

Human Protein Arginine Methyltransferase 7 (PRMT7) Is a Type III Enzyme Forming ω - N^G -Monomethylated Arginine Residues*[§]

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Background: PRMT7 is a poorly characterized member of the family of protein arginine methyltransferases (PRMTs) that catalyze the modification of a wide group of proteins.

Results: A bacterially expressed fusion protein of human PRMT7 produces only ω -monomethylated arginine (ω -MMA) derivatives.

Conclusion: PRMT7 is a type III PRMT distinct from type I and type II PRMTs characterized to date.

Significance: ω -MMA may be a distinct post-translational modification.

Full-length human protein arginine methyltransferase 7 (PRMT7) expressed as a fusion protein in *Escherichia coli* was initially found to generate only ω - N^G -monomethylated arginine residues in small peptides, suggesting that it is a type III enzyme. A later study, however, characterized fusion proteins of PRMT7 expressed in bacterial and mammalian cells as a type II/type I enzyme, capable of producing symmetrically dimethylated arginine (type II activity) as well as small amounts of asymmetric dimethylarginine (type I activity). We have sought to clarify the enzymatic activity of human PRMT7. We analyzed the *in vitro* methylation products of a glutathione *S*-transferase (GST)-PRMT7 fusion protein with robust activity using a variety of arginine-containing synthetic peptides and protein substrates, including a GST fusion with the N-terminal domain of fibrillarin (GST-GAR), myelin basic protein, and recombinant human histones H2A, H2B, H3, and H4. Regardless of the methylation reaction conditions (incubation time, reaction volume, and substrate concentration), we found that PRMT7 only produces ω - N^G -monomethylarginine with these substrates. In control experiments, we showed that mammalian GST-PRMT1 and Myc-PRMT5 were, unlike PRMT7, able to dimethylate both peptide P-SmD3 and SmB/D3 to give the expected asymmetric and symmetric products, respectively. These experiments show that PRMT7 is indeed a type III human methyltransferase capable of forming only ω - N^G -monomethylarginine, not asymmetric ω - N^G , N^G -dimethylarginine or symmetric ω - N^G , N^G -dimethylarginine, under the conditions tested.

Protein arginine methyltransferases (PRMTs)² are enzymes that transfer methyl groups to the arginine residues of peptides

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[§] This article contains supplemental Figs. S1–S6.

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² The abbreviations used are: PRMT, protein arginine methyltransferase; ω -MMA, ω - N^G -monomethylarginine; [³H] ω -MMA, [³H] ω - N^G -

and proteins and play functional roles in transcriptional control, splicing, DNA repair, and signaling (1–4). One of the less well characterized members of this family is PRMT7. This protein is expressed in mouse embryonic stem cells and germ cells in a pattern similar to pluripotency markers, suggesting that PRMT7 may contribute to cellular differentiation (5). PRMT7 activity also seems to increase with the mammalian testis-specific CTC-binding factor-like protein in genetic imprinting (6). In *Drosophila melanogaster*, deletion of the PRMT7 ortholog *Dart7* results in pupal lethality (7). Like PRMT5, PRMT7 also seems to mediate RNA splicing events by methylating Sm proteins, small nuclear ribonucleoproteins (snRNPs) that comprise the spliceosome (8). In addition, changes in PRMT7 expression may indicate cellular toxicity. In adult mice treated with doxorubicin to induce nephropathy, *Prmt7* gene expression was about 8-fold lower and confined to the nephrogenic zone, where newly formed epithelia are induced (9). Similarly, several studies have implicated PRMT7 as a modulator of DNA damage response. PRMT7 expression is associated with either hypersensitivity to or protection from the effects of the topoisomerase I inhibitor camptothecin and the topoisomerase II inhibitors 9-hydroxyellipticine and etoposide (10–13). Finally, the human *PRMT7* gene is in a region of the genome known to have high copy number aberrations in metastasizing tumors, suggesting a role in metastasis (14).

Human protein arginine methyltransferases are known to generate three different products: ω - N^G -monomethylarginine (ω -MMA), asymmetric ω - N^G , N^G -dimethylarginine (ADMA), and symmetric ω - N^G , N^G -dimethylarginine (SDMA) (1, 4, 15). PRMT1, PRMT2, PRMT3, CARM1/PRMT4, PRMT6, and PRMT8 have type I activity, forming both ω -MMA and ADMA (1, 4, 15, 16). PRMT5 has type II activity, forming both ω -MMA

monomethylarginine; ADMA, asymmetric ω - N^G , N^G -dimethylarginine; [³H]ADMA, asymmetric [³H] ω - N^G , N^G -dimethylarginine; SDMA, symmetric ω - N^G , N^G -dimethylarginine; [³H]SDMA, symmetric [³H] ω - N^G , N^G -dimethylarginine; AdoMet, *S*-adenosyl-L-methionine; MBP, myelin basic protein; GAR, glycine and arginine-rich domain of human fibrillarin.

PRMT7 Is a Type III PRMT

and SDMA (17). Enzymes that form only ω -MMA have type III activity. The only fully characterized type III enzyme to date has been found in the protozoan *Trypanosoma brucei* (18).

Although seven of the eight mammalian PRMTs characterized thus far are clearly type I or type II enzymes, PRMT7 has proven to be more difficult to characterize. Initially, Miranda and co-workers (19) found that a preparation of human GST-PRMT7 expressed in *Escherichia coli* could methylate small arginine-containing synthetic peptides but not common PRMT protein substrates such as GST-GAR, histones, and myelin basic protein. Furthermore, acid hydrolysis of the methylated peptides followed by cation-exchange chromatography revealed only the presence of ω -MMA (19) suggesting a type III designation for this enzyme. However, in a second study, Lee and colleagues (20) characterized PRMT7 as a type II enzyme capable of forming symmetric dimethylarginine residues. Recombinant GST-PRMT7 expressed in *E. coli* was reported to be able to dimethylate small peptides corresponding to sequences found in histone H4, myelin basic protein (MBP), and P-SmD3 (a truncated version of pre-mRNA splicing protein SmD3). A preparation of immunopurified FLAG-PRMT7 from HeLa cells was shown to actively methylate histones, MBP, GST-GAR, spliceosomal protein SmB, and peptides P-MBP and P-SmD3. Amino acid analysis via thin layer chromatography (TLC) suggested that the modifications produced were predominantly ω -MMA and SDMA, with some formation of ADMA, indicating not just type II activity, but type I activity as well (20).

To resolve this controversy, human recombinant GST-PRMT7 constructs have been purified from *E. coli* and used to methylate substrates under conditions described in both previous studies (19, 20). Despite using preparations with more robust enzymatic activity than those used by Miranda *et al.* (19) under a variety of reaction conditions, we were unable to show that GST-PRMT7 catalyzed the formation of either ADMA or SDMA, suggesting that detection of these products by Lee *et al.* (20) may have been due to contamination. One particular problem is that the antibody previously used to immunopurify the FLAG-PRMT7-tagged protein from HeLa cells (20) also recognizes endogenous PRMT5 (21). To our knowledge, PRMT5 continues to be the only mammalian type II methyltransferase, whereas PRMT7 is capable of forming only monomethylated arginine derivatives in both peptide and protein substrates and is thus the first mammalian type III methyltransferase.

EXPERIMENTAL PROCEDURES

Purification of GST Fusion Proteins of PRMT7, PRMT1, and GAR—Human PRMT7 was expressed as a GST fusion protein from either the construct prepared by Miranda *et al.* (19) or Lee *et al.* (20). Prior to expression, both plasmids were confirmed by DNA sequencing. Plasmids expressing rat GST-PRMT1 (22) and GST-GAR (containing the first 148 amino acids of human fibrillarilin) (23) have been described previously. GST-PRMT7 was expressed in either *E. coli* DH5 α or BL21 Star (DE3) cells (Invitrogen, catalog number C601003); GST-PRMT1 and GST-GAR were expressed in BL21 Star (DE3) cells. Cells containing GST-PRMT7 were grown to an optical density at 600 nm of 0.4–0.8 and expression was induced at 18 °C overnight with a

final concentration of 0.4–0.8 mM isopropyl β -D-thiogalactopyranoside, whereas the cells containing GST-PRMT1 or GST-GAR were induced for 4 h at 37 °C with 0.4 mM isopropyl β -D-thiogalactopyranoside. Cells containing GST-PRMT7 were lysed in a phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 100 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) containing 100 μ M phenylmethylsulfonyl fluoride with seven 30-s sonicator pulses (50% duty; setting 4) on ice with a Sonifier cell disrupter W-350 (Smith-Kline Corp.), whereas those containing GST-PRMT1 or GST-GAR were lysed with seven 20-s sonicator pulses. The cell lysates were centrifuged for 50 min at 23,000 $\times g$ at 4 °C. The proteins were then purified from extracts by binding to glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer's instructions and eluted with 30 mM glutathione, 50 mM Tris-HCl, 120 mM NaCl, and 4% glycerol at pH 7.5. Protein was quantified by a Lowry assay after trichloroacetic acid precipitation as described previously (22). Final protein concentrations of 0.5–1.1 mg/ml for GST-PRMT7 and PRMT1 and 0.8–1.0 mg/ml for GST-GAR were obtained.

Peptide Substrates—The following peptides were a kind gift from Dr. Sidney Pestka (UMDNJ, Robert Wood Johnson Medical School, Piscataway, NJ): GRG-amide; P-SmD3, AGRGRGKAAILKAQVAARGRGRGMGRGN-amide; M1, SGRGKGGKGLGKGGAKRHRK-amide; M8, SGAGKGGKGLGKGGAKAHAK-amide (M1 with all arginines substituted with alanine); and MM, SGR^{Me}GKGGKGLGKGGAKRHRK-amide (R^{Me}, monomethylarginine). Their synthesis has been previously described (20). All of these peptides have free N termini. We verified the mass of each peptide by MALDI-TOF analysis (data not shown). Peptide R1 (GGFGRRGGFG-amide)³ (free N terminus) and peptide R2 (*N*-acetyl-GGRGG-amide) were purchased from California Peptide Research, Inc. and have been previously described (19).

