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Accessibility of the Carbohydrate Moiety of Membrane-Bound Rhodopsin to Enzymatic and Chemical Modification

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Galactose was specifically inserted into the carbohydrate moiety of rhodopsin by incubating retinal disk membranes with UDP-galactose: N-acetylglucosamine galactosyltransferase. The stoichiometry of labeling ranged from 1.2 to 1.8 (average = 1.5) residues of galactose per molecule of rhodopsin, indicating that some or all of the oligosaccharide chains of membrane-bound rhodopsin are readily accessible to enzymatic modification. These modified membranes were treated with galactose oxidase to generate an aldehyde at the C-6 position of the inserted galactose units. The enzymatically-oxidized membranes were then reacted with dansyl hydrazide to yield a fluorescent hydrazone which is sufficiently stable to permit spectroscopic analysis. This procedure for the specific attachment of a spectroscopic probe should be applicable to a wide variety of membrane glycoproteins.

Key words: rhodopsin, retinal disk membranes, galactosyl transferase, fluorescent probes, carbohydrate unit, enzymatic modification

The use of fluorescence and other spectroscopic techniques to elucidate aspects of the structure and dynamics of biological macromolecules often depends on the introduction of a suitable probe at a specific site. The carbohydrate moiety of glycoproteins is a poten-

Abbreviations: UDP-Galactose – uridine diphosphogalactose; DH – dansyl hydrazide (1-dimethylaminonaphthalene-5-sulfonyl hydrazine); Gal-rhodopsin – rhodopsin in which the oligosaccharide side chain has been enzymatically modified by the addition of galactose; DH-Gal-rhodopsin – Gal-rhodopsin which has been fluorescently labeled with DH at the C-6 position of the terminal galactose after enzymatic oxidation with galactose oxidase; DTAB – dodecyl trimethlyammonium bromide; ALO – Ammonyx-LO; Con A – concanavalin A

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tial site for specific labeling. One approach has been to use periodate to oxidize vicinal diols to aldehydes, which then react with fluorescent hydrazides (1–3). The procedure for oxidizing the glycerol side chain of sialic acid residues is gentle and highly specific (4). However, periodate oxidation is not an ideal labeling procedure for other sugars because several sugar residues in the oligosaccharide chain are usually extensively modified. Also, periodate oxidation may result in side reactions in amino acid residues (5). Reactive aldehydes can be generated enzymatically in a more selective way if galactose or N-acetylgalactosamine is present. Galactose oxidase specifically converts the C-6 CH_2OH group of terminal galactosyl residues or N-acetylgalactosyl residues to an aldehyde (6). Penultimate galactose residues in proteins such as transferrin can be labeled by this sequence of reactions after the terminal sialic acid residues are enzymatically removed by neuraminidase (Shaper and Stryer, to be published).

We report here a novel method for specifically labeling the carbohydrate moiety of membrane bound glycoproteins that lack sialic acid and galactose but contain N-acetylglucosamine. We have applied this approach to rhodopsin, the photoreceptor protein in the disk membranes of retinal rod outer segments. Heller and Lawrence (7) reported that rhodopsin contains a single oligosaccharide chain consisting of 3 mannose and 3 N-acetylglucosamine residues. Subsequent analyses have shown that there are 9 moles of mannose and 5 moles of N-acetylglucosamine per mole of rhodopsin (8). Hargrave and Fong (9) have recently found that rhodopsin contains 2 oligosaccharides which are linked to amino acid residues 2 and 13. The presence of N-acetylglucosamine suggested that it might be feasible to enzymatically insert a galactose residue into rhodospin by reacting disk membranes with UDP-galactose in the presence of UDP-galactosyl transferase, an enzyme known to catalyze the formation of a 1,4-glycosidic bond between galactose and N-acetylglucosamine (10). In fact, we have incorporated 1.2–1.8 (average = 1.5) galactose residues per rhodopsin in this way. The inserted galactose residues were then oxidized with galactose oxidase, and dansyl hydrazide was coupled to the resulting aldehyde. This series of reactions is shown on Fig. 1.

