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Current Protein and Peptide Science, 2020, *21*, 000-000

REVIEW ARTICLE

Toward Understanding Molecular Recognition between PRMTs and their Substrates

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ARTICLE HISTORY

Received: August 17, 2019
Revised: October 08, 2019
Accepted: December 04, 2019
DOI:
10.2174/1389203721666200124143145

Abstract: Protein arginine methylation is a widespread eukaryotic posttranslational modification that occurs with as much frequency as ubiquitinylation. Yet, how the nine different human protein arginine methyltransferases (PRMTs) recognize their respective protein targets is not well understood. This review summarizes the progress that has been made over the last decade or more to resolve this significant biochemical question. A multipronged approach involving structural biology, substrate profiling, bioorthogonal chemistry and proteomics is discussed.

Keywords: PRMT, arginine methylation, substrate specificity, PRMT molecular recognition, arginine methylome, target recognition.

1. INTRODUCTION

Protein arginine methylation of histones sparked a great deal of attention when it was introduced as one of the many histone modifications that help control gene expression [1-5]. Little did anyone realize at the time that the role of protein arginine methylation in the cell extends far beyond histone function. We know now that arginine methylation is as prevalent as ubiquitinylation in the cell [6] and impacts numerous biological activities [7-12].

Over several years, the family of enzymes responsible for arginine methylation has been identified: nine protein arginine methyltransferase (PRMT) isoforms in humans catalyze the S-adenosyl L-methionine (AdoMet or SAM)-dependent methylation of select arginyl groups on their target proteins (Fig. **1**). Somewhat akin to their lysine methyltransferase cousins, the PRMT isoforms differ in what kind of methylated product is formed (monomethylarginine, MMA; asymmetric dimethylarginine, ADMA, and symmetric dimethylarginine, SDMA), giving rise to a potential combinatorial code for downstream recognition [13, 14]. Multiple methylations on the same protein target are common; e.g., SAM68 is methylated at 7 sites [15] and nucleolin is methylated at 10 sites [16]. Although few methylation events have been studied at the biophysical level, it is generally thought that arginine methylation alters protein-protein and/or protein-nucleic acid interactions [17-20]. More recent observations indicate that arginine methylation can be used to control the formation of membrane-less organelles by regulating liquid-liquid phase transitioning (reviewed in [21]). Additionally, arginine methylation has been associated with changes in subcellular localization, activity, and binding partners in the cell (reviewed in [22]). These observations correspond well with the widespread cellular consequences of arginine methylation that have been observed [7-12, 23]. With the significance of arginine methylation well-established, one might have predicted that the substrate specificity, that is, how and why PRMTs target particular proteins for methylation, would have been laid out years ago. However, the PRMTs have been reluctant to divulge their biochemical priorities for target recognition. This review is a summary of the various approaches that have been taken to understand PRMT targeting and their respective results.

2. APPROACHES TO DEFINE PRMT SUBSTRATE SPECIFICITY

Why is substrate specificity of the PRMTs so important? Understanding the molecular rules used by the PRMTs to recognize substrates will aid in the development of better prediction tools, help identify new pathways affected by arginine methylation, and present novel avenues for possible substrate-specific inhibitors. The biological and pharmaceutical significance of these rules has driven the field to use several different, but complementary strategies for understanding PRMT substrate specificity including structural biology efforts to identify active site space and pinpoint atomic interactions between substrates and the PRMTs, substrate profiling to systematically alter amino acid sequence and quantify residue preferences, bioorthogonal approaches to tag isoform-specific substrates *in vivo*, and proteomic studies to identify the cellular arginine methylome. Each

