and unmodified bO indicates that ETC modifies amino acids between positions 1−71 and 72−118 in the sequence. However, when [^{14}C]ETC was used, almost all the radioactivity eluted in the column flow-through. The conditions of the CNBr reaction (70% formic acid, 24 h) may promote cleavage of one of the −CO−NR− bonds of the 1-ethyl-[3-(3-trimethylamino)propyl]urea (ETU) substituent. Consequently, we sought conditions for peptide fragmentation at neutral pH with solubilized bacterioopsin. We found that a 30-min digestion of [^{14}C]ETC-bO with proteinase K (which is active in relatively high concentrations of dodecyl sulfate) released all ^{14}C in fractions containing small peptides (data not shown).

A proteinase K digest was applied directly to a reverse-phase C_8 HPLC column (Figure 1). Labeled peptides eluted at two different solvent polarities, which we refer to as K1 (2−5 min) and K2 (40−48 min). These fractions contained 19% and 62% of the eluted radioactivity, respectively. The total eluting radioactivity was 65% of the applied radioactivity. K1 eluted in the flow-through of the C_8 column. K1 was pooled and rechromatographed on a column of LH-20 equilibrated with 10 mM TFA in 50% CH_3CN/water (v/v) prior to acid hydrolysis and amino acid analysis. K1 was also HPLC-purified on a shallower gradient on the C_8 column (70% yield; not shown) prior to sequence analysis.

K2 was further analyzed by running the complete proteinase K digest on the C_8 column with a shallower gradient, revealing a cluster of a number of closely spaced radioactive peaks (Figure 1, inset). These were denoted K2a, K2b, and K2c. The amounts of K2a, K2b, and K2c varied somewhat from one digest to another, but K2b was always the largest peak. K2b was found to contain too much dodecyl sulfate for the Applied Biosystems sequencer, so a separate proteinase K digest was purified on a column of LH-60 prior to separation on the C8 column (not shown) for sequence analysis.

Amino Acid Sequence Analysis of Proteinase K Fragments Containing [^{14}C]ETC. Sequence analysis was performed on HPLC fractions from K1, K2a, K2b, and K2c. The results are shown in Table 1, interpreted in terms of the known amino acid sequence of bO. Eight Edman cycles on K1 showed two sequences: XAPEPSAG (residues 234−241, where X is a missing residue) and LIGSE (residues 190−194). These peptides contain possible ETC labeling sites at Glu 234, Glu 237, and Glu 194. Seven Edman cycles on K2a gave the sequence GVSDPXA (residues 33−39). Two possible ETC labeling sites are at Asp 36 and Asp 38. Fraction K2b was subjected to 10 Edman cycles, and the sequence was MVPFGGXQNP (residues 68−77). This sequence contains a single ETC-reactive site, Glu 74. Additional support for the presence of ETC at the Glu 74 site was obtained from measurements of released radioactivity collected from each Edman cycle. Cycle 7, which corresponds to sequence position 74, contained negligible amounts of Glu but showed a significant release of radioactivity. A fraction from K2c was sequenced for six Edman cycles, but no unambiguous sequence could be deduced.

