Association and release of the major intrinsic membrane glycoprotein from peripheral nerve myelin

Joseph F. PODUSLO and Jeffrey K. YAO

Membrane and Lipid Biochemistry Laboratories, Peripheral Nerve Center, Neurology and Biochemistry, Mayo Clinic and Mayo Foundation, Rochester, MN 55905, U.S.A.

(Received 19 September 1984/6 December 1984; accepted 21 January 1985)

Hypo-osmotic homogenization of the endoneurium from the adult-rat sciatic nerve and subsequent evaluation of the 197 000g aqueous supernatant by sodium dodecyl sulphate pore-gradient electrophoresis (SDS-p.g.e.) revealed a release of the major glycoprotein (P_0) (29 000 M_r) from peripheral nerve myelin. Immunological verification of the presence of this asparagine-linked glycoprotein in the aqueous supernatant was obtained by immune overlay after SDS-p.g.e. and electrophoretic transfer to nitrocellulose using anti- P_0 γ -globulin followed by autoradiographic detection with ^{125}I -protein A. A comparison of successive hypo- and iso-osmotic extractions of the endoneurium revealed that the hypo-osmotic extraction released increasing amounts of P_0 into the supernatant fraction, whereas the iso-osmotic treatment revealed lower levels of P_0 extracted from the myelin and lesser amounts with each successive extraction. Three successive hypo-osmotic extractions resulted in a 2.0-, 2.9-, and 9.5-fold increase in the amount of P_0 released compared with the successive iso-osmotic extractions. Although these results suggest that this major myelin glycoprotein has properties similar to those of extrinsic membrane proteins, temperature-dependent phase-partitioning experiments with Triton X-114 revealed that this glycoprotein is recovered in the detergent-enriched lower phase. These results indicate that this major myelin glycoprotein is an amphipathic integral membrane protein with a distinct hydrophobic domain and yet has solubility characteristics typical of an extrinsic membrane protein. P_0 labelled *in vitro* with [^3H]mannose could be immunoprecipitated from the aqueous supernatant with anti- P_0 γ -globulin by centrifugation at 197 000g without the addition of second antibody or protein A. Analysis of such an immune precipitate after incorporation *in vitro* with [^{14}C]acetate to label endoneurial lipids revealed that all major endoneurial lipid classes contained radioactive label, as determined by fluorography after high-performance t.l.c. The mechanism of release of this intrinsic glycoprotein from the myelin membrane, therefore, involves the osmotic-dependent formation of mixed micelles or membrane vesicles with endogenous membrane lipids.

Membrane proteins are classified according to thermodynamic constraints which maximize their hydrophobic or hydrophilic interactions (Tanford, 1980). Two categories of proteins bound to membranes have been devised which depend on a capacity to overcome these constraints and to extract the proteins from membrane structures according to their solubility properties (Singer & Nicolson,

1972; Vanderkooi, 1972). Extrinsic or peripheral proteins are dislodged from the membrane by relatively mild procedures, such as by changes in ionic strength of the medium or the addition of chelating agents. In contrast, intrinsic or integral proteins require more drastic methods to dissociate them from the membrane, such as treatment with detergents, bile acids, protein denaturants or organic solvents. Such membrane proteins are amphipathic molecules, whereby they are highly asymmetric with regard to the hydrophilic and hydrophobic domains in their structure (Lenard &

Abbreviations used: SDS-p.g.e., sodium dodecyl sulphate pore-gradient electrophoresis; h.p.t.l.c., high-performance thin-layer chromatography.

Singer, 1966; Wallach & Zahler, 1966; Segrest *et al.*, 1973). The hydrophilic parts of these proteins are likely to be exposed to the external aqueous environment, whereas the hydrophobic regions are sequestered from the aqueous environment and likely interact with the lipid bilayer in an apolar environment. A highly hydrophobic sequence or configuration of amino acids facilitates such interaction (Segrest & Feldmann, 1974). From the studies of Von Heijne (1981), the most important determinant for the amino acid composition of membrane-penetrating segments of integral proteins is the overall hydrophobicity of that segment; therefore, amino acid compositional analysis of intrinsic membrane proteins that gives the proportion of polar to non-polar amino acids generally does not indicate the type of membrane protein or its location in the membrane (Quinn & Chapman, 1980). A number of intrinsic membrane proteins have been sequenced and found to contain long (19 or more residues) sequences of hydrophobic amino acid residues which span the bilayer (for recent review, see Warren, 1981).

During our studies evaluating the techniques used to isolate myelin from the peripheral nerve, we were struck by the low yields of purified myelin in a preparation whose myelin percentage of area is 36.6% of the total fascicular area as evaluated by serial morphometric evaluation (Poduslo *et al.*, 1985). Analysis of the non-myelin fractions, particularly the supernatants from the osmotic shocks, revealed high concentrations of proteins characteristic of the myelin membrane. A prominent myelin component in these fractions is the major myelin glycoprotein, which comprises 50–60% of the total myelin protein (Greenfield *et al.*, 1973). This 28000–32000- M_r glycoprotein has been isolated (Brostoff *et al.*, 1975; Kitamura *et al.*, 1976; Roomi *et al.*, 1978; Mezei & Verpoorte, 1981) and found to have a carbohydrate content of 6.3% (w/w) (Roomi *et al.*, 1978) consisting of a single branch that is *N*-glycosidically linked to asparagine-14 (Ishaque *et al.*, 1980). It has been hypothesized that P_0 is an amphipathic membrane glycoprotein that penetrates the lipid bilayer and may play an important structural role in myelin compaction (Ishaque *et al.*, 1980). The present study addresses the solubility properties of this glycoprotein and the capacity to extract this protein from myelin under iso- and hypo-osmotic conditions. In addition, the hydrophobic domain of this glycoprotein is evaluated by temperature-dependent phase-separation experiments in Triton X-114 solutions (Bordier, 1981), as well as its capacity for immunoprecipitation after incorporation *in vitro* of the endoneurium with [3H]mannose and [^{14}C]acetate.

