Characterization of the Substrate Specificity and Mechanism of Protein Arginine Methyltransferase 1

Whitney Lyn Wooderchak
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CHARACTERIZATION OF THE SUBSTRATE SPECIFICITY AND MECHANISM
OF PROTEIN ARGinine METHYLTRANSFERASE 1

by

Whitney Wooederchak

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSPHY

in

Biochemistry

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UTAH STATE UNIVERSITY
Logan, Utah

2009
ABSTRACT

Characterization of the Substrate Specificity and Mechanism of Protein Arginine Methyltransferase 1

by

Whitney Wooderchak, Doctor of Philosophy

Utah State University, 2008

Major Professor: Joan M. Hevel, Ph.D.
Department: Chemistry and Biochemistry

Protein arginine methyltransferases (PRMTs) posttranslationally modify protein arginine residues. Type I PRMTs catalyze the formation of monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA) via methyl group transfer from S-adenosyl methionine onto protein arginine residues. Type II PRMTs generate MMA and symmetric dimethylarginine. PRMT-methylation affects many biological processes. Although PRMTs are vital to normal development and function, PRMT-methylation is also linked to cardiovascular disease, stroke, multiple sclerosis, and cancer.

Thus far, nine human PRMT isoforms have been identified with orthologues present in yeast, plants, and fish. PRMT1 predominates, performing an estimated 85% of all protein arginine methylation in vivo. Yet, the substrate specificity and catalytic mechanism of PRMT1 remain poorly understood.

Most PRMT1 substrates are methylated within repeating ‘RGG’ and glycine-arginine rich motifs. However, PRMT1 also methylates a single arginine on histone-H4.
that is not embedded in a glycine-arginine motif, indicating that PRMT1 protein substrates are not limited to proteins with ‘RGG’ sequences. In order to determine if PRMT1 displays broader substrate selectivity, I first developed a continuous spectrophotometric assay to measure AdoMet-dependent methyltransferase activity. Using this assay and a focused peptide library based on a sequence derived from the in vivo PRMT1 substrate fibrillarin, we observed that PRMT1 demonstrates amino acid sequence selectivity in peptide and protein substrates. PRMT1 methylated eleven substrate motifs that went beyond the ‘RGG’ and glycine-arginine rich paradigm, suggesting that the methyl arginine proteome may be larger and more diverse than previously thought.

PRMT1 methylates multiple arginine residues within the same protein to form protein-associated MMA and ADMA. Interestingly, ADMA is the dominant biological product formed and is a predictor of mortality and cardiovascular disease. To understand why PRMT1 preferentially forms ADMA in vivo, we began to 1) probe the mechanism of ADMA formation and 2) examine the catalytic role of certain active site residues and their involvement in ADMA formation. We found that PRMT1 dissociatively methylated several peptide substrates and preferred to methylate mono-methylated substrates over their non-methylated counterparts. Methylation of a multiple arginine-containing substrate was systematic (not random), a phenomenon that may be important biologically. All in all, our data help explain how PRMT1 generates ADMA in vivo.
To my grandmother, Ethel Jane Wooderchak
ACKNOWLEDGMENTS

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Whitney Woorderchak
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<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
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<tr>
<td>AdoHcy</td>
<td>$S$-adenosyl homocysteine</td>
</tr>
<tr>
<td>AdoMet</td>
<td>$S$-adenosyl methionine</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
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<td>EDTA</td>
<td>[Ethylenedinitrilo]-tetraacetic acid</td>
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<td>eIF4A</td>
<td>eukaryotic initiation factor 4 alpha</td>
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<td>H4</td>
<td>histone 4</td>
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<tr>
<td>Hepes</td>
<td>$N$-(2-hydroxyethyl)piperazine-$N'$-2-ethanesulfonic acid</td>
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<td>HPLC</td>
<td>high-performance liquid chromatograph</td>
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<td>IPTG</td>
<td>isopropyl-$\beta$-D-thiogalactopyranoside</td>
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<td>LB</td>
<td>Luria-Bertani</td>
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<td>MMA</td>
<td>monomethyl arginine</td>
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<tr>
<td>MTAN</td>
<td>5’-methylthioadenosine nucleosidase</td>
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<tr>
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<td>nitric oxide synthase</td>
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<td>PRMT</td>
<td>protein arginine $N$-methyltransferase</td>
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<tr>
<td>SAH</td>
<td>$S$-adenosyl homocysteine</td>
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<tr>
<td>SAM</td>
<td>$S$-adenosyl methionine</td>
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<tr>
<td>SDMA</td>
<td>symmetric dimethyl arginine</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
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<td>wild type</td>
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CHAPTER 1  
INTRODUCTION

Protein arginine methyltransferase (PRMT) enzymes are involved in a wide variety of cellular communication in eukaryotes. In addition to phosphorylation, acetylation, and glycosylation, protein arginine methylation is one more post translational modification that enables organisms to expand upon their limited genome. PRMT-methylation affects many biological processes including RNA processing, signal transduction, DNA repair, transcriptional regulation (reviewed in [1]), viral infection [2], chromatin remodeling [3], and neuronal cell differentiation [4]. The molecular basis for these events lies in the fact that methylation of protein arginine residues has the ability to enhance or disrupt certain protein-protein interactions, protein-DNA interactions, and protein-RNA interactions (described further in Chapter 2).

Thus far, eleven PRMT isoforms have been identified in humans (PRMT1-11) with orthologs present in yeast, protozoa, *Caenorhabditis elegans*, *Drosophila melanogaster*, plants, and fish. The breadth of these enzymes indicates the vital biological importance of arginine methylation across the plant and animal kingdoms. The significance of protein arginine methylation has also been demonstrated in PRMT1 null mice that died early during embryonic development [5] and PRMT4 null mice that showed incorrect cell differentiation [6]. Although PRMTs are vital to survival, protein arginine methylation has been linked to carcinogenesis [7], viral pathogenesis [2], multiple sclerosis [8], spinal muscular atrophy [9], lupus [10], cardiovascular disease [11], and stroke [12]. Therefore, the elucidation of the regulation and mechanism of these enzymes will prove invaluable.
PRMTs transfer a methyl group from \textit{S}-adenosyl methionine (AdoMet/SAM) onto a positively charged arginine residue in the protein substrate, and \textit{S}-adenosyl homocysteine (AdoHcy/SAH) is produced. Type I PRMTs (PRMT1, 3, 4, 6, and 8) catalyze the formation of monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA) via the transfer of a methyl group from \textit{S}-adenosyl methionine (AdoMet/SAM) onto protein arginine residues. PRMT5 and 7, both type II enzymes, generate MMA and symmetric dimethylarginine (SDMA). Opposing biological consequences result from whether a protein has been asymmetrically or symmetrically dimethylated [reviewed in 1].

Although much research has been performed on PRMTs since their cloning a little over ten years ago, the substrate specificity of PRMT1 (the most predominant PRMT isoform that performs over 85\% of all protein arginine methylation) [13], remains unclear. Few \textit{in vivo} PRMT1 protein substrates have been identified, and the mechanism of PRMT1 remained uncharacterized until recently [14]. Importantly, in order to understand how PRMTs impact biology, we must first understand how these enzymes select certain substrates over others and perform catalysis. The purpose of this dissertation is to add to the overall understanding of PRMT catalysis and substrate specificity by characterizing the mechanism and substrate selection of the most predominant PRMT isoform, PRMT1.

In Chapter 3, I helped develop the first continuous spectrophotometric assay for AdoMet-dependent methyltransferases and demonstrated the utility of this assay with PRMT1. Prior to this assay, PRMTs were assayed with peptide substrates and radioactively-labeled AdoMet. Radioactively-methylated peptide was then hydrolyzed.
and analyzed by HPLC [15]. Because this method is laborious and expensive, it is not practical for screening many peptides. In order to perform the types of kinetic analyses needed to address PRMT1 substrate specificity, we needed a more robust, higher throughput assay. In collaboration with Dr. Sunny Zhou, we developed a continuous spectrophotometric coupled assay (see Chapter 3) to measure AdoMet-dependent methyltransferase activity. Our assay employs two recombinant coupling enzymes, AdoHcy nucleosidase and adenine deaminase. AdoHcy nucleosidase hydrolyzes AdoHcy, a product and inhibitor of methyltransfer, into adenine and S-ribosyl homocysteine. Adenine is then converted into hypoxanthine via adenine deaminase which results in a decrease at 265 nm that can be monitored continuously with time. This assay was used repeatedly in several chapters of this dissertation.

In Chapter 4, we investigated the substrate specificity of PRMT1. Before 2007, little was known regarding the substrate specificity of PRMT1 other than the fact that PRMTs methylate arginine residues in glycine/arginine rich areas of proteins within ‘RGG’ and ‘RXR’ motifs. From recent studies, it is now known that 1) certain negatively charged surface residues of PRMT1 are important for substrate binding [16], 2) substrate positive charge positioned near the active site is important for PRMT1 catalysis [17], and 3) the N-terminus of PRMT1 influences substrate specificity [18]. Our study advanced the current understanding of PRMT1 substrate selection by examining the primary amino acid sequence determinants of PRMT1 substrates. Using the continuous assay (discussed in Chapter 3) and a focused peptide library based on a sequence derived from the in vivo PRMT1 substrate fibrillarin, we showed that PRMT1 displays substrate sequence selectivity at the amino acid level (see Chapter 4). We report
that PRMT1 has the ability to methylate eleven motifs that go beyond the ‘RGG’ and ‘RXR’ glycine/arginine rich paradigm suggesting that the methyl arginine proteome may be larger and more diverse than previously thought.

In Chapter 5, we tried to understand why PRMT1 predominantly forms ADMA in vivo by investigating how PRMT1 dimethylates substrate arginine residues mechanistically. Although PRMT1 is capable of forming both MMA and ADMA, most PRMT1 substrates are found in vivo in the dimethylated state with multiple dimethylated arginines on the same protein. Numerous studies have shown a relationship between elevated ADMA concentration and cardiovascular disease [19], and PRMT1 has been shown to be overexpressed in myocardial tissue taken from coronary heart disease patients [20]. Two possible ways to explain the predominance of ADMA formation are 1) preference for the mono-methylated substrate over the nonmethylated substrate or 2) processive methylation. Our data indicate that PRMT1 dissociatively methylates single arginine-containing peptides despite any increase in peptide length. PRMT1 preferentially methylated monomethylated substrates over unmodified substrates, a preference that would inevitably increase the amount of ADMA. Interestingly, methylation of a multiple arginine-containing substrate was shown to be systematic wherein one arginine was preferentially methylated over another. This is important because the biological response may change depending on which protein arginine residue is methylated first. Finally, PRMT1 was shown to dissociatively methylate a multiple arginine-containing substrate. Based on these results, the predominance of dimethylated protein substrates in vivo is likely due to the ability of PRMT1 to preferentially methylate
monomethylated versus unmodified arginine-containing proteins (see Chapter 5), a phenomenon that may depend on substrate sequence.

In Chapter 6, we investigated certain PRMT1 active site residues and their catalytic roles. Cheng and coworkers proposed that a conserved methionine residue among type I PRMTs (M155 in the PRMT1 active site) may ensure the formation of ADMA due to steric hindrance afforded by a bulky methionine residue [21]. Type II PRMTs such as PRMT5 have a smaller alanine residue at this site and form SDMA. When analyzing the methylation products formed from M155A-PRMT1, a mutant PRMT1 that alleviated any steric hindrance afforded by this active site methionine residue, no SDMA was present. This suggests that Met155 does not affect asymmetric versus symmetric dimethylation formation (see Chapter 6). Another conserved active site methionine residue (M48 in PRMT1) adjacent to AdoMet and the substrate arginine in the PRMT1 crystal structure was shown to be very important in catalysis. M48A-PRMT1 was nearly catalytically inactive while M48L-PRMT1 demonstrated a catalytic efficiency approximately half that of WT-PRMT1. These results indicated that the length of this amino acid residue is important in PRMT catalysis based on its proximity to the substrate arginine (see Chapter 6). Further product analysis from an extended reaction with M48L-PRMT1 revealed that this mutant was also not capable of generating SDMA. Thus, removing steric bulk afforded by Met155 or Met48 in the PRMT1 active site is not by itself sufficient to transform PRMT1 into a Type II PRMT.

Chapter 7 includes experimental results from several projects that have yet to be completed. In one project, we found that the amino acid sequence of certain peptide substrates directly affects the extent that a particular substrate is mono- and dimethylated.
Furthermore, we noticed that substrate inhibition of PRMT1 occurred at high substrate concentrations when using an ‘RGA’-containing fibrillarin-based peptide versus one housing an ‘RGG’ sequence. Together, these data indicate how the composition of the substrate sequence can alter the extent that a particular substrate is methylated. Next, I examined the importance of an interaction between the substrate and the conserved THW loop among PRMTs by mutating two residues (H293 and W294 to an alanine on PRMT1) and assessing the activity of each with several substrates. In another project, I showed how the AdoMet/AdoHcy ratio present in healthy and diseased individuals affected PRMT1 activity. Finally, I determined the $K_i$ for a fibrillarin-based peptide containing ADMA.

REFERENCES


CHAPTER 2
LITERATURE REVIEW

METHYLATION

After DNA is transcribed into mRNA in the nucleus, processed mRNA can be translated into amino acids by the ribosomal complex in the cytosol. After the emerging string of amino acids from the ribosome is folded into a three-dimensional protein, this protein can then be post translationally modified. While an organism’s genome has the ability to code for a variety of proteins, post translational modifications such as phosphorylation, acetylation, glycosylation, and methylation enable organisms to expand upon their limited genomes.

Methylation requires the presence of 1) a methyl donor such as S-adenosyl methionine (AdoMet/SAM) or tetrahydrofolate (THF) and 2) a methyl acceptor. AdoMet is the second most widely used enzyme substrate in the cell [1]. The preference for AdoMet over other methyl donors such as THF results from the favorable energetics of the AdoMet-dependent methyltransferase reaction (-17 kcal/mol). This amount of energy is more than twice the amount released during ATP hydrolysis. This energy does not come without a cost. The de novo biosynthesis of AdoMet costs the cell twelve equivalents of ATP making AdoMet the most expensive metabolic compound on a per carbon basis [2]. (Note, recycling AdoMet from AdoHcy costs the cell only 1 ATP.)

Thus far, five families of AdoMet-dependent methyltransferases have been identified (Classes I through V) suggesting five independent evolutionary paths to methyltransferases [3]. Classes I and V contain most of the protein methyltransferases
while the other classes are comprised of small molecule, DNA, and RNA methyltransferases. Although enzymes from the different families have diverse structural requirements for the way in which they bind AdoMet, they all share the same basic mechanism (Fig. 2-1). First, the positively charged sulfur moiety of AdoMet causes the S-CH$_3$ bond to be polarized. This polarization facilitates the nucleophilic attack of the "+CH$_3$" group by an electron rich nitrogen, oxygen, or carbon target atom. Ultimately, the electrons push towards the sulfur moiety in AdoMet resulting in the formation of the methylated target atom and S-adenosyl homocysteine (AdoHcy/SAH) [4].

![Fig. 2-1. General reaction mechanism for AdoMet-dependent methyltransferases. A general base (B:) abstracts a proton from the target atom (X=O, N, or C) prior to or during the course of the S$_{N}$2 reaction mechanism. A transition state is forms from the nucleophilic attack of the polarizable target atom onto the methyl group carbon of AdoMet. Ultimately, electrons push towards the sulfur moiety in AdoMet resulting in the formation of AdoHcy and X-CH$_3$.](image-url)
Among protein methyltransferases, nitrogen is the most dominant nucleophile. N-methylation of exposed lysine and arginine residues does not alter the positive charge of the amino acid, but it does increase the hydrophobicity and steric bulk of the residue which can disturb the way in which the modified protein interacts with certain biological molecules. Lysines can be mono, di, or tri-methylated, whereas the guanidino nitrogens of arginine can be mono- or dimethylated. In addition to lysine and arginine, the imidazole ring of histidine and the side chain amide nitrogens of glutamine and asparagine can be methylated. The methylation of protein histidine, glutamine, and asparagine residues is not readily reversible under physiological conditions. On the other hand, methyl groups transferred onto the nitrogens of DNA bases are readily removed through enzymatic oxygenation resulting in the formation of formaldehyde and the starting base [5].

In addition to nitrogen, proteins can also be methylated on oxygen atoms to form methyl esters. O-methylation of surface protein aspartates and glutamates neutralizes the negative charge on the carboxylate side chain and adds hydrophobicity to the protein surface. This modification is readily reversible, and the hydrolysis of protein methyl esters is often used as a signal during bacterial chemotaxis [6].

Although nitrogen and oxygen containing amino acid side chains are most commonly methylated, certain Archaeal methanogenic bacteria methylate proteins on electron-rich carbon and sulfur atoms. To date, methanogenic bacteria are the only organisms on earth that have the ability to C-methylate protein arginyl and glutamyl side chains. The methanogenic enzyme methyl-coenzyme M reductase, which helps catalyze the last step in the formation of methane, was shown crystallographically to contain C-
methylated arginine and glutamine side chains, an N-methylated histidine, and an S-methylated cysteine residue [7]. The function of these peculiar modifications in methanogens is unknown.

PROTEIN ARGININE METHYLATION

Arginine residues were first shown to contain methyl groups in 1967 [8]. Since then, arginine methylation has been shown to be a vital, relatively abundant posttranslational modification. Two percent of all protein arginine residues are asymmetrically dimethylated in rat liver nuclei [9], and approximately 12% of all arginine residues isolated from heterogeneous nuclear ribonucleoproteins (hnRNPs) are asymmetrically dimethylated [10].

The enzymes responsible for methylating protein arginine residues in both the nucleus and cytoplasm are the protein arginine methyltransferases (PRMTs). Thus far, eleven PRMT isoforms have been identified in humans with homologues present in yeast, protozoa, Caenorhabditis elegans, Drosophila melanogaster, plants, and fish [11]. The breadth of these enzymes indicates the vital biological importance of arginine methylation across the plant and animal kingdoms. The significance of protein arginine methylation has also been demonstrated in PRMT1 null mice that died early during embryonic development [12] and PRMT4 null mice that showed incorrect cell differentiation [13].

PRMTs perform the general S_N2 reaction mechanism characteristic of most AdoMet-dependent methyltransferases where an active site glutamate residue is proposed to abstract a proton from the substrate arginine residue (see Fig. 2-1). Methylation of
positively charged protein-arginine residues occurs via the attack of the terminal nucleophilic guanidino nitrogen onto the methyl group of AdoMet forming monomethyl arginine (MMA) and AdoHcy (Fig. 2-2). PRMTs can transfer another methyl group onto MMA resulting in the dimethylation of certain protein-arginine residues. Type I PRMTs (PRMT1, 3, 4, 6, and 8) transfer a second methyl group onto the previously modified guanidino nitrogen to form asymmetric dimethylarginine (ADMA). Type II PRMTs (PRMT5 and 7) transfer another methyl group to the unmodified guanidino nitrogen creating symmetric dimethylarginine (SDMA).

Fig. 2-2. Reactions catalyzed by PRMTs. PRMTs catalyze the S-adenosylmethionine (AdoMet) dependent methylation of a protein substrate arginine residue to form monomethyl arginine (MMA). Type I methyltransferases, PRMT1, 3, 4, 6, 8, and 10 dimethylate arginine asymmetrically to form asymmetric dimethyl arginine (ADMA). Type II methyltransferases, PRMT5, 7, and 9 catalyze the formation of symmetric dimethyl arginine (SDMA) where each guanidino nitrogen is modified.
PROTEIN ARGinine METHYL-TRANSFERASE FAMILY

Despite the discovery of protein arginine methylation thirty years ago, the PRMTs were not cloned until recently. Thus far, eleven PRMT isoforms have been identified (see Fig. 2-3). The most predominant mammalian type I methyltransferase, PRMT1, was discovered based on its homology to the yeast arginine methyltransferase Hmt1/Rmt1 [14]. PRMT2 was recognized due to its homology to PRMT1 [15] while PRMT3 was isolated as a PRMT1 binding partner in a yeast two-hybrid screen [16]. PRMT4/CARM1 was identified in a yeast two-hybrid screen bound to the p160 steroid receptor coactivator GRIP1 [17]. PRMT5 was cloned as a Jak2-binding protein [18]. PRMT6 and PRMT8 were identified based on their homology to other type I PRMTs [19 and 20], and PRMT7 was discovered in a genetic screen looking for suppressor elements conferring resistance to a topoisomerase II inhibitor [21]. PRMT9 was identified through a protein database search using a consensus query sequence corresponding to the conserved PRMT AdoMet binding domain. However, PRMT9 is structurally distinct from all other known PRMTs [22]. PRMT10, a type I PRMT, was identified in plants and was shown to preferentially asymmetrically dimethylate histone H4 in vitro [23]. PRMT11 was identified most recently through a yeast two-hybrid screen bound to the methyl-DNA-binding 7 (MBD7) protein in plants [24].
Fig. 2-3. Protein arginine methyltransferases. PRMT1-PRMT11 are aligned based upon their characteristic THW loop (in black) and AdoMet methyltransferase motifs I, post I, post II, and post III (also in black). PRMT2 has an SH3 domain while PRMT3 has a Zn\(^{2+}\) finger domain. PRMT7 and PRMT10 contain a repeat of the methyltransferase motifs. PRMT11 has an F box domain near the N-terminus.

**PRMT SUBSTRATES**

With PRMTs present throughout the plant and animal kingdoms, the significance of protein arginine methylation is evident. Yet, the methyl arginine proteome has only recently begun to be characterized using mass spectrometry techniques [25], and the substrate specificities of the different PRMT isoforms remain ill-defined. In general, PRMTs methylate arginines located in glycine arginine rich (GAR) areas of proteins in ‘RGG’ or ‘RXR’ motifs. Type I methyltransferases (PRMT1, 3, 6, and 8) mainly methylate arginines located within GAR sequences. PRMT4, on the other hand, displays a different degree of specificity and does not typically methylate GAR sequences. Type II methyltransferases, PRMT5 and 7, methylate isolated arginines as well as arginines within GAR motifs. PRMT9 has been shown to symmetrically dimethylate maltose
binding protein (MBP) [22] which is also a substrate of PRMT5 (Fig. 2-4). PRMT10 is a type I PRMT that asymmetrically dimethylates histone H4 in vitro [23]. Finally, a cell line lacking PRMT11 mRNA exhibited reduced levels of proteins with asymmetrically dimethylated arginines, suggesting that PRMT11 is a type I PRMT [24].

<table>
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<tr>
<th>PRMT1</th>
<th>PRMT3</th>
<th>PRMT6</th>
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<tr>
<td>CIRP</td>
<td>Fibrillarin</td>
<td>Fibrillarin</td>
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<tr>
<td>EWS</td>
<td>PABPN1</td>
<td>HIV tat</td>
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<tr>
<td>FGF-2</td>
<td>rpS2</td>
<td>PRMT6</td>
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<tr>
<td>Fibrillarin</td>
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<td>Histone H4</td>
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<td>hnRNP A1</td>
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<td>hnRNP A2</td>
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<td>hnRNP K</td>
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<td>TAFI168</td>
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<td>TLS/FUS</td>
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<td>ZF5</td>
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<th>PRMT7</th>
<th>PRMT8</th>
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<tr>
<td>Histone H3</td>
<td>Fibrillarin-</td>
<td>GAR peptide</td>
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<td>HuR</td>
<td>peptide</td>
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<td>PABP1</td>
<td>Sm proteins</td>
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<td>p300/CBP</td>
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<td>Sm B/B'</td>
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<td>TARPP</td>
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<th>PRMT9</th>
<th>PRMT10</th>
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<td>MBP</td>
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<td>Histone H2A</td>
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<td>MBD7</td>
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Fig. 2-4. PRMT substrates. Protein substrates are listed underneath the PRMT isoform that performs the modification. Many arginine methylated proteins have been identified since using mass spectrometry [25]; however, the enzyme responsible for the methylation remains unknown. Abbreviations are as follows: CIRP, cold inducible RNA binding protein; EWS, Ewing Sarcoma; ILF3, interleukin enhancer binding factor 3; TLS/FUS, translocated in liposarcoma; ZF5, Zn\(^{2+}\) finger 5; SAMT1, substrate of arginine methyltransferase 1; TARPP, thymocyte cyclic AMP-regulated phosphoprotein; LSm4, Sm-like protein 4; MBP, maltose binding protein, MBD7, methyl-DNA binding protein 7. PRMT2 has no reported activity.
Of the eleven mammalian PRMT isoforms, PRMT1 predominates performing an estimated 85% of all protein arginine methylations \textit{in vivo} [26]. Most PRMT1 substrates are methylated at multiple sites within the same glycine arginine rich region of proteins such as fibrillarin [27], heterogeneous nuclear ribonuclear proteins (hnRNPs) [28], and Sam68 [29]. In addition to ‘RGG’, ‘RXR’ motifs located on the surface of the Poly(A)-binding protein II (PABPII) are also modified by PRMT1 [30]. Although the predominant feature of known PRMT1 protein substrates is a glycine and arginine rich region, PRMT1 has also been shown to specifically methylate a single arginine residue on the histone H4 tail that is not embedded in an ‘RGG’ or ‘RXR’ motif [31], indicating that PRMT1 substrates are not limited to proteins bearing ‘RGG’ and ‘RXR’ sequences.

Several recent studies have helped identify how PRMT1 recognizes its substrates. First, acidic surface residues along the substrate binding groove of PRMT1 have been shown experimentally to be important for PRMT1 substrate binding through a pull down assay [32]. Complementing this finding, positively charged residues on the substrate distal from the active site were shown to be important for PRMT1 catalysis [33]. The N-terminus of PRMT1 was also shown to affect substrate specificity and activity [34]. Finally, eleven new PRMT1 substrate motifs that go beyond the typically methylated ‘RGG’ and ‘RXR’ motifs were identified using a fibrillarin-based peptide library screen [35]. This suggests that the PRMT substrate proteome is larger than previously thought.

\textbf{STRUCTURE OF PRMT1}

What is it about the PRMT1 active site that allows for the select modification of certain arginines but not others? In 2003, rat His-PRMT1 was co-crystallized with
AdoHcy and R3, a 19 amino acid peptide substrate derived from fibrillarin (GGRGGFGGRGGFGGRGGFG) [36] (Fig. 2-5, A). Due to a lack in observable electron density for R3, few inferences could be made concerning the substrate specificity of PRMT1. The crystal structure depicted three acidic substrate binding grooves (P1, P2, and P3) on the surface of PRMT1 that have the ability to interact with substrate arginine residues (Fig. 2-5, B). Except for the substrate arginine in the active site substrate binding groove (P1), the electron density of the side chains from all other 18 amino acids of the R3 peptide was not observed and any important PRMT1-peptide side chain interactions went undetected (Fig. 2-5, A). Fortunately, the majority of the carbon-nitrogen peptide backbone of R3 was resolved. Because of this, the authors stated that the substrate could be fitted equally well in either linear orientation into the active site. Electron densities from the four glycines that flank the central arginine were not observed and created a sequence gap in the model. This lack in observable substrate electron density suggests that the sequence surrounding the substrate arginyl group does little to govern PRMT1 substrate selection. Our recent studies suggest otherwise [35].

Importantly, the crystal structure provided much insight into the reaction mechanism. First, the structure revealed that two active site glutamates (E144 and E153) stabilize the substrate arginine guanidino nitrogen through hydrogen bonding. Upon mutating these residues to non-acidic glutamines, PRMT1 activity reduced 3,000 fold. Second, this study revealed that PRMT1 dimerization was essential for AdoMet binding and that enzymatically active PRMT1 exists as a homodimer consisting of two 42 kDa subunits.
Fig. 2-5. The Crystal Structure of Rat PRMT1. (A) PRMT1, shown in gray, was crystallized as a ternary complex with R3 (hot pink) and AdoHcy (red). The guanidino nitrogens of the substrate arginine residue (shown in ball and sticks) are oriented toward the sulfur moiety in AdoHcy (yellow). While most of the R3 peptide backbone was observed in the crystal structure, the electron densities from the other R3 peptide side chains were not observed. (B) This surface representation of PRMT1 highlights the negatively charged surface amino acid residues (green) located along the substrate binding grooves P1, P2, and P3. The P2 and P3 grooves are found on one protein face while the P1 groove is on the opposite face of PRMT1. The R3 peptide backbone (colored pink) is situated in the P1 substrate binding groove. PDB ID=10R8
LOCALIZATION OF PRMT1

PRMT1 is found in the nucleus, the cytoplasm, and has most recently been proposed to localize to the plasma membrane. Interestingly, PRMT1 has the ability to heterodimerize with PRMT8, an isoform that is highly homologous in sequence to PRMT1. PRMT8 has a myristolation site and has been shown to localize to the cell membrane when modified by a fatty acid chain. Therefore, PRMT1 which has already been shown to shuttle between the nucleus and the cytoplasm [37] has now been proposed to localize to the plasma membrane upon the myristolation of its heterodimeric counterpart, PRMT8 [20]. Clearly, the cellular distribution of PRMT1 and the diversity of PRMT1-methylated protein substrates emphasize its physiological role as the dominant PRMT in the cell.