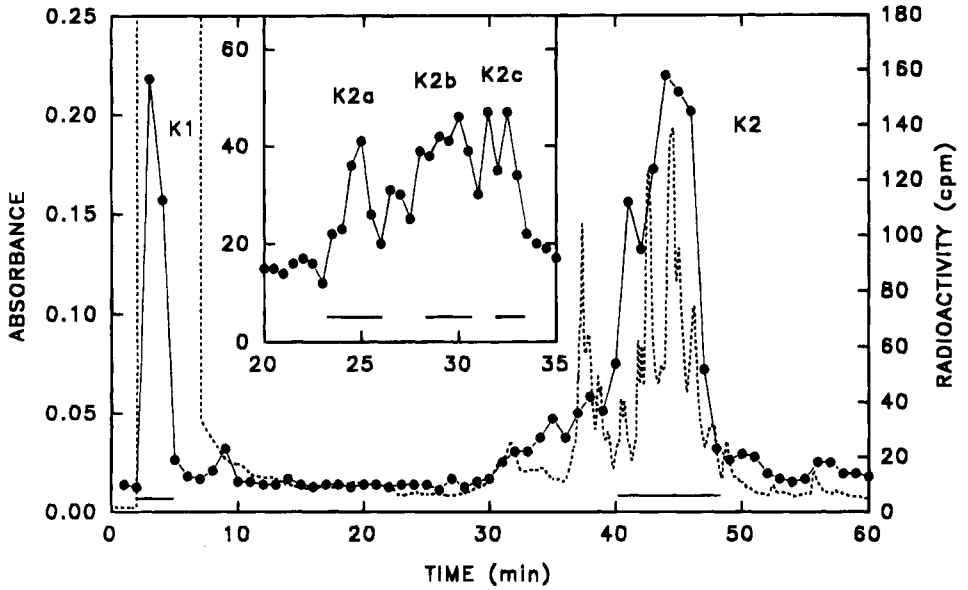


FIGURE 1: Reverse-phase chromatography of proteinase K fragments of ^{14}C-ETC-modified bacterioopsin: low-resolution gradient. The dried residue from proteinase K digest (130 nmol of bacterioopsin) was dissolved in 0.5 mL of 88% formic acid, and 0.46 mL was injected into the C8 column. Solvents: A = 10 mM TFA in water; B = 10 mM TFA in CH_3CN. Initial conditions: 20% solvent B. The column was eluted with a linear gradient from 20% to 80% solvent B over 90 min. The absorbance was monitored at 220 nm. Fraction size: 1 mL, of which 50-μL aliquots were removed for scintillation counting. ^{14}C-Labeled fractions eluting from 2 to 5 min are referred to as K1, and ^{14}C-labeled fractions at 40−48 min are referred to as K2. Inset: Similar conditions, except the C8 column was initially equilibrated with 30% solvent B. The column was eluted with a linear gradient from 30% to 50% solvent B over 40 min. Fraction size: 0.5 mL, of which 20-μL aliquots were removed for liquid scintillation counting. Fractions 47−52 (23.5−26 min) were denoted K2a, fractions 56−61 K2b, and fractions 62−67 K2c. Sequence analysis of K2a and K2b is given in Table 1.

Table 1: Labeling Stoichiometry of Proteinase K Fragments

fraction	sequence	peptide[a] (nmol)	ETC[b] (nmol)	ETC/peptide[c]
K1	LIGSE	0.9	0.95	(0.4)
	XAPEPSAG	0.6		(1)
K2a	GVSDPXA	1.0	1.0	1.0
K2b	MVPFGGXQNP	2.0	2.2	1.1
	Edman cycle 7	0.5	0.67	1.3

[a] Peptide quantitation: extrapolation of log of PTH-amino acid recovery to cycle 0. [b] ETC quantitation: liquid scintillation counting of sample applied to sequencer. [c] ETC/peptide ratio was calculated from the ratio of these quantities, except for K1, where the ratio from the amino acid analysis sample of XAPEPSAG peptide was used to estimate the amount of ETC on LIGSE. Quantitation of peptide at Edman cycle 7 for K2b was estimated by interpolation between cycles 6 and 8.

Amino acid analyses of the peak fractions of K1 and K2 gave compositions corresponding to peptides 234−244 (or 233−243) and 68−82. Because slightly different column purifications were used, the amino acid analysis of K1 did not show the LIGSE peptide found by sequence analysis.

The logarithms of the PTH-amino acid yields from each Edman cycle were plotted against cycle number and linearly extrapolated back to "cycle 0" to estimate the initial amount of peptide present in the sequencer. This quantity is compared with the measured amount of ETC present in the sample (based on radioactivity) in Table 1. The results show that the apparent labeling stoichiometries for the peptides are approximately 1 mol of ETC/mol of peptide for K2a and K2b. Also, the labeling stoichiometry for K2b based on the measured radioactivity yield at cycle 7 and the interpolated peptide concentration from the log concentration plot is about 1 mol of ETC/mol of peptide. We can attribute to the 234−244 (233−243) peptide all of the ETC found in the K1 sample used for amino acid analysis (0.8 mol/mol). Assuming 1 mol of ETC/mol on the XAPEPSAG peptide in the K1 sample that was sequenced, the maximum amount of labeling on the LIGSE peptide is 0.4 mol/mol.

Fragmentation of [^{14}C]ETC-Bacteriorhodopsin with Papain and Chymotrypsin. Because of the complexity of the proteinase K fragments, we wanted to view the ETC labeling pattern under more limited proteolytic conditions. Papain (Ovchinnikov et al., 1979) and chymotrypsin (Gerber et al., 1979) may be used under conditions where cleavage occurs at single sites. Papain cuts bR between residues 231 and 232, and chymotrypsin cuts between 71 and 72. Thus, the ETC site near the carboxyl terminus can be specifically removed by treatment with papain, and the ETC site at Glu 74 site can be separated from the site between residues 33 and 39 by treatment with chymotrypsin.

After reacting papain-cleaved purple membrane with [^{14}C]-ETC, we found only 2.8 mol of ETC was incorporated into bacteriorhodopsin that was truncated at residue 231, compared with the 3.5 mol of ETC bound when the C-terminal 17 amino acids were present (Table 2), which is consistent with the removal of one ETC site near the C-terminus.

When native purple membrane is treated with chymotrypsin, the yield of fragments is poor. However, when the retinal chromophore is first bleached with hydroxylamine, the yield is more than 90% (Gerber et al., 1979). Treatment of papain-cleaved [^{14}C]ETC-modified purple membrane with hydroxylamine resulted in the removal of about half of the radioactivity, but no significant losses of label occurred after reaction with chymotrypsin (Table 2). The chymotrypsin fragments were separated by LH-60 chromatography (Figure 2). About 30% of the radioactivity came off the protein under the acidic conditions of the chromatographic separation. Of the remaining radioactivity, 74% was in the first peak, containing a mixture of uncleaved bO and the large chymotryptic fragment, C1′ (residues 72−231), and 26% was

Table 2: Modification of Bacteriorhodopsin with ETC

sample	mol of ETC/mol of bR[a]
ETC−PM[b]	3.5 ± 0.1
ETC-papain-PM[b]	2.8 ± 0.1
after NH_2OH treatment[c]	1.5 ± 0.2
after chymotrypsin[c,d]	1.4 ± 0.2
C1′	1.0 ± 0.04
C2	0.4 ± 0.05

[a] Entries are the averages of three separate experiments ± the relative error. [b] Radioactivity was measured by scintillation counting. Moles of bR were measured by absorbance at 570 nm. [c] The radioactivity released into the supernatants after NH_2OH treatment and chymotrypsin cleavage was used to estimate the loss of ^{14}C, assuming no significant loss of protein. [d] The 1.4 mol/mol found after chymotrypsin cleavage were apportioned between C1′ and C2 according to the ratios calculated from the elution profile in Figure 2.

