

Automethylation of Protein Arginine Methyltransferase 8 (PRMT8) Regulates Activity by Impeding *S*-Adenosylmethionine Sensitivity*

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Background: Protein arginine methyltransferase (PRMT) 8 is negatively regulated by its N terminus, which contains two automethylation sites.

Results: Preventing automethylation increased sensitivity to AdoMet. Mimicking methylation had the opposite effect.

Conclusion: Automethylation of PRMT8 negatively regulates activity by decreasing affinity for AdoMet.

Significance: Selective reduction of AdoMet affinity for PRMT8 by automethylation is a unique regulatory mechanism among protein arginine methyltransferases.

Protein arginine methyltransferase (PRMT) 8 is unique among the PRMTs, as it has a highly restricted tissue expression pattern and an N terminus that contains two automethylation sites and a myristoylation site. PRMTs catalyze the transfer of a methyl group from *S*-adenosylmethionine (AdoMet) to a peptidylarginine on a protein substrate. Currently, the physiological roles, regulation, and cellular substrates of PRMT8 are poorly understood. However, a thorough understanding of PRMT8 kinetics should provide insights into each of these areas, thereby enhancing our understanding of this unique enzyme. In this study, we determined how automethylation regulates the enzymatic activity of PRMT8. We found that preventing automethylation with lysine mutations (preserving the positive charge of the residue) increased the turnover rate and decreased the K_m of AdoMet but did not affect the K_m of the protein substrate. In contrast, mimicking automethylation with phenylalanine (*i.e.* mimicking the increased hydrophobicity) decreased the turnover rate. The inhibitory effect of the PRMT8 N terminus could be transferred to PRMT1 by creating a chimeric protein containing the N terminus of PRMT8 fused to PRMT1. Thus, automethylation of the N terminus likely regulates PRMT8 activity by decreasing the affinity of the enzyme for AdoMet.

Arginine methylation is a post-translational modification catalyzed by the protein arginine methyltransferase (PRMT)² family (1–3). PRMTs catalyze the transfer of a methyl group from *S*-adenosylmethionine (AdoMet) to a peptidylarginine on a target protein substrate (1–3). The PRMTs methylate a large

variety of substrates and are involved in many biological processes from DNA repair to signal transduction (1–3). PRMT1, the predominant member, is responsible for 85% of the arginine methylation in the cell, is ubiquitously expressed, and is embryonically lethal when genetically deleted (4). In contrast, much less is known about its closely related family member, PRMT8. PRMT8 is >84% identical to PRMT1 in the methyltransferase core but has a unique N terminus that is known to inhibit its activity (5, 6). The N terminus of PRMT8 contains two automethylation sites and a myristoylation site, which localizes PRMT8 to membranes within the cell, and is the only PRMT known to have this localization (5, 6). Additionally, in contrast to all other PRMT family members, PRMT8 is the only family member with a highly restricted expression pattern; PRMT8 is expressed only in the central nervous system (5, 7, 8).

PRMT catalysis requires AdoMet as a methyl donor, and PRMTs are divided into three groups based on their methylation activity: Type I PRMTs (PRMT1, PRMT3, PRMT4 (CARM1), PRMT6, and PRMT8) dimethylate arginine asymmetrically (9–14); Type II PRMTs (PRMT5) dimethylate symmetrically (10, 12, 15, 16); and Type III PRMTs (PRMT7) monomethylate arginine (17). All PRMTs contain highly conserved regions involved in enzymatic activity but vary widely at the N and C termini, whose sequences can provide substrate specificity, define subcellular localization, regulate activity, and control interactions with binding partners (1–3). In the catalytic core of the PRMTs, a conserved α -helix provides stability for AdoMet binding and enzymatic activity (18–21). Although there is variability among the substrate specificity for PRMTs and no exclusive methylation target motif sequence is known, some Type I PRMTs have been found to methylate regions rich in glycine and arginines (10, 22, 23), whereas others like CARM1 prefer arginine surrounded by proline, glycine, and methionine (24).

For PRMTs, enzyme kinetics can reveal many particular aspects of catalysis, such as substrate recognition motifs and the order of substrate binding and product release (25–31). The kinetics of PRMT1-catalyzed methylation are well character-

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² The abbreviations used are: PRMT, protein arginine methyltransferase; AdoMet, *S*-adenosylmethionine.

ized, and it was determined that the kinetic mechanism proceeds in a random sequential fashion, where AdoMet and the protein substrate bind in a random fashion (26).

A wide range of factors are known to regulate the activity of PRMTs (9, 32–35). The formation of homodimers is essential for the function of PRMTs, and heterodimers have been found to also regulate activity (18, 20, 21, 33, 36–41). Most PRMTs also have distinct regions on their N terminus that confer substrate specificity and regulate activity (1, 3, 32, 34, 35, 42). For example, PRMT3 contains a zinc finger domain that is required for methylation of RNA-associated substrates (36). Among post-translational modifying enzymes, self-modification often alters the activity and/or substrate specificity of the enzyme (43, 44). For example, automethylation of the lysine methyltransferase G9a, while not affecting its methyltransferase activity *in vitro*, allows it to bind to other proteins, and mutating this site prevents its specific localization within the cell (43). Similarly, mutation of an automethylation site on CARM1 impairs CARM1-activated transcription and mRNA splicing (45). Automethylation of PRMT6 increases stability of the enzyme and regulates its anti-HIV-1 activity (46). Currently, the mechanism by which the N terminus inhibits the enzymatic activity of PRMT8 is unknown. In this study, we made specific mutants of the two automethylation sites, either to a lysine to preserve charge but prevent automethylation or to a phenylalanine to mimic automethylation, and examined the effect of self-modification on PRMT8 activity. Herein, we report that automethylation regulates AdoMet binding to PRMT8, thereby inhibiting its methyltransferase activity.

