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Enzymic methylation of myelin basic protein in myelin

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Myelin fractions with different degrees of compaction were isolated from bovine brain, and post-translational methylation of membrane-associated proteins was studied. When the purified myelin-basic-protein-specific protein methylase I and *S*-adenosyl-L-[*methyl*-^{14}C]methionine were added exogenously, the most compact myelin fraction exhibited higher methyl-accepting activity than the less compact dense fractions. The methylated protein was identified as myelin basic protein (18.4 kDa) exclusively among the several myelin proteins from all membrane fractions, by SDS/PAGE/radioautography of *methyl*-^{14}C-labelled membrane proteins. The *methyl*-^{14}C-labelled amino acid residue in the basic protein was identified by h.p.l.c. as N^G-methylarginine, indicating the high degree of specificity for the arginine residue as well as the myelin basic protein in the intact myelin membranes. The possibility of a charge alteration of myelin basic protein resulting from its arginine methylation was investigated by using the purified component 1 of myelin basic protein. The methylated component was shown to be less cationic than the unmethylated component by Bio-Rex 70 cation-exchange chromatography, since the former preceded the latter. However, in the presence of the denaturant (guanidinium chloride), the two species were co-eluted, indicating that the charge difference between methylated and unmethylated myelin basic protein can only be shown under the renatured condition.

INTRODUCTION

Most axons are ensheathed by a myelin membrane to increase the velocity of nerve impulse. Myelin basic protein (MBP) is one of the major and well-studied proteins of myelin, and is strongly bound to the phospholipid bilayer, giving rise to multilamellar myelin membranes. It has been shown that the myelin membrane exists at various levels of compactness, possibly owing to the different stages of transition between the oligodendroglial plasma membrane and multilamellar myelin (Matthieu *et al.*, 1973; Zimmerman *et al.*, 1975) or to the degeneration of the sheath in diseased conditions (Whitaker & Snyder, 1985).

Compact myelin (also referred to as myelin) has more highly repetitive multilamellar structure than the less compact myelin fractions and can be distinguished morphologically as well as biochemically (Cruz & Moscarello, 1985). Fractions of the myelin and the less compact myelin-like membranes have been isolated from the white matter of brain by density-gradient centrifugation (Cruz & Moscarello, 1985). The less compact (or dense) myelin has been shown to be composed of fewer lamellae, which correlates with the relative decrease in the cationic-charge isomer of the protein (Moscarello *et al.*, 1986).

The major species of bovine or human MBP is a single polypeptide of 18.4 kDa (Eylar, 1970), but it exhibits micro-heterogeneity on CM-52 chromatography, yielding several charge isomers (Chou *et al.*, 1976). This multiplicity of the MBP molecule was ascribed to several post-translational modifications such as phosphorylation, deamidation (Chou *et al.*, 1976; Martenson *et al.*, 1983), methylation (Baldwin & Carnegie, 1971; Brostoff & Eylar, 1971), glycosylation (Persaud *et al.*, 1988) and *C*-terminal arginine loss (Deibler *et al.*, 1975; Chou *et al.*, 1977). Most of these modifications occur between threonine-95 (by glycosylation) and arginine-107 (by methylation), the region where a double-chain hair-pin structure could possibly occur *in vivo* via the triproline sequence of residues 99–101 (Brostoff & Eylar, 1971; Boggs *et al.*, 1982).

Methylation of MBP at arginine-107 has been shown to be a site-specific modification catalysed by MBP-specific protein methylase I (PMI) (*S*-adenosylmethionine:protein-arginine *N*-methyltransferase, EC 2.1.1.23), *in vivo* and *in vitro*, yielding N^G-monomethyl- (MMeArg) and $N^GN'^G$-dimethyl-arginine (DiMeArg) (Baldwin & Carnegie, 1971; Brostoff & Eylar, 1971; Deibler & Martenson, 1973; Ghosh *et al.*, 1988; Kim *et al.*, 1990). Studies from our and other laboratories have demonstrated that the level of the methylase activity is correlated temporally with myelination during brain development (Miyake, 1975; Crang & Jacobson, 1982; Amur *et al.*, 1984; Chanderkar *et al.*, 1986) and depressed in dysmyelinating jimpy-mutant-mouse brains (Kim *et al.*, 1984), suggesting a possible role of the modification in the early stages of myelination.

In the present paper, we studied the status of methylation with the intact myelin fractions of different degrees of compaction, using purified MBP-specific PMI (Ghosh *et al.*, 1988), and of charge/hydrophobicity alterations on MBP subsequent to its arginine methylation.

EXPERIMENTAL

Materials

S-Adenosyl-L-[*methyl*-^{14}C]methionine (specific radioactivity 56 mCi/mmol) was obtained from ICN Radiochemicals. Phenylmethanesulphonyl fluoride (PMSF), Mops and CM-52 were purchased from Sigma Chemical Co. All other chemicals were of the highest reagent grade available. Calf brain was freshly obtained from a local slaughterhouse, kept at 4 °C and used within 2 h.

