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INVESTIGATION OF THE ORGANISATION OF THE MAJOR PROTEINS IN BOVINE MYELIN MEMBRANES

USE OF CHEMICAL PROBES AND BIFUNCTIONAL CROSSLINKING REAGENTS

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Bovine myelin was incubated with a variety of bifunctional reagents and chemical probes. The use of a photosensitive hydrophobic compound, 1-azido-[^{125}I]iodobenzene, led to the suggestion that the proteolipid protein is deeply intercalated into the hydrophobic milieu of the membrane, but did not support the contention that regions of the basic protein behave in a similar fashion. Crosslinking studies indicated that both polypeptides may be present in the membrane as homodimers and these dimers may be part of much larger assemblies. These results give rise to a somewhat different model for the structural organisation of myelin to that proposed earlier.

Introduction

Myelin, as found in bovine central nervous system white matter, is a specialised multilamellar membrane system that surrounds the nerve axons. This lipid-rich membrane has both its extracellular and cytoplasmic faces in close apposition, thereby forming many concentric bilayers. The system contains only 20–30% by dry weight of protein, represented largely by the hydrophobic proteolipid protein and the basic protein. It has been proposed that at least the basic protein and perhaps both are involved in maintaining the unique folded structure of the myelin membrane [1–4].

In order to determine the organisation and disposition of the two major species in the membrane and help elucidate any role they may play in the structure of myelin, use has been made of a variety of chemical probes. As part of this study, we report here results obtained when the myelin membrane was exposed to several bifunctional crosslinking agents and to hydrophilic and hydrophobic probes.

Materials

All chemicals were of the best grade available and purchased from British Drug Houses (Poole, Dorset, U.K.) and Sigma (Poole, Dorset, U.K.) unless otherwise stated. Dimethylsuberimidate dihydrochloride, dimethyladipimidate dihydrochloride, dithiobis(succinimidyl) propionate, and methyl 4-mercaptobutyrimidate were purchased from Pierce and Warriner (Chester, Cheshire, U.K.) and stored at 4°C in evacuated desiccators. Na^{125}I was obtained from Amersham International,

Abbreviations: DIABS, 3,5,diiodo-4-azidobenzene sulphonate; DDISA, diazotized 3,5-diiodosulphanilic acid; DTSP, dithiobis(succinimidyl) propionate; DMS, dimethylsuberimidate dihydrochloride; DMA, dimethyladipimidate dihydrochloride; SDS, sodium dodecyl sulphate.

Amersham, Bucks., U.K.). PAGE blue was purchased from British Drug Houses (Poole, Dorset, U.K.).

Methods

Synthesis of chemical probes

1-Azido-4-[^{125}I]iodobenzene. 4-Iodoaniline was first radiolabelled with Na^{125}I and iodine monochloride using the isotope-exchange reaction of Helmkamp and Sears [5] and then converted to 1-azido-4-[^{125}I]iodobenzene as described by Smith and Brown [6]. The final product was dissolved in ethanol at a specific activity of 10 Ci/mol and stored at −20°C in the dark. Its purity was confirmed by thin-layer chromatography on silica gel plates using either hexane or petroleum ether (40–60)/diethyl ether/acetic acid (20 : 20 : 1) as solvents.

Diazotized 3,5-diiodosulphanilic acid. Sulphanilic acid was converted to diiodosulphanilic acid, radiolabelled with Na^{125}I and iodine monochloride, and diazotized all by the methods of Helmkamp and Sears [5]. The probe was stored as a solid at −20°C in the dark and had a specific activity of 10 Ci/mol. The identity and purity of the product was established by elemental analysis (%I_2 = 59.5) and thin-layer chromatography on silica gel G plates using ethyl acetate/methanol/25% ammonia solution (17 : 6 : 5) as solvent.

3,5-[^{125}I]Diiodo-4-azidobenzene sulphonate. This was prepared by reacting the diazonium salt, DDISA, with NaN_3 using the method described by Smith and Brown [6]. The probe was stored at −20°C in the dark as an aqueous solution. The specific activity of [^{125}I]DIABS was 10 Ci/mol.

Purification of myelin membranes

A crude myelin fraction was obtained by homogenising bovine brain white matter from freshly slaughtered animals, in 0.32 M sucrose. This was then subjected to the purification scheme described by Rumsby et al. [7] using density gradient centrifugation and osmotic shocking to obtain a pure myelin membrane preparation.

