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Articles

Constraints on the Flexibility of Bacteriorhodopsin's Carboxyl-Terminal Tail at the Purple Membrane Surface†

Robert Renthal,* Nancy Dawson, John Tuley, and Paul Horowitz

ABSTRACT: A fluorescent probe, dansylhydrazine, was coupled to the purple membrane with a water-soluble carbodiimide. Fluorescence spectroscopy before and after proteolysis with papain indicated that the label was attached to the carboxyl-terminal tail of bacteriorhodopsin (residues 232–248). Reaction with [^{3}H]dansylhydrazine showed incorporation of 0.7 mol/mol of bacteriorhodopsin in this region. A minor site (0.3 mol/mol) was also labeled in the reaction, but its fluorescence was almost completely quenched by energy transfer to retinal. The conformation and flexibility of the C-terminal tail was studied by proteolysis and fluorescence polarization. Papain removes the C-terminal tail from the purple membrane in two steps, suggesting a segmented structure. At pH 8, 25 °C, the first-order rate constants were $k_1 = 0.23$ min^{-1} and $k_2 = 0.011$ min^{-1} for unmodified membrane. The cleavage mechanism was found to be sequential removal of the two tail segments. The labeled membrane had a similar mechanism but the first step was much slower: $k_1 = 0.026$ min^{-1} and $k_2 = 0.009$ min^{-1}. The steady-state polarization of the dansyl fluorescence on the tail was 0.24 at 25 °C, indicating a rigid environment. During proteolysis, the polarization decreased to 0.10 after 4 h. The time course of polarization decrease during proteolysis closely matched the rate of release of the inner tail segment. A model was derived for the polarization change during papain cleavage, on the basis of the assumption of only two tail states: covalently bound to bacteriorhodopsin and free in solution. This model gave a good fit to the proteolysis kinetics. Thus, the release of the outer segment does not affect the polarization, and hence the flexibility, of the inner segment. Time-resolved fluorescence anisotropy indicated no tail motion over two excited-state lifetimes. We conclude that the labeled region of the C-terminal tail of bacteriorhodopsin is rigidly held at the membrane surface.

Progress in diffraction and spectroscopic methods will provide increasingly detailed information about transmembrane proteins. However, the membrane surfaces are often the sites where membrane-mediated processes begin and end. Therefore, structural information must also be obtained about the surface regions of membrane proteins. So far, two approaches have provided information about membrane protein surfaces. Large extramembrane functional domains, of proteins such as cytochrome b_5, have been studied after removal from the membrane by proteolysis (Mathews et al., 1972; Spatz & Strittmatter, 1971). Exposed surface regions of proteins such as bacteriorhodopsin have been identified by proteolysis (Ovchinnikov et al., 1979) and chemical modification (Henderson et al., 1978; Dumont et al., 1981). Yet, little is known about the behavior of small surface segments of membrane-bound proteins. Undoubtedly, some membrane proteins will be found to utilize short surface segments in mediating effects between the surface and interior or between two surface sites on the same side of the membrane. We would like to know whether a membrane surface can provide special constraints on the folding of surface regions. In particular, are the amino and carboxyl termini (which are usually found to extend beyond the membrane surface) free to assume many conformations, or is their motion restricted in some way? The purple membrane is a useful model system for studying the dynamics of surface segments of membrane proteins. It contains only a single protein, bacteriorhodopsin, which functions as a light-induced proton pump [for a recent review, see Stoeckenius (1980)]. The amino acid sequence (Ovchinnikov et al., 1979; Khorana et al., 1979) and low-resolution crystal structure (Henderson & Unwin, 1975) have been determined. Although about 80% of bacteriorhodopsin is buried within the lipid bilayer, approximately 20 amino acids at the carboxyl

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terminus are thought to extend out from the cytoplasmic surface. The 17 C-terminal residues are removed by brief treatment of the membrane with papain or other proteolytic enzymes (Gerber et al., 1977; Ovchinnikov et al., 1979). They are not essential for proton-pump activity (Abdulaev et al., 1977), although the pump appears to work at a different rate without them (Govindjee et al., 1982). The diffraction techniques used to solve the membrane structure were not sufficiently sensitive to resolve all surface features (Henderson & Unwin, 1975; Agard & Stroud, 1982). Thus, the three-dimensional structure of the C-terminal tail is not known. Nor is any information available about its flexibility.

Previous work in this laboratory demonstrated that the bacteriorhodopsin C-terminal tail is accessible for cross-linking with a water-soluble carbodiimide (Renthal et al., 1979). We have now used the carbodiimide reaction to couple a fluorescent dye to the C-terminal region in order to probe the conformation and motion of this surface segment by proteolysis and fluorescence spectroscopy.

Materials and Methods

Purple membrane sheets were isolated by the method of Oesterhelt & Stoeckenius (1974) from *Halobacterium halobium* S_9. Purple membrane stock solutions were stored at a concentration of 0.1 mM bacteriorhodopsin at 4 °C in a stoppered flask sealed with parafilm. Under these conditions, we saw no loss of C-terminal tail by endogenous or bacterial protease for 1 month. Some samples stored for up to 4 months showed no changes, in contrast to findings in other laboratories (Govindjee et al., 1982). Bacteriorhodopsin concentration was determined by measuring the absorbance at 570 nm (Oesterhelt & Hess, 1973). Papain was obtained from Sigma (type IV; 2× crystallized; lot 19C-8095 was used for all experiments). Papain activity was assayed by measuring the rate of acid production during hydrolysis of benzoyl-L-arginine ethyl ester (BAEE)[1] (Smith & Parker, 1958). Dansylhydrazine (DH) [Sigma or Molecular Probes (Plano, TX)] and ethyl-[(dimethylamino)propyl]carbodiimide (EDC) (Sigma) were used as obtained from the supplier without further purification.

