CHARACTERIZATION OF THE PRODUCT SPECIFICITY AND KINETIC MECHANISM OF PROTEIN ARGinine METHYLTRANSFERASE 1

by

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in

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ABSTRACT

Characterization of the Product Specificity and Kinetic Mechanism of Protein Arginine Methyltransferase 1 (PRMT1)

by

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Protein arginine methylation is an essential post-translational modification catalyzed by protein arginine methyltransferases (PRMTs). Type I PRMTs transfer the methyl group from $S$-adenosyl-$L$-methionine (AdoMet) to the arginine residues and catalyze the formation of monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA). Type II PRMTs generate MMA and symmetric dimethylarginine (SDMA). PRMT-catalyzed methylation is involved in many biological processes and human diseases when dysregulated. As the predominant PRMT, PRMT1 catalyzes an estimated 85% of all protein arginine methylation in vivo. Nevertheless, the product specificity of PRMT1 remains poorly understood. A few articles have been published regarding the
kinetic mechanism of PRMT1, yet with controversial conclusions.

To gain more insights into the product specificity of PRMT1, we dissected the active site of PRMT1 and identified two conserved methionines (Met-48 and Met-155) significant for the enzymatic activity and the product specificity. These two methionines regulate the final product distribution between MMA and ADMA by differentially affecting the first and second methyl transfer step. Current data show that Met-48 also specifies ADMA formation from SDMA. To further understand the kinetic mechanism of PRMT1, we developed a double turnover experiments to conveniently assay the processivity of the two-step methyl transfer. Using the double turnover experiments, we observed that PRMT1-catalyzed dimethylation is semi-processive. The degree of processivity depends on the substrate sequences, which satisfies the controversy between the distributive or partially processive mechanisms previously reported. We are using transient kinetics and single turnover experiments to further investigate the mechanism of PRMT1. Interestingly, during these studies, we found that PRMT1 may incur oxidative damage and the histidine affinity tag influences the protein characteristics of PRMT1. These studies have given important insights into the product specificity and kinetic mechanism of PRMT1, and provided a strong foundation for future studies on PRMT1.
Investigation of the chemical properties and cellular function of PRMTs

Protein enzymes perform a vast array of functions within living organisms, catalyzing various metabolic reactions including DNA replication, DNA repair, protein synthesis, etc. In order to maintain proper cellular functions, enzymes need to be accurately regulated under different circumstances. Specifically, enzymes can be modified after their creation to give them additional functions. These modifications can do a variety of things including activating (turning on) or inactivating (turning off) an enzyme, changing what proteins or molecules can interact with the enzyme, changing the enzyme’s location in the cell, and/or targeting the enzyme for destruction. This dissertation focuses on a single class of enzymes, protein arginine methyltransferases (PRMTs), which transfer one or two methyl groups to a specific amino acid, arginine, in the target protein (substrate).

Arginine methylation is a small but significant modification involved in cellular processes such as transcriptional regulation, DNA repair, subcellular localization, signal transduction, and nuclear transport. Moreover, irregular expression and malfunction of PRMTs, which lead to altered amount and/or type of the methylation products, are broadly observed in cancer and cardiovascular disease. Thus, detailed study of PRMTs is essential for the development of therapeutic drugs for diseases associated with arginine methylation. This dissertation presents continuous studies with broad insight into the product specificity and catalytic mechanism of PRMT1 by addressing how PRMT1 is regulated to maintain its specificity and activity to generate the desired amount and type of methylation products.
To my grandfather Yufu Gao I dedicate this dissertation.
I would like to take this opportunity to thank my major advisor, Dr. Joanie Hevel, who has mentored, supported, and encouraged me throughout my studies at Utah State University. I also would like to thank my committee members, Dr. Alvan C. Hengge, Dr. Lance C. Seefeldt, Dr. Sean J. Johnson, and Dr. Tim Gilbertson, for their support and assistance throughout the entire process.

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CHAPTER 1
INTRODUCTION

In eukaryotic cells, protein arginine methylation is an essential post-translational modification that enables organisms to expand upon their limited genome in addition to phosphorylation, acetylation, and glycosylation. Protein arginine methyltransferases (PRMTs), which catalyze arginine methylation, are involved in a wide variety of fundamental cellular pathways including RNA processing, signal transduction, DNA repair, transcriptional regulation (reviewed in (1-3)), chromatin remodeling (4), and neuronal cell differentiation (5). Dysregulation of PRMT expression and/or activity has been observed in numerous diseases, including carcinogenesis (6), viral pathogenesis (7), multiple sclerosis (8), spinal muscular atrophy (9), lupus (10), cardiovascular disease (11, 12) and stroke (13). Overall, PRMTs play a crucial role in many biological processes.

Although the biological importance of PRMTs has become well accepted, current understanding of the fundamental biochemistry of these enzymes remains limited, partially due to the complexity of the system. Thus far, nine PRMT isoforms have been identified in mammalian cells (PRMT1-9). PRMTs transfer a methyl group from \textit{S}-adenosyl-\textit{L}-methionine (AdoMet/SAM) onto a positively charged arginine residue in the protein substrate, generating monomethylarginine (MMA) and \textit{S}-adenosyl-\textit{L}-homocysteine (AdoHcy/SAH). Type I PRMTs (PRMT1-4, 6, and 8) can further catalyze the formation of asymmetric dimethylarginine (ADMA); the type II enzyme, PRMT5, generates MMA and then symmetric dimethylarginine (SDMA), while PRMT7, the type III enzyme, only generates MMA.
PRMTs show broad and high substrate specificity, with different products correlating with specific biological outputs. For instance, PRMT4 methylates histone H3R2, H3R17, and H3R26 (14, 15), while PRMT1 and PRMT5 specifically target H4R3 and H3R8 (16, 17). Different dimethylation statuses (ADMA or SDMA state) of the same substrate can lead to distinct transcriptional outputs (18, 19). Importantly, MMA is not only the intermediate of dimethyl arginine formation, but also a physiologically relevant methylation status, which has been shown displaying a different transcriptional output from ADMA (20). Therefore, it is of great significance to understand the regulation of product specificity of PRMTs.

Many important questions about product specificity of PRMTs remain to be answered. For instance, it is unknown why Type I PRMTs specifically generate ADMA instead of SDMA and vice versa, even when the two types of PRMTs target the same substrates. How do PRMTs govern which arginyl residues are modified and which state of methylation is achieved? Currently, none of these aspects is clearly understood. To gain mechanistic insight about product specificity of PRMTs, we chose to start with PRMT1, the predominant PRMT isoform that performs over 85% of all protein arginine methylation in vivo (21). The purpose of this dissertation was to understand how PRMT1 modulates its product specificity by dissecting the PRMT1 active site and characterizing the kinetic mechanism of PRMT1.

In Chapter 3, I followed the discovery of Dr. Whitney Wooderchak-Donahue, a previous Ph.D. in the Hevel Lab, and further identified that two strictly conserved methionine residues (Met48 and Met155) in the PRMT1 active site play significant roles in regulating substrate recognition and product formation of MMA versus ADMA (22).
From the crystal structure of PRMT1, M155 (23) and M48 (22) were hypothesized to control ADMA formation instead of SDMA due to steric hindrance afforded by their bulky side chains. By direct site mutagenesis and product analysis, we found that the M48L, M48A and M155A mutants showed a decreased enzymatic activity, yet still generated ADMA instead of SDMA. However, these mutants change the distribution of final mono- and dimethylated products. To understand the mechanistic basis of the altered product formation, I developed single turnover experiments for PRMT1 based on the similar concepts done with DNA methyltransferases (24), which examined each methyl transfer step separately in the dimethylation process. Single turnover experiments reveal that M48L transfers the second methyl group much slower than the first one, especially for arginine residues located in the center of the peptide substrate where turnover of the monomethylated peptides is negligible. Thus, altered mono- and dimethyl product distribution in M48L results from the different effect of the mutation on the two-step methylation rates. Characterization of the two active-site methionines reveals for the first time how the active site of PRMT1 is engineered to modulate the product specificity.

Following the study of active site residues, we further examined what leads to the specific methylation patterns of PRMT1 with different substrates *in vivo* (25-27). In Chapter 4, we investigated the methylation preference of PRMT1 among multi-arginine substrates, as well as the determinants for the final methyl status on the targeted arginine. Most protein substrates of PRMT1 contain multiple arginines in close proximity. We found out that PRMT1 methylates a multisite peptide substrate in a non-stochastic manner with an N-terminal preference, consistent with the methylation patterns observed *in vivo*. With a single targeted arginine, we showed that the final methylation status is
affected by the amino acid sequence context. Another approach to regulate the proportion of mono- and dimethylation would be through regulation of dissociative or processive dimethylation. To conveniently study the processivity of PRMT1, a double turnover experiment was developed which revealed PRMT1-catalyzed dimethylation in a semi-processive manner. The degree of processivity is regulated by substrate sequences, which explained the controversial observations between the distributive and partial processive mechanism of PRMT1 (28, 29). Our results recognize a novel substrate-induced mechanism for modulating PRMT1 product specificity (30).

In Chapter 5, to further understand the semi-processive mechanism, single turnover experiments and pre-steady state kinetic studies were performed to examine the microscopic rates in the arginine dimethylation process. In 2011, a transient kinetics study (31) was performed with PRMT1 and a fluorescein-labeled peptide, of which the results were controversial to the previous steady-state kinetic studies (28, 32, 33). Herein, we chose the underivatized eIF4A1 peptide pair for further mechanistic investigations due to its relatively high degree of processivity shown in Chapter 4. Results from the single turnover experiments showed that PRMT1 is slower to bind AdoMet and generate a productive complex compared to the peptide substrates. Under single turnover conditions, PRMT1 has a slightly higher preference to the unmodified peptide substrates. Stopped-flow rapid mixing experiments exposed that AdoMet binding to PRMT1 appears to be a two-step process. Most surprisingly, a role for the reductant dithiothreitol (DTT) in substrate binding was revealed in my studies. Data from rapid mixing with PRMT1 and DTT indicated a transient change in protein conformation and/or its oligomeric state, possibly due to the histidine-tag within the PRMT1 construct. Due to the limited access
to the stopped-flow equipment (UC, San Francisco), not enough data were collected to draw a solid conclusion on the mechanism of PRMT1-catalyzed methylation.

Following the discovery of the effect of DTT on His-tagged rat PRMT1, in Chapter 6, we generated a tagless construct of rat PRMT1 to investigate the influences of affinity tags and the reductant DTT. The His$_6$-tag was once reported to have a drastic effect on the substrate specificity of human PRMT1 (34). Although we didn’t observe this with the hypomethylated cell lysates we used, the His$_6$-tag was found to lower the activity of rat PRMT1 compared to the tagless construct, and lead to a distinct oligomeric pattern from the tagless PRMT1, which can be partially recovered by the addition of DTT. Surprisingly the enzymatic activity of the tagless PRMT1 is barely affected under the same DTT treatment. Our results indicate that small affinity tags, such as the histidine-tag, can significantly affect the protein characteristics of PRMT1, which can be partially recovered by reducing agents. The effect of DTT suggests that His-rat PRMT1 may incur oxidative damage. Further experiments will be performed to understand more about the possible oxidative damage of PRMT1 and their influences on the enzymatic activity.

In Chapter 7, results from RNA aptamer development were summarized. RNA aptamers are small pieces of RNAs which have high binding affinity and specificity targeting selected molecules. As free ADMA becomes a biomarker for cardiovascular diseases (35), it is of great significance to develop a convenient and accurate method to detect and quantitate free ADMA. Unlike antibodies, aptamers are readily available and can be easily modified. Herein, we aimed to develop RNA aptamers targeting ADMA. Systematic evolution of ligands by exponential enrichment (SELEX) was performed against ADMA-coated agarose beads. The binding affinity and specificity of the RNA
pools generated in these SELEX cycles were tested via two well-accepted binding assays, an affinity chromatography and a filter-based binding assay. However, the binding results from these two assays did not agree with each other. Therefore, another novel scintillation proximity assay is under development to screen the selected RNAs and investigate the sequence and structural properties of the RNA aptamers.

Chapter 8 includes experimental results from several ongoing projects and their possible future directions. We previously discovered that the processivity of PRMT1-catalyzed dimethylation is substrate dependent (30). To further investigate the influence of peptide substrates on PRMT1 processivity, single turnover experiments were performed with series of peptide substrates to measure the methylation rate of the 1\textsuperscript{st} and 2\textsuperscript{nd} methylation step. Results showed that the methylation rate is uncoupled with the degree of processivity. More factors are involved in the substrate-dependent processivity. In another experiment, the M48F-PRMT1 mutant was found to generate a small amount of SDMA, which changes the product specificity of PRMT1 into Type I/II PRMT. Further experiments are focusing on computing the differences of the active sites of wild type PRMT1 and M48F-PRMT1 and their influence on achieving the product specificity. In summary, this dissertation provides more insights into the product specificity and kinetic mechanism of PRMT1. The in-depth mechanistic analysis as well as the preliminary experiments from the ongoing projects provides a strong basis for future studies.

References


Biological Methylation

In biological systems, S-adenosyl-L-methionine (AdoMet or SAM, Figure 2-1A) is the second most widely used enzyme substrate following adenosine triphosphate (ATP) (1). AdoMet can be utilized in a multitude of reactions including transsulfuration, aminopropylation, and transmethylation. The majority of AdoMet-dependent reactions involve methyl group transfer. After AdoMet is generated by methionine adenosyltransferase from methionine and ATP, the methyl group of methionine is activated (2), and can be transferred to diverse substrates by AdoMet-dependent methyltransferases (MTases) leaving the product S-adenosyl-L-homocysteine (AdoHcy). The huge preference for AdoMet over other methyl group donors (such as N⁵-methyltetrahydrofolate) reflects favorable energetics from the charged methylsulfonium center. The ΔG° for (AdoMet + homocysteine → AdoHcy + methionine) is very low (-17 kcal/mol), more than twice the amount released from ATP hydrolysis (1). AdoMet is also the most expensive metabolic compound made by cells on a per carbon basis. The de novo biosynthesis of AdoMet costs twelve equivalents of ATP in this process (3). However, the generation of AdoMet is also beneficial to the cell, because in the reactions involving AdoMet, the breakdown products of AdoMet can all be used in vivo (4).

In the structure of AdoMet (Figure 2-1A), the methyl group (-CH₃) attached to the methionine sulfur atom is chemically reactive and can be transferred to a methyl group
acceptor. Methylation substrates include small molecules like arsenite (5, 6) and sterol (7, 8), as well as macromolecules such as DNA, RNA, and proteins (reviewed in (9, 10)). The atomic target for the MTases can be carbon, oxygen, nitrogen, sulfur, and halides (10-13). With different methyl group acceptors, a wide variety of mechanisms are utilized to generate a catalytically active nucleophile. However, all MTases are thought to perform a fundamental S_N2-like mechanism (Figure 2-1B), transferring the methyl group to the substrate with an inversion of symmetry (14, 15).

**AdoMet-Dependent Protein Methylation**

Following transcription and translation process, most proteins are then chemically modified at some point. These post-translational modifications (PTMs) extend the range

![Figure 2-1](image)

**Figure 2-1.** (A) Structure of S-adenosyl-l-methionine, which includes the methionine moiety and the adenosine moiety. The chemically reactive methyl group attaches to the methionine sulfur atom making the sulfur atom positively charged. (B) A general reaction mechanism for AdoMet-dependent methyltransferases. A general base (:B) abstracts a proton from the target atom (X=C, N, O, or S) leading to methyl group transfer from AdoMet to the target atom.
of structures and functions of the proteins by attaching other biochemical functional
groups (such as phosphate, acetate, alkyl groups, lipids, etc.), changing the nature of
amino acid side chains (e.g. deimination), or making structural changes (e.g. disulfide
bridges). Specific PTMs serve numerous functions including enzyme regulation and
cellular signaling.

It has been predicted that over 1% of genes in the mammalian genome encode
methyltransferases (16). Protein methylation is one of the post-translational modifications
involving one methyl group transfer mostly from AdoMet. Among protein
methyltransferases, most methylations occur on nitrogen (N-methylation) and oxygen
(O-methylation), and to a lesser extent on carbon (C-methylation) and sulfur
(S-methylation) atoms on amino acids.

Nitrogen is the most common nucleophile for protein MTases, and N-methylation
occurs on the side chains of lysine, arginine, histidine, glutamine and asparagine (17).
N-methylation on lysine and arginines, which are the most common targets, does not
change the positive charge of the amino acid side chains, but it does increase the steric
bulk of residues as well as the hydrophobicity, which in turn influences the
protein-protein and protein-nucleic acid interactions (17). Lysine residues can be mono-,
di- and then tri-methylated at the ε-aminogroup, whereas arginine residues can only be
mono-, and then asymmetrically or symmetrically dimethylated on the guanidine moiety
(18, 19). Besides lysine and arginine residues, the imidazole ring of histidine can also be
methylated to 1-methylhistidine (20) and 3-methylhistidine (21), which are both found in
muscle protein and in human urine. Moreover, the side chains of glutamine and
asparagine can be methylated as well, yielding N⁵-methylglutamine (22) and
Unlike lysine and arginine methylation, glutamine or asparagine can only be monomethylated, which alters the chemical property of the amide acid side chain, disturbing its hydrogen bonding potential and significantly enhancing its hydrophobic character (24). The protein N-methylation in most cases is considered irreversible except for lysine, of which the demethylation can be catalyzed by the lysine-specific histone demethylase 1 (LSD1) family and the Jumonji C (JmjC) family (25, 26).

In addition to nitrogen methylation, proteins can also be methylated on oxygen atoms resulting in methyl esters (27). O-methylation occurs on the side chain carboxylate of glutamate and aspartate, which neutralizes the negative charge of the carboxylate group and adds hydrophobicity to the protein. Hence, O-methylation of glutamate and aspartate completely disturb the protein-protein and protein-nucleic acid interaction. This modification is a reversible process in cell, which appears to be hydrolyzed by simple esterases and these enzymes have been isolated from chemotactic bacteria (27).

To a lesser extent, the electron-rich carbon and sulfur atoms can also be methylated in methanogenic bacteria. The enzyme methyl-coenzyme M reductase was shown crystallographically containing C-methylated arginyl and glutamine side chains, an N-methylated histidine, and an S-methylated cysteine residue (28).

**Protein Arginine Methylation**

Protein arginine methylation is a common post-translational modification in all eukaryotic cells. Although arginine residues were first discovered to contain methyl groups in 1967 (29), arginine methylation was underappreciated until the mid-1990s, yet
later on shown to be an essential and relatively abundant post-translational modification.

Protein arginine methylation is catalyzed by protein arginine methyltransferases (PRMTs). A family of nine PRMT enzymes has been identified in mammals (Figure 2-2). Type I and type II PRMTs catalyze the formation of monomethylarginine (MMA), which can be further converted to asymmetric dimethylarginine (ADMA) by the type I PRMTs (PRMT1, 2, 3, 4, 6, and 8), while type II PRMTs (PRMT5) catalyze the formation of symmetric dimethylarginine (SDMA) (reviewed in (30, 31)). PRMT7 is found to generate MMA predominantly, and is thus classified as a type III PRMT (32). PRMT9 has no reported activity as yet.

![Figure 2-2. Mono- and dimethylation of arginine catalyzed by PRMTs. Type I, II, and III PRMTs catalyze the addition of a monomethyl group to one of the terminal \((\omega)\) guanidine nitrogens of arginine residue, generating MMA and AdoHcy. Type I and Type II PRMTs can further methylete MMA forming ADMA and SDMA, respectively. The second molecules of AdoMet and AdoHcy are omitted for clarity.]

**Protein Arginine Methyltransferase Family**

The PRMT family harbors a common catalytic core for methyltransferases, containing the set of four signature motifs and the highly conserved THW loop (Figure
2-3). Although all PRMT isoforms have a conserved AdoMet-binding site, their N-termini differ in length and the presence or absence of additional motifs, which facilitate the unique function of each PRMT isoform.

PRMT1 is the predominant mammalian type I enzyme, catalyzing 85% of total protein arginine methylation in vivo (33). PRMT1 localizes to both the cytoplasm and the nucleus (34). In both cellular compartments, PRMT1 has numerous substrates and primarily methylates the glycine-arginine-rich domain in RNA-binding proteins (35). In the human genome, seven variants of PRMT1 have been found resulting from alternative splicing (36). These variants have different N-terminal sequences and tissue localization, with distinct activity, substrate specificity, and subcellular localization. PRMT2 was then discovered by sequence similarity to PRMT1 (37). PRMT2 can directly interact with PRMT1 and stimulate PRMT1 activity in cells (38). A novel feature of PRMT2 is that it harbors a SH3 domain at its N-terminus (37, 39), which is essential for protein-protein interactions of PRMT2 with the proline-rich proteins (40, 41). PRMT3 was identified as a PRMT1 binding protein two years after the discovery of PRMT1 (42). PRMT3 is the only type I PRMT that does not display a nuclear location (34, 42). It is found that PRMT3 influences ribosomal biosynthesis by catalyzing the dimethylation of the 40S ribosomal protein, which is dependent on the zinc-finger domain at its N-terminus (43).

PRMT4 was identified in a yeast two-hybrid screen to associate within the p160 family proteins (44). As a transcriptional coactivator (45), PRMT4 functions synergistically with PRMT1 (46) and the histone acetyltransferases (HATs) (47). PRMT5 was cloned as a Jak2-binding protein and shown to be able to generate SDMA (48, 49). PRMT5 is a type II PRMT (48), which is generally regarded as a transcriptional corepressor (50). PRMT6
is another type I PRMT with a nuclear restricted pattern of location (34). PRMT7 harbors two putative AdoMet-binding motifs (51) and is the only PRMT which is classified as a type III PRMT generating only MMA (32, 51). PRMT8 was identified due to its high degree of homology with PRMT1 (52), and is capable of automethylation (41), similar to PRMT6 (34). The N-terminal myristoylation anchors PRMT8 to the plasma membrane. PRMT9 was discovered through a database search based on sequence homology to the conserved PRMT AdoMet-binding motif (53). PRMT9 contains an F-box motif at its N-terminus and a zinc-finger motif at its C-terminus, localized in both the nucleus and cytoplasm. PRMT9 was shown to generate MMA, ADMA, as well as SDMA (53).

Figure 2-3. Schemes of the nine canonical members of the human PRMT family. The highly conserved MTase core regions (grey) present in all PRMTs are indicated. Note that PRMT7 has a duplication of these motifs. PRMT2 and PRMT3 have an N-terminal SH3 domain or a zinc-finger domain, respectively. The N-terminal myristoylation tethers PRMT8 to the plasma membrane. PRMT9 has an F-box at its N-terminus and a zinc-finger domain at the C-terminus. The size of individual PRMT is indicated at each C-terminus.
Structures of the PRMT Family

A. Overall Structures of PRMTs

So far, crystal structures of PRMTs have been solved for rat PRMT1 (54), yeast RMT1/Hmt1 (55), rat PRMT3 (56), mouse PRMT4 (57), PRMT5 from *Caenorhabditis elegans* (58), and plant-specific PRMT10 from *Arabidopsis thaliana* (59). The overall monomeric structures of all PRMT structures contain three conserved parts (Figure 2-4): AdoMet binding domain (light green), β barrel (light yellow) and the dimerization arm which is embedded in the β barrel (light blue). The AdoMet binding domain has the consensus fold conserved in other AdoMet-dependent methyltransferases (9, 16, 56, 60), whereas the β barrel domain is unique to the PRMT family (54, 56). The type I PRMTs all have a helical N-terminus (Figure 2-4 A), yet PRMT5 contains an unexpected TIM barrel domain (Figure 2-4 B, light pink). Notably all PRMTs exist as a homodimer in the crystal structure. The dimerization is briefly shown to be essential for PRMT activity (54, 55). Different PRMTs contain various lengths of dimerization arms. AtPRMT10 has a significantly longer dimerization arm (12-20 residues longer than PRMT structures elucidated previously) and leads to a larger central cavity in the dimeric form than PRMT1 (59).

B. The Active Site of Type I and II PRMTs

For all type I PRMTs, the active sites shown in the crystal structures are very similar. As the predominant PRMT *in vivo*, the crystal structure of rat His-PRMT1 was solved in 2003 with AdoHcy and R3, a 19 amino acid peptide substrate derived from fibrillarin (GGRGGFGRGGFGRGGGFRG) (54). Until now, this is the only crystal structure of
Figure 2-4. (A). Overall monomeric structures of Type I PRMT, rat PRMT1 (PDB: 1ORI), rat PRMT3 (PDB: 1F3L), mouse PRMT4 (PDB: 2V74), and AtPRMT10 (PDB 3R0Q). (B) The overall monomeric structure of Type II PRMT, *C. elegans* PRMT5 (PDB: 3UA3). (C) The dimer structure of rat PRMT1. The N-terminal helix is shown in pink in type I PRMTs as well as the TIM-barrel at the N-terminus of PRMT5. The AdoMet binding domains in all structures are shown in light green, the β barrel structure in light yellow, and the dimerization arm in light blue. The bound AdoHcy is shown in a stick mode in dark grey. In PRMT1 dimer structure, the other monomer is shown in light grey.
PRMTs co-crystallized with a peptide substrate. Even though the electron density for the R3 peptide cannot be clearly observed, this crystal structure provided much important insight into the reaction mechanism. Two conserved active site glutamates (E144 and E153 in rat PRMT1, Figure 2-5A), called the “double-E” loop, stabilize the substrate arginine guanidino nitrogen through hydrogen bonding. Mutating these glutamate residues causes a dramatic reduction in PRMT1 activity (54).

Our research as well as other groups identified that two methionine residues (M48 and M155) sit very close to the guanidino group of the target arginine residue, regulating the product formation (further discussed in Chapter 3) (58, 61). The hydrophobic methylene groups of the target arginine lie parallel to the aromatic ring of Y148. In type II PRMT, the “double-E” loop is also conserved (E499 and E508, Figure 2-5B) in the

Figure 2-5. The active site structures of type I and II PRMTs. (A) The active structure of rat His-PRMT1 (PDB: 1OR8, pink) is shown with important amino acid residues shown in stick model. Substrates AdoHcy and arginine residues are shown in green. (B) Superimposition of the active site of PRMT1 and PRMT5 (PDB: 3UA3, grey). The amino acids of PRMT5 are labeled.
PRMT5 structure and is definitely required for enzymatic activity (58). One important difference with the PRMT5 active site is that F379, which is conserved among PRMT5 proteins, replaced the M48 residue in rat PRMT1. Mutating the F379 back to a methionine resulted in a more active enzyme, generating both SDMA and ADMA (58). Moreover, I also discovered that an M48F mutation in rat PRMT1 makes PRMT1 generating MMA, ADMA, and SDMA (Chapter 8). These observations indicate the significant role of M48 in PRMT1 in specifying the type of dimethyl arginine generation.