Isolation of myelin and myelin-containing fractions

Myelin and myelin-containing fractions were prepared from calf white matter as described by Lowden *et al.* (1966). The white matter (20 g) dissected from freshly obtained brain was immediately homogenized in 10 vol. of buffer A (10 mM-Mops/1 mM-PMSF, pH 7.4) containing 0.25 M-sucrose and the homogenate centrifuged at 600 *g* for 10 min. The supernatant

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; MBP, myelin basic protein; PMI, protein methylase I; PMSF, phenylmethanesulphonyl fluoride; MMeArg, N^G-monomethylarginine; DiMeArg, $N^GN'^G$-dimethylarginine.

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was further centrifuged at 20000 *g* for 20 min. The pellet was resuspended in 0.88 M-sucrose and divided into several tubes, after which 0.25 M-sucrose solution was layered on top. Centrifugation was performed at 100000 *g* for 60 min. The white fluffy membrane obtained at the interface between 0.25 M- and 0.88 M-sucrose was compact myelin, and the pellet (P_3) contained the less compact denser myelin fractions (Cruz & Moscarello, 1985). P_3 was resuspended in 60 ml of 0.25 M-sucrose and fractionated further on a discontinuous gradient, consisting of 0.80 M-, 1.0 M- and 1.2 M-sucrose, by centrifugation at 100000 *g* for 90 min. The materials present at the interfaces of 0.25 M-/0.80 M- and 0.80 M-/1.0 M-sucrose were designated P_3A and P_3B respectively (Cruz & Moscarello, 1985). All the membrane preparations were separately collected, washed a few times with distilled water and stored in buffer A at −20 °C until use.

Methylation of myelin fractions by MBP-specific PMI

Membranes were incubated at 45 °C for 60 min in a mixture containing 0.1 M-potassium phosphate (pH 7.2), 40 μM-Ado[*methyl*-^{14}C]Met (123 d.p.m./pmol), myelin membrane (5–10 μg of MBP) and an indicated amount of MBP-specific PMI. The reaction was stopped by placing the tube in an ice bucket, and the mixture was centrifuged at 12000 *g* for 10 min in an Eppendorf centrifuge. Ado[*methyl*-^{14}C]Met that had not reacted was removed by washing the pellets three times with buffer A. The pellets were then treated with diethyl ether for 30 min and the mixture was centrifuged (12000 *g* for 10 min). Finally, the membrane proteins were extracted with a solution containing 0.063 M-Tris/HCl, 2% SDS, 5% mercaptoethanol, 0.005% Bromophenol Blue and 10% glycerol (pH 6.8) at 100 °C for 10 min, and subjected to SDS/PAGE.

Methylation of extracted MBP from myelin by MBP-specific PMI

The isolated myelin and myelin-like membranes (P_3A and P_3B) were freeze-dried and the basic proteins were extracted with 5.0 ml of 0.2 M-H_2SO_4 overnight at 4 °C. The extracts were dialysed against water, freeze-dried, suspended in a minimum volume of water and centrifuged at 15000 *g* for 10 min to remove insoluble material. The purity of the basic protein was analysed on SDS/PAGE.

The methylation of MBP was carried out as described previously (Paik & Kim, 1968; Ghosh *et al.*, 1988). Briefly, a total volume of 0.25 ml containing 0.1 M-potassium phosphate (pH 7.2), 40 μM-Ado[*methyl*-^{14}C]Met, 7 μg of MBP and 3 μg of MBP-specific PMI was incubated at 45 °C for 60 min. The reaction was terminated by addition of 15% trichloroacetic acid and the *methyl*-^{14}C incorporation into MBP was analysed as described previously.

Purification of MBP-specific PMI

The MBP-specific PMI was purified from calf brain as described previously (Ghosh *et al.*, 1988). Briefly, the high-speed supernatant obtained from brain homogenate in sucrose buffer at 39000 *g* was successively purified by $(NH_4)_2SO_4$ fractionation, chromatography on DE-52, Sephadex G-200, hydroxyapatite and a second Sephadex G-200 chromatography. The enzyme activity was assayed as described by Ghosh *et al.* (1988).

Protein concentrations were determined by the BCA protein assay reagent (Pierce Chemical Co., no. 23225) or by determination of absorbance at 280 nm.

PAGE

SDS/PAGE was carried out by the method of Laemmli (1970) with 15% acrylamide in the presence of 0.1% SDS. Electrophoresis was performed at a constant current of 8 mA/slab until the dye front reached the bottom of the gel. The gel was stained with Coomassie Blue R-250, destained, and developed for radioautography.

Amino acid analysis of *methyl*-^{14}C-labelled protein

The enzymically *methyl*-^{14}C-labelled myelin membrane was first extracted with 0.2 M-H_2SO_4 as described above and hydrolysed in 6 M-HCl at 110 °C for 24 h. Amino acid analysis of the hydrolysate was performed on a Waters h.p.l.c. apparatus as described previously (Park *et al.*, 1986), by using the *o*-phthaldialdehyde derivative-formation method. Fractions (1.0 min) were collected and counted for radioactivity with ACS II scintillator.

