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TIME DEPENDENCE OF THE METHYLATION OF MYELIN BASIC PROTEIN FROM BOVINE BRAIN; EVIDENCE FOR PROTEIN-METHYLARGININE DEMETHYLATION

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SUMMARY: In the presence of excess *S*-adenosylmethionine (AdoMet), the extent of methylation of myelin basic protein (MBP) by partially purified AdoMet:MBP methyltransferase is a non-linear function of time, reaching a limiting value as available MBP is depleted and then decreasing monotonically. This decrease is not caused by proteolytic cleavage of MBP nor by effects related to substrate or product instability under the incubation conditions and is not observed in heat-inactivated samples. *S*-Adenosylhomocysteine is not required for the demethylation to occur, and with purified enzyme, the decrease is not observed. The data strongly suggest that the decrease in methyl content represents an enzyme-catalyzed demethylation reaction. This would represent the first report of an enzyme which catalyzes protein-methylarginine demethylation.

In previous work we have described the purification and kinetic properties of the enzyme *S*-adenosylmethionine:myelin basic protein (arginine-108) *N*-methyltransferase from bovine brain (1). This enzyme utilizes S-adenosylmethionine (AdoMet) to specifically methylate a single arginine residue in myelin basic protein (MBP), producing monomethyl- or N^G, N'^G- dimethylarginine. Values for the ratio of these forms and the percentage of *unmethylated* Arg-108 in the native protein have been reported to vary between 0.18 - 0.8 mole/mole for monomethylarginine and 0.12 - 0.20 mole/mole for N^G, N'^G- dimethylarginine (2,3,4), giving values between 0.3 and 1.0 for total moles of methylated residue per mole of protein. Unsymmetrical dimethylarginine has been reported to be a minor component of MBP by Brostoff, et al., but this report has not been confirmed by other workers (5). Using HPLC analysis (6), we have recently reported a total of 0.92 moles of total methylarginine per mole of protein with an isomer distribution of 0.61 mole/mole of monomethylarginine and 0.31 mole/mole protein of N^G, N'^G-dimethylarginine; no traces of the unsymmetrical isomer were found. The source of the variability in the literature data is

Abbreviations: HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; MBP, myelin basic protein.

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unknown but may be due to differences in the sample preparation, in measurement techniques, or may represent actual differences in the extent of methylation of MBP in the sample. In this communication, we report that the content of methylarginine in MBP *decreases* upon extended incubation with partially purified AdoMet:MBP methyltransferase, consistent with the presence of a contaminating methylarginine *demethylase* activity.

MATERIALS AND METHODS

Materials: (^{3}H-methyl)-S-Adenosylmethionine with a specific activity of 52 mCi/mmole was from Amersham and was diluted to give 100-150 dpm/pmole. Myelin basic protein was prepared from bovine brain white matter as previously described (7) and homogenity confirmed by SDS gel electrophoresis; protein concentrations were measured by the Bradford method (8). AdoMet:MBP methyltransferase was isolated from bovine brain as described previously (1) and was used in highly purified form or at the "ammonium sulfate precipitate" stage. Nitrocellulose membrane filters impregnated with carboxymethyl (CM) cellulose were from Schleicher & Schuell. A purified "nonspecific" adenosine deaminase was obtained from *Aspergillus oryzae* by the method of Sharpless and Wolfenden (9), purified 250-fold, relative to crude extract, and stored frozen in 10 mM MOPS buffer, pH 7.2, containing soybean trypsin inhibitor at a concentration of 1 mg/ml (added to inhibit proteolysis of MBP by an impurity in the deaminase preparation). Activity of the nonspecific deaminase was measured spectrophotometrically (9).

Assay Methods. The methylation of MBP by AdoMet was assayed by adsorbing MBP from the assay mixture onto nitrocellulose filters coated with carboxymethyl cellulose (1). This assay system relies upon the selective removal of myelin basic protein from the assay mixture by adsorption to the resin; MBP has a high affinity for CM cellulose, while the enzyme mixture, having an isoelectric point < 7.0, is not adsorbed. AdoMet slightly adsorbes to the resin, but can be quantitatively removed by extensive washing (400 ml) on a vacuum manifold. The standard assay contained MBP (1.0 mg), 0.2 ml methyltransferase enzyme preparation, 50 μM AdoMet (typically 110 dpm/pmole) and sufficient MOPS buffer (10 mM, pH 7.2) to give an assay volume of 0.5 ml. Assays were performed in 1.5 ml polyethylene micro- centrifuge tubes suspended in a thermostated water bath at 37° C for 20 minutes. The reaction was terminated by the addition of 1.0 ml of ice cold MOPS buffer followed by filtration of the mixture through the CM filter on a vacuum manifold. The filter was washed with 400 ml of buffer to remove retained AdoMet and then transferred to a scintillation vial. The protein was eluted from the membrane by the addition of 1.0 ml of 1 M HCl. Finally, 15 ml of scintillation fluid was added to the vial to dissolve the membrane coating. Radioactive incorporation due to the formation of methyl esters by contaminating protein methylase II was assayed by the procedure of Terwillinger et al. (10) and corrected for the assay efficiency. Liquid scintillation counting was performed using a Packard Tri-Carb system Model B-2450; internal standards were used to calculate quench corrections; external standards were run with every set of samples and observed counts per minute were corrected for quenching and tritium decay. The pH values of solutions were determined using a Corning pHM 130 pH meter with a combined glass electrode and a calomel junction. Glass distilled water was used throughout.

