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Identification of Frog Photoreceptor Plasma and Disk Membrane Proteins by Radioiodination†

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ABSTRACT: Several functions have been identified for the plasma membrane of the rod outer segment, including control of light-dependent changes in sodium conductance and a sodium–calcium exchange mechanism. However, little is known about its constituent proteins. Intact rod outer segments substantially free of contaminants were prepared in the dark and purified on a density gradient of Percoll. Surface proteins were then labeled by lactoperoxidase-catalyzed radioiodination, and intact rod outer segments were reisolated. Membrane proteins were identified by polyacrylamide gel electrophoresis and autoradiography. The surface proteins labeled included rhodopsin, the major membrane protein, and 12 other proteins. Several control experiments indicated that the labeled proteins are integral membrane proteins and that label is limited to the plasma membrane. To compare the protein composition of plasma membrane with that of the internal disk membrane, purified rod outer segments were lysed by hypotonic disruption or freeze–thawing, and plasma plus disk membranes were radioiodinated. In these membrane preparations, rhodopsin was the major iodinated constituent, with 12 other proteins also labeled. Autoradiographic evidence indicated some differences in protein composition between disk and plasma membranes. A quantitative comparison of the two samples showed that labeling of two proteins, 24 kilodaltons (kDa) and 13 kDa, was enriched in the plasma membrane, while labeling of a 220-kDa protein was enriched in the disk membrane. These plasma membrane proteins may be associated with important functions such as the light-sensitive conductance and the sodium–calcium exchanger.

The retinal rod photoreceptor is an elongated sensory receptor cell which transduces a light stimulus to a change in membrane potential, ultimately resulting in neurotransmitter release at the synaptic end of the cell. The portion specialized for phototransduction, the rod outer segment (ROS), consists of 1000–2000 flattened membranous disks physically separate from the surrounding plasma membrane. In the dark, a current of sodium ions flows through cation channels into the outer segment (the "dark current") and is balanced by a flow of potassium ions into the rod inner segment [reviewed in Matthews & Baylor (1981)]. Initial steps in the light response involve proteins associated with the disk membrane: photoisomerization of the retinal chromophore of rhodopsin, the major disk membrane protein; activation by rhodopsin of a GTP binding protein; and activation by the GTP binding protein of a cyclic GMP phosphodiesterase, which in turn hydrolyzes cGMP [see Chabre (1985) and Stryer (1986) for recent reviews]. Cation channels in the plasma membrane appear to be regulated by cGMP (Fesenko et al., 1985; Yau & Nakatani, 1985; Zimmerman et al., 1985).

Constituents of the plasma membrane, which include a sodium–calcium exchanger (Schnetkamp, 1980; Yau & Nakatani, 1984) as well as the light-regulated cation channel, have been difficult to isolate and study since the plasma membrane represents less than 2% of the total membrane in the amphibian ROS. In addition to regulating light-dependent ion permeability, the plasma membrane also probably contains recognition sites controlling phagocytosis of disks shed at the apical tip of the ROS (Hall, 1978), and may also contain receptors for renewal of the retinal destroyed by photobleaching.

Several different approaches have been taken to identifying plasma membrane constituents. Rhodopsin has been localized in the plasma membrane by using anti-rhodopsin antibodies (Dewey et al., 1969; Jan & Revel, 1974; Papermaster et al., 1978a). Surface labeling by radioiodination has identified three proteins on bovine ROS in addition to rhodopsin (Clark & Hall, 1982). A purification method for plasma membranes based on binding of surface glycoproteins to concanavalin A coated beads has yielded a membrane fraction which differs from total ROS membrane in both protein and fatty acid composition (Kamps et al., 1982).

This study has aimed toward identifying the constituents of the frog ROS plasma membrane labeled by lactoperoxidase-catalyzed radioiodination. To ensure that labeling was surface specific, purified and intact ROS were separated before and after labeling from leaky ROS and retinal contaminants by a method utilizing a discontinuous density gradient of Percoll (Biernbaum & Bownds, 1985).

This study has also examined whether any proteins are unique to the plasma or disk membrane by comparing the surface-labeled proteins with those labeled in ROS lysed by hypotonic disruption and by freeze–thawing, where disk membrane and also plasma membrane were accessible to labeling. Although the plasma and disk membranes appear to have different functions in vivo, some experimental evidence suggests their complements of functional proteins may not be different. Cyclic GMP dependent cation fluxes have been identified in disk membranes (Caretta & Cavaggioni, 1983; Koch & Kaupp, 1985; Caretta, 1985) as well as in the plasma membrane. Also, sodium–calcium exchange activity has been observed in intact ROS, presumably reflecting activity on the plasma membrane, and in leaky ROS, reflecting disk membrane activity (Schnetkamp et al., 1977; Schnetkamp, 1980). Disk membranes appear to be continuous with and probably formed from plasma membrane at the site of the connecting cilium (Young & Droz, 1968; Peters et al., 1983; Papermaster et al., 1982). If the protein compositions are different, a

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mechanism for specifically localizing proteins to one membrane or the other would be implicated.

Materials and Methods

Materials. Percoll was from Pharmacia, Inc. Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) was from Pierce Chemical Co. [^{32}P]ATP and Na^{125}I (carrier free) were from New England Nuclear. All other chemicals were from Sigma Chemical Co.

