Accessibility of the Carbohydrate Moiety of Rhodopsin†

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ABSTRACT: Rhodopsin contains a covalently attached carbohydrate moiety (Heller, J., and Lawrence, M. A. (1970), *Biochemistry 9*, 864). The location of this carbohydrate unit in the intact disk membrane was investigated by using concanavalin A (Con A) as a specific macromolecular probe. Con A is known to bind mono- and polysaccharides that contain terminal nonreducing α-mannosyl and α-glucosyl residues. Fluorescein-labeled Con A binds to disk membranes with a dissociation constant of 2×10^{-7} M. At saturation, one Con A monomer is bound per retinal. The binding of Con A to disk membranes is specific since it is inhibited by α-methyl D-mannoside. Bleaching of the rhodopsin in disk membranes has little effect on the binding of Con A. The accessibility of the carbohydrate moiety of rhodopsin to Con A suggests that this group is located on the surface of the disk membrane. Rhodopsin can be separated from some other constituents of the rod outer segment by affinity chromatography on a Con A–agarose column. Rhodopsin solubilized by cetyltrimethylammonium bromide binds strongly to a column consisting of Con A covalently attached to agarose. The bound rhodopsin is eluted from this column by 0.1 M D-glucose in detergent buffer.

Rhodopsin contains a covalently attached carbohydrate moiety consisting of three *N*-acetylglucosamine and three mannose residues (Heller and Lawrence, 1970). It has been suggested that this carbohydrate unit serves as a hydrophilic marker that orients rhodopsin in retinal disk membranes. We have investigated the location of the carbohydrate moiety of rhodopsin in intact disk membranes by using concanavalin A (Con A)[1] as a specific macromolecular probe. Con A, a lectin from jack beans (Sumner and Howell, 1936), specifically binds α-D-mannosyl and α-D-glucosyl residues at the nonreducing termini of mono-, oligo-, and polysaccharides (Goldstein *et al.*, 1965). Below pH 6, Con A is a dimer of identical subunits (Kalb and Lustig, 1968). Each 27,000-dalton subunit contains one carbohydrate binding site (Becker *et al.*, 1971; Hardman and Ainsworth, 1972). Con A has been used to monitor structural changes in the plasma membrane of transformed cells (Inbar and Sachs, 1969; Burger and Noonan, 1970). Ferritin-labeled Con A has served as an electron microscopic stain to visualize the distribution of certain oligosaccharides on cell surfaces (Nicolson and Singer, 1971).

We report here studies of the binding of Con A to bovine retinal disk membranes. Mixtures of varying amounts of Con A and disk membranes were centrifuged, and the supernatant was analyzed to determine the concentration of unbound Con A. Con A was labeled with fluorescein to facilitate the analysis. We found that Con A binds specifically to disk membranes. In fact, one Con A monomer is bound per retinal, which suggests that the carbohydrate moiety of rhodopsin is located on the surface of the disk membranes. We have taken advantage of the accessibility of the carbohydrate moiety of rhodopsin to Con A to carry out affinity chromatography of rhodopsin on a column consisting of Con A covalently attached to agarose.

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[1] The abbreviations used are: Con A, concanavalin A; F-Con A, fluorescein-labeled concanavalin A; Con A–agarose, agarose containing covalently attached concanavalin A.

Materials and Methods

Preparation of Retinal Disk Membranes. Rod outer segments (ROS) were isolated from bovine retinas (G. Hormel Co., Austin, Minn.) as previously described (Matthews *et al.*, 1963; Waggoner and Stryer, 1971). These ROS were suspended in deionized water, vortexed for about 15 sec, and centrifuged at 27,000g for 40 min. This procedure was repeated three times. The preparation at this stage consisted of disk membranes in the form of closed vesicles that had an average diameter of 0.4 μ (electron microscopic observations of glutaraldehyde-fixed, osmium-stained membranes by J. Pober). These washed disk membranes were then suspended in the standard buffer used in this study, which consisted of 10^{-3} M $CaCl_2$ and 10^{-3} M $MnCl_2$ in 0.05 M sodium acetate buffer, pH 5.0. The concentration of rhodopsin (expressed in terms of the concentration of its 11-*cis*-retinal prosthetic group) in the suspension of disk membranes was determined spectrophotometrically. Disk membranes (1 ml) suspended in standard buffer were mixed with 1 ml of 1.4% cetyltrimethylammonium bromide in standard buffer and vortexed for a few seconds. The absorbance of this solution was measured at 500 nm before and after bleaching. The concentration of rhodopsin was calculated from the difference in absorbance at 500 nm using an extinction coefficient of 40,000 cm^{-1} M (Heitzmann, 1972). All procedures involving disk membranes and rhodopsin were carried out at 3° under dim red light unless otherwise indicated.