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1389-2037/20 $65.00+.00

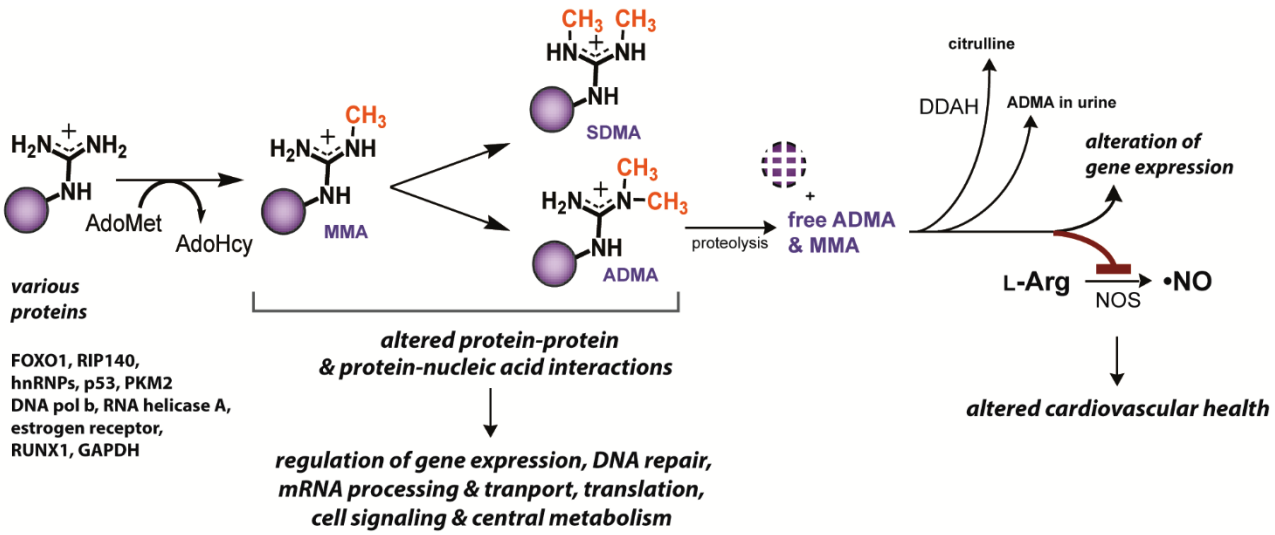

Fig. (1). PRMTs catalyze the AdoMet-dependent methylation of arginine residues within proteins and function to regulate many aspects of cell biology. A plethora of different proteins (e.g., histones, transcription factors, enzymes, receptors) are targeted by nine human PRMTs. Methylated proteins directly take part in altering biochemical signaling using altered protein-protein and /or protein-nucleic acid interactions. Proteins methylated at arginine groups also serve as the endogenous source of free ADMA and free MMA, which are both naturally occurring inhibitors of nitric oxide synthase (NOS). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

strategy presents unique benefits, as well as limitations. When used together, they give us insights into how PRMTs choose their substrates.

2.1. Structure

All known PRMT structures are defined by a central catalytic core, consisting of an AdoMet-binding Rossman fold, a β-barrel domain that appears to be unique to the PRMTs, and a dimerization arm consisting of a small helix-loop-helix motif inserted into the β-barrel domain (Fig. **2**). The active site is at the interface of the Rossman fold domain and the β-barrel domain. For mammalian PRMT1, 2, 3, 4, 6, and 8, the dimerization arm of one monomeric subunit interacts with residues on the Rossman fold domain of a second subunit to mediate the formation of a head-to-tail dimer with a central pore. PRMT5 also forms a head-to-tail dimer with a pore, but it does so with the aid of an additional domain at the N-terminus. PRMT7 [24] and 9 (PDB ID: 6PDM, associated publication not yet released) form a pseudodimeric construct from a single peptide which mimics the dimeric structure of the other PRMTs. The angle at which the two domains in PRMT7 and 9 interact essentially closes off the back face of the protein and forms a deep bowl. Some isoforms (e.g., PRMT2, 3, 4, 5 and 9) also have N-terminal and/or C-terminal domain extensions [25], but the locations of these extensions in the structure with respect to the central core are largely unknown.

2.1.1. Sterics of the Active Site

The dimerization of the two U-shaped PRMT monomers forms a doughnut-like structure with a central pore which varies in size from ~8 Å x 21 Å to ~17 Å x 20 Å in PRMT1, 2, 3, 4, 6, and 8 [29-35]. The active site is on the inner surface of this pore, roughly halfway between the front and back faces (think of the inside rim of a tire) (Fig. **2**). Without a significant conformational change, an alpha helix of a substrate protein may have difficulty fitting into the active site of PRMT1 (Fig. **3**), especially for substrates bearing large side chains. For reference, the diameter of a polyglycine helix is ~6 Å, while the diameter of a polyserine helix is ~ 10-12 Å. For some PRMTs, this would favor the methylation of arginine residues present on a loop or flexible termini. Flexibility and disorder at the target site is a characteristic of the arginine-glycine (RG)-rich protein substrates that are historically associated with PRMTs, especially PRMT1. On the other hand, PRMT6 has a much larger pore (Fig. **3**), suggesting that this isoform could accommodate substrate sequences in alpha helices with larger side chains. Entry through the center of the pore in a perpendicular fashion is only one way for substrates to gain access to the active site, but is supported by structures showing electron density of the peptide substrate threaded through the central pore of PRMT1 [29]. Helices or β-sheets could bind across the face of the enzyme; however, significant motions (discussed below) would need to occur for the targeted arginine to reach the active site.

If the dimeric PRMT structure is dynamic, either by dimer dissociation or by other protein dynamics (as discussed below), the size of the pore could change dramatically. In fact, the CREB-binding proteins (CBP)/p300 are methylated on an alpha helix by PRMT4 [36]. However, docking [37-39] of just the helical element of p300 into the active site of PRMT4 shows numerous steric clashes. This suggests that we have more to learn regarding conformational rearrangements required to accommodate substrate binding.

2.1.2. No Consensus Sequence for Substrates?

There are currently at least six studies [26, 29, 40-43] which together describe a small handful of peptide-bound PRMT structures. These include PRMT1 bound to RGG-based peptides [29], along with structures of PRMT4 bound

