Orientation of Membrane Glycoproteins in Sealed Rod Outer Segment Disks†

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ABSTRACT: The orientation of rhodopsin and the 220 000 molecular weight glycoprotein ROS 1.2 was studied by comparing the binding of [^{125}I]concanavalin A (Con A), succinyl-[^{125}I]Con A, and ^{125}I-labeled wheat germ agglutinin (WGA) to sealed and unsealed bovine rod outer segment (ROS) disks. Freshly prepared sealed disks isolated on a 5% Ficoll solution bound low quantities of lectin (less than 0.03 μg of succinyl-[^{125}I]Con A and [^{125}I]WGA and less than 0.07 μg of [^{125}I]Con A per μg of disk protein). Disks that were partially disrupted with 0.015% Triton X-100, but not completely solubilized, bound 25–35 times more succinyl-[^{125}I]Con A and [^{125}I]WGA and over 16 times more [^{125}I]Con A. Similar increases were found for disks treated with cholate. Disks which had been frozen and thawed showed a 16-fold increase in succinyl-Con A binding over unfrozen sealed disks but less than a 2-fold increase in WGA binding. Over 85% of the disks which had been frozen no longer floated on 5% Ficoll, indicating that most disks were no longer osmotically sealed. Scatchard analysis of the binding curves suggested the existence of two classes of binding sites. Approximately half of the total Con A binding sites on disks had an apparent dissociation constant (K_d) of 2×10^{-8} M for [^{125}I]Con A and succinyl-[^{125}I]Con A and the other half had an apparent K_d of 3×10^{-7} M. The two classes of WGA binding sites exhibited apparent K_d values of 3×10^{-7} and 3×10^{-6} M. Treatment of sealed disks with trypsin and *Streptomyces griseus* protease did not alter the low level of lectin binding, indicating that these enzymes do not expose additional lectin sites or make the disks permeable to macromolecules. These proteolytic enzymes, however, did degrade rhodopsin and ROS 1.2 glycoproteins to smaller carbohydrate-containing membrane fragments as previously reported. From these results we conclude that the carbohydrate chains on rhodopsin are oriented toward the interior of the disks where they are inaccessible to Con A or WGA in sealed disks. Low levels of lectin binding in sealed disk preparations are most likely due to a small contaminating population of unsealed or inside–outside disks or other membrane vesicles. Polypeptide segments of rhodopsin, including the carboxyl-terminal peptide, however, are exposed on the exterior of the disk (interdisk surface) since they are cleaved by various proteolytic enzymes in sealed disks. From this evidence we conclude that rhodopsin spans the ROS disk membrane. Indirect evidence that ROS 1.2 is also a transmembrane glycoprotein is presented.

Rod outer segments (ROS)[1] of vertebrate retinal rod cells function in the absorption of light and its transduction into electrical signals. This organelle consists of hundreds of closed, stacked disks which are surrounded by a plasma membrane. Analysis of ROS and ROS disks indicates that rhodopsin constitutes over 85% of the total membrane protein (Papermaster & Dreyer, 1974; Daemen et al., 1972; Smith et al., 1975; Krebs & Kühn, 1977). Bovine rhodopsin of apparent molecular weight 34 000 has been shown to be a glycoprotein containing mannose and *N*-acetylglucosamine residues (Heller & Lawrence, 1970; Plantner & Kean, 1976). The lectins concanavalin A (Con A) and wheat germ agglutinin (WGA) bind to these sugar residues on rhodopsin (Steineman & Stryer, 1973; Molday & Molday, 1979). Recently, Hargrave (1977) has reported that rhodopsin contains two oligosaccharide chains which are covalently bonded to asparagine residues located within a 16 amino acid peptide derived from the blocked amino-terminal segment. In addition to rhodopsin, bovine disk membranes contain a high molecular weight protein (Papermaster et al., 1976) designated as ROS 1.2 which also binds both Con A and WGA (Molday & Molday, 1979).

The orientation of rhodopsin in ROS disk membranes has been studied by using Con A as a probe for the carbohydrate chains. Röhlich (1976) has shown by transmission electron microscopy that ferritin–Con A binds only to the intradisk side of disk membranes. However, Nir (1978), using similar techniques, observed that Con A binds to both the interdisk (cytoplasmic) and the intradisk surface of frog and rat retinal ROS. Disk structures released from bovine ROS were also observed to bind Con A–microsphere reagents by scanning electron microscopy (Molday, 1976). Biochemical analysis has also yielded differing results. Steineman & Stryer (1973) have reported that one fluorescein-labeled Con A bound per retinal group of rhodopsin in bovine disk membranes. Yariv et al. (1974) have also reported that [^{60}Co]Con A binds to ROS disk membranes, but the extent of binding was lower by over 1 order of magnitude. More recently, Adams et al. (1978) have reported that isolated disks do not bind to Con A–Sepharose columns.

