Organization of Rhodopsin in Photoreceptor Membranes. 2. Transmembrane Organization of Bovine Rhodopsin: Evidence from Proteolysis and Lactoperoxidase-Catalyzed Iodination of Native and Reconstituted Membranes†

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ABSTRACT: Proteolysis of reconstituted membranes with papain and thermolysin reveals the existence of two rhodopsin populations: one susceptible to proteolysis and the other protected. The susceptible population corresponds to rhodopsin molecules with the same orientation as rhodopsin in the native membrane, while the protected population corresponds to "inverted" rhodopsin molecules only found in reconstituted membranes. Using an iodination enhancement probe, we demonstrate that lactoperoxidase catalyzes iodination of rhodopsin exclusively on the external surface of these sealed reconstituted vesicles. Furthermore, we find that both rhodopsin populations in reconstituted membranes (normal and inverted) are readily iodinated by lactoperoxidase, providing definitive evidence that the rhodopsin polypeptide spans the membrane thickness. Additional conclusions from these experiments are discussed in terms of a model for the folding of the rhodopsin polypeptide in the membrane.

In the preceding paper of this issue, the papain-cleavage pattern of rhodopsin in the native membrane was examined, and the distribution of rhodopsin sulfhydryl groups among the proteolytic fragments was determined. In the present paper, it will be shown how these results, together with lactoperoxidase-catalyzed iodination and "iodination enhancement probes", can be applied to a comparative study of native and reconstituted rod outer segment membranes to demonstrate that rhodopsin spans the thickness of the membrane. The combined results of this and the preceding paper will be discussed in terms of the polypeptide folding pattern of rhodopsin in the native membrane.

Experimental Section

Materials. Phosphatidylcholine (PC)[1] was purified from hen eggs according to Singleton et al. (1965). Phosphatidylethanolamine (PE) was purified from crude egg phospholipid extracts by chromatography on a silica gel column as described by Hanahan et al. (1958). Cholic acid was decolorized with activated charcoal and then recrystallized from ethanol containing 10% (v/v) water.

Bovine ROS membranes were prepared, and protein content was assayed as previously described (Fung and Hubbell, 1978). Sulfhydryl groups were assayed with 4-PDS as described by Chen and Hubbell (1978).

Preparation of Reconstituted Membranes. Egg PC (10 mg) and egg PE (10 mg) were solubilized in 5 mL of 0.2 M sodium cholate (pH 7.5). This lipid–detergent mixture was added directly to a pellet of ROS membranes containing 20 mg of protein and homogenized gently until the membranes were completely solubilized as judged by the absence of light scattering. Small amounts of insoluble materials were removed by centrifugation at 43 000*g* for 15 min, and the supernatant was then dialyzed against a 125-fold excess of a buffer containing 0.2 M NaCl and 10 mM Mops (pH 7.5). In order to prevent oxidation of unsaturated phospholipids, the dialysis buffer was deoxygenated with nitrogen gas and the dialysis carried out under a blanket of nitrogen. The dialysis buffer was changed four times over a period of 2 days. After this time, the concentration of NaCl in the dialysis buffer was reduced to 10 mM to facilitate efficient removal of the remaining detergent, and dialysis was continued for another 3 days with six changes of buffer solution. The reconstituted membrane vesicles were collected by centrifugation at 73 000*g* for 1 h and then resuspended by gentle homogenization in 10 mM NaCl, 10 mM Mops (pH 7.2).

The internal volume of the vesicles was determined using [^{3}H]sucrose according to published methods (Kornberg and McConnell, 1971). The permeability of the vesicles to D-glucose was determined by monitoring the efflux of the [^{14}C]-glucose from the internal space of the vesicles. For this determination, vesicles were loaded with [^{14}C]glucose by passive equilibration over a period of 24 h in a medium containing 10 mM radioactive glucose and 0.1 M sodium phosphate buffer (pH 7.2). The external [^{14}C]glucose was separated from the vesicles by passing the membrane suspension over a column of Bio-Gel A-0.5 m (1 × 30 cm) preequilibrated with the above buffer without glucose. The vesicles were collected at the void volume and immediately dialyzed against a 200-fold excess of the same buffer without glucose in a rapid dialysis system (Englander and Crowe, 1965). Dialysis buffer was changed every 2 h. Aliquots of the vesicles were removed as a function of time and measured for radioactivity. Permeabilities were calculated from the half-times of escape using mean vesicle dimensions measured by electron microscopy.

Proteolysis of the Reconstituted Membranes with Papain. Reconstituted membrane vesicles were subjected to proteolysis

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[1] Abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; ROS, rod outer segments; 4-PDS, 4,4′-dithiodipyridine; $NaDodSO_4$, sodium dodecyl sulfate; HPEM, *N*-(*p*-hydroxyphenylethyl)maleimide; NEM, *N*-ethylmaleimide; Mops, 3-(*N*-morpholino)-propanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid.

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with papain using the procedure described in the preceding paper of this issue (Fung and Hubbell, 1978). In experiments where lactoperoxidase-catalyzed iodination followed proteolysis, the reconstituted membrane vesicles were digested with papain (weight ratio papain/rhodopsin, 1:50) at room temperature for 12 h. At the end of this period, the cleaved membranes were washed once with buffer containing 10 mM Mops and 10 mM NaCl (pH 7.2), followed by gel filtration on a Bio-Gel A-0.5 m agarose column (1 × 30 cm) preequilibrated in 0.1 M sodium phosphate buffer in order to remove trace amounts of papain.

