Organization of Rhodopsin in Photoreceptor Membranes. 1. Proteolysis of Bovine Rhodopsin in Native Membranes and the Distribution of Sulfhydryl Groups in the Fragments†

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ABSTRACT: Papain and thermolysin are shown to cleave bovine rhodopsin in native membranes in two temporally distinct steps at room temperature. The final product of the proteolysis consists of two membrane-bound fragments of molecular weights 27 000 (Rh_{27}) and 12 500 (Rh_{12}). The molecular weights are not changed by reduction with dithiothreitol. The two fragments remain closely associated in both the membrane and nondenaturing detergents before and after bleaching and can be selectively cross-linked with carbodiimides. The sulfhydryl chemistry of the cleaved protein is nearly indistinguishable from native rhodopsin, and of the total of six sulfhydryl groups, two are located on Rh_{12} and four on Rh_{27}. In the membrane-bound protein, two sulfhydryl groups are accessible for modification, one on Rh_{12} and the other on Rh_{27}. The sulfhydryl on Rh_{12} is particularly reactive and may be selectively labeled with maleimides. Continuous irradiation with white light induces additional sulfhydryl reactivity on Rh_{27}.

The spatial arrangement of rhodopsin in rod outer segment (ROS)[1] disk membranes has been the subject of intensive investigation, and numerous models of the structure of rhodopsin have been formulated (Worthington, 1974; Wu and Stryer, 1972; Yeager, 1975). Even so, it is not yet known with certainty whether the rhodopsin polypeptide spans the thickness of the bilayer. This information is of considerable importance for understanding the role of rhodopsin in visual transduction and would place important constraints on models for its structure and function. Chen and Hubbell (1973), on the basis of freeze-fracture analysis, showed that rhodopsin is asymmetrically oriented in isolated disk membranes and provided suggestive evidence for a transmembrane organization of rhodopsin. It is an objective of this and the following paper in this issue to provide firm experimental evidence for this feature. Several other important conclusions regarding the polypeptide folding pattern and localization of reactive side chains will also be presented.

Our experimental approach is based on comparative proteolytic fragmentation and lactoperoxidase-catalyzed iodination of rhodopsin in native and reconstituted disk membranes. The details of the proteolytic fragmentation pattern of rhodopsin and the distribution of the rhodopsin sulfhydryl groups on the fragments are of central importance to our final conclusions and are the subject of this paper. In the following paper of this issue, these results, together with those from lactoperoxidase-catalyzed iodination, are applied to a comparative study of the native and reconstituted photoreceptor membranes.

A number of reports have already appeared dealing with proteolysis of rhodopsin in the native membranes (Bonting et al., 1974; Saari, 1974; Trayhurn et al., 1974a,b; Pober and Stryer, 1975; Van Breugal et al., 1975; Klip et al., 1976; Sale et al., 1977; Towner et al., 1977; Fong and Hargrave, 1978). Pober and Stryer (1975) reported that proteolysis with papain or thermolysin produces two membrane-bound polypeptides. More recently, Sale et al. (1977) observed papain to produce three polypeptide fragments, one of which is reduced further in molecular weight by reduction with mercaptoethanol. Fong and Hargrave (1978) report that thermolysin proteolysis proceeds in two temporally distinct steps to produce final fragments similar to those observed by Pober and Stryer (1975). In the present communication, we demonstrate that papain proteolysis also occurs in two temporally distinct steps. More importantly, we have determined the distribution of the rhodopsin sulfhydryl groups on the proteolytic fragments and the chemical reactivity of certain of these groups in the proteolytically modified protein. Finally, evidence will be presented to indicate that the proteolytic fragments remain closely associated and can be separated only in denaturing detergents.

Experimental Procedures

Materials. Papain and thermolysin were purchased from Sigma Chemical Co. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide, NEM, 4-PDS, and iodoacetamide were obtained from Aldrich Chemical Co., Inc. [^{3}H]NEM was a product of New England Nuclear Corp. 2,2,6,6-Tetramethylpiperidinyl-1-oxy-4-maleimide was purchased from Syva Research Chemicals. Frozen bovine retinas were obtained from George Hormel Co., Austin, Minn.

Preparation of ROS Membranes. Unless otherwise specified, all procedures involving rhodopsin preparations were done under dim red light at 4 °C. ROS membranes were isolated in a Ringer's solution (155 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Mops buffer, pH 7.2) according to the procedure described by Hong and Hubbell (1973). The final pellet of ROS membrane was washed twice

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[1] Abbreviations used are: ROS, rod outer segment; NEM, *N*-ethylmaleimide; 4-PDS, 4-dithiodipyridine; [^{3}H]NEM, *N*-[^{3}H]ethylmaleimide; Mops, 3-(*N*-morpholino)propanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PAS, periodic acid-Schiff; TrTAB, tridecyltrimethylammonium bromide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EPR, electron paramagnetic resonance.