These significant differences in Con A binding have led us to reinvestigate the accessibility of lectin binding sites in intact, sealed disks and disks treated with detergents or subjected to freezing and thawing. Our results on lectin binding to sealed and disrupted disk membranes and proteolysis studies on sealed disks (Molday & Molday, 1979) support the view that rhodopsin is a transmembrane protein with its oligosaccharides oriented toward the interior of the disks. Indirect evidence favoring the transmembrane disposition of ROS 1.2 is also discussed.

Experimental Procedures

Materials

Con A, ovomucoid, α-methyl mannoside, *N*-acetylglucosamine, and *Streptomyces griseus* protease (lot 114C-0168) were from Sigma Chemical Co. TPCK–trypsin was from Worthington, Ficoll 400 was from Pharmacia, ^{125}I was from Amersham/Searle, and WGA was purchased from Mann-

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[1] Abbreviations used: ROS, rod outer segments; Con A, concanavalin A; WGA, wheat germ agglutinin; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; BSA, bovine serum albumin; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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heim-Boehringer or prepared as described.

Methods

Preparation of ROS Disk Membranes. ROS fragments were prepared under dim red light at 4 °C from freshly dissected or frozen (Hormel) retinas by the procedure of Papermaster & Dreyer (1974). Disks were isolated by the method of Smith et al. (1975). Sealed disks which floated on 5% Ficoll solution were washed in distilled water and finally made to a concentration of 1–3 mg of protein per mL in distilled water. Disk protein concentration was determined by the assay of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard. The Lowry method gave similar values to those obtained by spectrophotometric analysis (Chen & Hubbell, 1978; Shichi, 1970). Ultraviolet–visible spectra of disks solubilized in cetyltrimethylammonium bromide yielded A_{280}/A_{500} and A_{400}/A_{500} ratios of 2.3–2.6 and 0.22–0.28, respectively. Disk suspensions were kept at 4 °C in the dark and used within 5 days.

Phosphorylation of Disks. ROS fragments were phosphorylated as previously described (McDowell & Kühn, 1977; Shichi & Somers, 1978). Briefly, ROS from 25 retinas were suspended in 0.01 M Tris, pH 7.4 (hereafter referred to as Tris buffer), and frozen at −20 °C. Immediately after thawing, we bleached the ROS suspension (0.8 mL) and incubated it with 100 μL of buffer containing 10 mM [γ-^{32}P]ATP (262 mCi/mmol), 10 mM $MgCl_2$, and 20 mM Tris, pH 7.4, at 37 °C for 1.5 h. The ROS were washed 2 times in 35 mL of 0.1 M phosphate buffer, pH 7.0, by centrifugation at 20000 rpm in a Sorvall SS-34 rotor at 4 °C and once in distilled water. Disks were then prepared as previously described. Ninety percent of the ^{32}P was collected on Millipore filters after precipitation with cold 10% trichloroacetic acid. NaDodSO_4–polyacrylamide gel electrophoresis indicated that approximately 80% of the ^{32}P migrated with rhodopsin.

Preparation of [^{125}I]Lectins. WGA was prepared by the method of Marchesi (1972), and succinyl-Con A was prepared by the method of Gunther et al. (1973). Lectins were labeled with ^{125}I by the lactoperoxidase method (Morrison et al., 1974): lectin (10 mg) was dissolved in 2.0 mL of 0.1 M sodium phosphate, pH 7.0; then 40 μL of 8 μM lactoperoxidase and approximately 500 μCi of iodide-125 were added. Hydrogen peroxide (10 μL of 0.03%) was added every 3 min until 10 additions had been made, and then the reaction was stopped after another 3 min with 10 μL of 1 M sodium azide. The iodinated Con A and succinyl-Con A were affinity purified by applying the iodinated reaction mixture to a 1.4 × 6 cm column of Sephadex G-200 equilibrated with Tris–Con A buffer (150 mM NaCl, 20 mM Tris, 1 mM $CaCl_2$, and 0.1 mM $MnCl_2$, pH 7.4). The column was then washed with Tris–Con A buffer and eluted with 0.1 M α-methyl mannoside in buffer. Finally, the eluted Con A was dialyzed against Tris–Con A buffer for several days. [^{125}I]WGA was purified on an ovomucoid–Sepharose column or by extensive dialysis against PBS. Final lectin concentrations of 0.6–1.0 mg/mL and specific activities of approximately (6–10) × 10^4 cpm/μg were obtained. Iodinated lectins stored at −20 °C were centrifuged before use to remove any aggregates. Electrophoresis on NaDodSO_4–polyacrylamide gels showed no differences in lectins before and after iodination.