EXPERIMENTAL PROCEDURES

Reagents—Histone H4 was purchased from New England Biolabs (M2504S). [³H]AdoMet (specific activity of 78 Ci/mmol; 0.4 mM in 9 parts sulfuric acid (pH 2.0) to 1 part ethanol) was purchased from PerkinElmer Life Sciences (NET155H001MC). [¹⁴C]AdoMet (specific activity of 55 mCi/mmol; 1.8 mM in 9 parts sulfuric acid (pH 2.0) to 1 part ethanol) was purchased from American Radiochemicals (ARC 0344).

Cell Culture—HeLa cells were grown in high-glucose DMEM (HyClone) supplemented with 1 mM HEPES (Mediatech), 0.5 μg/ml penicillin/streptomycin (HyClone), and 10% FBS (Atlanta Biologicals).

Immunofluorescence—HeLa cells were plated onto glass coverslips and transfected using Lipofectamine LTX (Invitrogen) following the manufacturer's instructions. The cells were washed with PBS, stained for 15 min with Alexa Fluor 488-conjugated wheat germ agglutinin (Invitrogen) at 0.5 μg/ml, washed with PBS, fixed in 4% paraformaldehyde, and stained with DAPI. The coverslips were mounted onto slides and imaged by fluorescence microscopy. Images were analyzed using ImageJ and analyzed with JACoP (47).

Cloning—Human full-length PRMT1, full-length PRMT8, and ΔN-PRMT8 (lacking residues 1–60 of PRMT8) cDNAs were subcloned into the pET29a(+) vector (Novagen). The N-terminal domain of PRMT8 was generated by amplifying residues 1–69 of PRMT8 and subcloning into the pET29a(+) vector. Ch8-1 was created by ligating portions of PRMT1 and PRMT8 cDNAs to create a chimeric molecule containing

amino acids 1–69 of PRMT8 fused to amino acids 29–325 of PRMT1. Human full-length PRMT8 subcloned into pEGFP-N1 and pGEX-6P-1 were gifts from Dr. Mark Bedford. All pET29 and pEGFP-N1 constructs contain C-terminal tags; pGEX-6P-1 constructs contain N-terminal tags. Additional linkage amino acids between the cDNA of the enzyme and tag are as follows: for pET29(+), PRMT8, ΔN-PRMT8, the PRMT8 N-terminal domain and KLAAALE-His₆ tag; for pET29(+), PRMT1 and Ch8-1 and LE-His₆ tag; for pEGFP-N1, PRMT8 and VPRARDPPVAT-EGFP tag; and for pGEX-6P-1, PRMT8 and GST tag-LGSPE.

Site-directed Mutagenesis—Mutagenesis was performed with the QuikChange Lightning site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions.

Recombinant Protein Expression and Purification—Over-Express C41(DE3)pLysS chemically competent cells (Lucigen) were transformed with the above constructs and grown on LB medium + antibiotic agar plates. Selected colonies were grown in 2× YT medium to $A_{600} = 0.8$ and induced with 0.25 mM isopropyl β-D-thiogalactopyranoside for 4 h at 37 °C. Cell pellets were resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole) supplemented with 1 mg/ml lysozyme (Sigma), sonicated, and centrifuged at 17,000 × *g* for 30 min. The clarified lysates were collected and incubated overnight with nickel-nitrilotriacetic acid slurry (Qiagen) at 4 °C. The nickel-nitrilotriacetic acid beads were collected at 1000 × *g* for 5 min and washed twice with lysis buffer + 30 mM imidazole for 15 min. Protein was eluted three times with lysis buffer + 300 mM imidazole for 1 h at 4 °C. PD-10 columns (GE Healthcare) were used to size-exclude contaminants and exchange the buffer to PBS, and protein-containing fractions were concentrated using an Amicon centrifugal filter (Millipore). Protein concentration was determined using the BCA kit (Pierce). The purification yielded >95% purity (by SDS-PAGE). GST-human fibrillar (residues 1–148) fusion protein (designated GST-GAR) was purified in a similar fashion using PBS as a lysis buffer, GST slurry (Thermo Scientific) to capture the protein, and 20 mM glutathione to elute.

In Vitro Methylation Assays—Recombinant protein (0.8 μM) was incubated overnight at room temperature in standard methylation buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 0.4 mM EDTA) with 1 μg of substrate protein (histone H4 or GST-GAR) and 2 μl of [³H]AdoMet (2 μCi). Reactions were quenched with 6× SDS loading buffer, boiled for 5 min, and separated by SDS-PAGE. Gels were stained with Coomassie stain (0.25% Coomassie Brilliant Blue, 50% MeOH, and 10% acetic acid) and destained with 50% MeOH and 10% acetic acid. After a 30-min incubation in Amplify (GE Healthcare), gels were dried and exposed to HyBlot CL film at –80 °C.

Steady-state Kinetics Assays—Reactions were performed in methylation buffer for kinetic studies (50 mM HEPES (pH 8.0), 1 mM EDTA, 50 mM NaCl, and 0.5 mM dithiothreitol) with 30 μM [¹⁴C]AdoMet and varying concentrations of histone H4 or vice versa. Mixtures were preincubated at 37 °C for 10 min. Enzyme was then added (0.4 μM for PRMT1, ΔN-PRMT8, and Ch8-1 or 0.8 μM for PRMT8 and point mutants), and reactions were incubated for 15 min (within steady state for PRMT1, ΔN-PRMT8, and Ch8-1) (data not shown) or 60 min (within

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steady state for PRMT8 and mutants) (data not shown) before being quenched with 6× SDS-PAGE loading buffer and boiled at 95 °C for 5 min. Samples were separated by SDS-PAGE (Bio-Rad), stained with Coomassie stain, and destained overnight. After rinsing in H₂O, gels were incubated in Amplify for 30 min, and dried. Incorporated radioactivity was measured using a PhosphorImager, and values were fit to Equation 1 using GraFit v7.

$$v = V_{\max}[S]/(K_m + [S]) \quad (\text{Eq. 1})$$

Co-immunoprecipitation Assays—Samples were prepared in standard methylation buffer. GST-tagged PRMT8 or PRMT8-E194Q (0.1 μM) was incubated overnight with either His-tagged PRMT8 or PRMT8-E194Q (0.1 μM) with 3 μl of anti-GST antibody (Cell Signaling 2624) and 20 μl of protein G slurry (Amersham Biosciences) at 4 °C. Beads were washed three times with PBS, separated on a SDS-polyacrylamide gel, transferred to PVDF membrane, and probed with anti-PRMT1 antibody (Upstate 07-404), which cross-reacts with PRMT8 via immunoblotting.

