Development of Novel Methods and their Utilization in the Analysis of the Effect of the N-terminus of Human Protein Arginine Methyltransferase 1 Variant 1 on Enzymatic Activity, Protein-protein Interactions, and Substrate Specificity

Brenda Bienka Suh-Lailam
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Development of Novel Methods and their Utilization in the Analysis of the Effect of the N-terminus of Human Protein Arginine Methyltransferase 1 Variant 1 on Enzymatic Activity, Protein-protein Interactions, and Substrate Specificity

by

Brenda Bienka Suh-Lailam, Doctor of Philosophy

Utah State University, 2010

Major Professor: Dr. Joan M. Hevel
Department: Chemistry and Biochemistry

Protein arginine methyltransferases (PRMTs) are enzymes that catalyze the methylation of protein arginine residues, resulting in the formation of monomethylarginine, and/or asymmetric or symmetric dimethylarginines. Although understanding of the PRMTs has grown rapidly over the last few years, several challenges still remain in the PRMT field. Here, we describe the development of two techniques that will be very useful in investigating PRMT regulation, small molecule inhibition, oligomerization, protein-protein interaction, and substrate specificity, which will ultimately lead to the advancement of the PRMT field. Studies have shown that having an N-terminal tag can influence enzyme activity and substrate specificity. The first protocol tackles this problem by developing a way to obtain active untagged recombinant PRMT proteins. The second protocol describes a fast and efficient method for quantitative measurement of AdoMet-dependent methyltransferase activity with protein substrates. In addition to being very sensitive, this method decreases the processing time for the analysis of PRMT activity to a few minutes compared to weeks by traditional methods, and generates 3000-fold less radioactive waste. We then used these methods to investigate the effect of truncating the
The N-terminal (NT) of human PRMT1 variant 1 (hPRMT1-V1) has been studied for its influence on enzyme activity, protein-protein interactions, and substrate specificity. Our studies show that the NT of hPRMT1-V1 influences enzymatic activity and protein-protein interactions. In particular, methylation of a variety of protein substrates was more efficient when the first 10 amino acids of hPRMT1v1 were removed, suggesting an autoinhibitory role for this small section of the N-terminus. Likewise, as portions of the NT were removed, the altered hPRMT1v1 constructs were able to interact with more proteins. Overall, my studies suggest that the sequence and length of the NT of hPRMT1v1 is capable of enforcing specific protein interactions.
To my great grand mother Anna Bienka Awantu
ACKNOWLEDGMENTS

I would like to take this opportunity to thank my major advisor, Dr. Joanie Hevel, for her constant guidance, support, assistance, and encouragement throughout my studies at Utah State University. I appreciate her willingness and patience to help me grow as a scientist and teacher, and for taking the time to train me. I also thank her for making the lab a very lively, pleasant environment to work in. I also would like to thank my committee members, Dr. Brett Adams, Dr. Scott Ensign, Dr. Robert Brown, and Dr. Sean Johnson, for their advice and suggestions.

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<td>asymmetric dimethylarginine</td>
</tr>
<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>EDTA</td>
<td>[Ethylenedinitrilo]-tetraacetic acid</td>
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<td>H4</td>
<td>histone 4</td>
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<td>Hepes</td>
<td>N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<td>MMA</td>
<td>monomethyl arginine</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>MTAN</td>
<td>5'-methylthioadenosine nucleosidase</td>
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<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<tr>
<td>PRMT</td>
<td>protein arginine N-methyltransferase</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosyl homocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
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<tr>
<td>SDMA</td>
<td>symmetric dimethyl arginine</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>WT</td>
<td>wild type</td>
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CHAPTER 1
INTRODUCTION

Protein arginine methylation is one of over 200 posttranslational modifications that increase the structural diversity of proteins and enable organisms to expand upon their limited genome. Methylation of arginine residues in polypeptide chains affects protein-protein, protein-RNA, and protein-DNA interactions [1]. As a result of this effect on molecular interactions, protein arginine methylation is involved in the regulation of numerous cellular processes including signal transduction, transcription, DNA repair, mRNA splicing and nuclear/cytoplasmic shuttling [2]. Protein arginine methylation has also been implicated in certain disease conditions such as cancer, cardiovascular disease, spinal muscular atrophy, multiple sclerosis, and viral pathogenesis [2, 3]. In this dissertation, I focus on the family of enzymes that catalyze the transfer of methyl groups to arginine residues in target proteins, known as, protein arginine methyltransferases (PRMTs). We use human PRMT1 variant 1 (hPRMT1-V1) as our model. Here, we contribute to the advancement of the PRMT field by providing solutions to two challenges in the PRMT field: (i) how to obtain tagless PRMTs, and (ii) how to quantitatively measure methyl transfer to protein substrates quickly and efficiently. We also use the tools we developed above to address some important questions in the PRMT field.

The field of protein arginine methylation has only recently emerged from the shadows of other posttranslational modifications such as phosphorylation and glycosylation. Despite considerable advancement in the field, it is still unclear how PRMTs choose substrates. PRMTs have a wide range of protein substrates found both in the nucleus and in the cytoplasm. It is therefore important to study how PRMTs achieve substrate specificity. A large part of the studies on substrate specificity thus far have been done with either peptide substrates [4-7] or pieces of proteins attached to large tags such as GST [8-10], and very little with protein substrates. This has mainly been due to the fact that most of the available methods for the analysis of protein
methylation by PRMTs are time-consuming, and laborious. This dissertation addresses this issue by developing a time efficient and less laborious method for the analysis of the methylation of protein substrates [11]. This method impacts not only the PRMT field, but all AdoMet-dependent methyltransferases as well. Also, most of the studies on PRMTs have been done using recombinant PRMTs purified with tags. As much as these studies have given a glimpse as to how PRMTs work, recent studies have shown that the presence of tags at the N-terminus of PRMTs affects their activity and substrate selectivity. It is therefore essential that when studying PRMTs and their substrate selectivity, tagless PRMTs should be employed. This dissertation also addresses this issue by developing a protocol for the cleavage of N-terminal tags off of PRMTs, leaving them tagless [12]. It also goes further to investigate the influence of the PRMT1 N-terminus on enzymatic activity, protein-protein interactions, and substrate specificity.

In Chapter 3, we developed a protocol for the effective cleavage of N-terminal tags off of PRMTs [12]. Before now, cleavage of tagged PRMTs has been problematic, and there is need for tagless PRMTs as current studies have shown that the presence of an N-terminal tag affects both the activity and substrate specificity of PRMTs [8-10]. In this protocol, a TEV cleavage site is introduced in between the tag and the PRMT N-terminus. After the PRMT has been expressed and purified, TEV protease is added to the protein. TEV protease cleaves the tag leaving only one glycine residue at the end of the PRMT protein. In developing this protocol, we found that in order to get efficient cleavage by TEV, a gentle method of cell lysis is required, and so we employed lysis by osmotic shock. This method may also be useful for cleaving other challenging target proteins that have the TEV protease recognition site. This method was then used to purify and cleave all the PRMT constructs used in other chapters of this dissertation.

In Chapter 4, we developed a fast and efficient method for the quantitative measurement of s-adenosyl-l-methionine-dependent methyltransferase activity with protein substrates [11]. Before this assay, the rate of methyl transfer was generally measured by two ways; either by
measuring the by-product, AdoHcy or by measuring the methylated product directly. Many assays are available for the measurement of methyl transfer based on both approaches, but these methods are either limited to using peptide substrates, require the use of coupling enzymes, laborious, or incredibly time-consuming [4, 7, 8, 13-15]. Since the most common way of measuring methyl transfer of protein substrates is by measuring the rate of transfer of a radiolabeled methyl group from [3H]-AdoMet to the protein, the accuracy and length of time employed in this measurement hinges on the ability to separate unreacted [3H]-AdoMet form radiolabeled protein. In this chapter, we report a novel approach for conducting AdoMet-dependent protein methylation assays (patent-filed) using ZipTip®C4 pipette tips. The time-consuming step of separating unreacted [3H]-AdoMet from radiolabeled protein products has been drastically shortened. This method is highly suitable for measuring initial rates under saturating conditions, it also has the ability to use nanomolar enzyme concentrations and low protein substrate concentrations. A further benefit of this protocol is the reduction in the volume of radioactive waste by more than ~3000-fold.

In Chapter 5, we investigated the influence of the human PRMT1 variant 1 (hPRMT1-V1) N-terminus on enzymatic activity, protein-protein interactions and substrate selectivity. It has previously been suggested that the N-terminus of PRMTs might influence substrate specificity [4]. In this chapter, we employed both truncated and chimeric constructs of hPRMT1-V1 and a variety of PRMT1 protein substrates to test for the effect of truncating the N-terminus and switching the PRMT1 N-terminus with that of PRMT6, on hPRMT1-V1 enzyme activity, protein-protein interaction, and substrate specificity. This study will enhance the current understanding of the role of the N-terminus of hPRMT1-V1 and by extension, that of the other PRMT family members. The results from this study confirmed reports that the N-terminus influences enzymatic activity and protein-protein interactions with certain protein substrates. It also identified a piece of the N-terminus consisting of the first 10 amino acid residues, which
might be involved in regulating enzyme activity and what proteins hPRMT1-V1 interacts with in vivo. Truncating the N_T of hPRMT1-V1 at methionine 10 did not affect its substrate specificity towards the two recombinant proteins tested nor the endogenous proteins from mouse PRMT1^{−/−} ES (embryonic stem) cells. However, truncating at glutamate 27 did affect substrate specificity towards Histone H4 protein. Also, truncating the N_T did not affect the oligomerization of hPRMT1-V1.

In Chapter 6, I describe experimental data and results from completed and uncompleted projects. I also report two interesting observations that need to be further investigated. In the first project I purified six of the seven PRMT1 variants which have C-terminal His₆-tags and assessed their activity with a peptide substrate. I found that the best way to get clean C-terminal His₆-tagged variants was by using a combination of Nickel resin purification followed by heparin column purification. In the second project, we are collaborating with the Kamp Lab (Northwestern University) to look at the change in the methyl arginine proteome when A549 lung cells are treated with crocidolite asbestos versus cells treated with particulate matter. So far, we have noticed a few differences, but more work still has to be done for conclusions to be reached. In the third project, we show that under our experimental conditions, the N_T of hPRMT1-V1 is not involved in protein-protein interactions with the PRMT1 regulator hCAF1. However, more pulldown experiments still need to be done under more stringent experimental conditions before definite conclusions can be made. The fourth project looks at the interesting observation that no linear rate is obtained when the protein substrate GST-MRE11 is methylated in an assay by hPRMT1-V1-E27. Lastly, I discuss an observation where using either Hepes buffer or sodium phosphate buffer in an assay alters the activity of hPRMT1-V1 constructs towards certain protein substrates.
References


**S-Adenosyl-L-Methionine**

*S*-Adenosyl-*L*-methionine (AdoMet/SAM) is a common co-substrate involved in methyl group transfers (Fig. 2-1). AdoMet is the most widely used enzyme substrate, second only to ATP [1]. Methionine adenosyltransferase makes AdoMet from methionine and adenosine triphosphate (ATP) [2]. AdoMet is the most expensive metabolic compound made by cells, as twelve equivalents of ATP are used in this process [3]. This emphasizes the importance of AdoMet in the cell. Several metabolic pathways use AdoMet as a substrate, including transsulfuration, aminopropylation, and transmethylation. It is also advantageous to the cell to make AdoMet because all its breakdown products can be used by the cell [4]. Although AdoMet is utilized throughout the body, most of it is made and consumed in the liver [2]. The chemical reactivity of the methyl group attached to the methionine sulfur atom in AdoMet allows for its donation to an acceptor substrate in transmethylation reactions.

![Figure 2-1](image)

**Figure 2-1.** Structure of *s*-adenosyl-*L*-methionine. This shows the transferable methyl group attached to the methionine sulfur making the sulfur atom positively charged.
There are over 40 metabolic reactions that involve a methyl group transfer from AdoMet to a variety of substrates such as lipids, nucleic acids, and proteins. AdoMet is preferred over other methyl donors such as tetrahydrofolate (THF) due to the favorable energetics resulting from the AdoMet-dependent methyltransfer (-17 kcal mol$^{-1}$), which is more than twofold that from ATP hydrolysis [1].

AdoMet is essential for cellular growth and repair, and is involved in the biosynthesis of numerous mood affecting hormones and neurotransmitters such as dopamine and serotonin. It is sold as a nutritional supplement to help fight depression, liver disease, and osteoarthritic pain. In bacteria, AdoMet is involved in the regulation of genes concerned with methionine or cysteine biosynthesis.

$\textit{S}$-Adenosyl-$L$-Methionine Dependent Protein Methylation

Following translation, most proteins go through some form of modification. These posttranslational modifications (PTMs) permit organisms to expand upon the function of their limited genomes. PTMs (glycosylation, phosphorylation, acetylation, methylation, etc.) serve numerous functions, one of them being that they serve as signals of cellular communication.

Protein methylation is a PTM which involves the transfer of a methyl group from a methyl donor, predominantly AdoMet, to a methyl acceptor such as protein. Since most protein methylations use AdoMet as the methyl donor of choice, a majority of protein methylations are therefore AdoMet-dependent.

Most methylations occur on nitrogen (N-methylation) and oxygen (O-methylation) atoms and to a lesser extent on carbon (C-methylation) and sulfur (S-methylation) atoms of amino acids. N-methylation occurs on the nitrogens of lysine, arginine, histidine, glutamine and asparagine, with lysine and arginine being the most common targets [5]. When the side chains of lysine and arginine are N-methylated, the positive charge of the amino acid is not altered but hydrophobicity and steric bulk are increased, which in turn affect protein-protein interactions [5]. While the $\varepsilon$-
amine of lysine can be mono-, di-, or trimethylated, the guanidino moiety of arginine can only be mono or dimethylated. The enzyme Lysine-specific histone demethylase 1 (LSD1) has been reported as being able to catalyze the demethylation of lysine residues on histones [6]. Some studies suggest that arginine methylation is also reversible [7], however despite many efforts by scientists’, this reversibility catalyzed by the Jumonji domain–containing 6 protein (JMJD6) enzyme has not been reproduced. N-methylation of the imidazole ring of histidine and the side chain amide nitrogens of asparagine and glutamine, are not known to be readily reversible under physiological conditions. Conversely, methylation on the nitrogens of DNA is dynamic [8].

O-methylation occurs on the side chain carboxylate of glutamate and aspartate, resulting in methyl esters. O-methylation of these residues, conceals the negative charge on the carboxylate side chain, and increases hydrophobicity. O-methylation is readily reversible with the modified residues hydrolyzed back to glutamate and aspartate. In bacterial chemotaxis, hydrolysis of protein methyl esters usually serves as a signal [9].

C- and S-methylations on electron-rich carbon and sulfur atoms have been shown to occur in methanogenic bacteria. These bacteria have the ability to C-methylate arginine and glutamine side chains, and also S-methylate the thiolate side chain of cysteine. X-ray crystallographic analysis of the methanogenic bacterial enzyme, methyl-coenzyme M reductase, revealed the presence of C-methylated arginine and glutamine, S-methylated cysteine and N-methylated histidine residues [10].

AdoMet-dependent methylation is catalyzed by AdoMet-dependent methyltransferases. AdoMet-dependent methyltransferases have been grouped into five classes based on the structure of the fold that binds AdoMet and catalyzes methyl group transfer to the bound substrate [11]. Most of the AdoMet dependent protein methyltransferases belong to Classes I and V. Class I, which is the largest class, has a characteristic seven-stranded β-sheet structure and the protein arginine methyltransferases belong to this class. Class V is dominated by the SET lysine
methyltransferases. Even though AdoMet dependent methyltransferases differ structurally, the mechanism by which they catalyze methyl transfer is similar (Fig. 2-2). Methyl transfer from AdoMet to substrate is thought to proceed by an S_N2 reaction mechanism (Fig. 2-2). This reaction depends on the polarizability of the target atom which is typically an electron rich oxygen, carbon or nitrogen atom, and the strong electrophilic character of the AdoMet methyl group. There is a nucleophilic attack of the methyl group by the target atom. A proton is abstracted from the target atom before, during or following methyl transfer. This eventually leads to the release of the AdoMet sulfur moiety forming s-adenosyl-L-homocysteine (AdoHcy/SAH) and the methylated product [12, 13] (Fig. 2-2). The AdoHcy formed is hydrolyzed by SAH hydrolase to adenosine and homocysteine [14]. Homocysteine has two possible recycle paths; it can either be methylated by methionine synthase to methionine or converted to glutathione [15].

Figure 2-2. A proposed mechanism of methyl transfer by AdoMet dependent methyltransferases. A nucleophile (target atom) attacks the –CH_3 group of AdoMet, and a general base abstracts a proton from the target atom leading to methyl transfer from AdoMet to target atom.
Protein Arginine Methylation

Protein arginine methylation (PRM) is a common post-translational modification that is found across the plant and animal kingdoms. PRM like other post-translational modifications increases the structural diversity of proteins and expands upon the function of the limited genome of organisms. Since its discovery 42 years ago [16], protein arginine methylation has been shown to be a vital post-translational modification that is used in the control of diverse cellular processes.

Arginine residues within proteins are positively charged and facilitate hydrogen bonding and amino-aromatic interactions. In protein arginine methylation, monomethyl or dimethyl groups are added to the guanidino nitrogen atoms of arginine [17]. This results in three main species; monomethyl arginine (MMA), asymmetric dimethylarginine (ADMA) or symmetric dimethylarginine (SDMA) (Fig. 2-3). Arginine methylation does not change the positive charge on the arginine, but increases its bulkiness, its hydrophobicity and obstructs hydrogen bonding [18]. Arginine residues also undergo cleavage by the enzyme peptidylarginine deiminase (PAD) generating citrulline [19]. Another enzyme dimethylarginine dimethylaminohydrolase (DDAH) converts free methylarginines to ornithine [20].

Protein arginine methyltransferases (PRMTs) are the enzymes that catalyze protein arginine methylation. Of the eleven PRMT isoforms that have been identified in humans, six are type I (PRMT1, 2, 3, 4, 6 and 8) and three are type II (PRMT5, 7 and 9) [21, 22], neither PRMT10 nor PRMT11 have been classified. Both type I and II PRMTs form MMA by transferring a methyl group from AdoMet to one of the terminal (or ω) guanidino nitrogens of arginine. AdoHcy is a byproduct produced during protein arginine methylation. Type I PRMTs add another methyl group to the previously methylated guanidino nitrogen in MMA to form ADMA (Fig. 2-3). Type II PRMTs add a second methyl group to the unmethylated terminal guanidino nitrogen forming SDMA [17]. PRMT7 has been reported to show type III activity in
Trypanosoma brucei where it only catalyzes monomethylation of a terminal (ω) guanidino nitrogen [23] (Fig. 2-3). In yeast, a type IV enzyme that only catalyzes the monomethylation of the internal guanidino nitrogen (or δ) has been reported [24] (Fig. 2-3). PRMTs are ubiquitous, with homologues found in yeast, mold, Drosophila melanogaster, Caenorhabditis elegans, sea squirt, protozoa, fish and plants [25, 26]. To demonstrate the importance of PRMTs, two mouse knockouts were made; PRMT1 null mice died shortly following implantation [27], while PRMT4 null mice showed impaired cell differentiation [28]. PRMTs play a vital role in cellular processes and their span across plant and animal kingdoms indicates an important role for PRMTs in biological systems.