Binding of [^{125}I]Lectins to Disk Membranes. Binding experiments were carried out in duplicate in 300-μL polystyrene microtiter plates with V-shaped bottoms (Cooke Engineering Co.) in a total volume of 100 μL for 1.5 h at 25 °C. Reagents were added as follows: 10 μL of bleached disks (10–30 μg), 10–80 μL of Tris–Con A buffer, and 10–80 μL of [^{125}I]lectin (1–200 μg/mL final concentration). Controls for Con A and succinyl-Con A contained 50 mM α-methyl mannoside, while WGA controls contained 50 mM *N*-acetylglucosamine and 5 mg/mL ovomucoid (Cohen et al., 1977) as specific inhibitors. When binding was carried out in the presence of 0.015% Triton X-100, all solutions used were made to that concentration of detergent, but only 1.0 μg of disks was used.

The effect of Triton X-100 concentration on the binding of lectin to disk membranes was studied by adding 10 μL of disks (1.0–9.5 μg) to 90 μL of lectin (15 μg) in Triton X-100 in the presence and absence of the inhibitor. The disks therefore were never exposed to higher than the final Triton X-100 concentration, which varied over the range 0–0.4%. The effect of cholate on lectin binding was studied over a cholate concentration of 0–0.8%.

In some experiments, disks were quickly frozen by immersing a glass tube of suspended disks in dry ice–ethanol and thawed prior to lectin binding.

Separation of bound from free lectin was carried out by a modification of the method of Phillips & Furmanski (1976). Routinely, 75 μL of the incubation mixture was layered onto 375 μL of 5% (w/v) BSA in PBS (8.0 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, and 2.17 g of $Na_2HPO_4 \cdot 7H_2O$ per L) in a 400-μL Eppendorf polypropylene micro test tube. In controls, the BSA–PBS solution also contained the appropriate lectin inhibitor. Six tubes were then placed in a Spinco SW-27 polycarbonate tube containing just enough water to float the micro test tubes and centrifuged at 20000 rpm (72000*g*) for 15 min at 15 °C. Centrifugation for 5 min at 20000 rpm or at 39000 rpm (270000*g*) for 1 min in a Spinco SW-41 gave similar results. The bottom 5 mm of the micro test tubes containing the sedimented disks was cut off with a hot scalpel and placed into individual 10 × 75 mm disposable glass culture tubes, and ^{125}I was determined in a γ counter. ^{32}P-Labeled disks were assayed for ^{32}P by counting Cerenkov radiation in distilled water in a liquid scintillation counter, a procedure which does not detect ^{125}I.

Greater than 90% of the disks were recovered by this centrifugation procedure as measured by ^{32}P recovery. Measurements on the rate of dissociation indicated that less than 10% of bound succinyl-Con A dissociated from the disks in 9 h. The slow rate of dissociation is in agreement with studies on Con A binding to neuroblastoma cells (Rosenberg & Charalampous, 1977).

Recentrifugation of Disks on 5% Ficoll 400. Immediately after preparation, disks were quickly frozen 1 or 10 times (or not at all) and recentrifuged in a Spinco SW-41 rotor at 35000 rpm for 0.5 h at 4 °C on 5% Ficoll 400 underlaid with 1 mL of 40% sucrose (w/w) to test for unsealing. Gradients were exposed to light, and 0.6-mL fractions were added to 0.6 mL of 0.1 M phosphate buffer, pH 7.0, containing 0.1 M cetyltrimethylammonium bromide and 1 mM hydroxylamine. The refractive index and absorptions at 280 and 365 nm were measured.

Proteolytic Treatment of Disks. Typically, 50–100 μL of disks was mixed with an equal volume of trypsin solution (0.02 mg/mL in Tris buffer prepared from 1 mg/mL frozen stock) or *S. griseus* protease (0.02 mg/mL in Tris buffer prepared from 1 mg/mL frozen stock) or Tris buffer and incubated at room temperature for 2 h. Trypsin was then inhibited with 10 μL of soybean trypsin inhibitor (2 mg/mL) and 2 mL of Tris buffer; protease was inhibited with 2 mL of 2.5 μL of phenylmethanesulfonyl fluoride (28 mg/mL in methanol) per mL of Tris buffer; 2 mL of Tris buffer was added to the control. Disks were centrifuged for 20 min at 15000 rpm in