C. The N-terminal Structures of PRMTs

The N-termini of PRMTs vary a lot with specific functional domains for different PRMTs (Figure 2-3). It has been predicted that the N-terminus of human PRMT1 is probably involved in protein-protein interactions and substrate recognition (36). However, in the crystal structure of PRMT1, the electron density for the N-terminus was completely missing. From this structure, AdoHcy and the arginine residue are exposed on the PRMT1 surface (Figure 2-6 left panel) indicating that AdoHcy or the peptide can be released during methylation reaction without conformational change. However, by superimposing the PRMT1 structure with PRMT3, of which the structure is almost identical to PRMT1 yet with the N-terminal helix crystallized, I discovered that the additional N-terminal helix folded right above the substrate binding pocket and trapped AdoHcy inside (Figure 2-6 right panel). This observation implicates that polypeptide motion or conformational change might be involved in the methylation process in order to release AdoHcy and bind another AdoMet for the next turnover. Indeed, research from the Zheng Group (62) and my pre-steady state kinetic studies (Chapter 5) identified a
Figure 2-6. Conformational change might be required for PRMT1 in methylation process. (Left) Solvent accessible molecular surface of PRMT1 shown in gray with bound AdoHcy and Arg shown in stick models (PDB: 1OR8). (Right) The N-terminal helix of the PRMT3 structure (PDB: 1F3L) shown in pink was superimposed onto PRMT1, which trapped AdoHcy and the substrate Arg in the active site pocket.

critical precatalytic step which might be a conformational transition induced by substrate binding.

**PRMT Substrates and Product Specificity**

PRMT-catalyzed methylation is a relatively abundant post-translational modification *in vivo*. 2% of all protein arginine residues are asymmetrically dimethylated in rat liver nuclei (63). Within the nuclear compartment, heterogeneous nuclear ribonucleoprotein (hnRNP) contains about 12% of the arginine residues being asymmetrically dimethylated (35, 63).

With numerous substrates *in vivo*, the product specificity of PRMTs includes not only recognizing the protein substrates, but also targeting specific arginine residues in the multiple methylation sites. It is known that PRMTs have a sequence preference to the proteins harboring glycine- and arginine-rich (GAR) motifs in “RGG” or “RXR”
canonical sequences (64). Although substrate profiling of PRMT1 reveals more amino acid sequences that extend beyond the “RGG” paradigm (65), most PRMTs have such preference. PRMT4, on the other hand, displays different substrate specificity and does not methylate the GAR motif.

Additionally, histone proteins are also common substrates for all PRMTs. Histone H3 can be methylated by PRMT4 (44, 66), -5 (32, 67), and -6 (68), and H4 protein can be methylated by PRMT1 (44, 69), -2 (70), -3 (42), -5 (49), -6 (68), -7 (32), -9 (53), and -10 (59). PRMTs demonstrate site preference to histone proteins. For instance, on histone H3, Arg17 and Arg26 are the preferred methylation targets for PRMT4 (44, 45), while Arg8 is methylated by PRMT5 (67). Methylation of histone arginines by PRMTs is largely involved in transcriptional regulation. Histone H4 Arg 3 is targeted by PRMT1 and PRMT5 in vivo, generating ADMA and SDMA, respectively. And these two methyl states of H4R3 lead to opposite transcription consequences (50, 69, 71-73). Moreover, it has long been considered that MMA is simply an intermediate of ADMA or SDMA generation. However, the Kouzarides Group showed that MMA is a methylation state that occurs in vivo on histone H3 Arg 2 in yeast nucleosomes, leading to a distinct transcriptional output from ADMA state on H3R2 (74). Thus, regulation of the product specificity of PRMTs is significant in proper cellular transmission of chemical information.

**Kinetic Mechanism of PRMTs**

In order to understand the product specificity of PRMTs, efforts have been put in exploring the kinetic mechanism of PRMTs. Among a handful of publications on the
mechanism of PRMT1, the conclusion is still controversial, especially on whether PRMT1-catalyzed dimethylation is a distributive (monomethylated species released from the enzyme before rebinding) or processive (mono- and dimethylation occur sequentially without releasing monomethyl species) mechanism. As a distributive mechanism would produce a higher concentration of MMA than the enzyme concentration, a processive mechanism will have an obligated dimethyl arginine formation.

In 2007, Thompson and coworkers first elucidated the mechanism of PRMT1 being partially processive using histone H4-derived peptides due to the results that PRMT1 generates MMA and ADMA containing peptides in approximately equal amounts (75). Such partially processive methylation has been observed for a number of protein lysine methyltransferases (76). Later in 2008, they further reported that human PRMT1 utilizes a rapid equilibrium random mechanism from initial velocity and inhibition experiments, which is consistent with the partially processive mechanism (77). However, Wahle and coworker, in 2009, stated that both PRMT1 and PRMT3 act distributively, i.e. with intermittent release of the MMA intermediate using a peptide substrate derived from the PABPN1 protein (78). To solve this disagreement, my research with various peptide substrates found out that PRMT1-catalyzed dimethylation is semi-processive. The degree of processivity is dependent on substrate sequences (Chapter 4) (79). Such fine-tuned semi-processive mechanism allows varied final amounts of MMA and ADMA with different substrates. Moreover, important active-site residues to product specificity in PRMT1 were also identified. As stated previously, our research (Chapter 3) and the Thompson Group discovered that mutation with smaller amino acids at M48 and M155 positions in rat PRMT1 influences the MMA/ADMA product formation but is not
responsible for the regiospecific formation of ADMA (61, 80). However, intriguing results were observed with M48F-PRMT1 mutation, which catalyzes a small amount of SDMA formation along with ADMA formation (Chapter 8).

Similar to the argument in the research of PRMT1 kinetic mechanism, PRMT6 was also previously reported to proceed via an ordered sequential mechanism in which AdoMet binds to the enzyme first and the methylated product dissociate first then AdoHcy, based on product inhibition studies, so that the distributive mechanism is guaranteed in dimethylation process (81). Four years later, another kinetic study found that PRMT6 follows a rapid equilibrium random mechanism and has limited processivity to the peptide substrates (82). Our findings about the semi-processive mechanism of PRMT1 may also contribute to understanding PRMT6 kinetic mechanism. Additionally, the resolution to these discrepancies might require some structural evidence. In the structure of PRMT4, a conformational change was observed upon AdoHcy binding, which then facilitates peptide substrate binding (57). This observation strongly argues that release of peptide is required to allow further AdoHcy/AdoMet exchange for the next methylation event, which would be incompatible with a random partially-processive mechanism.

**Regulation of Product Specificity in Protein Methylation**

Besides the kinetic mechanism of enzymes, the amino acid residues in the active site, regulators in vivo, as well as other post-translational modifications can all influence the product specificity of PRMTs. As an analogous phenomenon to arginine methylation, the elegant studies in the lysine methyltransferase field can strongly support the investigation
of product specificity of arginine methylation.

A. Regulation by Amino Acid Residues in Enzyme Active Site

Regulation of product specificity for both lysine and arginine methyltransferases is drawing more attention due to the distinct biological outputs from different methylation states of histones (83). It has been shown that the product specificity of protein lysine methyltransferases (PKMTs) could be controlled in part by the existence of specific residues at the active site (84-87). One of the best examples is a Phe/Tyr switch found in many PKMTs (87, 88). As lysine can be mono-, di-, then tri-methylated, different PKMTs generally have different product specificity. In SET7/9 and SET8, a tyrosine residue in the active site occupies the position and only allows for monomethyl lysine formation (85, 89, 90), and in DIM-5 (84), a phenylalanine in that position makes the PKMT a di- or tri-methyltransferase. Moreover, the substitution at the Phe/Tyr switch position could lead to changed product specificity, i.e. the F→Y mutation for DIM-5 altered the enzyme from a tri-methyltransferase to a mono/di-methyltransferase (84). Such a regulation switch might also exist in protein arginine methyltransferases. Two methionine residues are found highly conserved in the type I PRMT active site (61). Although mutating these methionines in PRMT1 into alanine does not change its ADMA-specificity (61, 80), an M→F mutation changed PRMT1 into type I/II enzyme (Chapter 8). Mutating the corresponding phenylalanine in type II PRMT5 back to a methionine resulted in an active enzyme generating both SDMA and ADMA (58).

B. Regulation by Interacting Proteins

Protein methylation has been shown to be regulated by proteins that bind to them.
The interaction of the regulator proteins can result in activation, inhibition or modulation of substrate specificity of protein methyltransferases. Histone H3 Lys 4 (H3K4) trimethylation is commonly considered as a transcriptional activation signal recruiting chromatin remodeling and modifying complexes (91-93). The PKMTs which catalyze this modification, SET1 and MLL1, however, display very weak methylation activity by themselves, and require additional four proteins, WDR5, RbBP5, ASH2L, and DPY30 to form a complex to achieve maximal activity (94-96).

The activity and substrate specificity of PRMTs are also regulated by interactions with other binding partners. PRMT1 activity was found to be stimulated by BTG1 and TIS2/BTG2 proteins towards tested substrates (97). Another protein, hCAF1, also interacts with BTG1, regulating PRMT1 activity in a substrate-dependent manner (98). Moreover, the substrate specificity of PRMTs is influenced by interacting proteins. PRMT4 is found endogenously in the nucleosomal methylation activator complex (NUMAC), which switched the methylation specificity from free core histone H3 to nucleosomal histones (99). Similar to PRMT4, a nuclear protein CORP5 tightly binds to PRMT5 in vivo, strongly recruits it to histone H4 protein, so that the substrate specificity of PRMT5 is modulated by CORP5 to preferentially methylate H4R3 when compared with H3R8 (100).

C. Regulation by Post-Translational Modifications

Besides interacting proteins in vivo, post-translational modifications on enzymes or substrates can also modulate the product formation of protein methylation. For example, PRMT4 was found to be phosphorylated at Ser217 which disrupts its hydrogen bond with
a close-by tyrosine and subsequently abolishes its methylation activity \(^{(101)}\). Moreover, other post-translational modifications close to the methylation site can also mask the methylation motif. This phenomenon is commonly seen on histone tails. It is found that PRMT5-catalyzed H3R8 methylation is inhibited by H3K9 acetylation \(^{(67)}\). Also H3K4 trimethylation catalyzed by an MLL complex and H3R2 dimethylation by PRMT6 are mutually exclusive \((66, 102, 103)\). In our recent studies, PRMT1 is suggested to incur oxidative modification, which also regulates the catalytic activity (Chapter 6). These modifications could serve as a molecular switch for controlling protein methylation.

D. Is Arginine Methylation Reversible?

Post-translational modifications can also be regulated through the counter reactions. Unlike phosphorylation or acetylation which is dynamically added or removed, methylation seems to be stable. Histone lysine methylation is shown being removable by histone-Lys-specific demethylase (LSD1). LSD1 catalyzes the demethylation of mono- or dimethyl histone H3 lysine 4 using FAD as a cofactor, generating hydrogen peroxide \((\text{H}_2\text{O}_2)\) and formaldehyde \((\text{CHO})\) as byproducts \(^{(104)}\). The observation of histone arginine demethylase was also reported in 2007 about the Jumonji domain-containing 6 protein (JMJD6), which demethylates histone H3 at arginine 2 (H3R2) and H4 at arginine 3 (H4R3) in both biochemical and cell-based assays \(^{(105)}\). However, this observation cannot be reproduced by other labs. In 2010, the crystal structure of JMJD6 was solved indicating that JMJD6 can catalyze C-hydroxylation rather than N-demethylation \(^{(106)}\). So far, the existence of arginine demethylase remains unclear.

**Biological Importance of PRMTs**
The list of PRMTs and PRMT substrates has rapidly expanded in recent years, implicating arginine methylation in a wide variety of cellular processes. Proteins known to be arginine methylated are mainly nucleic-acid-binding proteins and proteins involved in transcriptional regulation. The guanidino group of arginine residue is positively charged and capable of hydrogen bonding, which facilitates interactions with the phosphodiester backbones of RNAs and DNAs (107, 108). After arginine residues are methylated, molecular modeling suggests that the hydrogen-bonding capability is restricted and the bulkiness and hydrophobicity of the residue is increased (108). Although methyl arginine maintains the positive charge, methylation of hnRNP A1 \textit{in vitro} showed a lowered pI, indicating a decreased basicity of the protein (109). These changes in PRMT substrate proteins may influence the ability of arginine residues interacting with other proteins or nucleic acids, and further lead to different biological outputs.

A. Transcriptional Regulation

Protein arginine methyltransferases have been involved in transcription regulation through their methylation of many transcriptional factors and histone proteins. PRMT1 and -4 are often considered as transcriptional activators and have been reported to cooperatively enhance gene expression (110-112), while PRMT5 activity generally results in transcriptional repression (50, 67, 73). PRMTs are implicated in the regulation of numerous transcription factors, such as p53 (113), p300 (114), and YY1 (115).

Histone proteins are methylated by different PRMTs both \textit{in vivo} and \textit{in vitro} (Figure 2-7). The amino-terminal tail domains of these proteins can be heavily decorated with
covalent post-translational modifications including acetylation, ubiquitylation, methylation and phosphorylation. The combinatorial nature of histone modifications are further referred to as “histone code” (Figure 2-7). PRMT1 catalyzes H4R3 asymmetric dimethylation which further recruits lysine acetylation leading to transcriptional activation (69, 71, 72). This modification can be blocked by PRMT5 which symmetrically dimethylates H4R3 and recruits DNA methyltransferase DNMT3A leading to transcriptional silencing (73). Due to the distinct biological consequences of different methylation states, the product specificity of PRMTs is drawing more and more attentions.

![Figure 2-7. Post-translational modifications on the N-terminal tails of histone H3 and H4 proteins. Methylation sites for PRMTs are indicated (116, 117). P: phosphorylation; Me: methylation; Ac: acetylation.](image)

B. Signal Transduction

Protein arginine methyltransferases have been shown to play a significant role in cell signaling. PRMT1 was found to interact with the interferon α/β (IFN α/β) reporter (118).
The signal transducer and activation of transcription (STAT) family protein, STAT1, was suggested to be methylated by PRMT1 and involved in interferon signaling (119). Both PRMT1 and PRMT5 are involved in T-cell signaling through the nuclear factor of activated T-cell (NF-AT) transcription factor. PRMT1 methylates the NF-AT-interacting protein NIP45 both in vitro and in vivo, facilitating the binding event and resulting in stimulation of cytokine gene expression (120). PRMT5 also plays a positive role in regulating IL-2 (interleukin 2) gene expression in T lymphocytes (121).

Moreover, PRMTs are found to be involved in cell cycle and morphological control. The PRMT5 analog in yeast, Hsl7, degrades the kinase Swe1 which targets the cyclin-dependent kinase (CDK) Cdc28 to prevent entry into mitosis (122, 123). Recently PRMT6 is also shown regulating cell cycle progression through repression of CDK inhibitors (124).

C. Pre-mRNA Splicing and mRNA Transport

In the RNA processing procedures, numerous RNA binding proteins are involved, many of which are substrates of PRMTs. PRMT5 was found to dimethylate arginine residues in the spliceosomal proteins SmD1, SmD3, and SmB, which are the major epitopes for autoantibodies for lupus (125-127). Type I PRMTs may be involved in splicing or spliceosome assembly, due to the fact that nuclear SmB, D1/D2, and D3 contain ADMA residues (128).

In eukaryotic cells, mature mRNAs needs to be exported through the nuclear pore after processing. In yeast, Hmt1 (the predominant yeast type I PRMT) methylates Npl3 (129, 130) and Hrp1 (131, 132), both of which are implicated in mRNA transportation.
These proteins shuttle between the nucleus and cytoplasm, and the deletion of Hmt1 inhibits the nuclear export of both Npl3 and Hrp1 (131, 133).

D. DNA Damage Repair

The DNA repair protein MRE11 was identified containing ADMA residues (134) and later found to be methylated by PRMT1 both in vitro and in vivo (135). Methylation of the GAR motif of MRE11 does not affect the assembly of the MRE11/Rad50/NBS11 complex, which initially process the double-strand DNA breaks (135). And methyltransferase inhibition leads to the failure of MRE11 to localize to DNA damage sites (136). Another important protein involved in DNA repair is the tumor suppressor protein p53. Following DNA damage, activated p53 binds DNA and activates expression of target genes, such as CDK inhibiter p21 which promotes cell cycle arrest (137). PRMT5 was shown to directly bind and methylate p53, and regulate the target gene specificity of p53 (113). Recently PRMT6 was also reported to interact with the p53 promoter and negatively regulates p53 gene expression (138).

**Protein Arginine Methylation and Diseases**

PRMTs have a variety of protein substrates which are involved in many fundamental cellular pathways. Therefore considering the diverse roles of PRMTs, dysregulation of these enzymes would result in the appearance of some disease conditions.

A. Cancer

The connections between arginine methylation and cancer were noted since 1970s. Histones from patients with chronic erythremic myelosis show an increased methyl
arginine concentration \((139)\). Elevated mRNA levels of PRMT1 was observed in certain breast cancer cell lines compared to normal controls \((36)\) and high expression level of PRMT4 has been identified in prostate and breast cancer tumors \((140, 141)\). Recently, PRMT1 and PRMT6 are found to have a significantly higher expression level in cancer cells of various tissues than in non-neoplastic cells \((142)\). Furthermore, the serum ADMA levels of various cancer cases are considerably higher than those of non-tumor control cases \((142)\). PRMT5 levels have also been shown to be increased in leukemia and lymphoma cells \((143)\), as well as in gastric cancer \((144)\). Also, the tumor suppressor protein p53, implicated in over 50% of cancers, can be regulated by PRMT5 \((113)\) and PRMT6 \((138)\). Due to the overexpression of PRMTs and high ADMA levels found in many cancer cases, PRMTs are becoming novel potential therapeutic targets, and specific inhibitors of PRMTs \textit{in vivo} is becoming a large interest in drug development \((145)\).

B. Cardiovascular Diseases

Free MMA and ADMA in plasma is a potent inhibitor of nitric oxide synthase (NOS) generated from the degradation of methylated proteins \((146)\). The plasma concentration of ADMA was found to be elevated in hypertension \((147)\), hypercholesterolemia \((148)\), and atherosclerosis \((149)\), suggesting that type I PRMTs may involve in cardiovascular diseases. The free plasma levels of MMA and ADMA can be controlled by dimethylarginine dimethylaminohydrolase (DDAH), which metabolizes free MMA and ADMA. The inhibition of DDAH or the overexpression of PRMTs can both lead to an increase in the MMA and ADMA pool \((150)\). Although SDMA was unable to block the enzymatic activity of NOS directly \((151)\), it did have an indirect effect competing for
arginine transport mediated by a cationic amino acid transporter (152).

C. Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is a recessive genetic disorder resulting from lesions in the survival motor neuron (SMN) gene (153). The SMN proteins containing Tudor domains which bind to both ADMA and SDMA motifs can interact with numerous PRMT4 and PRMT5 products (154, 155). A point mutation (E→K) was discovered in the Tudor domain in SMA patients, which influences binding the RG domains of Sm proteins (156). However, mechanisms that may link the spliceosome assembly to SMN function remain unclear.

D. Viral Pathogenesis and Host Interaction

Numerous viral proteins have been found heavily methylated including HIV-1 proteins, hepatitis proteins, and Epstein-Barr viral proteins (157). PRMT6 methylates HIV transactivator protein Tat in vivo, which inhibits the stimulation of viral transcription, indicating a negative role for PRMT6 in HIV replication (158). Also it has been shown that PRMT1 methylates the small hepatitis delta antigen in vitro, and appears to facilitate antigenomic replication (159). Epstein-Barr virus nuclear antigen 2 (EBNA2) has been found to be methylated by PRMT5, which appears to be required for EBNA2 binding to the host protein SMN (160). The EBNA2-SMN interaction plays a significant role in viral infection (160).

CONCLUSION

Protein arginine methylation is a pervasive posttranslational modification in all
eukaryotic cells. It is involved in various cellular pathways. The diverse roles of PRMTs come from the numerous protein substrates in vivo, ranging from histones, RNA binding proteins to signaling proteins and many other enzymes. The dysregulation of PRMT activity and specificity leads to a number of diseases including cancer, cardiovascular diseases, lupus, and viral pathogenesis. Although the biological functions of PRMTs are well-accepted, many biochemical bases for arginine methylation remain unclear. We focused on the predominant PRMT in vivo, PRMT1, aiming to understand how product specificity of PRMT1 is achieved. This dissertation provides increased understanding of the detailed mechanism and product specificity of PRMT1, which will contribute to the PRMT research field, as well as PRMT-targeted drug development.

References


CHAPTER 3

INVESTIGATION OF THE MOLECULAR ORIGINS OF PRMT1 PRODUCT SPECIFICITY REVEALS A ROLE FOR TWO CONSERVED METHIONINE RESIDUES

ABSTRACT

Protein arginine methyltransferases (PRMTs) aid in the regulation of many biological processes by methylating specific arginyl groups within targeted proteins. The varied nature of the response to methylation is, in part, due to the diverse product specificity displayed by the PRMTs. In addition to site location within a protein, biological response is also determined by the degree (mono-/dimethylation) and type of arginine dimethylation (asymmetric/symmetric). Here we have identified two strictly conserved methionine residues in the PRMT1 active site that are not only important for activity, but also control substrate specificity. Mutation of Met155 or Met48 results in a loss in activity and a change in distribution of mono- and dimethylated products. The altered substrate specificity of M155A and M48L mutants is also evidenced by automethylation. Investigation into the mechanistic basis of altered substrate recognition led us to consider each methyl transfer step separately. Single turnover experiments reveal that the rate of transfer of the second methyl group is arginine residues located in the center of the peptide much slower than transfer of the first methyl group in Met-48L, especially for substrate where turnover of the monomethylated species

is negligible. Thus, altered product specificity in M48L originates from the differential effect of the mutation on the two rates. Characterization of the two active-site methionines provides the first insight into how the PRMT1 active site is engineered to control product specificity.

INTRODUCTION

Protein methylation is a significant post-translational modification in eukaryotic organisms. Protein arginine residues can be methylated on the guanidino nitrogens by protein arginine methyltransferases (PRMTs), which use S-adenosyl-L-methionine (AdoMet, SAM) as a methyl group donor. This post-translational modification is important in a wide variety of fundamental biological processes including transcription, RNA splicing, signal transduction, DNA repair, viral replication (reviewed in (1)), and chromatin remodeling (2). In recent years, the significance of PRMTs in human diseases has been increasingly studied, especially in cardiovascular disease (3) and cancer (4). In all, PRMTs play a crucial role in many biological processes.

Although the biological importance of PRMTs has become well accepted, the current knowledge of the fundamental biochemistry of these enzymes is limited, due in part to the complexity of the system. So far, 11 PRMT isoforms have been identified. In mammalian cells, nine PRMTs catalyze monomethyl arginine (MMA) formation and they can be categorized into two major types; PRMT1, -2, -3, -4 -6, and -8 additionally catalyze asymmetric dimethyl arginine (ADMA) formation, demonstrating type I activity; whereas PRMT5, -7, and -9 catalyze symmetric dimethyl arginine (SDMA) formation, demonstrating type II activity (Figure 3-1A). PRMT10 and -11 were identified as
putative PRMT genes with no methylation activity shown as yet (5). As with other enzyme families that post-translationally modify protein substrates, accurate substrate recognition by the PRMTs is critical for the proper transmission of biochemical information. However, very little information is available to explain how or why any of the PRMT isoforms target their cognate protein substrates, and more so, what determines which arginine within a protein should be methylated; i.e., a consensus sequence has not been identified.

Adding to the complexity of the PRMT field, different methylation statuses (MMA, ADMA or SDMA state) of the same substrate can lead to distinct biological outputs. One of the most striking examples is Arg-3 of histone H4 (H4R3), which can be either asymmetrically dimethylated by PRMT1 or symmetrically dimethylated by PRMT5, resulting in antagonistic effects on gene regulation (reviewed in (6)). Moreover, the two methylation products of Type I PRMTs, MMA and ADMA, can also have distinct biological functions. In yeast, MMA present in histone H3 at Arg-2 correlates to active transcription, while the ADMA state contributes to transcriptional repression (7). In contrast to the idea that MMA is simply an intermediate for ADMA generation, the current data point to a bona fide signaling role for MMA residues in much the same way that mono-, di-, and trimethylation of lysine residues differentially affect signaling (8,9). A key to understanding the biological function of the PRMTs is to understand how product specificity may be regulated, in terms of governing which arginyl residues are modified and which state of methylation is achieved.

In order to gain mechanistic insight regarding the product specificity of the PRMTs, we have initiated mutational studies of rat PRMT1, a ubiquitously expressed member of
Figure 3-1. (A) 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 clarity. (B) Sequence alignment for rat PRMT1, rat PRMT3, rat PRMT4, yeast RMT1/Hmt1 and rat PRMT6. The conserved methionine pair is highlighted in dark red in light grey boxes. (C) The rat PRMT1:AdoHcy:peptide substrate complex shows the positioning of two strictly conserved methionine residues. The active site of rat PRMT1 (PDB code 1OR8) is shown with AdoHcy in light orange (sulfur in dark orange), methionines 155 (left) and 48 (right) in dark red, and the substrate arginine group of the R3 peptide (ac-GGRGGFGGGGFGGRGGGFGG) in blue (terminal guanidino nitrogens are shown in dark blue).

the PRMT family that has been estimated to perform ~85% of arginine methylations in mammals (10). Structural studies of PRMT1 (11) and PRMT3 (12), both Type I methyltransferases, have identified the component residues of the Type I active site and have provided a basis from which to probe product specificity. In particular, it has been noted that all the type I PRMTs contain an active site methionine (position 155 in rat PRMT1), which has been hypothesized to dictate the synthesis of ADMA over SDMA by
preventing the binding of MMA in a configuration conducive for SDMA formation (12,13). The idea that a single residue can alter product specificity is reminiscent of the Phe/Tyr “switch” model that is used to explain product specificity in lysine methyltransferases (13-15). Interestingly, we noted a second active site methionine (position 48 in rat) that is also strictly conserved in Type I methyltransferases (Figure 3-1B). Together these two methionines are observed to sandwich the incoming substrate arginy1 group in the Type I methyltransferase active site (Figure 3-1C), and thus, be positioned to affect the type and degree of methylation. Here we report that these two active site methionine residues are essential for catalytic activity and product specificity of PRMT1. Our studies show that substitution of Met-48 does not promote the formation of SDMA, ruling out a methionine switch model. Our data are consistent with Met-48 functioning differentially in the methylation of arginine to MMA versus the methylation of MMA to ADMA. Surprisingly, our data also show that Met-48 plays a crucial role in specifying which peptidyl arginine is targeted by PRMT1.