![Figure 2-3. Mono- and dimethylation of arginine by PRMTs. Type I, II and III PRMTs catalyze the addition of a monomethyl group to one of the terminal (ω) guanidino nitrogens of arginine forming MMA and AdoHcy. Type I PRMTs catalyze the addition of another methyl group to the same guanidino nitrogen forming ADMA, while type II PRMTs add another methyl group to the unmodified nitrogen forming SDMA. Type IV PRMTs catalyze the addition of a monomethyl group to the internal (δ) guanidino nitrogen forming MMA.](image-url)
**Protein Arginine Methyltransferase Family**

PRMT activity was first described over forty years ago in nuclear thymus extracts from calf [16]. Following this, diverse PRMTs were discovered and purified from rat liver, calf brain and a variety of cell lines [29-32]. Even though the PRMTs were discovered over forty years ago, it wasn’t until twenty nine years later that cDNAs were isolated and cloned [33, 34]. Since their discovery, several isoforms of this family have been identified through homology studies, database searches, and sequence similarity [26, 35]. So far, eleven PRMT isoforms have been identified in humans (Fig. 2-4). PRMTs contain a common catalytic methyltransferase domain which includes a highly conserved core region, and motifs essential for binding to AdoMet and to the substrate [36-38]. Although PRMTs have a conserved core, they differ at their N-termini which are of variable lengths and contain different domain motifs (Fig. 2-4).

PRMT1 was the first PRMT to be discovered and isolated. PRMT1 is the most predominant PRMT in mammalian cells and is responsible for 85% of total protein arginine methylation in cells [39]. PRMT1 was first identified as a protein interacting with the leukemia-associated BTG1 protein and the mammalian intermediate-early TIS21 protein, or with the intracytoplasmatic domain of the IFNα receptor [34, 40]. After this, PRMT1 was then identified by sequence homology to the yeast arginine methyltransferase Hmt1/Rmt1 [41]. PRMT1 is found both in the nucleus and cytoplasm. The human genome has been shown to have seven splice variants of PRMT1 resulting from alternative splicing [42]. These variants differ mainly at their N-terminal sequence and tissue localization. PRMT2 was discovered second by sequence similarity to PRMT1 [41, 43] and has been shown to have two splice variants in humans which differ only in the second exon [21]. PRMT2 is localized in the nucleus and cytoplasm. An interesting feature of PRMT2 is that it has an SH3 domain at its N-terminus. Two years after the discovery of PRMT1, PRMT3 was identified in a yeast two-hybrid screen as a PRMT1 binding partner [44]. To date, only one variant of PRMT3 has been isolated, and has been shown to
localize to the cytoplasm [36, 44]. PRMT3 harbors a zinc-finger (ZnF) domain at its N-terminus and has been shown to use this in substrate recognition (Fig. 2-4). PRMT4 (CARM1) was also identified in a yeast two-hybrid screen interacting with GRIP1 which is a part of the p160 family of proteins [45]. One variant of PRMT4 has been isolated and is localized in the nucleus[21]. PRMT5 was identified as a Janus tyrosine kinase (Jak2)-binding protein in a two-hybrid search [46]. PRMT5 has two variants which differ in the first exon [21]. PRMT5 is localized in the cytoplasm [47]. PRMT6 was identified by a database search for proteins which harbor the characteristic methyltransferase motif [48]. PRMT6 is the smallest member of the PRMT family and its subcellular localization is in the nucleus [48]. PRMT6 has been shown to automethylate itself [48]. PRMT7 was found in a genetic screen for susceptibility to chemotherapeutic cytotoxicity [49]. Unlike other PRMTs, PRMT7 has two catalytic domains [50] and is localized both in the nucleus and the cytoplasm [51]. PRMT8 was discovered by a database search for proteins which harbor the characteristic methyltransferase motif [52]. PRMT8 has over 80% sequence identity to PRMT1 and is localized at the plasma membrane. It has a myristoylation (Myr) motif at its N-terminus which facilitates interaction with membrane lipids (Fig. 2-4). PRMT8, just like PRMT6, has the ability to automethylate itself [53]. PRMT9 was identified through a database search using the sequence of the conserved PRMT methyl donor binding domain [54]. PRMT9 harbors an F-box motif at its N-terminus and a ZnF motif at its C-terminus. It is localized in both the nucleus and cytoplasm (Fig. 2-4). Four splice variants have been identified for PRMT9 [21, 54]. PRMT10 was identified by sequence homology to PRMT7 [26]. Like PRMT7, PRMT10 has two catalytic domains, but also has an additional TPR repeat at its N-terminus. So far, no catalytic activity or substrates have been shown for PRMT10 [26, 35]. PRMT11 was identified by sequence similarity search using the sequence of PRMT9 [26, 35]. PRMT11 is the longest member of the PRMT family described. It contains an F-box
motif at its N-terminus and a nitrous oxidase accessory protein (NosD) motif at its C-terminus (Fig. 2-4). Just like PRMT10, no biochemical functionality has been reported for PRMT11 [35].

Figure 2-4. The PRMT methyltransferase family. Eleven isoforms of human PRMTs aligned based on their core domain and AdoMet methyltransferase binding motifs (black). The subcellular localizations for each isoform is shown; N (nucleus), C (cytoplasm), and PM (Plasma membrane). Localizations marked N/A have not yet been identified. * Denotes the automethylation site on the isoform. PRMT 1 and 4 have also been reported to be automethylated but the site of automethylation is not known.

PRMT Splice Variants

In the PRMT family, PRMT1 is the major PRMT present in mammalian cells and tissues, and is expressed in all cell types investigated [39, 43, 55]. PRMT1 is localized on chromosome 19q13.3 of the human genome [21], and has been shown to have seven alternatively spliced variants [42]. These variants have a conserved catalytic core domain and only differ at their N-termini. They show distinct subcellular localization; variants 1 and 7 are found in the nucleus, variant 2 in the cytoplasm, and variants 3, 4, 5, and 6 in both cellular compartments. The exclusive cytoplasmic localization of variant 2 can be explained by the presence of a nuclear
export sequence at its N-terminus. Whilst variants 1, 2, and 3 are expressed ubiquitously, variants 4, 5, 6, and 7 are expressed in singular tissues such as pancreas, heart, or muscle. Significantly, the relative occurrence of these spliced variants differs when normal and cancerous breast tissues are compared. This makes PRMT1 alternatively spliced variants useful as diagnostic markers in breast cancer [56]. Interestingly, it has been suggested that PRMT1 splice variants have distinctive activity and substrate specificity even though they only differ at their N-terminal sequence [42]. However, this study was done using C-terminal His$_6$-tagged enzymes, which in collaboration with our lab, were shown to have very low activities. Also, it used pieces of proteins attached to large tags such as GST, which does not correctly reflect physiological substrates. Nonetheless, some variance was observed under these conditions. Our goal was to develop the necessary techniques to test this in the right context, with untagged PRMT enzymes and full length proteins.

PRMT2 has two splice variants, with the second exon being the only difference between them [21]. Unlike PRMT1, both variants encode for the same protein, which is 433 amino acids long. PRMT3 only has one variant described, which encodes for a 531 amino acid long protein [21]. Like PRMT3, PRMT4 only has one variant which encodes for a 608 amino acid long protein [21]. PRMT5 has two variants described. These variants vary in the first exon and encode for two proteins which differ in length [21]. Only one splice variant has been described for PRMT6 and it is predominantly expressed in the kidney, testis and brain. PRMT7 has one variant expressed mainly in dendritic cells, the thymus, and reproductive system. PRMT8 also has only one variant which encodes for a protein which is 394 amino acids in length. Second to PRMT1 is PRMT9 which has four variants described in the human genome. All four variants differ in length and all contain the AdoMet binding domain and an F-box domain at the N-terminus, except for the shortest isoform which lacks the latter feature. In addition, the longest isoform harbors a ZnF motif at the C-terminal region. It is only for the shortest isoform of
PRMT9 that direct enzymatic activity with peptides and proteins has been described [21].

The Structure of PRMTs

The crystal structures of both PRMT1 and PRMT3 have been solved [57, 58]. PRMT1 was successfully co-crystallized with AdoHcy and a 19 amino acid peptide substrate, R3 (GGRGGFGRGGFGGRGGFG), which originates from fibrillarin (Fig. 2-5A). However, no electron density was observed for most of the R3 peptide, mainly that for the substrate arginine was seen (Fig. 2-4A). On the other hand, the PRMT3 structure only had AdoHcy bound (Fig. 2-5B).

From the solved crystal structures, it can be seen that PRMTs have a two domain structure composed of an AdoMet-binding domain and a barrel-like domain [57, 58]. The active site of PRMTs is situated between these two domains. The crystal structure of PRMT1 also revealed three substrate binding grooves (P1, P2, and P3) which are all acidic and located on the surface of PRMT1. The acidic nature of these grooves gives them the ability to interact with substrate arginine residues, hence the possibility that substrates can bind to PRMT1 from different directions.

Although both PRMT1 and PRMT3 structures are available, they have done little to improve on the understanding of PRMT substrate preference. This is partly due to the fact that in solving the crystal structure of PRMT1, no electron density was obtained for the N_T (only the residues after amino acid 40 of the 47 N_T residues were observed) even though the substrate peptide was crystallized bound to the enzyme, suggestive of a disordered N-terminal tail [57]. This suggests that the N_T does not interact with short substrate peptides nor does it take part in placing the substrate arginine in the active site. However, by employing pieces of proteins the N_T was shown to affect substrate specificity [42]. Therefore as previously proposed, the N_T is probably involved in protein-protein interactions and substrate recognition at a site on the protein substrate away from the active site.
Figure 2-5. Crystal structures of PRMT1 and 3 showing bound cofactor and peptide backbone. Structures (A) and (B) show: the X (Blue) and Y (Red) helices which are part of the conserved PRMT core, the C-terminus (Magenta), a part of the N-terminal loop (Yellow), $s$-adenosyl homocysteine (AdoHcy) (Orange) and the rest of the PRMT protein (Green). (C) PRMT1 crystal structure showing the P1, P2 and P3 binding grooves with bound peptide backbone. The P1 (blue peptide bound framing the groove) and P2 (blue peptide bound) binding grooves are parallel to the X and Y helices respectively and are close to the active site. P3 (pink peptide bound) is perpendicular to the Y helix and is further away from the active site. The C-terminus (Magenta), a part of the N-terminal loop (Yellow), $s$-adenosyl homocysteine (AdoHcy) (Orange) and the rest of the PRMT protein (Green) are also shown.
PRMT Protein Substrates

PRMTs are ubiquitous, present across plant and animal kingdoms and are known to be involved in many cellular processes. It is therefore only reasonable that they have many protein substrates that they act on. However, it is only in recent years that PRMT protein substrates are being identified (Fig. 2-6). Most of these substrates were identified using proteomic-based mass spectrometric techniques, and focused in vitro substrate screens [59]. With such a limited array of PRMT substrates, it is therefore not surprising that what determines substrate specificity among the different PRMT isoforms is still unclear.

Typically, PRMTs target proteins containing glycine arginine rich (GAR) motifs with the methylated arginines mainly found in ‘RGG’ or ‘RXR’ regions. This is the case with PRMT1, PRMT3, PRMT6 and PRMT8 which generally methylate arginines in GAR motifs. PRMT4 however deviates from the GAR motifs and displays a higher degree of specificity. Since there is no apparent motif recognized by PRMT4, it is therefore hard for possible substrates to be predicted by database searches. The type II PRMTs, PRMT5 and PRMT7, on the other hand
methylate isolated arginine residues as well as arginines located in GAR motifs. PRMT9 has been identified as a type II PRMT, symmetrically dimethylating MBP (maltose binding protein) [54].

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**Figure 2-6.** PRMT protein substrates grouped according to cellular function. Protein substrates are shown for PRMT1-7; substrates are listed below the PRMT responsible for their methylation. The grey regions are to help distinguish between substrates on adjacent lines. Substrates for PRMT8-11 are not shown, mainly because their substrates have either not been well characterized or not yet known. The abbreviations stand for: PGC1α, peroxisome proliferator activated receptor-γ-coactivator 1α; ZF5, Zn2+ finger 5; RIP140, Nuclear receptor-interacting protein 1; CIRP, cold inducible RNA binding protein; EWS, Ewing Sarcoma; ILF3, interleukin enhancer binding factor 3; ZF5, Zn2+ finger 5; TARPP, thymocyte cyclic AMP-regulated phosphoprotein; EBNA1(2), Epstein-Barr virus nuclear antigen 1(2); 53BP1, p53-binding protein 1; CBP, CREB binding protein; AIB1, Amplified in breast cancer 1; CA150, phosphoCTD-associating protein; U1C, U1 snRNP C.
No catalytic activity or substrates have been shown for PRMT10 in humans, but in plants, PRMT10 demonstrates type I activity, dimethylating Histone H4 in vitro [60]. Due to its similarity to PRMT9, it is predicted that PRMT11 might be a type II PRMT, but so far no biochemical data or functional information have been reported. Among the eleven PRMTs discussed thus far, PRMT1 has the largest number of substrates already identified. This is not surprising since it is the predominant PRMT in mammalian cells and accounts for the majority of PRMT activity in vivo [55, 61] (Fig. 2-6).

**Biological Significance of PRMTs**

PRMTs are increasingly gaining attention as more and more substrates with central roles in cellular processes are being discovered. With the discovery of these new substrates comes the knowledge of the vast number of PRMT-regulated cellular processes. Some of the cellular processes PRMT activity has been implicated in include: signal transduction, DNA repair and transcriptional regulation. Protein arginine methylation primarily regulates cellular processes by altering biomolecular interactions like protein-protein interactions [62-69], protein-RNA interactions [70-72], and protein-DNA interactions [73-75]. Importantly, the significance of PRMTs was established by studies with PRMT knockout mice. For instance, PRMT1 null mice died early in embryonic development suggesting that PRMT1 is required for early post implantation mouse development [27, 76], while PRMT 4 null mice which were smaller than their wild-type counterparts died shortly after birth [28] and PRMT4 null embryos demonstrated delayed endochondral ossification [77]. Both PRMT2 and PRMT3 null mice were viable, but the PRMT2 null embryos had fibroblasts which showed increased NF-KappaB activity and more resistance to apoptosis than wild type embryos displayed [78]. PRMT3 null embryos were smaller than wild type [79].
**Signal Transduction**

Signal transduction is generally directed by posttranslational modifications. However, protein arginine methylation was not considered to be one of them as for a long time, it was thought to be irreversible. Recent findings show that PRMTs play an important role in signal transduction.

PRMT1 was the first PRMT to be linked to signal transduction [40]. PRMT1 interacts with the cytoplasmic region of the type I interferon receptor, where its methyltransferase activity leads to an antisense-mediated reduction of PRMT1 levels. This gives the cells the ability to resist cell growth inhibition by IFN. PRMT1 has also been implicated in the NFAT signaling pathway. Here, PRMT1 methylation of NIP45 facilitates its binding to NFAT, resulting in the stimulation of cytokine gene expression [65]. Arginine methylation of FOXO1 by PRMT1 acts as an inhibitory modification where it blocks phosphorylation by Akt, as such promotes the nuclear localization of FOXO1 [80]. Also, there is evidence that suggests that PRMT1 is involved in NGF (Nerve growth factor) receptor signaling [81], insulin signaling and glucose metabolism [56]. It has also been suggested that PRMT8 could be involved in signaling due to the fact that it has a proline-rich domain that could bind to SH3 domains of numerous proteins [53], and a myristoylation motif that facilitates its association with the plasma membrane, positioning it to possibly impact a signaling pathway near its start [52].

**DNA Repair**

The MRE11/Rad50/NBS1 (MRN) complex in mammals plays a vital role in the repair of DNA double strand breaks (DSBs). Methylation of the GAR motif of MRE11 by PRMT1 does not affect the assembly of the MRN complex but prevents its recruitment to DSBs [82, 83]. Another protein, 53BP1 which is also involved in DSB repair has also been shown to be methylated by PRMT1, but the effect of this methylation is not yet established [84, 85]. The tumor suppressor protein p53 controls crucial cellular processes including DNA repair.
Following DNA damage, several signals are generated which lead to the stabilization of p53 which then functions by activating DNA repair proteins and also inducing the transcription of p21 which promotes cell cycle arrest. Cell cycle arrest will give the repair proteins time to carry out DNA repair for continuous survival of the cell or apoptosis to get rid of the damaged cell. Recently, p53 was reported to be methylated by PRMT5 [86]. PRMT5 is important for p53 expression as the knockdown of PRMT5 was shown to inhibit p53 protein synthesis [87]. Lack of p53 protein during DNA damage will seriously handicap the DNA repair response.

Transcriptional Regulation

Transcription of proteins can either be activated or repressed, allowing for their regulation by several factors. The ability of PRMTs to methylate histones gives them a way to regulate gene expression. PRMTs have been implicated in the regulation of numerous transcription factors including NF-κB, PPARγ, RUNX1, YY1, p53, and E2F1 [88, 89]. Both PRMT1 and PRMT4 act as general transcriptional coactivators, activating numerous transcription factors [61]. PRMT1 and PRMT4 have been reported to cooperatively enhance steroid hormone receptor-mediated reporter gene expression [25]. To further support their synergy, knockdown of PRMT1 and PRMT4 resulted in the down regulation of a set of STAT5-controlled genes [90]. Individually, PRMT1 methylates the transcription factor RUNX1, this prompts the transcription repressor, SIN3A, to dissociate promoting RUNX1 transcriptional activity [89].

PRMT5 activity generally results in transcriptional repression, and has been shown to function with many repressor complexes such as BRG1, hBRM, BLIMP1 and SNAIL [91-95]. It is thought that PRMT5 mediates transcriptional repression by symmetrically dimethylating H3R8 and H4R3, which will prevent the asymmetric dimethylation of H4R3 by PRMT1, which is generally recognized as a transcriptional activating mark [61, 96]. Methylation of H3R2 by PRMT6 prevents the methylation of H3K4 as such blocking the recruitment of transcriptional activators to the methylated H3K4 mark [97, 98].
Biological Regulation of Protein Arginine Methylation

Protein arginine methylation is a vital posttranslational modification that is involved in diverse cellular processes; therefore it is important that this modification be regulated. Since PRMTs show different subcellular localizations, their activity is limited by this compartmentalization. Also PRMTs in vivo can be regulated by PRMT-binding proteins, posttranslational modifications and enzymes that counteract their activity.

Regulation by PRMT-binding proteins

PRMT activity has been shown to be regulated by proteins that bind to them (Fig. 2-7). The binding of such proteins can result in the activation, inhibition, or even change in the substrate specificity of the PRMT. At least nine of such proteins that interact with PRMTs have been described. PRMT1 activity is stimulated by the related proteins BTG1 and TIS2/BTG2 towards tested substrates [34]. Another protein hCAF1 which also interacts with BTG1 regulates PRMT1 activity [75]. PRMT3 activity is inhibited by the binding of the tumor suppressor DAL-1 [99]. Recombinant PRMT4 would rather methylate free histone H3, but when in the nucleosomal methylation activator complex (NUMAC), PRMT4 acquires the capability to methylate nucleosomal histone H3. Like PRMT4, PRMT5 preferentially methylates H4R3 over H3R8 which it is in association with the histone-binding protein COPR5 [100]. PRMT5 activity is elevated when it is complexed with hSWI/SNF chromatin remodelers, BRG and BRM [96]. Likewise, PRMT7 activity is enhanced when it is bound to the CTCFL testis-specific factor [101] (Fig. 2-7).
**Figure 2-7.** Regulation of PRMT activity by PRMT-binding proteins. The interaction of PRMT5 with the proteins BRG and BRM, and PRMT7 with CTCFL protein, enhance PRMT activity, while the binding of PRMT3 to DAL-1 decreases PRMT activity. Other interactions like that of PRMT1 with BTG1 and hCAF1, and PRMT4 with the complex NUMAC alter PRMT specificity.

*Regulation by posttranslational modifications*

A wide array of proteins is regulated by posttranslational modifications and PRMTs are most likely not an exception. PRMT4 homodimerization is inhibited and its catalytic activity decreased as a result of phosphorylation by an unknown kinase during mitosis [102]. This is the first evidence that PRMTs are regulated by posttranslational modifications. Also PRMT1, PRMT4, PRMT6, and PRMT8 have been shown to be automethylated, but the effect of this modification is yet to be revealed [53].