Labelling of membrane proteins

Myelin membranes were suspended in 0.1 M sodium phosphate buffer (pH 7.4) to give a protein concentration of 5 g·l^{-1}. [^{125}I]DDISA (1.3 mM) or [^{125}I]DIABS (40 mM) were added to the membrane suspension as aqueous solutions to give final concentrations of 0.5 mM and 10 mM, respectively. [^{125}I]DIABS-containing samples were incubated for 30 min at room temperature in the dark before being irradiated for 60 min on ice using a 300 W Philips Ultraphil lamp. [^{125}I]DDISA-containing samples were incubated for 30 min at room temperature with no irradiation. Sonication was performed, using a type 7530 Branson-Dawe Soniprobe, for 3 × 10 s (power level 3) at 4°C immediately after addition of the probe to the membrane suspension. The samples were then incubated as above. 1-Azido-4-[^{125}I]iodobenzene (40 mM) was added to the membrane suspension from a stock solution in ethanol to give a final concentration of 1 mM. The samples were incubated for 30 min at room temperature in the dark before being irradiated for 1 h as described above.

The membranes were finally washed three times with 0.1 M sodium phosphate buffer (pH 7.4)/100 mM histidine to remove free probe before being solubilised in gel buffer for SDS-polyacrylamide gel electrophoresis.

Crosslinking experiments

Membrane suspensions were added to 25 mM sodium phosphate/1 mM magnesium chloride buffer (pH 8.0) to give a final protein concentration of 2 g·l^{-1}. Cross linking experiments were performed essentially as described by Brett and Findlay [8]. Dithiobis(succinimidyl) propionate (DTSP) was added from a freshly prepared stock solution of 20 g·l^{-1} in dimethyl sulphoxide, to give a working concentration range of 0.1–1.25 mM. The incubation time varied between 5 and 30 min at 20°C and 0°C and the reaction was stopped by the addition of 50 µl of 1 M ammonium bicarbonate/ml of reaction mixture. Dimethyl suberimidate (DMS) and dimethyl adipimidate (DMA) were freshly made up in cold phosphate/magnesium chloride buffer and used at a working concentration range of 2–40 mM. The reaction time was 1 h at 20°C and the reaction was stopped with ammonium bicarbonate as above. Glutaraldehyde was also made up in cold phosphate/magnesium chloride buffer and used at a working

concentration range of 1–5 mM. The reaction time was 15 min at 20°C and the reaction was again terminated with ammonium bicarbonate. Copper/*o*-phenanthroline was prepared by dissolving 6.8 mM *o*-phenanthroline in 3.4 mM $CuSO_4$ solution. The working concentrations were 0.125 mM and 0.25 mM. Samples were incubated for 30 min at 20°C before the reaction was terminated with 50 μl of iodoacetamide (20 $g \cdot l^{-1}$) per ml membrane suspension.

SDS-polyacrylamide gel electrophoresis

All samples were prepared for electrophoresis by the addition of SDS to give a 2% (w/v) solution. The samples were then diluted 1 : 1 with a mixture of 0.1 M Tris-HCl (pH 6.8), 2% (w/v) SDS and 20% (v/v) glycerol to give a protein concentration of 1–2 $g \cdot l^{-1}$. Bromophenol blue was added as the marker dye. Up to 100 μg of protein were loaded into one track of a slab gel.

Electrophoresis was performed in a 10–18% gradient acrylamide slab gel with 0.8% methylene bisacrylamide as crosslinker, using the method of Laemmli [9]. A 4% stacking gel was used and samples were electrophoresed for 16–18 h at 60 V in 0.02 M Tris/glycine buffer (pH 8.3).

Gels were stained for at least 4 h in 0.25% (w/v) PAGE Blue dissolved in acetic acid/methanol/H_2O (7 : 40 : 53, v/v). The stained gels were destained over a few days in acetic acid/methanol/water (7 : 40 : 53, v/v), and stored in 7% (v/v) acetic acid.

Where necessary, gel tracks were sliced into 1-mm or 2-mm segments using razor blades fitted in a Bio-Rad gel slicer. The gel slices were counted in an Intertechnique CG4000 counter.

Protein assay

Protein in the 10–50 μg range was determined by the manual ninhydrin assay described by Hirs [10]. Bovine serum albumin was used as a protein standard. The identity of the basic and proteolipid proteins were confirmed by amino acid analysis using a Rank Hilger Chromaspek J180.