A preliminary account of this work has appeared in abstract form (Poduslo, 1984c).

Experimental procedures

Endoneurial isolation and fractionation

Adult Sprague–Dawley rats (200g) were anaesthetized intraperitoneally with sodium pentobarbital. The entire length of the sciatic nerve, including the tibial, peroneal and sural branches, was removed and processed. The endoneurium was removed from the perineurium and epineurium by the microdissection technique of Dyck *et al.* (1970).

Hypo- and iso-osmotic extraction of the endoneurium

The endoneurium was fractionated as described previously by Poduslo (1984a). Briefly, the endoneurium (wet wt. 14–23mg) was homogenized under hypo- or iso-osmotic conditions with a ground-glass homogenizer (vol. size 250 μ l). In some experiments, a Dounce homogenizer with A and B pestles was used for homogenization. Similar results were obtained with the Dounce homogenizer; however, the extent of disruption of the multilamellar myelin, and hence the amount of P_0 glycoprotein extracted, was significantly less when the Dounce homogenizer was used. The hypo-osmotic extraction involved homogenization with deionized distilled water. The iso-osmotic extraction involved homogenization in phosphate buffer (0.109M-phosphate buffer, or 0.01M-phosphate buffer containing 0.150M-NaCl, pH 7.4). A uniform suspension was obtained which was then placed in a 30° A-100 rotor for centrifugation in a Beckman Airfuge ultracentrifuge at 197000g at 4°C for a sufficient time period to sediment 30S particles (ribosomal subunits). The supernatant was removed and designated 'S-I'. To the precipitate, 10% (w/v) SDS was added, followed by sonication for 1 h in a Branson B-220 ultrasonic cleaner. The sample was then spun in the Airfuge at 197000g to obtain an SDS-solubilized supernatant designated 'S-II'. The remaining precipitate ('ppt. fraction') was then hydrolysed in 1M-NaOH. Portions were taken from all fractions for protein determination by the procedure of Lowry *et al.*, (1951) as modified by Hess & Lewin (1965). Monomer standard bovine serum albumin (Miles) was used for the protein determination in the range 5–10 μ g with least-squares linear-regression analysis. In some experiments, the S-I precipitate was successively homogenized under either the hypo- or iso-osmotic conditions and then respun in the Airfuge. After the third extraction (S-Ia, S-Ib, S-Ic), the remaining precipitate was then treated with detergent for solubilization and subsequent centrifugation to give the S-II supernatant fraction.

SDS-p.g.e.

The endoneurial proteins from the S-I fraction

were dialysed against water to remove the phosphate-buffered saline and freeze-dried. Proteins were solubilized and separated by SDS-PAGE on a linear gradient with a gel concentration of 10–20% T [total gel concn. (acrylamide plus cross-linking agent, w/v)] and 1% C [gel cross-linking (percentage of cross-linking reagent/percentage total gel concentration)], prepared by using a programmable LKB gradient maker according to procedures described previously by Poduslo (1981a). Protein samples of approx. 8–32 µg were subjected to electrophoresis for 3.75 h at constant power (10W) with a Buchler model 3-1500 constant-power supply at a constant cooling temperature of 0°C maintained by the NES Lab RTE-8 refrigerated circulating bath. After electrophoresis, the gradient slab gels were fixed in methanol/water/acetic acid (9:9:2, by vol.) and stained for 2 h in 0.2% (w/v) Coomassie Blue in fixative.

M_r estimation by SDS-PAGE.

The relationship log *M_r* versus log (%T) for the *M_r* standards was used to determine the *M_r* of *P*₀ by a least-squares linear-regression analysis (Poduslo & Rodbard, 1980). *M_r* standards included myosin (200 000), β-galactosidase (116 350), phosphorylase *b* (92 500), albumin (66 200), catalase (60 000), ovalbumin (45 000), lactate dehydrogenase (36 000), carbonic anhydrase (30 100), trypsin inhibitor (20 100), and α-lactalbumin (14 400) (Bio-Rad, Pharmacia). Appropriate statistical evaluations of the calibration line and the *M_r* estimate were also performed (Poduslo & Rodbard, 1980). The accuracy of *M_r* estimates for glycoproteins as evaluated by SDS-PAGE has also been investigated (Poduslo, 1981a).

Quantification of P₀ glycoprotein after SDS-PAGE.

The dye-binding capacity for *P*₀ glycoprotein was evaluated by densitometrically scanning the positive images of negative photographs of Coomassie Blue-stained gels using the Beckman DU-8 spectrophotometer. Both the *P*₀ peak area and the *P*₀ percentage of total area were determined. Only adjacent lanes on the same electrophoretic run were evaluated for dye-binding capacity. The *P*₀ peak area was normalized with respect to the total protein applied to the gel.

P₀ antibody overlay after nitrocellulose transfer

After the SDS-PAGE, proteins from the endoneurial fractions were transferred from the acrylamide gel on to nitrocellulose (0.45 µm pore size; Schleicher & Schuell) by using the Hoefer Scientific Instrument TE-42 Transphor in 25 mM-Tris base/192 mM-glycine (pH 8.3)/20% (v/v) methanol for 18 h at 30 V. After transfer, the nitrocellulose was blocked with 2% bovine serum

albumin (Miles) in phosphate-buffered saline for 2 h. The nitrocellulose sheets were then incubated with rabbit anti-*P*₀ chick γ-gobulin at a dilution of 1:4000 (kindly provided by Dr. Catherine Mezei, Dalhousie University, Nova Scotia, Canada). Production of the anti-*P*₀ antibody and the characterization of its specificity have been investigated (Nunn & Mezei, 1984). After 18 h incubation, the nitrocellulose paper was washed for 7 h in phosphate-buffered saline and then overlaid with ¹²⁵I-protein A (Pharmacia) (radioiodinated by using the Iodogen procedure) containing 1 × 10⁶ c.p.m. in phosphate-buffered saline with 2% bovine serum albumin for 18 h. The nitrocellulose paper was then washed in phosphate-buffered saline, dried, and exposed to X-ray film (Kodak X-Omat AR or S).