Protein Substrates and Myc-tagged PRMT5—Recombinant human protein complex SmB/D3 purified from *E. coli* was generously provided by Dr. Christian Kambach (Paul Scherrer Institut, Switzerland) and Dr. Utz Fischer (Theodor-Boveri-Institut, Biocenter, University of Wuerzburg, Germany) and has been previously described (24). Histones H2A (M2502S), H2B (M2505S), H3.2 (M2506S), and H4 (M2504S) are human recombinant species from New England BioLabs, and myelin basic protein from bovine brain was obtained from Sigma (M1891). Purified Myc-PRMT5 from HEK293 cells was a kind gift from Dr. Jill Butler (University of Texas MD Anderson Cancer Center, Houston, TX).

In Vitro Labeling and Acid Hydrolysis of Peptide and Protein Substrates—Enzymatic reactions were initiated by the addition of *S*-adenosyl-L-[methyl-³H]methionine ([³H]AdoMet) at a final concentration of 0.7 μ M (PerkinElmer Life Sciences, 75–85 Ci/mmol, from a stock of 0.55 mCi/ml in 10 mM H₂SO₄; EtOH (9:1, v/v)) in a final reaction volume of either 30 or 60 μ l in 6 \times 50-mm glass test tubes. The incubation mixture was buffered in 50 mM potassium HEPES, 10 mM NaCl, pH 8.0, and 1 mM DTT and the reactions were allowed to proceed at 37 °C

³The sequence of the R1 peptide was erroneously given as GPGRRGGPG-amide by Miranda *et al.* (19).

for 1–25 h. Where proteins or the P-SmD3 peptide were used as substrates, 20 μg of bovine serum albumin was added in 2 μl as a carrier protein and the methylation reaction was then quenched with an equal volume of 25% (w/v) trichloroacetic acid. After incubation at room temperature for 30 min, the precipitated protein was collected by centrifugation at $4000 \times g$ for 30 min at 25 $^{\circ}\text{C}$, and the pellet was allowed to air dry. For the remaining peptide substrates, the reaction mixtures were dried in a vacuum centrifuge. Acid hydrolysis of all dried reaction mixtures (both peptides and proteins) was then carried out in a Waters Pico-Tag Vapor-Phase apparatus *in vacuo* for 20 h at 110 $^{\circ}\text{C}$ using 50 μl of 12 N HCl.

Amino Acid Analysis of Methylated Species by High Resolution Cation Exchange Chromatography—To determine the products of methylation, vacuum-dried hydrolyzed samples were resuspended in 50 μl of water and mixed with 1.0 μmol of the following standards: ω -MMA (acetate salt, Sigma, M7033), SDMA (di(*p*-hydroxyazobenzene)-*p*'-sulfonate salt, Sigma, D0390), and ADMA (hydrochloride salt, Sigma, D4268) for amino acid analysis by high-resolution cation-exchange chromatography. The hydrolyzed samples were diluted with 500 μl of citrate buffer (0.2 M Na^+ , pH 2.2), loaded onto a column (0.9 \times 8–12 cm) of PA-35 sulfonated polystyrene beads (6–12 μm ; Benson Polymeric Inc., Sparks, NV), and eluted with sodium citrate buffer (0.35 M Na^+ , pH 5.27) at 1 ml/min and 55 $^{\circ}\text{C}$. The column was regenerated with 0.2 N NaOH for 20–25 min and equilibrated with sodium citrate buffer for 20–25 min prior to each run.

Elution positions of the amino acid standards were determined by a ninhydrin assay (25, 26). The ninhydrin reagent is composed of 20 mg/ml of ninhydrin and 3 mg/ml of hydrindantin in a solvent of 75% (v/v) dimethyl sulfoxide and 25% (v/v) 4 M lithium acetate, pH 4.2. Aliquots (100 μl) of 1-ml column fractions were mixed with 600 μl of water and 300 μl of the ninhydrin solution, heated at 100 $^{\circ}\text{C}$ for 15 min and the absorbance measured at 570 nm (1-cm path length cuvette), or 50- μl aliquots of 1-ml column fractions were mixed with 100 μl of the ninhydrin solution, heated at 100 $^{\circ}\text{C}$ for 15 min, and the absorbance measured at 570 nm using a SpectraMax M5 microplate reader. Radioactivity in column fractions was quantitated using a Beckman LS6500 counter and expressed as an average of three 30-min counting cycles after mixing 900–950 μl of the column fractions with 400 μl of water and 10 ml of fluor (Safety Solve, Research Products International, 111177). For fractions from GST-PRMT1 reaction mixtures, only 200 μl was counted.

RESULTS

Because recombinant protein arginine methyltransferases purified from eukaryotic cells may be contaminated with endogenous PRMT family members, we used only GST-PRMT7 fusion protein expressed in *E. coli* cells lacking endogenous PRMT activity (27) to characterize its activity. The addition of an N-terminal GST protein has allowed for the rapid purification of several active members of the PRMT family and has not interfered with their activity (16, 18, 21, 22, 28, 29). Our GST-PRMT7 preparation was found to contain a major 102-kDa polypeptide of the size expected from the fusion of the 27-kDa GST and the 78-kDa human PRMT7 (Fig. 1). We

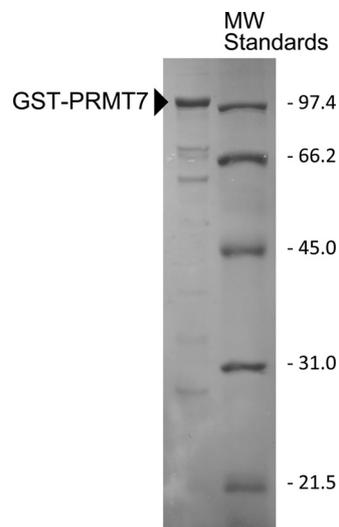


FIGURE 1. Purification of human GST-PRMT7. SDS-12.6% polyacrylamide gel electrophoresis of GST-PRMT7 following purification as described under "Experimental Procedures." GST-purified protein (3 μg) was analyzed in the left lane; molecular mass standards (Bio-Rad low range containing phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; and trypsin inhibitor, 21.5 kDa) were analyzed in the right lane. Following electrophoresis, the gel was stained with Coomassie Blue and dried.

assayed this enzyme using the buffer system recommended by Lakowski and Frankel (16), who found that replacement of the traditional phosphate buffers with HEPES buffers in the presence of DTT allowed them to detect the *in vitro* methylation activity of PRMT2 for the first time. In preliminary experiments, we found that DTT markedly enhanced the activity of GST-PRMT7 (supplemental Fig. S1).

To determine whether GST-PRMT7 catalyzed only ω -MMA formation or could also add additional methyl groups to generate SDMA or ADMA residues, we first used the R1 peptide (GGFGGRGGFEGG-amide) shown to be a substrate by Miranda *et al.* (19). Using the same concentrations we incubated the R1 peptide with [^3H]AdoMet and GST-PRMT7 in the new buffer system, acid hydrolyzed the samples, and purified the amino acids by high-resolution cation-exchange chromatography. Liquid scintillation counting of the column fractions identified ω -MMA as the only radiolabeled methylated residue (Fig. 2A). No radioactivity was detected at the elution positions of ADMA or SDMA standards as detected by ninhydrin analysis. The radioactive peak eluting just before the standard of ω -MMA shows the isotope effect expected for a tritiated methyl group on an arginine residue (22, 26, 30, 31, 32). Control experiments in which either the peptide substrate or GST-PRMT7 was omitted from the reaction mixture did not show formation of this product (Fig. 2A). To determine whether the initial 1-h incubation was insufficient to generate ADMA or SDMA, we then allowed the reaction to proceed for 5 and 20 h like the extended incubation times used by Lee *et al.* (20). Even under these conditions, however, we were unable to detect any radiolabeled ADMA or SDMA (Fig. 2B and inset).

We then considered the possibility that the R1 peptide might simply be a poor substrate for dimethylation. We note that only ω -MMA formation was found in an initial study with the R1 peptide and GST-PRMT1, even though this enzyme is capable