Enzymatic Iodination of ROS and Reconstituted ROS Membranes. The procedure for the enzymatic iodination of ROS and reconstituted ROS membranes using lactoperoxidase and H_2O_2 generated by a glucose oxidase–glucose system is essentially that described by Hubbard and Cohn (1972). Glucose oxidase and lactoperoxidase were assayed at room temperature according to the Worthington Enzyme Manual (Worthington Biochemical Corp., Freehold, N.J.). Both enzymes were stored at concentrations of 0.7 unit/mL in 0.1 M of sodium phosphate buffer (pH 7.2) at 4.0 °C. The activities of the enzyme in solution were checked every few weeks and were found to be stable over a period of 3 months. Iodination was routinely performed by incubating a suspension of vesicles (2 mg/mL protein) in 0.1 M sodium phosphate buffer (pH 7.2) containing 10 mM D-glucose, 2×10^{-3} mM NaI, 300 μCi/mL ^{125}I, 35 milliunits/mL lactoperoxidase, and 35 milliunits/mL glucose oxidase. After a 30-min incubation at room temperature in the dark, the reaction was terminated by adding 3 volumes of sodium phosphate buffer (pH 7.2) containing 0.1 mM $Na_2S_2O_3$ followed by centrifugation to pellet the membranes. To remove excess enzymes and ^{125}I, the membranes were washed twice by centrifugation at 45 000*g* with 0.1 M sodium phosphate buffer alone. In some cases, the membrane vesicles were then passed through a Bio-Gel A 0.5-m gel-filtration column (1 × 30 cm) to remove the last trace of unincorporated ^{125}I.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out as previously described (Fung and Hubbell, 1978). For radioactive samples, no loss of radioactivity from the protein bands was detected throughout the staining and destaining procedure. Densitometric traces of the protein bands stained with Coomassie blue were obtained by scanning the gels for absorbance at 550 nm. For samples containing ^{125}I, the gels were cut into 1-mm slices, and the amount of radioactive iodide incorporated into each protein band was determined with a gamma radiation spectrometer (Nuclear Chicago Mark II).

To identify the iodinated amino acids, opsin was eluted directly from $NaDodSO_4$–polyacrylamide gels (Castle and Palade, 1978) and digested with Pronase in 0.1% $NaDodSO_4$–detergent solution. After 48 h of enzymatic hydrolysis at 50 °C, the iodinated amino acids and other radioactive materials were fractionated by cellulose thin-layer chromatography (5 × 20 cm) using BuOH–HOAc–H_2O (3:1:1) as the developing solvent. The locations of the amino acids were first detected with ninhydrin. The cellulose thin-layer sheet was then cut into 2-mm strips, and the amount of radioactivity in each strip was determined.

Synthesis of N-(p-Hydroxyphenylethyl)maleimide (HPEM). Tyramine (3 g, 21.8 mmol) was dissolved in 65 mL of dioxane. With stirring, the above solution was added dropwise to 2.1 g (21.4 mmol) of maleic anhydride in 6 mL of dioxane. After the addition was complete, the mixture was allowed to react for another 2 h at room temperature. The light-yellow precipitate was collected, washed with a small amount of dioxane, and dried.

The above product (2.5 g), sodium acetate (0.5 g), and acetic anhydride (100 mL) were heated in a boiling water bath for 3 h. At the end of the period, the acetic anhydride was removed by rotary evaporation under reduced pressure. The resulting solid was dissolved in a small volume of absolute ethanol at 50 °C, filtered hot, and cooled to −20 °C. The light-yellow leaflike crystals of *N*-(*p*-acetoxyphenylethyl)maleimide were collected by filtration. The melting point of this compound was 93 °C, and one spot was observed on a silica gel thin-layer chromatograph developed with $CHCl_3$–CH_3OH (2:1, v/v). Infrared spectra showed the characteristic carbonyl absorption of the maleimide (1710 cm^{-1}) and carbonyl absorption of the phenyl acetate (1780 cm^{-1}). Anal. Calcd for $C_{14}H_{13}NO_4$: C, 64.86; H, 5.05; N, 5.40. Found: C, 64.69; H, 5.11; N, 5.34.

To deacylate the phenol, a catalytic amount of *p*-toluenesulfonic acid monohydrate was added to 0.26 g of *N*-(*p*-acetoxyphenylethyl)maleimide in 50 mL of anhydrous methanol, and the mixture was refluxed for 9 h. After cooling, an equal volume of ether was added, and the mixture was extracted three times, each with 3 volumes of distilled water. The ether phase was dried with Na_2SO_4, and the solvent was removed under reduced pressure to yield a yellow solid (mp 141 °C, uncorrected). Deacylation was confirmed by the disappearance of the infrared absorption at 1780 cm^{-1}. Anal. Calcd for $C_{12}H_{11}NO_3$: C, 66.35; H, 5.10; N, 6.45. Found: C, 66.26; H, 5.18; N, 6.48.

Modification of Rhodopsin with HPEM. The fast-reactive sulfhydryl group of rhodopsin was selectively modified by HPEM by the following procedure: 30 molar equiv of HPEM in ethanol with respect to rhodopsin (2.6 mg in 40 μL) was injected rapidly into a suspension of ROS membranes (15 mg at 4 mg/mL) in a buffer containing 10 mM Mops and 10 mM NaCl (pH 6.7). After 45 min at room temperature, the reaction was terminated and unreacted HPEM removed by washing the membranes with ice-cold buffer three times by centrifugation. The modified membranes were then iodinated according to the procedures described above.

Results

Rhodopsin solubilized in 0.2 M sodium cholate detergent solution is very stable at room temperature in the dark (Henselman and Cusanovich, 1974). When PC and PE were added to the solubilized ROS disk membrane solution and the solution was exhaustively dialyzed to remove the sodium cholate, membrane vesicles containing rhodopsin, ROS lipids, and added phospholipids spontaneously re-formed. The absorption spectra of rhodopsin before and after the reconstitution are identical, and the spectral ratio A_{278}/A_{498} is typically 2.6 to 2.8. Approximately 85% of the rhodopsin in the reconstituted membranes is regenerable with 11-*cis*-retinal after bleaching. All six sulfhydryl groups of rhodopsin are accessible to modification by 4-PDS when the reconstituted membranes are solubilized in 1% $NaDodSO_4$ solution, thus demonstrating that rhodopsin is not oxidized during the course of the dialysis.

