A Kinetic Study of Human Protein Arginine N-Methyltransferase 6 Reveals a Distributive Mechanism*

Received for publication, December 13, 2007, and in revised form, January 15, 2008. Published, JBC Papers in Press, February 8, 2008, DOI 10.1074/jbc.M710176200

Ted M. Lakowski and Adam Frankel1
From the Division of Biomolecular & Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.

Human protein arginine N-methyltransferase 6 (PRMT6) transfers methyl groups from the co-substrate S-adenosyl-L-methionine to arginine residues within proteins, forming S-adenosyl-L-homocysteine as well as ω-N6-monomethylarginine (MMA) and asymmetric dimethylarginine (aDMA) residues in the process. We have characterized the kinetic mechanism of recombinant His-tagged PRMT6 using a mass spectrometry method for monitoring the methylation of a series of peptides bearing a single arginine, MMA, or aDMA residue. We find that PRMT6 follows an ordered sequential mechanism in which S-adenosyl-L-methionine binds to the enzyme first and the methylated product is the first to dissociate. Furthermore, we find that the enzyme displays a preference for the monomethylated peptide substrate, exhibiting both lower $K_m$ and higher $V_{max}$ values than what are observed for the unmethylated peptide. This difference in substrate $K_m$ and $V_{max}$ as well as the lack of detectable aDMA-containing product from the unmethylated substrate, suggest a distributive rather than processive mechanism for multiple methylations of a single arginine residue. In addition, we speculate that the increased catalytic efficiency of PRMT6 for methylated substrates combined with lower $K_m$ values for native protein methyl acceptors may obscure this distributive mechanism to produce an apparently processive mechanism.

Human protein arginine N-methyltransferases (PRMTs)2 are a family of enzymes that transfer methyl groups from the co-substrate S-adenosyl-L-methionine (AdoMet) to the terminal nitrogen atoms on the guanidino groups of arginine residues within proteins, forming S-adenosyl-L-homocysteine (AdoHcy) and methylarginine residues in the process (1). Two types of PRMT activities have been identified for this enzyme family; both include the formation of an ω-N6-monomethylarginine (MMA) residue as an intermediate to the formation of an asymmetric ω-N6,N6'-dimethylarginine (aDMA) residue in Type I activity, or the formation of a symmetric ω-N6,N6'-di(dimethylarginine (sDMA) residue in Type II activity. To date mammalian enzymes that exhibit Type I activity include PRMT1 (2), PRMT3 (3), coactivator-associated arginine methyltransferase 1 (CARM1/PRMT4) (4, 5), PRMT6 (6), and PRMT7 has been shown to catalyze the formation of MMA residues on a peptide substrate (10), but in another study it was shown to form MMA, aDMA, and sDMA residues on protein substrates (11). A structurally unrelated protein to the highly conserved PRMT family (12–15) is F-box only protein 11 (FXO11), which has been reported to catalyze the formation of MMA, aDMA, and sDMA residues on proteins (16).

The functional consequence of arginine methylation within proteins is both chemically subtle and biologically profound, because the hydrogen-bonding capacity of an arginine residue is altered and its electrostatic charge is unchanged with the addition of one or two methyl substituents (17). Nevertheless, several reviews highlight the integral involvement of arginine methylation in a variety of cellular processes and pathologies, including signal transduction, transcription, RNA processing, chromatin remodeling, DNA repair, viral replication, and cancer (reviewed in Refs. 18–23).

The biological role of PRMT6 has yet to be clearly defined, but evidence suggests that its activity affects gene regulation primarily by modifying protein-nucleic acid interactions. PRMT6 localizes exclusively to the cell nucleus, exhibits auto-methylation, and methylates in vitro glycine- and arginine-rich (GAR) sequences in proteins (6). Despite its overlapping substrate specificity with PRMT1, some PRMT6-specific cellular targets do not contain the GAR consensus sequence (1, 24), including high mobility group proteins HMG1a and HMG1b (25, 26), DNA polymerase β (27), HIV-1 trans-activator of transcription (Tat) protein (28), HIV-1 regulator of virion (Rev) protein (29), HIV-1 nucleocapsid protein (30), and histone H3 (31–33). Methylation of polymerase β by PRMT6 was shown to increase its repair activity of damaged DNA, implicating PRMT6 as a regulator of base excision repair (27). In the case of HIV-1 substrate methylation, the role of PRMT6 activity appears to antagonize viral replication by attenuating interactions between arginine-rich motifs and RNA (28–30).
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34). Most recently several groups have demonstrated that PRMT6 methylation of Arg-2 on histone H3 in vivo exists in an exclusive relationship with Lys-4 methylation, thus adding to the mounting evidence that PRMT6 is a negative regulator of cellular as well as viral transcriptional activation (31–33). PRMT6 and other PRMTs are now a focus of attention for the development of inhibitors targeting methyl transfer activity (20, 29, 30, 35–37).