Results

When myelin membranes were dissolved in SDS-containing buffers and electrophoresed on a 10–18% gradient gel (Fig. 1), several stained bands could be detected. These include the basic protein (M_r 18 000), proteolipid protein (M_r 25 000), the Wolfgram protein doublet (M_r 55 000 and 56 000) together with a range of higher molecular weight protein species present in low concentrations. An intermediary protein band was also observed running between the basic and the proteolipid proteins which had many properties in common with the proteolipid protein.

A number of bifunctional crosslinking reagents with varying chemical specificities and chain lengths were then used to generate protein oligomers in the myelin membrane.

Crosslinking with DTSP

DTSP was added to the myelin membrane suspension in a solution of dimethylsulphoxide. The final concentration of the solvent (under 5%) did not affect the gel patterns.

The crosslinking of the proteins of myelin with DTSP was found to be dependent on the time and temperature of the reaction and on the concentration of reagent. Similar results were obtained at room temperature using reaction times of 5 and 10 min to those obtained with the same concentration of reagent at 4°C for 20 and 30 min. Greater degrees of cross-linking of all proteins occurred at higher concentrations of reagent. At 20°C with a concentration of 0.2 mM DTSP, the monomeric forms of the Wolfgram and higher molecular weight proteins were completely crosslinked to yield large molecular weight aggregates, even with reaction times as short as 5 min.

With a reaction time of 5 min, a temperature of 20°C and a DTSP concentration of 0.2 mM, the amounts of monomeric proteolipid and basic proteins were significantly reduced and two dimeric bands of molecular weights 40 000 and 48 000 appeared (Fig. 1). Some aggregated material running at the top of the gel was also seen. With increasing incubation times, the concentration of the dimeric species was gradually reduced until no dimer was seen, all the material appearing as high-molecular-weight oligomers. The small amount of intermediary protein that electrophoresed between the basic and the proteolipid protein also appeared to be crosslinked, but its fate could not be followed by these methods. At lower concentrations

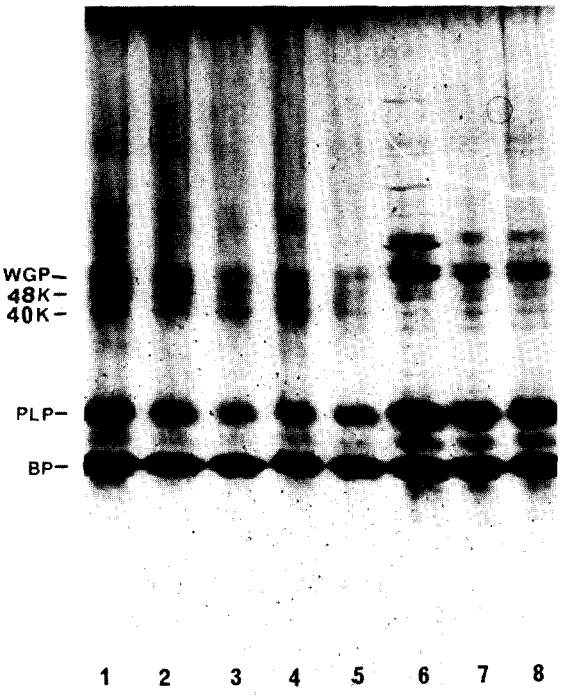

Fig. 1. SDS 10–18% polyacrylamide gradient gels of myelin proteins crosslinked with DTSP. (1) DTSP-treated, 0.1 mM, 5 min; (2) DTSP-treated, 0.1 mM, 10 min; (3) DTSP-treated, 0.1 mM, 20 min; (4) DTSP-treated, 0.2 mM, 5 min; (5) DTSP-treated, 0.2 mM, 10 min; (6) Untreated protein; (7) DTSP-treated, 0.1 mM, 10 min with the addition of 5 mM dithiothreitol; (8) DTSP-treated, 0.2 mM, 10 min with the addition of 5 mM dithiothreitol. Proteins (P): WG, Wolfgram; PL, proteolipid; B, basic.

of reagent (0.1 mM) a similar pattern to that described above was obtained. An interesting and perhaps informative observation was that trimeric and tetrameric species were seen at best only in very small quantities, even when radiolabelled proteins were followed. Instead, there seems to be a sharp transition from dimers to large oligomers.