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with 1 mM NaCl, 2 mM Mops (pH 7.2) by sedimentation at 45 000*g* and then resuspended in 10 mM NaCl, 10 mM Mops (pH 7.2). The rhodopsin and total opsin content of the membranes were estimated as described by Chen and Hubbell (1978). Typically, 20% of the total opsin content was bleached rhodopsin, the remaining 80% being native rhodopsin. In this paper, the total amount of rhodopsin plus bleached rhodopsin will be referred to as opsin. Samples of the ROS membrane suspension were prepared for freeze-fracture analysis according to Chen and Hubbell (1973).

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. Eight percent polyacrylamide gels in 0.5% $NaDodSO_4$ were prepared according to Fairbanks et al. (1971). Samples for electrophoresis were prepared by mixing membrane suspensions containing 2 mg/mL opsin with an equal volume of sample buffer (80 mM Tris, 40 mM sodium acetate, 8 mM EDTA, 2% $NaDodSO_4$, 20% glycerol, 80 mM DTT, and a trace amount of bromophenol blue, pH 7.4). After 20 min of incubation at room temperature, 20–40 μL (20–40 μg of opsin) of the sample mixture was applied to the gels. Care was taken not to heat the samples after solubilization in $NaDodSO_4$, as this procedure causes irreversible aggregation of opsin. Electrophoresis was carried out under constant voltage of 6 V/cm at room temperature until the bromophenol blue tracking dye had migrated to within 0.5 cm of the end of the gels. Gels were stained for protein with Coomassie blue, and carbohydrates were detected with PAS reagent as described by Fairbanks et al. (1971). Densitometric traces of the protein bands were obtained by scanning the gels at 550 nm for those proteins stained with Coomassie blue and at 560 nm for those stained with PAS reagent. Fluorescent profiles of gels were obtained using a RFT scanning densitometer (Model 2950, Transidyne General Corp.) equipped with a 300–400-nm band-pass primary filter (no. 5840) and a high-pass (400-nm cutoff) photomultiplier filter (no. 2E). The area under each protein band in densitometric traces was determined by cutting out the peak and weighing the paper. The apparent molecular weights of opsin and its proteolytic cleavage products were estimated by comparing parallel gels using bovine serum albumin dimer and monomer (mol wt 136 000 and 68 000), liver alcohol dehydrogenase (mol wt 42 000), and lysozyme (mol wt 14 400) as polypeptide standards.

Proteolysis of ROS Membranes. Unless otherwise specified, all ROS membrane suspensions used in proteolytic reactions contained 4 mg/mL total opsin protein, and the weight ratio of the proteolytic enzymes to total opsin was 1:50, except in the kinetic measurement (see below).

Proteolytic enzyme solutions (1 mg/mL) were freshly prepared immediately before each experiment. Concentrations of papain and thermolysin were estimated from their 280-nm extinction coefficients (Arnon, 1970; Matsubara, 1970). Papain was activated by incubating the enzyme in a buffer containing 10 mM Mops, 10 mM NaCl, 10 mM cysteine, and 1 mM EDTA (pH 7.2). Proteolysis of the ROS membrane suspension with papain was carried out at room temperature (22 °C) in the same buffer described in the enzyme-activation procedure. Proteolysis with thermolysin was carried out in 10 mM NaCl, 10 mM Mops, 1 mM $CaCl_2$ (pH 7.2). The proteolytic reactions were terminated after 3 h by washing the membranes three times with 10 mM NaCl, 10 mM Mops (pH 7.2). All subsequent labeling experiments were carried out using membranes prepared by this method. Before the membranes were solubilized for $NaDodSO_4$–polyacrylamide gel electrophoresis, proteolytic enzyme activities were further inhibited by adding an equal volume of 0.1 M iodoacetamide (for papain) or 0.1 M EDTA (for thermolysin), both at pH 7.2. Total inhibition of the enzyme activity was verified by adding an aliquot of bovine serum albumin solution (4 mg/mL) to an identical sample approximately 1 min after the addition of the inhibitor. No cleavage of the bovine serum albumin could be detected by $NaDodSO_4$–polyacrylamide gel electrophoresis. This result confirmed that the inhibitors terminated all proteolytic enzyme activities.

When the time course of proteolysis of the membranes was to be studied, the following procedure was routinely used. ROS membrane suspensions were incubated with the specified concentration (see figure legends) of the proteolytic enzymes in the required buffer solution at room temperature. Aliquots were removed at various reaction times, and proteolysis was immediately terminated by adding an equal volume of the appropriate inhibitor as described above. Since the inhibitors were found to terminate all enzyme activities under these conditions, no attempt was made to remove the inactivated enzymes in these kinetic measurements. After 15 min of incubation with the inhibitor, the samples were prepared for electrophoresis as described above.

Reduction of the Chromophore of Papain-Cleaved Rhodopsin by Sodium Borohydride. The procedure for the reduction of the retinal in the papain-cleaved rhodopsin by sodium borohydride was essentially the same as that described by Bonting et al. (1973). Complete reduction of the cleaved rhodopsin was confirmed by measuring the characteristic 330-nm absorption of *N*-retinylopsin extracted in 100 mM TrTAB detergent solution. The reduced rhodopsin fragments were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis, and the location of the protein band containing the retinal binding site was detected by the fluorescence of *N*-retinylopsin.

