# Protein Arginine Methyltransferase 5 Catalyzes Substrate Dimethylation in a Distributive Fashion

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# Protein Arginine Methyltransferase 5 Catalyzes Substrate Dimethylation in a Distributive Fashion

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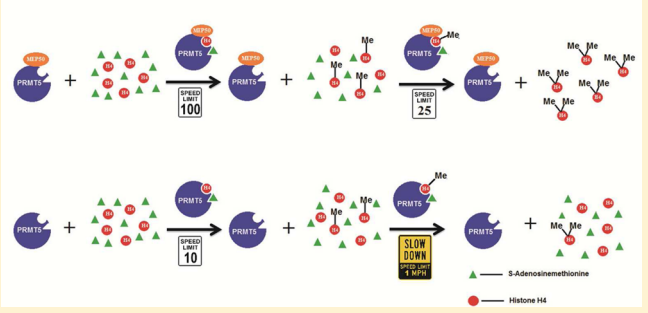
Supporting Information

**ABSTRACT:** Protein arginine methyltransferase 5 (PRMT5) is a histone-modifying enzyme whose activity is aberrantly upregulated in various cancers and thereby contributes to a progrowth phenotype. Indeed, knockdown of PRMT5 leads to growth arrest and apoptosis, suggesting that inhibitors targeting this enzyme may have therapeutic utility in oncology. To aid the development of inhibitors targeting PRMT5, we initiated mechanistic studies geared to understand how PRMT5 selectively catalyzes the symmetric dimethylation of its substrates. Toward that end, we characterized the regiospecificity and processivity of bacterially expressed *Caenorhabditis elegans* PRMT5 (cPRMT5), insect cell-expressed human PRMT5 (hPRMT5), and human PRMT5 complexed with methylosome protein 50 (MEP50), i.e., the PRMT5·MEP50 complex. Our studies confirm that arginine 3 is the only site of methylation in both histone H4 and H4 tail peptide analogues and that sites distal to the site of methylation promote the efficient symmetric dimethylation of PRMT5 substrates by increasing the affinity of the monomethylated substrate for the enzyme. Additionally, we show for the first time that both cPRMT5 and the hPRMT5·MEP50 complex catalyze substrate dimethylation in a distributive manner, which is assisted by long-range interactions. Finally, our data confirm that MEP50 plays a key role in substrate recognition and activates PRMT5 activity by increasing its affinity for protein substrates. In total, our results suggest that it may be possible to allosterically inhibit PRMT5 by targeting binding pockets outside the active site.




Histone post-translational modifications (PTMs) play essential roles in DNA transcription, replication, and repair, and are often dysregulated, in human diseases such as cancer.[1,2] Among the various histone-modifying enzymes, the protein arginine methyltransferases have emerged as interesting therapeutic targets for a range of ailments, including cardiovascular disease and multiple cancers.[1−6] In humans, the PRMTs catalyze the transfer of a methyl group from *S*-adenosylmethionine (SAM or AdoMet) to an arginine residue to generate monomethylarginine (PRMT7), asymmetric dimethylarginine (PRMT1−4, -6, and -8), and symmetric dimethylarginine (PRMT5). As the only source of SDMA (Figure 1), PRMT5 plays essential roles in gene transcription, kinase signaling, RNA splicing, DNA repair, and control of the cell cycle.[1,2,7]

PRMT5 is a particularly attractive anticancer target because this enzyme is overexpressed in cancers of the brain, breast, bladder, blood, colon, and stomach.[3,8−11] Additionally, coexpression of PRMT5 and programmed cell death 4 (PDCD4) in an orthotopic breast cancer model promotes tumor growth.[9] Importantly, siRNA knockdown of PRMT5 inhibits tumor cell growth,[8] suggesting that PRMT5 inhibitors hold therapeutic promise. How PRMT5 contributes to tumorigenesis remains unclear; however, recent studies have revealed some clues about the underlying mechanisms. For example, PRMT5 levels are elevated in human lymphoid cancer cells, and silencing of PRMT5 reduces the level of methylation of histones H3 and H4, which correlates with the increased level of expression of RBL2, an inhibitor of cellular proliferation.[11] Similar effects were observed in colon cancer cells, where Han et al. found that PRMT5 dimethylates the p65 subunit of NF-κB.[10] This modification enhances the recruitment of NF-κB to the promoter of a number of target genes (e.g., TNFα, IL1A, and TRAF1), which in turn stimulates cell proliferation.[10]

In contrast to the other PRMTs, PRMT5 functions as a complex and principally associates with methylosome protein 50 (MEP50).[2] MEP50 is thought to activate and expand the substrate scope of PRMT5[12,13] such that the hPRMT5·MEP50 complex methylates a variety of histone and non-histone proteins, including histones H2A, H3, and H4, as well as epidermal growth factor receptor (EGFR), p53, and NF-κB.

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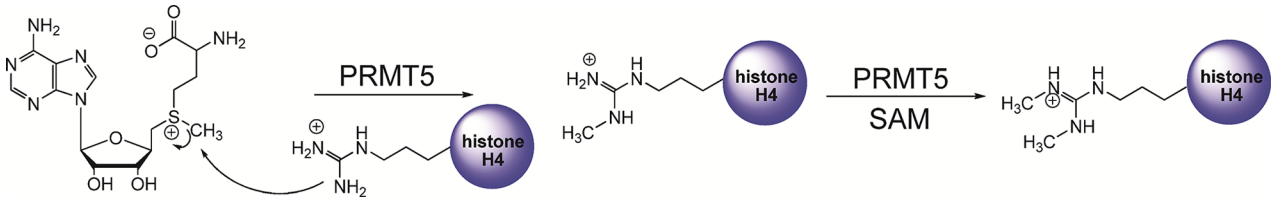


**Figure 1.** PRMT5-catalyzed methylation reactions.

**Table 1. Histone H4-Based Peptide Derivatives**

| substrate | sequence | expected mass (Da) | observed mass (Da) |
|---|---|---|---|
| AcH4−14 | Ac-SGRGKGGKGLGKGG | 1256 | 1257 |
| AcH4−18 | Ac-SGRGKGGKGLGKGGAKRH | 1749 | 1750 |
| AcH4−21 | Ac-SGRGKGGKGLGKGGAKRHRKV | 2132 | 2133 |
| AcH4−21R3MMA | Ac-SGR$^{Me}$GKGGKGLGKGGAKRHRKV | 2146 | 2147 |
| AcH4−26 | Ac-SGRGKGGKGLGKGGAKRHRKVLRDNI | 2744 | 2745 |
| AcH4−26R3MMA | Ac-SGR$^{Me}$GKGGKGLGKGGAKRHRKVLRDNI | 2758 | 2759 |
| AcH4−26N25A | Ac-SGRGKGGKGLGKGGAKRHRKVLRDAI | 2700 | 2701 |
| AcH4−26N25AR3MMA | Ac-SGR$^{Me}$GKGGKGLGKGGAKRHRKVLRDAI | 2714 | 2715 |