Usually more than one posttranslational modification occurs on the same protein substrate and sometimes neighboring modifications can mask arginine methylation motifs. Examples of this are the histone tails which are rich in posttranslational modifications, and the combination of these modifications give rise to the histone code. The methylation of H3R8 by PRMT5 is inhibited by H3K9 acetylation [96]. Also, H3K4 trimethylation partially blocks H3R2 methylation by PRMT6 [98, 103].
Regulation by enzymes that counteract PRMT activity

Arginine residues in proteins can be deaminated by peptidyl arginine deiminases (PADs) giving rise to citrulline (Fig. 2-8). PADs only deaminate arginines and not MMA or DMA (dimethyl arginine) as earlier thought [104]. Sometimes the activity of PADs results in the deamination of PRMT substrate arginines as such interfering with potential methylation. Therefore by converting arginine to citrulline, arginine methylation is blocked.

Figure 2-8. Enzyme activities that counteract protein arginine methylation. PRMTs dimethylate arginines forming ADMA and SDMA. PAD4 deaminates arginine residues, converting them to citrulline, preventing arginine methylation. JMJD6 is the first arginine demethylase described; it targets both ADMA and SDMA on histone tails.

Recently, it was reported that the enzyme JMJD6 (Jumonji domain-containing 6 protein) can demethylate dimethylation on H3R2 and H4R3 [7]. It seems that both ADMA and SDMA on H4R3 can be demethylated by this enzyme. This is the first arginine demethylase to be reported; hence it is possible that more demethylases of this sort remain to be identified.

Protein Arginine Methylation and Disease

Protein arginine methylation has been implicated in a variety of diseases including cancer, cardiovascular disease, viral pathogenesis, spinal muscular atrophy and multiple sclerosis. PRMTs have a wide range of protein substrates which are found both in the nucleus and
cytoplasm. Therefore considering how widely spread the activities of the PRMTs are, incorrect regulation of these enzymes will most likely lead to the emergence of certain disease conditions.

**Cancer**

Recently, it was demonstrated that mRNA levels for PRMT1 are elevated in certain breast cancer cell lines when compared to normal controls [42]. When PRMT1 or its substrate, Sam68, is fused directly to the mixed lineage leukemia (MLL) oncogenic protein, in place of its normal binding partner EEN, there is an activation of the oncogenic predisposition of MLL. This suggests that the arginine methylation of Sam68 is important in this process [105]. High levels of PRMT4 have been identified in resistant prostate and aggressive breast cancer tumors [106, 107]. It has also been shown that PRMT4 is vital for the estrogen-induced proliferation of the MCF-7 breast cancer cell line [108]. PRMT5 levels have also been shown to be increased in leukemia and lymphoma cells [109], and also in gastric cancer [110].

**Viral Pathogenesis**

Numerous viral proteins have been shown to be heavily methylated including several HIV-1 proteins, Epstein-Barr viral proteins, hepatitis proteins and adenoviral proteins [25]. Most of these proteins are substrates of either PRMT1 or PRMT6. It has been shown that inhibition of general PRMT activity increases HIV-1 gene expression and infectivity [111]. Also, it has been shown that PRMT activity is important for effective adenovirus infection [112]. Furthermore, it has been shown that replication of hepatitis delta is prevented by inhibition of arginine methylation [113]. All of this shows that methylation is very important for normal viral functioning.
Spinal Muscular Atrophy

When certain deletions or loss of function occurs in the SMN1 gene, the individual suffers from spinal muscular atrophy (SMA) which is an autosomal recessive disease. SMN is required for the assembly of proteins that are involved in RNA metabolism. This process requires that these proteins be arginine methylated. The Tudor domain of the SMN protein can bind to both SDMA and ADMA motifs. It also interacts with numerous PRMT4 and PRMT5 substrates [114, 115]. When the Tudor domain of SMN was examined, point mutations were present in SMA patients showing a link between the methylarginine-binding ability of SMN and SMA [116].

Cardiovascular Disease

The enzyme nitric oxide synthase (NOS) catalyzes the formation of nitric oxide (NO), a known vasodilator, from L-arginine. NO plays multiple roles in the cardiovascular system, and is very important for the proper functioning of this system. When methylated proteins are degraded by the proteasome, free MMA, ADMA and SDMA are released. MMA and ADMA inhibit NOS contributing to the development of atherosclerosis [117]. The generation of these NOS inhibitors is controlled by the enzymes dimethylarginine dimethylaminohydrolases (DDAHs) which metabolize free MMA and ADMA. It is therefore important that these enzymes (DDAHs) be well regulated. If DDAH is misregulated or PRMT activity is dysfunctional, this might lead to an imbalance in the MMA and ADMA pool which might increase the risk of cardiovascular disease [118]. This is supported by the fact that Ddah1 knockout mice displayed elevated blood pressure and developed vascular pathophysiology [119]. Also, it has been shown that patients with the following diseases displayed elevated levels of ADMA; hypercholesterolemia, hyperhomocysteinemia, peripheral arterial occlusive disease, hypertension, chronic heart failure and coronary artery disease [120]. These observations show that there is a link between high levels of ADMA and cardiovascular disease. To solidify the argument that ADMA concentration
plays a role in cardiovascular disease, a study revealed that at the early onset of atherosclerosis, ADMA levels were increased. These studies suggest that ADMA is a potential marker for cardiovascular disease [121].

Multiple Sclerosis

Multiple sclerosis (MS) is a disease of the central nervous system which involves demyelination of the myelin sheaths that surround and provide protection to the nerve axons. Myelin basic protein (MBP) which is one of the main proteins that make up the myelin sheath is a PRMT5 substrate. There is reason to believe that the methylation of MBP is linked to the development of MS as MBP methylation was found to be increased in MS patients [122]. It is suggested that methylated MBP might serve as an autoantigen, as is the case with methylated Sm and coilin proteins in lupus erythematosus [123].

Detection of Methyl Transfer and Measurement of PRMT Activity

A significant amount of what is currently known about PRMTs was acquired by studying the property of recombinant PRMTs. However, most of these studies have been done using recombinant PRMT proteins expressed as tagged fusion proteins. Several studies have shown that, the identity of the N-terminal fusion tag affects the substrate selectivity of PRMTs [53, 54, 124]. Therefore, to accurately study substrate recognition, it is imperative that a tagless PRMT be used. Cleavage of the fusion tag from PRMTs has proven to be extremely challenging, but recently we described a method for the purification of tagless PRMTs for in vitro studies [125].

The use of peptide substrates is the most common approach employed in enzymatic studies of PRMTs. Generally used in combination with novel assay systems [126-130], these studies have provided a first peek of the catalytic mechanism [131-135]. However, there is need to translate or confirm these findings with real protein substrates of the protein methyltransferases in vivo. There are three main approaches commonly employed to measure the rate of
methyltransfer; the first method measures the by-product, AdoHcy, the second directly measures the methylated protein, and the third measures the amount of MMA, and ADMA or SDMA in hydrolysed methylated proteins. These methods are all suitable for the detection of methyl transfer but not all are appropriate for the quantitative measurement of PRMT activity.

Recently, numerous novel assays have been developed geared at measuring AdoHcy formation [126-129]. When analyzing methylated proteins, radiolabeled AdoMet (S-adenosyl-L-[methyl-\(^3\)H]-methionine; \([^{3}\text{H}]\text{-AdoMet}\)) is commonly used. Here, unreacted \([^{3}\text{H}]\text{-AdoMet}\) is separated from radiolabeled protein product by SDS-PAGE. Another commonly used method is by using phosphorimaging/fluorography to detect radiolabeled proteins either in-gel or post-transfer to a membrane [42, 53]. These methods are good but quite laborious, tedious and time consuming. We recently described a novel method for the analysis of protein methylation which is efficient, time-saving, sensitive, and generates less radioactivity compared to other methods [136].

**Conclusion**

Protein arginine methylation is a widespread posttranslational modification that is involved in the regulation of a variety of cellular processes. PRMTs have diverse protein substrates ranging from RNA binding proteins, to proteins involved in signaling. PRMT knockout studies show that PRMT activity is vital for cell survival. However, PRMT activity has also been implicated in a number of diseases such as cancer, cardiovascular disease, spinal muscular atrophy, and multiple sclerosis. Even though the understanding of PRMTs has advanced in the past few years, there is still little understanding of how substrate specificity is achieved among the PRMTs. Also fascinating is how a single PRMT can recognize numerous substrates with such high specificity. Increased understanding of how this happens will greatly impact not only the PRMT field, but biology in general as it will facilitate the discovery of more PRMT substrates and PRMT-regulated processes.
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CHAPTER 3

A PROBLEMATIC TEV-PRMT1 CONSTRUCT IS CLEAVED WHEN OSMOTIC SHOCK IS EMPLOYED

Abstract

Protein arginine methyltransferases (PRMTs) are enzymes which are involved in many biological processes. Several studies have shown that the identity of the N-terminal fusion tag affects the substrate selectivity of PRMTs. Therefore, to accurately study substrate recognition, it is imperative that a tagless PRMT be used. However, cleavage of tagged PRMTs has been problematic. We have developed a successful method by which untagged PRMTs can be made using a TEV cleavage site at the N-terminal domain. This method may be useful for cleaving other challenging target proteins that have the TEV protease recognition site.

Introduction, Results and Discussion

Protein arginine methyltransferases or PRMTs are enzymes which catalyze the $S$-adenosyl methionine (SAM)-dependent methylation of protein arginine residues. PRMT activity has been implicated in the regulation of numerous cellular processes including sub-cellular localization of proteins [1], transcriptional regulation [2] and DNA repair [3], and in certain disease conditions such as carcinogenesis [4] and viral pathogenesis [5]. The question of how differential substrate selection is achieved between eleven different human PRMT isoforms is a major issue in the PRMT field that cannot be answered with recombinant proteins bearing fusion tags. Although they are convenient for affinity-based purification schemes, fusion tags interfere with the regulation of enzyme activity and substrate specificity that is controlled by the unique N-terminal domain of the PRMTs [6-8]. Differential methylation of some PRMT1 substrates is

observed when the N-terminal domain of human PRMT1 (hPRMT1) is altered [6]. More importantly, studies with both PRMT8 and PRMT9 show that the type of fusion tag used at the N-terminus (GST, FLAG, HA, or HIS) differentially affects methylation of certain protein substrates [7, 8]. Unfortunately, moving the tag to the C-terminus results in decreased enzyme activity (data not shown). In view of these findings, it is imperative that untagged PRMTs be employed to study PRMT substrate selection and enzyme activity. However, cleavage of the fusion tag from PRMTs has proven to be extremely challenging (this paper and personal communications). Here we describe a method to obtain untagged PRMTs starting with recombinant protein bearing a tobacco etch virus (TEV) cleavable affinity tag.

Many proteases are available commercially for cleaving fusion tags from proteins, for example Precision Protease (GE Healthcare), Thrombin and TEV protease. TEV protease is attractive because it can be easily expressed and purified in the lab (making it cost-effective), only one alanine residue is left at the end of the protein after TEV cleavage, and the conditions for cleavage are mild. However, we found that in order to get efficient cleavage by TEV, a gentle method of cell lysis is required.

*Escherichia coli* BL21 (DE3) cells overexpressing HIS$_6$-TEV-hPRMT1 were lysed by osmotic shock in the following manner. Cells (6.5 g) and 10 small glass marbles were added to a 40 ml centrifuge tube. Glycerol solution (4 M Glycerol, 50 mM Hepes, pH 8.0) was added to yield a total volume of 26 ml. The centrifuge tubes were mixed for 10 min in a shaker to resuspend the cells. Cells were centrifuged at 8000g for 20 min. The supernatant was removed by slowly decanting. A few flakes of DNAase and 1ml of a 100 mg/ml lysozyme solution were added to the tube and a spatula used to break the cells off the wall of the centrifuge tube. Extraction solution (50 mM Hepes, pH 8.0, 0.5 mM phenylmethylsulfonyl fluoride) was added to give a final volume of 27 ml. Tubes were quickly capped and vigorously shaken to rupture the cells. Cells were allowed to sit for 10 min on ice and then centrifuged at 20,000g for 20 min.
The supernatant was collected and incubated with Ni Sepharose™ 6 Fast Flow (GE Healthcare) overnight at 4°C. HIS₆-TEV-hPRMT1 was eluted with a stepwise imidazole gradient from 5-500 mM imidazole in 20 mM Hepes, pH 8.0 and 100 mM NaCl. Fractions were analyzed by SDS-PAGE to determine the presence of PRMT1. Fractions containing the PRMT1 were dialyzed into buffer containing 100 mM Hepes at pH 8.0, 200 mM NaCl, 1 mM DTT, 2 mM EDTA, and 10% glycerol for 2 hours. HIS₆-TEV protease [9, 10] was added to HIS₆-TEV-hPRMT1 at a ratio of 50 OD₂₈₀ of TEV to 100 OD₂₈₀ of HIS₆-TEV-hPRMT1. This mixture was dialyzed against dialysis buffer containing 5% glycerol overnight at 4°C. SDS-PAGE analysis showed that HIS₆-TEV-hPRMT1 was cleaved efficiently (Figure 1A). Generally, the amount of TEV required for a particular cleavage is empirically determined using a range of target protein:protease ratios of 100:1 to 5:1 [10]. TEV protease used in a ratio of 100 OD₂₈₀ of HIS₆-TEV-hPRMT1 to 1 OD₂₈₀ of TEV protease resulted in very little cleavage. We also tried ratios of target protein:protease of 10:1 and 2:1. Approximately 80% of the target protein was cleaved using a 10:1 ratio overnight at 4°C (data not shown). Further cleavage could be obtained if the incubation period was lengthened. The ratio of 2:1 was optimal for efficient cleavage in a 24 hour period at 4°C (Figure 3-1A).

The cleaved protein/TEV protease mixture was dialyzed into buffer containing 20 mM Hepes, 100 mM NaCl, 5 mM Imidazole, 0.5 mM PMSF and 10% glycerol. This protein mixture was then applied to a Ni sepharose column and incubated overnight at 4°C. Both protein purification steps can be done on the same Ni sepharose column. Flow through containing cleaved protein was collected and elution buffer containing 20 mM Hepes at pH 8.0, 100 mM NaCl, and 150 mM imidazole was used to elute cleaved protein interacting with the Ni resin. SDS-PAGE analysis was used to determine the presence of the target protein in both fractions. Under these conditions, neither HIS₆-TEV nor HIS₆-TEV-hPRMT1 was observed in either the flow through or the elution. Fractions were dialyzed into the same dialysis buffer described
above with 10% glycerol. Glycerol was added to the cleaved protein solution to a final concentration of 15% and concentrated using an Amicon Centricon plus-20 centrifugal filter device with a molecular weight cutoff of 10 kDa. Protein concentration was determined spectrophotometrically using the predicted extinction coefficient of 56435 M⁻¹ cm⁻¹ and the protein yield was 0.4 mg/L of cell culture.

**Figure 3-1.** Human PRMT1 variant 1 truncated at methionine 11 (hPRMT1v1 M11) before and after treatment with TEV protease. Coomassie stained SDS-PAGE showing HIS₆-TEV-hPRMT1v1 M11 before (lane 1) and after (lane 2) treatment with TEV protease overnight at 4°C. In (A), the cells were lysed by osmotic shock. The arrow indicates cleaved hPRMT1v1 M11. In (B) the cells were lysed by sonication. Cleavage of HIS₆-TEV-hPRMT1v1 M11 was not observed. In (C) the cells were lysed by French Press. Cleavage of HIS₆-TEV-hPRMT1v1 M11 was not observed.
The activity of the untagged protein was assessed using a continuous spectrophotometric assay previously described in [11] (Figure 3-2). The assay utilized various concentrations of R3 peptide (acetylGGRGGFGGGRGGFG), 4 μM hPRMT1, 10 nM AdoHcy/MTA nucleosidase, 0.02 μM Adenine Deaminase, 10 nM MnSO₄, 250 μM S-Adenosylmethionine, and 50 mM sodium phosphate buffer pH 7.1. R3 peptide was used to initiate the reactions and a decrease in absorbance at 265 nm was measured. The \( K_{m, \text{app}} \) obtained for the hPRMT1 construct was 38 ± 12 and the \( V_{\text{max}} \) was 3.2 ± 0.29. This shows that the untagged construct is active and can be used in PRMT studies.

![Figure 3-2. Michealis-Menten plot showing the rate of methylation of the R3 peptide by untagged hPRMT1v1 M11. Activity was assessed using a continuous spectrophotometric assay [11].](image)

The method described above consistently yielded cleaved protein with three hPRMT1 variant 1 constructs (full length hPRMT1 variant1, a truncated construct starting from Methionine 11, and a truncated construct starting from Serine 31) (Figure 3-1A and data not shown).
Osmotic shock generally yields less total protein than many other lysis methods, such as French press and sonication, which are more commonly used. However, lysing the cells by either French press or sonication resulted in very little if any cleavage of HIS6-TEV-hPRMT1 (Figure 3-1B and 3-1C).

In conclusion, we have developed a method by which untagged active PRMTs can be successfully obtained and used to study substrate recognition and processing. When cells containing HIS6-TEV-hPRMT1 are lysed by either French press or sonication, no TEV cleavage is observed. Therefore lysing cells by osmotic shock is the key to getting a tagless PRMT. Tagless PRMTs can be used in a wide range of studies without worrying about the effect of N-terminal or C-terminal tags. We note that in the crystal structure of rat PRMT1, no electron density was obtained for the N-terminus suggesting that the N-terminal tail is disordered [12]. It is possible that French Press and sonication result in conformations of the N-terminus that are inaccessible to TEV protease. This hypothesis is supported by recent studies [13-15], which documented conformational changes in proteins as a result of sonication that can lead to aggregation. Hence for other problematic TEV fusion constructs that show possibly disordered N- or C-termini, osmotic shock may facilitate cleavage.

References


CHAPTER 4

A FAST AND EFFICIENT METHOD FOR QUANTITATIVE MEASUREMENT OF S-ADENOSYL-L-METHIONINE-DEPENDENT METHYLTRANSFERASE ACTIVITY WITH PROTEIN SUBSTRATES

Abstract

Modification of protein residues by S-adenosyl-L-methionine (AdoMet)-dependent methyltransferases impacts an array of cellular processes. Here we describe a new approach to quantitatively measure the rate of methyl transfer that is compatible with using protein substrates. The method relies on the ability of reverse phase resin packed at the end of a pipette tip to quickly separate unreacted AdoMet from radiolabeled protein products. Bound radiolabeled protein products are eluted directly into scintillation vials and counted. In addition to decreasing analysis time, the sensitivity of this protocol allows for the determination of initial rate data. The utility of this protocol was shown by generating a Michaelis-Menten curve for the methylation of hnRNP K protein by human protein arginine methyltransferase 1, variant 1 (hPRMT1v1) in just over an hour. An additional advantage of this assay is the >3000-fold reduction in radioactive waste over existing protocols.

Introduction

S-Adenosyl-L-methionine (AdoMet)-dependent methyltransferases are a widespread class of enzymes present in both prokaryotes and eukaryotes. Small molecule, nucleic acid and protein methyltransferases play important roles in fundamental cellular processes such as replication and repair of DNA, transcription, translation, and signal transduction, reviewed

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in [1-4]. Although small molecule and nucleic acid methyltransferases have been well studied, the biochemical characterization of protein methyltransferases has advanced more slowly. The use of peptide substrates is the most common approach employed in enzymatic studies of the latter [5-10]. Used in conjunction with novel assay systems [8, 10-13], these studies have provided a first glimpse of the catalytic mechanism [6, 7, 9, 14, 15]. However, the ability to translate or confirm these findings with authentic protein substrates of the protein methyltransferases is required to address questions about substrate recognition and specificity in vivo.