Estimation of the Sulfhydryl Groups in Protease-Cleaved Rhodopsin. The number of reactive sulfhydryl groups on rhodopsin in various detergent solutions and in the membrane were determined spectrophotometrically by titration with 4-PDS according to the procedure given by Chen and Hubbell (1978). The distribution of the sulfhydryl groups in proteolytic fragments was determined by labeling the cleaved rhodopsin with [^{3}H]NEM of specific activity 25 Ci/mol. In this procedure, a 50-fold molar excess of [^{3}H]NEM with respect to rhodopsin was added to a suspension of thermolysin- or papain-treated ROS membranes (4 mg/mL) in 10 mM NaCl, 10 mM Mops, 1 mM EDTA (pH 6.7). The mixture was divided into four portions, and each portion was incubated under nitrogen atmosphere at room temperature according to the following specified conditions: (1) The first portion of the mixture was allowed to react in the dark. Aliquots of the mixture were removed after 45 min and after 8 h, and the reaction was quenched by diluting the mixture with 10 volumes of ice-cold buffer. Excess [^{3}H]NEM was then removed by washing the membranes three times by centrifugation. (2) A second portion of the mixture was processed by exactly the same procedures given in (1), except that the reaction was carried out under *continuous* room-light illumination for 8 h. (3) The cleaved rhodopsin in the third portion was *bleached* completely, followed by 8 h of *incubation in the darkness* under the same conditions as the above two samples. (4) A 10% solution of $NaDodSO_4$ was added to the last portion until the membranes were completely solubilized and the final concentration of $NaDodSO_4$ in the mixture reached 1%. The reaction mixture was incubated under continuous room-light illumination for 8 h. Excess [^{3}H]NEM was removed by dialysis.

At the end of each reaction, the concentration of protein in the sample was determined and the proteolytic fragments were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis.

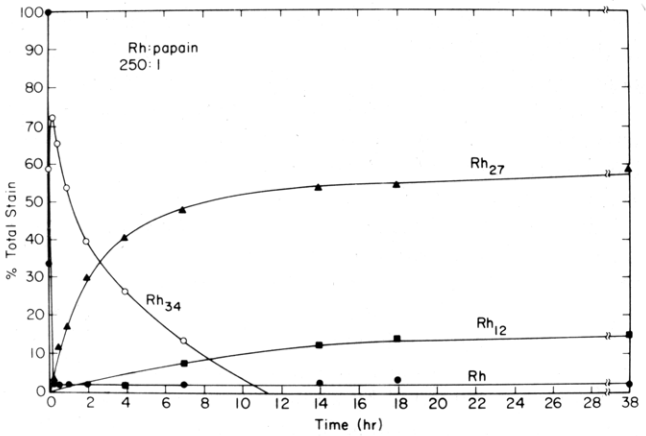

FIGURE 1: Kinetics of papain proteolysis. The percent of total Coomassie blue stain contained in the gel bands representing rhodopsin (Rh) and the proteolytic fragments (Rh_{34}, Rh_{27}, Rh_{12}) is plotted vs. time of proteolysis. The amount of stain in each band is estimated by the area of the absorption band in the densitometric scan at 550 nm. Each data point is an average of three different gels.

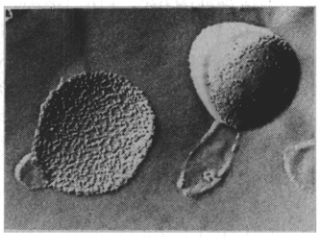

FIGURE 2: Freeze-fracture electron micrography of ROS membrane vesicles treated with papain. The intramembranous particles remain asymmetrically distributed; the concave fracture face (cytoplasmic face) contains most of the particles, while the convex face (intradiscal face) contains very few particles.

The gels were subjected to the staining and destaining procedures described above. No loss of radioactivity from the protein bands was detected throughout these procedures. Densitometric traces of the protein bands were obtained, the gels were cut into 1-mm slices, and each slice was incubated with 0.4 mL of 20% H_2O_2 in a glass counting vial at 60 °C for 12 h. When the gel slices were completely solubilized, 10 mL of scintillation fluid (Aquasol, New England Nuclear Corp., Inc.) was added and the vials were counted in a Beckmann LS-230 scintillation counter. After correcting for the counting efficiency and quenching, the number of sulfhydryl groups in each fragment modified by [^{3}H]NEM was calculated from the amount of opsin applied to each gel and the specific activity of the label.

Cross-linking of the Proteolytic Fragments of Rhodopsin in the Membrane and in Detergent Solutions. Papain-treated ROS membranes were suspended in 10 mM NaCl, 10 mM Mops (pH 5.0) to a final concentration of 1 mg/mL opsin. Solid 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide was then added to these membrane suspensions with continuous stirring until its final concentration reached 100 mM. The pH of the suspensions was continuously adjusted to 5.0 by addition of dilute HCl throughout the whole reaction. At various time intervals, 0.5-mL aliquots of the suspension were removed, and 0.05 mL of 1 M Tris–acetate (pH 8.5) was added to stop the reaction. The membranes were then washed free of carbodiimide with 10 mM NaCl, 10 mM Mops (pH 7.2) by centrifugation, and the extent of the cross-linking was examined on $NaDodSO_4$–polyacrylamide gel electrophoresis. Exactly the same procedure was followed with rhodopsin fragments solubilized in various detergent solutions, except that the unreacted carbodiimide was removed by dialysis.