When 2-mercaptoethanol or dithiothreitol was added to the DTSP-treated membranes to break the thiol group in the bifunctional reagent, the dimeric and oligomeric species disappeared, concomitant with almost total recovery of the monomeric forms of the major proteins (Fig. 1). This suggests that the pattern seen arose from DTSP induced specific crosslinking and not from non-specific aggregation.

Crosslinking with other bifunctional reagents

DMS, DMA and glutaraldehyde. The results of crosslinking experiments with DMS and DMA depended greatly on the concentration of the reagent used. At lower concentrations (1–10 mM), little or no crosslinking of any protein was observed. However, at a higher concentration of 40 mM, a large proportion of both the basic and the proteolipid protein were crosslinked to produce very high molecular weight species which did not penetrate the gradient gel upon electrophoresis. Only very small amounts of the dimeric species found upon DTSP treatment were observed.

Glutaraladehyde, even at concentrations as low as 1 mM, completely aggregated all of the membrane proteins.

Copper / o-phenanthroline. Exposure of myelin to [^{14}C]iodoacetamide indicates that several sulphydryl groups are available for modification on the proteolipid protein. No such residue is present in the basic protein (Fig. 2). Copper/*o*-phenanthroline (0.25 mM, 30 min at 20°C), however, was not able to induce the proteolipid protein to form dimers or higher molecular weight species. It must therefore be concluded that these

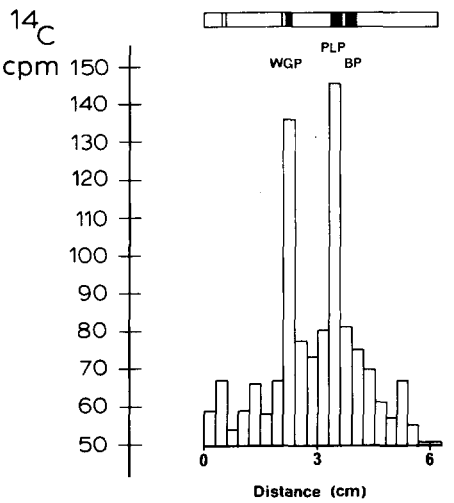

Fig. 2. SDS-polyacrylamide gel electrophoresis of myelin proteins labelled with iodo[^{14}C]acetamide. Myelin membranes were suspended in 50 mM Tris (pH 8.5) to which iodo[^{14}C]acetamide ($4 \cdot 10^{-2}$ μCi/μM) was added. The mixture was sonicated for 5 s on ice with a probe sonicator then incubated for 30 min at room temperature. Aliquots were then electrophoresed in a 7.5% resolving polyacrylamide gel. Abbreviations as for Fig. 1.

sulphydryl groups are not suitably oriented for intermolecular disulphide bond formation (Fig. 3).

The amino groups can react with methyl 4-mercaptobutyrimidate through its imidate function. The reagent also contains an -SH group which can be oxidised to form disulphide crosslinks. When myelin was treated with this compound (10 mM) for 30 min at 4°C followed by exposure to copper/*o*-phenanthroline (0.25 mM) for 30 min at 20°C, the proteolipid protein was totally crosslinked into high molecular weight oligomers with no intermediary forms (Fig. 3). Some of the basic protein was also crosslinked, while the Wolfgram and higher-molecular-weight proteins disappeared from the gel. When the crosslinked species were treated with 5 mM dithiothreitol, the monomeric forms of all proteins were observed. Crosslinking was abolished if the membrane were dissolved in SDS after reaction with mercaptobutyrimidate but before exposure to the copper/*o*-phenanthroline complex.

Labelling of myelin proteins with chemical probes

Hydrophobic probe (azido[^{125}I]iodobenzene). Membrane suspensions were incubated with azido[^{125}I]iodobenzene for 30 min at 20°C to allow complete equilibration of probe with the bilayer before the irradiation treatment. These times are based on the work of Wells and Findlay [11,12]