Notably, the symmetric dimethylation of histone H2A at R3, H3 at R2 and R8, and H4 at R3 is associated with the decreased level of transcription of a number of genes, including RB, ST7, and NM23, with consequent effects on cellular proliferation and differentiation.[11,14] Conversely, symmetric dimethylation of H3R2 was reported to be important for maintaining the euchromatic state at specific genomic loci, indicating that PRMT5-mediated methylation is differentially interpreted in a context-dependent manner.[15] PRMT5 also dimethylates p53 at R333, R335, and R337 to facilitate the response to DNA repair by promoting the formation of a DNA damage-regulated coactivator complex composed of Strap, p300, and JMY.[16,17] Overall, the studies mentioned above highlight the broad range of PRMT5 substrates and processes regulated by this enzyme.

In our recent work, we demonstrated that *Caenorhabditis elegans* PRMT5 (cPRMT5) uses a rapid equilibrium random kinetic mechanism with dead-end EAP and EBQ complexes and that long-range interactions are important for substrate recognition by cPRMT5.[18] We also provided evidence to support the notion that dimethylation occurs in a distributive fashion and showed that the rate of the dimethylation reaction is much slower than the rate of the initial monomethylation step.[18] This result implies that substrate dimethylation is a function of both the MMA peptide concentration and the affinity of the enzyme for the MMA peptide, as well as competition for the active site with the unmodified peptide.[18] By contrast, PRMT1 catalyzes substrate dimethylation in a partially processive manner.[19−21] Given our findings and the fact that most PRMT5 substrates are symmetrically dimethylated, it is unclear how monomethylated substrates are further processed *in vivo*. To address this issue, we extended our previous studies to the human hPRMT5·MEP50 complex and confirm that both enzymes use a distributive mechanism to catalyze the symmetric dimethylation of their substrates. We also provide evidence that long-range interactions may facilitate the rebinding of a monomethylated substrate to PRMT5 to promote the transfer of the second methyl group.

## EXPERIMENTAL PROCEDURES

**Reagents.** pFastbac HTA vector, Sf9 cells, and sf-900 II medium were purchased from Invitrogen Life Technologies. *N*-α-Fmoc-protected amino acids and preloaded Wang-based resins were purchased from Novabiochem. $^{14}$C-labeled bovine serum albumin (BSA), *S*-(5′-adenosyl)-L-methionine (SAM), and piperidine were purchased from Sigma-Aldrich. Protease inhibitors were purchased from Roche. [$^{14}$C-*methyl*]SAM was purchased from PerkinElmer Life Sciences. *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), Tricine, and dithiothreitol (DTT) were purchased from Research Products International Corp. Tris(hydroxymethyl)-aminomethane (Tris) was purchased from Bio-Rad. Ethylenediaminetetraacetic acid (EDTA) and trifluoroacetic acid (TFA) were from EMD. Lys-C was purchased from Promega. Histone H4 and H4R3K were purified as described previously.[19] *C. elegans* PRMT5 (cPRMT5) was purified as previously described.[18] The hPRMT5·MEP50 complex was purified as previously described with slight modification (see below).[22]

**Site-Directed Mutagenesis.** The histone H4 R3K mutant was constructed using the QuikChange mutagenesis kit (Stratagene) using the following primers: 5′-catatgtctggtaaag-gtaaaggtggtaaag-3′ (forward) and 5′-ctttaccacctttacctttaccagac-atatg-3′ (reverse). The mutant construct was verified by DNA sequencing.

**Expression and Purification of hPRMT5 and the hPRMT5·MEP50 Complex.** Full length human PRMT5 and MEP50 were cloned into the pFastbac HTA vector with a His tag on the N-terminus. Baculoviruses for both hPRMT5 and MEP50 were generated by using a standard bac-to-bac protocol. hPRMT5 and the hPRMT5·MEP50 complex proteins were expressed in Sf9 cells by infecting 250 mL of a 2 × 10$^6$ cells/mL culture with baculovirus while it was being stirred at 28 °C for 72 h. Cells were harvested and resuspended in 20 mL of lysis buffer [50 mM Tris (pH 7.5), 300 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and 1 tablet of protease inhibitors][22] and stirred at 4 °C for 30 min. The resulting cell lysate was centrifuged at 16000*g* for 20 min at 4 °C, and the supernatant was applied to a nickel-NTA column. The column was washed with a stepwise elution buffer containing 0 to 0.5 M imidazole in 50 mM Tris (pH 7.5), 300 mM NaCl, 10% (v/v) glycerol, and 1% (v/v) Triton X-100. Fractions were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and the concentration was determined using the Bradford method.