MECHANISM OF PRMT1

In September 2008, the kinetic mechanism of PRMT1 was elucidated by Thompson and coworkers [38]. They reported that human PRMT1 utilizes a rapid equilibrium random sequential mechanism to methylate the histone H4 peptide. In this type of reaction mechanism, the order of substrate (AdoMet and the unmodified peptide) binding to the enzyme and product (AdoHcy and the methylated peptide) release from the enzyme does not matter.

Next, the mechanism was examined further. Does PRMT1 1) perform dissociative methylation wherein the monomethylated species is released before rebinding and being dimethylated, or 2) perform processive methylation wherein the substrate binds and is mono- and dimethylated sequentially without releasing the
intermediate? Interestingly, PRMT1 was found to catalyze the dimethylation of the histone H4 peptide in a partially processive manner [38]. PRMT1 catalyzed the production of MMA and ADMA containing H4 peptides in approximately equimolar amounts, even in the presence of excess unmethylated peptide substrate. This ruled out a fully processive mechanism in which the concentration of the monomethylated intermediate does not rise above the concentration of the enzyme. Additionally, the monomethylated histone H4 peptide was not preferred over the unmodified peptide [33].

**BIOLOGICAL IMPORTANCE OF PRMTs**

PRMTs play important roles in biology, yet the biological consequence of protein arginine methylation is not fully understood. Studies have shown that PRMTs are important not only for the proteins they methylate, but also for the response that the modified protein elicits in the cell. PRMT activity has been implicated in RNA processing, signal transduction, DNA repair, transcriptional regulation (reviewed in [11]), viral infection [39], chromatin remodeling [40], and neuronal cell differentiation [41].

Arginine methylation also regulates transcription initiation and elongation. Specifically, PRMT4 methylates the transcription factor CBP/p300 [42] which inhibits its interaction with the p160 coactivator glucocorticoid receptor interacting protein (GRIP1) *in vitro* and *in vivo* [43]. Also, methylation of the transcription elongation factor SPT5 by PRMT1 and PRMT5 regulates its interaction with RNA polymerase II and may be involved in regulating the transcriptional elongation properties of SPT5 in response to viral and cellular factors [44]. Although PRMT1-PRMT11 have unique biological functions (summarized in Table 2-1), arginine methylation ultimately regulates these and
other biological processes at the molecular level by altering biomolecular interactions such as 1) protein-protein interactions, 2) protein-RNA interactions, and 3) protein-DNA interactions [45].

<table>
<thead>
<tr>
<th>PRMT</th>
<th>Biological Function</th>
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<tbody>
<tr>
<td>PRMT1</td>
<td>Transcriptional activation (H4R3), transcriptional elongation, DNA repair, signaling, chromatin remodeling, neuronal cell differentiation</td>
</tr>
<tr>
<td>PRMT2</td>
<td>Viral infection, apoptosis, transcriptional coactivator, nuclear retention</td>
</tr>
<tr>
<td>PRMT3</td>
<td>Ribosome assembly</td>
</tr>
<tr>
<td>PRMT4</td>
<td>Transcriptional activation (H3R2, H3R17, H3R26), chromatin remodeling, muscle differentiation, T cell development, tumorigenesis</td>
</tr>
<tr>
<td>PRMT5</td>
<td>Transcriptional repression (H3R8 and H4R3), transcriptional elongation, RNA processing, signaling, mitosis, muscle and germ cell differentiation, tumorigenesis, chromatin remodeling</td>
</tr>
<tr>
<td>PRMT6</td>
<td>HIV replication, DNA repair</td>
</tr>
<tr>
<td>PRMT7</td>
<td>Imprinting in male germ cells (H4R3)</td>
</tr>
<tr>
<td>PRMT8</td>
<td>Involved in the somatosensory and limbic systems</td>
</tr>
<tr>
<td>PRMT9-11</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Arginine (R) residues modified by each PRMT on either histone H3 or histone H4 are indicated in parenthesis.

1. Protein-Protein Interactions

First, protein arginine methylation alters protein-protein interactions by either blocking or facilitating contact between proteins [46]. In particular, arginine methylation can disrupt the formation of Asp-Arg salt bridges between proteins. For example, an arginine residue in Fyn (a protein involved in signal transduction and host cell activation) hydrogen bonds to an aspartate on the human immunodeficiency virus-1 (HIV-1) Nef protein stabilizing the interaction between the two proteins. Arginine methylation of Fyn disrupts the Asp-Arg salt bridge and blocks further interactions between the two proteins.
Another Asp-Arg salt bridge is disrupted when the adaptor protein Sam68 is methylated by PRMT1. Arginine methylated Sam68 can no longer associate with sarc homology 3 (SH3) domain containing proteins; however, it can still interact with proteins with WW (two tryptophan) domains [48]. Finally, arginine methylation also has the ability to disrupt key pi-cation interactions. Methylation of an arginine residue on the KIX domain of the CREB-binding protein (CBP)/p300 blocks a key pi-cation interaction and ultimately CREB activation by disrupting the interaction between the KIX domain of CBP and the kinase inducible domain (KID) of CREB [49].

Other protein interactions are facilitated by arginine methylation. PRMT1 methylates NIP45, a nuclear factor of activated T-cells (NF-AT) coactivator, and facilitates its interaction with NF-AT. This interaction promotes cytokine gene expression [50] and helps mediate the immune response. Arginine methylation also strengthens interactions between the spinal muscular atrophy gene product, SMN, and certain small nuclear ribonucleoproteins (SmB/B’, SmD1, and SmD3) [51]. When the C-terminus of SmB and SmD are methylated, their association with SMN is enhanced [52]. In fact, the symmetric dimethylation of arginine residues in Sm proteins by PRMT5 and PRMT7 facilitates the assembly of small nuclear ribonucleoprotein particles [53].

2. Protein-RNA Interactions

Second, protein arginine methylation affects interactions between proteins and ribonucleic acids. The glycine arginine rich hnRNP proteins A1, A2, K, R, and U that assist in the processing and folding of RNA during mRNA translation are highly modified by the PRMTs [28]. Arginine methylation of hnRNP A1 decreases its ability to
bind RNA [54]. In addition to the hnRNPs, Sam68 and Hu antigen protein R (HuR) have arginines that hydrogen bond to the secondary and tertiary structures of RNA. Arginine methylation has the ability to disrupt certain protein-RNA interactions by preventing hydrogen bonding and/or sterically hindering van der Waals interactions [55]. Other studies have shown that arginine methylation does not significantly affect protein-RNA interactions [56]. So far, the effect of arginine methylation on protein-RNA interactions remains unclear.

3. Protein-DNA Interactions

In 1967, it was recognized that histones were substrates of methyltransferases [8], and now it is understood that both types of PRMTs methylate histones. In the nucleus, DNA is packaged around histone protein octamers forming higher ordered structures known as chromatin. Histone tails that point away from the protein octamer can be highly post-translationally modified by methylation of arginines; acetylation, methylation, and ubiquitination of lysine residues; and phosphorylation of serine and threonine residues. These modifications regulate the tightness of the histone-DNA interaction which ultimately mediates dynamic changes in gene function and expression [57]. Therefore, histone-DNA interactions and the proteins that modify histones are vital to the genetic stability of the organism. Rett syndrome and Rubinstein-Taybi syndrome, inherited developmental disorders characterized by physiological deformities and a loss of developmental skills, result from the deregulation of gene transcription caused by altered histone-DNA interactions [58].
Both type I and type II PRMTs have been shown to methylate histones \textit{in vitro}. PRMT4 methylates three arginine residues on the histone H3 tail while PRMT5 methylates a single arginine (Arg 3) on both the histone H2A and H4 tails [59]. PRMT1 has also been shown to dimethylate arginine 3 in H4 both \textit{in vitro} and \textit{in vivo} [40]. In fact, arginine methylated H4 has been shown to be a better substrate than unmodified H4 for the histone acetyltransferase p300. Thus, arginine methylation of H4 facilitates lysine acetylation of H4 by p300 which ultimately stimulates gene transcription [60].

In addition to histones, other protein-DNA interactions are modulated by arginine methylation. High mobility group proteins (HMGs) modulate chromatin structure and transcription. HMGA1a is a PRMT1 substrate that has three positively charged motifs or AT hooks that bind DNA. These AT hooks are rich in RG sequences that once methylated may disrupt the interactions between the HMG protein and DNA [61]. An increase of HMGA1a arginine methylation could be related to heterochromatin and chromatin remodeling of apoptotic cells [62].

\textbf{REGULATION OF PRMTS AND ARGinine METHYLATION}

1. Regulation of PRMTs

PRMT activity is regulated by a variety of factors. Several isoforms of the PRMT family have different mRNA splice variants that when translated can lead to a set of proteins with unique activities and/or substrate specificities. PRMT1, for example, has seven N-terminal splice variants in humans [15] that have different activity and substrate specificity [34]. In addition to splice variants, the cellular localization of the PRMTs and the availability of their protein substrates dictate the level of protein arginine methylation.
that can occur. Finally, PRMT methylation is regulated by protein interaction partners. For example, PRMT1 methylation is stimulated towards select substrates *in vivo* by the binding of its two protein interaction partners, BTG1 and TIS21/BTG2 [14]. Another protein that interacts with BTG1, hCAF1 (CCR4-associated factor 1), has been shown to regulate PRMT1 activity in a substrate-dependent manner [63].

PRMTs are also regulated by their oligomeric status. Monomeric PRMTs combine to form biologically active homodimers that can bind AdoMet and methylate protein substrates [36]. In addition, PRMT1 and PRMT8 have the unique ability to heterodimerize. Depending on the abundance of the PRMT1-PRMT8 heterodimer *in vivo*, the concentration of available PRMT1 monomers and the amount of active PRMT1 homodimers in the cell would be reduced. Although this could be a very important regulatory mechanism of PRMT1, it remains unclear how the PRMT1-PRMT8 heterodimer affects the substrate selection or activity of PRMT1.

2. Regulation of arginine methylation

Once a protein is arginine methylated, can it then be demethylated? Protein arginine demethyliminase 4 (PAD4) has been shown to demethyliminate methylarginine residues in histones and reverse the biological consequence of methylation [64]. PAD4 is not a true demethylase, however, because it converts MMA into citrulline instead of arginine (Fig. 2-6). Recently, a true arginine demethylase was discovered. The Jumonji domain-containing 6 (JMJD6) protein is a JmjC-containing iron- and 2-oxoglutarate-dependent dioxygenase that was shown to reverse the effects of protein arginine methylation on histones H3 and H4 in both biochemical and cell-based assays [65].
Since JMJD6 acts on mono- and dimethylated histone proteins only, is the effect of protein arginine methylation reversible on other methyl-arginine containing proteins in the cell? The characterization of JMJD6 will help define how arginine methylation is regulated on histone proteins and may lead to the identification of other true protein demethylases.

Fig. 2-6. Reversing the effects of arginine methylation. PRMTs methylate protein arginine residues to form MMA (shown here), ADMA, and SDMA. PAD4, a protein arginine demethylaminase, converts MMA into citrulline but not back into arginine. JMJD6 is a true protein demethylase that targets methylated arginine residues on histone proteins. \( R=(CH_2)_3CH(NH_3^+)COO^- \)

**PROTEIN ARGinine METHylation & DISEASE**

Although PRMTs are vital to normal development and function [12], arginine methylation also leads to disease. Arginine methylation has mainly been implicated in the progression of 1) cardiovascular disease [66] and stroke [67], 2) multiple sclerosis, and 3) spinal muscular atrophy [11], and 4) cancer [68]. Fortunately, the cellular concentration of the PRMT inhibitor AdoHcy regulates methyltransferase activity. PAD4 and JMJD6 reverse the effects of protein arginine methylation by demethylating methylated protein arginine residues. Finally, the activity of dimethylarginine dimethylaminohydrolase (DDAH) helps keep free MMA and ADMA levels in check.
1. Cardiovascular Disease

Several studies have shown that free ADMA, a downstream product of the PRMTs, has a clear role in cardiovascular disease (reviewed in [66]) and stroke [67] by serving as an endogenous inhibitor of nitric oxide synthase [69]. When proteins that have been modified by the PRMTs are sent to the proteasome and degraded, free MMA, SDMA, and ADMA are released. MMA and ADMA specifically inhibit inducible nitric oxide synthase (iNOS). Since the nitric oxide produced by NOS acts as a vasodilator maintaining cardiovascular health, the inhibition of NOS contributes to atherosclerosis [70]. Free MMA and ADMA levels are controlled by dimethylarginine dimethylaminohydrolases (DDAHs). Although DDAHs keep the methylarginine levels in check, misregulation of DDAH or the hyperactivity of PRMTs may contribute to cardiovascular disease [71]. In fact, PRMT1 has been shown to be overexpressed in myocardial tissue taken from coronary heart disease patients [72].

Numerous studies have shown a relationship between elevated ADMA concentration and cardiovascular disease (CVD). Elevated ADMA concentration is highly prevalent in hypercholesterolemia, hyperhomocysteinemia, diabetes mellitus, peripheral arterial occlusive disease, hypertension, chronic heart failure, and coronary artery disease (reviewed in [66]). Although these studies were case studies and did not provide insight into exactly how ADMA was involved in promoting CVD, the observation that ADMA levels increase early in the development of atherosclerosis suggests that ADMA has the potential to be not only a marker but a mediator of vascular lesions [73]. In addition to CVD, elevated levels of ADMA increase the risk for ischemic stroke in the elderly [67].
2. **Multiple Sclerosis**

PRMT5 methylation of myelin basic protein (MBP) is involved in the progression of multiple sclerosis (MS), a demyelination disease of the central nervous system that targets myelin sheaths that surround and protect nerve axons. The demyelination of this protective coat contributes to the pathogenesis of MS. A recent study showed that myelin basic protein, which accounts for 35% of all protein in the myelin sheath, is a PRMT5 substrate. Arginine 107 on all three MBP isoforms was found to be mono- and symmetrically dimethylated in MS patients, and increased arginine methylation of MBP was shown to precede symptoms of MS in mice [74]. In addition, methylated MBP may also serve as an autoantigen, as is the case with methylated Sm and collin in lupus erythematosus [75].

3. **Spinal Muscular Atrophy**

Arginine methylation also plays a role in spinal muscular atrophy (SMA), an autosomal recessive disease that results from deletions or loss-of-function mutations within the SMN1 gene. A lack in the survival motor neuron (SMN) protein leads to motor neuron axonal guidance failure contributing to the progression of SMA [76]. SMN has been shown to be required for proper ribonucleoprotein assembly, a process that also requires arginine methylation [77]. Recently, point mutations were identified in the SMN Tudor domain in patients with SMA. This domain normally associates with SDMA on spliceosomal Sm proteins D1, D3, and B/B’ [50] aiding in the proper localization of the Sm proteins. Mutations in the Tudor domain in SMA patients cause the mislocalization of the Sm proteins and the improper assembly of ribonucleoproteins [78].
4. Cancer

PRMTs have also been directly linked to oncogenesis and may act as novel therapeutic targets in human cancer. PRMT1 has been shown to be an essential component of the mixed lineage leukaemia (MLL) oncogenic transcriptional complex that has both histone acetylation and H4-R3 methylation activities. An oncogenic MLL fusion complex that mediates step-wise histone modifications may facilitate an open chromatin structure for active gene expression and promote oncogenic transformation. In fact, the direct fusion with MLL to PRMT1 or SAM68, a bridging molecule in the complex for PRMT1 interaction, enhanced self-renewal of primary haematopoietic cells. Furthermore, the specific knockdown of SAM68 or PRMT1 expression suppressed MLL-mediated transformation. Taken together, PRMT1 plays an essential role in a novel protein arginine methyltransferase-dependent oncogenic pathway [68].

PRMTs are also known coactivators for nuclear receptors and are likely to be overexpressed in hormone-dependent cancers such as prostate and breast cancer. In fact, an increase in PRMT4 expression correlates with androgen independence in human prostate carcinoma [79]. Importantly, however, small molecules that inhibit both PRMT1 and PRMT4 suppress estrogen and androgen receptor-mediated transcriptional activation [80].

CONCLUSION

Protein arginine methyltransferases play an important role in a diverse set of biological processes. PRMTs methylate a variety of protein substrates including histones, RNA-binding proteins, and various enzymes. Protein arginine methylation
alters protein-protein, protein-RNA, and protein-DNA interactions. PRMT1 performs over 85% of all protein arginine methylation and is critical for survival. Yet, PRMTs have been linked to heart disease, spinal muscular atrophy, multiple sclerosis, and cancer. While PRMT biology has remained in the forefront, much of the biochemistry for this class of enzymes has received little attention until recently. Once the substrate specificity and mechanism of PRMT1 become clear, more specific, novel therapeutic targets can then be developed. Inhibiting PRMT activity could potentially slow the progression of cardiovascular disease and cancer in certain patients. It will be interesting to see how an increased biochemical understanding these enzymes will impact biology and medicine.

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CHAPTER 3

AN ENZYME-COUPLED CONTINUOUS SPECTROPHOTOMETRIC ASSAY FOR 
S-ADENOSYLMETHIONINE-DEPENDENT METHYLTRANSFERASES

ABSTRACT

Modification of small molecules and proteins by methyltransferases impacts a wide range of biological processes. Here we report an enzyme-coupled continuous spectrophotometric assay to quantitatively characterize S-adenosyl-L-methionine (AdoMet/SAM)-dependent methyltransferase activity. In this assay, S-adenosyl-L-homocysteine (AdoHcy/SAH), the transmethylation product of AdoMet-dependent methyltransferases, is hydrolyzed to S-ribosylhomocysteine and adenine by recombinant S-adenosylhomocysteine/5’-methylthioadenosine nucleosidase (SAHN/MTAN, EC 3.2.2.9). Subsequently, adenine generated from AdoHcy is further hydrolyzed to hypoxanthine and ammonia by recombinant adenine deaminase (EC 3.5.4.2). This deamination is associated with a decrease in absorbance at 265 nm that can be monitored continuously. Coupling enzymes are recombinant and are easily purified. The utility of this assay was shown using recombinant rat protein arginine N-methyltransferase 1 (PRMT1, EC 2.1.1.125) which catalyzes the mono- and dimethylation of guanidino nitrogens of arginine residues in select proteins. Using this assay, the kinetic parameters of PRMT1 with three synthetic peptides were determined. An advantage of this assay is the destruction of AdoHcy by AdoHcy nucleosidase, which alleviates AdoHcy product

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feedback inhibition of \( S \)-adenosylmethionine-dependent methyltransferases. Finally, this method may be used to assay other enzymes that produce AdoHcy, 5’-methylthioadenosine, or compounds that can be cleaved by AdoHcy nucleosidase.

**INTRODUCTION**

\( S \)-Adenosyl-L-methionine (AdoMet/SAM)-dependent methyltransferases play an important role in biological systems, including signal transduction, protein repair, biosynthesis, chromatin regulation, and gene silencing [1,2]. Small molecule, RNA, DNA, lipid, and protein methyltransferases exist [2-5]. More recently, data supporting the idea that protein arginine methylation plays a more dynamic role in the histone code has been put forth [6-9]. Defining how the protein methyltransferases work and what determines which proteins/residues will become methylated is pivotal for understanding the role these enzymes play in biology.

The majority of methyltransferase activity assays currently used are based on radioactive labeling using the AdoMet substrate labeled with \(^{14}\text{C}\) or \(^{3}\text{H}\) [10-12]. This is because there is very little detectable spectral change between the AdoMet substrate and its common transmethylation product, \( S \)-adenosylhomocysteine (AdoHcy/SAH). However, radioactive assays require subsequent separation of the product and substrate, which is often time-consuming. Another problem associated with this technique is that in many cases, the AdoHcy product acts as a potent feedback inhibitor to the methyltransferase, adding to the overall margin of error experienced in determining its kinetic parameters [13-16]. Two recently developed discontinuous assays make use of recombinant coupling enzymes that hydrolyze AdoHcy to homocysteine, which is detected by either chromogenic [15] or fluorescent [17] thiol-reactive reagents.
We report here a continuous enzyme-coupled photometric assay for AdoMet-dependent methyltransferases. As shown in Figure 3-1, AdoHcy can be converted to S-ribosylhomocysteine and adenine by AdoHcy nucleosidase. Earlier studies demonstrated that AdoHcy nucleosidase effectively cleaves the AdoHcy transmethylation product [15,16], eliminating the error associated with product inhibition. The adenine product of the reaction is then converted to hypoxanthine by adenine deaminase, resulting in an absorbance decrease at 265 nm that can be easily detected by UV spectrometry. The rapid and continuous detection of the conversion of substrate to product also helps to improve the accuracy over discontinuous assaying. We demonstrate the utility of this assay by characterizing the activity of recombinant rat PRMT1, a protein methyltransferase known to methylate a variety of proteins including histone 4, fibrillarin and RNA binding proteins (for a review, see [18]).

**Fig. 3-1.** Continuous assay scheme used to detect AdoMet-dependent methyltransferase activity. AdoHcy is converted into adenine and S-ribosylhomocysteine via AdoHcy nucleosidase. The deamination of adenine into hypoxanthine by adenine deaminase is associated with a decrease in absorbance at 265 nm, which can be monitored continuously using a spectrophotometer. Nu, nucleophile.
MATERIALS AND METHODS

Materials

AdoMet p-toluenesulfonate salt and AdoHcy were purchased from Sigma. Hypoxanthine was purchased from the California Foundation for Biochemical Research. Amylose resin was purchased from New England BioLabs. Adenine was purchased from Acros Organics. Recombinant AdoHcy nucleosidase containing a histidine tag was expressed and purified as previously described [19]. All other chemicals were of ACS grade or better.

Expression and purification of MBP-adenine deaminase

The DNA encoding *Bacillus subtilis* adenine deaminase was PCR-amplified from the pHH1010 plasmid [20] and ligated into a pMAL-c2x plasmid vector (New England BioLabs) between EcoRI and SalI sites. *Escherichia coli* TB-1 cells were transformed with the resulting plasmid and grown aerobically in 1 L LB broth at 37°C for 11 hours. Expression of maltose binding protein (MBP)/adenine deaminase fusion protein was induced with 0.8 mM IPTG for 10 hours. Cells were harvested by centrifugation and resuspended in ~30 mLs column buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, and 0.2 mM DTT). Cells were lysed via sonication using four 2 min discontinuous cycles on ice using, and the cell debris and unbroken cells removed by centrifugation at 55,000 x g, 4°C for 25 minutes. The supernatant was filtered through a 0.45 μm filter and incubated with 10 mL amylose resin slurry (New England BioLabs) at 4°C for 90 min with gentle agitation. After washing the resin with 30mL column buffer, MBP-adenine deaminase was eluted in 5 mL fractions with column buffer containing 10
mM maltose. Fractions demonstrating >95% purity by SDS-PAGE were concentrated by Centricon-Plus Concentrators (30,000 MWCO, Amicon) and the buffer exchanged to column buffer as per the manufacturer’s instructions. The purified protein was stored at -80°C in 25% glycerol. Protein concentration was determined spectrophotometrically using ε_{280nm}=85,770 M^{-1}cm^{-1}. Approximately 18 mg of purified protein was obtained from 1L of broth.

**Purification of His-PRMT1**

The DNA encoding rat PRMT1 was PCR amplified from the GST-PRMT1 vector with *Pfu* polymerase and ligated into a pET28b vector (Novagen) to yield an N-terminal His-tagged PRMT1 construct. *E. coli* BL21(DE3) cells carrying the pET28b/PRMT1 plasmid were grown in LB broth at 37°C. Protein expression was induced with 1.2 mM IPTG for 5 h. Cells were harvested by centrifugation and resuspended in wash buffer (50 mM sodium phosphate pH 7.5 and 20 mM imidazole). Cells were lysed by sonication using three 15s cycles and centrifuged at 100,000 x g at 4°C for 1 h. The resulting crude supernatant was incubated with Ni Sepharose™ High Performance resin (Amersham Biosciences) for 4 h at 4°C. The slurry was loaded into a 1.7 x 13 cm column, and the flow through was collected. The column was washed with 65 mL wash buffer, and the protein was eluted with 10 mL of wash buffer containing 250 mM imidazole. The eluate was concentrated in Centricon-Plus Concentrators (30,000 MWCO, Amicon), the buffer exchanged to 50 mM sodium phosphate buffer pH 7.5, and 10% glycerol was added to the pure protein before storing it at -80°C. Approximately 5.4 mg pure protein was obtained from 1 L of broth and was >95% pure by SDS-PAGE.
Procedure for the enzyme-coupled photometric assay

Assays were performed in thermostatted 1cm quartz cuvettes at 37°C. Manganese sulfate (MnSO₄) was added to a final concentration between 10 to 1050 µM. Between 10 to 1050 µM of manganese, the same activity was observed. Manganese or other divalent ions (e.g., zinc) are required for the deaminase activity ([21] and Dorgan & Zhou, unpublished results). The assay involving the conversion of adenine to hypoxanthine was run using adenine at various concentrations, 1050 µM MnSO₄, and 0.02 µM adenine deaminase buffered in 200 mM Tris pH 8.0. The assay monitoring the conversion of AdoHcy to hypoxanthine contained 54.3 µM AdoHcy, 1050 µM MnSO₄, 0.02 µM adenine deaminase, and 17.3 nM AdoHcy nucleosidase buffered in 200 mM Tris pH 8.0. Between 3.0 to 20.0 nM adenine deaminase, the same rate was observed. Measurement of PRMT1 activity was performed in 50 mM sodium phosphate pH 7.0 with 168 µM AdoHcy nucleosidase, 0.02 µM adenine deaminase, 10-1050 µM MnSO₄, and various concentrations of PRMT1. Use of AdoHcy nucleosidase at concentrations ranging from 10 nM - 168 µM yielded the same methyltransferase rate. Reactions were initiated with differing amounts of peptide substrates as indicated in the figures.

Radioactive assay used to determine PRMT1 activity

Methyltransferase assays were equilibrated at 37 °C for 15 minutes before initiating with 211 µM R3 peptide. Each 65 µL reaction contained S-adenosyl-L-[methyl-³H] methionine (specific activity 79 µCi/µmol, Amersham Biosciences), 4 µM PRMT1, 250 µM AdoMet, 100 µM MnSO₄, 0.02 µM adenine deaminase, 168 µM
AdoHcy nucleosidase, and 100 mM sodium phosphate pH 7.0. Aliquots of 10 µL were spotted directly onto P81 paper (Whatman) under vacuum in a S&S Minifold® I Slot Blot System (Schleisher & Shuell) at specific time points and washed three times with 500 µL 50 mM sodium phosphate buffer pH 7.5. After the filter paper dried, each piece was placed in 5 mL Scintisafe™ cocktail (Fisher) and counted (Beckman LS 6500).

Internal tritium standards were used initially to examine counting efficiency; however, we determined that the presence of protein had a profound effect on counting efficiency. Instead, various volumes of the control reaction, which did not contain peptide substrate, were spotted onto P81 membranes and counted. Linearity between observed cpm's and volume spotted was demonstrated. Counting efficiency was 4-6% and all calculations were adjusted accordingly. Data were plotted against time and fitted using linear regression.

**High-performance liquid chromatograph (HPLC) analysis of reaction products**

HPLC analysis of the hypoxanthine product formed during peptide methylation by PRMT1 was performed on an Apollo C-18 reverse-phase column (4.6 mm x 25 cm, Alltech, Deerfield, Il). The column was eluted with an isocratic mixture from 0-3 minutes of 100 mM ammonium bicarbonate (A) at pH 7.8 (90%) and methanol (B) (10%). A gradient mixture was used from 3-8 minutes where the composition changed from 90% A and 10% B to 10% A and 90% B. From 8-10 minutes a gradient mixture was used and changed the composition from 10% A and 90% B to 90% A and 10% B. Finally, an isocratic mixture was used from 10-15 minutes. A 1.0 mL/min flow rate was
used and the analytes (AdoMet, AdoHcy, adenine, and hypoxanthine) were monitored at 245 nm.

