Demonstration of five major glycoproteins in myelin and myelin subfractions

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Myelin was found to contain five major glycoproteins with molecular weights of 120 000, 95 000, 88 000, 43 000 and 38 000. Light myelin contained only 5–7% of the amount of these glycoproteins in whole myelin, whereas heavy myelin and the membrane fraction contained amounts nearly identical with whole myelin. Since all the major and minor glycoproteins, with the exception of 120 000-mol.wt. glycoprotein, were detected only after treating the myelin membrane with neuraminidase, *N*-acetylneuraminic acid is a terminal sugar residue in these glycoproteins.

Myelin is a specialized multilamellar membrane characterized ultrastructurally by the presence of a major dense line and an intraperiod line. Central-nervous-system-myelin membrane contains several major Coomassie Blue-staining proteins (Agrawal & Hartman, 1979) and a major myelin-associated glycoprotein (Quarles *et al.*, 1972, 1973). In recent years a number of workers have prepared myelin subfractions from purified myelin (Banik & Davison, 1969; Matthieu *et al.*, 1973; Benjamins *et al.*, 1973; Agrawal *et al.*, 1970, 1974). These subfractions differ from each other both morphologically and in their relative amounts of lipids, proteins and enzymes (Zimmerman *et al.*, 1975; Benjamins *et al.*, 1976; Fujimoto *et al.*, 1976; Waehndt *et al.*, 1977). The function of these myelin subfractions and their relationship to each other and with oligodendroglial cells is poorly understood.

The present investigation utilizes the enzyme galactose oxidase and NaB^3H_4 to detect the presence of glycoproteins with terminal galactose residues in whole myelin and myelin subfractions. In addition, a large number of glycoproteins that contain a terminal sialic acid residue was detected by incubating the myelin membrane first with neuraminidase followed by galactose oxidase (Mena *et al.*, 1978).

This approach has been used successfully for the detection of glycoproteins in several biological membranes (Gahmberg & Hakomori, 1973, 1975; Chui & Babitch, 1979; Mena & Moore, 1977).

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Materials and methods

Chemical and enzymes

All reagents used were analytical grade. NaB^3H_4 (sp. radioactivity 5 Ci/mmol) was purchased from Amersham-Searle, Arlington Heights, IL, U.S.A. Galactose oxidase type VI (EC 1.1.3.9) and neuraminidase type IX (EC 3.2.1.18) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Purification of galactose oxidase

Proteinase activity was not detected in the neuraminidase (Reimerdes & Klostermeyer, 1976). However, the commercial galactose oxidase preparation was heavily contaminated with proteinase activity. Since treatment with phenylmethanesulphonyl fluoride or heat by the method of Steck & Dawson (1974) was unsuccessful, the following procedure was used to eliminate this proteinase activity. Galactose oxidase (1 mg) was dissolved in 1 ml of 20 mM-potassium phosphate buffer, pH 7.0. Sephadex A-50 (300 μl) in 20 mM-potassium phosphate buffer, pH 7.0, was added to galactose oxidase. After centrifugation, the supernatant fluid was removed and the Sephadex A-50 was re-suspended in the potassium phosphate buffer (0.5 ml). The supernatant fluids were combined and phenylmethanesulphonyl fluoride was added to a final concentration of 100 μM . This purification procedure results in no detectable proteinase activity

remaining with 97% preservation of galactose oxidase activity.

Isolation of myelin and myelin subfractions

Myelin and myelin subfractions were prepared from brains of 28–30-day-old rats as described previously (Agrawal *et al.*, 1972, 1974).

Incorporation of ^3H into membrane glycoproteins

Neuraminidase (0.1 i.u.) was allowed to react with 200 μg of membrane protein (1 mg/ml) in phosphate-buffered saline (0.15 M-NaCl/10 mM-potassium phosphate buffer, pH 7.4) for 15 min at 25°C. Phosphate-buffered saline (5 ml) was added and the sample was centrifuged at 45 000 *g* for 15 min at 0°C. The membranes were prerduced by adding 2 ml of 10 mM- NaBH_4 in 100 mM-sodium borate buffer, pH 8.5, for 10 min at 25°C. The sample was then rinsed three times with phosphate-buffered saline. Purified galactose oxidase (0.5 i.u.) in 20 mM-potassium phosphate buffer, pH 7.0, was added to the sample and allowed to react for 10 min at 25°C. After three washes with phosphate-buffered saline, 200 μl of 0.1 M-sodium borate containing 5 mCi of NaB^3H_4 was added. After 10 min at 25°C the sample was diluted with 2 ml of the sodium borate buffer containing non-radioactive 10 mM- NaBH_4 and washed with phosphate-buffered saline. In some experiments the incubation with neuraminidase was omitted. In addition, treating the membranes with galactose oxidase in the presence of exogenous galactose (100 μM) completely blocked the incorporation of radioactivity into all glycoproteins detected.