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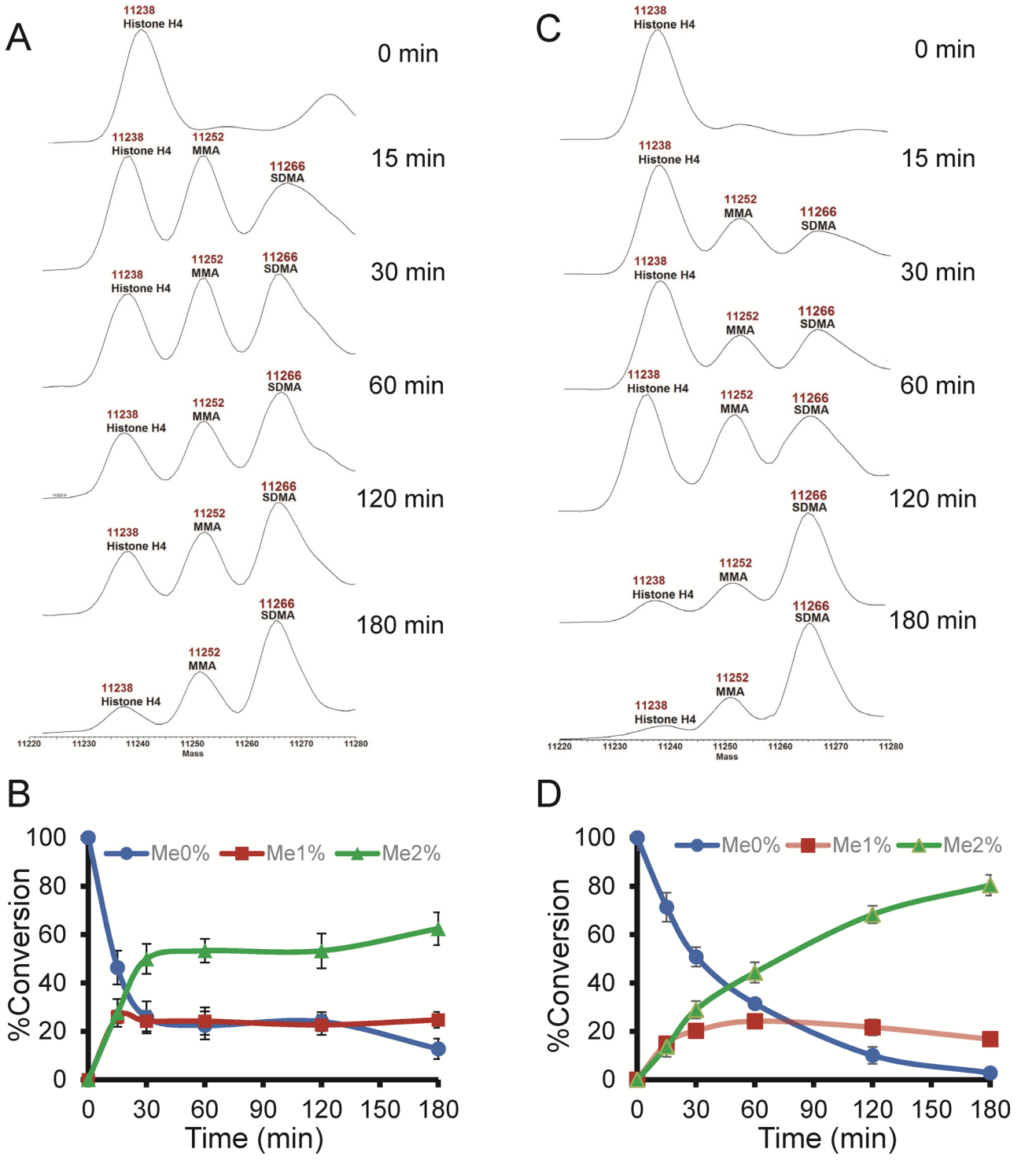


**Figure 2.** (A) ESI-MS-monitored multiple-turnover methylation of histone H4 catalyzed by cPRMT5. The reaction, including 200 $\mu$M SAM, 10 $\mu$M histone H4, and 1 $\mu$M cPRMT5, was conducted at 25 °C over 3 h. (B) Quantification of panel A. (C) ESI-MS-monitored multiple-turnover methylation of histone H4 catalyzed by the hPRMT5·MEP50 complex. The reaction, including 200 $\mu$M SAM, 10 $\mu$M histone H4, and 0.5 $\mu$M hPRMT5·MEP50 complex, was conducted at 37 °C over 3 h. (D) Quantification of panel C. Unmethylated (Me0), monomethylated (Me1), and dimethylated (Me2) histone H4 were quantified by mass spectrometry.

**Peptide Synthesis.** Peptides (AcH4−14, AcH4−18, AcH4−21, AcH4−21R3MMA, AcH4−26, AcH4−26R3MMA, AcH4−26N25A, and AcH4−26N25AR3MMA) were synthesized on a Rainin PS3 automated peptide synthesizer using preloaded Wang-based resins and Fmoc chemistry. After being synthesized, peptides were cleaved from the resin and purified by reverse phase high-performance liquid chromatography. The structures of the peptides were confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The sequences of the synthesized histone H4 peptides as well as the corresponding expected and observed masses are listed in Table 1.

**MALDI Mass Spectrometry Measurements.** Multiple- and double-turnover methylation reactions were monitored by


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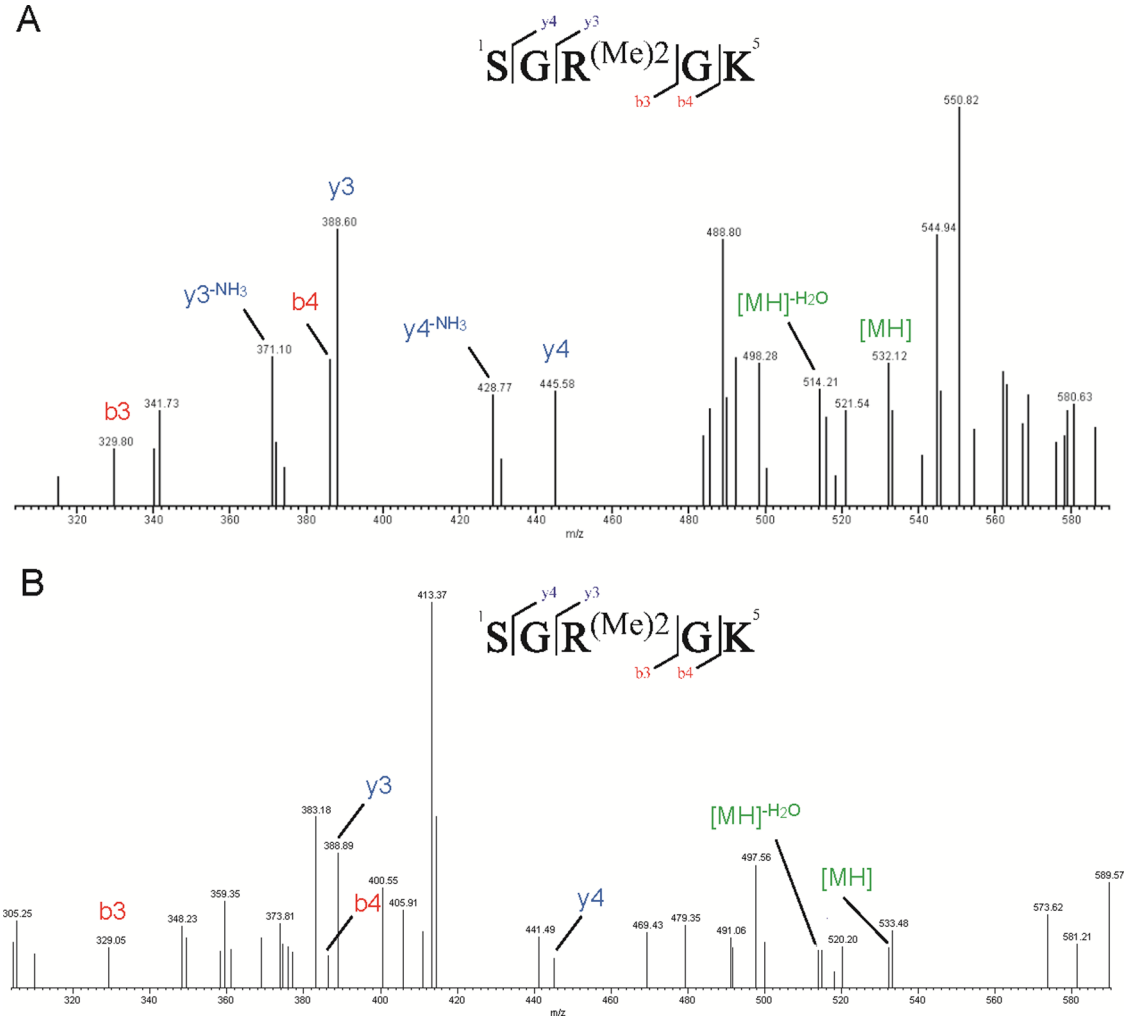