Despite efforts to understand various PRMT activities within a cellular context, very little is known about the mechanism of sequential methyl transfer for this family of enzymes. PRMTs are part of a larger family of AdoMet-dependent methyltransferases that use a Bi-Bi enzyme mechanism to produce two products from two reactants (38, 39). This mechanism is complicated with respect to PRMTs, because the product of a single methylation (MMA) can be the substrate for a second reaction to form the final dimethylated product. Another layer of mechanistic complexity arises when a protein substrate contains multiple arginine residues such as GAR- or arginine-rich motif-containing sequences, thereby increasing the combinations of potential products. Early enzyme preparations of PRMT were purified from animal tissue and often catalyzed the formation of MMA complexes supports the sequential ordered Bi-Bi reaction mechanism in which AdoMet was determined to bind prior to the protein substrate (40). Recent structural evidence from different PRMT-AdoHcy complexes supports the sequential ordered Bi-Bi reaction mechanism (13–15). All of these structures show a disordered N-terminal helix that resolves upon AdoHcy binding, suggesting a mechanism similar to a trap door that locks AdoMet into place. This helix forms a ridge along which the arginine-containing substrate can bind, implying that AdoMet must bind first to form the binding site for the methyl acceptor (13–15). Interestingly, PRMT dimerization is a requirement to stabilize the enzyme-AdoMet complex, and it places two active sites in close proximity to one another that may contribute to the production of aDMA residues in a processive manner (12–15). Therefore, a model for PRMT activity may involve both monomers loaded with AdoMet and each responsible for one of two methyl transfers. Perhaps this model explains the observation that endogenous PRMT substrates almost exclusively contain aDMA residues (41–47).

Previous analysis of PRMT6 methylation of GST-GAR (a recombinant protein expressed in *Escherichia coli* consisting of glutathione S-transferase fused to the first 148 amino acids of human fibrillarin (3)) revealed that formation of aDMA residues over time closely follows formation of MMA residues, suggesting a processive mechanism for multiple methyl transfers (6). Indeed, apparent processivity in GST-PRMT1 and GST-PRMT3 methylations of a GAR-like peptide substrate containing four arginine residues has also been observed (48). It has also been speculated that PRMT1 methylates with a partially processive mechanism whereby aDMA production does not occur in an obligate fashion relative to MMA formation, yet it still accumulates in significant amounts (49).

In the present study we elucidate the kinetic mechanism of His-tagged PRMT6 (H6-PRMT6) using a series of “minimal” peptide substrates bearing either a single arginine residue (R1), an MMA residue (R1(MMA)), or an aDMA residue (R1(aDMA)). Using the R1 and R1(MMA) peptides we describe the kinetic mechanism of single and multiple methylation events by PRMT6 on one arginine residue. A UPLC mass spectrometry (MS) method for directly measuring the rate of methylation is described. We find that PRMT6 follows an ordered sequential mechanism in which AdoMet binds to the enzyme first, and the methylated product is the first product to dissociate. Furthermore, we find that the enzyme displays a preference for the methylated peptide substrate, exhibiting both lower $K_m$ and higher $V_{max}$ values than those observed for the unmethylated peptide. This difference in substrate $K_m$ and $V_{max}$ along with the lack of detectable aDMA-containing product from the R1 peptide substrate suggests a distributive rather than processive mechanism for multiple methylations of a single arginine residue.

**EXPERIMENTAL PROCEDURES**

**PRMT6 Enzyme**—The construct for His-tagged PRMT6 (H6-PRMT6) was generated by subcloning the gene from the pGEX-6P-1 vector (a gift from Dr. Steven Clarke at UCLA) into the pET28a+ vector using the restriction enzymes BamHI and XhoI. H6-PRMT6 was induced in BL21 Star (DE3) with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h, and the cells were harvested by centrifugation. Cells were lysed by sonication and purified by a 1-ml His-Trap affinity column (GE Healthcare) according to established methods (50). The partially purified H6-PRMT6 was desalted using a HiPrep 26/10 desalting S300 strong cation exchange semi-prep high-performance liquid chromatography column. The column was washed with 20 ml of 50 mM NaH2PO4, pH 7.5, and H6-PRMT6 was eluted with 80 ml of a linear 0 to 100% gradient of 50 mM NaH2PO4, pH 7.5, with 1 mM NaCl over 20 min. Fractions containing H6-PRMT6 were desalted as above and applied to a 5-ml HiTrap Q HP (GE Healthcare) column. The column was washed with 30 ml of 50 mM Tris- HCl, pH 7.5, and pure H6-PRMT6 was eluted with 90 ml of a linear 0 to 100% gradient of 50 mM Tris- HCl, pH 7.5, with 1 mM NaCl over 30 min. Pure H6-PRMT6 was concentrated with an Ultra 15 10k NMWCO centrifugal filter (Amicon) and resuspended in 50 mM HEPES, pH 7.6, 100 mM KCl, 10 mM MgCl, 30% glycerol, and 7 mM mercaptoethanol for storage at –80 °C (50).

