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Substrate-Induced Control of Product Formation by Protein Arginine Methyltransferase 1

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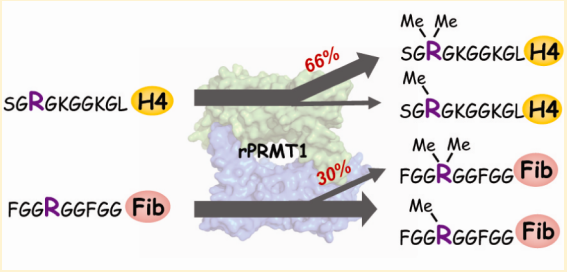
Supporting Information

ABSTRACT: Protein arginine methyltransferases (PRMTs) aid in the regulation of many biological processes. Accurate control of PRMT activity includes recognition of specific arginyl groups within targeted proteins and the generation of the correct level of methylation, none of which are fully understood. The predominant PRMT in vivo, PRMT1, has wide substrate specificity and is capable of both mono- and dimethylation, which can induce distinct biological outputs. What regulates the specific methylation pattern of PRMT1 in vivo is unclear. We report that PRMT1 methylates a multisite peptide substrate in a nonstochastic manner, with less C-terminal preference, consistent with the methylation patterns observed in vivo. With a single targeted arginine, PRMT1 catalyzed the dimethylation in a semiprocessive manner. The degree of processivity is regulated by substrate sequences. Our results identify a novel substrate-induced mechanism for modulating PRMT1 product specificity. Considering the numerous physiological PRMT1 substrates, as well as the distinct biological outputs of mono- and dimethylation products, such fine-tuned regulation would significantly contribute to the accurate product specificity of PRMT1 in vivo and the proper transmission of biochemical information.



Protein arginine methylation has emerged as a major mechanism for regulating protein function in eukaryotic cells.[1−8] This post-translational modification (PTM) is catalyzed by a family of enzymes called protein arginine methyltransferases (PRMTs). Nine human PRMT isoforms form monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) on targeted proteins (Figure 1). Type I PRMTs (such as PRMT1) form MMA and/or ADMA and represent the majority of identified PRMTs. Type III enzymes produce only MMA. Like the type I and III enzymes, type II PRMTs catalyze monomethylation; however, a second round of turnover results in the biologically distinct SDMA.

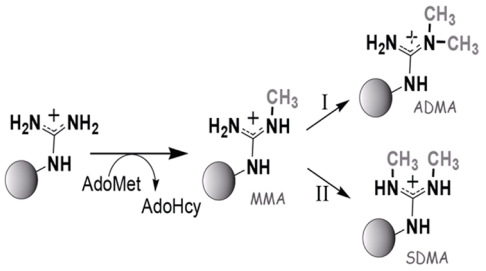


Figure 1. Methylation reactions catalyzed by PRMTs. Type I and type II PRMTs make MMA. Type I PRMTs may then go on to make ADMA, while type II PRMTs produce SDMA. The second molecules of AdoMet and AdoHcy have been omitted for the sake of clarity.

Each of the methylated arginines (MMA, ADMA, and SDMA) can induce different biological responses in the cell.[9−12] It follows that controlling the type of methylation, the amount of methylation (many proteins contain multiple arginines that are methylated in various patterns), and what proteins are methylated is necessary for a healthy cell. In fact, PRMT1 knockouts are lethal in mice,[13] and dysregulated arginine methylation has been associated with heart,[14−16] lung,[17,18] and kidney[19] pathologies, cancer etiology,[20] and other diseases.[21−24] Consequently, the exogenous control of PRMT activity by small molecule inhibitors is one of the newest targets in medicinal and chemical biology.[25−32] Despite the pivotal role in human biology and health, little to no mechanistic information about how the deposition of methyl marks is regulated, especially between MMA and ADMA, is available.

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Table 1. Methylation Status of Npl3, hnRNP K, and Sam68 in Vivo[a]

	Npl3	hnRNP K	Sam68
ADMA in vivo	^{290}**R**GGF**R**GGF**R**GGF**R**GG	^{254}PM**R**G**R**GG	
	^{307}RGGFGGP**R**GGF	^{265}PPG**R**GG	^{45}S**R**GGGGGSR
	^{321}RGGYGGYS**R**GG	^{294}PG**R**GG**R**GG	^{52}S**R**GGGGGSG**R**GGAR
MMA or partial ADMA in vivo			^{310}GVGPP**R**GALVR
	^{344}**R**GGYDSP**R**GGY	^{285}PR**R**GP	^{315}GALV**R**GTPVR
	^{351}RGGYDSP**R**GG	^{301}GS**R**AR	^{320}GTPV**R**GAITR
	^{377}RGSYGGS**R**GGY		^{325}GAIT**R**GATVTR

[a]Methylated arginines are highlighted in bold. The methylation statuses listed here were determined by mass spectrometry.[33−35]

PRMT1 is capable of both mono- and dimethylating target arginine residues. Contrary to the idea that MMA is simply an intermediate for further ADMA generation, studies have shown that MMA residues display a bona fide signaling role in vivo. Kirmizis and co-workers showed that distinct transcriptional consequences in yeast are correlated to the monomethyl and asymmetric dimethyl states of histone H3 at Arg-2.[12] Moreover, because of the technical advances in detecting the physiological PTM status, PRMT1 was shown to methylate numerous protein substrates in a distinctive MMA−ADMA pattern in vivo, including heterogeneous nuclear ribonucleoprotein K (hnRNP K),[33] hnRNP-like protein Npl3,[34] and Src substrate associated in mitosis of 68 kDa (Sam68)[35] (Table 1). Considering the existence of multiple methylation sites in various protein substrates, such specificity of MMA and ADMA deposition catalyzed by PRMT1 suggests a high level of endogenous regulation. Thus, it is of great interest and fundamental importance to elucidate the molecular basis of PRMT1 product specificity, in terms of selecting methylation site(s) in multi-arginine-containing substrates and determining the final methylation states on the targeted arginine.