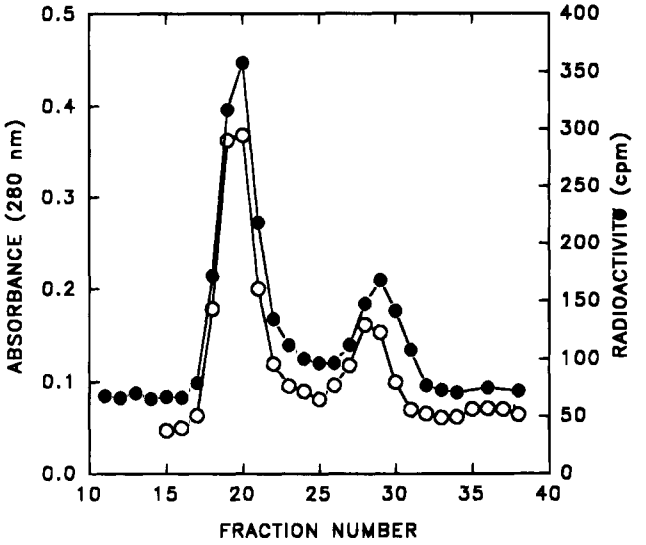


FIGURE 2: LH-60 chromatography of chymotrypsin fragments. Purple membrane (100 nmol of bR) from which the C-terminal tail had been removed by treatment with papain was modified with ETC and then bleached and cleaved with chymotrypsin. The lyophilized membrane was dissolved in 0.3 mL of 88% formic acid, diluted with 0.7 mL column buffer [$CHCl_3$:CH_3OH (1:1) containing 0.1 M ammonium acetate], and applied to a 2.5- × 90-cm column of LH60 equilibrated with column buffer. Approximately 2-mL fractions were collected. The absorbance was measured at 280 nm (filled circles) and then each entire fraction was diluted with scintillation fluid and the radioactivity was measured (open circles). Residues from positions 72−231 (C1′) elute in the first peak and 1−71 (C2) in the second peak. Absorbance is higher in C1′ because of its 2.5-fold larger extinction coefficient.

in the second peak, containing the small chymotryptic fragment, C2 (residues 1−71). Polyacrylamide gel electrophoresis of the first peak showed that the ratio of C1′ to uncleaved bO was 4:1. Assuming the same distribution of ^{14}C in the uncleaved bO as in the recovered fragments, the overall ratio of label on C1′:C2 was 1.0:0.4.

Additional samples of chymotryptic fragments of papain-cleaved, ETC-modified bR were separated by polyacrylamide gel electrophoresis and transferred to PVDF film. The bands of ETC-modified C1′ and unmodified C1′ were cut out and subjected to automated Edman degradation. The terminal sequences are compared in Figure 3. Clearly, glutamic acid at position 74 is diminished by about 80% in the ETC-modified sample compared with the unmodified control. Therefore, in papain-cleaved, hydroxylamine-treated ETC-PM, the major site of ETC modification is at Glu 74.

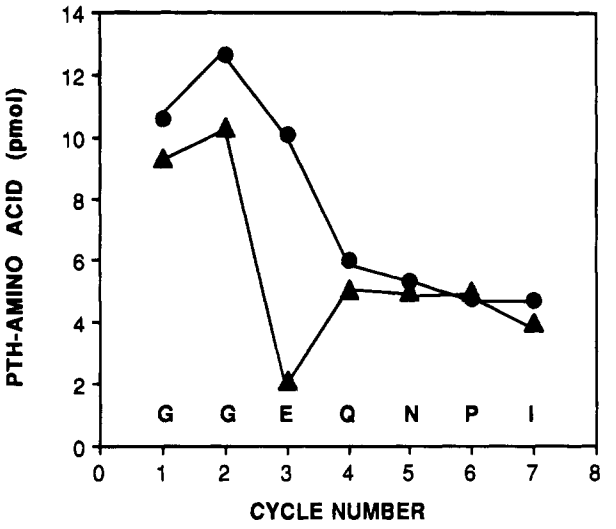


FIGURE 3: Sequence analysis of ETC-modified chymotrypsin fragment C1′. Samples of ETC-modified, papain-cleaved purple membrane and unmodified, papain-cleaved purple membrane were subjected to proteolysis with chymotrypsin (Figure 2) and then separated by polyacrylamide gel electrophoresis. The peptides were electrophoretically transferred to Immobilon and stained with Coomassie blue. The C1′ fragments were excised and sequenced. The yield of the major PTH-amino acid found in each round of automated Edman degradation is plotted in the Figure. Circles, control purple membrane; triangles, ETC-modified purple membrane.

Purple-to-Blue Transition. Polyacrylamide gels containing PM or ETC-PM were equilibrated with buffers at various pH values. We assume that the ETC modifies the same functionally important sites when the reaction occurs in gels or in free solution, since the products show identical functional effects. Confirming our previous results on PM reacted with EDC (Renthal & Wallace, 1980), carbodiimide modification lowers the apparent p*K* of the purple-to-blue transition by about 1 unit, from 3.2 to 2.3 (Figure 4). As we noted previously (Renthal & Wallace, 1980), the cooperativity of the transition is diminished by the carbodiimide modification. The Hill coefficient for the titration changes from 1.8 to 1.3. We can distinguish the individual effects of the three major sites of ETC modification as follows.

