THE ACCESSIBILITY OF BOVINE RHODOPSIN IN PHOTORECEPTOR MEMBRANES

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ABSTRACT
Bovine photoreceptor membranes have been treated with proteases to determine the accessibility of rhodopsin to these large, water soluble molecules. The polypeptides that remain associated with the membranous structure after proteolysis were detected by sodium dodecyl sulfate gel electrophoresis. Thermolysin and chymotrypsin degraded rhodopsin (apparent mol wt 35,000-36,000) to fragments of 29,000 and 23,000 apparent mol wt, respectively, without affecting the chromophoric absorption of the molecule or removing the region of the polypeptide carrying carbohydrate. The two fragments were isolated and their amino acid compositions were determined. They do not appear to be more hydrophobic than rhodopsin. Subtilisin, at low concentration and temperature, produced a fragment with the same molecular weight as that produced by thermolysin. At higher concentrations, subtilisin yields major fragments of mol wt 23,000 and 20,000 without affecting the chromophoric absorption. Two intermediate fragments of apparent mol wt 29,000 and 26,000 were detected during the course of this degradation. Carbohydrate is retained by all but the smallest fragment. Bleaching of the photoreceptor pigment did not appreciably alter any of the fragmentation patterns. Trypsin did not alter the molecular weight of rhodopsin under the conditions of this study. Approximately 35-45% of rhodopsin appears to be accessible to the aqueous environment and can be removed without affecting the chromophoric properties of the retinaldehyde-carrying region which remains bound to the membrane.

Rhodopsin is an intrinsic membrane protein found in the disk membrane of the photoreceptor rod cell. The function of rhodopsin, aside from its role as a light receptor, is not yet known although there is evidence to suggest that it plays a role in regulating ion flux across the disk membrane (for a recent review, see reference 1). The structure of this membrane and specifically the location of rhodopsin has been the subject of intensive investigation (2). There is now general agreement that rhodopsin is situated at least in part within the hydrophobic membrane interior (3-5). Other regions of polypeptide must be exposed to the aqueous environment since they are accessible to water-soluble reagents (6-10). In this paper the domains of rhodopsin structure are assessed through the use of macromolecular probes (proteases), assuming that the action of these enzymes will be limited to exposed regions of protein. It will be shown by this criterion that about 40% of rhodopsin is accessible.

MATERIALS AND METHODS
Dark-adapted bovine retinas were obtained from the George A. Hormel Co., Austin, Minn. Thermolysin was obtained from Daiwa Kasei K. K., Osaka, Japan,
Nagarse (subtilisin) from Teikoku Chemical Industry, Osaka, Japan, and α-chymotrypsin A and TPCK-1-treated trypsin from Worthington Biochemical Corp., Freehold, N. J. Hexadecyltrimethylammonium bromide and dodecyltrimethylammonium chloride were obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. 9-cis retinal was purchased from Sigma Chemical Co., St. Louis, Mo. Concanavalin A-Sepharose was prepared as described by Steineman and Stryer (8). All other reagents were the highest grade commercially available.