Coupling of Dansylhydrazine to Purple Membrane. For a typical reaction, two solutions were prepared. (A) A total of 12 mL of purple membrane (10^{-4} M bacteriorhodopsin suspended in deionized water) was added to 60 mL of 0.05 M NaCl. The pH of the membrane suspension was adjusted to 5.5 with 0.1 N NaOH or 0.1 N HCl as needed. Then 4.8 mL of DH (0.01 M in ethanol) was added, and the mixture was cooled on ice. (B) A 6.6 mM EDC–HCl solution was adjusted to pH 5.5 and then placed on ice. After it was cooled, 4.8 mL of solution B was added to solution A to start the reaction. After 1 h on ice, approximately 100 mL of ice-cold 0.05 M NaCl was added, and the solution was centrifuged at 40000*g* for 20 min at 5 °C. The supernatant was discarded, and the pellet was resuspended in about 200 mL of ice-cold 0.05 M NaCl and then centrifuged again. Two more resuspension and centrifugation steps were done with 0.015 M NaCl. The final pellet was resuspended in 12 mL of deionized water. For spectroscopic studies, an aliquot of 1–2 mL was centrifuged and resuspended in 1–2 mL of 0.05 M Tris, pH 8.0. After incubation for 1 h at 37 °C, the sample was centrifuged and the supernatant inspected with an ultraviolet light. If traces of fluorescence were found, the sample was resuspended in Tris. The process was continued until no fluorescence was detected in the supernatant (usually three washes).

Reaction of Purple Membrane with [^3H]Dansylhydrazine. [^{3}H]Dansylhydrazine was synthesized from [*methyl*-^{3}H]dansyl chloride (New England Nuclear) and hydrazine as follows: A dry sample of [^{3}H]dansyl chloride (36.7 Ci/mmol) was diluted with pentane to a concentration of 1.9 μg/mL. A 20-μL sample was pipetted into a 0.5-mL reaction vial, evaporated with N_2, and then dissolved in 20 μL of acetone. To this was added 20 μL of hydrazine monohydrate (under a fume hood), and the reaction was allowed to proceed for 45 min. Larger scale reactions showed the formation of dansylhydrazine was complete by this time. The reaction vial was placed in a side-arm flask attached to a freeze-drier and evaporated to dryness. The residue was taken up in 20 μL of acetone and dried again. Thin-layer chromatography showed that all traces of hydrazine are removed by this procedure, that the product migrates with authentic dansylhydrazine, and that no dansyl chloride remains.

The residue of [^{3}H]dansylhydrazine was solubilized in 20 μL of acetone and then diluted with 0.45 mL of nonradioactive dansylhydrazine (10^{-2} M in ethanol). Reaction with the purple membrane was done as described above, except that the diluted radioactive dansylhydrazine was used. Radioactivity in the product was counted after the following precipitation procedure: a 1.5-mL aliquot of the reaction mixture was added to 0.3 mL of 88% formic acid. Then, 5 mL of acetone and 0.9 mL of 30% ammonia were added. The sample was briefly sonicated in a bath sonicator. Then, 1.25 mL of ethanol was added, and the sample was centrifuged. After the pellet was washed a second time with acetone, the pellet was dried in air and dissolved in 2.5 mL of 0.2% sodium dodecyl sulfate/10 mM phosphate, pH 7.0. Aliquots of this (1 mL) were counted with 10 mL of Aquasol-2 (New England Nuclear) in a scintillation counter. Aliquots of the dansylhydrazine stock solution and the reaction mixture were also counted to determine the specific radioactivity of the dansylhydrazine.

Reaction of Purple Membrane with Papain. A typical reaction mixture contained 2 mL of purple membrane (10^{-4} M in bacteriorhodopsin) or EDC/DH purple membrane and 6 mL of 0.05 M Tris, pH 8.0. Papain was activated prior to the reaction by incubation of 0.8 mL of activation solution (0.02 M EDTA, 0.05 M cysteine, pH 8.0) with 0.48 mL of papain (0.05 mg/mL; sp act. measured as 10 BAEE units/mg) for 10 min at 25 °C. The purple membrane/Tris solution was then added to the activated papain to initiate the reaction. At each time point, 0.4 mL of the reaction mixture was withdrawn and immediately added to 0.1 mL of 88% formic acid with vortexing. The samples were stored at −20 °C until all time points were obtained. The samples were immediately thawed and centrifuged 20 min at 40000*g*. The pellets were resuspended in 5 mL of water, centrifuged again, resuspended again in water, and centrifuged. The final pellets were resuspended in 0.5 mL of water and lyophilized. A zero-time sample (to check for papain cleavage during the centrifugation and subsequent electrophoresis steps) was prepared as follows: 0.06 mL of activated papain was added to 0.1 mL of 88% formic acid; then 0.34 mL of the purple membrane/Tris solution was added.

Polyacrylamide Gel Electrophoresis. So that we could measure the cleavage of bacteriorhodopsin, the lyophilized samples that had been reacted with papain for various times

[1] Abbreviations: BAEE, benzoyl-L-arginine ethyl ester; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DH, [[5-(dimethylamino)-1-naphthalenyl]sulfonyl]hydrazine; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; EDC/DH purple membrane, fluorescent-labeled purple membrane, prepared by EDC coupling of DH; BT_1T_2, bacteriorhodopsin with C-terminal tail intact; T_2, outer tail segment; T_1, inner tail segment.

(see above) were subjected to electrophoresis in polyacryamide gels. The method of Weber & Osborn (1969) (10% acrylamide) was used for measuring the rate of cleavage, and the method of Swank & Munkres (1971) was used to estimate the size of the fragmented bacteriorhodopsin.