One of the most common protein methylations takes place on the nitrogens of lysine, and arginine, with histidine, asparagine, and glutamine being additional, but rare targets [16]. Although arginine methylation nearly hid in the shadows for more than 30 years [17, 18], a rapid succession of reports documenting the biological impact of protein arginine methylation has catapulted interest in this posttranslational modification [19-27]. Methylation of the terminal guanidino nitrogens of arginine occurs via the protein arginine methyltransferases (PRMTs), a family of eleven human enzymes that target both nuclear and cytosolic proteins [28]. The diverse set of substrates poses questions about how each PRMT isoform selects substrates. Two approaches to measuring the rate of methyl transfer are to measure the by-product, S-adenosylhomocysteine (AdoHcy) or to directly measure the methylated product. Several novel assays have been developed in recent years to measure AdoHcy formation [8, 11-13]. However, many of them are limited to using peptide substrates or require the use of coupling enzymes. Moreover, many are not compatible when the investigator wishes to determine the inhibitory effect of AdoHcy on enzyme activity.

The most commonly used method for analyzing methylated proteins is by using radiolabeled AdoMet (S-adenosyl-L-[methyl-³H]-methionine; [³H]-AdoMet). SDS-PAGE is used to separate unreacted [³H]-AdoMet from radiolabeled protein products. Although this separation
is facile, the detection of the radiolabeled proteins can be quite time consuming, especially if the specific activity of the $[^3\text{H}]$-AdoMet is low or few residues of the protein are modified. Two methods are described in the literature. In the first, protein bands in acrylamide are crushed, the protein is extracted, and radiolabel associated with the proteins is detected by liquid scintillation counting. This method is quite laborious and tedious, taking on the order of >12 hours to obtain efficient retrieval of the proteins from the acrylamide [29]. It is also difficult to assess the efficiency of the extraction. In the second method, radiolabeled proteins are detected, either in-gel or post-transfer to a membrane, by phosphorimaging/fluorography. Depending on the number of protein residues that can be methylated and the specific activity of the $[^3\text{H}]$-AdoMet, this step can be several weeks to month long [29, 30]. A standard curve that relates band intensity to moles of $[^3\text{H}]$-methyl groups must also be done in order to provide quantitative results. These methods are outlined in Scheme 1.

Here we report a novel approach for conducting AdoMet-dependent protein methylation assays (patent-pending) using ZipTip® C4 pipette tips. The time-consuming step of separating unreacted $[^3\text{H}]$-AdoMet from radiolabeled protein products has been drastically shortened (Scheme 1). Unlabeled AdoMet can be added to reactions to furnish saturating concentrations of AdoMet while maintaining sufficient detection sensitivity, even at short reaction times. Coupled with the ability to use nanomolar enzyme concentrations and low protein substrate concentrations, this protocol is also highly suitable for measuring initial rates under saturating conditions. An added benefit of this protocol is the reduction in the volume of radioactive waste by more than ~3000-fold. We demonstrate the utility of this protocol by rapidly generating a Michaelis-Menten curve for the methylation of hnRNP K protein by hPRMT1v1 and evaluating the effect of a GST tag on PRMT6.
Scheme 1. Comparison of currently used protocols for measuring protein methylation with the ZipTip protocol. The total time (*) for analysis by method I reflects conditions used to monitor a single end-point, usually under conditions of long enzyme incubation times. Short reaction times required to collect initial rate data necessitate much longer exposure times as shown in this paper. The separation of unreacted AdoMet and methylated protein is extremely fast when using the ZipTip protocol.

### Materials and Methods

#### Materials

ZipTipC4 pipette tips were purchased from Millipore. The ZipTipC4 is a pipette tip with 0.6 μL of reversed-phase (C4; silica, 15 μm, 300 Å pore size) chromatography resin fixed at the tip. As noted by the manufacturer’s specifications, these tips have a binding capacity of ~3.3 μg protein per tip. Tagless human PRMT1 variant 1 (hPRMT1v1) was expressed and purified as described previously [31]. Histone H3.3 was purchased from New England Biolabs. His-hnRNP K was expressed and purified according to [32]. S-adenosyl-L-[methyl-3H] methionine (specific
activity 67.3 Ci/mmol, 0.55 μCi/μl) was purchased from Perkin Elmer. S-Adenosyl-L-methionine is more stable in acidic conditions and so is usually provided in dilute acid. For instance, S-adenosyl-L-[methyl-3H] methionine purchased from Perkin Elmer is provided in 10 mM sulfuric acid: ethanol (9:1). Care should be taken to store AdoMet under acidic conditions and at low temperatures to minimize hydrolysis and decomposition.

Expression and purification of His-PRMT6

E. coli BL21 (Codon plus) cells overexpressing His-PRMT6 were grown in 1 L Luria-Bertani (LB) broth at 37°C to an OD600 of 0.6. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 22°C for 24 hours. Cells were harvested by centrifugation and resuspended in 30 ml loading buffer (50 mM sodium phosphate, 300 mM NaCl and 10 mM Imidazole; pH 7.4). Cells were lysed by sonication and cell debris was removed by centrifugation at 12,000 g at 4°C for 10 min. The supernatant was incubated with 3 ml packed HisPur cobalt resin (Pierce) at 4°C with end-over-rotation for 3 hours. The resin was washed with ~300 ml of loading buffer. Bound His-PRMT6 was eluted using a linear imidazole gradient from 10–150 mM imidazole in loading buffer. Fractions showing the presence of His-PRMT6 by SDS-PAGE analysis were concentrated using an Amicon Ultra centrifugal filter device with a 30 kDa molecular weight cutoff. Buffer was exchanged to buffer containing 50 mM sodium phosphate, pH 8.0 according to the manufacturer’s instructions. His-PRMT6 was further purified using a MonoQ column (GE healthcare). His-PRMT6 was loaded on to the column in 50 mM sodium phosphate, pH 8.0. The column was washed with loading buffer until the absorbance at 280 nm approached the baseline. Bound protein was then eluted using a stepwise salt gradient from 0 to 1.0 M NaCl. Fractions were analyzed by SDS and fractions containing His-PRMT6 were pooled and concentrated as above and buffer exchanged to loading buffer. Protein was stored at -80°C in 10% glycerol. Approximately 70 μg of purified protein was obtained from 1 L of LB broth.
Expression and purification of GST-PRMT6

E. coli BL21 (Codon plus) cells overexpressing GST-PRMT6 were grown in 1 L LB broth at 37°C to an OD₆₀₀ of 0.6. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 22°C for 24 hours. Cells were harvested by centrifugation and resuspended in 60 ml ice-cold phosphate buffered saline (PBS). Cells were pelleted by centrifugation at 3000 g at 4°C for 10 min, the supernatant was discarded and cells resuspended in 60 ml ice-cold PBS. Cells were lysed by sonication and cell debris was removed by centrifugation at 12,000 g at 4°C for 10 min. The supernatant was incubated with 4 ml of GST resin slurry (GenScript) at 4°C with end-over-rotation for 3 hours. The resin was washed with ~200 ml of PBS and bound GST-PRMT6 eluted using a linear gradient from 0–10 mM reduced glutathione in 50 mM Tris-HCL, pH 8.0. Fractions containing GST-PRMT6 were concentrated using an Amicon Ultra centrifugal filter device with a 30 kDa molecular weight cutoff. Buffer was exchanged to buffer containing 50 mM sodium phosphate, pH 8.0 according to the manufacturer’s instructions. GST-PRMT 6 was further purified using a MonoQ column (GE healthcare). GST-PRMT6 was loaded on to the column in 50 mM sodium phosphate, pH 8.0. The column was washed with loading buffer until the absorbance at 280 nm approached the baseline. Bound protein was then eluted using a stepwise salt gradient from 0 to 1.0 M NaCl. Fractions were treated as described above. Protein was stored at -80°C in 10% glycerol. Approximately 100 μg of purified protein was obtained from 1 L of LB broth.

Composition of the methyltransferase reaction for the determination of PRMT activity

Assays were performed at 37°C, in a final volume of 125 μl. Each assay contained 1.0 μM unlabeled S-adenosyl-L-methionine (AdoMet), 0.06 μCi/μl S-adenosyl-L-[methyl-³H]methionine (specific activity 67.3 Ci/mmol, 0.55 μCi/μl, Perkin Elmer), 100 mM HEPES, 0.38 μM bovine serum albumin (BSA), 10% glycerol, 10 nM AdoHcy Nucleosidase (MTAN),
and 1.7 μM protein substrate; pH of reaction was 7.6. The addition of unlabeled AdoMet is to ensure that saturating conditions for enzymatic activity are met. Each reaction was equilibrated at 37°C for 3 min and then initiated with 100 nM PRMT. Samples (10 μL) were removed at specified times and were analyzed using either SDS-PAGE or ZipTips as discussed below. Sampling the reaction over time is preferable because it results in several data points to calculate the rate of methyltransfer instead of a single end-point. It also allows the investigator to demonstrate linearity of the response over time.

**Separation of [3H]-AdoMet and methylated proteins by SDS-PAGE**

A 10 μL aliquot of the reaction mixture was transferred to 5 μl 4x SDS sample buffer to quench the reaction. Unreacted [3H]-AdoMet was separated from the methylated protein products by SDS-PAGE. Protein was transferred to a PVDF membrane. Transfer efficiency was monitored to completion by using prestained protein molecular weight markers that were run in adjacent lanes. Methylation was detected in one of two ways. The membrane was sprayed with EN3HANCE (Perkin Elmer) according to the manufacturer’s instructions, and methylation was detected by fluorography [30] after incubating with film for 10 weeks. Multi Gauge software V2.3 (FujiFilms) was used to quantitate band intensity. Alternatively, protein bands were cut out of the membrane and counted in scintillation cocktail on a liquid scintillation counter (LSC).

**Separation of [3H]-AdoMet and methylated proteins by ZipTips**

ZipTips were used with solvent compositions and volumes according to the manufacturer’s instructions. Briefly, a 10 μL aliquot of the reaction mixture was transferred to 6 μl of sample preparation buffer (8 M Guanidine-HCL in 2.5% trifluoroacetic acid (TFA)), making the final concentrations 3 M Guanidine-HCL and 0.94% TFA in the mixture. Guanidine-HCl denatures the protein and TFA makes the reaction very acidic so that PRMT activity is
quenched. A ZipTipC₄ (ZipTip), one for each time point, was then prewetted in wetting buffer (75% acetonitrile in deionized H₂O) by pipetting up and down twice. The ZipTip was equilibrated by washing twice with equilibration buffer (0.1% TFA in deionized H₂O). Using the pre-equilibrated ZipTip, each quenched reaction was pipetted up and down 20 times, so as to bind the protein in the reaction to the C4 resin. The ZipTip was washed 17 times with 10 μl wash buffer (0.1% TFA in deionized water) to remove unreacted [³H]-AdoMet. Bound protein was eluted by aspirating and dispensing 10 μl of elution buffer (75% acetonitrile in 0.1%TFA) 10 times in the same tube. Eluate was dispensed into 20 ml of scintillation cocktail (Fisher Scientific) and counted in a LSC.

**Binding and elution efficiencies**

The binding efficiency of hnRNPK to the ZipTips was determined in the following manner. A methylation reaction as described above was incubated at 37°C for 1.5 hours. This sample was rapidly desalted on a DG-10 column (Bio-Rad) to remove most of the unreacted AdoMet and then further dialyzed in buffer containing 6 M Guanidinium hydrochloride and 100 mM Hepes at pH 8.0. The dialysis buffer (1L) was changed three times. Volumes (3, 6, and 10 μL) of the dialyzed protein were counted directly in scintillation cocktail (representing the total amount of methylated protein). Additional volumes (3, 6, and 10 μL) were applied to three separate ZipTips. Methylated protein eluting from the ZipTips was counted as described above (representing the amount of methylation protein retrieved from the column).

**Results and Discussion**

*Separation of unreacted [³H]-AdoMet from incorporated tritium using ZipTipC₄*

Innovation of the current methodology hinges on accelerating the separation of radiolabeled-methylated protein products from unreacted radiolabeled AdoMet. In order to meet
this criterion, we turned our attention to reverse phase (RP) chromatography. RP resins have been used for more than a quarter of a century for the separation of peptides and proteins [33]. Under denaturing conditions which promote protein unfolding, exposed hydrophobic regions of proteins interact with the RP resin. Although HPLC is a common format when using RP chromatography, not all laboratories are equipped with this type of instrument. Instead we focused on an RP-based format which is easy to use and easily obtainable. The ZipTip houses a small amount of C4 RP resin at the end of a pipette tip and is commonly used to desalt proteins prior to mass spectroscopy. To determine if free [³H]-AdoMet can be separated from the radiolabeled protein using the ZipTips, a methyltransferase assay was divided into two equal parts; one part was initiated with PRMT1 while the other half was initiated with water (control). Both reactions were incubated at 37°C for 12 minutes and then were terminated. ZipTips with C4 resin were used to bind the protein in the reaction and then washed and eluted as described in the Materials and Methods section. When the bound protein was eluted, a radioactive peak was observed in the reaction initiated with hPRMT1v1 (Fig. 4-1A) and not with water (Fig. 4-1B). The background binding of [³H]-AdoMet or its breakdown products was less than 4.5 % of the signal under these conditions (Fig. 4-1C). Additionally, the same amount of background radioactivity was observed when the control was performed without protein substrate (data not shown). Baseline separation of the two peaks of radioactivity was observed using 17 washes (10 μL each). These results demonstrate that ZipTips afford a rapid and easy way to separate unreacted AdoMet from methylated protein products.
Figure 4-1. Elution profiles of radioactivity showing the separation of unreacted [3H]-AdoMet from incorporated 3H-methyl using ZipTips. 1.7 μM hnRNP K was incubated with 1.0 μM unlabeled AdoMet, 0.6 μCi/μl [3H]-AdoMet, 100 mM Hepes, 0.38 μM BSA, 10% glycerol, 10 nM MTAN and 100 nM hPRMT1v1, at 37°C for 12 min. A 10 μL aliquot was loaded onto a ZipTip using a pipetman. Unreacted [3H]-AdoMet was removed using 17 washes and the methylated protein eluted using organic solvent. Panel (A) shows the initial peak of radioactivity in fractions 1 and 2 representing unreacted [3H]-AdoMet followed by methylated protein in fractions 5 and 6. Panel (B) shows the profile from the control reaction demonstrating a lack of radioactivity in fractions 5 and 6. When the control reaction was run without protein substrate, the profile was the same (data not shown). Panel (C) represents a zoomed plot of fractions 5 and 6 from plots (1A) and (1B).
Binding and elution efficiencies

In order to determine the binding and elution efficiencies of hnRNPK to the ZipTips, we first prepared a sample of radiolabeled, methylated hnRNPK as described in the materials and methods section. Extensive dialysis was used to remove unreacted AoMet from the reaction mixture. The amount of methylated protein was determined by direct measurement using scintillation counting and plotted as a function of sample volume (Figure 4-2). The same volumes of methylated protein were applied to three separate ZipTips, the tips treated as described above, and the eluted proteins analyzed by scintillation counting. Figure 4-2 shows that all the radiolabeled protein applied to the column was retrieved from the ZipTips. The data also demonstrate that recovery is linear over a range of protein concentrations. Additionally, the current protocol uses a 75 % acetonitrile in 0.1 % TFA solution to elute protein products from the ZipTip (as suggested by the manufacturer). In order to determine if complete elution of protein products was obtained, an additional elution step using 100 % acetonitrile in 0.1 % TFA was performed. No additional radioactivity was observed in this additional elution step (data not shown). This result suggests that the conditions described in the materials and methods section are sufficient for complete binding and elution of hnRNPK protein products.

Comparison of the ZipTip assay to currently used protocols

We compared commonly used methods for measuring methylation of proteins to the ZipTip method. Three identical methyltransferase assays were set up as described in the methods, but different approaches were employed in the separation of unreacted \(^{3}\text{H}\)-AdoMet and/or the detection of methylation. When the reaction products were separated by SDS, transferred to a membrane and the membrane incubated with film, it took 10 weeks for methylation to be detected by fluorography (Fig. 4-3A). A two week exposure resulted in a blank film (data not shown). This shows that the sensitivity of this method is low. Higher sensitivity can be obtained by increasing the specific activity of the \(^{3}\text{H}\)-AdoMet but one is limited by the
acid that the AdoMet is supplied in, the cost of the radiolabel, and the desire to maintain saturating levels of AdoMet in the reaction. It should be noted that the data in Figure 4-3A were obtained using a saturating concentration of protein substrate. This suggests that reactions performed using sub-saturating concentrations of protein substrates would likely take months to analyze.

Figure 4-2. Measuring the binding and elution efficiencies of hnRNP K protein on ZipTipC4. [3H]-Methylated hnRNP K protein, free of any unreacted [3H]-AdoMet, was prepared as described in the materials and methods section. The amount of radioactivity associated with hnRNP K was measured and plotted as a function of volume (open circles). The same volumes of hnRNP K were processed using the ZipTip protocol. The amount of radioactivity associated with the eluted protein is shown by the closed circles.
Figure 4-3. Monitoring methylation by SDS-PAGE-based methods. Two identical assays were performed and SDS-PAGE used to separate unreacted $[^3]$H-AdoMet from methylated protein products. Reaction mixtures contained $1.0 \, \mu$M unlabeled AdoMet, $0.6 \, \mu$Ci/$\mu$l $[^3]$H-AdoMet, $100 \, \text{mM}$ Hepes, $0.38 \, \mu$M BSA, 10% glycerol, 10 nM MTAN, and $1.7 \, \mu$M hnRNP K protein; pH 7.6 in a final volume of 125 µl. Reactions were carried out at 37°C. Each reaction was equilibrated at 37°C for 3 min and then initiated with 100 nM hPRMT1v1. Samples (10 µL) were removed at specified times and reactions halted with 5 µL 4X SDS sample buffer. After SDS-PAGE analysis, proteins were transferred to a PVDF membrane. In (A) the membrane was sprayed with EN$^3$HANCE and exposed to film for 10 weeks. In (B) the protein bands at the appropriate molecular weight were cut from the membrane and counted in scintillation cocktail on an LSC. Plotted values have background counts associated with a $t = 0$ timepoint subtracted from them.

We found we could decrease the analysis time substantially by measuring the radiolabel associated with the PVDF membrane directly by liquid scintillation counting. After electrophoretic transfer, small pieces of the PVDF containing the protein of interest were cut from the membrane and transferred to liquid scintillation cocktail and counted on an LSC. Figure 4-3B
shows that this method yielded a linear, time-dependent increase in the amount of counts representing an increase in methylated protein with time. Although this modification decreased the amount of time required to analyze protein methylation by several weeks, large amounts of contaminated electrophoresis buffers were generated. Furthermore, the number of assays analyzed was limited by the number of gels that could be run.

With the third reaction, we employed the ZipTip method as described above and in ~38 minutes obtained a rate curve (Fig. 4-4A). Rates observed with the ZipTip protocol were comparable to the modified electrophoresis-based protocol (9.6 nMCH₃/min vs. 6.1 nMCH₃/min). Increased counts using the ZipTip protocol are expected because membrane-bound radiolabeled product is subject to sample adsorbent shielding during counting. A striking difference between the assays was the amount of radiolabeled waste generated; less than 0.3 mL of radiolabeled waste was produced with the ZipTip assay versus 1,100 mL of contaminated electrophoresis buffers. To further support the time efficiency of this method, we were able to generate an entire Michaelis-Menten curve of the methylation of hnRNP K protein by hPRMT1v1 in ~70 minutes (Fig. 4-4B).