RESULTS AND DISCUSSION

Conversion of AdoMet to hypoxanthine using the coupling enzymes

In order to yield valid kinetic parameters in the coupled assay, the coupled enzymes used should not be rate limiting, so that the measured rate is determined solely by the methyltransferase activity. The kinetics for the conversion of adenine to hypoxanthine via adenine deaminase were investigated first. Adenine absorbs maximally at 260 nm with an extinction coefficient of 13,400 M\(^{-1}\) cm\(^{-1}\) [22]. Upon adding adenine deaminase, an absorbance decrease at 265 nm was observed as adenine was converted to hypoxanthine rapidly in a stoichiometric fashion (Fig. 3-2). The \(k_{\text{cat}}\) for adenine deaminase in 100mM Tris pH 8.0 was 35.2 ± 0.92 sec\(^{-1}\). The difference spectrum shown in Fig. 3-2B shows the maximal change in absorbance at 265 nm. In the presence of 13.3 μM adenine, concentrations of hypoxanthine ranging from 3.5 μM to 142 μM did not inhibit adenine deaminase (data not shown). Complete conversion of AdoHcy to hypoxanthine using both the coupling enzymes, AdoHcy nucleosidase and adenine deaminase, was accompanied by a similar absorbance change (data not shown). The reaction, like that of adenine deaminase was found to be very rapid. The \(k_{\text{cat}}\) for AdoHcy nucleosidase in 100mM Tris, pH 8.0 was 4.12 ± 0.10 sec\(^{-1}\). In comparison, most AdoMet-dependent methyltransferases display \(k_{\text{cat}}\) in the 0.016 sec\(^{-1}\) (1 min\(^{-1}\)) range. The two coupling enzymes are over 100-fold more active than most of the methyltransferases, and thus are well suited for kinetic analysis described in this paper. The relationship
between AdoHcy concentration and absorbance change at 265 nm was linear and yielded a $\Delta \varepsilon_{265} \text{ of } 6,700 \pm 150 \text{ M}^{-1}\text{cm}^{-1}$ based on the $\varepsilon_{260}$ of $15,400 \text{ M}^{-1}\text{cm}^{-1}$ for AdoHcy [22] (Fig. 3-3).

Fig. 3-2. (A) Absorbance change associated with the conversion of adenine to hypoxanthine catalyzed by adenine deaminase over 5 minutes. The reaction mixture contained 54.3 µM adenine, 1050 µM MnSO$_4$, and 0.02 µM adenine deaminase buffered in 200 mM Tris pH 8.0 and 37 ºC. The reaction was completed in 5 minutes. The arrow indicates the decrease in absorbance at 265 nm with time. In (B) the spectrum of the original solution of adenine (dotted line), the reaction at completion (bold solid line) and difference spectrum (thin solid line) are shown.
Investigation of PRMT1 Activity Using the Coupled Assay

We next investigated the proposed coupled methyltransferase assay using protein arginine N-methyltransferase 1 (PRMT1) as our test enzyme and a peptide corresponding to a 19-amino acid stretch of the *in vivo* PRMT1 protein substrate fibrillarin [23]. Initiation of the reaction with R3 peptide resulted in a decrease in absorbance at 265 nm as in the coupling enzyme control reactions (data not shown). Figure 3-4 demonstrates that the reaction rate was dependent upon methyltransferase concentrations. The rate obtained with 2 μM PRMT1 using this continuous spectrophotometric assay with 211 μM R3 (5.1 ± 0.2 μM AdoHcy formed/min) was verified by following [³H] incorporation from S-adenosyl-L-[methyl-³H] methionine into the R3 peptide. The rate observed using the radioactive assay was 4.9 ± 0.6 μM AdoMet consumed/min. Furthermore, the overall
reaction rates were independent on the coupling enzyme concentrations under our assays conditions. For instance, using a different preparation of PRMT1, the rates of PRMT1 catalyzed methylation of 200 μM R3 peptide using 10 nM, 100 nM, and 1μM, 168 μM AdoHcy nucleosidase were 8.71 ± 0.58, 9.05 ± 0.10, 9.15 ± 0.16, and 8.90 ± 0.09, respectively.

Fig. 3-4. PRMT1 activity with R3 peptide is dependent upon PRMT1 concentration. Reaction mixtures contained 59 μM AdoMet, 1050 μM MnSO₄, 0.02 μM adenine deaminase, 168 μM AdoHcy nucleosidase, and 211 μM R3 peptide in 50 mM potassium phosphate pH 7.0. Reactions also contained 0 (closed circles) 3.9 (open circles), 7.7 (closed squares), 11.4 (open squares), and 14.9 (open triangles) μM PRMT1, respectively. The inset shows that activity (∆Abs 265 nm/min) is a function of protein concentration.

Formation of hypoxanthine and the lack of intermediate build-up during methylation of R3 by PRMT1 were confirmed using HPLC. Standards of 80 μM hypoxanthine, AdoMet, AdoHcy, and adenine were used for comparison, and were found
to elute at 6.0, 8.5, 9.7, and 10.1 minutes, respectively. The formation of hypoxanthine with a corresponding disappearance of AdoMet was observed in the assay, with no detectable accumulation of the adenine or AdoHcy intermediates (Fig. 3-5).

Fig. 3-5. HPLC chromatogram to confirm reaction products. The top trace shows an HPLC chromatogram of a mixture of authentic 80 µM samples of AdoMet, AdoHcy, adenine (Ado), and hypoxanthine (Hxan). The middle trace is an HPLC chromatogram of an aliquot of the reaction mixture prior to the addition of R3 peptide. The bottom trace is the chromatogram of an aliquot of the reaction mixture when there was a change at 265 nm. An impurity from AdoMet (I) is seen in the bottom two traces. All traces were monitored at 245 nm.

Using this assay we investigated the kinetic parameters of PRMT1 with R3 peptide from fibrillarin, an R3 analog peptide containing only one substrate arginine residue (JMH1), and H4 peptide from histone 4 (Table 3-1). The R3 peptide contains three possible arginine methylation sites, each capable of being mono and/or dimethylated (6 potential methylation events). The JMH1 peptide lacks the additional 2
substrate arginine residues of R3 but maintains the positive charge at these positions. Although $V_{\text{max}}$ for R3 and JMH1 were similar, the $K_m$ for R3 could only be estimated to be under 10 $\mu$M with this assay. A few peptide substrates have previously been used to study native PRMT1 activity and have yielded values for $K_m$ of 0.2 - 60 $\mu$M [24, 25]. We believe that the dramatic increase in $V/K$ for R3 is a result of processive methylation at multiple arginine residues on the same peptide substrate and are currently investigating this further. Compared to the JMH1 peptide, the H4 peptide also contains only one arginine residue and demonstrated a $K_{m,\text{app}}$ of $745 \pm 70$ $\mu$M and $V_{\text{max}}$ of $5.7 \pm 0.2$ $\mu$M min$^{-1}$. These results are consistent with reports that substrates containing the RGG repeats, such as hnRNPA1 and fibrillarin, are better (catalytic efficiency) PRMT1 substrates than histone 4 [26].

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>$K_m$ ($\mu$M)</th>
<th>$V_{\text{max}}$ ($\mu$M min$^{-1}$)</th>
<th>#Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3</td>
<td>AcGG</td>
<td></td>
<td>&lt;10</td>
<td>10.7 ± 0.8</td>
</tr>
<tr>
<td>JMH1</td>
<td>AcKG</td>
<td></td>
<td>450 ± 90</td>
<td>14.8 ± 1.7</td>
</tr>
<tr>
<td>H4</td>
<td>AcSG</td>
<td></td>
<td>745 ± 70</td>
<td>5.7 ± 0.2</td>
</tr>
</tbody>
</table>

Table 3-1  Kinetic parameters for peptide substrates of PRMT1

Reactions were performed at 37° C in 50 mM potassium phosphate, pH 7.0, using 250 $\mu$M AdoMet, 4 $\mu$M PRMT1, 1050 $\mu$M MnSO$_4$, 168 $\mu$M AdoHcy nucleosidase, 0.02 adenine deaminase, and various amounts of peptide. Initial rates (< 10% product formation) were plotted and the data fit with the Michaelis-Menton equation using non-linear regression. $V_{\text{max}}$ is expressed as $\mu$M AdoHcy min$^{-1}$.

This study shows that this continuous assay can be used to characterize PRMT1 enzyme activity and is currently being amended to other AdoMet-dependent-methyltransferases (personal communication, Zhou). This assay system is similar to that
described by Coward and co-workers in 1973 for catechol-O-methyltransferase [27] which uses adenosine deaminase from *Aspergillus oryzae* to convert AdoHcy to S-inosylhomocysteine. However, the fungal adenosine deaminase has not been cloned, so purification of the enzyme from Taka-Diastase is achieved with a multi-step/multi-column procedure [28]. In comparison the present assay utilizes recombinant fusion-tagged coupling enzymes that are easily purified in large amounts in a single day. More importantly, the structures of S-inosylhomocysteine and S-adenosylhomocysteine only differ by one atom (an oxygen versus a nitrogen), thus are very similar to each other. Hence, the use of MTA nucleosidase and adenine deaminase avoids potential product inhibition by S-inosylhomocysteine [29], the product of the fungal adenosine deaminase reaction.

Despite the versatility of the assay, there are some potential limitations. For instance, if the methyltransferase substrates strongly absorb around 265 nm, a narrow range of absorbance changes will be available for activity measurement, and analysis will be subject to the detection limits of the spectrophotometer. However, even with 1 mM each of dATP, dCTP, dGTP, dTTP we were still able to detect the change of 35 μM of AdoHcy to hypoxanthine (data not shown). Use of protein substrates exhibiting a strong UV absorbance may not be feasible. However, we were able to monitor methyltransferase activity using the small *in vivo* PRMT1 protein substrate histone 4 (H4) (Fig. 3-6). Several methyltransferases such as catechol O-methyltransferase (COMT) display for the methyl acceptor of ~100 μM [27,31], but some enzymes display much lower K_m values. Determination of K_m values lower than 10 μM may be limited by the small absorption changes at low substrate concentrations, but may be performed using
progress curve analyses [32]. In any case, the assay can be used to determine maximum rate. Finally, since AdoMet contributes to the background absorbance, concentrations of AdoMet should be kept at or below 250 μM or smaller pathlength cuvettes should be used to keep the total absorption around 265 nm within the linear range of the spectrophotometer for accurate measurement.

Fig. 3-6. The continuous assay monitors methylation of histone 4 (H4) protein. Reactions containing 250 μM AdoMet, 10 μM MnSO4, 10 nM AdoHcy nucleosidase, 0.02 μM adenine deaminase, and 0 (open circles) or 5.0 μM of purified [30] H4 protein (closed circles) were equilibrated to 37 ºC for 10 minutes and initiated with 4 μM PRMT1. The decrease in absorbance associated with the methylation of H4 was monitored at 265 nm.

The potential for this assay goes further than AdoMet-dependent-methlytransferases. The AdoHcy/MTA nucleosidase displays broad substrate specificity cleaving not only AdoHcy and MTA, but also structural analogs with hydrophobic
residues at the C5 position [19,33] (Cornell, Dorgan, Zhou unpublished results). In addition, the adenine deaminase also shows broad substrate specificity for purine analogs [34,35] (Dorgan, Zhou unpublished results). Therefore we predict that the assay will be applicable to a number of other enzymes whose products can be cleaved by AdoHcy/MTA nucleosidase to generate adenine or adenine analogs that can be used by adenine deaminase. Two examples include polyamine synthesis and acylhomoserine lactone synthesis, both of which produce MTA [36,37]. Other enzymes can be found in a recent review on AdoMet utilizing this enzyme [38].

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CHAPTER 4

SUBSTRATE PROFILING OF PRMT1 REVEALS AMINO ACID SEQUENCES THAT EXTEND BEYOND THE ‘RGG’ PARADIGM

ABSTRACT

Protein arginine methyltransferase 1 (PRMT1) catalyzes the mono- and dimethylation of certain protein arginine residues. Although this posttranslational modification has been implicated in many physiological processes, the molecular basis for PRMT1 substrate recognition is poorly understood. Most modified arginine residues in known PRMT1 substrates reside in repeating ‘RGG’ sequences. However, PRMT1 also specifically methylates Arg3 of histone H4 in a region that is not glycine-arginine rich, suggesting that PRMT1 substrates are not limited to proteins bearing ‘RGG’ sequences. Because a systematic evaluation of PRMT1 substrate specificity has not been performed, it is unclear if the ‘RGG’ sequence accurately represents the consensus target for PRMT1. Using a focused peptide library based on a sequence derived from the in vivo substrate fibrillarin we observed that PRMT1 methylated substrates that had amino acid residues other than glycine in the ‘RX₁’ and ‘RX₁X₂’ positions. Importantly, eleven additional PRMT1 substrate sequences were identified. Our results also illustrate that the two residues on the N-terminal side of the modification site are important and need not both be glycine. PRMT1 methylated the eukaryotic initiation factor 4A1 (eIF4A1) protein, which has a single ‘RGG’ sequence. Methylation of eIF4A1 and the similar

eIF4A3 could be affected using single site mutations adjacent to the modification site, demonstrating the importance of amino acid sequence in PRMT1 protein substrates. Dimethylation of the parent library peptide was shown to occur through a dissociative mechanism. In summary, PRMT1 selectively recognizes a set of amino acid sequences in substrates that extend beyond the ‘RGG’ paradigm.

**INTRODUCTION**

Protein arginine methyltransferase (PRMT) enzymes perform an important biochemical reaction resulting in the methylation of many cellular proteins. PRMTs transfer a methyl group from S-adenosyl methionine (AdoMet or SAM) onto the terminal guanidino nitrogen of a protein arginine residue resulting in the formation of monomethyl arginine (MMA) and S-adenosyl homocysteine (AdoHcy or SAH). Type I methyltransferases catalyze a second methylation event onto the previously methylated guanidino nitrogen of MMA to form asymmetric dimethylarginine (ADMA) (Scheme 4-1). Type I PRMTs include PRMT1 [1], PRMT3 [2], coactivator-associated arginine methyltransferase 1 (CARM1/PRMT4) [3, 4], PRMT6 [5], and PRMT8 [6]. Type II methyltransferases catalyze the formation of symmetric dimethylarginine (SDMA) whereby the second methyl group is transferred onto the unmodified guanidino nitrogen of MMA. Jak-binding protein 1 (JBP1/PRMT5) exhibits Type II activity [7, 8], but conflicting reports have been made for PRMT7. PRMT7 has been shown to generate MMA with a peptide substrate [9] and both asymmetric and symmetric dimethylarginine residues on protein substrates [10]. The F-box only protein 11 (FBXO11/PRMT9)
catalyzes the formation of MMA, ADMA, and SDMA on protein arginine residues [11]. No methyltransferase activity has been found for PRMT2.

Scheme 4-1. Reactions catalyzed by PRMT1. All PRMT isoforms catalyze the S-adenosylmethionine (AdoMet)-dependent methylation of protein arginine residues to form monomethyl arginine (MMA). PRMT1, a type I PRMT, transfers a second methyl group from AdoMet onto the previously modified guanidino nitrogen to form asymmetric dimethylarginine (ADMA). S-Adenosyl homocysteine (AdoHcy) is a product and inhibitor of this AdoMet-dependent methyltransferase reaction.

PRMT enzymes participate in a variety of cellular processes in eukaryotes. PRMT activity has been implicated in development [12, 13], RNA processing, transcriptional regulation, signal transduction, DNA repair, and chromatin remodeling [14, 15, 16]. In fact, the recent discovery of a histone protein arginine demethylase, Jumonji domain-containing 6 protein (JMJD6), validates that protein arginine methylation is a reversible post translational modification on certain histone tails [17]. Thus far, eleven putative PRMT isoforms (PRMT1-11) have been identified in humans with orthologs present in yeast [18], protozoa, Caenorhabditis elegans, Drosophila melanogaster [19], plants [20], and fish [21]. The breadth of these enzymes indicates the biological importance of arginine methylation across the plant and animal kingdoms.
Although PRMTs are vital to normal development and function, protein arginine methylation has also been linked to the progression of carcinogenesis, viral pathogenesis, multiple sclerosis, spinal muscular atrophy, lupus, and cardiovascular disease (reviewed in 14). Overall, PRMTs play important roles in biology, yet much of the basic biochemistry for this class of enzymes remains unexplored. Unlike the protease and kinase fields where substrate specificity has been studied in detail [22, 23], the molecular basis for substrate recognition by the PRMTs is poorly understood.

Of the eleven mammalian PRMT isoforms, PRMT1 predominates, performing an estimated 85% of all protein arginine methylations in vivo [18]. The prevailing observation is that PRMT1 methylates a variety of proteins that are glycine and arginine rich within repeating ‘RG’ or ‘RGG’ motifs such as fibrillarin (GGRRGGG)\textsuperscript{2} [24] and the heterogeneous nuclear ribonuclear proteins or hnRNPs (GGRGGGS) [25]. The ‘RGG’ motif is a recognized RNA-binding domain and is characterized by a variable number of closely spaced arginine-glycine-glycine repeats interspersed with other amino acids [26]. It is also common to observe a ‘GG’ sequence flanking the N-terminal side of the modification site. Many of these methylated proteins have been initially identified using commercially available anti-methyl arginine antibodies that recognize MMA or ADMA within the tandem ‘RGG’ context. Although the predominant feature of known PRMT1 protein substrates is a glycine/arginine rich region, PRMT1 has also been shown to specifically methylate several arginines within ‘RXR’ sequences on the surface of the Poly(A)-binding protein II (PABPII) [27] and the ‘MSG\textsuperscript{RGKG}’ sequence on the histone H4 tail [28]. It should be noted, that methylated histones are not detected by the commercially available anti-methyl arginine antibodies (e.g. Asym24 and Asym25 from
Upstate are directed against peptides with ADMA-glycine or ADMA-glycine-glycine repeats, respectively) [29], most likely due to the specificity of antibodies for arginine–glycine repeats [30]. Consequently, only a sub-set of methylated proteins can be recognized by anti-methyl arginine antibodies. This suggests that there may be a diversity in PRMT1 protein substrates that has previously gone unnoticed.

The crystal structure of PRMT1 complexed with AdoHcy and the R3 peptide substrate (GGRGGFGGRGGFGGRGGFG) provided insight into the reaction mechanism [31]; however, due to a lack in electron density afforded by the peptide substrate, few inferences could be made concerning the substrate specificity of PRMT1. Additionally, the electron densities from the four glycines that flank the substrate arginine of the R3 peptide substrate were not observed and created a gap in the linear sequence of R3. The lack in observable substrate electron density suggests that the substrate specificity of PRMT1 may be much broader than the typical ‘RGG’-containing substrate.

Together, these results prompted us to investigate the substrate specificity of PRMT1. The goals of this work were 1) to determine if PRMT1 displays substrate selectivity at the amino acid sequence level and 2) to identify novel PRMT1 substrate sequences. A focused directional peptide library based on the in vivo substrate fibrillarin was used to determine which peptide sequences are methylated by PRMT1. Our results show that PRMT1 demonstrates substrate selectivity at the amino acid sequence level in peptide substrates. We found that the selection of PRMT1 protein substrates is more complex, wherein the amino acid sequence surrounding the substrate arginyl group of the protein substrate is one important factor. In summary, our data show that PRMT1 has the
ability to discriminate between peptide and protein substrates at the amino acid sequence level. Furthermore, substrate profiling indicated that PRMT1 can methylate sequences that go beyond the ‘RGG’ paradigm.

MATERIALS AND METHODS

All chemicals used were of ACS reagent grade or better. AdoMet was purchased from Sigma as a chloride salt (≥80%, from yeast). The custom PepScreen peptide library was ordered from Sigma Genosys using AcKGGFGGRGGFGGGKW as the parent peptide. The two glycines C-terminal to the arginine (underlined) were substituted with all other amino acids except arginine one at a time to give rise to 36 unique peptides. The tryptophan on the C-terminus was added in order to accurately determine the concentration of each peptide in solution by UV-vis spectrometry. Peptides were analyzed by MALDI mass spectrometry to confirm their molecular weight and sampled by HPLC to assess purity.

Peptide Library Kinetics

A continuous spectrophotometric assay for AdoMet-dependent methyltransferases (see Chapter 3) was used to assay PRMT1 with peptides from the peptide library. Briefly, recombinant AdoHcy nucleosidase (MTAN) was used to hydrolyze the AdoHcy generated from methyltransfer. The resulting adenine was deaminated by recombinant adenine deaminase. Importantly, this strategy alleviates any product inhibition that could occur from AdoHcy. Each 110 µL reaction contained 4 µM His-PRMT1 (purified as described in Chapter 3), 0.02 µM adenine deaminase (purified as described in Chapter 3), 250 µM AdoMet, 10 nM MTAN (purified as in [32]), 100 µM MnSO₄, and 50 mM
sodium phosphate buffer pH 7.1. Reactions were equilibrated at 37 °C for 10 minutes before they were initiated with various amounts of peptide. The decrease in absorbance at 265 nm was monitored continuously using a Cary 300 Bio UV-visible spectrophotometer. Initial rate data representing no more than 10% of product formation were fit to the Michaelis-Menten equation [33] to obtain $k_{\text{cat, app}}$ values. Mass spectrometry results with the parent peptide (Fig. 4-1) indicate that the time frame utilized in fitting the raw spectrophotometric data is representative of a single methylation event. Apparent kinetic parameters are listed due to technical limitations of the spectrophotometric assay; that is, concentrations of AdoMet >250 μM were not feasible due to high background absorbance in the assay (see Chapter 3). However, the AdoMet concentration in the assay did not support $V_{\text{max}}$ conditions ($K_{m, \text{AdoMet}} = 38 \pm 12$ μM when using the parent peptide as the methylacceptor). Each reaction was performed at least in duplicate, and at least two enzyme preparations were used to confirm trends in substrate utilization. The limit of detection for the spectrophotometric assays was 0.1 μM CH₃/min corresponding to a $k_{\text{cat}}$ of 0.025 min⁻¹. A radioactive assay (see Chapter 3) was also used to confirm the methylation rates of peptides at saturating conditions. The formation of MMA or ADMA was also confirmed using amino acid analysis [34]. Briefly, peptides were hydrolyzed in 6 M HCl, derivatized with AccQ-Fluor™ (Waters Corporation) and separated by reverse phase HPLC. This method provides base-line separation of MMA, ADMA, and SDMA.
**Construction of FLAG-eIF4A1 and His-eIF4A1 plasmids**

N-terminal FLAG epitope eIF4A1 fusion proteins were constructed by amplifying the DNA encoding mouse eIF4A1 from pc4A1 [35] using a sense primer 5’GGCGCCATATGTCTGCGAGTCA3’ and an antisense primer 5’CCCCGAATTCAATGAGGTCAGC3’. The FLAG-eIF4A1 (pST020 in Table 4-1) was created when this PCR product was digested with NdeI and EcoRI and inserted into the pAED4FLAG vector [36]. Mutation of the eIF4A1 Gly363 codon was accomplished by PCR amplification [37] with complementary oligonucleotide primers (see supplementary table 1) spanning the desired site of mutation using pST020 as template. In particular, G363Sforward and G363Sreverse were the primers used along with the pST020 template to give FLAG-eIF4A1(G363S) (pST202 in Table 4-1).

**Table 4-1  eIF4A1 Plasmid Constructs**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pST020</td>
<td>FLAG-eIF4A1 in pAED4FLAG</td>
</tr>
<tr>
<td>pST202</td>
<td>FLAG-eIF4A1(G363S) in pAED4FLAG</td>
</tr>
<tr>
<td>pST210</td>
<td>6XHis-eIF4A1(G363A) in pET20b</td>
</tr>
<tr>
<td>pST211</td>
<td>6XHis-eIF4A1(G363H) in pET20b</td>
</tr>
<tr>
<td>pST212</td>
<td>6XHis-eIF4A1(G363Y) in pET20b</td>
</tr>
<tr>
<td>pMA300</td>
<td>6XHis-eIF4A1 (wt) in pET20b</td>
</tr>
<tr>
<td>pMA301</td>
<td>6XHis-eIF4A1(G363S) in pET20b</td>
</tr>
</tbody>
</table>

The eIF4A1 constructs used in this work are listed followed by their descriptions. The pET20b vector was used to create the N-terminal histidine tagged eIF4A1 constructs, and the pAED4FLAG vector was used to generate the N-terminal FLAG tagged eIF4A1 constructs.
Construction of the N-terminal His-eIF4A1 and His-eIF4A1(G363S) plasmids (pMA300 and pMA301, respectively in Table 1) was performed by PCR amplifying pST202 with the following primers: sense= CCCATATGCATCATCATCATCATCATCACATGTCTGCGAGTCAG GATTC and antisense= GGGATCCTCAAATGAG GTCAGCAACGTTGAGGGGCA. PCR products were digested with NdeI and BamHI and ligated into the pET20b vector (Novagen) to yield N-terminal His-tagged eIF4A1 constructs. Next, N-terminal His-eIF4A1(G363A), His-eIF4A1(G363H), and His-eIF4A1(G363Y) constructs were obtained using pMA301(G363S) as the template and the corresponding primers. In particular, the G363H and G363Y mutations were made simultaneously using a sense oligo with YAT mismatch replacing WT G363 to create either a His or Tyr codon. The complementary antisense primer had a purine (A/G) at the position corresponding to the C/T in the sense primer. All plasmid inserts were sequenced in their entirety to confirm insert junctions and desired base substitutions.

**Purification of FLAG-eIF4A1 proteins**

FLAG-eIF4A1 proteins expressed in BL21(DE3) CodonPlus cells (Stratagene, La Jolla, CA) were incubated overnight at 4 °C with 0.5 μg M2 antibody (Sigma-Aldrich, St. Louis, MO) in radio-immunoprecipitation assay (RIPA) buffer [38]. Immune complexes were collected by incubation with protein G-agarose (Pierce Chemicals, Rockford, IL) for 1 hou at room temperature. Unbound proteins were removed by resuspension in RIPA buffer followed by brief centrifugation to collect bound proteins.
Samples were washed with RIPA buffer two more times followed by a single wash in 1X HMT (20 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 0.4 mM EDTA).

**Fluorography**

Methylation was performed by adding $1.1 \mu$Ci $[^3H]$-AdoMet (76.4 Ci/mmol, Perkin-Elmer, Boston, MA) to the adsorbed FLAG-eIF4A proteins (0.8 μg) and 0.5 μg His-PRMT1 in a total liquid volume of 30 μL HMT buffer. Reactions were allowed to incubate for 1 hour at 37 °C and were stopped by adding sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Laemmli sample buffer. Proteins were resolved in 12.5% polyacrylamide gels, treated with Enlightning (Perkin-Elmer), dried and exposed to X-ray film (RPI, Mt. Prospect, IL) for 2-7 days as indicated.

**His-eIF4A1 protein purification**

*E. coli* BL21(DE3) cells carrying the wild type (WT) and mutant eIF4A1 plasmid vectors were grown in Luria-Bertani (LB) broth at 37 °C. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 hours, and cells were harvested by centrifugation and re-suspended in wash buffer (50 mM sodium phosphate [pH 7.5] with 20 mM imidazole). Cells were lysed by sonication, and the crude supernatant was obtained by centrifugation. His-eIF4A1 was purified using Ni$^{2+}$-sepharose high performance resin (Amersham Biosciences) according to the manufacturer’s instructions. Purified protein was eluted with wash buffer containing 250 mM imidazole, and the eluate was concentrated in a Centricon-Plus Concentrator (30,000 MWCO, Amicon). The buffer was exchanged to 50 mM sodium phosphate buffer (pH
7.5) and the pure protein was stored at -80 °C. Proteins were >95% pure as judged by SDS-PAGE.