The gels were stained with Coomassie blue and scanned at 600 nm on a McPherson spectrophotometer with a gel scanner accessory with a 0.05-mm slit, which was masked in order to scan only the center of the gels. Although the bands were resolved on gels, the scanner reduced the resolution. Thus, it was necessary to graphically resolve the bands. This was done by linear extrapolation of tangents to the sides of the peaks. Integration was done gravimetrically. Unstained gels (without tracking dye) were scanned for fluorescence immediately after electrophoresis, on a Helena R & D gel scanner (excitation at 366 nm).

Fluorescence Spectroscopy. Polarization of dansylhydrazine fluorescence was measured on an SLM ratio recording spectrofluorometer. The excitation wavelength was selected at 321 nm with a double monochromator. The emission wavelength was selected with Schott cut-off filters of 370 and 418 nm. In experiments with nonpolarized light, the fluorescence intensity was measured on a Farrand Mark I ratio recording spectrofluorometer (excitation 320 nm, emission 500 nm, selected with single monochromators).

Fluorescence anisotropy decay was measured at the Center for Fast Kinetics Research, University of Texas at Austin. Excitation pulses (40 ps) were from a frequency-tripled Nd:YAG laser (Quantel) (5 mJ at 353 nm). Filtered emission was monitored above 450 nm with a fast-response photomultiplier tube (600-ps rise time). Detector output was recorded on a Tektronix 7912 digitizer and stored in a PDP-11/34 computer. Parallel and perpendicular components of the emission were measured from separate pulses with a polarizing filter. The emission decay curves were normalized to the same relative excitation intensity. Ten separate pulses were averaged for each orientation of the polarizer. The anisotropy $A(t)$ was calculated at a particular time t after the light pulse as

$$A(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)}$$

where $I_{\parallel}(t)$ and $I_{\perp}(t)$ represent the normalized intensity of the parallel and perpendicular components of the emission, respectively, averaged from ten pulses. The intensity measurements appear to contain a systematic error of ±15%, possibly due to the pulse-amplitude determination.

Results

Fluorescent Labeling of Purple Membrane with Dansylhydrazine. Purple membrane was reacted with EDC in the presence of dansylhydrazine (DH). After the membrane was washed free of noncovalently bound label, dansyl fluorescence was found remaining attached to the membrane. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed that all the remaining fluorescence migrated with bacteriorhodopsin (Figure 1C,D). The fluorescence emission spectrum of EDC/DH purple membrane is shown in Figure 2A, upper curve. The EDC reaction also causes a small amount of cross-linking (Figure 1A), approximately 20% of the bacteriorhodopsin (10% dimer and 10% trimer). The fluorescence scan of the gels shows that the amount of DH is only slightly diminished in the dimers and trimers with respect to that in the monomer (7% of the fluorescence in dimers and 7% trimers) (Figure 1C).

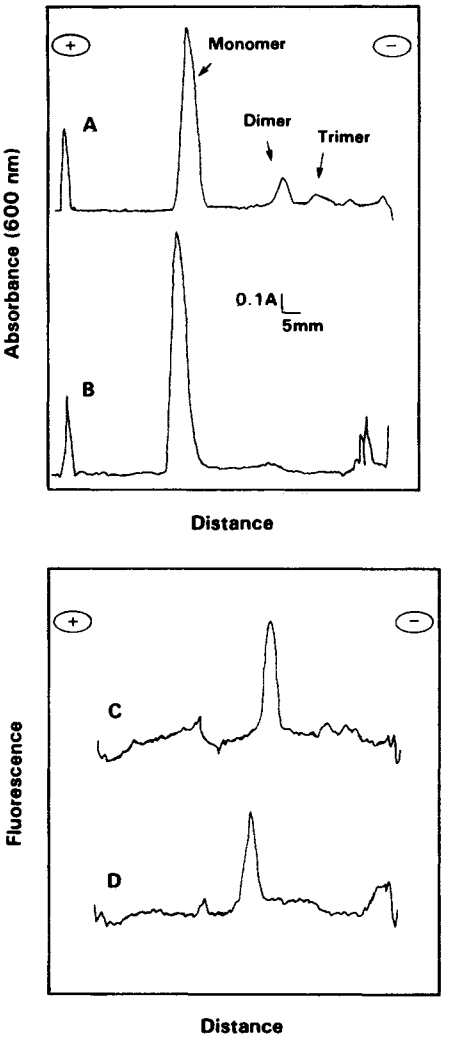
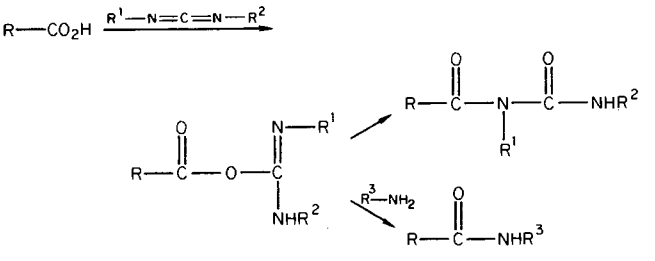


FIGURE 1: Polyacrylamide gel electrophoresis of fluorescent-labeled bacteriorhodopsin. Purple membrane was labeled with dansylhydrazine via a carbodiimide reaction (see text). Membrane samples were dissolved in sodium dodecyl sulfate, and electrophoresis was run under the conditions of Weber & Osborn (1969). (A and B) Gels stained with Coomassie blue and scanned at 600 nm; (C and D) unstained gels scanned for dansyl fluorescence; (A and C) labeled, intact bacteriorhodopsin; (B and D) after removal of C-terminal tail (residues 232–248) by treatment with papain (same conditions as described under Materials and Methods except reaction was at 37 °C for 2 h).

The labeled groups are likely to be carboxyl groups, since carbodiimides are well-known as activating agents for protein carboxyl groups (Carraway & Koshland, 1972). The probable reactions of bacteriorhodopsin carboxyl groups at pH 5.5 are



where R^1 and R^2 = ethyl or (dimethylamino)propyl and R^3NH_2 = protein amino groups or dansylhydrazine; the former would result in cross-linking, while the latter would give a fluorescent-labeled product.