Other methods that are used to directly detect methylated protein products include protein hydrolysis and amino acid analysis of released N-methyl amino acids [29], detection by specific antibodies [34, 35], and mass spectroscopy [29, 32]. These methods are either labor intensive, limited to commercially available antibodies, or require specialized equipment needing technical expertise. Our results show that the ZipTip protocol is superior to current protocols for measuring the rate of AdoMet-dependent protein methylation.
Figure 4-4. Quantitative measurement of the rate of hPRMT1v1-catalyzed methylation of hnRNP K protein using the ZipTip protocol. Reaction mixtures contained 1.0 μM unlabeled AdoMet, 0.6 μCi/μl [³H]-AdoMet, 100 mM Hepes, 0.38 μM BSA, 10% glycerol, and 10 nM MTAN; pH 7.6 in a final volume of 125 μl. Reactions were carried out at 37°C. Each reaction was equilibrated at 37°C for 3 min and then initiated with 100 nM hPRMT1v1. Samples (10 μL) were removed at specified times and processed using the ZipTip C4. Plotted values have background counts associated with a t = 0 timepoint subtracted from them. Background counts at t = 0 were similar to counts obtained from control reactions having either no protein substrate or no enzyme (data not shown). (A) The rate of methylation of 1.7 μM hnRNP K protein by hPRMT1v1. (B) Michealis-Menten plot showing the methylation of hnRNP K protein by hPRMT1v1 as a function of hnRNP K concentration.

Comparison of His-PRMT6 and GST-PRMT6 activities using the ZipTip protocol

The ease with which the ZipTip assay is performed will now allow for a more thorough investigation of AdoMet-dependent protein methylation. In particular, we sought to quickly investigate the effect of the GST tag on the activity of PRMT6. Although the GST tag is a commonly used fusion tag in PRMT studies, investigations with PRMT1, PRMT8, and PRMT9 suggest that the N-terminus is involved in regulating methylation activity [29, 36, 37]. When we employed the ZipTip protocol to measure the rate of methylation of Histone H3.3 protein by His-
PRMT6 and GST-PRMT6, the activity of the two fusion proteins differed greatly. Two identical assays were initiated with His-PRMT6 and GST-PRMT6. We found that His-PRMT6 had 5-fold higher activity compared to GST-PRMT6 (Fig. 4-5) at 500 nM enzyme. The GST tag therefore affects the activity of PRMT6. This might be due to the fact that GST can form dimers which may affect the oligomeric structure of PRMT6 [36].

**Figure 4-5.** A comparison of the methylation activities of GST-PRMT6 and His-PRMT6 using the ZipTip protocol. 1.7 μM Histone H3.3 was incubated with increasing amounts (0.03 - 0.5 μM) of GST-PRMT6 (open circles) or His-PRMT6 (closed circles), and 1.0 μM unlabeled AdoMet, 0.6 μCi/μl [3H]-AdoMet, 100 mM Hepes, 0.38 μM BSA, 10% glycerol, and 10 nM MTAN; pH 7.6 in a final volume of 125 μl. Reactions were carried out at 37°C. Each reaction was equilibrated at 37°C for 3 min and then initiated with the appropriate amount of PRMT6. The reactions were processed by ZipTip and counted in scintillation cocktail. Plotted values have background counts associated with a t = 0 timepoint subtracted from them. Background counts at t = 0 were similar to counts obtained from control reactions having either no protein substrate or no enzyme (data not shown).
Considerations

Despite the usefulness and speed of this assay, there are limitations. For instance, this assay is sensitive to reproducible pipetting. Also attention should be paid to the fact that pipetting has to be done slowly since the pipette tips have resin at their ends and this gives a very small back pressure. Pipetting slowly will make sure that buffer moves effectively through the packed resin bed. To save more time when processing many samples, we tried to use a multichannel pipette with the ziptips but the pipette did not pull up buffer in all tips at the same speed. This resulted in some samples being washed more than others causing the resulting counts to be skewed.

Although a large majority of proteins are expected to bind to C4 reverse phase resin, small protein and peptide substrates may require the use of C18 reverse phase resin which is also available in the ZipTip format. Millipore quotes the capacity of ZipTip

\[ \text{C4} \] at \(~3.3\ \mu\text{g}; therefore care should be taken not to load greater than 3.3 \mu g when setting up an assay. The binding and elution efficiency for each protein substrate/product can easily be determined as described in this paper. Additionally, because the ZipTip does not offer any separation between different proteins, this method is not suitable when a mixture of protein substrates is used. However, the latter case can be addressed using the modified SDS-PAGE protocol and direct counting as described in this paper.

Conclusions

This study shows that the ZipTip

\[ \text{C4} \] protocol can be used to quantitate AdoMet-dependent protein methylation as exemplified by the PRMT family of enzymes used in this work. It is also highly probable that this assay will work for other AdoMet-dependent protein methyltransferases such as lysine methyltransferases and carboxylmethyltransferases. Three characteristics of this protocol differentiate it from currently used methods: 1) Separation of unreacted AdoMet and methylated protein is extremely fast. Scheme 1 compares previous protocols to the ZipTip
protocol. 2) Because the methylated protein is directly measured using liquid scintillation counting, the ability to detect small amounts of radiolabel is increased. Detection of small amounts of methyltransfer during the early part of the reaction allows steady state kinetics to be measured with this protocol. Given the current conditions of the reaction, the lower limit of detection of this assay is 0.12 nM CH₃/min. The detection limit could be increased by increasing the specific activity of the [³H]-AdoMet in the assay or by using C14-labelled AdoMet. 3) The volume of radiolabeled waste generated is decreased by >3000-fold. Furthermore, given the commercial ability of C4 loaded multiwell formats, this protocol could easily be optimized for high through-put screens. The absence of coupling enzymes in this protocol will reduce false positive hits, making this protocol highly suitable for screening for modulators of protein methyltransferase activity.

References


CHAPTER 5

ANALYSIS OF THE EFFECT OF THE N-TERMINUS OF hPRMT1-V1 ON ENZYMATIC ACTIVITY, PROTEIN-PROTEIN INTERACTIONS, AND SUBSTRATE SPECIFICITY

Abstract

PRMT1 is the predominant PRMT in mammals performing over 85% of the total arginine methylation. It is essential for early embryonic development in mice, and has also been implicated in cellular processes such as signal transduction, DNA repair, and transcription regulation. It has previously been suggested that the N-terminus (N$_T$) of PRMTs is involved in enzymatic activity, protein-protein interaction, and substrate specificity. However, some of these studies were done using C-terminal tagged PRMTs (which have low activity) and pieces of proteins attached to large tags such as glutathione-S-transferase (GST) which do not correctly reflect physiological substrates. Here we describe the effect of truncating the N$_T$ of human PRMT1 variant 1 (hPRMT1-V1) on enzymatic activity, protein-protein interactions, substrate specificity, and oligomerization, using untagged hPRMT1-V1 truncation constructs, chimera and full length proteins. Our results suggest that a small piece of the N$_T$ (10 amino acids long) is involved in the negative regulation of the enzymatic activity of hPRMT1-V1. Also, our results suggest that the N$_T$ is involved in protein-protein interactions and that the 10 amino acid piece at the N$_T$ might also be involved in regulating hPRMT1-V1 interaction with certain proteins. Furthermore, we show that the first 10 amino acids of the N$_T$ are not involved in hPRMT1-V1 oligomerization.

Introduction

The human genome is predicted to have about 30,000 genes, but only about 1.5% of the genome codes for proteins [1, 2]. Surprisingly, the proteome contains about two to three orders of magnitude more protein isoforms than the number of genes in the genome. This ability of cells
to expand on their limited genome is due to their utilization of processes like alternative splicing and posttranslational modification (PTM). PTMs are chemical modifications that occur on proteins after their translation. More than 5% of human genes encode for the enzymes that catalyze these modifications. Examples of these enzymes include protein kinases, acetylases, glycosyl transferases, hydroxylases, ligases, prenyl transferases and methyl-transferases.

Considering the fact that PTMs alter protein structure and function, the substrate specificity and activity of these modification enzymes is crucial for cell function. However, not very much is currently understood about the substrate specificity of most modification enzymes and also how their activity is regulated. That is the case with protein arginine methyltransferases (PRMTs) which are enzymes that catalyze the addition of monomethyl or dimethyl groups to the guanidino nitrogen atoms of arginines in protein substrates. The activity of PRMTs results in the formation of either monomethyl arginine (MMA), asymmetric dimethylarginine (ADMA) or symmetric dimethylarginine (SDMA) in their protein substrates. A total of nine PRMTs have been reported in humans, classified as either type I or type II. Type I PRMTs (PRMT1, -3, -4, -6, and -8) catalyze the formation of MMA and ADMA, while type II PRMTs (PRMT5, 7, and 9) catalyze the formation of MMA and SDMA [3]. PRMTs are increasingly gaining attention as more and more substrates with central roles in cellular processes such as signal transduction, DNA repair, RNA processing, and transcriptional regulation are being discovered [4, 5].

However, several questions still remain unanswered in this emerging field. For example, it is still unknown how the activity of PRMT1 (the principal PRMT in mammalian cells) towards numerous proteins is regulated. Although a few studies have shed light on this issue [6, 7], a lot still remains to be done for it to be better understood. Another question of great interest in the field of arginine methylation is how substrate specificity is achieved amongst the PRMTs. A consensus sequence for PRMT-dependent methylation has not yet been identified. Typically, PRMTs target proteins containing glycine arginine rich (GAR) motifs with the methylated
arginines mainly found in ‘RGG’ or ‘RXR’ regions. However, some PRMT substrates deviate
from this model and recent studies have shown that PRMTs also recognize other amino acid
sequences outside the “RGG” paradigm [8]. So how do PRMTs which have a highly conserved
active site demonstrate such differences in substrate specificity?

PRMTs possess a homologous core domain of about 310 amino acids which contains
both protein substrate and s-adenosyl-L-methionine (AdoMet) binding sites. Each PRMT
isoform has a unique N-terminal (N_T) extension, while PRMT4 and -7 have additional C-terminal
extensions. The role of this unique N_T is not well understood, but several studies suggest that it is
involved in substrate specificity and enzymatic activity. For instance, it has been suggested that
distinct substrate specificities exist amongst the seven spliced variants of human PRMT1 which
only differ at their N_T in both sequence and length [9]. Also when the N_T of PRMT3 is deleted its
enzyme activity decreases and its substrate specificity is altered [10, 11]. Likewise, the
oligomerization of the yeast arginine methyltransferase, Hmt1 is considerably hindered and its
enzymatic activity decreased when its N_T is deleted [12].

From the crystal structures of PRMT1 and PRMT3, it was determined that these two
isoforms possess X and Y motifs which are important for activity as truncations in the X helix of
PRMT1 resulted in inactive proteins [13, 14] and mutation of the conserved methionine 155 in
the Y helix abolished methyltransferase activity in PRMT1 [13]. The secondary structures of
PRMT1, 3, 4 and 6 show the presence of the X, Y and Z motifs. By using sequence alignments
(CLUSTAL) and prediction algorithms (PSIPRED), the secondary structures of the PRMTs
without crystal structures can be obtained while that of those with crystal structures (PRMT1 and
PRMT3) have already been determined (Fig. 5-1). Since the PRMTs have similar core regions
and X helices, it is therefore possible that any portion of the N_T which influences substrate
preference is N-terminal to the X helix where their sequences differ. Therefore if the part of the
N_T that affects enzymatic activity and/or substrate recognition is either absent or substituted,
differences in substrate preference would be observed.

**Figure 5-1.** Sequence and structural homologies between Type I PRMTs. Secondary structure was predicted using Psipred from the Expasy.org website. Strictly conserved residues amongst all PRMTs are shown in bold face and exist in grey boxes. Arrows indicate residues that were observed interacting with the co-substrates in the crystal structures.

In this chapter, we investigate the effect of; (i) truncating the N_T of human PRMT1 variant 1 (hPRMT1-V1) and, (ii) switching the N_T of PRMT1 with that of PRMT6 in a protein chimera, on methyltransferase activity, protein-protein interactions, substrate specificity, and oligomerization of hPRMT1-V1. In doing this, both the X and Y helices of all the constructs were kept intact (Fig. 5-2). Overall we observed changes in both activity and substrate specificity dependent upon where in the N_T the enzyme was truncated. Also we can deduce from our data that the N_T of hPRMT1-V1 does not play a crucial role in oligomer formation. Our study also suggests that the first 10 amino acids of the N_T of hPRMT1-V1 may function in negatively
regulating enzymatic activity and potentially protein-protein interactions with certain protein substrates.

**Figure 5-2.** Sequence alignment of hPRMT1-V1 truncation constructs (hPRMT1-V1-M1, hPRMT1-V1-M11, hPRMT1-V1-E27, and hPRMT1-V1-S31) and PRMT6 N:\ hPRMT1-V1-E27 chimera. hPRMT1-V1-M1 is the full length protein starting with methionine 1. hPRMT1-V1-M11 is truncated beginning with methionine 11 and so is hPRMT1-V1-E27 which begins with glutamate 27. The entire N:\ is deleted in hPRMT1-V1-S31 which begins with serine 31. The first 41 amino acids of the PRMT6 N:\ hPRMT1-V1-E27 chimera corresponds to the N:\ of PRMT6 and the second half contains hPRMT1-V1 core starting at glutamate 27. All the constructs contain the X, Y and Z helices.

**Materials and Methods**

**Materials**

ZipTip\textsuperscript{c}4 pipette tips were purchased from Millipore (described in Ref. [15]). Histone H4 and Histone H3.3 were purchased from New England Biolabs. Untagged hPRMT1-V1 (accession number: AAF62895) constructs (hPRMT1-V1-M1, hPRMT1-V1-M11, hPRMT1-V1-E27, and hPRMT1-V1-S31) and His-hnRNP K (accession number: NP_002131) were expressed and purified as described previously [15]. GST-MRE11 (accession number:...
NP_005581) was obtained from our collaborator Jocelyn Cote (University of Ottawa, Ontario, Canada). S-adenosyl-L-[methyl-³H] methionine (specific activity 70.8 Ci/mmol, 0.55 μCi/μl) was purchased from Perkin Elmer. S-Adenosyl-L-methionine is more stable in acidic conditions and so is usually provided in dilute acid. For instance, S-adenosyl-L-[methyl-³H] methionine purchased from Perkin Elmer is provided in 10 mM sulfuric acid: ethanol (9:1). Care should be taken to store AdoMet under acidic conditions and at low temperatures to minimize hydrolysis and decomposition.

The making and purification of the PRMT6 N-terminus: hPRMT1-V1 core chimera

The PRMT6 NT (accession number: NP_060607; residues 1-39) was PCR amplified using downstream primers which added an Xho1 restriction site to the 3’ end. In amplifying the hPRMT1-V1 core (residues 27-356), the upstream primers added an Xho1 site to the 5’ end while the downstream primers added a Pst1 site to the 3’ end. The resulting PCR products were digested with an Xho1 restriction enzyme and ligated using the rapid ligation kit from Fermentas. The ligation mixture was resolved on a 0.8% agarose gel and the ligation product at the correct molecular weight (1.2 Kbp) was gel extracted, using a gel extraction kit from Qiagen. This DNA ligation product was then PCR amplified with the addition of both a Kpn1 restriction site and a TEV cleavage site to the 3’ end of the PRMT6 segment of the construct. This DNA construct was then ligated into a pET 45b vector and E. coli XL-1 blue cells were transformed with the vector containing the insert and used to make more copies of the vector. The sequence of the chimera was confirmed by DNA sequencing. E. coli BL21 (DE3) cells overexpressing His-TEV-PRMT6 NT: hPRMT1-V1-E27 core chimera were grown in 0.5L of LB broth at 37°C to an OD₆₀₀ of 0.4. Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 22° for 24 hours. Cells were harvested by centrifugation and lysed by osmotic shock. The protein chimera was then purified and cleaved as described in [16].
In vitro methylation of \textit{hnRNP K} in the determination of the composition of the methyltransferase reaction

Assays were performed at 37°C, in a final volume of 125 µl. Each assay contained 500.0 µM unlabeled \textit{S}-adenosyl-L-methionine (AdoMet), 0.6 µM \textit{S}-adenosyl-L-[methyl-\textsuperscript{3}H]methionine, 100 mM Hepes buffer pH 8.0, 3.03 µM bovine serum albumin (BSA), 10% glycerol, 10 nM AdoHcy Nucleosidase (MTAN), and 1.7 µM \textit{hnRNP K}. Reactions were each equilibrated at 37°C for 3 min and then initiated with 6.25 pmol hPRMT1-V1-M1. The concentrations of BSA and AdoMet were then varied as discussed in the results section. Samples (10 µL) were removed at specified times and analyzed by SDS-PAGE. The methylated protein was then transferred to a Polyvinylidene Fluoride (PVDF) membrane and the protein bands cut out and counted in scintillation cocktail using a liquid scintillation counter.

\textit{Composition of the ZipTip methyltransferase reaction for the determination of PRMT activity}

Assays were performed at 37°C, in a final volume of 125 µl. Each assay contained 1.0 µM unlabeled \textit{S}-adenosyl-L-methionine (AdoMet), 0.06 µCi/µl \textit{S}-adenosyl-L-[methyl-\textsuperscript{3}H]methionine (specific activity 70.8 Ci/mmol, 0.55 µCi/µl, Perkin Elmer), 100 mM Hepes, 0.38 µM bovine serum albumin (BSA), 10% glycerol, 10 nM AdoHcy Nucleosidase (MTAN), and indicated concentration of protein substrate; pH of reaction was 8.0. Reactions were each equilibrated at 37°C for 3 min and then initiated with 70 nM PRMT. Samples (10 µL) were removed at specified times and were analyzed using ZipTips as discussed previously [15].

\textit{In vitro methylation of endogenous protein substrates from PRMT\textsuperscript{-/-}ES cells}

The preparation of the \textit{PRMT}\textsuperscript{-/-}Embryonic stem (ES) cells and the endogenous protein substrates (total proteins) from them have previously been described [9]. The obtained cell extracts were heat-inactivated at 70°C for 10 min. The methylation reactions were performed at 37°C, in a final volume of 150 µl. Each reaction was composed of 15 µg of total protein (ES\textsuperscript{-/-})
cell lysate), 0.582 μM $^3$H-[AdoMet], and 70 nM PRMT, in 50 mM sodium phosphate buffer, pH 8.0. Reactions were each equilibrated at 37°C for 3 min and then initiated with the PRMT. Samples (30 μL) were removed at specified times and the methylated proteins resolved by SDS-PAGE. Proteins were then transferred from gel to PVDF membrane. The membrane was sprayed with EN3HANCE (Perkin Elmer) according to the manufacturer’s instructions, and methylated proteins were detected by fluorography. Fluorographs were exposed for four days at -80°C.

**Results**

*Determination of the reaction composition of a PRMT assay*

To determine the reaction composition of our PRMT assay, we looked at the literature and found that different studies used different reagent compositions in their PRMT assays, but no mention was made as to the selection criteria for each component [9, 17-19]. These reagents included dithiothreitol (DTT), detergent (e.g. Nonidet P-40 (NP-40)), potassium acetate (KOAc) and bovine serum albumin (BSA). To find out which of these reagents were actually vital for the activity and stability of the PRMT during the reaction, we removed each of these reagents one at a time from the reaction and observed the effect on the reaction rate. We found that the absence or presence of KOAc did not make a difference in the reaction rate (data not shown). It was also observed that in the absence of NP-40 or DTT, the reaction rate increased by 2-fold or 1.5-fold respectively. On the other hand, the reaction rate decreased by 2-fold when BSA was absent in the reaction mixture (Figure 5-3A). We concluded that BSA is an important component in the reaction mixture for the PRMT assay.

We then set out to find the lowest concentration of BSA that supports high PRMT activity. Because BSA is only one protein amongst a few other proteins in the reaction mixture, it is important to keep its concentration low so that there will be enough room for each protein to bind to the ZipTip which has a binding capacity of 3.3 μg. The effects of three concentrations of
BSA (1.52 µM, 0.76 µM, and 0.38 µM) lower than the concentration of BSA (3.03 µM) used in the previous reaction (Fig. 5-3A) were assessed in a reaction mixture that lacked KOAc, NP-40, and DTT. The effect of the absence of BSA on the reaction rate was also assessed. The lowest concentration of BSA tested, 0.38 µM, resulted in a reaction rate comparable to those shown by the higher BSA concentrations used (Fig. 5-3B). We therefore decided to use 0.38 µM BSA in the PRMT assays.