Inhibitor Kinetic Assays—Prior to the 15-min incubation at 37 °C, PRMT1 or ΔN-PRMT8 was incubated with the isolated N-terminal domain (amino acids 1–69) of PRMT8 for 1 h at room temperature. The subsequent steps were performed as described above. Values for incorporated radioactivity were fit to competitive inhibition (Equation 2), mixed inhibition (Equation 3), noncompetitive inhibition (Equation 4), and uncompetitive inhibition (Equation 5). Appropriate fit was determined by reduced χ² analysis.

$$v = V_{\max}[S]/([S] + K_m(1 + [I]/K_{is})) \quad (\text{Eq. 2})$$

$$v = V_{\max}[S]/([S](1 + [I]/K_{ii}) + K_m(1 + [I]/K_{is})) \quad (\text{Eq. 3})$$

$$v = V_{\max}[S]/([S](1 + [I]/K_{ii}) + K_m(1 + [I]/K_{i})) \quad (\text{Eq. 4})$$

$$v = V_{\max}[S]/([S](1 + [I]/K_{ii}) + K_m) \quad (\text{Eq. 5})$$

RESULTS

Removal of the PRMT8 N Terminus Results in Increased Enzymatic Activity—The activity and/or substrate specificity of the PRMTs is often regulated by the N terminus of the enzyme (1, 3, 32, 34, 35, 42). Previously, Clarke and co-workers (6) showed that the N terminus of PRMT8 negatively regulates its activity because recombinant PRMT8 lacking the first 60 residues (ΔN-PRMT8) has significantly more activity compared with the full-length enzyme. Indeed, PRMT1 and ΔN-PRMT8 can be regarded as very similar enzymes because they are >84% identical. Because this work was done using an N-terminal GST tag, we determined the kinetic activity of ΔN-PRMT8 with a C-terminal His tag compared with C-terminal His-tagged versions of PRMT1 and full-length PRMT8 under steady-state conditions. As shown in previously published studies (5, 26), PRMT1 has much higher activity than PRMT8. For example, the k_{cat}/K_m value for AdoMet is 210-fold higher than that obtained with PRMT8 (Fig. 1 and Table 1). Consistent with the increase in activity seen by Clarke and co-workers, both $k_{\text{cat(AdoMet)}}$ and $k_{\text{cat(H4)}}$ were dramatically increased (between 10- and 20-fold) upon deletion of the N terminus of PRMT8

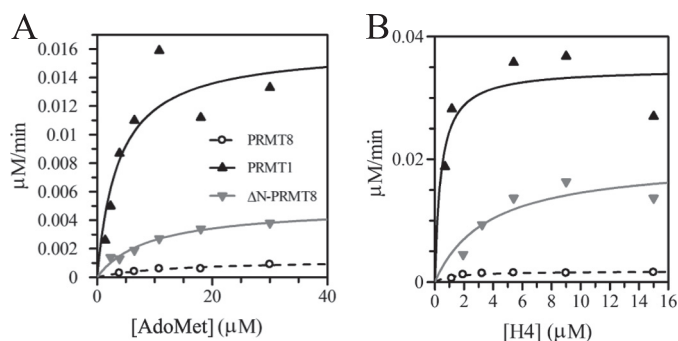


FIGURE 1. AdoMet sensitivity is restricted in PRMT8 compared with PRMT1. A, steady-state kinetic velocities for PRMT8, PRMT1, and ΔN-PRMT8 with a constant H4 concentration (6 μM) and varying [¹⁴C]AdoMet concentrations. Reactions were performed for 15 min (PRMT1 and ΔN-PRMT8; 0.4 μM enzyme) or 1 h (PRMT8; 0.8 μM enzyme) at 37 °C. Methylation was detected by phosphor screen. B, steady-state kinetic velocities as in A except with a constant [¹⁴C]AdoMet concentration (30 μM) and varying H4 concentrations. Data are representative of two independent experiments. K_m and k_{cat} values are listed in Table 1.

TABLE 1
Kinetic parameters of PRMTs and mutants

The steady-state kinetic values for recombinant enzymes are given. Values represent the means ± S.D. from at least two independent experiments.