Removal of Some Fluorescent Labeling Sites by Treatment with Papain. Exposure of EDC/DH purple membrane to papain removes nearly all the fluorescence from the membrane

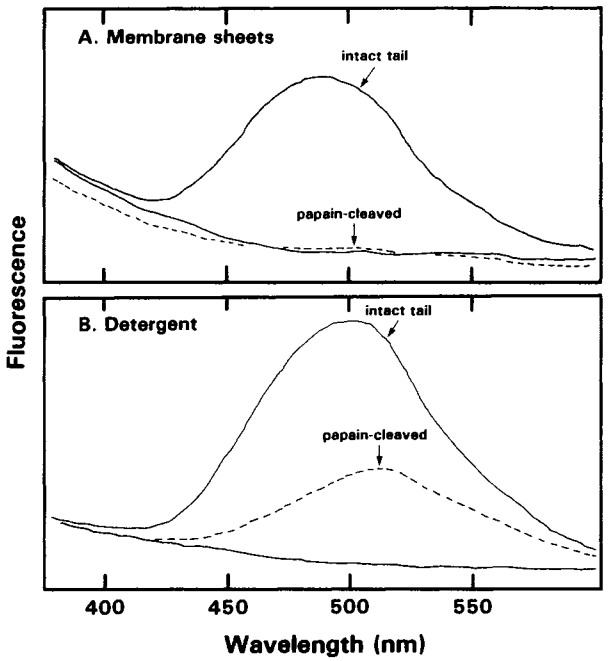


FIGURE 2: Fluorescence emission spectra of dansylhydrazine-labeled purple membrane: excitation wavelength 330 nm; bacteriorhodopsin concentration 0.8 μM. (A) Membrane sheets suspended in water: (upper curve) before treatment with papain; (lower curve, dashed line) after removal of C-terminal tail with papain (37 °C, 2 h); (lower curve, solid line) unmodified purple membrane. (B) Same samples as (A) except dissolved in 0.2% sodium dodecyl sulfate, which bleaches the retinal chromophore.

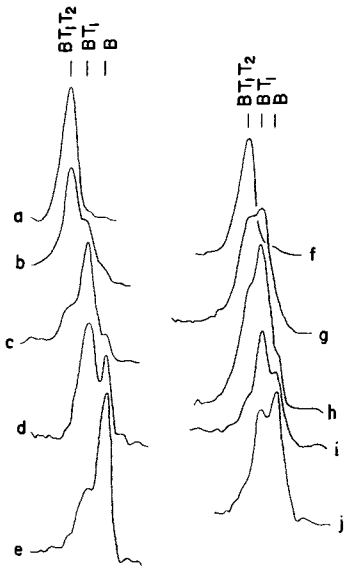


FIGURE 3: Densitometer scans of stained polyacrylamide gels of proteolytic cleavage of bacteriorhodopsin by papain. Decreasing molecular weight is to the right. (Scans a–e) Unmodified bacteriorhodopsin; (scans f–j) bacteriorhodopsin labeled with dansylhydrazine. After various times of cleavage, aliquots were acidified with formic acid, frozen, lyophilized, and then applied to gels. Times, in minutes after addition of papain: (control) (a) 0, (b) 2, (c) 6, (d) 20, (e) 120; (EDC/DH purple membrane) (f) 0, (g) 30, (h) 45, (i) 75, (j) 120. Lines above gel traces mark three forms of bacteriorhodopsin observed: (BT_1T_2) bacteriorhodopsin with intact C-terminal tail; (BT_1) bacteriorhodopsin after removal of outer tail segment; (B) bacteriorhodopsin after removal of entire tail. Proteolysis was at 25 °C.

(Figure 2A, lower curve). The cross-links are also quantitatively removed (Figure 1B). Papain was shown to cleave the C-terminal 17 residues from bacteriorhodopsin (Abdulaev et al., 1977). This region of bacteriorhodopsin contains five carboxyl groups, and thus, it is a likely labeling site for DH. However, additional DH sites could be present that are not susceptible to papain cleavage but that have low fluorescence quantum yields. Low quantum yields could arise, for example, from quenching by singlet energy transfer from dansyl to retinal. That this must be the case is shown in Figure 2B. The same samples as in Figure 2A were dissolved in 1% sodium dodecyl sulfate, which bleaches the retinal chromophore. By contrast with Figure 2A, the papain-cleaved protein is now seen to have about one-third the fluorescence of the uncleaved membrane. Thus, bleaching of the chromophore reveals additional DH sites that are quenched in the native membrane.

Quantitiation of labeling by reaction with [^{3}H]dansylhydrazine showed that 1.08 mol of DH were incorporated per mol of bacteriorhodopsin. When EDC was excluded from the reaction mixture, only 0.04 mol of DH were incorporated per mol of bacteriorhodopsin. After papain cleavage, 0.35 mol of DH/mol of bacteriorhodopsin remained, while a membrane sample that was reacted with DH in the absence of EDC and then cleaved with papain showed only 0.04 mol of DH/mol of bacteriorhodopsin. Thus, the C-terminal tail contains 0.7 mol of DH/mol of bacteriorhodopsin, while the papain-resistent site(s) contains (contain) 0.3 mol/mol of bacteriorhodopsin. The latter sites appear to be between residues 72 and 118 in the bacteriorhodopsin amino acid sequence (Renthal, 1981).

