Biochem. J. (1992) 287, 929–935 (Printed in Great Britain)

Studies on N^G-methylarginine derivatives in myelin basic protein from developing and mutant mouse brain

Nenoo RAWAL, Yong-Ju LEE, Woon Ki PAIK and Sangduk KIM*
Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA 19140, U.S.A.

The amounts of N^G-methylarginine derivatives in myelin basic protein (MBP) purified from dysmyelinating mutant and different stages of normal myelinating mouse brains have been studied by using h.p.l.c. with a highly sensitive post-column *o*-phthaldialdehyde derivative-formation method. All three naturally occurring derivatives {N^G-monomethylarginine (MeArg), $N^GN'^G$-dimethylarginine [Me_2(sym)Arg] and N^GN^G-dimethylarginine [Me_2(asym)Arg]} were found in MBP; however, their relative concentrations varied significantly with the age of the animal. The amounts of MeArg and Me_2(sym)Arg in MBP increased as a function of the age of the brain, whereas that of Me_2(asym)Arg decreased. MBP from early-myelinating mouse brain was shown to contain a high proportion of Me_2(asym)Arg, which was hardly detectable in older brain MBP. This derivative, Me_2(asym)Arg, was also absent from MBP embedded in the most compact multilamellar myelin, but was present in MBP in the least compact myelin (P_3B). Comparing the extent of total methylation *in vivo* (sum of all three arginine derivatives), MBP extracted from less-compact myelin (P_3A and P_3B) showed a level approx. 40% higher than that from compact myelin. MBPs isolated from dysmyelinating mutant mouse brains, such as jimpy (jp/y) and quaking (qk/qk), contained a much higher level of Me_2(asym)Arg relative to the other two methyl derivatives and also in comparison with those levels in the mother brain MBP. SDS/PAGE analysis of MBPs extracted from the mutant (both jp/y and qk/qk) as well as young normal (6–13 days old) mouse brains indicated the presence of a high-molecular-mass isoform of MBP (about 32 kDa), but this isoform was not found in adult brains. These results therefore indicate that structural integrity of myelin membrane in which MBP is embedded appears to play a pivotal role in determining the extent and the kind of Me_2Arg formation in MBP at the post-translational level.

INTRODUCTION

Myelin basic protein (MBP) is one of the major myelin proteins, and constitutes 30–40% of the total myelin protein (reviewed by Campagnoni, 1988). This basic protein has been shown to exhibit both size and charge isomers, depending on the species and stages of myelination (Martenson & Gaitonde, 1969; Barbarese *et al.*, 1977; Agrawal *et al.*, 1986; Chanderkar *et al.*, 1986). Baldwin & Carnegie (1971*a*) and Brostoff & Eylar (1971) have shown that bovine MBP contains methylated arginine at position 107 as a mixture of monomethylarginine (MeArg) and $N^GN'^G$-dimethylarginine [Me_2(sym)Arg]. Soon thereafter, Deibler & Martenson (1973) reported that the amount of these methylarginines in MBPs varied depending on the species of brain. Subsequently the methylated arginines have been found in other naturally occurring proteins, such as nuclear/nucleolar proteins (Karn *et al.*, 1977; Lischwe *et al.*, 1982, 1985; Williams *et al.*, 1985), muscle proteins (Reporter & Corbin, 1971) and ribosomal proteins (Chang *et al.*, 1976). In these cases, however, the dimethylated arginine was shown to be the asymmetric isomer N^GN^G-dimethylarginine [Me_2(asym)Arg], but not the symmetric one. Since MeArg is the putative common precursor for both dimethyl derivatives, this preferential occurrence of a specific isomer in certain proteins has been an enigma to us.

The post-translational methylation of arginine residues in proteins is catalysed by a group of protein methylases I [PMI; *S*-adenosylmethionine (AdoMet):protein-arginine *N*-methyltransferase, EC 2.1.1.23), utilizing AdoMet as the methyl donor (Paik & Kim, 1980; Kim *et al.*, 1990). Recently, MBP- and histone-specific methylases have been highly purified from bovine brain; their molecular, catalytic and immunological properties were characterized (Ghosh *et al.*, 1988). When MBP (18.5 kDa) was used as the methyl-acceptor protein *in vitro*, the former enzyme yielded Me_2(sym)Arg and MeArg, but not Me_2(asym)Arg.

In view of the fact that methyl deficiency in animal induces neuropathy (Crang & Jacobson, 1982; Small *et al.*, 1981), a condition similar to subacute combined degeneration in humans (Russell *et al.*, 1900), several investigators have studied the regulation of protein methylase I during brain development/myelination (Miyake, 1975; Crang & Jacobson, 1982; Amur *et al.*, 1984; Chanderkar *et al.*, 1986; Rawal *et al.*, 1991) and found a temporal correlation between MBP-specific PMI activity and myelination (but not with histone-specific PMI). We have shown that MBP methylase activity was depressed in the brains from dysmyelinating mutant mice compared with that from the normal littermate (Kim *et al.*, 1984; Rawal *et al.*, 1991). However, the methylase product under these conditions has not been studied.