Preparation of Intact Rod Outer Segments. Retinas were isolated from dark-adapted bullfrogs (*Rana catesbeiana* or *Rana grylio*) and rinsed in a Percoll–Ringer's solution: 105 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 5% Percoll, 5 mM glucose, and 5 mM $NaHCO_3$, at pH 7.6. Details of dissection and preparation of the Percoll solution are given in Biernbaum and Bownds (1985). Retinas were shaken gently in a 5% Percoll–Ringer's; any large pieces of material were allowed to settle. The rod outer segment (ROS) suspension was layered onto a preformed discontinuous gradient of Percoll–Ringer's with layers of 70%, 45%, and 5% Percoll in a 3.5-mL plastic centrifuge tube, and the gradient was centrifuged for 4 min at 4000 rpm. Intact ROS were recovered from the 70–45% interface, while broken or permeabilized ROS sedimented to the 45–5% interface. Light microscopic examination showed that the intact ROS preparation was free of contamination by other cell types, and contamination by subcellular particles was unlikely because they do not enter the Percoll gradient (Hamm & Bownds, 1985). The intact ROS preparation was then washed in a Ringer's solution lacking Percoll (115 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.6) and divided into aliquots for different procedures. To determine intactness, a 5-μL aliquot of ROS was mixed on a microscope slide with 5 μL of 50 μM didansylcystine and observed under epifluorescent illumination (Yoshikami et al., 1974). Leaky ROS absorb the dye and are easily differentiated from intact ROS. In some experiments, as few as 1% of the ROS were "leaky" by this assay. The proportion of ROS to which the inner segment remained attached was also quantitated at the same time by using phase microscopy. The percent of inner segment contamination varied between 2% and 50%; the inner segment content in a given experiment is noted under Results. All procedures were carried out in darkness under infrared illumination. Rhodopsin concentration was measured by difference spectroscopy (Bownds et al., 1971). The yield of rhodopsin, which represents about 70% of the total purified ROS protein, was typically about 0.1–0.2 mg/retina.

Iodination of Rod Outer Segments. (*A*) *Iodination of Intact ROS.* Intact ROS were iodinated by using two different catalyst systems which covalently attach iodide to tyrosyl residues. The two catalysts are lactoperoxidase (Hubbard & Cohn, 1972; Cook & Lilien, 1982), which is too large to cross the cell membrane, and Iodogen (Fraker & Speck, 1978), an insoluble compound coated on the sides of the reaction vessel. For the lactoperoxidase method, intact ROS (between 0.1 and 1.0 mg of total protein) were resuspended in lactoperoxidase (1 mg/mL), 0.2 mCi of Na^{125}I, and 50 μM H_2O_2 in Ringer's. Reaction conditions which resulted in a low level of labeling (10^{-3} mol of I/mol of rhodopsin) were used to minimize damage to ROS membranes. In some experiments, 10% Percoll was included to minimize osmotic disruption of ROS; this did not affect the iodination results. ROS were iodinated for 6–7 min at room temperature in dim red illumination, with periodic gentle resuspension, and then washed in Ringer's containing 1 mM KI. To separate intact ROS from those which became leaky during iodination and washing, intact ROS were then repurified on a second discontinuous Percoll gradient to ensure that only surface proteins were iodinated. Between 70% and 90% of the ROS were recovered from the intact band on the second Percoll gradient. The percentage of leaky ROS, and ROS with inner segments in the intact sample, was assayed as above. The ROS sample was then pelleted and solubilized for gel electrophoresis. Intact ROS incubated under identical iodination conditions but without lactoperoxidase showed no detectable labeled proteins on autoradiograms. This also supported the specificity of the iodination reaction to proteins on the ROS exterior, since the impenetrability of lactoperoxidase is the basis of the surface nature of the labeling (Morrison & Schonbaum, 1976). As an alternate method, intact ROS in 100 μL of Ringer's were transferred to 1-mL glass centrifuge tubes coated with 10 μg of Iodogen and incubated with 0.2 mCi of Na^{125}I in Ringer's. The reaction was stopped by removing ROS from the reaction vessel, and all other procedures were the same.

(*B*) *Preparation of ROS Membranes.* A portion of intact ROS was pelleted and resuspended in 150 μL of distilled water, or water containing 3 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), to facilitate solubilization of peripheral membrane proteins (Kühn, 1981), 0.1 mM phenylmethanesulfonyl fluoride (PMSF) to retard proteolysis, and 2 mM dithiothreitol (DTT). The ROS were drawn 10–15 times through a 1-mL syringe fitted with a 25-gauge needle to break open the ROS and disks and solubilize nonmembrane proteins. The membranes were then pelleted by centrifugation for 15 min at 100000*g* (Beckman Airfuge). This process was repeated 3 times to remove nonmembrane proteins. During iodination (same as above), ROS membranes were drawn in and out of the syringe to increase access of reactants to membranes. ROS membranes prepared by this technique are referred to as lysed or washed ROS membranes in the text. This lysate was prepared for electron microscopic observation by allowing the lysate to adsorb to formvar-coated copper grids, fixing in 2.5% glutaraldehyde, staining with 1% uranyl acetate, and drying by the critical point procedure of Anderson (1951). Alternatively, ROS were resuspended in iodination reactants and subjected to three cycles of freezing and thawing to break ROS and disk membranes (Adams et al., 1982). These are referred to as freeze–thawed ROS membranes in the text.

