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Enzymatic Carboxyl-Methylesterification of Myelin Basic Protein

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Protein Methylase II 에 의한 Myelin 염기성단백질의 Carboxyl-methyl esterification.

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=국문초록=

소뇌의 myelin 염기성 단백질(MBP 또는 AI 염기성단백질)이 in vitro에서 S-adenosylmethionine : protein carboxyl-O-methyltransferase(protein methylase II; EC 2.1.1.24)에 대하여 우수한 기질이었으며 K_m 값은 4.0×10^{-5}M이었다. Protein methylase II에 의해 methyl화된 MBP-[*methyl*-^{14}C]를 pepsin으로 소화시켜 peptide mapping 한 결과 진한 방사성 반점 1개와 다른 하나의 흐린 반점이 나타났다. 이 실험결과 protein methylase II가 MBP에 대하여 비교적 강한 부위특이성을 나타내고 있음을 알수 있었다. 이효소는 MBP를 최대로 4.9 mole % methyl화 시킬수 있었으며 Jimpy mutant mice의 뇌에 있는 protein methylase II의 양은 protein methylase I과는 달리 정상적인 다른 형제들이 갖고있는 효소량과는 차가 없었다.

INTRODUCTION

Myelin basic protein (MBP) constitutes approximately 30% of the total protein found in the myelin sheath, and occurs in at least four forms with different molecular weights, ranging between 14,000 and 21,500[1]. The function of MBP is generally considered to be involved in the structural capacity of myelin[2].

MBP has been shown to be modified posttranslationally by two types of modification reactions; phosphorylation of serine or threonine residues[3,4,5] and N-methylation of arginine residue-107[6,7,8]. Since my research interests during the past two decades have been primarily concerned with the elucidation of the biochemical function of protein methylation in general, it was felt worthwhile to study MBP-carboxyl O-methylation and its functional significance. This is the topic of this communication.

MATERIALS AND METHODS

Materials

S-Adenosyl-L-[*methyl*-^{14}C]methionine (specific activity, 59mCi/mmol) was obtained from Amersham, Arlington Heights, IL. Myelin basic protein from bovine brain was purified according to the method published[9] or purchased from Calbiochem-Behring, La Jolla, CA. The colonies of all of the mice strains (C57Bl) used in this experiment were maintained in the Department of Chemistry, University of Maryland, College Park, MD., and the brains were immediately frozen after sacrifice of the mice. The brains in their frozen state were transported to Philadelphia where they were homogenized and served for enzyme assays. Pepsin, ACTH and γ-globulin were obtained from Sigma Chemical Co., St. Louis, MO. The remaining reagents were purchased from various commercial sources and were of the highest grade available.

Protein methylase II [S-adenosylmethionine : protein-carboxyl O-methyltransferase; EC 2.1.1.43] was purified by the method previously published[10], using S-adenosylhomocysteine affinity chromatography. This purified preparation was found to be devoid of contaminating protein methylase I [EC 2.1.1.23] or protein methylase III [EC 2.1.1.43] activity.

Protein methylase II assay with mice brains

Frozen mice brains (at −70°C) were individually homogenized in 4 volumes of ice-cold water[11], and the homogenates centrifuged at 27,000×*g* for 15 minutes. The supernatants were then decanted and assayed for protein methylase II activity, using bovine MBP or calf thymus histone type II-A (Sigma Chemical Co.; a mixture of various histone subfractions) as the protein substrate. Protein methylase II activity was assayed by the method of Kim and Paik (1978)[12]. Protein concentration was determined by the method of Lowry[23] et al. (1951), using bovine serum albumin as the standard.

Peptide mapping analysis of [*methyl*-^{14}C]-MBP

For analysis of pepsin-digested [*methyl*-^{14}C]-MBP, rabbit brain MBP (Calbiochem-Behring) was first methylated by protein methylase II with S-adenosyl-L-[*methyl*-^{14}C]methionine as the methyl donor according to the method described previously[14]. Briefly stated, 5.0mg of rabbit brain MBP was incubated with 660nmol of EDTA,

200nmol of S-adenosyl-L-[*methyl*-^{14}C]methionine, 600 units of purified bovine brain protein methylase II (approximately 0.12mg) and citrate-phosphate buffer (pH 6.0) in a total volume of 1.0ml at 37°C for 1 hour. The reaction was terminated by adding 0.010 ml of glacial acetic acid, and immediately applying the mixtures onto a column of Sephadex G-75 (0.8 cm×120cm), which had previously been equilibrated with 0.1M acetic acid. The column was eluted with 0.1M acetic acid, and the fractions containing [*methyl*-^{14}C]-MBP were pooled and lyophilized.