Electron microscopy

Freshly prepared membrane fractions were immediately washed with distilled water and centrifuged in an Eppendorf centrifuge for 1 min, followed by fixation with 2.5% glutaraldehyde in 0.1 M-sodium phosphate buffer (pH 7.4) for 1 h at 4 °C. It was noted that several washes of membrane with distilled water resulted in the destruction of the myelin structure. The samples were rinsed with the phosphate buffer and post-fixed with 1% OsO_4 in 0.1 M-phosphate buffer at 4 °C for 1 h. After being rinsed with the phosphate buffer, the membranes were completely dehydrated and embedded in EM bed 812 epoxy resin, followed by staining with uranyl acetate and lead citrate, sectioned, and viewed in a Philips 300 electron microscope.

Preparation of methylated component-1 MBP

The component-1 MBP was isolated from calf brain as previously described (Deibler *et al.*, 1972; Chou *et al.*, 1976; Chanderkar *et al.*, 1986) by successive treatment of delipidation, acid extraction, DE-52 treatment and finally CM-52 cation-exchange column chromatography. The pooled component 1 was rechromatographed on the CM-52 in order to remove contaminating component 2 completely (Chou *et al.*, 1976). Component 1 (5.5 mg) was methylated as described above in a total volume of 0.5 ml with 1.1 units of purified MBP-specific PMI. The reaction was stopped by the addition of 40 μl of 3 M-HCl, and the mixture was immediately applied to a Sephadex G-25 column (0.7 cm × 87 cm) equilibrated in 0.01 M-HCl, to remove unchanged Ado[*methyl*-^{14}C]Met. Methylated MBP was eluted at the void volume (20 ml), and the Ado[*methyl*-^{14}C]Met that had not reacted was eluted at around 30 ml. The pooled *methyl*-^{14}C-labelled MBP fractions (about 10 ml) were freeze-dried.

Bio-Rex 70 chromatography of methylated component-1 MBP

Methylated component-1 MBP was dissolved in 15 ml of 0.08 M-glycine/NaOH buffer (pH 9.5) containing 1 mM-PMSF (buffer B). The first chromatography (Fig. 4) was carried out on a Bio-Rex 70 column (1.0 cm × 6.6 cm) equilibrated in buffer B in the presence and absence of guanidinium chloride using a linear NaCl gradient (0–1.0 M). The second chromatography (Fig. 5) was carried out similarly by using both the *methyl*-^{14}C-labelled and native unlabelled component 1 in the absence of the denaturant on a Bio-Rex 70 column (0.7 cm × 42 cm) by using a shallower NaCl gradient (0.1–0.6 M). Further details of the procedures are given in the appropriate Figure legends.

RESULTS

Morphological and protein profiles of different myelin fractions

Calf brain white matter was fractionated by discontinuous sucrose-density-gradient centrifugation to obtain myelin fractions of different degrees of compaction. Three subfractions

