External Labeling of Galactose in Surface Membrane Glycoproteins of the Intact Myelin Sheath

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The molecular organization of surface galactose residues in glycoproteins of the intact myelin sheath was investigated using the enzymatic membrane probe, galactose oxidase. Rat spinal cords treated under physiological conditions with this nonpermeant probe were labeled specifically in galactose residues by reduction with tritiated sodium borohydride. The enzymatically modified proteins from isolated myelin were analyzed electrophoretically and their specific radioactivities determined. Results indicated tritium label associated with a surprising variety of high molecular weight proteins. The most extensively labeled peak corresponded to the major myelin glycoprotein as indicated by the coincidence of tritium label with that of [14C]fucose used as an internal marker for the glycoproteins. The radioactivity associated with this protein was 1.1 to 2.7 times higher after treatment with galactose oxidase when compared to reduction in the absence of the enzyme and 1.4 to 4.8 times higher when oxidized and reduced after prior treatment with neuraminidase. The results suggest a complex heterogeneity of minor glycoproteins associated with isolated myelin. It is concluded that from this complexity of glycoproteins, a major glycoprotein is at least partially localized on the external surface of either the intact myelin sheath or the closely associated oligodendroglial plasma membrane. Such a localization of this glycoprotein and the probable localization of the other glycoproteins enhances their potential role in specific interactions in the process of myelination or myelin maintenance.

Understanding the molecular architecture of biological membranes requires precise information concerning the localization of the individual membrane components. Such localizations may not only facilitate an understanding of the functional properties of the various components within the membrane but may also provide insight as to their potential role in various pathological processes.

Myelin is one such biological membrane whose structural understanding at the molecular level should provide insight as to the mechanism of demyelination seen in multiple sclerosis and other neurological diseases. In a recent publication, Poduslo and Braun (1) have initiated an investigation designed to elucidate the molecular organization of myelin. In this study the arrangement of the membrane proteins on the outer surface of the intact myelin sheath was explored using the lactoperoxidase iodination technique. Several high molecular weight proteins were iodinated predominantly by this nonpenetrating enzyme which indicates their localization on the outer membrane surface. In contrast, basic protein was not labeled by this technique, and, therefore, evidence was presented which localized basic protein on the inner surface of the membrane corresponding to the major dense line of myelin. The present communication investigates more closely the nature and localization of the high molecular weight proteins found in isolated myelin.

Covalent labeling of surface membrane glycoproteins was achieved by specific modification of galactose and related sugars by the enzyme, galactose oxidase. It has been shown by Avigad et al. (2) that oxidation of β-galactose and related sugars by galactose oxidase occurs at the C-6 position with the formation of β-galactohexodialdose. This resulting aldehyde group can then be labeled by reduction back to the primary hydroxyl group with tritiated sodium borohydride. Such a procedure has been used by Radin et al. (3) and Suzuki and Suzuki (4) for labeling isolated glycosphingolipids and by Morell et al. (5) for labeling isolated glycoproteins. More recently this procedure has been used for determining the accessibility of a variety of sugar-bearing macromolecules on the surface of cell membranes. Steck and Dawson (6, 7) and Gahmberg and Hakomori (8) have investigated the external labeling of glycolipids and glycoproteins in the erythrocyte membrane. In addition, Gahmberg and Hakomori (9-12), as well as Critchley (13), have examined the surface carbohydrates of hamster fibroblasts. The rationale for this approach is that galactose oxidase is impermeant to the membrane; hence, only those sugar residues that are accessible to the enzyme will be oxidized. Since possible penetration of the tritiated sodium...
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RESULTS

Myelin Isolation and Preparation for Electrophoresis—Myelin was isolated according to the standard procedure described by Norton and Poduslo (19). The isolated myelin was delipidated partially in ethyl ether/absolute ethanol (3/2, v/v), and the insoluble residue dissolved in a sodium dodecyl sulfate-mercaptoethanol system as described previously by Poduslo and Braun (15).