The removal of the C-terminal 17 amino acids by papain cleavage removes the ETC site at Glu 234. Papain cleavage alone has essentially no effect on the p*K* of the purple-to-blue transition (Figure 4). However, PM subjected to ETC modification after papain cleavage shows about the same p*K* shift and diminished cooperativity as ETC modification of non-papain-cleaved PM (Figure 4). Therefore, neither the p*K* shift nor the diminished cooperativity is due to the modification of the Glu 234 site on the carboxyl terminus.

Treatment of ETC-modified purple membrane with hydroxylamine leaves Glu 74 almost fully modified by ETC, but most of the remaining ETC is removed (Table 2 and Figures 2 and 3). We find that the purple-to-blue transition of hydroxylamine-treated PM is essentially the same as native purple membrane. The purple-to-blue transition of ETC-modified PM subsequently treated with hydroxylamine retains both the p*K* shift and the cooperativity loss observed in ETC-modified PM not treated with hydroxylamine (Figure 4). Thus, we can attribute most of the effect of ETC on the purple-to-blue transition to modification of Glu 74.

Chromophore Regeneration. The rate of regeneration of the purple chromophore was measured by adding *all-trans-*

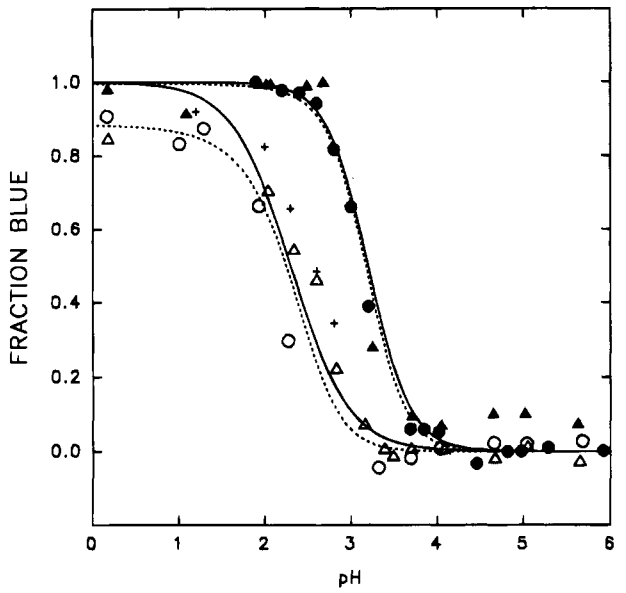


FIGURE 4: Purple-to-blue transition. The fraction of blue pigment, f_B, was calculated from absorbance spectra of purple membrane samples polymerized in polyacrylamide gels (see eq 1). Filled circles, Unmodified purple membrane; filled triangles, papain-cleaved purple membrane; open circles, ETC-modified purple membrane; open triangles, ETC-modified papain-cleaved purple membrane; Crosses, ETC-modified purple membrane subsequently reacted with 0.2 M hydroxylamine for 2.5 h. Solid lines were calculated from $f_B = [H^+]^n/(10^{-npK} + [H^+]^n)$ with $pK = 3.2$ and $n = 1.8$ for unmodified purple membrane and $pK = 2.3$ and $n = 1.3$ for ETC-modified purple membrane. Dashed lines were calculated from eq 4 (see text).

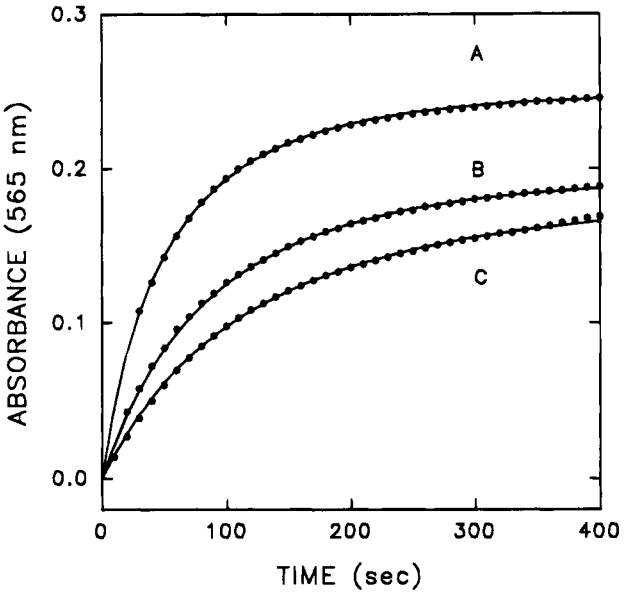


FIGURE 5: Regeneration of hydroxylamine-treated bacteriorhodopsin in purple membrane sheets. Purple membrane was bleached with hydroxylamine. The samples were washed free of hydroxylamine and then resuspended at a bO concentration of approximately 4 μM in 0.1 M sodium phosphate buffer, pH 7.0. Chromophore regeneration was initiated by adding *all-trans*-retinal (final concentration 6.5 μM). Absorbance (filled circles) was monitored at 565 nm, 25 °C. Curve A, bleached purple membrane; curve B, bleached ETC-modified, papain-cleaved purple membrane; curve C, bleached ETC-modified PM. Lines were calculated from eq 3 as described in the text.