Spin-Labeling of ROS Membranes. Papain-treated ROS membrane suspensions containing 4 mg/mL opsin in 10 mM NaCl, 10 mM Mops (pH 6.5) were incubated with a tenfold molar excess of 2,2,6,6-tetramethylpiperidinyl-1-oxy-4-maleimide for 8 h at room temperature (22 °C) in the dark. At the end of the reaction, excess unreacted maleimide spin-label was removed by washing the membranes three times with the same buffer. Electron paramagnetic resonance spectra of the spin-labeled rhodopsin in the membrane and in various detergent solutions were recorded at ambient temperature with a Varian E-109 EPR spectrometer.

Results

Proteolysis of Rhodopsin in ROS Membrane Vesicles. $NaDodSO_4$–polyacrylamide gels of the ROS disk membranes show that the opsin band contains approximately 90% of the total Coomassie blue stain (Papermaster and Dreyer, 1974). The apparent molecular weight of opsin, obtained by reference to the migration of proteins of known molecular weight, is 37 500 ± 500. The remaining 10% of the stain is associated with numerous minor protein bands, all of which have apparent molecular weights higher than that of opsin.

Papain cleavage of rhodopsin (or bleached rhodopsin) occurs in two successive stages, similar to those observed by Fong and Hargrave (1978) for thermolysin proteolysis. After the addition of papain, opsin (initially at 37 500 molecular weight) is quantitatively transformed to a species of ~34 000 molecular weight (Rh_{34}). Rh_{34} is a transient intermediate in the proteolysis and is further cleaved to produce two fragments of apparent molecular weights 27 000 ± 500 (Rh_{27}) and 12 500 ± 500 (Rh_{12}). The kinetics of the proteolytic reactions are summarized in Figure 1.

The proteolysis has no effect on either the 500-nm absorption band of rhodopsin or the regeneration yield of rhodopsin from 11-*cis*-retinal and bleached rhodopsin. The Rh_{27} and Rh_{12} fragments remain membrane bound even after repeated washings with urea (2 M), DTT (40 mM), or EDTA (50 mM), in the dark or after bleaching, and thus can be considered as integral membrane fragments.

Freeze-fracture images of the papain-treated ROS membranes containing Rh_{27} and Rh_{12} (Figure 2) show an asymmetric particle distribution similar to that of the native ROS membrane vesicles (Chen and Hubbell, 1973), with very little apparent change in particle density, distribution, or size both before and after bleaching. Thus, most of the freeze-fracture particles remain preferentially with the outer concave fracture face, with only a few percent of the total particles found on the inner convex fracture face. Therefore, it can be concluded that the asymmetry of rhodopsin is maintained after proteolysis.

The above observations refer to proteolysis of membrane-bound rhodopsin, but we have found that papain (and thermolysin) produces an identical cleavage pattern when the membranes are solubilized in Triton X-100 (1%) or sodium cholate (100 mM) prior to enzyme treatment.

Pober and Stryer (1975) have already shown that the carbohydrate content of rhodopsin is associated with the high-molecular-weight fragment produced by thermolysin proteolysis, while the chromophore-attachment site is on the low-molecular-weight fragment. Since the polypeptide fragments produced by thermolysin and papain are indistinguishable on the basis of electrophoretic mobility on $NaDodSO_4$–polyacrylamide gels, it is anticipated that similar results will be found for the papain fragments. This is, in fact, the case, and we find over 90% of the PAS stain for carbohydrates to be associated

TABLE I: Sulfhydryl Content of Native and Papain-Cleaved ROS Membranes under Various Conditions.

ROS preparation	reaction conditions	reaction times	no. of sulfhydryl groups per opsin[a] native membrane	papain-cleaved membrane
dark adapted	dark	3 h	2.4 ± 0.2	2.1 ± 0.2
bleached	light	3 h	4.5 ± 0.3	3.8 ± 0.2
bleached	dark	3 h	2.4 ± 0.2	2.3 ± 0.2
solubilized in 2% Triton X-100	light	15 min		5.6 ± 0.2
solubilized in 100 mM TrTAB	light	15 min	6.0 ± 0.1	5.6 ± 0.2
solubilized in 100 mM sodium cholate	light	15 min	6.1 ± 0.1	5.8 ± 0.2
solubilized in 1% $NaDodSO_4$	light	15 min	5.9 ± 0.3	5.6 ± 0.2

[a] The error limits are determined as the standard deviation of two to five independent determinations using 4-PDS.

with Rh_{27} and virtually all of the retinylopsin fluorescence obtained by sodium borohydride reduction of the papain-treated membranes to be associated with Rh_{12} (Figure 3).

