A specific protein carboxyl methylesterase that demethylates phosphoprotein phosphatase 2A in bovine brain

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ABSTRACT Phosphoprotein phosphatase 2A (PP2A) is one of the four major protein serine/threonine phosphatases found in all eukaryotic cells. We have shown that the 36-kDa catalytic subunit of PP2A is carboxyl methylated in eukaryotic cells, and we have previously identified and purified a novel methyltransferase (MTase) that is responsible for this modification. Here, we describe a novel protein carboxyl methylesterase (MEase) from bovine brain that demethylates PP2A. The enzyme has been purified to homogeneity as a monomeric 46-kDa soluble protein. The MEase is highly specific for PP2A. It does not catalyze the demethylation of other protein or peptide methylesters. Moreover, MEase activity is dramatically inhibited by nanomolar concentrations of okadaic acid, a specific inhibitor of PP2A. From these results, we conclude that PP2A methylation is controlled by two specific enzymes, a MTase and a MEase. Since PP2A methylation is highly conserved in eukaryotes ranging from human to yeast, it is likely that this system plays an important role in phosphatase regulation.

The vast majority of protein phosphoserine and phosphothreonine groups are hydrolyzed by a relatively limited number of phosphoprotein phosphatases. These enzymes fall into four major classes, Types 1, 2A, 2B, and 2C (1–3). Phosphoprotein phosphatase 1 (PP1) is controlled by distinct targeting subunits (4), and PP2B (also termed calcineurin) is regulated by Ca^{2+}/calmodulin (5). The mechanism of regulation of PP2A, however, remains to be established. PP2A has been purified from a number of different cell types as a heterodimer, composed of a 36-kDa catalytic C subunit that is homologous to the catalytic subunits of PP1 and PP2B, and an unrelated 65-kDa A subunit (1–3). The PP2A heterodimer is generally found associated with a third, variable subunit termed B (6–8). The A and B subunits are thought to provide regulatory functions.

The catalytic subunit of PP2A is subject to methyl esterification of its C terminus (9–11). This reaction is catalyzed by a specific S-adenosylmethionine (AdoMet) dependent methyltransferase (MTase) (9). Most protein methylation occurs at nitrogens in lysine, arginine, and histidine side chains, or at N-terminal α-amino groups. These are essentially irreversible posttranslational events. In contrast, methylesters are potentially subject to hydrolysis through the action of esterases, and protein methyl esterification can, therefore, function as a reversible regulatory modification analogous to protein phosphorylation. Reversible methylation at specific glutamates in membrane receptor proteins provides an essential adaptive function in bacterial chemotaxis (12), and reversible methylation at C-terminal prenylcysteine residues may regulate signal transduction pathways that involve G proteins and Ras-related low-molecular-weight GTP-binding proteins in vertebrate cells (13).

Here we report the identification and purification of an enzyme (or enzymes) that specifically catalyzes the demethylation of PP2A. This is the first eukaryotic protein methylesterase (MEase) to be isolated and characterized, and its

activity provides the final essential component of a reversible methylation system that specifically modifies PP2A. The reversible nature of the methylation reaction was confirmed in a reconstituted system composed of only MTase, MEase, and PP2A in which more than 40 rounds of methylation and demethylation occurred over a 24-hr period with concomitant production of methanol from AdoMet.

MATERIALS AND METHODS

Materials. Phenyl-Toyopearl TSK 650M and DEAE-Toyopearl TSK 650M were from Supelco. Superose 6 HR 10/30, Superose 12 HR 10/30, Superdex-75 HR 10/30, Mono Q HR 5/5, and NICK Spin columns were from Pharmacia. DEAE-Cellulose (DE-52) and Whatman 3 MM paper were from Whatman. Centricon-10 and -30 and Centriprep-10 and -30 ultrafiltration devices were from Amicon. Frozen bovine brains were from RJO Biologicals (Kansas City, MO). Ado-[*methyl-*^{3}H]Met [5–80 Ci/mmol (1 Ci = 37 GBq)] was from DuPont/NEN. AdoMet, leupeptin, aprotinin, pepstatin, and okadaic acid were from Boehringer Mannheim. The peptides corresponding to the C-terminal 4 or 10 residues of the PP2A catalytic subunit (DYFL and VTRRTPDYFL) and the carboxyl methylated tetrapeptide (DYFL-Me) were synthesized by the Princeton University Core Protein Facility.