Enzyme	AdoMet		
	K_m μM	k_{cat} min ⁻¹	k_{cat}/K_m M ⁻¹ min ⁻¹
PRMT1 ^a	2.2 ± 0.4	0.047 ± 0.009	2.1 × 10 ⁴
PRMT8 ^a	19.7 ± 0.5	0.0015 ± 0.0005	76
ΔN-PRMT8 ^a	13 ± 5	0.018 ± 0.005	1.4 × 10 ³
Chimeric PRMT8/1 ^a	18 ± 4	0.019 ± 0.006	1.1 × 10 ³
R58K ^a	11 ± 6	0.0052 ± 0.0005	4.7 × 10 ²
R73K ^a	4 ± 1	0.0038 ± 0.0011	9.5 × 10 ²
R58K/R73K ^a	2.8 ± 1.1	0.0026 ± 0.0004	9.3 × 10 ²
R58F ^a	4 ± 3	0.0004 ± 0.00006	1.0 × 10 ²
R73F ^a	5.6 ± 0.3	0.0004 ± 0.00000	71
R58F/R73F ^a	6 ± 3	0.0028 ± 0.0004	4.7 × 10 ²
Histone H4			
PRMT1 ^b	1.8 ± 1.6	0.090 ± 0.008	5.0 × 10 ⁴
PRMT8 ^b	1.5 ± 0.3	0.0024 ± 0.0003	1.6 × 10 ³
ΔN-PRMT8 ^b	2 ± 2	0.044 ± 0.0012	2.2 × 10 ⁴
Chimeric PRMT8/1 ^b	6 ± 5	0.015 ± 0.002	2.5 × 10 ³
R58K ^b	2.4 ± 0.2	0.0077 ± 0.0011	3.2 × 10 ³
R73K ^b	1.9 ± 0.0	0.0053 ± 0.0001	2.8 × 10 ³
R58K/R73K ^b	10 ± 1.1	0.006 ± 0.004	6.0 × 10 ²
R58F ^b	1.1 ± 0.4	0.0009 ± 0.0008	8.2 × 10 ²
R73F ^b	0.9 ± 0.1	0.0008 ± 0.0007	8.9 × 10 ²
R58F/R73F ^b	3 ± 1	0.0045 ± 0.0008	1.5 × 10 ³

^a [histone H4] = 6 μM.

^b [AdoMet] = 30 μM.

(compare the values for ΔN-PRMT8 versus full-length PRMT8) (Fig. 1 and Table 1). Thus, our C-terminal His-tagged versions of PRMT8 behaved as expected. Furthermore, the lack of the first 60 residues of the N terminus did not result in a change in $K_m(\text{H4})$. In contrast, the $K_m(\text{AdoMet})$ was decreased in the truncated version of PRMT8 compared with full-length PRMT8 (Table 1), suggesting that the N-terminal domain of PRMT8 regulates the affinity of PRMT8 for the AdoMet cofactor but not the protein substrate.

Automethylation Occurs in cis—Although the enzyme core amino acid sequence of PRMT8 is >84% identical to PRMT1, the 80-residue N terminus of PRMT8 is unique among PRMTs (5, 6). It contains a myristoylation site at Gly-2 and two automethylation sites, *i.e.* Arg-58 and Arg-73 (5, 6). Because dimer formation is necessary for PRMT activity (18, 20, 37) and mutations in the dimerization arms of multiple PRMTs drastically

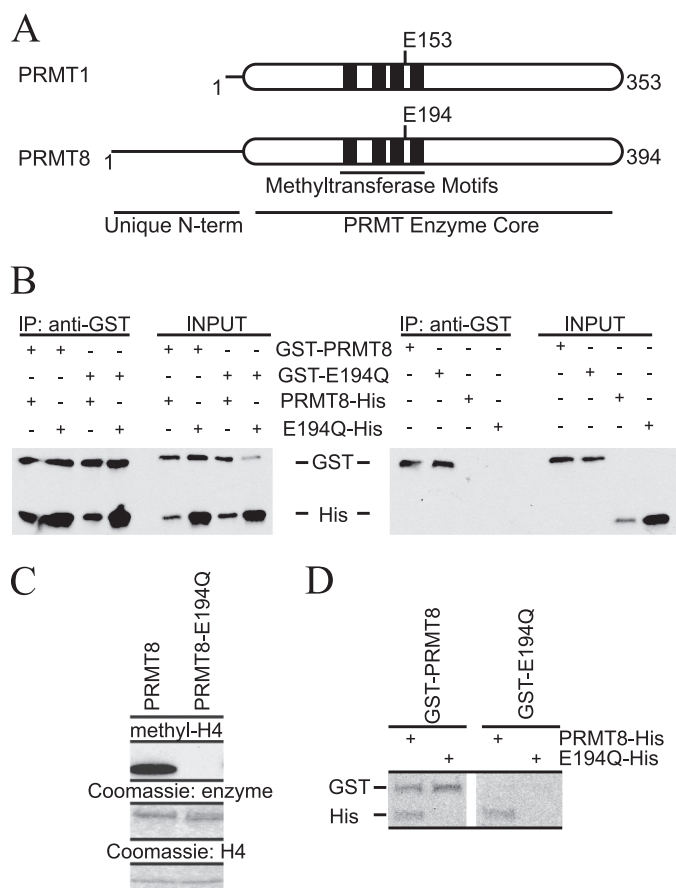


FIGURE 2. Automethylation occurs in cis. *A*, schematic of PRMT1 and PRMT8 with homologous arginine-binding glutamate residues highlighted. *Black bars* denote methyltransferase motifs conserved across the PRMT family. *N-term*, N terminus. *B*, immunoblot of co-immunoprecipitated GST- or His-tagged PRMT8 and PRMT8-E194Q after overnight anti-GST immunoprecipitation (IP) with input-only controls. *C*, autoradiograph of methylated H4 after 1 h of incubation with recombinant PRMT8 or PRMT8-E194Q and [³H]AdoMet and Coomassie Blue-stained loading controls. *D*, autoradiograph of automethylation with mixed enzymes: GST-tagged PRMT8 or PRMT8-E194Q with His-tagged PRMT8 or PRMT8-E194Q. Data are representative of two independent experiments.

decrease enzymatic activity (18, 20), we sought to determine whether automethylation of PRMT8, which is also known to dimerize (6), occurs in *cis* or *trans*. To distinguish between these two possibilities, we utilized GST- or His-tagged recombinant versions of PRMT8 to track automethylation. In addition, we created a catalytically impaired PRMT8 mutant, *i.e.* PRMT8-E194Q (Fig. 2*A*), and confirmed that this mutant lacked significant activity (Fig. 2*C*). The corresponding mutation in PRMT1 decreased activity by 30–100-fold (20, 48). Additionally, we confirmed that heterodimers formed between differently tagged enzymes by pulldown assays (Fig. 2*B*). For these experiments, PRMT8-His and GST-PRMT8-E194Q were incubated together, as well as the reverse combination, *i.e.* GST-PRMT8 and PRMT8-E194Q-His, for 1 h with [³H]AdoMet with no additional methyl-accepting substrate present in the reaction. Automethylation was detected only on the wild-type PRMT8 subunit and never on PRMT8-E194Q (Fig. 2*D*). Thus, automethylation occurs in *cis* on the PRMT8 homodimer.