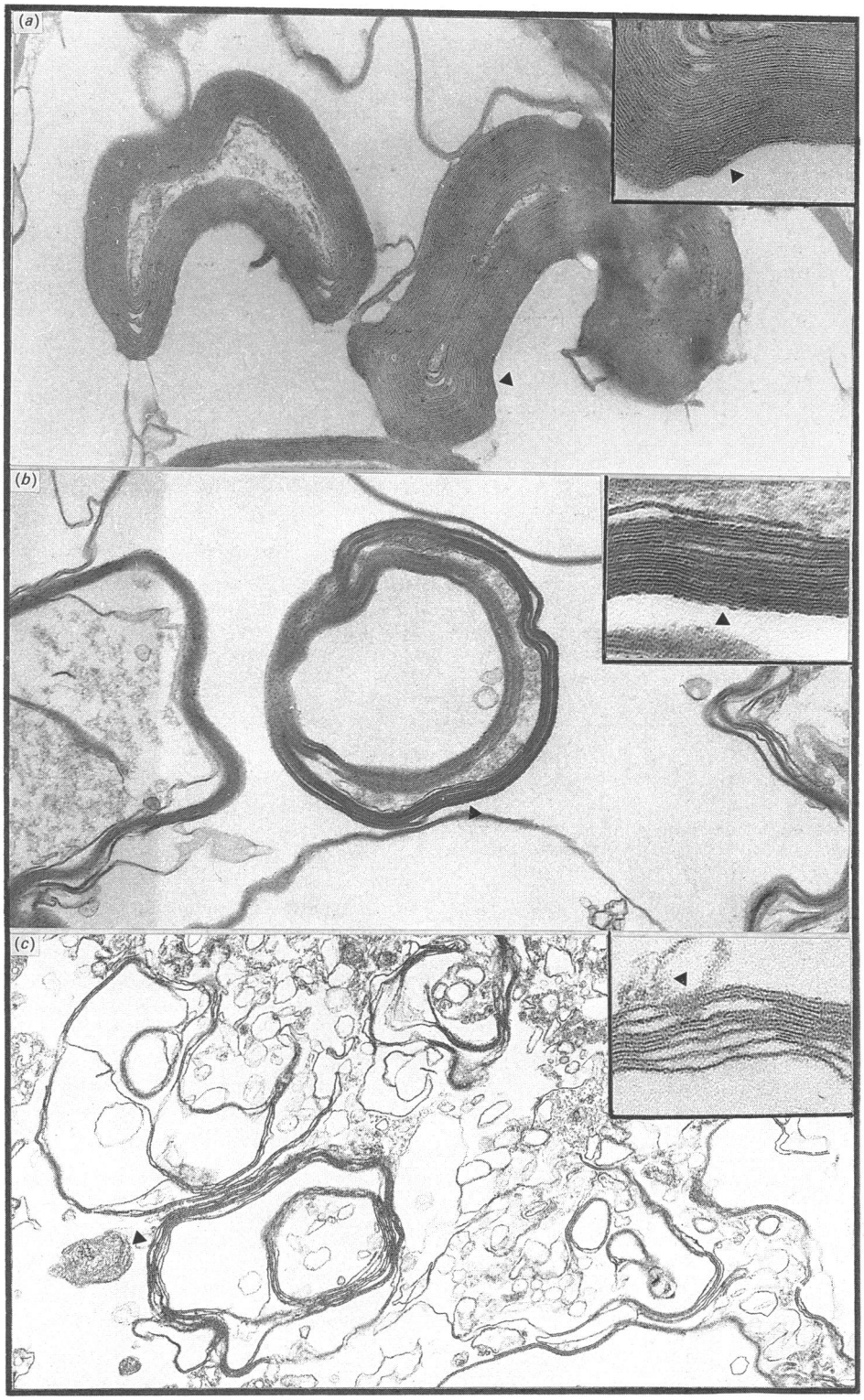

Fig. 1. Electron micrographs of myelin and myelin-like membranes obtained by density-gradient fractionation

Calf brain white matter was fractionated by discontinuous sucrose-density-gradient fractionation. The membranes were briefly washed once with distilled water before fixation. (*a*) Myelin fraction at 0.25 M-/0.88 M-sucrose interface. (*b*) P_3A fraction at 0.25 M-/0.80 M-sucrose interface. (*c*) P_3B fraction at 0.80 M-/1.0 M-sucrose interface. Magnification: lower, ×22000; higher, ×110000 (inset).

thus separated were examined by electron microscopy. The light and compact myelin fraction (Fig. 1*a*) contained a high degree of multilamellar structure, whereas the less compact and dense subfractions, P_3A and P_3B (Figs. 1*b* and 1*c*), contained fewer lamellae.

The proteins associated with these membrane fractions were extracted and analysed by SDS/PAGE (Fig. 2*a*). Marked differences in the relative proportion of MBP to the other higher-molecular-mass membrane proteins (such as proteolipid protein and Wolfgram protein) are seen depending on the density of the

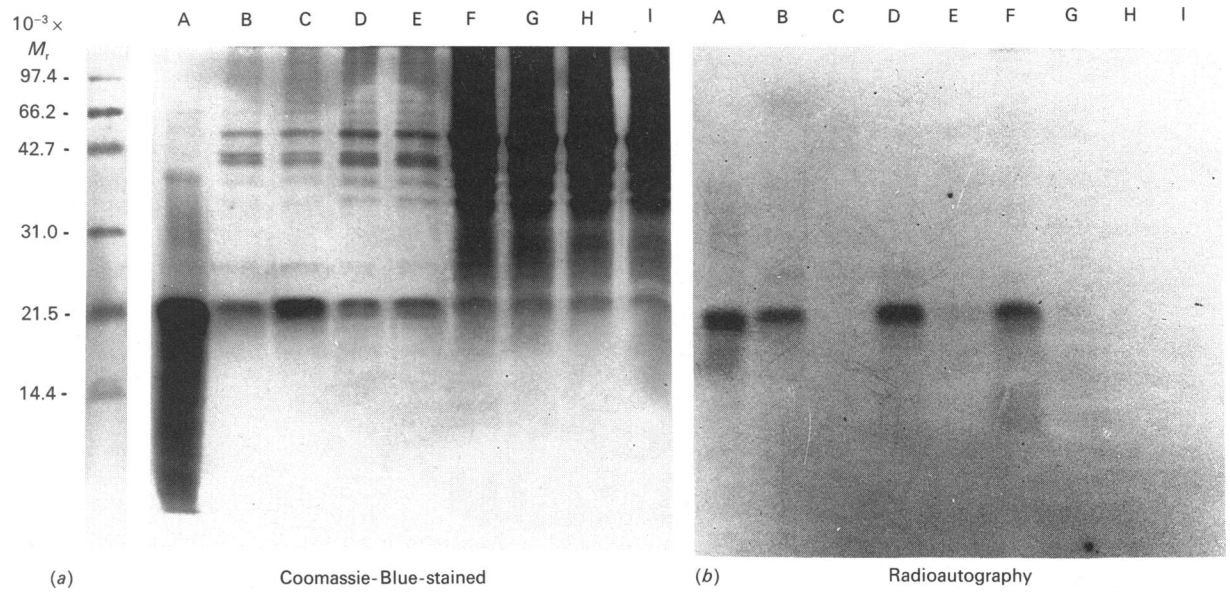

Fig. 2. SDS/PAGE and radioautogram of methylated myelin and myelin-like membranes by MBP-specific PMI