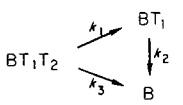
Kinetics of Papain Cleavage of Native Purple Membrane. Purple membrane was treated with papain in Tris buffer, pH 8.0. Aliquots were removed at various times during the reaction and analyzed by polyacrylamide gel electrophoresis to observed the progress of degradation of bacteriorhodopsin. It

Table I: Kinetic Constants for Papain Cleavage of Bacteriorhodopsin Carboxyl-Terminal Peptide

sample	k_1 (min^{-1})	k_2 (min^{-1})
purple membrane	0.23	0.011
DH-labeled purple membrane	0.026	0.009

is immediately clear from the gels (Figure 3a–e) that bacteriorhodopsin is cleaved by papain in two steps to slightly lower molecular weight products. The reaction is more than 70% complete in 2 h at 25 °C, but the native bacteriorhodopsin disappears very quickly. Nearly all of it is degraded in 6 min (Figure 3c). In addition to the C-terminal cleavage (Abdulaev et al., 1977), papain also acts on other sites at longer times (Ovchinnikov et al., 1979). We also ran the papain-cleaved bacteriorhodopsin on urea gels (data not shown) and found no detectable amounts of cleavage products from the long reaction time sites (i.e., at positions 65–66, 72–73, and 161–162). Thus, the two-step cleavage suggests that there are two papain-sensitive sites in the C-terminal region. The mechanism of removal of the C-terminal tail could be either sequential (eq 1) or simultaneous (eq 2). In eq 1 and 2, BT_1T_2

$$BT_1T_2 \xrightarrow{k_1} BT_1 \xrightarrow{k_2} B \quad (1)$$



(2)

represents intact bacteriorhodopsin. BT_1 is bacteriorhodopsin with part of the tail peptide removed by papain cleavage. B is bacteriorhodopsin with the entire tail removed. The solution of the kinetic equations (Appendix, part I) indicates that the cleavage mechanism is best fit by eq 1 (calculated lines, Figure

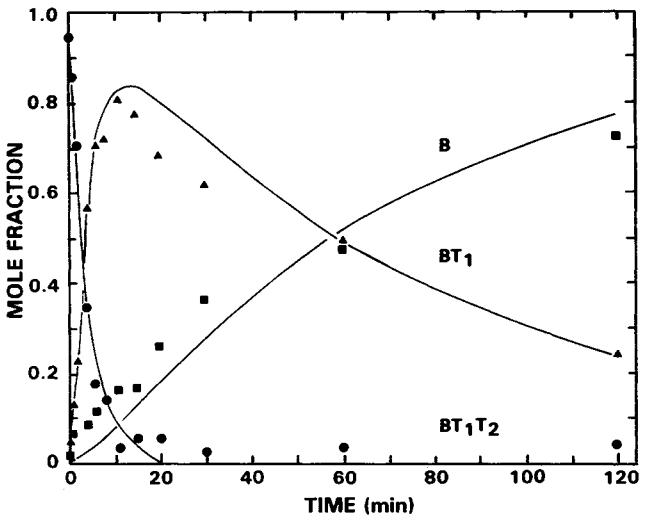


FIGURE 4: Rate of papain cleavage of C-terminal tail of unlabeled purple membrane. Curves show disappearance of bacteriorhodopsin, BT_1T_2 (circles), formation and decay of an intermediate with part of the tail removed, BT_1 (triangles), and formation of bacteriorhodopsin lacking the entire C-terminal tail, B (squares). Lines were calculated from the sequential rate equations given under part I of Appendix and the rate constants given in Table I, with the data from integration of gel scans in Figure 3a–e and additional gels at other times.

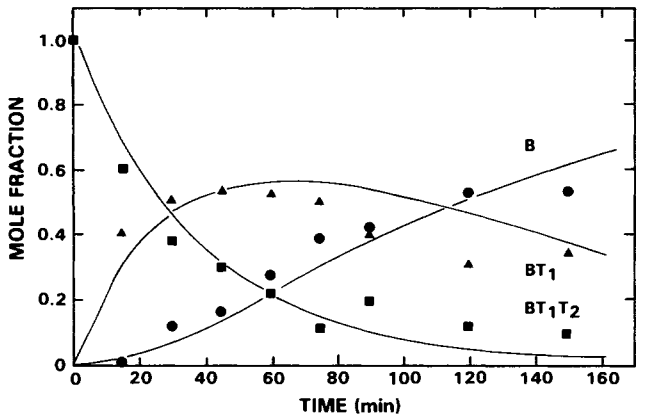


FIGURE 5: Rate of papain cleavage of C-terminal tail of dansylhydrazine-labeled purple membrane. Symbols are the same as in Figure 4. Lines calculated as described in Figure 4 from the integration of gel scans in Figure 3f–j and additional gels at other times.

4). That is, the papain-sensitive sites are attacked sequentially. The rate constants are given in Table I.

Kinetics of Papain Cleavage in EDC/DH Purple Membrane. Purple membrane modified by EDC/DH was treated with papain under identical conditions with the experiment with native membrane. As measured from polyacrylamide gels, the result (Figure 3f–j and Figure 5) appeared to be the same sequential two step mechanism as that for native membrane. However, the first kinetic constant (k_1) is 10 times smaller than that for native membrane (Table I).

The carbodiimide coupling reaction also causes a small amount of cross-linking between bacteriorhodopsin molecules (Figure 1A). A bacteriorhodopsin dimer band is prominent in the first few gels (not shown). The rate of disappearance of dimers closely matches the rate of disappearance of BT_1T_2 (intact bacteriorhodopsin).

Fluorescence Polarization. The DH label on the C-terminal tail was used as a probe to study the rotational motion of this portion of the molecule. In 0.05 M Tris, pH 8.0, the polarization was 0.24. This value is near the limiting polarization reported for some dansyl proteins (Metzger et al., 1966).