retinal to hydroxylamine-bleached PM (Figure 5 and Table 3). ETC-modified PM shows a 2-fold reduction in the second-order rate constant for regeneration. The inhibitory

Table 3: Chromophore Regeneration of Hydroxylamine-Bleached PM

sample	rate constant k (M^{-1} s^{-1})	relative fraction regenerated
Bacterioopsin plus Retinal		
PM	3600	1.00
ETC-modified PM	1700	0.92
ETC-modified, papain-cleaved PM	1250	0.79
Bacterioopsin plus Retinol		
PM	8800	
ETC-modified PM	5200	

role of the ETC site at Glu 234, near the carboxyl terminus, was examined by measuring the regeneration rate of ETC-modified, hydryoxylamine-bleached PM from which the C-terminal 17 amino acids had been cleaved by papain. The regeneration rate was similar to that of intact ETC-PM (Figure 5 and Table 3), showing that the inhibitory effect of ETC is not due to the Glu 234 site. Thus, the inhibitory effect of ETC on the regeneration rate appears to result primarily from the modification at Glu 74.

Chromophore regeneration involves two steps, retinal binding and Schiff base formation. These steps can be separated by studying the interaction of *all-trans*-retinol with bleached purple membrane (Schreckenbach et al., 1978). Retinol cannot form a Schiff base, but upon binding to bO, the UV absorbance of retinol is enhanced near 350 nm. Thus, the rate of binding to bO may be examined in the absence of Schiff base formation. The rate constant for retinol binding to ETC-modified PM is only 59% of the rate constant for unmodified PM (Table 3). Therefore, the step affected by ETC appears to be binding to bO, rather than Schiff base formation.

A similar inhibition of the rate of regeneration by ETC was observed with purified bO solubilized in detergent micelles, except that about half of the ETC-modified bO failed to regenerate (data not shown), presumably because of an inhibition of refolding.

DISCUSSION

Identification of ETC-Modified Sites. After reaction of purple membrane with [^{14}C]ETC at neutral pH, 3.5 mol of ETC were incorporated/mol of bR. Nearly all of the radioactivity was associated with bR, and essentially none was found in extracted lipids. On the basis of our analysis of fragments of the modified bO, stoichiometric labeling of Asp 38, Glu 74, and Glu 234 accounts for three of these sites. Additional less specific labeling totaling 0.5 mol/mol may be present at other carboxyl groups or on tyrosines. We can rule out appreciable intramolecular cross-linking by considering the CNBr and proteolytic cleavage patterns, along with the three-dimensional molecular model of bR. The ETC sites at Glu 74 and Glu 234 are probably the same as the fluorescent labeling sites we previously reported for reaction of PM with EDC in the presence of dansyl hydrazine (Renthal et al., 1983, 1987).

The electron crystallography studies of Henderson et al. (1990) do not provide much information about the structures of the surfaces of purple membrane. However, their results identify which segments of the polypeptide chain connect surface loops between the seven transmembrane helices (Figure 6). The two surfaces of the protein clearly have many carboxyl groups available for reaction with ETC. The

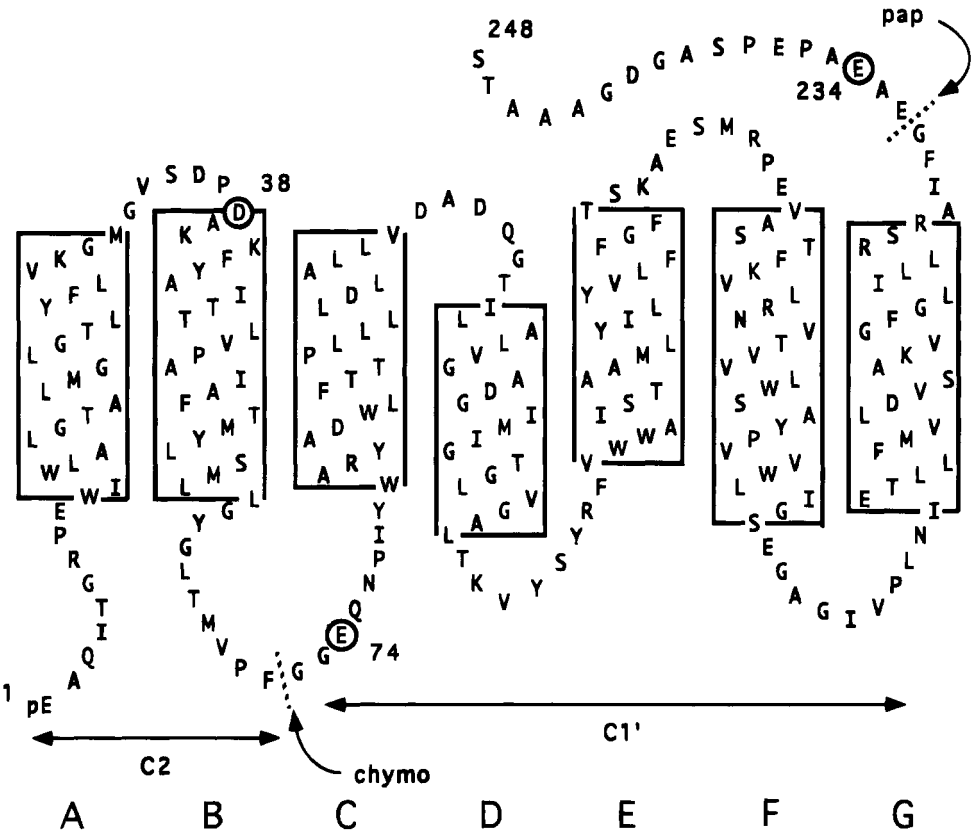


FIGURE 6: ETC-modified sites in bacteriorhodopsin. The amino acid sequence of bacteriorhodopsin (Khorana et al., 1979) is shown. The transmembrane helices identified by Henderson et al. (1990) are shown in boxes and labeled A−G. The cytoplasmic side of the protein is on the top of the Figure. Amino acids found to be modified by ETC are circled. Chymotrypsin (chymo) and papain (pap) cleavage sites are indicated with dashed lines. The chymotrypsin fragments C1′ and C2 are indicated. The N-terminal amino acid of bR is pyroglutamic acid, pE.