EXPERIMENTAL PROCEDURES

Materials

AdoMet was purchased from Sigma as a chloride salt (≥80%, from yeast). [methyl-³H]AdoMet was purchased from Perkin Elmer. All the peptides were synthesized by the Keck Institute and purified to ≥95%. ZipTip® C4/C18 pipette tips were purchased from Millipore. His-hnRNP K was expressed and purified according to (16).

Expression and purification of mutant PRMT1 proteins
PRMT1 mutant proteins were generated using the QuikChange® Site-Directed Mutagenesis kit (Stratagene) with sets of complementary oligonucleotide primers spanning the desired site of mutation. For each PCR reaction, the pET28b vector (Novagen) containing the gene that codes for N-terminal histidine tagged rat WT-PRMT1 plasmid (pET28b-PRMT1) (17) was used as a template. Desired mutations (M155A, M48A, M48L, M48F, M48Y, and M48W) were confirmed through DNA sequencing. Mutant proteins were purified using the same methods used to express and purify wild type His-PRMT1 (described in (17)). Purified proteins were ≥95% pure by SDS-PAGE. Mutant protein sequences were verified using mass spectrometry.

**Reverse phase (RP)-HPLC analysis of methylated amino acids**

Assays containing 4 μM WT- or mutant-PRMT1 proteins, 800 μM AdoMet, 10 nM AdoHcy nucleosidase (MTAN, purified as in (18)), and 50 mM sodium phosphate buffer (pH 7.5) were equilibrated at 37 ºC for 3 minutes. Reactions were initiated with 200 μM R3 peptide and were terminated after 3 hours with 10% (v/v, final concentration) trichloroacetic acid (TCA). TCA-precipitated protein was removed through centrifugation, and the supernatant (containing the peptide) was added to a glass vial. An equivalent volume of 12 M HCl was added to each vial. Vials were sealed and heated to 110 ºC for approximately 24 hours for a complete acid hydrolysis. Hydrolyzed amino acids from WT-PRMT1 and the mutant-catalyzed reactions were separated using o-phthalaldehyde (OPA) derivatization (19) with a Gemini® 3 μm C18 110 Å LC Column 75 x 4.6 mm (Phenomenex). Mobile phase A consisted of 40 mM sodium phosphate buffer (pH 7.8), and mobile phase B was acetonitrile/methanol/H₂O
(45/45/10, v/v/v). The HPLC gradient conditions are not shown. To verify the presence and peak times of the methylated arginine products, sample reactions were spiked with 2.6 μM \[^{3}H\]AdoMet (specific activity of 2.02 mCi/μmol). Fractions (83.3 μl) were collected and radioactivity was counted in 5 ml scintillation cocktail (Fisher Scientific). MMA, ADMA, or SDMA standard amino acids were used to verify the identity of the methylated products generated. The detection limit for this method is ~10 pmole of methylated arginine in a 20 μl sample.

**Continuous spectrophotometric kinetic assays of PRMT1 mutants**

A continuous spectrophotometric assay for AdoMet-dependent methyltransferases (17) was used to assay PRMT1 mutants 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 avoids any 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 (20) to obtain \(K_{m,\text{app}}\) and \(k_{\text{cat, app}}\) values. Each reaction was performed at least in duplicate. The limit of detection for this assay was 0.1 μM CH₃/min (which corresponds to a \(k_{\text{cat}}\) of 0.025 min⁻¹).

**Discontinuous kinetic assays of PRMT1 mutants**

Another discontinuous but more sensitive assay with ZipTipC₄/C₁₈ was used in testing the enzymatic activity under steady-state conditions (21). Unless noted otherwise in the text, enzyme catalytic activity were tested with 100 nM wt-PRMT1 or mutants, 1 μM AdoMet and 1 μM \[^{3}H\]AdoMet, initiated by 200 μM peptide substrates or 1.7 μM
hnRNP K protein substrate at 37 °C. At different time points, samples were removed from reactions and processed with ZipTip_{C4/C18} pipette tips (for protein or peptide substrates, respectively) to separate the unreacted $[^{3}$H]AdoMet and the radiolabelled product.

**Dissociation constant measurement by intrinsic fluorescence quenching**

An RF-5301PC spectrofluorophotometer (Shimadzu) was used for fluorescence measurements. For R3 peptide and AdoMet affinity determinations, an excitation wavelength of 290 nm was used and emission spectra from 300-420 nm were collected. The change in fluorescence intensity at the maximum emission (333 nm) was monitored. The excitation and emission slit was 5 nm and the scan speed was 100 nm/min using 1325 µL containing 1.4 µM PRMT1 in 150 mM sodium phosphate buffer pH 7.1. Increasing concentrations from 1 to 50 µM peptide ligand (or AdoMet) were added at 2-3 min intervals. Data from at least two titrations were averaged and analyzed using the modified Stern Volmer (22) plots. Data were evaluated by nonlinear regression analysis using SigmaPlot in order to obtain the dissociation constant ($K_D$) using the following equation: $F_c = F(10^\frac{\varepsilon cd}{2})$ where $F_c$ is the corrected fluorescence, $\varepsilon$ is the extinction coefficient of AdoMet, $c$ is the concentration of AdoMet, and $d$ is the pathlength. $F_{\text{initial}}/(F_{\text{initial}}-F_c)$ was then plotted against $1/[\text{AdoMet}]$ and the data was fit to a line where the $y_{\text{intercept}}=1/\varepsilon$ $a$, the slope=$1/\varepsilon*K_Q$, and the $K_Q=1/K_D$.

**Protein crystallization**

A truncated construct of M48L-PRMT1 was made which lacked the first thirteen amino acids of the native sequence. Previous crystallographic studies reported better
diffracting crystals using the truncated proteins (11). Histidine-tagged, truncated M48L PRMT1 was expressed and initially purified as described previously (17). In addition to immobilized metal chromatography, M48L was further purified by anion exchange (MonoQ) and gel filtration chromatography as described previously (11). Purified fractions were concentrated to 10-20 mg/mL and incubated with 600 µM AdoHcy and 1mM peptide (acGGRmeGGFGGGKGGFGGKW) for 10-30 minutes before placing in crystallization trays. Crystallization was performed using sitting drop vapor diffusion. Crystals were grown at room temperature (~22 °C) in 0.1 M Tris pH 9.0 and 1.62 M ammonium phosphate monobasic at a 1:1 protein:well drop ratio. Crystals were flash frozen in a cryo solution containing 0.1 M Tris pH 9.0, 1.62 M ammonium phosphate monobasic, 0.5mM peptide, 300 µM AdoHcy, and 20% glycerol.

Data collection, structure determination and refinement

Diffraction data were collected using a home source generator and detector (Rigaku RU-200/Raxis IV++). Data were indexed and processed using d*TREK in the program Crystal Clear (23). Molecular replacement was performed using Phaser (24) from the CCP4 suite (25,26). The search model was wild-type rat PRMT1:AdoHcy:peptide ternary complex (Protein Data Bank entry 1OR8). PHENIX (27) was used to perform positional, b-factor, and TLS refinement. The TLS groups were generated by the TLS Motion Determination server (28). Coot (29) and MolProbity (30) were used for model building and structure validation. The model was refined to an R/Rfree of 20.1%/24.5% with no Ramachandran outliers (data not shown). AdoHcy was observed bound to the active site (data not shown). No compelling electron density was observed for the peptide substrate.
Density that could accommodate ~3 covalently bonded atoms was observed in the active site near Glu-144. The density is inconsistent with solvent molecules included in the crystallization buffer and may correspond to a portion of the peptide substrate. However, since the density could not be confidently assigned, it was not included in the final model. Structure factors and coordinates have been deposited in the Protein Data Bank (PDB ID 3Q7E). Figures were generated with PyMOL (31).

Mass spectrometry peptide methylation analysis and De Novo sequencing study

A reaction with 4 µM PRMT1, 800 µM AdoMet, and 100 nM MTAN in 50 mM sodium phosphate buffer (pH 7.5) was initiated with 200 µM R3 peptide. At various time points, 10 µL aliquots were quenched with TFA (10% v/v final) and analyzed by LC/MS followed by MS/MS of the desired peaks. 4uL of reaction solution was injected onto a reverse phase column (75 µm × 150 mm, BEH C18-nanoACQUITY column, Waters, Manchester, UK). Peptides with and without methylation were separated at 40 °C with a nonlinear gradient (data not shown). Solvent A was 0.1% formic acid in water and Solvent Bs was 0.1% formic acid in acetonitrile. Monomethylated peptides ([M+H]\(^{3+}\)=575.9) were then analyzed using a tandem mass spectrometer (Synapt Q-Tof, Waters, Manchester, UK) to determine the position of monomethylation.

Single turnover experiments

Single turnover experiments were carried out by mixing a solution containing the pre-incubated complex of 20 µM wild type or M48L-PRMT1, 20 µM AdoMet (along with 1 µM of \(^{3}\)H]-AdoMet), and 10 nM MTAN in 50 mM sodium phosphate buffer (pH 7.5) at 23 °C for 3 minutes. Commercially available AdoMet was purified as described
(32,33) before use. Reactions were initiated with 200 μM peptide substrates. Radiolabel incorporation over time was measured using the discontinuous ZipTip® assay described previously. Briefly, 10 μL reaction samples of different time points were taken out and quenched by 6 M guanidine HCl solution and processed with ZipTip®C18 assay to quantitate the amount of methylated product (21). The resulting time course of [3H]AdoMet incorporation was fit into single exponential curve $y=a(1-e^{-bx})$ to determine the parameters, $a$ (maximum product concentration) and $b$ (kchem).

RESULTS

Analysis of the products formed by M48A- and M48L-PRMT1

Previous analysis of the PRMT1 crystal structure suggested that the conserved Met at position 155 may be important for governing ADMA formation over SDMA formation by providing steric bulk in the active site and preventing free rotation of the MMA intermediate (12,13). A closer examination of the PRMT1 structure identified Met-48, a similarly conserved active site residue located opposite Met-155. Given the potential for either methionine to influence substrate geometry in the active site, we investigated whether these residues are involved in specifying ADMA formation versus SDMA.

Met-48 and Met-155 were individually mutated to alanines. We analyzed whether ADMA or SDMA was formed in the mutant-catalyzed reaction by amino acid analysis of the acid-hydrolyzed peptide products, using [3H]AdoMet as a tracer. M48A produced only MMA and ADMA, albeit with a higher MMA:ADMA ratio compared to wt-PRMT1 (Figure 3-2A,B). Because of the low activity of M48A observed in the 3-hour reaction, Met-48 was further mutated to leucine in order to determine if activity could be rescued
by reintroducing more steric bulk. Like M48A, M48L also generated only MMA and ADMA, but in a much larger amount, with an even higher ratio of MMA:ADMA than that of M48A (Figure 3-2C). No SDMA was detected. Similar results were observed with the M155A mutant, consistent with the recent report that Met-155 is not involved in SDMA formation (34). Standards of MMA, ADMA, SDMA, as well as other amino acids in the R3 peptide (Gly, Arg and Phe) were analyzed by the same method to verify the methylated product (data not shown). Our results show that removing the steric bulk afforded by Met-155 or Met-48 in the PRMT1 active site was not enough to transform the Type I PRMT into a Type II PRMT. Thus, Met-48 or Met-155 do not, by themselves, dictate ADMA formation over SDMA formation.

**Steady-state methylation activities of PRMT1 mutants**

The altered product ratios obtained for M48L and M48A in the previous experiments suggested that the activity of the mutants may be impaired. In order to determine if Met-48 has an effect on the ability of PRMT1 to catalyze peptide methylation, the rate of methylation was measured with wt-PRMT1, M155A and M48A. The activity was followed using an enzyme-coupled continuous spectrophotometric assay and the R3 peptide, a common peptide substrate used in arginine methylation (ac-GGRGGFGGRGGFGGRGGFGG) (Table 3-1) (17). The R3 peptide is a good substrate for wt-PRMT1 with a catalytic efficiency \( \left( \frac{k_{\text{cat}}}{K_m} \right) = 4460 \text{ M}^{-1}\text{s}^{-1} \). When the two methionines in question were mutated to small amino acid residues, i.e. alanine, mutants still utilized R3 as a peptide substrate but at varying efficiencies (Table 3-1 and data not shown). Substitution of Ala for Met at position 155 resulted in a 90% decrease in \( \frac{k_{\text{cat}}}{K_m} \).
The M155A mutation did not affect turnover velocity, but $K_m$ increased by a factor of ten compared to wt-PRMT1. Substitution of Ala for Met-48 also decreased catalytic efficiency. However an increase in $K_m$ was coupled with a 10-fold drop in $k_{cat}$, leaving this mutant with just under 2% of WT activity. These results suggest that Met-48 might be more important for activity than Met-155. Increasing the size of the residue at position 48 from Ala to Leu restored 40% of wild type activity with the R3 peptide. The activity results indicate that the Type I conserved Met residues at positions 48 and 155 play important roles in enzymatic activity. Furthermore, the size of Met 48 is critical for maintaining the catalytic activity of PRMT1.

To further explore the effect of the residue size at position 48 on the catalytic activity, we further mutated the methionine to amino acids with larger-size side chains, such as phenylalanine, tyrosine, and tryptophan. The same kinetic assay was employed to measure the activity of these three mutants with R3 peptide; however the turnover rate was very slow and beyond the limit of detection for this method (data not shown). We turned to a more sensitive method that we recently reported on (21) and examined mutant activity on a protein substrate, heterogeneous nuclear ribonucleoprotein K (hnRNP K) (data not shown). With all larger-sized mutations, the activity was severely impaired. M48F had the highest activity among these three mutants, displaying no more than 3% of WT activity. Mutants with the even bigger amino acid tryptophan lost >95% activity. Overall the kinetic analyses of the mutants show that residues smaller or larger than methionine at position 48 result in less efficient methyl group transfer.
Figure 3-2. Analysis of M48 mutants-catalyzed methylation products by amino acid analysis. Products from reactions catalyzed by (A) wild type, (B) M48A, or (C) M48L-PRMT1, were analyzed using reverse phase HPLC. [3H]AdoMet was used as a tracer to verify the presence of the methylated species in each reaction (open circles). MMA, ADMA, and SDMA standard amino acids were used to identify the methylated species in each sample (solid line).
Table 3-1. Steady-state kinetic activity of PRMT1 mutants with small-sized side chains with R3 peptide via an enzyme-coupled continuous spectrophotometric assay

<table>
<thead>
<tr>
<th>PRMT1</th>
<th>( K_m (\mu M) )</th>
<th>( k_{cat} (s^{-1}) )</th>
<th>( k_{cat}/K_m (\text{M}^{-1}\text{s}^{-1}) )</th>
<th>% activity(^a) of wt-PRMT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>&lt;10</td>
<td>0.045 ± 0.003</td>
<td>4500 ± 330</td>
<td>100</td>
</tr>
<tr>
<td>M155A</td>
<td>120 ± 30</td>
<td>0.054 ± 0.005</td>
<td>460 ± 120</td>
<td>10</td>
</tr>
<tr>
<td>M48A</td>
<td>300 ± 210</td>
<td>0.0040 ± 0.0020</td>
<td>13 ± 13</td>
<td>1.4</td>
</tr>
<tr>
<td>M48L</td>
<td>18 ± 2.7</td>
<td>0.030 ± 0.0009</td>
<td>1700 ± 260</td>
<td>37</td>
</tr>
</tbody>
</table>

\(^a\) The percentage activity is based on the catalytic efficiency \( k_{cat}/K_m \) of mutants compared to wild type.

**Substrate binding affinity of PRMT1 mutants**

The decrease in activity observed with mutations of either Met-155 or Met-48 could be a result of impaired substrate binding or an inability to catalyze methyl transfer. In order to discern if Met-155 or Met-48 mutations affect the binding of either AdoMet or peptide substrates, we measured the dissociation constants of PRMT1, both wild type and mutants, with AdoMet and the R3 peptide substrate. The crystal structure of PRMT1 indicates that there are two tryptophans at positions 145 and 294 which lie in the catalytic region near the substrate arginine residue and opposite the AdoMet binding region (11). The intrinsic fluorescence from these residues was exploited in a fluorescence-quenching assay to determine the dissociation constants for the R3 peptide and AdoMet with PRMT1. Lehrer’s modified Stern-Volmer plot (22) was used to analyze the fluorescence-quenching data for wt-PRMT1 (data not shown) and the mutants. Dissociation constants of either AdoMet or R3 peptide with wt-PRMT1 and all the mutants were similar to each other in the low micromolar range (Table 3-2). The observed fluorescent quenching was consistent with the number of active site tryptophans relative to the total number of tryptophans. These results indicate that the decrease in
Table 3-2. Binding affinity of R3 peptide substrate and AdoMet with intrinsic fluorescence quenching.

<table>
<thead>
<tr>
<th>PRMT1</th>
<th>$K_D$, R3 (µM)</th>
<th>Fluorescent quenching (%)</th>
<th>$K_D$, AdoMet (µM)</th>
<th>Fluorescent quenching (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.7 ± 0.52</td>
<td>28 ± 1.9</td>
<td>4.2 ± 1.5</td>
<td>17 ± 0.13</td>
</tr>
<tr>
<td>M48A</td>
<td>3.4 ± 0.32</td>
<td>37 ± 1.3</td>
<td>7.1 ± 1.8</td>
<td>15 ± 0.17</td>
</tr>
<tr>
<td>M48L</td>
<td>2.4 ± 0.29</td>
<td>28 ± 1.5</td>
<td>7.1 ± 0.94</td>
<td>29 ± 4.2</td>
</tr>
<tr>
<td>M48F</td>
<td>4.9 ± 0.38</td>
<td>30 ± 1.4</td>
<td>4.4 ± 0.93</td>
<td>23 ± 3.1</td>
</tr>
<tr>
<td>M155A</td>
<td>3.9 ± 0.53</td>
<td>34 ± 1.3</td>
<td>3.8 ± 0.87</td>
<td>19 ± 2.6</td>
</tr>
</tbody>
</table>

activity observed with the methionine PRMT1 mutants is not due to impaired substrate binding in the enzyme active site.

**Automethylation activity of PRMT1 mutants**

In addition to the methylation of peptide and protein substrates, automethylation of some PRMTs has been documented. For example, automethylation has been observed with PRMT6 and PRMT8 (35,36). Additionally Frankel and coworkers recently reported that histidine-tagged human PRMT1 also catalyzes automethylation (37). Although we have never observed automethylation with his-tagged rat PRMT1, we hypothesized that the methionine mutants of rat PRMT1 may show altered substrate recognition, and hence, altered product formation. Therefore, the ability of each of the PRMT1 mutants to catalyze automethylation was tested using $[^3]$H]AdoMet. Under our reaction conditions, wt-PRMT1 showed no tritium incorporation after 4 hours incubation. Unlike wt-PRMT1, the M48L and M155A mutants clearly showed a strong capacity for automethylation (Figure 3-3). We quantified the amount of automethylation using our ZipTip® C4 assay (21). A sample of 15 µM M48L and M155A incorporated 2.14 ± 0.59 µM (14.3%) and
0.68 ± 0.24 μM (4.5%) methyl groups in a 4-hour period, respectively. We also confirmed the product of the automethylation by digesting the methylated M48L and resolving the derivatized amino acids by HPLC. The presence of radiolabel was associated with ADMA (data not shown). These results show that mutation of either Met-155 or Met-48 gives rise to a PRMT1 enzyme capable of automethylation. We conclude that both Met-155 and Met-48 are important for substrate recognition.

![SDS-PAGE and Autoradiograph](image)

**Figure 3-3.** Automethylation of PRMT1 mutants. Reactions were carried out at 37 °C for 4 hrs with 15 μM PRMT1 (wt- or mutants), 8.57 μM AdoMet (0.9 μM of [3H]-AdoMet added) in 50 mM sodium phosphate buffer, pH 7.5. The top panel is an SDS polyacrylamide gel stained with coomassie blue to detect total protein, and the bottom panel is an autoradiograph to detect radiolabel incorporation. The gel was exposed to film for 5 days.

**Analyzing for changes in protein structure**

In order to confirm that mutations of the conserved methionine had no deleterious effect on the PRMT1 structure, we determined the crystal structure of the M48L mutant. Using conditions described for the crystallization of wt-PRMT1, we obtained a 2.2 Å structure of M48L bound to AdoHcy (Figure 3-4). No significant differences were observed between the wild-type and mutant structures (RMS deviation of 0.23 Å over
299 residues), consistent with the kinetic data showing similar binding of AdoMet to WT and M48L.

![Image of superposition of overall structure and active site of M48L PRMT1 and wild type PRMT1](image)

Figure 3-4. Superposition of the overall structure (A) and the active site (B) of M48L (blue) and wild type PRMT1 (grey). Met-48/Leu-48, Met-155 and AdoHcy are shown in sticks with wt-PRMT1 residues colored tan and M48L residues colored orange.

**The positional preference of M48L-PRMT1**

Although the crystal structure of M48L indicates that there are no significant changes in the structure of the M48L mutant, the automethylation experiments suggested that substrate recognition is altered in M48L. To further explore the effect that the M48L mutation has on substrate specificity, we asked whether M48L strongly prefers substrate arginines at certain positions and compared the results to wt-PRMT1. We employed the R3 peptide in which all three arginines can be methylated, with one arginine at the N-terminus, one in the middle, and one at the C-terminus. The short-time methylation products of R3 peptide were analyzed by tandem mass spectrometry (MS/MS) to find out which arginine was first methylated by wt-PRMT1 and M48L (data not shown). MS/MS
showed that the N-terminal arginine is strongly preferred by both wt-PRMT1 and M48L, with 55% and 65% of the first methylation located on the N-terminal arginine, respectively (Table 3-3). The middle arginine is the second most preferred, however the percentage for M48L was reduced by almost 50% compared to the wt-PRMT1. These results suggest that although recognition of the N-terminal arginine of the R3 peptide appears unaltered in M48L, recognition and/or catalysis at the central Arg of R3 is impaired by M48L.

Table 3-3. The first methylation position in the R3 peptide catalyzed by wt-PRMT1 and M48L from MS/MSMS analysis

<table>
<thead>
<tr>
<th>PRMT1</th>
<th>1st R</th>
<th>2nd R</th>
<th>3rd R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt-PRMT1</td>
<td>55</td>
<td>39</td>
<td>6</td>
</tr>
<tr>
<td>M48L</td>
<td>65</td>
<td>21</td>
<td>14</td>
</tr>
</tbody>
</table>

Peptide substrate specificity change for M48L-PRMT1

As a way of complementing the above studies, we also tested the activity of M48L with a series of peptides that differ in the location of the targeted arginine within the peptide and methylation status (listed in Table 3-4) with the M48L-PRMT1 mutant. All the peptides listed could be easily methylated by wild type PRMT1 (Table 3-4). M48L was able to methylate R3 at a relatively lower rate compared to wild type enzyme. We tested peptides containing a single arginine along with its monomethylated counterpart. M48L could methylate the RKK peptide pair (single arginine at N-terminus) at a rate close to the R3 peptide. Surprisingly, even though the sequence of RKK and KRK peptides is the same around the targeted arginylic group (GGRGG), the ability of M48L to
methylate the centrally-located Arg in KRK was severely hampered. We conclude that the inactivity observed with M48L and KRK peptide is most likely due to the central position of the arginine in KRK peptide pair.

The previous experiments predict that the M48L mutant would be unable to stoichiometrically methylate all arginine residues in the R3 peptide. In other words, the amount of methyl groups transferred by M48L is expected to be lower, while wt-PRMT1 can fully methylate the R3 peptide, transferring six methyl groups to one peptide. When allowed to react with 10 µM, 20 µM, and 30 µM R3 and excess AdoMet (60 µM, 120 µM, and 180 µM total methyl group transfer expected, respectively), M48L catalyzed the transfer of 35 µM, 48 µM, and 83 µM methyl groups, respectively (data not shown). This indicated that each peptidyl arginine was not being fully dimethylated by M48L. Given our current data, it is likely that M48L-PRMT1 methylated the R3 peptide on the first terminal arginine but was unable to fully access, or got aborted at the central arginine residue. Overall wt-PRMT1 and M48L mutant strongly prefer the N-terminal arginine and the methylation activity of M48L decreased dramatically with a single arginine in the middle position.

**Single turnover experiments with M48L-PRMT1**

In order to investigate why the KRK pair cannot be methylated well by M48L-PRMT1, we set up single turnover experiments that would allow us to evaluate the effect of the M48 mutation on the conversion of arginine to MMA, and the conversion of MMA to ADMA in separate experiments. Reactions were conducted under conditions where the AdoMet concentration was the same as or slightly higher than the enzyme
Table 3-4. Peptide substrate specificity of wt-PRMT1 and M48L under steady-state conditions

<table>
<thead>
<tr>
<th>Peptide substrate</th>
<th>Sequence</th>
<th>Activity of wt-PRMT1 (s⁻¹)</th>
<th>Activity of M48L (s⁻¹)</th>
<th>% activity of wt-PRMT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3</td>
<td>acGGRGGFGGRGGGFGGRGG</td>
<td>0.045</td>
<td>0.029</td>
<td>65.9</td>
</tr>
<tr>
<td>RKK</td>
<td>acGGRGGFGKKGGFGGGKW</td>
<td>0.0051</td>
<td>0.0026</td>
<td>50.5</td>
</tr>
<tr>
<td>RKK-CH₃</td>
<td>acGGRₘₘGGFGGKGFFGGKW</td>
<td>0.011</td>
<td>0.0053</td>
<td>47.9</td>
</tr>
<tr>
<td>KRK</td>
<td>acKGGFGGRRGGFGGGKW</td>
<td>0.0071</td>
<td>0.000075</td>
<td>1.06</td>
</tr>
<tr>
<td>KRK-CH₃</td>
<td>acKGGFGGRRₘₘGGFGGGKW</td>
<td>0.0039</td>
<td>0.000023</td>
<td>0.577</td>
</tr>
</tbody>
</table>

*The methylation velocity is tested under one saturating condition for both AdoMet and peptide substrates, representing the activity of enzymes. The percentage activity is calculated based on the methylation velocity.