Triton X-114 phase partitioning

The procedure of Bordier (1981) was followed for the temperature dependent phase separation of integral membrane proteins using Triton X-114. The endoneurium was homogenized at a protein concentration of 2 mg/ml in 10 mM-Tris/HCl (pH 7.4)/150 mM-NaCl with the 250 µl ground-glass homogenizer. Triton X-114 (Fluka AG, Buchs, Switzerland) [recondensed to eliminate hydrophilic molecules as described by Bordier (1981)] was added to the homogenate to give a 2% (v/v) concentration. The homogenate was incubated for 30 min at 4°C with stirring, followed by centrifugation for 30 min at 4°C at 10 000g. To the precipitate, 10% (w/v) SDS was added and, after sonication, portions were removed for protein determination and SDS-PAGE analysis. Portions were also removed from the supernatant for protein determination and for SDS-PAGE analysis of the Triton X-114-solubilized components. The remaining supernatant was then incubated at 4°C with occasional mixing for 5 min, overlaid on 1.0 ml of a cushion of 6% (w/v) sucrose in 10 mM-Tris/HCl (pH 7.4)/150 mM-NaCl, incubated at 30°C for 5 min, and then spun in the centrifuge at 300g for 5 min. The sample partitioned into two phases, and the upper phase was separated from the lower phase. Triton X-114 (0.5%) was then added to the upper phase, mixed, reincubated at 4°C, and then overlaid on to the original lower phase followed by incubation at 30°C and spinning at 300g. The upper phase was removed and then re-extracted. Triton X-114 was removed from the upper phase, lower phase and the Triton X-114-solubilized sample before electrophoresis by using the chloroform extraction procedure as described by Horikawa & Ogawara (1979). Portions were removed for protein determination, and samples were electrophoresed by using the SDS-PAGE system.

Lectin overlay after SDS-p.g.e.

After electrophoresis, gel fixation, and gel washing in phosphate-buffered saline, radioiodinated lectins were applied to the slab gel to detect lectin-binding glycoproteins (P_0) in the Triton X-114 phase-partitioned samples as described by Poduslo (1981b). Wheat-germ agglutinin (*Triticum vulgaris*) and concanavalin A (*Canavalia ensiformis*) were obtained from E-Y Laboratories. Lectin binding was assessed autoradiographically using Kodak X-Omat S-film with Du Pont Cronex Lightning-Plus intensifying screens with variable exposure times to reveal the bands optimally.

Incubation in vitro of the endoneurium with [^3H]-mannose and [^{14}C]acetate

Endoneurial slices from 35-day-transected sciatic nerves of Sprague-Dawley rats (250 g) were incubated for 2 h in 0.5 ml of modified Krebs mammalian Ringer solution containing 250 μCi of D-[2,6- ^3H]mannose (32 Ci/mmol) (Amersham) as described previously (Poduslo, 1984b). In another series of experiments, the sciatic-nerve endoneurium from 4-week-old Sprague-Dawley rats was incubated in 1.5 ml of modified Krebs' solution (Pleasure & Towfighi, 1972) for 2 h containing 2.5 μCi of [1- ^{14}C]acetate (60 mCi/mmol) (Amersham) as described by Yao & Cannon (1983). After incorporation, the endoneurium was fractionated into the S-I and S-II fractions as described above.

Immunoprecipitation

Portions from the S-I fraction of the [^3H]-mannose-incorporated endoneurium (24.5 μg total protein; 244 000 c.p.m.) and the [^{14}C]acetate-incorporated endoneurium (25.6 μg total protein; 51 400 c.p.m.) were incubated with 7.5 μl of rabbit anti-(chick P_0) γ -globulin in a final volume of 225 μl . After overnight incubation at 4°C, the immunoprecipitates were obtained by centrifugation at 197 000 g in the Airfuge ultracentrifuge at 4°C. Control experiments to verify the specificity of the immunoprecipitate were performed with an equivalent amount of rabbit γ -globulin (Cappel; 0.12 mg). No precipitate formed, although it was important to maintain the hypo-osmotic conditions of the original S-I portion. The precipitates were washed twice with water, and the [^3H]-mannose-labelled proteins of the immunoprecipitate were solubilized in the solubilizing solution and subjected to SDS-p.g.e. After electrophoresis, the gels were fixed in methanol/water/acetic acid (9:9:2, by vol.) for 0.5 h, followed by detection of the [^3H]mannose-labelled glycoproteins by fluorography using 22% (w/v) diphenyloxazole in acetic acid (Pulleyblank & Booth, 1981) and Kodak X-Omat AR or S film with Du Pont Cronex

Lightning-Plus intensifying screens. ^{14}C -methylated M_r markers were obtained from Amersham and consisted of myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and lysozyme.

H.p.t.l.c.

Radiolabelled lipids from [^{14}C]acetate incorporation were extracted from the whole endoneurium, portions of the endoneurial fractions (S-I, S-II) or the immunoprecipitate as described previously (Yao *et al.*, 1981). Separation of total endoneurial lipids into cerebrosides, sulphatides, phospholipids and other non-polar lipids was achieved by h.p.t.l.c. using the method of Yao (1984). Lipid standards and whole endoneurial lipids were detected by charring the thin-layer plate at 180°C for 10 min after spraying with 3% (w/v) cupric sulphate in 8% (w/v) phosphoric acid (Touchstone *et al.*, 1983). The ^{14}C -labelled lipids were detected by fluorography after spraying the plate with EN 3 HANCE (New England Nuclear Corp.), followed by exposure to Kodak X-Omat AR or S X-ray film utilizing Du Pont Cronex Lightning-Plus intensifying screens.