Preparation of Fluorescein-Labeled Concanavalin A. Con A (grade 4) and fluorescein isothiocyanate (isomer I) were obtained from Sigma Chemical Co. Fluorescein isothiocyanate adsorbed on Celite (0.2 mg of reagent/mg of Celite) was prepared as described (Rinderknecht, 1962; Gennis *et al.*, 1972). Fluorescein-labeled Con A (F-Con A) was prepared by addition of 66 mg of fluorescein isothiocyanate–Celite to 200 mg of Con A dissolved in 25 ml of sodium acetate buffer, pH 6.8, containing 10^{-3} M $CaCl_2$ and 10^{-3} M $MnCl_2$. The mixture was very gently stirred overnight at 3° and then centrifuged at 10,000g for 15 min. The supernatant was passed through a Sephadex G-10 column equilibrated with standard buffer containing 1 M NaCl. The unreacted labeling reagent was adsorbed by the column. The effluent containing F-Con

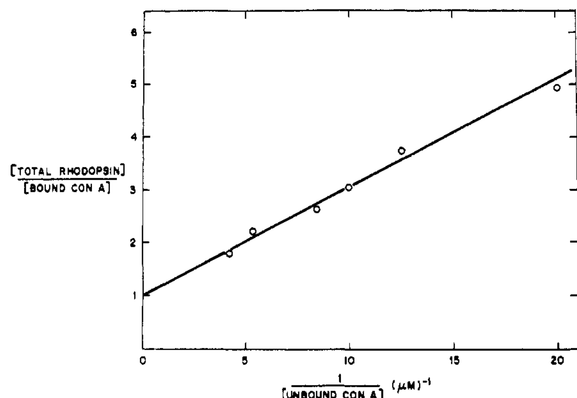

FIGURE 1: Determination of the stoichiometry and dissociation constant of the complex between F-Con A and disk membranes. The total concentration of disk membranes (expressed in terms of the rhodopsin concentration) was 1.8×10^{-6} M. The concentration of F-Con A was varied in this experiment. The intercept of 0.96 indicates that disk membranes bind one Con A monomer per retinal at saturation. The dissociation constant calculated from the slope and intercept is 2×10^{-7} M.

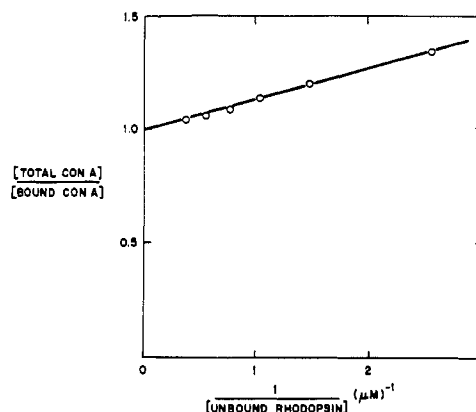

FIGURE 2: Determination of the binding activity of F-Con A to disk membranes. The total concentration of F-Con A was 5.6×10^{-7} M. The concentration of disk membranes (expressed in terms of the rhodopsin concentration) was varied in this experiment. The intercept of 1.0 indicates that this preparation of F-Con A had full binding activity.

A was then purified by affinity chromatography on Sephadex G-100 (Agrawal and Goldstein, 1967). The effluent from the G-10 column was transferred to a 1.5×29 cm column of Sephadex G-100 equilibrated with standard buffer containing 1 M NaCl. A small proportion of the fluorescent-labeled material was eluted by this buffer. The rest formed a colored band at the top of the column. Active F-Con A was eluted by standard buffer containing 0.1 M D-glucose and 1 M NaCl. This solution of F-Con A could be stored at 3° for at least 1 week without loss of activity. Aliquots were taken from this stock solution and glucose was removed by dialysis against standard buffer.