(*C*) *Iodination of Disrupted ROS.* To compare with labeling of intact ROS, a portion of intact ROS was disrupted by repeated pipetting before iodination. About 90% of the ROS became leaky as a result. These ROS were iodinated as above, washed 3 times in Ringer's plus 1 mM KI, and solubilized for gel electrophoresis without a second Percoll purification step.

Separation of Soluble and Integral Membrane Fractions. To examine whether internal proteins became iodinated in intact ROS, soluble plus peripheral membrane proteins were separated from integral membrane proteins by disruption of ROS in distilled water containing EGTA, PMSF, and DTT as above. The first supernatant was retained as the soluble plus peripheral membrane protein sample for gel electrophoresis, and the two subsequent washes were discarded. The membrane pellet then represented the integral membrane protein fraction.

Trypsin Digestion of Intact Rod Outer Segments. To confirm that the proteins labeled by radioiodination were on the ROS surface, iodinated intact ROS were exposed to

proteolytic digestion by trypsin. ROS (about 200 μg of protein) were resuspended in 250 μL of 0.2 mg/mL trypsin (bovine pancreas) in Ringer's and incubated for 15 min at room temperature. The reaction was stopped by 50 μL of 10 mg/mL trypsin inhibitor, and the ROS were washed in Ringer's and pelleted for gel electrophoresis.

Phosphorylation of Rod Outer Segments. The ROS suspension in Ringer's was incubated with 20 μCi of [^{32}P]ATP, and 10 mM cyclic GMP for 8 min, and the reaction was quenched with 3 volumes of cold 10% trichloroacetic acid. The pellet was then washed in Ringer's and solubilized for gel electrophoresis.

Polyacrylamide Gel Electrophoresis and Autoradiography. ROS pellets or soluble ROS proteins were prepared for electrophoresis by solubilization to a concentration of 0.5–1.0 mg/mL in 62.5 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 6.8, 2.3% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, and 2% bromphenol blue. Proteins were separated on 5–15%, 8–20%, or 10–20% continuous gradient polyacrylamide gels [Laemmli, 1970; as described in Polans et al. (1979)]. The molecular weight standards were rabbit muscle myosin, phosphorylase *b*, bovine albumin, ovalbumin, aldolase, glyceraldehyde-3-phosphate dehydrogenase, pepsin, carbonic anhydrase, α-chymotrypsinogen, β-lactoglobulin, lysozyme, and cytochrome *c*. Dried gels were exposed to X-ray film (Kodak X-omat R) at −80 °C with an intensifying screen for 1 h to 3 days.

Quantitation of Radioiodine Incorporation. To quantitate the amount of ^{125}I label incorporated into ROS proteins, lanes of proteins separated by gel electrophoresis were sliced into 1- or 2-mm slices and counted in a γ counter (Model 1290, TM Analytic). The counting efficiency was 95% and the accuracy within 1%.

Results

Proteins Labeled in Lysed and Intact Rod Outer Segments. The Coomassie-stained proteins in washed ROS membranes (Figure 1, lane a) are predominantly rhodopsin and rhodopsin oligomers (arrows at right) and a barely visible protein of approximately 220K molecular weight. The intact ROS preparation (lane b) retains soluble and peripheral membrane proteins as well, including the phosphodiesterase doublet at approximately 92 kDa (Miki et al., 1975; Baehr et al., 1979), a 48-kDa protein (Kühn, 1980), and others. Plasma membrane proteins were identified on the basis of their accessibility to surface-specific radioiodination. Intact purified ROS were labeled by iodination and purified again on a Percoll gradient, and the proteins were separated by gel electrophoresis and autoradiographed. Proteins labeled by iodination in intact ROS (lane e) include rhodopsin as the predominant species and 12 other proteins, with molecular weights of approximately 220K (three bands; see below), 60K, 56K, 53K, 45K, 33K, 27K, 24K, 22K, and 13K (lines). Although some of the proteins are not easily visible in this autoradiogram, these proteins were reproducibly radioiodinated in intact ROS in 25 experiments. The same proteins were labeled in ROS exposed to room light for 1–3 min prior to iodination.

To investigate possible differences between the protein composition of the plasma membrane with disk membranes, washed ROS membranes, including both plasma and disk membranes, were iodinated. The pattern of labeled proteins (Figure 1, lane c) differed in several bands from the plasma membrane pattern. Rhodopsin and oligomers of rhodopsin were the major proteins labeled. The 220-kDa protein and 11 other proteins (lines) having molecular weights of approximately 55K, 50K, 45K, 33K, 30K, 23K (two bands), and