The myelin membrane normally exists in different degrees of compactness: the most compact myelin is the highly multilayered membrane and forms the bulk of myelin, whereas the less compact myelin (P_3A and P_3B fractions) consists of fewer lamellae (Cruz & Moscarello, 1985). The heterogeneity observed is due to the different regions of transition between the oligodendroglial plasma membrane and the multilamellar myelin (Matthieu *et al.*, 1973; Zimmerman *et al.*, 1975). It may also result either from the newly formed myelin of the internodal regions or from the degeneration of the sheath in diseased conditions (Whitaker &

Abbreviations used: MBP, myelin basic protein; MeArg, N^G-monomethylarginine; Me_2(sym)Arg, $N^GN'^G$-dimethylarginine; Me_2(asym)Arg, N^GN^G-dimethylarginine; MeLys, ϵ-*N*-monomethyl-lysine; Me_2Lys, ϵ-*N*-dimethyl-lysine; Me_3Lys, ϵ-*N*-trimethyl-lysine; AdoMet, *S*-adenosyl-L-methionine; OPA, *o*-phthaldialdehyde; PMI, protein methylase I.

* To whom correspondence should be addressed.

Snyder, 1985). Our recent studies *in vitro* carried out with isolated membranes of different compactness demonstrated a differential methyl acceptability of MBPs associated with these different subfractions (Ghosh *et al.*, 1991). To understand further the structure–function relationship of MBP methylation, we undertook a systematic study to measure *in vivo* the amount of methylarginines in MBP under the various conditions described above. Accordingly, we have developed a highly sensitive h.p.l.c. procedure with post-column treatment with *o*-phthaldialdehyde (OPA) which efficiently separates all three naturally occurring N^G-methylarginine derivatives, thus enabling us to measure a minute quantity of methylarginines in the basic protein. The results of these analyses have been presented in the paper.

EXPERIMENTAL

Materials

L-Arginine hydrochloride and L-lysine hydrochloride were purchased from Pierce, Rockford, IL, U.S.A. MeArg, Me_2-(sym)Arg and OPA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Me_2(asym)Arg, ϵ-*N*-dimethyl-lysine (Me_2Lys) and ϵ-*N*-trimethyl-lysine (Me_3Lys) were from Calbiochem (San Diego, CA, U.S.A.), and ϵ-*N*-monomethyl-lysine (MeLys) was from Nutritional Biochemical Corp. Other reagents used were from various commercial sources and of the highest grade available.

CD-1 Swiss mice, Fischer rats and rabbits were from Charles River Laboratories (Wilmington, MA, U.S.A.), and mutant mice, jimpy (jp/y and jp/+) and quaking (qk/qk and qk/+), were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). All animals were maintained in the Temple University animal care facility. Human brain was obtained from the Department of Neuropathology, Temple University Hospital, and was a 6 h *post-mortem* sample.

Purification of MBP

MBP was isolated from whole brain of different species by the procedure described previously (Deibler *et al.*, 1972; Chou *et al.*, 1977; Chanderkar *et al.*, 1986; Ghosh *et al.*, 1991). Briefly, the brain homogenate in methanol/chloroform was subjected to successive treatments of delipidation, acid extraction, DE-52 treatment, CM-52 chromatography and finally gel-filtration on Sephadex G-25 for desalting.

For the purification of MBP from myelin subfractions, first the various myelin membranes were prepared from freshly excised bovine brain by the method of Lowden *et al.* (1966). The membrane was then freeze-dried and stirred overnight in 0.2 M-H_2SO_4 at 4 °C to extract MBP. The acid extract containing MBP was dialysed against distilled water and centrifuged at 15000 *g* for 10 min.

H.p.l.c.

Amino acid analysis was performed on a h.p.l.c. instrument (Waters Associates) equipped with a model 721 programmable system controller, a model 730 data module, a model 510 pump and a Rheodyne injector model 7125, utilizing a strong cation Amino Acid Analysis Column (0.4 cm × 25 cm; Waters). Separation of basic methylated amino acids was performed by an isocratic elution with 0.3 M-sodium citrate (pH 3.8) at 60 °C and a flow rate of 0.5 ml/min during a period of 400 min. The amino acids eluted were converted into derivatives with OPA in a Beckman post-column reactor (model 231) at a flow rate of 0.5 ml/min. The OPA adducts were monitored with a fluorescence detector (model 420-AC; Waters) with excitation at 338 nm and emission at 424 nm, with a magnification factor of 64.

OPA solution was prepared as follows (Method 4; Waters Associates Manual No. 07124): OPA (210 mg) and 60 μl of 2-mercaptoethanol were first dissolved completely in 3 ml of methanol by vortex-mixing and added to 300 ml of the stock borate solution containing 0.3 ml of aq. 30% Brij 35. It should be noted that the OPA solution was prepared daily, filtered through a membrane (0.2 μM; Gelman Sciences) and degassed before use. The stock borate solution was prepared by mixing boric acid (123.6 g) and KOH (105 g) in 4 litres of double-distilled water, adjusted to pH 10.4 and stored in an amber glass bottle.

Preparation of MBP for amino acid analysis

The MBP was hydrolysed in 6 M-HCl *in vacuo* for 24 h at 110 °C. The hydrolysate was dried and washed at least three times with double-distilled water to remove HCl completely. It was then dissolved in 1.0 ml of distilled water and centrifuged in a Centrex disposable microfilter (Schleicher and Schuell) at 1500 *g* for 10 min. The filtrate was again passed through a Sep-Pak C_{18} cartridge, freeze-dried and suspended in 500 μl of double-distilled water. Routinely, two separate h.p.l.c. runs were performed for each sample: one for quantification of the methylated arginine derivatives, which requires overloading of the sample with respect to Arg (equivalent to 150 μg of MBP), and the other for arginine, with a limited amount (9 μg of MBP).