RESULTS AND DISCUSSION

The time dependence of the methylation of myelin basic protein by excess S-adenosyl-methionine, as catalyzed by highly purified AdoMet:MBP methyltransferase, is shown in Figure 1 (■). Addition of excess MBP to the assay mixture results in an increase in the extent of total methyl incorporation after two hours, suggesting that MBP is limiting under the assay conditions. The molar quantity of methyl groups incorporated (≈10% of the total MBP) agrees well with our

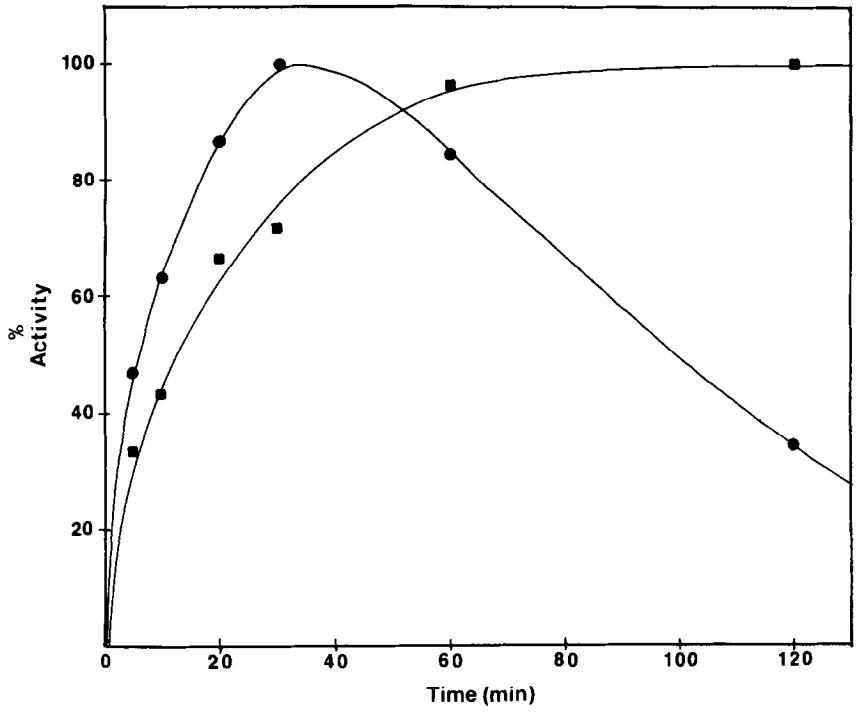

Figure 1. Dependence on time of the rate of methyl group incorporation from ^{3}H-methyl-*S*-adenosylmethionine into myelin basic protein by highly purified (■) and crude (●) AdoMet:MBP methyltransferase. Assay mixtures contained 0.5 mg MBP, 10 μM ^{3}H-AdoMet, 0.02 mg adenosine deaminase and 0.6 mg enzyme in 0.5 mL MOPS buffer, pH 7.2, 37° C; 20 minute assay.

previous determination of ≈8% unmethylated Arg-108 in "native" MBP (6). The fact that a *limiting* value is reached at the 10% level suggests that the purified enzyme does not methylate, or methylates very slowly, the ≈30% of MBP with monomethylarginine at position 108 since this would show up as a higher fraction incorporation or as a biphasic increase to a higher limiting value.

Methylation of MBP by less highly purified methyltransferase preparations, however, results in an initial increase to the apparent limiting value followed by a *decrease* in the extent of methyl incorporation (Figure 1 (●)). This decrease is not caused by proteolytic cleavage of MBP to fragments which might not be assayed by the filter method since assay samples examined electrophoretically showed no diminution in the intensity of the MBP band with time and protease inhibitors did not effect the time dependence observed. Autoradiography of these gels showed radioactivity present in MBP at the early stages of the incubation, but diminishing with time, consistent with the curve shown in Figure 1. The apparent loss of methyl groups is not an artifact of the assay system since incubation of (Enzyme + MBP) and (Enzyme + AdoMet) had no effect