**Preparation of Photoreceptor Membranes**

Photoreceptor membranes were isolated by two procedures. All operations were carried out under dim red illumination (Kodak Safelight, Watten filter 1A) at a temperature between 0 and 5°C. Method A: 100 frozen bovine retinas were thawed and ground to a smooth paste in a chilled mortar. 100 ml of ice-cold 10 mM Tris-acetate buffer, pH 7.5, containing 40% sucrose (wt/vol) was added and the retinas were briefly ground. This suspension was centrifuged three times at 2000 g for 20 min or until no further precipitate was obtained. The supernate was diluted with 2 vol of 10 mM Tris-acetate, pH 7.5 and centrifuged at 25,000 g for 20 min. The pellet from this step was suspended in buffer containing sucrose (40%, wt/vol) by brief homogenization in a motor-driven Teflon-glass homogenizer (two passes), and layered beneath an equal volume of the buffer without sucrose. Centrifugation at 25,000 g for 20 min concentrated the outer segment membranes at the interface. This deep red band was removed with a Pasteur pipette along with the upper buffer layer and the combined fractions were centrifuged at 25,000 g for 20 min. The flotation step was suspended in buffer containing sucrose (40%, wt/vol) by brief homogenization in a motor-driven Teflon-glass homogenizer (two passes), and layered beneath an equal volume of the buffer without sucrose. Centrifugation at 25,000 g for 20 min concentrated the outer segment membranes at the interface. This deep red band was removed with a Pasteur pipette along with the upper buffer layer and the combined fractions were centrifuged at 25,000 g for 20 min. The flotation step was repeated and the membranes were washed twice with 10 mM Tris-acetate. The final pellet was resuspended in this buffer (concentration of rhodopsin was about 3 mg/ml) and stored frozen in small portions at -20°C, wrapped in aluminum foil. Method B: the procedure of Papermaster and Dryer (11), recommended for use with frozen bovine retinas, was followed except that the final step utilized a continuous linear gradient (0.77 M - 1.14 M sucrose) instead of their discontinuous gradient. The membranes were stored as described for method A.

**Gel Electrophoresis**

SDS gels (0.6 x 8 cm) were prepared according to Weber and Osborn (12) except that the final SDS concentration was 0.2%. All the gels illustrated were 10% in acrylamide except for the experiment shown in Fig. 2 A in which 7.5% gels were used. Samples for electrophoresis were dissolved by the addition of an equivalent volume of a 10 mM sodium phosphate buffer, pH 7.2; 7% in SDS, 0.1% in β-mercaptoethanol, and 20% in sucrose. A few drops of 0.2% bromophenol blue were added to serve as a tracking dye. When gels were used for the determination of apparent molecular weights, samples were heated at 100°C for 2 min before application to the gel. Electrophoresis was carried out for about 6 h at room temperature and 5 V/cm or until the dye marker had migrated to within 1 cm of the end of the gel. The protein in the gel was fixed for 1–1.5 h by the method of Fairbanks et al. (13). The gels were then soaked in Coomassie blue stain 1 (13) and destained by soaking in 5% methanol-7.5% acetic acid. Staining for carbohydrate with PAS 1 reagent was also done according to Fairbanks et al. (13).

The apparent molecular weight of the fragments produced upon proteolysis was obtained by calibration of the gels with proteins of known molecular weight. The following marker proteins (and molecular weights) were used: ovalbumin (43,000), lactate dehydrogenase (36,000), pancreatic deoxyribonuclease (30,072), α-chymotrypsinogen (25,700), and myoglobin (17,200).

**Limited Proteolysis of Photoreceptor Membranes**

All the analytical experiments in which limited proteolysis was employed were performed in essentially the same manner. All operations were carried out under dim red illumination unless specifically indicated. The concentration of rhodopsin in these experiments was 3.5 mg/ml; the ratio of protease to rhodopsin is specified in the text or in the legends to the figures. During the proteolysis, tubes containing photoreceptor membranes were wrapped in aluminum foil. A sample of membranes (200–500 μl) was centrifuged and resuspended in 200–500 μl of 10 mM Tris-acetate buffer, pH 7.5, by repeatedly drawing the suspension into a Pasteur pipette. After withdrawal of an initial sample, an aliquot of a fresh protease solution was added and the tubes were kept at room temperature, approximately 23°C, unless otherwise specified. Portions of 20–50 μl were removed at various reaction times and proteolysis was stopped by the addition of phenylmethane sulfonyl fluoride (final concentration 1 mM in the experiments with subtilisin and chymotrypsin) or EDTA (final concentration 5 mM when thermolysin was used). These portions were stored frozen until analysis. The experimental details of the larger-scale proteolysis procedures used to isolate fragments Th-1 and Ch-3 are described later.