To determine the recovery of arginine derivatives, known amounts of authentic Me_2(asym)Arg, Me_2(sym)Arg, MeArg or Arg (100 nmol each) were added to BSA (150 μg). The mixture was then subjected to acid hydrolysis, followed by filtration through a Centrex microfilter and a Sep-Pak C_{18} cartridge as described above. A sample was subjected to h.p.l.c. to determine the recovery by the post-column OPA derivative-formation method. The percentage recovery of arginine derivatives was 90–98%.

PAGE

SDS/PAGE was performed by the method of Laemmli (1970). MBP purified by CM-52 ion-exchange chromatography as described above was subjected to electrophoresis on 15% acrylamide for the running gel and 3% for the stacking gel at a constant current of 35 mA for 5 h.

Alkali-urea-gel electrophoresis

This was carried out by the method of Deibler *et al.* (1972) on a slab gel rather than a tube gel, in a Hoefer Scientific Instruments apparatus. Briefly, the gel was prepared with 5% acrylamide, 8 M-urea and 0.01 M-sodium glycinate (pH 10.6 or 11.0), and electrophoresed in the glycinate buffer for 8 h at the specified current (20 or 30 mA). Before sample application, the gel was routinely pre-electrophoresed overnight at 5 mA

Western immunoblotting analysis

Purified MBP was first subjected to SDS/PAGE and then electrophoretically transferred to nitrocellulose paper by the method of Towbin *et al.* (1976) as described previously (Ghosh *et al.*, 1988). Rabbit anti-(bovine MBP) IgG was used as the primary antibody, whereas goat (anti-rabbit IgG) IgG was used as the secondary antibody. The protein bands were detected by the method of Glass *et al.* (1981).

Other analytical methods

Protein concentrations were determined with the Coomassie Blue reagent (Pierce), based on the Bradford (1976) method, with BSA as the standard. Concentrations of authentic amino acid solutions were estimated by the ninhydrin reaction (Moore &

Stein, 1948) with L-arginine or L-lysine as the reference amino acid.

RESULTS

Separation of methylated basic amino acids on h.p.l.c.

The elution pattern of the methylated lysine and arginine derivatives by the post-column OPA derivative-formation method on h.p.l.c., utilizing a strong cation exchanger, is shown in Fig. 1. Retention times for Me_2(asym)Arg, Me_2(sym)Arg, MeArg and Arg were 228 min, 256 min, 331 min and 365 min respectively, whereas those for Me_3Lys, Me_2Lys and Lys were 120 min, 153 min and 169 min respectively. Under the present conditions, MeLys co-eluted with Lys, although it was occasionally observed that MeLys had a tendency to precede Lys. As noted in Fig. 1, the differences in retention times of the methylated arginine derivatives were large enough to accommodate overloading of the sample on the column, thus enabling us to quantify a minute amount of MeArg together with a large amount of Arg present in most biological materials.

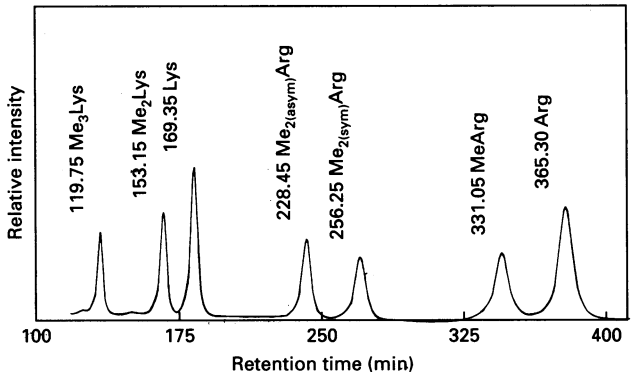

Fig. 1. Separation of standard basic amino acids by h.p.l.c. using a strong cation exchanger

Standard amino acids [Arg (3.5 nmol), MeArg (2.0 nmol), Me_2-(sym)Arg (1.8 nmol), Me_2(asym)Arg (2.5 nmol), Lys (5.0 nmol), Me_3Lys (2.4 nmol) and Me_3Lys (1.0 nmol)] were applied on an h.p.l.c. apparatus equipped with a strong cation-exchanger amino acid analysis column. The details of the experimental conditions are as described in the Materials and methods section.

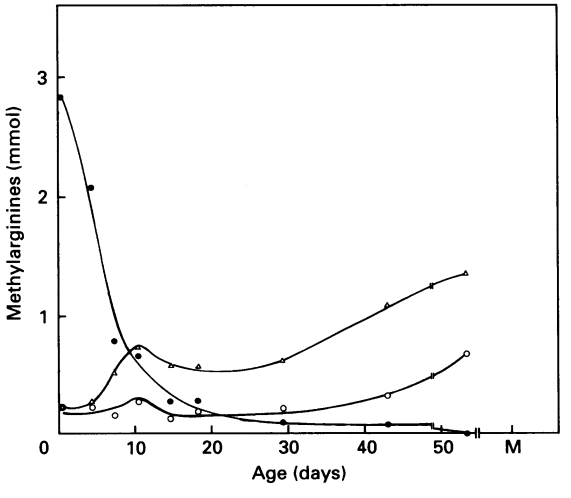

Fig. 2. Analysis of methylated arginine derivatives in MBP during brain development of normal mice

Data are taken from Table 1. Symbols: ●, Me_2(asym)Arg; ○, Me_2(sym)Arg; △, MeArg. Abbreviation: M, mother.