Isolated membranes were methylated with purified MBP-specific PMI and Ado[*methyl*-^{14}C]Met at 45 °C for 60 min as described in the Experimental section. After removal of radioactivity that had not reacted, the membrane was treated with diethyl ether and the total protein fractions were electrophoresed on SDS/15%-polyacrylamide gels. Lanes of Coomassie Blue staining (*a*) and radioautography (*b*) are shown. The purified MBP (lane A; overloading and degradation of MBP is noted), the compact myelin (total protein of 68 μg for B and C), P_3A (60 μg, for D and E) and P_3B (300 μg, for F–I) were electrophoresed. Lanes: A, B, D, F and H, with enzyme; C, E, G and I, without enzyme; H and I, with 400 μM of *S*-adenosyl-L-homocysteine. The radioautogram was overexposed.

Table 1. Methylation of membrane-bound and extracted MBP

In all experiments, 7 μg of MBP (membrane-bound or extracted) was incubated with 3.0 μg of purified MBP-specific PMI at 45 °C for 60 min. The *methyl*-^{14}C incorporation into MBP was analysed by the trichloroacetate-precipitation method as described previously (Ghosh *et al.*, 1988). Data are means ± S.E.M. of three independent experiments. The values in parentheses are percentages of activity with myelin.

	methyl-^{14}C incorporation (pmol)	
Fraction	Membrane-associated	Extracted
Myelin	15.1 ± 1.6 (100)	28.5 ± 1.7 (100)
P_3A	5.3 ± 0.59 (35.1)	16.6 ± 1.1 (58.2)
P_3B	6.0 ± 0.42 (39.7)	5.9 ± 0.42 (20.7)

membrane, as has also been reported by others (Cruz & Moscarello, 1985). The lighter and compact myelin membrane (lanes B and C) contained a higher proportion of MBP than of the high-molecular-mass membrane proteins which migrated at around 40–60 kDa. The relative amount of MBP to the total membrane proteins in each membrane subfraction was measured by densitometric analysis of the gel. Myelin, P_3A and P_3B contained MBP (approx. 37%, 28% and 1.8% of the total protein respectively), indicating a close relationship between the number of lamellae (Fig. 1) and amount of MBP.

Methylation of MBP embedded in membranes of different degrees of compaction

The methyl acceptability of MBP embedded in membranes of different levels of compaction has been measured in both the membrane-associated and the extracted forms. Since Arg-107 in MBP is partially methylated *in vivo* (Deibler & Martenson, 1973), it is quite possible to methylate the monomethylated as well as the unmethylated residue further. The relative methyl acceptability of MBP in membranes from different levels of compaction was studied under the condition in which MBP was saturated with respect to enzyme. As shown in Table 1, MBP from the most compact myelin, as either the membrane-bound or the extracted protein, showed higher methyl acceptability compared with that from the less compact fractions, P_3A and P_3B. However, the extracted MBP generally showed higher methyl acceptability than its membrane-bound counterpart, except in the case of the P_3B fraction, which is the least compact, with fewer lamellae. This strongly suggests that the presence of the membrane component hinders free interaction between MBP which is embedded in the membrane and the 500 kDa MBP-specific PMI during reaction.

Identification of the *methyl*-^{14}C-labelled myelin protein and amino acid residues

To identify the membrane protein methylated, the membranes were incubated with exogenous MBP-specific PMI and Ado[*methyl*-^{14}C]Met, and analysed on SDS/PAGE. As shown in Fig. 2(*b*), among several molecular species of membrane proteins (detected by Coomassie Blue staining), only those that co-migrated with the standard MBP were *methyl*-^{14}C-labelled, as evidenced by radioautography. This MBP-specific methylation is shown in all membrane preparations (Fig. 2*b*, lanes B, D and F). The absence of methylation from the high-molecular-mass region clearly demonstrates the high degree of membrane-bound substrate specificity for MBP-specific PMI. Moreover, the methylation was completely inhibited by the presence of *S*-adenosyl-L-homocysteine, a potent product inhibitor for AdoMet-dependent methyltransferases (Zappia *et al.*, 1969), in the incubation mixture (lanes H and I, Fig. 2).