Reactivity of the Sulfhydryl Groups in the Cleaved Rhodopsin. The number of reactive sulfhydryl groups in papain-treated rhodopsin has been determined under different conditions by titration with 4-PDS, and the results are summarized in Table I. There are several interesting points to be made regarding these data. First, the number of titratable sulfhydryl groups in the papain-treated membranes under various conditions is similar to that of the native membranes but consistently less by an average of about 0.3 group. If the ROS membranes are labeled with either 4-PDS or [^{3}H]NEM *prior* to proteolysis, we find that 0.2–0.3 labeled group is released into solution *during* proteolysis. This indicates that the small decrease in reactive groups after proteolysis is due to actual removal of the groups rather than oxidation during the treatment. The second interesting result presented in Table I involves an unusual and rather unexpected reaction first oberved by Chen and Hubbell (1978) in native ROS membranes. To appreciate this result, a careful distinction must be made between completely bleached membranes reacting with 4-PDS in the *dark* and completely bleached membranes reacting with 4-PDS under *continuous illumination* with room lighting. In particular, the data of Table I show that no additional reactive sulfhydryl groups appear in the membrane after bleaching in either native *or* cleaved rhodopsin, as long as the titration itself is carried out in the *dark* following the initial bleach. On the other hand, additional reactive groups *do* appear in the membrane for both native and cleaved rhodopsin if the titration is carried out under room light. The nature of this unusual light-driven reaction has been discussed by Chen and Hubbell (1978), and our objective here is simply to emphasize that all details of the sulfhydryl chemistry characteristic of the native protein are conserved in the cleaved protein.

Finally, when cleaved membranes are solubilized in Triton X-100, TrTAB, $NaDodSO_4$, or sodium cholate detergent solutions and bleached, 5.6 or 5.8 sulfhydryl groups per cleaved opsin are accessible to modification by 4-PDS. The total number and the reactivity of the sulfhydryl groups of thermolysin-cleaved rhodopsin are essentially identical to those of the papain-cleaved rhodopsin.

Distribution of the Sulfhydryl Groups. The distribution of sulfhydryl groups in the proteolytic fragments can be determined by treating the cleaved rhodopsin with [^{3}H]NEM for 8 h in the presence of 1% $NaDodSO_4$. Under these denaturing conditions, complete modification of all sulfhydryl groups is achieved (De Grip et al., 1975). Figure 4 shows the resulting distribution of radioactivity on the fragments after separation on polyacrylamide gels. About 90% of the total radioactivity

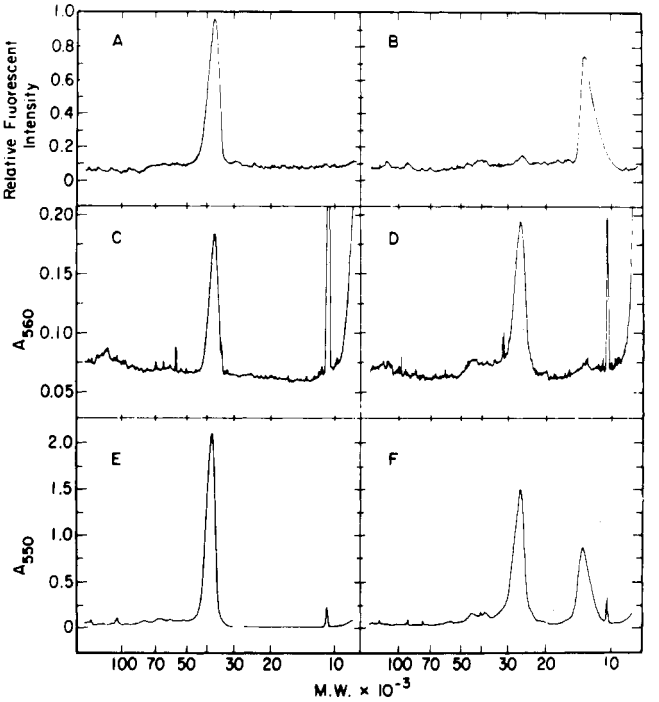

FIGURE 3: Locations of the carbohydrates and the retinal binding site of rhodopsin in the proteolytic fragments. Top Panel: Fluorescence intensity profiles of $NaDodSO_4$–polyacrylamide gel electrophoresis gels of $NaBH_4$-treated ROS membranes showing the location of the retinyl polypeptides: (A) ROS membranes and (B) ROS membranes treated with papain. The fluorescent intensity is not calibrated and is given in arbitrary units. Virtually all the *N*-retinyl fluorescence is associated with the Rh_{12} fragment. Center panel: Densitometric scan at 560 nm of PAS-stained $NaDodSO_4$–polyacrylamide gel electrophoresis gels of ROS membranes showing the location of the carbohydrates: (C) ROS membranes and (D) ROS membranes treated with papain. Ninety percent of the PAS-staining material (carbohydrates) is associated with the Rh_{27} fragment. The PAS-positive materials migrating ahead of the tracking dye are probably free retinals and glycolipids. Bottom panel: Densitometric scans at 550 nm of the same gels shown in the center panel after Coomassie blue staining: (E) ROS membranes and (F) ROS membranes treated with papain.

on the gel coincides with the Rh_{27} and Rh_{12} bands, while 5% coincide with the small amount of undigested opsin. The remaining 5% radioactivity is associated with minor protein bands migrating behind Rh_{27}. Thus, close to 95% of the radioactivity is associated with opsin and its fragments. From the amount of [^{3}H]NEM incorporated into each band, it is estimated that Rh_{27} contains a total of 3.9 sulfhydryl groups, while Rh_{12} contains a total of 1.8.