Table 1. Levels of N^G-methylarginine derivatives in MBP during brain development

MBP was extracted and purified from CD-1 Swiss-mouse brains of different ages, as described in the Materials and methods section. MBP hydrolysates (150 μg for methylarginine derivatives, and 9.0 μg for arginine) were applied to h.p.l.c. Data are expressed as nmol of amino acid per 150 μg of MBP: n.d., not determined.

Age (days)	Me_2(asym)Arg	Me_2(sym)Arg	MeArg	Arg
6	2.88	0.28	0.28	n.d.
10	2.24	0.28	0.32	39.0
13	0.84	0.20	0.56	37.2
17	0.72	0.32	0.78	56.3
20	0.32	0.18	0.62	32.6
24	0.33	0.24	0.73	35.8
35	0.15	0.26	0.76	32.6
49	0.12	0.37	1.14	53.1
Mother (6 months)	0.04	0.72	1.40	50.6

The standard curves for estimating concentrations of Arg and Lys derivatives were established by tracing, cutting and weighing the standard amino acid peak areas. This method was found to be highly accurate and could easily quantify the methylated derivatives in the concentration range of 0.5–5.0 nmol (results not shown). The percentage recovery of arginine and its derivatives by this method was 90–98 %.

Changes in the pattern of N^G-methylarginine derivatives in MBP during brain development

We have systematically studied the amounts of three methylarginine derivatives in MBP extracted from brains of CD-1 Swiss mice during development. As shown in Fig. 2 and Table 1, the level of Me_2(asym)Arg in MBP from young mice (6–10 days old) was about 10 times those of Me_2(sym)Arg and MeArg. This initial high concentration of Me_2(asym)Arg decreased precipitously as the brain matured (after 13 days of life), whereas the other two derivatives increased concomitantly during the same period (Fig. 2). The MBP from adult brain contained an almost negligible amount of Me_2(asym)Arg. Fig. 2 also indicates small but significant peaks of both MeArg and Me_2(sym)Arg at day 17. This time period corresponded to the highest MBP methylase activity in mouse brain (Chanderkar *et al.*, 1986; Rawal *et al.*, 1991); however, it is not obvious at present whether these methylarginine peaks are due to an increase in the enzyme activity.

Amounts of each N^G-methylarginine derivatives in MBPs from young and old brains of rabbit and rat were also compared (Table 2). In all cases, younger brain MBPs contained a markedly high amount of Me_2(asym)Arg, which was barely detectable in the older brains of the respective species. These results further indicate the predominant occurrence of Me_2(asym)Arg in MBP during the early phase of myelination. The total arginine content of MBP from whole brain of rodents increased with age (Tables 1 and 2) (see the Discussion section for a possible explanation of this increase).

Amounts of methylated arginines in MBP purified from different myelin fractions

It is known that normal brain contains myelin membranes of different degrees of compaction, where the most compact myelin exhibits the highest level of multilamellar structure (Cruz &

Table 2. N^G-Methylarginine derivatives in MBPs from brains of different species

Experimental conditions are same as for Table 1. Data are expressed as nmol of amino acid per 150 μg of MBP: ND, not detectable.

Species	Age	Me_2(asym)Arg	Me_2(sym)Arg	MeArg	Arg
Human	56 years	0.20	2.72	0.96	43.2
Bovine	4 months	0.09	1.89	1.20	59.6
Rabbit	12 days	0.84	0.56	0.45	44.2
	2 years	ND	2.40	0.98	39.7
Rat	8 days	0.96	0.10	0.08	16.2
	Mother (10 months)	ND	0.90	1.12	38.4
Mouse	10 days	2.24	0.28	0.32	39.0
	Mother (6 months)	0.04	0.72	1.40	50.6

Table 3. N^G-Methylarginine derivatives in MBP from myelin of different degrees of compaction

MBP was purified from different myelin subfractions as described in the Materials and methods section. MBP hydrolysate (110 μg) was analysed for methylated arginine derivatives on h.p.l.c. Fractionation of myelin and less compact myelin (P_3A and P_3B) was carried out by the method of Lowden *et al.* (1966). Data are expressed as nmol of amino acid per 110 μg of MBP: N.D., not detectable.

Source of MBP	Me_2(asym)Arg	Me_2(sym)Arg	MeArg	Me_2(asym)Arg + Me_2(sym)Arg + MeArg
Compact myelin	N.D.	0.93	0.79	1.72
P_3A	N.D.	1.57	0.76	2.33
P_3B	0.36	1.24	0.83	2.43

Moscarello, 1985). Our previous studies on the methylation *in vitro* of MBP extracted from the various myelin subfractions showed MBP from the compact myelin to exhibit the highest methyl-group acceptability compared with that from the P_3A and P_3B subfractions, suggesting that the level of methylation *in vivo* of MBP associated with the compact myelin is less than for the MBP from the less compact myelin fractions, P_3A and P_3B (Ghosh *et al.*, 1991). Therefore, in order to investigate whether the MBPs associated with the different fractions of myelin exhibit any differences in their levels of methylarginines *in vivo*, MBPs were extracted from the three different myelin membrane subfractions (myelin, P_3A, P_3B) and analysed for their contents of N^G-methylarginines. As seen in Table 3, Me_2(sym)Arg and MeArg were present in MBPs from all the different fractions (myelin, P_3A, P_3B), but Me_2(asym)Arg was present only in the MBP extracted from the least compact myelin (P_3B). Furthermore, the sum of all three methylarginines (column 5, Table 3) shows the lowest level in MBP extracted from the compact myelin, whereas higher levels were observed in that from the less compact myelin membranes (P_3A and P_3B). These results support our previous findings (Ghosh *et al.*, 1991) and further suggest that the structural integrity of MBP and/or its topographical position in the myelin membrane play an important role in the extent of methylation as well as the type. It is also noteworthy that the differences in the total amount of N^G-methylarginines are mainly reflected by the quantity and the kind of Me_2Arg, whereas the amount of MeArg remained almost constant in MBP.