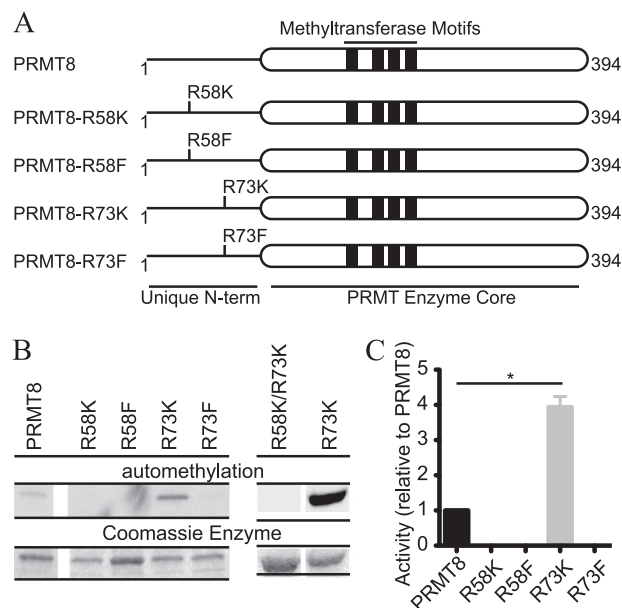


FIGURE 3. Arg-73 automethylation is dependent on methylation at Arg-58. *A*, schematic of PRMT8 and point mutants. *Black bars* denote methyltransferase motifs conserved across the PRMT family. The PRMT enzyme core is the region of high identity (84%) to PRMT1. *N-term*, N terminus. *B*, autoradiograph of automethylation by PRMT8 and point mutants of recombinant enzymes after 1 h of incubation with [³H]AdoMet. *C*, densitometry analysis of *B* (means \pm S.E.). Data are representative of at least three independent experiments. *, $p < 0.05$.

Automethylation of Arg-73 Is Dependent on Arg-58—To determine whether automethylation of PRMT8 regulates methyltransferase activity, we used site-directed mutagenesis to create point mutants that either mimicked or prevented automethylation. The use of these mutants allowed for the control of the methylation status of the bacterially purified wild-type enzyme, which is likely a heterogeneously methylated population. Initially, Arg-58 and Arg-73 were individually mutated to lysine to generate the PRMT8-R58K and PRMT8-R73K mutants; lysine was used because it preserves the positive charge while preventing methylation (Fig. 3*A*). PRMT8-R58F and PRMT8-R73F were also generated because phenylalanines are often used to mimic the increased hydrophobicity of a methylated arginine (Fig. 3*A*) (49).

To establish whether the arginine methylation states of Arg-58 and Arg-73 are interdependent, we examined the automethylation activity of the PRMT8-R58K and PRMT8-R73K mutants using [³H]AdoMet as the methyl donor. Fig. 3*B* shows that the R58K mutant did not automethylate, whereas the R73K mutant did (6). Interestingly, PRMT8-R73K showed a higher level of Arg-58 automethylation compared with wild-type PRMT8 (Fig. 3*C*). To confirm that the automethylation detected with the PRMT8-R73K mutant is located on Arg-58, we examined the automethylation of the double lysine mutant, PRMT8-R58K/R73K. In this case, no automethylation was detected (Fig. 2*B*), strongly suggesting that Arg-58 is the site of automethylation on the PRMT8-R73K mutant. There are at least two interpretations of the above results, including the possibility that Arg-58 is the major site of automethylation and that its modification requires a positively charged residue at position 73. Alternately, automethylation of Arg-58 may occur

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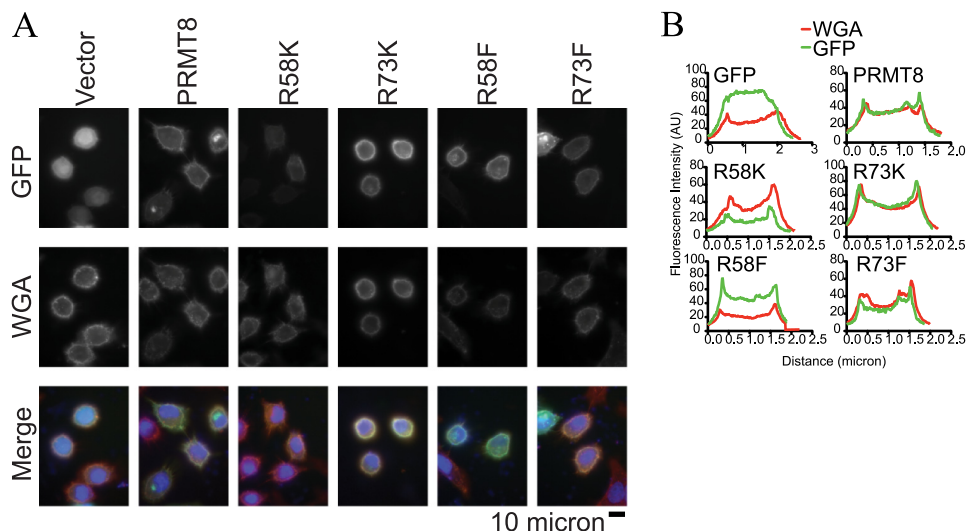