Table I: Lectin Binding to Disks. Summary of Scatchard Analysis

lectin	Triton X-100 concn (%)	high affinity K_d (M)[a]	high affinity bound[b]	low affinity K_d (M)[a]	low affinity bound[b]	total bound[b]
Con A	0	1.2×10^{-8}	0.036	2.9×10^{-7}	0.038	0.074
	0.015	1.3×10^{-8}	0.81	1.5×10^{-7}	0.41	1.22
succinyl-Con A	0	3.3×10^{-8}	0.0072	2.8×10^{-7}	0.0048	0.012
	0.015	1.5×10^{-8}	0.17	2.8×10^{-7}	0.17	0.34
WGA	0	4.0×10^{-7}	0.0055	4.2×10^{-6}	0.020	0.025
	0.015	2.1×10^{-7}	0.18	1.4×10^{-6}	0.46	0.64

[a] Dissociation constants are based on molecular weights of 110 000 for Con A, 55 000 for succinyl-Con A, and 23 000 for WGA. [b] Micrograms of lectin bound per microgram of disk protein.

2.5-mL Sorvall SS-34 centrifuge tubes and then washed twice in Tris buffer. The final pellet was resuspended in a volume of distilled water equal to the original volume of disks used.

When testing for integrity of membranes after proteolytic digestion, we assayed the disks for protein concentration; the Con A and WGA binding was measured for 1 μg of disk protein in the absence and presence of 0.015% Triton X-100.

Cleavage products were examined by electrophoresis on 9% $NaDodSO_4$–polyacrylamide slab gels using the buffer system of Laemmli (1970).

Results

Binding of [^{125}I]Lectins to ROS Disks. Binding of [^{125}I]Con A, succinyl-[^{125}I]Con A, and [^{125}I]WGA to osmotically active sealed disks and Triton X-100 treated disks, as exemplified by succinyl-Con A in parts A and B of Figure 1, increased sharply over a lectin concentration of 1–50 μg and saturated at a lectin concentration greater than 100 μg/mL. Measurements of lectin binding to sealed disks, however, required a substantially higher disk protein concentration than when 0.015% Triton X-100 was present. This reflects a low level of lectin binding to freshly prepared disks compared to that for disks treated with Triton X-100. Specificity of binding was confirmed in control experiments in which binding was reduced by over 90% in the presence of the appropriate lectin inhibitor.

Nonlinear Scatchard plots (Scatchard, 1949) were obtained from the lectin binding data. With the assumption that there is no interaction between sites, analysis of the Scatchard plots indicates that at least two major classes of lectin binding sites are present in disk membrane preparations. Apparent dissociation constants and quantitation of Con A, succinyl-Con A, and WGA binding sites are summarized in Table I.

Disks treated with 0.015% Triton X-100 consistently showed a much greater amount of lectin binding compared to sealed disks. From the data in Table II, it can be seen that detergent-treated disks bound over 16 times more Con A, 28 times more succinyl-Con A, and 25 times more WGA than untreated, sealed disks. There was some variation in the absolute value of lectin bound to different disk preparations. For example, total Con A binding to disks in the absence of Triton X-100 and measured at a saturating Con A concentration varied from 0.026 to 0.13 μg of Con A per μg of disk protein. Con A binding was always greater than succinyl-Con A binding on a weight to weight basis. This is in part due to the tetrameric nature of Con A and the dimeric nature of succinyl-Con A (Gunther et al., 1973). Con A also showed greater nonspecific binding than succinyl-Con A.

Although the number of accessible lectin binding sites was greatly affected by Triton X-100, the affinity was not (Table I). The apparent dissociation constant (K_d) of 1.2×10^{-8} M for the binding of Con A (molecular weight 110000) and 2×10^{-8} M for the binding of succinyl-Con A (molecular weight 55000) to the high-affinity sites on disk membranes was comparable to the value of 1.6×10^{-8} M as reported by Yariv et al. (1974) for [^{60}Co]Con A (molecular weight 55000). The apparent K_d value of 2.8×10^{-7} M for the binding of Con A to the low-affinity site is similar to that reported by Steineman & Stryer (1973). WGA was found to bind less strongly than Con A.