Table 4. Levels of N^G-methylarginine derivatives in MBP from mutant mouse brain

Brains from jimpy (23-day) and quaking (28-day) mice were used for extraction of MBP as described in the Materials and methods section. Other experimental procedures are the same as for Table 1. Data are expressed as nmol of amino acid per 150 μg of MBP.

Mutant		Me_2(asym)Arg	Me_2(sym)Arg	MeArg	Arg
Jimpy	(jp/y)	2.06	0.28	0.18	33.9
	(jp/+)	0.89	0.40	0.90	38.7
	mother	0.28	1.72	2.76	59.5
Quaking	(qk/qk)	0.82	0.48	0.64	36.5
	(qk/+)	0.26	0.32	0.70	39.7

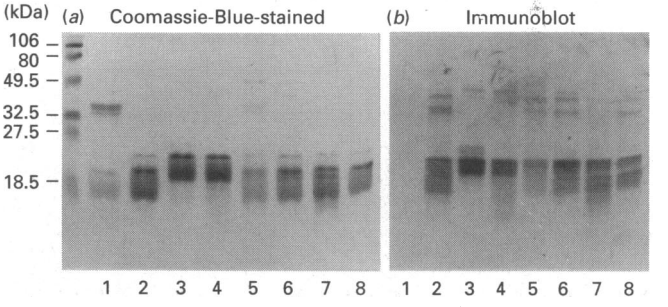

Fig. 3. SDS/PAGE of MBP from rat, rabbit and mouse brains at different ages

MBP (20 μg) purified from brain was analysed on SDS/PAGE, followed by immunoblotting against anti-bovine MBP. Details of the experimental conditions are described in the Materials and methods section. Lanes of Coomassie Blue-stained (*a*) and immunoblot (*b*) are shown: 1, 8-day-old rat; 2, mother rat (10 months); 3, 12-day-old rabbit; 4, 3-year-old rabbit; 5–8, 10-, 17- and 49-day-old and mother (6 months) CD-1 Swiss mouse respectively. Prestained protein standards (low range; Bio-Rad) were used: phosphorylase *b* (106 kDa), BSA (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa).

Analysis of methylarginine derivatives in MBP isolated from mutant mouse brains

We have previously reported that brains of hemizygous jimpy mutant mice contain a significantly depressed level of MBP PMI activity compared with the control heterozygous brains (Kim *et al.*, 1984; Rawal *et al.*, 1991). However, the level *in vivo* of the enzymic product in the mutant MBP has not been analysed. As seen in Table 4, analyses of the three methylarginine derivatives in jimpy mutant mouse brain MBP indicated the highest level of Me_2(asym)Arg in the hemizygous (jp/y) and the lowest in the mother, whereas the situation was completely reversed with MeArg. Interestingly, the heterozygous mouse (jp/+) showed an intermediate level of both compounds. MBP from quaking mutant mouse also showed a similar pattern of arginine derivatives (Table 4); the level of Me_2(asym)Arg was higher in the homozygous (qk/qk) than in the heterozygous (qk/+).

SDS/PAGE and immunoblot analyses of MBPs from different animal species

Analysis of MBP on SDS/PAGE followed by Coomassie Blue staining showed that, in addition to the major 18.5 kDa molecular form, a high-molecular-mass (about 32 kDa) isoform was present in the MBPs from young developing brains (Fig. 3*a*, lanes 1, 5 and 6); however, it decreased with age, as evidenced by the faint

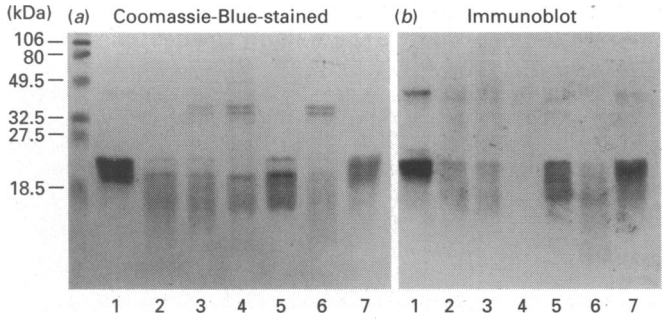

Fig. 4. SDS/PAGE of MBP from bovine, human and mutant mouse brains

All experimental conditions are the same as in Fig. 3. Lanes are: 1, 18-week-old bovine; 2, jimpy mother mouse; 3, 23-day-old heterozygous jimpy mouse (jp/+); 4, 23-day-old hemizygous jimpy mouse (jp/y); 5, 28-year-old heterozygous quaking mouse (qk/+); 6, 28-day-old homozygous quaking mouse (qk/qk); 7, 56-year-old man.