**Determination of Rhodopsin Concentration**

Samples of disk membrane suspensions (usually 50 μl) were removed before and after proteolysis and added to 1 ml of 40 mM hexadecyltrimethylammonium bromide, 0.067 M in phosphate, 0.25 M in NH₄OH, pH 7.0. After the absorption at 500 nm had been measured, the sample was bleached under normal room illumination until the

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1 Abbreviations used in this paper: SDS, sodium dodecyl sulfate; PAS, periodic acid-Schiff; Th, thermolysin; Ch, chymotrypsin; S, subtilisin; TPCK, N-tosyl-L-phenylalani
dimechloromethyl ketone.
Asoo reached a constant value. The concentration of rhodopsin was determined from the change in the absorption at 500 nm assuming an extinction coefficient of 40,600 (14, 15).

**Regeneration of Absorption after Bleaching**

The procedure of Hubbard et al. (14) for the formation of isorhodopsin was used to assess the extent to which proteolysis modified the capacity of visual pigment to regenerate its original absorption spectrum after bleaching. A sample of disk membranes (before and after proteolysis) was suspended in 10 mM Tris-acetate, pH 7.5 and bleached under room illumination until the absorption at 500 nm reached a constant value. Small aliquots of 9-cis retinal, dissolved in methanol, were added under dim red illumination to a molar concentration approximately twofold over opsin (final concentration of methanol was ca. 5%). After 30 min to 1 h, at room temperature, the concentration of bleachable isopigment in the solution was determined as described above.

**Amino Acid Analysis**

The amino acid compositions of rhodopsin and proteolytic degradation fragments Th-1 and Ch-3 were obtained after hydrolysis of samples at 110°C for 24 h in evacuated, sealed tubes in 6 N HCl containing 0.1% (vol/vol) mercaptoacetic acid, 0.2% (wt/vol) phenol. The HCl was removed by rotary evaporation at 30°C. Samples were analyzed using either a microbore amino acid analyzer (16) or a Durrum D-500 amino acid analyzer (Durrum Instrument Corp., Palo Alto, Calif.). Values for serine and threonine were not corrected for decomposition.

**Electron Microscopy**

For thin sectioning, samples were fixed for 2 h at room temperature in 2.5% buffered glutaraldehyde (0.1 M cacodylate buffer, pH 7.3), washed briefly in buffer, postfixed in buffered 1% OsO4, stained en bloc with uranyl acetate, and embedded in Epon (17). For freeze-fracturing, membranes were fixed for 20-30 min in the above buffered aldehyde mixture at room temperature, rinsed with buffer, rapidly frozen in liquid Freon 22 (chlorodifluoromethane), and stored in liquid nitrogen. Freeze-fracturing was performed with a Balzers apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) (18, 19).

**RESULTS**

**Preparation of Photoreceptor Membranes**

The two methods of preparation used in this study represent variations of procedures in common use in other laboratories (20, 21, 22, 11). Method A utilizes a more vigorous homogenization of the retinas before flotation, whereas in method B the retinas are gently agitated in buffer to shear off the outer segments with minimum disruption of the tissue. No striking differences were noted when the two preparations were compared by SDS gel electrophoresis. For example, gel 1, Figs. 2 A and B and 4 A, show material isolated using method A, while gel 1, Fig. 3 A, depicts material isolated using method B. Identical proteolysis patterns were produced using either preparation.

Electron microscope examination of photoreceptor membranes prepared by either method shows that homogenization and perhaps hypotonicity (23) have produced a considerable disruption of the outer segment (Fig. 1). The plasma membrane which normally surrounds the outer segment appears to have been completely ruptured. The disks exist, for the most part, free from one another and appear to be intact. The membrane preparation was also examined by the technique of freeze-fracture electron microscopy (Fig. 1). All images examined displayed a characteristic distribution of intramembranous particles uniquely associated with the outer leaflet (fracture face A). This same distribution of particles was noted by Clark and Branton (24) in intact guinea pig retina and thus indicates that no inversion of the membranes has occurred during isolation.