The concentrations of Con A and of the covalently attached fluorescein chromophore were determined from the absorption spectrum of a solution of F-Con A in pH 6.8 buffer. These extinction coefficients were used: 55,000 cm^{-1} M^{-1} at 495 nm and 19,800 cm^{-1} M^{-1} at 280 nm for fluorescein; 30,500 cm^{-1} M^{-1} at 280 nm for Con A. This molar extinction coefficient for Con A (Agrawal and Goldstein, 1968) refers to the 27,000-dalton subunit that contains one saccharide binding site. The preparations of F-Con A used in this study contained about 0.2 fluorescein per Con A monomer.

Preparation of Concanavalin A–Agarose. Con A was covalently attached to agarose that was activated by reaction with cyanogen bromide (Porath *et al.*, 1967; Lloyd, 1970; Edelman *et al.*, 1971). A suspension of 75 ml of Sepharose 4B (Pharmacia) in an equal volume of H_2O was brought to pH 10.5 by the addition of 0.2 M NaOH. Cyanogen bromide (Eastman), 1.5 g in 30 ml of H_2O, was added to this suspension. The mixture was stirred gently at room temperature, and the pH was maintained at 10.5 by addition of 0.2 N NaOH. After 10 min, the suspension was transferred to a Büchner funnel and washed with 750 ml of cold H_2O, followed by 750 ml of cold 0.1 M sodium acetate buffer, pH 6.8. The tip of the Büchner funnel was then sealed with parafilm and 150 mg of Con A in 75 ml of 0.1 M sodium acetate buffer, pH 6.8, containing 0.15 M NaCl was added to the activated agarose. The mixture was transferred to a beaker and gently stirred overnight at 3°. Unattached Con A was removed by washing the suspension with 1 l. of 1 M NaCl containing 10^{-3} M $CaCl_2$ and 10^{-3} M $MnCl_2$. The Con A–agarose preparation was stored at 3° in standard buffer containing 1 M NaCl.

Determination of Binding. Standard buffer was used in the binding studies. Experiments were carried out at 3° unless otherwise indicated. In a typical experiment, 0.5 ml of a disk membrane suspension (10^{-5} M in retinal), 2 ml of a 2×10^{-6} M solution of F-Con A, and 3 ml of buffer were mixed in a test tube and vortexed for about 20 sec. A set of tubes containing varying amounts of disk membranes and F-Con A were centrifuged at 23,000*g* for 30 min. The supernatant contained unbound F-Con A, whereas the pellet contained disk membranes and bound F-Con A. The concentration of F-Con A in the supernatant was determined from the intensity of the fluorescein fluorescence. The excitation wavelength was 480 nm. A Corning 3-69 filter was used to isolate the fluorescein emission.

Results

Stoichiometry and Affinity of Binding of F-Con A to Disk Membranes. F-Con A binds strongly to disk membranes. The stoichiometry and affinity of binding were determined by mixing a fixed amount of disk membranes with varying amounts of F-Con A. After centrifugation, the concentration of F-Con A in the supernatant (which is equal to the concentration of unbound F-Con A) was measured. The resulting linear plot of [total rhodopsin]/[bound F-Con A] *vs.* 1/[unbound F-Con A] is shown in Figure 1. The mean intercept derived from six binding experiments was 0.96 ± 0.04. This result indicates that disk membranes bind one Con A monomer per retinal at saturation. The ratio of the slope to the intercept of this double reciprocal plot (Figure 1) gives the dissociation constant K of the complex, which is 2×10^{-7} M. There was some variation in the binding constants of different preparations of disk membranes. K for six preparations ranged from 1.1×10^{-7} M to 3.3×10^{-7} M.

Binding Activity of F-Con A. The above analysis assumes that the F-Con A preparation was fully active. The actual proportions of active molecules in preparations of F-Con A used in this study were determined by mixing a fixed amount of F-Con A with varying amounts of disk membranes. The resulting linear plot of [total F-Con A]/[bound F-Con A] *vs.* 1/[unbound rhodopsin] is shown in Figure 2. The intercept of 1.00 ± 0.02 indicates that this preparation of F-Con A was fully active in binding disk membranes. The dissociation