**Figure 3.** MS/MS spectrum of the Lys-C-digested peptide SGRGK derived from (A) the hPRMT5·MEP50 complex and (B) cPRMT5-methylated histone H4. The dimethylated precursor peptide (MH) is highlighted in green.

MALDI-MS spectrometry. Briefly, under multiturnover conditions, enzyme (1 $\mu$M cPRMT5 or 0.5 $\mu$M hPRMT5·MEP50) and SAM (200 $\mu$M) were preincubated at 25 or 37 °C, respectively, for 10 min in 50 mM HEPES (pH 8.0), 50 mM NaCl, 1 mM EDTA, and 0.5 mM DTT. For double-turnover experiments, SAM (20 or 4 $\mu$M) and enzyme (10 $\mu$M cPRMT5 or 2 $\mu$M hPRMT5·MEP50) were preincubated at 25 or 37 °C for 10 min in 50 mM HEPES (pH 8.0), 50 mM NaCl, 1 mM EDTA, and 0.5 mM DTT. The reactions were subsequently initiated by the addition of the peptide substrate (final concentration of 20 $\mu$M). The reaction was quenched at various times with 3 $\mu$L of 50% TFA in dd$H_2O$. The samples were then desalted with C-18 ZipTips (Millipore), mixed with a saturated solution of $\alpha$-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 50% water, and 0.1% TFA and spotted onto a MALDI plate. Each assay was conducted in duplicate. MS spectra were obtained in the positive ion mode and analyzed using Flex Analysis. The percentage turnover was calculated, as previously described,[19] by dividing the intensity of the modified substrate by the sum of the intensity of the substrate and product. As previously described, the unmodified and mono- and dimethylated peptides ionize to a similar extent.[19]

**ESI Mass Spectrometry Measurements.** The cPRMT5-catalyzed methylation of histone H4 was monitored by ESI-MS in multiple- and double-turnover methylation experiments. Briefly, under multiple-turnover conditions, histone H4 (10 $\mu$M) and SAM (100 $\mu$M) were preincubated at 25 °C for 10 min in 50 mM HEPES (pH 8.0), 50 mM NaCl, 1 mM EDTA, and 0.5 mM DTT. The reaction was initiated by the addition of 1 $\mu$M cPRMT5. For double-turnover experiments, 10 $\mu$M histone H4 and 20 $\mu$M SAM were preincubated at 25 °C for 10 min in 50 mM HEPES (pH 8.0), 50 mM NaCl, 1 mM EDTA, and 0.5 mM DTT. The reaction was initiated by the addition of 5 $\mu$M cPRMT5. Reactions were then quenched at various times with 0.1% formic acid (FA) at the appropriate time and the mixtures loaded immediately into the LTQ mass spectrometer (Thermo-Fisher Scientific). Proteins were analyzed in positive ion mode. Each assay was conducted in duplicate, and the raw data, comprising multiple charged H4 species, were deconvoluted and deisotoped using MaqTran, and the percentage turnover was calculated on the basis of the intensity values of relevant peaks using the methodology described above.

**MS/MS Analysis To Identify the Site of Modification.** To determine the methylation site in histone H4, methylated histone H4 (10 $\mu$g) was separated by SDS−PAGE and the corresponding bands were excised from the gel. The resultant bands were then washed and dehydrated with water and acetonitrile twice. Proteins were digested with Lys-C (Promega, V1071) at a protease:protein ratio of 1:10 (w:w) overnight at 37 °C. The enzymatic reaction was quenched with 1% TFA,


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Table 2. Masses of Fragments Derived from the Methylated Histone H4 Peptide SGR$^{(Me)_2}$GK

| | | | | | hPRMT5·MEP50 | | cPRMT5 | |
|---|---|---|---|---|---|---|---|---|
| amino acid | B ion label | Y ion label | calcd B ions | calcd Y ions | observed B ions | observed Y ions | observed B ions | observed Y ions |
| G | 2 | 4 | 145.06 | 445.29 | N/A | 445.58 | N/A | 445 |
| $R^{(Me)_2}$ | 3 | 3 | 329.19 | 388.27 | 329.80 | 388.60 | 329.05 | 388.89 |
| G | 4 | 2 | 386.21 | 204.13 | 386.10 | N/A | 386.40 | N/A |

and the resulting peptides were desalted and concentrated using C18 ZipTips (Millipore). Dried peptides were reconstituted in 0.1% formic acid. All samples were analyzed by liquid chromatography−tandem MS (LC−MS/MS) using a linear ion trap-equipped LTQ mass spectrometer (ThermoFisher Scientific).

**Gel-Based Activity Assay.** PRMT5 methyltransferase activity was determined using a previously described discontinuous gel-based assay.[18] Briefly, assay buffer [50 mM HEPES (pH 8.0), 50 mM NaCl, 1 mM EDTA, and 0.5 mM dithiothreitol], containing a peptide substrate and $^{14}$C-labeled SAM, was incubated for 10 min at 25 °C for cPRMT5 and 15 min at 37 °C for human PRMT5 as well as the hPRMT5·MEP50 complex. The reaction was then initiated by the addition of cPRMT5 (final concentration of 200 nM), the hPRMT5·MEP50 complex (final concentration of 100 nM), or hPRMT5 (final concentration of 300 nM) and quenched with 6× Tris-Tricine gel loading dye. Samples were then loaded onto 16.5% Tris-Tricine polyacrylamide gels to separate methylated protein/peptide from unreacted SAM. The gels were dried, and the incorporated radioactivity was quantified by phosphorimage analysis using a Typhoon scanner. The assays were conducted in duplicate, and the initial rates were fit to eq 1

$$v = V_{max}[S]/([S] + K_M) \quad (1)$$

where $v$ is the initial rate, $V_{max}$ the maximal velocity, [S] the substrate concentration, and $K_M$ the Michaelis constant. $k_{cat}$ was calculated from the ratio of $V_{max}$ and enzyme concentration.