The morphology of the reconstituted membrane vesicles was examined by various techniques of electron microscopy, and three important features of the vesicles are recognized: (1) All reconstituted vesicles are single walled and topologically closed. This feature is seen in the thin-section electron micrograph of Figure 1a. (2) The sizes of the reconstituted membrane vesicles are quite uniform, with most of the vesicles having diameters between 400 and 700 Å. The size distribution of the reconstituted membrane vesicles determined from electron micrographs of a platinum-shadowed preparation (Figure 1b) gives an average diameter and variance of 599 and 230 Å, respec-

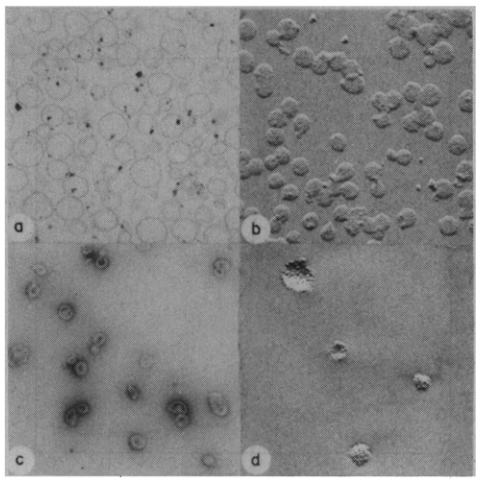

FIGURE 1: Electron micrographs of reconstituted membrane vesicles: (a) thin-section preparation, ×36 800; (b) platinum-shadowed preparation, ×36 800; (c) negatively stained preparations (uranyl acetate), ×36 800; (d) freeze-fracture, ×36 800.

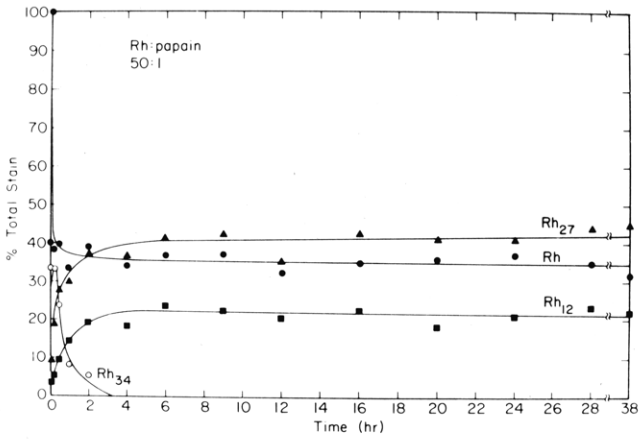

FIGURE 2: Kinetics of production of proteolytic fragments of reconstituted membranes. The percent Coomassie blue stain contained in Na-DodSO$_4$-polyacrylamide gel electrophoresis gel bands representing rhodopsin (Rh) and the proteolytic fragments (Rh_{27}, Rh_{12}) is plotted against time of proteolysis. The amount of stain in each band was estimated by the area of the absorption band in the densitometric scan at 550 nm. This value is expressed as a percentage of the area under the rhodopsin band at time zero. Each data point is an average of three different gels.

tively. (3) In contrast to the native ROS membranes in which the freeze-fracture particles only reside on the concave fracture face (Chen and Hubbell, 1973), particles can be seen on *both* the concave and the convex fracture faces of the reconstituted membranes (Figure 1d). This result demonstrates that the vectorial orientation of rhodopsin in the native membranes is lost during the reconstitution process. Therefore, if rhodopsin is a transmembrane protein, both the cytoplasmic and intradiscal surfaces of the protein should be exposed at the outer-membrane surface of the reconstituted membrane vesicles.

The internal volume of the vesicles as measured by [^{3}H]-sucrose entrapment is approximately 76% of that estimated from the size distribution of the vesicles, assuming a perfectly spherical shape. This may indicate that the vesicles are, in fact, somewhat "flattened" but could also reflect an excluded volume due to the presence of rhodopsin. The glucose permeability of the vesicles is very low, with a permeability of approximately 2×10^{-11} cm/s. This value is comparable in magnitude to that of pure lipid vesicles (Lossen, 1972). Thus, the reconstituted vesicles are "sealed" as well as topologically closed.

Proteolysis of the Reconstituted Membrane Vesicles with Papain. Rhodopsin is vectorially oriented in the native ROS membranes, and in the preceding paper of this issue we have shown that the entire population of rhodopsin molecules is accessible to papain proteolysis (Fung and Hubbell, 1978). Since the vectorial orientation is not preserved in the reconstituted membrane vesicles (Figure 1d), it is anticipated that the proteolytic cleavage pattern of rhodopsin in the reconstituted membranes by papain may be quite different from that of the native disk membranes.

Figure 2 shows the time course of the cleavage of rhodopsin when reconstituted membranes were incubated with papain. It may be seen that ~61% of the rhodopsin is first rapidly cleaved by papain into Rh_{34} (apparent molecular weight 34 500), followed by another much slower cleavage which converts this intermediate into two fragments, Rh_{27} (apparent molecular weight 27 000) and Rh_{12} (apparent molecular weight 12 500). Thus, the cleavage pattern in this population of rhodopsin is exactly the same as that in the native ROS membranes, as discussed in the preceding paper of this issue. The remaining ~39% of the rhodopsin in the reconstituted membrane, however, is inaccessible to papain cleavage even after prolonged enzymatic treatment. The total integrated Coomassie blue staining intensity of the rhodopsin band after the initial rapid drop of 61% remains fairly constant over a period of 38 h of proteolysis. Proteolytic cleavage of rhodopsin does not lead to rupture of the vesicles, since the glucose permeability does not change dramatically during proteolysis. Vesicles containing cleaved rhodopsin have a glucose permeability of approximately 4.5×10^{-11} cm/s, compared to 2.0×10^{-11} cm/s for uncleaved controls. Thus, it is reasonable to assume that the vesicles remain impermeable to the high-molecular-weight papain during the time course of proteolysis.