Gel electrophoresis and fluorography

Electrophoresis with 6–20%-linear-polyacrylamide-gradient slab gels (28 cm long) was carried out as described previously (Mena *et al.*, 1980). The gels were fluorographed by the techniques of Bonner & Laskey (1974).

Radioactive measurements

The radioactivity in the gel was quantified by cutting out the desired region of the gel by direct comparison with the X-ray film. The sections were digested with 0.5 ml of 30% (v/v) H_2O_2 , 10 ml of scintillation fluid (Scintiverse; Fisher Chemical Co., St. Louis, MO, U.S.A.) was added and the samples were counted in a Packard Tri-Carb scintillation spectrometer. The counting efficiency was 21%.

Results

Glycoprotein of rat brain myelin

When myelin membranes were incubated with galactose oxidase, and then labelled with NaB^3H_4 , the radioactivity was predominantly associated with

one glycoprotein with an apparent molecular weight of 120 000 and some labelling of 95 000- and 88 000-mol.wt. glycoproteins was observed after prolonged fluorography (Fig. 1b, sample 2). Prior treatment of myelin membranes with neuraminidase followed by oxidation with galactose oxidase and reduction with NaB^3H_4 clearly revealed the presence of at least four additional glycoproteins (mol.wts. 95 000, 88 000, 43 000 and 38 000) (Figs. 1b and 1c, sample 1). The radioactivity associated with the glycoprotein band at mol.wt. 43 000 appears to migrate at the same position as a major Coomassie Blue-staining myelin polypeptide. Some non-specific labelling occurred in the portion of the gel where lipids would be expected to migrate in spite of the pre-treatment with non-radioactive NaBH_4 (Fig. 1).

Exposing the same gel for 3 days revealed an additional 10–15 radioactive bands (Fig. 1c, sample

Fig. 1. The glycoproteins of whole myelin revealed by NaB^3H_4 reduction of myelin as described in the Materials and methods section

Sample 1, myelin was incubated with neuraminidase followed by oxidation with galactose oxidase and reduction with NaB^3H_4 ; sample 2, myelin was treated with galactose oxidase and then reduced with NaB^3H_4 ; sample 3, myelin was incubated with NaB^3H_4 without treatment with neuraminidase or galactose oxidase. All samples were subjected to electrophoresis and fluorographed as described in the Materials and methods section. (a) shows the Coomassie Blue-staining patterns obtained with the three samples; (b) and (c) show the pattern of radioactivity obtained after fluorography for 1 day and 3 days respectively.

1). These include a number of glycoproteins with molecular weight greater than 120 000 in addition to glycoproteins detected with apparent mol.wts. of 25 000 and 27 000 (Fig. 1c, sample 1). Furthermore, a neuraminidase-dependent increase in radioactivity was detected in a band migrating just slower than the myelin-specific proteolipid protein.

Glycoproteins of myelin subfractions

The glycoproteins of myelin subfractions were examined to determine if there was a difference in the distribution of glycoproteins among the light and heavy myelin, and membrane fractions. The myelin subfractions were incubated with galactose oxidase and reduced with NaB^3H_4 with or without pretreatment with neuraminidase. The five major myelin glycoproteins (mol.wts. 120 000, 95 000, 88 000, 43 000 and 38 000) were detected in all of the myelin