Preparation of [^{3}H]Methylated Protein Substrates. To prepare [^{3}H]methylated PP2A, 1.0 μM purified AC heterodimer (9) was mixed with 0.2 μM purified MTase (9) in a final volume of 100 μl containing 50 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)-Na (pH 7.2), 5.0 mM DTT, 1.0 mM EDTA, 1.0 mg/ml of BSA, and either 1.0 or 10 μM [^{3}H]AdoMet (50,000 and 5,000 cpm/pmol, respectively). After 30 min at 32°C, unlabeled AdoMet was added to a final concentration of 100 μM, and the reaction mixture was immediately subjected to gel filtration on a NICK Spin column (Sephadex G-50, 7 × 75 mm) pre-equilibrated in buffer containing 50 mM Mops-Na (pH 7.2 or pH 6.0), 1.0 mM DTT, 1.0 mM EDTA, 0.20 M NaCl, and 5.0% glycerol. Fractions of 50 μl were collected, and an aliquot of each was analyzed by liquid scintillation counting. The fractions containing [^{3}H]methylated PP2A were combined and stored at 4°C. The yield of the reaction was typically around 20 pmol of [^{3}H]methylated PP2A, which was stable for at least 2 weeks at 4°C.

To prepare methylated bacterial chemoreceptor proteins, an *Escherichia coli* membrane preparation enriched for the chemoreceptor Tar (50 μl, 350 μg total protein) was incubated in a final reaction volume of 100 μl containing 10 μM [^{3}H]AdoMet (5.5 Ci/mmol), 50 mM Tris·HCl (pH 7.5), 1.0 mM EDTA, 1.0 mM DTT, 10% glycerol, and 0.40 μM pure CheR MTase (14). After 1 hr at 32°C, the reaction mixture was centrifuged at 4°C for 5 min, and the resulting pellet was washed with 3 × 1 ml of buffer

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containing 50 mM Mops-Na (pH 7.2), 1.0 mM EDTA, 1.0 mM DTT, 5.0% glycerol, and 0.20 M NaCl.

To prepare protein methylated on isoaspartyl residues, 750 µg of ovalbumin was methylated in a final reaction volume of 200 µl containing 10 µM [³H]AdoMet (5.5 Ci/mmol), 50 mM Mops-Na (pH 6.0), 1.0 mM EDTA, 5.0 mM DTT, and 250 µg of partially purified bovine brain isoaspartyl MTase obtained from the flow-through fraction off a DE-52 column (15). After 30 min at 32°C, unlabeled AdoMet was added to the reaction mixture to a final concentration of 100 µM, and [³H]methylated ovalbumin was separated from free AdoMet on a NICK Spin column as described above for [³H]methylated PP2A, except that all procedures were performed at pH 6.0. Fractions containing [³H]methylated ovalbumin were combined and chromatographed on a Superose 12 HR 10/30 column equilibrated in the same buffer as above.

To prepare methylated Ras, recombinant human p21$^{Ki-ras-2B}$ was purified and modified as described (16). After methylation, the membrane-bound [³H]methylated Ras was separated from unincorporated AdoMet as described above for the [³H]methylated *E. coli* chemoreceptor Tar.

Assays for MEase Activity. Unless otherwise indicated, reaction mixtures contained 50 mM Mops-Na (pH 7.2), 1.0 mM EDTA, 1.0 mM DTT, 1.0 mg/ml of BSA, ≈2000 cpm of [³H]methylesterified protein substrate, and a source of MEase, in a total volume of 20 µl. After incubation at 32°C for the indicated times, reaction mixtures were spotted onto 12 × 12 mm Whatman 3 MM filter papers that were then dried *in vacuo* at 45°C for 20 min to remove [³H]methanol produced by protein demethylation. The papers were then assayed for radioactivity in a liquid scintillation spectrometer. When crude preparations of [³H]methylesterified protein were used as substrates, the samples were analyzed by SDS/PAGE, and the presence of carboxyl methyl groups on the substrate proteins were quantitated by the methanol diffusion assay (17).