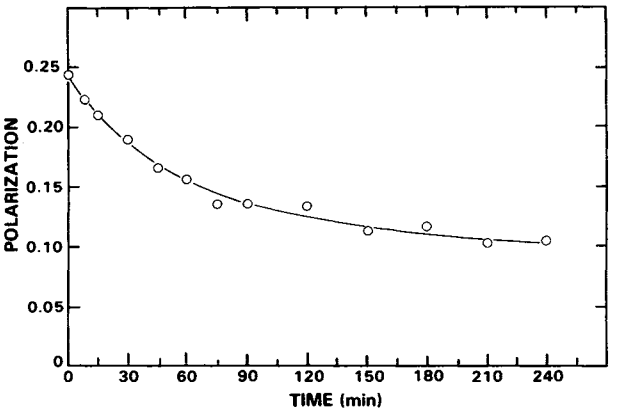


FIGURE 6: Decrease in polarization of dansyl fluorescence on C-terminal tail after addition of papain. See text for conditions. Immediately after polarization was measured, an aliquot was removed from the proteolysis reaction mixture, acidified, and frozen. These samples were then subjected to electrophoresis to obtain the data in Figure 3f–j and Figure 5.

However, when papain is added, the polarization begins decreasing, reaching a value of 0.10 after 200 min (Figure 6). This decrease appears to represent the release of the dansyl C-terminal peptide from the membrane. The supernatant after centrifugation of the papain cleavage reaction mixture was found to have a polarization of 0.026. Thus, not all of the dansyl label has been removed after 200 min, in agreement with the finding (Figure 5) that not all of the C-terminal peptide has been removed.

In order to compare the kinetics of cleavage with the kinetics of polarization decrease, we derived an expression for the fraction F_b of the dansyl group left attached to the membrane at a time after addition of papain, in terms of the measured polarization P at that time:

$$F_b = \frac{n(P_f/P - 1)}{1 - P_b/P - n(1 - P_f/P)} \tag{3}$$

where $n = (I_v + I_h)_f/(I_v + I_h)_b$, the ratio of the sums of vertical (I_v) and horizontal (I_h) intensities for the dansyl group released by papain (f) and the dansyl group bound to the membrane (b); P_b is the initial polarization (before addition of papain), and P_f is the polarization of the supernatant containing fragments of the dansyl-labeled C-terminal tail after centrifugation to remove dansyl membrane (see Appendix, part II). In Figure 7 (triangles), F_b is plotted, calculated from the measured values of P from Figure 6, with $P_b = 0.24$ and $P_f = 0.026$. The value of n was measured as 1.54.

How does this result compare with the kinetics of papain cleavage (Figure 5)? If one assumes the dansyl label is uniformly distributed over the tail, then

$$F_b = \frac{2(BT_1T_2) + (BT_1)}{2[(BT_1T_2) + (BT_1) + (B)]} \tag{4}$$

However, if the label is confined entirely to T_1, then

$$F_b = \frac{(BT_1T_2) + (BT_1)}{(BT_1T_2) + (BT) + (B)} \tag{5}$$

Values of F_b are plotted in Figure 7 from the data in Figure 5, with either eq 4 (filled circles) or eq 5 (open circles). It is clear that only eq 5 is consistent with the polarization data. Thus, the dansyl sites must be clustered entirely on T_1.

Since the emission from DH is somewhat quenched by energy transfer when bound to the membrane, we also were able to measure the increase in fluorescence as the tail is

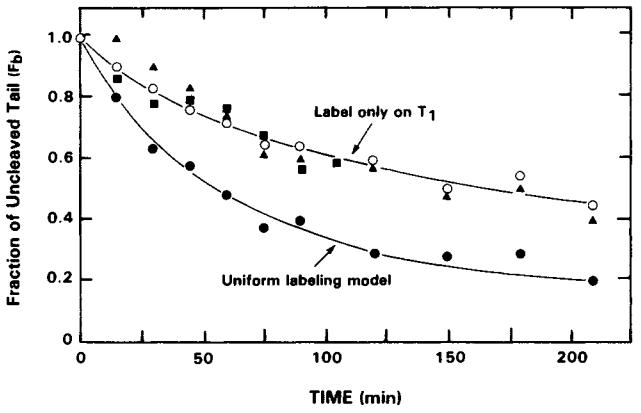


FIGURE 7: Calculation of the amount of label remaining on the membrane during papain cleavage (F_b): (open circles) calculated from cleavage kinetics (Figure 5) assuming all the label is on the inner tail segment (T_1) (eq 5); (filled circles) calculated from cleavage kinetics (Figure 5) assuming the label is uniformly distributed over the entire tail (T_1 and T_2) (eq 4); (triangles) calculated from polarization data (Figure 6) assuming only two tail states (eq 3), membrane-bound and free in solution; (squares) calculated from change in fluorescence quantum yield during proteolysis (eq 6).

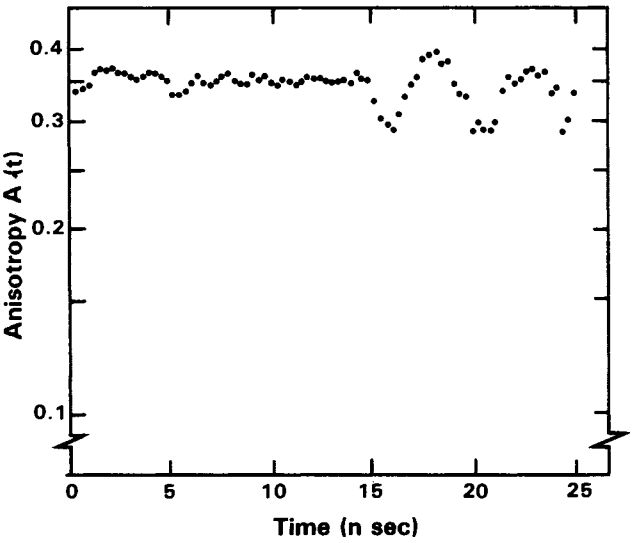


FIGURE 8: Time-resolved fluorescence anisotropy of dansylhydrazine-labeled purple membrane. Excitation was with a frequency-tripled Nd-YAG laser (353 nm). Parallel and perpendicular components of emission were normalized to constant excitation intensity and averaged (10 pulses each).

released. The emission E at a particular time is related to the fraction of bound DH at that time by

$$F_b = (E - E_f)/(E_b - E_f) \quad (6)$$

where E_f and E_b are the fluorescence emission from released tail and from bound tail, respectively. The fluorescence emission intensity result [Figure 7 (squares)] agrees well with the kinetics measured from polarization [Figure 7 (triangles)].