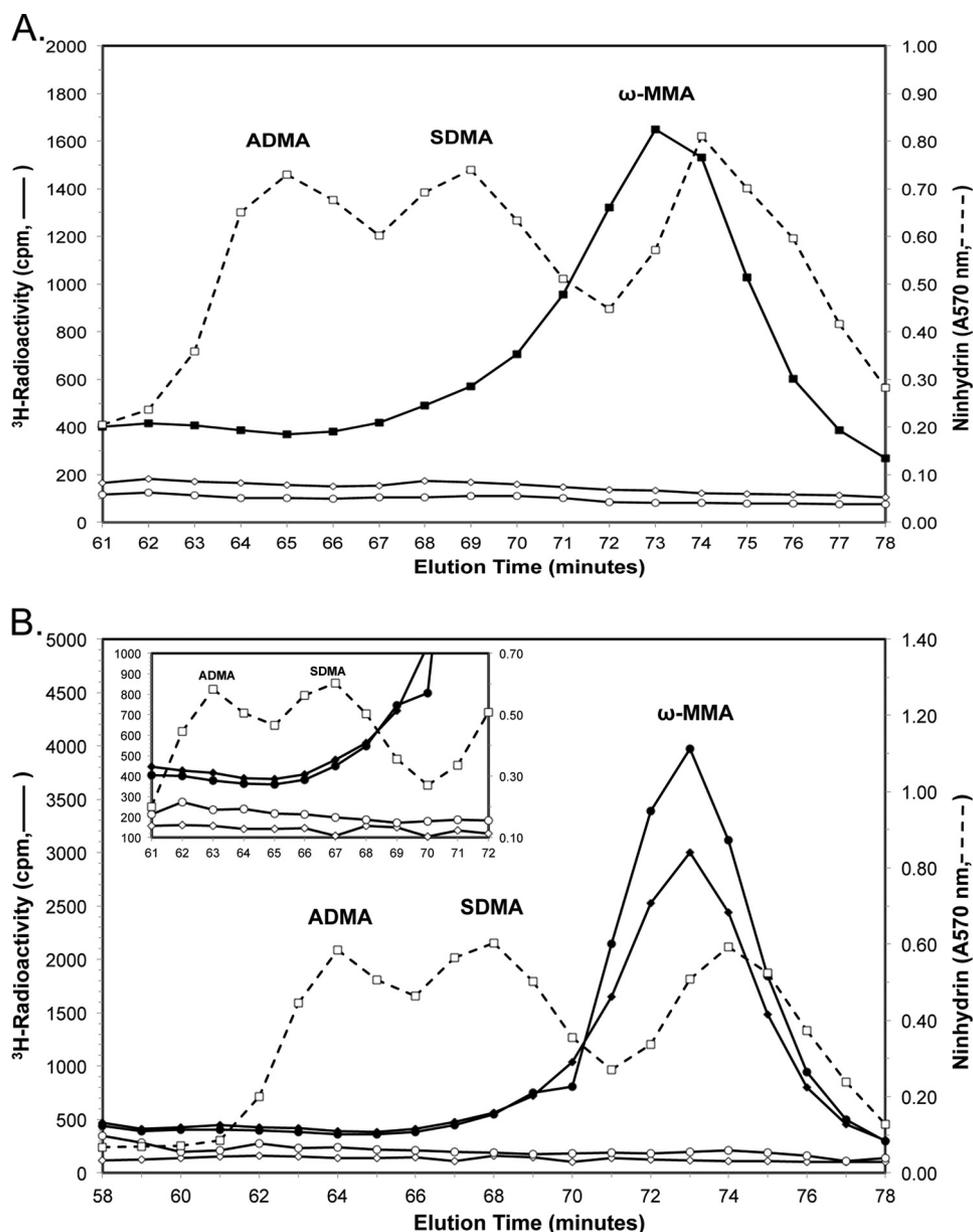


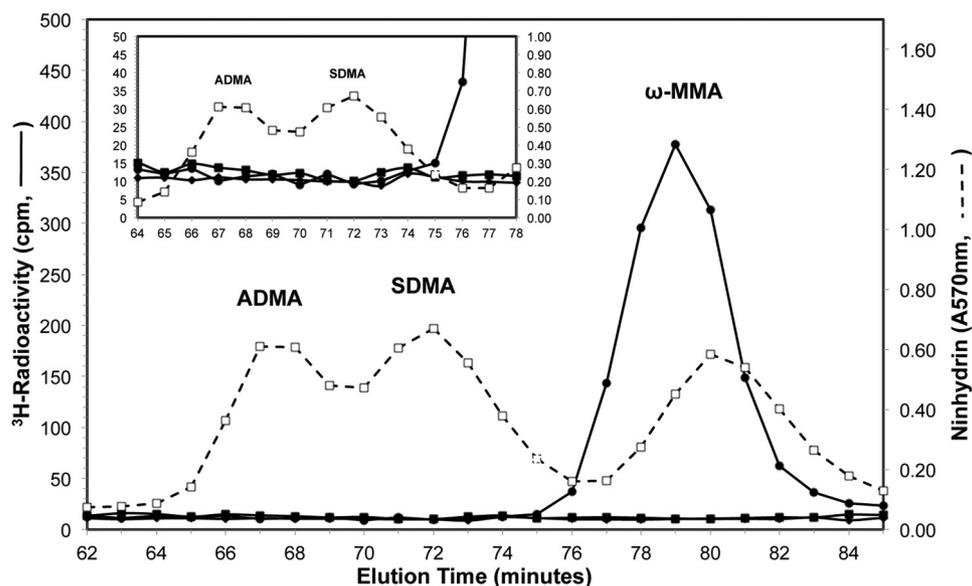
FIGURE 2. GST-PRMT7 can monomethylate peptide R1 (GGFGGRGGFGG-amide). *In vitro* methylation reactions in a final reaction volume of 60 μl were carried out with GST-PRMT7 (6 μg ; 0.95 μM) in the presence of 0.7 μM [^3H]AdoMet with peptide substrate R1 (52 μg ; 1.0 mM) as described under "Experimental Procedures." After acid hydrolysis, methylated amino acid derivatives were analyzed by high-resolution cation-exchange chromatography as described under "Experimental Procedures." Radioactivity (solid lines) from a 1- (\blacksquare , panel A), 5- (\blacklozenge , panel B), and 20-h (\bullet , panel B) incubation is shown for complete reaction mixtures. Control incubations were also performed in the absence of peptide substrate (\circ) or PRMT7 enzyme (\diamond) for 1 (panel A) or 20 h (panel B). Radioactivity and ninhydrin color of the methylated arginine standards (100 μl analyzed; \square , dashed lines) were determined as described under "Experimental Procedures." Due to a tritium isotope effect, the ^3H -methyl derivatives of ADMA, SDMA, and ω -MMA elute on high-resolution cation-exchange chromatography columns slightly earlier than the non-isotopically labeled standards (22, 26, 30, 31, 32). The inset in panel B shows a magnification of the radioactivity in the elution region of ADMA and SDMA.

of forming ADMA in protein substrates (22). We confirmed that only marginal ADMA formation with GST-PRMT1 and the R1 peptide could be detected using our own reaction conditions described above (supplemental Fig. S2). Therefore, we tested the ability of GST-PRMT7 to methylate a 28-residue fragment of the splicing protein SmD3 (P-SmD3). This longer peptide was used by Lee *et al.* (20) to provide evidence that SDMA and ADMA could be formed after 16 h of incubation with 0.1–1 μg of PRMT7 and 5 μg of peptide P-SmD3. We further extended the assay by incubating 8.4 μg of this peptide for 20 h with a much larger amount of GST-PRMT7 (4 μg) (Fig.

3A). Although we found a substantial peak of radioactivity in the expected position of [^3H] ω -MMA, we detected no incorporation of radioactivity in the positions expected for [^3H]ADMA and [^3H]SDMA. Negative control reactions in the absence of GST-PRMT7 or the P-SmD3 peptide did not show incorporation of radioactivity into arginine residues (Fig. 3A). A positive control using GST-PRMT1 showed that peptide P-SmD3 can be readily dimethylated to form [^3H]ADMA (as well as [^3H] ω -MMA) (Fig. 3B).

To determine whether arginine residues in a full-length protein could be dimethylated by PRMT7, purified spliceosomal

A. PRMT7



B. PRMT1

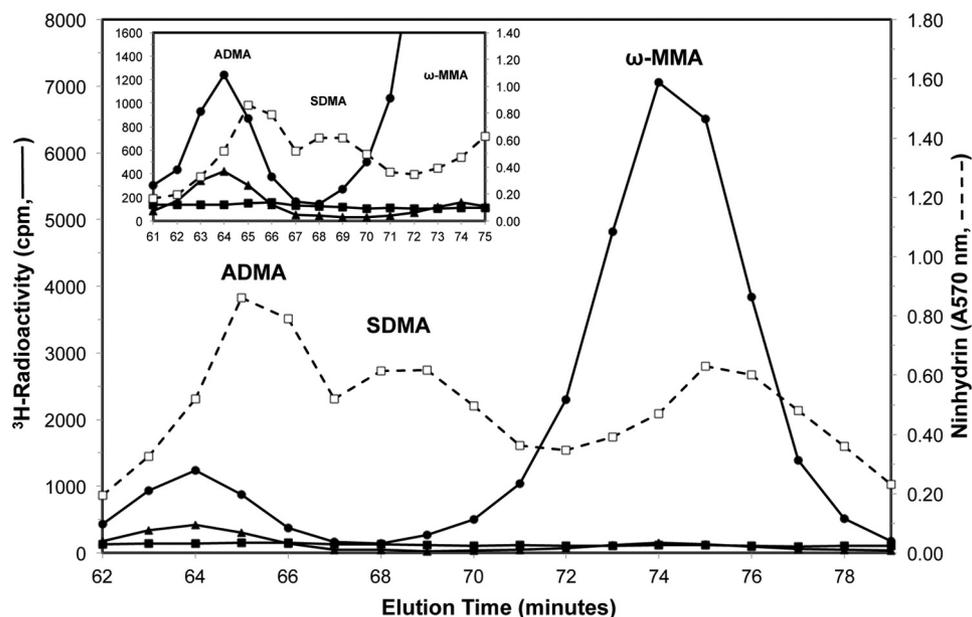


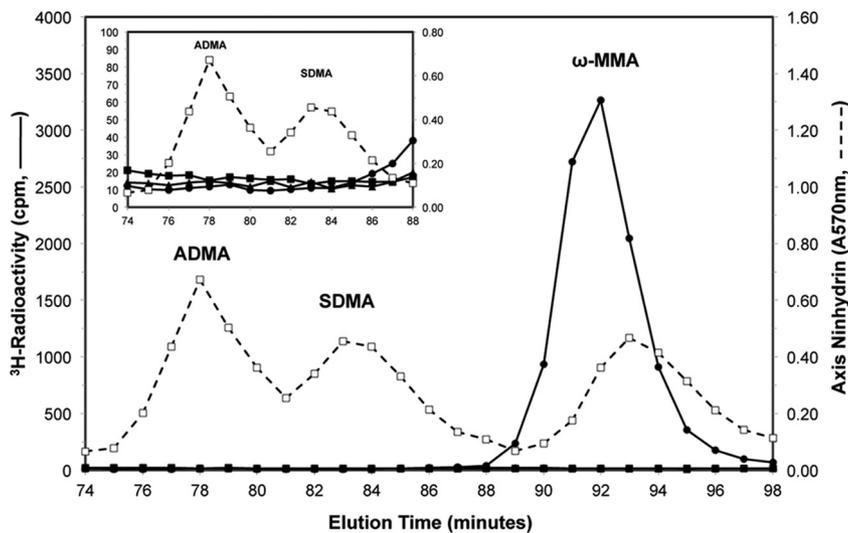
FIGURE 3. **GST-PRMT7 fails to dimethylate peptide P-SmD3.** *In vitro* methylation reactions were carried out for 20 h in a final volume of 60 μ l with the substrate P-SmD3 (AGRGRGKAAILKAQVAARGRGRGMGRGN-amide) (8.4 μ g; 50 μ M) in the presence of 0.7 μ M [3 H]AdoMet and GST-PRMT7 (4 μ g; 0.63 μ M) (●, panel A) or GST-PRMT1 (4 μ g; 0.98 μ M) (●, panel B) as described under "Experimental Procedures." Enzyme alone controls (▲, PRMT7, panel A, or ▲, PRMT1, panel B), and substrate alone controls (■, peptide P-SmD3) were carried out under the same conditions. Chromatography of the hydrolyzed reaction products was carried out as described under "Experimental Procedures" using 100 μ l for ninhydrin analysis of the standards (□, dashed lines). Radioactivity is shown by solid lines. The inset in both panels shows a magnification of the radioactivity in the elution region of ADMA and SDMA.