MATERIALS AND METHODS

Materials

Rod outer segments were prepared from bovine retinas (Hormel) as previously described (11, 12) and stored in the dark at −20°C until used. Galactosyl transferase (UDP-galactose:N-acetylglucosamine galactosyl transferase) was isolated from bovine milk by affinity chromatography on UDP-Sepharose and α-lactalbumin-Sepharose 4B was prepared as described by Barker et al. (13). Galactosyl transferase activity was assayed by following the transfer of uniformly labeled [^{14}C]galactose from UDP-[^{14}C]-D-galactose to N-acetylglucosamine as described by Trayer and Hill (14). Crude galactose oxidase from Polyporus circinatus was purchased from Sigma and partially purified by chromatography on DEAE as indicated by Morell and Ashwell (15). Homogeneous galactose oxidase was generously provided by Dr. D. J. Kosman of the State University of New York at Buffalo. The galactose oxidase, supplied as a solution in 1 M $(NH_4)_2$ SO_4, pH 7.0, was dialyzed at 4°C against 2 changes of Buffer A and stored at 4°C until used. The oxidase was purified, assayed, and the units of activity defined as described by Kosman et al. (16). UDP-[^{14}C]galactose, uniformly labeled and [methyl-^{3}H]-5-dimethyl-amino-1-naphthalenesulfonyl chloride (dansyl chloride) were obtained from New England Nuclear

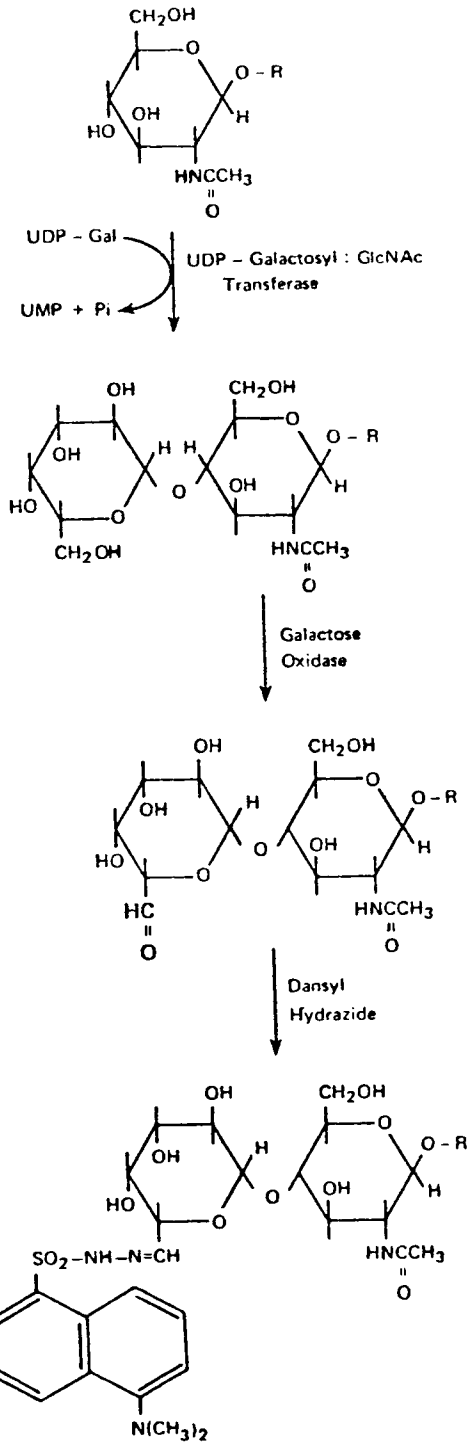

Fig. 1. The sequence of enzymatic and chemical reactions employed to introduce a fluorescent probe into the nonreducing terminal residue of a carbohydrate unit of rhodopsin.

Corporation. Unlabeled UDP-galactose was purchased from Sigma. Unlabeled dansyl chloride and dansyl hydrazide (1-dimethylaminonaphthalene-5-sulfonyl hydrazine) were obtained from Pierce. Dansyl hydrazide was recrystallized from warm absolute ethanol before use.