In our recent work, we probed the active site of rat PRMT1 and identified key residues for substrate recognition and the differential regulation of mono- and dimethylation.[36] To follow up on this study and understand the specific methylation patterns observed in vivo, we have further examined the product specificity of PRMT1 from a substrate-centered perspective. From the observed methylation status of Npl3, hnRNP K, and Sam68 protein substrates in vivo (Table 1), we hypothesized that different local amino acid sequences or the location of a targeted arginine in the protein substrate may help to govern the specific products of PRMT1. To test this hypothesis, we designed and characterized several series of peptides, including multi- and single-arginine-containing peptides with varied amino acid sequences and arginine locations (Table 2). All peptides used had similar catalytic efficiencies, regardless of the length and the amino acid sequence of the peptides. Our results show that PRMT1 methylates a multi-arginine substrate in a nonstochastic fashion, with a weaker preference for the C-terminal arginine. When a single arginine is targeted, PRMT1-catalyzed dimethylation occurs in a semiprocessive manner; however, the degree of processivity observed is substrate-dependent. Our results provide a mechanistic explanation for developing the patterns of specific MMA and ADMA deposition on the targeted arginine residues in vivo, which reveals a previously unidentified mechanism for regulating arginine methylation.

Table 2. List of Peptide Substrates[a]

peptide	sequence	category
R3	GG**R**GGFGG**R**GGFGG**R**GGFG	fibrillarin-based multi-Arg
R2	GG**R**GGFGGKGGFGG**R**GGFG	
RKR-CH_3	GG**R**GGFGGKGGFGG**R***GGFG	
RKK	GG**R**GGFGGKGGFGGKW	R3-based single-Arg (for R location)
RKK-CH_3	GGR*GGFGGKGGFGGKW	
KRK	KGGFGG**R**GGFGGKW	
KRK-CH_3	KGGFGG**R***GGFGGKW	
eIF4A1	YIHRIG**R**GGR	eukaryotic initiation factor 4A-1 (eIF4A1)-based (for sequence)
eIF4A1-CH_3	YIHRIG**R***GGR	
eIF4A1-Y	YIHRIG**R**YGR	
eIF4A1-S	YIHRIG**R**SGR	
H4	SG**R**GKGGKGLGKGGAKR	histone 4-based, single-Arg

[a]R* indicates monomethylated arginine.

EXPERIMENTAL PROCEDURES

Materials. AdoMet was purchased from Sigma as a chloride salt (≥80%, from yeast). [^{3}H-*methyl*]AdoMet was purchased from Perkin-Elmer.

Recombinant Proteins. Full-length His-tagged PRMT1 (residues 1−353) was expressed and purified as described previously.[36] Purified proteins were ≥95% pure as judged by SDS−PAGE. Protein concentrations were determined by UV spectroscopy using the theoretical absorption coefficients and by the Bradford assay with bovine serum albumin as a standard.

Synthetic Peptides. All the peptides were synthesized by the Keck Institute (Yale University, New Haven, CT) with acetylated N-termini and free carboxyl termini and purified to ≥95%. The lyophilized peptides were dissolved in water. Their concentrations were determined by mass and/or by UV spectroscopy using their theoretical absorption coefficients. Even though some of the peptides used in this study are less than 21 amino acids long, the k_{cat}/K_m values for all the peptides used in this study are within 1 order of magnitude of the previously characterized 21-amino acid peptide derived from histone 4 (H4−21).

Mass Spectrometry Analysis of Peptide Methylation and De Novo Sequencing Study. Conditions of methylation reactions were as published previously.[36] If not stated otherwise, the methylation reaction mixture contained 4 μM

PRMT1, 250 μM AdoMet, and 100 nM AdoHcy nucleosidase (MTAN, purified as described in ref 37) in 50 mM sodium phosphate buffer (pH 7.1). Independent reactions were initiated with each peptide substrate at 200 μM. At various times points (2.5, 5, 10, 12.5, 15, 20, and 30 min), 10 μL aliquots were quenched with trifluoroacetic acid (TFA) (final concentration of 10%) and analyzed by liquid chromatography and mass spectrometry followed by tandem mass spectrometry (MS/MS) of the desired peaks. Samples that showed a maximal amount of monomethylation yet no or a minimal amount of enzymatically formed dimethyl species were further analyzed via LC−MS/MS. The time point used for each peptide shown in Table 3 was 20, 10, or 10 min for the R2, RKRm, or R3 peptide, respectively. Peptides with monomethylation and without methylation were analyzed using nano-LC−MS-MS on a Q-Tof Primer tandem mass spectrometer (Waters, Manchester, U.K.). Peptide samples were loaded (3 μL) using a NanoACQUITY Sample Manager (Waters) onto a trapping column (Symmetry C18, 180 μm × 20 mm) (Waters). Samples were washed with 99% H_2O and 1% TFA for 1 min at 15 μL/min to a waste container and then eluted with a 30 min gradient (1 to 4% B over 0.1 min, 4 to 60% B over 20 min, 60 to 85% B over 3 min, 85 to 1% B over 1 min, and 1% B for 6 min where A consists of 0.1% formic acid in water and B consists of 0.1% formic acid in acetonitrile) at a rate of 800 nL/min using a NanoACQUITY UPLC system (Waters) over a 100 μm × 100 mm BEH 130 C18 column. MS survey and product ion MS/MS scan times were 1.0 s. The collision offset was automatically determined on the basis of precursor mass and ion charge state. MS/MS data were used for the de novo sequencing of methylated peptides by Waters BioLynx (Waters).

For the eIF4A1 peptide samples shown in Figure 3, reaction mixtures were diluted to 2 μM using an acetonitrile/water/TFA mixture [50/50/0.05 (v/v/v)]. Diluted peptide samples (0.5 μL) were spotted on a polished standard 192-well stainless steel MALDI sample plate followed by the addition of an α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution {0.4 μL, 10 mg/mL in an acetonitrile/water/TFA mixture [50/50/0.05 (v/v/v)]}. The resulting mixtures were air-dried and analyzed using an AB 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Framingham, MA). MS and MS/MS spectra were acquired in reflector positive mode. Typically, 2500 shots per spectrum accumulated in the MS mode and 5000 shots per spectrum in the MS/MS mode. A standard peptide, human angiotensin I ($[M + H]^+$ 1296.67), was used for the external calibration, and the data were analyzed using Data Explorer version 4.6. The sequences of peptides were analyzed using ProteinProspector (an online program, version 5.10.2). To construct a reaction time course (Figure 3), the relative height of each peak (naked, monomethylated, and dimethylated species) was calculated by dividing the individual peak height by the sum of peak heights from all peptide species (nonmethylation, monomethylation, and dimethylation) observed in mass spectra at each time point. The intensity of each peak from the nonmethylated peptide or the methylated peptide was based on peak height.