**Rates of in vitro methylation of eIF4A1 protein**

Reactions were performed using the same conditions that were used to test the peptides from the peptide library with the following modifications: 50 nM His-PRMT1, 500 μM AdoMet with 0.3 μM [H³]-AdoMet (Specific activity=83 μCi/μmol), and 50 mM sodium phosphate buffer (pH 8.0). After equilibrating at 37 °C for 10 minutes, reactions were initiated with 25 μM WT or mutant histidine tagged eIF4A1 protein. Various time point aliquots were taken out and quenched with an equal volume of cold 20% trichloroacetic acid (TCA). These time points were then loaded onto glass fibers (1.2 μm) in a UniFilter-96 well plate using a filter mate harvester (Perkin Elmer) and washed to rid the samples of remaining [H³]-AdoMet. Radiolabeled eIF4A1 protein was counted using Ultima Gold F scintillation cocktail and a TopCount NXT (Perkin Elmer). Rates of protein methylation were corrected by subtracting the background counts associated with the control reaction that lacked eIF4A1.

**Analysis of products by mass spectrometry**

A reaction prepared as described in the peptide kinetics methods was initiated with 300 μM WT-eIF4A1 peptide (YIHRIGRGGGR), 300 μM eIF4A1-S peptide (YIHRIGRSGGR), or 400 μM eIF4A1-Y peptide (AcYIHRIGRYGR). After 120 minutes, the reactions were terminated 1:1 with 20% TCA. Precipitated protein was removed by centrifugation, and the peptide samples were analyzed for methyl incorporation using MS and MS/MS analysis. For MALDI analysis, peptide samples were diluted in HPLC grade
water to a final concentration of 0.5-5 µM. Diluted peptide samples (0.5 µL) were spotted on a polished standard 192-well stainless steel MALDI sample plate followed by an alpha-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. A peptide standard, human angiotensin I ([M+H]^+ =1296.67), was used for the external calibration. Data were analyzed using Data Explorer software 4.6. In addition, unmodified, mono, and dimethylated peptides were analyzed by LC/MS using a Surveyor HPLC system coupled to a LCQ Deca XP mass spectrometer (Thermo Fisher, Waltham, MA). The peptides were diluted to 1 µM in 0.1% formic acid and a 2 µL aliquot was injected into a self-packed reversed phase column (75 µm i.d. × 15 cm, Magic C18 resin, 3 µm particle size, 200 Å pore size, Michrom Bioresources, Auburn, CA) and eluted at 200 nL/min with a linear gradient from 99:1 solvent A:B to 5:95 solvent A:B. Solvent A was water containing formic acid, 0.1%, v/v. Solvent B was acetonitrile containing formic acid (0.15, v/v). Data were processed using Xcalibar Data System 2.0 (Thermo Fisher, Waltham, MA).

RESULTS

Design of the peptide library

In order to determine if PRMT1 displays substrate selectivity at the amino acid sequence level, and if PRMT1 can methylate peptidyl arginine residues that are not present in either ‘RGG’ or ‘RXR’ sequences, we constructed a peptide library based on a
peptide (R3 in the literature) derived from the *in vivo* substrate fibrillarin. Although the R3 peptide has been widely used to study PRMTs and has been co-crystallized with PRMT1 [31], it contains three arginine residues, each of which can be methylated. In order to simplify the kinetics, the two flanking arginines of the R3 peptide were replaced with positively charged lysines. This fibrillarin-based peptide (KGGFGGRGGFGGKG) displayed saturation kinetics (data not shown) and was both mono- and dimethylated in a dissociative fashion as deduced by the lag in dimethylated product formation (Fig. 4-1). In order to allow for more accurate peptide quantification, a tryptophan was added to the C-terminus. The resulting peptide, AcKGGFGGRGGFGGKW, was used as the parent substrate in the peptide library. This peptide demonstrated saturation kinetics (data not shown).

![Fig. 4-1. Time-dependent formation of monomethylarginine and dimethylarginine by PRMT1. A reaction was performed with 100 μM fibrillarin-based peptide (KGGFGGRGGFGGKG), 250 μM AdoMet, and 2.5 μM PRMT1. At 0.2, 2, 4, 12, and 16 minutes, 120 μL aliquots were terminated with TFA (0.8% final) and analyzed by LC/MS. Substrate and product peak intensities were normalized for ionization differences between time points using an internal standard peptide with a similar amino acid composition as the fibrillarin-based peptide. The ratio of the intensity of peptide product(s) peak(s)/substrate peak is plotted as a function of time. Solid lines connecting (●) or (○) indicate ratios of mono- and dimethylated peptide product/substrate, respectively. The data support a distributive, or dissociative, mechanism.](image-url)
shown). As an initial step to ascertain the importance of the amino acid sequence adjacent to the methylation site, two residues C-terminal to the arginyl group (underlined in the sequence above) were independently altered to all of the other amino acids except arginine to generate 36 peptides. Of the 36 peptides in the library, 26 peptides were soluble in sodium phosphate buffer and were tested for their methyl acceptor activity with PRMT1. Such a screen is important for determining the ability of PRMT1 to methylate sequences outside the ‘RGG’ paradigm.

**PRMT1 demonstrates amino acid sequence selectivity with peptide substrates**

Soluble peptides were incubated with PRMT1 and AdoMet to determine if methylation could occur at sequences other than ‘RGG’ and ‘RXR’. Peptides were examined kinetically using PRMT1 and a continuous spectrophotometric assay for AdoMet-dependent methyltransferases (see Chapter 3). Peptides that were methylated by PRMT1 displayed a linear decrease in absorbance at 265 nm which was monitored continuously with time. Initial rates (first 10% of product formation) were used to obtain Michaelis-Menten kinetic parameters, which are shown graphically in relation to the parent peptide which had a $K_m,\text{app} = 89 \pm 35 \, \mu M$ and a $k_{cat,\text{app}} = 3.0 \pm 0.5 \, \text{min}^{-1}$ (Fig. 4-2). For comparison, the R3 peptide (GGRGFGGGRGGFGGRGGFG) had a $K_m = <10 \, \mu M$ and a $k_{cat} = 2.7 \pm 0.2 \, \text{min}^{-1}$ (see Chapter 3).

Table 4-2 and Fig. 4-2 summarize the kinetic data ($k_{cat,app}$ and $k_{cat}/K_m$ values) obtained when PRMT1 was assayed with peptides modified from the parent peptide in the $X^1$ (Fig. 2, A) or the $X^2$ (Fig. 4-2, B) positions. Overall, PRMT1 was capable of methylating less than half of the peptides tested that possessed an amino acid other than
glycine in the $X^1$ position (Fig. 4-2A). Peptides containing ‘RLG’, ‘RYG’, ‘RFG’, ‘RTG’, and ‘RKG’ sequences were PRMT1 substrates (see Table 4-2). Importantly, the maximum velocity for each peptide substrate and incorporation of a methyl group was confirmed using a $[^3]H$-AdoMet radioactive assay [21] (data not shown).

![Graph A](attachment:graph_a.png) ![Graph B](attachment:graph_b.png)

Fig. 4-2. PRMT1 methylates sequences that extend beyond ‘RGG’. $k_{cat, app}$ values were obtained for soluble peptides in the peptide library. The library was constructed based on a peptide sequence ($KGGFGGRGFGKW$) derived from the in vivo PRMT1 substrate, fibrillarin. Kinetic parameters were obtained using a continuous spectrophotometric assay. Reactions containing 4 µM PRMT1 and 250 µM AdoMet were initiated with 25-2000 µM peptide. PRMT1 substrates displayed a linear decrease in absorbance at 265 nm that was monitored continuously with time. Initial rate data was fit to the Michaelis-Menten equation to determine kinetic constants. (A) $k_{cat, app}$ values for peptides that varied in the $X^1$ position (X in ‘RXG’), (B) $k_{cat, app}$ values for peptides that varied at $X^2$ are represented in bar graphs. The letter under each bar indicates the amino acid that ‘X’ is equivalent to in either the $X^1$ or $X^2$ position. Peptides containing boxed amino acid residues in either position were very poor PRMT1 substrates. Peptides with residues in the $X^1$ or $X^2$ positions labeled (*) were not methylated by PRMT1.

We also confirmed that MMA was formed by performing amino acid analysis on acid-hydrolyzed peptide products [34]. Using reverse phase HPLC, all PRMT1 peptide
substrates displayed a peak that co-eluted with authentic MMA (data not shown). Peptides bearing an aspartate, alanine, glutamate, or asparagine directly C-terminal to the substrate arginine were classified as very poor PRMT1 substrates. The maximum velocity exhibited by PRMT1 for each of these peptides using radiolabel incorporation was only slightly above the control reaction rate. The limit of detection for the radioactive assay was 0.05 \( \mu \text{M CH}_3/\text{min} \) corresponding to a \( k_{\text{cat}} \) of 0.013 min\(^{-1}\). Methylation of peptides bearing serine or histidine in the \( X^1 \) position was not detected using either kinetic assay.

Table 4-2  Peptide Library Kinetics

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>( k_{\text{cat}}/K_m ) (M(^{-1})sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-X(^1)</td>
<td>KGGFGGRRGFGGKW</td>
<td>560</td>
</tr>
<tr>
<td>L-X(^1)</td>
<td>KGGFGGGLGFGGKW</td>
<td>34</td>
</tr>
<tr>
<td>Y-X(^1)</td>
<td>KGGFGGRYGFGGKW</td>
<td>62</td>
</tr>
<tr>
<td>F-X(^1)</td>
<td>KGGFGGRFGFGGKW</td>
<td>130</td>
</tr>
<tr>
<td>T-X(^1)</td>
<td>KGGFGGRGTFGGKW</td>
<td>710</td>
</tr>
<tr>
<td>K-X(^1)</td>
<td>KGGFGGGKGFGGGKW</td>
<td>160</td>
</tr>
<tr>
<td>G-X(^2)</td>
<td>KGGFGGGGGFGGKW</td>
<td>560</td>
</tr>
<tr>
<td>A-X(^2)</td>
<td>KGGFGGGRAGFGGGKW</td>
<td>430</td>
</tr>
<tr>
<td>L-X(^2)</td>
<td>KGGFGGGRGLFGGGKW</td>
<td>160</td>
</tr>
<tr>
<td>F-X(^2)</td>
<td>KGGFGGRRGF GGKW</td>
<td>50</td>
</tr>
<tr>
<td>T-X(^2)</td>
<td>KGGFGGRGTFGGKW</td>
<td>95</td>
</tr>
<tr>
<td>K-X(^2)</td>
<td>KGGFGGGKGKF GGKW</td>
<td>1.0 x 10(^3)</td>
</tr>
<tr>
<td>S-X(^2)</td>
<td>KGGFGGGGSFGGGKW</td>
<td>220</td>
</tr>
</tbody>
</table>

Peptide names are listed followed by their sequences and catalytic efficiencies \( (k_{\text{cat}}/K_m \) (M\(^{-1}\)s\(^{-1}\)))

PRMT1 was also able to methylate peptides with amino acids other than glycine in the \( X^2 \) position. The \( k_{\text{cat, app}} \) values for PRMT1 with peptides modified in the \( X^2 \) position (X in ‘RGX’) are shown in Fig. 4-2, B. Although PRMT1 preferentially
methylated the ‘RGG’ containing parent peptide over all other X^2 position modified peptides, new PRMT1 substrate sequences were identified. Peptides harboring leucine, phenylalanine, threonine, lysine, alanine, or serine in the X^2 position were shown to be PRMT1 substrates (also see Table 4-2). Peptides with ‘RGP’, ‘RGI’, ‘RGW’, ‘RGQ’, and ‘RGE’ sequences were very poor PRMT1 substrates; i.e., k_{cat} values were only slightly above the limit of detection. Finally, PRMT1 did not tolerate tyrosine, aspartate, or methionine in the X^2 position (k_{cat} values were lower than the limit of detection). Overall, the data from the peptide library shows that 1) PRMT1 methylates sequences that go beyond the glycine arginine rich paradigm and 2) PRMT1 prefers certain amino acids over others in either the X^1 or X^2 positions.

**PRMT1 demonstrates amino acid sequence selectivity with a protein substrate**

In order to determine if PRMT1 demonstrates the same sequence selectivity at the protein level as it does at the peptide level, a model protein substrate was chosen and the sequence C-terminal to the substrate arginyln group was modified. Since many known PRMT1 protein substrates contain multiple arginine residues that can be methylated, we searched for a protein substrate that was methylated by PRMT1 at a single arginine residue to use as a model protein substrate. We observed that eIF4A was specifically methylated by PRMT1 (Bochinski et al., in preparation). The eukaryotic initiation factor 4A (eIF4A) family of DEAD-box proteins, of which several structures have been solved [39], has seven motifs characteristic of nucleic acid helicases, including a 14 amino acid, arginine-rich motif VI located towards the C-terminus. eIF4A1, an eIF4A isoform that has an ‘RGG’ sequence located in motif VI (ENYIHRIGRGGR) of the DEAD-box
domain, was chosen as our model protein substrate. Wild-type eIF4A1 incorporated tritium from [3H]-AdoMet when incubated with PRMT1 (Fig. 4-3) in a time-dependent manner (data not shown). An R362K-eIF4A1 mutant did not incorporate radiolabel from [3H]-AdoMet in the presence of PRMT1 (data not shown). These results suggest that eIF4A1 is methylated at a single arginine residue and will serve as a good model substrate.

In order to examine if the amino acid sequence surrounding the substrate arginyl group affects the ability of PRMT1 to methylate protein substrates, mutants of eIF4A1 at the X1 position were examined as PRMT1 substrates. Mutations were selected based on either the results of the fibrillarin-based library or forthcoming experiments as discussed below. Mutants bearing ‘RYG’ (G363Y), ‘RAG’ (G363A), and ‘RSG’ (G363S) sequences were incubated with PRMT1 and [3H]-AdoMet. As Fig. 4-3 shows, none of the mutant proteins incorporated radiolabel. These results show that amino acid sequence in both peptides and proteins is an important factor dictating PRMT1 substrate selection.

Fig. 4-3. Effect of single site mutations in the PRMT1 protein substrate eIF4A1. 0.83 µM eIF4A1 protein was incubated with 0.69 µM [3H]-AdoMet and 5 µM PRMT1. Reactions were quenched with SDS loading dye and run on an SDS-PAGE gel that was stained with Coomassie (CB panel) and later exposed to film (3H panel). Lanes contain the following proteins: WT, G363A, G363S, and G363Y-eIF4A1, respectively. Lanes containing G363S-eIF4A1 and G363Y-eIF4A1 were purposely overloaded in this particular gel but no radiolabel incorporation was observed in these mutants.
Discrimination of eIF4A isoforms by PRMT1

A sequence alignment was performed for eIF4A1 against other DEAD-box domain containing proteins. Two human eIF4A isoforms, eIF4A2 and eIF4A3, were most similar in sequence to eIF4A1, with eIF4A2 and eIF4A1 sharing 90% sequence identity. Although eIF4A3 shares 66% sequence identity with eIF4A1, eIF4A1 and 3 share over 80% structural similarity (blue residues in Figure 4-4). The single amino acid difference between eIF4A1 and 3 in motif VI (boxed amino acids in Figure 4-5A) occurs at G368 in eIF4A1 (S368 in eIF4A3), directly C-terminal to the methylation site. We asked if PRMT1 could methylate the eIF4A3 protein, which has an ‘RSG’ instead of an ‘RGG’ sequence at the same position.

Fig. 4-4. Sequence conservation of eIF4A isoforms. The crystal structure of the yeast eIF4A1 protein (PDB accession 1FUU_1) highlights the proposed site of PRMT1-methylation, Arg362, shown in ball and stick representation on a flexible loop. Yeast eIF4A1 was aligned with human eIF4A3 sequence (NCBI accession# P38919) using the Dali Server. Strictly conserved residues are shown in blue while functionally conserved residues are shown in dark gray. Functionally distinct, non-conserved residues are shown in orange. The conserved arginine is blue while the non-conserved amino acid directly C-terminal to it is orange.
Based on previous results with the G363S mutant of eIF4A1, we predicted that eIF4A3 would not be a good PRMT1 substrate. No tritium incorporation into eIF4A3 protein was observed upon incubation with \(^3\text{H}\)-AdoMet and PRMT1 (Fig. 4-5, B) indicating that the eIF4A3 protein is not a PRMT1 substrate. However, an S368G-eIF4A3 mutant did incorporate tritium when incubated with \([\text{H}^3]\)-AdoMet and PRMT1 (data not shown). Thus, despite the high sequence and structural similarity between the two isoforms, the eIF4A1 protein is a PRMT1 substrate while the eIF4A3 protein is not. Furthermore, a single change in the amino acid residue adjacent to a substrate arginyl group dictates whether or not the eIF4A1/3 proteins are methylated.

Fig. 4-5. Comparison of eIF4A1 and eIF4A3 isoforms as PRMT1 substrates. (A) The sequences from the human eIF4A1 (accession# NP_001407) and eIF4A3 (accession# P38919) isoforms were aligned using CLUSTALW and were shown to be 66% identical. Residues 352-365 from motif VI (boxed residues) of the eIF4A1 DEAD box domain are shown. The proposed site of PRMT1-methylation is bolded. Position 363, the glycine residue immediately C-terminal to the eIF4A1 substrate arginine residue (R362), is indicated by an arrow. Instead of glycine at this position in motif VI, eIF4A3 has a serine. In (B), eIF4A3 protein was examined as a PRMT1 substrate using conditions described in Fig. 4-2.
Effect of substrate sequence context on PRMT1 activity

The amino acid sequence surrounding the substrate arginine residue is different between the G363Y eIF4A1 protein (…YIHRIGRYGR…) and the fibrillarin-based ‘RYG’ peptide (AcKGGFGGGRFFGGKW). It is possible that the inability of PRMT1 to methylate the ‘RYG’ sequence in the eIF4A1 mutant could be due to sequence context. That is, amino acids in one position affect whether or not amino acids in a second position are favored. We therefore examined PRMT1 catalyzed methylation of peptides which were based on the wild-type and mutant eIF4A1 sequences. PRMT1 was capable of methylating all eIF4A1-peptides tested (Table 4-3). The eIF4A1-Y peptide was methylated ($k_{cat}/K_m$, app = 80 ± 18 M$^{-1}$s$^{-1}$) albeit at a much slower rate than wild-type ($k_{cat}/K_m$, app = 610 ± 18 M$^{-1}$s$^{-1}$). This result suggests that the inability of PRMT1 to methylate the G363Y eIF4A1 protein was not due to sequence context. Additionally, these results show that while the sequence surrounding the reactive arginyl group is important (e.g., eIF4A1 mutant proteins which are not PRMT1 substrates), other factors also contribute to protein substrate selection. For example, we note that the loop in which eIF4A1 is methylated consists of only six amino acid residues. This loop is restrained by a beta sheet and alpha helix at its termini and may display limited flexibility compared to a peptide substrate. The ability of peptides corresponding to the loop region or single amino acid mutants of the loop region to be PRMT1 substrates while the corresponding proteins were not PRMT1 substrates may be related to decreased flexibility within the protein loop.
Table 4-3  eIF4A peptide kinetics with PRMT1

<table>
<thead>
<tr>
<th>Name</th>
<th>Peptide sequence</th>
<th>$K_{m, \text{app}}$ (μM)</th>
<th>$k_{\text{cat}}/K_{m, \text{app}}$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4A1</td>
<td>YIHRIGRGGR</td>
<td>39 ± 2.4</td>
<td>610 ± 99</td>
</tr>
<tr>
<td>eIF4A1-Y</td>
<td>YIHRIGRYGR</td>
<td>250 ± 28</td>
<td>80 ± 18</td>
</tr>
<tr>
<td>eIF4A1-S (eIF4A3)</td>
<td>YIHRIGRSGR</td>
<td>47 ± 3.9</td>
<td>170 ± 22</td>
</tr>
</tbody>
</table>

Peptide sequences and kinetic parameters ($K_{m, \text{app}}$ and $k_{\text{cat}}/K_{m, \text{app}}$) are listed for peptides derived from eIF4A1 and eIF4A3. The peptide arginine residue that gets methylated by PRMT1 is bolded. Kinetics were performed as described in Fig. 4-2.

We also noted that the ‘RSG’ sequence presented within the fibrillarin context (AcKGGFGGGRSGFGGKW) was not a PRMT1 substrate while the same sequence presented within the eIF4A1 context (YIHRIGRSGR) was a PRMT1 substrate. The eIF4A1-S peptide was in fact, a better substrate than the eIF4A1-Y peptide (Table 4-3). This suggests that PRMT1 substrate specificity is contextual with respect to the amino acid sequence surrounding the substrate arginyl residue.

Because the eIF4A1 peptides have three arginine residues, it was possible that changing the peptide sequence shifted the methylation site. In order to address this issue, we determined the methylation site in each peptide by MS/MS. Analysis of the WT-eIF4A1 peptide after incubation with PRMT1 and AdoMet showed that only the arginine within the ‘RGG’ sequence (bolded R7 in AcYIHRIGRGGR) was both mono and dimethylated (Fig. 4-6 and Fig. 4-7, A).

The eIF4A1-S peptide, AcYIHRIGRSGR, with an ‘RSG’ sequence was also methylated at R7 (data not shown). Mass spectrometry analysis of the eIF4A1-Y mutant
peptide, AcYIHRIGRYGR, housing an ‘RYG’ sequence showed a +14 peak corresponding to monomethylation, which could not be ascribed to R4 or R10 methylation (Fig. 4-7, C). The presence of MMA in PRMT1 reaction mixtures using each of the three eIF4A1-derived peptides was confirmed by amino acid analysis via HPLC (data not shown). Importantly, these results suggest that the methylation site did not shift when the eIF4A1 peptide sequence was altered. In summary, all eIF4A1-derived peptides were methylated by PRMT1.

Fig. 4-6. MALDI-TOF/TOF-MS/MS spectra of (A) 1184.68 (1184.68 calculated) [M+H]+ precursor ion corresponding to the unmodified WT-eIF4A1 peptide, (B) 1198.62 (1198.69 calculated) [M+H]+ precursor ion corresponding to the monomethylated WT-eIF4A1 peptide, confirms the modification site to be Arg7, and (C) 1212.64 (1212.71 calculated) [M+H]+ precursor ion corresponding to the dimethylated WT-eIF4A1 peptide, confirms the modification site to be Arg7. The peptide sequence is labeled with observed b and y ions.
Fig. 4-7. MALDI-MS spectra of methylation reactions of (A) WT-eIF4A1, (B) eIF4A1-S and (C) eIF4A1-Y peptides. Labeled peaks are for the unmodified (native), mono-methylated (+14 Da) and dimethylated (+28 Da), sodium adduct (+22 Da), disodium adduct (+44 Da), and dimethylated plus sodium adduct (+50 Da) peptides, respectively.
DISCUSSION

**PRMT1 substrate selectivity goes beyond the ‘RGG’ paradigm**

When PRMT1 was crystallized with the R3 peptide and AdoHcy, the electron density for the two amino acid residues C-terminal to the fully resolved substrate arginine was not observed. Because no stabilizing interactions were observed in the enzyme-substrate complex between PRMT1 and substrate side chains, one might predict that the active site residues of PRMT1 have little influence over which substrate sequences can interact with the enzyme. The results from the peptide library show that PRMT1 substrate selectivity exists (Fig. 4-2, the X\(^1\) and X\(^2\) positions); however, the variety of sequences that are methylated extends beyond the ‘RGG’ paradigm.

Our results show that the ‘RGG’ sequence is not a necessity of a PRMT1 substrate. We confirmed the PRMT1-dependent methylation of several novel sequences using a coupled spectrophotometric assay, radiolabel incorporation, and amino acid analysis. The ability to methylate a variety of peptide sequences indicates that PRMT1 has evolved a certain degree of flexibility with regard to substrate binding and/or catalysis. The arginine of all PRMT1 substrates must be recognized, and PRMT1 residues that fulfill this function can be seen in the crystal structure [31]. This recognition appears strict as even a peptide where the arginine has been replaced with a citrulline, (where one of the guanidino nitrogens has been replaced with a carbonyl group) does not compete with substrate peptides (data not shown), suggesting that citrulline does not bind in the active site. Of the eleven peptide sequences that were methylated by PRMT1, the amino acid residues allowed in either the X\(^1\) or X\(^2\) positions
possess a diverse range of chemical properties including residues with aromatic rings and/or hydroxyl groups, hydrophobic amino acids, and positively charged residues.

Even though the experimental design of this study was geared toward substrate profiling using a peptide screen, certain observations from the screen are worthy of noting. In addition to the ‘RGG’ and ‘RXR’ PRMT1-methylation sequences identified prior to this study, peptides containing leucine, phenylalanine, threonine, or lysine in either the $X^1$ or $X^2$ positions were methylated by PRMT1 (Fig. 4-2). Surprisingly, alanine was not favored by PRMT1 in the $X^1$ position despite its resemblance to glycine in size and charge but was favored in the $X^2$ position. Interestingly, tyrosine and phenylalanine, both larger amino acids, were allowed in the $X^1$ position.

Serine was favored in the $X^2$ position (‘RGS’) within the context of the fibrillarin sequence. We note that the R3 peptide substrate which was complexed with PRMT1 in the crystalline state could be modeled into the substrate binding groove in either orientation. Therefore, our data is consistent with the possibility that the fibrillarin-based ‘RGS’ peptide and a peptide derived from histone H4 that is methylated by PRMT1 in an ‘SGR’ sequence [40] bind PRMT1 in opposite orientations. This data suggests that a hydrogen bond afforded by serine in this position (‘RGS’ or ‘SGR’) may contribute to binding/catalysis. However, in order for us to be able to describe specific PRMT1-substrate interactions between the newly discovered substrate sequences and PRMT1, more structural information about positions of substrate side chain residues and directionality of the peptide substrate are needed.

Our study reveals that directional peptide libraries are useful to begin characterizing the substrate selectivity of PRMT1, but they are not the end-all approach
because PRMT1 displayed contextual recognition of peptide substrates. Specifically, the ‘RSG’ containing peptide modeled from eIF4A3 was a PRMT1 substrate while the ‘RSG’ containing fibrillarin-based peptide was not. Compensatory interactions have also been observed in the deacetylase SIRT1 [41]. Alternatively, the ability of PRMT1 to differentially methylate peptides containing the ‘RSG’ sequence (eIF4A3 peptide versus fibrillarin-based peptide) may reside in the ability of PRMT1 to bind peptide substrates in either an N\textsubscript{T} to C\textsubscript{T} or C\textsubscript{T} to N\textsubscript{T} orientation. This characteristic would be consistent with the crystal structure. Since it is expected that the binding mode of protein substrates would be fixed in one orientation, it would follow that not all peptide sequences (N\textsubscript{T} to C\textsubscript{T}) which are PRMT1 substrates may be PRMT1 protein substrates.

**Selection of PRMT1 protein substrates**

Our results with the eIF4A proteins show that even at the protein level, the amino acid sequence of the substrate protein surrounding the reactive arginine group is one factor that dictates PRMT1 substrate selectivity. Even proteins bearing remarkable sequence and structural similarity can be distinguished by a single amino acid difference near the substrate arginyl group. Comparison of the eIF4A1 proteins and corresponding peptides as PRMT1 substrates illustrates that recognition of protein substrates is not governed by amino acid sequence alone. Nonetheless, the peptide data suggest the possibility of a more diverse methylarginine proteome than the previously recognized ‘RGG’-containing proteins. Using the MSDmotif server at EMBL-EBI (http://www.ebi.ac.uk/msd-srv/msdmoi///), the pdb was queried for structures harboring potential PRMT1 methylation sites. A PSI-BLAST on loop sequences yielded several
proteins with ‘GRYG’, ‘GRFG’, and ‘GRGF’ sequences (Table 4-4). Human protein and human pathogens with diverse functions are represented. The structures of these proteins confirm that the target arginine is present on an accessible region of the protein. Presently, a more diverse PRMT1 recognition sequence is also supported by the methylation of histone 4 and HNF4 [42] in ‘RGK’ and ‘RYG’ sequences, respectively.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>PDB ID</th>
<th>UniProt ID</th>
<th>Protein Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRFG</td>
<td>2CS5</td>
<td>P29074</td>
<td>Hydrolase, Tyrosine phosphatase, pdz domain</td>
</tr>
<tr>
<td>GRYG</td>
<td>2YQP</td>
<td>Q5T1V6</td>
<td>Hydrolase, RNA helicase ddx59</td>
</tr>
<tr>
<td>GRYG</td>
<td>2HTV</td>
<td>Q6XV46</td>
<td>Hydrolase, influenza A virus</td>
</tr>
<tr>
<td>GRYG</td>
<td>1DTD</td>
<td>P48052</td>
<td>Hydrolase, Carboxypeptidase a2.</td>
</tr>
<tr>
<td>GRGF</td>
<td>2PIE</td>
<td>O76064</td>
<td>Ligase, E3 ubiquitin-protein ligase rnf8.</td>
</tr>
<tr>
<td>GRGF</td>
<td>1W8K</td>
<td>O61130</td>
<td>Antigen, <em>Plasmodium vivax</em></td>
</tr>
<tr>
<td>GRGF</td>
<td>1X4L</td>
<td>Q14192</td>
<td>Metal-binding portein, lim domain</td>
</tr>
<tr>
<td>GRGF</td>
<td>1N9D</td>
<td>P01236</td>
<td>Hormone, prolactin</td>
</tr>
<tr>
<td>GRGF</td>
<td>2C35</td>
<td>P62487</td>
<td>Polymerase, rpb7</td>
</tr>
</tbody>
</table>

The sequences indicated were queried against the pdb using the MSDmotif server as discussed in the text. All protein listed are either human or are human pathogens.