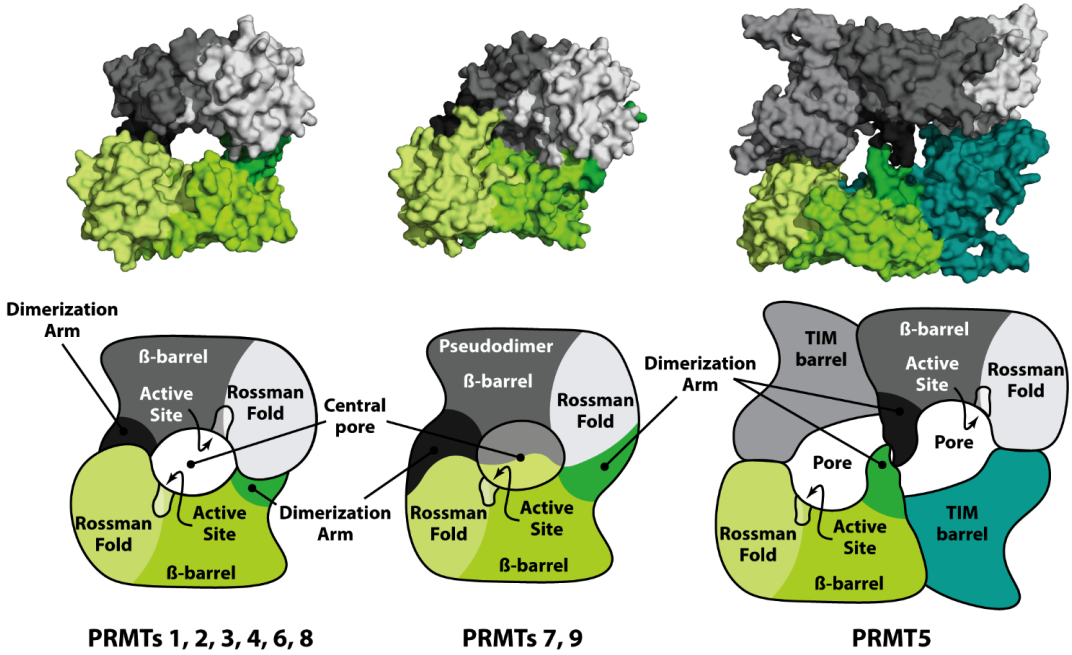

Fig. (2). General structure of the PRMT enzymes. Structures from PRMT1 (1Or8), PRMT7 (4C4A) and PRMT5 (4GQB) are shown on the top and are color-coded to match the cartoon structures below. The human PRMT5 structure is shown [26]. We note that the first PRMT5 structures show the *C. elegans* homolog that has a slightly different architecture [27]. For a more detailed discussion of the PRMT structures and methyltransferase mechanism, we recommend recent reviews by Ho [28] and Ferreira de Freitas [25]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

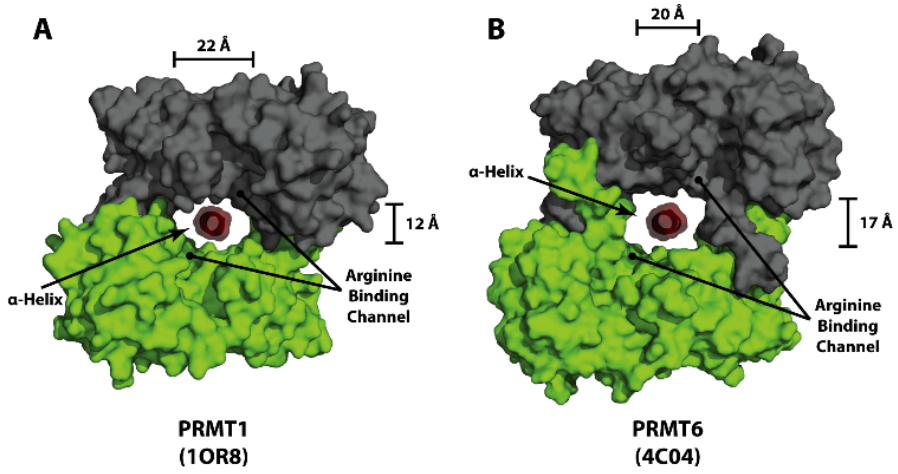

Fig. (3). Comparing the size of a polyglycine alpha helix to the pore size in PRMT1 and 6. Measurements indicate the approximate dimensions of the central pore. The PDB IDs of the structures used are indicated in parenthesis. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

to Histone H3-based [41] and PABP1-based peptides [41, 42], and PRMT5 bound to Histone H4-based peptides [26, 43].

The first structures of a PRMT bound to a substrate showed PRMT1 bound to RGG-based peptides (R3: Ac-GGRGGFGGRGGFGGRGGFG, and R1: Ac-GGFGGRGG FG) [29]. The structures revealed electron density (attributed to the substrate peptide) in three different shallow grooves along the PRMT1 surface. However, none of the grooves individually accounted for the entire length of the peptide substrate. More importantly, the density was insufficient to resolve any substrate side chains except for the modified arginine in the active site. These results suggest that specific interactions between the PRMT1 enzyme and the side chains of the substrate do not directly contribute to binding specificity. The observation of substrate electron density draped over both the back and front faces (in three different grooves) is unique to PRMT1, and could be the basis for the ability of PRMT1 to methylate such a diverse set of substrates (different substrates could utilize different binding grooves [13]).

Among all of these structures, specific interactions between the enzyme and a peptide side chain can be observed

for just four substrate residues other than the methylated arginine. These involve Asn 449 and Glu 448 of a PABP-based peptide with PRMT4 (PDB IDs: 5LGP and 5LGR [42]), Lys 14 of an H3 based peptide bound to PRMT4 (PDB ID: 5DWQ [41]), and Lys 5 of a Histone H4 based peptide bound to PRMT5 (PDB ID: 4GQB [26]). In one PRMT4 study [41], the authors reported 5 structures of PRMT4 bound to peptides based on PABP1 and Histone H3. The authors describing these structures note conserved interactions with the backbone of substrate residues in specific positions relative to the bound arginine, and they also observed that the substrate residues within the central pore all have a similar conformation (Fig. **4**) [41]. This suggests that perhaps, at least for PRMT4, the ability to take on a conformation complementary to the enzyme surface is a more important factor for substrate selection than the identity of specific side chains.

2.1.3. Mobile Elements and Accessory Domains

Although we now have a very good idea of what the core of the PRMT structures look like, there are still some parts of the structures that are missing, or that vary considerably in conformation. Inconveniently, these regions are near the active site (Fig. **5**). In particular, there is a 6 to 20 amino acid stretch that resides immediately upstream of the Rossman fold that is only visible in some structures. This region is part of a regulatory element that is used in many Rossman fold methyltransferases to control AdoMet binding [23]. When resolved in the PRMT structures, this region can act as a lid to the active site. It is widely believed that this region undergoes a conformational change upon AdoMet binding [15, 32, 35, 44] and is part of the chemically competent active site [29]. What is really interesting is the observation that in the majority of PRMT structures where this lid is present, it occurs in one of three distinct conformations that we define as *Up*, *Down*, or *Across*.