To identify the amino acid residue methylated, the *methyl*-^{14}C-labelled myelin protein was hydrolysed in 6 M-HCl and subjected to h.p.l.c. As shown in Fig. 3, over 90% of the radioactivity was

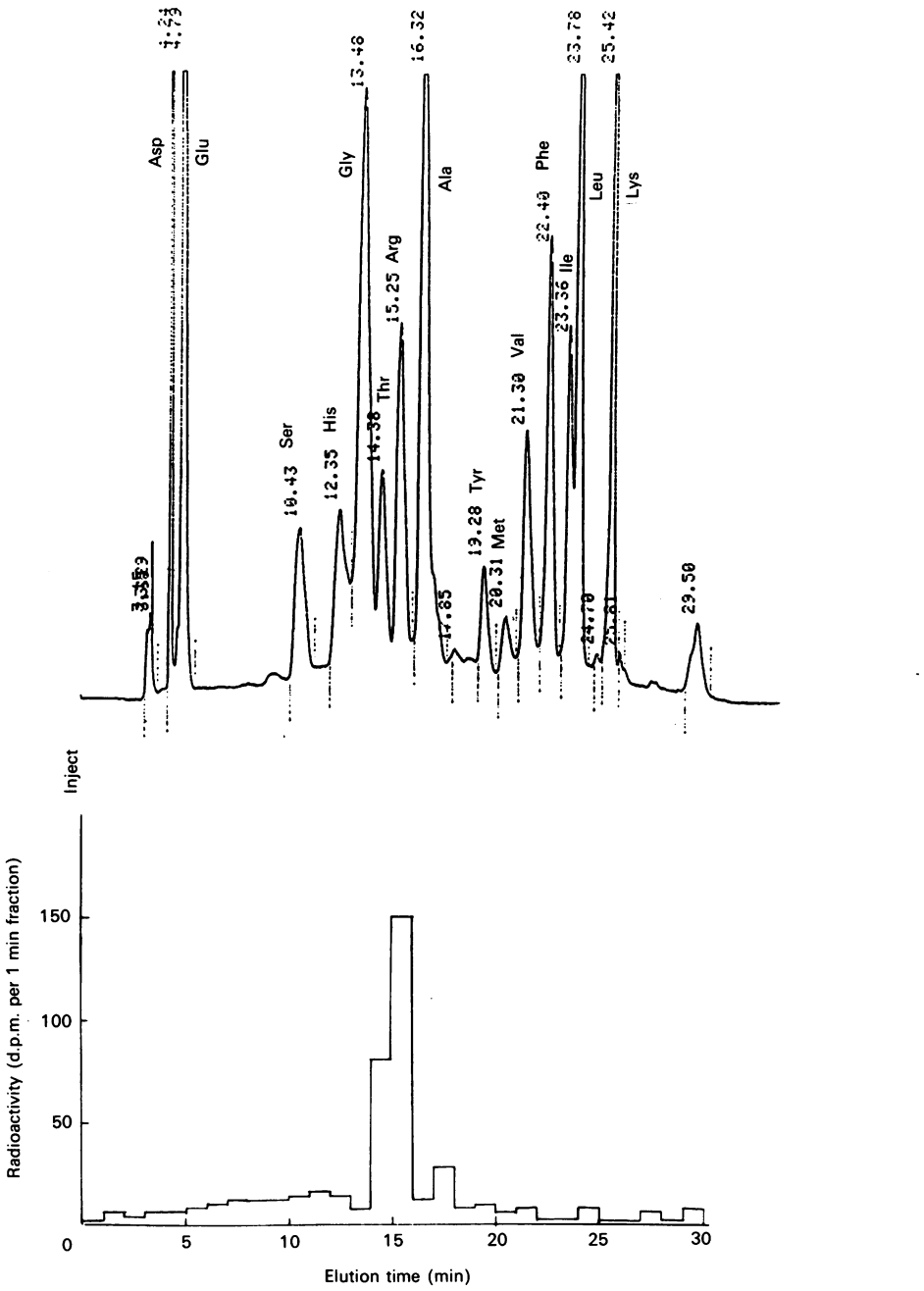

Fig. 3. Amino acid analysis of methylated myelin protein

The *methyl*-^{14}C-labelled myelin was extracted successively with diethyl ether and H_2SO_4. The extract was hydrolysed in 6 M-HCl at 110 °C for 24 h. The hydrolysate was then analysed by h.p.l.c. as described in the text. Fractions (1.0 min) were collected and counted for radioactivity. The standard arginine derivatives were eluted: MMeArg at 15 min and DiMeArg at 16 min.

eluted between 14 and 16 min. Some 30% of MMeArg (15 min) and 60% of DiMeArg (16 min) were estimated on the basis of the retention time of authentic methylated compounds (Park *et al.*, 1986).

Analysis of *methyl*-^{14}C-labelled component-1 MBP on Bio-Rex 70 chromatography

Since bovine MBP is known to contain several charge isomers which are eluted sequentially on cation-exchange chromatography (Chou *et al.*, 1976), a possibility of charge alteration of MBP subsequent to arginine methylation was studied with purified component-1 MBP, the unmodified native form (Chou *et al.*, 1976).