As has been noted by others (20, 25, 11), SDS gel electrophoresis of photoreceptor membranes produces only a single major band that stains with Coomassie blue (for example, see Fig. 2 A, gel 1). That this band represents rhodopsin is indicated by several criteria: (a) coelectrophoretic migration on SDS gels with chromatographically purified rhodopsin; (b) an amino acid composition similar, if not identical, to that of purified rhodopsin; (c) the presence of markers known to be associated with rhodopsin such as carbohydrate, 32P after incubation of photoreceptor membrane preparations with [γ-32P]ATP (25, 26) and the retinyl moiety after reduction with NaBH4. Major bands of higher molecular weight are occasionally seen (for example, Fig. 3 B, gel 1) which are polymers of the most rapidly migrating band. Polymerization of rhodopsin can be induced by freezing or boiling of the
FIGURE 1  A. Low magnification electron micrograph of the photoreceptor membrane enriched fraction. The material was isolated according to method A, described in the text. The preparation contains both disk-shaped photoreceptor membrane as well as vesicular elements not derived from rod outer segments. × 17,100. B. Electron micrograph of the photoreceptor membrane fraction at higher magnification. × 31,500. C. Freeze-fracture appearance of the isolated photoreceptor membranes. The membranes maintain an asymmetric distribution of intramembranous particles; the outer fracture face (concave fracture face) contains most of the particles, while the inner fracture face contains very few. These features imply that there is no inversion of the membranes during isolation. × 62,500.
photoreceptor membrane preparations in the presence of SDS. The apparent molecular weight of rhodopsin, obtained by reference to the migration of proteins of known mol wt, is 35,000–36,000, a value in agreement with recently published results (20, 25). However, this value should be accepted with caution since the migration of rhodopsin is dependent on the prior treatment of the sample. For instance, if the outer segment material is dissolved in the sample application buffer containing SDS, but not heated, the apparent mol wt is 38,000–40,000. Heating at 100°C for 2 min before electrophoresis or pipetting the sample directly into hot SDS-mercaptoethanol (27) yields, for reasons unknown, a mol wt of 35,000–36,000. Such treatment does not affect the migration of the proteins used in standardization of the gels. A similar finding was noted by Frank et al. (25).

Proteolysis of Rhodopsin

In order to measure the accessibility of rhodopsin to large, water-soluble reagents, isolated membranes were treated with several proteases. The extent of reaction at various times was measured by SDS gel electrophoresis. Portions of the reaction mixture were removed, mixed with protease inhibitor, and diluted directly into the sample buffer.
application buffer. No attempt was made to wash the membranes in these kinetic experiments. In other experiments, however, the membrane pellet was extensively washed with buffer before gel electrophoresis to verify that the bands detected were indeed associated with the membrane and not removed by washing. No differences were detected in major band patterns.

As a control, photoreceptor membranes were allowed to stand for 21–24 h without added protease. No degradation of rhodopsin was apparent. In addition, gel electrophoresis of a sample containing protease but no outer segment material revealed no bands, probably because the amount of protease present was below the level of detection.

Proteolysis by Thermolysin

Fig. 2 A illustrates the course of the reaction of photoreceptor membranes with thermolysin during which rhodopsin is nearly completely converted to an insoluble product (Th-1) with an apparent mol wt of 29,000. The weight ratio of thermolysin to rhodopsin in this experiment was 1:65. This fragment still retains the carbohydrate of the original rhodopsin (see Figure 2 B) and is not bleached during proteolysis (see Table I). Changes in temperature or the ratio of thermolysin to rhodopsin affected only the velocity of proteolysis and not the electrophoretic behavior of the final products. Bleaching of the outer segments before proteolysis did not appreciably alter the pattern. An additional band of lower molecular weight (approx. 17,000) was also evident. It is faintly visible below Th-1 in Fig. 2 A, gels 6, 7, and 8 but was more prominent in other experiments. Fragment Th-1 remains associated with the disk membranes since repeated washing with buffer does not release it into the aqueous phase. The soluble portion of polypeptide chain released from rhodopsin has not been identified. However, preliminary experiments with 32P-labeled rhodopsin suggest that this portion is initially released as a large, water-soluble fragment which is subsequently degraded to low molecular weight peptides (unpublished experiments). The presence of Ca++ (2 mM) appeared to enhance the specificity of cleavage by thermolysin and was included in the experiment shown in Fig. 2. Without exogenous Ca++ an additional cleavage product was seen with an apparent mol wt of 23,000, whereas in the presence of Ca++ this product was absent or present in only very small amounts relative to Th-1.