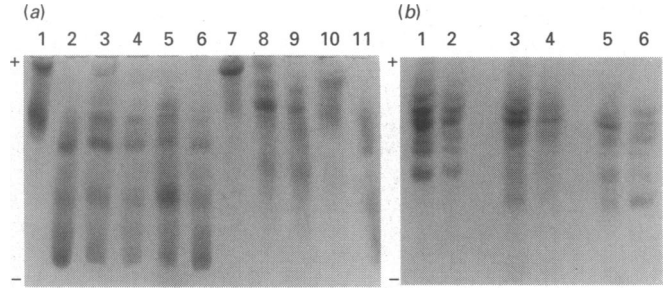

Fig. 5. Alkaline-urea-gel electrophoresis of MBP from brains of various species

The gel was pre-run overnight at 5 mA and then electrophoresed with MBP (20 μg unless otherwise specified) purified from brains as described in the text by CM-52 chromatography. (*a*) Electrophoresis was carried out at pH 10.6 for 8 h at 20 mA. Lanes indicate: 1, 8-day-old rat brain; 2, mother rat; 3–5, 17-, 24-, and 49-day-old CD-1 Swiss mouse respectively; 6, normal mother CD-1 mouse; 7, 23-day-old hemizygous jimpy mouse (jp/y); 8, 23-day-old jimpy (jp/+); 9, heterozygous jimpy mother; 10, 28-day-old homozygous quaking mouse (qk/qk); 11, 28-day-old heterozygous quaking littermate. (*b*) Electrophoresis was carried out at pH 11.0 for 8 h at 30 mA. Lanes indicate: 1 and 2, with 40 μg and 20 μg of MBP from 18-week-old bovine; 3 and 4, 40 μg and 20 μg from 56-year-old man; 5, 20 μg from 3-year-old rabbit; 6, 20 μg from 12-day-old rabbit.

or nearly undectable Coomassie Blue staining in some tracks. The high-molecular-mass was also detected in MBPs from the hemizygous jimpy (jp/y) and the homozygous quaking (qk/qk) mutant mouse (Fig. 4, lanes 4 and 6). However, the heterozygous jimpy MBP (from 21-day-old brain) showed a lesser amount of the 32 kDa species than did that from the hemizygous jimpy MBP of the same age (Fig. 4*a*, lane 4). It should be noted that the presence of the high-molecular-mass isoform of MBP has been previously reported only in young mouse brains (Barbarese *et al.*, 1977; Chanderkar *et al.*, 1986) and in jimpy mouse brain (Fannon & Moscarello, 1990).

Subsequently, immunoreactivity of these PAGE-separated various MBP species has been studied against rabbit anti-(bovine MBP) IgG. As seen in Fig. 3(*b*), most of the MBP species from normal brain showed positive immunoreactivity against both size isomers, except the 8-day-rat brain MBP, in which both species are negative (Fig. 3*b*, lane 1). It is also noted that, although detected by immuno-blot, the high-molecular-mass species were not uniformly shown by Coomassie Blue staining. This is most likely due to a high sensitivity of the immunoreactivity. MBP from mutant mice, in which myelin is loosely formed, similarly to that in young developing brain, showed negative immunoreactivity in the hemizygous jimpy mouse (Fig. 4*b*, lane 4), whereas a weak response was observed at the lower-molecular-mass region of quaking (qk/qk) mouse MBPs (Fig. 4*b*, lane 6). These findings are consistent with the previous report by Fannon & Moscarello (1990), who showed poor immunoreactivity of MBPs from jp/y and early myelinating normal mice on reaction with antibodies prepared against MBP (rat, chicken, bovine and monkey) as well as against the C8 isomer (McLaurin & Moscarello, 1990).

Separation of MBP charge isomers by alkaline-urea-gel electrophoresis

Because of poor immunoreactivity of MBPs from young and mutant rodent brains, alkaline-urea PAGE was carried out to verify further the nature of these MBP species. The alkaline-gel electrophoresis has been widely used to separate highly basic proteins such as MBP, based on its charge difference (Deibler *et al.*, 1972; Moscarello *et al.*, 1986; Fannon & Moscarello, 1990, 1991). Fig. 5(*a*) shows the profile of MBP charge isomers from whole brains of animals of different ages. Thus MBP from an 8-day-old rat (Fig. 5*a*, lane 1) exhibited two slow-moving less cationic species (corresponding to C6 and C7), whereas that from the mother rat (Fig. 5*a*, lane 2) showed several fast-moving species (C1–C5). Quite similar profiles are seen in MBPs from brains of mice of all ages (Fig. 5, lanes 3–6), except the presence of an additional faint slow-moving band from 17-day-old mouse MBP, which is quite prominent in the MBP from a 10-day-old mouse brain (results not shown). In contrast with the profiles of normal mice, MBPs from mutant mice (both jimpy and quaking) showed mostly slow-moving species similar to those seen in the 8-day-old rat. Hemizygous jimpy mouse brain MBP showed the highest amount of the least cationic species. These results therefore indicate that the less cationic MBP isomers from mutant and young brains appear to exhibit poor immunoreactivity on the blot. In fact, Fannon & Moscarello (1990) have also reported a poor immunoresponse of the less cationic C8 (in their nomenclature) charge isomer of MBP in the jimpy mutant and in a 6-day-old mouse, as well as for the C8 isomer from human. It is noted in the present study that an alkaline-urea gel of MBP charge isomers from human, bovine and rabbit (Fig. 5*b*) did not show as much differences between their isomers as was observed with mouse (Fig. 5*a*). These differences may be attributed to the differences inherent in the respective species of MBP, since Fannon & Moscarello (1991) have also reported that mouse MBP is more cationic than that of human.