FIGURE 4. **Membrane localization of PRMT8 is independent of automethylation.** *A*, fluorescence microscopy images of GFP-tagged PRMT8 and mutants (Pearson's coefficients of 0.91 ± 0.02 for PRMT8, 0.93 ± 0.02 for R58K, 0.98 ± 0.01 for R73K, 0.94 ± 0.02 for R58F, and 0.96 ± 0.01 for R73F) compared with empty vector (Pearson's coefficient = 0.76 ± 0.09) transfected into HeLa cells and co-stained with Alexa Fluor 555-conjugated wheat germ agglutinin (WGA; membrane) and DAPI (nucleus). Images are representative of multiple fields. *B*, fluorescence intensity analysis of cell cross-sections of *A*. AU, arbitrary units.

independently of Arg-73 methylation, but Arg-58 methylation is a prerequisite for methylation of Arg-73. In contrast to the results obtained for the lysine mutants, neither of the phenylalanine mutants, *i.e.* PRMT8-R58F and PRMT8-R73F, automethylated (Fig. 1, *B* and *C*). This result is consistent with the notion that Arg-58 is the preferred site of automethylation and that its methylation requires a positively charged residue, either lysine or arginine, at position 73.

Automethylation Does Not Alter Membrane Targeting of PRMT8—In eukaryotic cells, PRMT8 is modified by myristoylation at Gly-2, which promotes membrane localization of the enzyme (5). Because the myristoylation modification occurs cotranslationally, modification of PRMT8 by myristoylation must occur prior to automethylation (50, 51). However, it is possible that automethylation regulates the association of myristoylated PRMT8 with the cell membrane. To determine whether any of the automethylation mutants affect the subcellular localization of PRMT8, we transiently transfected HeLa cells with GFP-tagged PRMT8 variants. Transfected cells were also stained with fluorescently labeled wheat germ agglutinin (Alexa Fluor 555-labeled) to identify the cell membrane and with DAPI as a nuclear stain (Fig. 4*A*). Cells with GFP-tagged PRMT8 were imaged by microscopy, and colocalization was analyzed by determining the Pearson's coefficient (47). Additionally, colocalization was visualized by determining fluorescence intensity across the diameter of the cell. As expected, wild-type PRMT8 and point mutants all displayed a similar level of colocalization (Pearson's coefficient of 0.91–0.98) compared with GFP alone (Pearson's coefficient of 0.76) (Fig. 4, *A* and *B*). In total, these results indicate that the membrane localization of PRMT8 is independent from its automethylation activity.

Blocking Automethylation Increases Sensitivity to AdoMet—To determine whether automethylation regulates enzymatic activity, we next investigated whether prevention of automethylation using the PRMT8-R58K or PRMT8-R73K mutant changes the kinetic parameters of the PRMT8 enzyme. As wild-

type PRMT8 purified from bacteria likely consists of a heterogeneous population of automethylated and unautomethylated enzymes due to the presence of AdoMet within the bacterial cells, the use of the lysine and phenylalanine mutants allowed for the control of the automethylation status of PRMT8. Because the biological substrates of PRMT8 are currently unknown, we used a histone H4 peptide containing a single arginine (Arg-3), which can be methylated by PRMT1 and PRMT8 *in vitro* (6). As depicted in Table 1, when either automethylation site was blocked by lysine mutations (R58K or R73K), in kinetic assays with constant H4 and varying AdoMet concentrations, the turnover rate ($k_{\text{cat(AdoMet)}}$) was increased by 1.5–3-fold compared with wild-type PRMT8, and the $K_{m(\text{AdoMet})}$ was decreased by 2-fold. In contrast, when the AdoMet concentration was held constant and the H4 concentration was varied, the turnover rate also increased, but there was no corresponding decrease in $K_{m(\text{H4})}$ (Table 1).

These data led us to postulate that a further increase in catalytic activity could be achieved by generating the R58K/R73K double mutant. As expected, the $k_{\text{cat}}/K_{m(\text{AdoMet})}$ for the R58K/R73K enzyme was increased to a level that was comparable to the enzyme lacking the entire N terminus ($\Delta\text{N-PRMT8}$) (Table 1). Because the increase in $k_{\text{cat}}/K_{m(\text{AdoMet})}$ was largely driven by a decrease in $K_{m(\text{AdoMet})}$ with little to no effect on the kinetic parameters for histone H4, these data suggest that automethylation likely regulates either the conformation of the AdoMet-binding pocket or the ability of AdoMet to access this site.

Mimicking Automethylation Decreases Sensitivity to AdoMet—Because blocking methylation increased both turnover rates and decreased $K_{m(\text{AdoMet})}$, we predicted that a mimic of methylation, phenylalanine, would have the opposite effect, *i.e.* decrease the turnover rate and increase the $K_{m(\text{AdoMet})}$. As expected, the $k_{\text{cat(AdoMet)}}$ was reduced for both of the phenylalanine single mutants, corresponding to a decrease in $k_{\text{cat}}/K_{m(\text{AdoMet})}$ (Table 1). Importantly, although a similar decrease in activity was seen in the $k_{\text{cat(H4)}}$, no corresponding decrease was detected in the $K_{m(\text{H4})}$ (Table 1). Thus, automethylation of

PRMT8 most likely affects R58K/R73K sensitivity but not the affinity of the enzyme for H4.

Likewise, we sought to determine whether the double methylation mimic would also enhance the differences seen with the individual phenylalanine mutants. Interestingly, in contrast to the single mimic mutants PRMT8-R58F and PRMT8-R73F, the activity of the double phenylalanine mutant, PRMT8-R58F/R73F, was dramatically increased relative to wild-type PRMT8 (Table 1). Although the reason for this discrepancy is unclear, it likely relates to the fact that phenylalanine is an imperfect mimic of a methylarginine because it lacks positive charge and is therefore incapable of forming the idealized cation- π interactions that are characteristic of methylarginine- and methyllysine-binding domains (52). Regardless, the data for the single mutants provide compelling evidence for the hypothesis that methylarginines bind to a site on the enzyme that alters the affinity of the AdoMet-binding pocket for AdoMet.