## RESULTS

**PRMT5 Dimethylates Histone H4.** PRMT5 is reported to symmetrically dimethylate both histone and non-histone protein substrates to regulate a variety of cellular processes.[1,2,7] Histones H2A, H3, and H4 are prototypical PRMT5 substrates, and these proteins are methylated at Arg3 in both H2A and H4 and at arginines 2 and 8 in histone H3.[15,23] Previously, we established that histone H4 is an excellent substrate for cPRMT5 and that histone tail analogues are preferentially monomethylated by this ortholog; addition of the second methyl group is kinetically slow.[18] To further investigate the mechanism of PRMT5-catalyzed histone H4 dimethylation and extend these studies to human PRMT5 (hPRMT5), we used LTQ-MS to monitor the methylation of intact histone H4 protein. For these studies, substrate methylation was monitored as a function of time over 3 h. The hPRMT5·MEP50 complex reactions were performed at 37 °C, whereas the cPRMT5-catalyzed reactions were conducted at 25 °C because cPRMT5 loses activity when incubated at higher temperatures. As depicted in panels A and B of Figure 2, histone H4 is rapidly mono- and dimethylated under the assay conditions by cPRMT5, with the amounts of MMA and SDMA reaching maximal levels of 25 and 60%, respectively. Notably, the amount of dimethylated histone H4 does not increase significantly at the later time points (i.e., 60, 120, and 180 min), which may be caused by either nonspecific inactivation of cPRMT5 or product inhibition by SAH ($K_i$ = 5.6 μM).[18] Similar results were obtained for the hPRMT5·MEP50 complex (Figure 2C,D). The fact that we observe intermediate levels of the MMA-containing protein suggests that both cPRMT5 and the hPRMT5·MEP50 complex process their substrates in a distributive fashion.

**Arg3 Is Dimethylated in Histone H4.** To establish that arginine 3 is the main site of dimethylation in histone H4, the protein substrate was incubated with either cPRMT5 or the hPRMT5·MEP50 complex for 3 h. Under these conditions, ∼80% of histone H4 exists as the dimethylated species (Figure S1 of the Supporting Information). Methylated histone H4 was subjected to SDS−PAGE and then visualized by Coomassie Blue staining. The band corresponding to histone H4 was then excised from the gel and subjected to in-gel digestion with Lys-C. The digested peptides were then analyzed by LC−MS, and the peptide containing the dimethylation site was further fragmented by CID. The resulting MS/MS spectrum of this peptide is shown in Figure 3. The expected and observed masses are listed in Table 2. The results of this analysis are consistent with the dimethylation of histone H4 at arginine 3. The fact that both cPRMT5 and the hPRMT5·MEP50 complex yielded similar results indicates that both enzymes preferentially dimethylate histone H4 at arginine 3.

To further confirm these findings, we expressed and purified the histone H4R3K mutant and then evaluated its ability to act as a substrate for both cPRMT5 and the hPRMT5·MEP50 complex. The results of these studies indicate that neither enzyme efficiently processes the H4R3K mutant; the $k_{cat}/K_M$ values are reduced by more than 600-fold (Table 3). Overall, these data confirm that arginine 3 is regiospecifically dimethylated by both orthologues.

**Double-Turnover Methylation of Histone H4.** In our previous study, we provided evidence suggesting that PRMT5 dimethylates the AcH4−21 peptide in a distributive manner wherein the methylated protein or peptide substrate is released from the enzyme and must compete for rebinding with an unmethylated substrate.[18] To further investigate the processivity of PRMT5, double-turnover experiments were developed for both cPRMT5 and the hPRMT5·MEP50 complex. Briefly, for these experiments, the protein or peptide substrate is kept in excess and there are 2 equiv of SAM relative to the enzyme concentration. Thus, if the methylated product is released from the enzyme (i.e., a distributive mechanism), it must then compete with the unmodified substrate to rebind and be dimethylated. By contrast, for a processive mechanism, the dimethylated species will accumulate regardless of the concentration of the peptide substrate. Hevel and colleagues have previously used this assay to show that PRMT1 uses a partially processive mechanism.[20]

For the cPRMT5-catalyzed methylation of histone H4, the reaction mixtures contained 10 μM SAM, 10 μM histone H4, and 5 μM cPRMT5. For the hPRMT5·MEP50 complex-catalyzed reaction, mixtures contained 2 μM SAM, 10 μM


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Table 3. Substrate Specificities