Results

Hypo-osmotic homogenization of the endoneurium and extraction of P_0 glycoprotein

The SDS-p.g.e. profile after Coomassie Blue staining of the two endoneurial fractions is shown in Fig. 1. P_0 glycoprotein was identified, and an M_r estimate of 29 000 for P_0 was obtained after an evaluation of the relationship $\log M_r$ versus $\log (\%T)$ (Poduslo & Rodbard, 1980), utilizing the protein standards and the indicated M_r values. P_0 glycoprotein is the major component in the S-II fraction. Since this glycoprotein comprises 50–60% of total myelin protein (Greenfield *et al.*, 1973), and since the percentage fascicular area having myelin (myelin percentage of area) in transverse sections of the sciatic nerve is 36.6% (Poduslo *et al.*, 1985), this glycoprotein should be the prominent protein when evaluated by SDS-p.g.e. This glycoprotein can also be found in the aqueous supernatant fraction (S-I) of the endoneurium. This fraction represents a preparation enriched in soluble proteins, including proteins of the endoneurial fluid, soluble cytoplasmic proteins and extrinsic membrane proteins that can be dissociated from membranes by this hypo-osmotic homogenization procedure. The SDS-solubilized fraction (S-II) represents a preparation enriched in integral membrane proteins and glycoproteins. Fig. 2 illustrates an SDS-p.g.e. Coomassie Blue-stained gel after successive hypo-osmotic extractions of the endoneurium. S-Ia shows a similar

Fig. 1. SDS-p.g.e. analysis of endoneurial fractions after hypo-osmotic homogenization

Gels were stained with Coomassie Blue. The linear polyacrylamide gradient contained 10–20%T and 1%C (see the text). Gels were run for 3.75 h at 0°C at constant power of 10 W. The buffer was Tris/glycine, pH 8.5. The following-sized samples were run: S-I, 29.8 μ g of protein; S-II, 30.4 μ g of protein. The M_r estimate for glycoprotein P_0 was 29000. The expected coefficient of variation was $\pm 5.6\%$ and the 95% confidence limit $\pm 12.9\%$. Linear-regression analysis gave r (correlation coefficient) = 0.9982 and $S_y \cdot x =$ (residual variance) $^{\frac{1}{2}}$ = root-mean-square error = 0.0226. The M_r estimate was determined from the centroid of the peak after the densitometric scan.

profile to that observed in the S-I fraction of Fig. 1. Subsequent hypo-osmotic homogenization and extraction of the precipitate from the S-Ia fraction shows an increase in the amount of P_0 in the S-Ib fraction. An additional extraction of the precipitate from the S-Ib fraction as shown in S-Ic reveals an equivalent amount of P_0 extracted from the endoneurium. Since myelin is a multilamellar structure, these successive hypo-osmotic extractions are likely fragmenting the myelin membrane, resulting in the release of increasing amounts of P_0 glycoprotein into the aqueous supernatant compartment. Because of the large amount of myelin present, there is no suggestion of depletion of P_0 in this S-II fraction. Protein distribution expressed as a percentage of the total in a typical successive

Fig. 2. Successive hypo-osmotic homogenization of the endoneurium and extraction of P_0 glycoprotein

Coomassie blue stain was used, and the gel conditions described in Fig. 1 were employed. S-I (a–c) are supernatants after successive hypo-osmotic homogenization and extraction. S-II is SDS-solubilized material after successive extraction (S-Ia–c). The total protein applied to the gel is given in Table 1.

hypo-osmotic extraction of the endoneurium was 20.9, 10.4, 3.3, 55.7 and 9.7% for the S-Ia, S-Ib, S-Ic, S-II, and Ppt fractions respectively.

Immunological verification of P_0 glycoprotein in the aqueous supernatant fraction

Because of the multiplicity of bands that are found in the S-I fraction, it was important to determine if the band that was found with M_r 29000 was indeed P_0 glycoprotein. Fig. 3 illustrates an autoradiogram of nitrocellulose paper containing samples that were electrophoresed as in Fig. 2 (S-Ia, b, c; S-II), followed by incubation of the nitrocellulose paper with rabbit anti-(chick P_0) γ -globulin fraction followed by 125 I-protein A. P_0 glycoprotein is identified in the S-II fraction as well as in the successively extracted S-I fractions. The amount of P_0 in the S-Ia fraction is less than that in the S-Ib or S-Ic fraction, which corresponds to the observations made with Coomassie Blue stain (Fig. 2). When equivalent amounts of protein

Fig. 3. Immunological identification of P_0 in the S-I and S-II fractions using antibody overlay after electrophoretic transfer to nitrocellulose

Electrophoretic conditions were the same as those described in Fig. 1. Transfer conditions involve electrophoretic transfer to nitrocellulose using 30 V for 18 h, followed by blockage with 2% bovine serum albumin in phosphate-buffered saline. The rabbit anti-(chick P_0) γ -globulin (1:4000) in 2% bovine serum albumin in phosphate-buffered saline was overlaid on to the nitrocellulose. Excess antibody was washed off and the nitrocellulose then incubated with 10^6 c.p.m. of ^{125}I -protein A in 2% bovine serum albumin in phosphate-buffered saline. Kodak AR film was exposed for 5 h at -80°C to the nitrocellulose. Protein amounts applied to the gel were $2\mu\text{g}$ for each lane. Abbreviation used: ^{125}I -PS, ^{125}I -labelled protein standards.

are electrophoresed, substantial amounts of P_0 can be found in the S-Ib and the S-Ic fraction. This experiment, therefore, positively identifies P_0 glycoprotein in the aqueous supernatant fractions after hypo-osmotic extraction.

Fig. 4. Successive iso-osmotic homogenization of the endoneurium and extraction of P_0 glycoprotein. The SDS-p.g.e. slab gel was stained with Coomassie Blue. Total protein applied to the gel is given in Table 1.