fractions. However, they are greatly enriched in the membrane fraction and heavy myelin subfraction when compared with the light myelin (Fig. 2). The specific radioactivity of the major glycoproteins (i.e. mol.wts. 120 000, 95 000, 88 000, 43 000 and 38 000) in whole myelin and in the myelin subfractions is shown in Fig. 2. When myelin subfractions were treated only with galactose oxidase, the 120 000-mol.wt. glycoprotein incorporated the greatest amount of radioactivity per μg of protein (Fig. 2a). The 95 000-mol.wt. glycoprotein incorporated less radioactivity, whereas the radioactivity incorporated into the 88 000-, 43 000- and 38 000-mol.wt. glycoproteins was barely detectable. The specific radioactivity of the individual glycoproteins in the whole myelin, heavy myelin and membrane subfractions was virtually identical (Fig. 2a). However, light myelin contained only 5–7% of the radioactivity incorporated into the glycoproteins when compared with that found in the whole myelin, heavy myelin and membrane subfractions. Pretreatment of these membranes with neuraminidase before the addition of galactose oxidase and NaB^3H_4 increased the incorporation of ^3H into the glycoproteins with mol.wts. 95 000, 88 000, 43 000 and 38 000 by factors of from 3 to 10 (Fig. 2b). However, neuraminidase pretreatment had little effect on the radioactivity incorporated into the 120 000-mol.wt. glycoprotein in whole myelin or the myelin subfraction (Fig. 2b).

Discussion

The results presented here demonstrate that myelin contains several major glycoproteins in addition to the 120 000- and the 27 000-mol.wt. glycoproteins originally described by Quarles and his co-workers (Quarles *et al.*, 1972, 1979). Four additional major glycoproteins (mol.wts. 95 000, 88 000, 43 000 and 38 000) and many minor glycoproteins are revealed only after the treatment of isolated myelin membrane with neuraminidase before oxidation with galactose oxidase and subsequent reduction with NaB^3H_4 . The possibility of spurious labelling of glycoproteins of myelin and myelin subfractions with neuraminidase/galactose oxidase was eliminated by blocking experiments (results not shown).

The myelin-associated glycoproteins were greatly enriched in the heavy-myelin and membrane subfractions when compared with light myelin. Furthermore, these glycoproteins have also been identified in whole myelin isolated from animals up to 90 days old (Mena, 1978). The present results provide further support for the hypothesis that the various subfractions are distinct biochemical entities that may be derived from oligodendroglial plasma membrane or mixtures of membrane derived from inner

Fig. 2. Myelin and myelin subfraction treated with galactose oxidase (a) or neuraminidase followed by galactose oxidase (b) and then reduced with NaB^3H_4 . After electrophoresis and fluorography the areas of gel that corresponded to the five major glycoproteins were sectioned and the radioactivity was determined ($n = 3$). The results are expressed as the total c.p.m. in each section per μg of protein applied to the gel. All values were within 10% in three separate experiments. A value of zero implies that the radioactivity detected was not significantly above background values. The glycoproteins were designated as follows: 1, mol.wt. 120 000; 2, mol.wt. 95 000; 3, mol.wt. 88 000; 4, mol.wt. 43 000; 5, mol.wt. 38 000. Abbreviations used: W, whole myelin; M, membrane subfraction; H, heavy myelin; L, light myelin.

or outer lamellae of myelin membrane (Agrawal *et al.*, 1970; Quarles, 1979).

There is some concern regarding the origin of the minor polypeptide and glycoprotein components of myelin. Although they may indeed be integral components of myelin, these proteins may be derived from either the pieces of the axolemma that remain attached to the myelin (Matthieu *et al.*, 1978) or oligodendroglial plasma membrane. Our results indicate that these glycoproteins are associated with highly purified myelin fractions. Immunohistochemical techniques must be used to determine if the glycoproteins described in the present paper and in other reports (Poduslo *et al.*, 1980; Lane & Fagg, 1980) are in fact integral parts of the myelin membrane. Our results do suggest, however, that these five glycoproteins are closely associated with each other or at least located on membranes of similar densities, since the relative amounts of these glycoproteins remain constant in each of the myelin subfractions, whereas the total amount of these glycoproteins differs greatly among the myelin subfractions.

Glycoproteins have been implicated in a number of membrane-mediated events (Gottlieb *et al.*, 1974). The glycoproteins of myelin demonstrated in the present study and in other studies (Zanetta *et al.*, 1977; McIntyre *et al.*, 1979; Quarles *et al.*, 1979; Lane & Fagg, 1980; Poduslo *et al.*, 1980) may have roles in the formation and maintenance of this membrane structure or in the axonal-myelin interaction. The large number of glycoproteins associated with central-nervous-system myelin suggests an unsuspected complexity in the interactions involved in the development and maintenance of this plasma membrane.