Polyacrylamide Gel Electrophoresis—A similar discontinuous gel electrophoresis system was used for the separation of myelin proteins as described previously (1). A 6% (w/v) acrylamide spacer gel was used in conjunction with a 10% (w/v) acrylamide separating gel. The electrophoresis was run at a constant current of 0.6 mA per tube for 16 to 18 hours. Approximately 200 to 220 μg of protein as determined by the procedure of Lowry et al. (16) using human serum albumin as a standard were applied to the gels. This system maximized the separation of the high molecular weight proteins found in isolated myelin since the basic proteins, proteolipid protein, and a protein intermediate between them ran off the gel. The electrophoresis buffer was not circulated between the upper and lower reservoirs. After the run, the gels were removed from the tubes by breaking the glass and fixed overnight in MeOH/H2O/HOAc (45/45/10, v/v/v). The gels were then stained in 0.2% Coomassie blue dissolved in fixative for 2 hours at 37°C. Destaining was accomplished either by transverse electrophoresis for 20 min in a Canalco gel destainer with fixative as the solvent or by diffusion. A Gilford linear transport system was used to scan the stained gels at 555 nm in fixative. The gels were sliced transversely with a razor blade in a Canalco slicer template into sections of approximately 1.5 mm thick. The sections were placed in scintillation vials, and 100 μl of hydrogen peroxide (30%) (v/v) were added to cover the sections. The vials were capped tightly and incubated overnight at 30°C. The depolymerized sections were then cooled and scintillation fluid was added for counting by liquid scintillation spectrometry in an LS-220 liquid scintillation system (Beckman Instruments, Fullerton, Calif.). The scintillation mixture contained 42 ml of Spectrafluor (Amersham/Searle) per liter of toluene mixed with methyl Cellosolve (ethylene glycol monomethyl ether) (Pierce Chemical Co.) in a ratio of 5/3 (v/v). For counting of the solubilized gel slices, 10 ml of the above mixture containing 2% NCS (Amersham/Searle) and a radioactive quench calibration was performed on each sample using the combined external standard and channels ratio method. Maximum counting efficiencies using chloroform as a quenching agent were 20.3% tritium and 64.1% for carbon-14.

Carbohydrate Analysis—Lyophilized myelin was obtained from rat spinal cords that were first labeled in vivo with [14C]fucose and then surface labeled with galactose oxidase and tritiated sodium borohydride after neuraminidase pretreatment. This material was extracted with chloroform/methanol (2/1, v/v) containing 5% water (0.5 mg of myelin protein/ml of solvent). Dispersion of the myelin was accomplished by sonication in a water bath, and the insoluble residue was collected by centrifugation at 30,000 × g for 30 min. The precipitate was washed twice with chloroform/methanol, and during the third wash aliquots were removed for counting and protein determination. The chloroform/methanol-insoluble residue was then dried under N2 and hydrolyzed in 2 N HCl in sealed vials for 4 hours at 100°C. The hydrolysates were then analyzed by gas chromatography in conjunction with a 10% (w/v) acrylamide separating gel. The eluent mixture contained 2% NCS (Amersham/Searle) were added. Automatic quench calibration was performed on each sample using the combined external standard and channels ratio method. Maximum counting efficiencies using chloroform as a quenching agent were 20.3% tritium and 64.1% for carbon-14.

Computer Data Analysis—The DEC system-10 computer facilities at the Division of Computer Research and Technology at the National Institutes of Health were used for all data analysis. A SAIL program was designed to analyze and graphically display the data using a PDP-10 processor. Either a DEC 340 graphics display terminal or a Tektronix 4010 terminal was used for the interactive computer graphics display. A Calcomp plotter was used to obtain a hard copy of the graphs appearing in this publication.

Carbohydrate Analysis—Lyophilized myelin was obtained from rat spinal cords that were first labeled in vivo with [14C]fucose and then surface labeled with galactose oxidase and tritiated sodium borohydride after neuraminidase pretreatment. This material was extracted with chloroform/methanol (2/1, v/v) containing 5% water (0.5 mg of myelin protein/ml of solvent). Dispersion of the myelin was accomplished by sonication in a water bath, and the insoluble residue was collected by centrifugation at 30,000 × g for 30 min. The precipitate was washed twice with chloroform/methanol, and during the third wash aliquots were removed for counting and protein determination. The chloroform/methanol-insoluble residue was then dried under N2 and hydrolyzed in 2 N HCl in sealed vials for 4 hours at 100°C. The hydrolysate was dried completely and dissolved in chloroform/ methanol/water 10/10/3 (v/v/v). The hydrolyzed sugars were then applied on Whatman No. 1 chromatography paper and separated in an ethyl acetate/pyridine/acetic acid/water 5/5/1/3 (v/v/v/v) solvent system. Mixtures of standard sugars were chromatographed on the sides of the paper. After a 17-hour ascending run, the paper was cut into strips, and the chromatogram was scanned for radioactivity. The strips were then counted and the radioactive peaks correlated with the positions of the standard monosaccharides on the chromatogram localized by visualization with the silver nitrate/NaOH procedure.