Although the amino acid sequence of a protein substrate may play a role in PRMT1 substrate recognition and catalysis, PRMT1 substrate selectivity may not be limited to the PRMT1 active site and its ability to distinguish between different sequences. It has already been shown that peptide length and the presence of positively
charged peptidyl residues distant from the PRMT1 active site chemistry affect substrate binding and catalysis [40]. This feature is also illustrated in the eIF4A1 methylation site which is flanked by five positive residues. In addition to interactions within the PRMT1 active site, PRMT1 may also take advantage of specific protein-protein interactions distal from the methylation site, a type of “proximity-induced catalysis” that has been observed in ERK2 [43].

**Mono- and dimethylation of substrates**

Using mass spectrometry, we demonstrated that the dimethylation of a fibrillarin-based peptide substrate occurs through a dissociative mechanism (Scheme 4-2) based on an observable lag in the formation of the dimethylated product (Fig. 4-1). Our results are consistent with Frankel and co-workers [44], but are different from the findings of Thompson and co-workers [40] who defined a partially processive dimethylation of a histone 4 peptide by PRMT1. Because the mechanism of methyltransfer is dissociative, formation of the dimethylated peptide product depends upon how easily PRMT1 can recapture the monomethylated product. Frankel and co-workers [44] have reported that the monomethylated peptide substrate is a better substrate for PRMT6 than the naked peptide. We have observed similar results with PRMT1 and a fibrillarin-based peptide (Wooderchak and Hevel, manuscript in preparation) and suggest that sequence differences between a fibrillarin-based peptide and a histone 4-based peptide may be the basis for the different observations.
Conclusion

Limited studies and selective tools have promoted the idea that PRMT1 substrates conform to an ‘RGG’ sequence with a few rare exceptions [25, 27, 28, 45]. Importantly, our data clearly show that PRMT1 is capable of methylating sequences that go beyond the ‘RGG’ paradigm. Additionally, our results suggest that residues N-terminal to the arginyl modification site are also important in substrate recognition. This suggests that the methyl arginine proteome may be larger and more diverse than previously thought. It will be interesting to see if any of the candidate proteins identified in our study are indeed methylated by PRMT1.

REFERENCES


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[40] T.C. Osborne, O. Obianyo, X. Zhang, X. Cheng, P.R. Thompson, Protein arginine methyltransferase 1: positively charged residues in substrate peptides distal to the site of methylation are important for substrate binding and catalysis, Biochemistry 46 (2007) 13370-13381.


CHAPTER 5

EXAMINING THE FORMATION OF ADMA BY PRMT1

ABSTRACT

Protein arginine methyltransferase 1 (PRMT1) performs over 85% of all protein arginine methylation in the cell having the ability to both mono- and asymmetrically dimethylate protein arginine residues. Interestingly, many PRMT1 protein substrates are dimethylated instead of monomethylated on multiple arginines of certain proteins such as the hnRNPs, SAM68, and fibrillarin. Consequently, we sought to understand how PRMT1 preferentially asymmetrically dimethylates protein arginine residues in vivo using kinetic and mass spectrometry analysis. PRMT1 may have evolved a way to ensure the formation of a dimethylated product through 1) preference for the monomethylated substrate over the nonmethylated substrate or 2) processive methylation. We show here that PRMT1 did not processively methylate a single arginine-containing peptide substrate. PRMT1 preferred to methylate a peptide substrate that had already been monomethylated over an unmodified peptide substrate. A multiple arginine-containing peptide was found to be dissociatively dimethylated at a single arginine residue using a modified pulse-chase experiment analyzed by mass spectrometry. Interestingly, methylation of a multiple arginine-containing peptide was systematic (not random). Based on these results, the predominance of dimethylated protein substrates in vivo is likely due to PRMT1 preferentially methylating monomethylated substrates over naked substrates.

Coauthored by W.L. Wooderchak, D. Chen, J.M. Hevel, Examining the formation of ADMA by PRMT1, Biochemistry (2009), manuscript in preparation.
INTRODUCTION

Protein arginine methyltransferases (PRMTs) post-translationally modify a number of protein arginine residues. Targeted methylation of these proteins affects a variety of cellular processes including RNA processing, transcriptional regulation, signal transduction, and DNA repair (reviewed in [1]). Type I PRMTs (PRMT1, 3, 4, 6, and 8) catalyze the formation of monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA) via the transfer of a methyl group from S-adenosyl methionine (AdoMet/SAM) onto protein arginine residues (Scheme 5-1) [2-7]. Type II methyltransferases (PRMT5 and PRMT7) catalyze the formation of symmetric dimethylarginine (SDMA) where the second methyl group is transferred onto the unmodified guanidino nitrogen of MMA [8, 9].

Scheme 5-1. Reactions catalyzed by PRMTs. PRMTs catalyze the S-adenosylmethionine (AdoMet) dependent methylation of a protein substrate arginine residue to form monomethyl arginine (MMA). Type I methyltransferases, PRMT1, 3, 4, 6, and 8 dimethylate arginine asymmetrically to form asymmetric dimethyl arginine (ADMA). Type II methyltransferases, PRMT5 and PRMT7 catalyze the formation of symmetric dimethyl arginine (SDMA).
Thus far, nine PRMT isoforms (PRMT1-11) have been identified in humans with orthologs present in yeast [10], protozoa, Caenorhabditis elegans, Drosophila melanogaster [11], plants [12], and fish [13]. Of the eleven isoforms, PRMT1 predominates, performing an estimated 85% of all protein arginine methylation in vivo [10]. PRMT1 has the ability to methylate protein substrates at multiple sites within a single protein. Even though PRMT1 is capable of forming both MMA and ADMA, most PRMT1 substrates are found dimethylated in vivo. Fibrillarin [14], hnRNPs [15], and Sam68 [16] are asymmetrically dimethylated by PRMT1 at multiple sites within the same protein.

Although PRMTs are vital to normal development and function [17, 18], protein arginine methylation has also been linked to carcinogenesis [19], viral pathogenesis [20], multiple sclerosis [21], and spinal muscular atrophy (reviewed in [1]). In addition, several studies have shown that free ADMA, a downstream proteolytic product of PRMT substrates, has a clear role in cardiovascular disease [22] and stroke [23] by serving as an endogenous inhibitor of nitric oxide synthesis [24]. ADMA competitively inhibits nitric oxide synthase which generates the vascular smooth muscle relaxant, nitric oxide. Only 20% of ADMA is excreted in urine while approximately 80% of ADMA is cleared by dimethylarginine dimethylaminohydrolases (DDAHs). DDAHs convert ADMA to citrulline and dimethylamine. However, the misregulation of DDAHs or the hyperactivity of PRMTs may contribute to cardiovascular disease [22]. In fact, PRMT1 has been shown to be overexpressed in myocardial tissue taken from coronary heart disease patients [25]. In addition to contributing to cardiovascular ailments,
pathophysiological concentrations of ADMA have also been shown to elicit significant changes in coronary artery endothelial cell gene expression [26].

It was discovered early on that ADMA was the most abundant of the three possible PRMT modifications. Two percent of all protein arginine residues are asymmetrically dimethylated in rat liver nuclei [27], and approximately 12% of all arginine residues isolated from heterogeneous nuclear ribonucleoproteins (hnRNPs) are asymmetrically dimethylated [28]. Most methylated proteins derived from cellular extracts have a far greater amount of dimethylated arginine residues than monomethylated arginine residues. Yet, the mechanism that the Type I PRMTs use to ensure ADMA formation is unclear. Because ADMA is an important molecule that mediates a variety of cellular processes [26], it is important to understand how ADMA is formed.

In order to understand how ADMA is the major biological product of PRMT1-methylation, we investigated the enzymatic mechanism of PRMT1. We hypothesized that this biological phenomenon is achieved by the nature of PRMT1 preferentially methylating monomethylated substrates rather than naked substrates. Alternatively, PRMT1 could perform processive arginine methylation wherein the substrate binds to the enzyme and is not released until the arginine residue is fully dimethylated (see Scheme 5-2). We show here that PRMT1 dissociatively dimethylates peptides containing a single arginine residue using mass spectrometry (MS) and HPLC analysis. Further kinetic analysis revealed that PRMT1 prefers to methylate monomethylated peptide substrates over their non-methylated counterparts. Methylation of a multiple arginine-containing peptide (R2) was systematic (not random), and the analysis of the R2 peptide using a
modified pulse-chase experiment and MS revealed that PRMT1 dissociatively methylates fibrillarin-based peptides.

Scheme 5-2. Dissociative versus processive methylation. Dissociative and processive mechanisms are depicted for PRMT1. In a dissociative mechanism, PRMT1 (or E=enzyme) monomethylates the arginine-containing peptide and releases it. When the monomethylated species re-binds, another methyl group is transferred from AdoMet onto the monomethylated peptide to yield the fully dimethylated product. In a processive mechanism, PRMT1 mono- and dimethylates the single arginine-containing peptide without releasing the monomethylated species from the active site.

**MATERIALS AND METHODS**

All chemicals used were of ACS reagent grade or better. AdoMet was purchased from Sigma as a chloride salt (≥80%, from yeast). JMH1-long (GGKGGFGGRGGFGKGGFG) was synthesized by Sigma Genosys. N-terminally acetylated peptides based on the *in vivo* PRMT1 substrate fibrillarin, JMH1 (AcKGGFGGRGGFGGK), JMH1W (AcKGGFGGRGGFGGKW), JMH1W-CH3
(\text{AcKGGFGGGR}_{\text{methyl}}GGFGGKW), and \text{R2} (\text{AcGGGGFGGKGGRGFG}), were synthesized and purified by the Keck Institute (Yale University). Two peptides based on the \textit{in vivo} PRMT1 substrate eukaryotic initiation factor 4A1 (eIF4A1), the eIF4A1 peptide (YIHRIGRGGR) and the eIF4A1-\text{CH}_3 peptide (YIHRIGRGGR_{\text{methyl}}GGR), were synthesized and purified by the Keck Institute. All peptides used in this study are listed in Table 5-1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>JMH1</td>
<td>KGGFGGRRGGFGGK</td>
</tr>
<tr>
<td>JMH1-long</td>
<td>GGKGGFGGRRGGFGGKGGFG</td>
</tr>
<tr>
<td>JMH1W</td>
<td>KGGFFGRGGFGGKW</td>
</tr>
<tr>
<td>JMH1W-\text{CH}_3</td>
<td>KGGFGG(R_{\text{CH}}_3)GGFGGKW</td>
</tr>
<tr>
<td>eIF4A1</td>
<td>YIHRIGRGGR</td>
</tr>
<tr>
<td>eIF4A1-\text{CH}_3</td>
<td>YIHRIGR(CH_{\text{3}})GGR</td>
</tr>
<tr>
<td>\text{R2}</td>
<td>GGRRGGGKGGGGFRGFGGFG</td>
</tr>
</tbody>
</table>

Peptide names with their corresponding sequences are listed. Arginine residues that are methylated by PRMT1 are shown in bold.

**HPLC analysis of a single arginine-containing peptide**

HPLC analysis was used to analyze the formation of MMA and ADMA with time [29]. Briefly, a reaction containing 2.5 µM PRMT1, 250 µM AdoMet, 100 nM AdoHcy nucleosidase (purified as in [30]) in 50 mM sodium phosphate buffer (pH 7.5) was initiated with 100 µM eIF4A1 peptide (see Table 5-1) at 37 ºC. Time points were taken out and quenched in 10% TFA (final). Precipitated protein was removed through centrifugation. Soluble eIF4A1 peptide in the supernatant was hydrolyzed in 6 M HCl, derivatized with the AccQ-Fluor™ reagent (Waters Corporation), and separated by
reverse phase HPLC. This method provides base-line separation of arginine, MMA, ADMA, and SDMA [29]. The area under the MMA and ADMA peaks were integrated. Standard curves of known MMA and ADMA concentrations were used to calculate the amount of MMA and ADMA in each sample.

**Mass spectrometry analysis of a single arginine-containing peptide**

A reaction with 2.5 µM PRMT1, 250 µM AdoMet, and 100 nM MTAN in 50 mM sodium phosphate buffer (pH 7.5) was initiated with 100 µM JMH1-long peptide (see Table 5-1). At various times points, 10 µL aliquots were quenched with TFA (10% final) and analyzed by LC/MS. The intensities of substrate and product peaks were normalized for ionization differences between time points using an internal standard peptide with a similar amino acid composition as the fibrillarin-based peptide.

**Kinetic analysis**

A continuous spectrophotometric assay for AdoMet-dependent methyltransferases (see Chapter 3) was used to assay PRMT1 with several fibrillarin-based methyl-accepting peptides and two eIF4A1-based peptides (eIF4A1 and eIF4A1-CH₃). Briefly, recombinant AdoHcy nucleosidase (MTAN) was used to hydrolyze the AdoHcy generated from methyltransfer. The resulting adenine was deaminated by recombinant adenine deaminase. This strategy alleviates any product inhibition that could occur from AdoHcy. Each 110 µL reaction contained 4 µM His-PRMT1 (purified as in Chapter 3), 0.02 µM adenine deaminase (purified as in Chapter 3), 250 µM AdoMet, 10 nM MTAN (purified as in [30]), 100 µM MnSO₄, and 50 mM sodium phosphate buffer pH 7.1.
Reactions were equilibrated at 37 °C for ten minutes before they were initiated with various amounts of peptide. The decrease in absorbance at 265 nm was monitored continuously using a Cary 300 Bio UV-visible spectrophotometer. All rates were well above the limit of detection of 0.1 μM CH₃/min corresponding to a k_{cat} of 0.025 min⁻¹, and the time frame utilized in fitting the raw spectrophotometric data was representative of a single methylation event (see Chapter 4).

For calculation of kinetic constants, the concentration of peptide was varied at several fixed concentrations of AdoMet (40 μM - 250 μM). The average initial velocity representing no more than 10% of product formation was obtained from two measurements for each pair of substrate concentrations and was used to construct Michaelis-Menten plots. The data were graphed as Lineweaver-Burk plots, and linear regression was employed to construct slope and intercept replots [31]. These replots were linear and were used to calculate the values of the kinetic parameters.

**Intrinsic fluorescence**

An RF-5301PC spectrofluorophotometer (Shimadzu) was used for fluorescence measurements. For peptide 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 of 0.7 μM PRMT1 in 150 mM sodium phosphate buffer pH 7.1. Increasing concentrations from 1 to 50 μM peptide ligand were added at 2-3 min intervals. Data from at least two titrations were averaged and analyzed using a Stern-Volmer plot [32]. The linearity of
the Stern-Volmer plot suggests that cofactor binding is the dominant fluorescence-quenching phenomenon over the range of concentrations tested. Data were evaluated by least squares linear regression analysis to obtain the slope which corresponds to the dissociation constant ($K_D$).

**HPLC analysis of multiple arginine-containing peptides**

HPLC analysis was used in order to determine the time frame of the mass spectrometry pulse-chase experiment and to quantify the amount of MMA produced at various time points over the course of the reaction [29]. Reactions containing various concentrations of PRMT1, 1200 µM AdoMet, 100 nM AdoHcy nucleosidase in 150 mM sodium phosphate buffer (pH 7.1) were initiated with 200 µM R2 peptide on ice. Time points were taken out and quenched in 10% TFA (final), and precipitated protein was removed through centrifugation. The R2 peptide in the resulting supernatant was hydrolyzed in 6 M HCl, derivatized with the AccQ-Fluor™ reagent (Waters Corporation), and the individual amino acids were separated by reverse phase HPLC [29]. The area under the MMA peak was integrated, and a standard curve of known MMA concentrations was used to calculate the amount of MMA formed in each reaction mixture.

**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 7.1) was initiated with 200 µM R2 peptide. At various times points, 10 µL aliquots were quenched with TFA (10% final) and analyzed by
LC/MS followed by MS/MS of the desired peaks. Peptides with 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% acetonitrile 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 = 99.9% H2O, 0.1% formic acid and B = 99.9% acetonitrile, 0.1% formic acid) at 800nL/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).

**Modified pulse-chase experiment to test processivity of mono- to dimethylated R2**

A reaction with 4 µM PRMT1, 1200 µM AdoMet, and 100 nM MTAN in 50 mM sodium phosphate buffer (pH 7.1) was initiated (or pulsed) with 200 µM R2 peptide on ice. At t=5.5 minutes, a 10 µL time point was quenched with TFA (10% final) to verify that only monomethylation of R2 had taken place. At t=6 minutes, the reaction was chased with 1 mM JMH1W-CH3 peptide. Note, a methylated chase peptide was used instead of an unmodified peptide in order to design a fair competition experiment. To determine if this portion of the reaction was processive, subsequent time points (t=6.5, 9,
Precipitated protein was removed, and soluble R2 and JMH1W-CH$_3$ peptides (and their methylated products) were analyzed using the MS analysis techniques described previously. The sum of all peak intensities associated with the R2 peptide was used to normalize each product peak intensity; e.g. the data point plotted for trimethylated R2 = peak intensity of trimethylated R2/sum of all peaks associated with R2.

**Modified pulse-chase experiment to test processivity of di- to trimethylated R2**

A reaction with 4 µM PRMT1, 1200 µM AdoMet, and 100 nM MTAN in 50 mM sodium phosphate buffer (pH 7.1) was initiated (or pulsed) with 200 µM R2 peptide at 37 °C. At t=59.5 minutes, a 10 µL time point was quenched with TFA (10% final) to verify that only mono- and dimethylation of R2 had taken place. At t=60 minutes, the reaction was chased with 1 mM JMH1W-CH$_3$ peptide. To determine if this portion of the reaction was processive, additional time points (t=60.5, 70, 80, 100, 120, 150, and 200 minutes) were taken out and quenched in TFA (10% final). Precipitated protein was removed, and the soluble R2 peptide and JMH1W-CH$_3$ peptide (and their methylated products) were analyzed using the MS and data analysis techniques described previously.

**RESULTS**

**PRMT1 dissociatively methylates a single arginine-containing peptide**

Many PRMT1 protein substrates are observed to have multiple methylated arginines. Yet, it is unclear why or how the enzymatic conversion of all designated arginines within a protein substrate is accomplished without generating diversity. If all
designated arginines are required to be dimethylated for the biological effect, how does PRMT1 accomplish this? One way to ensure a dimethylated product is to perform processive methylation. Previously, we showed by mass spectrometry analysis that PRMT1 methylates a single arginine-containing fibrillarin-based peptide substrate ($\text{AcKGGFGG\text{R}GGFGGK}$) in a dissociative manner (see Chapter 4). Here we show data that suggests that PRMT1 dissociatively methylates another arginine-containing peptide ($\text{YIHRIG\text{R}GGR}$) based on the eukaryotic initiation factor 4A1 (eIF4A1) protein based on an initial lag in the formation of the dimethylated product (Fig. 5-1, A). Kinetic analysis revealed that $4.7 \pm 1.0 \mu\text{M} \text{AdoHcy}$ was generated in 45 seconds, a time in which mass spectrometry results suggest no presence of dimethylated peptide. This amount was higher than the concentration of enzyme (2.5 $\mu$M). In a processive mechanism, the concentration of MMA would never exceed the concentration of enzyme in the reaction. These data suggest that PRMT1 methylates the eIF4A1 peptide in a dissociative manner. Note, only the central arginine (R7) of the eIF4A1 peptide is methylated by PRMT1 (see Chapter 4).

Previous studies [33, 34] have shown that PRMT1 activity increases with peptide length. In order to determine if peptide length also influences the manner in which the substrate is methylated, we next increased the length of the dissociatively methylated fibrillarin-based peptide (see Chapter 4) by six amino acids ($\text{AcKGGFGG\text{R}GGFGGK} \rightarrow \text{AcGGKGGFGG\text{R}GGFGGKGGFG}$) to see if the longer peptide could be processively methylated. Despite an increase in length, mass spectrometry analysis of a reaction with the longer peptide displayed an obvious lag in the formation of dimethylated product (Fig. 5-1, B) suggesting that methylation was dissociative. Kinetic analysis revealed that
3.6 ± 1.1 μM AdoHcy was generated in 1 minute, a time in which mass spectrometry results suggest no presence of dimethylated peptide. Because this amount was higher than the concentration of enzyme (2.5 μM), the results support a dissociative mechanism.

Fig. 5-1. Time-dependent formation of mono- and dimethylarginine. Reactions containing 250 μM AdoMet and 2.5 μM PRMT1 in 50 mM sodium phosphate buffer (pH 7.5) were initiated with 100 μM eIF4A1 peptide (YIHRIGRGGGR) in (A) or 100 μM fibrillarin-based peptide (GGKGGFGGRIGGFGGGKGGFGF) in (B). Aliquots were quenched in TFA (10% final) at various times, analyzed by LC/MS, and peak intensities of peptide product peaks were plotted as a function of time. Substrate and product peak intensities were normalized for ionization differences using an internal standard peptide with a similar amino acid composition as the fibrillarin-based peptide. Solid (●) and dashed (○) lines indicate ratios of mono- and dimethylated peptide product/substrate, respectively. The data support a distributive, or dissociative, mechanism.
PRMT1 preferentially methylates monomethylated substrates

As an alternative to processive methylation, another way to ensure the formation of a dimethylated product biologically is if PRMT1 were to preferentially methylate a substrate that has already been methylated. To test this, we compared how well PRMT1 could methylate the monomethylated peptide substrates (JMH1W-CH$_3$=AcKGGFGGR$_{methyl}$GGFGGKW; eIF4A1-CH$_3$=YIHR1GR$_{methyl}$GGGR) versus the naked peptide substrates (JMH1W=AcKGGFGGRGGFGGKW; eIF4A1=YIHR1GRGGGR) using a continuous spectrophotometric assay for AdoMet-dependent methyltransferases (see Chapter 3). Kinetic data were collected and analyzed as described in the methods section. Michaelis-Menten plots for the JMH1W-CH$_3$ and JMH1W peptides with PRMT1 show that the peptides were able to saturate PRMT1 at a variety of fixed AdoMet concentrations (data not shown). A Lineweaver Burk plot of the data indicated that PRMT1 performs a sequential reaction mechanism with the JMH1W-CH$_3$ and JMH1W peptides (data not shown). This type of reaction mechanism is consistent with the PRMT6 reaction mechanism [34, 35]. PRMT1 preferentially methylated the monomethylated substrate, JMH1W-CH$_3$ (see Table 5-2). The $K_m$ was smaller ($K_m$, peptide$= 5.78 \mu$M) and the catalytic efficiency was higher ($k_{cat}/K_m= 4890 \text{ M}^{-1}\text{s}^{-1}$) for the monomethylated substrate in comparison to the values obtained for the unmodified JMH1W peptide ($K_m$, peptide$= 54.7 \mu$M; $k_{cat}/K_m= 1640 \text{ M}^{-1}\text{s}^{-1}$).

The same trend was observed with an additional peptide substrate pair (Table 5-2) based on the PRMT1 protein substrate eIF4A1 (see Chapter 4). Michaelis-Menten plots for the eIF4A1-CH$_3$ and eIF4A1 peptides with PRMT1 showed that peptides were able to
saturate PRMT1 at a variety of fixed AdoMet concentrations. A Lineweaver Burk plot of the eIF4A1-CH3 and eIF4A1 peptide data indicated that PRMT1 performs a sequential reaction mechanism with each peptide (data not shown). PRMT1 preferred to methylate the monomethylated eIF4A1 substrate ($k_{cat}/K_m = 2610 \text{ M}^{-1}\text{s}^{-1}$) versus the unmodified substrate ($k_{cat}/K_m = 1960 \text{ M}^{-1}\text{s}^{-1}$) (see Table 5-2). Such a preference for arginine residues that have already been monomethylated by PRMT1 would lead to an increasing amount of asymmetrically dimethylated protein arginine residues.

Table 5-2  Kinetic constants for PRMT1 with naked and monomethylated peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide Sequence</th>
<th>$K_m_{\text{SAM}}$ (μM)</th>
<th>$K_m_{\text{peptide}}$ (μM)</th>
<th>$V_{\text{max}}$ (μM CH3/min)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JMH1W</td>
<td>KGGFGGRRGGFGGKW</td>
<td>117</td>
<td>54.7</td>
<td>21.5</td>
<td>1640</td>
</tr>
<tr>
<td>JMH1W-CH3</td>
<td>KGGFGGR(CH3)GGFGGKW</td>
<td>15.1</td>
<td>5.78</td>
<td>6.79</td>
<td>4890</td>
</tr>
<tr>
<td>eIF4A1</td>
<td>YIHRIGRGGR</td>
<td>35.3</td>
<td>52.4</td>
<td>21.4</td>
<td>1960</td>
</tr>
<tr>
<td>eIF4A1-CH3</td>
<td>YIHRIGR(CH3)GGR</td>
<td>49.1</td>
<td>28.2</td>
<td>17.7</td>
<td>2610</td>
</tr>
</tbody>
</table>

All kinetic constants were obtained using a continuous spectrophotometric assay for AdoMet-dependent methyltransferases (see Chapter 3). Substrate arginine residues are bolded.

**Peptide binding**

In order to discern if the preference for PRMT1 to modify monomethylated arginine residues was also due to preferential binding, we measured the dissociation constants between PRMT1 and the eIF4A1 peptide substrate pair. The PRMT1 crystal structure depicts two tryptophans at positions 145 and 294 which lie in the catalytic region near the substrate arginine residue opposite the AdoMet binding region [36]. The intrinsic fluorescent-properties of PRMT1 were exploited in a fluorescence-quenching
assay to determine the dissociation constants for the eIF4A1 and eIF4A1-CH$_3$ peptides with PRMT1. The Stern-Volmer plot [32] was used to analyze the quenching data (Fig. 5-2, A). A Stern-Volmer plot for the eIF4A1 and eIF4A1-CH$_3$ peptides (Fig. 2, B) revealed that both the methylated and nonmethylated eIF4A1 peptides had nearly the same affinity for PRMT1 as judged by the similar $K_d$ values (Table 5-3). These results indicate that preference for the monomethylated substrate is not due to binding.

Fig. 5-2. Naked and monomethylated substrates bind similarly to PRMT1. (A) Steady-state fluorescence emission spectra of PRMT1 with the eIF4A1-CH$_3$ peptide where buffer (○), enzyme (●), 1 μM peptide (▲), and 15 μM peptide (▲) are shown. (B) Stern-Volmer plot of quenching of PRMT1 fluorescence by the eIF4A1-CH$_3$ peptide.
Table 5-3. Peptide dissociation constants.

<table>
<thead>
<tr>
<th>Peptide Ligand</th>
<th>$K_D$ (nM)</th>
<th>Fluorescence quenching (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4A1</td>
<td>51 ± 7.2</td>
<td>30 ± 2.0</td>
</tr>
<tr>
<td>eIF4A1-CH₃</td>
<td>41 ± 5.0</td>
<td>25 ± 4.7</td>
</tr>
</tbody>
</table>

Dissociation constants were obtained using intrinsic fluorescence quenching assays (performed at least in duplicate) as described in the methods section. The quenching observed was consistent with the number of active site tryptophans relative to the total number of tryptophans.