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References

- Agrawal, H. C. & Hartman, B. K. (1979) in *Biochemistry of Brain* (Kumar, S., ed.), pp. 583–613, Pergamon Press, London
- Agrawal, H. C., Banik, N. L., Bone, A. H., Davison, A. N., Mitchell, R. F. & Spohn, M. (1970) *Biochem. J.* **120**, 635–642
- Agrawal, H. C., Burton, R. M., Fishman, M. A., Mitchell, R. F. & Prenskey, A. L. (1972) *J. Neurochem.* **19**, 2082–2039
- Agrawal, H. C., Trotter, J. L., Burton, R. M. & Mitchell, R. F. (1974) *Biochem. J.* **140**, 99–109
- Banik, N. L. & Davison, A. N. (1969) *Biochem. J.* **115**, 1051–1062
- Benjamins, J. A., Miller, K. & McKhan, G. M. (1973) *J. Neurochem.* **20**, 1589–1603
- Benjamins, J. A., Gray, M. & Morell, P. (1976) *J. Neurochem.* **27**, 571–575
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Chui, T. & Babitch, J. (1979) *Biochim. Biophys. Acta* **510**, 112–123
- Fujimoto, K., Roots, B. I., Burton, R. M. & Agrawal, H. C. (1976) *Biochim. Biophys. Acta* **426**, 659–668
- Gahmberg, C. A. & Hakomori, S. (1973) *J. Biol. Chem.* **248**, 2135–2142
- Gahmberg, C. A. & Hakomori, S. (1975) *J. Biol. Chem.* **250**, 2447–2451
- Gottlieb, D. I., Merrell, R. & Glaser, L. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1800–1802
- Lane, J. D. & Fagg, G. E. (1980) *J. Neurochem.* **34**, 163–171
- Matthieu, J. M., Quarles, R. H., Brady, R. O. & Webster, H. DeF. (1973) *Biochim. Biophys. Acta* **329**, 305–317
- Matthieu, J. M., Webster, H. deF., DeVries, G. H., Carthay, S. & Koellreuther, B. (1978) *J. Neurochem.* **31**, 91–102
- McIntyre, L. J., Quarles, R. H. & Brady, R. O. (1979) *Biochem. J.* **183**, 205–212
- Mena, E. E. (1978) Ph.D. Thesis, Washington University School of Medicine, St. Louis
- Mena, E. E. & Moore, B. W. (1977) *Trans. Am. Soc. Neurochem.* **8**, 215
- Mena, E. E., Hagen, S., O'Connell, K., Agrawal, H. C. & Moore, B. W. (1978) *Trans. Am. Soc. Neurochem.* **9**, 121
- Mena, E. E., Hooser, C. A. & Moore, B. W. (1980) *Brain Res.* **188**, 207–231
- Poduslo, J. F., Hartman, J. L. & McFarlin, D. E. (1980) *J. Neurochem.* **34**, 1733–1744
- Quarles, R. H. (1979) in *Biochemistry of Brain* (Kumar, S., ed.), pp. 81–102, Pergamon Press, London
- Quarles, R. H., Everly, J. L. & Brady, R. O. (1972) *Biochem. Biophys. Res. Commun.* **68**, 491–497
- Quarles, R. H., Everly, J. L. & Brady, R. O. (1973) *J. Neurochem.* **21**, 1177–1191
- Quarles, R. H., McIntyre, L. J. & Posnok, C. F. (1979) *Biochem. J.* **183**, 213–221
- Reimerdes, E. H. & Klostermeyer, H. (1976) *Methods Enzymol.* **45**, 26–28
- Steck, T. L. & Dawson, G. (1974) *J. Biol. Chem.* **249**, 2135–2142
- Waehndt, T. V., Matthieu, J. M. & Neuhoff, V. (1977) *Brain Res.* **138**, 29–43
- Zanetta, J. P., Sarlieve, L. L., Mandel, P., Vincendon, G. & Gombos, G. (1977) *J. Neurochem.* **29**, 827–838
- Zimmerman, A. W., Quarles, R. H., Webster, H. DeF., Matthieu, J. & Brady, R. O. (1975) *J. Neurochem.* **25**, 749–757