Iso-osmotic homogenization of the endoneurium and successive extraction of P_0 glycoprotein

Fig. 4 illustrates the SDS-p.g.e. profile of a Coomassie Blue-stained gel after successive iso-osmotic homogenization of the endoneurium in an iso-osmotic phosphate buffer at pH 7.4. As can be seen from the electrophoretic profile, the amount of P_0 extracted in the S-Ia fraction is substantially less than that from the hypo-osmotic extraction (Figs. 1 and 2). Successive iso-osmotic extractions of the endoneurium resulted in decreased amounts of P_0 glycoprotein in the S-Ib and S-Ic fractions. Although the protein distribution was lower with each successive iso-osmotic extraction (28.9, 4.5, 1.3, 59.0 and 6.3% for the S-Ia, S-Ib, S-Ic, S-II and ppt. fractions respectively), it does not reflect the decreased levels of P_0 extracted into the S-Ia, S-Ib or S-Ic fractions (see below). The decrease in the amount of P_0 in the S-Ib and S-Ic fractions may reflect different structural and functional compartments of myelin-related membranes with differing susceptibilities to extraction under hypo- or iso-osmotic conditions.

A quantitative comparison was made of the

amount of P₀ extracted after the successive hypo- and iso-osmotic homogenizations of the endoneurium (Table 1). The amount of P₀ extracted in the S-Ia fraction after hypo-osmotic treatment was approx. 43% of that found in the S-II fraction. The amount extracted in the S-Ib and S-Ic fractions was nearly equivalent to the amount found in the S-II fraction. In contrast, the amount of P₀ found in the S-Ia fraction after iso-osmotic treatment was approx. 17% of that found in the S-II fraction. The amount of P₀ in the S-Ib fraction was approx. 30% of that found in the S-II fraction, whereas the amount in the S-Ic fraction fell substantially to a value that was 8% of that found in the S-II fraction. As illustrated in Fig. 5, direct comparison of the iso-osmotic with the hypo-osmotic extraction pro-

Fig. 5. Quantification of P₀ glycoprotein after successive hypo-osmotic and iso-osmotic homogenization and extraction of the endoneurium

Concentrations were determined by densitometry after SDS-p.g.e. for Coomassie Blue stain dye-binding capacity. ▨, Hypo-osmotic, ■, iso-osmotic.

cedures indicated a 2-, 2.9-, and 9.5-fold increase in the amount of P₀ extracted in the S-Ia, S-Ib, and S-Ic fractions respectively for the hypo-osmotic as compared with the iso-osmotic extractions.

Triton X-114 phase partitioning of P₀ glycoprotein

The ease of extraction of P₀ from the myelin membrane under hypo-osmotic extraction conditions suggested that this glycoprotein may be an extrinsic-type membrane protein. This proved not to be the case, on the basis of temperature-dependent phase partitioning of Triton X-114 solutions. Fig. 6 shows a Coomassie Blue-stained gel after SDS-p.g.e. of Triton X-114 temperature-dependent phase separation of Triton X-114-solubilized endoneurium. P₀ was identified in the protein sample that had been solubilized using the Triton X-114 at 4°C. After temperature-dependent phase partitioning, SDS-p.g.e. analysis of the lower phase showed P₀ glycoprotein as the predominant protein in the lower phase. Since P₀ was the major component in the lower phase, complete extraction of P₀ into this phase was suggested. Several minor bands, however, were observed in the P₀ region in the upper phase. In order to verify that these minor bands were not residual P₀ glycoprotein, lectin overlay-experiments were performed on the Triton X-114 phase-separated samples. Previous results have shown that P₀ glycoprotein binds to ¹²⁵I-concanavalin A and ¹²⁵I-labelled wheat-germ agglutinin after SDS-p.g.e. electrophoresis (Poduslo, 1984b). Fig. 7 shows the lectin overlays after Triton X-114 phase separation. The binding of ¹²⁵I-concanavalin A and ¹²⁵I-labelled wheat-germ agglutinin to P₀ are shown in the Triton X-114-solubilized protein. After the temperature-dependent phase partition-

Table 1. Extraction of P₀ glycoprotein after successive hypo-osmotic and iso-osmotic homogenization of the endoneurium

Treatment	Endoneurial fraction	P ₀ peak area*	P ₀ area (as % of total area)	Protein applied to gel (μg)†	Peak area
					applied protein (area/mg of protein)
Hypo-osmotic	S-Ia‡	2.415	9.2	29.4	82.1
	S-Ib	4.155	21.7	21.2	196.0
	S-Ic	3.811	23.8	21.1	180.6
	S-II	5.738	23.5	29.8	192.6
Iso-osmotic	S-Ia‡	1.179	4.8	29.1	40.5
	S-Ib	1.621	10.0	23.7	68.4
	S-Ic	0.166	9.5	8.7	19.1
	S-II	7.102	21.1	30.6	232.1

* The peak area for P₀ was assessed by densitometric scans of transparencies (positive image) of Coomassie Blue-stained gels as shown in Fig. 3; adjacent lanes on the same electrophoretic run were evaluated for dye-binding capacity; λ = 500 nm.

† The total protein sample was applied to an 8 cm × 25 mm well in volumes of 25–45 μl.

‡ S-I: supernatants after hypo-osmotic or iso-osmotic successive homogenization and extraction (a–c); S-II: SDS-solubilized material after successive S-I extraction; extraction: centrifugation at 197000g at 4°C).

Fig. 6. Temperature dependent phase separation of P_0 glycoprotein by Triton X-114. Coomassie Blue stain was used, and a total of 30–31 μ g of protein was applied to the gel.

Fig. 7. Radioiodinated lectin overlay after SDS-PAGE of Triton X-114 phase-separated endoneurial samples. The autoradiogram exposure time was 3 days for the wheat-germ agglutinin (WGA) and 6 days for the concanavalin A (Con A); Kodak S film was used.

ing of this glycoprotein, lectin binding to P_0 glycoprotein is only observed in the lower phase. No lectin binding was observed in the upper phase, suggesting that all the P_0 glycoprotein could be found in the detergent-enriched lower phase.

Immunoprecipitation analysis

Direct immunoprecipitation of P_0 with anti- P_0 γ -globulin could be achieved by ultracentrifugation at 197000g. Fig. 8 illustrates such an immunoprecipitation after incubation of the endoneurium with the [3 H]mannose precursor. A wide variety of [3 H]mannose-labelled glycoproteins are observed after fluorography in both the S-I and S-II

Fig. 8. Fluorogram of an immunoprecipitate (IP) of [3 H]mannose-labelled P_0 from the permanently transected nerve at 35 days after transection.