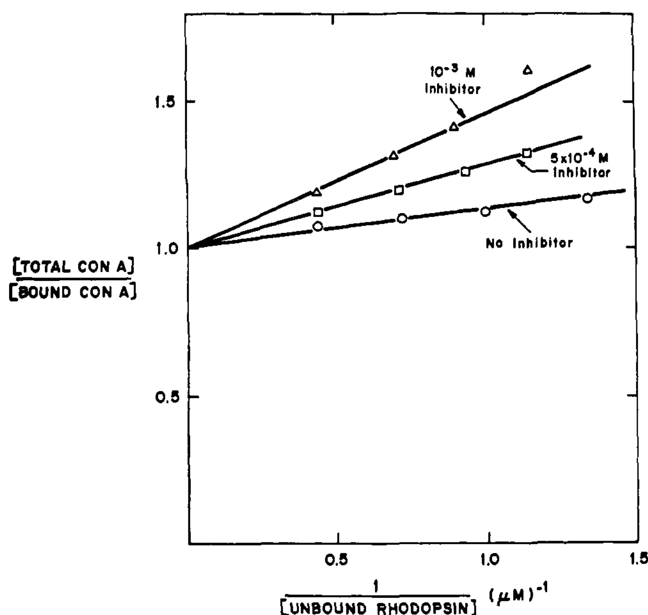

FIGURE 3: Inhibition of the binding of F-Con A to disk membranes by α-methyl D-mannoside. The total concentration of F-Con A was 5.3×10^{-7} M. The apparent dissociation constant of the complex between F-Con A and disk membranes is 2.9×10^{-7} M and 4.6×10^{-7} M in the presence of 5×10^{-4} and 10^{-3} M α-methyl D-mannoside, respectively. The dissociation constant in the absence of this competing sugar is 1.4×10^{-7} M.

constant given by the slope of this plot is 2.2×10^{-7} M, which agrees with the value determined from binding experiments of the type shown in Figure 1. Solutions of F-Con A in standard buffer retained full binding activity for at least 1 week when stored at 3°. In contrast, solutions of F-Con A in 0.1 M sodium phosphate buffer, pH 6.8, lost about half of their disk binding activity when stored at 3° for 1 week. The degree of binding of fresh F-Con A to disk membranes is nearly the same at pH 5 and 6.8.

Inhibition of Binding by α-Methyl D-Mannoside. The effect of α-methyl D-mannoside on the binding of F-Con A to disk membranes was investigated to determine whether this interaction is mediated by the saccharide binding site of F-Con A. α-Methyl D-mannoside binds strongly to the saccharide binding site of Con A (Goldstein *et al.*, 1965). As anticipated, α-methyl D-mannoside is a competitive inhibitor of the binding of F-Con A to disk membranes (Figure 3). The apparent dissociation constants K' of the complex between F-Con A and disk membranes are 2.9×10^{-7} M and 4.6×10^{-7} M in the presence of 5×10^{-4} M and 10^{-3} M α-methyl D-mannoside, respectively. The inhibition constant K_i for α-methyl D-mannoside can be calculated from the observed dissociation constant in the absence of this inhibitor ($K = 1.4 \times 10^{-7}$ M) and in its presence (K') at a concentration [I]: $K' = K(1 + [I]/K_i)$. The K_i calculated from this equation is 4.5×10^{-4} M.

Effect of Temperature on the Binding Affinity. The binding affinity changed by a factor of only 1.5 in going from 2 to 27°. The dissociation constants determined for a single preparation of disk membranes were 1.1×10^{-7} M at 2°, 1.3×10^{-7} M at 18°, and 1.7×10^{-7} M at 27°. Measurement of the binding constant at higher temperatures was not feasible because F-Con A aggregates under those conditions.

Effect of Bleaching on the Binding Affinity. Bleaching of the rhodopsin in disk membranes had only a small effect on the

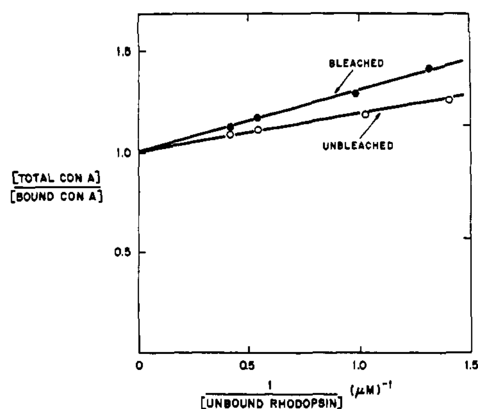

FIGURE 4: Effect of bleaching on the binding of F-Con A to disk membranes. The dissociation constant was 1.6×10^{-7} M for unbleached disk membranes and 2.7×10^{-7} M for bleached disk membranes. The total concentration of F-Con A was 5.6×10^{-7} M.

binding of F-Con A. The dissociation constant for bleached disk membranes was 2.7×10^{-7} M, whereas an unbleached aliquot of the same preparation of disk membranes exhibited a binding constant of 1.6×10^{-7} M (Figure 4). There was no detectable difference in the number of binding sites for F-Con A in bleached and unbleached disk membranes. In the experiments involving bleached disk membranes, F-Con A was added after bleaching was completed.