RESULTS

Identification and Purification of PP2A MEase. To identify the MEase activity that is responsible for demethylation of PP2A, we developed a simple and reliable assay. Purified PP2A was methylated *in vitro* by PP2A-specific MTase in the presence of [³H]AdoMet. Unincorporated [³H]AdoMet was removed from the reaction mixture by gel filtration, and the [³H]methyl PP2A was used as a substrate in demethylation reactions. Since all of the radioactivity present could be recovered in the pellet after trichloroacetic acid precipitation, or converted to volatile radioactivity after treatment at high pH, we assumed that all radioactivity is associated with the base-labile methylester group on PP2A. The hydrolysis of methylesters from PP2A could, therefore, be easily detected by monitoring the decrease in radioactivity associated with the protein substrate, or conversion of radioactivity to a volatile product, [³H]methanol. Using this assay, the MEase activity was purified by standard chromatographic procedures (Fig. 1).

The purification involved hydrophobic interaction chromatography on a Phenyl-Toyopearl column followed by ion exchange chromatography on a DEAE-Toyopearl column. The MEase was further fractionated by size exclusion HPLC using a Superdex-75 HR column (Fig. 2*A*), and activity (shaded area) was observed in fractions eluting with retention volumes characteristic of 40- to 50-kDa globular proteins. The active fractions were combined, concentrated, and rechromatographed on a Superdex-75 column under the same conditions. The MEase eluted with a retention time corresponding to an apparent molecular weight of ≈46,000. This material was then fractionated on an ion exchange Mono Q HR column using an NaCl gradient (Fig. 2*B*). Activity was observed in two peaks eluting with 300 mM NaCl (peak I) and 310 mM NaCl (peak II). SDS/PAGE analysis revealed that both peaks
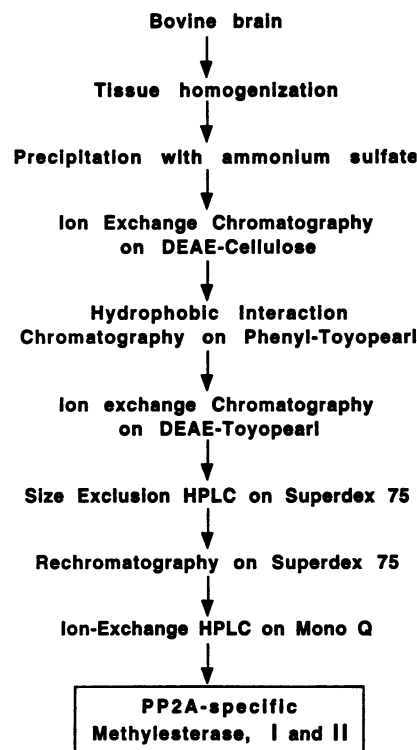

FIG. 1. Purification scheme for bovine PP2A-specific MEase. The preparation of initial material for purification of MEase (including tissue homogenization and extraction, and DEAE-Cellulose and Phenyl-Toyopearl chromatography) was performed as described (9). MEase activity from extract prepared from two brains was separated from PP2A during chromatography on Phenyl-Toyopearl and was found in fractions eluting between 430 and 220 mM ammonium sulfate. These fractions were combined, dialyzed against buffer A (50 mM Mops-Na (pH 7.2)/1.0 mM EDTA/1.0 mM DTT/0.50 µg/ml each of leupeptin, pepstatin, and aprotinin), and loaded onto a DEAE-Toyopearl TSK 650M column (1.6 × 12 cm) pre-equilibrated in buffer A. MEase was eluted between 124 mM and 185 mM NaCl using a linear salt gradient from 0 to 300 mM NaCl in 300 ml of buffer A and was concentrated to a final volume of 1.0 ml by ultrafiltration using a Centriprep-10. The resulting material was chromatographed in two 0.50-ml portions on a Superdex-75 HR 10/30 column pre-equilibrated in buffer A containing 0.20 M NaCl at a flow rate of 0.30 ml/min at room temperature. Fractions containing MEase activity were combined, concentrated as described above, and rechromatographed on a Superdex-75 HR 10/30 column. Fractions containing MEase were combined (≈1 mg of total protein), concentrated, and re-equilibrated with buffer A by ultrafiltration using a Centricon-10. The protein sample was loaded at 0.70 ml/min at room temperature onto a Mono Q 5/5 HR column equilibrated with buffer A, and eluted at 0.70 ml/min with consecutive linear gradients of NaCl in buffer A from 0 to 160 mM in 7.0 ml, 160 to 360 mM in 28 ml, and 360 to 400 mM in 7.0 ml. The enzyme resolved into two peaks that eluted at 300 mM (peak I) and 310 mM (peak II) NaCl. The two peaks were concentrated separately by ultrafiltration using a Centricon-10 and stored at −20°C in 40% glycerol. Peaks I and II contained 56 and 44 µg, respectively. Protein concentrations were determined by the method of Bradford (18) using BSA as a standard.