Isolated PRMT8 N-terminal Domain Competitively Inhibits AdoMet—Because these data suggested that the N terminus of PRMT8 inhibits AdoMet binding, we sought to determine the mechanism by which the N-terminal domain of PRMT8 acts as an inhibitor. For these studies, we expressed and purified the N-terminal domain (residues 1–69) as a His-tagged fusion protein and used it in kinetic studies with Δ N-PRMT8.

To determine whether the N-terminal domain can inhibit Δ N-PRMT8 activity in *trans*, we also determined the steady-state kinetic parameters for these enzymes in the presence of increasing concentrations of this domain. Based on visual inspection of the double-reciprocal plots and a comparison of the reduced χ^2 values, the N-terminal domain acted as a competitive inhibitor of AdoMet for Δ N-PRMT8 ($K_{i(\text{AdoMet})} = 6.0 \pm 1.3 \mu\text{M}$) (Fig. 5A). This suggested that the isolated N-terminal domain of PRMT8 would act as a substrate for Δ N-PRMT8. Unfortunately, methylation of the N-terminal domain by Δ N-PRMT8 was below the limit of detection (data not shown). Thus, we isolated point mutants of the N-terminal domain of PRMT8 to mimic the methylated or unmethylated states. The R58F point mutant of the N-terminal domain of PRMT8 acted as a competitive inhibitor of AdoMet for Δ N-PRMT8 ($K_{i(\text{AdoMet})} = 2.2 \pm 0.5 \mu\text{M}$) (Fig. 5B), supporting the hypothesis that automethylation of the N-terminal domain inhibits AdoMet binding. In contrast, the R58K point mutant of the N-terminal domain acted as a noncompetitive inhibitor of AdoMet for Δ N-PRMT8 ($K_{i(\text{AdoMet})} = 5.4 \pm 1.3 \mu\text{M}$) (Fig. 5C), suggesting that there are additional mechanisms by which the N-terminal domain regulates PRMT8 activity that do not involve AdoMet binding. This conclusion seems likely because the N terminus acts as both a substrate and an inhibitor of AdoMet binding.

The results obtained for AdoMet were in contrast to the effect of the N-terminal domain of PRMT8 on H4 kinetics, where this domain acted as a noncompetitive inhibitor of H4 ($K_{i(\text{H4})} = 8.2 \pm 1.4 \mu\text{M}$) (Fig. 5D). In total, these data, combined with our above studies, suggest that the N terminus of PRMT8 impairs the AdoMet/PRMT8 interaction.

Chimeric PRMT1 with the N-terminal Domain of PRMT8 Has Decreased AdoMet Sensitivity—Finally, to confirm that inhibition is an intrinsic property of the N-terminal domain of

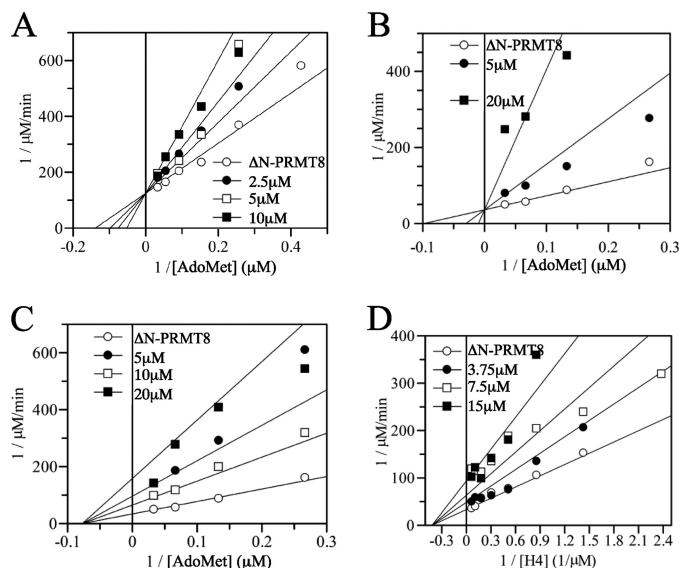


FIGURE 5. Isolated N-terminal domain of PRMT8 competitively inhibits AdoMet. A, Lineweaver-Burke plot for Δ N-PRMT8 (0.4 μM) with a constant H4 concentration (6 μM), varying [^{14}C]AdoMet concentrations, and the N-terminal domain of PRMT8 (amino acids 1–69; 2.5, 5, or 10 μM). The data were fit to a competitive inhibition model (Equation 2). Reactions were performed for 15 min at 37 $^{\circ}\text{C}$. Methylation was detected by phosphor screen. Data were analyzed by reduced χ^2 analysis for best fit. B, the conditions were similar to those described for A except that the R58F point mutant of the N-terminal domain of PRMT8 was used at 5, 10, or 20 μM . The data were fit to a competitive inhibition model (Equation 2). C, the conditions were similar to those described for A except that the R58K point mutant of the N-terminal domain of PRMT8 was used at 5, 10, or 20 μM . The data were fit to a noncompetitive inhibition model (Equation 4). D, Lineweaver-Burke plot as described for A with a constant [^{14}C]AdoMet concentration (30 μM), varying H4 concentrations, and the N-terminal domain of PRMT8 (3.75, 7.5, or 15 μM). The data were fit to a noncompetitive inhibition model (Equation 4). Data are representative of two independent experiments.

PRMT8, we generated a chimeric enzyme containing the PRMT1 core and the N-terminal domain of PRMT8 (referred to as Ch8-1). In steady-state kinetic studies, the $K_{m(\text{AdoMet})}$ of Ch8-1 was increased to a similar level compared with full-length PRMT8, whereas the $K_{m(\text{H4})}$ remained similar to that of PRMT1 (Table 1). The $k_{\text{cat}(\text{AdoMet})}$ and $k_{\text{cat}(\text{H4})}$ of Ch8-1 were also decreased by 2–4-fold compared with PRMT1 (Table 1). Additionally, automethylation was detected on Ch8-1 at similar levels compared with PRMT8, whereas PRMT1 and Δ N-PRMT8 did not have detectable levels of automethylation (Fig. 6). Thus, automethylation of the N-terminal domain of PRMT8 reduces the activity of the enzyme by increasing the concentration of AdoMet necessary for the catalytic reaction to proceed but does not greatly affect the access and binding of the protein substrate.