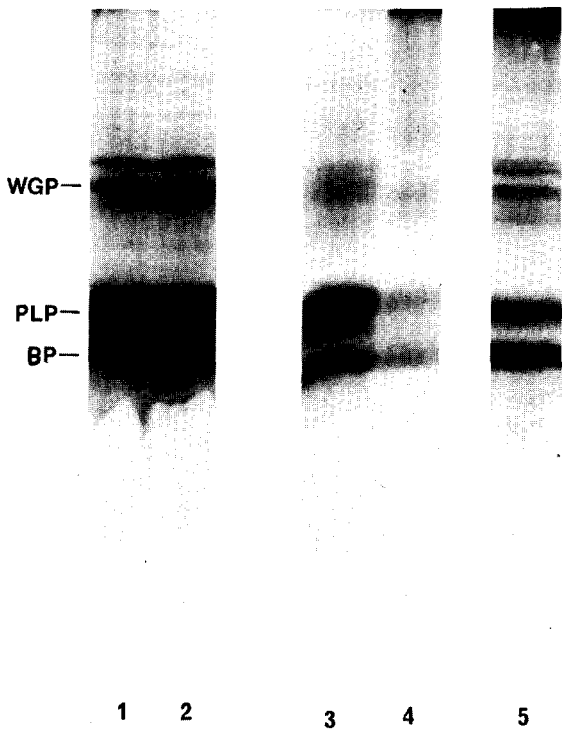

Fig. 3. SDS 10–18% polyacrylamide gradient gels of myelin proteins crosslinked with methyl-4-mercaptobutyrimidate and copper/*o*-phenanthroline. (1) 0.25 mM copper/*o*-phenanthroline in absence of SDS; (2) 0.25 mM copper/*o*-phenanthroline in presence of SDS; (3) 10 mM mercaptobutyrimidate and 0.25 mM copper/*o*-phenanthroline in the presence of SDS; (4) 10 mM mercaptobutyrimidate and 0.25 mM copper/*o*-phenanthroline in absence of SDS; (5) 10 mM mercaptobutyrimidate, 0.25 mM copper/*o*-phenanthroline and 5 mM dithiothreitol. Abbreviations as for Fig. 1.

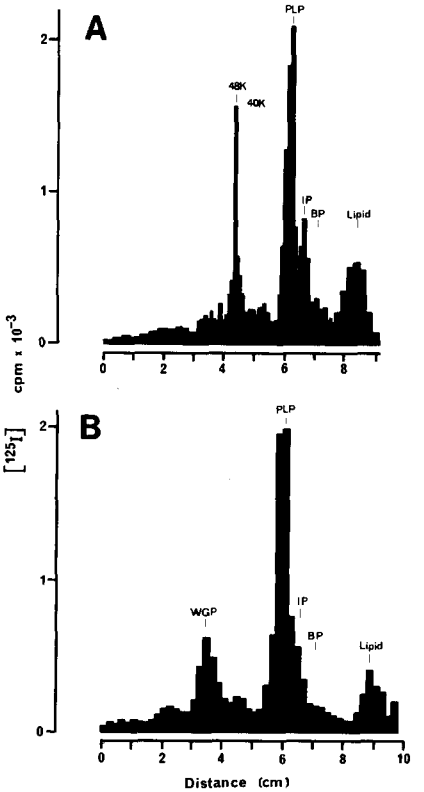

Fig. 4. SDS-polyacrylamide gel electrophoresis of myelin proteins labelled with the hydrophobic probe, 1-azido-4-[^{125}I]iodobenzene. The proteins were labelled and electrophoresed on a 10–18% gradient gel as described in Methods. The gels were stained in PAGE blue, destained and sliced into 1-mm or 2-mm segments prior to counting. PLP, proteolipid protein; BP, basic protein; WGP, Wolfgram protein; IP, intermediary protein. (A) Azido[^{125}I]iodobenzene-labelled proteins crosslinked with 0.2 mM DTSP for 10 min. (B) Azido[^{125}I]-labelled proteins, control without DTSP present.

using erythrocyte membranes in which they showed that complete partitioning of azidoiodobenzene was obtained well within the 30 min preincubation period. Photoactivation of the probe was maximal by 1 h [11,12].

After irradiation, the myelin membranes were extensively washed with buffer containing 100 mM histidine to remove any free or unreacted probe. At this stage, some samples were treated with the crosslinking reagent DTSP as described above. The membranes were then solubilised in 2% (w/v) SDS and electrophoresed in a 10–18% gradient gel.

It was found with the control systems where no crosslinking reagent was used, that the major band to be labelled corresponded to the proteolipid protein with some labelling of the intermediary protein band. The Wolfgram protein was also heavily labelled with this probe but, significantly, the basic protein contained little or no radioactivity (Fig. 4).

When the labelled proteins were crosslinked with 0.2 mM DTSP for 10 min at 4°C, the two dimeric bands, of molecular weights of 40 000 and 48 000, appeared. Only the 48 kDa dimer was found to be labelled with azido[^{125}I]iodobenzene (Fig. 4). High molecular weight oligomers which did not enter the gel were also labelled.