A portion (24.5 μ g; 244000 c.p.m.) from the S-I fraction was incubated with rabbit anti-(chick P_0) γ -globulin at 4°C for 20 h, followed by ultracentrifugation at 197000g. The immunoprecipitate was directly solubilized in solubilizing solution followed by analysis by SDS-PAGE. Samples from the S-I and S-II fractions (10 μ g total protein) were also analysed by SDS-PAGE. Arrows indicate P_0 glycoprotein and a [3 H]mannose-labelled glycoprotein (3 H-MLG) in the immunoprecipitate with an approximate M_r of ~225000. The fluorographic exposure time was 3 days on Kodak X-Omat AR film. Abbreviation used: 14 C-PS, 14 C-labelled protein standards.

endoneurial fractions. [^3H]Mannose-labelled P_0 is found in both endoneurial fractions, although it has a greater abundance in the S-I fraction. Further analysis of the carbohydrate moieties of this glycoprotein in the permanently transected nerve have been described (Poduslo, 1984b; Poduslo, 1985). After an overnight incubation of a portion of the S-I fraction with anti- P_0 γ -globulin, followed by centrifugation at 197000g, fluorographic analysis of the immunoprecipitate is shown in the adjacent lane. [^3H]Mannose-labelled P_0 is observed as the major immunoprecipitated component. In addition, a high- M_r mannose-labelled glycoprotein ($M_r \sim 225000$; arrow) is also observed after immunoprecipitation.

On the basis of its capacity to immunoprecipitate P_0 from the aqueous supernatant fraction by centrifugation at 197000g, further analysis of the immune precipitate for possible lipid components was performed in order to evaluate the mechanism by which P_0 is released into the supernatant as an apparent soluble protein. H.p.t.l.c. analysis of the

lipid extracts from the immunoprecipitate after [^{14}C]acetate incorporation into the endoneurium is seen in Fig. 9. A wide variety of endoneurial lipids are identified in the immunoprecipitate, including triacylglycerol, non-esterified fatty acid, cholesterol, monogalactosyldiacylglycerol, non-hydroxy- and hydroxy-cerebrosides, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine and sphingomyelin. These immunoprecipitated radiolabelled lipids are similar to the [^{14}C]acetate-incorporated lipids found in the S-I fraction, the S-II fraction, and to that found in the whole endoneurium. Charring of the t.l.c. plate for analysis of the endoneurial lipids reveal the additional presence of both non-hydroxy- and hydroxy-sulphatide. It is likely that these sulphatides are also present in the immunoprecipitate; however, they are not labelled by [^{14}C]acetate with this incubation procedure. These data therefore indicate that the immunoprecipitation of P_0 from the aqueous supernatant with anti- P_0 γ -globulin also results in the precipitation of a family of

Fig. 9. High-performance thin-layer chromatograms of lipids detected by fluorography (a) and charring (b). Preabsorbent silica-gel thin-layer plates (10 cm $\times$ 20 cm) were first washed overnight in a chloroform/methanol (2:1, v/v) solvent system. The washed plates were predeveloped in solvent system I [methyl acetate/propan-1-ol/chloroform/methanol/0.25% KCl, 25:25:25:10:9, by vol. (Vitiello & Zanetta, 1978)] immediately before sample application and then air-dried completely. Phospholipids and neutral glycolipids were first separated in solvent system I. The solvent front is allowed to migrate a distance of 4.8 cm above the origin. The plates were air-dried and then developed to full length in solvent system II (hexane/diethyl ether/acetic acid, 75:23:2, by vol.) to separate non-polar lipids. Finally, the plates were developed to full length in hexane solvent (system III) to separate any hydrocarbons from non-polar lipids. Plate (a) was exposed to Kodak X-Omat S film for 3 days at -70°C after spraying with EN 3 HANCE. From left to right: [^{14}C]acetate-labelled endoneurial lipids after immunoprecipitation (IP) of combined S-Ia and S-Ib fractions (25.6 μg of total protein; 51400 c.p.m.) with rabbit anti-(chick P_0) γ -globulin; [^{14}C]acetate-labelled lipids extracted from the S-I, S-II and whole endoneurium. Approx. 3800 c.p.m. were spotted on the t.l.c. plate for each sample, except endoneurial lipids (7600 c.p.m.). Plate (b) was charred at 180°C for 10 min by spraying with 3% (w/v) cupric sulphate in 8% (w/v) phosphoric acid. Similar amounts of endoneurial lipids to those used in plate (a) were used for charring. Abbreviations used: SF, solvent front; CE, cholesterol ester; ME, fatty acid methyl esters; TG, triacylglycerol; NEFA, non-esterified fatty acid; C, cholesterol; MGDG, monogalactosyldiacylglycerol; Ce, cerebroside; Ce-OH, hydroxycerebroside; Su, sulphatide; Su-OH, hydroxysulphatide; PE, phosphatidylethanolamine; CL, cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; Sp, sphingomyelin; LPC, lysophosphatidylcholine; O, origin.

membrane lipids as a mixed micelle or membrane vesicle containing P_0 as the principal protein component.

Discussion

The mechanism of release of the major myelin glycoprotein involves the formation of mixed micelles or membrane vesicles with endoneurial membrane lipids. This observation was dependent on the capacity of the multivalent polyclonal anti- P_0 antibody to immunoprecipitate this micelle/vesicle. The composition of the immunoprecipitated lipids was similar to that found in the whole endoneurium. It is possible that other myelin proteins might also be found in this mixed micelle/membrane vesicle population which may have an important relationship to P_0 (see Fig. 8).