<table>
<thead>
<tr>
<th>Protease employed</th>
<th>Fragments*</th>
<th>ΔA100</th>
<th>Regenerability†</th>
</tr>
</thead>
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<tr>
<td>Thermolysin</td>
<td>ca. 90% Th-1</td>
<td>97</td>
<td>60</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>ca. 90% Ch-3</td>
<td>93</td>
<td>60</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>ca. 50% S-3, 50% S-4</td>
<td>92</td>
<td>Not determined.</td>
</tr>
</tbody>
</table>

* Estimated from SDS gels after staining with Coomassie blue.
† Bleachable rhodopsin relative to the control which was not treated with the protease.
§ Percent regeneration of original absorbance at 500 nm after addition of 9-cis retinal to bleached photopigment membranes.

Proteolysis by Subtilisin

At 0°C, low concentrations of subtilisin (subtilisin to rhodopsin ratio of about 1:1,500 wt/wt) degrade rhodopsin to a fragment (designated S-1) with an apparent molecular weight similar to that produced by thermolysin (see Fig. 3 A). Further proteolysis of this fragment occurs only very slowly under these conditions. Analogous to the reaction with thermolysin, a band of about 17,000 mol wt was also present.

At room temperature and higher concentrations of subtilisin (subtilisin to rhodopsin ratio of about 1:100 wt/wt), proteolysis is much less specific and proceeds through the production of several intermediates which have been termed S-1, S-2, S-3, and S-4, and are shown in Fig. 3 B. These bands represent insoluble material associated with the membranous structure. The nature of the soluble material released from the rhodopsin during proteolysis has not been examined. Presumably after prolonged proteolysis, only relatively low molecular weight fragments will be obtained.

The apparent molecular weights of the fragments are presented in Table II. Carbohydrate is present in all species except the lowest molecular weight band, S-4 (see Fig. 3 B). A kinetic analysis of the reaction suggests that rhodopsin is rapidly degraded to yield S-1 and that this is degraded further to S-2. It is not apparent, however, if S-3 and S-4 are, in turn, part of an orderly progressive degradation or if they are formed by more complex
proteolytic processes. As shown in Table I, rhodopsin is only 5–10% bleached during the course of the proteolysis which leads to roughly 50% S-3 and 50% S-4. Upon prolonged incubation with subtilisin, a mixture of S-3 and S-4 (plus undefined low molecular weight products) was usually obtained in which S-3 was the prominent species. Bleaching of the outer segments before proteolysis did not change the pattern of fragments obtained.

Proteolysis by Chymotrypsin

Proteolysis with α-chymotrypsin also proceeded through a series of intermediates of apparent molecular weights similar to those of the fragments produced by subtilisin (see Fig. 4 B). However, a band of mol wt 26,000 was never present in significant concentrations and was only observable after short reaction times. Proteolysis seems to be arrested after production of a 23,000 mol wt band (designated Ch-3). As shown in Fig. 4 B, the major insoluble product retains carbohydrate. No appreciable bleaching is observed during proteolysis (Table I).

Proteolysis by Trypsin

Trypsin was without effect on rhodopsin at weight ratios as high as 1:10 and at temperatures between 0 and 40°C. Bleaching of the photoreceptor membranes did not render them susceptible to tryptic degradation.