One-tenth of the above [*methyl*-^{14}C]-MBP was subsequently digested by pepsin [EC 3.4.23.1] in 0.2ml of 0.1M HCl at 37°C for 1 hour. The pepsin to MBP ratio was 1:10[14]. The digest was lyophilized, dissolved in 0.010ml of water, and the sample (approximately 28,000 cpm) was applied on Whatman 3MM paper (0.33mm thickness) for peptide mapping analysis. Paper electrophoresis was used for the first dimension and carried out in collidine/acetic acid buffer, pH 6.0, at 350 volts for 6 hours[14], subsequently followed by chromatography in *n*-butanol/acetic acid/pyridine/water (61:19:94.5:75.5) for 8 hours. The dried map was autoradiographed using X-Omat AR film for 13 days at −70°C. The film was then developed to locate the radioactive peptide(s) and the dried paper was sprayed with 0.1% ninhydrin in ethanol for visualization of the peptides.

RESULTS

Efficiency of various proteins as *in vitro* substrates for protein methylase II

Fig. 1 illustrates the substrate-efficiency of various proteins for protein methylase II which has been highly purified from bovine brain. Comparing the initial enzyme activity at low substrate-protein concentrations, ACTH appears to be the best substrate, followed by MBP and calmodulin (CaM). It is, however, of interest to note that independent of the protein species employed the maximum rate of enzyme activity was achieved at higher substrate concentrations. The K_m values were calculated from the results shown in Fig. 1 by the Lineweaver-Burk reciprocal plot, and are listed in Table I.

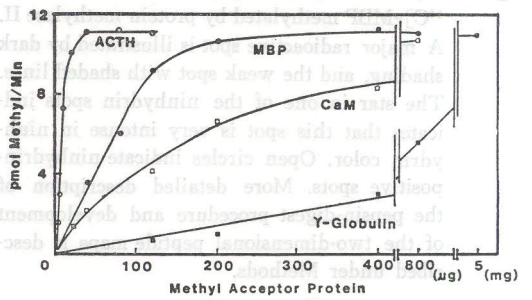

Fig. 1. *In vitro* methyl-acceptability of various proteins for protein methylase II.
ACTH, MBP and CaM represent adrenocorticotropic hormone, myelin basic protein (bovine) and calmodulin, respectively. One μg of purified bovine brain protein methylase II was used in a total volume of 0.125ml. Detailed description of the assay conditions of protein methylase II is described under methods.

Table I. K_m Values and Some of the Properties of Proteins used in Fig. 1.

	Molecular weight	No. of basic amino acid residues*	No. of acidic amino acid residues	Isoelectric point(pI)	K_m** ($\times 10^{-5}$M)
ACTH	4,500	8	5	6.6	1.2
MBP	18,300	40	10	>12	4.0
γ-Globulin***	160,000	159	234	6.6	5.7
Calmodulin	16,800	14	50	3.9~4.3	10.0

* Basic amino acids include lysine, arginine and histidine, and acidic amino acids include glutamic and aspartic acid.

** As shown in Fig. 1, V_{max} values for various proteins of protein methylase II were identical; 20μmoles of methyl transferred/min/mg enzyme protein.

*** Bovine serum γ-globulin was used for determination of both K_m and V_{max} values. However, due to the lack of reported values on this protein, the amino acid composition described herein comes from the value of human γ-globulin.

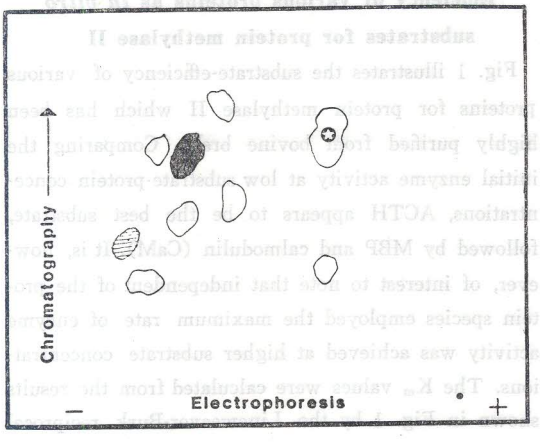

Fig. 2. Peptide mapping of pepsin-digested [*methyl*-^{14}C]-MBP methylated by protein methylase II. A major radioactive spot is illustrated by dark shading, and the weak spot with shaded lines. The star in one of the ninhydrin spots indicates that this spot is very intense in ninhydrin color. Open circles indicate ninhydrin-positive spots. More detailed description of the pepsin-digest procedure and development of the two-dimensional peptide maps is described under Methods.