The release of this glycoprotein from myelin is dependent on the osmotic conditions of the extraction procedure as well as the method of homogenization. The ease of extraction of P_0 from the myelin membrane is facilitated by the hypo-osmotic disruption of the myelin membrane. Reversible swelling and disruption of the myelin sheath by hypo-osmotic treatment is a well-known phenomenon that has been evaluated by X-ray diffraction and electron microscopy (Finean & Millington, 1957; Robertson, 1958; Finean & Burge, 1963; Worthington & Blaurock, 1969; Blaurock, 1971, 1978; Caspar & Kirschner, 1971; Lalitha & Worthington, 1975; McIntosh & Robertson, 1976; Rand *et al.*, 1979). In contrast, maintenance of the iso-osmotic environment preserves the integrity of the myelin and limits the extraction of P_0 from the membrane. This becomes an important parameter which should be considered in designing procedures for isolating myelin from the peripheral nerve.

The major glycoprotein of peripheral-nerve myelin was found to aggregate with the detergent Triton X-114 in the lower phase after temperature-dependent phase partitioning. It is therefore concluded that P_0 is an integral membrane protein with a distinct hydrophobic domain. The Triton X series of non-ionic detergents have been used effectively for the selective solubilization and characterization of membrane proteins under conditions that do not appreciably alter the properties of the proteins. As shown by Bordier (1981), further analysis of the Triton X-114-solubilized protein by temperature-dependent phase partitioning permits the separation of intrinsic amphipathic membrane proteins from the extrinsic hydrophilic membrane proteins. This separation is based on the convenient cloud-point temperature (20°C) of Triton X-114, which permits the reversible condensation and separation of the

detergent under conditions which are compatible for isolating membrane proteins. Conditions which promote the condensation of the Triton X-114 permit the formation of micelles between the detergent and amphipathic integral membrane proteins which aggregate in the detergent phase while the hydrophilic proteins are recovered in the aqueous phase. Since hydrophilic proteins are recovered exclusively in the aqueous phase and were excluded from the detergent phase after sedimentation through a sucrose cushion, repeated extraction of the aqueous phase with fresh Triton X-114 permits complete extraction of the hydrophobic proteins as is evidenced by the lectin-overlay experiments (Fig. 7).

Recently, Piacentini *et al.* (1983) described the release of three integral enzymic activities (NADH: and NADPH: cytochrome *c* reductase and 5'-nucleotidase) from washed rat liver microsomal membranes after incubation at 37°C in aqueous media. Their data suggest that the released enzymes are solubilized from the microsomal membranes in the form of mixed micelles mostly formed by free fatty acids and integral membrane proteins. Such a formation of mixed micelles containing these intrinsic membrane proteins are in support of our present observations regarding P_0 glycoprotein. Our experiments have not distinguished whether the lipid released from myelin by hypo-osmotic homogenization is in the form of a mixed micelle or small membrane vesicles. In either case, such formation may result from simple pinching off of membranes during homogenization or, alternatively, small endogenous amphipathic molecules may exhibit detergent-like properties which could result in a perturbation of membrane structure and the extraction of significant amounts of integral membrane proteins. Such membrane perturbation and extraction of membrane components have been demonstrated with exogenous small amphipathic molecules added to red-blood-cell membranes under iso-osmotic conditions (Maher & Singer, 1984).

Topographical analysis of P_0 has shown that at least one-third of the glycoprotein is accessible in the myelin membrane (Roomi & Eylar, 1978; Ishaque *et al.*, 1980). Trypsin digestion of purified myelin preparations results in the accumulation of a 19000- M_r product whose relationship to P_0 has been demonstrated by immunochemistry, peptide mapping, amino acid composition and amino acid sequencing. These investigators have also shown that the action of trypsin on myelin cleaves the Lys-Met linkage of P_0 , resulting in the formation of a 27-amino-acid peptide with Asn-14 containing the *N*-linked oligosaccharide chain. This glycopeptide contains proportionally more polar, and fewer hydrophobic, amino acids than its parent protein;

hence it is likely to exist outside the myelin bilayer where it is accessible to trypsin and capable of interacting with antibody after micelle/vesicle formation.

It is interesting to compare the two major proteins from central- and peripheral-nervous-system myelin. Central-nervous-system myelin contains a predominant protein, designated 'proteolipid protein', which has an M_r of 24000 and constitutes about 40–50% of total myelin protein (Norton, 1981). This particularly hydrophobic integral membrane protein has been operationally defined by Folch & Lees (1951) by its solubility in chloroform/methanol mixtures. It has about 60% non-polar amino acids and 40% polar amino acids, readily forms large aggregates in aqueous solution, and is relatively resistant to proteolysis. In contrast, P_0 glycoprotein, which comprises 50–60% of total peripheral-nervous-system myelin protein, is not soluble in chloroform/methanol (J. F. Poduslo, unpublished work) and is glycosylated. This 29000- M_r glycoprotein has about 27% non-polar amino acid residues and approx. 52% polar amino acid residues, although species differences in amino acid composition have been reported (Mezei & Verpoorte, 1981). Proteolipid protein has been very difficult to solubilize, and high concentrations of SDS are usually required. As reported in the present paper, P_0 can be extracted from the myelin membrane under hypo-osmotic conditions without the presence of exogenous detergents. These observations suggest that the myelin integrity is quite different between the peripheral and central nervous systems. This extends the variety of structural, compositional and quantitative differences already reported (for review, see Norton, 1981). Peripheral-nervous-system myelin may be more susceptible to environmental insults characterized by localized changes in osmotic conditions with resulting damage to the myelin membrane. These observations may have important implications for the mechanism of the demyelinating process associated with a variety of demyelinating peripheral neuropathies.

This investigation was supported by a National Institutes of Health grant from the National Institute of Neurological and Communicative Disorders and Stroke (NS 14304) and by the Borchard Fund. The expert technical assistance of Carole T. Berg and the secretarial assistance of Lorraine Vander Schaaf are gratefully appreciated. We also thank Dr. Catherine Mezei for the gift of anti-(glycoprotein P_0) γ -globulin.