Isolation of Proteolysis Products Th-1 and Ch-3

Both thermolysin and chymotrypsin degrade rhodopsin to insoluble, carbohydrate-bearing bands (Th-1 and Ch-3, respectively) with little remaining residual rhodopsin. These products were isolated using the affinity chromatography system originally described by Steineman and Stryer (8) for the isolation of rhodopsin. In separate experiments, 1 ml of membrane suspension (containing approximately 5 mg of rhodopsin) was treated at 35°C with thermolysin or chymotrypsin. Additions of protease were made initially and after 6 h to give a final weight ratio of 1:50 (wt/wt). After 24 h the suspensions of membranes were washed with buffer containing protease inhibitors in order to remove soluble fragments and to stop the reaction. In experiments with chymotrypsin, the mixture was centrifuged and washed once with 10 mM Tris-acetate, pH 7.5, 1 mM in phenylmethane sulfonly fluoride and once with the same buffer 1 mM in diisopropyl phosphofluoridate. The suspension of membranes which had been treated with thermolysin was washed three times with 10 mM

<table>
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<th>Designation of fragments</th>
<th>Apparent mol wt</th>
<th>Carbohydrate</th>
</tr>
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<tbody>
<tr>
<td>Th-1 Ch-1 S-1</td>
<td>29,000</td>
<td>+</td>
</tr>
<tr>
<td>S-2</td>
<td>26,000</td>
<td>+</td>
</tr>
<tr>
<td>Ch-3 S-3</td>
<td>23,000</td>
<td>+</td>
</tr>
<tr>
<td>S-4</td>
<td>20,000</td>
<td>–</td>
</tr>
</tbody>
</table>

* Ch, chymotrypsin; Th, thermolysin; S, subtilisin.
† Estimated from SDS gel electrophoresis of proteins of known molecular weight.
§ Indicates the presence of carbohydrate material staining positively with PAS stain.

Figure 3: Proteolysis of photoreceptor membranes by subtilisin. A. Reaction at 0°C and low concentrations of subtilisin. Gel 1. Membrane protein components before addition of protease. Gel 2. Protein components after 24 h of reaction with subtilisin at 0°C. The concentration of rhodopsin in this experiment was 3 mg/ml. The weight ratio of subtilisin to rhodopsin was 1:1,000. B. Reaction at room temperature. Gel 1. Protein components of the membrane preparation before addition of subtilisin. The major, high molecular weight components are aggregates of rhodopsin induced by heating the sample before electrophoresis. Gel 2. Protein components present after 16 h of reaction. Gel 3 represents a gel comparable to gel 2 which has been stained with PAS reagent. The ratio of subtilisin to rhodopsin in this experiment was 1:65.
Tris-acetate, pH 7.5 which was 10 mM in EDTA (28). The membrane material was solubilized in 50 mM sodium acetate buffer, pH 5, 1 mM in CaCl₂, 1 mM in MnCl₂, and 1.4% in hexadecyltrimethylammonium bromide (8), centrifuged, and the supernate applied to a column (0.8 × 16 cm) of concanavalin A-Sepharose. After elution at room temperature, of a fraction absorbing at 280 nm, the material absorbing at 500 nm was eluted with the same buffer 0.1 M in glucose. The elution profile was similar to that obtained by Steineman and Stryer (8) and is not shown here. Appropriate fractions were pooled, dialyzed, and concentrated with an Amicon microultrafiltration system (Model 8MC, Amicon Corp., Scientific Sys. Div., Lexington, Mass.). Detergent was removed by extraction with CHCl₃. Fragment Th-I emerged from the column with a spectral ratio $A_{280}/A_{500}$ of 1.9–2.0, which is similar to that of unmodified rhodopsin isolated in the same way. Fragment Ch-3, however, was substantially bleached during the isolation procedure ($A_{280}/A_{500} = 5$), perhaps reflecting lower stability of the fragment in the presence of detergent. The amino acid compositions of the isolated fragments and purified rhodopsin are reported in Table III.