To determine the purification of H6-PRMT6 away from contaminating proteins that can act as potential substrates we performed methylation reactions after each purification step. Samples were incubated with methylation buffer (50 mM HEPES, pH 8.0, with 10 mM NaCl and 1 mM dithiothreitol) and 112.5 μM (9.25 KBq) S-adenosyl-L-[14C]methionine ([14C]AdoMet) at 37 °C for 90 min. Following incubation, the reactions were precipitated with 12% trichloroacetic acid, dried, and mixed with 2× SDS-PAGE sample dilution buffer and applied to a 10% SDS-PAGE gel prepared according to established methods (51) using a mini-Protein 3 electrophoresis apparatus (Bio-Rad). The dried gel was exposed to a storage
phosphor screen (Amersham Biosciences) for 72 h. The concentration of H₄PRMT6 was determined by total acid hydrolysis, and amino acid analysis was performed at the Hospital for Sick Children, Toronto, Canada, and confirmed by UV spectrometry using the calculated extinction coefficient of ε₂₈⁰ nm = 59275 M⁻¹ cm⁻¹ (52).

**Peptide Substrates**—Peptide substrates R1, R1(MMA), R1(aDMA), and R1(sDMA) shown in Fig. 1 were synthesized by Sigma Genosys. The concentrations of the peptides were determined by total acid hydrolysis, and amino acid analysis as above. Peptide concentrations were confirmed with UV spectrometry using the calculated extinction coefficient of ε₂₈⁰ nm = 13980 M⁻¹ cm⁻¹ (52).

**Mass Spectrometer Assay**—The activity of H₄PRMT6 was measured using a UPLC mass spectrometer (UPLC-MS) assay. A Waters Acquity UPLC BEH C18 column (2.1 × 100 mm) was used at a flow rate of 0.3 ml/min at 40 °C. The mobile phases 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B) were used in a step gradient of 99% A for 2.3 min and 85% A for 2.4 to 11.5 min. A Quatro PremierXE electrospray mass spectrometer (Micromass MS Technologies) was operated in positive ion mode with a cone voltage of 20 V selected ion recording for ions with m/z 385, 651.9, 658.9, and 665.9 corresponding to AdoHcy⁺¹ R1⁺², R1(MMA)⁺², and R1(aDMA)⁺², respectively. The standards AdoHcy, R1(MMA), and R1(aDMA) were used at concentrations of 0.06 to 3 μM, and all standards were separated by at least 0.5 min (supplemental Fig. S1).

Unless stated otherwise enzyme assays were performed with 0.035 μg/μl H₄PRMT6 (0.77 μM final concentration) in the methylation buffer (described above) in a total assay volume of 40 μl at 37 °C for 90 min. All reactions were stopped by incubating the samples at 80 °C for 3 min followed by flash freezing in liquid nitrogen. To establish a linear range of enzyme activity, 50 μM R1 or R1(MMA) substrates were incubated with 200 μM AdoMet at 37 °C for 0–300 min. To determine the type of bi-substrate reaction (ping-pong or sequential) we performed enzyme reactions with 25, 50, 75, 150, and 300 μM R1 peptide or 25, 50, 75, 100, and 150 μM R1(MMA) peptide with constant AdoMet concentrations of 5, 10, 25, and 150 μM. Assays with the product inhibitor AdoHcy at concentrations of 2, 5, and 15 μM were performed with constant (100 μM) or variable (25, 50, 75, and 100 μM) R1(MMA) and constant (20 μM) or variable (10, 20, 30, and 50 μM) AdoMet. Complementary assays with the product inhibitor R1(aDMA) at concentrations of 20, 50, 100, and 300 μM were performed with constant (50 μM) or variable (25, 50, 75, and 100 μM) R1(MMA) and constant (20 μM) or variable (10, 20, 30, and 50 μM) AdoMet. For the R1 peptide substrate, assays with the product inhibitor AdoHcy at concentrations of 2, 5, and 15 μM were performed with constant (75 μM) or variable (50, 100, 150, and 200 μM) R1 and constant (20 μM) or variable (10, 20, 30, and 50 μM) AdoMet. Complementary assays with the product inhibitor R1(aDMA) at concentrations of 50, 100, and 300 μM were also performed with the same constant or variable concentrations of R1 and AdoMet as above. For the assays in which AdoHcy was the product inhibitor the reaction was monitored for ions with m/z = 658.9 (R1(MMA)⁺²) or 665.9 (R1(aDMA)⁺²) for the substrates R1 and R1(MMA), respectively. Where R1(aDMA) was used as the product inhibitor the reaction was monitored by measuring the accumulation of the ion m/z = 385 corresponding to AdoHcy⁺¹.