When reconstituted vesicles were disrupted with either 100 mM cholate or 2% Triton X-100 and treated with papain under the same conditions, *all* rhodopsin was degraded to Rh_{27} and Rh_{12}, suggesting that the membrane barrier protects the population of undigested rhodopsin in the reconstituted vesicles (neither cholate nor Triton X-100 denature rhodopsin in the dark). The simplest interpretation of these results when compared with those obtained from proteolysis of the native ROS vesicles is that the "inverted" population of rhodopsin in the reconstituted vesicles is refractory to proteolysis, while the "normal" orientation is susceptible, yielding the same proteolytic fragments as found in the native ROS vesicles. Further evidence in support of this interpretation will be discussed below. The fact that the population of rhodopsin with the inverted orientation remains intact during proteolysis does not necessarily imply that the molecule does not span the membrane thickness, since the inverted population may present a portion of the polypeptide on the external surface that simply lacks a proteolytically sensitive site. These results do provide, however, a convenient method for distinguishing the normal and inverted orientational populations of rhodopsin in the reconstituted membrane. This capability, together with the use of membrane-impermeable labeling reagents, has allowed us to determine whether or not rhodopsin is transmembrane, since *both* orientational populations will be labeled on the exterior surface with impermeable reagents if and only if the polypeptide spans the bilayer. For this purpose, we have chosen the lactoperoxidase-catalyzed iodination procedure originally devised by Morrison (Phillips and Morrison, 1970, 1971). We have employed impermeable fluorescent diazonium labels with similar results (Fung, 1977), but only the lactoperoxidase system will be discussed here.

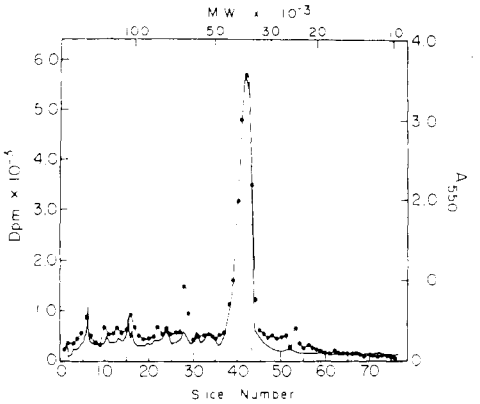

FIGURE 3: Absorbance (—) and radioactivity (●) profiles of a NaDodSO$_4$-polyacrylamide gel of ROS membranes after lactoperoxidase-catalyzed iodination with ^{125}I.

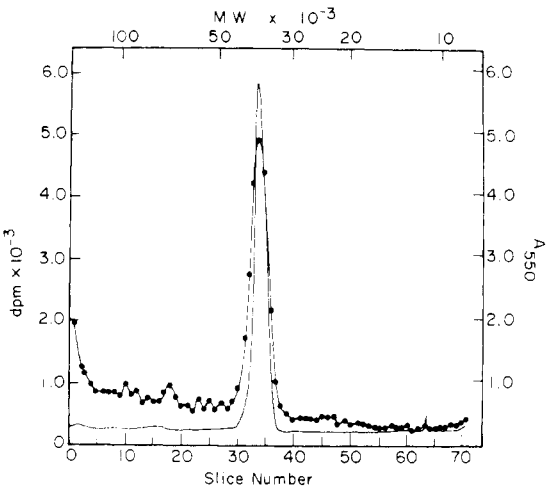

FIGURE 4: Absorbance (—) and radioactivity (●) profiles of a NaDodSO$_4$-polyacrylamide gel of reconstituted membranes after lactoperoxidase-catalyzed iodination with ^{125}I. The major peak in both profiles corresponds in molecular weight to opsin.

Lactoperoxidase-Catalyzed Iodination of ROS Membranes. In the presence of H_2O_2, lactoperoxidase catalyzes the iodination of tyrosyl residues in proteins (Morrison and Schoubaum, 1976). Under proper conditions, it has been shown that the iodination reaction occurs via an enzyme-substrate complex between the protein substrate and the lactoperoxidase (Morrison and Bayse, 1970; Morrison and Schoubaum, 1976). Since lactoperoxidase is relatively large (molecular weight 78 000), it is unable to penetrate intact cells (Hubbard and Cohn, 1972, 1975; Phillips and Morrison, 1971) or closed subcellular organelles (Kreibich et al., 1974; Huber and Morrison, 1973; Huber et al., 1975). Thus, the iodination reaction is ideally limited to those membrane surfaces exposed to lactoperoxidase and should provide information on the asymmetric disposition of the membrane proteins. In the following sections, we present experimental results obtained using this technique to probe the accessibility of rhodopsin in native ROS and reconstituted membranes. It is expected that the reconstituted membrane vesicles should be impermeable to lactoperoxidase, since papain, a protein considerably smaller than lactoperoxidase, apparently cannot cross the reconstituted membranes. During the course of this investigation, it was observed that any excess H_2O_2 present during the iodination reaction caused oxidation of both rhodopsin and phospholipids, resulting in irreversible formation of protein aggregates. Therefore, a mild iodination procedure using glucose oxidase and glucose as an H_2O_2 generating system was used (Hubbard and Cohn, 1972, 1975). With this system, oxidation of rhodopsin was greatly reduced, and no aggregation of protein was found. Several different factors which influence the efficiency of the iodination reaction of ROS disk membranes have been examined (Fung, 1977). From these results, the routine procedure given under the Experimental Section for the iodination of rhodopsin has been adopted. Figure 3 shows a densitometric scan of a polyacrylamide gel of ROS membranes after enzymatic iodination using Na^{125}I. Only the major band corresponding to opsin is stained heavily with Coomassie blue. There is no change in the electrophoretic mobility of opsin before and after iodination, and the apparent molecular weight of labeled opsin remains the same as the native protein. When the same gel was examined for radioactivity, the majority of the applied radioactivity was found to be associated with the opsin band (Figure 3). Approximately 10% of the radioactive label migrated with lipid and free iodide in front of the tracking dye. Of the 90% radioactive iodide incorporated into the proteins, 55% of the radioactivity was associated with the opsin band, while the remaining label was distributed among various minor bands. Under the conditions used here, approximately 1.5 × 10^{-4} mol of iodide was incorporated/mol of rhodopsin. When papain-treated ROS membranes were iodinated with lactoperoxidase and the fragments resolved on NaDodSO$_4$-polyacrylamide gel electrophoresis, it was found that both Rh_{27} and Rh_{12} were radioactively labeled (not shown). Deleting either lactoperoxidase, glucose, or glucose oxidase from the iodination system consistently reduced the amount of radioactive iodide incorporation by greater than 95%, indicating that the iodination reaction is enzyme specific. Furthermore, if the disk membrane suspensions were separated by a piece of dialysis membrane from the total iodination system, no iodination of the membrane occurred. This result eliminates the possibility of nonspecific iodination of the membranes due to the formation of long-lived, diffusable species generated by the enzyme and capable of reaction with rhodopsin.