Continuous Spectrophotometric Kinetic Assays of PRMT1. A continuous spectrophotometric assay for AdoMet-dependent methyltransferases[38] was used to assay PRMT1 with arginine-containing peptides. Briefly, two coupling enzymes, MTAN and adenine deaminase, were used to hydrolyze and deaminate the AdoHcy generated from methyl group transfer, respectively. This assay minimized product inhibition that could occur from AdoHcy. Initial rate data representing no more than 10% of product formation were fit to the Michaelis−Menten equation[39] to obtain $K_{m,app}$ and $k_{cat,\ app}$ values. Each reaction was performed in at least duplicate. The limit of detection for this assay was 0.01 μM methyl group transfer.

Double-Turnover Experiments. A reaction containing 20 μM PRMT1, 40 μM AdoMet [2.6 μM [^{3}H]AdoMet (specific activity of 2.02 mCi/μmol)], and 100 nM MTAN in 50 mM sodium phosphate buffer (pH 7.5) was initiated with 200 μM peptide substrates at 37 °C. After 1 h, proteins were precipitated with 10% TFA (final) and removed via centrifugation. An equivalent volume of 12 M HCl was added to each mini vial (Deltaware). Vials were then sealed and heated to 110 °C for approximately 24 h for complete acid hydrolysis. Because of the small inner space, sample oxidation is minimized.[36] The methylation statuses of hydrolyzed peptide products were analyzed by HPLC and MS.

Identification and Quantification of Methylated Arginines by HPLC. Hydrolyzed amino acids were neutralized by an equivalent volume of 6 M NaOH and then separated using *o*-phthaldialdehyde (OPA) derivatization[40] with a Gemini 3 μm C18 110 Å LC Column (75 mm × 4.6 mm, Phenomenex). Mobile phase A consisted of 40 mM sodium phosphate buffer (pH 7.8), and mobile phase B was an acetonitrile/methanol/H_2O mixture [45/45/10 (v/v/v)]. Fractions (83 μL) were collected, and radioactivity was counted in 5 mL of scintillation cocktail (Fisher Scientific). MMA and ADMA standard amino acids were used to verify the identity of the methylated products generated. The detection limit for this method is ∼10 pmol of methylated arginine in a 20 μL sample.

RESULTS AND DISCUSSION

PRMT1 Methylation of a Multiple-Arginine-Containing Substrate Is Nonstochastic. Many PRMT protein substrates harbor repetitive "RGG" or "RGG-like" regions that are targets for arginine methylation.[1,33−35,41] For instance, the C-terminus (C_T) of Npl3 contains an extensive arginine-glycine rich domain composed of 15 RGG tripeptide repeats, of which nine consecutive N-terminal (N_T) RGG motif arginines (R290−R337) were found exclusively dimethylated. On the other hand, variable levels of methylation were associated with the C-terminal RGG motif arginines (Table 1). Such cumulative methylation of Npl3 is important for protein function, especially in the N-terminal RGG motifs.[34] However, the molecular origins of such distinct methylation patterns remain unclear. To understand whether particular patterns of methylation can be preferentially deposited in such repetitive regions, we investigated whether PRMT1 functions as a systematic (preferred sites) or stochastic (random) modifier. We examined the sequential methylation of a simple multi-arginine-containing peptide, the R2 peptide (GG**R**GGFGGKGGFGG**R**GGFG).[a] Because both arginine residues exist within the same amino acid context (GG**R**GG), we avoided the variability that local sequences might induce[42] and instead were able to test whether PRMT1 displays a regional (N_T or C_T) preference in a multi-arginine substrate.

Methylation of the R2 peptide over time was analyzed by MS/MS. MS/MS analysis showed that the N-terminal arginine of the R2 peptide was first mono- and then dimethylated (Figure S1A,B of the Supporting Information). No methylation of the C-terminal arginine of the R2 peptide was detected until the N_T arginine was fully dimethylated. The catalytic

competency of both arginine residues was confirmed in an extended reaction (8 h) where MS/MS analysis demonstrated the presence of the tetramethylated R2 peptide (Figure S1C of the Supporting Information). Figure 2 summarizes the series of

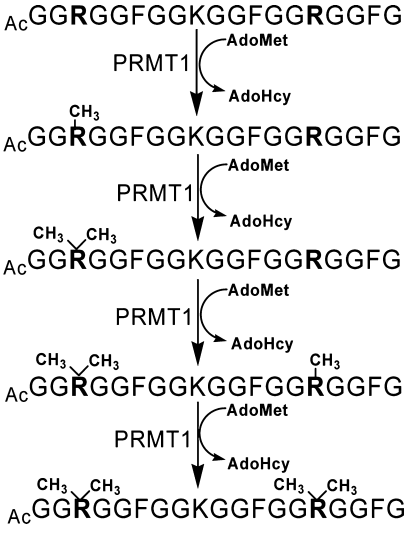

Figure 2. Methylation events observed on the R2 peptide. Time-dependent MS/MS product analysis showed a systematic methylation of the R2 peptide.

methylation events observed on the R2 peptide. These results demonstrate that the PRMT1-catalyzed methylation of a multi-arginine substrate is not stochastic. PRMT1 preferentially methylates one arginine over another, suggesting a systematic or regulated recognition of a specific target arginine in multi-arginine substrates.

PRMT1 Exhibits Regioselectivity in Multi-Arginine Substrates. In the previous experiment, we showed that PRMT1 displays a strong preference for an N-terminally located arginine compared to a C_T arginine in the R2 peptide. We further questioned whether such a preference still exists under more challenging situations, i.e., when either a monomethylated arginine or more arginine residues exist within the peptide.

