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The Intricate Effects of alpha-Amino and Lysine Modifications on Arginine Methylation on the N-terminal Tail of Histone H4

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Abstract

Chemical modifications on the DNA and nucleosomal histones tightly control the gene transcription program in eukaryotic cells. The “histone code” hypothesis proposes that the frequency, combination, and location of post-translational modifications (PTMs) on the core histones compose a complex network of epigenetic regulation. Currently, there are at least 23 different types and over 450 histone PTMs discovered, and the PTMs on lysine and arginine residues account for a crucial part of the histone code. Although significant progress has been achieved in recent years, the molecular basis for the histone code is far from being fully understood. In this study, we investigated how naturally occurring N-terminal acetylation and PTMs on histone H4 lysine-5 (H4K5) affect arginine-3 methylation catalyzed by both type I and type II PRMTs at the biochemical level. Our studies found that acylations of H4K5 resulted in decreased arginine methylation by PRMT1, PRMT3, and PRMT8. In contrast, PRMT5 exhibits increased arginine methylation upon H4K5 acetylation, propionylation, and crotonylation, but not upon H4K5 methylation, butyrylation, or 2-hydroxyisobutyrylation. Methylation of H4K5 did not affect arginine methylation by PRMT1 or PRMT5. There was a small increase in arginine methylation by PRMT8. Strikingly, a marked increase in arginine methylation was observed for PRMT3. Finally, N-terminal acetylation reduced arginine methylation by PRMT3, but had little influence on PRMT1, 5, and 8 activity. These results together highlight the underlying mechanistic differences in substrate recognition among different PRMTs and pay the way for the elucidation of the complex interplays of histone modifications.

TOC image

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ACCESSION CODES

H. sapiens PRMT1 (UniProt ID: Q99873), *H. sapiens* truncated PRMT3 (residues 211–531, UniProt ID: O60678), *H. sapiens* PRMT8 (UniProt ID: Q9NR22), *H. sapiens* PRMT5 (UniProt ID: O14744), and *H. sapiens* MEP50 (UniProt ID: Q9BQA1).

Supporting Information.

Additional tables and figures are available free of charge on the ACS website.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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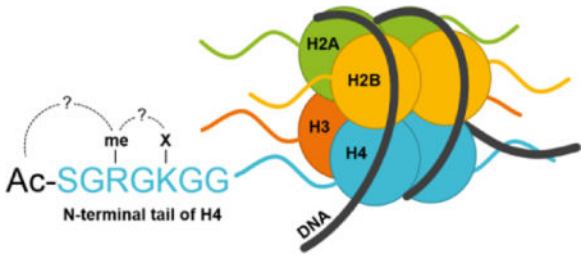

Keywords

PRMT; arginine methylation; histone H4; acylation; acetylation; propionylation; butyrylation; crotonylation; 2-hydroxyisobutyrylation; N-terminal; cooperativity

INTRODUCTION

Post-translational modifications (PTMs) on the nucleosomal core histones are important epigenetic mechanisms during the orchestration of chromatin dynamics.[1] There are at least 450 modifications reported with respect to the site and modification type.[2] The frequency, combination, and location of histone modifications are hypothesized to form a complex network of information proposed as the "histone code".[3, 4] With the fact that most of the histone modifications identified are located on lysine and arginine residues,[2] this has spurred great interest in understanding the enzymatic mechanisms and functions of lysine and arginine methyltransferases. Notably, arginine methylation and protein arginine methyltransferases (PRMTs) are involved in cancer, aging, cardiovascular, metabolic and neurodegenerative diseases.[5, 6] Therefore, it is important to understand the regulation of arginine methylation by PRMTs for unraveling the molecular basis of the histone code combinations as well as the implications in disease.

In 1968, Paik and Kim discovered a protein extract that used the cosubstrate S-adenosylmethionine (SAM) to catalyze arginine methylation and named it "protein methylase I."[7] By 1977, the products of protein methylase I were identified as ω-N^G-monomethylarginine (MMA), ω-N^G,N^G-asymmetric dimethylarginine (ADMA), and ω-N^G,N'^G-symmetric dimethylarginine (SDMA).[8] With the advancement of molecular biology in the 1990s, it was made clear that were at least nine enzymes in mammals, now named protein arginine methyltransferases (PRMTs) that install the arginine methylation mark in proteins.[9, 10] These PRMT proteins are classified into type I, II, or III based on the methylated arginine products they catalyze.[11] As observed by Paik and Kim, PRMTs use SAM as the cosubstrate to catalyze methylation of the terminal guanidino nitrogens (ω-N^G) on arginine residues; however, the PRMTs diverge in the capacities to produce the methylated arginine products.[8] While all PRMT types catalyze MMA, only type I catalyze ADMA and type II catalyze SDMA.[11] The major PRMTs exhibit type I activity, and these include PRMT 1, 2, 3, 4 (CARM1), 6, and 8.[11, 12] PRMT9 and PRMT5 exhibit type II activity; however, PRMT5 is the representative type II PRMT, and it is considerably more active when in complex with MEP50.[13–15] Finally, type III activity (MMA only) has been observed with PRMT7.[13, 16] From this group, PRMT1 has been observed to be responsible