Decay of Fluorescence Anisotropy. The kinetics of anisotropy decay (Figure 8) show essentially no reorientation of the dansyl probe for 25 ns after excitation.[2] Using the same fluorescence kinetics measuring system with the emission polarizer set at 54°, we found the emission lifetime of the labeled membrane to be 13 ns. Thus, there appears to be no motion of the labeled tail over two lifetimes.

[2] Similar results were obtained on a single photon counting nanosecond fluorometer (J. Yguerabide, unpublished experiments).

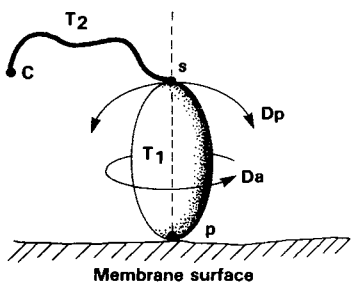


FIGURE 9: Rotatory motions of the C-terminal tail: (C) carboxyl terminus (residue 248); (s) first papain cleavage site (possibly between residues 239 and 240); (p) second papain cleavage site (between residues 231 and 232); (T_2) outer tail segment, which is apparently unlabeled and flexible; (T_1) inner tail segment, which contains the dansylhydrazine labeling site(s) and appears to be rigid; (dashed line) major ellipsoid axis of T_1; (D_p and D_a) rotational diffusion coefficients for the motions indicated by arrows.

Discussion

What rotational correlation times are expected for motions of the C-terminal tail of bacteriorhodopsin, protruding from the purple membrane surface? The tail peptide may be considered a prolate ellipsoid with one end fixed. Two types of rotatory motion of the tail could occur (Figure 9). Rotation toward the membrane surface and away from it, about the fixed point p, will contribute to a diffusion coefficient D_p. Rotation about the ellipsoid major axis will contribute to a diffusion coefficient D_a. We have used the theoretical treatment of Wegener et al. (1980) to estimate expected diffusion coefficients due to these two motions. We assume that the purple membrane may be regarded as essentially immobile and that eq 32 and 33B of Wegener et al. (1980) describe the diffusion coefficients D_a and D_p, respectively. Separate rotational correlation time components due to these motions are assumed to equal $1/(6D)$.

The shape of the C-terminal tail is not known. The crystal structure (Henderson & Unwin, 1975; Agard & Stroud, 1982) does not reveal a large C-terminal surface peptide, possibly because of conformational disorder or low scattering density. Our results suggest that the tail is segmented. The outer part is cleaved by papain first and the inner part in a subsequent slower step (Figures 3–5). The most probable site of cleavage of the outer segment is between Ser-239 and Ala-240. Conformational analysis (Chou & Fasman, 1978) of the tail sequence indicates a high probability for a β bend involving residues 238–241. The active site of papain requires a bent conformation for the peptide backbone of its substrate (Berger & Schechter, 1970). Moreover, this site was identified by Ovchinnikov et al. (1979) as sensitive to proteolysis by trypsin. Ovchinnikov et al. (1979) identified the inner papain site between residues 231 and 232.

The C-terminal tail contains five possible sites for dansylhydrazine labeling: Glu-232, Glu-234, Glu-237, Asp-242, and the C-terminal Ser-248. The kinetics of papain cleavage (Figures 5 and 7) suggest that all the label is on T_1; that is, Glu-232, Glu-234, and/or Glu-237. The selectivity of the dansylhydrazine labeling reaction is somewhat surprising, in view of the large number of carboxyl groups (twelve) placed at the surface of the membrane by the folding model of Engelman et al. (1980). The selectivity of carbodiimide reactions is thought to reflect the pK of the reacting carboxyl group (Carraway & Koshland, 1972). Thus, Glu-232, -234, and/or -237, as well as the internal labeling site(s) between residues 72 and 118, are likely to have somewhat higher pKs than the average bacteriorhodopsin carboxyl group. The reactive carboxyl groups must also be more accessible to the dis-

placement of the intermediate isourea by dansylhydrazine.

The papain cleavage kinetics (eq 3 and 5; Figure 7) also suggests that the fluorescence polarization of the inner segment of the tail is unaffected by removal of the outer segment. In other words, P_b in eq 3 behaves as a time-independent constant. If the outer segment is much more flexible than the inner segment, it should not greatly affect the rotational motion (and polarization) of the inner segment. For the calculations of expected minimal rotational correlation times, we have assumed that the outer segment is completely flexible and that the motions of the inner segment are not affected by the outer segment.

Two different calculations were done. The inner segment was first considered to consist of 15 α-helical residues (for example, Arg-225–Ser-239), resulting in an "ellipsoid" with major axis a = 22.5 Å and minor axis b = 10 Å. This gives rotational correlation times of about 1 ns for rotation toward and away from the membrane and 0.2 ns for rotation about the major axis. The inner segment was then considered to have an extended conformation (15 residues of β sheet), resulting in an ellipsoid with a = 50 Å and b = 10 Å. This results in rotational correlation times of about 9 ns for rotation toward and away from the membrane and 0.5 ns for rotation around the major axis. The measured anisotropy decay (Figure 8) shows no evidence of components with rotational correlation times on the order of 0.2–0.5 or 1–9 ns. Instead, the anisotropy is essentially unchanged at 0.35 for 25 ns. Thus, the motion of the inner segment is very restricted. It is conceivable that the carbodiimide modification has altered the structure of the membrane surface and that the native inner segment is in fact very flexible. The kinetic constant for removal of the outer segment is indeed 10 times slower after the fluorescent labeling reaction (Table I; Figures 3–5). However, the rate of removal of the inner segment is essentially unchanged. Thus, enzymatic accessibility to the outer segment does appear to be affected by chemical modification, but the inner segment is unaffected. Rates of proteolysis are often sensitive measures of protein conformation. The results indicate that the average conformation of the inner segment is not distorted by the modification reaction. Moreover, the outer segment does not appear to significantly restrict the motion of the inner segment, since the fluorescence polarization is unchanged after removal of the outer segment (see Figure 7 and discussion above).