contained homogeneous polypeptides with identical electrophoretic mobility corresponding to an M_r of 46,000 (Fig. 2*C*). MEase I and II exhibited similar specific activities. Protein microsequencing of a pool of MEase I and II indicates that there is no identity to any species whose sequence has been deposited in currently available data bases.

Characterization of PP2A MEase. The MEase activity had a broad pH optimum between 6.0 and 9.0. In the absence of MEase, the [³H]methylester of PP2A was stable for up to 40 min at pH values ranging from 4.0 to 9.0, indicating that the cleavage of methylester at a pH of >8.0 in the presence of MEase
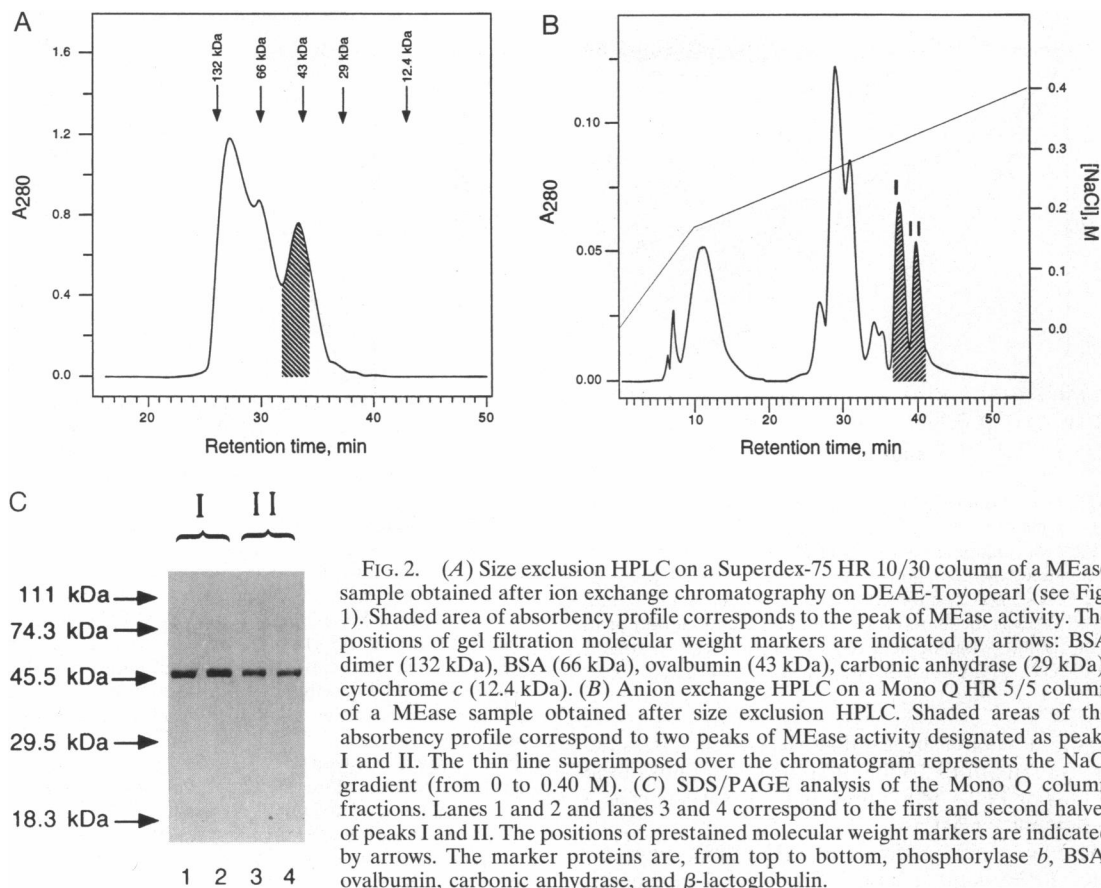
FIG. 2. (*A*) Size exclusion HPLC on a Superdex-75 HR 10/30 column of a MEase sample obtained after ion exchange chromatography on DEAE-Toyopearl (see Fig. 1). Shaded area of absorbency profile corresponds to the peak of MEase activity. The positions of gel filtration molecular weight markers are indicated by arrows: BSA dimer (132 kDa), BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa). (*B*) Anion exchange HPLC on a Mono Q HR 5/5 column of a MEase sample obtained after size exclusion HPLC. Shaded areas of the absorbency profile correspond to two peaks of MEase activity designated as peaks I and II. The thin line superimposed over the chromatogram represents the NaCl gradient (from 0 to 0.40 M). (*C*) SDS/PAGE analysis of the Mono Q column fractions. Lanes 1 and 2 and lanes 3 and 4 correspond to the first and second halves of peaks I and II. The positions of prestained molecular weight markers are indicated by arrows. The marker proteins are, from top to bottom, phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, and β-lactoglobulin.