The distribution of the *accessible* sulfhydryl groups (as opposed to total groups) in the *membrane-bound fragments*

TABLE II: Distribution of Reactive Sulfhydryl Groups in Native and Papain-Cleaved Rhodopsin under Various Conditions.

ROS preparation	reaction conditions	reaction times	no. of sulfhydryl groups[a]		
			Rh_{27}	Rh_{12}	$Rh_{27} \pm Rh_{12}$
dark adapted	dark	45 min	0.3 ± 0.1	1.0 ± 0.1	1.3 ± 0.1
dark adapted	dark	8 h	1.1 ± 0.1	1.0 ± 0.1	2.1 ± 0.1
bleached	dark	8 h	1.2 ± 0.1	1.1 ± 0.1	2.3 ± 0.1
bleached	light	8 h	2.8 ± 0.2	1.5 ± 0.1	4.3 ± 0.2
solubilized in 1% $NaDodSO_4$	light	8 h	3.9 ± 0.2	1.8 ± 0.1	5.7 ± 0.2

[a] The error limits for the data in rows 2 and 4 are the standard deviations from two independent determinations. Due to the long times required for these experiments, the data presented in rows 1, 3, and 5 were obtained from a single experiment, and the error limits are confidence limits based on the precision of the individual experiment.

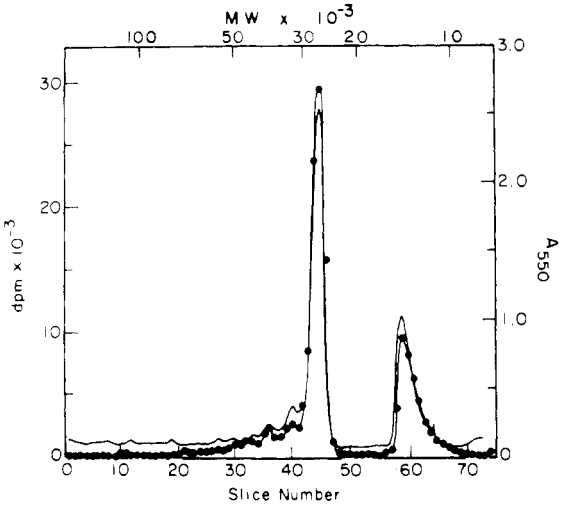

FIGURE 4: Coomassie blue stain and radioactivity profiles of thermolysin-cleaved ROS membranes. The Coomassie blue stain (—) and radioactivity profile (●) of a $NaDodSO_4$-polyacrylamide gel of thermolysin-cleaved rhodopsin labeled in 1% $NaDodSO_4$ with [^{3}H]NEM. Thermolysin rather than papain was used in this particular experiment because the iodoacetamide needed to inhibit papain in $NaDodSO_4$ would also react with rhodopsin sulfhydryl groups.

can also be determined using the same approach. The cleaved ROS membranes were incubated with [^{3}H]NEM under various conditions corresponding to those of Table I, and the number of labeled sulfhydryl groups in the Rh_{27} and Rh_{12} fragments was calculated from the radioactivity of the protein bands after separation on $NaDodSO_4$-polyacrylamide gel electrophoresis. The conditions and results are summarized in Table II. Both Rh_{27} and Rh_{12} contain one accessible sulfhydryl group in the dark-adapted membrane. The reactivity of these sulfhydryl groups, however, is very different. [^{3}H]-NEM reacts rapidly with the sulfhydryl group in the Rh_{12} fragment, and the reaction is essentially complete in 45 min. On the other hand, [^{3}H]NEM reacts more slowly with the sulfhydryl group in the Rh_{27} fragment. Upon bleaching the cleaved membrane using *continuous* illumination in the presence of [^{3}H]NEM, a large increase in radioactivity of Rh_{27} is observed, corresponding to the modification of 1.8 *additional* sulfhydryl groups. In contrast, continuous irradiation only induces a small increase in the number of reactive sulfhydryls in Rh_{12}. If the reaction of the cleaved opsin with [^{3}H]NEM is carried out in the *dark* following an initial complete bleach, approximately one reactive sulfhydryl group is found in each fragment, the same as for dark-adapted membranes.