RESULTS

Short Term Fucose Incorporation into Glycoproteins of Purified Myelin—A typical electrophoretic pattern of a 10%
acrylamide gel showing $[^{14}C]fucose incorporation into the high molecular weight proteins of myelin isolated from the rat spinal cord is illustrated in Fig. 1. This is the result of an intracisternal injection 18 to 22 hours prior to the killing of the animal. A surprising variety of radioactive fucose peaks is observed. The predominant radioactive peak (Sample Numbers 11 to 15) corresponds to the location of the major glycoprotein found in isolated myelin which accounts for 24% of the recovered counts from the gel. This profile of fucose label has been observed consistently in a large variety of experiments suggesting that the minor fucose peaks may indeed be real. The absolute amount of fucose incorporation does vary considerably; however, this is most likely a reflection of the injection technique (see also Fig. 2 and 3).

External Labeling of Surface Glycoproteins of Intact Spinal Cord Preparation—A densitometric scan at 555 nm of the high molecular weight proteins from rat spinal cord myelin stained with Coomassie blue on a polyacrylamide gel is shown in Fig. 2A. The basic protein, an intermediate protein, and the proteolipid protein have migrated off the gel to allow maximal separation of the high molecular weight proteins. The major staining band corresponds to the predominant high molecular weight protein, sometimes designated as the major Wolfram protein. A large number of minor staining bands are also evident, suggesting a complex heterogeneity of high molecular weight proteins. The sharp band near the top of the gel (Sample Numbers 4 to 5) represents the interface between the 6% acrylamide spacer gel and the 10% separating gel. The reason for this sharp peak is presumably because of optical distortion as a result of light scattering (1).

The results of short term $[^{14}C]fucose incorporation into the high molecular weight proteins of myelin isolated from two different spinal cords are seen in Fig. 2, B and C (right-hand scale, dashed line). The profile for Fig. 2B is similar to that of Fig. 1, although the levels of fucose incorporation were less. In contrast, there was little incorporation in the experiment shown in Fig. 2C; however, the peak seen corresponds exactly to the major fucose peak in the above experiment which is indicative of the high degree of accuracy with which the major myelin glycoprotein can be localized on the acrylamide gel.

Reduction of the intact spinal cord preparation by tritiated sodium borohydride after pretreatment with galactose oxidase is illustrated in Fig. 2B (left-hand scale, solid line). Fig. 2C shows the levels of nonspecific reduction as a result of treatment in the absence of the galactose oxidase. There is a distinct stimulation of tritium incorporation into a variety of surface glycoproteins by the oxidase reaction. The most striking enhancement of label is seen in the major myelin glycoprotein as indicated by the coincidence of a tritium peak with that of radioactive fucose used as an internal marker for the glycoprotein. This observation is seen only after prior treatment with galactose oxidase. This finding is illustrated further in Fig. 3B which shows the tritium incorporation by galactose oxidase after correcting for nonspecific reduction. Again the most striking peak is that of the major glycoprotein. A variety of other minor tritium peaks are observed; some of which overlap the fucose peaks; whereas, others do not. The peak near the top of the gel (Sample Numbers 4 to 5) represents material at the interface between the 6 and 10% acrylamide gels.

Partial Characterization of Labeled Product—In order to determine the extent to which the tritium radioactivity was associated with galactose or related sugars, the chloroform-methanol-insoluble residue of labeled myelin which includes the high molecular weight proteins (17-19) was hydrolyzed under acidic conditions. The results of the paper chromatogram of the intact spinal cord preparation after reduction with the tritiated reducing agent in the absence of galactose oxidase is illustrated in Fig. 4. The locations of the standard monosaccharides on the paper chromatogram are indicated on the top of the figure. The predominant radioactive peak is that of the $[^{14}C]fucose indicating that nearly all of the $^{14}C$ radioactivity was in intact fucose. In contrast, there was essentially no tritium label associated with any sugar residues indicating that nonspecific reduction of sugars did not occur. Fig. 5 shows the results of the chromatogram of the spinal cord preparation after prior treatment with galactose oxidase followed by treatment with the reducing agent. Again the $[^{14}C]fucose radioactive peak corresponds to the location of the fucose standard. In addition a predominant tritium peak is seen which corresponds to the location of the galactose standard. This labeled galactose moiety accounted for 43% of the recovered counts and, therefore, confirms the specificity of the oxidase reaction. There is no apparent label associated with galactosamine. The other tritium peaks have not been identified although the slower moving peak may be the result of insufficiently hydrolyzed di- or trisaccharides while the faster moving peaks may represent breakdown products.