The RKR-CH_3 peptide (GG**R**GGFGGKGGFGG$\mathbf{R}_{Me}$GG-FG) contains two arginine residues where the C_T arginine is monomethylated. Our previous studies[36] and data supplied herein using single-arginine peptide substrates suggested that a monomethylated arginine in R2 would be a better substrate (based on catalytic efficiency, k_{cat}/K_m) than the unmodified one. The first methylation event in the RKR-CH_3 peptide was mapped using MS/MS (Figure S2A of the Supporting Information). Although methylation of the C_T arginine was observed (13%), PRMT1 still showed a strong preference for the N-terminal arginine site (Table 3).

We also tested PRMT1 substrates that contain more than two arginines. We mapped the first methylation event in the R3 peptide (GG**R**GGFGG**R**GGFGG**R**GGFG), which contains three potential target sites (Figure S2B of the Supporting Information). Compared to that of the R2 peptide, the distance between the terminal arginine residues is maintained, but an additional arginine replaces a positively charged lysine residue in the middle of the peptide. Mapping the initial methylation event in the R3 peptide demonstrated three things. (1) As observed above, the majority of the first methylation again

Table 3. Locations of the First PRMT1-Catalyzed Methylation Events[a]

		% of the first methylation position[b]		
peptide	peptide sequence	N_T-R	center R	C_T-R
R2	GG**R**GGFGGKGGFGG**R**GGFG	100	–	0
RKR-CH_3	GG**R**GGFGGKGGFGG**R***GGFG	87	–	13
R3	GG**R**GGFGG**R**GGFGG**R**GGFG	55	39	6

[a]**R*** indicates monomethylated arginine. [b]The standard error of the percentage was typically less than 5%.

occurred at the N_T arginine (55%). (2) The internal arginine was targeted to a significant extent (39%). (3) Minimal methylation (6%) occurred on the C_T arginine (Table 3). These in vitro observations are consistent with the physiological methylation status of Npl3 and Sam68 proteins. The repeated N-terminal RGG motif arginines of Npl3[34] and Sam68[35] were found to be exclusively dimethylated, and evidence showed monomethylation or partial dimethylation associated with the C-terminal RGG motif arginines (Table 1).

The preference of PRMT1 for the N_T arginines might be seen as a consequence of a high degree of flexibility at this position and easy accessibility. However, the internal arginine in the R3 peptide, which is less structurally flexible than the N_T arginine, is also targeted to a significant degree for the first methylation. Therefore, the basis of the preference is not the immediate flanking amino acid context or the flexibility afforded by an N-terminal arginine. One hypothesis that would be consistent with our observations would entail using the C_T arginine of the peptide substrate in a distal binding event. Osborne and co-workers[43] showed that positively charged residues distal to the methylation site (11−14-amino acid interval) are very important for PRMT1 substrate binding and catalytic activity. Interestingly, the peptides used in the Osborne study were based on histone 4, where the targeted arginine is located at the N_T and the necessary positive charges that were identified were all located downstream (C-terminal) from the arginine.

When more than one arginine is present in the targeted protein, the distance between them may also influence processing. Many proteins that contain RGG repeats have been identified;[44] however, whether they are all PRMT1 substrates and what the in vivo pattern of methylation looks like are unknown. The limited studies of in vivo methylation patterns of PRMT1 show that the common distance between targeted arginines is either two or three amino acids (e.g., **R**GG**R**GG in hnRNP K) or five to seven amino acids (e.g., **R**GGFGGP**R**GG in Npl3) within the repetitive arginine-glycine rich region. In such repetitive RGG regions, extensive and consecutive dimethylation is often observed. Because of the five amino acids between the arginine residues in the R3 peptide, we would classify the R3 peptide as an "RGG-repeat" substrate. The spacing of the RGG repeats possibly correlates to a high percentage of methylation occurring in both the N-terminal and the center-located arginines. In contrast, the R2 peptide contains a longer interval (11 amino acids) between the two arginines, which may cause the distal RGG motif to be considered as a discrete substrate. In most physiological methylation patterns of which we are aware, methylation of the C-terminal arginine in the arginine rich domain is infrequently observed.[33−35]

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Overall, our results show that PRMT1 methylates multi-arginine substrates in a nonstochastic and regioselective manner wherein more extensive methylation is predicted at the N-terminal or internal arginines. The center and/or the C-terminal arginines (or positive charges) are necessary for the N-terminal preference of PRMT1, which is consistent with the importance of the distal positive charges in the substrates shown by Osborne and co-workers.[43] We also note that the distance between arginine residues in the substrate may affect the process of methylation-site targeting and await the analyses of additional in vivo PRMT1 substrates to test this hypothesis.

Although we have shown that PRMT1 can preferentially target specific arginine residues in multi-arginine substrates, it is unclear how product specificity at the targeted arginine (mono- or dimethylation) is governed. Because of the potential distinct biological effects of MMA and AMDA,[12] it is important to understand the determinants or regulators of the final methylation status at a particular arginine residue. We started to explore the mechanistic basis of this control by (1) probing the efficiency of methylation (as defined by k_{cat}/K_m) for a variety of peptide sequences and (2) probing the possibility of regulated processive dimethylation. Because comparisons of synthetic peptides with full-length proteins from which they were derived indicate that, at least in some cases, both substrates are methylated with similar kinetic efficiencies,[43,45] peptide substrates were used thereafter.

The Catalytic Efficiency for a Single-Arginine-Containing Peptide Increases with Monomethylation. Although previous steady-state kinetic studies of PRMT1 have shown a slight or no preference for a monomethylated arginine against an unmodified substrate, all the experiments were based on single-peptide substrates, derived from histone 4 or PABPN1.[46,47] Our data have shown that different characteristics of the target arginine, for example, the location of the arginine residue within the peptide and the flanking amino acid sequence,[42] influence the activity of PRMT1. To more thoroughly understand whether PRMT1 displays a kinetic preference for the naked or monomethylated arginine residue, steady-state kinetic experiments were performed using three different peptide pairs as a primary comparison of arginine residue location and peptide sequence (Table 4). Two fibrillarin-based peptides with identical amino acid sequences denoted KRK and RKK represent the alternation of the target arginine position in the peptides. The eIF4A1 peptide (YIHRIG**R**GGR), derived from eukaryotic initiation factor 4A-I, bears an entirely different peptide sequence versus that of the fibrillarin-based peptides. Although eIF4A1 peptides contain three arginines, we previously showed that the central arginine in the eIF4A1 peptide (YIHRIG**R**GGR) was the only one modified by PRMT1.[42] Hence, this peptide pair is still considered a single-arginine substrate with the same center-located arginine residue as the KRK peptides. These three peptide pairs serve as a start for systematically probing whether PRMT1 methylates both naked and monomethylated substrates with the same catalytic efficiency.