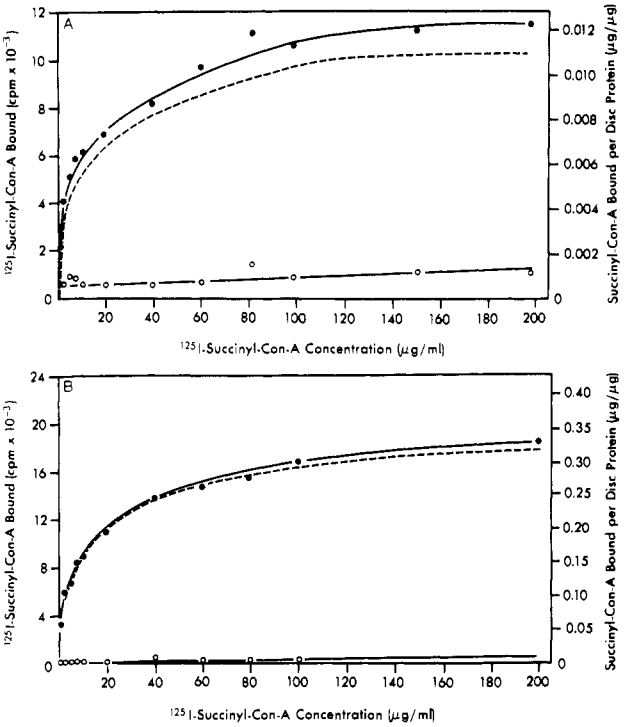

FIGURE 1: Binding curves of succinyl-[^{125}I]Con A to sealed disks and disks in the presence of 0.015% Triton X-100. Net lectin bound (---) is total lectin bound in the absence of inhibitor (●) less the lectin bound in the presence of inhibitor (○). Protein concentrations were 190 μg/mL for sealed disks and 10 μg/mL for disks in the presence of 0.015% Triton X-100. Binding to sealed disks (A); binding to disks with Triton X-100 (B).

Table II: Lectin Binding to Various Disk Preparations

lectin	Triton X-100 concn (%)	no. of disk prepn	av[a] of lectin found
Con A	0	11	0.067 ± 0.033
	0.015	4	1.12 ± 0.10
succinyl-Con A	0	5	0.013 ± 0.0053
	0.015	4	0.46 ± 0.12
WGA	0	10	0.025 ± 0.012
	0.015	6	0.62 ± 0.13

[a] Micrograms of lectin bound per microgram of disk protein. The standard deviation is given.