An autofluorographic analysis of pepsin-digested [*methyl*-^{14}C]-MBP

Carboxyl methyl esters of proteins formed enzymatically have been shown to be highly alkaline-labile[15]. Therefore, both the digestion of [*methyl*-^{14}C]-MBP by pepsin and subsequent separation of the peptides by two-dimensional mapping were carried out in acidic buffer media. As shown in Fig. 2, one major and one minor radioactive spots are detectable on autofluorography. This result strongly indicates that bovine brain protein methylase II is quite site-specific towards MBP.

Assuming that a single major site of the MBP molecule is methyl-esterified by protein methylase II, prolonged incubation of MBP with protein methylase II and S-adenosyl-L-[*methyl*-^{14}C]methionine indicated that the maximum amount of [*methyl*-^{14}C] incorporated into MBP was 4.9% (data not shown).

Protein methylase II activity in jimpy mutant mice brains

Jimpy mice are one of the dysmyelinating mutants in which the synthesis of MBP and its incorporation into myelin are severely reduced. Recently, we have demonstrated that MBP-specific protein methylase I which specifically methylates residue-107 arginine-guanidino group of MBP was drastically reduced in jimpy mice brains[11]. Thus, it was felt worthwhile to examine whether or not protein methylase II activity in these mutant mice brains was also affected. As shown in Table II, the protein methylase II activity in jimpy mice remained unchanged from their normal littermates.

DISCUSSION

In this communication, we demonstrated that bovine brain MBP serves as an excellent substrate for highly purified bovine brain protein methylase II.

Table II. Protein Methylase II Activity in Jimpy Mutant Mice Brains

Age (Days)	Enzyme activity (pmoles methyl/min/mg enzyme protein)		
	Normal*	jp/y	jp/+
12	8.97±0.30**	8.52±0.61	7.73±0.01
15	9.58±0.86	9.68±0.57	8.96±0.82
18	10.18±0.97	10.99±0.65	9.14±0.46
21	9.20±0.28	8.88±0.83	8.63±0.46

* C57Bl strain mice was used. The normal, jp/y and jp/+ represents normal littermates, jimpy homozygous and hemizygous (phenotypically normal), respectively. The gene that causes jimpy mice is sex-linked and recessive.

** At least 3 mice brains were individually determined. For the assay in this series of experiments, histone type II-A (Sigma Chemical Co.) was used as a substrate. However, bovine brain MBP as a substrate had the same enzyme activity.

A K_m value of 4.0×10^{-5}M for bovine brain MBP is among the lowest values reported for a protein-substrate of this enzyme; porcine follicle-stimulating hormone has one of 0.77×10^{-5}M, ACTH 1.2×10^{-5}M, and neurophysin 1.55×10^{-5}M[7]. The efficiency of proteins as *in vitro* substrates for the enzyme does not seem to be dependent on their charge(pI values) nor on the size of the protein molecules (Table II). However, in general, the K_m values for the polypeptide substrates for protein methylase II are inversely proportional with the chain length; namely, the shorter the length of the peptide, the larger the K_m values[16].

It is generally accepted that aspartyl residues of protein-substrate are the methylation site for the enzyme in higher organisms whereas glutamyl residues are the site in lower organisms[17,18]. Although there are 11 aspartyl residues in rabbit brain MBP[19] (same number in bovine brain MBP), one major and one minor radioactive spot were detected on autofluorography of pepsin-digest map of [*methyl*-^{14}C]-MBP methylated by protein methylase II (Fig. 2). This indicates that protein methylase II has a relatively strong site-specificity towards this protein-substrate. Assuming that MBP accepts a single methyl group per molecule, approximately 5mol% of MBP is maximally methylated by protein methylase II. This value is quite close to the reported values of pituitary polypeptide hormones[20] (lutropin and ACTH with 6.6 and 4.8mol%, respectively). However, this is much less than the value of 78mol% with deaminated ACTH[21].

Methyl-esterification neutralizes the negative charges associated with free carboxyl groups ef cellular proteins with the introduction of chemically labile methyl esters. Its role in bacterial chemotaxis has well been established[22,23]. Recently, methyl-esterification of erythrocyte membrane proteins has been postulated to play an important role in the repair of aged membrane proteins[24]. In addition, protein-carboxyl methylation has been suggested to be involved in the release of neurotransmitters, exocytosis, storage and release of pituitary hormones, and locomotion of sperm[7]. What role, if any, the enzymatic methyl-esterification of MBP by protein methylase II plays in the formation and maintenance of myelin is not clear at present, since the enzyme activity in jimpy mutant mice brains remained unchanged from those of normal littermates (Table II). In these mutant mice brains, synthesis and incorporation of MBP into myelin is severely hampered[1], and our recent studies have demonstrated that protein methylase I is drastically reduced[11].

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