protein complex SmB/D3 was tested as a methyl-accepting substrate. Indirect evidence has been presented for a role of PRMT7 in the modification of these proteins (8). We incubated GST-PRMT7 with protein SmB/D3 and [3 H]AdoMet and analyzed the products after acid hydrolysis by high resolution cation-exchange chromatography (Fig. 4A). Although we observed robust formation of [3 H] ω -MMA, we detected no formation of [3 H]ADMA or [3 H]SDMA, even under conditions where a peak corresponding to just 0.3% of the [3 H] ω -MMA peak could have readily been detected if it was present (see inset

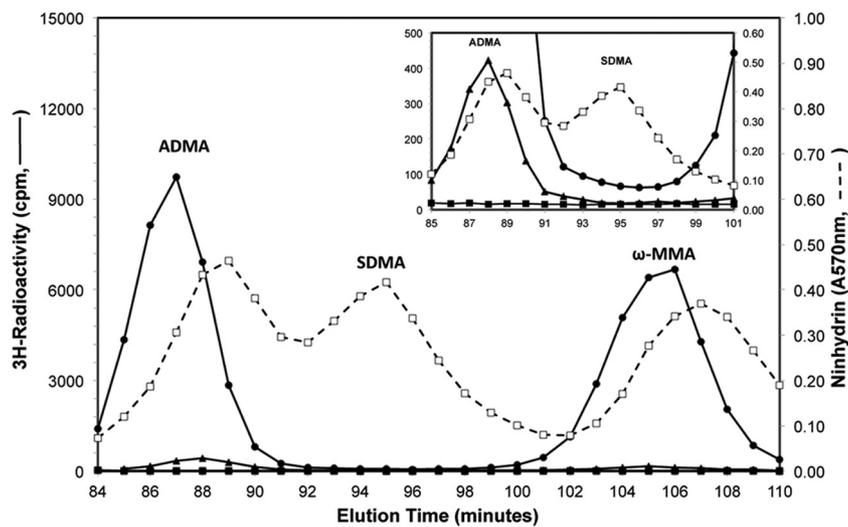
of Fig. 4A). Positive controls were performed in which GST-PRMT1 or Myc-PRMT5 was substituted for GST-PRMT7 under the same incubation conditions. We found that GST-PRMT1 catalyzed the formation of [3 H]ADMA as the major product (Fig. 4B), and that Myc-PRMT5 catalyzed a significant production of [3 H]SDMA (Fig. 4C). Thus, under conditions where ADMA and SDMA are formed by PRMT1 and PRMT5, respectively, only ω -MMA is formed with PRMT7.

To determine whether substrate concentration plays a role in the formation of ADMA or SDMA, methylation reac-

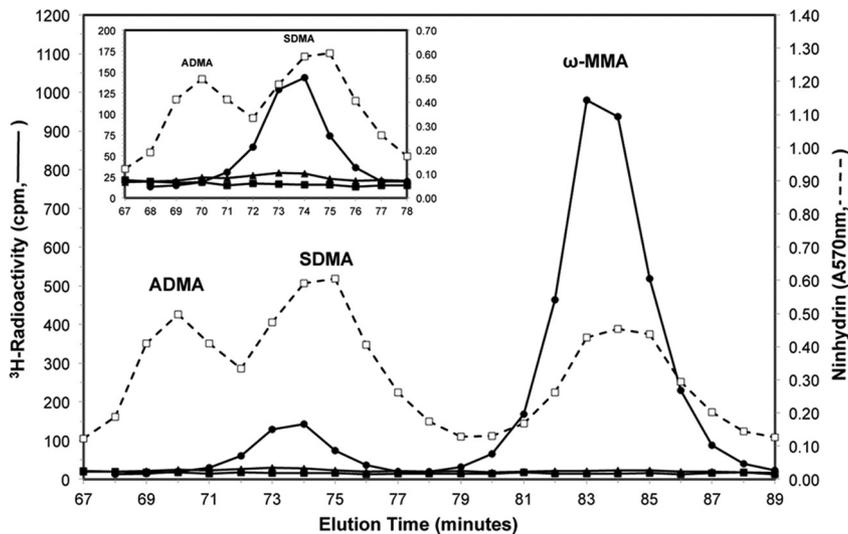
A. PRMT7



B. PRMT1



C. PRMT5



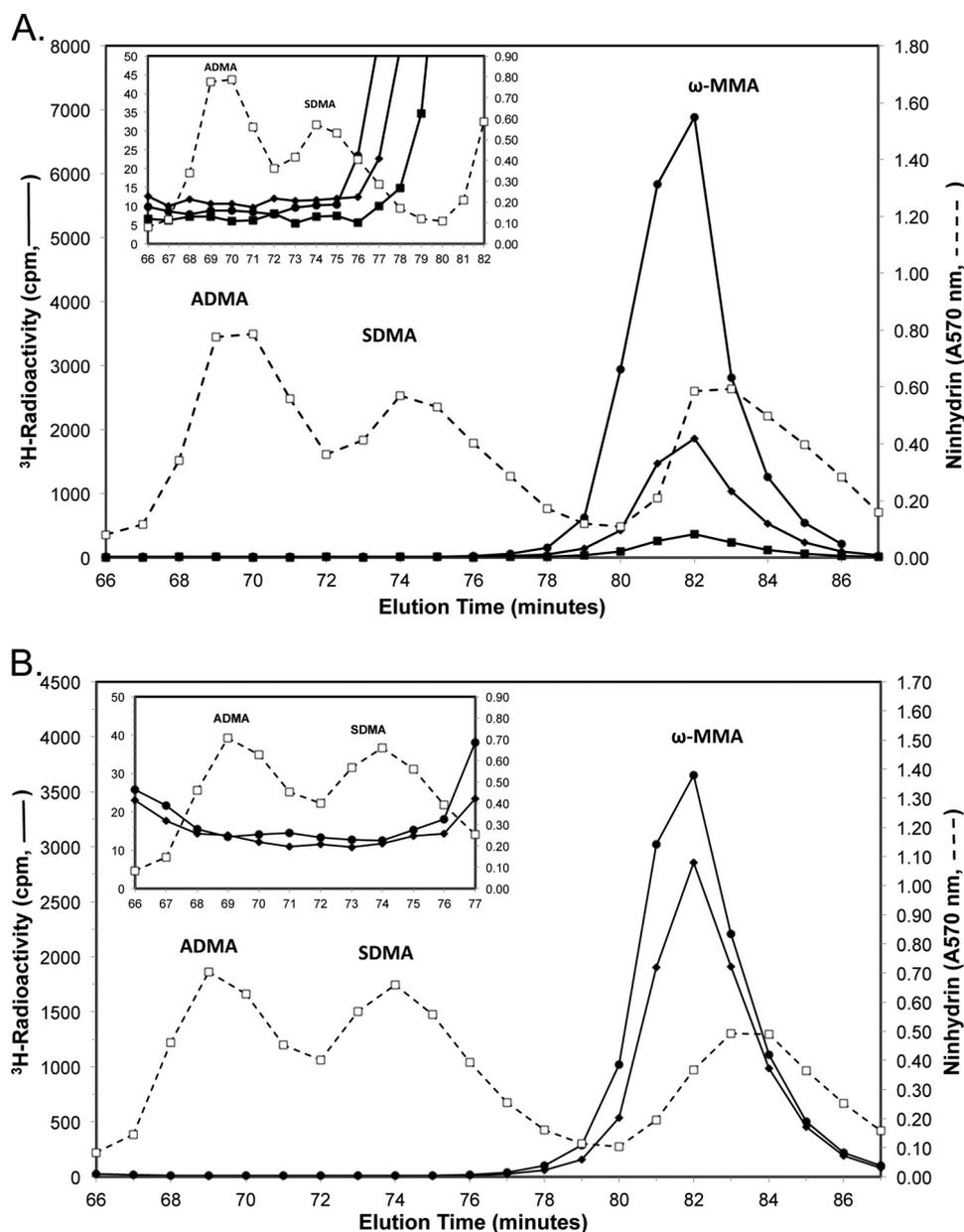


FIGURE 5. GST-PRMT7 fails to form ADMA or SDMA with increased substrate concentrations. *In vitro* methylation reactions (60 μ l) of GST-PRMT7 (2 μ g; 0.32 μ M) with peptide P-SmD3 (1 μ g (■), or 5 μ g (◆), or 50 μ g (●) (6.0, 30, and 300 μ M, respectively)) were carried out for 21 h in the presence of 0.7 μ M [3 H]AdoMet as described under "Experimental Procedures" (panel A). *In vitro* methylation reactions of GST-PRMT7 (2 μ g; 0.32 μ M) with peptide P-SmD3 (8.3 μ g; 50 μ M) were performed for 21 h in the presence of either 0.7 μ M [3 H]AdoMet (◆) or 1.4 μ M [3 H]AdoMet (●) (panel B). Chromatography of the hydrolyzed reaction products was carried out as described under "Experimental Procedures" using 100 μ l for ninhydrin analysis of the standards (□, dashed lines). The insets show a magnification of the radioactivity in the elution region of ADMA and SDMA.

tions were carried out with GST-PRMT7 and various concentrations of the peptide P-SmD3. Increasing the peptide concentration by 5- and 50-fold greatly increased the production of [3 H] ω -MMA but failed to produce any dimethylated product (Fig. 5A). To determine whether the methyl donor AdoMet was limiting, we doubled the concentration of [3 H]AdoMet to 1.4 μ M but again saw no evidence for

[3 H]ADMA or [3 H]SDMA under conditions where we could detect these compounds at 0.1% of the level of the [3 H] ω -MMA formed (Fig. 5B, inset). In an independent experiment, we measured [3 H] ω -MMA formation with concentrations of [3 H]AdoMet ranging from 0.35 to 2.1 μ M. We found approximate half-maximal methylation at the lowest concentration (supplemental Fig. S3), consistent with K_m values of 0.35 and

FIGURE 4. GST-PRMT7 fails to dimethylate protein complex SmB/D3. *In vitro* methylation reactions were carried out for 1 h in a final volume of 60 μ l with the protein substrate SmB/D3 (7 μ g; 5.8 μ M) in the presence of GST-PRMT7 (15 μ g; 2.4 μ M) (●, panel A), GST-PRMT1 (2 μ g; 0.49 μ M) with 7.5 μ g of SmB/D3 (5.4 μ M) (●, panel B) or Myc-PRMT5 (4 μ g; 0.81 μ M) with 13 μ g of SmB/D3 (10.8 μ M) (●, panel C) as described under "Experimental Procedures." Enzyme alone controls (▲) and substrate alone controls (■) were carried out under the same conditions for each enzyme. Chromatography of the hydrolyzed reaction products was carried out as described under "Experimental Procedures" using 100 μ l for ninhydrin analysis of the standards (□, dashed lines). The insets show a magnification of the radioactivity in the elution region of ADMA and SDMA.