Fig. 4 shows the analysis of *methyl*-^{14}C-labelled component 1 on Bio-Rex 70 chromatography in the presence or absence of guanidinium chloride. There are two major differences in the elution profile under these conditions. First, in the presence of the denaturant, the proteins are eluted before 0.1 M-NaCl concentration (Fig. 4*a*), whereas in its absence they are eluted between 0.2 and 0.35 M (Fig. 4*b*), indicating that the overall charge status of the unfolded component-1 MBP is much less cationic than the native form. Secondly, the elution pattern of the methylated species (indicated by radioactivity) overlapped identically with that of protein in the presence of the denaturant (Fig. 4*a*). However, in the absence of the denaturant, the methylated species was eluted before the unmethylated (Fig. 4*b*). To

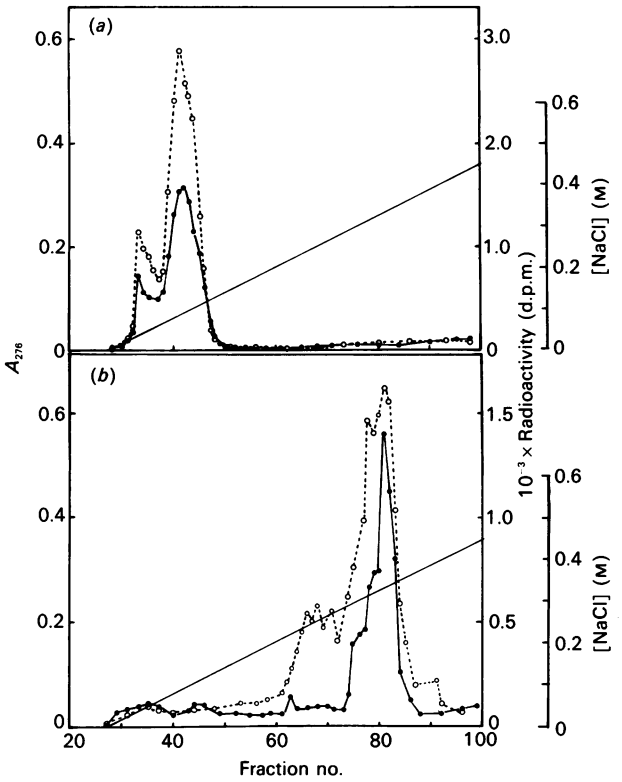

Fig. 4. Bio-Rex 70 chromatography of methylated component-1 MBP in the presence of guanidinium chloride

(*a*) The *methyl*-^{14}C-labelled component-1 preparation was applied to a column (1.0 cm × 6.6 cm) of Bio-Rex 70 equilibrated with buffer B containing 0.05 M-guanidinium chloride at 4 °C. Elution was carried out at a flow rate of 2 ml/h with a linear gradient of 0–1.0 M-NaCl (——; 75 ml each). fractions (1 ml each) were collected, and A_{276} (●) and radioactivity (○; 0.05 ml portion from each fraction) were measured. (*b*) Fractions between 30 and 50 in (*a*) were pooled, freeze-dried and desalted, and then chromatographed on a Bio-Rex 70 column under conditions identical with those above except that guanidinium chloride was omitted from all buffers.

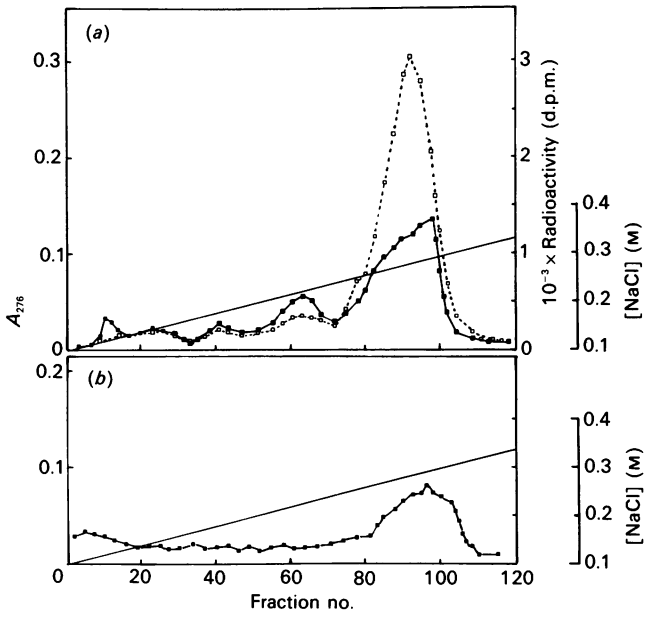

Fig. 5. Separation of methylated and unmethylated component-1 MBP by Bio-Rex 70 chromatography

The component-1 MBP (5.5 mg, a mixture of methylated and unmethylated) was applied to a Bio-Rex 70 column (0.7 cm × 42 cm) equilibrated with buffer B at 4 °C. Elution of the protein was effected by a linear gradient of 0.1–0.6 M-NaCl (——; 100 ml each) in buffer B with a flow rate of 2 ml/h. Fractions (1 ml each) were collected and A_{276} (■) was determined. Also a portion of each fraction was counted for radioactivity (□). (*a*) The *methyl*-^{14}C-labelled component-1 methylated *in vitro* (100000 d.p.m.); (*b*) the native form of component 1 which had not been labelled with *methyl*-^{14}C *in vitro*.

confirm this further, the chromatography was carried out on a thinner and longer column (0.7 cm × 42 cm) with a shallower salt gradient (0.1–0.6 M-NaCl). As shown in Fig. 5(*a*) the methylated (identified as radioactivity) and unmethylated component 1 (represented by A_{276}) have now been clearly separated (fractions 92 versus 99). It should be noted that, although it had not been methylated *in vitro*, the component 1 in Fig. 5(*b*) is a mixture of naturally methylated and unmethylated species. The nearly overlapping unmethylated peak fractions in Figs. 5(*a*) and 5(*b*) (no. 99 versus 98) further confirm the validity and reproducibility of the currently applied chromatography. In conclusion, the cationic property of the bovine MBP has been shown to be significantly diminished after methylation of Arg-107.