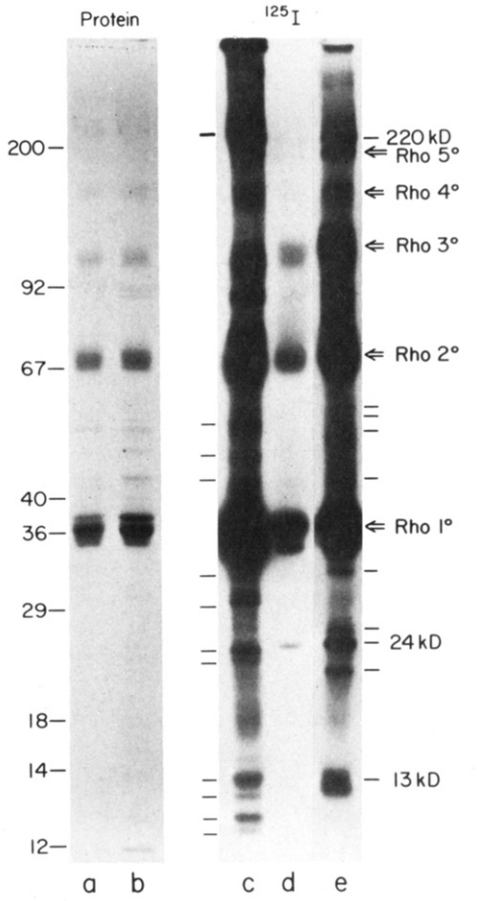

FIGURE 1: Iodinated proteins in intact rod outer segments and membranes. Proteins were radioiodinated, separated by sodium dodecyl sulfate (SDS) gradient gel electrophoresis, and autoradiographed. Equal amounts of total protein (20 μg of rhodopsin) were loaded on gels of both ROS membranes (lane a) and intact ROS (lane b) and stained with Coomassie blue. In this preparation, the inner segment content was 40%, and there was less than 6% leaky ROS. In the autoradiogram of ROS membranes (lane c), labeling could be observed in 11 proteins (lines) in addition to rhodopsin and its oligomers (arrows, Rho 1°, Rho 2°, etc.) and the 220-kDa protein. For comparison, an equally exposed autoradiogram of labeled intact ROS is shown (lane d). In a longer exposure to visualize minor proteins (lane e), nine proteins were labeled in addition to rhodopsin and the 220-kDa protein.

12–13K (four bands) were also labeled. Since the disk membrane surface area in frog ROS is approximately 50-fold greater than the plasma membrane surface, these heavily labeled bands probably reflect labeled proteins from the disk membranes. It is also likely that trace amounts of peripheral membrane proteins remained after washing of the membrane preparation and were iodinated. One such protein, the cyclic GMP phosphodiesterase of 92K molecular weight, is visible after iodination (lane c) although not by Coomassie staining (lane a). The relative contribution of labeled plasma membrane proteins to the total ROS membrane labeling profile can be shown by comparing similarly exposed autoradiograms of surface-iodinated intact ROS (lane d) and ROS membranes (lane c) having the same total protein content. (Lane e is identical with lane d but has been exposed longer for visualization of minor labeled proteins.) Quantitative differences in labeling between proteins in ROS membrane and intact preparations are evaluated below under Differences between Plasma and Disk Membrane Proteins.

When proteins were analyzed on gel systems containing a lower percentage of acrylamide (Figure 2), two additional high molecular weight proteins were visualized in autoradiograms

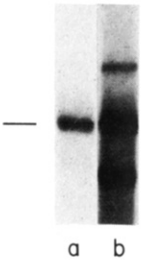

FIGURE 2: Three high molecular weight proteins are labeled in intact ROS. In autoradiograms of 5–15% acrylamide gradient gels, three labeled bands at, below, and above 220 kDa are resolved in intact preparations (lane b) while the major Coomassie-stained protein (line) is the primary protein labeled in the lysed ROS membrane preparation (lane a). Autoradiogram b was exposed longer than autoradiogram a, since incorporation in the lysed sample was greater.

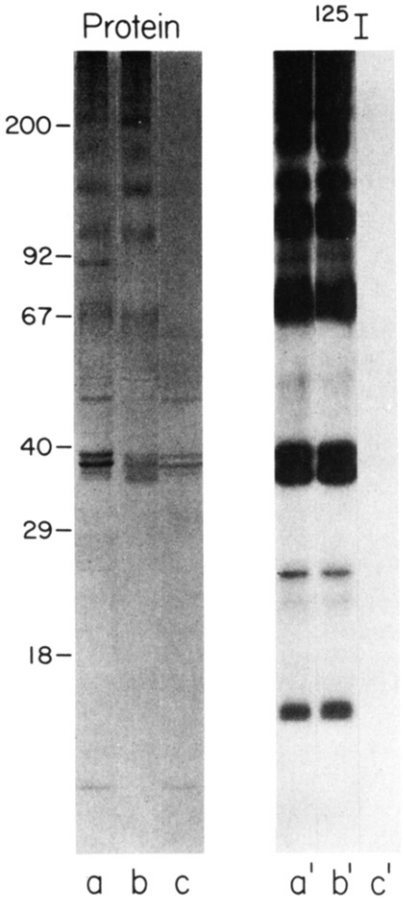

FIGURE 3: Internal proteins of intact rod outer segments are not iodinated. When intact ROS are radioiodinated (lane a′), only the integral membrane proteins (lane b′) are labeled, and the soluble and peripheral membrane proteins (lane c′) appear not to have incorporated any label. In this preparation, the inner segment content was 19%, and less than 5% of the ROS were leaky after iodination. The corresponding Coomassie-stained gels are shown in lanes a–c. On lanes a and b, 20 μg of rhodopsin was loaded, and on lane c, an amount of protein was loaded corresponding to 20 μg of rhodopsin.

of intact ROS (lane b) above and below the 220-kDa protein, also labeled in the lysed ROS membrane preparation (lane a).