In the *Up* conformation, the lid extends up from the Rossman fold of one monomer and interacts with the dimerization arm and β-barrel domain on the second monomer (observed in PRMT6 [33], *T. brucei* PRMT7 [40] and a *S. cerevisiae* PRMT1 [45]) (Fig. **5A**). In this conformation, the active site would be solvent accessible, and residues that have previously been shown to be important for catalysis would not be positioned near the active site [29]; therefore, it would seem that this conformation represents a form of the enzymes that would not be catalytically competent, but demonstrates that the lid is mobile. In the *Down* conformation, the lid covers the AdoMet binding pocket but leaves the central pore open (observed in PRMTs 2, 3 and 6 [30, 31, 33, 34] (Fig. **5B**). In the *Across* conformation, the lid folds down and covers the AdoMet binding pocket, but also folds across the central pore and forms a septum which closes the pore off from the front (observed in one PRMT1 structure [46] and PRMT4 [32]) (Fig. **5C**). In both the *Down* and the *Across* conformations, the lid residues form surfaces adjacent to the active site that could play an important role in substrate selection (Fig. **5C**) [25].

In all the peptide-bound structures of PRMT4 [41, 42], the *Across* conformation is observed and the peptide is bound to the back face of the enzyme, which is unsurprising given that access to the active site from the front side is blocked by the septum. In PRMT4 structures bound to the H3 peptide, Arg 16 is bound in the active site and the acetylated N-terminus of the peptide can be observed near the front of the central pore. In one subunit of one of the structures, the side chain of Lys 14 interacts with residues that form the septum (PDB ID: 5DWQ [41]). In some PRMT4 structures bound to peptides from PABP1, the peptide chain enters the central pore from the back side and wraps around the inside of the active site, across the second monomer, then out the back side of the central pore (see PDB IDs: 5LGP and 5LGR [42]). Thus, it is likely that the specific residues

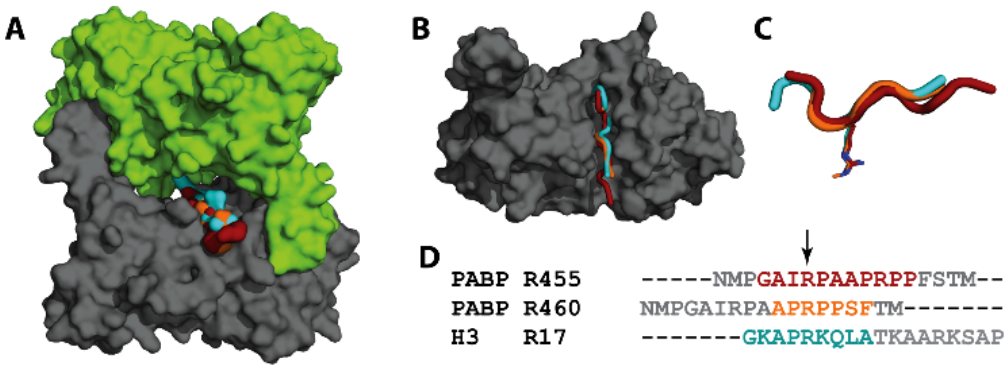

Fig. (4). Substrate peptides show conserved conformation bound to PRMT4. [41] **A**: Peptides with arginine residues in three distinct sequence contexts bound to PRMT4 (PDB ID: 5DX1). Note that the enzyme is viewed from the back face of the dimer and that while peptides are bound to both monomeric subunits, only peptides bound to one of the subunits are shown. The surface of the peptides are shown, and the sequences are from Histone H3 when R16 is bound in the active site (cyan, PDB ID: 5DX0), or from PABP when either R455 is in the active site (red, PDB ID: 5DX1) or when R460 is in the active site (orange, PDB ID: 5DXA). **B**: Top down view of the three peptides bound to a monomeric subunit, with the peptide backbones represented as tubes. **C**: Side view of the peptide conformations. The bound arginine from each peptide is shown as a stick representation. **D**: The three sequence contexts aligned at the bound arginine (denoted by the arrow). The full sequence of each peptide is shown, with residues that are not visible in the crystal structures colored in grey. The visible residues are color-coded to the corresponding peptides in A, B and C. Also note that the histone peptide is acetylated on the N-terminus while the PABP peptides harbor N-terminal biotin and aminohexanoic acid. This figure was inspired by reference [41]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