Affinity Chromatography of Rhodopsin on Con A–Agarose. Solubilized rhodopsin binds to Con A that is covalently attached to an insoluble agarose matrix. Disk membranes were solubilized in detergent buffer (1.4% cetyltrimethylammonium bromide in standard buffer) and loaded on a Con A–agarose column in dim red light. The elution profile is shown in Figure 5. Almost no rhodopsin emerged when the column was washed with detergent buffer, as evidenced by the virtual absence of 500-nm absorbing material in the first set of fractions. This first peak contained about 14% of the protein and nearly all of the phosphorus that was loaded on the column. The amino acid analysis of the peak was different

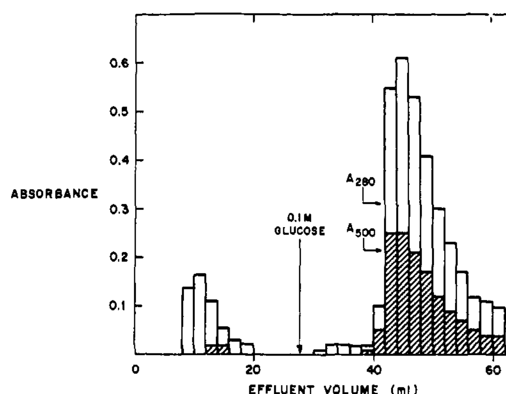

FIGURE 5: Affinity chromatography of rhodopsin on a Con A–agarose column. Disk membranes containing 70 nmol of rhodopsin were solubilized by the addition of 2 ml of detergent buffer (1.4% cetyltrimethylammonium bromide in standard buffer). This solution was loaded on a 1 × 9 cm column of Con A–agarose equilibrated with detergent buffer (effluent from 8 to 20 ml). The rhodopsin bound to the column was eluted by 0.1 M D-glucose in detergent buffer (effluent from 38 to 62 ml). The absorbance of the effluent was measured at 280 nm (top of unfilled bars) and at 500 nm (top of crossed bars).

from that of rhodopsin. The identity of this material has not yet been established. Virtually all of the rhodopsin loaded on the column was bound to it. The bound rhodopsin was then eluted from the column by addition of 0.1 M D-glucose in detergent buffer. Glucose displaced rhodopsin from the saccharide binding site of Con A. The recovery of rhodopsin, based on the absorbance at 500 nm, was 90%. The capacity of the column was 14 nmol of rhodopsin/ml of Con A–agarose.

Discussion

Concanavalin A binds tightly to disk membranes. The dissociation constant of the complex is 2×10^{-7} M. Con A binds to carbohydrate groups on disk membranes, as evidenced by the inhibition of the interaction by α-methyl D-mannoside. The linearity of the binding plots (Figures 1 and 2) indicates that there is a single class of binding sites. The stoichiometry of one Con A binding site per retinal strongly suggests that Con A binds to the carbohydrate moiety of rhodopsin molecules in disk membranes. *We conclude that the carbohydrate unit of rhodopsin is located on the surface of disk membranes where it is accessible for interaction with Con A.* We have recently observed that wheat germ agglutinin (which is specific for *N*-acetylglucosamine residues) also binds specifically to disk membranes. These findings support the proposal that the carbohydrate group acts as a hydrophilic surface marker to orient rhodopsin in the assembly of the disk membrane (Heller and Lawrence, 1970). The present study does not reveal whether the carbohydrate moiety of rhodopsin is located on the inner, outer, or both surfaces of intact disks. Electron microscopic studies of the interaction of rod outer segments with ferritin-labeled or peroxidase-labeled Con A should answer this important question.

It is significant that the Con A–agarose column had full binding activity in 1.4% cetyltrimethylammonium bromide. Disk membranes are solubilized by this high concentration of detergent. Affinity chromatography on Con A–agarose appears to be a promising means of separating rhodopsin from some other rod outer segment proteins (Figure 5). The purification of rod outer segment enzymes such as adenylyl cyclase (Bitensky *et al.*, 1971) and rhodopsin kinase (Kühn and Dreyer, 1972; Bownds *et al.*, 1972) may be facilitated by the use of Con A–agarose and other lectin affinity columns.

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