Proteins Labeled in Intact ROS Are Plasma Membrane Proteins. Control experiments confirmed that radioiodination of intact ROS resulted in labeling of only integral membrane proteins and that soluble and peripheral ROS proteins were not labeled. Figure 3 shows preparations separated by gel electrophoresis of iodinated intact ROS (lane a) which were subsequently separated by low ionic strength washes and

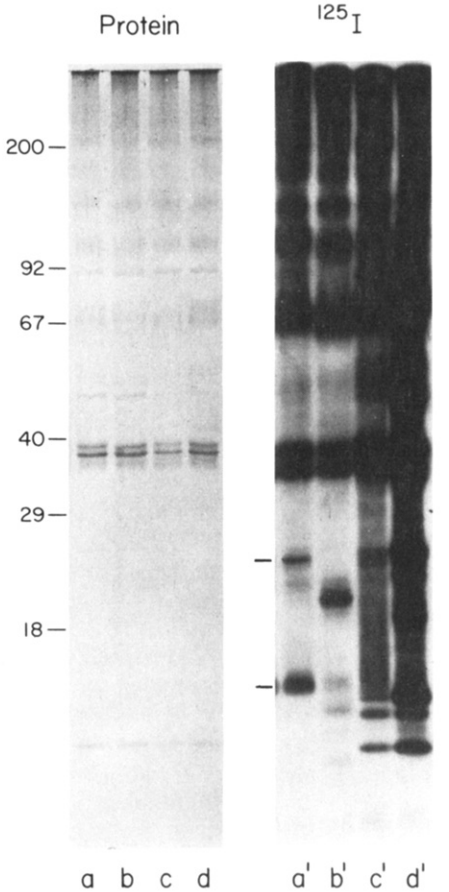

FIGURE 4: Effects of trypsin digestion and of induced leakiness on labeling. Labeling of the 24- and 13-kDa proteins (lines) in intact iodinated ROS (lane a′) was removed by incubation with trypsin (lane b′). ROS which became leaky during the radioiodination procedure and were separated from intact ROS on a Percoll gradient following iodination (lane c′) show more labeled proteins than the intact ROS (lane a′). ROS disrupted by repeated pipetting (lane d′) show extensive labeling of proteins. The intact preparation, used as the starting material for all these subsequent treatments, is the same as in Figure 3. Equal amounts of rhodopsin were loaded on all the lanes, and exposure time was equal.

centrifugation (Kühn, 1981) into an integral membrane fraction (lane b) and a soluble plus peripheral membrane protein fraction (lane c). Major proteins of the integral membrane fraction are rhodopsin monomer and oligomers, and the 220-kDa protein, while in the soluble plus peripheral membrane fraction, the major proteins visible are the approximately 39- and 37-kDa G protein α and β subunits and the 48-kDa protein (Godchaux & Zimmerman, 1979; Kühn, 1980; Fung et al., 1981). The corresponding autoradiograms are shown in lanes a′, b′, and c′. The patterns of labeling in the intact (lane a′) and integral membrane (lane b′) fractions were identical, indicating that the surface-labeled proteins were integral membrane proteins. Proteins of 13 and 24 kDa were prominently labeled. No label was visible in the soluble plus peripheral membrane fraction (lane c′), showing that internal proteins were not labeled.

When leaky ROS were radioiodinated, more proteins were labeled than in the intact preparation. In Figure 4, proteins labeled in intact ROS (lane a′) are compared with the greater number of proteins labeled in ROS which became leaky during the iodination reaction (lane c′). As described under Materials and Methods, following the iodination reaction intact ROS were separated from leaky ROS on a Percoll density gradient. When ROS were made leaky by repeated pipetting and then radioiodinated, more extensive labeling was observed (Figure

4, lane d'), including peripheral membrane proteins and those soluble proteins retained in the ROS.

To determine whether the exposure of ROS to Percoll affected labeling, possibly by blocking access of reactants to the surface, ROS were also iodinated before Percoll purification. Several additional proteins were labeled in the unpurified preparation; however, after Percoll purification, the pattern of labeled proteins was identical with that of ROS purified before iodination (data not shown). This suggests that the additional labeled proteins in the crude preparation were not of ROS origin and that Percoll did not affect labeling.

An independent indication that the 24- and 13-kDa proteins are surface proteins is that they were sensitive to proteolytic digestion by trypsin. Figure 4, lane b', shows that these two proteins were completely (24 kDa) or substantially (13 kDa) removed after trypsin treatment, while rhodopsin remained unaffected. A labeled protein fragment at approximately 20 kDa appeared to have been generated by proteolytic digestion of one or more larger polypeptides, possibly the 24-kDa protein. Other surface-labeled proteins were not visibly affected by trypsin, although rhodopsin and its oligomers obscured some areas of the gel.

Differences between Plasma and Disk Membrane Proteins. In the absence of biochemical techniques to physically isolate the two membrane systems and analyze their components, the availability of a labeling method specific for plasma membrane proteins was utilized to determine whether any proteins are unique to the plasma or the disk membrane. For analysis of disk membrane components, ROS were treated by two different methods for lysing disks to make disk membrane proteins accessible to radioiodination: repeated cycles of freeze–thawing; resuspension in hypotonic solution followed by repeated passage through a syringe needle (see Materials and Methods). Electron microscopic observation showed that the lysed membrane preparation contained vesicles 40–500 nm in diameter (mean, 210 nm; SD, 101 nm), indicating that disk vesicles were disrupted by this technique (data not shown). The total incorporation in the hypotonic-lysed ROS membrane preparation was about 13-fold greater than in the intact preparation, and in the freeze–thawed membrane preparation about 26-fold greater, presumably because of the increased surface area accessible for labeling.