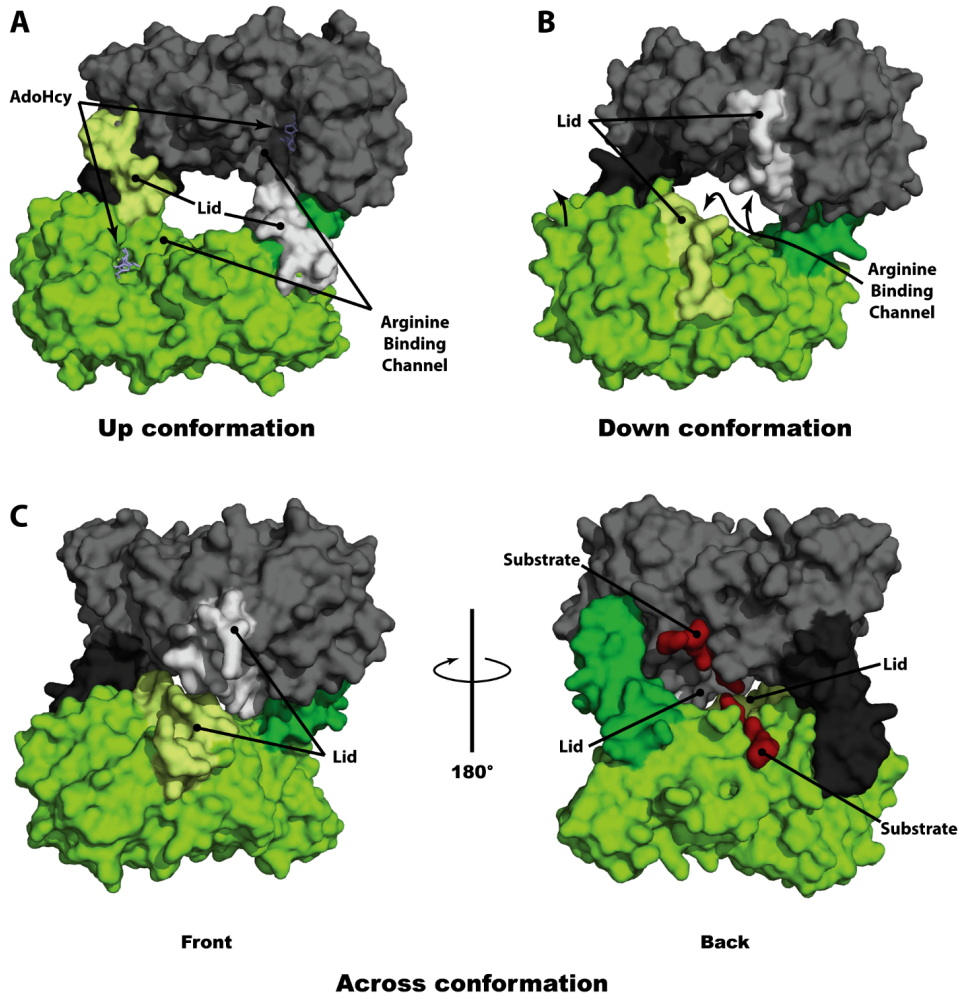

Fig. (5). A lid structure is observed in three conformations. A: The *Up* lid conformation leaves the AdoMet binding pocket exposed to solvent and the central pore open. The structure shown is of PRMT6 (PDB ID: 4C04). The *Up* conformation is also observed in *T. brucei* PRMT7 (PDB ID: 4M38), and in *S. cerevisiae* PRMT1 (PDB ID: 1G6Q). B: The *Down* conformation covers the AdoMet binding pocket but leaves the central pore open. The structure shown is of PRMT3 (PDB ID: 1F3L). The *Down* conformation is also observed in PRMT2 (PDB ID: 5JMQ) and PRMT6 (PDB ID: 4C03). C: The *Across* lid conformation covers the AdoMet binding pocket and also occludes the active site from the front, forcing peptides to approach the active site from the back side of the dimer. Note that residues of the lid region form a potential interaction surface for peptide substrates entering from the back face. The structure shown is for PRMT4 (PDB ID: 5DX1). An *Across* conformation is also observed in PRMT1 (PDB ID: 6NT2). In all currently available structures of PRMTs 8 and 9, the lid region is disordered. In PRMT5 the lid conformation is most similar to the down conformation. In human PRMT5 the pore is open. However in *C. elegans* PRMT5, due to an inserted sequence in the dimer arm, the pore is mostly closed. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

which form the lid, as well as its conformation, strongly influence substrate selection both by interacting with bound substrates, and by controlling access to the active site from different binding grooves.

In addition to the lid, several PRMTs harbor N-terminal accessory domains that could be used to select protein substrates. The pleckstrin homology (PH) domain of PRMT4 (CARM 1) was crystallized by itself, but did not provide electron density when expressed as the natural fusion to the PRMT4 central core [32]. Although we know what the PH domain looks like, we do not know how or where it is positioned relative to the central core. Interestingly, recent studies show that the PH domain is indeed important for substrate selection [47]. PRMT2 has an N-terminal SH3 domain which was shown to be required for full activity with the protein substrate RSF1 [30], but it is currently not clear if the SH3 domain alters activity or specificity (or both). The zinc-finger of PRMT3 is necessary and sufficient for the interaction with Ribosomal protein S2 (rpS2), but the global effect on substrate selectivity was not tested [48]. In PRMT5, the TIM barrel domain is important for dimerization and also for recruiting binding partners such as MEP50. MEP50 is involved in substrate binding and greatly enhances PRMT5 catalytic activity [26, 27, 49, 50]. Interestingly, the structure of the *Xenopus* PRMT5:MEP50 complex shows a narrow channel on the back face of the Rossman fold that extends from solvent to the active site [50]; this opening is not apparent in the human [26] or *C. elegans* [27] structures. The authors suggest that protein substrates could bind to MEP50 and be presented to the active site of PRMT5 through this

channel in what they coined a "cross-dimer" binding mode. However, if one considers the fact that only the *S, S* isomer of AdoMet is likely to be used, it is not quite clear how a substrate accessing the active site from this channel would position an arginine in a chemically competent S_{N2}-reactive geometry. These results suggest, yet again, that significant protein dynamics may be associated with substrate binding.