| substrate | cPRMT5 | | | hPRMT5 | | | hPRMT5·MEP50 | | |
|---|---|---|---|---|---|---|---|---|---|
| | $K_M$ ($\mu$M) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_M$ ($M^{-1}$ $min^{-1}$) | $K_M$ ($\mu$M) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_M$ ($M^{-1}$ $min^{-1}$) | $K_M$ ($\mu$M) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_M$ ($M^{-1}$ $min^{-1}$) |
| histone H4[a] | 22 ± 3[d] | 0.21 ± 0.01[d] | (9.5 ± 1.3) × $10^3$[d] | 25 ± 3 | 0.12 ± 0.01 | (4.8 ± 0.6) × $10^3$ | 2.4 ± 0.4 | 0.13 ± 0.01 | (5.4 ± 0.9) × $10^4$ |
| histone H4R3K[a,c] | | | <15 | | | <33 | | | <28 |
| histone H3[a,c] | | | <40 | | | <50 | 26 ± 3 | 0.043 ± 0.001 | (1.6 ± 0.2) × $10^3$ |
| AcH4−14 | | | <22.3[d] | | | <23 | 65 ± 4 | 0.21 ± 0.01 | (3.2 ± 0.2) × $10^3$ |
| AcH4−18 | 310 ± 40[d] | 0.27 ± 0.01[d] | (8.7 ± 1.1) × $10^2$[d] | 61 ± 7 | 0.15 ± 0.01 | (2.5 ± 0.3) × $10^3$ | 10 ± 2 | 0.20 ± 0.01 | (2.0 ± 0.4) × $10^4$ |
| AcH4−21[a] | 13 ± 2[d] | 1.2 ± 0.01[d] | (9.2 ± 1.4) × $10^4$[d] | 18 ± 4 | 0.14 ± 0.02 | (7.8 ± 1.8) × $10^3$ | 1.6 ± 1 | 0.18 ± 0.01 | (1.1 ± 0.1) × $10^5$ |
| AcH4−21R3MMA[a] | 390 ± 50[d] | 0.22 ± 0.01[d] | (5.7 ± 0.7) × $10^2$[d] | 184 ± 19 | 0.06 ± 0.001 | (3 ± 0.3) × $10^2$ | 5.8 ± 0.5 | 0.045 ± 0.001 | (7.8 ± 0.7) × $10^3$ |
| AcH4−26[a] | 8.9 ± 1 | 1.3 ± 0.01 | (1.5 ± 0.2) × $10^5$ | 36 ± 7 | 0.16 ± 0.01 | (4.4 ± 0.9) × $10^3$ | 1.5 ± 0.2 | 0.23 ± 0.01 | (1.5 ± 0.2) × $10^5$ |
| AcH4−26R3MMA[a] | 37 ± 2 | 0.2 ± 0.01 | (5.4 ± 0.3) × $10^3$ | 50 ± 4 | 0.08 ± 0.01 | (1.6 ± 0.1) × $10^3$ | 2.7 ± 2 | 0.056 ± 0.001 | (2.1 ± 0.2) × $10^4$ |
| AcH4−26N25A[a] | 26 ± 3 | 1.1 ± 0.01 | (4.2 ± 0.1) × $10^4$ | 19 ± 5 | 0.14 ± 0.01 | (7.4 ± 2) × $10^3$ | 1.8 ± 2.4 | 0.21 ± 0.01 | (1.1 ± 0.2) × $10^5$ |
| AcH4−26N25AR3MMA[a] | 102 ± 9 | 0.2 ± 0.01 | (2.0 ± 0.2) × $10^3$ | 91 ± 9 | 0.09 ± 0.001 | (9.9 ± 0.9) × $10^2$ | 3.1 ± 0.4 | 0.054 ± 0.001 | (1.7 ± 0.2) × $10^4$ |
| SAM[b] | 5.3 ± 0.2 | 1.1 ± 0.01 | (2.1 ± 0.1) × $10^5$ | 12 ± 1 | 0.14 ± 0.01 | (1.2 ± 0.1) × $10^4$ | 1.1 ± 0.1 | 0.23 ± 0.01 | (2.1 ± 0.2) × $10^5$ |

[a]At 15 $\mu$M SAM. [b]At 100 $\mu$M AcH4−21. [c]The amount of product formed was too small to accurately measure the kinetic parameters for this peptide substrate. Therefore, an estimation of $k_{cat}/K_M$ was made by dividing the maximal observed rate by the concentration of substrate. [d]Data from a previous report.[18]

histone H4, and 1 $\mu$M hPRMT5·MEP50 complex. PRMT5 and SAM were mixed and incubated at 25 °C for 10 min to ensure the binding of SAM to the enzyme. The reaction was then initiated by the addition of histone H4 at 25 °C for cPRMT5 and 37 °C for the hPRMT5·MEP50 complex. Under these conditions, the amount of SAM is sufficient for only two turnovers. Thus, for a distributive mechanism, one would predict that monomethylated histone H4 would be the only product because histone H4 must be released before rebinding to facilitate the second methylation event. By contrast, if the enzyme follows a processive mechanism, a fraction of the product should be the dimethylated species. The experimental data show that both cPRMT5 and the hPRMT5·MEP50 complex generate MMA as the only product over the entire time scale of the experiment (Figure 4). Thus, both cPRMT5 and the hPRMT5·MEP50 complex catalyze substrate dimethylation in a distributive fashion. In addition to our previous mechanistic studies, we propose that MMA is released after the first methyl group is transferred to the substrate. MMA then rebinds to facilitate the transfer of the second methyl group to the unmethylated $\omega$-nitrogen. Because this process competes with the unmodified substrate, the rate of dimethylation is dictated by both the MMA peptide concentration and the affinity of the enzyme for the MMA peptide, as well as competition for the active site with the unmodified peptide. This model rules out the need to invoke the energetically unfavorable rotation of the guanidinium within the active site, which is required for a processive dimethylation mechanism.

**PRMT5 Dimethylates Histone Tail Analogues.** Similar results were obtained for the multiple-turnover methylation experiments with the AcH4−21 peptide; AcH4−21 is a histone H4 tail analogue whose sequence corresponds to the first 21 amino acids of this protein (Figures 5A and 6A). Specifically, our data show that both cPRMT5 and the hPRMT5·MEP50 complex generate intermediate levels of the MMA-containing peptide and that the SDMA-containing peptide is detected only after the concentration of the monomethylated species exceeds that of the unmodified peptide (Figures 5A and 6A, respectively). This result is entirely consistent with the fact that the AcH4−21R3MMA peptide is a significantly poorer substrate for both cPRMT5 (161-fold) and the hPRMT5·MEP50 complex (14-fold) based on a comparison of $k_{cat}/K_M$ values. We also confirmed that this peptide is processed in a distributive fashion using double-turnover methylation assays (Figures 5B and 6B).

Intriguingly, however, the rate of SDMA formation is significantly higher when histone H4 is used as the substrate (compare the progress curves in Figure 2 to those observed in Figures 5A and 6A), suggesting that the binding affinity of monomethylated histone H4 may be higher than that of the AcH4−21 peptide. Considering the larger size of the full length protein, we synthesized a longer 26-mer peptide, AcH4−26, and evaluated its ability to act as a substrate for both cPRMT5 and the hPRMT5·MEP50 complex. Initially, we performed the same set of multiple- and double-turnover methylation experiments and confirmed that the AcH4−26 peptide is methylated in a distributive fashion by both cPRMT5 and the hPRMT5·MEP50 complex (Figures 5C,D and 6C,D). We next determined the steady-state kinetic parameters for the AcH4−26 and AcH4−26R3MMA peptides. In accord with our hypothesis, the results show that the AcH4−26 peptide is at least as efficiently processed as both the AcH4−21 peptide and histone H4 for both cPRMT5 and the hPRMT5·MEP50