...concentration, so as to observe only one turnover of the methylation reaction (Figure 3-5).

Maximal product concentration and $k_{chem}$ for each reaction were obtained (Table 3-5).

Wt-PRMT1 fully methylated the KRK peptide with the maximum product concentration equal to 20 μM (Table 3-5). With the KRK-CH₃ peptide, the total methyl group transfer decreased slightly, but the turnover rate (0.029 s⁻¹) was very close to the naked peptide (0.020 s⁻¹). With M48L, the reaction only went to 20% completion with the KRK peptide, with the turnover rate reduced by 50%. However, when the M48L reaction was initiated with the monomethylated peptide, hardly any methylation occurred, with the maximum product concentration of around 0.16 μM, and the turnover rate $k_{chem}$ close to 0 s⁻¹. Control experiments were set up under the same conditions without the peptide substrates, to rule out radiolabel incorporation as a result of M48L automethylation. Our results show impaired monomethylation of the internal arginine by M48L PRMT1, and nearly abolished dimethylation.
The lack of activity that was observed with M48L and KRK-CH$_3$ could be due to the inability of the peptide to bind the mutated active site. To determine whether the KRK monomethylated peptide binds to M48L, we set up a competitive reaction using the RKK-CH$_3$ peptide as a substrate with or without the KRK peptide pair. The concentration of RKK-CH$_3$ peptide was close to its $K_m$ and KRK-CH$_3$ peptide was one third the RKK-CH$_3$ concentration. With KRK added into the reaction, the methyl group transfer rate was close to the one without KRK (0.0012 and 0.0011 $\mu$M/s, respectively) (Figure 3-6). However with the monomethylated peptide KRK-CH$_3$ added into the pre-incubation solution, the reaction rate was decreased to half of the original rate (0.0012 and 0.0006 $\mu$M/s, respectively). These data suggest that the KRK-CH$_3$ peptide was actually a PRMT1 inhibitor in the methylation reaction. With the KRK peptide in the pre-incubation mixture, the rate was slightly decreased, possibly because the KRK peptide would bind to M48L but could not be methylated as fast as the RKK-CH$_3$ peptide, as shown in Table 3-5. The fact that KRK-CH$_3$ could inhibit the methylation of RKK-CH$_3$ suggests that the KRK-CH$_3$ peptide can bind M48L-PRMT1, but is not turned over by the enzyme in appreciable amounts.

We also observed impairment in the rate at which M48L dimethylated argininyl groups located at the Nt of the peptide, albeit to a lesser extent. Although dimethylation by wild type was 26% slower than monomethylation, dimethylation by M48L was 60% slower than monomethylation of the RKK peptide (Table 3-5). Clearly, substitution of methionine 48 with leucine in PRMT1 results in differential effects on the conversion of arginine to MMA versus the conversion of MMA to ADMA. Furthermore, the ability of M48L mutants to covalently transfer methyl groups to all targeted arginines in a substrate
Figure 3-5. Single turnover kinetics of wt-PRMT1 (A) and M48L (B) with KRK and KRK-CH$_3$ peptide pair. Filled circles and open circles indicate the KRK peptide and the monomethylated counterpart KRK-CH$_3$ peptide, respectively.

Table 3-5. Pre-steady state kinetic parameters of M48L with wt-PRMT1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>peptide</th>
<th>Maximal product concentration (μM)</th>
<th>$k_{chem}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 μM wt-PRMT1</td>
<td>KRK</td>
<td>20.31 ± 0.25</td>
<td>0.020 ± 0.0008</td>
</tr>
<tr>
<td></td>
<td>KRK-CH$_3$</td>
<td>14.96 ± 1.15</td>
<td>0.029 ± 0.0082</td>
</tr>
<tr>
<td></td>
<td>RKK</td>
<td>14.53 ± 1.82</td>
<td>0.073 ± 0.037</td>
</tr>
<tr>
<td></td>
<td>RKK-CH$_3$</td>
<td>13.08 ± 0.85</td>
<td>0.054 ± 0.016</td>
</tr>
<tr>
<td>20 μM M48L</td>
<td>KRK</td>
<td>3.85 ± 0.11</td>
<td>0.0087 ± 0.0006</td>
</tr>
<tr>
<td></td>
<td>KRK-CH$_3$</td>
<td>0.16 ± 0.084</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>RKK</td>
<td>4.71 ± 0.20</td>
<td>0.044 ± 0.008</td>
</tr>
<tr>
<td></td>
<td>RKK-CH$_3$</td>
<td>9.37 ± 1.43</td>
<td>0.018 ± 0.008</td>
</tr>
</tbody>
</table>

ND stands for “not detectable”.
is impaired. We conclude that the Type I-conserved methionine 48 in rat PRMT1 plays a significant role in determining product specificity.

Figure 3-6. Competitive methylation experiments of the KRK-CH₃ peptide. The reactions were carried out with 100 nM M48L, 60 μM RKK-CH₃ peptide as a substrate, 1 μM AdoMet (1 μM of [³H]AdoMet added) in 100 mM HEPES buffer at 37 °C, pH 8.0. 20 μM KRK-CH₃ peptide were tested as an inhibitor. The filled circles indicate the control group without the KRK-CH₃ peptide, and the open circles for the test group with KRK-CH₃ added.

**DISCUSSION**

In mammals, the PRMT family of enzymes is capable of generating three different methylated arginyl species within protein targets, MMA, ADMA, and SDMA. Coupled with the observation that many PRMT targets have multiple arginines that are methylated (38,39), the diversity of potential protein products is large, and suggests the possibility for a methylarginine code. The biological relevance of such a code has most recently been validated as part of the complex set of posttranslational histone modifications
affecting gene expression (2,6,7). Yet, the molecular details of how each of the PRMTs selectively controls the position and degree of methylation have remained elusive. In this work we provide the first insight into how the Type I arginine methyltransferase active site is engineered for product specificity.

The absence of a methionine switch to specify ADMA and SDMA formation

When the first structures (11,12) of Type I arginine methyltransferases were solved, a strictly conserved methionine (Met-155 in the rat PRMT1 sequence) was located very close to the substrate arginine. Given the fact that this residue is strictly conserved in all Type I methyltransferases, but much smaller (e.g., alanine or serine) in Type II methyltransferases, a hypothesis was put forth that the methionine provided enough steric bulk to block the binding of monomethylated arginine in a conformation that would allow symmetric dimethylation (12,13). Likewise, we questioned whether Met-48, which resides on the opposite side of the active site (Figure 3-1C) could function in the same manner. The idea of a single residue switch to control product specificity was supported by the elegant studies in the lysine methyltransferase field, which showed that a Phe/Tyr switch in the active site regulates the ability of the SET domain lysine methyltransferases to perform successive methylations (13-15). Surprisingly, M155A and M48A mutants generated only MMA and ADMA after three-hour reactions. Even at the picomole detection limit of our monitoring system, no SDMA was detected (Figure 3-2). Therefore, removing the steric bulk afforded by either Met-155 or Met-48 in the active site of PRMT1 is not by itself sufficient to transform PRMT1 into a Type II PRMT, and rules out the idea of a simple methionine switch. How the Type I arginine
methyltransferases discriminate between ADMA and SDMA is more complex than previously thought, and will likely not be answered until a Type II PRMT structure becomes available.

**Met-155 and Met-48 are important for enzyme activity**

The kinetic results presented here show that both conserved methionines are important for catalytic activity of PRMT1 but in different ways (Table 3-1). The similarity between the WT and M48L crystal structures (Figure 3-4) indicates that the impaired activity of the mutants is not due to global changes in protein structure. Similar binding affinity of both AdoMet and R3 peptides for wt-PRMT1 and all the mutants (Table 3-2) illustrates that decreased activity in the mutants is also not due to changes in binding affinity.

As a third possibility to explain the altered activity of the methionine mutants, we considered the mechanism of methyl transfer. Given that methyl transfer occurs by way of an $S_N2$ reaction (40), the orientation of the arginine substrate and the thioether moiety of AdoMet in the active site becomes critical. Mutation of the methionines to smaller residues increases the space in the active site, and potentially allows more orientations to be sampled by the incoming arginyl substrate, not all of which may be productive or as productive as in wild type. Mutation of the methionines may change the ability of the mutant enzyme to undergo compression during catalysis (41) or affect the pKa of the guanidino nitrogen of arginine as it approaches the positively charged sulfonium of AdoMet (42). Consistent with the idea that Met-48 is important for catalysis, our single turnover experiments (Table 3-5) showed that $k_{chem}$ is decreased in the M48L mutant.
With the larger aromatic amino acid mutants, the positioning of the substrate arginine is likely fixed in an orientation which results in slow methyl transfer. We propose that the two type I methionines may work as molecular tweezers to align arginyl substrates at proper orientations. Interestingly, this methionine clamp is not only conserved in Type I PRMTs, but also exists in some other methyltransferases, such as salicylic acid carboxyl methyltransferase (SAMT), isoflavone O-methyltransferase (IOMT) and caffeic acid O-methyltransferase (COMT) (43-45), suggesting a common mechanism for optimizing methyl transfer among certain AdoMet-dependent methyltransferases.

**Met-48 influences product specificity**

Accurate product specificity by PRMT1 is a combination of targeting the correct arginine within a protein, as well as catalyzing the correct number of methylations on a particular residue. The work presented here shows that Met-48 plays a role at both levels. Curiously, substitution of Met-48, as well as Met-155, caused the enzyme to recognize a new substrate, observed as automethylation (Figure 3-3). On the other hand, several experiments (Table 3-4, Table 3-5, Figure 3-5) provided results which indicate that the Met-48 mutation differentially impaired the ability of the mutant to dimethylate arginine residues, especially those located near the center of the peptide substrate. Although it is difficult to tease out which step in arginine methylation is hindered in M48L using steady-state data, our single turnover experiments measuring $k_{chem}$ limit the choices to peptide binding or the actual chemical transfer step. When the peptide is already monomethylated, one or both of these steps is hampered. When the monomethylated
residue is presented in the center of the peptide substrate, very little if any substrate appears to be bound in the M48L mutant in a catalytically-productive manner.

CONCLUSION

Product specificity as it relates to PRMT1 is a multifaceted. Site selection within a protein (sequence and location), degree of methylation (mono- or dimethylation) and number of methylations in each target can produce a dizzying number of different products. How the enzyme is designed to control each one of these aspects is a challenging question that is likely to lead to overlapping/interdependent mechanisms. In the work presented here, we provide the first insight into product specificity in PRMT1. We show that the conserved methionine 48 is important for activity and accurate site selection, which also impacts the degree of methylation in the final product.

References


CHAPTER 4

SUBSTRATE-INDUCED CONTROL OF PRODUCT FORMATION BY PROTEIN ARGinine METHYLTRANSFERASE 1 (PRMT1)

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 also 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 non-stochastic 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 semi-processive 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.

1S. Gui, W.L. Wooderchak-Donahue, T. Zang, D. Chen, M.P. Daly, Z.S. Zhou, and J.M. Hevel, Substrate-induced control of product formation by protein arginine methyltransferase 1 (PRMT1), Biochemistry (in press)
INTRODUCTION

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 4-1). Type I PRMTs (such as PRMT1) form MMA and/or ADMA, and represent the majority of identified PRMTs. Type III enzymes only produce 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.

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 is available about how the deposition of methyl marks is regulated, especially between MMA and ADMA.

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,
Figure 4-1. 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 clarity.

studies have shown that MMA residues display a bona fide signaling role in vivo. Kirmizis and coworkers showed that distinct transcriptional consequences in yeast are correlated to the mono- and asymmetric dimethyl states of histone H3 at Arg-2 (12). Moreover, due to 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 4-1). 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 4-1), 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 4-2).

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 non-stochastic fashion, with less preference towards the C-terminal arginine. When a single arginine is targeted, PRMT1-catalyzed dimethylation occurs in a semi-processive manner; however, the degree of processivity observed is substrate-dependent. Our results provide a mechanistic explanation for developing the

| Table 4-1. The methylation status of Npl3, hnRNP K and Sam68 in vivo*. Methylated arginines are highlighted in bold. |
|---|---|---|
| Npl3 | hnRNP K | Sam68 |
| ADMA in vivo | RGGFRGGFRGGFRGGG | 254 PMRGRGG | 45 SRGGGGGSR |
| | RGGFGGPRGGF | 265 PPGRGG | 352 SRGGGGGSGRGGAR |
| | RGYYGGYSRG | 294 RG | 285 PRGG |
| | 290 RG | 254 PMRGRGG | 301 GSRAR |
| | 307 RG | 265 PPGRGG | 310 GVGPPRGALVR |
| MMA or partial ADMA in vivo | 315 GALVRGTPVR | 320 GTPVRGAIITR |
| | 325 GAITRGATVTR | 335 GAI |

*The methylation statuses shown above were determined by mass spectrometry (33-35).
### Table 4-2. List of peptide substrates (R* indicates monomethylated arginine)

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Peptide Sequence</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3</td>
<td>GGRGGFGGRGGFGGRGGFGG</td>
<td>Fibrillarin-based multi-Arg</td>
</tr>
<tr>
<td>R2</td>
<td>GGRGGFGGGKGFGGRGGFGG</td>
<td></td>
</tr>
<tr>
<td>RKR-CH₃</td>
<td>GRRGGFGGKGFGGRGGFGG</td>
<td></td>
</tr>
<tr>
<td>RKK</td>
<td>GGRGGFGGKGFGGKW</td>
<td>R3-based single Arg</td>
</tr>
<tr>
<td>RKK-CH₃</td>
<td>GGRGGFGGKGFGGKW</td>
<td></td>
</tr>
<tr>
<td>KRK</td>
<td>KGGFGGGKGGFGGGKW</td>
<td></td>
</tr>
<tr>
<td>KRK-CH₃</td>
<td>KGGFGGGKGGFGGGKW</td>
<td></td>
</tr>
<tr>
<td>eIF4A1</td>
<td>YIHRIGRGGR</td>
<td></td>
</tr>
<tr>
<td>eIF4A1-CH₃</td>
<td>YIHRIGRGGR*GGR</td>
<td>eukaryotic initiation factor 4A-1 (eIF4A1)-based</td>
</tr>
<tr>
<td>eIF4A1-Y</td>
<td>YIHRIGRYGR</td>
<td></td>
</tr>
<tr>
<td>eIF4A1-S</td>
<td>YIHRIGRSGR</td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td>SGRGKGGKGLGKGGAKR</td>
<td>Histone 4-based single Arg</td>
</tr>
</tbody>
</table>

patterns of specific MMA and ADMA deposition on the targeted arginine residues in vivo, which reveals a previously unidentified mechanism for regulating arginine methylation.

**Experimental Procedures**

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

*Recombinant Proteins*— Full length his-tagged PRMT1 (residues 1-353) was expressed and purified as described (36). Purified proteins were ≥95% pure 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 standard.

*Synthetic peptides*— All the peptides were synthesized by the Keck Institute (Yale
University) 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 an 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 (36). If not stated otherwise, the methylation reaction contained 4 μM PRMT1, 250 μM AdoMet, and 100 nM AdoHcy nucleosidase (MTAN, purified as in (37)) in 50 mM sodium phosphate buffer (pH 7.1). Independent reactions were initiated with 200 μM of each peptide substrate. At various times points (2.5, 5, 10, 12.5, 15, 20, 30 min), 10 μL aliquots were quenched with trifluoroacetic acid (TFA) (10% final) and analyzed by LC/MS followed by MS/MS of the desired peaks. Samples that showed a maximum amount of monomethylation yet no or minimal amount of the enzymatically formed dimethyl species were further analyzed with LCMS/MS. The time point used for each peptide shown in Table 4-3 was 20 min, 10 min, and 10 min for the R2, RKRm and 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, UK). Peptide samples were loaded (3 μL) using a NanoACQUITY Sample Manager (Waters, Manchester, UK) onto a trapping column (Symmetry® C18, 180μM x 20mm) (Waters, Manchester, UK). Samples were washed with 99% H₂O and 1% TFA for one minute at 15 μL/min to a waste container and then eluted with a 30 min gradient.
(1-4% B in 0.1 minutes, 4-60% B in 20 minutes, 60-85% B in 3 minutes, 85-1% B in 1 minute, and 1% B for 6 minutes where A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile) at 800 nL/min using an NanoACQUITY UPLC (Waters, Manchester, UK) over a 100 μm x 100 mm BEH 130 C18 column. MS survey and product ion MS/MS scan times were 1.0 second. The collision offset was automatically determined based on precursor mass and ion charge state. MS/MS data was used for the De Novo sequencing of methylated peptides by Waters BioLynx software (Waters, Manchester, UK).

For the eIF4A1 peptide samples shown in Figure 4-3, reactions were diluted to 2 μM using acetonitrile/water/TFA (v:v:v, 50:50:0.05). Diluted peptide samples (0.5 μL) were spotted on a polished standard 192-well stainless steel MALDI sample plate followed by the addition of α-cyano-4-hydroxy cinnamic acid (CHCA) matrix solution (0.4 μL, 10 mg/mL in a mixture of acetonitrile/water/TFA, v:v:v, 50:50:0.05). 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/spectrum were accumulated in the MS mode and 5000 shots/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 software 4.6. The sequences of peptides were analyzed using ProteinProspector (an online program, version 5.10.2). In order to construct a reaction time course (Figure 4-3), the relative height of each peak (naked, monomethylated, and dimethylates species) was calculated by dividing the individual peak height by the sum of peak heights from all peptide species (non-methylation,
mono-methylation and di-methylation) observed in mass spectra at each time point. The intensity of each peak from non-methylated peptide or 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 $K_{m,\text{app}}$ and $k_{\text{cat,\text{app}}}$ values. Each reaction was performed at least in duplicate. The limit of detection for this assay was 0.01 $\mu$M methyl group transfer.

**Double turnover experiments**— A reaction containing 20 $\mu$M PRMT1, 40 $\mu$M AdoMet (2.6 $\mu$M $[^{3}\text{H}]$AdoMet (specific activity of 2.02 mCi/$\mu$mol)), 100 nM MTAN in 50 mM sodium phosphate buffer (pH 7.5) was initiated with 200 $\mu$M peptide substrates at 37 ºC. After 1 hour, proteins were precipitated with 10% TFA (final) and removed through centrifugation. An equivalent volume of 12 M HCl was added to each mini vial (Deltaware). Vials were then sealed and heated to 110 ºC for approximately 24 hours for complete acid hydrolysis. Due to the small inner space, sample oxidation is minimized (36). The methylation status of hydrolyzed peptide products were analyzed by HPLC and MS.

**Identification and quantification of methylated arginines by HPLC**— Hydrolyzed amino acids were neutralized by 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 x 4.6 mm (Phenomenex). Mobile phase A consisted of 40 mM sodium phosphate buffer (pH 7.8), and mobile phase B was acetonitrile/methanol/H₂O (45/45/10, v/v/v). Fractions (83 μL) were collected and radioactivity was counted in 5 mL 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 pmole of methylated arginine in a 20 μL sample.

**RESULTS AND DISCUSSION**

**PRMT1 methylation of a multiple arginine-containing substrate is non-stochastic.**

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 (Cₜ) of Npl3 contains an extensive arginine-glycine rich domain composed of 15 ‘RGG’ tripeptide repeats, of which nine consecutive N-terminal (Nₜ) ‘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 4-1). Such cumulative methylation of Npl3 is important for protein function, especially at the N-terminal ‘RGG’ motifs (34). However, the molecular origins of such distinct methylation patterns remain unclear. In order 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 (GGRGGFGGKGGFGGRGFG). Because both arginine residues exist
within the same amino acid context (GGRGG), 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 tandem mass spectrometry (MS/MS). MS/MS analysis showed that the N-terminal arginine of the R2 peptide was first mono-, then dimethylated (data not shown). 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 hours) where MS/MS analysis demonstrated the presence of the tetramethylated R2 peptide (data not shown). Figure 4-2 summarizes the series of 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

![Diagram](Image)

**Figure 4-2.** Methylation events observed on the R2 peptide. Time-dependent MS/MS product analysis showed a systematic methylation of the R2 peptide.
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-CH₃ peptide (GGRGGFGGKGGFGGGRMeGGFGG) 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-CH₃ peptide was mapped using MS/MS (data not shown). Although methylation of the C T arginine was observed (13%), PRMT1 still showed a strong preference for the N-terminal arginine site (Table 4-3).

We also tested PRMT1 substrates that contain more than two arginines. We mapped the first methylation event in the R3 peptide (GGRGGFGGRRGGFGGRGGFGG), which contains three potential target sites (data not shown). Compared to 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 occurred at the N T arginine (55%), 2) the internal arginine was targeted in a significant amount (39%) and 3) minimal methylation (6%) occurred on the C T arginine (Table 4-3). 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 mono- or partial dimethylation associated with the C-terminal ‘RGG’ motif arginines (Table 4-1).

The preference of PRMT1 for the N\textsubscript{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 has less structural flexibility compared to the N\textsubscript{T} arginine, is also targeted in a significant amount 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\textsubscript{T} arginine of the peptide substrate in a distal binding event. Osborne and coworkers (43) showed that positively charged residues distal to the methylation site (11-14 amino acids 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\textsubscript{T} and the necessary

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide Sequence</th>
<th>% of the 1\textsuperscript{st} methylation position\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N\textsubscript{T}-R</td>
</tr>
<tr>
<td>R2</td>
<td>GGRGGFGGKGGFGRGGFG</td>
<td>100</td>
</tr>
<tr>
<td>RKR-\textsubscript{CH}_3</td>
<td>GGRGGFGGGKGGFGRGGFG\textsuperscript{*}GGFG</td>
<td>87</td>
</tr>
<tr>
<td>R3</td>
<td>GGRGGFGRGGFGGRGGFG</td>
<td>55</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The standard error of the percentage was typically less than 5\%.
positive charges that were identified were all located downstream (C-terminal) to the arginine.

When more than one arginine is present in the targeted protein, the distance between them may also influence processing. Many proteins have been identified that contain ‘RGG’ repeats (44); however, whether they are all PRMT1 substrates and what the in vivo pattern of methylation looks like is unknown. The limited studies of in vivo methylation patterns of PRMT1 show that the common distance between targeted arginines is either 2-3 amino acids (e.g. RGG in hnRNP K) or 5-7 amino acids (e.g. RGGFGGGPRGG in Npl3) within the repetitive arginine-glycine rich region. In such repetitive ‘RGG’ regions, extensive and consecutive dimethylation is often observed. Due to the 5 amino acids between the arginine residues in the R3 peptide, we would classify the R3 peptide as an ‘RGG-repeats’ 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 we are aware of, methylation of the C-terminal arginine in the arginine-rich domain is infrequently observed (33-35).

Overall our results show that PRMT1 methylates multi-arginine substrates in a non-stochastic 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 coworkers (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. Since 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 hereinafter.

**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. In order 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 4-4). Two fibrillarin-based peptides with the identical amino acid sequences denoted KRK and RKK represent the alternation of the target arginine position in the peptides. The eIF4A1 peptide (YIHRIRGGGR), derived from eukaryotic initiation factor 4A-I, bears an entirely different peptide sequence from the fibrillarin-based peptides. Although eIF4A1 peptides contain three arginines, we previously showed that the central arginine in the eIF4A1 peptide (YIHRIRGGGR) was the only one modified by PRMT1 (42). Hence, this peptide pair is still considered a single-arginine containing substrate with the same center-located arginine residue as the KRK peptides. These three peptide pairs serve as a start to systematically probe whether PRMT1 methylates both naked and monomethylated substrates with the same catalytic efficiency.

Data for the KRK and KRK-CH₃ peptides with PRMT1 showed saturation kinetics at a variety of fixed AdoMet concentrations (data not shown). Similar results were observed with eIF4A1 and RKK peptide pairs (data not shown). Under saturating concentrations of AdoMet, the apparent catalytic efficiency ($k_{cat}/K_m$) for the eIF4A1-CH₃ peptide was nearly double that of the eIF4A1 peptide (Table 4-4). The RKK peptide pair showed similar results. Although the effect was minimal, the KRK-CH₃ peptide also showed a higher value of $k_{cat}/K_m$ than the KRK peptide (Table 4-4). 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 4-3) which showed that monomethylation of the C₇ 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 $k_{\text{cat}}/K_m$ between non-modified 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 coworkers (48) reported a higher catalytic efficiency with the monomethyl H4 peptide ($k_{\text{cat}}/K_m = 1.6 \pm 0.3 \, \mu\text{M}^{-1}\text{min}^{-1}$) than the unmodified counterpart ($k_{\text{cat}}/K_m = 0.9 \pm 0.1 \, \mu\text{M}^{-1}\text{min}^{-1}$). On the other hand, Kölbel and coworkers (47) found no significant changes in catalytic efficiency ($k_{\text{cat}}/K_m$) of PRMT1 based on one MMA-peptide derived from PABPN1, Importantly, this PABPN1-derived peptide substrate bears an “RXR” amino acid paradigm (FYSGFNSRPRGRVYATSWY) 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

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>$K_m, \text{peptide*} (\mu\text{M})$</th>
<th>$k_{\text{cat}*} \times 10^{-2} \text{ s}^{-1}$</th>
<th>$k_{\text{cat}}/K_m \times 10^{2} \text{ M}^{-1}\text{s}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4A1</td>
<td>72 ± 9.6</td>
<td>9.0 ± 0.3</td>
<td>13 ± 1.7</td>
</tr>
<tr>
<td>eIF4A1-CH$_3$</td>
<td>26 ± 6.2</td>
<td>5.8 ± 0.2</td>
<td>23 ± 5.5</td>
</tr>
<tr>
<td>KRK</td>
<td>105 ± 4.4</td>
<td>6.0 ± 0.1</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>KRK-CH$_3$</td>
<td>32 ± 7.5</td>
<td>2.5 ± 0.2</td>
<td>7.6 ± 1.8</td>
</tr>
<tr>
<td>RKK</td>
<td>21 ± 6.7</td>
<td>3.7 ± 0.2</td>
<td>17 ± 5.5</td>
</tr>
<tr>
<td>RKK-CH$_3$</td>
<td>52 ± 16.8</td>
<td>14 ± 1.1</td>
<td>27 ± 8.9</td>
</tr>
</tbody>
</table>

*Apparent $K_m$ and $k_{\text{cat}}$ are reported using a single saturating concentration of AdoMet (250 \mu\text{M}).
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 to 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 higher 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 than MMA.