was not due to spontaneous alkaline hydrolysis. MEase activity was not affected by 1.0 mM concentrations of either phenylmethylsulfonyl fluoride or diisopropyl fluorophosphate, or by a combination of aprotinin, leupeptin, and pepstatin at concentrations of up to 100 μg/ml. The activity we have isolated is, therefore, distinct from the serine esterase/protease activity recently observed by Xie and Clarke (19) in bovine brain extracts that also removes methyl groups from PP2A, since their activity was sensitive to phenylmethylsulfonyl fluoride.

There are three distinct types of protein carboxyl methylation reactions that are known to occur in prokaryotic and eukaryotic cells: methylation of side chains on D-aspartyl and L-isoaspartyl residues (20); methylation of carboxyl-terminal, prenylated cysteine residues on proteins such as Ras (21); and methylation of glutamyl side chains in bacterial chemoreceptor proteins (22). We examined the substrate specificity of purified PP2A MEase by measuring its activity toward protein substrates that are modified by each of these types of methylation reactions. To minimize the spontaneous hydrolysis of ovalbumin isoaspartyl methylesters, [³H]methyl-ovalbumin was prepared and assayed at pH 6.0. Our results show that [³H]methyl-PP2A is highly susceptible to the action of MEase, whereas none of the other known methylesterified proteins were affected (Fig. 3). To exclude the possibility that the crude preparations of methylated protein substrates might contain an inhibitor of the PP2A MEase, PP2A demethylation assays were performed in the presence of each of the other potential [³H]methylated protein substrates. The reaction products were then separated by SDS/PAGE, and the radioactivity remaining on PP2A was determined by methanol diffusion assay. No inhibition of PP2A demethylation was observed in the presence of any other methylated protein substrates. Thus, we conclude that MEase specifically demethylates PP2A.

Incubation of MEase with a 600-fold molar excess of unmethylated PP2A resulted in 50% inhibition of demethylation

compared with the standard reaction condition, in which there is typically about a 4-fold molar excess of unmethylated over methylated PP2A (Fig. 4). However, two synthetic peptides corresponding to the C-terminal 4 or 10 residues of the PP2A catalytic subunit (DYFL and VTRRTPDYFL, respectively) did not affect the demethylation of PP2A even when present in 10⁶-fold molar excess over [³H]methyl-PP2A. Moreover, under the same conditions, the carboxyl methylated tetrapeptide corresponding to the C terminus of methylated PP2A, DYFL-Me, neither inhibited the hydrolysis of [³H]methyl-PP2A nor served as a substrate for MEase (data not shown).
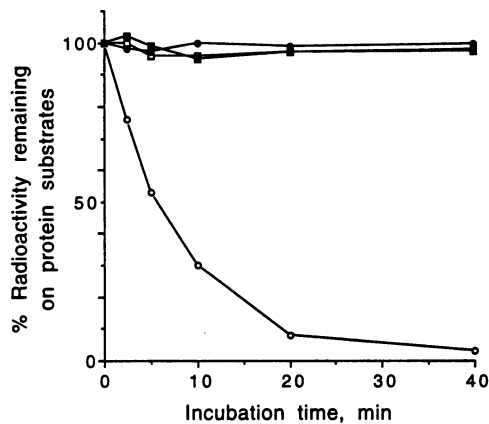

