서울特別市 保健環境研究院報 第24卷(1988)
Report of S.I.H.E., 24 : 59~65 (1988)

Myelin Basic Protein-Specific Protein Methylase I Activity in Shiverer Mutant Mouse Brain

Toxicopharmaceutical Division,
**Fels Research Institute, Temple University*
Myunghee Kim and Woon Ki Paik*

Shiverer Mutant Mouse뇌중의 MBP-Specific Protein Methylase I의 활성도

독성약품과, 템플대학교, Fels 연구소*
김 명 희 · 백 운 기*

=국문초록=

MBP-specific protein methylase I의 activity는 같은배로 태어난 정상적인 mouse에 비하여 homozygous shiverer mutant mouse에서 myelination 초기에는 현저하게 증가되었다. 그러나 myelination이 진행되는 시기동안에는 정상적인 mouse 뇌에서는 이 효소의 activity가 크게 증가하나 mutant mouse에서는 현저히 감소되었다. 이러한 결과는 다른 dysmyclinating mutant jimpy mouse에서 나타난 결과와는 정반대 현상이었다. 정상군과 shiverer mutant mouse군의 뇌의 무게와 단백질함량은 연령에 따라 차이가 없었으며, histone-specific protein methylase I의 activity 도 영향을 받지 않았다.

INTRODUCTION

The existence of myelin basic protein (MBP)-specific protein methylase I (S-adenosyl-L-methionine:protein-arginine N-methyltransferase) has now been well established.[1-4]

We recently observed that the activtty of this enzyme was significantly lower in the brains of hemizygous jimpy mutant mice (*jp*/Y) than in the brains of their normal littermates during the period of myelination.[5] Since the shiverer mutant is an autosomal mutation characterized by reduced synthesis of MBP[6] and severe hypomyelination in homozygous (*shi/shi*) animals[7-9] as in jimpy mutant mice, it was deemed worthwhile to investigate the activity of MBP-specific protein methylase I in these mutant mice brains; hopefully to find some common and/or different features between these two dysmyeli nating mutants. The activity of the MBP-specific protein methylase I is compared to that of the histone-specific protein methylase I, which should not be altered by the degree of myelination in the brain. The results of this investigation are presented below.

MATERIALS AND METHODS

Materials

Histone (calf thymus, type II-A) was purchased from Sigma Chemical Co. (St. Louis). Myelin basic protein of rabbit brain was obtained from Calbiochemical, Hoechst (La Jolla, CA). S-adenosyl-L-(*methyl*-^{14}C)methionine (specific activity, 59mCi/mmol) and S-adenosyl-L-[*methyl*-^{3}H] methionine (specific activity, 78Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Phenylmethyl sulfonyl-fluoride (PMSF), leupeptin, and pepstain were from Sigma Chemical Co. The remaining reagents were obtained from various commercial

sources and were of the highest grade available.

Wild-type mice (+/+) are the F3 of B6C3 hybrid breeder pairs. The shiverer mutation is maintained in the same B6C3 hybrid background. The shiverer mice (*shi/shi*) are the pups of homozygous breeder pairs. Mice colonies were maintained in the Department of Chemistry, University of Maryland and in the Center for Laboratory Animal Care, University of Connecticut Health Center, Farmington. At different ages, the animals were sacrificed, and the brains were removed and immediately frozen in liquid nitrogen. The brains were kept frozen until the enzyme assay was carried out.

Enzyme Assay

Detailed conditions for the homogenization and enzyme assay are described elsewhere.[5,10] Frozen brains (at −70°C) were individually homogenized in four volumes of ice-cold water using a 2-ml Broeck tissue grinder (ten strokes). A 0.5-ml aliquot of the whole brain homogenate was then taken for the enzyme assay. The remainder of the whole homogenate was centrifuged at 27,000g for 15min to obtain the "supernatant" fraction.