The final methylation status is affected by 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 specific arginine residues in the protein substrates, such as hnRNP K and Sam68 (Table 4-1). 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, respectively. As stated previously, among all three arginine residues, only the central arginine is methylated by PRMT1, so 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 more than an 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 4-3A). However, the eIF4A1 peptides bearing the ‘RSG’ and ‘RYG’ sequences demonstrated incomplete methylation in the reaction (Figure 4-3B, 3C). Surprisingly, less than 10% turnover was observed with eIF4A1-S/Y samples from even longer reaction time periods (400 or 600 min, data not shown). In order 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 the mono- and dimethylated arginine products observed was quite different in the three eIF4A1 peptides (Figure 4-3). After the 105-min reaction, the ‘RGG’ containing WT-eIF4A1 peptide was mostly dimethylated (97%) while the ‘RSG’ and ‘RYG’ containing peptides were mainly
Figure 4-3. Effect of flanking amino acid sequence on methylated product distribution. Reactions were prepared as described in steady-state kinetics and were initiated with WT-eIF4A1 in (A), eIF4A1-S in (B), and eIF4A1-Y in (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.
monomethylated (7% and 4%, respectively). The presence of MMA in the reaction 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. In order to investigate the processivity of PRMT1-catalyzed dimethylation, we designed double turnover experiments containing 20 μM PRMT1, 40 μM AdoMet ([3H]-AdoMet added) and an excess of peptide substrate. With the concentration of AdoMet higher than 10-fold $K_{D(AdoMet)}$, the majority of the enzyme is bound with AdoMet when the reaction is initiated with peptide substrate. Assuming the reaction is completely distributive, monomethylated peptides will be released after the first turnover, and replaced by bound non-modified peptides. As 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 reactions products were analyzed, both MMA and ADMA were clearly observed (Figure 4-4A, 4B). The same result was obtained using MS (data not shown). Quantification of the radioactivity present in both the MMA and ADMA peak showed that the reaction had gone to completion (data not shown).

The formation of both MMA and ADMA indicates a semi-processive mechanism where 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 semi-processive 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 to quantify 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 of eIF4A1, 17.1 μM MMA and 11.4 μM ADMA were generated from 40 μM of AdoMet. Quantification was based on radiolabel incorporation 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 current double turnover experiment, we can deduce that the amount of MMA that was generated in the 1st turnover, but remained bound to PRMT1 and was subsequently dimethylated, is equal
to the final ADMA concentration (11.4 μM). We can further approximate the amount of MMA released from PRMT1 after the first turnover (20 - 11.4 = 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 4-4C).

**Figure 4-4.** 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 eIF4A1 peptide showing both the fluorescence (black lines) from amino acid derivatization and radioactivity (grey 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 based on the calculation from eIF4A1 peptide discussed in the text.

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2 Approximations assume that the small difference observed in $k_{cat}/K_m$ between the naked and monomethylated peptide substrates in the steady state do not alter product partitioning by an appreciable amount in the double turnover experiments.
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, due to the similar $K_D$ of the eIF4A1 and eIF4A1-CH$_3$ peptides (data not shown), the presence of excess eIF4A1 peptide should make MMA rebinding negligible.

The degree of the processivity varies depending on the sequence of the peptide substrate. We performed the double turnover reactions using three other single-arginine containing peptide substrates to investigate processivity. With all the peptides we tested, both MMA and ADMA were observed (data not shown), suggesting a general semi-processive mechanism. Remarkably, the proportion of MMA and ADMA generated from the double turnover experiments are distinct among certain peptide substrates (Table 4-5). These results were further confirmed with MS, and the reactions were shown to go to completion (data not shown).

Similar to calculating the percentage of MMA processed distributively or processively, the ratio of MMA:ADMA is directly related to the amount of MMA released and the amount that remains bound to PRMT1 (respectively), 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 smaller ratio of MMA:ADMA, and *vice versa*. As can be seen from Table 4-5, the eIF4A1 and H4 peptides, which have a small ratio of MMA:ADMA (close to 1:1), demonstrate a much higher degree of processivity than the KRK or RKK peptides (MMA:ADMA close to 5:1). Such distinct ratios from the double-turnover experiments indicate that certain peptide sequences are processed through a processive mechanism, leading
Table 4-5. The degree of PRMT1-catalyzed processive dimethylation as represented by the MMA/ADMA ratio using different peptide substrates.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MMA:ADMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4</td>
<td>SGRGKGKGLGKGGAKR</td>
<td>0.967 : 1</td>
</tr>
<tr>
<td>eIF4A1</td>
<td>YIHRIGRGGGR</td>
<td>1.50 : 1</td>
</tr>
<tr>
<td>KRK</td>
<td>KGGFGGRRGGFGGKW</td>
<td>4.10 : 1</td>
</tr>
<tr>
<td>RKK</td>
<td>GGRGGFGGGKGFGGKW</td>
<td>4.73 : 1</td>
</tr>
</tbody>
</table>

to a dimethylated product, while other sequences partition more frequently through a distributive mechanism where both monomethyl- and dimethylated products are possible. To investigate what leads to the different ratios of MMA:ADMA, we tested the binding affinity of the naked and monomethylated eIF4A1 peptides to PRMT1 using intrinsic fluorescence quenching (data not shown). The $K_D$s 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 semi-processive 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 Kölbl 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 of Obianyo and co-workers (43, 46). We also note that the semi-processive dimethylation we observe is consistent with the rapid equilibrium random mechanism proposed by Obianyo and coworkers (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, semi-processive 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 to instill such complex deposition codes. Therefore, a fine-tuned degree of processivity is required, such as the substrate-induced control described in the current 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 controlling the substrate specificity in epigenetics. Such fine-tuned
processivity in posttranslational modification reactions is also observed with multisite phosphorylation (reviewed in (65, 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 (1, 68)) highly suggests that the sequential arginine methylation (Figure 4-2) may also serve as a fundamental switch for protein function.

On a final note, a tunable semi-processive 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, as of yet 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 semi-processive 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 insuring the proper transmission of biochemical information.

References


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CHAPTER 5

SINGLE TURNOVER AND PRE-STEADY STATE KINETIC STUDIES OF PRMT1 MECHANISM

ABSTRACT

Protein arginine methylation is a wide-spread eukaryotic posttranslational modification catalyzed by a family of enzymes known as protein arginine methyltransferases (PRMTs). PRMTs play a critical role in fundamental cellular pathways and the maintenance of a healthy cellular state, and contribute to disease when dysregulated. Regulation of arginine methylation not only includes correct target recognition and activity, but also correct product deposition (monomethylarginine, MMA; asymmetric dimethylarginine, ADMA; and symmetric dimethylarginine, SDMA), neither of which are fully understood. We previously discovered that PRMT1, the predominant PRMT in vivo, catalyzes arginine dimethylation semi-processively. The degree of processivity is in a substrate-dependent manner, which can contribute to determine the major final product to be MMA or ADMA (Chapter 4). We further explored the semi-processive dimethylation via single turnover experiments and pre-steady state kinetics. Both approaches provide us insight into the microscopic rate constants for each step in the mechanism. From single turnover experiments we found that the monomethyl peptide substrate has a lower methylation rate compared to the unmodified peptide, but a higher catalytic efficiency under the steady state. Although the complete mechanism is not fully understood, the methodologies I developed and discussed in this chapter are shown to be useful for investigating the detailed mechanism of PRMT1. Further research
will be needed to fully understand the substrate-dependent semi-processive
dimethylation.

**INTRODUCTION**

Over the last decade, protein arginine methyltransferases (PRMTs) have been
implicated in various biological pathways, such as transcriptional regulation, signal
transduction, RNA metabolism, and DNA damage repair (1-3). The expression and
enzymatic activity of PRMTs are found to be dysregulated in many human diseases
including cardiovascular diseases (4-6) and cancer (7-9). As the predominant PRMT
catalyzing 85% of arginine methylation *in vivo* (10), PRMT1 is capable of transferring
the methyl group from *S*-adenosyl-*L*-methionine (AdoMet or SAM) to the guanadino
group of the arginyl residue generating monomethylarginine (MMA) as well as the
byproduct *S*-adenosyl-*L*-homocysteine (AdoHcy or SAH) (Figure 5-1). MMA can be
further dimethylated by PRMT1 forming asymmetric dimethylarginine (ADMA). For
decades, MMA was simply considered to be the intermediate for generating
dimethylarginines (11). However, the biological significance of MMA became clear after

Figure 5-1. Methylation reactions catalyzed by PRMT1. PRMT1 catalyzes the methyl
group transfer from AdoMet to protein arginine residues generating AdoHcy and
monomethylarginine (MMA). MMA can be further methylated to asymmetric
dimethylarginine (ADMA).
MMA was detected *in vivo* as a physiologically relevant modification on yeast and mammalian histones (12, 13). Moreover, MMA induces distinct transcriptional outputs from ADMA when deposited on the same arginine residue (13). Thus it is of great importance to investigate the product specificity of PRMT1 and the regulation of MMA and ADMA generation.

As a foundation for the development of pharmaceutical inhibitors/activators of PRMT activity, the kinetic mechanism of PRMT1 has been studied (11, 14-17). Steady state data from Thompson and colleagues (16) suggests that PRMT1 follows a partially processive mechanism, with the production of ADMA occurring in significant amounts, but not in an obligate fashion. A fully processive mechanism would produce only ADMA without the release of MMA, and a distributive mechanism would require free MMA rebound to PRMT1 for further dimethylation (Scheme 5-1). Thompson further proposed that partially-processive dimethylation could result from the occasional fast dissociation of AdoHcy and AdoMet binding prior to the release of MMA, and thereby allow the transfer of a second methyl group (Scheme 5-1). On the other hand, a recent transient

![Scheme 5-1](image.png)

Scheme 5-1. The processive versus distributive mechanism of PRMT1-catalyzed dimethylation. (E: PRMT1; SAM: AdoMet; SAH: AdoHcy; pep: monomethyl peptide; pep^me2: dimethyl peptide)
kinetic study (18) suggests that methylated peptide release is much faster than the chemical transfer of the methyl group during turnover. Although both studies utilized a peptide substrate derived from the H4 protein substrate, the steady state study was performed with underivatized peptides while the transient kinetic study was performed with peptides containing a fluorescein molecule to monitor binding events. These two kinetic studies are inconsistent with each other and provide an unsatisfactory understanding of the mechanism for PRMT1.

Our most recent work showed that modifying the peptide sequence can alter the partitioning between distributive and processive dimethylation mechanisms (19). This suggested to us that some of the disparity observed between the two published kinetic studies may be based on the use of the fluorescein-labeling of the peptide substrate in the transient kinetic study. Additionally, we hypothesized that our results showing varying degrees of processive dimethylation with varying peptide sequences (19) could be explained by varying dissociation rates for monomethylated peptides of differing sequences.

To understand more about the mechanism of PRMT1, single turnover experiments and stopped-flow rapid mixing were utilized to explore the microscopic rates which contribute the distributive or processive pathway (Scheme 5-1). In this chapter, the underivatized eIF4A1 and eIF4A1-CH$_3$ peptides are used as the substrates, due to their relatively high degree of processivity (see Table 4-4). The same experiments will be performed with other peptide substrates in order to understand the substrate-dependent processivity. Although solid conclusions about the substrate-dependent processivity of PRMT1 cannot be drawn yet, my research showed that single turnover experiments and
the stopped-flow measurement can both be used as powerful tools in the pre-steady state kinetic research of PRMTs. Our results are consistent with the rate-limiting step in PRMT1-catalyzed methylation being the chemical step of –CH₃ transfer, as shown in the previous transient kinetic study (18). The binding and orienting process of AdoMet is slower than that of peptide substrates. Data from stopped-flow experiments showed that AdoMet binding appears to be a two-step process. Most surprisingly, a role for a reductant in substrate binding was revealed in my studies.

MATERIALS AND METHODS

AdoMet was purchased from Sigma as a chloride salt (≥80%, from yeast). [methyl-³H]AdoMet was purchased from Perkin Elmer. All the peptides were synthesized by the Keck Institute and purified to ≥95%. ZipTip® C₁₈ pipette tips were purchased from Millipore.

Expression and purification of mutant PRMT1 proteins

His-tagged rat PRMT1 was expressed and purified as described (17). Purified proteins were ≥95% pure 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 standard.

Dissociation constant measurement by intrinsic fluorescence quenching

An RF-5301PC spectrofluorophotometer (Shimadzu) was used for fluorescence measurements. For peptide and AdoMet affinity determinations, an excitation wavelength of 290 nm was used and emission spectra from 300-420 nm were collected.
The change in fluorescence intensity at the maximum emission (333 nm) was monitored. The excitation and emission slit was 5 nm and the scan speed was 100 nm/min using 1325 µL containing 1.4 µM PRMT1 in 150 mM sodium phosphate buffer pH 7.8. Increasing concentrations from 1 to 50 µM peptide ligand (or AdoMet) were added at 2-3 min intervals. Data from at least two titrations were averaged and analyzed using the modified Stern Volmer (20) plots. Data were evaluated by nonlinear regression analysis using SigmaPlot in order to obtain the dissociation constant ($K_D$) using the following equation $F_c = F (10^\frac{\epsilon c d}{2})$ where $F_c$ is the corrected fluorescence, $\epsilon$ is the extinction coefficient of AdoMet, $c$ is the concentration of AdoMet, and $d$ is the pathlength. $F_{\text{initial}}/(F_{\text{initial}}-F_c)$ was then plotted against $1/[\text{AdoMet}]$ and the data was fit to a line where the $y_{\text{intercept}}=1/fa$, the slope=$1/fa*K_Q$, and the $K_Q=1/K_D$.

**Single turnover experiments**

Single turnover experiments were performed to evaluate the rate of substrate incorporation and the chemical catalysis step. Hence the single turnover reactions were initiated with either peptide substrate or AdoMet in order to find out the differences in rates of peptide and AdoMet incorporation. Reactions initiated by peptides were carried out by mixing a solution containing the pre-incubated complex of 21 µM wild type PRMT1, 20 µM AdoMet (along with 1 µM of $[^{3}\text{H}]$-AdoMet), and 10 nM MTAN in 50 mM sodium phosphate buffer (pH 7.5) at 23 °C for 3 minutes. Commercially available AdoMet was purified as described (21, 22) before use. Reactions were initiated with 200 µM peptide substrates. Reactions initiated by AdoMet were performed with the same 20 µM wild type PRMT1, 200 µM peptide, and 10 nM MTAN in 50 mM sodium phosphate buffer.
buffer (pH 7.5) at 23 °C for 3 min. 50 μM AdoMet (along with 1 μM of [³H]-AdoMet) was used to initiate the reaction. Radiolabel incorporation over time from each reaction was measured using the discontinuous ZipTip® assay described previously. Briefly, 10 μL reaction samples of different time points were taken out and quenched by 6 M guanidine HCl solution and processed with ZipTip®C18 assay to quantitate the amount of methylated product (23). The resulting time course of [³H]AdoMet incorporation was fit into single exponential curve

\[ y = A_o \times (1 - \exp(-b\times x)) \]

(1)
to determine the parameters, where \( A_o \) represents the amplitude of the exponential phase and \( b(k_{chem}) \) is the observed rate constant associated with that process.

**Stopped-flow fluorescence measurement**

The transient-state kinetics of AdoMet association with PRMT1 was determined by stopped-flow fluorescence assays using a HiTech SF-61DX2 instrument (Hi-Tech Ltd., Salisbury, U.K. at University of California, San Francisco). The transient fluorescence signal change was detected at 23 °C on stopped-flow equipment using an excitation wavelength of 280 nm and an emission wavelength at 300-350 nm. Each reaction was at least triplicated. A high concentration of PRMT1 (5 μM) was used in the fluorescent measurement in order to provide sufficient fluorescent signals. PRMT1 was pre-incubated with 1 mM DTT to achieve a reliable quenching curve. PRMT1 solution was then mixed with increasing concentration (50 μM, 100 μM, and 250 μM) of AdoMet in the reaction buffer (50 mM sodium phosphate, pH 7.6). After averaging the shot data, the association time courses were fitted to a double-exponential function:
\[ F = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + C \]  

(2)

\( F \) is the fluorescence intensity at time \( t \), \( A \) is the amplitude of the fluorescence change, and \( k \) is the rate constant. The observed rate constants were plotted against AdoMet concentration, and the data were fit to the following equation:

\[ k_{\text{obs}} = k_{\text{on}} [\text{AdoMet}] + k_{\text{off}} \]  

(3)

to derive the association rate constants \( k_{\text{on}} \) as well as the dissociation rate \( k_{\text{off}} \). The dissociation constant \( K_D \) can then be calculated as

\[ K_D = \frac{k_{\text{off}}}{k_{\text{on}}} \]  

(4)

RESULTS

Similar binding affinity of the eIF4A1 and eIF4A1-CH\textsubscript{3} peptides with PRMT1

In the semi-processive mechanism, if the binding of monomethyl peptides is highly preferred, it may lead to a pseudo-processive observation in which the MMA species is first released from PRMT1 but quickly rebinds to the enzyme. Thus the binding affinity of PRMT1 with the eIF4A1 and eIF4A1-CH\textsubscript{3} peptides was first tested via the intrinsic fluorescence quenching experiments (Figure 5-2). The modified Stern-Volmer plot was applied to analyze the quenching data (20). The \( K_D \) values of both peptides are very similar to each other in low \( \mu \)M range (Table 5-1) revealing that PRMT1 binds the unmodified and the monomethylated peptides with comparable affinity.

Single turnover experiments of the eIF4A1 peptide pair

To gain more information about the microscopic rates of PRMT1-catalyzed methylation, single turnover experiments were conducted where the chemistry step \( (k_{\text{chem}}) \) could be isolated from substrate binding or product release steps (Scheme 5-2).
Additionally, single turnover experiments allow us to analyze the two-step methylation separately with unmodified and monomethyl peptides, and thus ascertain the intrinsic reactivity of the 1st versus the 2nd methyl group transfer (17). The single turnover experiments were conducted at 22 °C with a slight excess of PRMT1 (21 μM) pre-incubated with a limiting amount of AdoMet (20 μM) (~10-fold higher than $K_D$). Reactions were initiated with saturating peptide substrates (200 μM). Samples from different time points were taken out and analyzed via ZipTip assays (Figure 5-3). The maximal product concentration ($A_0$) and $k_{chem}$ for peptide incorporation and methylation

![Figure 5-2. Modified Stern-Volmer plot showing the intrinsic fluorescence quenching of PRMT1 by the eIF4A1 and eIF4A1-CH$_3$ peptides. The data was fit to a line with nonlinear regression where the $y_{intercept}=1/ fa$, the slope=$1/ fa*K_Q$ and the $K_Q=1/K_D$.](image)

Table 5-1. Peptide dissociation constants from the modified Stern-Volmer plots.

<table>
<thead>
<tr>
<th>Peptide ligand</th>
<th>Peptide sequence</th>
<th>$K_D$ (μM)</th>
<th>Fluorescent quenching (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4A1</td>
<td>YIHRIGRGGR</td>
<td>1.3 ± 0.1</td>
<td>30 ± 2.0</td>
</tr>
<tr>
<td>eIF4A1-CH$_3$</td>
<td>YIHRIGR$_{Me}$GGR</td>
<td>2.6 ± 0.2</td>
<td>27 ± 4.7</td>
</tr>
</tbody>
</table>
Pre-steady state kinetics:

\[ \begin{align*}
E + A & \rightleftharpoons EA^B \quad k_{chem} \\
EAB & \rightleftharpoons EAB' \\
EAB' & \rightarrow EPQ \\
EPQ & \rightarrow EQ + P \\
EQ + P & \rightarrow E + Q
\end{align*} \]

Steady state kinetics:

\[ k_{cat}/K_m \quad k_{cat} \]

Scheme 5-2. Meaning of the kinetic parameters under the pre-steady state and the steady state of a simplified methyltransfer reaction. The expressions for \( k_{chem} \), \( k_{cat} \) and \( k_{cat}/K_m \) include steps within the brackets in each case. E: enzyme (PRMT1), A and B: two substrates (AdoMet and peptide substrate for PRMT1); EAB': a transient state of methylation; P and Q: two products (AdoHcy and methyl peptide generated by PRMT1).

were obtained (Table 5-2). The eIF4A1 peptide shows a slightly higher \( k_{chem} \) (3.3 ± 0.6 x 10⁻² s⁻¹) than the eIF4A1-CH₃ peptide (2.6 ± 0.2 x 10⁻² s⁻¹). We further calculated the pre-steady state efficiency constant \( k_{chem}/K_D \), which is a similar concept to \( k_{cat}/K_M \) in steady state kinetics (24). The unmodified eIF4A1 peptide has a 2.5-fold higher \( k_{chem}/K_D \) than the monomethyl counterpart (Table 5-2), indicating that PRMT1 has a slightly higher preference for the naked peptide under single turnover conditions.

As a bi-substrate reaction, PRMT1-catalyzed methylation involves substrate binding steps of both AdoMet and the peptide substrate. The rate of AdoMet forming a productive complex also contributes to the degree of processivity. Therefore, single turnover experiments were also performed with limited amount of peptides. 21 μM of PRMT1 was pre-incubated with limited peptides (20 μM) with reactions initiated by saturating AdoMet (50 μM, 1 μM ³H-AdoMet included), so that the process of ternary complex formation and methyl group transfer was monitored with AdoMet initiation (single turnover plots not shown). For eIF4A1 and eIF4A1-CH₃ peptides, the methylation rate \( k_{chem} \) and the pre-steady state catalytic efficiency \( k_{chem}/K_D \) were very similar (1.5 x 10⁻² s⁻¹).
and $3.5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, respectively, Table 5-2). These observations showed that pre-incubating PRMT1 with the peptide substrates eliminated the differentials in $k_{\text{chem}}/K_D$ previously observed between unmodified or monomethylated arginine residues.

Reactions initiated with the eIF4A1 or eIF4A1-CH$_3$ peptides (PRMT1 pre-incubated with AdoMet) eliminate the AdoMet binding and/or orienting step, providing a 7-fold or 3-fold higher $k_{\text{chem}}/K_D$ compared to the AdoMet-initiation reactions. Because the mechanism of PRMT1 was previously described as a random equilibrium mechanism (16), the rate of methyl group transfer (the EAB’→EPQ step in Scheme 5-2) should be the same with the same peptide substrate, no matter whether the reaction is initiated with AdoMet or peptides. Therefore, the lower $k_{\text{chem}}/K_D$ in AdoMet-initiation reactions indicates that a slower step is involved in AdoMet binding and/or forming the productive ternary complex (EAB’) before the methyl group transfer. This novel observation is essential in understanding the methylation catalyzed by PRMT1, due to the fact that in a fully processive dimethylation (Scheme 5-1), AdoMet is the substrate that binds to the PRMT1- eIF4A1-CH$_3$ complex and initiates the methyl group transfer.

Figure 5-3. Single turnover experiments of PRMT1 initiated with peptide substrates. Data were fitted with equation (1).
Table 5-2. Pre-steady state parameters of PRMT1 with unmodified and monomethylated eIF4A1 peptide under different initiation orders

<table>
<thead>
<tr>
<th>Peptide initiation</th>
<th>$A_0$ (μM)</th>
<th>$k_{chem} \times 10^{-2}$ s$^{-1}$</th>
<th>$k_{chem}/K_D \times 10^3$ M$^{-1}$s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4A1</td>
<td>16.6 ± 0.5</td>
<td>3.3 ± 0.6</td>
<td>25.4</td>
</tr>
<tr>
<td>eIF4A1-CH$_3$</td>
<td>13.0 ± 0.3</td>
<td>2.6 ± 0.2</td>
<td>10.0</td>
</tr>
<tr>
<td>AdoMet initiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eIF4A1</td>
<td>8.2 ± 0.7</td>
<td>1.5 ± 0.4</td>
<td>3.7</td>
</tr>
<tr>
<td>eIF4A1-CH$_3$</td>
<td>12.9 ± 1.6</td>
<td>1.5 ± 0.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Comparison of the eIF4A1 and eIF4A1-CH$_3$ peptides under steady-state and single turnover conditions

Results from both steady-state kinetics and single turnover experiments were combined together to analyze the two-step methyl group transfer. Since the single turnover experiments were all performed under room temperature, parameters in steady-state kinetics were further collected at 22 °C instead of 37 °C as tested previously. Unsurprisingly, the apparent $k_{cat}$ under room temperature is much lower than at 37 °C for both unmodified and monomethyl peptides (Table 5-3). The eIF4A1-CH$_3$ peptide is barely a better substrate under steady-state kinetics (a slightly higher value of $k_{cat}/K_m$).

Under single turnover conditions, the eIF4A1-CH$_3$ peptide has a slightly lower methylation rate ($k_{chem}$). Along with the higher $K_D$, the monomethyl peptide has a lower pre-steady state catalytic efficiency ($k_{chem}/K_D$) than the unmodified one. Although these differences are modest at best, the different preference of PRMT1 to the naked or monomethylated peptides under steady-state and single turnover conditions provides further insights into the mechanism of PRMT1-catalyzed dimethylation, which will be further discussed.
Table 5-3. Comparison of the steady-state and single turnover kinetic efficiency of the eIF4A1 peptide pair. The catalytic efficiency under steady-state kinetics or single turnover conditions is highlighted in bold face.