Effect of Detergents on Lectin Binding. Figure 2 shows the

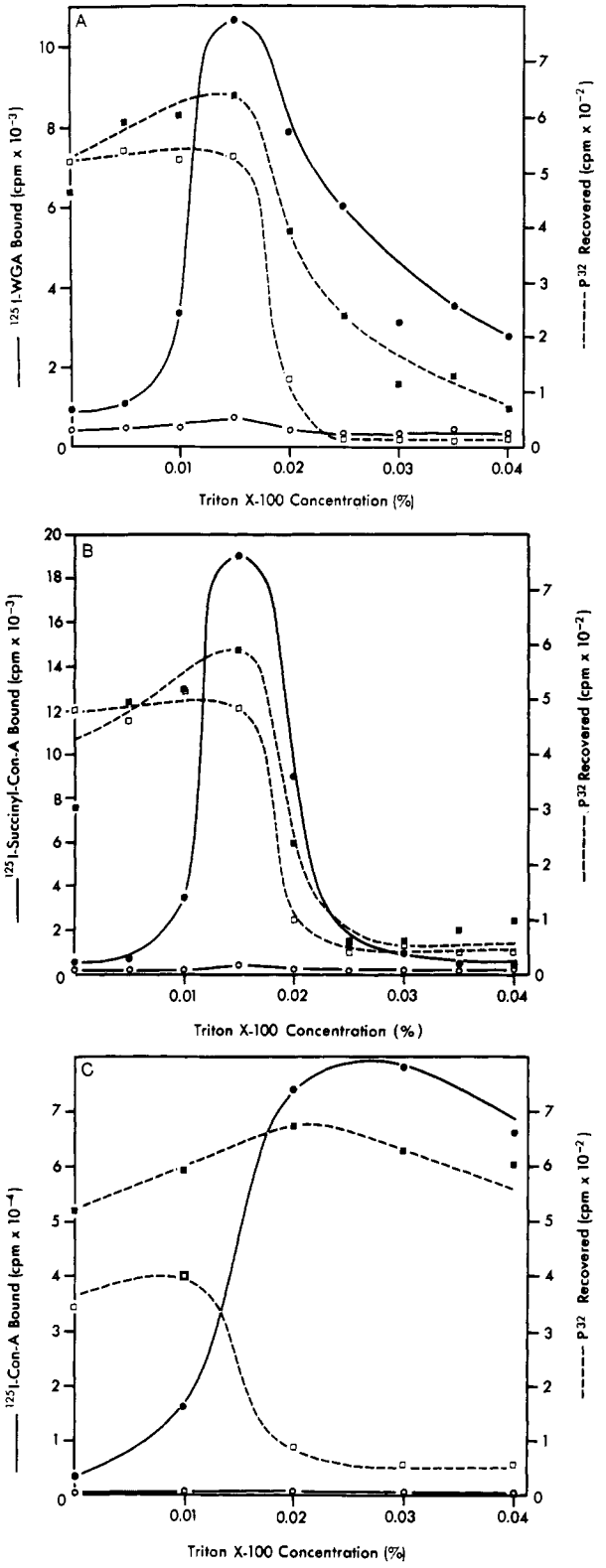

FIGURE 2: Effect of Triton X-100 on the binding of [^{125}I]lectins and the recovery of disk membranes. ^{32}P-Labeled disks (10 μg/mL) were incubated for 1.5 h with 150 μg/mL [^{125}I]lectins in the indicated Triton X-100 concentration in the presence or absence of inhibitor. Disks were then separated from unbound lectin by centrifugation. [^{125}I]-Lectin bound in the absence (●) and presence of inhibitor (○) was determined in a γ counter, and ^{32}P recovery in the absence (■) and presence (□) of inhibitor was determined in a liquid scintillation counter. (A) WGA; (B) succinyl-Con A; (C) Con A.

effect of Triton X-100 concentration on lectin binding and membrane solubilization of disk membranes. A large increase in WGA, succinyl-Con A, and Con A binding is evident with

Table III: Comparison of Lectin Binding to Unfrozen and Frozen-Thawed Disks

lectin	bound to disks[a] unfrozen	frozen once
Con A	0.063 ± 0.029	0.21 ± 0.09
succinyl-Con A	0.0096 ± 0.0048	0.16 ± 0.07
WGA	0.032 ± 0.011	0.048 ± 0.035

[a] Micrograms of lectin bound per microgram of disk protein. Average of four to six experiments ± standard deviation.

increasing Triton X-100 concentration. For a disk suspension having a protein concentration of 10 μg/mL, maximum binding for WGA and succinyl-Con A was reached at 0.015% Triton X-100. The disk membranes did not appear to be solubilized at this detergent concentration or stabilized by the lectins since they were quantitatively sedimented in the presence or absence of the lectin inhibitor as measured by ^{32}P recovery. At Triton X-100 concentrations greater than 0.015%, however, a decrease in succinyl-Con A and WGA binding paralleled the decrease in ^{32}P recovery. This apparent decrease in binding can be attributed to solubilization of the membrane.

In contrast, maximum binding of Con A at a disk protein concentration of 10 μg/mL is reached at 0.03% Triton X-100. On the order of 2 μg of Con A is bound per μg of disk. At this detergent concentration disks are largely solubilized as shown by the low ^{32}P recovery in the presence of α-methyl mannoside. In the absence of inhibitor, however, ^{32}P recovery and lectin binding are high at 0.03% Triton X-100 and fall only gradually at higher Triton X-100 concentrations (Figure 2C). High lectin binding and ^{32}P recovery are also obtained if Triton X-100 is added to disks prior to the addition of Con A. Thus, tetrameric Con A is not stabilizing the membrane but is apparently cross-linking detergent-solubilized disk glycoproteins. The large aggregates which form are readily sedimented under the conditions used in the assay. WGA and succinyl-Con A, which are divalent, also display this effect but to a much smaller extent (parts A and B of Figure 2).

The effect of disk concentration on WGA binding and disk solubilization as a function of Triton X-100 concentration was also investigated. As the disk concentration is increased, maximal WGA binding and disk solubilization as measured by ^{32}P recovery occur at a higher Triton X-100 concentration. At disk concentrations of 19, 38, and 95 μg/mL, maximal binding occurred at 0.018, 0.02, and 0.03% Triton X-100, respectively. The ratio of WGA bound to disk protein at maximum binding, however, was independent of disk protein concentration.

A similar bell-shaped curve as found with Triton X-100 was also observed when lectin binding was measured as a function of cholate concentration. Binding of the lectins increased sharply above 0.2% cholate. Maximum binding of 0.3 and 0.4 μg of succinyl-[^{125}I]Con A and [^{125}I]WGA per μg of disk protein was reached at a cholate concentration of 0.45–0.50% when a disk protein concentration of 10 μg/mL was used. For [^{125}I]Con A maximum binding was reached at 0.6% cholate where approximately 1.4 μg of Con A was bound per μg of disk protein. At higher cholate concentrations an apparent decrease in lectin binding was observed due largely to solubilization of the disk membrane.