**Methylation of a multiple arginine-containing substrate is systematic**

Most PRMT1 protein substrates harbor regions that contain multiple sites of arginine methylation. It is unknown which of these sites undergoes methylation first. Is one site preferentially methylated on a substrate containing multiple arginine residues (systematic methylation), or is there no preference for which arginine on a multiple arginine-containing substrate gets methylated first (random methylation)? To examine how PRMT1 methylates substrates containing multiple arginine residues (random versus systematic methylation), we designed another fibrillarin-based peptide substrate, R2=AcGGRGFGGKGFGGGFG. The R2 peptide was first assessed by mass spectrometry to assess purity (bottom spectrum, Fig. 5-3, A). The R2 peptide was then purified (top spectrum, Fig. 5-3, A) and used in all experiments thereafter.

First, we analyzed the product formation of the purified R2 peptide with time using mass spectrometry. This technique allowed us to identify the methylated products at various time points and distinguish which arginine residue that had been modified. A reaction containing 4 µM PRMT1, 800 µM AdoMet, and 10 nM MTA-nucleosidase was initiated with 200 µM purified R2 peptide. The bottom mass spectrum of Fig. 5-3, B shows that R2 (561.9 m/z) was monomethylated (566.6 m/z) within seconds of initiating
the reaction. MS/MS analysis was used to further analyze the dimethylated peptide product peak (571.3 m/z) (top spectrum, Fig. 5-3, B). We observed that only the N-terminal arginine of the R2 peptide had been di-methylated. MS/MS sequencing data shown in Fig. 5-3, C gave the following sequence: GGR\(_{\text{2-methyl}}\)GGFGGKKGGFGGRGGFG. No methylation of the C-terminal arginine of R2 was detected (Fig. 5-3, C). This indicates that the N-terminal arginine of R2 was first mono- and then dimethylated. After an extended reaction (8 hours), MS/MS analysis was used to confirm the location of methylation of the tetramethylated R2 peptide. MS/MS analysis of the tetramethylated peptide peak (581 m/z) (MS data not shown) revealed that R2 had also been dimethylated on the C-terminal arginine (Fig. 5-3, D). MS/MS sequencing data shown in Fig. 5-3, D gave the following sequence: GGR\(_{\text{2-methyl}}\)GGFGGKKGGFGGR\(_{\text{2-methyl}}\)GGFG. Scheme 5-3 summarizes the series of methylation events and indicates that methylation of R2 was systematic and not random. Like R2, preliminary MS/MS data suggests that the R3 peptide that has an additional arginine residue is also methylated at its N-terminal arginine first GGR\(_{\text{methyl}}\)GGFGGRGGFGRGGRGGFG (data not shown). Altogether, these results demonstrate that 1) one arginine is preferentially methylated over another, 2) both arginyl groups in R2 can be converted to ADMA, and 3) methylated product species can be effectively separated and detected using MS.
Fig. 5-3. MS data shows that methylation of a multiple arginine-containing substrate is systematic. (A) Mass spectrums of the R2 peptide (561.9 m/z) before (bottome) and after purification (top). (B) A reaction containing 4 μM PRMT1 and 800 μM AdoMet was initiated with 200 μM R2 (pure). The modified peptide was analyzed by MS. The 0.5 minute time point (bottom) depicts the formation of monomethylated R2 at 566.66 m/z (mono). The 24.5 minute sample (top) shows the formation of dimethylated R2 at 571.3 m/z (di). (C) MS/MS was used to confirm the location of methylation of the dimethylated R2 peptide. (D) After an extended reaction (8 hours), MS/MS analysis was used to confirm the location of methylation of the tetramethylated R2 peptide peak (581 m/z).
Scheme 5-3. Methylation of R2 is systematic, not random. The N-terminal arginine of R2 (AcGGGGFGGKGGFGRGGFG) is monomethylated first. Dimethylation of this modified arginine occurs next followed by the mono- and subsequent dimethylation of the C-terminal arginine.

**Methylation of a multiple arginine-containing substrate is dissociative**

One way to ensure dimethylated product formation would be to perform processive methylation. Although we have shown that PRMT1 methylates substrates containing a single arginine residue in a dissociative manner (Fig. 5-1 and Chapter 4), it is possible that multiple arginine-containing substrates are processed differently. Because most PRMT1 protein substrates are found to be dimethylated on multiple arginine residues within the same protein, we examined how PRMT1 methylates a multiple arginine-containing substrate (processive versus dissociative methylation) at two different levels. First, examined how a single arginine residue in a multiple arginine-
containing substrate was methylated. Second, we examined if methylation from one arginine residue to the next arginine residue on the same substrate was processive.

Using the R2 peptide, which we have shown is first mono- and then dimethylated at the N-terminal arginine (Fig. 5-3), we tested if PRMT1 could processively dimethylate the N-terminal arginine residue ($GG^RGGFGGKGGFGG^RGGFG \rightarrow GG^{Rdimethyl}GGFGGKGGFGG^RGGFG$). First, reverse phase HPLC analysis [29] of the acid-hydrolyzed product R2 peptide was used to verify the reaction time in which only monomethyl arginine was present (Fig. 5-4). This method along with a standard curve of MMA was then used to quantify the amount of MMA generated up until the time that the reaction was quenched with TFA. At 20 minutes, approximately 4.2 $\mu$M MMA had been formed. This amount was greater than the 10 nM enzyme in the reaction. Since R2 is monomethylated at the N-terminal arginine first followed by the dimethylation of that same arginine residue (and not the monomethylation of the C-terminal arginine residue), a higher concentration of MMA than enzyme in the reaction indicates that methylation is dissociative. If the mechanism had been processive, the concentration of MMA (monomethylated-R2) would have never exceeded the concentration of enzyme.

A modified pulse-chase experiment (Fig. 5-5, A) coupled to mass spectrometry analysis was used to verify that the dimethylation of the N-terminal arginine ($GGRGGFGGKGGFGG^RGGFG \rightarrow GGR^{Rdimethyl}GGFGGKGGFGG^RGGFG$) was dissociative. A reaction containing 4 $\mu$M PRMT1 and 1200 $\mu$M AdoMet was pulsed with 200 $\mu$M R2 peptide substrate at 37 °C. At 6 minutes, a time point where only the monomethylated product peak was detected by mass spectrometry (bottom spectrum in
Fig. 5-4. HPLC chromatogram verifies the presence of arginine and MMA only. A reaction containing 10 nM PRMT1, 1200 µM AdoMet, and 10 nM MTA-nucleosidase in 150 mM sodium phosphate buffer pH 7.1 was initiated with 200 µM R2 peptide on ice. Time points were taken out and quenched 1:1 in 20% TFA. Precipitated protein was removed through centrifugation, and the resulting supernatant containing the modified peptide was hydrolyzed with concentrated HCl. Samples were derivatized with the AQFluor reagent and analyzed by HPLC. The HPLC chromatogram indicates that only arginine (Arg) and monomethylated arginine (MMA) at 24.5 min and 31.7 min, respectively, were present after a 5 minute reaction on ice (bold line). MMA and ADMA standards injected separately are shown in gray.

Fig. 5-5, B), the reaction was chased with an excess amount (1 mM) of JMH1W-CH₃ peptide (KGGFGGGR(CH₃)GGFGGKW). Time points were taken out at 6.5, 9, 12, 15, and 20 minutes (Fig. 5-5, C) and compared to time points from a control reaction that had been pulsed with R2 and chased with water at 6 minutes (data not shown). Fig. 5-5, C shows that the chase peptide (475.88 m/z) was dimethylated (479.9 m/z) with time. On the other hand, the peak intensity of the dimethylated R2 peptide product (571.29 m/ z) did not increase as much with time when the reaction was chased with the JMH1W-CH₃ peptide (Fig. 5-5, D) when compared to the control reaction containing the R2 pulse only.
Importantly, a sample of the chase peptide alone verified that the dimethylated chase peptide peak was not present in the JMH1W-CH$_3$ peptide standard (data not shown).

Furthermore, the peak intensities obtained from the raw mass spectrometry data were plotted against time (as described in the methods section) in Fig. 5-5, E for the R2 only control reaction (left panel) and the chase reaction (right panel). Dimethylation of R2 was clearly influenced by the chase peptide. The dimethylated R2 peak increased 0.08 from 6.5-20 minutes in the control reaction but only increased 0.03 from 6.5-20 minutes in the chase reaction (Fig. 5-5, E). Because the chase peptide was methylated almost immediately in this experiment, the monomethylated R2 peptide had to have been released from the PRMT1 active site. These results indicate that dimethylation of a single arginine residue is dissociative on a substrate containing multiple arginine residues.

Finally, we examined if methylation from one arginine to the next arginine residue on the same substrate was processive. In a separate modified pulse chase mass spectrometry experiment (Fig. 5-6, A), we tested if the mechanism going from dimethylated-R2 (at the N-terminal arginine) to trimethylated-R2 ($GGR_{\text{dimethyl}}GGFGGGFGGFGGRGFGRGGFGGR_{\text{methyl}}GGFG$) was processive. A reaction containing 4 µM PRMT1 and 1200 µM AdoMet was pulsed with the 200 µM R2 peptide substrate at 37 °C. At 60 minutes, a time point where only the mono- and dimethylated product peaks were detected by mass spectrometry (see bottom trace of Fig. 5-6, B), the reaction was chased with an excess amount of JMH1W-CH$_3$ peptide (1 mM). Later time points were quenched and analyzed by MS. The data showed that the chase peptide was dimethylated with time (479.9 m/z in Fig. 5-6, C).
Fig. 5-5. PRMT1 performs dissociative methylation of a single arginine residue on a multiple arginine-containing substrate. (A) Modified pulse chase mass spectrometry experimental design. PRMT1 was allowed to methylate the R2 peptide (pulse) for a period of time wherein the first methylation event could occur. A time point is taken out to verify the presence of the monomethylated species. The reaction is then chased with an excess of JMH1W-CH3 peptide. Subsequent time points are taken out and analyzed by mass spectrometry to identify if the chase peptide gets methylated immediately, a result indicative of a dissociative mechanism. (B) MS samples of the R2 peptide control reaction at 6, 9, 12, and 15 minutes where the native (R2), monomethylated (mono), and dimethylated (di) R2 peaks are labeled at 561.9 m/z, 566.6 m/z, and 571.3 m/z, respectively. (C-D) At 6 minutes, a reaction that had been pulsed with 200 µM R2 was chased with 1 mM JMH1W-CH3 peptide. Subsequent time points (6.5, 9, 12, 15, and 20 minutes) were quenched and analyzed by MS to identify if the chase peptide (475.88 m/z) or the R2 peptide was dimethylated. (C) Mass spectrums reveal that the chase peptide was dimethylated (di-chase=479.9 m/z) with time while the R2 peptide was not dimethylated (R2-di=571.29 m/z) with time (in D). The bottom mass spectrum from the 59.5 minute time point (in both C and D) was taken out before the chase peptide was added. (E) Time dependent formation of mono- and dimethylated R2 from the R2 only control reaction (left panel) and chase reaction (right panel). Peak intensities associated with each product were normalized as discussed in the materials and methods section and were plotted as a function of time. Filled circles (●), open circles (○), and triangles (▲) represent monomethylated-R2, dimethylated-R2, and the dimethylated-chase peptide, respectively.
D

R2, chased, GFP 200 fmol/ul 6.5 min, A

Whitney-R2_080912-Chase-20-1 383 (8.966) Sm (SG, 1x5.00); Cm (361:456)
1: TOF MS ES+ 9.80e3

R2 and chase peptide reaction time: 20 minutes

Whitney-R2_080912-Chase-15-1 381 (8.929) Sm (SG, 1x5.00); Cm (361:456)
1: TOF MS ES+ 9.02e3

R2 and chase peptide reaction time: 15 minutes

Whitney-R2_080912-Chase-12-1 363 (8.966) Sm (SG, 1x5.00); Cm (361:456)
1: TOF MS ES+ 8.79e3

R2 and chase peptide reaction time: 12 minutes

Whitney-R2_080912-Chase-9-1 388 (9.077) Sm (SG, 1x5.00); Cm (361:456)
1: TOF MS ES+ 6.65e3

R2 and chase peptide reaction time: 9 minutes

Whitney-R2_080912-Chase-6p5-1 386 (9.040) Sm (SG, 1x5.00); Cm (361:456)
1: TOF MS ES+ 7.14e3

R2 and chase peptide reaction time: 6.5 minutes

Whitney-R2_080912-Chase-5p5-1 300 (9.111) Sm (SG, 1x5.00); Cm (361:456)
1: TOF MS ES+ 538

R2 and chase peptide reaction time: 5.5 minutes
The peak intensities of trimethylated-R2 grew slower (Fig. 5-6, D) than the peak intensities from the R2 only pulse reaction (raw data not shown). The ratios of the peak intensities obtained from the raw MS data were plotted against time (as described in the methods section) in Fig. 5-6, E for the R2 only control reaction (left panel) and chase reaction (right panel). Both the di- and trimethylated R2 peak intensities did not increase as much with time when the chase peptide was added. PRMT1 preferentially methylated the chase peptide over both the mono- and dimethylated R2 peptides. In fact, the dimethylated R2 peak increased 0.27 from 60-200 minutes in the control reaction but increased only 0.02 during the chase reaction (Fig. 5-6, E). The trimethylated R2 peak increased 0.14 from 60-200 minutes in the control reaction but only increased 0.02 from 60.5-200 minutes in the chase reaction (Fig. 5-6, E). Our results indicate once again that the catalytic event going from mono- to dimethylated R2 is dissociative \( \text{GGR}_{\text{methyl}} \text{GGFGKGFGGGRGGFG} \rightarrow \text{GGR}_{\text{dimethyl}} \text{GGFGKGFGGGRGGFG} \).
However, these results do not definitively prove that going from di- to trimethylated

\[ \text{GGR}_{\text{dimethyl}} \text{GGFGGKGGFGGRGGFGG} \rightarrow \text{GGR}_{\text{methyl}} \text{GGFGGKGGFGGR} \]

\( \text{GGR}_{\text{dimethyl}} \text{GGFGGKGGFGGR} \) is dissociative. In order to prove whether this event was truly dissociative, the reaction was chased at 200 minutes instead of 60 minutes wherein a larger buildup of the dimethylated-R2 product had been generated. Unfortunately, the activity of the enzyme used to perform this chase experiment had decreased upon storage at 4 °C for days. The buildup of dimethylated-R2 product was comparable to the previous 60 minute chase experiment. Despite any experimental complications, the preliminary data suggest a dissociative mechanism wherein PRMT1 releases the N-terminally dimethylated-R2 product before it rebinds and monomethylates the C-terminal arginine.

**DISCUSSION**

**PRMT1 preferentially methylates monomethylated substrates dissociatively**

Previously, we demonstrated that the dimethylation of a single arginine-containing fibrillarin-based peptide substrate occurs through a dissociative mechanism based on an observable lag in the formation of the dimethylated product using mass spectrometry (see Chapter 4). Mass spectrometry analysis suggests that the eIF4A1 peptide was also dissociatively methylated by PRMT1 (Fig. 5-1, A). Our results are consistent with Frankel and coworkers who also observed dissociative methylation of a minimal peptide \( \text{WGGYSRGGYGWW} \) by PRMT6 [35], but are different from the findings of Thompson and co-workers who defined a partially processive dimethylation of a histone 4 peptide \( \text{AcSGRGKGGKGLGKGGAKRHRKV} \) by PRMT1 [33, 34].
Fig. 5-6. PRMT1 may dissociatively dimethylate multiple arginine residues. (A) Modified pulse chase mass spectrometry experimental design. PRMT1 methylates the R2 peptide (pulse) for a period of time wherein only two methylation events have occurred. The reaction is chased with JMH1W-CH₃ peptide in excess. Subsequent time points are quenched and analyzed by MS. If the chase peptide gets methylated immediately (before R2 trimethylation), PRMT1 is dissociative. (B) MS samples of the R2 peptide control reaction at 59.5, 60, 70, 80, 100, 120, 150, and 200 minutes where the native (R2), monomethylated (mono), dimethylated (di), and trimethylated (tri) R2 peaks are labeled at 561.9 m/z, 566.6 m/z, 571.3 m/z, and 576.3 m/z respectively. Only the top spectrum is labeled for clarity. (C-D) At 60 minutes, the reaction with 200 µM R2 was chased with 1 mM JMH1W-CH₃ peptide. Time points (60.5, 70, 80, 100, 120, 150, and 200 minutes) were quenched in TFA (10% final). Mass spectrums revealed that the chase peptide was dimethylated (di-chase=479.9 m/z) with time in (C) while the R2 peptide underwent little trimethylation (R2-tri=576.3 m/z) (D). The 59.5 minute sample (bottom spectrum in C and D) was taken out before the chase peptide was added. (E) Time dependent formation of mono-, di-, and trimethylated R2 from the R2 only control reaction (left panel) and chase reaction (right panel). Peak intensities associated with each product were normalized as discussed in the materials and methods section and were plotted as a function of time. The mono- (●), di- (○), and trimethylated (▲) R2 peptides along with the dimethylated chase peptide (□) are shown.
Because the fibrillarin-based substrates (see Chapter 4 and [35]) and the eIF4A1-based substrate (Fig. 5-1, A) were 12-13 amino acids long and the histone 4 peptide was 21 amino acids long, substrate length could influence the PRMT1 mechanism. However, despite an increase in peptide length, our results suggest that the longer fibrillarin-based peptide substrate was dissociatively dimethylated as indicated by a lag in the formation of the dimethylated product (Fig. 5-1, B). In a dissociative mechanism, the formation of the dimethylated peptide product depends upon how easily PRMT1 can recapture the monomethylated product. Frankel and co-workers have reported that the monomethylated peptide substrate is a better substrate for PRMT6 than the naked peptide [35]. We observed similar results with two fibrillarin-based peptides (JMH1W and JMH1W-CH₃) (Table 5-2). PRMT1 preferentially methylated the monomethylated substrate versus the naked peptide 3 to 1.

In order to understand if the sequence differences between a fibrillarin-based peptide and a histone 4-based peptide may be the basis for the different mechanistic
observations, we examined the kinetic parameters of an additional peptide substrate pair (eIF4A1 and eIF4A1-CH$_3$) derived from eIF4A1, another PRMT1 protein substrate. This time, we observed that the monomethylated peptide substrate was a better substrate for PRMT1 than the naked peptide (Table 5-2), but the preference for the monomethylated eIF4A1 substrate versus the naked peptide was not as significant (1.33 to 1). The mechanistic basis for the observed preference for the monomethylated substrate is not due to binding as evidenced by the similar dissociation constants between eIF4A1 and eIF4A1-CH$_3$ (Table 5-3). A slight preference for the monomethylated substrate must be due to other factors in the kinetic mechanism.

Perhaps the methylated peptides examined adopt a conformation in the active site that facilitates a better S$_{N}2$ attack of the methyl group on AdoMet than the nonmethylated peptide. Alternatively, the central position of the substrate arginine in the peptides tested may structurally constrain the arginine only allowing for a more restricted mechanism where the peptide has to dissociate from the enzyme before dimethylation can occur. An arginine located towards the N or C-terminal end of the peptide (as in the case of the H4 peptide [33, 34]) may have more flexibility allowing a second methyl group to transfer to the substrate arginine resulting in more of a semi-processive mechanism. A crystal structure of PRMT1 with a monomethylated peptide would allow us to visualize how a monomethylated substrate interacts with PRMT1 and may help us understand the mechanism.
PRMT1 methylation of substrates containing multiple arginine residues

PRMT1 methylation of substrates containing multiple modification sites was shown to be systematic (not random) using mass spectrometry (Fig. 5-3). Monomethylation of the R2 peptide occurred first at the N-terminal arginine residue. This monomethylated arginine was then dimethylated, a result that is consistent with the fact that PRMT1 prefers to methylate arginine residues that have already been monomethylated versus non-methylated arginine residues (Table 5-2). PRMT1 then went on to trimethylate and then fully methylate R2 at the C-terminal peptidyl arginine. PRMT1 systematically methylated arginine residues in a multiple arginine-containing substrate. Preliminary MS/MS evidence suggests that the N-terminal arginine of R3 (GGRGGFGGRGGFGGGRGGFG) is also methylated first suggesting that methylation does not occur randomly (data not shown). Although these results were obtained using peptide substrates, PRMT may also methylate multiple arginine residues in protein substrates in a systematic manner. PRMT1 methylation of multiple arginine residues on a protein substrate may result in different biological responses depending on the order in which a series of arginines is methylated.

Next, using two carefully designed modified-pulse chase experiments, we found that the methylation of a substrate containing multiple arginine residues was dissociative. First, we found that going from monomethylated to dimethylated R2 at the N-terminal arginine

\[
\text{GGR}_{\text{naked}}\text{GGFGGKGGFGGGRGGFG} \quad \rightarrow \quad \text{GGR}_{\text{dimethyl}}\text{GGFGGKGGFGGGRGGFGG}
\]

was dissociative when the reaction containing R2 was flooded with chase peptide. Because the chase peptide was methylated shortly
after its addition (Fig. 5-5, E), the R2 peptide had to have been released from PRMT1. Also supporting a dissociative mechanism was the fact that the concentration of MMA was greater than the concentration of enzyme in the reaction when no ADMA was present (Fig. 5-4). If the mechanism were processive, the concentration of MMA (monomethylated-R2) could never exceed the concentration of enzyme. A dissociative mechanism is consistent with what we (see Fig. 5-1 and Chapter 4) and others [35] had already determined for peptides containing a single arginine residue. Although the presence of another arginine residue in the peptide sequence could have altered how PRMT1 performed catalysis, the dissociative mechanism remained the same.

Finally, preliminary results suggested that the methylation of one arginine to the methylation of another arginine residue on the same peptide substrate was dissociative (\( \text{GGR}_{\text{monomethyl}} \text{GGFGKGFG} \rightarrow \text{GGR}_{\text{dimethyl}} \text{GGFGKGFG} \)). The reaction was chased at a time (60 minutes) in which most of the R2 peptide was not mostly dimethylated. When this reaction was flooded with chase peptide, the chase peptide was methylated before the R2 peptide could be di- or trimethylated (Fig. 5-6, E). Both R2 dimethylation and trimethylation were substantially impeded. These results 1) verify that R2 monomethylation to dimethylation follows a dissociative mechanism and 2) suggest that PRMT1 releases the dimethylated peptide product before it can rebind and monomethylate the C-terminal peptidyl arginine residue. In order to definitively prove if the mechanism from di- to trimethylated-R2 is dissociative, however, the reaction was chased at a later time point (200 minutes) wherein R2 should have been mostly dimethylated. Although some trimethylation of R2 will have already occurred by this
time, the correct mechanistic event (methylation of one arginine to another on the same peptide) will be probed. Unfortunately, the PRMT1 used to perform this experiment was nearly inactive due to multiple freeze/thaw cycles and the data was not usable. If PRMT1 is found to methylate multiple arginine residues in a dissociative manner, the following scheme will be proposed (Scheme 5-4).

Scheme 5-4. Suggested reaction mechanism scheme of PRMT1 with the R2 peptide. PRMT1 (E=enzyme) interacts with AdoMet and the R2 peptide. The first methylation event takes place, and both products (AdoHcy and R2 mono-CH₃) dissociate from PRMT1. Monomethylated-R2 and AdoMet re-bind to PRMT1. Dimethylation occurs, and both products are released. Dimethylated-R2 and another molecule of AdoMet re-bind to PRMT1 and trimethylated-R2 is generated. AdoHcy and R2 tri-CH₃ are released. Finally, R2 is fully methylated when the trimethylated peptide and another molecule of AdoMet bind to PRMT1. Fully methylated-R2 and AdoHcy are released from the PRMT1 active site. Note, because the order of substrate binding and product release is not known, the rebinding event of the peptide products are not shown for simplicity.
We have shown that PRMT1 methylates arginine-containing peptide substrates in a systematic manner preferring to methylate substrates that have already been monomethylated. These results may be extended to help explain how PRMT1 methylates protein substrates; however, the structural surface of a protein substrate may be more restrictive (less flexible) than a peptide substrate. Based on the fact that PRMT1 did not methylate multiple arginines within the same substrate randomly, PRMT1 may exhibit directional methylation of protein substrates or even utilize a type of scanning mechanism. All in all, methylation of multiple arginine residues on a protein substrate by PRMT1 has the potential to result in different biological responses depending on the order in which a series of arginines is methylated.

Conclusion

Many PRMT1 protein substrates such as fibrillarin, the hnRNPs, and SAM68 are asymmetrically dimethylated at multiple arginine residues. In order to explain the biological predominance of ADMA, we investigated the catalytic mechanism of PRMT1. Although some methyltransferases have been shown to catalyze processive methylation [37-40], an enzymatic feature that would help explain the predominance of ADMA, PRMT1 does not catalyze processive methylation of fibrillarin-based peptides. Instead, we have shown that PRMT1 methylates single arginine-containing peptides in a dissociative manner. Methylation of a single arginine in a multiple arginine-containing peptide was shown to be dissociative. PRMT1 preferentially monomethylated substrates versus the unmodified peptide substrates, a phenomenon that may result from substrate length, sequence, and/or the location of the substrate arginine within the substrate. Based
on these results, the predominance of dimethylated protein substrates *in vivo* is likely due to the PRMT1 mechanism and the preference for monomethylated proteins.

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CHAPTER 6

DETERMINANTS OF PRMT1 PRODUCT FORMATION: ROLES OF ACTIVE SITE RESIDUES

ABSTRACT

Protein arginine methyltransferases (PRMTs) play important roles in biology. Type I methyltransferases, PRMT1, 3, 4, 6, and 8, catalyze the formation of monomethyl arginine (MMA) and asymmetric dimethylarginine (ADMA) on protein arginine residues via methyl group transfers from $S$-adenosyl methionine (AdoMet/SAM). PRMT5, a Type II methyltransferase performs symmetric dimethylation generating symmetric dimethylarginine (SDMA). Encoded in each isoform is the necessary active site geometry and chemical functionalities to generate SDMA or ADMA and regulate how much MMA versus ADMA is made. Using the crystal structure of ratPRMT1 as our guide, we analyzed the effect of several active site mutations on the rate of methylation and the ability of PRMT1 to selectively perform asymmetric dimethylation. Met155 has been proposed to play a direct role in dictating ADMA formation. M155A-PRMT1 was assayed with R3, a 19 amino acid peptide derived from the $in vivo$ PRMT1 substrate fibrillarin and exhibited approximately one tenth the activity of wild type PRMT1. Peptide products were hydrolyzed and analyzed by fluorescent derivatization via RP-HPLC. Only MMA and ADMA were produced. Thus, removing steric bulk afforded by Met155 in the PRMT1 active site is not by itself sufficient to transform PRMT1 into a

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$^1$Coauthored by W.L. Wooderchak, J.M. Hevel, Determinants of PRMT1 product formation: Roles of active site residues, Biochemistry (2009), manuscript in preparation.
Type II PRMT. Other PRMT1 mutants (M48A, M146A, S154A, W145A, Y148S, and M48L-PRMT1) displayed altered methyltransferase activity generating only MMA and ADMA. Importantly, we show that Met48 plays a critical role in PRMT1-catalysis.

INTRODUCTION

Protein arginine methyltransferases (PRMTs) catalyze the transfer of a methyl group from S-adenosyl methionine (SAM/AdoMet) to one of the terminal guanidino groups of arginine generating monomethyl arginine (MMA) and S-adenosyl homocysteine (SAH/AdoHcy). Type I methyltransferases, PRMT1, 3, 4, 6, and 8, asymmetrically dimethylate arginine on the same guanidino nitrogen and form asymmetric dimethylarginine (ADMA) [1-6]. Type II methyltransferases, PRMT5 and PRMT7, perform symmetric dimethylation wherein the guanidino nitrogens are modified to produce symmetric dimethylarginine (SDMA) (Scheme 6-1) [7, 8]. Selective formation of ADMA by PRMT1 has many biological implications [9]; specifically, Arg 3 of histone H4 can be asymmetrically dimethylated by PRMT1 or symmetrically dimethylated by PRMT5 resulting in antagonistic biological consequences (reviewed in [10]). Because of this, we wanted to study why PRMT1 selectively forms ADMA versus SDMA.