References

- Blaurock A. E. (1971) *J. Mol. Biol.* **56**, 35–52
 Blaurock A. E. (1978) *Biochim. Biophys. Acta* **510**, 11–17
 Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607
 Brostoff, S. W., Karkhanis, Y. D., Carlo, D. J., Reuter, W. & Eylar, E. H. (1975) *Brain Res.* **86**, 449–458
 Caspar, D. L. D. & Kirschner, D. A. (1971) *Nature (London) New Biol.* **231**, 46–52
 Dyck, P. J., Ellefson, R. D., Lais, A. C., Smith, R. C., Taylor, W. F. & VanDyke, R. A. (1970) *Mayo Clin. Proc.* **45**, 286–327
 Finean, J. B. & Burge, R. E. (1963) *J. Mol. Biol.* **7**, 672–682
 Finean, J. B. & Millington, P. F. (1957) *J. Biophys. Biochem. Cytol.* **3**, 89–94
 Folch, J. & Lees, M. B. (1951) *J. Biol. Chem.* **191**, 807–817
 Greenfield, S., Brostoff, S., Eylar, E. H. & Morell, P. (1973) *J. Neurochem.* **20**, 1207–1216
 Hess, H. H. & Lewin, E. (1965) *J. Neurochem.* **12**, 205–211
 Horikawa, S. & Ogawara, H. (1979) *Anal. Biochem.* **97**, 116–119
 Ishaque, A., Roomi, M. W., Szymanska, I., Kowalski, S. & Eylar, E. H. (1980) *Can. J. Biochem.* **58**, 913–921
 Kitamura, K., Suzuki, M. & Uyemura, K. (1976) *Biochim. Biophys. Acta* **445**, 806–816
 Lalitha, S. & Worthington, C. R. (1975) *J. Mol. Biol.* **96**, 625–639
 Lenard, J. & Singer, S. J. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1828–1835
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 Maher, P. & Singer, S. J. (1984) *Biochemistry* **23**, 232–240
 McIntosh, T. J. & Robertson, J. D. (1976) *J. Mol. Biol.* **100**, 213–217
 Mezei, C. & Verpoorte, J. A. (1981) *J. Neurochem.* **37**, 550–557
 Norton, W. T. (1981) in *Basic Neurochemistry* (Siegel, G. J., Albers, R. W., Agranoff, B. W. & Katzman, R., eds.), 3rd edn., pp. 63–92, Little, Brown and Co., Boston
 Nunn, D. J. & Mezei, C. (1984) *J. Neurochem.* **42**, 158–165
 Piacentini, M., Spinedi, A., Beninati, S. & Autuori, F. (1983) *Biochim. Biophys. Acta* **731**, 151–160
 Pleasure, D. E. & Towfighi, J. (1972) *Arch. Neurol.* **26**, 289–301
 Poduslo, J. F. (1981a) *Anal. Biochem.* **114**, 131–139
 Poduslo, J. F. (1981b) *J. Neurochem.* **36**, 1924–1931
 Poduslo, J. F. (1984a) in *Peripheral Neuropathy* (Dyck, P. J., Thomas, P. K., Lambert, E. H. & Bunge, R., eds.), vol. 3, pp. 494–509, W. B. Saunders, Philadelphia
 Poduslo, J. F. (1984b) *J. Neurochem.* **42**, 493–503
 Poduslo, J. F. (1984c) *Trans. Am. Soc. Neurochem.* **15**, 92
 Poduslo, J. F. (1985) *J. Neurochem.* **44**, in the press
 Poduslo, J. F. & Rodbard, D. (1980) *Anal. Biochem.* **101**, 394–406
 Poduslo, J. F., Dyck, P. J. & Berg, C. T. (1985) *J. Neurochem.* **44**, 388–400
 Pulleyblank, D. E. & Booth, G. M. (1981) *J. Biomed. Biophys. Methods* **4**, 339–346
 Quinn, P. J. & Chapman, D. (1980) *CRC Crit. Rev. Biochem.* **8**, 1–117
 Rand, R. P., Fuller, N. L. & Lis, L. J. (1979) *Nature (London)* **279**, 258–260

- Robertson, J. D. (1958) *J. Biophys. Biochem. Cytol.* **4**, 349–364
- Roomi, M. W. & Eylar, E. H. (1978) *Biochim. Biophys. Acta* **536**, 122–133
- Roomi, M. W., Ishaque, A., Khan, N. R. & Eylar, E. H. (1978) *Biochim. Biophys. Acta* **536**, 112–121
- Segrest, J. P., Kahane, I., Jackson, R. L. & Marchesi, V. T. (1973) *Arch. Biochem. Biophys.* **155**, 167–183
- Segrest, J. P. & Feldmann, R. J. (1974) *J. Mol. Biol.* **87**, 853–858
- Singer, S. J. & Nicolson, G. L. (1972) *Science* **175**, 720–731
- Tanford, C. (1980) *The Hydrophobic Effect*, 2nd edn., Wiley-Interscience, New York
- Touchstone, J. C., Levin, S. S., Dobbins, M. F., Matthews, L., Beers, P. C. & Grabbe, S. G. (1983) *Clin. Chem.* **29**, 1951–1954
- Vanderkooi, G. (1972) *Ann. N.Y. Acad. Sci.* **195**, 6–15
- Vitiello, F. & Zanetta, J. P. (1978) *J. Chromatogr.* **166**, 637–640
- Von Heijne, G. (1981) *Eur. J. Biochem.* **120**, 275–278
- Wallach, D. F. H. & Zahler, P. H. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1552–1559
- Warren, G. (1981) in *Membrane Structure* (Finean, J. B. & Michell, R., eds.), vol. 1, pp. 215–257, Elsevier, Amsterdam
- Worthington, C. R. & Blaurock, A. E. (1969) *Biochim. Biophys. Acta* **173**, 427–435
- Yao, J. K. (1984) in *Peripheral Neuropathy* (Dyck, P. J., Thomas, P. K., Lambert, E. H. & Bunge, R., eds.), vol. 3, pp. 510–530, W. B. Saunders, Philadelphia
- Yao, J. K. & Cannon, K. P. (1983) *Biochim. Biophys. Acta* **753**, 331–338
- Yao, J. K., Dyck, P. J., Vanloon, J. A. & Moyer, T. P. (1981) *J. Neurochem.* **36**, 1211–1218