Effect of Freezing and Thawing on ROS Disks. When sealed disks were frozen, succinyl-Con A binding increased 16-fold (Table III). Binding of tetrameric Con A to disks which had been frozen was also greater, but relatively high backgrounds, i.e., Con A binding in the presence of α-methyl

Table IV: Binding of Succinyl-Con A to Disks Treated with Proteolytic Enzymes

proteolytic treatment	succinyl-Con A bound[a]		increase[b] in binding
	sealed	Triton X-100	
untreated	0.021	0.57	27.1×
trypsin	0.014	0.43	30.7×
protease	0.014	0.51	36.4×

[a] Micrograms of succinyl-Con A bound per microgram of disk protein. Disks were incubated in buffer (untreated), trypsin, or *S. griseus* protease for 2 h, washed by repeated centrifugation, and assayed for lectin binding in the absence (sealed) or in the presence of 0.015% Triton X-100. [b] Ratio of lectin bound with Triton X-100 present to lectin bound in the absence of Triton X-100 (sealed).

mannoside, made the data more variable. In general, however, binding was less than that observed for Triton X-100 treated disks. WGA, on the other hand, showed only a slight increase in binding to frozen and thawed disks.

The effect of freezing on the sedimentation properties of disks on 5% Ficoll was also studied. Whereas essentially all freshly prepared disks washed in distilled water were found to refloat on Ficoll after centrifugation, only 15% of the disks floated after they had been frozen 1 time. Disks which were repeatedly frozen and thawed 10 times all sedimented through the Ficoll layer. Repeated freezing and thawing were also found to cause the disks to aggregate.

Effect of Proteolytic Enzymes on Lectin Binding to Disks. Disks treated with trypsin or *S. griseus* protease exhibited the same low level of succinyl-Con A and WGA binding as that observed for untreated disks (Table IV). Enzyme-treated disks, however, do contain lectin binding sites, since these disks bind large quantitites of lectins when assayed in the presence of 0.015% Triton X-100.

$NaDodSO_4$ gel electrophoresis of enzyme-treated disks confirms the fact that essentially all the rhodopsin, the high molecular weight glycoprotein ROS 1.2, and several minor components are cleaved by trypsin and *S. griseus* protease in sealed disk preparations. Rhodopsin is degraded to a fragment of apparent molecular weight 32000 by trypsin and a fragment of apparent molecular weight 26000 by *S. griseus* protease as previously reported (Molday & Molday, 1979). In this study these fragments have been shown to bind Con A. Additional lower molecular weight fragments are also produced by *S. griseus* protease treatment. ROS 1.2 is observed to be degraded to a fragment of apparent molecular weight 110000 by trypsin. Treatment of ROS membranes with thermolysin also has been shown to produce membrane-bound peptides of rhodopsin which contain Con A binding sites (Pober & Stryer, 1975).

Discussion

The results reported here show that osmotically active, sealed disk preparations bind only small quantities of Con A and WGA, whereas disks which have been treated with low concentrations of detergent or subjected to freezing and thawing bind significantly larger quantities of these lectins. We interpret these findings to indicate that rhodopsin, the major lectin binding component in ROS (Steineman & Stryer, 1973; Molday & Molday, 1979), is oriented with its oligosaccharide chains toward the interior of sealed disks where they are inaccessible to the lectins unless the disks are unsealed. This orientation is consistent with the electron microscope studies of Röhlich (1976) which showed that Con A binding sites are accessible only on the intradisk membrane surface and the more recent studies of Adams et al. (1978), who demonstrated that intact disks do not bind to Con A–Sepharose columns or to ferritin–Con A markers. It is also consistent with the localization of lectin binding sites on the extracellular surface of the ROS plasma membrane by electron microscopy (Molday, 1976; Hall & Nir, 1976) and the observation that disk structures are derived from the ROS plasma membrane by an invagination and pinching off of this membrane (Moody & Robertson, 1960).

Calculations based on the lectin binding data in Table II and on the assumption that only one succinyl-Con A (M_r 55000), one tetrameric Con A (M_r 110000), or one WGA (M_r 23000) binds to one rhodopsin molecule (M_r 34000; 90% of disk protein) reveal that on the order of 0.01 mol of succinyl-Con A, 0.02 mol of Con A, or 0.04 mol of WGA binds per mol of rhodopsin in sealed disk preparations. These values are, at best, rough estimates since one multivalent lectin may bind to more than one rhodopsin molecule or, conversely, two lectin molecules may bind to one rhodopsin molecule, i.e., each of the carbohydrate chains (Hargrave, 1977), in the absence of steric hindrance. Furthermore, the Lowry method may result in some error in the protein concentration determination (Downer & Englander, 1977), and the molecular weight of WGA is not firmly established (Nicolson, 1974; Goldstein & Hayes, 1978).