<table>
<thead>
<tr>
<th>Condition</th>
<th>eIF4A1</th>
<th>eIF4A1-CH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 °C</td>
<td>$k_{cat}$ ($x10^2$ s⁻¹)</td>
<td>9.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>$K_{m,peptide}$ (μM)</td>
<td>72 ± 9.6</td>
</tr>
<tr>
<td></td>
<td>$k_{cat}/K_{m}$ ($x10^2$ M⁻¹s⁻¹)</td>
<td>13 ± 1.7</td>
</tr>
</tbody>
</table>

| 22 °C   | $k_{cat,app}$ ($x10^2$ s⁻¹) | 0.62ᵃ | 0.50ᵃ |
|          | $K_{m}$ (μM) | NDᵇ | ND |
|          | $k_{chem}$ ($x10^2$ s⁻¹) | 3.3 ± 0.6 | 2.6 ± 0.2 |
| 22 °C   | $K_D$ (μM) | 1.3 ± 0.1 | 2.6 ± 0.2 |
|          | $k_{chem}/K_D$ ($x10^3$ M⁻¹s⁻¹) | 25.4 | 10.0 |

ᵃ The standard error for the $k_{cat,app}$ at 22 °C is about 10-15%.
ᵇ ND stands for ‘not determined’.

**Measurement of the substrate binding rates of PRMT1**

In the single turnover experiments, both naked and monomethylated peptide reaction that were initiated with AdoMet had equally low $k_{chem}/K_D$ compared to the peptide-initiated reactions. To further investigate the slower step in AdoMet initiation, stopped-flow rapid mixing experiments were performed to explore the substrate binding rates via measuring the intrinsic fluorescence quenching of PRMT1 by AdoMet. Due to the relatively low fluorescent signal given by PRMT1, a high concentration of enzyme (5 μM) was used to monitor the binding event. Binding reactions were carried out with both AdoMet and peptide substrates under saturating concentrations. However, no reliable binding curve could be obtained, especially with peptide substrates. Quite surprisingly, it was not until PRMT1 was pre-incubated with a reductant dithiothreitol (DTT), that AdoMet was capable of quenching the intrinsic fluorescence of PRMT1. Measurements were then carried out as described in Materials and Methods.
The stopped-flow traces from various concentrations of AdoMet binding experiments (Figure 5-4) fit well to the double exponential equation (eq. 2) with two observed rate constants. Plots of the fast phase rates versus the AdoMet concentration (eq. 3) are linear with y-intercepts equal to the dissociation rate \( k_{off} \) (8.40 s\(^{-1}\)), and the slope representing the association rate \( k_{on} \) (4.7x10\(^4\) M\(^{-1}\)s\(^{-1}\)). The plot gave an average \( K_{D(AdoMet)} \) equal to \( k_{off}/k_{on} = 0.18 \) mM. Notably, the value of \( K_{D(AdoMet)} \) measured by the stopped-flow experiment is significantly larger than the one measured by fluorescent quenching method without the presence of DTT \((17)\). This may be due to the fact that the HPLC-purified AdoMet was used in the previous \( K_D \) measurements (intrinsic fluorescence quenching), but not in the stopped-flow measurements. Also the presence of 1 mM DTT in the stopped-flow measurement could possibly influence the binding affinity of AdoMet.

Although not much information can be gained yet from the \( k_{on} \) and \( k_{off} \) of AdoMet or peptide substrates, it suggests a potential two-step binding event, possibly including a conformational change. Our observation is consistent with the previous transient kinetic study based on a fluorescence-labeled peptide substrate \((18)\). Additionally, the requirement for a reductant to observe reliable binding curves poses new questions about PRMT1 and the mechanism which are explored in more details in the next chapter of this dissertation.

**DISCUSSION**

In all eukaryotic cells, PRMT1 is the predominant PRMT *in vivo* generating MMA and ADMA within protein targets \((1, 10)\). Coupled with the observation that MMA
Figure 5. Stopped-flow measurements of AdoMet association with PRMT1. Fluorescence changes of PRMT1 (5 μM) were shown upon mixing with different concentrations of AdoMet at room temperature. All the curves are fit with eq 2. The bottom right panel shows the observed association rate constant of AdoMet (phase 1 shown in squares and phase 2 in triangles) plotted against the concentration of AdoMet. The data were fit to linear eq 3.

and ADMA modifications in histone H4 Arg 3 are capable of inducing distinct transcriptional consequences, the determinants for PRMT1 generating mono- or dimethyl arginines become essential to understand the specific *in vivo* methylation pattern of PRMT1. We previously showed that PRMT1-catalyzed dimethylation is semi-processive and the degree of processivity is in a substrate-dependent manner ([19]). Hence the
substrates of PRMT1 intrinsically participate in encoding the MMA/ADMA product formed. To further understand the determinants of the semi-processive mechanism, single turnover experiments and stopped-flow rapid mixing were performed to elucidate the microscopic rate constants in the dimethylation process. Combined with the steady-state kinetic studies (Table 5-3), the detailed mechanism of PRMT1 catalysis was studied based on the eIF4A1 peptide pair chosen as the studying model due to its high degree of processivity (see Table 4-4).

Kinetic parameters from steady-state and pre-steady-state kinetics can tell us stories bracketing different steps in the whole methylation process. As shown in Scheme 5-2, the expression of $k_{\text{cat}}$ includes all first order steps including the conformational changes (EAB$\rightarrow$EAB$'$), the chemistry step (EAB$'$→EPQ), and product dissociation (EPQ$\rightarrow$E+P+Q), while the $k_{\text{cat}}/K_m$ expressions include all steps from binding of substrate through the first irreversible step (25-27). For methyltransferases, the first irreversible step is methylation (the chemistry step). Under the steady-state kinetic conditions, multiple turnovers occur including substrate binding and product release. From the steady state kinetic study of the eIF4A1 peptide pair (Table 5-3), $K_m$ of the peptide substrates (>20 μM) is 10 fold higher than the value of $K_D$ (~2 μM), indicating that the rate of product release is fast. Due to the fact that $K_m = k_{\text{release}}/k_{\text{capture}}$ (25), the lower $K_m$ for the eIF4A1-CH$_3$ peptide shows that the monomethylated peptide has either a larger $k_{\text{capture}}$ (faster to be captured by PRMT1) and/or a smaller $k_{\text{release}}$ (slower to release from PRMT1), in another word, “stickier” than the unmodified peptide substrate. Also, the eIF4A1-CH$_3$ peptide shows a slightly smaller $k_{\text{cat}}$ than the eIF4A1 peptide, demonstrating a longer chemistry step and/or product release process from Scheme 5-2.
Overall, the monomethyl eIF4A1-CH$_3$ peptide shows a larger $k_{\text{cat}}/K_m$ indicating a higher catalytic efficiency and a better substrate under steady state kinetics.

With pre-steady state kinetics, $k_{\text{chem}}$ obtained from single turnover experiments includes steps from generation of the EAB complex and potential conformational changes to methyl group transfer (EAB$\rightarrow$EAB'$\rightarrow$EPQ in Scheme 5-2). For both unmodified and monomethyl eIF4A1 peptide substrates, similar $k_{\text{chem}}$ was observed (Table 5-3), showing that both peptides have similar rates of EAB complex formation, and methyl group transfer. However, when the pre-steady state catalytic efficiency $k_{\text{chem}}/K_D$ was calculated, the eIF4A1 peptide showed a higher $k_{\text{chem}}/K_D$, indicating that the naked peptide is a more efficient substrate under single turnover conditions. The altered preference of PRMT1 under steady state and single turnover conditions may be due to the different concentrations and oligomeric forms of PRMT1 under single turnover (high enzyme, high peptide) and steady state (low enzyme, high peptide) conditions. Additionally, the differences at the two temperatures (22 °C in single turnover, 37 °C in steady state studies) could affect the protein-peptide interaction and lead to a different preference.

On a final note, the semi-processive mechanism indicates that the groove on the surface of PRMT1 active site (28), which is a common structure for enzymes performing processive turnover (29), provides binding capacity to the monomethyl product to some extent, but not enough to hold it through the AdoHcy–AdoMet exchange (Scheme 5-3). To further investigate the mechanism of PRMT1-catalyzed dimethylation, the rate of AdoMet–AdoHcy exchange ($k_{\text{on}}$(AdoMet) and $k_{\text{off}}$(AdoHcy)) with the presence of the monomethyl peptide will need to be tested. The measurement of $k_{\text{on}}$(eIF4A1), $k_{\text{on}}$(eIF4A1me),
and $k_{\text{off(eIF4A1me)}}$ will be necessary in order to understand the semi-processivity of PRMT1 by comparing the peptide release rate with the AdoHcy-AdoMet exchange velocity. Moreover, $k_{\text{on}}$ for other peptide substrates with and without the presence of AdoMet will also be desirable in order to find out whether the difference in $k_{\text{on}}$ is the reason for the substrate-dependent processivity.

As a brief summary, the stopped-flow fluorescent measurement and the single turnover experiments have been optimized and can be used as very powerful tools to investigate the detailed mechanism of PRMT1-catalyzed dimethylation. Due to the limited access to the stopped-flow rapid mixing equipment (Dr. Susan Miller at UC San Francisco), further experiments will be required to measure the unknown association or dissociation rates and understand the semi-processive mechanism of PRMT1 (Scheme 5-3). Additionally, during the research with the stopped-flow fluorescent measurement, I found out that no reliable binding event can be detected unless PRMT1 was preincubated.

Scheme 5-3. The proposed semi-processive mechanism of PRMT1-catalyzed dimethylation. The important yet unknown $k_{\text{on}}$, $k_{\text{off}}$ and $K_S$ are indicated in red. (E: PRMT1; SAM: AdoMet; SAH: AdoHcy; eIF4A1$^{\text{me}}$: the eIF4A1-CH$_3$ peptide; eIF4A1$^{\text{me2}}$: the asymmetric dimethyl eIF4A1 peptide)
with the reductant DTT. Data from rapid mixing with PRMT1 and DTT indicated a transient change in protein conformation and/or its oligomeric state (data not shown), which may at least partially result from the histidine$_6$-tag flanking our PRMT1 construct. We generated a tagless construct of PRMT1 and further experiments were carried out exploring the effect of DTT and the affinity tags on the protein characteristics of PRMT1 (Chapter 6).

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CHAPTER 6
EFFECTS OF AFFINITY TAGS AND REDUCING AGENTS ON THE PROTEIN CHARACTERISTICS OF RAT PRMT1

ABSTRACT

Protein arginine methylation is a significant post-translational modification catalyzed by protein arginine methyltransferases (PRMTs). PRMTs are widely involved in various cellular pathways and their dysregulation lead to many human diseases. Although PRMTs have been studied for more than 10 years, the research of PRMTs mainly uses recombinant proteins, of which the affinity tags may significantly influence the protein characteristics. Herein, we report that the small affinity tag, the histidine-6 tag, impairs the enzymatic activity of rat PRMT1 and displays a distinct oligomerization pattern from the tagless rat PRMT1. Surprisingly, the reductant dithiothreitol (DTT) greatly increases the enzymatic activity of His-rPRMT1 and reduces the His-PRMT1 aggregation, while it does not influence the oligomerization form of the tagless rPRMT1. A caveat of these experiments lies in the instability of the tagless construct, suggesting that rPRMT1 expressed without tags may incur damage that is not recoverable by an exogenous reductant. Due to the effect of DTT, we hypothesize that rPRMT1 suffers from potential oxidative damage, possibly on one or more cysteine residues. In all, our results show that the small His-tag can affect the protein characteristics of PRMT1 and lead to misinterpreted conclusions. Further experiments will be performed to find out the possible oxidative sites and the cause of PRMT1 oxidation. Future research will be focused on the possible oxidative regulation of PRMT1 in vitro and in vivo.
INTRODUCTION

In all eukaryotic cells, protein arginine methylation is one of the abundant post-translational modifications in vivo catalyzed by protein arginine methyltransferases (PRMTs). PRMTs transfer the methyl group from S-adenosyl-L-methionine (AdoMet) to a targeted arginine and generate monomethylarginine (MMA), asymmetric and symmetric dimethyl arginine (ADMA/SDMA). PRMT activity has been associated with a variety of biological pathways (reviewed in (1-3)), and has most recently garnered interest as a pharmaceutical target due to its involvement in various human diseases (4-8).

In most publications about PRMTs, recombinant proteins with peptide or protein affinity tags were examined in kinetic or product specificity assays. Although affinity tags are highly efficient in purifying recombinant proteins from the crude extracts, these additional amino acids may affect the inherent characteristics of PRMTs. It is shown that the N-terminal sequences of PRMTs (either from the affinity tags or different native isoforms) can alter the enzymatic activity of PRMT1, -7, and -8 (9-11). In one short report, the commonly used hexa-histidine (His6) tag was shown to alter the product specificity of human PRMT1 (12).

The concern over using affinity tags extends beyond just the PRMT field into the general biochemistry community. Small peptide tags such as Strep tag II or His6 tag are shown to influence the protein expression (13), protein dynamics (14) and crystallization (15). Although the effects of affinity tags are drawing increasing attention, His6-tagged or glutathione S-transferase (GST)-tagged PRMTs are still the most commonly-used PRMT constructs, with little understanding about the effects of affinity tags on protein features.

During our previous transient kinetic studies (Chapter 5), His-tagged rat PRMT1 did
not show any reliable binding curve data until it was pre-incubated with dithiothreitol (DTT). Stopped-flow rapid mixing measurement indicates a protein environment change of His-PRMT1 in the presence of DTT. Additionally, DTT was reported to significantly enhance the in vitro activity of GST-PRMT2 (16) and GST-PRMT7 (17). These observations bring us to question how the histidine-tag influences PRMT1, and whether the effect of DTT is on the intact PRMT protein or actually an artifact from the affinity tags. Herein, we report the studies based on the effects of the His-tag and DTT using the tagged and tag-free rat PRMT1 as our model protein. Our results indicate that the His-tag lowers the enzymatic activity and changes the oligomeric state of rat PRMT1, which can be partially recovered by the reducing agent DTT. PRMT1 is shown to play significant roles in oxidative-stress-induced apoptosis (18) and PRMT1 activity is upregulated with hydrogen peroxide or reactive oxygen species (ROS) (18, 19). PRMT1 may be regulated through oxidation under oxidative stress. Further experiments will focus on identifying the potential oxidative damages on rPRMT1, as well as the changes on downstream effects due to PRMT1 oxidation.

EXPERIMENTAL PROCEDURES

Materials

AdoMet was purchased from Sigma as a chloride salt (≥80%, from yeast). [methyl-3H]AdoMet was purchased from Perkin Elmer. All the peptides were synthesized by the Keck Institute (Yale University) and purified to ≥95%. The lyophilized peptides were dissolved in water. Peptide concentrations were determined by mass or by UV
spectroscopy ($\varepsilon_{280\text{ nm}} = 5,690 \text{ M}^{-1}\text{cm}^{-1}$). ZipTip® C4/C18 pipette tips were purchased from Millipore.

**Recombinant proteins**

His-tagged rat PRMT1 was expressed and purified as described (20). The plasmid expressing tagless rat PRMT1 was constructed by subcloning the rPRMT1 sequence into an NdeI and BamHI site within the pGEMEX-2-Nde1 vector (21). The tagless rPRMT1 was expressed similarly to His-rPRMT1 and purified as follows. Frozen cells were resuspended in 3x cell mass volume of lysis buffer (50 mM sodium phosphate pH 7.6 with 1 mg DNase) and subjected to freeze and thaw lysis 3 times. Soluble proteins were clarified by centrifugation at 20,000 RPM and the filtered supernatant was applied to a 5.0 mL HiTrap MonoQ anion exchange column (GE Healthcare). The fractions containing the tagless rPRMT1 were applied to a 1.0 ml HisTrap column (GE Healthcare), and finally a 1.0 mL HiTrap MonoQ anion exchange column (GE Healthcare) for additional separation and concentration. Purified proteins were ≥95% pure by SDS-PAGE. His-hnRNP K protein was expressed and purified as described previously (22). Protein concentrations were determined by UV spectroscopy and by the Bradford assay with bovine serum albumin as standard.

**Kinetic assays of PRMT1 constructs**

A sensitive methylation assay using ZipTip C4/C18 pipette tips was used in testing the enzymatic activity under steady-state conditions (22). Unless noted otherwise in the text, enzyme catalytic activity was tested with 100 nM PRMT1 constructs, 2 μM AdoMet (1 μM [$^3$H]AdoMet), and 10 nM MTAN (5′-methylthioadenosine /S-adenosylhomocysteine
nucleosidase) in 50 mM NaPO₄ (pH 7.6), initiated by 200 μM peptide substrates or 2 μM protein substrate at 37 °C. The effect of DTT was tested by pre-incubation for 10 min prior to reaction initiation with peptide or protein substrates. At different time points, samples were removed from reactions and processed with ZipTip_C4/C18 pipette tips (for protein or peptide substrates, respectively) to separate the unreacted [³H]AdoMet and the radiolabelled product.

Size exclusion chromatography

Samples (500μL at 1 mg/mL) of His-rPRMT1 and tagless rPRMT1 were analyzed in 50 mM sodium phosphate and 150 mM NaCl at 0.5 mL/min on a 24 mL gel filtration column. The effect of DTT on oligomerization was tested by pre-incubating the enzymes with the appropriate concentration of DTT for 30 min prior to loading.

RESULTS

DTT significantly enhances the enzymatic activity of His-rPRMT1

Dithiothreitol (DTT) is a common reducing agent used during purification and/or protein arginine methylation assays (16, 17), but the requirement by PRMT1 has never been assessed. To rationalize the need for DTT in PRMT1 methylation reactions, we first tested its effect on the enzymatic activity of His-rat PRMT1 (rPRMT1). Pre-incubation of His-rPRMT1 with 1 mM DTT increased methyl transferase activity by 13-17-fold with the R3 peptide substrate (acGGRGGFGGRGGFGGRGGFG) (Figure 6-1A). DTT-treated His-rPRMT1 also showed a 5-fold increase in activity with the hnRNP K protein substrate (data not shown). To understand such significant enhancement of His-rPRMT1 activity by DTT, we first examined the duration of this effect by removing DTT in the
reaction buffer with a desalting step following the 10 min pre-incubation. The activity of the desalted His-rPRMT1 was increased only by 2-fold using the R3 peptide (Figure 6-1A), indicating that the effect of DTT is transient.

We further studied the relationship between enzymatic activity and the concentration of DTT by comparing His-rPRMT1 activity using a wide range of DTT concentrations from 0.1 mM to 100 mM (Figure 6-1B). Our results indicate that the effect of DTT is concentration-dependent, achieving maximal methyl transferase enhancement at 1.5-2 mM DTT (apparent $k_{cat} = 1.94 \text{ min}^{-1}$) (Figure 6-1B).

**Maximum His-rPRMT1 activity requires a reductant**

By analyzing the structure of DTT and AdoMet (Figure 6-2A), DTT could increase the His-rPRMT1 activity through the sulfhydryl groups acting as an intermediate.

![Figure 6-1](image_url). The effect of DTT on the enzymatic activity of His-rPRMT1. (A) The effect of DTT on the enzymatic activity of His-rPRMT1 in the absence of DTT (closed circles), in the presence of 1mM DTT (closed squares), pre-incubated in 1mM DTT followed by a desalting step (open circles), and a control reaction with no substrates (open squares). (B) The apparent $k_{cat}$ was measured as a function of DTT concentration.
acceptor molecule as part of the methyl group transfer process. Methylation of sulfhydryl groups in DTT was previously observed with small molecule plant O-methyltransferases (23). Although we did not detect any DTT methylation in the control experiments (Figure 6-1A, open squares), we further tested this hypothesis with an alternative thiol-free reducing agent, Tris(2-carboxyethyl)phosphine (TCEP). When the activity of His-rPRMT1 in 1 mM TCEP was examined with the R3 peptide (Figure 6-2B), the observed rate enhancement in the presence of TCEP (1 mM) was identical to that observed in the presence of DTT (1 mM). Hence, the rate enhancement of His-rPRMT1 is due to the reducing power of the DTT or TCEP additives.

Figure 6-2. The effect of reducing agents on the ability of His-rPRMT1 to methylate the R3 peptide. (A) Structures of DTT, TCEP, and S-adenosyl methionine. (B) The comparison of enzymatic activity with 1 mM DTT (closed squares) and 1 mM TCEP (open circles), together with methylation rates measured with no DTT (closed circles) and no substrates (open squares).
DTT does not affect the enzymatic activity of tagless rPRMT1

In order to understand the observed rate enhancement of His-rPRMT1 by reducing agents, we questioned whether the effect of DTT was truly due to PRMT1, or was observed because of the addition of the His$_6$ affinity tag. To answer these questions, we generated and purified the tagless rPRMT1 construct (expressed with no tag). In the absence of DTT, the enzymatic activity of freshly prepared tagless rPRMT1 is higher than that of His-rPRMT1 with the R3 peptide substrate (shown as the apparent $k_{cat}$ in Figure 6-3). Therefore, despite of the small size of His-tag, the attached histidine-tag reduced the enzymatic activity of rPRMT1.

We further examined the effect of DTT on the catalytic activity of the tagless rPRMT1. Surprisingly, the activity of the tagless rPRMT1 did not show significant rate changes upon addition of 0.5 mM and 1 mM DTT (Figure 6-3). Such differences between His-rPRMT1 and tagless rPRMT1 indicate that the His$_6$-tag changes the protein characteristics of rPRMT1, possibly the oxidative states, which can be recovered by DTT or other reducing agents. A caveat to these experiments lies in the observed instability of the rPRMT1 expressed as the tagless construct; this recombinant protein could not be dialyzed or frozen without losing nearly all activity. Additionally, it is noted that the activity of tagless rPRMT1 is eclipsed by His-rPRMT1 when DTT is present in the reaction. This may suggest that PRMT1 expressed as a tagless construct incurs damage that is not recoverable by an exogenous reductant. Nonetheless, the differential effects that DTT has on His$_6$-tagged and tagless rPRMT1 enzymes led us to further investigate what protein characteristic(s) the His$_6$-tag alters to induce these observed effects.
Figure 6-3. The differential effects of DTT on the enzymatic activity of His-rPRMT1 (grey bars) and tagless rPRMT1 (black bars) with increasing DTT concentration.

Oligomerization of PRMT1 is influenced by the His$_6$-tag

DTT was previously found to alter the oligomerization pattern of FLAG-PRMT1 (24), but it is unclear whether or not the effect was due to the FLAG tag or an inherent quality of PRMT1. In order to investigate the reason for the different responses of His$_6$-tagged and tagless rPRMT1 to DTT, we first examined the oligomeric states of His$_6$-tagged and tagless rPRMT1 constructs via size-exclusion chromatography. A standard curve for the size-exclusion column was first achieved with high correlation efficiency (data not shown). In the absence of DTT, the tagless rPRMT1 elutes as a single peak at the same retention time as would be expected for a solution containing mostly the dimeric form (Figure 6-4A), while the His-tagged rPRMT1 eluted from gel filtration at an ultra-high molecular weight greater than 2000 kDa and various oligomers ranging from monomers through icosomers (20-mer) (Figure 6-4C). These distinct chromatograms of His-rPRMT1 and tagless rPRMT1 show that the His$_6$ tag, a tiny addition on PRMT1,
substantially alters the oligomeric state of rPRMT1.

**Oligomerization of His-rPRMT1 is influenced by DTT**

We further examined whether DTT influenced the oligomerization patterns of the His-tagged and tagless rPRMT1 differently. 1 mM or 10 mM DTT was pre-incubated with the proteins for 30 min. In the presence of DTT, the higher-order oligomers of His-rPRMT1 were dissociated and shifted towards lower molecular weight oligomers (Figure 6-4D, 4E). However, size-exclusion chromatogram of the tagless rPRMT1 in 1 mM DTT showed no change in the single peak retention time (Figure 6-4B). Our results indicate that His-tag leads to a distinct oligomerization pattern of His-rPRMT1 from the tagless rPRMT1, and such difference can be recovered to some extent by addition of DTT.
DISCUSSION

In this chapter, we investigated the effect of the histidine affinity tag and the reducing agent DTT. Overall, our results showed that the His-tag impairs activity of PRMT1 (Figure 6-3). Reductants (DTT or TCEP) increased the enzymatic activity of His-tagged rat PRMT1, but not the untagged rPRMT1. His-rPRMT1 shows a distinct oligomerization pattern with a large amount of high molecular weight oligomers which can be considered as protein aggregation (Figure 6-4). The aggregation of His-rPRMT1 can be partially removed by DTT. Yet the tagless PRMT1 mainly exists in the dimeric form, which does not change with the DTT treatment. Although the addition of affinity tags greatly eases the purification steps, it may significantly alter the protein characteristics, such as enzymatic activity and oligomerization patterns.

Our observations indicate an effect of the oligomeric states of PRMT1 on the enzymatic activity. A negative correlation was suggested between the high molecular weight oligomers of His-rPRMT1 (>2000 kD) and its enzymatic activity; and the dimeric tagless PRMT1 has higher activity than the multimers forms of His-PRMT1. Consistently, the dimeric form of tag-labeled PRMT1 was reported to be the active form of PRMT1 in vitro and in vivo (25, 26). Interestingly, the recent transient kinetic study of His-rPRMT1 showed that high-order His-rPRMT1 multimers (~250 kD) is the most active form in the range of 0-0.5 μM PRMT1 (27). Together with our observations, different oligomeric states of His-rPRMT1 are shown to correlate with distinct methylation activity. Notably, the size-exclusion chromatography in our research was performed under a high PRMT1 concentration (1 mg/ml, or 23.8 μM), which is significantly higher than the enzyme concentration used in the activity assays (0.1 μM). Due to the fact that the
oligomerization states can be influenced by the protein concentration, we also performed cross-linking experiments to determine the oligomeric state at 0.1 μM PRMT1 (data not shown). Similar effects of DTT were observed with His-PRMT1 in cross-linking experiments with the dimer form increasing as more DTT was added into the solution. Thus, our results indicated a connection between the oligomeric form of PRMT1 and its enzymatic activity.

We also noted that the enzymatic activity of His-rat PRMT1 continues to increase over that of the tagless rPRMT1 when higher DTT concentrations are present (Figure 6-3). This may in part be due to the observed low stability of rat PRMT1 purified as a tagless construct (data not shown). In fact, it prompted us to look at the human construct which is more easily expressed and purified with a His-tag that can be cleaved afterwards. With human PRMT1, the His-tag also decreases the enzymatic activity and appears to be associated with a change in oligomeric state, some of which can be restored by DTT (data not shown). Preliminary data suggested that DTT still has some effect on the cleaved hPRMT1 enzyme (data not shown) implying that some oxidative event may still exist the tagless enzyme which is associated with a decrease in activity, some of which can be recovered with reductant.