**DISCUSSION**

A knowledge of the function of a biological membrane requires a thorough understanding of its structure including a description of its components, their interactions, movements, and locations. The disk membrane of the rod cell holds the promise of being one of the more amenable systems for such a study since the membrane can be readily isolated in adequate amounts and has a relatively simple complement of proteins, rhodopsin accounting for approximately 80–90% of the protein present.

![Figure 4](image-url)
Considerable controversy exists, however, as to the exact position of rhodopsin in the disk membrane (9) and the literature is replete with models for the structure of this system (2, 3, 10, 29, 30). Most authors agree that a portion of the polypeptide chain extends within the hydrophobic interior of the membrane. The arguments in favor of this arrangement stem from X-ray diffraction measurements (3, 5), electron microscopy (24), the extremely hydrophobic nature of rhodopsin [as reflected in its amino acid composition (31)], and the interaction of the pigment with lipid hydrocarbon side chains in a reconstituted phospholipid vesicle system (32).

At the same time, chemical modifications have demonstrated that sulfhydryl (6, 33), amino (7, 9, 23), and carbohydrate (8) residues are available for reaction with water-soluble reagents. Wu and Stryer (6) have attempted quantitatively to describe these domains of structure using energy transfer from artificially introduced fluorescent donors to the naturally occurring acceptor chromophore, 11-cis retinaldehyde. They concluded that rhodopsin was a cigar-shaped molecule, at least 75 Å in length, sufficient to span the membrane structure if suitably oriented. They were unable to label rhodopsin with markers closer than about 40 Å to the 11-cis retinal chromophore, suggesting that this was the limit of rhodopsin accessible to water-soluble reagents.

The results of the present investigation demonstrate that the major polypeptide of the disk membrane can be readily degraded by exogenous proteases from a mol wt of 36,000 to approximately 23,000 without bleaching the photopigment. The large size and water solubility of the proteases is assumed to confine their action to those regions of protein exterior to the membrane barrier. By this criterion, about 36% of the rhodopsin molecule is accessible to the bulk aqueous phase.

The lowest molecular weight major species detected in this study was the fragment designated S-4 with a mol wt of 20,000. Production of this fragment, however, was never complete in that mixtures of S-3 and S-4 were generated. Since the latter fragment was not isolated, there is no direct evidence that this material contains bound retinaldehyde, and its designation as a sequential intermediate in the degradation of rhodopsin must await further confirmation.

Recent reports by Trayhurn et al. (34) and Bonting et al. (35) demonstrate that segments of rhodopsin can be removed by proteolysis without affecting the absorption spectrum of the molecule. Previous studies by Radding and Wald (36) were performed using digitonin-solubilized photoreceptor membranes and are hence not directly comparable to the studies described here. They do, however, suggest that rhodopsin is more susceptible to proteolytic degradation after solubilization.

Photoreceptor membranes, treated with either thermolysin or chymotrypsin, were capable of regenerating about 60% of their original absorption at 500 nm after bleaching and addition of 9-cis retinaldehyde (see Table I). The control, which had not been treated with proteases, regenerated about 85% of its original absorption under the same conditions. This result further indicates that the retinal binding region is little affected by the removal of regions of protein accessible to these proteases.

Both thermolysin and subtilisin (at low concentration and temperature) remove a segment of rhodopsin of about 6,000 mol wt, leaving the product Th-1 or S-1 associated with the membrane. Evidently this portion of the polypeptide chain, near one end of the rhodopsin molecule, is particularly susceptible to proteolysis. Phosphate incorporated during phosphorylation of rhodopsin by the endogenous protein kinase (26) appears to be uniquely associated with this region (unpublished experiments). In addition, chymotrypsin and subtilisin produce similar degradation patterns suggesting that their action is confined by the structure of rhodopsin to similar regions of the molecule.