cytoplasmic surface has 11, and the extracellular surface has 4. The fact that we observe far fewer groups reacting with ETC is due, in part, to the reaction conditions (time, temperature, and ETC concentration), which were selected for a limited reaction (Renthal et al., 1979, 1987). But the results must also reflect differences in the chemical environments of the surface carboxyl groups. There are three main reasons why surface carboxyl groups may have different reactivity. (1) Some carboxyl groups may be ion-paired and thus not available for reaction. Although bR has many surface carboxyl groups, it also has many surface amino groups. Experimental measurement of the net surface charge of bR in neutral detergent micelles (Alexiev et al., 1994) shows only −2.5 charges on the cytoplasmic side and −1.8 charges on the extracellular side. (2) The reaction was done at neutral pH. Carboxyl modification by carbodiimides requires proton transfer to one of the carbodiimide nitrogens, either from the protonated carboxyl or from the medium. At neutral pH, local acidity or neighboring proton donors can contribute selectivity to the reaction. (3) ETC might act as a cationic affinity label. The quaternary ammonium ion of ETC could bind to a cation binding site on the protein surface and then form a covalent bond with a carboxyl group located about 1 nm away. Evidence for this comes from the ionic strength dependence of the reaction. The reaction of EDC with purple membrane was previously shown to be inhibited by high ionic strength, and the reaction was particularly sensitive to Ca^{2+} (Renthal et al., 1979). We also find that the Glu 74 ETC site is only partially modified if the purple membrane is not first deionized (presumably removing divalent metal ions).

Protein carboxyl side chains are modified by ETC to form 1-acyl-1-ethyl-3-[3-(trimethylamino)propyl]ureas (ETU) of carbodiimides in the absence of added nucleophiles (Hoare & Koshland, 1967). The ETU linkage to proteins was previously reported to be alkaline-labile (Millett et al. 1983), complicating the process of identifying the sequence positions of the modified residues. Fragmentation of bO with CNBr resulted in loss of the ^{14}C label. Fragmentation with proteinase K in neutral detergent solution preserved the labeling and yielded a complex mixture of small peptides. The complexity is probably due in part to the nonspecific nature of proteinase K cleavage, which can generate a series of similar peptides differing by only a few amino acids.

Treatment of ETC-modified, papain-cleaved purple membrane with hydroxylamine removed about half of the ETC labeling (Table 2). Two of the major reaction products of carbodiimides with proteins are known to be sensitive to nucleophilic displacement: *N*-acylureas (Nau & Riordan, 1975) and *O*-arylisoureas (Grouselle & Pudles, 1977). Thus, the hydroxylamine cleavage of ETC labeling is expected. What is surprising is the apparent selectivity of this reaction. After cleavage of the hydroxylamine-treated bO with chymotrypsin and separation of the two fragments (Figures 2 and 3 and Table 2), we found that labeling at the Glu 74 site is virtually untouched, whereas the ETC bound to one (or more) of the other sites is substantially decreased. We cannot determine from the present data whether the hydroxylamine-sensitive sites include Asp 38 or are entirely tyrosine

side chains. In any case, tyrosine isoureas displaced by hydroxylamine will revert to tyrosine, and *N*-acylureas displaced by hydroxylamine will initially form hydroxamic acids, which will hydrolyze to carboxylic acids when excess hydroxylamine is removed (Jencks et al., 1963). Thus, the treatment with hydroxylamine yields bO with about 1 mol of Glu 74 modified/mol and with less than 0.5 mol/mol modification between residues 1 and 71, presumably at Asp 38.

Functional Effects of ETC Modifications. The cooperativity of the acid-induced purple-to-blue transition suggests that several titratable groups interact to cause the transition (Mowery et al., 1979). Paradoxically, the mutagenesis of a single group, Asp 85, produces a permanently blue pigment (Mogi et al., 1988; Subramaniam et al., 1990; Otto et al., 1990). It has been assumed that the titration of Asp 85 is influenced by one or more other groups to cause the cooperativity (Varo & Lanyi, 1989; Balashov et al., 1993). The removal of the Arg 82 side chain by mutagenesis shifts the p*K* of the purple-to-blue transition to about 7 and eliminates the cooperativity (Brown et al., 1993; Balashov et al., 1993). The ETC modification of PM has the opposite effect on the purple-to-blue transition and also eliminates the cooperativity of the transition (Figure 4). Although our results are not as clear-cut as mutagenesis experiments, we have shown that the ETC effect on the purple-to-blue transition is primarily due to modification of Glu 74.