Effects of Neuraminidase Treatment of Intact Spinal Cord—Removal of terminal sialic acid residues by neuraminidase should result in the exposure of additional galactose residues of the surface glycoproteins and hence a stimulation of labeling by galactose oxidase. Table I displays the changes in the specific activity of the myelin high molecular weight proteins taken collectively compared to the major myelin glycoprotein as a result of neuraminidase pretreatment. In each case the

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**FIG. 1.** Pattern of $[^{14}C]fucose in a 10% polyacrylamide-sodium dodecyl sulfate gel as a result of short term incorporation into the high molecular weight proteins of myelin isolated from the rat spinal cord. The major glycoprotein found in isolated myelin is located in sample numbers 11 to 15; the major molecular weight protein that is predominantly stained with Coomassie blue is located in sample numbers 28 to 32.
specific activities, defined as the disintegrations per min per μg of protein applied to the electrophoresis gel, are corrected for nonspecific reduction in the absence of galactose oxidase. The ratio of the changes in specific activities as a result of neuraminidase pretreatment showed a 3.4 to 6.1-fold stimulation of labeling by galactose oxidase for the high molecular weight proteins when observed collectively. When the similar

FIG. 2. Internal and external labeling of glycoproteins of the intact rat spinal cord preparation. A, densitometric scan at 555 nm of the high molecular weight proteins from isolated myelin on a polyacrylamide gel stained with Coomassie blue; B, radioactivity associated with the high molecular weight proteins of myelin isolated from the intact spinal cord preparation as a result of reduction by tritiated sodium borohydride after treatment with galactose oxidase (Gal Oxidase) C, radioactivity associated with the same group of proteins as a result of reduction by borohydride in the absence of galactose oxidase. Internal labeling of glycoproteins with [¹⁴C]fucose (- - -); external labeling of surface glycoproteins with NaBH₄, (-----).

FIG. 3. Tritium incorporation by galactose oxidase into surface glycoproteins of the intact rat spinal cord preparation after correcting for nonspecific reduction. A, densitometric scan at 555 nm of a Coomassie blue stain gel of the high molecular weight proteins; B, change in levels of reduction by NaBH₄ (-----) as a result of treatment with galactose oxidase. C, radioactivity associated with the high molecular weight proteins of myelin isolated from the intact spinal cord preparation as a result of treatment with galactose oxidase (Gal Oxidase) and reduction by tritiated sodium borohydride. Internal labeling of glycoproteins with [¹⁴C]fucose (- - -); external labeling of surface glycoproteins with NaBH₄, (-----).
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FIG. 4. Chromatogram of the labeled carbohydrates from the surface glycoproteins of myelin isolated from the intact rat spinal cord preparation as a result of reduction by NaB{sub 3}H{sub 4} in the absence of galactose oxidase. The chromatogram of the standard monosaccharides is indicated at the top of the figure. ['C]Fucose from internally labeled glycoprotein (—). Nonspecific reduction by NaB{sub 3}H{sub 4} (—). GALN, galactosamine; GAL, galactose; FUC, fucose.

FIG. 5. Chromatogram of the labeled carbohydrates from the intact spinal cord surface glycoproteins found in isolated myelin as a result of reduction by NaB{sub 3}H{sub 4} in the presence of galactose oxidase. ['C]Fucose from internally labeled glycoproteins (—). GALN, galactosamine; GAL, galactose; FUC, fucose.

ratio for the major myelin glycoprotein was examined, a higher stimulation of labeling (7.1 to 12.0-fold) was observed.

DISCUSSION

The asymmetrical localization of carbohydrate residues on the outer surface of plasma membranes has prompted considerable speculation concerning their potential roles in cell surface interactions. The involvement of such glycosylated macromolecules on cell surfaces may provide the basis for the formation and maintenance of the intricate neuronal-glial relationships seen in the nervous system. A necessary prerequisite for pursuing such speculation, however, is first the identification and localization of glycosylated membrane components on the membrane surface and second the characterization of the membrane as to its cellular origin.

The utilization of galactose oxidase is a highly sensitive method by which it is possible to identify partially those glycoproteins which are located on the membrane surface. The alteration of galactose residues on these surface proteins proceeds stereospecifically with the primary hydroxyl group of galactose being the only absolute requirement for enzymatic activity. Chemical modification of positions 1, 2, and 3 does not affect the activity although modification of the 4-hydroxy group suggests a restricted size requirement for optimal enzyme interaction (20, 21).

In this investigation, a variety of high molecular weight proteins associated with isolated myelin were labeled by this technique. The most extensively labeled protein was the major myelin glycoprotein. These results suggest a heretofore unrecognized complexity associated with myelin-related membranes. Furthermore, the sensitivity of this powerful labeling technique over conventional periodic acid-Schiff staining of glycoproteins or standard sugar analysis may be sufficient to detect surface membrane proteins with both qualitative and quantitative variations in the levels of glycosylation.