To ascertain the products of the iodination reaction, we have examined the identity of iodinated amino acids of opsin according to the procedure described under the Experimental Section. Approximately 66% of the total radioactivity on the chromatogram was found to migrate at the position of a monoiodotyrosine standard, while ~30% was associated with the undigested polypeptides near the origin or with background counts distributed evenly over the profile. Thus, the majority of radioactivity is associated with monoiodotyrosine, and there is no evidence for iodination of other amino acids.

Iodination of Papain-Cleaved Reconstituted Membranes. Similar to the ROS disk membrane, rhodopsin in the reconstituted membrane can be enzymatically iodinated with ^{125}I using lactoperoxidase with a balanced amount of hydrogen peroxide generated by a glucose oxidase-glucose system. As shown in Figure 4, the majority of the radioactivity is found to comigrate with the opsin band when the labeled reconstituted membrane is analyzed by NaDodSO$_4$-polyacrylamide gel electrophoresis. Under identical conditions, the total amount of radioactive iodide incorporated into rhodopsin in reconstituted membranes is found to be 30–40% less than the amount incorporated into rhodopsin in native ROS membranes. Since the iodination reaction is not allowed to proceed to a stoichiometric end point, but stopped at a specific time, this effect is probably due to differences in the kinetics of the reaction due to different surface properties in the native and reconstituted membranes. Iodination of the papain-cleaved rhodopsin in reconstituted membranes does not alter the glucose permeability of the vesicles.

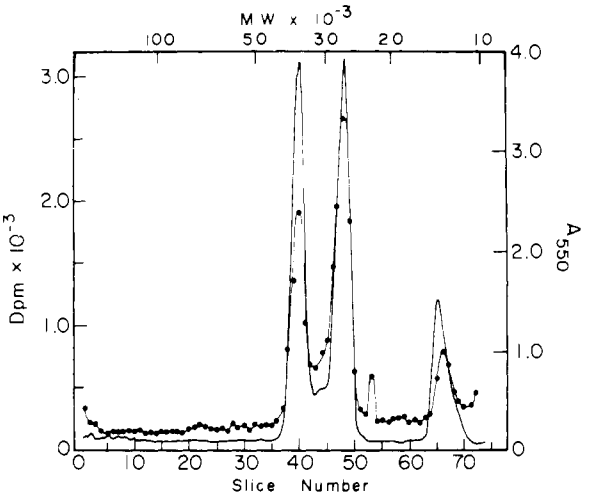

FIGURE 5: Absorbance (—) and radioactivity (●) profiles of a NaDodSO$_4$–polyacrylamide gel of reconstituted membranes following proteolysis and lactoperoxidase-catalyzed iodination with ^{125}I.

The important experiment is to iodinate reconstituted membrane vesicles after papain proteolysis so that the two orientational populations are distinguishable in NaDodSO$_4$–polyacrylamide gels.[2] Figure 5 shows the distribution of radioactivity on a NaDodSO$_4$–polyacrylamide gel of the reconstituted membrane following papain treatment and lactoperoxidase-catalyzed iodination with ^{125}I. Radioactive iodide is incorporated into both Rh_{27} and Rh_{12} polypeptide bands, indicating that both fragments are accessible to enzymatic iodination. In addition, the band containing uncleaved rhodopsin (i.e., those molecules having the *inverted orientation*) shows labeling to an extent of approximately 30% of the total radioactivity associated with rhodopsin and its cleavage products. The above results indicate that *both* orientational populations are exposed at the external surface of the vesicles. Thus, the polypeptide must be transmembrane. This conclusion, of course, is subject to verification that iodination occurs exclusively at the external surface of the vesicles.

Iodination Enhancement of Rhodopsin Using HPEM. To demonstrate that iodination is restricted to the external surface of the vesicles, the fast-reacting sulfhydryl group of rhodopsin located on Rh_{12} (Fung and Hubbell, 1978) has been modified with the iodination enhancement probe HPEM. The phenolic group of HPEM provides an additional site for iodination on the protein. The rationale of the experiment is straightforward and shown schematically in Figure 6. Each rhodopsin molecule is specifically labeled on the fast-reacting sulfhydryl group with HPEM in the native membrane *before* reconstitution. *During* reconstitution, the rhodopsin orientation is randomized. If HPEM is located on a region of rhodopsin exposed to the aqueous solution in the native membrane, some population of HPEM will now reside at the inner surface of the reconstituted membrane vesicles. If iodination by lactoperoxidase occurs exclusively at the external membrane surface, only one of the two orientational populations will show enhanced iodination as a result of the HPEM label on Rh_{12}. On the other hand, if lactoperoxidase or some reactive iodine species reaches the inner surface, both populations should show enhanced iodination. Since the orientational populations are easily resolved on NaDodSO$_4$–polyacrylamide gel electrophoresis after