The low level of lectin binding to sealed disk preparations is most likely due primarily to the presence of a small population (1–4%) of unsealed or inside-out disk vesicles. Although these disk preparations are highly purified (Smith et al., 1975; Molday & Molday, 1979), residual membrane contaminants may also contribute to the observed lectin binding. It would appear unlikely that a small percentage of the lectin binding sites are exposed on the exterior surface of disks since glycoproteins and glycolipids are asymmetrically oriented in biological membranes (Singer & Nicolson, 1972; Rothman & Lenard, 1977); i.e., oligosaccharide chains are exposed on only one of the membrane surfaces. Such a possibility, however, cannot be ruled out on the basis of these experiments. Studies by transmission (Nir, 1978) and scanning electron microscopy (Molday, 1976) which showed some Con A binding to the exterior surface of disks may be due to cytoplasmic glycoproteins or mucopolysaccharides which adsorb to the disk surface under conditions of sample preparation. In the latter study it is also possible that the observed binding is due to ROS plasma membrane which still surrounds the disks. The observed accessibility of carbohydrates on rhodopsin to fluorescent Con A (Steineman & Stryer, 1973) and enzymatic modification (Shaper & Stryer, 1977) is likely due to the use of unsealed ROS vesicle preparations in these studies.

Proteolytic studies on sealed disks (Molday & Molday, 1979) in conjunction with lectin binding studies described here lead to further insight into the properties of these disk preparations and the organization of ROS disk membrane glycoproteins. Treatment of sealed disks with trypsin or *S. griseus* protease is shown not to affect the extent of lectin binding. Enzyme-treated disks bound the same amount of lectin as untreated disks when assayed in the absence or presence of Triton X-100. $NaDodSO_4$ gel electrophoresis, however, showed that rhodopsin and ROS 1.2 were quantitatively degraded into smaller carbohydrate-containing fragments. Since additional lectin binding sites do not become accessible even after degradation and some loss in membrane protein by proteolysis (van Breugel et al., 1975; Pober & Stryer, 1975), it can be argued that these proteolytic enzymes do not unseal the disks to macromolecules. Furthermore, these results support the view that even masked or cryptic lectin binding

sites are most likely not present on the exterior of the disks.

The presence of proteolytic-sensitive regions of rhodopsin on the exterior of these sealed disks taken together with the presence of carbohydrate chains on the interior surface of the disk provides biochemical evidence for the transmembrane disposition of rhodopsin. Thus, rhodopsin is situated in ROS disk membranes with its carbohydrate-containing blocked amino-terminal segment (Hargrave, 1977) oriented toward the interior of the disk and a trypsin-sensitive carboxyl-terminal segment and other protease-sensitive regions exposed on the exterior of the cytoplasmic side of the disks. This is in agreement with the observed transmembrane disposition of rhodopsin in reconstituted vesicles as recently reported by Fung & Hubbell (1978). Proteolytic digestion also indicates that the ROS 1.2 polypeptide is partially exposed on the exterior side of the disk membrane. On the basis of indirect evidence, one can argue that the carbohydrate residues of ROS 1.2 are most likely exposed on the same side of the disk membrane as rhodopsin, i.e., the interior side. This is based on the fact that biological membranes are asymmetrical with respect to their glycoproteins as cited above and the finding of Adams et al. (1978), who failed to observe ferritin–Con A binding to the exterior surface of disks by electron microscopy. Thus, it would appear that ROS 1.2 is also a transmembrane glycoprotein in disks with its oligosaccharide moiety oriented toward the interior of the disk. However, without more direct measurements, this orientation must be considered tentative.

The increase in lectin binding to disks which have been frozen and thawed or treated with detergents can be attributed primarily to an unsealing of the disks which makes glycoproteins more available for lectin binding. This is supported by the observation that most of the disks which have been frozen and thawed no longer float on Ficoll and, therefore, are no longer osmotically intact. Disks which have been frozen were also observed by Adams et al. (1978) to bind ferritin–Con A markers. Disks disrupted with low concentrations of Triton X-100, but not solubilized, as measured by ^{32}P recovery after centrifugation, were found to bind more lectin than disks which had been unsealed by freezing. This was particularly true for WGA. Thus, it would appear that detergents not only unseal the disks but also cause structural changes which are manifested in a further increased accessibility of the glycoproteins to lectins and, in particular, WGA. Additional Con A binding sites become accessible when the disks become solubilized at higher Triton X-100 concentrations. Thus, carbohydrate chains of ROS glycoproteins and, in particular, rhodopsin appear to be only partially accessible to lectins in disrupted disk membranes as a result of steric constraints.

Finally, these experiments and other recent studies (Norisuye et al., 1976; Adams et al., 1978; Molday & Molday, 1979; Albert & Litman, 1978) indicate that sealed disks prepared by the method of Smith et al. (1975) provide a particularly useful system to study structural and functional properties of retinal rod photoreceptor membranes. Quantitative lectin binding studies serve as a valuable marker to assess the relative purity of sealed, right-side-out disks.

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