DISCUSSION

In the present paper, we describe the comparative methyl acceptability of MBP embedded in three different subfractions of myelin membranes: the MBP in the most compact multilayered myelin exhibited the highest methyl acceptability (Table 1), suggesting possible structural and/or topographical differences toward the methyl acceptabilities of MBP associated with different membrane subfractions. Burgisser & Matthieu (1989) have shown differential partition distribution properties of MBP from the unmyelinated oligodendrocyte and that from the myelin, implying that MBP could exist in different conformational states. Apparently, in the present studies, the conformation of MBP embedded in and/or extracted from the compact myelin was more amenable to enzymic methylation than that from less compact myelin (Table 1). Since MBP is partially methylated *in vivo* (Deibler & Martenson, 1973), it is expected that additional methylation can take place in the isolated MBP. Indeed, the newly methylated arginine was shown by analysis to be both MMeArg and DiMeArg, the latter being the major product (Fig. 4) which has been derived from its precursor MMeArg.

Recent studies from our laboratory indicated that protein substrate specificity of PMI resides not only at the amino acid side chain, but also at the level of the protein molecule (Ghosh *et al.*, 1988). In the present study, utilizing the intact myelin fractions as the substrate, we have for the first time unequivocally demonstrated MBP as the sole methyl acceptor of the myelin proteins from all subfractions (Fig. 2). Based on this high specificity of the enzyme, it is possible to use MBP-specific PMI as a marker to methylate and identify MBP from a mixed population of proteins.

The most cationic unmodified component-1 MBP (Chou *et al.*, 1976) was chosen to study possible charge alteration subsequent to its methylation. In the presence of denaturant (0.05 M-guanidinium chloride), the methylation by itself did not manifest any charge difference (Fig. 4*a*). However, upon the removal of denaturant, the renatured methylated MBP became less cationic relative to the unmodified (Fig. 4*b* and Fig. 5*a*). Martenson *et al.* (1981) have also observed a similar finding in which elution of rabbit MBP peptide from a CM-cellulose column was in the order DiMeArg, MMeArg and unmethylated Arg (cf. Fig. 4 of Martenson *et al.*, 1981). These results are quite contradictory to

a prediction based on the pI values of free methylated arginine derivatives (11.01 for DiMeArg versus 10.02 for Arg; Paik *et al.*, 1983). Interestingly, for yeast apocytochrome *c*, a decrease in pI value after trimethylation of Lys-72 has also been reported (Park *et al.*, 1988), even though the value for free trimethyl-Lys is 0.43 unit higher than that of Lys (Paik *et al.*, 1983). It is rather difficult to assess how Arg-107 methylation on MBP induced its conformational alteration to be less cationic. However, it is conceivable that, since an unusual Pro-Pro-Pro sequence located between residues 99 and 101 is very close to Arg-107, the bulky methyl group might have enhanced hydrophobicity of the region to lock the predicted double chain 'hair-pin' structure (Brostoff & Eylar, 1971) into a more stable form.

The mechanism by which MBP participates in the formation of multilayered myelin is not known at present; however, two lines of hypothesis have been proposed: (1) electrostatic interaction between the positively charged MBP and negatively charged group of the lipid and/or membrane proteins, and (2) hydrophobic interaction between these components. In respect to the first hypothesis, Cheifetz & Moscarello (1985) demonstrated that MBP charge isomers exhibited differential aggregation status which is correlated with multilayer myelin membrane structure. Thus component-1 MBP (the most cationic) is most efficient at producing vesicle aggregation (Moscarello *et al.*, 1986). In addition, Schulz *et al.* (1988) observed that the highly phosphorylated component was in greater amount in the less compact fraction than in the compact myelin fraction. The second hypothesis has emerged from several studies (Boggs *et al.*, 1981; Vadas *et al.*, 1981; Smith, 1982; Fraser & Deber, 1984) which showed that hydrophobic regions of MBP penetrate into the hydrophobic membrane interior. Edwards *et al.* (1989), using a ligand-blot overlay technique, have demonstrated that there was no difference in binding between phosphorylated (component 3) and non-phosphorylated (component 1) MBP to lipophilin. Nevertheless, it is possible that both mechanisms are not mutually exclusive. It is our long-standing interest to understand the effect of site-specific methylation of MBP in myelin formation. Certainly, an enzymic methylation of the critical regions of the MBP molecule should have a significant impact on the molecular organization, as clinical experiments clearly indicated the importance of a transmethylation reaction in normal nerve function (Russell *et al.*, 1900).

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