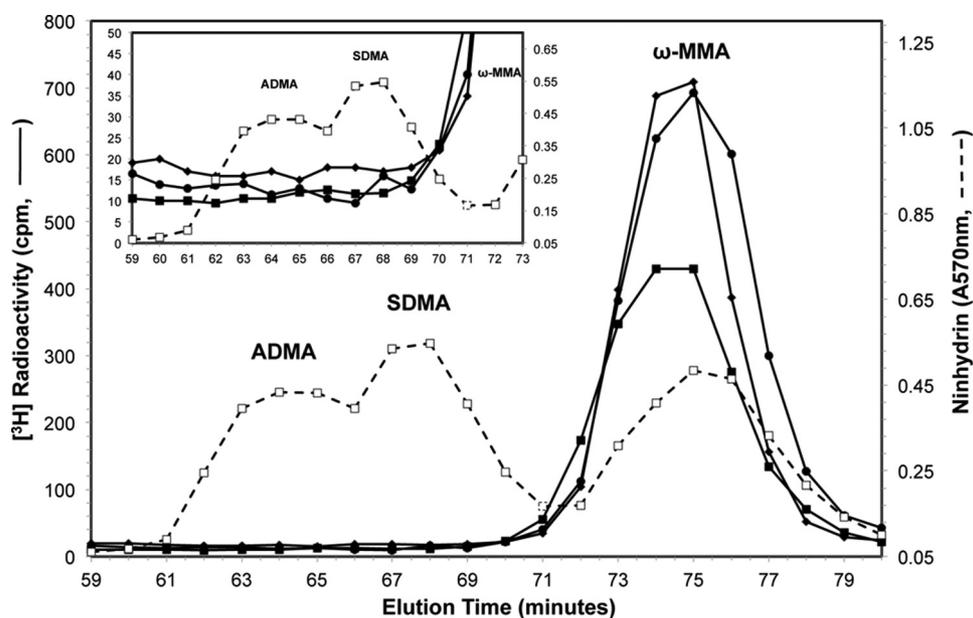


FIGURE 6. **GST-PRMT7 can methylate histone H2B producing only ω -MMA.** *In vitro* methylation reaction of GST-PRMT7 (15 μ g; 2.4 μ M) with histone H2B (10 μ g; 12.1 μ M) was carried out for 1 (■), 5 (◆), and 25 h (●) in a final volume of 60 μ l. Chromatography of the hydrolyzed reaction products was carried out as described under "Experimental Procedures" using 50 μ l for ninhydrin analysis of the standards (□, dashed lines). The inset shows a magnification of the radioactivity in the elution region of ADMA and SDMA.

TABLE 1
Peptide substrates for human GST-PRMT7

	Substrates	Methylation product	Reference
Peptide R1	GGFGGRGGFG-amide	ω -MMA	Fig. 2 and Ref. 19
Peptide R2	Acetyl-GGRGG-amide	ω -MMA	Supplemental Fig. S5A and Ref. 19
Peptide M1	SGRGKGGKGLGKGGAKRHRK-amide	ω -MMA	Supplemental Fig. S5B
Peptide MM	SGR ^{Me} GKGGKGLGKGGAKRHRK-amide (R ^{Me} , monomethylarginine)	ω -MMA	Supplemental Fig. 5C
Peptide Smd3	AGRGRGKAAILKAQVAARGRGRGMGRGN-amide	ω -MMA	Figs. 3 and 5 and supplemental Figs. S1 and S3
Non-substrates			
Peptide M8	SGAGKGGKGLGKGGAKAHAK-amide	None detected	Data not shown
Peptide GRG	GRG-amide	None detected	Data not shown

0.30 μ M previously measured for GST-PRMT1 and GST-(Δ 1–60)PRMT8, respectively (29).

Histone proteins are common substrates for PRMTs, and specific evidence has been presented for histone methylation by PRMT7 (6). To determine whether PRMT7 could dimethylate an arginine residue in a histone protein, methylation reactions were carried out at 1, 5, and 20 h using GST-PRMT7 with [³H]AdoMet and histone H2B. We found that PRMT7 produces only [³H] ω -MMA; neither [³H]ADMA nor [³H]SDMA was observed (Fig. 6). To address the possibility that the presence of the GST domain may change the specificity of PRMT7, we measured product formation of a thrombin-cleaved preparation. Once again, only [³H] ω -MMA was formed (supplemental Fig. S4).

Finally, we tested GST-PRMT7 with each of the remaining peptide and protein substrates previously observed by Lee *et al.* (20) to form ADMA and SDMA when methylated by PRMT7. In supplemental Fig. S5, we show that ADMA and SDMA are not formed in peptides R2, M1, or MM. These results are summarized in Table 1. Furthermore, under our conditions the protein substrates histone H2A, histone H3, histone H4, myelin basic protein, and GST-GAR, when incubated with [³H]AdoMet and GST-PRMT7, acquired ω -MMA but not ADMA or SDMA residues (supplemental Fig. S6).

Taken together, these results show that GST-PRMT7 has type III protein arginine methyltransferase activity because it monomethylates peptides and proteins to form ω -MMA but is not capable of adding a second methyl group to ω -MMA to form ADMA or SDMA. In this respect PRMT7 now differs from the known type I family members that form ADMA (PRMT1, PRMT2, PRMT3, CARM1/PRMT4, PRMT6, and PRMT8) and the known type II family member PRMT5 that forms SDMA. Although it is possible that a substrate yet to be identified can be dimethylated by PRMT7, it is also possible that the structural makeup of this enzyme may preclude such a reaction.

DISCUSSION

Our results supporting the type III catalytic activity of human PRMT7 are consistent with recent studies involving apparent orthologs of this protein in the protozoan *T. brucei* (18) and the nematode worm *Caenorhabditis elegans* (33). *In vitro* studies using several different protein substrates showed that these methyltransferases, like human PRMT7, only produce ω -MMA (18, 33). The identification of type III activity in these divergent eukaryotes indicates that the ω -MMA modification is not only an intermediate on a pathway to ADMA and SDMA formation. Rather, the ω -MMA

TABLE 2

Recognition by mammalian PRMTs of the protein methyl-accepting substrates utilized in this study (+, methylation observed; -, methylation not observed; representative references are in parentheses)

Protein Substrate	PRMT7	PRMT1	PRMT2	PRMT3	PRMT4/ CARM1	PRMT5	PRMT6	PRMT8
Histone H2A	+ This Study, Supp. Fig. 6; (6) - (19)	+ (22, 43, 44, 45)			+ (43, 59) - (45)	+ (17, 53, 63, 64) - (65, 66)	+ (45, 50)	+ (29)
Histone H2B	+ This Study, Fig. 6 and Supp. Fig. 4 - (6)	+ (43, 44) - (45)			+ (43, 45)	- (63, 65, 66)	+ (50) - (45)	
Histone H3	+ This Study, Supp. Fig. 6; (6)	+ (16, 30, 44, 46) - (43, 45)			+ (16, 30, 43-45, 47, 50, 51, 56, 59-62)	+ (66-68) - (63, 65)	+ (16, 45-47, 50, 62, 72)	- (29, 30)
Histone H4	+ This Study, Supp. Fig. 6; (6)	+ (16, 30, 43-52)	+ (16)		+ (16, 30, 43) - (45, 59)	+ (62-68)	+ (16, 45, 46, 50)	+ (29, 30)
Myelin basic protein (MBP)	+ This Study, Supp. Fig. 6 - (19)	+ (29, 53) - (22)			+ (30)	+ (17, 44, 53, 62, 63, 67-69)		+ (29)
SmB/D3	+ This Study, Fig. 4; (8)	+ This Study, Fig. 4			+ (56)	+ This Study, Fig. 4; (8, 68, 70, 71)		
GST-GAR	+ This Study, Supp. Fig. 6 - (19)	+ (16, 23, 28-30, 44, 52-55)	+ (16)	+ (23, 28, 44, 47, 52, 55-58)	- (30, 52, 54)	+ (17, 53, 68)	+ (28, 44, 52, 54, 56)	+ (29, 30, 44)

residue may be recognized as a signal in itself, perhaps as a "methyl mark" by protein interaction domains similar to the Tudor domain that recognizes ADMA (34) and SDMA residues (35) and the BRCT domain that recognizes ADMA (36). These results also indicate that the only member of the mammalian PRMT family with established type II activity forming SDMA is PRMT5 (17).

The structural basis for the inability of PRMT7 to further methylate peptide substrates with MMA is unknown. However, we note that there are two conserved amino acid differences between PRMT7 and the known type I and type II enzymes in the double-E and THW loops in the active site (19, 37). The replacement of a conserved glycine residue in the double-E loop and/or the replacement of the threonine residue in the THW loop with aspartyl residues in PRMT7 may limit the ability of the enzyme to productively bind MMA residues for a second methylation reaction. Additionally, we note that PRMT7, in common with the type I PRMTs, lacks the conserved phenylalanine residue in the pre-motif I region that has been shown to be important in PRMT5 specificity for SDMA (38).