**TLC Assay**—The activity of H₄PRMT6 was also measured by a TLC assay. Methylation reactions to establish a linear range for enzyme activity were repeated as above with 50 μM R1 or R1(MMA) substrate except that the final reaction volume was 20 μl, and 100 μM [¹⁴C]AdoMet (4.1 kBq per 20-μl reaction) was used. The reactions were stopped at times 0–300 min by the addition of 10 μl of a 70% suspension of nickel-nitrioltriacetic acid nickel affinity agarose (Qiagen) with 2 μl of 2.5 mM AdoHcy, and incubated for 10 min at ambient temperature. This reaction was centrifuged and the resulting 20-μl supernatant was added to Pyrex hydrolysis tubes, followed by the addition of 100 μl of acetone. The acetone/supernatant mixture was dried in a vacuum centrifuge and then hydrolyzed in vacuo with 6 M HCl at 110 °C for 24 h. The hydrolyzed material for each reaction was resuspended in 10 μl of water of which 1 μl was spotted onto glass-backed 5 × 20-cm silica TLC plates. Unlabeled arginine, MMA, aDMA, and sDMA (250 pmol each) were also spotted onto the plate as reference standards. No-enzyme controls were prepared as above except with 500 μM R1(MMA) and 180 μM [¹⁴C]AdoMet (3.6 kBq per 10-μl reaction). The plates were developed in 75% ethanol and 25% concentrated NH₄OH, and the dried plate was exposed to a storage phosphor screen along with 0.04–9 pmol spots of [¹⁴C]AdoMet as radioactive standards. The unlabeled standards were visualized using ninhydrin according to established methods (53).

**Data Analysis**—The data for the bi-substrate reaction was initially fit via non-linear least squares using SigmaPlot 8 (SYSTAT) to the Michaelis-Menten equation for a Uni-Uni reaction for each fixed and series of varied substrate concentrations. To derive the kinetic parameters the reciprocal apparent maximum reaction velocities (apparent Vₘₐₓ) for each fixed sub-

![Figure 1. The amino acid sequences of the R1 peptide series.](image-url)
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strate were plotted against the reciprocal of the varied substrate concentrations. We found that kinetic parameters derived in this way differ slightly (i.e. <10%) from traditional multiple linearized reciprocal plots for each fixed substrate concentration and subsequent re-plot of apparent $V_{\text{max}}$ for each fixed substrate according to established methods (54). Because the traditional reciprocal plots provide a convenient qualitative assessment of enzyme mechanism for both the initial plots of fixed and varied substrates and for the product inhibition studies, we used this method of analysis throughout.

RESULTS

A previous study has shown that GST-tagged PRMT6 co-purifies with several bacterial proteins during affinity purification (6). Accordingly, we have found that contaminating proteins of similar molecular weights are found in affinity-purified H$_6$PRMT6. These contaminating proteins are problematic, because they make it difficult to determine the concentration of H$_6$PRMT6 accurately and they also in some cases act as substrates. Both of these problems interfere with the rigorous determination of kinetic parameters; therefore, we further purified H$_6$PRMT6 using a combination of strong cation and anion exchange chromatography. A Coomassie-stained gel showing the relative purification of H$_6$PRMT6 after each step is shown in Fig. 2A. Fractions from each purification step were incubated with methylation buffer and $^{14}$CAdoMet. In agreement with previous studies on GST-PRMT6 we find that H$_6$PRMT6 methylates itself, and this activity can be used as a gauge for the relative activity of H$_6$PRMT6. We find that the multistep purification results in an increase in H$_6$PRMT6 purity from 0.4 to 97% from lysate to final product as measured by densitometry of Coomassie-stained bands at 45.5 kDa, whereas the nitritotriacetic acid-affinity purified H$_6$PRMT6 is only 40% pure (Fig. 2A, lanes 2 and 3). In addition to an increase in the overall homogeneity of H$_6$PRMT6, our purification scheme results in the removal of many contaminating bacterial proteins, some of which are substrates (Fig. 2B). Two of these substrates appear to co-purify with H$_6$PRMT6 under all of the conditions used. Any contaminants in enzyme preparations used in kinetic studies will invariably introduce an error in enzyme quantitation and kinetic parameters dependent on enzyme concentration (e.g. $V_{\text{max}}$ values), but relative to the Coomassie-stained band (Fig. 2A) and the automethylation band (Fig. 2B) for purified H$_6$PRMT6 these contaminants appear insignificant.