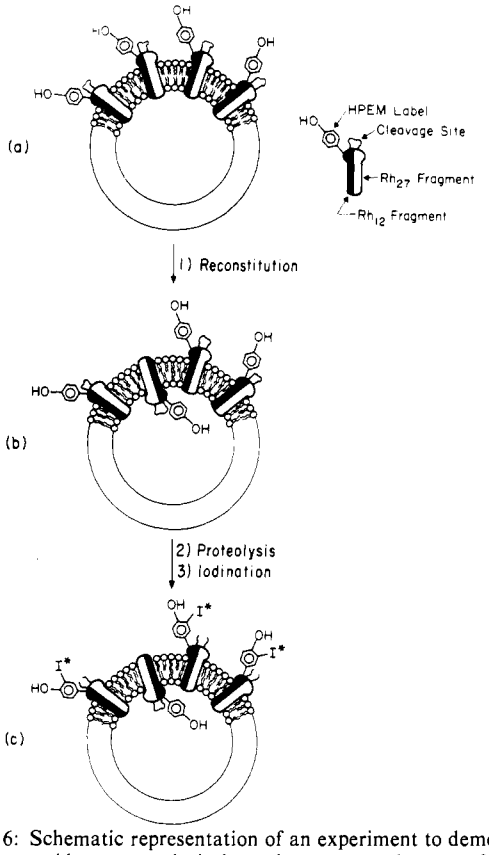

FIGURE 6: Schematic representation of an experiment to demonstrate that lactoperoxidase acts exclusively on the outer membrane surface. The top drawing represents a vesicle of native ROS membranes in which the rhodopsin has been labeled with HPEM (Ph-OH) on the Rh_{12} sulfhydryl group. The various features of the rhodopsin are indicated in the drawing of the molecule at the upper right. The first step in the experiment is reconstitution, which results in randomization of the protein orientation as shown in the center drawing. The second step is proteolysis: only the molecules with the same orientation as the native molecule will be susceptible to proteolysis, which is indicated schematically as a break in the loop connecting Rh_{27} and Rh_{12}. The final step is lactoperoxidase-catalyzed iodination. If iodination occurs exclusively at the external surface, the HPEM label on the *uncleaved* (inverted) population will not be iodinated.

papain proteolysis, it is straightforward to quantitate their relative iodination enhancements and apply the above logic to determine whether or not iodination occurs exclusively at the vesicle external surface. This scheme will be successful if the HPEM label is iodinated to an extent easily detectable above the normal iodination level of rhodopsin.

Before presenting the results of the experiment outlined in Figure 6, it is necessary to demonstrate the usefulness of the HPEM label as an iodination enhancement probe. When ROS membranes in 10 mM NaCl, 10 mM Mops (pH 6.7) were reacted with a 30-fold molar excess of HPEM with respect to rhodopsin for 45 min, approximately 1.4 sulfhydryl groups per rhodopsin were labeled as determined by titration with 4-PDS. In order to confirm that the majority of the HPEM is attached to the fast-reactive sulfhydryl group as is the case with *N*-ethylmaleimide (Fung and Hubbell, 1978), HPEM-labeled rhodopsin was cleaved by papain in the ROS membranes and iodinated, and the Rh_{12} and Rh_{27} fragments were resolved on NaDodSO$_4$–polyacrylamide gel electrophoresis. As a control, ROS membranes were also allowed to react with *N*-ethylmaleimide under identical conditions and subjected to proteolysis and iodination.[3] There was very little difference in the

[2] An important result of the preceding paper was that cleaved rhodopsin is essentially identical in properties to the native protein. Thus, results of the experiments presented here do not depend on whether iodination is performed prior to or after proteolysis. In most cases, proteolysis was carried out before iodination to minimize manipulation with the radioactive sample.

[3] Reaction of HPEM with rhodopsin could be blocked by prior labeling with NEM, indicating that NEM and HPEM react with the same sulfhydryl group.

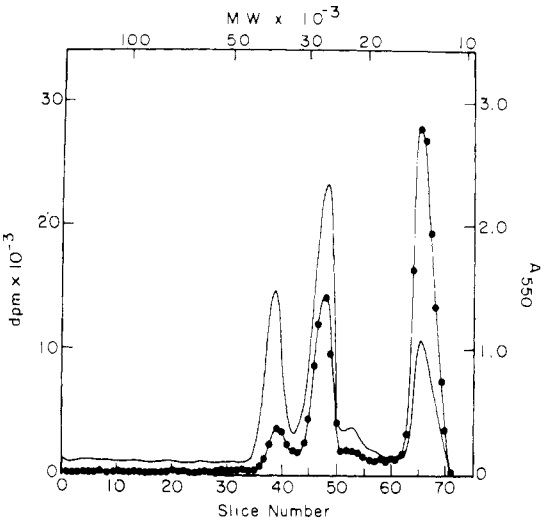

FIGURE 7: Absorbance (—) and radioactivity (O) profiles of a NaDodSO$_4$-polyacrylamide gel of HPEM-labeled reconstituted membranes after proteolysis and lactoperoxidase-catalyzed iodination. The radioactivity in the Rh_{12} fragment of the rhodopsin population with the normal (cleaved) orientation is tremendously enhanced relative to that in the inverted (uncleaved) population. The inverted population shows little or no enhancement of iodination even though it contains a HPEM label.

amount of radioactive iodide incorporation between native rhodopsin and rhodopsin labeled with NEM. In contrast, the amount of radioactive iodide incorporated into rhodopsin bearing a single HPEM label was four times greater than that labeled with NEM, demonstrating that HPEM-enhanced iodination is easily detectable above the normal level of labeling of rhodopsin. Furthermore, 64% of the radioactivity in the polyacrylamide gel was found to migrate at the position of the Rh_{12} fragment in the HPEM-labeled protein, whereas in the absence of HPEM only 29% of the total radioactivity resided at the Rh_{12} fragment. Thus, the increase in radioactive iodide incorporation into the Rh_{12} fragment must be due to the additional ^{125}I incorporated into the HPEM label.[4] There is also a small increase in the iodination of the Rh_{27} fragment in the HPEM-labeled protein. This is expected, since perfect selectivity in HPEM labeling of the fast-reactive group on Rh_{12} is not achieved, and some fraction of the slow-reacting group on Rh_{27} was also labeled with HPEM.

Rhodopsin labeled with HPEM in the ROS membrane can be reconstituted by the cholate dialysis method with a recovery greater than 90% based on the absorbance at 500 nm. For the actual test of exclusive external surface iodination, HPEM-labeled *reconstituted* membrane vesicles were digested with papain and iodinated with lactoperoxidase as diagrammed in Figure 6, and the resulting fragments were separated by NaDodSO$_4$-polyacrylamide gel electrophoresis. The cleavage pattern of the HPEM-labeled rhodopsin is found to be essentially identical to the unlabeled reconstituted membrane; however, the radioactivity distribution profile (Figure 7) indicates that ^{125}I incorporation into the Rh_{12} fragment of the rhodopsin with the normal orientation has increased tremendously relative to that of unlabeled reconstituted membranes treated in the same way (Figure 6). The total increase in radioactivity of the HPEM-labeled rhodopsin with the normal orientation is approximately seven times, with 69% of the radioactivity residing on the Rh_{12} fragment. There is also a small increase in radioactivity on the Rh_{27} fragment due to the incorporation of radioactive iodide into the small amount of HPEM that has reacted with Rh_{27}. Thus, rhodopsin with the *normal* orientation in reconstituted membranes behaves similarly to rhodopsin in native membranes with respect to HPEM-induced increases in radioactivity.