Interaction between Rh_{27} and Rh_{12} Fragments in Detergent Solutions. Pober and Stryer (1975) reported that the two proteolytic fragments produced by thermolysin digestion completely dissociated in the presence of Triton X-100 upon bleaching. In view of the fact that rhodopsin is not regenerable after bleaching in Triton X-100 (Johnson and Williams, 1970), it is possible that the photoinduced dissociation may in fact be due to denaturation of the protein. Using the procedures devised by Pober and Stryer (1975), we have found that the light-induced dissociation of Rh_{27} and Rh_{12} does indeed occur in solutions of Triton X-100 (2% w/v) and TrTAB (100 mM) but *not* in solutions of sodium cholate (100 mM) or digitonin (2% w/v). Thus, it appears that the microenvironment of the protein determines whether or not photodissociation occurs, and it is of interest to determine whether or not this phenomenon is observed in the native membrane. If Rh_{27} and Rh_{12} remain closely associated with each other in the membrane before and after bleaching, it should be possible to specifically cross-link the fragments together by suitable reagents. Table III lists the molecular weights of the cross-linked products produced by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in papain-treated ROS membranes and in various detergents along with the percent of total Coomassie blue associated with each product resolved on $NaDodSO_4$-polyacrylamide gel electrophoresis. In all cases, cross-linking of the Rh_{27} and Rh_{12} fragments in the dark produced a major polypeptide with an apparent molecular weight close to 33 000, presumably corresponding to Rh_{34}. Very little protein was found at molecular weights corresponding to multimers of either Rh_{12} or Rh_{27}. The 33 000 molecular weight product of cross-linking, however, is found to be very stable to proteolysis and cannot be converted back to Rh_{27} and Rh_{12} fragments by proteolysis even when high concentrations of papain are used. The yield of cross-linking of the fragments in membranes and in detergents was not the same; the highest yield was obtained from fragments solubilized in Triton X-100 in the dark. The difference in cross-linking efficiencies between the rhodopsin fragments in various environments probably reflects the difference in accessibility of the carbodiimide to the proteins. Bleaching has no effect on the percent cross-linking of the fragments in the ROS membranes and caused only small changes for rhodopsin fragments solubilized in digitonin. On the other hand, in Triton X-100 an irreversible aggregation of the entire population of fragments results upon bleaching during the cross-linking reaction. The product has an extremely high molecular weight that cannot enter 8% polyacrylamide gels.

In parallel experiments, electron paramagnetic resonance (EPR) spectra of papain-cleaved rhodopsin labeled with 2,2,6,6-tetramethylpiperidinyl-2-oxy-4-maleimide were used to detect conformational changes of the protein in various detergent solutions (Pontus and Delmelle, 1975). Illumination of the labeled, papain-cleaved membrane suspension or the same membrane suspension solubilized in 2% digitonin or 80 mM sodium cholate did not produce any change in the EPR line shape of the labeled protein. On the other hand, bleaching the cleaved rhodopsin in the presence of 50 mM TrTAB or 2%

TABLE III: Carbodiimide Cross-linking of Proteolytic Fragments.

papain-cleaved rhodopsin in	reaction conditions	% of total Coomassie blue stain[a] Rh_{27} (%)	Rh_{12} (%)	Rh_{34} (%)	$(Rh_{27})_2$ (%)	$(Rh_{34})_2$ (%)
ROS disk membrane	dark	30	19	34	7	0
ROS disk membrane	light	30	19	34	7	0
1% digitonin	dark	36	9	46	0	0
1% digitonin	light	44	10	30	0	0
2% Triton X-100	dark	5	1	87	0	7
2% Triton X-100	light	aggregation				

[a] One-hundred percent is taken as the total amount of stain associated with all the peptide bands. This value does not include background stain and stain associated with a small amount of protein material at the top of the gels. The percent stain associated with Rh_{27} and Rh_{12} is approximately 62 and 28%, respectively, before the cross-linking reaction.

Triton X-100 produced substantial changes in the spectral line shape, suggesting that the protein has undergone a conformation change which mobilizes the spin-labels. Similar results are found on bleaching labeled but uncleaved rhodopsin in these detergents (Pontus and Delmelle, 1975).

Discussion

As judged from the freeze-fracture analysis, the ROS membrane vesicles used in these experiments have the same surface orientation as the native disk membranes from which they are derived (Figure 2). The extremely rapid attack by papain (or thermolysin) on rhodopsin in these vesicles to produce Rh_{34} (Figure 1) suggests that this first cleavage site is readily accessible and resides on the external surface of the vesicles (the cytoplasmic surface of the native disk). More conclusive evidence regarding the locations of the proteolytic sites is given in the following paper of this issue (Fung and Hubbell, 1978). It is important, however, to note here that this fast cleavage which removes a 3000-dalton fragment (or fragments) in the production of Rh_{34} must contain a terminal sequence, otherwise more than one membrane-bound fragment would be produced. Hargrave (1977) has shown that the carbohydrates of rhodopsin are attached very close to the amino terminus of the polypeptide. Since all carbohydrates are retained in Rh_{27} after proteolysis, the fast cleavage thus must represent the removal of a carboxy-terminal sequence on the external (cytoplasmic) surface of the ROS membranes. Using histochemical labeling techniques, Röhlich (1976) has recently reported that the carbohydrates of rhodopsin are located exclusively at the interior surface of the disk. Hence, the amino-terminal sequence must be on the interior surface of the ROS membranes.