The linear range for the H$_6$PRMT6 catalyzed methylation of R1 and R1(MMA) was determined by both TLC and UPLC-MS methods (Fig. 3). For the TLC assay, the hydrolyzed methylation reactions of H$_6$PRMT6 with $^{14}$CAdoMet and the R1 or the R1(MMA) peptides are shown in Fig. 3, A and B, respectively. Fig. 3C shows that the unlabeled standards L-arginine, MMA, aDMA, and sDMA (whose $R_F$ values are 0.11, 0.16, 0.22, and 0.26, respectively) are completely separated from one another. Fig. 3 (A and B) shows an increase in intensity (by densitometry) with time for the spots at $R_F$ = 0.16 corresponding to MMA for which R1 is the substrate (Fig. 3A), and at $R_F$ = 0.22 corresponding to aDMA for which R1(MMA) is the substrate (Fig. 3B). In addition, the $R_F$ values corresponding to AdoMet, and the potential products of acid-catalyzed hydrolysis of AdoMet (55) are also indicated (Fig. 3, D and E). We note the presence of equal intensity spots consistent with MMA ($R_F$ = 0.16) in the reactions in which R1(MMA) is the substrate (Fig. 3B). This is likely due to incomplete removal of H$_6$PRMT6, which can be a source of MMA and aDMA as a result of auto-methylation (Fig. 3A, lane 1). A spot with an $R_F$ between that of MMA and aDMA is also visible. This may be a product of acid-catalyzed hydrolysis of AdoMet (as observed in Fig. 3, D and E), or it may also be tailing from the aDMA sample. The background spots in the TLC assay do not appear to affect the linearity curve (Fig. 3F), because it is a consistent error, and this background intensity does not change with incubation time (Fig. 3E, lanes 2 and 3).
Using the R1 substrate we find that the accumulation of its methylation product R1(MMA) is linear with respect to time up to at least 120 min with 0.77 μM enzyme (Fig. 3F). However, a striking difference between the two peptide substrates, differing only by a single methyl group, is that the reaction rate appears to be at least 3-fold greater for the R1(MMA) substrate than the rate for the R1 substrate.

Unexpectedly, as measured by both MS and TLC assays, the only major product of reaction with R1 appears to be R1(MMA). The R1(adMA) product is formed in detectable amounts at the 300-min incubation time by TLC and MS, but it is below the level of quantitation by MS (supplemental Fig. S3). These results argue against a processive mechanism in which the amount of R1(adMA) would be comparable to or exceed the level of R1(MMA) (56).

To determine the bi-substrate mechanism that H6PRMT6 catalyzes we incubated increasing concentrations of the R1 or R1(MMA) peptide substrates with increasing AdoMet concentrations. When either R1 or AdoMet concentrations were varied in the presence of a constant concentration of the other substrate the resulting double reciprocal plots exhibit an increasing slope with decreasing AdoMet concentrations, producing a pattern of intersecting lines (Fig. 4, A and B). The same pattern of lines is produced when R1(MMA) is the substrate (Fig. 4, C and D). Therefore, both substrates demonstrate that the activity of H6PRMT6 is indicative of a sequential rather than a ping-pong bi-substrate mechanism (54). The rate of reaction for this mechanism can be described using Equation 1 or its reciprocal form expressed as Equation 2 for which $K_m$ is the dissociation constant for the first substrate to bind, $K_{m}^A$ and $K_{m}^{ab}$ are the Michaelis constants for the first and second substrates to bind, respectively, $V_{max}$ is the maximum rate of the reaction, and $[A]$ and $[B]$ are the concentrations of the first and second substrates to bind, respectively.

$$
\frac{V}{V_{max}} = \frac{[A][B]}{K_{m}^A + K_{m}^{ab} + [A][B]}
$$

(Eq. 1)

$$
\frac{1}{V_{max}} = \frac{1}{V_{max}} \left( \frac{K_{m}^{ab}}{K_{m}^A} \right) + \frac{1}{V_{max}} \left( \frac{1}{K_{m}^{ab}} \right)
$$

(Eq. 2)

In agreement with the determination of the linear range for the substrates (Fig. 3F) the reaction rate with the R1(MMA) substrate is greater than the reaction rate for the R1 substrate at all AdoMet concentrations. The kinetic parameters for both R1 and R1(MMA) substrates determined by the double reciprocal slope and intercept re-plots are listed in Table 1. For the R1(MMA) substrate, the $V_{max}$ is greater and the $K_{m}$ is lower than the corresponding parameters for the R1 substrate, indicating a preference by H6PRMT6 for the monomethylated substrate. Accordingly, the catalytic efficiency ($k_{cat}/K_{m}$) of H6PRMT6 for the R1(MMA) substrate is 4.5-fold greater than the $k_{cat}/K_{m}$ for the R1 substrate.

Information derived from Equations 1 and 2 cannot differentiate among the random, ordered, or Theorell-Chance sequential Bi-Bi mechanisms (54). To determine the possible sequential bi-substrate reactions that H6PRMT6 may catalyze we performed a series of product inhibition assays using the constant and variable substrates AdoMet and R1(MMA), and the
product inhibitors R1(aDMA) and AdoHcy. The inhibition of H₆PRMT6 by AdoHcy with a constant non-saturating concentration of AdoMet (20 μM) and variable R1(MMA) concentrations is mixed (Fig. 5A). In contrast, the inhibition by AdoHcy with a constant non-saturating concentration of R1(MMA) (100 μM) and variable AdoMet concentrations is competitive, thus demonstrating direct competition for the enzyme between AdoMet and AdoHcy (Fig. 5B). This type of direct competition suggests an ordered sequential binding mechanism in which AdoMet is the first substrate to bind to H₆PRMT6. This behavior also argues against a random sequential bi-substrate mechanism: in such a mechanism no avenue for direct competition between AdoMet and AdoHcy for free enzyme exists. Mixed inhibition of H₆PRMT6 by R1(aDMA) is observed in Fig. 5C in which R1(MMA) and AdoMet are the variable and constant substrates, respectively. Such inhibition confirms an ordered sequential mechanism, but rules out a Thorell-Chance mechanism, as competitive inhibition would be expected in that case. Mixed inhibition is shown in Fig. 5D by R1(aDMA) at a constant concentration of R1(MMA) and variable AdoMet concentrations. This type of inhibition once again confirms the presence of an ordered sequential mechanism for H₆PRMT6. A summary of the product inhibition studies using R1(MMA) as the methyl acceptor is shown in Table 2.