At neutral pH, ETC modification of protein carboxyl groups substitutes an ETU group for one of the carboxyl oxygens. In ETU-Glu the carboxyl negative charge is replaced by a positive quaternary ammonium ion. Can the altered surface charge of the ETC-modified PM explain the shift in p*K* of the purple-to-blue transition? The purple-to-blue transition would be affected by a change in surface charge density, because the surface pH would change. We have analyzed the expected shift in the p*K* of the purple-to-blue transition due to the ETU modification. Using methods similar to those of Szundi and Stoeckenius (1989), we find that, over a wide range of surface charge densities, only small shifts can be attributed to surface charge. The maximum surface charge contribution from the ETC modification (amounting to about half of the observed p*K* shift) would occur if PM has zero surface charge below pH 2.5, a circumstance considered unlikely by Szundi and Stoeckenius (1989).

Most of the p*K* shift may be explained in terms of steric effects. According to the bR model of Henderson et al. (1990), Glu 74 is located at the purple membrane surface, with its carboxyl oxygens separated from those of Asp 85 by a distance of at least 2 nm. The exact position of Arg 82 is not known, since the electron density of its side chain is weak. Two conformations of Arg 82 have been suggested: one near Asp 85, Asp 212, and the Schiff base (the *in* position) and one pointing outward toward the extracellular surface (the *out* position). There has been some speculation that movement of Arg 82 may be involved in the mechanism of proton release by the proton pump (Braiman et al., 1988; Balashov et al., 1993; Brown et al., 1993). Even assuming Arg 82 is in the *out* position, the guanidinium nitrogens are no closer than 1.2 nm from the carboxyl oxygens of Glu 74. Thus, Glu 74 is too far from Asp 85 and Arg 82 to have a direct influence on the p*K* of the purple-to-blue transition. The ETU group, in the extended conformation, is about 1 nm long. Model-building indicates that an ETU group attached to Glu 74 could place the positive charge of the quaternary ammonium group near Arg 82. The quaternary ammonium ion of ETU-Glu 74 and the side-chain guanidinium group of Arg 82 in the *out* position nearly overlap in the model shown in Figure 7.

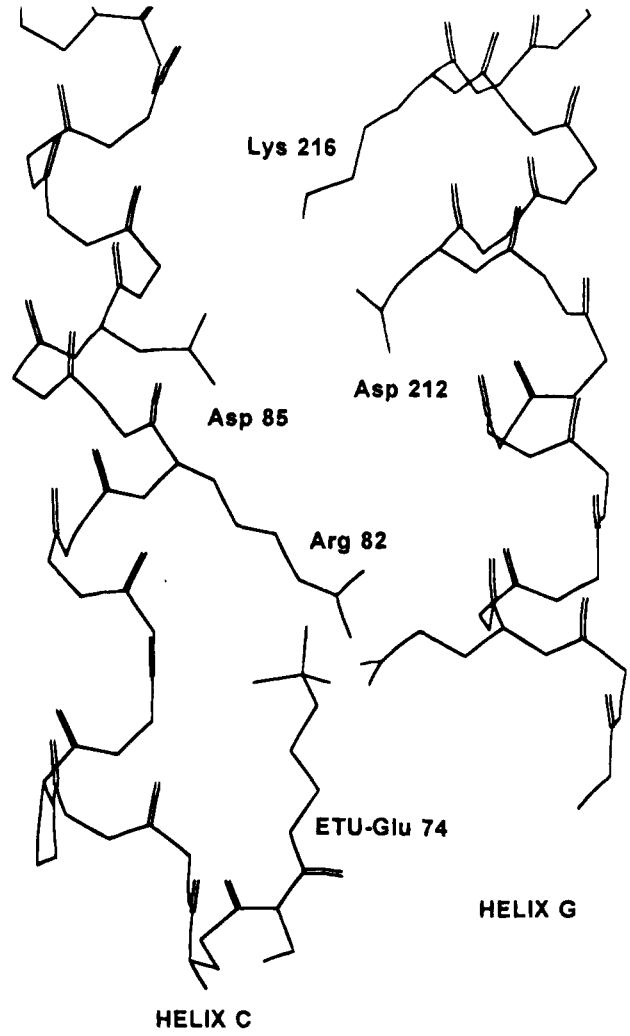
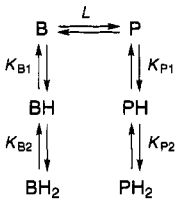


FIGURE 7: Distance between ETU group on Glu 74 and guanidinium group of Arg 82. Projection of the C and G helices of bR onto the *xz* plane (coordinates from Brookhaven Protein Data Bank) is shown. The quaternary ammonium group of the ETU modification of Glu 74 nearly overlaps the position of the guanidinium group of Arg 82 in the *out* position.

The structural model in Figure 7 suggests a mechanism for transmitting the effect of a membrane surface modification to the interior of the membrane. We assume that the guanidinium group of Arg 82 interacts electrostatically with Asp 85 and possibly Asp 212 (the *in* position) in the purple form of bR. This keeps the p*K* of Asp 85 low, since protonation will require additional energy to break the ion pair. Protonation of Asp 85 releases Arg 82 from its position near the Schiff base, and it moves to a new position closer to the membrane surface (the *out* position), which it occupies in the blue form of bR. Lacking the interaction with Arg 82, Asp 85 has a relatively high p*K* in the blue form of bR. However, if the quaternary ammonium group of ETU-Glu 74 occupies the guanidinium site of the *out* position of Arg 82, this will add the energy of displacing the ETU group to the titration of Asp 85, lowering its p*K*.