To compare the amount of each labeled protein in intact ROS and ROS membranes, gel lanes containing equal amounts of total protein were cut into slices, and the ^{125}I incorporated into protein was counted. For every protein, significantly more labeling was observed in the total ROS membrane preparations; thus, no unique plasma membrane proteins could be detected. Figure 5a shows that in a freeze–thawed preparation (solid line), greater incorporation occurred along the entire length of the gel compared to the intact (dotted line) preparation. This was also the case when labeling of lysed ROS membranes was compared with labeling of intact ROS. Significant differences were observed in the relative labeling of several proteins in total ROS membranes compared to ROS plasma membrane when the results were normalized with respect to the total amount of radiolabel incorporation in each preparation. Figure 5b compares the labeling of the freeze–thawed ROS membranes (solid line, same as 5a) with the labeling of the intact ROS (dotted line), while Figure 5c compares a lysed ROS membrane preparation with that of intact ROS. In both examples, labeling of proteins of approximately 13 and 24 kDa was greater in intact ROS, indicating enrichment of these two proteins in the plasma membrane. The 13-kDa protein incorporated about 5-fold

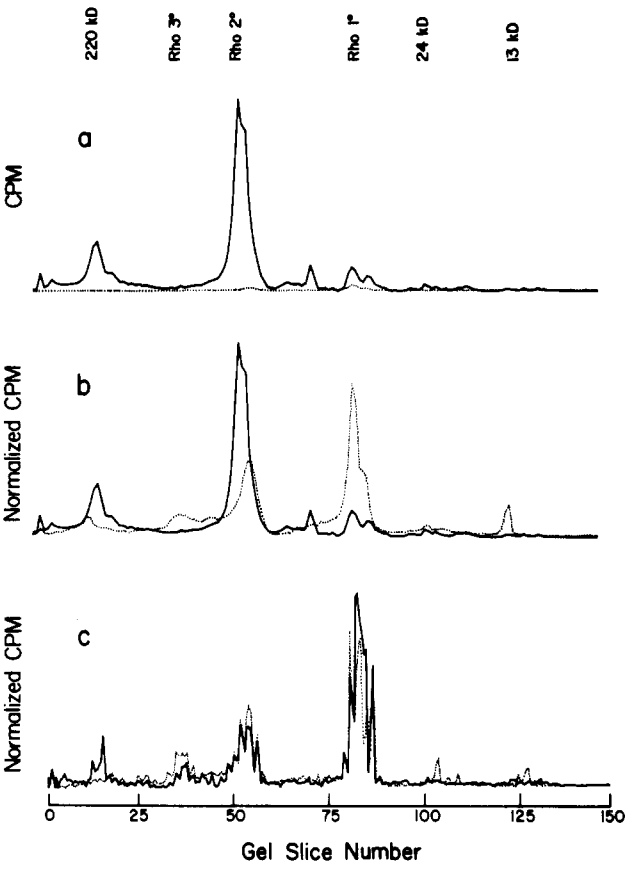

FIGURE 5: Quantitation of iodinated proteins in intact rod outer segments and membranes. The radiolabel incorporated by proteins is plotted against migration distance in SDS gels. (a) cpm of incorporated ^{125}I in intact ROS (dotted line) and freeze–thawed ROS membranes (solid line) is compared, when equal amounts of total protein were loaded on the polyacrylamide gel lane. (b) Data from experiment shown in lane a have been normalized with respect to the total cpm in the intact ROS sample (dotted line) and the freeze–thawed ROS membrane sample (solid line). The peak at gel slice number 69 corresponds to a protein of 48 kDa, which is a soluble protein and was not reproducibly found in the membrane fraction. (c) In a separate experiment, the relative incorporation of radiolabel in intact ROS proteins (dotted line) and hypotonic-lysed ROS membrane proteins (dotted line) was compared. The prominent peaks, 24 and 13 kDa, are slightly out of register with those above because the gradient gels ran somewhat differently. These traces are noisier because gel slices 1-mm thick were analyzed rather than 2-mm slices as in (a) and (b). The autoradiogram of the gels in (c) was shown in Figure 1.

more counts relative to total radioactivity in intact ROS than in freeze–thawed (Figure 5b) or lysed (Figure 5c) ROS membranes. Labeling of the 24-kDa protein was more variable, having 6-fold more counts relative to total radioactivity in intact ROS than in lysed ROS membranes (5c) and only 1.6-fold in freeze–thawed ROS membranes (5b). Neither the 24-kDa nor the 13-kDa iodinated band corresponds to a band detectable by staining with Coomassie brilliant blue. The lower limit for detectability by Coomassie was approximately 10–20 ng, which corresponds to less than 1 copy per 1000 rhodopsin molecules. Another difference between intact and disrupted ROS is that the 220-kDa protein showed 3–5-fold greater relative incorporation in total ROS membrane than in intact ROS, indicating an enrichment of this protein in disk membranes. On this gel system, the three proteins near 220 kDa labeled in intact ROS are not separated and appear as a single peak. Although the protein labeling patterns for intact ROS and ROS membranes appeared qualitatively different (Figure 1), suggesting that other proteins in the disk membrane are not found in the plasma membrane, other significant quan-

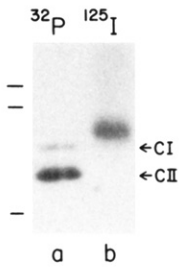

FIGURE 6: 13-kilodalton iodinated protein is not component I or II. The autoradiogram shows components I and II, phosphorylated in an ROS preparation incubated with [^{32}P]ATP (lane a) and the 13-kDa iodinated protein (lane b) from a separate preparation, run in adjacent lanes. The molecular weight markers indicated at left (lines) are the lysozyme doublet (about 13 kDa) and cytochrome *c* (about 12.5 kDa).

titative differences could not be measured.