For the assay of protein methylase I activity, a total incubation mixture of 0.25ml, containing 0.05 ml of 0.5M phosphate buffer, pH 7.2, 0.05ml of protein substrate (histone type II-A of Sigma or MBP, 30mg/ml), 0.05ml of enzyme preparation, 0.05ml of S-adenosyl-L-[*methyl*-^{14}C] methionine (5nmol), and 0.05ml of water were incubated at 37°C for 10min.[5,10]

The reaction was stopped by the addition of 0.25 ml of 15% trichloroacetic acid (TCA), and the acid-soluble fraction, nucleic acids, and phospholipids were removed by successive treatment of the assays with hot TCA and ethanol. One-half milliliter of 0.5M phosphate buffer (pH 8.0) was then added to the above-treated sample, and the mixture was heated at 80°C for 5min. This procedure removes the methyl-esters produced by the action of contaminating protein methylase II (S-adenosyl-L-methionine:protein-carboxyl O-methyltransferase). The proteins were precipitated once more with TCA, the precipitate was transferred quantitatively into a scintillation vial, and the radioactivity determined. Assays were carried out in duplicate, subtracting the blank values. Enzyme preparations that had been boiled for 5min served as the blanks. The protein concentration was determined by the method of Lowry et al.[11] using bovine serum albumin as the standard.

Analysis of In Vitro Methylated Protein (s) on Sodium Dodesyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE)

In order to identify the in vitro enzymatically methylated protein(s) on SDS-PAGE, 3mg of MBP was incubated in a total volume of 0.5ml (with or without protease inhibitors) as described earlier, except that S-adenosyl-L-[*methyl*-^{3}H] methionine (25μCi) was used in place of S-adenosyl-L-[*methyl*-^{14}C]methionine. After incubation for 20min at 37°C, the mixture was added to 0.5ml of 0.5M HCl and centrifuged in a table-top clinical centrifuge for 20min. The supernatant was saved, and the precipitate was resuspended in 1ml of 0.01M HCl and centrifuged again. Both supernatants were combined (approximately 2ml) and were loaded on a Sephadex G-25 column (1.0×80cm) that had been equilibrated in 0.01M HCl in order to separate unreacted S-adenosyl-L- [*methyl*-^{3}H] methionine from the methylated proteins. Elution was carried out with 0.01M HCl at a flow rate of 45ml/hr. One and one-half milliliter fractions were collected, protein was measured by absorbance at 280nm, and a small aliquot was used to determine the radioactivity. Protein fractions with radioactivity (elution between 35 and 45ml volume) were pooled, lyophilized, and dissolved in 0.5ml of deionized water; unreacted S-adenosyl-L-[*methyl*-^{3}H]methionine was eluted at around 65ml volume. In order to remove protein-[methyl-^{3}H]-esters, the pH of the above preparation was adjusted to 9 with 0.1M NaOH, heated at 80°C for 5min, and then lyophilized again.

SDS-PAGE was carried out according to Laemmli[12] with 15% acrylamide in running gel and 3% in stacking gel at a constant current of 35mA/slab at 4°C. Gels were stained with Coomassie Brilliant blue R-250 and destained by diffusion. Gels were then scanned by Bio-Rad Model 1650 transmittance/

reflectance densitometer. The individual slabs were cut into 40 pieces (3mm thick), dissolved in 0.3ml of "Protosol" (duPont), and radioactivity was counted by a Prias liquid scintillation spectrometer.

RESULTS

Protein Methylase I Activity in Shiverer (*shi/shi*) Mutant Mouse Brain During Myelination

The activity of protein methylase I in the whole homogenate (Table I) and 27,000g supernatant (Table II) of homozygous shiverer (*shi/shi*) mutants and wild-type (+/+) mouse brain was determined during the period of most active myelination. The specific activity (picomoles of [*methyl*-^{14}C] transferred/min/mg enzyme protein) of the histone-specific enzyme protein methylase I is much higher than that of the MBP-specific enzyme in both +/+ and (*shi/shi*) brains at all the time periods.

In normal mouse brain, the MBP-specific protein methylase I activity increases during the period of myelination (15~18 days of life)[6] and falls off thereafter, whereas the histone-specific protein methylase I activity declines during this period.