Table 4. Steady-State Kinetic Parameters of PRMT1 with Unmodified and Monomethylated Peptide Pairs

peptide	$K_{m,peptide}$[a] (μM)	k_{cat}[a] ($\times 10^{-2}$ s^{-1})	k_{cat}/K_m ($\times 10^2$ M^{-1} s^{-1})
eIF4A1	72 ± 9.6	9.0 ± 0.3	13 ± 1.7
eIF4A1-CH$_3$	26 ± 6.2	5.8 ± 0.2	23 ± 5.5
KRK	105 ± 4.4	6.0 ± 0.1	5.7 ± 0.3
KRK-CH$_3$	32 ± 7.5	2.5 ± 0.2	7.6 ± 1.8
RKK	21 ± 6.7	3.7 ± 0.2	17 ± 5.5
RKK-CH$_3$	52 ± 16.8	14 ± 1.1	27 ± 8.9

[a]Apparent K_m and k_{cat} values are reported using a single saturating concentration of AdoMet (250 μM).

Data for the KRK and KRK-CH$_3$ peptides with PRMT1 showed saturation kinetics at a variety of fixed AdoMet concentrations (Figure S3 of the Supporting Information). Similar results were observed with eIF4A1 and RKK peptide pairs (data not shown). The apparent catalytic efficiency (k_{cat}/K_m) for the eIF4A1-CH$_3$ peptide was nearly double that of the eIF4A1 peptide (Table 4). The RKK peptide pair showed similar results. Although the effect was minimal, the KRK-CH$_3$ peptide also showed a higher value of k_{cat}/K_m than the KRK peptide (Table 4). We note that the increased apparent catalytic efficiency is observed with the monomethylated substrate regardless of the location of the single arginine (KRK vs RKK peptide pairs) or the peptide sequences (KRK vs eIF4A1 pairs). Further support for this observation comes from our previous data (Table 3) that showed that monomethylation of the C_T arginine in the R2 peptide redirected 13% of the first methylation event to this arginine. Although the effect of monomethyl arginine is apparent in this study, the difference in k_{cat}/K_m between nonmodified and monomethylated substrates would likely need to be much greater to contribute to ADMA generation in vivo, especially in cases where excess unmodified substrates are present.

Consistent with our observations, Zheng and co-workers[48] reported a higher catalytic efficiency with the monomethyl H4 peptide (k_{cat}/K_m = 1.6 ± 0.3 μM^{-1} min^{-1}) than with the unmodified counterpart (k_{cat}/K_m = 0.9 ± 0.1 μM^{-1} min^{-1}). On the other hand, Kölbel and co-workers[47] found no significant changes in catalytic efficiency (k_{cat}/K_m) of PRMT1 based on one MMA peptide derived from PABPN1. Importantly, this PABPN1-derived peptide substrate bears an "RXR" amino acid paradigm (FYSGFNSRP**R**GRVYATSWY) instead of the canonical RGG sequence. The difference between the observations of Kölbel and our study is possibly due to the different amino acid sequences of peptide substrates used in the kinetic assays, which indicates a possibility that the higher catalytic efficiency of monomethyl peptides is substrate-dependent. Within the RGG substrate paradigm, we found a preference of PRMT1 for the monomethylated substrate compared to the unmodified ones regardless of the peptide origins or the location of the arginine residue. Although the effect is modest at best, such a preference of PRMT1 would contribute to making ADMA the favored product in certain substrates. Interestingly, PRMT1 has been shown to display substrate overlap and/or interactions with PRMT2,[49] -3,[50] -4,[51−53] -6,[54] and -8,[55,56] which gives rise to a possibility that PRMT1 can methylate the monomethyl products from other PRMTs.[12,57] With other PRMTs involved, the preference of PRMT1 for the MMA residues advances the probability of a more regulated system. In all, our results show a higher catalytic efficiency of PRMT1 with monomethylated substrates, which provides one explanation for the physiological higher percentage of ADMA versus that of MMA.

The Final Methylation Status Is Affected by the Amino Acid Sequence Context. Although ADMA appears to be the major product of PRMT1 in vivo,[58] under certain circumstances only monomethylated product is found at

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specific arginine residues in the protein substrates, such as hnRNP K and Sam68 (Table 1). While examining product deposition in these PRMT1 substrates, we noted that the final mono- or dimethylation status was loosely correlated to differences in the amino acid sequences flanking the targeted arginine. This led us to hypothesize that sequence context may inherently control product specificity. To determine whether amino acid context affects PRMT1 mono- or dimethylation, we selected a series of eIF4A1 peptide substrates based on our previous study:[42] wild type (WT) that harbors the RGG sequence, eIF4A1-S, and eIF4A1-Y with the RGG sequence changed to RSG and RYG. As stated previously, among all three arginine residues, only the central arginine is methylated by PRMT1, so members of the eIF4A1 peptide series are considered single-arginine substrates. Importantly, the steady-state kinetic parameters for these three peptides were previously determined[42] and do not vary by more than 1 order of magnitude from each peptide.

End products generated from all three eIF4A1 peptides were analyzed by MS as a function of time. After an extended reaction (105 min), the WT-eIF4A1 sequence (RGG) was stoichiometrically dimethylated (Figure 3A). However, the eIF4A1 peptides bearing the RSG and RYG sequences demonstrated incomplete methylation in the reaction (Figure 3B,C). Surprisingly, <10% turnover was observed with eIF4A1-S/Y samples from even longer reaction time periods [400 or 600 min (data not shown)]. To rule out the possibility that PRMT1 was inactivated or reaction conditions had been altered during the course of the eIF4A1-S/Y reactions, the R3 peptide was added into the reaction mixture after the 105 min reaction and monitored for methylation activity (data not shown). We observed a significant increase in activity, suggesting that PRMT1 and AdoMet were still viable during the extended reaction time period. Consistent with our former observations with eIF4A1/3 protein substrates,[42] the eIF4A1 peptides bearing the RSG and RYG sequences are poor substrates for PRMT1 and cannot be stoichiometrically methylated.