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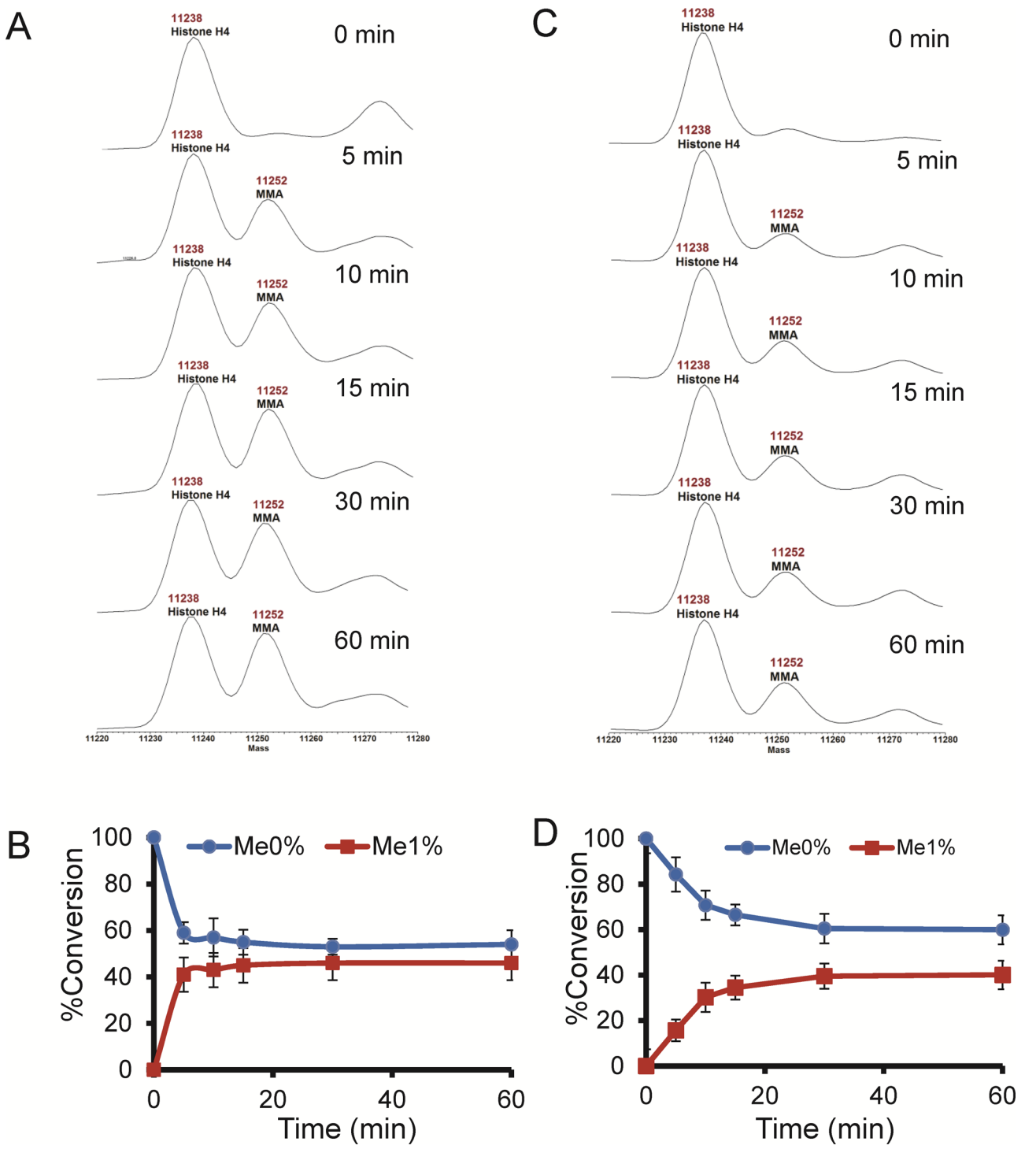


**Figure 4.** PRMT5 methylates histone H4 in a distributive fashion. (A) ESI-MS-monitored double-turnover methylation reaction catalyzed by cPRMT5. The reaction, including 10 $\mu$M SAM, 20 $\mu$M histone H4, and 5 $\mu$M cPRMT5, was conducted at 25 °C over 1 h. (B) Quantification of panel A. (C) The hPRMT5·MEP50 complex catalyzed double-turnover methylation of histone H4. The reaction, including 2 $\mu$M SAM, 10 $\mu$M histone H4, and 1 $\mu$M hPRMT5·MEP50 complex, was conducted at 37 °C over 1 h. (D) Quantification of panel C. Unmethylated (Me0) and monomethylated (Me1) histone H4 were quantified by mass spectrometry.

complex. The AcH4−26R3MMA peptide is, however, a much better substrate for cPRMT5; the $k_{cat}/K_M$ value is 9.5-fold higher than that obtained for the AcH4−21R3MMA peptide. This effect appears to be driven by an increase in the affinity of the enzyme for the AcH4−26R3MMA peptide as the $K_M$ for this peptide (37 $\mu$M) is 10-fold lower than that obtained for the AcH4−21R3MMA peptide, while the turnover number ($k_{cat}$ = 0.2 $min^{-1}$) is comparable to that obtained for the AcH4−21R3MMA peptide. These trends are at least partially recapitulated for the hPRMT5·MEP50 complex as illustrated by the observation that the $k_{cat}/K_M$ value obtained for the AcH4−26R3MMA peptide is 2.7-fold higher than that obtained for the AcH4−21R3MMA peptide.


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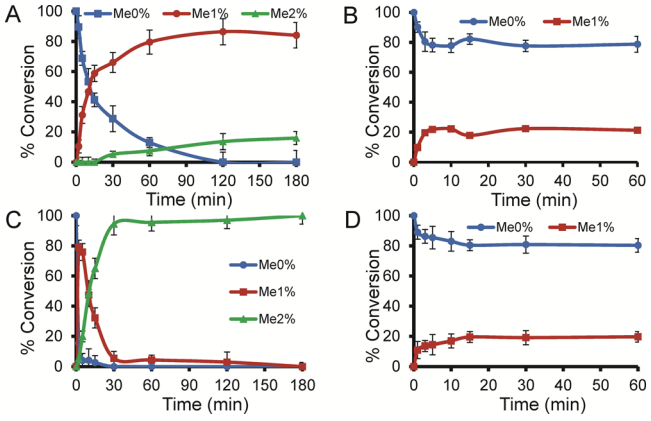


**Figure 5.** cPRMT5 methylates peptide substrates in a distributive fashion. (A) Multiple-turnover methylation of the AcH4−21 peptide. The reaction, including 200 $\mu$M SAM, 10 $\mu$M AcH4−21, and 1 $\mu$M cPRMT5, was conducted at 25 °C over 3 h. (B) Double-turnover methylation of the AcH4−21 peptide. The reaction, including 20 $\mu$M SAM, 100 $\mu$M AcH4−21, and 10 $\mu$M cPRMT5, was conducted at 25 °C over 1 h. (C) Multiple-turnover methylation of the AcH4−26 peptide. The reaction, including 200 $\mu$M SAM, 10 $\mu$M AcH4−26, and 1 $\mu$M cPRMT5, was conducted at 25 °C over 3 h. (D) Double-turnover methylation of the AcH4−26 peptide. The reaction, including 20 $\mu$M SAM, 100 $\mu$M AcH4−26, and 10 $\mu$M cPRMT5, was conducted at 25 °C over 1 h.