DISCUSSION

Regulation of enzymatic activity is critical to maintain cellular homeostasis and to allow for the proper functioning of signaling networks. Given that the PRMTs are generally thought to be constitutively active when purified from orthogonal sources, there must be mechanisms to negatively regulate and “turn off” their activity. For example, phosphorylation of CARM1 is known to decrease the activity of this isozyme (53). With respect to PRMT8, the unique N terminus of this enzyme is known to be myristoylated and automethylated, and these

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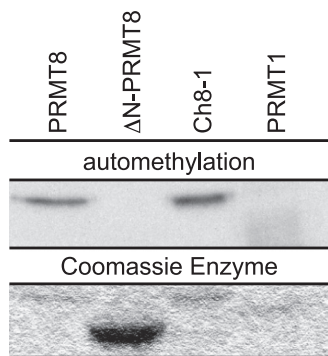


FIGURE 6. Fusion of the N-terminal domain of PRMT8 to PRMT1 induces automethylation. Shown is an autoradiograph of full-length PRMT8, Δ N-PRMT8, Ch8-1, and PRMT1 incubated overnight with [3 H]AdoMet to determine automethylation.

modifications appear to be important for regulating the subcellular localization and activity of this enzyme. In fact, deletion of the first 60 residues of the protein results in a large increase in activity (6). Here, we have shown that automethylation within the N-terminal domain of PRMT8 regulates its activity by altering the affinity of PRMT8 for its cofactor AdoMet.

On the basis of our data, we propose that the methyltransferase activity is regulated by the following mechanism (Fig. 7). PRMT8 is unmethylated in its open and active state. As PRMT8 progresses and methylates its endogenous protein substrate, this decreases the availability of unmethylated substrate. The lack of unmethylated substrate results in automethylation of PRMT8, causing the N terminus to bind to the enzyme core and blocking AdoMet access to the catalytic site, leading to downregulation of PRMT8 activity. The N terminus of PRMT8 extends from the conserved α -helix of the PRMTs, which is required for enzymatic activity and AdoMet binding (18–21). Automethylation of the N-terminal domain of PRMT8 may induce a conformational shift in the α -helix, preventing its ability to properly bind AdoMet, thus lowering activity.

In support of this model, we used two alternative mutations of the arginine automethylation sites, Arg-58 and Arg-73: lysine, which preserves the positive charge of the residue but is not methylated by PRMT8, and phenylalanine, which mimics the increased hydrophobicity of a methylated arginine (49). Interestingly, we found that neither of the Arg-to-Phe mutants, *i.e.* PRMT8-R58F and PRMT8-R73F, had detectable levels of automethylation, nor did the methylation-null mutant PRMT8-R58K. However, PRMT8-R73K had increased levels of automethylation, and the double lysine mutant PRMT8-R58K/R73K did not have detectable levels of automethylation. This suggests that Arg-58 is the automethylation site of the PRMT8-R73K mutant. It appears that Arg-58 is the “gateway” automethylation site, without which Arg-73 is not methylated. In addition, the presence of increased automethylation in PRMT8-R73K indicates that Arg-73 is the automethylation site that more strongly affects the methylation activity of PRMT8.

These observations played out when we examined the activity of PRMT8 methylating the substrate histone H4, which is methylated at Arg-3. In both cases of preventing automethylation, PRMT8-R58K and PRMT8-R73K, there was an increase in turnover rates and a decrease in $K_{m(\text{AdoMet})}$. The opposite was

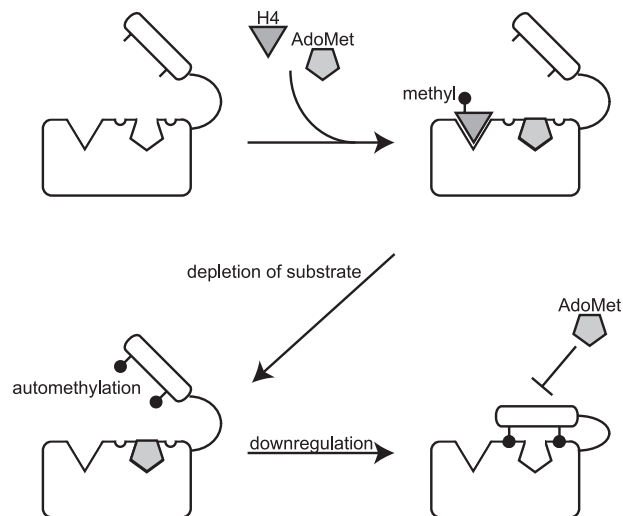


FIGURE 7. Model of regulation of PRMT8 by automethylation.

true for PRMT8-R58F and PRMT8-R73F, the methylation mimics; both had decreased methylation activity. Intriguingly, these effects could be transferred to a similar enzyme, PRMT1, by fusing on the N terminus of PRMT8, thus verifying that the N-terminal domain of PRMT8 directly decreases the ability of PRMT8 to utilize AdoMet.

In this study, we determined that automethylation of PRMT8 negatively regulates its methyltransferase activity. Because the biological function of PRMT8 is currently unknown, the involvement of these post-translational modifications in cellular pathways has yet to be determined. However, because automethylation appears to function as a negative regulator by lowering AdoMet affinity of PRMT8, myristoylation of the endogenous enzyme and subsequent membrane association may affect as a positive regulator of catalytic activity, potentially increasing enzyme velocity by increasing the affinity of AdoMet or the protein substrate for PRMT8. Future crystallography studies on the structure of PRMT8 will help provide insight as to how these residues interact with the rest of the protein to regulate activity.

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