In addition to the extra domains that some of the PRMT isoforms possess, alternative splicing and initiator codon usage (reviewed in [51]) can give rise to many different variants of some of the isoforms. In the case of PRMT1, Cote showed that the N-terminus of PRMT1 (which is represented in the proteome as seven different splice variants) affects substrate selection [52]. The same may be true for variants of other isoforms.

2.1.4. Structural Summary

In summary, the structural data provide an interesting picture for substrate selection: 1) If we set dynamics aside, spatial restrictions around the active site would suggest that disordered or small diameter alpha helical structures would be preferred by PRMT1. Certainly the disordered "RG" sequences fit well within this criteria, but many alternative disordered sequences could also be included. The larger pores observed for some PRMTs should in theory allow entry of helices harboring larger residues, as long as the arginine can be oriented into the active site. 2) A consensus sequence for arginine methylation may not exist. Bound peptides are often poorly resolved, and when resolved, often show few specific interactions between the enzyme and the side chains of the substrate. What may be more important is a consensus conformation that allows for the positioning of the arginine into the active site while maintaining the ability of the peptide/protein to enter and exit. Certainly, the identities of the side chains will influence the allowable conformations of the substrate. 3) The lid region and accessory domains of the PRMTs may be the most important pieces of the puzzle. Although it is unclear if the lid of a single isoform can adopt all three conformations and if the conformations exist in solution, the lid is in a position to act as a molecular ruler and/or selectivity filter for substrates. The ability of accessory domains to bind substrates or alter selectivity suggests that interactions far from the targeted arginine may be extremely important molecular recognition elements for the PRMTs.

2.2. Peptide and Protein Libraries Tell us that Context and Length Matter

Focused and combinatorial peptide/protein libraries have been extremely useful resources in the identification of amino acid sequence consensus sites for a variety of enzymes that perform posttranslational modifications (PTMs) [53, 54]. When used in conjunction with kinetic analyses, this approach has the ability to yield quantitative information about how individual changes in the peptide sequence affect the ability of the enzyme to catalyze the reaction. First applied to the PRMTs in 2008, our lab screened 36 different peptide sequences to find 11 substrates that did not fit the "RGG" paradigm, debunking the idea that all PRMT1 substrates require an "RGG" sequence [55]. We also saw evidence that the recognition of substrates by PRMT1 was contextual, meaning that residues in one position may influence residues allowed in other positions. This finding was confirmed in a later study [56]. The idea that PRMT1 may be somewhat promiscuous with respect to the amino acid sequence surrounding the targeted arginine was confirmed by larger libraries [57] and future proteomic studies (discussed below).

Certainly, there are amino acids that are not often found in PRMT1 substrates, but the rules regarding these amino acids are context-specific. For example, when considering k_{cat}/K_m values, a peptide sequence based on the PRMT1 protein substrate fibrillarin (Ac-KGGFGGRGGFGGKW) is methylated as well as a peptide based on eIF4A1 (Ac-YIHRIGRGGR). However, the substitution of the glycine residue just to the C-terminal side of the targeted arginine with a serine has very different effects on activity for the two peptides. In the case of the "RS" fibrillarin peptide, there was not enough product to quantify, but the eIF4a1-based "RS" peptide retained 30% of k_{cat}/K_m. However, when the S substitution was embedded in the full eIF4A1 protein sequence, the protein was no longer methylated [55]. In another study [56], the presence of proline near the arginine target site affected methylation, but only in a specific context of sequence. Each new attempt to find the elusive consensus sequence for PRMT1 methylation comes with a new set of sequences presented in a new context [57-59]. All of the data thus far suggest that other than the targeted arginine, there is no consensus sequence for PRMT1. Interestingly, the same may be true for PRMT5 [60], although there is much less data for this isoform.

That recognition by the PRMTs is contextual has important implications. It suggests caution when trying to discern consensus sequence rules originating from a single sequence context. For example, PRMT7 showed a preference for histone-based peptides that contained an RXR sequence [61], but fibrillarin-based peptides, which do not contain RXR, are better substrates for PRMT7 [62].

Instead of a consensus sequence, it seems that sufficient length, particular peptide conformations and positive charges in distal regions may be the key for PRMT substrates. Peptide length is a key factor for PRMT1, CARM1, PRMT5, and PRMT9 [63], suggesting the presence of some type of extended binding surface. Additionally, positive charges in the extended regions make peptides better substrates for both PRMT1 [64] and PRMT5 [60].

The idea that PRMT1 lacks a consensus sequence is consistent with observations that several proteins are methylated within an RXRXXS AKT recognition site by PRMT1. The introduction of a negative charge by phosphorylation of the serine, just three amino acids away from the PRMT1-methylated RXR motif, seemed to have no effect on the ability of PRMT1 to methylate these sites [65, 66] (and reviewed in [67]). On the other hand, one study of the Androgen Receptor suggests that phosphorylation of the serine in an AKT site blocks methylation by PRMT6 [68]. Overall, the substrate profiling data are not surprising based on what we know about the structures of the PRMTs. As noted above, few interactions between the enzyme and the substrate amino acid side chains were observed when peptide substrates were

co-crystallized with a PRMT. In order to capture PRMT recognition elements, the field may need to point its gaze a little farther away from the targeted arginine arginine.

2.3. Bioorthogonal Click-it Studies are in their Infancy

Unlike substrate profiling *in vitro*, the use of bioorthogonal reactions and click chemistry to specifically label molecules of interest can be used *in vivo* to capture substrate proteins from the natural cellular system [69, 70]. Several different AdoMet analogues have been synthesized that can be used by the PRMTs to transfer a clickable chemical functional group instead of the methyl group [71-74]. If made to specifically bind to a particular PRMT isoform (e.g., by mutating a specific PRMT isoform as part of a bump-and-hole strategy), isoform-specific substrates are labeled with a functional handle, allowing selective capture and characterization.