Surprisingly the carbohydrate-bearing portion of the protein is not readily removed by proteases even though experiments of Steineman and Stryer (8) suggest that carbohydrate is readily accessible to water-soluble reagents. As shown in Fig. 3 B, carbohydrate is retained in the insoluble proteolysis band of mol wt 23,000 and is not lost until the molecular weight falls below this value. Such a result would be obtained if the carbohydrate-bearing sequence occurred near the middle of the rhodopsin polypeptide chain or at an end of the molecule not undergoing immediate proteolytic attack. While the PAS stain will not determine whether all the carbohydrate of rhodopsin is present on the proteolytic fragments, this seems likely since there is only one site for fixation of carbohydrate (37, 38).
The procedure used for isolation of Th-1 and Ch-3 requires the complete degradation of rhodopsin to single carbohydrate-bearing fragments since Concanavalin A-Sepharose binds all fragments with \(\alpha\)-D-glucosyl or \(\alpha\)-D-mannosyl residues (39). The gels shown in Fig. 2 illustrate that this requirement was readily met during the analytical procedures in which thermolysin was used. Preparative procedures required raising the temperature to 35°C to obtain the same pattern. The preparative reaction conditions for chymotryptic digestion proved more difficult to control and the isolated fragment was invariably contaminated with a small amount of the next higher molecular weight band (designated Ch-1 in Fig. 4).

It has been suggested that rhodopsin is comprised of two domains of structure which differ in overall hydrophobicity and account for the amphipathic nature of the protein (6, 31, 40). The amino acid compositions presented in Table III do not indicate that the composition of rhodopsin becomes more hydrophobic as one removes the accessible regions. Indeed, the summed mole fractions of polar amino acids (polarity index) (31) are higher for Ch-3 than for rhodopsin and Th-1. This, however, does not rule out the possibility that rhodopsin is anchored to the membrane by interaction with a hydrophobic region of its structure. Such a mode of attachment to a membrane has been suggested for cytochrome \(b_5\) (41) and the major sialoglycoprotein from erythrocyte membranes (42) and remains a viable model for the interaction of rhodopsin and the disk membrane.

The fact that no rhodopsin remains after treatment with the three proteases suggests strongly that the entire population of rhodopsin molecules is accessible to these agents. Since electron microscopy shows this preparation of photoreceptor membranes to consist primarily of closed disk membranes retaining the sidedness of the intact retina, this result argues for an asymmetrical distribution of rhodopsin across the disk membrane, with the protein uniquely situated on the cytoplasmic side.

It has been suggested that, upon bleaching, rhodopsin sinks more deeply into the membrane (3). Such a mechanism would presumably require a considerable structural rearrangement to prevent the direct interaction of polar groups with the hydrophobic environment and might be expected to hinder the approach of the protease. No such burying phenomenon was observed in these experiments. A comparison of the proteolysis patterns of bleached and unbleached rod outer segments suggests that, if anything, rhodopsin is more readily proteolized by thermolysin after bleaching. The observed effect, however, could be simply due to decreased stability of bleached rhodopsin and not to any specific structural change.

The mobility of rhodopsin in the disk membrane is becoming well established. Both rotation and lateral displacements have been reported (4, 43, 44). Displacement in a vertical plane may also be possible, and it is important to note that such movements probably would not be detected by the techniques employed in this study. Thus, the approach will not distinguish between a rhodopsin which maintains a fixed position 50% buried within a membrane and a "bobbing" rhodopsin which alternates between a totally buried position and one that is 50% exposed.

Rhodopsin was completely refractory to the action of trypsin under the conditions of this study. Other proteases, however, were clearly capable of interacting with rhodopsin, and the result obtained with trypsin is most likely due to a lack of susceptible lysyl and arginyl residues in accessible regions. This example emphasizes that care must be used in judging the exposure of a membrane protein based on the use of a single probe.

The controlled proteolysis of rhodopsin described in these experiments, when used in conjunction with other probes of surface regions, should lead to a more detailed description of the position of rhodopsin in the disk membrane and will serve as a convenient starting point for other structural studies.

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