The fact that DTT changes the protein characteristics of rat and human PRMT1 leads us to hypothesize that PRMT1 incurs oxidative damage. Indeed, PRMT1 was identified as a target of the endogenous oxidative product 4-hydroxy-2-nonenal (HNE) (28); and HNE inhibited the catalytic activity of PRMT1 (29). The most likely oxidative damage occurs on cysteine residues, which can be oxidized to cystine generating a disulfide bond. Also the thiol group (-SH) in cysteine can be oxidized to the sulfenyl moiety (-SOH),
which can be reduced by many antioxidants in vivo, as well as DTT. The sulfenyl group can be further oxidized to the sulfinyl (-SO₂H), and sulfonyl moiety (-SO₃H), which cannot be reduced by DTT (reviewed in (30)). Thus, some of the 11 cysteine residues in His-PRMT1 may have been oxidized to Cys–SOH which further influences the enzymatic activity. Based on the crystal structure of PRMT1 (25), we hypothesize that Cys-101 may play a critical role in PRMT1 oxidation, which is located on the solvent accessible surface and makes direct contact with AdoMet. In a previous study, Cys-101 is suggested to be a potential oxidative site with heightened nucleophilic and reductive catalysis (or “hyper-reactive” cysteines) based on a C101A mutant (29). Site-directed mutagenesis is underway to generate a C101S mutant to investigate whether the DTT effect still occurs on C101S-PRMT1.

In all, our results showed that the affinity tag influences the enzymatic activity and protein oligomerization of His-PRMT1. These influences can be recovered by DTT, indicating possible oxidative damages on His-rPRMT1. Further experiments will be performed to find out the possible oxidative sites as well as the cause of the oxidative damages.

References


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CHAPTER 7
GENERATION AND SELECTION OF RNA APTAMERS TARGETING ASYMMETRIC DIMETHYLARGININE (ADMA)

ABSTRACT

Free asymmetric dimethylarginine (fADMA) is a potent inhibitor of nitric oxide synthase \textit{in vivo}. Increased plasma levels of fADMA are shown to be highly involved in cardiovascular and renal diseases. Recently, the concentration of plasma fADMA has been suggested as a novel cardiovascular risk factor to predict cardiovascular conditions in clinical trials \((1, 2)\). Thus, specific methods for quickly and quantitatively measuring the plasma fADMA level are valuable for diagnostic and therapeutic purposes. As an alternative to antibodies, RNA aptamers display high binding affinity and specificity against selected biological molecules. Herein, we aimed to develop RNA aptamers targeting ADMA. Systematic evolution of ligands by exponential enrichment (SELEX) was performed against ADMA-coated agarose beads. Binding specificity for the methylated guanidino moiety was controlled with the counter selection against arginine recognition. 20-30 selection cycles of SELEX were carried out. The binding affinity and specificity of the RNA pools generated in these cycles were tested via two well-accepted binding assays, an affinity chromatography and a filter-based binding assay. However, the binding results from these two assays did not agree with each other, with the possible reasons further discussed. Therefore, another novel scintillation proximity assay is under development in order to measure the ADMA-binding affinity and specificity of the RNA pools conveniently and quantitatively. Further experiments will be required to generate a
reliable binding assay for screening the selected RNAs and investigate the sequence and structural properties of the RNA aptamers.

**INTRODUCTION**

Free asymmetric dimethylarginine (ADMA), an analog of L-arginine, is an endogenous inhibitor of nitric oxide (NO) synthase (3) which is highly involved in cardiovascular and renal diseases (4-7). Free ADMA is released to the plasma after the arginine-methylated proteins are degraded *in vivo*. fADMA can be eliminated by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which hydrolyzes fADMA to dimethylamine and L-citrulline (8, 9). The reduction of DDAH activity under oxidative stress appears to be the main reason for increased ADMA accumulation (7, 10). Elevated plasma concentrations of fADMA have been observed in several clinical conditions including aging (11, 12), hypercholesterolemia (13, 14), hypertension (12, 15, 16), and diabetes (11, 17-19). A growing body of literature supports that the plasma fADMA level can be used as a cardiovascular risk factor to predict the cardiovascular outcome for patients (1, 2, 20-25).

Although the biological functions of both free and protein-bound ADMA are becoming well accepted, the analytical tools for quantitatively detecting ADMA remain limited. Often analysis of fADMA involves liquid chromatography/tandem mass spectrometry (LC-MS/MS), which is very precise and accurate for analyzing plasma or urine samples, but loses the sensitivity and specificity in small tissue samples (26). Also the process of LC-MS/MS analysis is time-consuming and laborious, which makes it difficult for clinical applications. HPLC analysis has also been used but is also
time-consuming and requires special equipment (27). A precise and sensitive method is required to handle a variety of sample matrices in a short amount of time.

Due to their unique and diverse conformations, RNAs are becoming important biopolymers for target recognition. The RNA aptamer technology has been broadly used for generating antibody-like molecules against selected targets (28). Compared to antibodies, RNA aptamers produced by chemical synthesis are conveniently available, of which the characteristics can be easily changed on demand by chemical modifications. Thus, aptamers become a convenient and valuable tool with high affinity and specificity for target molecules. Moreover, diagnostic and therapeutic approaches of RNA aptamers have been applied for diseases such as age-related macular degeneration (AMD) and inflammatory diseases (reviewed in (29)). Collectively, we decided to generate an RNA aptamer specifically targeting ADMA.

Aptamers are generated through the in vitro selection termed SELEX (Systematic Evolution of Ligands by EXponential enrichment) (30), which is a powerful tool to select nucleic acids with a desired property from libraries of synthetic DNA or RNA with up to $10^{12}$ different sequences and possible 3-dimensional structures (31). From the SELEX procedure, the initial library of oligonucleotides is screened by repeated selection and amplification based on the affinity and specificity for the target molecule so that selected aptamers are enriched after each cycle (Figure 7-1). The ADMA-binding affinity of the RNA pools was measured via the ADMA-resin binding reaction (Figure 7-1 Step c) as well as a modified filter-based binding assay. Both methods are commonly used in aptamer development. However, the binding percentage observed from the ADMA-affinity column did not agree with the binding affinity tested in the
membrane-based binding assay. Thus, we are developing a scintillation proximity assay (SPA) in order to minimize artifacts and measure the ADMA-binding affinity. The potential aptamers will be sequenced and further studied for their structural properties.

Figure 7-1. Scheme of the SELEX procedure. a) The initial ssDNA pool was transcribed into the RNA pool; b), specificity module (counter selection step) removes RNAs unspecifically binding to arginine residues and the agarose resin; c) affinity module (positive selection) collects RNAs bound to the ADMA resins; d-f) selected RNAs are recovered and reverse-transcribed to ssDNA, which is further amplified for the next cycle of selection.

**EXPERIMENTAL PROCEDURES**

**SELEX procedure: Target and Library Preparation**

N-hydroxysuccinimide (NHS)-activated Sepharose™ 4 Fast Flow resins (GE Healthcare) were used to immobilize the arginine or fADMA molecules. The coupling step was performed at 4 °C overnight in the coupling buffer (50 mM arginine or ADMA
in 0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3) according to the manufacturer’s procedure. The Arg- or ADMA-coated resins were packed into columns for further RNA selection.

The initial single strand DNA (ssDNA) libraries were purchased, or received as a generous gift from the Ellington Lab (UT Austin, TX). Each element of the ssDNA random library was composed of 35 or 40 randomized nucleotides flanked by 5’ and 3’ primer sites designated as N35 or N40 ssDNA library, respectively (i.e. 5’-CACAGCGGGACAGTTTAGC-[N35]-GGTAGGTGGGTGCGTCTAAA-3’). Each ssDNA library was amplified by PCR and then transcribed to generate the corresponding RNA random pools using an AmpliScribe™ T7-Flash™ Transcription Kit (Epicentre Biotechnologies, Madison, WI). *In vitro* transcription reactions took place at 42 °C for 2.5-3 hours or at 37 °C for 6 hours, and the DNA template was then digested using Turbo DNase I (Ambion, Austin, TX).

The RNA library was purified by urea-polyacrylamide gel electrophoresis (PAGE) followed by gel extraction and ethanol precipitation. The targeted RNA band in the urea-PAGE gel was visualized under a hand-hold UV illuminator on the top of a thin layer chromatography (TLC) plate, thanks to the fact RNAs block the fluorescence of TLC plate under UV light. The targeted RNAs were located and removed from the gel. The sliced gel pieces were then crushed in 400 µL of 300 mM NaOAc, stirred vigorously with Vortex Genie® 2 Shaker, and heated at 75 °C for 5-10 min for a sufficient RNA extraction. The gel pieces were then removed by centrifuging at 14,000 RPM for 3-5 min and washed with another 600-800 µL of 300 mM NaOAc. The combined supernatant from each washing step was further filtered with the centrifugal tube filters (2.2 μm). The
purified RNA was then precipitated from the filtered supernatant using 100% EtOH and trace amount of glycogene at -80 °C for >20 min, centrifuged at 14,000 RPM for 40 min. The RNA pellet was then washed with 70% EtOH and dried by Savant SpeedVac Concentrator (Thermo). The dried RNA pellet was further resuspended in 20 μL of diethyl pyrocarbonate (DEPC)-treated H₂O with the final RNA concentration determined by NanoDrop (Thermo Fisher Scientific).

**SELEX procedure: Affinity and specificity modules**

Both arginine and ADMA columns were rinsed with 2 mL of binding buffer (50 mM HEPES, 250 mM NaCl, 5 mM MgCl₂, pH 7.5). The resuspended RNA pool was denatured by heating at 65 °C for 10 min within 100 μL of binding buffer and then cooled to room temperature (RT) for recovery. The recovered RNAs were loaded onto the ADMA column followed by light agitation and incubation for 0.5-1 hour at RT. Unspecific binding events were mostly eliminated by several washing steps with binding buffer. Tightly binding RNAs were then eluted using 3 volumes of 6 mM fADMA elution buffer and concentrated by EtOH precipitation and resuspension. The RNA binding percentage of each cycle was then calculated based on the amount of RNA eluted from the ADMA column over the total amount of RNA loaded onto the column.

Every three cycles, the Arg-column was used as the specificity module, to avoid accumulating sequences that unspecifically bind to agarose beads or arginines (instead of the methylated guanidino moiety). In this negative selection, RNA pools from gel extraction were directly loaded on the Arg-column. Solution from flow-through and three washing steps was collected and processed through the ADMA-column for a following
positive selection as described above. After each counter selection step, the RNA binding percentage dropped dramatically. The eluted RNAs were then reversed transcribed with SuperScript® Reverse Transcriptase (Invitrogen) into ssDNA. Each SELEX cycle finished with a PCR amplification and EtOH clean-up.

**Filter-based binding assay development**

To confirm the RNA binding percentage obtained by affinity chromatography (the ADMA column binding), another approach was utilized, derived from a commonly-used membrane binding assay. RNA aptamers were labeled with radioactive $\alpha$-$^{32}$P-ATP during *in vitro* transcription. Peptides derived from the N-terminus of the histone H4 protein containing arginine, MMA or ADMA at Arg-3 (SGRGKGGKGLGKGGAKRHRKV) were selected as a bona fide substrate for binding assays (250 $\mu$M peptides in 100 $\mu$L of 50 mM sodium phosphate buffer pH 7.6). Due to the positively-charged characteristic of the H4 peptides, the labeled RNAs bound to the H4 peptides were separated from the unbound RNAs through a 0.45 $\mu$m pore-size negatively-charged nitrocellulose membrane (Millipore, MA) to capture the peptides together with the bound RNAs. Another piece of positively-charged nylon membrane was placed below the nitrocellulose membrane in order to seize all the unbound RNA as a positive control. All the binding assays were performed with a 48-well Bio-Dot SF Apparatus (Bio-Rad Laboratories). The membranes were then quickly washed three times with 1 mL of the TBE buffer, removed from the apparatus, and dried in the hood. The amount of radioactivity on the membrane was measured by autoradiograph.

**Cloning of the selected RNA**
Based on the binding assays stated previously, SELEX cycles N35-R10, N35-R14, and N40-R10 were selected for cloning and further sequencing. ssDNA pools from these cycles were processed with the NcoI restriction enzyme, and cloned into the *E. coli* XL1-Blue cells using the pGEM plasmid kit (Invitrogen) to create a DNA library. Three to five colonies from the transformation of each DNA pool were randomly picked. Selected aptamers were extracted and purified with the Qiagen Plasmid Mini Kit (Qiagen) for the future sequencing experiments.

**RESULTS AND DISCUSSION**

**Overview of the SELEX process**

In the SELEX process, two libraries of single strand DNA (ssDNA) molecules, N35 and N40, were used as the initial pools, consisting of a random 35- or 40-nucleotide region flanked by constant primer regions, respectively. Starting with the transcribed RNA pools, affinity chromatography, as a canonical method to select aptamers, was applied with the columns derivatized with ADMA or arginine. The ADMA column was used as affinity selection. The amount of RNA molecules eluted with ADMA was measured, based on which the RNA binding percentage of each cycle was calculated to monitor the aptamer enrichment. For every 2-3 rounds of affinity selection and amplification, counter selection was employed for binding specificity control with the arginine column. Non-binding RNAs in the flow-through of the arginine selection were directly loaded to the ADMA-affinity column in order to gain aptamers only binding to the methyl guanidino moiety of ADMA.

After 8 or 9 rounds of SELEX process, the population of the ADMA-specific
aptamers started to increase for both the N35 and N40 libraries (Table 7-1, data not shown for the first 6 or 8 rounds). In fact, even when the counter selection was employed, 7-8% of the applied RNAs eluted with ADMA at Round 10 for both N35 and N40 libraries. By reasoning that the relatively high ADMA concentration in the affinity column could lead to enrichment of unspecific or weak binding RNAs, the ADMA column was diluted by 10-fold to select aptamers with higher binding affinity. Such dilution leads to a dramatic drop in the ADMA-eluted fraction. The binding percentage never exceeded more than 2% in the following five rounds (Table 7-1).

As a commonly-used selection method in SELEX, affinity chromatography monitors the RNA binding affinity and specificity by measuring the RNA binding percentage after the positive and/or negative selection. In the growing body of literature about aptamers targeting various biological molecules, the RNA elution percentage was observed to be varied from as low as 2% (32) to more than 50% (33). Therefore, no rule of thumb can be

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*NA: Not available

Table 7-1. The binding percentage of RNA aptamers from each round with or without the counter selection with arginine-column.
concluded for the RNA binding percentage in order to judge whether the SELEX cycle has been performed enough times. Considering the high binding percentage after the negative selection, ssDNA pools from N35-R10 and N40-R10 (bold faced in Table 7-1) were selected as the top choices for further binding and sequencing experiments.

**Filter-binding assays with selected SELEX cycles**

From the SELEX process, RNA libraries were created in each cycle, of which the binding affinity and specificity were measured against fADMA. Due to the fact that the affinity chromatography only measures the concentrations of the applied and eluted RNA molecules based on UV absorbance, the accuracy of such measurement is questionable especially under the low binding affinity after Round 12 for both libraries. To better approximate the fraction of ADMA-bound aptamers after each round, we attempted to modify a conventional nitrocellulose-binding assay, which utilizes a piece of nitrocellulose filter membrane to lock the proteins, and therefore, bind the RNA/protein complexes with the unbound RNAs washed out.

In order to confirm that our peptides containing methylarginines would be locked by the nitrocellulose membrane, we first compared the binding efficiency of the nitrocellulose membranes with the well-developed ZipTip® assay (34). Methylation reactions were performed with the tritiated S-adenosyl-L-methionine (3H-AdoMet) along with the H4-21 peptide substrate, the histone H4 protein, and the hnRNP K protein. The radioactivity incorporation measured by the ZipTip® assay was used as the standard value to assess the binding efficiency of nitrocellulose membranes with different pore sizes and different washing procedures (Figure 7-2). Briefly, the amount of radioactivity retained
on the 0.45 μm nitrocellulose membrane with a more stringent washing procedure (light grey bars) was the closest to what obtained in the ZipTip® assay (crossing bars). Due to the positive charge on AdoMet, the nitrocellulose membrane tends to bind both peptides/proteins and the methyl group donor ³H-AdoMet, which probably leads to the higher radioactivity observed in all nitrocellulose binding assays than in the ZipTip® assay. Therefore, a more rigorous washing procedure was required to remove the bound ³H-AdoMet and lower the inaccurately high radioactivity (Figure 7-2, light vs dark grey bars). Moreover, the nitrocellulose membranes with smaller pore sizes (0.2 μm) also lead to a high degree of AdoMet binding which was difficult for further removal in the

Figure 7-2. The binding efficiency of nitrocellulose membranes with different pore sizes and different washing procedures. Separate methylation reactions were performed with ³H-AdoMet and the H4-21 peptide, the histone H4 and hnRNP K proteins. Peptides or proteins were isolated and analyzed with ZipTip® assays, and nitrocellulose membranes (0.2 μM or 0.45 μM). Two washing procedures were performed to remove bound ³H-AdoMet on the nitrocellulose membranes, one with 1 mL of 50 mM sodium phosphate buffer (pH 7.6) (0.45 μm-2, dark grey bars), the other with 2 mL of 50 mM sodium phosphate buffer (pH 7.6) with 100 mM NaCl (black and light grey bars).
washing steps. Therefore, the nitrocellulose membrane (0.45 μm) was chosen for further aptamer selection with a stringent step-wise washing procedure.

In our modified binding assay, RNA aptamers from selected SELEX cycles were labeled with α-32P-ATP in the \textit{in vitro} transcription step for accurate quantification. The negatively-charged nitrocellulose membrane was used to retain peptides or ADMA/arginine coated resins and isolate target-binding RNAs from non-binding RNA molecules. Moreover, a piece of positively-charged nylon membrane was placed underneath to capture the free RNA aptamers flowing through the nitrocellulose membrane in order to gain the total amount of RNA (Figure 7-3A). The 32P-labeled RNA library was incubated with the target peptides or resins on the nitrocellulose membrane. All binding assays were performed in a 48- or 96-well apparatus connected to the vacuum manifold (Figure 7-3A). Different from the SELEX process, solutions from \textit{in vitro} transcription were directly loaded onto the membrane without the gel-purification step in order to minimize the radioactive waste. Followed by the washing procedure, the radioactivity retained on both membranes was then detected and measured by autoradiography (Figure 7-3B).

From the autoradiogram of the nylon membrane, the amount of free 32P-RNAs can be estimated as a positive control to confirm that a similar amount of RNA molecules was generated in the \textit{in vitro} transcription step with ssDNA libraries from different SELEX cycles. The radioactivity retained on the nitrocellulose membranes identified the RNA libraries that bound to the specific targets. Figure 7-3B clearly showed that most RNAs directly flow through the nitrocellulose membrane without binding to the Arg- or ADMA-resins. RNAs from some SELEX cycles showed high binding affinity towards
Figure 7-3. Nitrocellulose filter binding assay. (A) Scheme of the filter-binding assay performed in the 48-well apparatus. (B) Autoradiographs of the nitrocellulose and nylon membranes with arginine- or ADMA-coated beads. The SELEX cycles tested are labeled on both membranes for the N35 and N40 libraries.
arginine instead of ADMA, such as Round 10, 12, and 16 from the N35 library. Surprisingly, RNAs from Round 7 with the N40 library showed a high ADMA-binding affinity and no arginine-binding property, indicating a high ADMA-binding specificity (circled in Figure 7-3B).

More filter-binding assays were performed with promising SELEX cycles to confirm the observed high ADMA-binding specificity. However, results from repeated assays were not clear or reproducible (data not shown). The radioactivity signals on the nitrocellulose membrane were difficult to distinguish from the background noise, especially when peptidyl-ADMA was used as the binding target instead of the ADMA-beads. More importantly, the binding affinity tested by the nitrocellulose-binding assay was different from the results of affinity chromatography. For example, Round 10 from both N35 and N40 libraries showed a high RNA binding percentage with the ADMA-column after the arginine selection (Table 7-1). However, the nitrocellulose membrane for these two SELEX cycles (Figure 7-3B) retained either little amount of radioactivity (N40-R10) or a large amount of $^{32}$P-RNA bound to arginine-beads (N35-R10). Such a difference between two binding assays might result from the unpurified RNA pools tested in the nitrocellulose-binding assay. Without the gel-purification step, solutions from in vitro transcription contain the RNA primers, initial DNA pools, and enzymes, which can potentially influence the RNA binding events. Moreover, the binding efficiency and specificity of the nitrocellulose membranes remains unclear, especially with peptides surrounded by a large amount of RNAs. Although it is shown the methylated H4-21 peptides could be captured by the nitrocellulose membrane (Figure 7-2), the existence of the RNA pool along with other components could
potentially mask the positive charges on the peptide leading to inefficient binding. Therefore, the binding affinity measured by the affinity chromatography might be more accurate, despite the fact that its RNA quantification method based on UV absorption is not as sensitive or accurate as radioactivity incorporation used in filter-binding assays.

**CONCLUSION**

As a significant metabolite, fADMA is involved in many biological pathways. Dysregulation of the plasma fADMA concentrations is observed in various human diseases. Therefore, it is important to develop a convenient analysis tool to quickly detect and measure the amount of fADMA in various samples. Herein, we determined to generate RNA aptamers specifically targeting ADMA. RNA libraries were generated from SELEX cycles, of which the binding affinity and specificity was analyzed by affinity chromatography and filter-binding assay. Despite of the technical difficulties in both methods, ssDNA pools from N35-R10, N35-R14 and N40-R10 were cloned for further sequencing.

To obtain a more accurate and reproducible binding result, the scintillation proximity assay (SPA) is currently under development with radiolabeled RNAs targeting the ADMA-coated SPA beads. With the SPA, we can spontaneously screen the binding affinity of RNA aptamers and measure the binding affinity of selected RNA pools. Future experiments will focus on obtaining promising RNA aptamer sequences and looking for the structural details of the binding event. Further investigations can be performed to generate a diagnostic or analysis tool based on the ADMA-targeted aptamer.

**References**


CHAPTER 8

SUMMARY AND FUTURE DIRECTIONS

For all types of post-translational modifications (PTMs), it is essential to control the specificity for accurately regulating the downstream effects. As one of the significant post-translational modifications, protein arginine methylation, catalyzed by protein arginine methyltransferases (PRMTs), is involved in a wide variety of fundamental cellular pathways and human diseases (reviewed in Chapter 2). As the predominant PRMTs in vivo (1), PRMT1 has broad substrate specificity, with many protein substrates containing multiple methylation sites. Moreover, PRMT1 was shown to generate a distinctive MMA−ADMA pattern in vivo, with some arginines fully dimethylated and others only mono- or partially dimethylated (2-4). Importantly, the biological consequence of arginine methylation is determined by the degree (mono- /dimethylation) and type of arginine dimethylation (asymmetric/symmetric). Thus, a fine-tuned regulation is required for the diverse, yet high product specificity of PRMT1; however the current knowledge of the fundamental regulation of PRMT product specificity remains limited.

A strong foundation has been established by this work in understanding the molecular origins of the product specificity of PRMT1 and the kinetic mechanism regulating product generation. For each project, summary and future directions along with existing preliminary data are described below.

The conserved methionines in the active site of PRMT1 specify MMA, ADMA, and SDMA formation.
In protein lysine methylation, the Phe/Tyr “switch” model has been well studied to regulate mono-, di-, and trimethylation in lysine methyltransferases (5-7), which leads us to believe that a similar model of a single residue altering product specificity could exist in arginine methyltransferases as well. By dissecting the active site of PRMT1, we identified that two conserved active site residues, Met-48 and Met-155, play a significant role in enzymatic activity and product specificity (Chapter 3). Both Met-48 and Met-155 regulate PRMT1 mono- versus asymmetric dimethylation (see Figure 3-2). Using single turnover experiments to analyze each methyl transfer step separately, Met-48 shows a differential effect on the first and second methylation rates, where the turnover of monomethyl peptides is severely impaired leading to the accumulation of final MMA products (see Figure 3-5). Thus investigation of the two conserved active site methionines provided the first insight into the intrinsic regulation of MMA versus ADMA formation (8).

Met-48 discriminates between ADMA and SDMA formation — Before the crystal structure of PRMT5 was solved, sequence alignment of Type I and II PRMTs indicates that the equivalent residue of Met-155 was serine, a smaller amino acid. Therefore, Met-155 was previously hypothesized to specify ADMA and SDMA formation between Type I and II PRMTs by providing steric hindrance in the active site (9-11). However, M48L-, M48A-, M155L-, and M155A-PRMT1 exclusively generate MMA and ADMA (8, 12). When the crystal structure of human PRMT5 was solved (13), Phe-397 was found at the same location in the active site of PRMT5 as Met-48 in rat PRMT1 (see Figure 2-5). By western blotting, a F397M-PRMT5 mutant was shown to generate both ADMA and SDMA (13). Although we did not observe SDMA formation with M48L or
M155A mutants previously, we further generated M48F-PRMT1 mutant and analyzed the product formation with trace amount of tritium incorporation by reverse phase HPLC. A small amount of the tritium-labeled products eluted at the retention time of SDMA (data not shown). We further confirmed SDMA formation by electron transfer dissociation (ETD) and orbitrap mass spectrometry (MS) with our new collaborator Dr. Qu (at SUNY Buffalo). ETD-MS is capable of accurately locating the methylation site and distinguishing between asymmetric and symmetric dimethylation (14). ETD-MS analyses proved that M48F-PRMT1 generates MMA, ADMA, and a very small amount of SDMA (Table 8-1). Thus, our results indicate that Met-48 dictates ADMA synthesis over SDMA. By characterizing the two conserved methionines in the PRMT1 active site, Met-48 and Met-155 are shown to govern the product formation of rat PRMT1 specifying MMA/ADMA generation, as well as ADMA/SDMA formation.

Table 8-1. Product analysis of M48F-PRMT1 by electron transfer dissociation and orbitrap mass spectrometry (the SDMA-peptide was squared).