Despite the improved enzymatic activity of human GST-PRMT7 in this study, and despite using a wide variety of reaction conditions and substrates, we were unable to confirm the previous report that human GST-PRMT7 could

dimethylate protein and peptide substrates to produce either ADMA or SDMA (20). In our study, we utilized all of the potential methyl-accepting peptide and protein substrates tested by Lee *et al.* (20) using similar incubation conditions. There are two possible explanations for these discrepancies. In the first, the FLAG-tagged PRMT7 preparation previously used may have been contaminated with endogenous PRMT enzymes present in the mammalian HeLa and COS cells used for its expression. The Sigma anti-FLAG M2 monoclonal antibody used by Lee *et al.* (20) to purify FLAG-tagged PRMT7 also immunoprecipitates endogenous PRMT5 that catalyzes SDMA formation (21, 39). Thus, the SDMA forming activity that Lee *et al.* (20) found for FLAG-PRMT7 may actually have resulted from the activity of endogenous PRMT5; contamination with other PRMTs may also explain the low levels of ADMA formation reported. Furthermore, using anti-FLAG M2 antibody to enrich for FLAG-tagged proteins may have compromised other studies such as the identification of FBXO11 as a type II methyltransferase (40) (*cf.* Ref. 41). A second explanation for the apparent formation of SDMA and ADMA with both the FLAG-tagged and GST fusion PRMT7 preparations is in the identification of these amino acid derivatives by TLC. In our experience, the use of TLC to analyze radiolabeled methylation products is inherently low resolution and can be compromised by the

presence of [³H]AdoMet breakdown products, especially when the peptide and protein products are not separated before analysis and when the standards are not mixed with the radiolabeled reaction products prior to chromatography.

In this study, we characterized the radiolabeled methylation products by high-resolution cation-exchange chromatography on sulfonated polystyrene spherical resins. This methodology allows for good separation of ω-MMA, ADMA, and SDMA without the need of derivatization or the need for analysis of breakdown products. Moreover, the resolution is so high that partial separation can be observed between the tritiated methylated arginine residues and the nonradiolabeled methylated standards. As confirmed in our experiments, tritiated methylated arginine derivatives reproducibly elute 1–2 min earlier due to a tritium isotope effect (22, 26, 30–32, 42). Unlike TLC, cation-exchange chromatography allows for large amounts of material to be separated without loss of resolution, providing for greater sensitivity in detecting methylated product.

We recognize, however, the possibility that PRMT7 may be able under some conditions to further modify ω-MMA to produce higher methylated species. Although we have used a wide variety of methyl-accepting substrates, one or more particular substrates may be found that would allow multiple methylation of arginine residues. It is also possible that proteins or other factors present in mammalian cells may affect the product specificity of PRMT7. There are a number of reports of interactions of distinct PRMT species (4). Specifically, a synergistic relationship between PRMT5 and PRMT7 has been suggested (8), although no evidence for a direct interaction has been found. We should emphasize, however, that we have found no evidence for any formation of SDMA by the bacterially expressed GST-PRMT7 fusion protein in this study that would justify a type II designation for this enzyme. Additionally, in preliminary experiments, we have purified a green fluorescent protein-human PRMT7 fusion protein expressed in human HEK293 cells and have not detected any SDMA formation using GST-GAR, P-SmD3, and MBP as substrates (data not shown). Finally, we find no SDMA formation in a commercial preparation of an N-terminal His-FLAG-tagged human PRMT7 that had been expressed in insect Sf9 cells (BPS Bioscience Inc., San Diego, CA, catalogue number 51054) with GST-GAR, P-SmD3, and histones H2A and H2B as substrates (data not shown).

In Table 2, we compare the specificities of members of the human PRMT family for the methyl-accepting protein substrates used in this study. Importantly, the substrates found to be methylated by PRMT7 in this study also appear to be substrates for PRMT1, -4, and -6. The PRMT2, -3, and -8 enzymes also share specificity with PRMT7 for GST-GAR, but have apparently not been tested with the other substrates. The only clear difference between PRMT7 and the other family members is that PRMT5 has not been shown to recognize histone H2B in several studies (Table 2). Thus, although PRMT7 cannot form dimethylated arginine derivatives, it can methylate many of the substrates recognized by the other members of the PRMT family.

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SUPPLEMENTAL MATERIALS FOR:

Human Protein Arginine Methyltransferase 7 (PRMT7) is a Type III Enzyme Forming ω - N^G -Monomethylated Arginine Residues

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SUPPLEMENTAL FIGURE 1: Addition of 1 mM DTT increases GST-PRMT7 activity. *In vitro* methylation reactions (30 μ l) of GST-PRMT7 (2 μ g) with peptide P-SmD3 (8.4 μ g) were carried out with [3 H]AdoMet as described in “Experimental Procedures” in the presence (●) or absence (◆) of 1 mM DTT for 1 h. The enzyme alone control (○) also contained 1 mM DTT. After hydrolysis, methylated amino acid derivatives were analyzed by high resolution cation-exchange chromatography as described in “Experimental Procedures.” Radioactivity (solid lines) and ninhydrin color of the methylated arginine standards (50 μ l aliquots of 1 ml fraction; □, dashed lines) were analyzed as described in “Experimental Procedures.” The inset shows a magnification of the radioactivity in the elution region of ADMA and SDMA.

SUPPLEMENTAL FIGURE 2: GST-PRMT1 can dimethylate peptide R1 producing ADMA. *In vitro* methylation reaction of GST-PRMT1 (2 μ g) with peptide R1 (52 μ g) was carried out for 1 h (◆) in a final reaction volume of 60 μ l. Chromatography of the hydrolyzed reaction products was carried out as described in “Experimental Procedures” using 50 μ l for ninhydrin analysis of the standards (□, dashed lines). Radioactivity is shown in solid line. The inset shows a magnification of the radioactivity in the elution region of ADMA and SDMA.

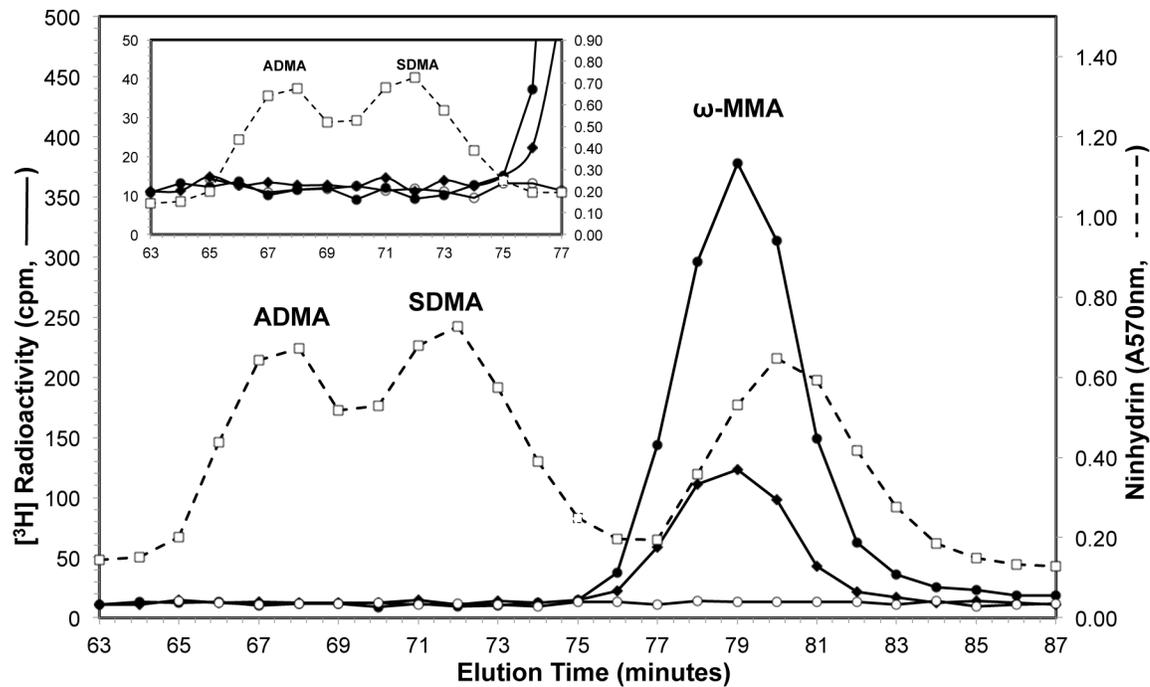
SUPPLEMENTAL FIGURE 3: GST-PRMT7 forms only MMA at various concentrations of AdoMet. *In vitro* methylation reactions (60 μ l) of GST-PRMT7 (2 μ g) with peptide P-SmD3 (8.4 μ g) were carried out for 1 h in the presence of either 0.35 μ M [3 H]AdoMet (panel A), 0.7 μ M [3 H]AdoMet (panel B), 1.4 μ M [3 H]AdoMet (panel C), or 2.1 μ M [3 H]AdoMet (panel D). After hydrolysis, methylated amino acid derivatives were analyzed by high resolution cation-exchange chromatography as described in “Experimental Procedures.” Radioactivity (950 μ l aliquots of 1 ml fractions; ◆, solid lines) and ninhydrin color of the methylated arginine standards (50 μ l aliquots of 1 ml fractions; ○, dashed lines) were analyzed as described in “Experimental Procedures.”

SUPPLEMENTAL FIGURE 4: Thrombin cleavage of GST-PRMT7 does not alter product specificity. Two samples of GST-PRMT7 were incubated in 50 μ l phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 100 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) in the presence (panel A) or absence (panel B) of thrombin (50 U thrombin from GE Healthcare in the same solution) for 5 h at 4 °C. Aliquots of 3 μ g protein were used to perform *in vitro* methylation reactions for 1 h with histone H2B (10 μ g) in a final reaction volume of 30 μ l. Chromatography of the hydrolyzed reaction products was carried out as described in “Experimental Procedures.” Radioactivity (950 μ l aliquots of 1 ml fractions; ◆, solid lines) and ninhydrin color of the methylated arginine standards (50 μ l aliquots of 1 ml fractions; ○, dashed lines) were analyzed as described in “Experimental Procedures.”