DISCUSSION

Methylation of an arginine residue in MBP is one of the several post-translational side-chain modification reactions (Paik & Kim, 1980; Kim *et al.*, 1990). Several investigators observed that MeArg and Me_2(sym)Arg are the only methylated derivatives found on Arg-107 of human/bovine MBP (Baldwin & Carnegie, 1971*b*; Brostoff & Eylar, 1971; Deibler & Martenson, 1973). However, our studies described herein indicate that the other dimethyl isomer, namely Me_2(asym)Arg, is equally detectable in MBP, depending on the status of the myelin: a large amount of the asymmetric isomer is present in MBP from early-myelinating brain, whereas only a negligible amount if detected from fully developed brain (Fig. 2, Tables 1 and 2). It is therefore obvious that Me_2(asym)Arg is not expected to be found in MBPs from older/matured brain. In most of the previous studies, the age of the brain used to extract MBP had not been specified. One

exception is the report by Brostoff *et al.* (1972), who described the presence of Me_2(asym)Arg in bovine brain MBP at a ratio of 1:2 [Me_2(asym)Arg:Me_2(sym)Arg], although the age of the animal is not known. In their study, an indirect method was used to characterize the chemical nature of Me_2Arg, where the release of dimethylamine upon alkaline hydrolysis of the dimethylarginine-containing sample was taken as the evidence. In contrast, the method used here is a direct one, utilizing a strong cation-exchanger column on h.p.l.c. and detecting the amino acids by OPA derivative-formation. This method is highly sensitive, detecting small amounts of MeArg in the presence of large amounts of arginine, since the difference in retention times between MeArg and the next-eluted Arg is approx. 30 min (Fig. 1).

Fig. 2 and Table 1 show that a large amount of Me_2(asym)Arg is present mainly in MBP isolated from young brains at early stages of myelination. In mouse brain, the onset of myelination starts at approx. day 6 postnatal, reaching a peak at around day 17. In addition, the jp/y mutant mouse, whose myelin is severely defective and loosely formed, owing to a mutation of the X chromosome and is similar to that in early-myelinating brains, contained a significantly higher amount of Me_2(asym)Arg in comparison with the control littermates (jp/+). Similarly, another dysmyelinating mutant, quaking mice, showed higher amounts of Me_2(asym)Arg (Table 4). Furthermore, only MBPs from early-myelinating and dysmyelinating mutant brain contained the less cationic charge isomers evidenced by slow mobilities on the alkaline-urea gel (Fig. 5, lanes 1, 3, 7, 8 and 10). It is intriguing to speculate that the less cationic MBP associated with non-matured myelin membrane favours the formation of Me_2(asym)Arg. This contention is further supported by the fact that MBP only in the least compact myelin (P_3B) contained this asymmetric isomer (Table 3). Fannon & Moscarello (1991) have reported the molar percentage of arginine in the less cationic C8 isomer of MBP in jimpy mice to be decreased by 6% (owing to citrulline substitution) compared with that of the normal adult mouse MBP, which is essentially comprised of the C1–C5 charge isomers. In the present studies, we observed that the arginine content of MBP in rodent brains increased as a function of age (Tables 1 and 2), suggesting that this increase may be partly due to the difference in the type of MBP isomers associated with age, although it is not known whether all MBPs in young and early-myelinating brains are associated with myelin.

Biochemically, Me_2Arg is presumably derived from MeArg. However, what factor(s) determines the next level of methylation to yield preferably one of the two dimethyl isomers (symmetric or asymmetric) is not known. Two lines of possibilities are conceivable, either due to the enzyme specificity or due to the structure of the methyl-accepting substrate. Although the first possibility has not been ruled out, our present studies strongly suggest that the tertiary structure of methyl-accepting MBP appears to play a key role in determining the kind of dimethylarginine formation, in other words, whether the MBP is associated with early-myelinating membrane or matured compact myelin. Fannon & Moscarello (1990) have indeed reported a similarity between early-myelinating and jimpy mutant brain MBP; both show a 32 kDa size-isomer (one of the C8 size isomers, by their nomenclature) on SDS/PAGE. We have also observed the 32 kDa isomer to be present in early-myelinating and dysmyelinating mutant (Fig. 3*a* and 4*a*), but not in adult, brain MBP (Chanderkar *et al.*, 1986). In this regard, it should be pointed out that de Rosbo *et al.* (1991) recently proposed that the large-size MBP occurring during the early phase of myelination may provide a signal for maturation of myelin which contains predominantly smaller MBP size isomers (18.5 kDa). It is therefore of interest to isolate separately size isomers of MBP and analyse for the kind of methylated arginines in order to correlate with the size as well as the status of myelination.

This work was supported in part by Grant RG1765-B-2 from the National Multiple Sclerosis Society, CA 12227 from the National Cancer Institute and DK09602 from the National Institute of Diabetes, Digestive and Kidney Diseases. Y.-J. L. was the recipient of the Kil Chung-Hee Fellowship Fund.