FIG. 3. Substrate specificity of MEase. Purified MEase (10 nM) was incubated with 250 fmol of [³H]methyl-Tar (□), 560 fmol of [³H]methyl-ovalbumin (■), 100 fmol of [³H]methyl-Ras (●), or 400 fmol of [³H]methyl-PP2A (○) in 20 μl. The radioactivity remaining on [³H]methyl-PP2A, [³H]methyl-Tar, and [³H]methyl-ovalbumin was measured by the filter paper adsorption assay (see *Materials and Methods*), and the radioactivity remaining on [³H]methyl-Ras was quantitated by the methanol diffusion assay (17).
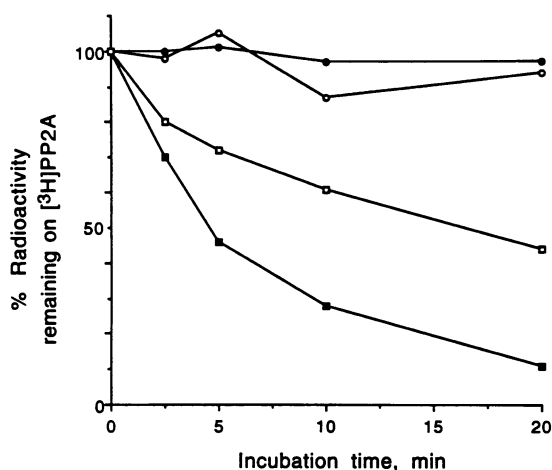
FIG. 4.   Inhibition of demethylation reaction by excess PP2A or by okadaic acid. To test for the effects of excess PP2A, 40 fmol of [³H]methyl-PP2A was incubated with buffer alone (●) or with MEase (10 nM) preincubated for 5.0 min in the presence of 24 pmol of unmethylated PP2A (□). To test for the effects of okadaic acid, 40 fmol of [³H]methyl-PP2A was incubated for 5.0 min on ice alone (■) or in the presence of 100 nM okadaic acid (○) followed by the treatment with MEase (10 nM). PP2A demethylation was assayed as described in the text.

Taken together, these data suggest that MEase recognizes the higher order structure of PP2A and that the C terminus of the catalytic subunit of PP2A is required but not sufficient as a recognition element for MEase.

Okadaic acid, a specific inhibitor of PP2A (23), quantitatively inhibited PP2A demethylation (Fig. 4). We have previously shown that okadaic acid also inhibits PP2A methylation (24). Since okadaic acid has a high affinity for PP2A (23), we suspect that binding of okadaic acid to PP2A in some way protects its carboxyl terminus from the action of both modification enzymes.

Methylester Turnover. The identification of a specific PP2A MEase establishes the final requisite component of a system for the reversible methylation of the phosphatase C terminus. To confirm this, we have shown that a reconstituted system composed of purified MTase and MEase can catalyze multiple rounds of PP2A methylation and demethylation (Fig. 5). Thus, over a 24-hr period, an amount of methanol equivalent to >40 times the total level of PP2A present in the reaction mixture was generated from AdoMet. Methanol production was completely dependent on the presence of all three protein components.