Table I. Protein Methylase I Activity in Shiverer Mutant and Normal Mouse Whole Brain Homogenates

Age (days)	Substrate proteins used	Specific activity[a]	
		Normal[b]	shi/shi
12	Histone	1.52±0.30[c]	1.82±0.02
	MBP	0.17±0.04	0.34±0.03
15	Histone	1.33±0.17	1.61±0.08
	MBP	0.20±0.07	0.38±0.04
18	Histone	1.16±0.07	1.25±0.07
	MBP	0.26±0.03	0.30±0.03
21	Histone	0.93±0.11	1.05±0.04
	MBP	0.16±0.04	0.21±0.03

[a] Expressed as pmol of (*methyl*-^{14}C) transferred/min/mg enzyme protein±SD.
[b] B6C3 strain normal littermates and shiverer mutant (*shi/shi*).
[c] Values are the average of six brains assayed independently.

Table II. Protein Methylase I Activity in Shiverer Mutant and Normal Mouse Brain Supernatant

Age (days)	Substrate proteins used	Specific activity[a]	
		Normal	shi/shi
12	Histone	2.51±0.21[b]	3.32±0.30
	MBP	0.32±0.08	0.72±0.06
15	Histone	1.96±0.05	2.53±0.35
	MBP	0.60±0.04	0.62±0.17
18	Histone	2.12±0.40	2.12±0.50
	MBP	0.51±0.09	0.37±0.06
21	Histone	1.92±0.15	1.76±0.17
	MBP	0.40±0.06	0.34±0.12

[a] pmol of S-adenosyl (*methyl*-^{14}C) methionine used/min/enzyme protein±SD.
[b] Valves are the average of six brains assayed independently.

However, in the shiverer mutant supernatants, the activity of the MBP-specific enzyme is more than two-fold higher than that of the normal animal at

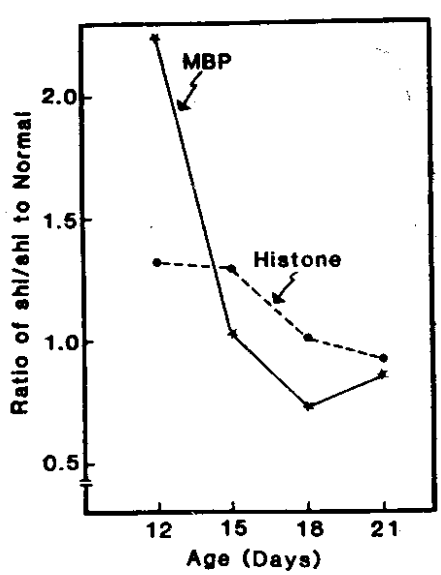

Fig. 1. Changes in MBP- and histone-specific protein methylase I activity in the shiverer (*shi/shi*) mutant and normal mouse brain during myelination. The ratio of protein methylase I activity between the shiverer (*shi/shi*) and normal mice of the same age was calculated from the results shown in Table II.

Table III. Protein Concentration in Normal and Shiverer Mouse Brains

Age(days)	Normal brain[a]			Shiverer brain[a]		
	Wet weight (g)	Protein (mg)	Protein/weight (mg/g)	Wet weight (g)	Protein (mg)	Protein/weight (mg/g)
12	0.145	21.6	149	0.120	21.6	180
15	0.231	23.8	103	0.195	26.0	133
18	0.231	31.3	136	0.204	29.3	144
21	0.198	29.0	147	0.210	27.9	133

[a] Values are average of two brains.

12 days (onset of myelination) and then decreases during the myelination period. In contrast, the histone-specific protein methylase I in the shiverer mutant brains shows the same pattern as that of the normal brains during the myelination period. Thus, when the ratio of MBP-specific protein methylase I activity in the shiverer mutant to that in the normal brain is plotted versus age (Fig. 1), the ratio drastically drops during the myelination period. On the other hand, the histone-specic protein methylase I shows only a slight decline in this ratio.