This model may be translated into quantitative terms as a Monod−Wyman−Changeux (MWC) allosteric transition (Monod et al., 1965):



B and P represent conformational states of bR in which Arg 82 is *out* or *in*, respectively. The states BH and BH_2 represent the protonation of two discrete sites in state B, and PH and PH_2 represent the protonation of the same sites in state P. One of the two sites is Asp 85. The second site has been postulated in various models [e.g., Varo and Lanyi (1989)]. Although its identity is unknown, it may correspond to Asp 212. The equilibrium constant between B and P is $L = [P]/[B]$. The letters B and P suggest that the B state tends to be blue and the P state purple, but it is the titration of Asp 85 that determines the actual color of the chromophore. Only BH_2 and BH with the Asp 85 site filled have a blue chromophore. The B state, and the form of BH with the proton on the other site, have purple chromophores. K_{B1}, K_{B2}, K_{P1}, and K_{P2} are the intrinsic dissociation constants of H^+ from the indicated species. For simplicity we assume that $K_{B1} = K_{B2} = K_B$ and that $K_{P1} = K_{P2} = K_B$. (The model can accommodate distinguishable values of K, but the data can be easily fit with indistinguishable intrinsic constants.) The fraction of bR in the blue form, f_B, is

$$f_B = ([H^+]/K_B + [H^+]^2/K_B^2)/\{(1 + [H^+]/K_B)^2 + L(1 + [H^+]/K_P)^2\} \quad (4)$$

We have used eq 4 to fit the data in Figure 4. The dashed lines are fit with the following constants: $pK_B = 7$, $pK_P = 2$, and $L = 4 \times 10^7$ for the unmodified membrane or 1.3×10^9 for the ETC-modified membrane.

There are several interesting aspects to this model. (1) The data for modified and unmodified membrane can be fit with identical intrinsic dissociation constants. That is, only the conformational change of Arg 82 contributes to the apparent pK shifts. (2) The model allows f_B to level off at less than 100% at low pH. This behavior is similar to the observations of Szundi and Stoeckenius (1988) for the purple-to-blue transition in purple membrane substituted with neutral lipids. (3) The removal of Arg 82 by mutagenesis can be modeled in eq 4 by setting $L = 0$ (i.e., in the absence of the guanidinium side chain, the aspartic acid groups near the Schiff base will be in a similar environment to the pure B state). When $L = 0$, eq 4 becomes $f_B = [H^+]/(K_B + [H^+])$, the equation for a simple single-site titration, which is exactly what is observed for the R82A and R82Q mutants, with $pK_B = 7$ (Balashov et al., 1993; Brown et al., 1993).

We previously reported that the modification of PM by EDC is inhibited by Ca^{2+} (Renthal et al., 1979). Jonas and Ebrey (1991) found that the purple-to-blue transition of deionized purple membrane can be reversed by a high-affinity Ca^{2+} binding site, which they postulated to be located near the Schiff base and Asp 85. ETC-modified purple membrane lacks the high-affinity Ca^{2+} binding site (R. Jonas, T. Ebrey, K. McMillan, and R. Renthal, unpublished experiment). This result suggests that the high-affinity Ca^{2+} site linked to the purple-to-blue transition may be the same site to which both the guanidinium group of Arg 82 and the quaternary ammonium group of ETU-Glu 74 bind.

We find that regeneration of the 570-nm chromophore is inhibited by the presence of the ETU group attached to Glu 74 (Figure 5 and Table 3). The inhibition of the binding of retinol (Table 3) suggests that it is the retinal binding step of chromophore regeneration, rather than Schiff base formation, that is affected. Like the effect of ETC on the purple-to-blue transition, this is a surface modification altering a process occurring in the membrane interior. Could the inhibition of chromophore formation also involve the conformation of Arg 82? This amino acid is positioned on the extracellular half of the membrane, in an aqueous channel leading from the extracellular surface to the Schiff base site in the membrane interior. One possible regeneration mechanism involving Arg 82 would require that retinal enters its binding site in the membrane interior through the extracellular side of the membrane. This mechanism would be interesting due to the analogy of the bR structure to rhodopsin (Schertler et al., 1993) and the adrenergic receptor family (Baldwin, 1993), where the agonist could enter the interior binding site through a position analogous to the bR extracellular water channel. Such a mechanism for retinal binding to bR is not completely absurd: though it lacks an outer membrane, *H. salinarium* does have a periplasmic space (Blaurock et al., 1976); and the aqueous solubility of retinal is about 0.1 μM (Szuts & Harosi, 1991). Nevertheless, it seems far more likely that retinal enters its binding site in the interior through the lipid domain of the membrane. A more plausible mechanism would involve a conformational change in which two transmembrane helices could separate slightly to allow the retinal into its site. Such a conformational change would very likely require a rearrangement of one or more surface loops connecting the helices. There is some evidence that such a rearrangement occurs in the binding of retinal. The BC loop (residues 65−75) contains a chymotrypsin cleavage site in bO that is absent in bR (Gerber et al., 1979). Deletion of the BC loop by mutagenesis results in slow regeneration (half-times 1.4−1.8 times longer than the wild type: Gilles-Gonzalez et al., 1991). This loop contains Glu 74, the major ETC-reactive site. If modification of Glu 74 with an ETU group locks the BC loop in a bO-like conformation, the result would be inhibition of the entry of retinal into its binding site.

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