Once a protein has been modified, it is eventually degraded in the proteasome and free MMA, SDMA, and ADMA are released. Interestingly, higher levels of free ADMA have been linked to cardiovascular disease (reviewed in [11]) and stroke [12]. ADMA is also second only to age as a predictor of mortality and cardiovascular events in chronic renal failure patients [13]. ADMA and MMA are endogenous inhibitors of nitric oxide.
synthase (NOS) [14] which catalyzes the formation of nitric oxide, a vasodilator that prevents atherosclerosis. Fortunately, free MMA and ADMA levels are controlled by dimethylarginine dimethylaminohydrolases (DDAHs), but the misregulation of DDAH or the hyperactivity of PRMTs may contribute to cardiovascular disease [15]. In fact, PRMT1 has been shown to be overexpressed in myocardial tissue taken from coronary heart disease patients [16]. Because PRMT1 performs an estimated 85% of all protein arginine methylation \textit{in vivo} [17], this isoform generates more ADMA than all other PRMT isoforms combined. Therefore, it is important to understand how PRMTs control Type I (asymmetric) versus Type II (symmetric) dimethylation.

\[ \text{Scheme 6-1. Reactions catalyzed by PRMTs. PRMTs catalyze the } S\text{-adenosylmethionine (AdoMet) dependent methylation of a protein substrate arginine residue to form monomethyl arginine (MMA). Type I methyltransferases, PRMT1, 3, 4, 6, 8, and 10 dimethylate arginine asymmetrically to form asymmetric dimethyl arginine (ADMA). Type II methyltransferases, PRMT5, 7, and 9 catalyze the formation of symmetric dimethyl arginine (SDMA).} \]
The crystal structure of PRMT1 [18] has been used to offer an explanation as
to why Type I PRMTs exclusively perform asymmetric dimethylation versus symmetric
dimethylation. Interestingly, all Type I PRMTs contain an active site methionine
(Met155 for rat PRMT1) which is not conserved among Type II PRMTs. Methionine
155, located at the end of an alpha helix positioned near the reactive arginine of the
substrate peptide (Fig. 6-1), has been proposed to exclude monomethylated arginine from
binding in a conformation that would allow its symmetric dimethylation [18]. The
residue corresponding to Met155 in the Type II PRMT5 is a serine (amino acid 446 in rat
PRMT5 and amino acid 474 in yeast PRMT5). PRMT7, another Type II PRMT, has an
alanine residue in place of the bulky methionine at this location. It was hypothesized that
the smaller bulk of the serine and alanine side chains at this location may enable the free
rotation of the substrate arginine about the terminal C-N bond resulting in the formation
SDMA formation.

Fig. 6-1. Active site methionines position the substrate arginine. The active site of rat
PRMT1 (PDB code 1OR8) is shown with AdoHcy (cyan) and the R3 peptide in orange.
The substrate arginine of R3 is depicted in ball and stick mode with the two guanidino
nitrogens pointing towards the sulfur atom (blue) of AdoHcy. Met 155 (left) and Met 48
(right) are shown in yellow as sticks with their electron densities depicted in dots.
In addition to Met155, we hypothesized that another active site methionine residue (Met48 in rat PRMT1) positioned near the reactive substrate arginine (Fig. 6-1) plays a role in selective ADMA formation. This methionine residue is conserved among all Type I enzymes but is replaced by a proline in the Type II PRMT5. The presence of a proline instead of a methionine at this position could open up the active site pocket enough to allow for the free rotation about the substrate arginine terminal C-N bond. In addition to controlling exclusive ADMA formation, Met 155 and Met 48 may serve another role. Mechanistically speaking, the pair of methionines (Met155 and Met48 depicted in Fig. 6-1) could act as a molecular tweezer aligning the guanidino nitrogen for the most efficient S_N2 methyl transfer among Type I PRMTs.

The crystal structures of ratPRMT3 [19] and ratPRMT1 bound to AdoHcy and substrate peptide [18] have offered much insight into the PRMT reaction. A mechanism has been proposed where the carboxylate of an active site glutamate (Glu153 in rat PRMT1) localizes the positive charge on one of the guanidino nitrogens, allowing the lone pair of electrons of the other guanidino nitrogen to become a better nucleophile. The residue required to deprotonate the guanidino nitrogen of the substrate arginine or the mono-methylated product has not been identified. Thompson and coworkers recently elucidated the kinetic mechanism of PRMT1 [20]. They report a different mechanism wherein PRMT1 utilizes a rapid equilibrium random sequential mechanism to methylate the histone H4 peptide. In this type of reaction mechanism, the order of substrate binding to the enzyme and product release from the enzyme does not matter. Although the mechanism for Type I PRMTs has become clearer, the mechanistic roles of certain PRMT1 active site residues have not been identified.
The goal of this work was to identify residues in the PRMT1 active site that are important for substrate binding and catalysis and may affect ADMA formation. We hypothesized that two active site methionine residues (Met155 and Met48) positioned near the reactive substrate arginine (Fig. 6-1) enable PRMT1 to selectively form ADMA instead of SDMA. M155A and M48A mutant proteins were characterized according to their ability to catalyze methylation of a multiple arginine-containing peptide based on the in vivo substrate fibrillarin. Removing the steric bulk afforded by each of these residues did not allow for the formation of SDMA. The mutation of Met48 to an alanine in the PRMT1 active site rendered the enzyme virtually inactive. When this residue was mutated to a leucine, activity was partially restored indicating that the length of Met48 is critical to PRMT1 catalysis. Our results indicate that removing the steric bulk afforded by either Met155 or Met48 in the active site of PRMT1 is not by itself sufficient to transform PRMT1 into a Type II PRMT. Additional PRMT1 mutants (M146A, S154A, W145A, and Y148S-PRMT1) displayed altered methyltransferase activity generating only MMA and ADMA. Interestingly, M48L-PRMT1 was capable of automethylation while wild type PRMT1 was not.

**MATERIALS AND METHODS**

AdoMet was purchased from Sigma as a chloride salt (≥80%, from yeast). The R3 peptide, acylGGRGFGGRGGFGGRGGFG; the JMH1W peptide, acylKGGFGGRGGFGGRGGKW; and the JMH1W-CH₃ peptide, acylKGGFGGRmethylGGFGGRGGKW were synthesized by the Keck Institute and purified to ≥95%.
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 wild type PRMT1 plasmid (pET28b-PRMT1) (see Chapter 3) was used as template. Desired mutations (M155A, M48A, M48L, S154A, M146A, Y148S, W145A, and M48P) were confirmed through DNA sequencing. Mutant proteins were expressed and purified using the same methods used to express and purify wild type His-PRMT1 (described in Chapter 3). Each mutant protein was expressed at the same level as wild type PRMT1 in cell culture except for the M48A mutant which exhibited slightly lower expression levels when compared to wild type. Purified proteins were ≥95% pure by SDS-PAGE. Mutant protein sequences were verified using mass spectrometry. The binding of AdoMet is consistent with a properly folded structure for all mutants.

Kinetic assays of PRMT1 mutants

A continuous spectrophotometric assay for AdoMet-dependent methyltransferases (described in Chapter 3) was used to assay PRMT1 mutants with arginine-containing peptides. Two coupling enzymes, AdoHcy nucleosidase (MTAN) and adenine deaminase, were used to hydrolyze and deaminate the AdoHcy generated from methyltransfer, respectively. This assay avoids any product inhibition that could occur from AdoHcy. Each 110 µL reaction contained 4 µM His-PRMT1 (WT or mutant), 0.02 µM adenine deaminase (purified as described in Chapter 3), 250 µM AdoMet, 10 nM
MTAN (purified as in [21]), 100 µM MnSO₄, and 50 mM sodium phosphate buffer pH 7.1. Reactions equilibrated at 37 ºC for ten minutes before they were initiated with various amounts of peptide. The decrease in absorbance at 265 nm was monitored continuously using a Cary300 Bio UV-visible spectrophotometer. Initial rate data representing no more than 10% of product formation were fit to the Michaelis Menten equation [22] to obtain Kₘ,app and kₖₐₜ,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 kₖₐₜ of 0.025 min⁻¹).

**HPLC analysis of methylated amino acids**

Assays containing 4 µM WT- or mutant-PRMT1 proteins, 1200 µM AdoMet, 10 nM MTAN, and 50 mM sodium phosphate buffer (pH 7.1) equilibrated at 37 ºC for 8 minutes. Reactions were initiated with 200 µM R3 and were terminated after 3 hours with 10% TCA final. TCA-precipitated protein was removed through centrifugation, and the supernatant (containing the peptide) was added to a glass vial. An equivalent volume of 12.1 M HCl was added to each vial. Vials were crimp-sealed and heated to 110 ºC for approximately 24 hours to carry out acid hydrolysis. Hydrolyzed amino acids from the mutant catalyzed peptide product were analyzed by fluorescent derivatization (AccQ●Fluor reagent kit, Waters) using a well-established RP-HPLC protocol [23]. This method uses a gradient of acetonitrile and a C18-Luna column (Phenomenex) to separate MMA, ADMA, and SDMA based on their hydrophobicity. To verify the presence and peak times of the methylated arginine products, subsequent runs of samples incubated with 0.6 µM [H³]-AdoMet (specific activity of 83 µCi/µmol) for 3 hours and analyzed via
HPLC [24]. Fractions (0.5 mL) were collected and radioactivity was counted in 4 mL scintillation cocktail (Fisher Scientific). MMA, ADMA, or SDMA standard amino acids were also used to verify the identity of the methylated products generated. Peak times were 37.5, 43.65, and 48 min for MMA, ADMA, and SDMA respectively. Shifting of the product peaks was observed from one run to another, so standards were run before and after each sample to accurately identify the presence of each arginine species.

**Intrinsic fluorescence**

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 0.7 µM PRMT1 in 150 mM sodium phosphate buffer pH 7.1. Increasing concentrations from 1 to 50 µM peptide ligand (or 1 to 50 µM AdoMet in a separate experiment) were added at 2-3 min intervals. Data from at least two titrations were averaged and analyzed using the Stern-Volmer [24] or modified Stern Volmer [25] plots. Data were evaluated by least squares linear regression analysis using Kaleidagraph in order to obtain the dissociation constant (K_d). For the modified Stern Volmer plots, the following equation was used: \( F_c = F \left( 10^{-\varepsilon c d/2} \right) \) 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 \( y_{\text{intercept}}=1/ka \), the slope=\( 1/kaK_Q \), and the \( K_Q=1/K_D \).
RESULTS

Active site methionines do not govern
strict ADMA formation

It was proposed [18] that Met155 helps determine the formation of ADMA
because of the increased steric bulk of the residue. If the length of Met155 is solely
responsible for the strict formation of ADMA in the PRMT1 active site, then the
mutation of this residue to a smaller amino acid might allow for the formation of SDMA.
To investigate this hypothesis, we mutated Met155 to an alanine residue. M155A-
PRMT1 was expressed, purified, and demonstrated saturation kinetics with a peptide
derived from the \textit{in vivo} substrate fibrillarin (R3=\textsubscript{acyl}GGRGGFGGGGFGGGRGGFGG)
(see Fig. 6-2). Compared to WT-PRMT1, this mutation did not affect \(k_{\text{cat}}\), but \(K_m\)
increased by a factor of ten (Table 6-1). As a result of this, the catalytic efficiency
(\(k_{\text{cat}}/K_m\)) of M155A-PRMT1 was only 10.3% the activity of WT-PRMT1 (Table 6-1)
indicating that Met155 is important to catalysis.

<table>
<thead>
<tr>
<th>PRMT1</th>
<th>(K_m) ((\mu\text{M}))</th>
<th>(V_{\text{max}}) ((\mu\text{M CH}_3/\text{min}))</th>
<th>(k_{\text{cat}}/K_m) ((\text{M}^{-1}\text{s}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>&lt;10</td>
<td>10.7 ± 0.803</td>
<td>4460 ± 333</td>
</tr>
<tr>
<td>M155A</td>
<td>118 ± 29.6</td>
<td>12.9 ± 1.12</td>
<td>457 ± 121</td>
</tr>
<tr>
<td>M48A</td>
<td>303 ± 214</td>
<td>0.959 ± 0.470</td>
<td>13.2 ± 13.1</td>
</tr>
<tr>
<td>M48L</td>
<td>17.6 ± 2.65</td>
<td>7.06 ± 0.209</td>
<td>1670 ± 255</td>
</tr>
<tr>
<td>M48P</td>
<td>439 ± 205</td>
<td>6.69 ± 1.49</td>
<td>63.4 ± 30.2</td>
</tr>
</tbody>
</table>

Activity was assessed using various concentrations of R3 peptide (25-1000 \(\mu\text{M}\)), 250 \(\mu\text{M}\)
AdoMet, 4 \(\mu\text{M}\) His-PRMT1 (WT or mutant), 10 nM MTAN, 10 \(\mu\text{M}\) MnSO\(_4\), and 50 mM
NaPO\(_4\) buffer pH 7.1 at 37 °C. Reactions were performed at least in duplicate, and
initial reaction rates were used to assess activity.
Fig. 6-2. M155A-PRMT1 methylates R3. Various concentrations of R3 peptide [25-1000 µM] were used to initiate reactions containing 4 µM M155A-PRMT1, 250 µM AdoMet, 10 nM MTA nucleosidase, 10 µM MnSO₄, and 50 mM NaPO₄ buffer pH 7.1 at 37 °C. Initial rates were used to plot the Michaelis-Menten curve.

Next, we analyzed the products generated from an extended reaction between M155A-PRMT1 and R3 to determine if mutating M155 to a smaller alanine residue converted PRMT1 into a Type II PRMT. Using a reverse phase-HPLC method that provides baseline separation between MMA, ADMA, and SDMA [23], we show that similar to wild type (Fig. 6-3, A), M155A-PRMT1 catalyzed the formation of only MMA and ADMA (Fig. 6-3, B). Interestingly, an additional peak whose identity is unknown was found to incorporate radioactively labeled AdoMet and eluted at approximately 39 minutes (between MMA and ADMA) in the M155A-PRMT1 sample. All in all, removing the steric bulk afforded by Met155 in the PRMT1 active site was not enough to transform the Type I PRMT into a Type II PRMT that is capable of catalyzing the formation of SDMA.
Fig. 6-3. M155A-PRMT1 catalyzes the formation of MMA and ADMA. Acid hydrolyzed amino acids from reactions that had been initiated with 200 µM R3 and catalyzed by wild type in (A), or M155A-PRMT1 in (B), were derivatized using the AccQFluor reagent. HPLC [23] was used to separate the methylated products based on their hydrophobic properties (solid lines). 3H-AdoMet was used as a tracer to verify the presence of the methylated species in each reaction (dashed lines). MMA, ADMA, and SDMA standard amino acids were used to identify the methylated species in each sample. MMA and ADMA are labeled appropriately according to the retention times of the standards. SDMA, which had a later retention time, was not generated.
Because Met155 was not responsible for the strict formation of ADMA versus SDMA, we next examined the role of another active site methionine residue (Met48) due to its proximity to the substrate arginine of the R3 peptide in the PRMT1 crystal structure (Fig. 6-1) [18]. To determine if the bulk of this methionine is responsible for aiding in the strict formation of ADMA in the PRMT1 active site, we mutated Met48 to a smaller amino acid (alanine). Unlike the M155A-PRMT1 mutant, M48A-PRMT1 demonstrated extremely low activity with the R3 peptide (Table 6-1 and Fig. 6-4, A). When an extended reaction with M48A-PRMT1 and R3 was analyzed by HPLC, methylated arginine species may have been formed but levels were only slightly above background when using radioactivity (Fig. 6-4, A). M48A-PRMT1 was not capable of methyllating two additional single arginine-containing fibrillarin-based peptides that wild type PRMT1 is capable of methyllating (\textit{acyl}KGGFGGRGGFGGGKW and its monomethylated counterpart \textit{acyl}KGGFGGR\textit{methyl}GGFGGGKW) (data not shown). Thus, mutating Met48 to an alanine obliterated PRMT1-activity altogether and eliminated the catalysis of both arginine and monomethylarginine-containing substrates.

Based on these findings, we proposed that the length of Met48 is critical to catalysis. Perhaps mutating Met48 was too drastic of a change in length. Therefore, we mutated Met48 to a leucine, a residue that was in between alanine and methionine in length. Interestingly, M48L-PRMT1 displayed saturation kinetics with R3 (data not shown). The catalytic efficiency of M48L-PRMT1 (1670 ± 255 M$^{-1}$s$^{-1}$) was approximately 40% of wild type (Table 6-1) indicating that the length of this active site residue is critical for catalysis. Further HPLC analysis of the products generated from an extended reaction with M48L-PRMT1 and R3 indicated that higher levels of MMA and
ADMA had been generated (Fig. 6-4, B) compared to the reaction with M48A-PRMT1. The shape of the ADMA peak was not as defined as in other samples due to sample overloading.

Fig. 6-4. M48A/L-PRMT1 mutants catalyze the formation of MMA and ADMA. Acid hydrolyzed amino acids from reactions that had been initiated with 200 µM R3 and catalyzed by M48A in (A) or M48L-PRMT1 in (B) were derivatized using the AccQFluor reagent. HPLC [23] was used to separate the methylated products based on their hydrophobic properties (solid lines). ³H-AdoMet was used as a tracer to verify the presence of the methylated species in each reaction (dashed lines). Standard amino acids were used to identify methylarginine products in the sample. MMA and ADMA are labeled appropriately according to the retention times of the standards. SDMA, which had a retention time of approximately 51 minutes, was not generated in (A).
Like M48A-PRMT1, M48L-PRMT1 was not capable of methylating two additional single arginine-containing fibrillarin-based peptides (acylKGGFGGRGGFGGGKW and its monomethylated counterpart acylKGGFGGR_methylGGFGGGKW) (data not shown). It is unclear why the Met 48 mutant proteins were unable to methylate these peptides. Based on these results, however, M48L-PRMT1 may not be able to methylate arginine residues located in the center of peptides.

**Peptide substrate and cofactor binding**

To further investigate the role of Met48, we measured the dissociation constants of WT, M48A, and M48L-PRMT1 for each substrate (R3 peptide and AdoMet). The PRMT1 crystal structure depicts two tryptophans at positions 145 and 294 which lie in the catalytic region near the substrate arginine residue opposite the AdoMet binding region [18]. The intrinsic fluorescent-properties of PRMT1 were exploited in fluorescence-quenching assays to determine the dissociation constants for the R3 peptide with WT and mutant PRMT1. Stern-Volmer plots [24] were used to analyze the quenching data. A representative Stern-Volmer plot for WT-PRMT1 with R3 is shown in Fig. 6-5, A. These plots revealed that WT and M48A/L-PRMT1 had nearly the same affinity for the R3 peptide as judged by the similar K_d values (Table 6-2). Lehrer’s modified Stern-Volmer plot [25] was used to interpret the fluorescence-quenching data from the titrations of WT and M48A/L-PRMT1 with AdoMet. A representative modified Stern-Volmer plot for WT-PRMT1 with AdoMet is shown in Fig. 6-5, B. The results in Table 6-2 indicate that the diminished activity of M48A-PRMT1 or M48L-PRMT1 is not
due to differences in AdoMet binding. The affinity of WT-PRMT1 and both mutants
for AdoMet was nearly the same. Importantly, results obtained from isothermal titration
calorimetry (ITC) were consistent with the dissociation constant values obtained using
intrinsic fluorescence. When WT-PRMT1 was titrated with AdoMet, a value of $4.2 \pm 1.5$
$\mu$M was obtained using intrinsic fluorescence (Table 6-2) versus $2.5 \mu$M from ITC (data
not shown).

Fig. 6-5. Stern Volmer and modified Stern Volmer plots. In (A), a Stern Volmer plot
is shown for the titration of WT-PRMT1 with R3. The slope of the line is equivalent to
the dissociation constant. In (B), a modified Stern Volmer plot is shown for the titration
of WT-PRMT1 with AdoMet. The data was fit to a line where the $y_{\text{intercept}}=1/\alpha$, the
slope=$1/\alpha \cdot K_Q$, and the $K_Q=1/K_D$. 
Table 6-2  Dissociation constants for mutant and WT-PRMT1 for AdoMet and R3

<table>
<thead>
<tr>
<th>PRMT1</th>
<th>$K_d$, peptide (nM)</th>
<th>Fluorescent quenching (%)</th>
<th>$K_d$, AdoMet (µM)</th>
<th>Fluorescent quenching (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>36 ± 11</td>
<td>36 ± 19</td>
<td>4.2 ± 1.5</td>
<td>17 ± 0.13</td>
</tr>
<tr>
<td>M48A</td>
<td>47 ± 8.2</td>
<td>47 ± 2.0</td>
<td>7.1 ± 1.8</td>
<td>15 ± 0.17</td>
</tr>
<tr>
<td>M48L</td>
<td>49 ± 18</td>
<td>39 ± 7.8</td>
<td>7.1 ± 0.94</td>
<td>29 ± 4.2</td>
</tr>
</tbody>
</table>

Dissociation constants were obtained using intrinsic fluorescence quenching assays (performed at least in duplicate) as described in the methods section.

Active site residues affect PRMT1 substrate processing

Because the active site methionine mutations did not govern the strict formation of ADMA versus SDMA, the roles of several additional active site residues that were conserved among Type I PRMTs but not among Type II PRMTs were evaluated. The following mutations to the PRMT1 sequence were verified by DNA sequencing: Y148S, S154A, W145A, and M146A-PRMT1. Proteins were overexpressed and purified to homogeneity before being assayed with the R3 peptide. All mutants demonstrated saturation kinetics with R3 (data not shown). However, each mutant PRMT1 protein resulted in a substantial decrease in catalytic activity ($k_{cat}/K_{m, app}$) when compared to wild type PRMT1 except for S154A-PRMT1 (Table 6-3).

Next, we analyzed the peptide product generated from extended reactions with the four mutant PRMT1 proteins and R3. Mutant proteins did not generate a detectable amount of SDMA but were able to catalyze monomethylation and asymmetric dimethylation of the R3 peptide (data not shown). These results are summarized in Table 6-3. Thus, the strict formation of ADMA versus SDMA in the PRMT1 active site is not governed by Tyr148, Ser154, Trp145, or Met146.
<table>
<thead>
<tr>
<th>PRMT1</th>
<th>Catalytic efficiency (M⁻¹s⁻¹)</th>
<th>MMA</th>
<th>ADMA</th>
<th>SDMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4458 ± 333 [22]</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>M155A</td>
<td>457 ± 121</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>M48A</td>
<td>13.19 ± 13.05</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>M48L</td>
<td>1668 ± 255</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Y148S</td>
<td>151 ± 39.5</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>S154A</td>
<td>4406 ± 1366</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>W145A</td>
<td>139 ± 20.8</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>M146A</td>
<td>463 ± 162</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>M48P</td>
<td>63.4 ± 30.2</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

Activity was assessed using various concentrations of R3 peptide (25-1000 µM), 250 µM AdoMet, 4 µM His-PRMT1 (WT or mutant), 10 nM MTAN, 10 µM MnSO₄, and 50 mM NaPO₄ buffer pH 7.1 at 37 °C. Reactions were performed at least in duplicate, and initial reaction rates were used to assess protein activity. HPLC analysis [23] and a radioactive tracer (³H-AdoMet) were used to confirm the presence or absence of MMA, ADMA, and SDMA formation for each PRMT1 protein reaction with the R3 peptide.

**DISCUSSION**

**PRMT1 mutants catalyze the formation of MMA and ADMA at varying efficiencies**

The purpose of this study was to identify a residue that was responsible for controlling the strict formation of ADMA (versus SDMA) in the active site of the Type I PRMT, PRMT1. First, Met 155 was probed because this residue had previously been hypothesized [18] to determine ADMA formation based on its steric bulk. Due to the proximity of this residue to the substrate arginine (Fig. 6-1) and the fact that it was conserved among Type I PRMTs but not Type II PRMTs, this residue was mutated to a smaller amino acid residue to see if SDMA could be generated. M155A-PRMT1 did not form SDMA (Fig. 6-2) and displayed a decrease in catalytic activity when compared to
WT-PRMT1 (Table 6-1). Thus, reducing the size of this methionine to an alanine at this active site location did not allow for the free rotation about the terminal C-N bond of the arginine residues in R3 and the formation of SDMA. Met 48, the methionine on the other side of the substrate arginine (Fig. 6-1), was shown by HPLC analysis not to alter Type I versus Type II product formation either (Fig. 6-4). In fact, all mutations made to the PRMT1 active site (M155A, M48A, M48L, M146A, W145A, S154A, and Y148S) did not result in a detectable amount of SDMA but did result in the formation of both MMA and ADMA (Table 6-3). When Met 48 was mutated to a proline, the corresponding residue found in the Type II PRMT5 active site, activity dropped dramatically ($k_{cat}/K_m = 63.4 \pm 30.2 \text{ M}^{-1}\text{s}^{-1}$) (Tables 6-1 and 6-3). No detectable amount of SDMA was detected by HPLC after an extended reaction (data not shown). Ultimately, these results indicate that transforming a Type I PRMT into a Type II PRMT is more complex than previously thought and may extend beyond the composition of the active site. Alternatively, the mutation of other residues or a combination of active site residues (such as an M48L/M155A-PRMT1 double mutant) could govern ADMA versus SDMA formation.

PRMT1 mutants utilized R3 as a peptide substrate but did so at varying efficiencies. All PRMT1 mutations resulted in a substantial decrease in catalytic activity ($k_{cat}/K_{m, \text{app}}$) when compared to wild type PRMT1 except for S154A-PRMT1 (Table 6-3). Interestingly, M48A-PRMT1 had very little to no activity with the R3 peptide, the most kinetically competent fibrillarin-based peptide substrate suggesting that this methionine is critical to catalysis (Table 6-1). Further kinetic analysis of Met 48 with the M48L-
PRMT1 mutant and R3 (Table 6-1) proved that the length of this amino acid residue is important to catalysis.

Surprisingly, the M48L-PRMT1 mutant methylated the R3 peptide with nearly half the activity of WT-PRMT1 but would not methylate two single arginine-containing peptides, acylKGGFGGRGGFGGKW and its monomethylated counterpart acylKGGFGGR_{methyl}GGFGGKW (data not shown). This inactivity may be due to the central position of the arginine in the substrate. Perhaps M48L-PRMT1 methylated the R3 peptide on the two terminal arginines in bold \((\text{acylGG} \text{RGGFGGRGGFGG} \text{RGGFG})\) but was unable to access the central arginine residue. MS/MS analysis of the R3 peptide product from this reaction will reveal if this hypothesis is true.

Although it is unclear how Met 48 affects PRMT1 catalysis, this residue may help position AdoMet or the substrate arginine so that a more direct $S_N2$ attack of the methyl group of AdoMet can be made (Fig. 6-6). The active site appears to ‘open up’ more when Met 48 is replaced with a leucine (Fig. 6.6). Because the M48L-PRMT1 mutant was unable to methylate two central single arginine-containing peptides but was able to methylate R3, Met 48 may play a significant role in substrate specificity. Interestingly, M48L-PRMT1 was also capable of automethylation (described further below). Further examination of this residue may provide crucial mechanistic details because of its close proximity to the substrate arginine.

**Automethylation of PRMT1**

M48L-PRMT1 was capable of automethylating itself when incubated with tritiated AdoMet (data not shown). This phenomenon was not observed for the wild type
Fig. 6-6. Methionine 48 positions PRMT1 substrates for catalysis. AdoMet (purple) was modeled into the PRMT1 active site (PDB ID, 1OR8). The AdoMet methyl group (carbon shown in red) is positioned for a direct $S_N2$ attack from the R3 peptide substrate arginine (yellow). The wild type active site (M48 in gray) is depicted in the top panel while the M48L mutant is depicted below (gray).

enzyme under the identical reaction conditions (data not shown). PRMT6 and PRMT8 have been shown to automethylate themselves [5, 26]. PRMT6 was found to be mono- and dimethylated at certain arginine residues [5]. PRMT8 automethylates two arginine residues (monomethylarginine 58 and dimethylarginine 73) on its N-terminal tail, an ability that is thought to regulate and possibly inhibit the methylation of other substrates [26]. Although PRMT1 and PRMT8 are highly identical in sequence, the methylated
arginine residues in PRMT8 do not align with arginines in PRMT1. In the same study, Clarke and coworkers detected that WT-PRMT1 had also underwent automethylation to a lesser degree [26]. Under our reaction conditions, however, we could not detect WT-PRMT1 automethylation. It will be interesting to see where M48L-PRMT1 has been methylated.

Conclusion

Our results indicate that transforming a Type I PRMT into a Type II PRMT is more complex than previously thought and may extend beyond the composition of the active site. Importantly, our study identified PRMT1 active site residues that are critical to catalysis. The mutation of Met 48 to an alanine in the PRMT1 active site rendered the enzyme virtually inactive. In particular, the length of Met 48 is critical to PRMT1 catalysis. Further examination of this residue may provide crucial mechanistic details.