REFERENCES

Agrawal, H. C., Agrawal, D. & Jenkins, R. P. (1986) Neurochem. Res. **11**, 375–382
Amur, S. G., Shanker, G. & Pieringer, R. A. (1984) J. Neurochem. **43**, 494–498
Baldwin, G. S. & Carnegie, P. R. (1971*a*) Science **171**, 579–581
Baldwin, G. S. & Carnegie, P. R. (1971*b*) Biochem. J. **123**, 69–74
Barbarese, E., Braun, P. E. & Carson, J. H. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 3360–3364
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Brostoff, S. & Eylar, E. H. (1971) Proc. Natl. Acad. Sci. U.S.A. **68**, 765–769
Brostoff, S. W., Rosegay, A. & Vandenheuvel, W. J. A. (1972) Arch. Biochem. Biophys. **148**, 156–160
Campagnoni, A. T. (1988) J. Neurochem. **51**, 1–14
Chanderkar, L. P., Paik, W. K. & Kim, S. (1986) Biochem. J. **240**, 471–479
Chang, F. N., Navickas, I. J., Chang, C. N. & Dancis, B. M. (1976) Arch. Biochem. Biophys. **172**, 627–633
Chou, C. H. J., Chou, F. C. H., Kowalski, T. J., Shapira, R. & Kibler, R. F. (1977) J. Neurochem. **28**, 115–119
Crang, A. J. & Jacobson, W. (1982) J. Neurochem. **39**, 244–247
Cruz, T. F. & Moscarello, M. A. (1985) J. Neurochem. **44**, 1411–1418
Deibler, G. E. & Martenson, R. E. (1973) J. Biol. Chem. **248**, 2387–2391
Deibler, G. E., Martenson, R. E. & Kies, M. W. (1972) Prep. Biochem. **2**, 139–165
de Rosbo, N. K., Tsang, S. & Bernard, C. C. A. (1991) Dev. Neurosci. **13**, 34–40
Fannon, A. M. & Moscarello, M. A. (1990) Biochem. J. **268**, 105–110
Fannon, A. M. & Moscarello, M A. (1991) NeuroReport **2**, 135–138
Glass, W. F., II, Briggs, R. C. & Hnilica, L. S. (1981) Science **211**, 70–72
Ghosh, S. K., Paik, W. K. & Kim, S. (1988) J. Biol. Chem. **263**, 19024–19033
Ghosh, S. K., Rawal, N., Syed, S. K., Paik, W. K. & Kim, S. (1991) Biochem. J. **275**, 381–387
Karn, J., Vidali, G., Boffa, L. C. & Allfrey, V. G. (1977) J. Biol. Chem. **252**, 7307–7322
Kim, S., Tuck, M., Kim, M., Campagnoni, A. T. & Paik, W. K. (1984) Biochem. Biophys. Res. Commun. **123**, 468–474
Kim, S., Chanderkar, L. P. & Ghosh, S. K. (1990) in Protein Methylation (Paik, W. K. & Kim, S., eds.), pp. 77–95, CRC Press, Boca Raton, FL
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lischwe, M. A., Roberts, K. D., Yeoman, L. C. & Busch, H. (1982) J. Biol. Chem. **257**, 14600–14602
Lischwe, M. A., Ochs, R. L., Reddy, R., Cook, R. G., Yeoman, L. C., Tan, E. M., Reichlin, M. & Busch, H. (1985) J. Biol. Chem. **260**, 14304–14310
Lowden, J. A., Moscarello, M. A. & Morecki, R. (1966) Can. J. Biochem. **44**, 567–577
Martenson, R. E. & Gaitonde, M K. (1969) J. Neurochem. **16**, 333–347
Matthieu, J. M., Quarles, R. H., Brady, R. O. & Webster, H. deF. (1973) Biochim. Biophys. Acta **329**, 305–317
McLaurin, J. & Moscarello, M. A. (1990) Anal. Biochem. **191**, 272–277
Miyake, M. (1975) J. Neurochem. **24**, 909–915
Moore, S. & Stein, W. H. (1948) J. Biol. Chem. **176**, 367–388
Moscarello, M. A., Brady, G. W., Fein, D. B., Wood, D. D. & Cruz, T. F. (1986) J. Neurosci. Res. **15**, 87–99
Paik, W. K. & Kim, S. (1980) in Protein Methylation (Meister, A., ed.), pp. 112–141, John Wiley and Sons, New York
Rawal, N., Paik, W. K. & Kim, S. (1991) J. Neurosci. Methods **37**, 133–140
Reporter, M. & Corbin, J. L. (1971) Biochem. Biophys. Res. Commun. **43**, 644–650
Russell, J. S. R., Batten, F. E. & Collier, J. (1900) Brain **23**, 39–110
Small, D. H., Carnegie, P. R. & Anderson, R. McD. (1981) Neurosci. Lett. **21**, 287–292

Towbin, H., Staehelin, T. & Gordon, J. (1976) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
Whitaker, J. N. & Snyder, S. D. (1985) CRC Crit. Rev. Clin. Neurobiol. **1**, 45–82
Williams, K. R., Stone, K. L., LoPresti, M. B., Merrill, B. M. & Planck, S. R. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 5666–5670
Zimmerman, A. W., Quarles, R. H., Webster, H. deF., Matthieu, J. M. & Brady, R. O. (1975) J. Neurochem. **25**, 749–757

Received 4 February 1992/6 May 1992; accepted 12 May 1992