**Figure 5-3.** Determining the composition of the PRMT assay. (A) The activity of 100 µM hPRMT1-V1-M1 was assessed in a reaction mixture composed of 1.7 µM hnRNP K protein, 10 nM MTAN, 1.0 µM AdoMet, 0.6 µM ³H-AdoMet and 100 mM Hepes buffer pH 8.0, 10% Glycerol, 0.01% NP-40, 0.5 mM DTT, and 3.03 µM BSA. The reaction mixture was then modified by removing NP-40, DTT, or BSA, and then assessing the activity of hPRMT1-V1-M1 as described in the materials and methods. The reaction was initiated with hPRMT1-V1-M1. (B) The same reaction mixture used in (A) but lacking NP-40, and DTT, was utilized, with different concentrations of BSA.
hPRMT1-V1 enzymatic activity and substrate specificity can be influenced by the N-terminus

To determine the effect of the N-terminus (N_{T}) of PRMT1 on enzymatic activity and substrate specificity, four untagged human PRMT1 variant 1 (hPRMT1-V1) constructs were made including: full length hPRMT1-V1 (hPRMT1-V1-M1), a truncated construct starting from Methionine 11 (hPRMT1-V1-M11), a truncated construct starting from glutamate 27 (hPRMT1-V1-E27), and a truncated construct starting from Serine 31 (hPRMT1-V1-S31). In addition, a protein chimera consisting of the PRMT6 N_{T} (Residues 1 – 39) and the hPRMT1-V1 core starting from residue E27 (PRMT6 N_{T}: hPRMT1-V1-E27) was made (Fig. 5-2). If the N_{T} is involved in substrate recognition, differences in substrate preference would be observed if the N_{T} is absent or substituted. Alternatively, the N_{T} may be important for overall activity of the enzyme. Therefore, the methyltransferase activity of each untagged hPRMT1-V1 construct was assessed in vitro with both peptide and protein substrates. First, the activity of each untagged PRMT was assessed with the R3 peptide (acetylGGRGFGGRGGFGGRGGFG), using the continuous spectrophotometric assay previously described in [20] (Fig. 5-4). The R3 peptide is a widely used PRMT1 substrate that corresponds to residues 13 to 31 of the PRMT1 protein.
substrate, fibrillarin [13]. The K_m, app, V_max and k_cat obtained for each of the hPRMT1 constructs are shown in Table 1. hPRMT1-V1-M1, -M11 and -E27 all demonstrated similar enzymatic activity with the R3 peptide (Fig. 5-4 A, B and C). hPRMT1-V1-S31 had about 11-fold less activity compared to hPRMT1-V1-M1, -M11 and -E27. Several attempts to obtain good data with the chimera using the continuous assay were unsuccessful due to a lot of scatter, but the chimera did show enzymatic activity higher than hPRMT1-V1-S31, but less than hPRMT1-V1-M1, -M11 and -E27 (data not shown).

To test if the activity observed with peptides can be extended to more physiologically relevant substrates, the methyltransferase activity of each hPRMT1-V1 construct was assessed with protein substrates in vitro, using the ZipTip assay (previously described in [15]). hPRMT1-V1-M11 showed the most activity with the protein substrates tested. hPRMT1-V1- was second with about 14% more activity than hPRMT1-V1-M1 with the hnRNP K protein E27 (fig. 5-5A and B). Due to the low activity demonstrated by hPRMT1-V1-S31 with peptide (Table 1), it was not used in the in vitro assays with protein substrates. The PRMT6 N_T: hPRMT1-V1-E27 chimera, when tested with hnRNP K protein, showed significantly less activity (~11 fold less activity when compared to M1, ~18 fold less activity to M11 and ~13 fold less activity to E27) than hPRMT1-V1-M1, -M11 and -E27 (fig 5-5A). The chimera was not tested with any other purified protein substrates due to the low activity.
Figure 5-4. Steady-state analysis of the methylation of the R3 peptide by untagged hPRMT1-V1 constructs. Activity was assessed using a continuous spectrophotometric assay (2). The assay utilized various concentrations of R3 peptide [25-200μM] (GRGGFGGRGFGGRGFG), 4 μM hPRMT1-V1-M1 (hPRMT1-V1-M11), 10nM AdoHcy/MTA nucleosidase (MTAN), 0.02μM Adenine Deaminase, 10 nM MnSO4, 250μM S-Adenosylmethionine (SAM), and 50mM sodium phosphate buffer pH 7.1. R3 peptide was used to initiate the reactions.
Table 5-1. Summary of kinetic parameters of the hPRMT1-V1 protein constructs when methyltransferase activity with the R3 peptide substrate is assessed using the continuous spectrophotometric assay.

<table>
<thead>
<tr>
<th>hPRMT1-V1 Construct</th>
<th>$V_{\text{max}}$ (μM CH₂/min)</th>
<th>$k_{\text{cat}}$ (s⁻¹)</th>
<th>$K_{m/\text{peptide}}$ (μM)</th>
<th>$k_{\text{cat}}/K_{m}$ (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPRMT1-V1-M1</td>
<td>4.3 ± 0.2</td>
<td>0.018 ± 0.008</td>
<td>48 ± 6.2</td>
<td>3.7 x 10²</td>
</tr>
<tr>
<td>hPRMT1-V1-M11</td>
<td>3.2 ± 0.3</td>
<td>0.013 ± 0.001</td>
<td>38 ± 12</td>
<td>3.4 x 10²</td>
</tr>
<tr>
<td>hPRMT1-V1-E27</td>
<td>5.6 ± 0.3</td>
<td>0.023 ± 0.001</td>
<td>43 ± 7.0</td>
<td>5.4 x 10²</td>
</tr>
<tr>
<td>hPRMT1-V1-S31</td>
<td>0.40 ± 0.04</td>
<td>0.0017 ± 0.0002</td>
<td>16 ± 7.8</td>
<td>1.1 x 10²</td>
</tr>
</tbody>
</table>

Figure 5-5. Steady-state analysis of the kinetics of methylation of protein substrates by untagged hPRMT1-V1 constructs. Activity was assessed using the previously described ZipTip assay (Chapter 4). The assay utilized various concentrations of protein [0.2-10.0 μM] (hnRNP K or Histone H4), 70 nM hPRMT1-V1 constructs, 10 nM AdoHcy/MTA nucleosidase (MTAN), 1.0 μM S-Adenosylmethionine (SAM), 0.06 μCi/μL ³H-AdoMet and 100 mM Hepes buffer pH 8.0. The reactions were initiated with the respective hPRMT1-V1 construct. (A) And (B) A plot of the $K_{\text{cat}}$ of hPRMT1-V1-M1, -M11, -E27, and PRMT6 N:\ hPRMT1-V1-E27 chimera versus the concentration of hnRNP K or Histone H4 protein respectively. The $k_{\text{cat}}$ was plotted instead of the velocity because the concentration of chimera used in this assay was different from that used for the other constructs.
PRMT3 has an N_T which is 194 amino acids long. This acidic amino acid rich N-terminal extension is absent in PRMT1 (fig. 5-6). Unlike PRMT3 where deletion of the N_T at residue 183 (fig. 5-6) resulted in a decrease in enzymatic activity and a change in protein substrate specificity [10-11], truncating the N_T of hPRMT1-V1 at residue 10 increased enzymatic activity (k_cat, Table 2) towards purified protein substrates used but did not alter substrate specificity.

However, truncating at residue 27 increased substrate specificity towards Histone H4 protein as evidenced by an order of magnitude increase in kcat/Km. This result is similar to that obtained with PRMT8 where cleavage of its N_T (amino acid residues 1 to 60, fig. 5-6) resulted in a significant increase in enzyme activity [21] towards the GST-GAR protein. Adding the PRMT6 N_T to E27 in the chimera significantly decreased the activity of the enzyme (k_cat, Table 5-2) towards hnRNP K (fig. 5-5A).
Figure 5-6. Protein sequences of human PRMT1-V1 (Accession #: AAF62895), PRMT3 (Accession #: NP_005779), and PRMT8 (Accession #: NP_062828). The above protein sequences are aligned based on the beginning of the core (highlighted in grey). The arrows indicate the amino acid residues where each enzyme was truncated. This figure shows where each PRMT construct was cut and the proximity of the cut to the core.

To test if these observations can be broadened to endogenous protein substrates, hypomethylated cellular extracts obtained from PRMT1-/-ES cells were utilized in an *in vitro* assay with each of the untagged hPRMT1-V1 constructs as described above in the methods section. The results obtained were consistent with our observations when using purified recombinant protein substrates. hPRMT1-V1-M11 showed the most activity toward endogenous protein substrates in both total and cellular fractions followed by hPRMT1-V1-E27 and lastly...
Interestingly, \([^3H]\)-methyl incorporation was not observed with certain substrates at 30mins when hPRMT1-V1-M1 was utilized (fig 5-7A); marked with asterisks, compare lanes 4, 8, and 12). When treated with hPRMT1-V1-S31, no \([^3H]\)-methyl incorporation was observed with either the total cell lysate or nuclear cell extract (data not shown). PRMT6 Nt: hPRMT1-V1-E27 chimera showed low activity towards the endogenous protein substrates (fig. 5-7A, lanes l3-16 and fig. 5-7B lanes 13-16). Overall, minor differences were observed in the ability of the full length and truncated constructs to methylate endogenous protein substrates. Also, when the nuclear lysate was methylated, hPRMT1-V1-M1 showed difficulties in methylating some lower molecular weight bands which hPRMT1-V1-M11 and –E27 seemed to methylate with relative ease.

Table 5-2. Summary of the Michaelis constants of each of the hPRMT1-V1 protein constructs when the rate of methyltransferase activity with protein substrates is assessed using the ZipTip assay.

<table>
<thead>
<tr>
<th>Protein Substrate</th>
<th>hPRMT1-V1 Construct</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(K_{m\text{protein}}) ((\mu\text{M}))</th>
<th>(k_{cat}/K_{m}) (M(^{-1})s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>hnRNP k</td>
<td>hPRMT1-V1-M1</td>
<td>0.042 ± 0.001</td>
<td>0.76 ± 0.04</td>
<td>5.5 x 10(^4)</td>
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<td>hPRMT1-V1-M11</td>
<td>0.065 ± 0.002</td>
<td>0.80 ± 0.09</td>
<td>8.2 x 10(^4)</td>
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<td>hPRMT1-V1-E27</td>
<td>0.048 ± 0.002</td>
<td>0.77 ± 0.08</td>
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<td>PRMT6Nt: hPRMT1-V1-E27 core Chimera</td>
<td>0.010 ± 0.001</td>
<td>0.31 ± 0.08</td>
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<td>Histone H4</td>
<td>hPRMT1-V1-M1</td>
<td>0.025 ± 0.002</td>
<td>0.31 ± 0.09</td>
<td>8.1 x 10(^4)</td>
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<td>hPRMT1-V1-M11</td>
<td>0.035 ± 0.003</td>
<td>0.41 ± 0.13</td>
<td>8.5 x 10(^4)</td>
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<td>hPRMT1-V1-E27</td>
<td>0.0250 ± 0.0001</td>
<td>0.120 ± 0.002</td>
<td>2.1 x 10(^5)</td>
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hPRMT1-V1 truncation constructs have different enzymatic activities but not substrate specificities towards endogenous proteins. A) 15 μg of total endogenous protein substrates from mouse PRMT1−/− ES cells were incubated with equal amounts of purified untagged hPRMT1-V1 truncation constructs in the presence of [3H]-AdoMet. The resulting reactions were separated by SDS-PAGE and proteins transferred to PVDF membranes and 3H-labeled proteins were visualized by fluorography. Exposure of fluorographs was at -80 ºC for 4 days. Bands at the astericks were not visible at 30 minutes by hPRMT1-V1-M1 and the chimera. B) Cell extracts from mouse PRMT1−/− ES cells were fractionated using the QProteome nuclear protein kit from Qiagen. The nuclear fraction was obtained and dialyzed against 50 mM sodium phosphate buffer, pH 7.5. 10 μg of nuclear proteins were used in in vitro methylation assays performed same as in (A).
Is the N-terminus of hPRMT1-V1 involved in protein-protein interactions?

To investigate the involvement of the N-terminus of hPRMT1-V1 in protein-protein interactions, each hPRMT1-V1 construct including the chimera was incubated with recombinant human proteins immobilized on a glass slide (Invitrogen Protoarray® Human Protein Microarray Version 3.0). The recombinant proteins were expressed using Baculovirus expression in insect cells. The same molar amount of each construct was used to probe the proteins bound to the array according to the manufacturer’s instructions. A PRMT1 antibody (primary) was used to detect the presence of bound hPRMT1-V1 constructs and a fluorescent labeled secondary antibody was used to probe for the bound PRMT1 antibody. Each array was scanned using a fluorescent microarray scanner and the results analyzed. PRMT6 NT: hPRMT1-V1-E27 chimera interacted with the highest number of proteins, 56, followed by hPRMT1-V1-M11 which interacted with 48 proteins. hPRMT1-V1-E27 interacted with even fewer proteins, 26 and hPRMT1-V1-M1 interacted with the least number of proteins, 13 (Table 5-3). As a control, western blot analysis was done using equal molar amounts of the hPRMT1-V1 constructs and the PRMT1 primary antibody to show that the antibody detected all the constructs (data not shown).

Table 5-3. Large scale protein-protein interaction screen with purified recombinant untagged hPRMT1-V1-M1, -M11, -E27, and PRMT6 NT: hPRMT1-V1-E27 chimera, using the Invitrogen Protoarray® Human Protein Microarray Version 3.0.

<table>
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<th>hPRMT1-V1 Construct</th>
<th>Reference Number</th>
<th>Protein Name</th>
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<td><strong>M1 and E27</strong></td>
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<td></td>
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Is the N-terminus of hPRMT1-V1 involved in the formation of higher order oligomers in PRMTs?

The functional unit of PRMTs is the dimer. It has been shown that dimer formation is important for the methyltransferase activity of PRMTs [13]. However, PRMTs have been shown to exist as higher order oligomers in solution, not dimers [13]. To test if truncating the N-terminus of hPRMT1-V1 will influence the formation of these higher order oligomers in solution, gel filtration analysis of both the full length hPRMT1-V1-M1 and hPRMT1-V1-M11 truncated at...
residue 10 was done. It was observed that both hPRMT1-V1-M1 and hPRMT1-V1-M11 still existed as higher order oligomers in solution at approximately 400 KDa and 350 KDa respectively. To test if the binding of AdoHcy will affect the size of oligomers formed by hPRMT1-V1-M1 and –M11 in solution, we incubated AdoHcy with each PRMT construct for one hour before gel filtration analysis. A slight but not so significant shift in oligomer size was observed with both hPRMT1-V1 constructs (fig. 5-8). Truncating the N_T of hPRMT1-V1 did not hinder the formation of higher order oligomers in solution.

Figure 5-8. Molecular weights of untagged PRMT1 constructs determined by gel filtration. 15μM of untagged PRMT protein in 50mM phosphate buffer, pH 7.5 was loaded on to a gel filtration column (Phenomenex). Untagged PRMT constructs incubated with S-adenosylhomocysteine (SAH) were also analyzed but no significant shift in peaks was observed. Untagged PRMT constructs form higher-order oligomers as shown by gel filtration chromatography.
**Discussion**

*Composition of the PRMT assay reaction*

PRMTs are known to exist as higher order oligomers in solution; however it is not known how oligomerization affects their activity. It is possible that adding NP-40 (a detergent) to the PRMT assay mixture resulted in the disruption of not only these oligomers but also some of the PRMT-protein substrate interactions. This will lead to a decrease in the number of substrate arginines methylated, explaining the decreased activity observed in the presence of NP-40. Although many proteins may be protected from oxidative damage by the addition of reducing agents, we observed that methylation activity decreased in the presence of DTT and conclude that this reagent should be omitted from PRMT1 reactions.

The serum albumin protein, BSA is widely used in biochemical applications, including DNA digestion where it is used to stabilize some enzymes and prevent enzyme adhesion to reaction tubes. It is therefore not very surprising that the presence of BSA in the PRMT assay mixture resulted in a significant increase in reaction rate. We conclude that BSA should be added to PRMT reactions to stabilize the enzyme. It is worth noting that BSA has not been reported to be methylated by any of the PRMTs.

Our observations thus suggest that the reaction composition that will yield a high activity for hPRMT1-V1-M1 is: low nM hPRMT1-V1-M1, protein substrate, 10 nM MTAN, 1.0 μM AdoMet, 0.06 μCi/μL ³H-AdoMet, 100 mM Hepes buffer pH 8.0, 10% Glycerol, and 0.38 μM BSA. This is the reagent composition used in all subsequent PRMT assays with protein substrates.

*The N_T of hPRMT1-V1 influences enzymatic activity*

Several studies suggest that the N_T of PRMTs is involved in enzymatic activity; deleting the N_T of both PRMT3 and yeast arginine methyltransferase, Hmt1 resulted in a decrease in
enzymatic activity [10-12], and more recently, an increase in enzyme activity was observed when the N_T of PRMT8 was removed [21] (Fig. 5-6). In this study, we investigated the effect of the N_T of hPRMT1-V1 on enzymatic activity with both peptide and protein substrates. Truncation studies showed that truncating the N_T up to residue E27 did not affect enzyme activity with peptide substrate. However, differences in enzyme activity towards protein substrates were observed when the N_T of hPRMT1-V1 was truncated.

Truncating the N_T of PRMT1 should not affect activity with peptide substrates given that previous studies suggest that the N_T does not interact with short substrate peptides nor does it take part in placing the substrate arginine in the active site [13]. hPRMT1-V1-M1, -M11 and -E27 show similar activity with the peptide substrate thus supporting the above proposal. However, hPRMT1-V1-S31 demonstrated extremely low activity (k_cat, fig. 5-9). In considering the location of Ser31 in the context of the crustal structure, it is possible that the N_T was cut too close to the core (just one amino acid from the start of the X helix). The N_T of PRMTs has been reported to assist in the constraint of bound AdoHcy [13]. Truncating the N_T up to amino acid 27 did not affect enzyme activity with peptide showing that AdoMet binding was not affected. Conversely, truncating at S31 probably did not leave a long enough N_T sequence to help in constraining AdoMet, resulting in the low activity of hPRMT1-V1-S31.
Figure 5-9. Summary of $N_T$ sequences and kinetic parameters of hPRMT1-V1 constructs with peptide and protein substrates. The PRMT6 NT sequence which is attached to hPRMT1-V1-E27 core is shown in grey and the linker residues are shown in a lighter grey (-LE-). Each column of kinetic parameters in the table corresponds to a hPRMT1-V1 construct obtained with either a peptide or protein substrate as indicated. The last row in the table shows the number of proteins each construct interacted with in the protein array screen described earlier.