Our kinetic measurements of the complete proteolysis process clearly demonstrate that the digestion of rhodopsin by papain is terminated when Rh_{27} and Rh_{12} fragments are produced. The results obtained after prolonged proteolysis with papain and thermolysin thus confirm those of Pober and Stryer (1975) and are different than those of other workers, Sale et al. (1977) in particular. The latter workers reported three membrane-bound fragments to be produced by papain proteolysis of rhodopsin in ROS vesicles. Furthermore, it was reported that one of the fragments (~23 000 daltons) could be further degraded to low-molecular-weight polypeptides with mercaptoethanol. We deliberately used high concentrations of DTT (up to 0.1 M) in the preparation of $NaDodSO_4$–polyacrylamide gel electrophoresis and have not been able to produce further fragmentation of either Rh_{27} or Rh_{12}.

Membrane-bound rhodopsin has been shown to have two reactive sulfhydryl groups in the native dark-adapted state (De Grip et al., 1973; Chen and Hubbell, 1978). The value of 2.4 sulfhydryl groups per rhodopsin presented in Table I was obtained with intact ROS membranes, and the 0.4 excess group may be attributed to nonopsin content of the membranes (De Grip et al., 1975; Chen and Hubbell, 1978). The fact that the sulfhydryl content per opsin molecule approaches 2 after proteolysis thus suggests two possibilities. First, the ~0.3 sulfhydryl group released from the membrane during proteolysis may originate from the minor protein population and not opsin itself. Second, a sulfhydryl group of rhodopsin may be located very close to papain cleavage sites, and in a subpopulation of rhodopsin this sulfhydryl group could be released by multiple, closely spaced cleavages. These possibilities cannot be distinguished on the basis of our data.

The results of the experiments presented here allow a classification of the reactive rhodopsin sulfhydryl groups with respect to both reactivity (De Grip et al., 1975; Chen and Hubbell, 1978) and location on the major proteolytic fragments. The first major class of sulfhydryls contains the two reactive groups in the dark-adapted membranes. Chen and Hubbell (1978) found that these groups react at different rates with 4-PDS, and we have found that this differential reactivity is also observed with respect to [^{3}H]NEM in the papain-cleaved protein. The more rapidly reacting group is located on Rh_{12}, while the more slowly reacting group is on Rh_{27}. Since Chen and Hubbell (1978) showed that bleaching increases the reactivity of both groups, we conclude that conformational changes occur in both Rh_{12} and Rh_{27} as a result of photon absorption.

An important result of the present experiments is that another major class of sulfhydryls (ca. two groups) in cleaved rhodopsin becomes available for reaction with [^{3}H]NEM under *continuous*, but not flash, irradiation. This interesting behavior was first observed by Chen and Hubbell (1978) in the native protein and is apparently conserved in the cleaved protein. These "light-activated" sulfhydryl groups are located on Rh_{27}. McDowell and Kühn (1977) have recently reported that the ability of bleached rhodopsin to be phosphorylated decays with time but may be restored by exposure to *continuous* irradiation. It is tempting to suggest that this effect and the increased sulfhydryl reactivity under continuous irradiation are expressions of the same conformational changes in the protein. It is evident from the data presented in this paper that the membrane-bound, cleaved protein has a sulfhydryl chemistry very similar to the native molecule, both in the dark and bleached state. In addition, the absence of any effect of papain proteolysis on the visible absorption spectra, the regeneration yields after bleaching, or the freeze-fracture images suggests that the measurable properties of the cleaved molecule in the membrane are virtually identical to those of native rhodopsin.

Pober and Styer (1975) have shown that illumination of thermolysin- or papain-cleaved rhodopsin in Triton X-100 results in a dissociation of the proteolytic fragments. There are several lines of evidence which suggest that this reaction may be due to a denaturation of the protein and not necessarily to a functionally significant process. Firstly, we have observed this phenomenon only for rhodopsin solubilized in detergents known to denature rhodopsin upon bleaching, i.e., Triton X-100 and TrTAB (Hong and Hubbell, 1973; Johnson and Williams, 1970). Secondly, the large changes observed in the EPR spectra of spin-labeled rhodopsin upon bleaching in solutions of these detergents support the notion that the rhodopsin polypeptide undergoes significant changes in conformation which are *not* observed in the native membrane or in solutions of sodium cholate or digitonin. The fact that these conformational changes are observed upon bleaching in Triton X-100 and TrTAB in the native as well as the cleaved protein indicates that the cleavage itself does not lead to instability and suggests an intrinsic property of the protein–detergent system. Thirdly, the dramatic polymerization of the protein bleached in Triton X-100 but not in digitonin upon cross-linking with carbodiimide further suggests denaturation of the molecule in the former detergent. In short, we have not succeeded in separating the fragments under nondenaturing conditions in detergent solutions. As expected, this conclusion apparently extends to the membrane-bound protein. If Rh_{12} and Rh_{27} dissociated upon bleaching in the membrane, one would expect the cross-linking reaction, if effective at all, to produce some amount of all possible products. Instead only Rh_{34} is produced, as shown in Table III. This suggests, but does not prove, that Rh_{12} and Rh_{27} remain closely associated both in the dark and bleached state in the membrane. Since the chromophore is bound to Rh_{12} and the light-induced sulfhydryl reactivity is on Rh_{27} in the cleaved protein, this reaction itself may be viewed as evidence for the close association of the fragments in the membrane after bleaching. The mechanism of this reaction, however, is not understood and such conclusions must await further study of this interesting process.

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