The proposed complete reaction mechanism for H₆PRMT6 with R1, as shown in Fig. 6, indicates that R1 is initially methylated by H₆PRMT6 to form R1(MMA) in one step, and this product is subsequently methylated by H₆PRMT6 to form R1(aDMA) in a second step. This model predicts that product inhibitor studies conducted as above, but with the R1 substrate, should produce similar results as in Fig. 5 (A and B) where AdoHcy is the product inhibitor. However, this model also predicts that uncompetitive inhibition should be observed where R1 is the substrate and R1(aDMA) is the product inhibitor. In the latter case, the R1(aDMA) product should bind to the E:AdoHcy complex, effectively reducing the pool of available enzyme for the first step of the reaction. R1(aDMA) does not, however, result in mixed inhibition since it does not affect the equilibrium between the E:AdoMet-R1 and E:AdoHcy-R1(MMA) complexes, because it is not a product of the single methyl transfer reaction (i.e. R1(aDMA) is not the product inhibitor of the R1 to R1(MMA) conversion).

Product inhibitor studies shown in Fig. 5 were repeated using R1 as the variable and constant substrate (Fig. 7). Unexpectedly, inhibition of H₆PRMT6 with AdoHcy at a constant non-saturating concentration of AdoMet (20 μM), and variable R1 concentrations produce a series of curves that intersect to the positive side of the 1/[R1] axis (Fig. 7A). Although some form of inhibition appears to be present, this pattern of

### TABLE 1

**Kinetic parameters for H₆PRMT6**

Unless otherwise indicated the values are listed as mean ± S.D. in parenthesis of two measurements made on separate days.

| Compound     | Type             | Kₘ (μM) | Kᵣ (μM) | Vₘₐₓ (nmol/min mg) | kₘ (×10⁻³ s⁻¹) | kₘ/kᵣ (s⁻¹) |
|--------------|-----------------|---------|---------|---------------------|-----------------|--------------|
| AdoMet       | Substrate       | 18.6 (3.3) | 7.5 (1.9) | 326 (113)²          | 2.5 (0.1)       | 134.8 (20.5) |
| R1           | Substrate       | 501 (86) | 1.8 (0.3) | 14.0 (0.4)          | 2.9 (0.9)       |              |
| R1(MMA)      | Substrate       | 183.7 (12.3) | 3.2 (0.2) | 2.4 (0.2)          | 13.1 (0.03)     |              |
| R1(aDMA)     | Product         | 1.4 (0.0) | 1.0 (0.1) | 7.0 (0.2)          | 8.0 (0.1)       |              |

¹ The Vₘₐₓ and kₘ for AdoMet were determined by using R1(MMA) as the substrate and measuring the initial rate of methylation by quantitation of R1(aDMA), so these values are similar to those measured for R1(MMA).
² The numbers represent an average (±S.D.) of all Kᵣ values (Table 2) determined using the R1(MMA) substrate.
³ The numbers represent an average (±S.D.) of Kᵣ/slope values determined using AdoMet and R1 or R1(MMA) at the constant substrates and AdoHcy as the product inhibitor.
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[14C]AdoMet or [3H]AdoMet as a methyl donor is probably the most common technique. It has the advantage of the low limit of detection afforded by radioactivity, and it provides a convenient method for performing product inhibitor studies by using unlabeled product inhibitor with radioactive AdoMet (58–60).

For the specific case of PRMTs, radioactively methylated protein products have been hydrolyzed, and the component-methylated arginine amino acids are separated by ion exchange chromatography (42), TLC (8), or reversed-phase high-performance liquid chromatography (5). Radioactively methylated proteins are also frequently separated by SDS-PAGE, and the resultant dried gels are exposed to film or storage phosphor screen for detection and quantitation (27, 30, 49, 61). The presence of radioactively methylated arginine, MMA, aDMA and sDMA, and some additional unidentified compounds that may be products of AdoMet hydrolysis (55) (Fig. 3, D and E). Therefore, these results demonstrate that determining the type of methylation activity (Type I or II) of a PRMT enzyme can be problematic using this TLC method. Finally, as we have noted above, incomplete removal of H6PRMT6 can lead to the presence of MMA and aDMA from auto-methylation of the enzyme, and this is also a potential confounding factor with this assay. For these reasons we abandoned this TLC assay in favor of the more simple and reliable MS assay.