On the other hand, the radioactivity incorporated into rhodopsin with an inverted (uncleaved) orientation is almost the same as the control and is substantially less than the radioactivity in the Rh_{12} fragment. Only 6% of the total radioactivity is associated with the intact rhodopsin band, although the Coomassie blue staining intensity is approximately 30% of the total. If iodination occurred inside the reconstituted membrane vesicles either by lactoperoxidase catalysis or by any other reactive species, the HPEM covalently bound to the rhodopsin with inverted orientation should be labeled, and the calculated amount of ^{125}I incorporated into rhodopsin with the inverted orientation should be at least as high as the Rh_{12} fragment. Since this is not the case, iodination must occur only at the external membrane surface. These data, together with the fact that rhodopsin with the inverted orientation is accessible to iodination, provide conclusive evidence showing that rhodopsin is a transmembrane protein in the reconstituted membrane.

Discussion

The results of our experiments presented above provide direct evidence that the rhodopsin polypeptide spans the thickness of the membrane. The only important assumption on which this conclusion rests is that the population of rhodopsin in reconstituted membranes protected from proteolysis is indeed an "inverted" population rather than, say, a denatured population. This latter possibility is an unlikely one, since at least 85% of the reconstituted rhodopsin can be regenerated after bleaching. Accepting regenerability as a criterion for the native structure, at most 15% could then be considered denatured, while our results indicate that 30–40% remains proteolytically insensitive. In addition, we find that the amount of bleached rhodopsin in the reconstituted membranes is unrelated to the population of protected protein, since similar cleavage patterns were obtained even though the entire population of rhodopsin is bleached prior to or after reconstitution (see Results). Perhaps the most convincing *chemical* evidence that the protected population corresponds to an inverted orientation is based on the fact that the disruption of the reconstituted membrane with nondenaturing detergents during proteolysis leads to complete degradation of all protein to the same proteolytic fragments found for rhodopsin in the native membrane. This suggests that the membrane barrier rather than a difference in protein conformation protects a fraction of the protein from cleavage. Furthermore, the fact that an HPEM label on the protected population of rhodopsin is inaccessible to lactoperoxidase is also consistent with the protected population being inverted. The freeze-fracture images of reconstituted membranes provide direct *physical* evidence for the existence of an inverted population. From the size distribution of the vesicles and the bilayer thickness, it is possible to estimate the expected fraction of protected rhodopsin. As a first approximation, it is reasonable to assume that the ratio of normal to inverted populations should stand in the same ratio as the surface areas of the outer and inner monolayers of the membrane. From the vesicle size distribution function and a bilayer thickness of 50 Å, we have estimated this ratio to be approximately 1.5. The ratio of cleaved to protected rhodopsin,

[4] Direct evidence has not been presented to demonstrate that HPEM is, in fact, iodinated by lactoperoxidase when attached to rhodopsin. However, it is well known that such labels are excellent substrates for lactoperoxidase and are commonly used to provide labels for the iodination of water-soluble proteins. The iodination of rhodopsin is not enhanced by NEM labeling, and it is very unlikely that HPEM simply perturbs the conformation of rhodopsin to produce the enhancement observed.

as measured from the staining intensity of the polyacrylamide gels, is typically 1.7, tolerably close to the ratio of surface areas. In view of all of the above evidence, we conclude that there exist "normal" and "inverted" rhodopsin populations in the reconstituted vesicles that are distinguished by their differential sensitivity to proteolysis.

At this point, it is helpful to restate the evidence leading to the conclusion that rhodopsin is transmembrane. The important result is that both the normal and inverted populations in the reconstituted membrane are iodinated by lactoperoxidase (Figure 5), which we have shown acts exclusively at the vesicle exterior surface; this can occur only if the rhodopsin polypeptide spans the membrane. For the sake of further discussion, it is expedient to adopt a nomenclature to describe the membrane topology of the rhodopsin molecule. Since the surface orientation of the membrane in the isolated ROS vesicles is the same as that in the disk membranes from which they are derived (Chen and Hubbell, 1973), the exterior surface of the ROS vesicles corresponds to the cytoplasmic surface of the native disk membranes. Hence, we will refer to the region of rhodopsin exposed on the exterior surface of the isolated ROS vesicles as the *cytoplasmic surface* or *C surface* of the protein. The region of rhodopsin exposed at the interior surface of the ROS vesicles corresponds to that at the intradiscal surface of the native disk and will be called the *intradiscal surface* or *I surface* of the protein.

Additional conclusions that may be drawn from the results of this and the preceding paper in this issue are most easily discussed in terms of a model for the rhodopsin polypeptide in the native disk membrane. This model is presented in Figure 8. The first feature of the model is that the rhodopsin polypeptide spans the membrane thickness, which was the principal result presented above. At present, we have no evidence to indicate whether or not *both* Rh_{12} and Rh_{27} are transmembrane. Rh_{27} clearly spans the membrane, since it bears the carbohydrate moiety, which is located, according to Röhlich (1976), at the I surface of rhodopsin but is produced by proteolytic action directed exclusively at the C surface (see below). We have drawn Rh_{12} to be transmembrane only as a suggestion.

As tentatively concluded in the preceding paper (Fung and Hubbell, 1978), it is now clear that both the first rapid cleavage that removes a terminal sequence and the second cleavage that produces two membrane-bound fragments must occur on the C surface of rhodopsin. This follows simply from the fact that the inverted population is not susceptible to proteolysis even though rhodopsin spans the thickness of the membrane. This implies that the portion of rhodopsin normally exposed on the intradiscal space simply has no accessible sites for proteolytic attack by papain. This conclusion is also consistent with the fact that disruption of the membrane barrier of ROS vesicles with Triton or sodium cholate detergents during proteolysis does not lead to any additional cleavage of Rh_{27} and Rh_{12} (Fung and Hubbell, 1978; Pober and Stryer, 1975).