The proportion of rhodopsin oligomers also varied between the preparations. The freeze–thawing treatment apparently generated more rhodopsin dimers (Figure 5a,b), while the lysed preparation showed somewhat more rhodopsin monomers than the intact preparation (Figure 5c). It should be noted that oligomerization of rhodopsin is an artifact of gel electrophoresis common to frog rod preparations and varied in different gels of the same preparation. The native state of rhodopsin, in both light and dark ROS, is apparently monomeric (Downer & Cone, 1985a,b). Incorporated radioactivity in rhodopsin was taken as the sum of radioactivity in monomer, dimer, and trimer. Rhodopsin (monomer plus oligomers) represented 54–67% of the total incorporated radioactivity in all samples.

The 13-kDa iodinated surface protein is not the same as the 13-kDa proteins, designated components I and II, reported to be phosphorylated in the dark and dephosphorylated in the light (Polans et al., 1979; Hermolin et al., 1982). As shown in the autoradiogram in Figure 6, the iodinated surface protein (lane b) can be distinguished from components I and II (lane a), which incorporate ^{32}P.

Inner Segment or Outer Segment Proteins? The preparations of intact and permeabilized ROS used for the labeling experiments had a content of ROS with attached inner segments of between 2% and 50%. The percentage of inner segments in Figure 1 and 2 was 40% and in Figures 3 and 4 was 5%. Given that the inner segment surface area is about 10% of the outer segment surface area, inner segment membrane represents from 0.2% to 5% of the membrane in intact preparations. Since the same complement of proteins was reproducibly labeled in over 25 experiments, irrespective of inner segment content in the preparation, it is more likely that they are of outer segment origin. However, due to the sensitivity of the iodination technique, the possibility that these proteins are from the inner segment cannot be ruled out.

DISCUSSION

This study has defined, by surface-specific radioiodination, 13 proteins in the plasma membrane of frog ROS. The lack of labeling of internal proteins, and the sensitivity of two of these proteins to trypsin digestion, confirms that they are surface proteins. The surface proteins are not likely to be components of the extracellular matrix, which is soluble and would be removed by washing (Adler & Klucznik, 1982). Radioiodination of lysed ROS, in which both plasma and disk membranes were labeled, showed a somewhat different labeling pattern with 13 disk membrane proteins predominantly labeled.

In intact bovine ROS, rhodopsin and proteins of approximately 226, 110, and 66 kDa were radioiodinated, and not extractable in hypotonic medium, indicating they were surface integral membrane proteins (Clark & Hall, 1982). While the 66-kDa polypeptide may correspond to one of the ones labeled here in frog (60 kDa), the region corresponding to 110 kDa was obscured in the frog preparation by rhodopsin oligomers. Kamps et al. (1982) found enrichment of proteins with molecular weights of 226K, 160K, 125K, 110K, 66K, 57K, 55K, 54K, and 33K in a bovine ROS membrane fraction which adhered to a concanavalin A affinity column. Independent evidence of the purity of this membrane fraction was not obtained; however, proteins of similar molecular weights were labeled in the present study, confirming the potential usefulness of such a purification method for the plasma membrane. Clark and Hall (1982) and Kamps et al. (1986) also found that their bovine plasma membrane protein of 226K was of slightly lower molecular weight than the more abundant protein which stains with Coomassie and has been localized to disk rims and incisures (Papermaster et al., 1978b). In this study, the abundant (designated 220 kDa) protein was labeled on the surface, in addition to two other less abundant proteins of similar molecular weight.

Does the Increased Labeling Reflect Protein Enrichment? A quantitative comparison of the labeling in intact, freeze–thawed, and lysed preparations showed that labeling of two proteins, of approximately 24 and 13 kDa, was enriched in the plasma membrane, relative to total labeling. Labeling of a protein of approximately 220K, the most abundant high molecular weight protein, was enriched in disk membranes. Several factors, however, affect the interpretation of the quantitative results. First, the amount of radioiodination of a protein depends on the number of accessible tyrosyl residues, which may vary for each protein and for portions of a protein on opposite sides of the membrane. Therefore, quantative comparisons between a protein labeled in the plasma membrane preparation, accessible only on the exterior surface, with that protein labeled in the ROS membrane preparation, accessible on both inner and outer surfaces, could reflect differences in the number of accessible groups per protein as well as differences in the quantity of the protein in the two different membrane preparations. Second, it is difficult to establish unequivocally that disks were broken and accessible to labeling on the interior surface. In this study, similar results were obtained when ROS were lysed by two different methods, and electron microscopic observation showed that disks were disrupted. In addition, a different pattern of protein labeling was observed in the membrane preparation. Therefore, we believe that the most obvious and likely interpretation of the increased labeling observed in the ROS membrane preparations (13-fold for hypotonic disruption and 26-fold for freeze–thawing) is that the inside as well as the outside of the disks was labeled and that the enrichment of labeling of the 13- and 24-kDa proteins and of the 220-kDa protein reflects their enrichment in plasma and disk membranes, respectively.