Furthermore, MS analysis also revealed that the proportion of mono- and dimethylated arginine products observed was quite different in the three eIF4A1 peptides (Figure 3). After the 105 min reaction, the RGG-containing WT-eIF4A1 peptide was mostly dimethylated (97%) while the RSG- and RYG-containing peptides were mainly monomethylated (7 and 4%, respectively). The presence of MMA in the reaction mixture of eIF4A1-S/Y was confirmed using amino acid analysis and HPLC (data not shown). These data show that, although these three peptide substrates showed similar catalytic efficiency under steady-state conditions,[42] sequence differences around the substrate arginyl group have a significant effect on whether mono- or dimethylation is the major end product in long-term course reactions and suggest that the amino acid sequence of a PRMT1 substrate could dictate how a protein arginine is modified in vivo.

Design of a Double-Turnover Experiment To Probe the Degree of Distributive versus Processive Dimethylation. Besides the effect of flanking amino acid sequences, another way to regulate the proportion of mono- and dimethylation products would be through regulation of dissociative or processive dimethylation. As several articles have been published about the kinetic mechanism of PRMT1,[43,46,47,59] two major views of this topic are that it is partially processive or completely distributive. To investigate the processivity of PRMT1-catalyzed dimethylation, we

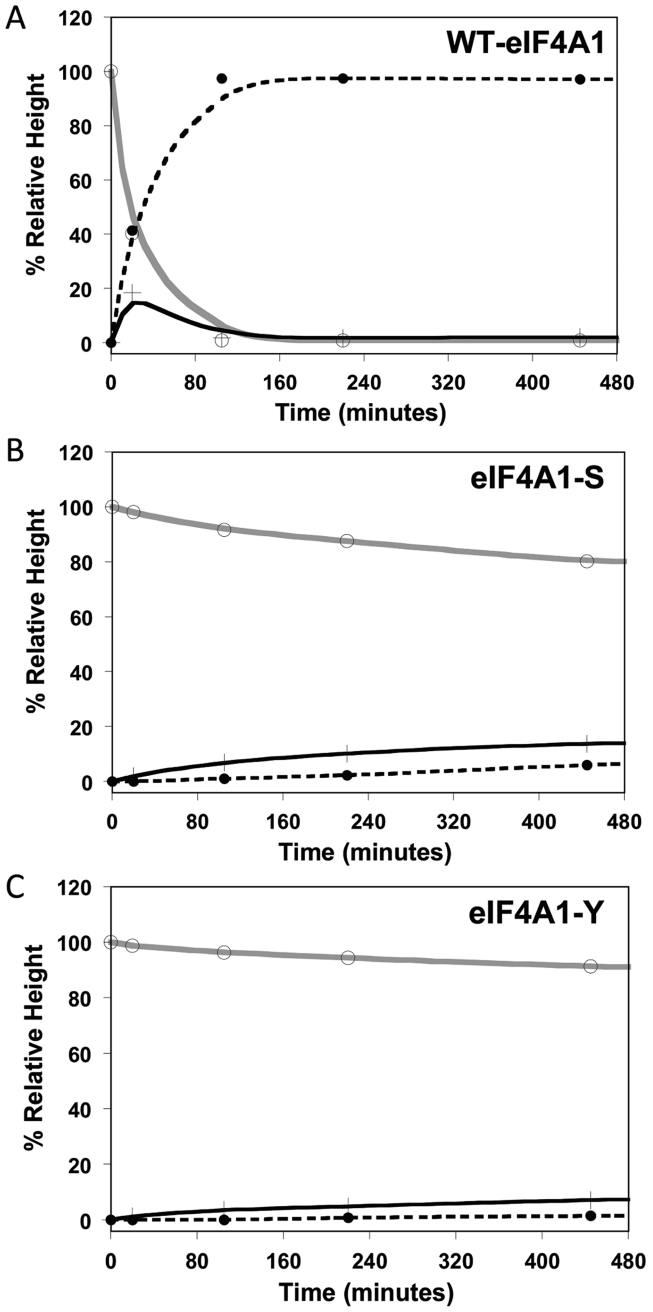

Figure 3. Effect of flanking amino acid sequence on methylated product distribution. Reaction mixtures were prepared as described for steady-state kinetics, and reactions were initiated with WT-eIF4A1 (A), eIF4A1-S (B), and eIF4A1-Y (C). The time-dependent modification of the peptides was monitored by MALDI mass spectrometry. The relative amount of the parent (gray lines), monomethylated (solid lines), and dimethylated (dashed lines) peptide species was determined using peak intensities of the individual species at various reaction times.

designed double-turnover experiments containing 20 μM PRMT1, 40 μM AdoMet ([^{3}H]AdoMet added), and an excess of the peptide substrate. With the concentration of AdoMet 10-fold higher than $K_{D(AdoMet)}$, the majority of the enzyme is bound with AdoMet when the reaction is initiated with the peptide substrate. Assuming the reaction is completely distributive, monomethylated peptides will be released after the first turnover and replaced with bound nonmodified peptides. As