It is apparent from this investigation that any comprehensive understanding of the molecular organization of myelin will have to involve a consideration of the minor components in addition to the major components. The high molecular weight proteins found in isolated myelin have been the subject of considerable controversy (see, for example, Refs. 22-26). Two possibilities concerning the cellular origin of these high molecular weight proteins have to be considered: (a) the possibility that they are myelin-related components; that is, myelin, per se, the axolemma, or the oligodendroglial plasma membrane, all of which may be present in the isolated myelin preparation; or (b) the possibility that they are not myelin-related but rather contaminants entrapped by the fragmented myelin during the isolation procedure.

A considerable amount of evidence has been presented in a series of publications indicating that the major glycoprotein found in isolated myelin is a myelin-related component rather than a contaminant. These include experiments with myelin fractions isolated from white and gray matter as well as from myelinated and unmyelinated brain by Quarles et al. (19), with myelin deficient mutant mice by Matthieu et al. (27), and with subfractionated myelin in which the major glycoprotein

| Experiment | − Neuraminidase | + Neuraminidase | % Increase |
|------------|-----------------|----------------|-----------|
| High Molecular Weight Proteins | 1 | 17.4 | 55.7 | 3.4 |
| | 2 | 34.1 | 123.3 | 3.9 |
| | 3 | 5.8 | 35.6 | 6.1 |
| Major Glycoprotein | 1 | 0.24 | 1.71 | 7.1 |
| | 2 | 0.36 | 4.32 | 12.0 |
| | 3 | 0.40 | 4.17 | 10.4 |
and a myelin-associated enzyme, 2',3'-cyclic nucleotide 3'phosphohydrolase follow similar distributions by Matthias et al. (28).

Evidence has also been presented which indicates that this major glycoprotein is not associated with the axolemma (28-30). Furthermore, since this glycoprotein is labeled predominantly by galactose oxidase and hence occupies a surface location coupled with the fact that the axolemma is exposed to only a limited extent relative to the available surface area of the myelin and oligodendroglial plasma membrane is supportive of a location other than the axolemma. In addition, the presence of glial-axonal junctions as described by Livingston et al. (31) presumably would block diffusion of the galactose oxidase and further limit the extent to which the axolemma is accessible. Furthermore, an autoradiographic study at the light microscope level of both the intact dorsal column of the cat and the spinal cord of the rat after iodination by lactoperoxidase revealed that only the outermost myelin sheaths had label associated with them. This suggests a general limit to the extent of diffusion of the membrane probes. The extent of diffusion through the lamellae of the myelin sheath via the intraperiod line is also very limited particularly in light of the observation by Mugnaini and Schnapp (32) of a continuous tight junction or zonula occludens which is present at the borders of each myelin segment.

From the results presented, it was not possible to localize this major glycoprotein further as to a myelin or oligodendroglial plasma membrane origin. The fact that this glycoprotein is the major staining protein on the acrylamide gel by periodic acid-Schiff stain and is also the major fucose-containing protein while, in contrast, represents less than 12% of the total label associated with all the high molecular weight proteins as a result of labeling with galactose oxidase, suggests additional less accessible locations for this major glycoprotein. It is possible that this protein may be distributed throughout the myelin sheath at the intraperiod line, a location which is contiguous with the outer membrane surface. This is supported by the finding of Matus et al. (33) who not only found concanavalin A receptors on the outer surface of the myelin sheath but also at the intraperiod line of the underlying lamella. This is only observed, however, when the outermost lamella is disrupted physically which again attests to the presence of a continuous tight junction that limits diffusion into the intact myelin sheath.

In addition, it is possible that this major glycoprotein may be concentrated in specific areas such as on the outer surface of the inner or outer cytoplasmic loops or the lateral cytoplasmic loops at the paranodal region. A location on the cytoplasmic loops certainly promotes speculation concerning the potential role of the protein in recognizing axolemma components in the process of myelination. The finding of coarse particles of approximately 10-nm diameter at the outer glial leaflets by Livingston et al. (31) supports such a localization of this glycoprotein, particularly in light of the association of similar particles with glycoproteins in erythrocyte membranes (34, 35).

It was not determined whether the other minor high molecular weight glycoproteins that were labeled by galactose oxidase plus tritiated borohydrate were associated with the myelin-related membranes or a contamination of the isolated myelin.

1 J. F. Poduslo and N. K. Gonatas, unpublished observations.

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