Proof of principle with a variety of different AdoMet analogues has been demonstrated, but these approaches have yet to yield the desired proteomic jackpot of PRMT substrates. Luo and co-workers produced a small initial proteomic set of 79 distinct PRMT3 substrates using a matched PRMT3 mutant (M233G) and the 4-propargyloxy-but-2-enyl AdoMet analogue [73]. HEK293T lysates containing the engineered PRMT3 were incubated with the AdoMet analogue, followed by the addition of a biotin probe, enrichment with streptavidin, and MS analysis. Interestingly, for two of the modification sequences, neither of the arginine sites were located in Arg- and Gly-rich domains (e.g., TCOF1 contains EEDSRSSSE and MYO18B contains RPRIRKENQD). The authors noted that their data suggests that PRMT3 can recognize sequence motifs other than Arg- and Gly-rich domains. Their conclusions may also highlight one of the benefits of this approach in studying substrate specificity; i.e., because the enrichment step does not use antibodies, there is no amino acid sequence bias introduced into the study.

A current limitation for using this approach is the inability to administer the positively charged AdoMet analogs to cells; instead, cell lysates are treated with the analogs. The inability to label proteins that are already methylated and the loss of cellular structure and natural complexes upon cell lysis all are likely to contribute to low yields. However, metabolic labeling *in vivo* has proven to be very powerful when applied to other systems [75], so it is likely that with time, we will see creative improvements in this approach.

2.4. Proteomic Studies have Yielded the Largest Database of Protein Substrates to Review

Proteomic methodologies to identify proteins containing methylarginines have evolved over the last ~15 years. As with many MS-based protocols, some type of enrichment in the desired molecule prior to MS is important. However, because methylation of arginyl residues does not change the pKa of the amino acid [76] and the addition of the methyl group changes the hydrophobicity in small increments, the most practical way to selectively capture peptides containing a methylarginine is through immunoprecipitation. In 2003, the Richard lab was the first to generate both SDMA- and ADMA-specific antibodies and use them in the first proteomic analysis to extract methylated proteins from HeLa cells [77]. One year later, the Mann lab made improvements in the protocol by applying stable isotope labeling by amino acids in cell culture (SILAC) [78]. In this technique, two sets of cultures are grown, one in a light media (non-deuterated methionine) and one in media containing deuterated methionine (deuterium in the methyl group), which are converted in the cell to deuterated AdoMet (CD_3-AdoMet). Methylated peptides (from methylated proteins) appear as pairs of peaks (SILAC pairs) separated by a predicted mass difference. The use of SILAC increased confidence in the identification of methylation sites and their relative quantities.

Both the initial proteomic studies used antibodies elicited from single sequence peptides containing ADMA or SDMA. Data sets achieved were mostly limited to the identification of those peptides containing a similar sequence to the peptide used to generate the antibodies. Specifically, many of the identified methylation sites resided in arginine-glycine sequences. This limitation was overcome with the production of pan-specific antibodies, generated using a peptide library housing either an MMA, ADMA, or SDMA residue (now sold by Cell Signaling Technology) [79]. In 2015, Acuto and co-workers [80] took advantage of these new pan-specific antibodies and added a twist to the SILAC approach. Noting that the incorporation of deuterated methionine into proteins would also generate SILAC pairs, the authors eliminated this ambiguity by using a combination of L-methionine-$^{13}C_4$ in one culture set and L-methionine-$^{13}CD_3$ in the other set and called the strategy "isomethionine methyl-SILAC" (iMethyl-SILAC). This study identified numerous methylated sequences (27%) that deviated from the well-known arginine-glycine rich sequence.

This finding was confirmed in a 2016 proteomic study by Nielsen and co-workers, who identified 8030 arginine methylation sites in proteins from human embryonic kidney 293 cells [6]. The increased sample size allowed the authors to reach three very important conclusions related to PRMT substrate specificity: 1) arginine methylation and phosphorylation localize to the same sequence regions in target substrates, supporting several studies noting crosstalk between the two PTMs, 2) the PRMTs catalyze methylation independent of the sequence surrounding the arginine, and 3) PRMTs target arginine-glycine and non-arginine-glycine sequences with equal preference. It is important to note that because this study did not distinguish between PRMT isoforms, any specificity exhibited by a particular PRMT isoform was likely masked.

Continued efforts have focused on identifying isoform-specific PRMT substrates, where it is expected that sequence preference can be teased out of the data and a set of isoform-specific recognition rules may be discovered. PRMT4 (CARM1) [47] and PRMT5 [81] substrates have been evaluated using knock out cell lines or inhibitors, respectively. Bioinformatic analyses showed that proline residues were enriched in CARM1 substrates, but proline was not required in CARM1 substrates [47]. Similar to what has been observed *in vitro* [82], CARM1 substrates were not enriched in glycine-arginine-rich (GAR) sequences. The authors of the PRMT5 study detected a preference of glycine in the -1 position. Although the authors did not discuss it, their data also showed that sequence recognition by PRMT5 is contextual;

e.g., mutation of G26 in the protein substrate CNBP to alanine abolished methylation by PRMT5, but adding a mutation of S31G restored methylation by PRMT5 [81].