Several methods of measuring the rate of methylation of PRMT enzymes exist that do not use radioactive AdoMet. One of these methods utilizes detection of methylated arginine products by antibodies (Abcam and Upjohn products) specific for methylated polypeptides (35, 41, 62–65), some of which are capable of distinguishing between MMA and aDMA residues. In addition, spectrophotometric assays (66) and several MS assays are now being used (48, 49, 67). With the aid of fragmentation some MS assays are even able to detect the presence of aDMA versus sDMA, thereby determining the type of enzyme activity (68).

In contrast to these efforts for measuring PRMT activity, we have developed a quantitative MS assay that can directly measure the amount of mono- and dimethylated peptides using peptide standards. We were able to detect the m/z+2 of each standard and achieve a baseline separation of the peptide substrates and products R1, R1(MMA), and R1(aDMA) by at least 0.5 min. In conjunction with the selected ion recording detection method, the LC portion of the assay makes the unique identification of each of the peptides unambiguous (supplemental Fig. S1). We were able to validate the results of the MS assay using the TLC assay (Fig. 3F), finding good agreement in the measurement of linearity of R1(MMA) and R1(aDMA) product formation from 0 to 120 min.

For the R1(aDMA) product inhibitor studies we measured the rate of methylation of the R1 and R1(MMA) substrates as a function of the rate of accumulation of AdoHcy. Unfortunately, the detec-
tion of AdoHcy is complicated by the auto-methylation of H₄PRMT6, which also produces AdoHcy. However, as the incubation time and amount of enzyme remain the same throughout all of our experiments, the additional AdoHcy formed does not appear to affect the increased rate of formation of AdoHcy with increasing peptide substrate concentration, nor the decrease in rate of AdoHcy formation in the presence of the product inhibitor R1(aDMA). Nevertheless, throughout all of the product inhibitor studies, we used peak areas as a surrogate for initial rate of enzyme activity rather than quantifying the amount of methyl groups transferred due to the effect of the AdoHcy produced by H₄PRMT6 auto-methylation.

**Enzyme Mechanism**—To the best of our knowledge the present study represents the first comprehensive kinetic analysis of a recombinant PRMT enzyme that includes a determination of all bi-substrate kinetic parameters and an analysis of the kinetic mechanism. However, studies on other AdoMet-dependent methyltransferase enzymes using similarly designed experiments can serve as comparisons. The mechanisms of these enzymes range from ordered sequential rapid equilibrium (69), ordered sequential (40, 58, 59), semi-ordered sequential (70), to Theorell-Chance mechanisms (60). In accordance with the consensus of these studies we find that the simplest mechanism that is consistent with the variable and fixed substrate data (Fig. 4) and the product inhibition data (Fig. 5) is a steady-state ordered sequential mechanism involving an enzyme bi-substrate complex in which AdoMet binds first and the methylated arginine product peptide is the first to dissociate (Fig. 6). This fits with the mechanism proposed in a recent structural study of PRMT4. This study revealed that substrate-free PRMT4 has an unfolded N terminus that, upon AdoMet binding, folds on top of AdoMet to lock it into place (13). In the newly formed enzyme-AdoMet complex, the resolved N-terminal helix forms a ridge along which the arginine-containing substrate can bind. This structural information implies that AdoMet must bind first to form the binding site for the methyl acceptor and that the methylated product must dissociate before AdoHcy (13).

The $K_m$ for AdoMet (Table 1) is comparable to other non-PRMT N-methyltransferases, which have values ranging from 1.7 to 10 μM (58, 59, 70). In contrast to studies with histamine N-methyltransferase, the dissociation constant ($K_{dh}$) for AdoMet from H₄PRMT6 is less than the $K_m$ indicating that the $K_m$ reflects more than just the true affinity of the enzyme for AdoMet (58, 59). As expected the $K_m$ for AdoHcy is much lower than the $K_m$ for AdoMet (Tables 1 and 2) and consistent with previously derived $K_m$ values of 1.1–2.7 μM for histamine N-methyltransferase (58, 59).

Several groups have attempted to derive $K_m$ and $V_{max}$ values for some PRMT enzymes with different substrates (48, 49, 61, 66). However, because none of these studies vary either AdoMet or methyl acceptor substrate concentrations, or attempt a non-linear least squares fit of the data to Equation 1, the values of constants derived are more properly considered apparent values. Nevertheless, it is instructive to compare the kinetic parameters we derived to these apparent values. Accordingly, previous studies show that the $K_m$ for peptides JM1H (66) and AcH4–13 (49), which are 13 residues long with a single arginine residue, are 450 and 466 μM for PRMT1, respectively. These values are consistent with the $K_m$ value of the R1 peptide in the present study (Table 1). Previous studies have also shown that the $k_{cat}$ for the AcH4–13 and a peptide corresponding to residues 676–692 of human nucleolin are 2.8 and 6.5 × 10⁻³ s⁻¹, respectively; both values are similar to the $k_{cat}$ for the R1 peptide (Table 1) (48, 49). In contrast to our results, a previous study on the methylation of partially methylated peptides containing MMA residues shows both a decrease in $K_m$ and $k_{cat}/K_m$ relative to their unmethylated counterparts (49).