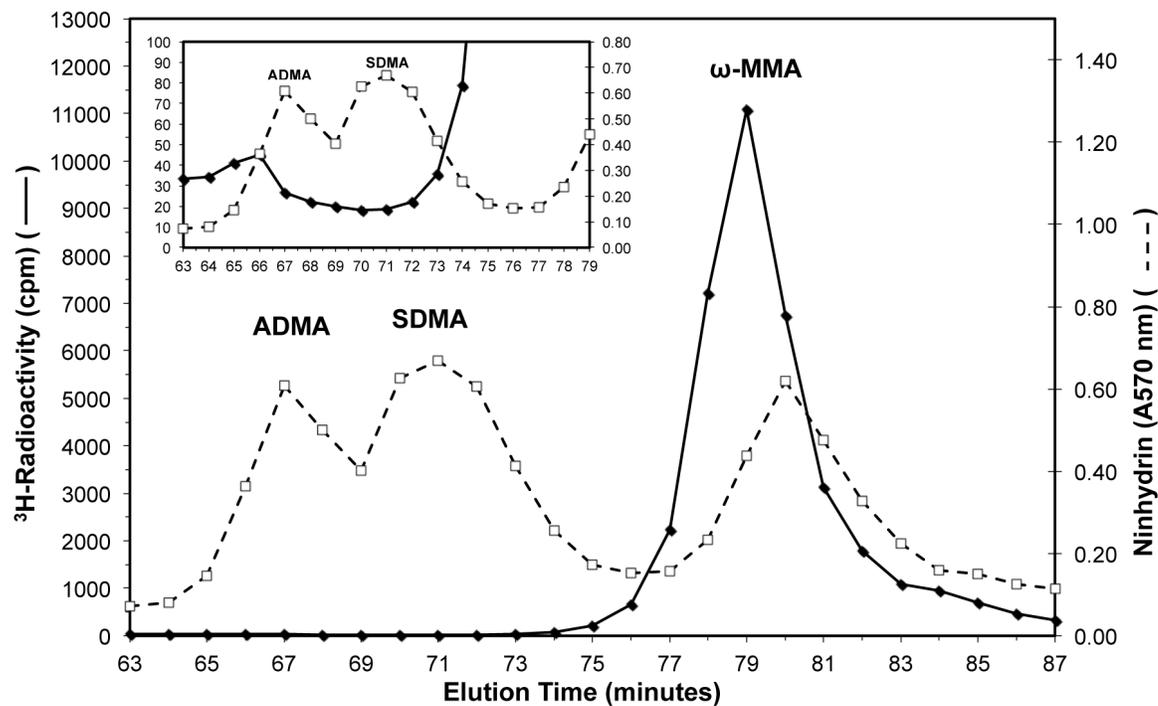
SUPPLEMENTAL FIGURE 5: GST-PRMT7 can monomethylate peptides R2, M1 and MM. One hour *in vitro* methylation reactions in a final volume of 60 μ l were carried out as described in “Experimental Procedures” with GST-PRMT7 (2 μ g) and the peptide R2 (26.5 μ g, panel A), or peptide M1 (10 μ g, panel B), or peptide MM (10 μ g, panel C). After acid hydrolysis and chromatography was carried out as described in “Experimental Procedures,” the elution position of the radioactive products (●, solid lines) was compared to those of methylated amino acid standards detected by ninhydrin analysis of 100 μ l of the fractions (□, dashed lines). In panel A, radioactivity and ninhydrin was measured in every other fraction. In each case, controls were performed in the absence of GST-PRMT7 (■).

SUPPLEMENTAL FIGURE 6: GST-PRMT7 can monomethylate histone proteins H2A, H3, H4 and myelin basic protein (MBP) and GST-GAR. One hour *in vitro* methylation reactions in a final volume of 60 μ l were carried out as described in “Experimental Procedures” with GST-PRMT7 (2 μ g) and histone H2A (10 μ g, panel A), histone H3 (5 μ g, panel B), histone H4 (10 μ g, panel C), MBP (10 μ g, panel D), and GST-GAR (1 μ g, panel E). After acid hydrolysis and chromatography as described in “Experimental Procedures,” the elution position of the radioactive products (●) was compared to those of methylated amino acid standards detected by ninhydrin analysis of 100 μ l of the fractions (□, dashed lines). In panel A, radioactivity and ninhydrin was measured in every other fraction. In each case, controls were performed in the absence of GST-PRMT7 (■). In panel A, a control reaction mixture lacking histone H2A is also shown (◆). In panel E, the radioactivity scale is expanded in the inset in the elution region of ADMA and SDMA.