Protein Concentration in Normal and Shiverer Mouse Brains

Since homozygous shiverer mutant (*shi/shi*) has a hypomyelination and protein methylase I activity is expressed presently on the basis of enzyme-homogenate protein, there is a possibility that changing patterns of MBP-specific protein methylase I activity during myelination period might have been a mere reflection of changes in the protein concentration in the mutant brains. As shown in Table III, the total protein concentration per brain increases gradually as the animals grow older. This is quite expected since the weight (wet) of brains also increases slowly during this period. When comparing the protein concentration per gram of brain (4th and 7th columns), there is no significant difference between thc normal and shiverer brains of the same ages.

Analysis of Protein(s) Methylated In Vitro by Protein Methylase I

In order to ascertain that MBP and not the other component(s) in the enzyme-homogenate preparation was [*methyl*-^{3}H]-labeled, total methylated proteins

Fig. 2. SDS-PAGE analysis of proteins methylated in vitro by MBP-specific protein methylase I. The [*methyl*-^{3}H]-labeled proteins were prepared in a total 0.5ml volume with 3mg of substrate MBP and 0.1ml of 27,000g supernatant (0.24mg protein) prepared from 15-day-old normal mouse brain. Detailed experimental conditions are described in "Methods." Lane A, rabbit MBP (10μg); lane B, [*methyl*-^{3}H]-protein prepared in a standard assay condition without inhibitor (45μg); lane C, [*methyl*-^{3}H]-protein prepared in the presence of 1.0mM PMSF (45μg protein); lane D, [*methyl*-^{3}H]-protein prepared in the presence of 5μg leupeptin/lm (45μg protein); lane E, [*methyl*-^{3}H]-protein prepared in the presence of 5μg pepstatin/ml (45μg protein). The molecular markers used were bovine serum albumin (66,000 daltons), ovalbumin (54,000 daltons), glyceraldehyde 3-phosphate dehydrogenase (36,000 daltons), carbonic anhydrase (29,000 daltons), trypsinogen (24,000 daltons), soybean trypsin inhibitor (20,100 daltons), and α-lactalbumin (14,200 daltons).

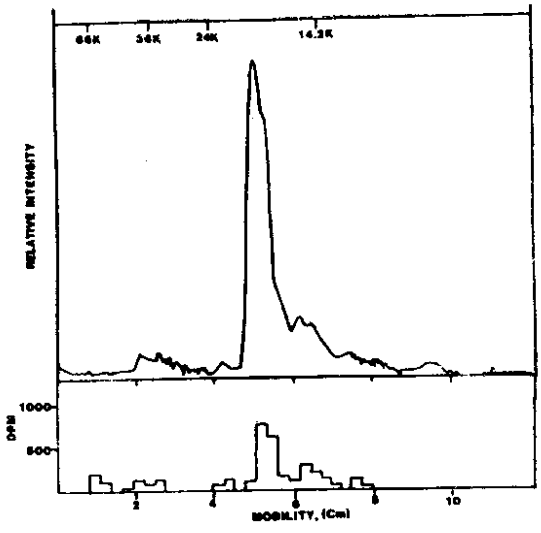

Fig. 3. Densitometric scan and measurement of radioactivity incorporated into lane B, Figure 2. Detailed experimental procedures are described in the text.

(prepared in the presence or absence of the protease inhibitors; see under "Methods") were analyzed on SDS-PAGE (Figs. 2, 3). First of all, it is evident in Figure 2 that the major protein in the control incubation mixture (lane B) migrated at the region of approximately 18kD, which is similar to the standard MBP (indicated in lane A). The inclusion of various protease inhibitors such as PMSF (lane C), leupeptin (lane D) and pepstatin(lane E) during the incubation showed similar electrophoretic pattern. Broadness of the 18-kD region in lanes B-E is due to the large amount of MBP used for electrophoresis; this was necessary for the radioactivity determination, illustrated in Figure 3. Regardless of the excess amount of MBP applied, no small molecular weight degradation products were produced during the enzymatic methylation in vitro.