Synthesis of [^{3}H] Dansyl Hydrazide

One mCi of [methyl-^{3}H]-dansyl chloride in acetone was transferred to a glass-stoppered flask, and dried under a stream of nitrogen. Four milliliters of hydrazine (95+%, Eastman) were rapidly added and the mixture was stirred for 15 min. An additional 100 mg of unlabeled dansyl chloride was added slowly to the hydrazine solution over a 30-min period. The reaction mixture was stirred for an additional 60 min and then transferred to a separatory funnel containing 50 ml ethyl acetate and 50 ml saturated sodium bicarbonate. The aqueous phase was removed and extracted twice more with ethyl acetate. The organic phases were combined and back extracted 3 times with saturated sodium bicarbonate, 3 times with water, and then dried over anhydrous magnesium sulfate. The ethyl acetate was removed on a rotary evaporater, and the residue dissolved in 2 ml of warm absolute ethanol. Upon storage at −10°C, crystallization of [^{3}H] dansyl hydrazide began after 6 h and was complete after 24 h. The product was collected and washed with a minimum amount of cold absolute ethanol (−40°C) and stored at −10°C. The product (mp 124–126°C) cochromatographed with commercially available dansyl hydrazide on polyamide thin layer sheets (Cheng Chen Trading Co., Taipai, Taiwan) in 2 solvent systems: 1.5% formic acid and benzene/glacial acetic acid (9:1). The specific activity of the labeled hydrazide obtained in different syntheses ranged between 1.5 and 2×10^9 cpm/mmole.

Enzymatic Modification of the Carbohydrate Moiety of Rhodopsin With UDP-Galactosyl Transferase

All reactions involving rhodopsin were carried out at room temperature under dim red light unless otherwise indicated. Buffers and other solutions were used immediately after being purged with N_2 for at least 30 min. Enzymatic reactions were carried out under N_2 in a glove box. To a preparation of rod outer segment membranes (disk membranes) derived from 50 retinas was added 0.05 ml of 0.5 M sodium cacodylate, pH 7.0, 1.0 ml of 1.14×10^{-2} M UDP-[^{14}C] galactose (2.4×10^9 cpm/mmole), 0.010 ml of 1 M $MnCl_2$, and 4.0 ml of the UDP-galactosyl transferase (0.15 mg/ml in 0.5 M sodium cacodylate, pH 7.0). The mixture was very gently stirred for 24 h, diluted to 25 ml with 0.05 M sodium cacodylate, pH 7.0 (Buffer A), and centrifuged at 12,100 × g for 10 min. The pellet of disk membranes was resuspended in 25 ml of 0.05 M sodium cacodylate, pH 7.0, and again centrifuged at 12,100 × g for 10 min. This last step was repeated 2 additional times. The supernatants were discarded after each step. The modified disk membranes containing galactose covalently attached to rhodopsin (Gal-rhodopsin) were dispersed into small aliquots and stored in the dark at −20°C until used.

Enzymatic Oxidation of the Inserted Galactosyl Residues of Gal-Rhodopsin With Galactose Oxidase

To UDP-galactosyl transferase modified disk membranes obtained from 10 retinas was added 0.2 ml of galactose oxidase (300 units/ml of Buffer A) and 0.1 ml of Buffer A. The reaction mixtures was gently agitated for 24 h, diluted to 25 ml with Buffer A, and centrifuged at 12,100 × g for 10 min. The disk membrane pellet was twice resuspended in

25 ml of 0.1 M sodium acetate, pH 5.6, and centrifuged at 12,100 × g for 10 min, before reacting it with dansyl hydrazide.

Labeling of the Enzymatically-Oxidized Galactosyl Residues of Gal-Rhodopsin With Dansyl Hydrazine

Enzymatically-oxidized disk membranes obtained from 10 retinas were suspended in 2.0 ml of 0.1 M sodium acetate buffer, pH 5.6 saturated with either dansyl hydrazine (DH) or [^{3}H] DH. Under these conditions the molar ratio of dansyl hydrazide to rhodopsin is about 20:1. The reaction mixtures were gently mixed for 6–8 h, diluted to 10 ml with distilled water, and centrifuged at 12,100 × g. The fluorescent labeled disk membranes were washed twice by suspending them in 0.015 M sodium phosphate buffer, pH 7.0, followed by centrifugation as before.

Hydroxylapatite Chromatography of Enzymatically-Modified and Fluorescent-Labeled Rhodopsin

Chromatography was carried out at 4°C in dim red light. Enzymatically-modified or fluorescent-labeled disk membranes from 10 retinas were suspended in 10 ml of 0.015 M sodium phosphate buffer, pH 7.0, pelleted at 12,100 × g, solubilized by the addition of 3–4 ml of 3% dodecyl trimethylammonium bromide (DTAB) in 0.015 M sodium phosphate, pH 7.0 (DTAB buffer), and recentrifuged. The detergent solution was applied to a column (0.9 × 10 cm) of hydroxylapatite previously equilibrated with DTAB buffer and chromatographed as suggested by Dr. Robert Renthal. Lipids were eluted with DTAB buffer containing 0.05 M NaCl. Modified or unmodified rhodopsin was eluted with DTAB buffer containing 0.25 M NaCl. Typical flow rates were 0.15–0.35 ml/min.