Hydrophilic probes: [^{125}I]DDISA and [^{125}I]-DIABS. Myelin membrane suspensions were preincubated for 30 min with [^{125}I]DDISA or [^{125}I]-DIABS to allow complete equilibration. [^{125}I]-DDISA does not require activation and reacted readily with proteins in the hydrophilic phase. [^{125}I]DIABS was photoactivated by irradiation for 1 h to achieve maximal labelling and, like DDISA, reacted with groups in the aqueous phase. Free probe was removed by washing the membranes with buffer containing 100 mM histidine.

Using these techniques, all the major proteins were labelled with both probes, the proteolipid protein much more than the basic protein in unsonicated preparations (Fig. 5). In labelled crosslinked myelin preparations that contained the dimeric species (M_r 40 000 and 48 000), both of these contained radioactivity. Upon sonicating membranes in the presence of the hydrophilic probes, there was at least a 2-fold increase in the labelling of both the proteolipid and basic proteins (Fig. 5).

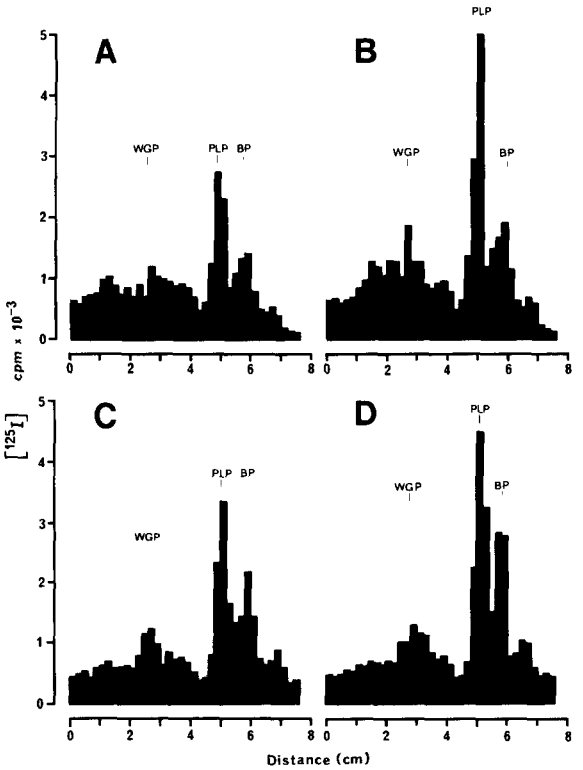

Fig. 5. SDS-polyacrylamide gel electrophoresis of myelin proteins labelled with the hydrophilic probes. [^{125}I]DDISA and [^{125}I]DIABS. The proteins were labelled and electrophoresed on a 10–18% gradient gel as described in Methods. The gel was stained in PAGE blue and destained before individual tracks were sectioned into 2-mm segments prior to counting. Abbreviations as for Fig. 4. (A) (unsonicated) and (B) (sonicated): preparation of [^{125}I]DDISA-labelled myelin proteins. (C) (unsonicated) and (D) (sonicated): preparation of [^{125}I]DIABS-labelled myelin proteins.

Discussion

Investigations into the role played by the major polypeptides in the unique structure of myelin has concentrated on the basic protein. The relative protection from proteolytic attack offered to certain stretches of this polypeptide by a lipid monolayer has been interpreted as suggesting that these regions are tightly associated with the bilayer [4,17]. The existence of hydrophobic associations has been inferred from spin-label [13] and calorimetry studies [14] and evidence has been put forward for hydrophilic interactions with acidic sulphated glycolipids [15]. Most of these interpretations tend to

be in conformity with a model for the organisation of the major proteins in myelin put forward by Crang and Rumsby [16]. Our approach has been to investigate this organisation using a variety of chemical probes and bifunctional reagents.

Studies with the hydrophilic probes are consistent with other reports [1,17] suggesting that the proteolipid protein traverses the bilayer whilst the basic protein is confined to the equivalent of the cytoplasmic face of the membrane. The small amount of radioactivity associated with the basic protein found after incubating unsonicated myelin presumably arises from unsealed lamellae.