In order to quantitate the radioactivity incorporation into MBP, individual slabs shown in Figure 2 were sliced into 3-mm-thick sections and counted for radioactivity (Fig. 3). As shown in the lower panel of Figure 3, over 80% of the radioactivity comigrated with MBP. Two minor radioactivity peaks at the larger M_r region are the uncharacterized proteins present endodenously in the cytosol fraction. These results, therefore, indicate that the presently employed assay condition for MBP-specific protein methylase I measures the majority of radioactivity incorporated into exogenously added MBP, and a possible proteolytic degradation of MBP during the incubation appears to be negligible.

DISCUSSION

What role, if any, the enzymatic methylation of MBP-arginine plays in the formation of myelin remains to be determined. However, strong indications that protein methylation plays a critical role in maintaining the integrity and maintenance of myelin come from a series of recent observations using animals with subacute combined degeneration (SCD). The disease can be induced experimentally in mice by exposing them to an atmospheric environment containing nitrous oxide,[12] however, when their diet is supplemented with methionine, they are free of any detectable clinical or microscopical changes in the spinal cord.[14] Furthermore, administration of cycloleucine to mice was shown to produce neurological changes that were histologically distinguinsihable from SCD.[13,15]

Cycloleucine is an analogue of methionine that inhibits the biosynthesis of S-adenosyl-L-methionine, one of the most important biological methyl donor.

An additional suggestion that the impairment of MBP-arginine methylation might be responsible for neurological damage caused by cycloleucine comes from the observations of Small et al.[16] In these experiments, cycloleucine was shown to strongly depress the formation of N^G-methylarginines in vivo. Furthermore, adequate doses of cycloleucine during the treatment of tumors in man resulted in signs and symptoms of demyelination in these patients.[17]

With the above considerations, earlier investigation of MBP-specific protein methylase I activity in brain of the dysmyelinating mouse mutant hemizygous jimpy (*jp*/Y) demonstrated that the enzyme activity in mutant brain was significantly lower in comparison with their normal littermate brain.[5]

Thus, reduction of the MBP-specific protein methylase I activity is common in both shiverer (*shi/shi*) and jimpy (*jp*/Y) mutant mice, which are characterized by hypomyelination in the central nervous system.[7]

However, there is a significant difference in the changing patterns of both MBP- and histone-specific protein methylase I activity in the brains of these two dysmyelinating mutants.[5] First, at the onset of myelination (12 days of age) both MBP- and histone-specific protein methylase I activity in the shiverer (*shi/shi*) brain is significantly higher than in brain from their normal littermates. This observation is highly reproducible, but the reason is not presently known. Secondly, the MBP-specific rpotein methylase I activity significantly decreased during the myelination period in the shiverer (*shi/shi*) brain, while the enzyme activity remained unchanged or failed to increase in jimpy (*jp*/Y) brain; in normal littermates, the enzyme activity increased in brain during myelination (15~18 days of age).[5]

Thus, although the MBP-specific enzyme activity is decreased in both mature shiverer (*shi/shi*) (Fig. 1) and jimpy (*jp*/Y) brains, the mechanism for the decrease appears to be quite different in each mutant.

The decrease of MBP-specific protein methylase I activity in shiverer mutant brain appears to be a highly specific phenomenon, not necessarily associated with hypomyelination, not owing to the change of total protein concentration (Table III), nor to the proteases presumably present in brain enzyme preparation (Fig. 2). Although this enzyme activity decreased during the myelination period in shiverer mutant brain, histone-specific protein methylase I activity did not differ from the normal littermate brain (Table II). Additionally, other marker enzymes such as 2′,3′-cyclic nucleotide 3′-phosphohydrolase and 5′-nucleotidase are shown to be normal in shiverer (*shi/shi*) brains.[18]

Before concluding this paper, some explanation for determining protein methylase I activity in both whole homogenate (Table I) and high speed supernatant (Table II) is in order. Protein methylase I is a cytosolic enzyme.