Alternatively, enzymatically-modified or fluorescent-labeled disk membranes were solubilized in 3% Ammonyx-LO (ALO) in 0.01 M imidazole, pH 7.0, centrifuged at 12,100 × g and applied to a column (0.9 × 15 cm) of hydroxylapatite previously equilibrated with 1% ALO in 0.01 M imidazole, pH 7.0. Chromatography was carried out essentially as described by Appelbury (17) except that a 2-step gradient was used instead of a continuous phosphate gradient (18). Lipids, free DH, and opsin were eluted with 1% ALO in 0.04 M sodium phosphate, pH 7.0. Rhodopsin and fluorescent-labeled rhodopsin were eluted with 1% ALO in 0.15 M sodium phosphate, pH 7.0. Typical flow rates were 0.05–0.75 ml/min.

Con A-Sepharose 4B was synthesized by the procedure of Steinemann and Stryer (19). Chromatography of detergent solubilized [^{14}C] Gal-rhodopsin and enzymatically-cleaved [^{14}C] Gal-rhodopsin on Con A-Sepharose 4B was performed as described by Pober and Stryer (20).

Partial Purification of a [^{14}C] Galactose-Glycopeptide

[^{14}C] Gal-disk membranes obtained from 30 retinas were solubilized in DTAB buffer and chromatographed on hydroxylapatite as described above. [^{14}C] Gal-rhodopsin fractions ($OD_{500} > 0.05$) were pooled and exhaustively dialyzed at 4°C against multiple changes of distilled water for 5 days. The [^{14}C] Gal-opsin which precipitated was collected by centrifugation (6,000 × g) and resuspended in 10% acetic acid. Peptic digestion of [^{14}C] Gal-opsin and the subsequent ion exchange chromatography of the soluble peptic peptide on a Dowex-50-X2 column (0.9 × 5 cm) were performed as described by Heller and Lawrence (7).

Analytic Procedures

Spectral measurements of enzymatically-modified and fluorescent-labeled rhodopsin in DTAB or ALO were made at 4°C. Rhodopsin concentrations were determined from the absorbance at 498 nm using an extinction coefficient of 40,000 cm^{-1} (21). The stoichiometry of [^{14}C] galactose and [^{3}H] DH incorporation into the oligosaccharide side chain of rhodopsin was determined on samples after chromatography on hydroxylapatite. Samples (0.25 ml) were dispensed into scintillation vials containing 15 ml of Aquasol (New England Nuclear Corporation), and counted in a liquid scintillation spectrometer (Unilux II-A). Electrophoretic analysis of modified disk membranes, [^{14}C] Gal-opsin and fluorescent-labeled [^{14}C] Gal-opsin on 5% and 7.5% SDS-polyacrylamide, was carried out according to standard procedures (22, 23). Fluorescent-labeled components were visualized on SDS-acrylamide gels with a short wave UV hand lamp prior to staining with Coomassie blue (24). Components labeled with [^{14}C] were detected by dissolving acrylamide gel slices in scintillation vials in 1.0 ml of 30% hydrogen peroxide at 60°C for 12–18 h. Nanosecond fluorescence kinetics were measured at 4°C with a single photon counting apparatus (25, 26).

RESULTS

Incorporation of Galactose Into Rhodopsin in Disk Membranes by UDP-Galactosyl Transferase

[^{14}C] Galactose was introduced covalently into the carbohydrate moiety of rhodopsin when retinal disk membranes were incubated with UDP-[^{14}C] galactose and UDP-galactosyl transferase. Three lines of evidence indicated that the linkage of galactose to rhodopsin was covalent: 1) The modified disk membranes were solubilized in ALO or DTAB and chromatographed on hydroxylapatite. The [^{14}C] galactose coeluted with the rhodopsin. 2) The same elution behavior was observed when the detergent-solubilized modified membranes were chromatographed on Con A-Sepharose 4B. 3)[^{14}C] Galactose migrated with opsin monomer and dimer on SDS-acrylamide gels. No radioactivity was found in other regions of the acrylamide gel. More than 90% of the [^{14}C] galactose incorporated into disk membranes was found in rhodopsin following hydroxyapatite chromatography.