3. DO PRMTS RECOGNIZE SECONDARY STRUCTURE?

While many of the well-characterized methylation sites (histones, RNA binding proteins) exist in disordered sequences, we wondered how often PRMTs might methylate arginine residues in secondary structural elements. To address this question, we retrieved the UniprotKB identifiers and corresponding methylation sites for the proteins identified in Nielsen's study [6]. We extracted sequences truncated to ± 50 residues from the methylarginine site and used the PROMALS3D server [83] and the JPred server [84] to screen these sequences for secondary structure. We were surprised to find 1499 sites (~20%) with the methylarginine present on a predicted α-helix, and 19 sites (~0.3% of those considered) on a β-sheet.

When a new study characterizes a new arginine methylation site, we often check if a structure of the methylation site is available. Often we find that even if a structure of the substrate protein is available, the region containing the methylation site is disordered or otherwise not present in the structure. However, we reasoned that if the sites identified in our bioinformatic analysis do indeed occur within a structured element, then some of the sites may be represented in the Protein Data Bank. Out of about 10 sites picked randomly from the dataset, we found two structures [85, 86] of a well-resolved arginine methylation site present on an alpha helix (Fig. **6**). Given that much of the available literature on PRMT substrate selection has focused on disordered RXR and RGG motifs, it is somewhat surprising that this analysis indicates that such a high proportion of methylarginine sites lie within defined structural elements. While this analysis should be considered a rough estimate, it opens the possibility that a significant fraction of PRMT methylation sites exist in defined structures. Importantly, because the proteomic dataset we used represents all PRMT substrates, it is possible that hidden in this data is a set of recognition motifs for a particular isoform of PRMT.

SUMMARY

Defining the substrate specificity of the PRMTs is no small task. The arginine methylome is large and there have been technical challenges to overcome. Substrate profiling (let's consider this a bottom-up strategy) quantitatively examines the sequences directly adjacent to the targeted arginine but misses the context of the entire protein and extended binding surfaces that are likely needed/used *in vivo*. Still, they accurately predicted a promiscuous PRMT1 that does not target substrates using a conventional consensus sequence. Proteomic and bioorthogonal studies (let's consider these top-down strategies) have done well to reveal the variety of methylation sites, but have yet to deliver on a proteome for each PRMT isoform. Importantly, caution must be applied to distinguish between high abundance substrates versus preferred substrates. The major points from the data collected from all of these approaches are summarized in

Table 1. Observations related to the substrate specificities of the PRMT isoforms.

Isoform	Substrate sequences/Observation[a]	Context	Reference
PRMT1	**R**G and **R**GG sequences		
	RGX and **R**XG sequences are substrates	fibrillarin library	[55]
	Recognition is contextual; no consensus		[55]
	Substrates rich in Phe and Arg; no consensus	unbiased library	[58]
PRMT2	GGT**R**VY and GGG**R**PYN	RSF1	[30]
PRMT3	**R**G sequences	rpS2	[48]
	LTC**R**LEK	GAPDH	[87]
	ESL**R**SHF	hnRNPA1	[88]
	EDS**R**SSS	TCOF	[73]
	PRI**R**KEN	MY18B	[73]
PRMT4	Enrichment in Pro-containing motifs	Immunoprecipitate	[82]
	QDL**R**(N/S)HL	P300/CBP	[36]
PRMT5	Histone 4-based > fibrillarin-based		[60]
	Distal + charges important	Histone 4	[60]
	G**R**G sequences are substrates; contextual	Immunoprecipitate	[81]
PRMT6	**R**G and **R**GG sequences	Fibrillarin, Npl3	[89]
	AVF**R**LLH	CRAF	[90]
PRMT7	RXR motifs or RXXR motifs, **R**G	Histone2B	[91]
PRMT9	CFK**R**KYL	SF3B2	[63]

[a]The targeted arginine is in bold face font.

Table **1**. Cracking the code to PRMT target recognition is going to require both quantitative and proteomic approaches and rely heavily on understanding the extended interface between protein substrates and the PRMTs.

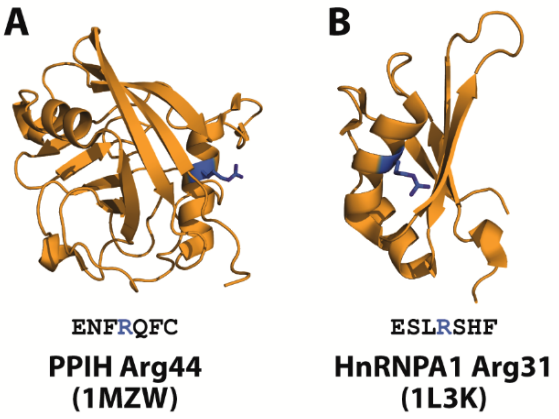

Fig. (6). Structures of **A**: PPIH (UniprotKB: O43447) and **B**: hnRNPA1 (UniprotKB: P09651). The reported methylation site is shown in light blue with a stick representation, with the sequence context written below. In the structure for hnRNPA1, only the structure for residues 8 - 91 is shown. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

LIST OF ABBREVIATIONS

ADMA	=	Asymmetric Dimethylarginine
AdoHcy	=	S-Adenosyl-L-Homocysteine
AdoMet	=	S-Adenosyl-L-Methionine
MMA	=	Monomethylarginine
PH	=	Pleckstrin Homology
PTM	=	Posttranslational Modification
PRMT	=	Protein Arginine Methyltransferase
SILAC	=	Stable Isotope Labeling by Amino Acids in Cell Culture
SDMA	=	Symmetric Dimethylarginine
TIM barrel	=	Triose Phosphate Isomerase Barrel

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation [CHE- 1412405 to JMH].

Both authors have contributed substantially to the writing of this review. The authors wish to thank the Hevel Lab members for critically evaluating the manuscript.

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