Therefore, when the enzyme activity is examined in the cytosolic fraction, much higher specific activity can be observed owing to removal of nonenzymic proteins. This is evident from Tables I and II, thus offering an advantage in magnifying the possible difference between the wild-type and shiverer mutant brain. In addition, the uniformity observed in the ratio of protein methylase I activities in the whole homogenate and in the supernatant throughout the myelination period strongly indicates that there is no endogenous inhibitor or stimulator involved that changes during this period of myelination.

The biochemical significance of the observed reduction of MBP-specific protein methylase I activity during myelination and the apparent difference in the mechanism of reduction are not clear at present. However, the observed reduction of MBP-specific protein methylase I during the myelination period may reflect the existence of an important role during myelination.

REFERENCES

1. Lee, H.W., Kim, S., Paik, W.K.: S-Adenosylmethionine:protein-arginine methyltransferase. Purification and mechanism of the enzyme. Biochemistry 16:78 (1977).
2. Miyake, M.: Methylases of myelin basic protein and histone in rat brain. J. Neurochem. 24:909 (1975).
3. Gupta, A., Jensen, D., Kim, S., Paik, W.K.: Histone-specific proteinarginine methyltransferase from wheat germ. J. Biol. Chem. 257:9677 (1982).
4. Crang, A.J., Jacobson, W.: The relationship of myelin basic protein(arginine) methyltransferase to myelination in mouse spinal cord. J. Neurochem. 39:244 (1982).
5. Kim, S., Tuck, M., Kim, M.-H., Campagnoni, A.T., Paik, W.K.: Studies on myelin basic protein-specific protein methylase I in various dysmyelinating mutant mice. Biochem. Biophys. Res. Commun. 17:468 (1984).
6. Campagnoni, A.T., Campagnoni, C.W., Bourre,

J.-M., Jacque, C., Baumann, N.: Cell-free synthesis of myelin basic proteins in norma and dysmyelinating mutant mice. J. Neurochem. 42: 733 (1984).
7. Biddle, R., March, E., Miller, J.R.: Mouse New Lett. 48:24 (1973).
8. Chernoff, G., March, E., Miller, J.R.: Provisional gene symbol. Mouse New Lett. 51:12 (1974).
9. Kirschner, D.A., Ganser, A.L.: Compact myelin exists in the absence of basic protein in the shiverer mutant mouse. Nature 283:207 (1980).
10. Durban, E., Lee, H.W., Kim, S., Paik, W.K.: Purification and characterization of protein methylase I (S-Adenosylmethionine: Protein-arginine methyltransferase; EC 2.1.1.23) from calf thymus. In Stein, G., Stein, J., Kleinsmith, L.J. (eds.): "Methods in Cell Biology," Vol. XIX. New York: Academic Press, p.59 (1978).
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265 (1951).
12. Laemmli, U.K.: Cleavage of structural proteins during the assembly of the new bacteriophage T4. Nature 227:680 (1970).
13. Jacobson, W., Gandy, G., abd Sidman, R.L.: Experimental subacute combined degeneration of the cord in mice. J. Pathol. 109:13 (1973).
14. Scott, J.M., Dinn, J.J., Wilson, P., Weir, D.G.: Pathogenesis of subacute combined degeneration: A result of methyl group deficiency. Lancet. 2:334 (1981).
15. Gandy, G., Jacobson, W., Sidman, R.: Inhibition of transmethylation reaction in the central nervous system—an experimental model for subacute combined degeneration of the cord. J. Physiol. (Lond) 233:1 (1973).
16. Small, D.H., Carnegie, P.R., Anderson, R.Mc. D.: Cycloleucine-induced vacuolation of myelin is associated with inhibition of protein methylation. Neurosci. Lett. 21:287 (1981).
17. Aust, J.B., Roux, K.: Phase I study of 1-aminocyclopentnecarboxylic acid (NSC-1026) in cancer patients. Cancer Chemother Rep. 49:93 (1965).
18. Hogan, E.L., Greenfield, S.: Animal models of genetic disorders of myelin. In Morrell, P. (ed.): "Myelin." New York: Plenum Press, p.489 (1984).