The higher methyltransferase activity consistently demonstrated by hPRMT1-V1-M11 both on purified recombinant ($k_{cat}$, fig. 5-9) and endogenous proteins (fig. 5-7) is possibly due to the absence of the first 10 amino acid residues at the $N_T$. It is possible that this 10 amino acid piece at the tip of the $N_T$ is involved in regulating the activity of hPRMT1-V1 in vivo. Most PRMT substrates have multiple arginines which make them positively charged. The substrate binding grooves of PRMT1 have been shown to be highly negatively charged due to a high incidence of acidic residues, this facilitates good interaction with the substrates. The first 10 amino acid piece of the $N_T$ mostly contains hydrophobic residues (70%), it only has 1 acidic residue (10%), and its polar residues make up the remaining 20% of its composition. Due to its
overwhelming hydrophobicity, it is very unlikely that this 10 amino acid piece will interact
with or bind positively charged regions of proteins. The 21 amino acid piece next to the first 10
amino acids on the N\textsubscript{T} is composed of 24\% acidic residues, 5\% positively charged residues, 24\%
hydrophobic residues and 47\% polar residues. Even though this 21 amino acid piece has an equal
amount of both acidic and hydrophobic residues, it has a considerable amount of acidic residues
for a small piece of protein and also a considerable amount of polar residues. Also, the
hydrophobic residues present in this 21 amino acid piece are scattered throughout this portion of
the protein and not concentrated in one place as with the 10 amino acid piece. It is therefore
possible that this 21 amino acid piece will interact with positively charged regions of proteins.
Consequently, I propose that this 21 amino acid piece at the N\textsubscript{T} binds to a positively charged
region on the protein substrate positioning it in the right orientation for binding and catalysis to
occur. On the other hand, the first 10 amino acid piece binds to a hydrophobic patch on the
substrate protein resulting in an orientation of the protein that is not very favorable for binding
and catalysis at the enzyme active site. Therefore on the full length protein (hPRMT1-V1-M1)
which has both peptide portions, there is a 50\% chance of either one binding first to the protein
substrate. If the 21 amino acid piece binds first, there will be methylation of the protein substrate.
But if the 10 amino acid piece binds first, no catalysis will occur and the protein will have to
dissociate and rebind in the right orientation for catalysis to occur (fig. 5-10). This will result in a
decrease in enzyme activity as observed with hPRMT1-V1-M1, since binding will only result in
catalysis part of the time. However, with hPRMT1-V1-M11 where the 10 amino acid piece is
lacking, the protein can only bind to the 21 amino acid piece, implying that every binding event
will result in catalysis consequently increased activity observed (fig. 5-10). When the first 16 of
the 21 amino acids at the N\textsubscript{T} of hPRMT1-V1-M11 are truncated (hPRMT1-V1-E27), most of the
acidic residues are removed as such correct binding and orientation of the protein at the active site
is no longer facilitated by the binding of the N-terminal 21 amino acid residue piece. Protein
substrates will therefore possibly take longer to bind in the right orientation at the active site, resulting in an observed lower enzyme activity. This 21 amino acid piece can also possibly be involved in stabilizing the protein substrate in the active site; as such deleting most of it will leave a small piece that can no longer carry out this function fully, resulting in fewer substrate arginines being methylated (fig. 5-10). It is not well understood why the chimera which is similar to hPRMT1-V1-E27 but for the additional PRMT6 N\textsubscript{T} showed lower enzymatic activity towards both purified and endogenous proteins. These results strongly suggest that the N\textsubscript{T} of hPRMT1-V1 influences methyltransferase activity. This is similar to what was observed with PRMT8 whereby, cutting off the N\textsubscript{T} resulted in an increase in activity [21].

![Figure 5-10](image)

**Figure 5-10.** Model for the N-terminal regulation of hPRMT1-V1 enzyme activity. A less active full-length hPRMT1-M1 contains an additional predominantly hydrophobic 10 amino acid piece at the tip of the N\textsubscript{T} (denoted by, oooo). Increase activity occurs when the 10 amino acid piece is removed in hPRMT1-V1-M11. *In vivo*, we suggest that the 10 amino acid piece regulates the activity of hPRMT1-V1 by some times binding to a hydrophobic region of the protein substrate (denoted by, oooo) leading to an orientation of the substrate which may bind the PRMT in a way that does not allow for catalysis (right). On the other hand, if a positively charged region of the protein substrate (denoted by, ++++) binds to the negatively charged 21 amino acid piece (denoted by, -----) which is next to the 10 amino acid piece, this will result in an orientation of the protein that will effectively bind the PRMT favoring catalysis (left). Therefore hPRMT1-V1-M11 is more active because every binding event results in catalysis, meanwhile with hPRMT1-V1-M1 only some of the binding events result in catalysis consequently lowers activity.
The effect of truncating the N_T of hPRMT1-V1 on substrate specificity is substrate dependent

Previous studies showed that deleting the N_T of PRMT3 (amino acid residues 1 to 183) resulted in the alteration of its substrate specificity [10, 11]. Also, a study of seven spliced variants of C-terminally Histidine tagged hPRMT1 which have different N-terminal lengths and sequences, suggested that a difference in substrate specificity exists amongst these variants [9]. Unlike PRMT3, truncating the N_T of hPRMT1-V1 did not alter its substrate specificity towards hnRNP K recombinant protein tested (k_{cat}/K_{m}, fig. 5-9). However, removal of the first 26 amino acids of hPRMT1-V1 resulted in an order of magnitude increase in k_{cat}/K_{m} for Histone H4 protein (k_{cat}/K_{m}, fig. 5-9).

When endogenous proteins from ES^{-/-} cells were methylated for a maximum of 30 minutes, some proteins were not visible with hPRMT1-V1-M1 and the chimera, but were very clear with both hPRMT1-V1-M11 and –E27. Also, hPRMT1-V1-M1 showed some difficulty in methylating lower molecular weight proteins in the nuclear extract, which both hPRMT1-V1-M11 and –E27 had no trouble methylating in 30 minutes. hPRMT1-V1 construct is slightly more restricted, additional experiments where longer incubations are tested will have to be performed. The difference we see might also be due to the difference in enzyme activity between the constructs; since hPRMT1-V1-M1 has the lowest activity, it might actually be taking more time to methylate what hPRMT1-V-M11 and –E27 methylate in a shorter time. We can therefore say that given our current experimental conditions, we did not observe significant differences in substrate specificity amongst the hPRMT1-V1 truncation constructs and bulk protein substrates.

The N_T of hPRMT1-V1 influences protein-protein interactions

When solving the crystal structure of PRMT1, no electron density was obtained for the N_T, indicating that it is disordered. The large scale screen for protein-protein interactions between the purified hPRMT1-V1 constructs and immobilized proteins described in the results
section showed that the $N_T$ can influence protein-protein interactions, as certain proteins only interacted with specific constructs and others interacted with two or more hPRMT1-V1 constructs. A similar trend to that observed with the enzyme activity of each hPRMT1-V1 construct toward the two protein substrates tested ($k_{cat}$, fig. 5-9), was observed with the number of proteins each construct interacted with. hPRMT1-V1-M1, which is the full length construct, interacted with the fewest number of proteins. The 10 amino acid piece present at the $N_T$ possibly gives hPRMT1-V1-M1 more specificity as to which proteins to interact with. hPRMT1-V1-M11 which lacks the first 10 amino acids at the $N_T$ interacts with almost four times more proteins than does hPRMT1-V1-M1. Strangely enough, truncating 16 more amino acids from the $N_T$ of hPRMT1-V1-M11 resulted in hPRMT1-V1-E27 interacting with almost 2-fold fewer proteins. This is a possible indication that this 16 amino acid fragment (residues 11 to 26) increases the ability of hPRMT1-V1 to interact with many proteins non-specifically. Suggesting that addition of the 10 amino acid piece (residues 1 to 10) at the $N_T$ of this 16 amino acid piece (as is the case with hPRMT1-V1-M1) serves to regulate it, increasing its specificity in protein-protein interactions, hence reducing the number of proteins it can interact with. This is most likely not the case with the PRMT6 $N_T$ as its addition to the core of hPRMT1-V1 considerably increased the number of proteins the PRMT6 $N_T$: hPRMT1-V1-E27 chimera interacted with by a little over two fold. It is therefore reasonable to say that the first 10 amino acids at the $N_T$ of hPRMT1-V1 serve as a regulatory piece, regulating the interaction of hPRMT1-V1 with certain proteins. I propose that the first 10 amino acids at the $N_T$ of hPRMT1 may also function in regulating the protein-protein interactions of hPRMT1 towards certain substrates \textit{in vivo}.

\textit{Truncating the $N_T$ of hPRMT1-V1 does not influence oligomer formation by hPRMT1-V1}

It has been previously observed that in solution PRMTs exist as higher order oligomers [13]. A study showed that the oligomerization of the yeast arginine methyltransferase, Hmt1 is
considerably hindered when its N\textsubscript{T} is deleted [12]. To test if the N\textsubscript{T} of hPRMT1-V1 is involved in oligomer formation, gel filtration analysis of solutions of hPRMT1-V1-M1 and hPRMT1-M11 were done. The results obtained show that truncating the N\textsubscript{T} of hPRMT1-V1 did not affect the oligomerization of hPRMT1-V1. This means that in the higher order oligomers of hPRMT1-V1, the PRMT dimers probably interact at sites other than the N\textsubscript{T}. The N\textsubscript{T} therefore does not significantly contribute to the formation of higher order oligomers by hPRMT1-V1 in solution.

**Conclusion**

It has long been postulated that the N\textsubscript{T} of PRMTs is involved in enzyme activity, substrate specificity, and protein-protein interactions, but very little is known on how this happens. In this chapter, using hPRMT1-V1 as a model, I show that the N\textsubscript{T} is involved in protein-protein interactions and also influences enzyme activity. On the other hand, truncating the N\textsubscript{T} either did or did not influence substrate specificity based on what protein substrate was used. Here I propose that the amino acid N-terminal sequence composition is what influences substrate specificity, not the length. I also suggest that the first 10 amino acids at the N\textsubscript{T} of hPRMT1 may function in regulating both the enzymatic activity and protein-protein interactions of hPRMT1-V1 in vivo. The N-terminus has previously been shown to function in regulating the activity of enzymes [10, 11, 21-23].

**References**


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CHAPTER 6
COMPLETED PROJECTS, PROJECTS IN PROGRESS, AND INTERESTING OBSERVATIONS

Project 1: Expression and Purification of C-terminal Histidine$_6$-tagged PRMT1 Variants

In order to study the role of the PRMT1 N-terminus we obtained six DNA vectors, each containing one of the first six human PRMT1 alternative splice variants from our collaborator Jocelyn Cote (University of Ottawa, Canada). PRMT1 variant 7 was not available. These variants have a similar core domain but differ at their N-termini. They also have a C-terminal Histidine$_6$ (His$_6$) – tag. To use these variants to determine if the difference in their N-terminal sequence affects their substrate preference, they had to be expressed and purified. All seven splice variants were expressed and purified similarly. DNA vectors were transformed into BL21 E. coli cells and single colonies obtained. Single colonies were used to inoculate start up cultures, which were used to inoculate 2.5 L of culture media. Cells were grown to an OD$_{280}$ of 600 0.6 at 37°C and then induced with 1mM IPTG for four hours. Cells were harvested by centrifuging at 10,000 RPM for 20 minutes. Cells were resuspended in 5 ml loading buffer (50 mM Na$_2$HPO$_4$, 300 mM NaCl, 10 mM imidazole, 20 mM 2-mercaptoethanol), and sonicated on ice. Cell debris was pelleted by centrifugation. The supernatant was incubated with 1.25 ml of 50 % slurry of Ni-NTA resin beads, overnight at 4°C. The resin with bound protein was first washed with 15 ml wash buffer (50 mM Na$_2$HPO$_4$, 10 mM imidazole, 1M NaCl), and then with 22.5 ml wash buffer (50 mM Na$_2$HPO$_4$, 10 mM imidazole, 300 mM NaCl). Proteins were eluted in 1ml fractions to a total of 5 ml of elution buffer (50 mM Na$_2$HPO$_4$, 300 mM NaCl, 250 mM imidazole). Fractions containing clean protein were pooled, concentrated and the buffer exchanged to a heparin column binding buffer (10 mM sodium phosphate, pH 7.0). The concentrated proteins were loaded onto a heparin column. The column was washed with 12.5 ml
binding buffer, and the bound proteins were eluted by a salt gradient, with the salt concentration increased in a stepwise manner from 0.2 M to 1.5 M NaCl (elution buffer: 10 mM sodium phosphate, pH 7.0, 1.5 M NaCl). Fractions were analyzed by SDS-PAGE and those containing clean proteins were pulled, concentrated and buffer exchanged to storage buffer (50 mM sodium phosphate, pH 7.0) (Fig. 6-1). Protein was frozen and stored at -80°C. The heparin column is the column that did the best job of cleaning up the proteins, as both cobalt resin and MonoQ columns did very little to purify the protein. In conclusion, a combination of Ni-NTA resin followed by a heparin column is the best way to purify the C-terminal His<sub>6</sub>-tagged human PRMT1 variants.

![Image of Coomassie stained gel of purified PRMT1 variants 1-6](image)

**Figure 6-1.** Coomassie stained gel of purified PRMT1 variants 1-6. Proteins were purified using Ni-NTA resin, followed by a heparin column. Arrows indicate each purified PRMT variant.

The purified proteins were then used in a continuous spectrophotometric assay previously described in chapters 3 and 4, to determine their activity with the peptide substrate R3 (acetylGGRGGFGGRGGFGGRGGFG), which is derived from fibrillarin. The N-terminus is not expected to affect PRMT1 activity with a peptide substrate, making this assay a good way to check the activities of these variants. The assay utilized 200 μM R3 peptide, 4 μM hPRMT1,
10 nM AdoHcy/MTA nucleosidase, 0.02 μM Adenine Deaminase, 10 nM MnSO₄, 250 μM S-Adenosylmethionine, and 50 mM sodium phosphate buffer pH 7.1. R3 peptide was used to initiate the reactions and a decrease in absorbance at 265 nm was measured. When the assay was performed, the enzymes were found not to be very active as the rate of the variants with 200 μM R3 peptide was barely over that of the control (Fig. 6-2). Also, there was much scatter observed in the obtained data points. This low activity is probably because the His₆-tag is attached at the C-terminus of the PRMT1 splice variants. In the solved PRMT1 structure, the C-terminus is seen to be very close to the active site. Therefore attaching a His₆-tag to the C-terminus might interfere with the substrate binding or activity of the active site. These results suggest that C-terminal tags should be avoided in PRMT isoforms that have a C-terminus near the active site.

**Figure 6-2.** The continuous assay monitors the methylation of the R3 peptide by the PRMT1 variants. The graphs show a decrease in absorbance at 265 nM over time. The control reaction is shown in black, while the reaction with 200 μM R3 is in blue. The rates for all six variants are shown; PRMT1 variant 1 (A), PRMT1 variant 2 (B), PRMT1 variant 3 (C), PRMT1 variant 4 (D), PRMT1 variant 5 (E), and PRMT1 variant 6 (F).
Project 2: Comparison of Methyl Arginine Proteome from Cells Treated with Particulate Matter vs. Cells Treated with Asbestos

This project is being done in collaboration with David W. Kamp, M.D. at Northwestern University Feinberg School of Medicine. The Kamp lab has previously reported that particulate matter (PM) induces p53-dependent apoptosis in primary human alveolar epithelial cells [1]. Prior to this, the Aust lab showed that crocidolite asbestos causes apoptosis of A549 cells (human epithelial lung cells) [2]. Our lab has previously observed a difference in the methyl arginine proteome between control and A549 cells treated with asbestos. We therefore set out to determine if the methyl arginine proteome change observed in crocidolite asbestos treated A549 cells, is also observed with particulate matter treated A549 cells and if this change is associated with apoptosis. To test this hypothesis, six conditions were set up;
1. A549 Control

2. A549- Crocidolite Asbestos 6 μg/cm² (24 h)

3. A549 - PM 6 μg/cm² (24 h)

4. A549-Bclxl overexpression

5. A549-Bclxl overexpression - Crocidolite Asbestos 6 μg/cm²

6. A549-Bclxl overexpression - PM 6 μg/cm² (24 h).

The Bclx1 protein inhibits apoptosis, therefore cells stably overexpressing the Bclx1 protein should not undergo apoptosis even when treated with asbestos or PM. We expected to see a change in the methyl arginine proteome when A549 cells are treated with asbestos and PM. If apoptosis is required for this proteome change to take place, no change in methyl arginine proteome will be observed when Bclx1 overexpressing cells are treated with either asbestos or PM. But if apoptosis is not required, then a change will be observed in both treatments.

The A549 cells were lysed in phosphate buffered saline (PBS) and their concentrations determined using the DC protein assay from BioRad. The concentration of the lysates was low and some were very low such that the same protein concentration could not be loaded for all of them.

Based on the western blot shown below (Fig. 6-3), there are six positions where we see potential differences, but no absolute conclusions can be made as of now. This is mainly due to the fact that the actin blot shows differences in the protein amounts loaded. The protein concentrations have to be re-determined and another western blot done before a conclusion can be reached.
Figure 6-3. Western blot of lysates obtained from A549 treated and untreated cells. (A) The top panel shows the western blot using Asym24 which is an antibody against asymmetric dimethylarginines. The amount of protein loaded is indicated for each lane. * Indicate observed differences. (B) This is an actin blot to determine if the same protein concentration was loaded for all lanes. This western was done using an anti-actin primary antibody.
Project 3: Is the N-terminus of hPRMT1-V1 Involved in Protein-Protein Interactions with the PRMT1 Regulator hCAF1?

Introduction

CCR4-associated factor1 (hCAF1), B-cell translocation gene 1 and -2 (BTG1 and -2) are the only proteins that have so far been shown to have the ability to regulate the activity of PRMT1 in cells [3-5]. hCAF1 regulates the activity of PRMT1 by complex formation, but what surfaces are involved in the interaction of these two proteins is unknown. To test if the N-terminus of hPRMT1-V1 is important for interaction with hCAF1 protein, we conducted pulldown experiments in an attempt to see if recombinant GST-hCAF1 would still interact with and pull down each truncated construct of hPRMT1-V1.

Method

Prewashed GST-resin (100 μL packed) was incubated with 410 μM (9.7 μg) GST-hCAF1 for three hours at 4°C with tumbling, to allow binding of protein to resin. The resin was then washed five times with 1000 μL 1xPBS each time to remove unbound hCAF1 protein. Resin was resuspended in 400 μL 1xPBS and protease inhibitors added. 100 μL of protein bound-resin suspension was put into four separate tubes and 154 μM of each hPRMT1-V1 construct added to individual tubes. Mixtures were incubated for four hours at 4°C with tumbling to allow binding of the hPRMT1-V1 construct to hCAF1. The resin was washed 12 times with 500 μL 1xPBS each time to remove unbound hPRMT1-V1 protein constructs. 15 μL of 4x SDS sample buffer was added to each resin with bound protein and boiled for eight minutes. Samples were resolved on 10 % SDS-PAGE gels and proteins transferred to PVDF membranes. The westerns were first probed with a GST antibody to check for the presence of GST-hCAF1. The membrane was then stripped and probed with an antibody against PRMT1 to find out which of the different hPRMT1-V1 proteins were pulled down by hCAF1.
Results, Discussion, and Conclusion

All the truncation constructs hPRMT1-V1-M1, -M11, -E27 and -S31 were pulled down by GST-hCAF1 (fig. 6-4). hCAF1 interacts with PRMT1 both in vivo and in vitro regulating its activity in a substrate dependent manner. Our results suggest that the N_T of hPRMT1 is not critical in its interaction with hCAF1 as all the truncation constructs formed complexes with hCAF1. However, a control experiment still needs to be done where a PRMT that is not known to interact with hCAF1 for instance, His-PRMT6, is incubated with hCAF1 under similar conditions and the outcome noted. If hCAF1 pulls down His-PRMT6, then maybe the current conditions are very favorable for protein-protein interactions, hence the entire pull down experiment should be repeated under more stringent conditions. For instance the new conditions can mimic the conditions used in the Protoarray protein-protein interaction screen done in chapter 5 (outlined in the manufacturer’s manual).

**Figure 6-4.** The hPRMT1-V1 N_T is not critical for protein-protein interaction with hCAF1. Equal amounts of the hPRMT1-V1 constructs were each incubated with the same amount of GST-hCAF1 pre-bound to GST-beads for 4 hours. After extensive washing of resin, 4X SDS sample buffer was used to elute the bound proteins by boiling for 8 mins. Eluted proteins were resolved on SDS-PAGE and transferred to PVDF membranes. A) Westerns were probed with GST antibody (Santa Cruz Biotechnology) to check for the presence of hCAF1. B) Membranes from (A) were stripped and reprobed with antibodies against PRMT1 (Upstate) to determine the presence of the hPRMT1-V1 constructs pulled down by GST-hCAF1.
Observation 1: When the Human PRMT1 Variant 1 E27 Construct is used in an Assay with Protein Substrate, the Enzyme could not be Saturated at the Substrate Concentration Used

When using the ZipTip assay to look at the steady state kinetics of the PRMT1 variant 1 E27 (hPRMT1-V1-E27) construct with the protein substrate GST-MRE11 B, 790 nM of this enzyme was mistakenly used instead of 70 nM. Analysis of the data from this experiment revealed that this PRMT construct at this high concentration did not show saturation (Fig. 6-5). However, when this experiment was repeated with enzyme at low concentration (70 nM), the data obtained could not be fitted into any particular kind of curve (Fig. 6-6A). Repetition of some of the data points which seemed to be questionable still resulted in a similar pattern.