Third, since labeling has been normalized to the total radioactivity, which is primarily accounted for by rhodopsin, it is necessary to establish whether rhodopsin density is the same in the plasma and disk membranes in order to determine whether a certain protein is enriched. In freeze–fractured ROS, the density of 10-nm-diameter intramembrane particles thought to represent rhodopsin aggregates was similar for plasma and disk membranes (Besharse & Pfenninger, 1980), suggesting that rhodopsin density is the same. If so, then the greater proportion of the 13- and 24-kDa proteins in the plasma membrane and of the 220-kDa protein in the disk membrane reflects real difference in density.

A fourth point concerns the presence of "open" disks and their effect on the labeling results. At the juncture of the ROS

with the inner segment, new disks are formed, apparently as outfoldings of the plasma membrane. These open disks soon close off, becoming separate flattened spheres which are moved upward in the ROS and after several weeks are phagocytized at the apical tip. Newly synthesized proteins, labeled in vivo by incorporation of [^{3}H]leucine, or rhodopsin, identified by immunocytochemistry, are probably inserted into forming disk membranes in this area (Young, 1968; Young & Droz, 1968; Basinger et al., 1976; Papermaster et al., 1975, 1982, 1985; Besharse & Pfenninger, 1980; Peters et al., 1983). In this study, ROS were prepared and labeled toward the end of the dark phase of the circadian cycle when the number of open disks would be at a minimum, since it was not known whether the protein composition of open disks resembles disk or plasma membrane. Besharse et al. (1977) found that after 4 h in darkness, an average of fewer than three open disks was observed in *Xenopus laevis* ROS. It was calculated that this would correspond to less than 25% of the total ROS plasma membrane. Since differences in the pattern of proteins labeled were observed in intact and lysed ROS preparations, it appears that the presence of open disks did not compromise the ability to distinguish the two membrane systems. However, if open disks resemble disk membrane rather than plasma membrane, it is possible that even greater differences exist between plasma and disk membranes than were observed here.

Are Disk and Plasma Membranes Different? Several sorts of experimental evidence have led to the notion that plasma and disk membranes may contain the same proteins. Since the disk and plasma membranes appear continuous during disk formation, and newly synthesized proteins inserted at the base of the ROS freely diffuse throughout the plasma membrane as well as the forming disks (Basinger et al., 1976), then similar protein compositions might be expected in the two membrane systems. The best-studied ROS protein, rhodopsin, is found in both plasma and disk membranes. The lectins concanavalin A and wheat germ agglutinin have been observed to bind to the carbohydrate segment of rhodopsin on the extracellular surface of ROS (Hall & Nir, 1976; Molday, 1976) and will bind not to closed disks but only to those opened by detergent treatment or freeze–thawing, suggesting that the carbohydrate moiety is intradiskal (Rohlich, 1976; Molday & Molday, 1979). This has lent substance to the idea that disks represent "inside-out" plasma membrane. Interestingly, some functional similarities between disk and plasma membrane protein components have also been observed, although their physiological significance is unclear. Cyclic GMP regulated cation channels have been observed in both patch-clamped plasma membrane and disk vesicle preparations (Fesenko et al., 1985; Zimmerman et al., 1985; Caretta & Cavaggioni, 1983; Caretta, 1985; Koch & Kaupp, 1985), and sodium–calcium exchange activity in plasma and disk membranes has been measured (Schnetkamp et al., 1977; Schnetkamp, 1980, 1986).

In many eukaryotic cells, certain proteins appear to be specifically localized in their functional regions in the membrane [see Kelly (1985) for a review]. Immunocytochemical and autoradiographic studies suggest that rhodopsin may be specifically localized in the outer segment, since it is greatly reduced in the rod inner segment plasma membrane compared to the outer segment plasma and disk membranes (Papermaster et al., 1985; Hicks & Molday, 1986). This study has observed differences in the proportion of several proteins in plasma compared to disk membranes, and although the existence of unique plasma or disk membrane proteins was not quantitatively supported, qualitative results from autoradiographic patterns suggested the presence of some proteins in the disk membrane not in the plasma membrane.

The two highly labeled plasma membrane proteins might be components of the light-dependent sodium channel or of the sodium–calcium exchanger. Both proteins were present in less than 10^6 copies per ROS. Estimates for the number of light- and cGMP-sensitive channels range from 200 to $1000/\mu m^2$ of plasma membrane or about 1 per 10^3–10^5 per ROS (Zimmerman et al., 1985; Zimmerman & Baylor, 1986; Haynes et al., 1986; Gray & Attwell, 1985). The 24- and 13-kDa proteins also might be structural components of the plasma membrane having a cytoskeletal role. For example, connections between disk and plasma membranes, and between edges of adjacent disks, have been observed in transmission electron micrographs of freeze-etched ROS (Roof & Heuser, 1982). Since some rod inner segments were also present in the preparation, it is possible, although less likely, that these proteins may be components of the inner segment membrane, including non-light-sensitive ionic conductances (Bader et al., 1982), a sodium–potassium ATPase (Stirling & Lee, 1980; Ueno et al., 1980), or receptors such as for interphotoreceptor retinal binding protein (Hollyfield et al., 1985). It will be the object of future studies to assign functional roles in the transduction process to these proteins.

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