The stoichiometry of incorporation in 4 independent experiments ranged from 1.2 to 1.8 (average = 1.5) galactose residues per rhodopsin. In the absence of the transferase, less than 0.02 galactose residues were incorporated per rhodopsin. It is evident that the insertion of galactose was enzyme catalyzed. The location of the inserted galactose residues was investigated in 2 ways. Pober and Stryer (20) have shown that thermolysin cleaves rhodopsin into 2 large membrane-bound fragments: F1 contains the carbohydrate moieties of rhodopsin, whereas F2 contains the N-retinyl group formed by borohydride reduction. We found that [^{14}C] galactose was located exclusively on the F1 fragment derived from Gal-rhodopsin. Information about the location of the attached galactose residues was also obtained by digesting detergent-free, hydroxylapatite purified [^{14}C] Gal-opsin with pepsin. About 75% of the [^{14}C] label was solubilized in this way. When the soluble peptic digest was chromatographed on Dowex-50-X2, all recoverable [^{14}C] label (43%) coeluted with the glycopeptide fraction described previously by Heller and Lawrence (7).

Oxidation of Gal-Rhodopsin by Galactose Oxidase and Coupling of Dansyl Hydrazide

Disk membranes containing Gal-rhodopsin were treated with galactose oxidase to

TABLE I. Stoichometry of Labeling of Gal-Rhodopsin or Rhodopsin With [^{3}H] Dansyl Hydrazide

	Experiment	Moles [^{3}H] DH/mole Gal-Rhodopsin
1	Gal-ROS disk membranes + galactose oxidase + [^{3}H] DH	0.35
2	Gal–ROS disk membranes + [^{3}H] DH	0.02
3	ROS disk membranes + galactose oxidase + [^{3}H] DH	0.05
4	ROS disk membranes + [^{3}H] DH	0.01

convert the C-6 CH_2OH group of the attached galactose residues to an aldehyde. The oxidized galactose residues were then reacted with [^{3}H] dansyl hydrazide to form DH-Gal-rhodopsin. The fluorescent dansyl group cochromatographed with rhodopsin on hydroxylapatite and it coelectrophoresed with opsin monomer and dimer on SDS-acrylamide gels, demonstrating that it was covalently attached to rhodopsin. The stoichiometry of labeling of Gal-rhodopsin treated with galactose oxidase was 0.35 moles [^{3}H] dansyl hydrazide per mole of rhodopsin. Control experiments were carried out in which galactosyl transferase or galactose oxidase (or both enzymes) was omitted (Table I). Only 0.02 moles of [^{3}H]-dansyl hydrazide were incorporated per mole of rhodopsin when the galactose oxidase was omitted. A slightly higher background level, 0.05 moles [^{3}H] dansyl hydrazide per mole of rhodopsin, was observed when galactose oxidase was retained but galactosyl transferase was omitted. Thus, the extent of specific labeling of rhodopsin by dansyl hydrazide was at least 7 times higher than that of nonspecific labeling. However, the degree of incorporation of dansyl hydrazide was four to fivefold less than expected on the basis of the number of galactose residues. Mn^{2+} has been reported to be required for the efficient coupling of methionine sulfone hydrazide to erythrocyte glycoproteins following oxidation by galactose oxidase (27). However, we found that 5×10^{-3} M Mn^{2+} had almost no effect on the degree of incorporation of [^{3}H] dansyl hydrazide into Gal-rhodopsin treated with galactose oxidase.

Commercially available galactose oxidase, partially purified by ion-exchange chromatography on DEAE (15) or preincubated at 50°C for 30 min (28) contained proteases that quantitatively cleaved membrane-bound Gal-rhodopsin within a few hours. Consequently, it was essential to use homogenous enzyme preparations for the enzymatic oxidation of Gal-disk membranes.

Membrane constituents other than rhodopsin were labeled by dansyl hydrazide, irrespective of whether the membranes had previously been treated with galactose oxidase. We were unable to remove from the membrane all of the dansyl hydrazide that was not covalently attached to rhodopsin. It was therefore necessary to purify the fluorescent-labeled rhodopsin by chromatography on hydroxylapatite before spectroscopic studies could be carried out. The absorption spectrum of purified enzymatically-modified rhodopsin is shown on Fig. 2. The retention of the 500-nm absorption band shows that the series of reactions used to introduce the fluorescent probe did not grossly alter the conformation of the bound retinal in rhodopsin. The nanosecond emission kinetics of the dansyl fluorescence of DH-Gal rhodopsin and of DH-Gal-opsin contained at least 2 components indicating that the attached dansyl probes were located in 2 or more distinct en-