<table>
<thead>
<tr>
<th>Sequence</th>
<th>m/z (3+)</th>
<th>Relative abundance (%)</th>
<th>S/Δcorr</th>
<th>Mass error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methylation product of M48f</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGR*GGFGRGFGGGGGFGF</td>
<td>575.949</td>
<td>8.19</td>
<td>0.95/6.46</td>
<td>1.70</td>
</tr>
<tr>
<td>GGRGGFGRG*GGFGRGGGFG</td>
<td>575.949</td>
<td>2.55</td>
<td>0.88/5.20</td>
<td>1.70</td>
</tr>
<tr>
<td>GGRG(a)GGFGRGFGGRGFGF</td>
<td>580.621</td>
<td>2.35</td>
<td>0.93/6.00</td>
<td>3.60</td>
</tr>
<tr>
<td><strong>GGRG(a)GGFGRGFGGRGFG</strong></td>
<td>580.621</td>
<td>0.10</td>
<td>0.93/4.20</td>
<td>3.60</td>
</tr>
<tr>
<td>GGRG(a)GGFGRGFGGRGFGF</td>
<td>585.293</td>
<td>0.10</td>
<td>0.80/6.18</td>
<td>6.45</td>
</tr>
<tr>
<td>GGRG(a)GGFGRGFGGRGFG</td>
<td>585.293</td>
<td>0.02</td>
<td>0.85/0.98</td>
<td>4.64</td>
</tr>
</tbody>
</table>

| **Methylation product of WT** |         |                        |          |                 |
| GGR*GGFGRGFGGGGGFGF  | 575.949  | 7.69                   | 0.94/6.42 | 1.80            |
| GGRGGFGRG*GGFGRGGGFG  | 575.949  | 3.96                   | 0.89/5.34 | 1.84            |
| GGRG(a)GGFGRGFGGRGFGF  | 580.621  | 0.12                   | 0.86/5.57 | 3.61            |
| GGRG(a)GGFGRGFGGRGFG  | 580.621  | 1.68                   | 0.93/6.05 | 4.10            |
| GGRG(a)GGFGRG*GGFGRGFGF  | 580.621  | 0.06                   | 0.87/5.30 | 4.24            |
| GGRG(a)GGFGRG*GGFGRGFG  | 585.293  | 0.11                   | 0.93/6.40 | 6.52            |

Only intact peptide has been counted; the Xcorr vs Charge State is 1.8(1+), 2.3(2+), 3.3(3+), 4.0(4+) for both CID and ETD activation. S/Δcorr = 0.85 for ETD, Probability = 0.05 for CID; *indicating mono-methylation; #indicating di-methylation, and (a) indicating aDMA, (s) sDMA.
Computer simulation to understand the energetics of the PRMT1 product specificity

To further understand the product specificity of PRMT1 as well as changes of the product specificities as a result of M48F mutation, we pursued computer simulations to examine the energy origin for the product specificity of wild type PRMT1 and the M48F mutant. This approach has been applied for studies with protein lysine methyltransferases (PKMTs) suggesting that, at least in some cases, the energetics of the methyltransfer reactions may determine the product specificity of PKMTs (6, 15-17). However, no such research has been done with PRMTs. We are collaborating with Dr. Acevedo (Auburn University) to perform the hybrid quantum mechanical/molecular mechanical (QM/MM) free energy simulations for methyl-transfer processes in PRMT1 and M48F-PRMT1. Due to the fact that the N-terminal helix may be significantly involved in catalysis, yet the current crystal structure of PRMT1 does not show electron density of that helix (see Figure 2-6), Dr. Acevedo and his student built in the N-terminal helix to supply residues to the active site (Figure 8-1). Also, the original crystal structure lacks the electron density of the whole peptide substrate, except for the single arginine bound in the active site (9). Therefore, modeling the entire peptide substrates in is currently underway for future simulation. After the preparation is finished, the energy barrier for Arg $\rightarrow$ MMA and MMA $\rightarrow$ ADMA will be calculated as well as the changes from M48F mutation in order to understand how the energy landscape contributes to the product specificity of PRMT1.

Identification of the automethylation site(s) in M48L-PRMT1 — We previously reported the automethylation characteristics of the M48L and M155A mutants (Figure 3-3), indicating a change in the PRMT1 product specificity. Due to the fact that PRMT1
Figure 8-1. The N-terminal helix of rat PRMT1 was built in the existing crystal structure (1OR8) for further investigation. The modeled region is shown in orange, with the original crystal structure of PRMT1 shown in cyan.

prefers arginine residues within the “RGG” or “RXR” canonical sequences in an unstructured loop (18-20), we hypothesize that Arg-353 is likely to be methylated as it is in the “RMR” sequence and the last amino acid on the C-terminus of PRMT1. To identify the position of automethylation, we generated M48L-R353K mutant to investigate whether automethylation of M48L disappeared with the R353K mutation. After M48L-R353K was generated, its enzymatic activity was first tested with the R3 peptide (acGGRGGFGGRGGFGGRGGFG), which showed about one third of the methylation rates compared to M48L (data not shown). Automethylation assays were then performed as described in Chapter 3. The amount of tritium incorporation reduced by >90% when Arg-353 was mutated to lysine (Figure 8-2A), indicating that Arg-353 is likely the automethylation site for M48L-PRMT1.

Product specificity changed with the R353K mutation in M48L-PRMT1 — We made the interesting observation that M48L-PRMT1 can barely methylate the arginine residue
at the center of the peptide substrate (Table 3-4), such as the KRK peptide (acKGGFGGRGGFGGKW). We further tested whether the M48L-R353K mutant maintains such substrate recognition ability with the KRK peptide and the RKK peptide (acGGRGGFGGGGGFGGKW). Surprisingly, M48L-R353K cannot methylate either the RKK peptide or the KRK peptide (see Figure 8-2B), suggesting that M48L-R353K cannot recognize the arginine residues on the N-terminus or at the center of the single-arginine containing peptide. Considering that M48L-R353K shows a relatively good activity with the R3 peptide, we hypothesize that M48L-R353K has a high preference to the C-terminal arginines, or the activity of M48L-R353K requires multiple arginine residues in the peptide substrates. To answer these questions, we ordered the KKR peptide with a single arginine on the C-terminus. Further experiments will be performed to find out the product specificity of M48L-R353K. If M48L-R353K does show a C-terminal preference, the product specificity of M48L-R353K is completely

![Graph](image)

Figure 8-2. Automethylation (A) and product specificity investigation (B) of M48L-R353K compared to M48L. Both the automethylation and the activity assays were performed the same as described in Chapter 3.
reversed from wild type PRMT1 or M48L, which has a strong preference to the N-terminal and center-located arginines (Table 3-3). A single mutant R353K-PRMT1 is also generated to investigate whether the change in product specificity is due to the R353K mutation or is a synergy from both the M48L and R353K mutations. From the crystal structure of PRMT1 (9), Arg-353 is ~17 Å away from Met-48 and the active site, and ~13 Å away from the putative peptide binding grooves. Our observations indicate that the distal residues in PRMT1 also have an effect on the enzymatic activity and substrate recognition.

**The kinetic mechanism of PRMT1-catalyzed dimethylation**

We previously showed that the conserved active site residues govern the deposition of methyl marks (MMA, ADMA, or SDMA); however it is not sufficient to explain the distinctive methylation pattern of PRMT1 *in vivo*, as well as substrate recognition of PRMT1 among multiple arginines. We further explored the product specificity of PRMT1 from a substrate-centered perspective (Chapter 4). With multi-arginine peptide substrates, we found that PRMT1 shows less preference to the C-terminal arginines, similar with the physiological methylation patterns observed *in vivo*. With a single targeted arginine, we identified that the amino acids flanking the substrate arginine influences the final MMA or ADMA status. To conveniently study the processivity of PRMT1-catalyzed dimethylation, which also influences the MMA and ADMA distribution, double turnover experiments were designed based on single turnover experiments. Results showed that PRMT1 dimethylates the peptide substrates in a semi-processive manner, consistent with the rapid equilibrium random mechanism
proposed by Obianyo and co-workers (21). Interestingly, the degree of processivity is dependent on the substrate sequences, which explained the controversial observations between the distributive and partial processive mechanism of PRMT1 based on two different peptide substrates (21, 22). Thus, our results recognize a novel substrate-induced mechanism for modulating PRMT1 product specificity (23).

*Single turnover experiments to understand the semi-processivity of PRMT1—* Among the handful of publications regarding kinetic mechanisms of PRMT1, our research (23) and steady state data from Thompson and colleagues (21) suggest a partially processive or semi-processive mechanism; while a recent transient kinetic study (24) proposed a completely dissociative mechanism (reviewed in Chapter 5). To understand the controversy, we further investigated the PRMT1 mechanism using transient kinetics and single turnover experiments to understand the microscopic steps in PRMT1-catalyzed methylation reactions (Chapter 5). Single turnover experiments with the eIF4A1 peptide pair indicate that the unmodified peptide is a slightly better substrate than the monomethyl substrate under single turnover conditions (see Table 5-2).

We previously observed that different peptide substrates have distinct degrees of processivity (see Table 4-5). What leads to the different degrees of processivity? Is it due to the methylation rates of the first and second methyl group transfer? If a peptide has a higher methylation rate of the MMA→ADMA step than the Arg→MMA step, it would probably show a higher degree of processivity. In the transient kinetic analysis of PRMT1 (24), the 2nd methylation step (MMA→ADMA) is shown to be much slower than the 1st step (Arg→MMA) with a fluorescein-tagged H4 peptide. However, in our studies, the untagged H4-21 peptide shows a high degree of processivity (see Table 4-5). To answer
these questions, the methylation rates of other peptide pairs were further tested with single turnover experiments. Same protocols were followed as stated in Chapter 5 with reactions initiated by peptide substrates. Two typical single turnover curves from the H4-21-CH₃ and RKK-CH₃ peptide were shown in Figure 8-3. Combined with previous data (Chapter 3, 4, and 5), Table 8-2 summarizes the maximal product concentration $A_0$ and methylation rate $k_{obs}$ of all four peptide pairs (except for the H4-21 peptide, of which

![Figure 8-3. Single turnover experiments of PRMT1 with monomethylated H4-21 and RKK peptide substrates. The data were fitted into equation: $y = A_0 \times (1 - \exp(-b^x))$.](image)

Table 8-2. Different peptide substrates lead to distinct chemistry rates for the first and second methylation steps.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Arg $\rightarrow$ MMA</th>
<th>MMA $\rightarrow$ ADMA</th>
<th>MMA: ADMA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_0$ (µM)</td>
<td>$k_{obs}$ (x10^2 s⁻¹)</td>
<td>$A_0$ (µM)</td>
</tr>
<tr>
<td>H4-21</td>
<td>/</td>
<td>/</td>
<td>15.2 ± 0.6</td>
</tr>
<tr>
<td>eIF4A1</td>
<td>10.8 ± 0.3</td>
<td>3.8 ± 0.1</td>
<td>13.0 ± 0.3</td>
</tr>
<tr>
<td>KRK</td>
<td>20.3 ± 0.3</td>
<td>2.0 ± 0.1</td>
<td>15.0 ± 1.1</td>
</tr>
<tr>
<td>RKK</td>
<td>14.5 ± 1.8</td>
<td>7.3 ± 3.7</td>
<td>13.1 ± 0.8</td>
</tr>
</tbody>
</table>
the tritium incorporation was too scattered to achieve a reliable single turnover curve). Based on the preliminary data here, we cannot find any clear clues of the relationship between the degree of processivity and the methylation rates of each methyl group transfer step. Thus, the substrate-dependent processivity is uncoupled with the methyl transfer rates, which is more complex than previously thought.

**Transient kinetic study using stopped-flow measurements to understand PRMT1-AdoMet and PRMT1-peptide interactions** — To gain more insight into the microscopic steps in PRMT1-catalyzed dimethylation, stopped-flow rapid mixing was applied to measure the rate of AdoMet or peptide binding based on the intrinsic fluorescence of PRMT1. Surprisingly, no reliable binding curve can be achieved in the absence of the reductant dithiothreitol (DTT) (Chapter 5, data not shown). In the presence of DTT, AdoMet binding process showed two phases, which may indicate the initial binding step and a conformational change after the PRMT1-AdoMet complex formed (see Figure 5-3). As we are now aware that the His₆-tag influences the oligomerization states of PRMT1, which can be partially recovered by DTT (Chapter 6), the same stopped-flow measurements will be performed with the tagless PRMT1 to find out the real $k_{on}$ and $k_{off}$ for AdoMet and peptide binding.

**The differential effects of DTT in steady-state kinetics and single turnover experiments** — To understand more about the DTT enhancement of the enzymatic activity of rat PRMT1 under steady state (see Figure 6-1), single turnover experiments with the addition of DTT was performed using the eIF4A1 peptide with His-rPRMT1 pre-incubated with 1 mM DTT. Surprisingly, barely any turnover can be detected in the presence of DTT under the single turnover conditions (data not shown). It is unknown
why DTT increases the activity under steady state (low enzyme, high peptide concentration), yet diminishes the methylation activity under single turnover conditions (high enzyme, high peptide). One hypothesis is that some PRMT1 inhibitors may co-purify with His-rPRMT1 which can be activated by the reductant DTT. Under low PRMT1 concentrations (0.1 µM), inhibitors may also be too dilute to impede the reaction. When high concentrations of PRMT1 (21.0 µM) were used in the single turnover experiments, the amount of inhibitors also greatly increased, sufficient to eliminate the enzymatic activity of PRMT1. Also, single turnover experiments were performed at 22 °C, while the steady state kinetic studies were at 37 °C. Different temperatures may also influence the effect of DTT and/or the inhibitors.

The oxidative damage in His-rPRMT1 — As the protein characteristics of PRMT1 can be partially revived by DTT, we hypothesize that His-rat PRMT1 incurs oxidative damage, which likely influences the enzymatic activity and protein oligomerization (discussed in Chapter 6). To further examine the protein oxidation, Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) was used to quantitate the free thiol groups in the protein (25). Based on the preliminary data, two (2) of the 11 cysteines in rat PRMT1 can be detected on the solvent accessible surface of His-rPRMT1 (data not shown). Further experiments will be performed to quantitate the sulfenyl groups (-SOH) in His-rPRMT1. Site directed mutagenesis is currently underway to target the role of single cysteine residue in rPRMT1 oxidation.

Overexpression of PRMT1 and enhancement of PRMT1 activity was observed under oxidative stress, correlated with the accumulation of intracellular reactive oxygen species (ROS), and eventually enhanced oxidative stress-induced apoptosis (26, 27). Oxidative
stress was suggested as one of the stimuli regulating PRMT1, of which the mechanism remains uncertain (28). Due to the fact that protein cysteine oxidation is a significant pathway for cellular signal transduction (reviewed in (29)), PRMT1 may be regulated by oxidative stress through selective oxidation of Cys-101 or other cysteine residues. Therefore, it will be of great significance to identify the oxidation sites of PRMT1 and the downstream effects of PRMT1 oxidation.

Other ongoing projects

_The amino acids flanking the substrate arginine influences the steady state kinetics and product formation of PRMT1_—We previously identified the effect of flanking amino acid sequence on methylated product distribution (see Figure 4-3). Peptides with the canonical “RGG” amino acids sequence were fully dimethylated, while the peptides with “RYG” or “RSG” were only monomethylated or partially dimethylated. Besides the effect on the end-product formation, the flanking amino acid sequences also influence the steady state characteristics of PRMT1. The RGA peptide (acKGGFGGRGAFGGKW) and RGA-CH₃ peptide (acKGGFGGRmeGAFGGKW) were tested to identify if the sequence context near the substrate arginine dictated how the substrate was methylated. The RGA-peptide (see Figure 8-4) and the RGA-CH₃ peptide (data not shown) both displayed substrate inhibition at high substrate concentrations (>600 μM), an observation which may prove to be biologically important. Although the mechanism of RGA-caused substrate inhibition is unknown, ignoring it and truncating the data can lead to mistaken estimates of kinetic parameters. Interestingly, DNA methyltransferase 1 (DNMT1), a maintenance methyltransferase, is subject to strong substrate inhibition by unmethylated
DNA (30, 31). The probable biological reason for such inhibition may be to guarantee the unmethylated region remaining unmethylated and ensure the faithful reproduction of DNA methylation patterns (32). The same biological reason may also apply to the RGA-induced substrate inhibition that is to maintain the substrate specificity of PRMT1. Our results indicate that protein substrates that are methylated in the context of sequences other than a glycine/arginine rich motif may undergo such inhibition in cells.

Figure 8–4. The RGA peptides (acKGGFGGGRGAFGGKW) caused substrate inhibition at high substrate concentrations. Reactions were performed following the same protocol as the continuous spectrophotometric kinetic assays stated in Chapter 3. The data was fitted into equation: $v = \frac{V_{max} [S]}{(K_s + [S] + [S]^2/K_i)}$, where $[S]$ is the substrate concentration, and $V_{max}$, $K_s$, and $K_i$ are constants independent of $[S]$.

Quantitation of the endogenous PRMT1 concentration in healthy and disease cell lines — For all the kinetic reactions performed in vitro, we always question whether the results are physiologically relevant and what is the endogenous concentration of PRMT1 in the cell. To answer these questions, we performed western blotting to estimate the
endogenous concentration of human PRMT1. Cell lysates from healthy lung and brain tissues and the corresponding tumor tissues were tested as well as lysates from A549 lung cancer cell, BT-20 breast carcinoma cells, and Hela cells (Figure 8-5). The tag-cleaved human PRMT1 was used for quantitation. We identified that 10 nM of PRMT1 is our detection limit (see Figure 8-5). Most tissue samples were beyond our detection limit, yet an overexpression of PRMT1 in lung tumor cells can be clearly observed, consistent with previous observations (33). We also tested heart and liver cell lysate as well as their tumor cell lysate, yet no obvious signal could be detected with western blotting (data not shown). Comparing the three cancer cell lines, A549 cells showed a higher PRMT1 concentration (42 nM) than BT-20 and Hela cells (~23 nM) quantitated from the hPRMT1 standards (Table 8-3).

In order to quantitate the endogenous PRMT1 concentrations, Hela cells were used for calculation as the bona fide model with an average cell mass of $3\times10^8$ cells/g, and an average cell volume of $\sim3000 \mu m^3$ (34). As the cell lysate samples contain a total of 500 $\mu g$ of protein, we estimate that the total sample comes from $1.7\times10^6$ cells, as each Hela cell approximately contains 300 pg of protein (35). Based on the PRMT1 concentration of Hela cells quantitated from Figure 8-4 (24 nM), the whole cell lysate contains 4.5
pmole of PRMT1 in $1.7 \times 10^6$ cells. Calculated from the individual cell volume, the endogenous PRMT1 concentration in Hela cells is 2.5 μM. The PRMT1 concentrations in A549 and BT-20 cells were then approximated assuming that these two cell lines share the same cellular parameters as Hela cells (see Table 8-3). Although quantitation from western blotting was not very accurate, it provides us the first insight into the physiological concentrations of PRMT1 in a low micromolar range, which is directive for future experimental designs.

<table>
<thead>
<tr>
<th></th>
<th>[PRMT1] in gel (nM)</th>
<th>Estimated endogenous [PRMT1] (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>42</td>
<td>4.7</td>
</tr>
<tr>
<td>Hela</td>
<td>24</td>
<td>2.5</td>
</tr>
<tr>
<td>BT-20</td>
<td>22</td>
<td>2.6</td>
</tr>
</tbody>
</table>

**Summary**

This dissertation focused on understanding the product specificity of PRMT1 and the kinetic mechanism that regulates the final distribution of methylation products. As part of the puzzle has been solved, many opportunities are available for further research with PRMT1, such as profiling the energetic barriers for methyl group transfer, investigating the oxidative damage in PRMT1, as well as the cause of the substrate inhibition from peptide substrates containing non-canonical “RGG” amino acid sequences. It is hoped that this dissertation can provide a foundation and direction for future research that will answer the significant remaining questions of PRMT1.
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APPENDIX
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October 9, 2012

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Whitney Wooderchak-Donahue
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Author: Shanying Gui, Whitney L. Wooderchak-Donahue, Tianzhu Zang, Dong Chen, Michael P. Daly, Zhaohui Sunny Zhou, and Joan M. Hevel

Publication: Biochemistry
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Permission Letter

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Dong Chen
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I would like to thank again you for leading the project and wish you the best for a bright career ahead.

Sincerely yours,
CURRICULUM VITAE

Shanying (Laurel) Gui
(Jan. 2013)

EDUCATION

- **Ph.D. in Biochemistry** (2013) Utah State University, Logan UT
  
  *Mentor: Joan M. Hevel, Ph.D.*
  
  Dissertation: *Characterization of the Product Specificity and Kinetic Mechanism of Protein Arginine Methyltransferase 1 (PRMT1)*

- **B.S. Biopharmaceutics**, (2007) Nanjing University, China
  
  (Joint program with China Pharmaceutical University)

RESEARCH EXPERIENCE

**Graduate Research Assistant** (2007 – present)

- Developed a reverse-phase HPLC-based amino acid analysis assay for analyzing different methyl arginines
- Designed and developed a radioactive assay for measuring the processivity of PRMTs
- Designed, expressed and purified specific PRMT constructs as tools for studying the mechanism of PRMT1-catalyzed dimethylation
- Extensively used fluorography to monitor the radiolabel incorporation by peptide and protein substrates in kinetic assays and product analysis
- Trained and supervised over 5 undergraduate and new graduate researchers
- Developing lab SOPs and assisting laboratory management
- Working knowledge of MS office, PyMOL, Kaleidagraph, SigmaPlot, ChemDraw, EndNote, Adobe Illustrator

**Undergraduate Research Assistant** (09/2006 – 06/2007)

- Organic synthesis of deoxybenzoin derivatives from genistein
- Testing antibacterial activity of synthesized derivatives

PUBLICATIONS

methyltransferase 1 (PRMT1). (2013) *Biochemistry* 8, 199-209


**MANUSCRIPTS IN PREPARATION**

1. **Gui, S.**, Tarbet, H.J., Nitzel, D.V., Hevel, J.M. Effects of different affinity tags and buffering systems on the protein characteristics of Protein Arginine Methyltransferase 1 (To be published)

2. **Gui, S.**, Acevedo, O., Li, J., Qu, J., Hevel, J.M. Met48-Phe mutation in Protein Arginine Methyltransferase 1 (PRMT1) switched PRMT1 from a Type I PRMT to a Type I/II PRMT. (To be published)

**RESEARCH PRESENTATIONS**

09/2012 2nd Annual Biochemistry Department Retreat, USU, Logan, UT

Research overview of the Hevel laboratory “Protein arginine methyltransferases: diverse biological roles, complex product formation and regulation”

09/2012 2nd Annual Biochemistry Department Retreat, USU, Logan, UT

**Gui, S.**, Wooderchak-Donahue, W.L., Hevel, J.M. “Multifaceted insights into the product specificity of PRMT1” (oral presentation)

08/2012 FASEB Summer Research Conference, Biological Methylation, Snowmass, CO

**Gui, S.**, Wooderchak-Donahue, W.L., Hevel, J.M. “Modulation of protein arginine methyltransferase 1 product formation”

03/2012 243rd ACS National Meeting & Exposition, San Diego, CA

**Gui, S.**, Hevel, J.M. “Insights into the molecular origins of PRMT1 product specificity: A tale of two conserved Met residues”

09/2011 1st Annual Biochemistry Department Retreat, USU, Logan, UT

Research overview of the Hevel laboratory “Protein arginine methyltransferases: diverse biological roles, complex product formation, and unsettled regulation”

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Gui, S., Wooderchak-Donahue, W.L., Hevel, J.M. “Insights into the molecular origins of PRMT1 product specificity: A tale of two conserved Met residues” (oral presentation)

07/2010 Gordon Research Conference, Enzymes, Coenzymes & Metabolic Pathways, Waterville Valley, NH
Gui, S., Hevel, J.M. “Modulation of protein arginine methyltransferase 1 product formation”

06/2010 FASEB Summer Research Conference on Biological Methylation, Carefree, AZ
Gui, S., Wooderchak-Donahue, W.L., Suh-Lailam, B.B., Hevel, J.M. “Modulation of protein arginine methyltransferase 1 product formation”

03/2010 Intermountain Graduate Research Symposium, Logan, UT
Gui, S., Wooderchak, W.L., Hevel, J.M. “Molecular dissection of the active site of Protein Arginine Methyltransferase 1: identification of residues which control substrate specificity and activity” (oral presentation, won 3rd place in the Biochemistry section)

03/2009 237th ACS National Meeting, Salt Lake City, UT
Gui, S., Wooderchak, W.L., Hevel, J.M. “Determinants of Protein Arginine Methyltransferase 1 (PRMT1)-catalyzed ADMA Formation”

03/2009 Intermountain Graduate Research Symposium, Logan, UT
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*Speaker of the presentation underlined

HONORS & AWARDS

Department of Chemistry/Biochemistry Travel Award – Utah State University 2012
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Department of Chemistry/Biochemistry Travel Award – Utah State University 2011
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GlaxoSmithKline Scholarship of China Pharmaceutical University, China 2007
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People’s Scholarship of Nanjing University, China 2004
MAJOR RESEARCH PROJECTS

1. Characterization of the active site of Protein Arginine Methyltransferase 1 (PRMT1)
2. Profiling the determinants of PRMT1 substrate recognition
3. Kinetic mechanism of PRMT1 and determinants of PRMT1-catalyzed asymmetric dimethylarginine (ADMA) formation
4. Explore the influences of different protein tags and buffering systems on the protein characteristics of PRMT1, especially on enzymatic activity and substrate specificity
5. Product analysis of M48F-PRMT1, a hybrid type of PRMT generating both asymmetric and symmetric dimethyl arginine.
6. Product analysis of TbPRMT7 and the determinants of PRMT7 generating only monomethylarginine.
7. Generating an ADMA-specific aptamer and developing a SPA-bead screening method for aptamer selection

TEACHING EXPERIENCE

Teaching Assistant – Biochemistry Laboratory (Spring 2009)
Teaching Assistant – Principles of Chemistry I Laboratory (Fall 2007 and 2008)
Teaching Assistant – Principles of Chemistry II Laboratory (Spring 2008)

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2010 – 2012 Graduate Advisor of Chemistry Club, Utah State University
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