Supplemental Figure 1

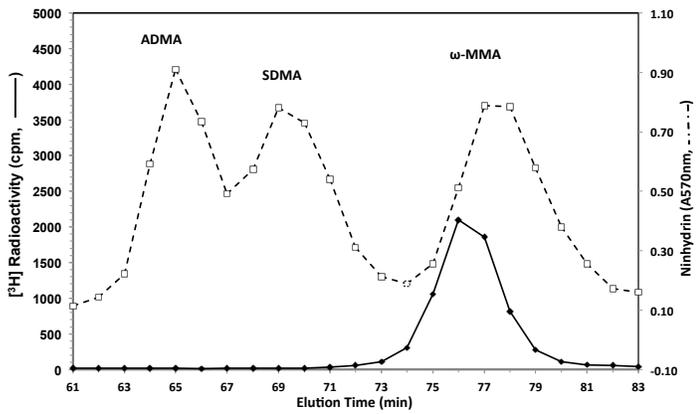


Supplemental Figure 2

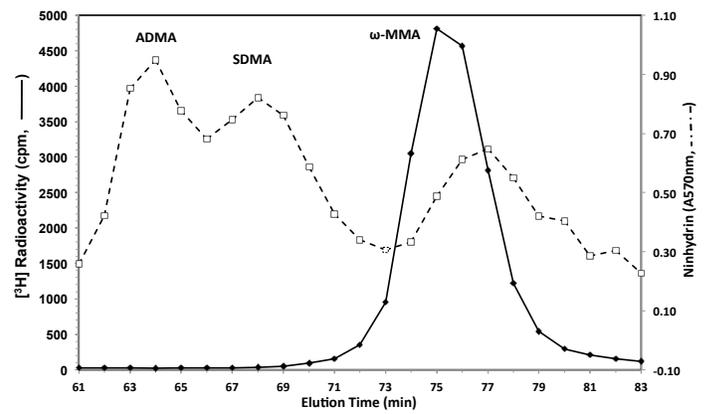


Supplemental Figure 3

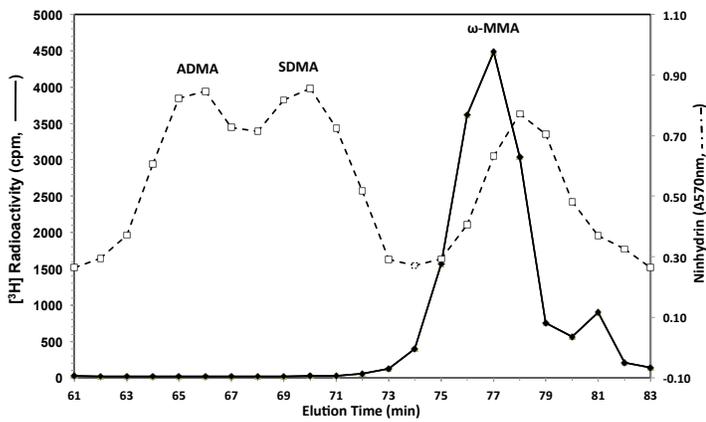
A. 0.35 μM AdoMet



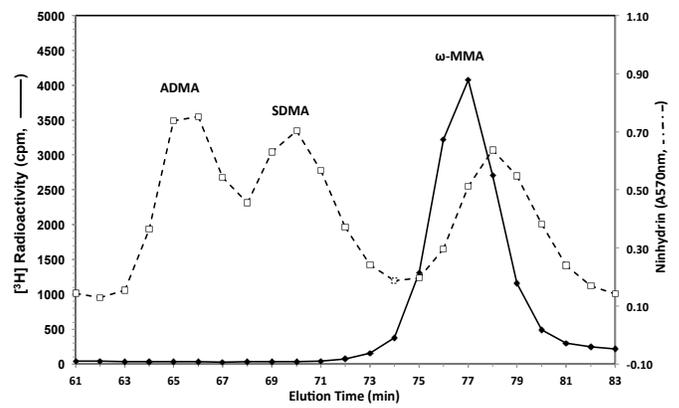
B. 0.7 μM AdoMet



C. 1.4 μM AdoMet

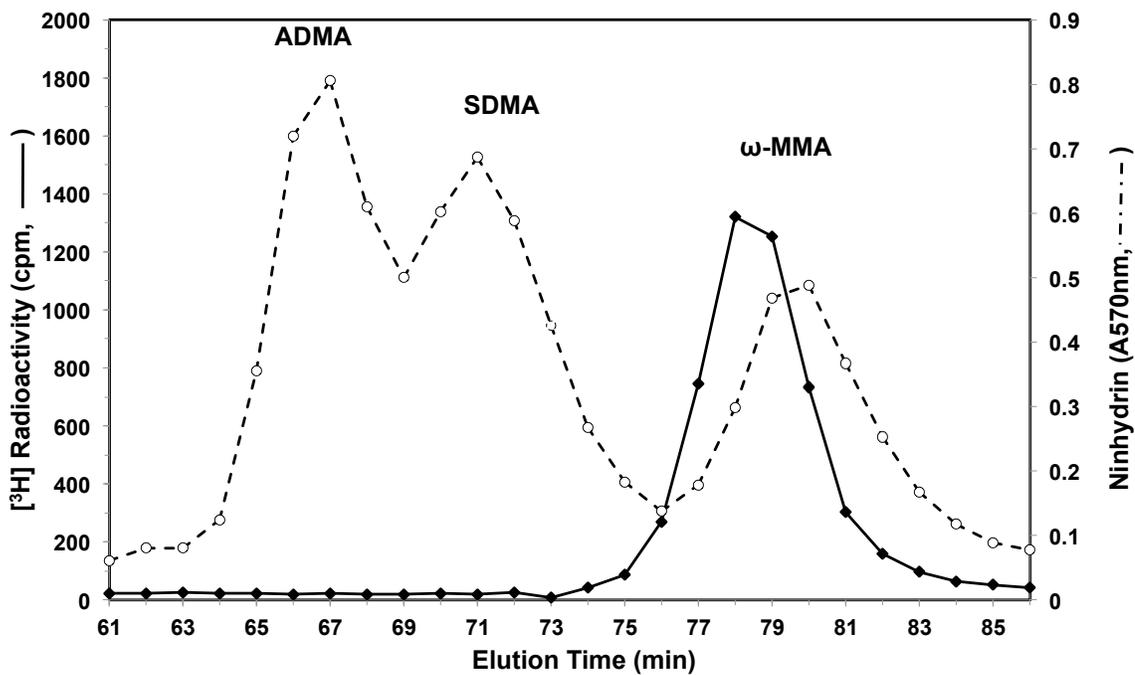


D. 2.1 μM AdoMet

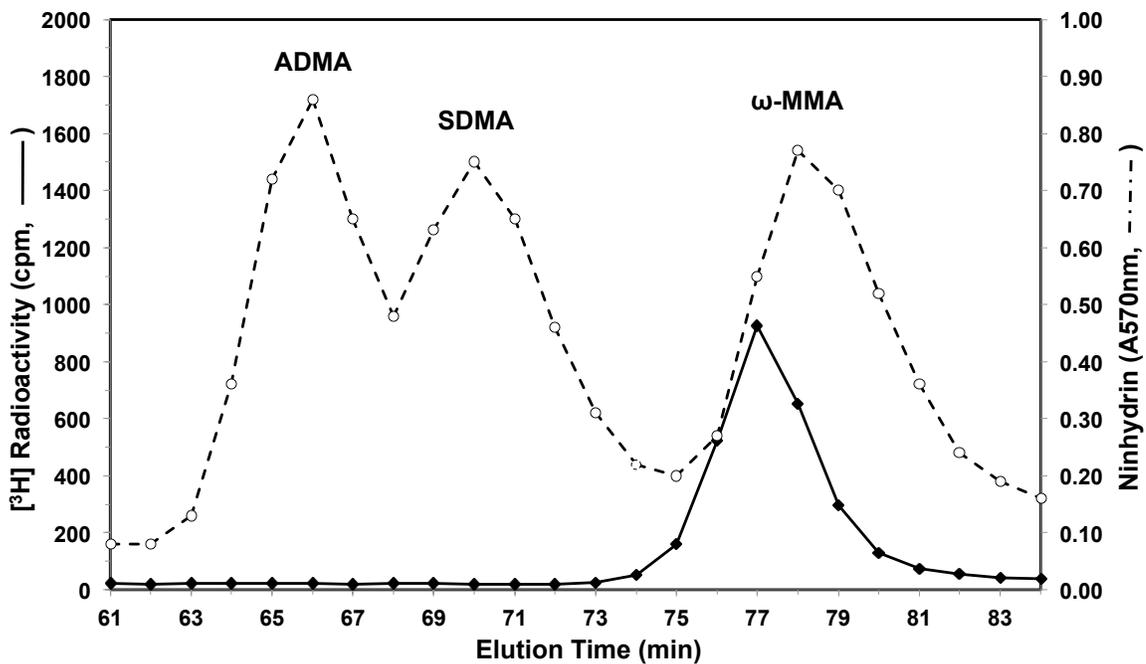


Supplemental Figure 4

A. Cleaved PRMT7

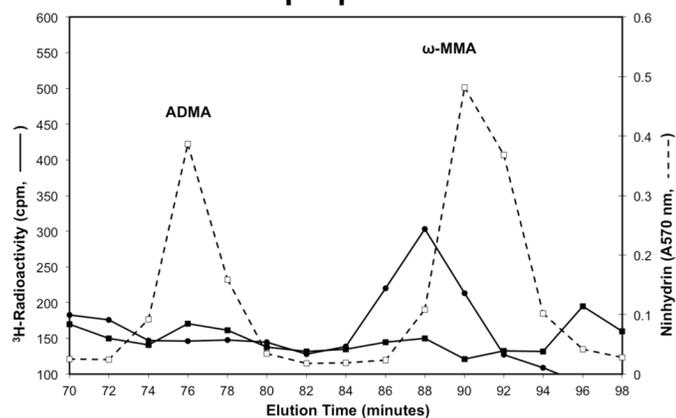


B. Non-cleaved PRMT7

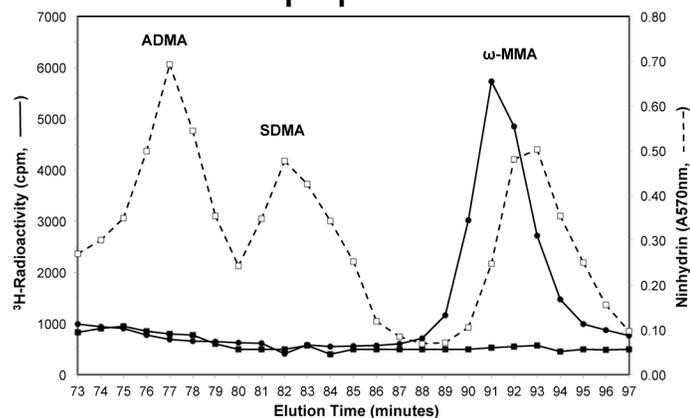


Supplemental Figure 5

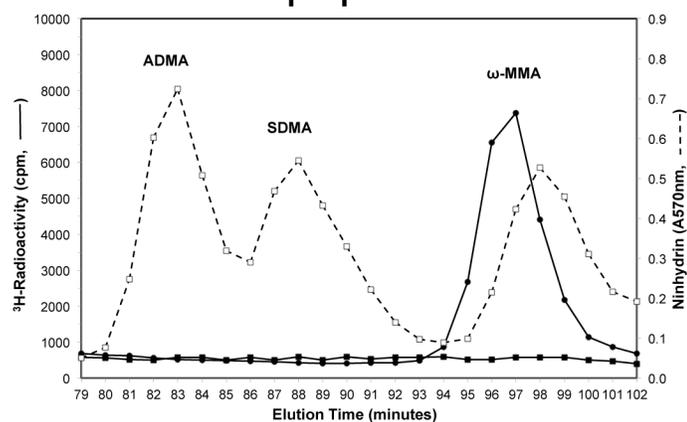
A. PRMT7 + peptide R2



B. PRMT7 + peptide M1

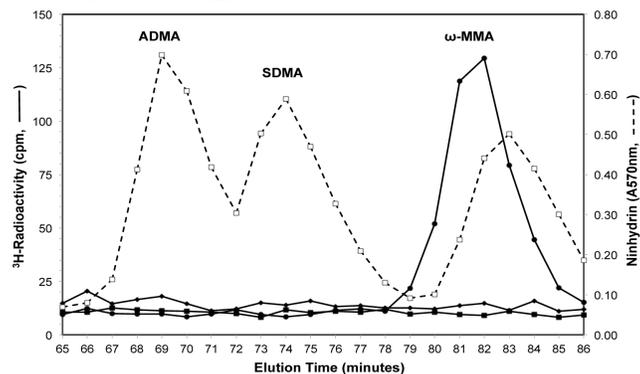


C. PRMT7 + peptide MM

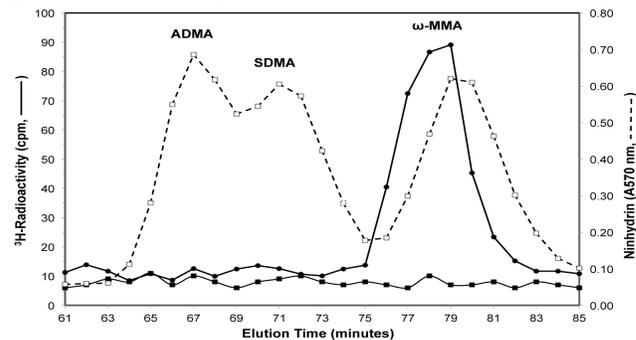


Supplemental Figure 6

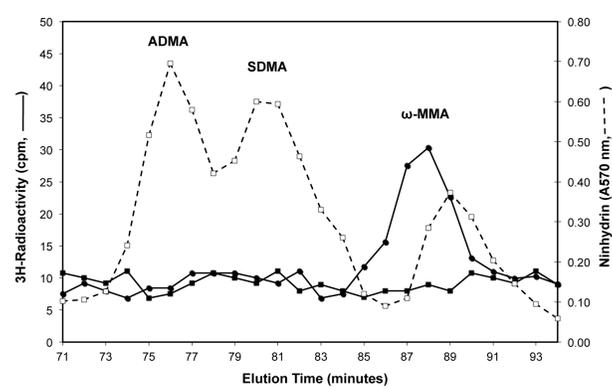
A. PRMT7 + H2A



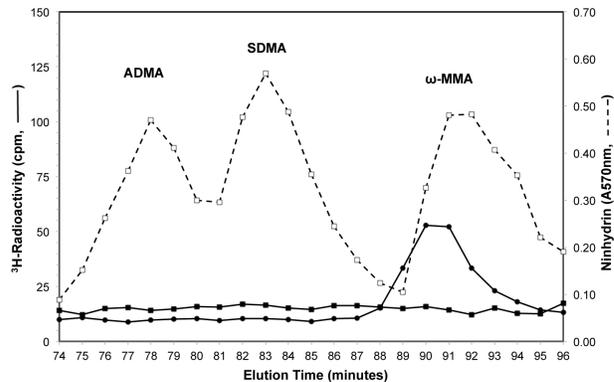
B. PRMT7 + H3



C. PRMT7 + H4



D. PRMT7 + MBP



E. PRMT7 + GST-GAR