The results with the hydrophobic probe also point to the quite different character of the two proteins. In contrast to the proteolipid protein which is heavily labelled with this probe, the basic protein contains no more radioactivity than is obtained when a water-soluble polypeptide (e.g., ribonuclease) is included in the system as a control. Under similar experimental conditions, a protein such as glycophorin, in the human erythrocyte membrane, which could well have as much of the same kind of amino acid side-chains buried in the bilayer as the basic protein, is much more heavily labelled than the basic protein [11,12]. We are forced to the conclusion, on this evidence, that although the basic protein probably associates very strongly with the lipid bilayer, this interaction does not to any significant extent consist of loops of polypeptide chain penetrating deeply into the non-polar regions of the membrane. It is just possible that such loops may be protected by association with the proteolipid protein but our crosslinking studies do not indicate a close spatial relationship between the two proteins. Any penetration of the membrane by the basic protein, therefore, may be largely confined to the more polar, headgroup regions of the bilayer. In contrast, the proteolipid protein traverses the membrane, exposing large areas of its structure to reaction with hydrophobic probes and therefore has a structure similar to the bacteriorhodopsin or rhodopsin type of integral membrane protein [18–20].

The crosslinking studies have been difficult, particularly because of the unique characteristic of both proteins in this membrane to inhibit a sharp transition from monomer to high-molecular-weight oligomers on exposure to crosslinking agents. Close attention to the experimental conditions, however, reveals the presence of basic protein species consistent with dimer formation, but trimers and tetramers were not recognisably detected, even using radioactive labels. Similar dimer formation of the basic proteins was seen in studies with detergent extractions of myelin and by others in purified basic protein preparations [2,21–23]. Hydrophobic and -SH-directed probes did not bind to the 40 kDa material or to the monomeric basic protein, indicating that the proteolipid protein was not present and therefore this 40 kDa species represented a homodimer of basic protein. Amino acid analysis of the protein eluted from the excised band confirmed this assignment, giving Leu : Glu and Thr : Ser ratios of 0.88 and 0.47 compared to 1.0 and 0.4 for the pure monomeric basic protein. If the basic protein were present as monomeric material, crosslinking should normally give all the oligomeric species but present in exponentially decreasing amounts such as is seen with rhodopsin [8]. Moreover, extraction from the membrane in non-denaturing detergent, which dramatically reduces the effective protein concentration, should eliminate oligomer formation. Since neither of these situations occurred, we believe that basic protein is present in myelin most intimately as dimer.

Since the second new band (48 kDa) formed upon crosslinking is heavily labelled with the hydrophobic probe and also reacts with sulphydryl-directed reagents, we conclude that it contains the proteolipid protein. The molecular weight of this protein species suggests that it, too, represents a dimer. The amino acid composition of the dimer eluted from excised bands supports this conclusion, since the Leu : Glu and Thr : Ser ratios of 1.78 and 1.18, respectively, were characteristic of those for the pure proteolipid protein, 1.85 and 1.1, and very different from the corresponding values of 1.0 and 0.4 for the pure basic protein. Moreover, assuming that the basic protein is present in situ as a dimer, any interaction with the proteolipid protein should yield heterotrimers or tetramers (rather than heterodimers), neither of which we see. Extraction of the proteolipid protein into mild non-denaturing detergent was not efficient and gave rise to crosslinking patterns sugges-

tive of large aggregates of the protein. We are unable, therefore, to carry out unambiguously the corollary experiment of crosslinking at relatively low protein concentrations such as was possible with the basic protein.

One final point concerns the facility with which high molecular weight oligomers were obtained on crosslinking myelin proteins, even under reaction conditions which, in the case of rhodopsin, yielded very little oligomer formation and where membrane fluidity was substantially reduced. This observation suggests the possibility that the dimeric species may associate in situ into patches or columns of protein which allow large assemblies to be readily crosslinked. Bacteriorhodopsin, for example, is present in the membrane of *Halobacterium halobium* as large 'islands' of protein, each comprising associations of large numbers of trimers. As a result of these investigations, the possibility is advanced that the presumed ability of the basic protein to facilitate myelin compaction is due to its assembly into dimers, one surface of which is responsible or dimer formation and the second interacting with the lipid bilayer. The proteolipid protein is also present as membrane-spanning dimers probably not in meaningful association with the basic protein but possibly capable of dimer-dimer interaction across the interbilayer gap. The transmembrane disposition of the proteolipid protein suggests, however, that the primary role of the protein may be related more to transport phenomena than solely bilayer adhesion.

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