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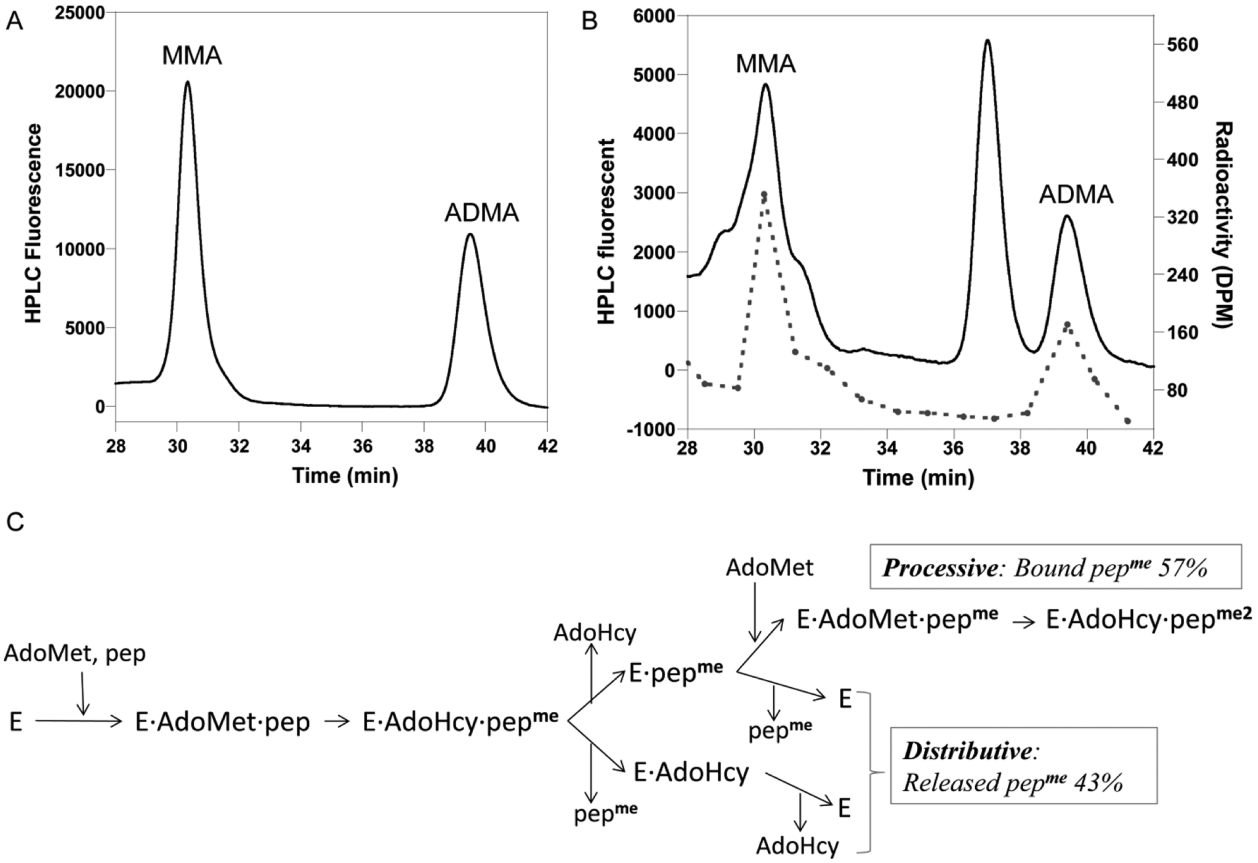

Figure 4. Reverse-phase HPLC analysis of methylation products of the eIF4A1 peptide from double-turnover experiments. (A) MMA and ADMA standard amino acids were used to identify the elution time for each methylated species under our HPLC conditions. (B) Product analysis from double-turnover reactions of the eIF4A1 peptide showing both the fluorescence (black lines) from amino acid derivatization and radioactivity (gray dots) from tritium in the methyl group. (C) Scheme showing the partitioning of reaction products through distributive and processive mechanisms by PRMT1. Proportions of distributive and processive dimethylation are labeled on the basis of calculation from the eIF4A1 peptide discussed in the text.

the amount of AdoMet is sufficient for only two turnovers, MMA would be detected as the final product. On the other hand, if the mechanism is fully processive, monomethyl peptides will remain bound and only ADMA would be generated after two turnovers. The double-turnover experimental design was used with the eIF4A1 peptide. MTAN was also added to prevent AdoHcy inhibition. When the reaction products were analyzed, both MMA and ADMA were clearly observed (Figure 4A,B). The same result was obtained using MS (data not shown). Quantification of the radioactivity present in both the MMA and ADMA peaks showed that the reaction had gone to completion (data not shown).

The formation of both MMA and ADMA indicates a semiprocessive mechanism in which some substrate molecules were processed through a dissociative dimethylation mechanism (MMA released before the second AdoMet binds to PRMT1) and others through a processive dimethylation mechanism (MMA remains bound after the second AdoMet binds to PRMT1). Such semiprocessive dimethylation provides a rationale for the observations that ADMA is the major product of PRMT1 in vivo but that the generation of ADMA is not obligatory.[46,59] Our experimental design strategy also provides us with a method for quantifying how much substrate was processed either distributively or processively by measuring the final amount of MMA and ADMA products in the double-turnover experiment. In the double-turnover experiment for eIF4A1, 17.1 μM MMA and 11.4 μM ADMA were generated from 40 μM AdoMet. Quantification was based on incorporation of the radiolabel into each of the MMA and ADMA peaks, and the ratio of MMA to ADMA was further verified using mass spectrometry. Previously,[36] we showed that 20 μM PRMT1−AdoMet bicomplex is fully functional and generates 20 μM MMA under single-turnover conditions. In the double-turnover experiment described here, we can deduce that the amount of MMA that was generated in the first turnover but remained bound to PRMT1 and was subsequently dimethylated is equal to the final ADMA concentration (11.4 μM). We can further approximate[b] the amount of MMA released from PRMT1 after the first turnover (20 μM − 11.4 μM = 8.6 μM). Therefore, we approximate that 57% of the bound peptide underwent processive dimethylation, while 43% was released after the first turnover, indicating distributive behavior (Figure 4C). Although such a calculation cannot discriminate the small amount of ADMA generated due to MMA rebinding, it is sufficient to estimate the partitioning between the two pathways. Moreover, because of the similar K_D values of the eIF4A1 and eIF4A1-CH_3 peptides (Figure S4 of the Supporting Information), the presence of excess eIF4A1 peptide should make MMA rebinding negligible.

The Degree of Processivity Varies Depending on the Sequence of the Peptide Substrate. We performed the double-turnover reactions using three other single-arginine-

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containing peptide substrates to investigate processivity. With all the peptides we tested, both MMA and ADMA were observed (data not shown), suggesting a general semiprocessive mechanism. Remarkably, the proportions of MMA and ADMA generated from the double-turnover experiments are distinct among certain peptide substrates (Table 5). These results were further confirmed with MS, and the reactions were shown to go to completion (data not shown).