To test if this was just a characteristic of hPRMT1-V1-E27 with this substrate or if the other constructs will demonstrate the same pattern, we did steady state kinetics of hPRMT1-V1-M1 with the same protein (GST-MRE11 B) at an enzyme concentration of 70 nM. Unlike hPRMT1-V1-E27, hPRMT1-V1-M1 showed a rapid linear increase in rate with increasing protein substrate, followed by saturation (Fig. 6-6B).

This suggests that the phenomenon observed with hPRMT1-V1-E27 and GST-MRE11 B, is not due to the substrate only, since the substrate shows normal steady state kinetics with
hPRMT1-V1-M1. No other protein substrate tested with hPRMT1-V1-E27 produced similar results. This is possibly a situation where hPRMT1-V1-E27 reacts with this particular substrate differently than hPRMT1-V1-M1. This observation needs to be further explored with different enzyme concentrations, different enzyme batches and different enzyme substrates.

![Graph](image)

**Figure 6-5.** A plot of the rate of methylation of GST-MRE11 B per min against the concentration of GST-MRE11 B shows that PRMT1 variant 1 E27 could not be saturated at a concentration of 790 nM. This ZipTip assay contained 1.88 μCi $^3$H-AdoMet, 100 mM Hepes buffer at pH 8.0, 0.38 μM BSA, 10 % Glycerol, 10 nM MTAN and GST-MRE11 B at varying concentrations. The reactions were initiated with 790 nM PRMT1 variant 1 E27, and 10 μl timepoints taken out and processed as previously described in chapter 4.
Figure 6-6. Kinetic analysis of the methylation of GST-MRE11 B by hPRMT1-V1-E27 and hPRMT1-V1-M1. (A) Rate of methylation (nMCH₃/min) versus GST-MRE11 B concentration catalyzed by hPRMT1-V1-E27. (B) Steady-state analysis of the methylation of GST-MRE11 B by hPRMT1-V1-M1. The assays were performed by ZipTip as previously described in chapter 4. 70 nM PRMT was utilized in these assays.
Observation 2: When *in vitro* Methylation Assays are done in HEPES Buffer versus Sodium Phosphate Buffer; Differences in PRMT Activity are observed.

Histidine-tagged eukaryotic translation initiation factor 4A, isoform 1 (His-eIF4a1) protein has previously been shown to be a substrate of PRMT1 [6]. However, several attempts at methylating purified recombinant (His-eIF4a1) protein with hPRMT1-M1, -M11, or E27, were unsuccessful. The reaction mixture consisted of 1.88 μCi ³H-AdoMet, 100 mM Hepes buffer at pH 8.0, 0.38 μM BSA, 10% Glycerol, 10 nM MTAN, 10 μM His-eIF4a1 and 100 nM of either hPRMT1-M1, -M11, or E27, which were used to initiate each reaction. Since His-eIF4a1 has been shown to be methylated before in sodium phosphate buffer, we decided to repeat the methylation reaction but this time use sodium phosphate buffer (100 mM, pH 8.0) instead of Hepes buffer. Interestingly, methylation of His-eIF4a1 was observed (Fig. 6-7) this time. These reactions were done at 37 °C for 1 hour. Each reaction was halted in 4X sample buffer and boiled for 5 minutes. They were then resolved on SDS-PAGE and the proteins transferred to a PVDF membrane. The membrane was dried and sprayed with En3Hance from Perkin Elmer according to the manufacturer’s directions and then incubated with film for 5 days. The methylated proteins were then viewed by fluorography.

This observation was further investigated by methylating endogenous protein obtained from PRMT1/- ES cells with PRMT6 N_T: hPRMT1-V1-E27 core chimera. We decided to reinvestigate the activity of the chimera to see if it was more active in Hepes buffer. The reaction mixtures were similar except for having either Hepes or sodium phosphate buffer, they consisted of 1.88 μCi [³H]-AdoMet, 100 mM Hepes or sodium phosphate buffer at pH 8.0, 0.38 μM BSA, 10% Glycerol, 10 nM MTAN, 15 μg total endogenous protein and 280 nM PRMT6 N_T: hPRMT1-V1-E27 core chimera. The reactions were treated the same way as the His-eIF4a1 methylation assay described above. The resulting fluorograph shows that the chimera is not as active in Hepes buffer as when in sodium phosphate buffer (Fig. 6-8). Most surprising is
that some proteins were only methylated in Hepes while others were only methylated in phosphate buffer.

Figure 6-7. Effect of buffer composition on hPRMT1 activity. Figure shows methylation of His-eIF4a1 in sodium phosphate buffer but not in Hepes buffer. Arrow shows methylated His-eIF4a1.

It is not understood why the methyltransferase activity of PRMTs differ in either Hepes buffer or sodium phosphate buffer. This effect of buffers on the activity of PRMTs has to be further investigated as this will greatly impact the PRMT field since certain labs exclusively use sodium phosphate buffer and others Hepes buffer in their study of PRMTs 	extit{in vitro}. Maybe some of the proteins that PRMTs have been reported as showing less preference for in sodium phosphate buffer will be more favored in Hepes buffer and vice versa. Therefore the PRMT field has to come to an agreement as to which buffer should be used for 	extit{in vitro} assays in the study of
PRMTs. Understanding how different buffer compositions selectively affect specific protein methylations may also reveal novel ways in which the PRMTs are regulated \textit{in vivo}.

\textbf{Figure 6-8.} Activity of PRMT6 N\textsubscript{T}: hPRMT1-V1-E27 core chimera towards endogenous proteins differs in Hepes buffer and sodium phosphate buffer. Stars (\textstar) show proteins that the chimera is less active towards in sodium phosphate buffer and the arrow shows the protein that the chimera is more active towards in sodium phosphate buffer.

\textbf{References}


Summary

The activity of most eukaryotic proteins is modulated by post-translational modifications (PTMs) that increase the structural and functional diversity of the proteome. PTMs like phosphorylation and acetylation of proteins have been widely studied and their roles in the regulation of cellular processes such as signal transduction and gene expression are well understood. Recently, the importance of another kind of PTM, known as methylation has begun to be recognized. Protein methylation can occur on amino acid residues such as lysine, histidine, asparagine, glutamine, proline, or arginine, some of them more frequent than others. Arginine methylation is a common methylation that is involved in numerous cellular processes and is catalyzed by a family of enzymes known as protein arginine methyltransferases (PRMTs). Although PRMTs have been around for a while, a lot of pertinent questions still remain in the field; how is substrate specificity achieved amongst the PRMTs; how is PRMT activity regulated by posttranslational modifications such as phosphorylation, acetylation, ubiquitination and trans- or automethylation; are there really enzymes that demethylate arginine residues; other than Tudor domains, are there additional modules that bind methylarginine?

In order to contribute to the advancement of the PRMT field, I decided to investigate claims that the PRMT N-terminus (NT) influences enzymatic activity, protein-protein interaction and substrate specificity. In this study, I used human PRMT1 variant 1 (hPRMT1-V1) as a model. hPRMT1 is the PRMT of choice in this study because it is the major PRMT in the cell methylating over 85% of all protein arginine residues and has also been implicated in a number of disease conditions such as cancer. hPRMT1 also has the highest number of variants as a result of alternative splicing (variants 1-7). In order to do this study, we first made untagged hPRMT1-V1 truncation constructs and a protein chimera consisting of a PRMT6 NT and a hPRMT1 core
starting from residue 27. These constructs were then used in the development of a fast and efficient assay for the quantitative analysis of \( s \)-adenosyl methionine dependent enzymes. Finally, we used this assay to analyze the effect the \( N_T \) of hPRMT1 on enzymatic activity, protein-protein interaction and substrate specificity.

The PRMT field has greatly advanced from when PRMTs were first discovered. Much of what is known about PRMTs was attained by studying the properties of recombinant PRMTs. However, these recombinant proteins are usually expressed as tagged fusion proteins, and it has been shown that the nature of the tag used at the \( N_T \) of PRMTs differentially affects methylation of certain protein substrates. To solve this problem, we developed a method of obtaining purified untagged recombinant PRMTs, which uses osmotic shock to lyse the bacteria cells harboring the expressed protein. In this method, we start by purifying a recombinant protein bearing a tobacco etch virus (TEV) cleavable affinity tag. TEV cleavage site is then cleaved using TEV protease leaving behind only a single glycine residue. Passage over a second affinity column achieves separation of the untagged PRMT and the TEV cleavage site linked to the affinity tag. Untagged PRMTs obtained by this method are active and can be used in a variety of studies in which the properties of PRMTs are being investigated without worrying about the effect of a tag. Importantly, this method can be used in the purification of other problematic TEV fusion constructs that show possibly disordered N or C termini.

For a longtime, one major hindrance to the advancement of the PRMT field has been the lack of fast and efficient ways to quantitatively measure PRMT activity with protein substrates, the existing techniques take anywhere from 10 weeks to 4 months to obtain a single data point. Meanwhile several fast techniques exist for measuring PRMT activity with peptides which do not work well if at all with proteins. However, in order to reflect what might be happening \textit{in vivo}, there is need to move from studying the properties of PRMTs with peptide substrates to protein substrates. As a major highlight of my research, we developed a fast and efficient assay for the
quantitative measurement of $s$-adenosyl-L-methionine (AdoMet)-dependent methyltransferase activity with protein substrates. This method employs the capability of a reverse-phase resin packed at the end of a pipette tip to bind radiolabeled protein products and not unreacted [$^3$H]-AdoMet as such quickly separating the two (Fig. 7-1). The radiolabeled protein products bound to the resin are then eluted into scintillation cocktail and counted in a liquid scintillation counter. This assay comes with several advantages over existing methods; (i) it decreases the analysis time, (ii) it is very sensitive allowing for the determination of initial rate data, (iii) the volume of radioactive waste is reduced by more than 3000-fold over existing protocols, (iv) it can be used to study the inhibitory effect of $s$-adenosylhomocysteine (AdoHcy), (v) it does not use coupling enzymes as such false-positive hits are reduced, thus making this assay highly suitable for use in screening modulators of protein methyltransferase activity, (vi) it can be optimized for high-throughput screens, and (vii) finally, this assay will work for other AdoMet-dependent protein methyltransferases including lysine methyltransferases, and carboxyl methyltransferases.

We then set out to use this assay together with protein arrays, the continuous spectrophotometric assay and other standard protocols to analyze the effect of the $N_T$ of hPRMT1-V1 on enzymatic activity, protein-protein interaction, and substrate specificity. This study confirmed reports that the $N_T$ influences enzymatic activity with protein substrates and protein-protein interactions. It also identified a piece of the $N_T$ which consists of the first 10 amino acid residues that might be regulating the activity and what proteins hPRMT1 interacts with in vivo. This is because truncation of this piece results in an increase in enzyme activity and in the number of proteins that the enzyme interacts with. This observation is similar to what has been reported in PRMT8 where cleavage of the $N_T$ resulted in an increase in enzyme activity (Chapter 5). However, truncating the $N_T$ of hPRMT1-V1 at Methionine 11 did not affect substrate specificity towards the recombinant proteins tested nor the endogenous proteins from
mouse PRMT1\textsuperscript{-/-} ES (embryonic stem) cells. Nevertheless, truncating at Glutamate 27 influenced substrate specificity towards Histone H4 protein. I propose that the difference in substrate specificity suggested between hPRMT1 variants which have the same core but different N-termini, is due to the difference in their N\textsubscript{T} sequences, not their lengths, since the absence of the hPRMT1-V1 did not significantly influence substrate specificity.

**Figure 7-1.** Summary of how the new fast and efficient assay for the measurement of S-adenosylmethionine-dependent protein methylation works. The main thing that makes this method different from others is its ability to separate unreacted [\textsuperscript{3}H]-AdoMet (\textsuperscript{*}AdoMet) from radiolabeled proteins (\textbullet) quickly and efficiently. Unlabeled proteins are shown in blue (\textcolor{blue}{\textbullet}).
**Future Work**

More work still has to be done in order to confirm the model proposed above and also to further this field.

Firstly, I suggest the utilization of site directed mutagenesis to mutate some of the residues in the first 10 amino acids of the N$_T$. This will make it possible to tell which residues at the N$_T$ are important for regulating the activity and protein-protein interactions of hPRMT1. It will also be advantageous to mutate some of the residues between residues 11 and 27, to find out which ones played an important role in the increase enzyme activity observed when the first 10 amino acids were truncated.

Secondly, I suggest doing a peptide competition assay using two peptides, one that corresponds to the first 10 amino acids of the hPRMT1 N$_T$ and another which corresponds to the first 27 amino acids of the N$_T$. Each peptide will be pre-incubated with the protein substrate in separate reactions for at least 30 minutes before the assay is initiated with the hPRMT1 enzyme. The assay will then be carried out in a similar manner as described in chapter 4. Analysis of these results will illustrate if either of the N-terminal peptides was able to bind to a certain portion of the protein thus hindering or delaying the binding of the added hPRMT1 enzyme to the protein. This will be observed as a decrease in enzyme activity when compared to a parallel assay reaction done in the absence of either N-terminal peptides.

Also, to confirm that there are no differences in the abilities of each of the hPRMT1-V1 constructs to bind AdoMet, I suggest that it is important for the $K_d$ (dissociation constant) of each construct be obtained. This can be done using intrinsic fluorescence which is a technique that our lab is familiar with. This technique is currently in active use in our lab and is not very time consuming compared to other methods that are usually used for this purpose.

Using stopped-flow fluorescence the movement of the N$_T$ can be monitored before and during catalysis. This has been done with DNA methyltransferase where the movement of a loop
was observed using this method. This will require the use of a stopped-flow reaction analyzer. Norbert Reich of the University of California, Santa Barbara, has this instrument, and this project can be done in collaboration with him. He has previously given a seminar at our department and is familiar with our work. To do this, one or two amino acid residues at the $N_T$ will have to be mutated to tryptophan (W); this can be done using the QuikChange kit from Stratagene. Already purified protein substrates such as hnRNP k and Histone H4 can be used. Also proteins which interact with hPRMT1-V1 but which are not substrates can be used, this will allow observation of what the $N_T$ does without a favorable spot to bind to. There are five other tryptophans in hPRMT1-V1, to make sure that only the movements of the tryptophans at the $N_T$ are being observed, wild type hPRMT1-V1 will be used as a control and the results from this will be subtracted as background from the reactions with the mutants. In this reaction, the enzyme is first preincubated with the protein substrate and mixed in the stopped-flow apparatus with cofactor (AdoMet or AdoHcy). Using AdoHcy allows one to examine binding, while using AdoMet allows one to view catalysis. This way, the change in conformation of the $N_T$ can be observed during binding and catalysis. The results gathered from this experiment will be differences in observed fluorescence intensity which indicate shifts in the amounts of the various $N_T$ tail conformers. No observable fluorescence change will suggest that there has been no significant change in amount, meaning no binding.

hPRMT1-V1 shuttles between the nucleus and the cytoplasm although it is mainly localized in the nucleus, but it neither has a nuclear localization signal nor a nuclear export signal. It is not known what allows it to localize to either the nucleus or cytoplasm. Since truncation constructs of hPRMT1-V1 and a chimera consisting of the $N_T$ of PRMT6 and hPRMT1-V1 core have already been made, it will be interesting to put these constructs into a GFP vector and transfect HEK293 cells. Confocal microscopy can then be used to see what part of the cell each construct will localize to. If the $N_T$ is important for either nuclear localization, then the constructs
lacking the $N_T$ will remain in the cytoplasm, but if it is important for cytoplasmic localization, then the construct lacking it will be localized only in the nucleus. PRMT6 localizes exclusively to the nucleus. If its $N_T$ is the reason for this localization, then the chimera bearing its $N_T$ will be localized exclusively to the nucleus.
February 5, 2010

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(March 2010)

EDUCATION

PhD, Biochemistry 2004-2010
Utah State University (USU), Logan, Utah, USA
Thesis: “Investigating the Role of the N-terminal tail of the Human Protein Arginine Methyltransferase 1 (PRMT1) in substrate recognition”
Advisor: Joan Hevel

B.S., Biochemistry June 2003
Minor: Medical Laboratory Technology
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RESEARCH EXPERIENCE

Graduate Student / Research Assistant 08/04 – 05/10
Department of Chemistry and Biochemistry, Utah State University
- Developed a fast and efficient assay to measure protein methylation
- Developed a protocol to efficiently cleave problematic TEV-PRMT 1 constructs
- Designed and developed a radioactive assay for measuring the activity of PRMTs
- Designed, expressed and purified PRMT truncation constructs as tools for studying the effect of the N-terminus on PRMT 1 substrate recognition.
- Extensively cultured both mammalian and bacterial cell lines
- Expressed and purified various PRMT protein substrates (some of which have not been previously described in the literature) using chromatographic techniques
- Extensively used fluorography to monitor radiolabel incorporation by protein substrates in kinetic studies

TEACHING EXPERIENCE

Teaching Assistant, Utah State University, Logan, UT 08/04 – 12/08
Teaching Assistant, Utah State University, Logan, UT 08/04 – 12/08
Teaching Assistant, Utah State University, Logan, UT 08/04 – 12/08
Taught undergraduate Chemistry and Biochemistry laboratories which included teaching, grading, preparing quizzes and holding office hours:
- General Chemistry Lab I (CHEM 1115)
- Chemistry Principles Lab I (CHEM 1215)
- Introduction Biochemistry Lab (CHEM 3710)
- Substituted for major professor to teach Principles of Biochemistry/General Biochemistry II / Advanced Biochemistry II (CHEM 5710)
- Volunteer CPR and First Aid Instructor at the Cache County American Red Cross 06/06 - present

Tutor / Grader, Utah State University, Logan, UT
- Graded Principles of Biochemistry (CHEM 5710).

PUBLICATIONS


PROFESSIONAL AFFILIATIONS

Memberships
- American Chemical Society (ACS)
- Golden Key International Honor Society

Leadership
- Graduate Student Senator 03/06 – 03/08 College of Science, Utah State University
- Supervised over 5 undergraduate student researchers
- Performed Chemistry demonstrations at local middle school with USU Chemistry club

AWARDS AND HONORS

- Center for integrated Biosystems research (CIBR) grant, Utah State University 06/08 – 09/09
- Award for the 2nd best poster presentation at the Intermountain Graduate Research Symposium, Utah State University April 2009
- USU Graduate Student Senate Travel Award, Utah State University March 2009
- Graduate student senate enhancement award, Utah State University March 2008
- Teaching Instructor Certificate, Utah State university September 2004
- Minister of Higher Education Award for top female student in science, University of Buea, Cameroon September 2002

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Suh B.B. and Hevel J.M. Investigating the Role of the N-terminal tail of the Human Protein Arginine Methyltransferase 1 on substrate recognition. FASEB Summer Research Conference on Biological Methylations, Carefree, AZ, 2008 (Poster)

Suh B.B. and Hevel J.M. Investigating the Role of the PRMT6 N-terminal Tail in Subcellular Localization and substrate processing. Intermountain Graduate Research Symposium, Utah State University, Logan, UT, 2007 (Poster)

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OTHER WORK EXPERIENCE

Medical Laboratory Technician Intern, Provincial Hospital Bamenda, Cameroon 07/03 – 09/03
Laboratory technician intern, analyzed patient samples in different sections of the laboratory including serology, biochemistry, parasitology and microbiology.