As discussed in the previous paper of this issue, the carbohydrate of rhodopsin, which is known to be located very near the amino terminus, is carried exclusively on the Rh_{27} fragment. Hence, the terminal fragment removed from the surface by the first fast cleavage must contain the carboxyl terminus. It is also evident that this fragment (or fragments) must be continuous with the Rh_{12} polypeptide before the cleavage. Since the carbohydrate is apparently on the intradiscal surface, the amino terminus must also be located at that surface (Hargrave, 1977). Thus, the carboxyl and amino termini are on opposite sides of the membrane as shown in the model. The fact that *both* Rh_{12} and Rh_{27} are membrane bound indicates that the polypeptide must cross the membrane surface more

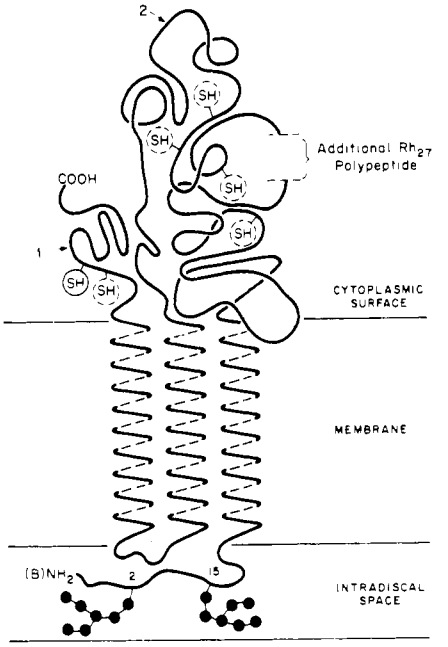

FIGURE 8: A model for the organization of rhodopsin in disk membranes. Only enough of the polypeptide chain is shown to illustrate the major topological features. The placement of the sugar residues (filled circles) with respect to the membrane surfaces is after Röhlich (1976). The position of the sugars relative to the N-terminus (blocked) is after Hargrave (1977). The sites of the fast (1) and slow (2) cleavage are marked by arrows. The Rh_{12} fragment is that portion of the polypeptide between arrows 1 and 2. The portion of Rh_{27} that is not shown is indicated by the break in the polypeptide. The fast-reacting sulfhydryl group is located at the cytoplasmic surface and is circled with a solid line. Other sulfhydryl groups circled with dotted lines represent those with an uncertain location relative to the membrane surface. They are shown only to indicate the distribution relative to Rh_{12} and Rh_{27}.

than once, otherwise no more than one membrane-bound product could be produced by proteolysis at the cytoplasmic surface. With the carboxy and amino termini on opposite sides of the membrane, the total number of crossings must be odd and greater than or equal to three. It is evident that the polypeptide of Rh_{12} must cross the cytoplasmic surface at least twice (as shown), since both proteolytic cleavages which produce the fragment occur at the cytoplasmic surface. The minimum requirement of two crosses of Rh_{12} is likely to represent the actual number as well, since the molecular weight of the peptide is too low to accommodate four or more crossings if the fragment is indeed transmembrane (the number of Rh_{12} crossings at the surface must be even in number). The number of transmembrane excursions of Rh_{27} cannot be deduced from our data, and we can only conclude that the crossings must be odd in number. However, considering the high molecular weight of this fragment and the large hydrophobic domain of the protein (as suggested in freeze-fracture images), it is likely that Rh_{27} crosses the membrane more than once.

The transmembrane stretches of polypeptide are shown here as α helical, this feature being speculative but reasonable. On thermodynamic grounds, only secondary structures with satisfied hydrogen-bonding capacity are expected to exist in the low dielectric membrane interior. Either α helices or β structures will satisfy the requirement. However, nonresonance Raman spectroscopy (Rothschild et al., 1976) and circular dichroism (Shichi et al., 1969; Shichi and Shelton, 1974; Stubbs et al., 1976) studies indicate extensive α-helical conformation but little or no β-structures. We are thus left to consider α-helices as the primary candidate for transmembrane secondary structures. Other evidence supporting the existence of oriented transmembrane α helices in rhodopsin has been

discussed in detail elsewhere along with the functional implications of this type of structure (Hubbell and Fung, 1978). The arguments will not be reproduced here, since our main objective is to illustrate the topological organization of rhodopsin as deduced from proteolysis and labeling experiments. However, we note that if the transmembrane segments are α helical the maximum number of transmembrane excursions will be constrained by the total α-helix content of rhodopsin and the thickness of the bilayer. Various estimates for the helix content of rhodopsin based on circular dichroism range from 47 to 60% (Shichi et al., 1969; Shichi and Shelton, 1974; Stubbs et al., 1976), and the membrane hydrophobic thickness is about 40 Å. This range of values would place an upper limit between six and eight transmembrane helical segments.

Rhodopsin has a total of six free sulfhydryl groups: four on Rh_{27} and two on Rh_{12} (Fung and Hubbell, 1978). In the dark-adapted membrane-bound protein, two sulfhydryl groups are reactive, one on Rh_{12} and one on Rh_{27}. The highly reactive group on Rh_{12} is located on the C surface of the protein, as indicated in Figure 8 by the heavy circle. This follows directly from the observation that an HPEM label attached to this sulfhydryl group is readily iodinated only in the population of rhodopsin susceptible to proteolysis in the reconstituted membrane, i.e., the normal orientation. We have not yet carried out experiments to locate the other sulfhydryl groups but have included them in the model only to show their distribution on the proteolytic fragments. The retinal chromophore is not included in the model, since our experiments give no information regarding its location. It is clear, however, that the chromophore is covalently linked to the Rh_{12} fragment.

It should be emphasized that the topological assignments presented here rely heavily on the correct assignment for the location of the sugar groups. This assignment is based on the experiments of Röhlich (1976), which represent the best information available at this time. If the sugars were located externally, the amino and carboxyl termini would both be placed on the external surface and the number of polypeptide surface crossings would be even in number.

The approach used in these experiments to obtain information on the topological organization of rhodopsin in membranes is quite general and should be applicable to other systems where reconstitution is feasible. It should be particularly useful in systems such as the ROS membranes where "inside-out" vesicles are not easily obtained.

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