The relatively high $K_m$ values derived for the R1 and R1(MMA) peptides likely reflect the “minimal” nature of this substrate series, and the lack of any positively charged flanking residues that have been shown to increase affinity of substrates for PRMTs by binding to acidic grooves on the enzyme (14, 49). However, a striking difference between the R1 and R1(MMA) peptides is that the $K_m$ for R1(MMA) is nearly one-third that of R1, and the $V_{max}$ for R1(MMA) is nearly twice as high as that for the R1 peptide. This leads to the R1(MMA) peptide having a $k_{cat}/K_m$ that is 4.5-fold higher than that for the R1 peptide. In the context of the peptides used in this study, these parameters show a consistent preference for the methylated substrate by H₄PRMT6. This preference may be mediated by the increased hydrophobicity of the mono-methylated substrate that has higher affinity for the enzyme. An alternate or complementary explanation is that the hyperconjugation from an additional methyl group makes the guanidino $\omega$-nitrogen atom a stronger Lewis base and possibly a better nucleophile, thereby increasing the inherent chemical reactivity of the mono-methylated substrate.

The product inhibition studies performed with the R1 peptide (Fig. 7 and Table 2), were expected to show similar results to the R1(MMA) peptide (Fig. 5 and Table 2) where AdoHcy was the product inhibitor. However, Fig. 7A shows a series of curves corresponding to increasing AdoHcy concentrations that converges on the positive side of the 1/[R1] axis. This pattern of intersecting lines does not correspond to a known form of inhibition. As a result this curve was not used to derive kinetic parameters or draw qualitative conclusions about enzyme behavior. Fig. 7B, on the other hand, exhibits competitive inhibition that confirms the inhibition pattern observed in Fig. 5B, and can be used to derive a $K_i$-slope for AdoHcy (Tables 1 and 2). It was predicted that competitive inhibition would be observed where R1 is the substrate and R1(aDMA) is the product inhibitor (Fig. 7, C and D), because the R1(aDMA) product should bind to the E-AdoHcy complex, effectively reducing the pool of available enzyme for the first step of the reaction while not affecting the equilibrium between the E-AdoMet·R1 and E-AdoHcy·R1(MMA) complexes. With the possible exception of low concentrations of R1(aDMA) in Fig. 7C (dashed lines), this inhibition pattern was not observed. Fig. 7D shows a pattern of lines similar to Fig. 7A, and for the reasons above was not used to derive kinetic parameters. The unexpected inhibition patterns observed in Fig. 7 may result from the high $K_m$ for the R1 peptide. Additionally, this behavior may be a result of the further complication of the two-step bi-substrate process of methylation when starting with R1 as opposed to a simple one-step bi-substrate reaction when starting with R1(MMA) as the substrate (Fig. 6).
**PRMT6 Enzyme Kinetics**

_Distributive Mechanism—_Arginine residues that are methylated by PRMT enzymes are frequently dimethylated, and many of these residues are part of protein substrates with multiple arginine residues that are also potential methyl acceptors. This type of substrate–enzyme relationship raises the possibility of processive or distributive mechanisms of methylation. In a processive mechanism the rate of release of the product is slower than the rate of the chemical reaction, and the result is that multiple chemical reactions occur for each enzyme-substrate binding event. A distributive mechanism is one in which an enzyme dissociates after each chemical reaction. In theory it is difficult to determine if an enzyme is processive. However, enzymes are frequently called processive if the rate of accumulation of products that have undergone multiple chemical reactions is as fast or faster than the rate of accumulation of products that have undergone a single chemical reaction. Therefore, with respect to PRMTs, an enzyme that catalyzes multiple methylations per binding event is processive. This processivity would manifest itself as a rapid accumulation of multiple methylated products relative to the formation of single methylated species. In the present study we find no evidence of processivity for H2PRMT6. In fact, the assays in which the R1 peptide is used would manifest itself as a rapid accumulation of multiple methylations per binding event is processive. This processivity is as fast or faster than the rate of accumulation of products than the rate of the chemical reaction, and the result is that apparent processivity observed for PRMTs.

A second mechanism exists for processivity of PRMTs with arginine residues that are methylated for PRMT6 Enzyme Kinetics. The explanation can be likely attributed to the substantial processive mechanisms. The explanation can be likely for H6PRMT6. In fact, the assays in which the R1 peptide is used would manifest itself as a rapid accumulation of multiple methylations per binding event is processive. This processivity is as fast or faster than the rate of accumulation of products that have undergone multiple chemical reactions is as fast or faster than the rate of the chemical reaction, and the result is that apparent processivity observed for PRMTs.

_Acknowledgment—_We thank Dr. Steven Clarke (UCLA) for the pGEX-6P-1 vector harboring recombinant human PRMT6 and for his critical reading of our manuscript.

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