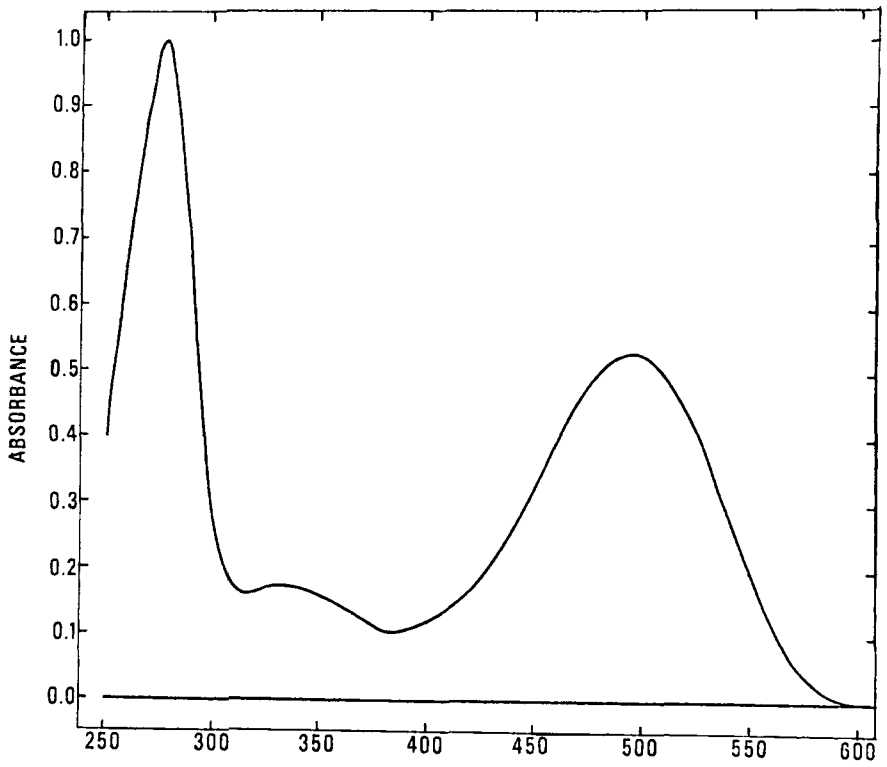

Fig. 2. Absorption spectrum of enzymatically-modified rhodopsin labeled with dansyl hydrazide. Modified disk membranes were solubilized in 3% ALO in 0.01 M imidazole and chromatographed on hydroxyapatite.

vironments. These 2 sites may correspond to the 2 oligosaccharide chains found by Hargrave and Fong (9). It will be necessary to ascertain the basis of the heterogeneity in lifetimes of the attached dansyl probes before energy transfer and emission anisotropy data for them can be interpreted unequivocally.

DISCUSSION

These experiments show that one or both oligosaccharide chains of membrane-bound rhodopsin can undergo a series of 3 reactions: 1) the insertion of galactose by UDP-galactosyl transferase; 2) the oxidation of the C-6 CH_2OH group of the inserted galactose residues to an aldehyde; and 3) the condensation of this aldehyde with dansyl hydrazide to form a fluorescent hydrazone. We do not have information concerning the polarity and permeability of the vesicles used in these labeling studies, and so we are uncertain as to whether sites on the intradisk or extradisk surface were labeled by galactose. Röhlich (29) recently reported that ferritin-labeled concanavalin A binds to the intradisk surface of disk membranes that were subjected to freezing and thawing. It would be interesting to visualize the location of the galactose residues in enzymatically-modified membranes using galactose-specific lectins.

The insertion of 1.5 ± 0.3 galactose residues per rhodopsin indicates that the labeling reaction catalyzed by UDP-galactosyl transferase was efficient. In contrast, the oxidation of Gal-rhodopsin membranes by galactose oxidase followed by the addition of dansyl hydrazide led to the introduction of only 0.35 moles of the fluorescent probe per retinal. It remains to be determined whether this reduced stoichiometry is due to incomplete oxidation or incomplete coupling with dansyl hydrazide.

The series of reactions described here for the insertion of a spectroscopic probe into the carbohydrate moiety of a glycoprotein should be applicable to a wide variety of membrane glycoproteins. One advantage of this approach for generating reactive aldehydes compared to periodate oxidation is its greater specificity. Furthermore, the insertion of a galactose residue and its subsequent enzymatic oxidation to an aldehyde should be a lesser perturbation than the cleavage of several bonds in an oligosaccharide unit by periodate.

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