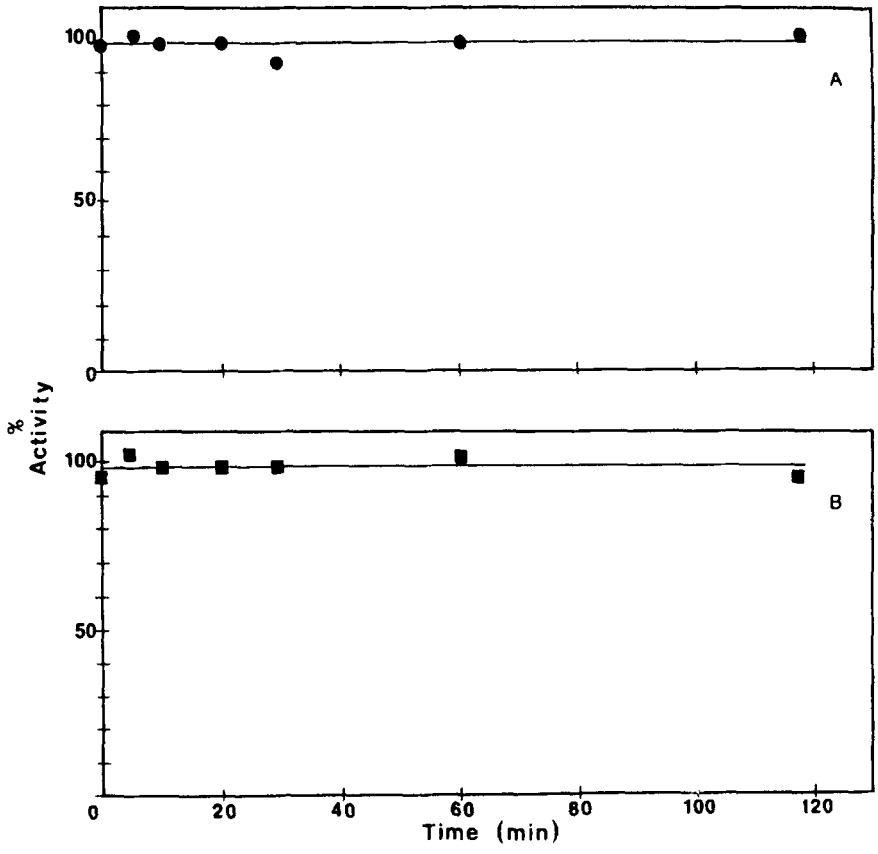

Figure 2. Dependence on preincubation time of the rate of methyl group incorporation from ^{3}H-methyl-*S*-adenosylmethionine into myelin basic protein by enzyme preincubated with MBP (A) or with AdoMet (**B**). Preincubation or final assay mixtures contained 0.5 mg MBP, 10 μM ^{3}H-AdoMet, 0.02 mg adenosine deaminase and 0.6 mg enzyme in 0.5 mL MOPS buffer, pH 7.2, 37° C; 20 minute assay.

on the observed time dependence (Figure 2, **A** and **B**, respectively). The simplest explanation for this loss of radioactivity from the protein with time is that a *demethylation* is occurring. This demethylation is not a result of the hydrolysis of protein methyl esters, since the radioactive methyl incorporation has previously been shown to be base-stable. Further, we (1) and others (11) have shown that the radioactivity is incorporated into a single tryptic peptide under these assay conditions and ^{1}H NMR spectra (200 MHz) of MBP before and after methylation by the enzyme clearly show an increase in the methylarginine resonance at 2.79 ppm (*N*-acetyl-Ala as internal standard; P. R. Young & F. Grynspan, unpublished data) with no evidence of lysine or carboxymethylation. Further, the observed demethylation must be an *enzyme catalyzed* reaction since the demethylation is heat sensitive and can be separated from the methylation activity during protein purification. *S*-Adenosylhomocysteine (AdoHcy) is not required for the demethylation

since the nonspecific adenosine deaminase (9) from *Aspergillus oryzae* (which rapidly deaminates AdoHcy but not AdoMet) has no effect on the observed time dependence.

Although methylarginine residues turnover very slowly *in vivo* (12), an enzyme responsible for methylarginine demethylation has not been described. There is precedent, however, for the enzymatic demethylation of ε-*N*-methyllysine residues (13); the enzyme (ε-alkyl-L-lysine:oxygen oxidoreductase; EC 1.5.3.4) catalyzes the oxidative removal of methyl groups yielding formaldehyde as the oxidation product. An enzyme catalyzing the hydrolysis of γ-glutamyl methyl ester residues of membrane-bound chemotactic proteins has also been observed (14). There is evidence that methylhistidine residues turnover *in vivo* (15) although an enzyme responsible for this reaction has not been isolated. The methyl acceptor in the present case and the enzyme catalyzing the demethylation are currently being investigated.

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