What is the cause of the inflexibility of the labeled C-terminal tail of bacteriorhodopsin? One possibility is a long-range interaction between the tail and other surface residues, either on the same or on a different bacteriorhodopsin molecules. This is unlikely, since it is not a sufficiently strong force to prevent rapid dissociation of the tail after proteolysis. Alternatively, the negatively charged tail could be repelled from the membrane surface by electrostatic interactions between carboxyl and phosphate groups. We have explored this possibility by measuring the polarization in the presence of 0.2 M NaCl, which should decrease electrostatic repulsion. We found little effect of added salt (data not shown). However, high ionic strength causes aggregation of purple membrane sheets, and this could mask any salt-induced flexibility of the tail by placing new restrictions on its motion.

We conclude that a short terminal sequence of an integral membrane protein can be held rigidly at the membrane surface. Similar conclusions were recently made by Low et al. (1982), who found the motion of the carbohydrate moiety of glycophorin to be restricted at the erythrocyte membrane surface. Many membrane-bound proteins have short sequences that protrude beyond the membrane surface, some of which are removed by proteolysis during membrane assembly (Rothman & Lenard, 1977; Wickner, 1979). Our results show that conformational flexibility of short protruding peptides can be limited by a membrane surface. This effect could regulate the processing of membrane proteins.

Added in Proof

An electron microscopy study of the location of bacteriorhodopsin's C-terminal tail has recently appeared (Wallace & Henderson, 1982). The C-terminal tail was reported to be completely disordered. A comparison between this result, obtained with samples dried in glucose, and our fluorescence experiments is not possible with the present data.

Acknowledgments

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Appendix

(I) Rate Equations for Sequential and Simultaneous Cleavage of Two Papain Sites in the C-Terminal Tail. For the sequential reaction

$$BT_1T_2 \xrightarrow{k_1} BT_1 \xrightarrow{k_2} B$$

the rate equations are

$$-d(BT_1T_2)/dt = k_1(BT_1T_2)$$

$$d(BT_1)/dt = k_1(BT_1) - k_2(B)$$

and

$$d(B)/dt = k_2(BT_1)$$

If the concentrations of BT_1T_2, BT_1, and B are expressed in mole fractions, then

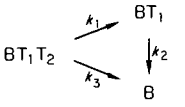
$$(BT_1T_2) = \exp(-k_1t)$$

$$(BT_1) = [\exp(-k_1t) - \exp(-k_2t)]k_1/(k_2 - k_1)$$

and

$$(B) = 1 - (BT_1T_2) - (BT_1)$$

For the simultaneous reaction



the rate equations are

$$-d(BT_1T_2)/dt = (k_1 + k_3)(BT_1T_2)$$

$$d(BT_1)/dt = k_1(BT_1T_2) - k_2(BT_1)$$

and

$$d(B)/dt = k_2(BT_1) + k_3(BT_1T_2)$$

Then

$$(BT_1T_2) = \exp[-(k_1 + k_3)t]$$

$$(BT_1) = (\exp[-(k_1 + k_3)t] - \exp(-k_2t))k_1/(k_2 - k_1 - k_3)$$

and

$$(B) = 1 - (BT_1T_2) - (BT_1)$$

The slope of a semilog plot of the rate of disappearance of BT_1T_2 (replotted from Figures 4 and 5) gives the constant k_1 (sequential mechanism) or $k_1 + k_3$ (simultaneous mechanism).

Similarly, the decay of BT_1 gives the constant k_2. The magnitude of k_3 can be estimated from the data for the rates of formation of BT_1 and B. In both sets of data (i.e., Figures 4 and 5) it was found that $k_3 \ll k_1$. In this case, nearly all of BT_1T_2 will have disappeared before the process k_3 can compete with k_2. Thus, the sequential model fits the results best.

(II) Calculation of Fraction of Fluorescent Label Bound to Membrane Surface during Papain Cleavage. The subscript b refers to fluorescent label still attached to the membrane, and f refers to label that has been released. Viewing the fluorescence intensity I with vertical (v) and horizontal (h) polarizers (excitation vertically polarized) will give a polarization before papain cleavage of

$$P_b = (I_v - I_h)_b/(I_v + I_h)_b \quad \text{(A1)}$$

After all the label has been released from the membrane, the polarization will be

$$P_f = (I_v - I_h)_f/(I_v + I_h)_f \quad \text{(A2)}$$

At some time during the papain cleavage reaction, when there is a mixture of free and bound label, the polarization will be

$$P = \frac{(I_v - I_h)_b F_b + (I_v - I_h)_f F_f}{(I_v + I_h)_b F_b + (I_v + I_h)_f F_f} \quad \text{(A3)}$$

where F_b and F_f are the respective mole fractions of bound and free label. The contribution from the internal labeling site(s) can be neglected, due to the low quantum yield (Figure 2A).

Substituting eq A1 and A2 into eq A3 yields

$$P = \frac{F_b P_b + n F_f P_f}{F_b + n F_f} \quad \text{(A4)}$$

where

$$n = (I_v + I_h)_f/(I_v + I_h)_b$$

Solving eq A4 for F_b gives

$$F_b = \frac{n(P_f/P - 1)}{1 - P_b/P - n(1 - P_f/P)}$$

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