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CHAPTER 7
ONGOING PROJECTS

INTRODUCTION

This chapter includes experimental results from several projects that have yet to be completed. In the first section, we found that the amino acid sequence of certain peptide substrates directly affects the extent that a particular substrate is mono- and dimethylated. We also found that two ‘RGA’-containing fibrillarin-based peptides demonstrated substrate inhibition at high substrate concentrations when incubated with PRMT1. This indicates that substrate sequence can regulate PRMT1 activity at a higher level by altering the amount of dimethylation that can occur physiologically. Together, these data show that the sequence around the substrate arginyln group affects how the substrate arginine is being modified and indicate that although many protein sequences may be PRMT1 substrates, the identity of the sequence and its abundance in the cell may dictate how the substrate arginine residue is mono- or dimethylated in vivo.

Next, I examined the importance of interactions between the substrate and the conserved THW loop among PRMTs by mutating two residues (H293 and W294 to an alanine on PRMT1) and assessing the activity of each with several substrates. I found that both residues were essential for PRMT1 catalysis. In another section, I investigated how the AdoMet/AdoHcy ratio present in healthy and diseased individuals affected PRMT1 activity. The AdoMet/AdoHcy ratio is higher in healthier individuals (~4-6) [1] and lower in patients with Type II diabetes [2], Alzheimer’s disease [3], and cystic fibrosis [4] (~1.4-3.5). Interestingly, the lower the AdoMet/AdoHcy ratio, the more
severe the disease [2]. As expected, I found that as the concentration of AdoHcy increased (meaning the AdoMet/AdoHcy ratio decreased), PRMT1 activity decreased. Because PRMT1 performs over 85% of all protein arginine methylation in the cell, a decrease in PRMT1 activity due to an alteration in the AdoMet/AdoHcy ratio could cause a decrease in total protein arginine methylation. This inhibition could have numerous biological ramifications that have yet to be fully explored. Finally in a related experiment, I determined the $K_i$ for a fibrillarin-based peptide containing ADMA.

RESULTS AND DISCUSSION

Effect of amino acid sequence context on activity and end-products

In Chapter 4, we identified that PRMT1 was capable of methylating a variety of peptide sequences that extended beyond the ‘RGG’ paradigm using a small fibrillarin-based peptide library. We also observed that methylation was contextual with the ‘RSG’ sequence. The ‘RSG’ sequence presented within the fibrillarin context was not a PRMT1 substrate while the same sequence presented within the eIF4A1 context was a PRMT1 substrate. This suggested that amino acids in one position affect whether or not amino acids in a second position are favored. To explore the effects of substrate sequence on PRMT1 activity further, we examined the end products generated with time from reactions that had been initiated with the WT-eIF4A1, eIF4A1-S, or eIF4A1-Y peptide. Because the eIF4A1 peptides contain three arginine residues, we employed tandem mass spectrometry first to prove that R7 (bolded in $\text{AcYIHRIGRGGR}$, $\text{AcYIHRIGRYGR}$, and $\text{AcYIHRIGRSGR}$, respectively) was the only modified arginine after incubating with PRMT1 and AdoMet (Fig. 4-6).
The MS analysis of the eIF4A1 peptides revealed that the proportion of the mono- and dimethylated arginine products observed was different in the eIF4A1 peptides (Fig. 7-1). The ‘RGG’ containing WT-eIF4A1 peptide was almost fully dimethylated (97.3%) while the eIF4A1-peptide containing the ‘RSG’ motif was mostly not modified after 105 minutes. Only 7.1% of the eIF4A1-S had been monomethylated after 105 minutes (Fig. 7-1). The eIF4A1-peptide containing the ‘RYG’ motif only underwent monomethylation (3.7%) after 105 minutes. After an extended reaction with PRMT1, the ‘RGG’ containing WT-eIF4A1 peptide was mostly dimethylated while the ‘RSG’ and ‘RYG’ containing eIF4A1 peptides were mainly monomethylated. PRMT1 and AdoMet were shown to be viable during the extended reaction time period (Fig. 7-2) indicating that the reaction conditions were such that the reaction should have gone to completion (i.e., peptides should have been fully dimethylated instead of partially monomethylated). Perhaps the reactions with eIF4A1-Y and eIF4A1-S peptides did not go to completion due to substrate inhibition (described more fully in the next section). These data suggest that sequence differences around the substrate arginyl group in peptide substrates may affect whether mono- or dimethylation is the major end-product and demonstrate that the amino acid sequence of a PRMT1 substrate may dictate how an arginine is modified. Thus, many sequences may be PRMT1 substrates, but the identity of the sequence may dictate how the substrate arginine residue is mono- or dimethylated \textit{in vivo}. 
Fig. 7-1. Effect of sequence on methylated product distribution. Reactions were prepared as described in Fig. 4-2 and were initiated with WT-eIF4A1 in (A), eIF4A1-S in (B), and eIF4A1-Y in (C). Mass spectrometry was used to identify the parent (gray lines), monomethylated (solid lines), and dimethylated (dashed lines) peptide products.
Fig. 7-2. PRMT1 and AdoMet are viable during extended reactions. Continuous spectrophotometric assays with 4 μM PRMT1, 250 μM AdoMet, and coupling enzymes in 50 mM sodium phosphate buffer (pH 7.1) that had equilibrated to 37 °C were initiated with 300 μM eIF4A1-Y peptide. At 105 minutes (A) and 405 minutes (B), reactions were spiked with 200 μM R3 peptide. When comparing this methylation rate to the rate from a reaction initiated with 200 μM R3 at t=0 (with no eIF4A1 peptide), PRMT1 was 92% active at 105 minutes and 89% active at 405 minutes.

**Substrate inhibition**

In another experiment, both the RGA-peptide (KGGFGGRGAFGGKW) and RGA-CH₃ peptide (KGGFGGRₚₘₐₓₚₖₚₚₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖ₆

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**Diagram**: 
- **A**: eIF4a1-Y + R3, 100 min incubation at 37 °C. 
- **B**: eIF4a1-Y + R3, 400 min incubation at 37 °C.
substrate inhibition. If dimethylation is required to elicit the biological response, then the biological response will not happen.

Fig. 7-3. Peptides caused substrate inhibition at high substrate concentrations. Reactions containing 4 μM PRMT1, 0.2 μM adenine deaminase, 10 nM AdoHcy nucleosidase, 1050 μM MnSO₄, and 250 μM SAM in 100 mM sodium phosphate buffer pH 7.1 were initiated with various concentrations of RGA-peptide (KGGFGGGRGAFGGKW). Higher peptide concentrations demonstrated substrate inhibition, an observation that may be found to be important biologically.

**THW loop mutations alter PRMT1 activity**

In Chapter 4, new substrate motifs were identified that went beyond the ‘RGG’ paradigm. Two of these motifs (‘RYG’ and ‘RFG’) surprisingly had large, ring-containing amino acids directly C-terminal to the substrate arginine residue. How could such large amino acid residues adjacent to the substrate arginine facilitate methylation when several smaller amino acids such as alanine were not tolerated in the X¹ position? Perhaps PRMT1 could tolerate phenylalanine and tyrosine in this position because they were ring containing and could make pi-pi stacking interactions with PRMT1. When
looking at the PRMT1 crystal structure [5] and sequence alignments among the PRMT family, a conserved THW loop positioned near the substrate X\(^1\) position could potentially make pi-pi stacking interactions the substrate. In order to test this hypothesis and further understand the role of the active site THW loop residues H293 and W294, these residues were mutated to an alanine.

H293A and W294A-PRMT1 proteins were purified and assessed for activity with the R3 peptide (GG\text{RGGFGGRGGFGRGGFG\text{R}}). H293A-PRMT1 utilized R3 (k\(_{\text{cat}}\) = 0.27 ± 0.11 min\(^{-1}\)) while W294A-PRMT1 did not (data not shown). W294A-PRMT1 was not active with either an ‘RYG’ or an ‘RFG’-containing fibrillarin-based peptide (data not shown). Further HPLC analysis of this protein revealed that AdoMet was still able to bind suggesting that the AdoMet binding pocket had not been disrupted by the mutation and that the protein was able to fold properly (data not shown).

The activity of H293A-PRMT1 was analyzed next with ‘RYG’ and ‘RFG’-containing peptides. Preliminary data suggests that H293A-PRMT1 was not capable of methylating the peptide housing the ‘RFG’ sequence very well as demonstrated by a low V\(_{\text{max}}\) (Fig. 7-4, A). These data suggest that the pi-pi interactions between enzyme and substrate at this location have been disturbed; however, further investigation is needed. Interestingly, H293A-PRMT1 was able to methylate the tyrosine-containing peptide (k\(_{\text{cat}}/K_m\) = 10.2 ± 8.01 M\(^{-1}\)s\(^{-1}\)) (Fig. 7-4, B). H293A-PRMT1 was also able to methylate the ‘RYG’-containing eIF4A1-Y peptide (YIHRIG\text{RYGR}) at a catalytic efficiency of 27 ± 13 M\(^{-1}\)s\(^{-1}\) (data not shown). Although the k\(_{\text{cat}}/K_m\) values were extremely low, the rate of methylation increased as the concentration of peptide increased suggesting that pi-pi stacking interactions are not by themselves responsible for PRMT1 activity with ‘RYG’-
containing peptides. Perhaps the ability of tyrosine to hydrogen bond to the enzyme via its hydroxyl group facilitates methylation.

Our main objective to determine if pi-pi interactions are important in substrate selection and catalysis remains somewhat elusive because I was unable to perform additional follow-up studies. Despite this, I was able to show that each peptide tested with either the H293A or W294A-PRMT1 mutant proteins was not methylated very efficiently. These preliminary results suggest that histidine and tryptophan from the conserved THW loop are necessary for PRMT catalysis.

Fig. 7-4. Mutations in the THW loop alter PRMT1 activity. Reactions containing 4 μM H293A-PRMT1, 0.2 μM adenine deaminase, 10 nM AdoHcy nucleosidase, 1050 μM MnSO₄, and 250 μM SAM in 100 mM sodium phosphate buffer pH 7.1 were initiated with various concentrations of ‘RFG’-peptide (KGGFGGRFGFGGKW) in (A) or ‘RYG’-peptide (KGGFGGTRYGFGGKW) in (B). Although the reactions were performed in duplicate, the data remained scattered in (A).
**AdoMet/AdoHcy ratio affects PRMT1 activity in vitro**

A measurement of the AdoMet/AdoHcy ratio reflects the wellness of an individual [1-4]. We were interested in determining if the biologically significant ratios that we chose to classify as healthy (4.0), diseased (2.0), and extremely sick/near death (1.0) had any effect on PRMT1 activity. We found that as the amount of AdoHcy present in the methylation reaction increased, PRMT1 activity decreased accordingly when initiating the reaction with an unmodified fibrillarin-based peptide substrate (Fig. 7-5, A) or a monomethylated fibrillarin-based peptide substrate (Fig. 7-5, B). In both (A) and (B), addition of any AdoHcy to the reaction reduced methyltransferase activity as expected. Despite the large standard deviations observed in the reactions containing peptide only, PRMT1 was clearly able to methylate peptides in these reactions more easily than in the reactions containing AdoHcy. Although it is more apparent with the monomethylated substrate (Fig. 7-5, B), increasing the amount of AdoHcy diminished PRMT1 activity in a dose dependent manner. Interestingly, both peptides were methylated by PRMT1 in AdoMet/AdoHcy conditions approaching death. Based on these preliminary results, the activity of PRMT1 is reduced by biologically relevant AdoHcy concentrations such as those found in healthy individuals. This implies that future *in vitro* PRMT activity studies may obtain a more biologically relevant measurement of kinetic parameters if a healthy AdoMet/AdoHcy ratio was included in the study.
Fig. 7-5. PRMT1 activity is reduced during disease states. PRMT1 activity was probed under various AdoMet/AdoHcy ratios using the JMH1W peptide (KGGFGGRRGGFGGKW) in (A) and the JMH1W-CH₃ peptide (KGGFGGRR₃methyGGFGGKW) in (B). Reactions containing 4 μM PRMT1, 0.3 μM [H₃]-AdoMet (specific activity of 83 μCi/μmol), 50 μM AdoMet (S-isomer only) in 100 mM sodium phosphate buffer pH 7.1 were initiated with peptide or a mixture of peptide and AdoHcy. Methylation was monitored discontinuously with time using a P81 membrane assay (as described in Chapter 3). Reactions initiated with peptide only (●) exhibited the highest activity. Reactions initiated with a mixture of peptide and AdoHcy decreased PRMT1 activity according to the amount of AdoHcy present. The data corresponding to the following AdoMet/AdoHcy ratios: 4 to 1, 2 to 1, and 1 to 1 represent individuals that are healthy, (○); diseased, (▲); and near death, (open triangles), respectively. Control reactions (x) containing only PRMT1 and AdoMet displayed little activity as expected. Reactions were performed in duplicate, and the standard deviations are shown.

Obtaining the Kᵢ for a fibrillarin-based peptide containing ADMA

In order to determine which type of mechanism PRMT1 performs (random sequential or ordered sequential); I looked at product inhibition profiles. First, I examined the JMH1W-ADMA peptide inhibitor (KGGFGGR₃methyGGFGGKW) kinetically because AdoHcy could not be examined using the continuous assay (see
Chapter 3). Each reaction containing 4 µM His-PRMT1 and 250 µM AdoMet in 50 mM sodium phosphate buffer pH 7.1 equilibrated at 37 ºC for 10 minutes before being initiated with various amounts of JMH1W-CH₃ peptide or a mixture of JMH1W-CH₃ and JMH1W-ADMA inhibitor peptide. For calculation of kinetic constants, the concentration of peptide was varied at several fixed concentrations of JMH1W-ADMA inhibitor peptide (0 µM - 150 µM). The average initial velocity representing no more than 10% of product formation was obtained from two measurements for each pair of substrate concentrations and was used to construct Michaelis-Menten plots (Fig. 7-6, A). The data were graphed as Lineweaver-Burk plots (Fig. 7-6, B) and linear regression was employed to construct slope and intercept replots (data not shown). These replots were used to calculate the value of the inhibition constant, K_{ii}. A larger K_{ii} of 462 µM was determined for the JMH1W-ADMA inhibitor peptide. Because this value is larger than both the K_m for JMH1W and JMH1W-CH₃, it is not a potent PRMT1 inhibitor. Although the Lineweaver-Burk plot rules out uncompetitive inhibition, we could not conclude whether the peptide inhibitor was a competitive or noncompetitive inhibitor without performing additional product inhibition profiles. Recently, the mechanism of PRMT1 was published in September 2008, so any further studies were aborted. Using peptides derived from the in vivo substrate histone H4, Thompson and coworkers concluded that PRMT1 performs a random sequential mechanism [6].
Fig. 7-6. (A) Velocity versus μM JMH1W-CH₃ plot. The following velocities were plotted against μM JMH1W-CH₃ substrate concentration to obtain Michaelis Menton parameters: 0 μM ADMA inhibitor (●), 25 μM ADMA inhibitor (♦), 50 μM ADMA inhibitor (crosses), 100 μM ADMA inhibitor (▲), and 150 μM ADMA inhibitor (■). (B) Lineweaver-Burke plot. The values for 1/velocity (μM CH₃/min) at different ADMA inhibitor concentrations were plotted against 1/substrate (μM JMH1W-CH₃) to obtain the slope and y-intercept values of each line at 0 μM ADMA inhibitor (●), 25 μM ADMA inhibitor (♦), 50 μM ADMA inhibitor (crosses), 100 μM ADMA inhibitor (▲), and 150 μM ADMA inhibitor (■).

Conclusion

The purpose of this chapter was to introduce several projects that were started and have yet to be fully developed. Importantly, I have shown preliminary data that suggests that sequence differences around peptide substrate arginyl groups may affect whether mono- or dimethylation is the major end-product. Although many sequences may be PRMT1 substrates, the identity of the sequence may dictate how the substrate arginine residue is mono- or dimethylated in vivo. I also found that certain substrates cause substrate inhibition at high concentrations, a factor that may have played a role in the inability of PRMT1 to fully methylate the eIF4A1-Y and eIF4A1-S peptides (Fig. 7-1).
Next, I showed that the histidine and tryptophan from the conserved THW loop are necessary for PRMT catalysis and that this made it difficult to probe specific pi-pi interactions that exist at this location between enzyme and substrate. Finally, I showed that PRMT1 activity decreased when AdoHcy or a peptide housing ADMA were added. Interestingly, AdoHcy was a more potent inhibitor than the ADMA-containing peptide because the addition of a biologically relevant concentration of it regulated PRMT1 activity. Altogether, these studies provide good starting points for the development of future interesting projects.

REFERENCES


Protein arginine methyltransferases (PRMTs) play an important role in a diverse set of biological processes. PRMTs methylate a variety of protein substrates including histones, RNA-binding proteins, and various enzymes. While PRMT biology has been in the forefront, much of the biochemistry for this class of enzymes has remained a mystery. The purpose of my study was to develop a biochemical understanding for how PRMT1 substrates are recognized and modified. I chose to characterize PRMT1 because it methylates over 85% of all protein arginine residues \textit{in vivo} and has been implicated in cardiovascular disease and cancer. Using a continuous spectrophotometric assay for AdoMet-dependent methyltransferases that I helped develop, we first investigated the substrate specificity of PRMT1. Next, in order to understand why most methylated proteins are found in the fully dimethylated state \textit{in vivo}, we analyzed the mechanism of asymmetric dimethylarginine (ADMA) formation of single and multiple arginine-containing peptide substrates. Finally, we probed a variety of amino acid residues in the PRMT1 active site to understand their role in catalysis, specifically their ability to control ADMA formation.

Defining how PRMT1 works and what the preferred substrates are is pivotal for understanding the role that PRMT1 plays in biology. A major milestone of my research was the development of the continuous spectrophotometric assay for AdoMet-dependent methyltransferases. Instead of assaying PRMT1 with radioactively labeled $[\text{H}^3]$-AdoMet, I helped develop a continuous assay that enabled me to quickly and inexpensively assess
PRMT1 activity with a variety of peptide substrates. In the continuous assay, AdoHcy, the transmethylation product of AdoMet-dependent methyltransferases, is hydrolyzed to S-ribosylhomocysteine and adenine by recombinant AdoHcy nucleosidase (SAHN/MTAN). Adenine generated from AdoHcy is further hydrolyzed to hypoxanthine and ammonia by recombinant adenine deaminase. This deamination is associated with a decrease in absorbance at 265 nm that can be monitored continuously with time.

The continuous spectrophotometric assay is robust, inexpensive, and amenable to high throughput when evaluating methyltransferases that have been targeted for drug discovery. One main advantage of this assay is the destruction of AdoHcy by AdoHcy nucleosidase, which alleviates AdoHcy product feedback inhibition of S-adenosylmethionine-dependent methyltransferases. In the absence of enzyme inhibition, more accurate measurements of kinetic parameters can be made. This method may also be used to assay enzymes that produce AdoHcy, 5’-methylthioadenosine, or compounds that can be cleaved by AdoHcy nucleosidase.

Using the continuous assay, we made the important discovery that PRMT1 selectively recognizes and methylates a set of amino acid sequences in substrates that extend beyond the ‘RGG’ paradigm. Prior to this study, PRMT1 was thought to methylate substrates only within glycine arginine rich motifs such as ‘RGG’ and ‘RXR’ with one exception, histone H4. Using a focused peptide library based on a sequence derived from the in vivo substrate fibrillarin, we observed that PRMT1 methylated eleven substrates that had amino acid residues other than glycine in the ‘RX1’ and ‘RX1X2’
positions (Fig. 8-1). Notably, our results illustrate that the two residues on the N-terminal side of the modification site are important and need not both be glycine.

Fig. 8-1. New model for PRMT1 substrate specificity. Substrate profiling of PRMT1 revealed that PRMT1 methylates a broad range of substrate motifs when compared to the old paradigm.

This study also demonstrated the importance of amino acid sequence in PRMT1 protein substrates. Methylation of two eukaryotic initiation factor 4A protein isoforms, eIF4A1 and the similar eIF4A3, could be affected using single site mutations adjacent to the modification site. After comparing eIF4A peptides and proteins, we concluded that recognition of protein substrates was not governed by amino acid sequence alone. Other factors such as global protein-protein surface interactions may dictate protein substrate recognition. Future crystallographic studies that examine specific PRMT1-protein substrate interactions will help identify important surface interactions.

Before examining the substrate specificity of PRMT1, limited studies and selective tools promoted the idea that PRMT1 substrates conform to an ‘RGG’ sequence with few rare exceptions such as histone H4. Our study clearly showed that PRMT1 is
capable of methylating sequences that go beyond the ‘RGG’ paradigm and suggests that residues N-terminal to the modification site are also important in substrate recognition. This suggests that the methyl arginine proteome may be larger and more diverse than previously thought. Hopefully, investigators that previously discounted arginine methylation as a regulatory factor in their system because the arginine of interest was not in a glycine arginine rich motif will now give this modification more thought.

PRMT1 has recently emerged as a potential new target for the development of a novel therapeutic for heart disease because it generates the majority of cellular asymmetrically dimethylated arginine (ADMA). Interestingly, most PRMT1 substrates are found dimethylated instead of monomethylated on multiple arginine residues within the same protein. In order to understand the biological prevalence of ADMA, we investigated why PRMT1 preferentially asymmetrically dimethylates protein arginine residues. Using kinetics and mass spectrometry analysis, we showed that PRMT1 preferentially methylated monomethylated peptide substrates versus their unmodified counterparts. PRMT1 did not processively methylate single arginine-containing peptide substrates. Interestingly, methylation of a multiple arginine-containing peptide was systematic (not random) and dissociative (not processive). Based on these results, the predominance of dimethylated protein substrates in vivo may be due to the preference that PRMT1 has for monomethylated substrates over unmodified substrates. It will be interesting to see if PRMT1 methylates multiple arginine-containing protein substrates in the same manner.

Finally, we investigated the role of several amino acid residues in the PRMT1 active site in order to understand their role in catalysis and if they specifically controlled
ADMA formation. We hypothesized that two active site methionine residues (Met155 and Met48) positioned near the reactive substrate arginine (Fig. 6-1) enable PRMT1 to selectively form ADMA instead of SDMA. M155A and M48A mutant proteins were characterized according to their ability to catalyze methylation of a multiple arginine-containing peptide based on the in vivo substrate fibrillarin. Removing the steric bulk afforded by each of these residues did not allow for the formation of SDMA. The mutation of Met48 to an alanine in the PRMT1 active site rendered the enzyme virtually inactive. When this residue was mutated to a leucine, activity was partially restored indicating that the length of Met48 is critical to PRMT1 catalysis. Most importantly, our results showed that removing the steric bulk afforded by either Met155 or Met48 in the active site of PRMT1 was not by itself sufficient to transform PRMT1 into a Type II PRMT. Therefore, something else about the active site of Type I PRMTs governs the specific formation of ADMA. Perhaps the way in which the monomethylated substrate is forced to orient itself in the active site due to strict geometric/structural constraints of the active site as a whole facilitates ADMA formation and prohibits SDMA formation.

Further analysis of the M48L-PRMT1 mutant revealed that it was capable of automethylation while wild type PRMT1 was not. This is not a unique, unheard of phenomenon because PRMT6 and PRMT8 are capable of automethylation. It will be interesting to identify which arginines of M48L-PRMT1 are methylated and how automethylation regulates PRMTs. Clearly, something about having a methionine residue at this active site location juxtaposed to the substrate arginine in wild type PRMT1 1) prohibits PRMT1 automethylation and 2) is essential to catalysis.
Overall, the research presented in this dissertation mainly focuses on the development of a paradigm for understanding how PRMT1 substrates are recognized and modified. First, we identified eleven new substrate motifs that are modified and go beyond the typically modified glycine arginine rich motifs (Fig. 8-1). Second, single and multiple arginine-containing peptide substrates are modified dissociatively, and PRMT1 prefers to methylate monomethylated substrates over their nonmethylated counterparts. We also showed that altering select active site residues one at a time did not convert PRMT1 into a Type II PRMT. Although Met 48 was shown to be critical to catalysis, its exact mechanistic role has yet to be determined.
September 18, 2008

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To Dr. W.B. Jakoby, Editor-in-Chief:

I am preparing my Ph.D. dissertation in the Department of Chemistry and Biochemistry at Utah State University. I hope to complete my degree in the fall of 2008. An article, K.M. Dorgan, W.L. Wooderchak, D. Wynn, E.L. Karschner, J.F. Alfaro, Y. Cui, Z.S. Zhou, J.M. Hevel, An enzyme-coupled continuous spectrophotometric assay for S-adenosylmethionine-dependent methyltransferases. Anal. Biochem. 350 (2006) 249-255, of which I am second author, and which appeared in your journal Analytical Biochemistry, reports an essential part of my Ph.D. research. I would like permission to reprint it as a chapter in my dissertation. (Reprinting the chapter may necessitate some revision.) Please note that USU sends dissertations to Bell & Howell Dissertation Services to be made available for reproduction. I will include an acknowledgment to the article on the first page of the chapter, as shown below. Copyright and permission information will be included in a special appendix. If you would like a different acknowledgment, please so indicate.

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To Dr. Richard M. Armstrong, Biochemistry Editor:

I am preparing my Ph.D. dissertation in the Department of Chemistry and Biochemistry at Utah State University. I hope to complete my degree in the fall of 2008. An article, W.L. Wooderchak, T. Zang, Z.S. Zhou, M. Acuña, S.M. Tahara, J.M. Hevel, Substrate profiling of PRMT1 reveals amino acid sequences that extend beyond the ‘RGG’ paradigm, Biochemistry 47 (2008) 9456-9466, of which I am first author, and which appeared in your journal Biochemistry, reports an essential part of my Ph.D. research. I would like permission to reprint it as a chapter in my dissertation. (Reprinting the chapter may necessitate some revision.) Please note that USU sends dissertations to Bell & Howell Dissertation Services to be made available for reproduction. I will include an acknowledgment to the article on the first page of the chapter, as shown below. Copyright and permission information will be included in a special appendix. If you would like a different acknowledgment, please so indicate.

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Publications


Research Presented
2008  W. Wooderchak, D. Chen, and J.M. Hevel. Understanding how Protein Arginine Methyltransferase 1 (PRMT1) substrates are Recognized and modified. American Society for Biochemistry and Molecular Biology (ASBMB) Post Translational Modification Meeting, Lake Tahoe, NV


2007  W. Wooderchak and J.M. Hevel. Substrates and mechanism of Protein Arginine Methyltransferase 1 (PRMT1). Graduate Student Research Symposium. Logan, UT

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<td>2006</td>
<td>W. Wooderchak and J.M. Hevel.</td>
<td>Investigation of PRMT1 Residues which Modulate Activity &amp; Control TypeI/TypeII Dimethylation. <em>American Society for Biochemistry and Molecular Biology (ASBMB) National Conference</em>. San Francisco, CA</td>
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**Awards and Honors**

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<th>Year</th>
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<tr>
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2007  D.A. Greenwood Memorial Award in Biochemistry
2006  Graduate Student Senate Travel Award
2006  Women and Gender Research Institute Travel Award
2004  Department of Chemistry and Biochemistry Fellowship

Western Illinois University
2003  Cecile A Christison Sterrett College Scholar in the College of Arts and Sciences Award
2003  National Dean’s List
2003  Suma Cum Laude
2003  Abraham Lincoln Laureate Award for Western Illinois University
2003  Hardin Chemistry Award
2003  Chemistry Departmental Scholar
2003  Natural Sciences and Mathematics Award
2003  Graduation Marshall
2002  Sheila and Paul Nollen Phi Kappa Phi Science Scholarship
2002  Currently Enrolled Student Award
2001-2002  ACS Organic Chemistry Student Award
2000-2001  Roy M. Salee Biology Award
2000-2001  Incoming Freshman Chemistry Award

Student and Professional Organizations
*American Chemical Society  *Phi Kappa Phi
*ASBMB  *Marching & Jazz Band
*Golden Key Club-volunteer coordinator  *Chemistry club-treasurer

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Cell biology:  mammalian cell culture, western blot, immunoprecipitation, microscopy
Microbiology:  microbial genetics, mutagenesis, qRT-PCR, microbial cell culture
Chemistry:  chemical synthesis, compound isolation, compound identification and analysis (NMR, IR, GC-MS, TLC, mass spectrometry)
Radioactivity:  trained to properly use radioactive substances