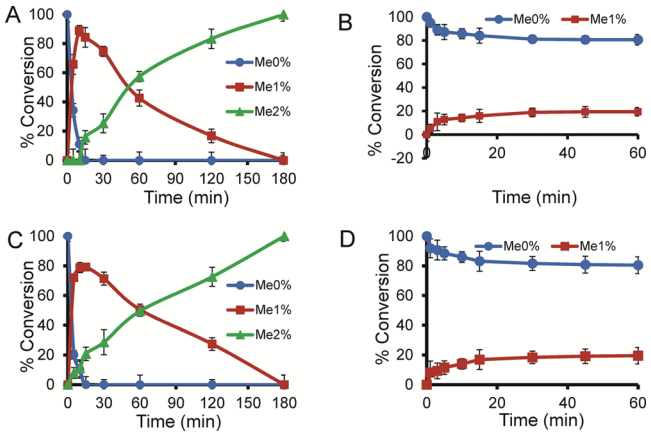


**Figure 6.** hPRMT5·MEP50 complex methylates peptide substrates in a distributive fashion. (A) Multiple-turnover methylation of the AcH4−21 peptide. The reaction, including 200 $\mu$M SAM, 10 $\mu$M AcH4−21, and 0.5 $\mu$M hPRMT5·MEP50 complex, was conducted at 37 °C over 3 h. (B) Double-turnover methylation of the AcH4−21 peptide. The reaction, including 4 $\mu$M SAM, 50 $\mu$M AcH4−21, and 2 $\mu$M hPRMT5·MEP50 complex, was conducted at 37 °C over 1 h. (C) Multiple-turnover methylation of the AcH4−26 peptide. The reaction, including 200 $\mu$M SAM, 10 $\mu$M AcH4−26, and 0.5 $\mu$M hPRMT5·MEP50 complex, was conducted at 37 °C over 3 h. (D) Double-turnover methylation of the AcH4−26 peptide. The reaction, including 4 $\mu$M SAM, 50 $\mu$M AcH4−26, and 2 $\mu$M hPRMT5·MEP50 complex, was conducted at 37 °C over 1 h. Unmethylated (Me0), monomethylated (Me1), and dimethylated (Me2) peptide substrates were quantified by mass spectrometry.

Because the steady-state kinetic data suggest that one or more residues located between positions 21 and 26 in histone H4 facilitate the rebinding of the monomethylated peptide, we generated the AcH4−26N25A and AcH4−26N25AR3MMA mutants to help confirm our hypothesis. The steady-state kinetic data show that the introduction of an alanine in the position of N25 reduces $k_{cat}/K_M$ 2.5−3.6-fold for PRMT5, which is consistent with our hypothesis. The effect on the hPRMT5·MEP50 complex is significantly less pronounced.

**Role of MEP50.** To further understand the role of MEP50 in PRMT5 catalysis, we also expressed and purified the individual enzyme from insect cells. Without coexpression of MEP50, the amount of purified hPRMT5 is ∼3 times smaller than when the two proteins are coexpressed, suggesting that MEP50 is important for protein folding and/or stability. Surprisingly, steady-state kinetic studies indicate that on its own, hPRMT5 behaves like cPRMT5 in that the $K_M$ values for the two orthologues are ≥6-fold higher than those obtained for the hPRMT5·MEP50 complex (Table 3). By contrast, the turnover numbers are more similar to those obtained for the hPRMT5·MEP50 complex, indicating that MEP50 mainly affects substrate recognition. Further supporting a role for MEP50 in substrate binding is the observation that the AcH4−14 and AcH4−18 peptides are methylated by the hPRMT5·MEP50 complex but show significantly reduced activity with either cPRMT5 or hPRMT5. Nevertheless, it is important to note that these shorter substrates are also less efficiently processed by the hPRMT5·MEP50 complex relative to histone H4 or the AcH4−21 and AcH4−26 peptides, indicating that sites distal from the site of methylation are important for substrate recognition. In total, those data suggest that MEP50 plays a key role in facilitating substrate binding.

## DISCUSSION

PRMT5 is unique among the PRMTs in that it is the only enzyme known to catalyze the symmetric dimethylation of arginines.[23] Given the links between dysregulated PRMT5 activity and tumorigenesis, we initiated kinetic studies to identify mechanistic features that can be employed to guide the development of specific PRMT5 inhibitors, focusing initially on the substrate specificity and processivity of the enzyme. Building on our work with the *C. elegans* orthologue, we confirmed that Arg3 is the only site of methylation in both histone H4 and peptide-based histone H4 tail analogues. We further showed that both cPRMT5 and the hPRMT5·MEP50 complex catalyze substrate dimethylation in a distributive manner and further confirmed that sites distal to the site of methylation are important for substrate recognition by cPRMT5, hPRMT5, and the hPRMT5·MEP50 complex. Importantly, our results also suggest that long-range interactions promote the efficient symmetric dimethylation of PRMT5 substrates by increasing the affinity of the monomethylated substrate for the enzyme. Comparisons of the substrate specificity of cPRMT5, hPRMT5, and the hPRMT5·MEP50 complex further showed that MEP50 is important for substrate recognition. Whether MEP50 directly interacts with these substrates or allosterically activates the enzyme is not known. Nevertheless, our studies will provide the basis for the future examination of this phenomenon. Interestingly, there does not appear to be a MEP50 ortholog in *C. elegans*, suggesting that some other factor may modulate the activity of this ortholog. Our results suggest that it may be possible to target binding pockets outside of the active site to allosterically inhibit PRMT5 activity.

## ASSOCIATED CONTENT

### Supporting Information

MS spectra (Figures S1−to S11). This material is available free of charge via the Internet at http://pubs.acs.org.


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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

PTM, post-translational modification; PRMT, protein arginine methyltransferase; CARM1, coactivator-associated arginine methyltransferase 1; MMA, monomethylarginine; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; PDCD4, programmed cell death protein 4; NF-κB, nuclear factor κB; MEP50, methylosome protein 50; SAM, *S*-adenosyl-L-methionine; CID, collision-induced dissociation.

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