DISCUSSION

We have identified, purified, and characterized an enzyme from bovine brain that specifically demethylates PP2A. The protein is monomeric with an apparent molecular weight of 46,000. Two forms of the enzyme that differed slightly in their chromatographic behavior but were indistinguishable by SDS/PAGE analysis and with respect to their specific activities toward methylated PP2A were obtained. These proteins may represent differently modified forms of the same enzyme, or two closely related isozymes. The MEase exhibited no activity toward protein substrates that are methylated by the three other known protein carboxyl MTases, the *E. coli* MTase CheR that methylates bacterial chemoreceptors (22), the MTase that methylates Ras and other similarly processed proteins (21), and the MTase that methylates damaged proteins at sites of aspartyl isomerization (20). These results, together with the fact that the demethylation reaction can be inhibited by unmethylated PP2A but not by much higher concentrations of methylated and unmethylated C-terminal peptides that correspond to the C
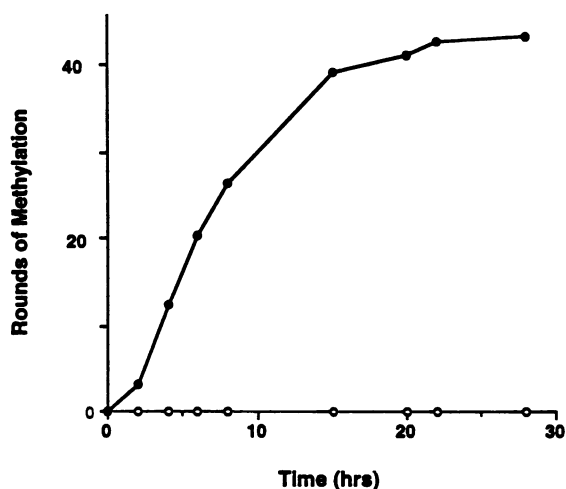

FIG. 5.   Multiple rounds of PP2A methylation and demethylation in the presence of both MEase and MTase. [³H]AdoMet (4.0 μM, 10,000 cpm/pmol) was incubated in the presence or absence of PP2A (4 pmol), MTase (1.25 pmol), and MEase (2.1 pmol) in 100 μl of buffer (50 mM Mops-Na, pH 7.2/5.0 mM DTT/1.0 mM EDTA/50 mM NaCl/1.0 mg/ml BSA) at 37°C in an open 1.5-ml Eppendorf tube placed in a sealed scintillation vial containing scintillation fluid. The production of [³H]methanol was assayed by measuring the appearance of radioactivity in the scintillation fluid. In a separate experiment, it was shown that the level of PP2A methylation under these conditions in the presence only of MTase was 3.6 pmol. Results are presented as minimal rounds of methylation and demethylation, i.e., pmol of methanol released/3.6 pmol (●). Each possible combination of one or two of the three required proteins was also tested (○). Without all three protein components, the amount of [³H]methanol produced did not exceed the background value (≈3 pmol/24 hr) obtained with AdoMet alone. This background was subtracted from the data shown in the figure.

terminus of the PP2A catalytic subunit, provide strong evidence that the PP2A MEase is highly specific for PP2A.

One other specific protein carboxyl MEase has been identified, the enzyme CheB that demethylates bacterial chemotaxis receptors such as Tar (22, 25). In bacterial chemotaxis, the MEase CheB is highly regulated by phosphorylation in response to chemotactic stimuli, whereas the activity of the MTase CheR appears to only depend on the level of AdoMet and the accessibility of protein substrate residues. Thus, the MEase plays a much more important role in chemotaxis regulation than the MTase. The structure of the catalytic domain of the chemotaxis MEase has recently been determined, and the active site has been shown to be composed of a Ser-His-Asp catalytic triad with a structural arrangement distinct from that found in protease/esterases such as trypsin and subtilisin (26). Despite the fact that it is clearly a serine esterase, the chemotaxis MEase is not inhibited by compounds such as phenylmethylsulfonyl fluoride or diisopropyl fluorophosphate.

Methylation has little effect on PP2A activity *in vitro* (11); therefore, it seems likely that this reversible modification functions indirectly to regulate PP2A. It has been shown that PP2A is phosphorylated and inactivated by tyrosine kinases such as the insulin receptor and v-*src* (27). Since the site of phosphorylation, Tyr-307, is just two residues from the methylated C terminus, Leu-309, methylation may very well modulate this aspect of PP2A regulation. Methylation may also function to control interactions between the AC heterodimeric form of PP2A and regulatory B subunits. These possibilities are particularly intriguing in light of changes in PP2A methylation that have been correlated with progression through the cell cycle (28). There is substantial evidence that PP2A plays an important role in the regulation of cell proliferation (29–36), and it seems likely that changes in methylation help orchestrate this process. Carboxyl methylation is a highly conserved feature of PP2A. The modification has been ob-

served in numerous different vertebrate tissues as well as in yeast (16, 24, 37). Therefore, it is highly probable that this modification apparatus involving two specific enzymes serves an essential function in the regulation of PP2A.

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