Table 5. Degrees of PRMT1-Catalyzed Processive Dimethylation As Represented by the MMA/ADMA Ratio Using Different Peptide Substrates

peptide	sequence	MMA/ADMA
H4	SGRGKGGKGLGKGGAKR	0.967/1
eIF4A1	YIHRIGRGGR	1.50/1
KRK	KGGFGGRGGFGGKW	4.10/1
RKK	GGRGGFGGKGGFGGKW	4.73/1

Similar to the calculation of the percentage of MMA processed distributively or processively, the MMA/ADMA ratio is directly related to the amount of MMA released and the amount that remains bound to PRMT1, so it can be viewed as an easier measurement of the degree of processive dimethylation. By definition, a higher degree of processivity leads to a larger proportion of ADMA compared to MMA and a lower MMA/ADMA ratio, and vice versa. As can be seen from Table 5, the eIF4A1 and H4 peptides, which have a low MMA/ADMA ratio (close to 1/1), demonstrate a much higher degree of processivity than the KRK or RKK peptides (MMA/ADMA ratio close to 5/1). Such distinct ratios from the double-turnover experiments indicate that certain peptide sequences are processed through a processive mechanism, leading to a dimethylated product, while other sequences partition more frequently through a distributive mechanism where both monomethylated and dimethylated products are possible. To investigate what leads to the different MMA/ADMA ratios, we tested the binding affinity of the naked and monomethylated eIF4A1 peptides to PRMT1 using intrinsic fluorescence quenching (Figure S4 of the Supporting Information). The K_D values for the eIF4A1 pair as well as an H4-based peptide pair[48] and another R3-based peptide pair were all similar (data not shown), indicating that the differences observed in processivity are not due to differences in binding affinity. Comparison between the RKK and KRK peptides indicates that the location of substrate arginine does not influence the degree of processivity between these two peptides. Instead, the general sequences differentiate the degree of dimethylation processivity among the substrates. While it is quite conceivable that the precise degree of processivity may be different with the intact protein substrates, our results clearly demonstrate that the semiprocessive nature of PRMT1 to dimethylate substrate arginines can be fine-tuned in a substrate-dependent manner.

We note that the use of single-peptide substrates in the previous research may be the cause of the conflicting conclusions. Indeed, Obianyo and co-workers[43,46] used a series of peptides based on the histone 4 protein, showing a partially processive mechanism, while Kölbel et al.[47] tested the dimethylation mechanism with one of the peptides very similar to the RKK and KRK peptides, demonstrating a completely distributive dimethylation. Our results from double-turnover experiments clearly revealed the degree of processivity is substrate-dependent. The H4 peptide showed a much higher processivity than fibrillarin-based peptides, consistent with previous studies by Obianyo and co-workers.[43,46] We also note that the semiprocessive dimethylation we observe is consistent with the rapid equilibrium random mechanism proposed by Obianyo and co-workers.[46] Lastly, we question whether conflicting observations regarding the processive nature of PRMT6[60,61] may also be partially due to changes in substrate-induced processivity.

Given a tunable, semiprocessive reaction for PRMT1, the degree of processivity will significantly affect the final product. Therefore, modulation of the processivity becomes essential in accurate product formation. In the case of PRMT1, modulation most likely results from a combination of factors.

For enzymes with broad substrate specificity like PRMT1, flux through a fully distributive or fully processive mechanism is possibly insufficient for instilling such complex deposition codes. Therefore, a fine-tuned degree of processivity is required, such as the substrate-induced control described in this study. Besides PRMT1, substrate-regulated processivity has also been described for two DNA methyltransferases, Dam and DNMT3A, where the inherent processivity depends on the flanking sequences of the DNA substrates[62,63] and the substrate DNA topology,[64] respectively. These mechanisms facilitate control of the substrate specificity in epigenetics. Such fine-tuned processivity in posttranslational modification reactions is also observed with multisite phosphorylation (reviewed in refs 65 and 66). Although originally considered as an all-or-none signal, studies now support a combinatorial regulation of switch properties, somewhat akin to how the histone code may function.[67] The similarity between the multisite phosphorylation and the repetitive RGG paradigm in PRMT1 substrates (reviewed in refs 1 and 68) strongly suggests that the sequential arginine methylation (Figure 2) may also serve as a fundamental switch for protein function.

On a final note, a tunable semiprocessive mechanism opens the door for a higher order of modulation using substrate-interacting partners or PRMT1-interacting partners to influence PRMT1 product formation. For example, during nuclear processing of pre-mRNAs, control of the poly(A) tail length requires regulating the processivity of poly(A) polymerase induced by two RNA-binding proteins.[69,70] These auxiliary proteins are known as processivity factors, such as DNMT3L, which enhances the DNMT3A processivity in de novo DNA methylation,[64] and Doc1, which is required for efficient substrate recruitment and processive ubiquitylation of the ubiquitin-protein ligase (E3) APC.[71−73] Such "higher-order" regulation of processivity modulates the enzymatic mechanism via specific interacting partners. Although interaction partners of PRMT1 have been documented,[74,75] the molecular basis for these regulators remains unclear.

■ CONCLUSIONS

In this study, we provide the first insight into the effect of the substrate on PRMT1 product specificity. We show that PRMT1 exhibits regional selectivity with multi-arginine-containing peptide substrates and utilizes a semiprocessive mechanism that is controlled in a substrate-dependent manner. Considering the broad substrate specificity of PRMT1, as well as the distinct biological outputs of MMA and ADMA, such a "substrate-induced" mechanism helps to explain how patterns of methylation can be deposited, thus ensuring the proper transmission of biochemical information.

■ ASSOCIATED CONTENT

Supporting Information

MS and LC−MS/MS analyses of methylation order in multi-arginine-containing peptide substrates and steady-state kinetic study and intrinsic fluorescence quenching results for the single-arginine-containing peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; ADMA, asymmetric dimethylarginine; eIF4A, eukaryotic initiation factor 4α; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hnRNP K, heterogeneous nuclear ribonucleoprotein K; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria-Bertani; MS, mass spectrometry; MTAN, methyl thioadenosine nucleosidase; MMA, monomethyl arginine; PRMT, protein arginine methyltransferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; WT, wild type.

■ ADDITIONAL NOTES

[a]Bold and underlined arginine residues (**R**) are methylation sites of PRMT1.

[b]Approximations assume that the small difference observed in k_{cat}/K_m between the naked and monomethylated peptide substrates in the steady state does not alter product partitioning by an appreciable amount in the double-turnover experiments.

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