for most of the asymmetric dimethylarginine marks in mammals.[12, 17] PRMT1 will methylate histone H4 Arg3 (H4R3me) *in vivo*,[18, 19] and this has been reproduced *in vitro* with recombinant PRMT1 and histone H4 peptide substrates H4(1-20) and H4(1-21).[20, 21] Also, further truncation of the H4 peptide sequence leads to reduced arginine methylation by PRMT1.[21] PRMT3, 5, and 8 can catalyze the methylation of an H4 peptide H4(1-24).[22, 23] While PRMT3 is found localized to the cytoplasm [24, 25] and is important for methylating 40S ribosomal protein S2 (RPS2),[26, 27] both PRMT1 and PRMT3 appear to methylate substrates near 14.4 kDa (near the molecular weight of H4) in yeast extracts.[24] Since the residues neighboring H4R3, Ser1 and Lys-5, are also subject to modification,[2] it raises the question of how neighboring PTMs affect H4R3 methylation by type I and type II PRMTs. Previous studies have investigated the impact of H4K5 acetylation and N-terminal acetylation on H4R3 methylation.[20, 28, 29] However, there has yet to be a study that dissects the impact of the diverse H4K5 PTMs and H4 N-terminal acetylation on type I and type II PRMTs.

Thus far, methylation (me), acetylation (ac), propionylation (pr), butyrylation (bu), crotonylation (cr), 2-hydroxyisobutyrylation (hib), have been observed on H4K5.[2] In yeast, acetylation is the predominant histone H4 PTM (approximately 88%), and about 32% of the H4 lysine acetylation exists on K5.[30] Propionylation and butyrylation of H4K5 occurs *in vivo* from nuclear extracts of HeLa cells in a proteomics study, and an *in vitro* biochemical study confirmed that histone acetyltransferases p300 and CBP are capable of introducing these modifications.[31] Crotonylation and 2-hydroxyisobutyrylation of H4K5 was observed in mouse and human cell lines.[32, 33] While p300 has shown cosubstrate promiscuity by accepting crotonyl-CoA in addition to propionyl-CoA, butyryl-CoA, and acetyl-CoA,[31, 34] it is not clear which enzyme is responsible for catalyzing crotonylation and 2-hydroxyisobutyrylation of H4K5. Since the rates of crotonylation, butyrylation, and propionylation by p300 are extremely low (3-fold to as much as 62-fold) in comparison to acetylation,[34] there may be a metabolic component that involves increasing short chain acyl-CoA concentrations and/or a non-enzymatic mechanism to regulate the diversity and levels of protein lysine acylations.[35, 36] Methylation of H4K5 exists *in vivo* and is catalyzed by the lysine methyltransferase SET5 in yeast and by the SET and MYND domain-containing protein 3 (SMYD3) in human.[37, 38] Moreover, while SMYD3 is able catalyze mono-, di-, and trimethylation of H4K5 as well as monomethylation of H4K12, the major product of SMYD3 is monomethylated H4K5.[38] Considering the structure of the naturally occurring H4K5 PTMs, these modifications set the stage for collecting a basic structure-activity relationship in addition to better understanding the impact of H4K5 PTM on arginine methylation by PRMT family members.

In this study, we sought to understand how the natural modifications on histone H4K5 and N-terminal acetylation affect arginine methylation by different type I and type II PRMTs from a biochemical basis (Figure 1). We designed and synthesized a library of unacetylated and N-α-acetylated histone H4 peptides (H4 N-terminal tail residues 1-20 or 1-21) that incorporated six naturally occurring PTMs on H4K5. Biochemical assays were performed to quantitate the steady-state activities of different PRMTs with the peptide library. Interestingly, while both type I and II PRMTs methylated the unmodified H4 peptide, the presence of H4K5 acylations led to different impacts on substrate recognition among the

PRMTs, which suggests a fine-tuning mechanism of arginine methylation among the PRMTs. Our findings provide a molecular-level understanding for the functional distinction of individual PRMTs in response to different biological cues and contexts.

METHODS AND MATERIALS

Chemical Reagents

The *N*-α-Fmoc-protected amino acids were purchased from either Novabiochem or ChemPep Inc. HPLC grade methanol and acetonitrile were purchased from either Sigma Aldrich or British Drug Houses (BDH). Phenylmethylsulfonyl fluoride (PMSF) was purchased from either Gold Biotechnology or Sigma Aldrich. Kanamycin, ampicillin, and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Gold Biotechnology. Unless otherwise stated, the remaining chemical reagents described were purchased from Fisher Scientific, Acros Organics, Sigma Aldrich, Alfa Aesar, BDH, Research Products International Corp., Macron Fine Chemicals, Bio-Rad, or J.T. Baker.

Protein Expression

Human recombinant, N-terminal flag-tagged PRMT5 (PRMT5, UniProt ID: O14744) and N-terminal His-tagged MEP50 (UniProt ID: Q9BQA1) was purchased as a complex through Reaction Biology Corporation (Product HMT-22-148). The PRMT5-MEP50 complex was co-expressed in a baculovirus/insect cell expression system, and the purified product was kept in a 50 mM tris-HCl (pH 8) buffer with 110 mM NaCl, 2.2 mM KCl, 3 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and 20% (v/v) glycerol. The human recombinant PRMT1 (PRMT1, UniProt ID: Q99873) was delivered in the pET28b(+) vector, truncated PRMT3 (PDB ID: 2FYT, residues 211–531, UniProt ID: O60678) in the pET28a-LIC vector, and PRMT8 (UniProt ID: Q9NR22) in the pET100 vector. All type I PRMTs were expressed with an N-terminal His-tag in *Escherichia coli*. In detail, the plasmids underwent heat shock transformation into BL21(DE3) cells (Stratagene), and then cultured overnight in 8 mL of LB media containing either kanamycin or ampicillin at 37 °C with constant shaking (225 rpm). The next day, the cultures were scaled up to 2 L cultures (8 mL culture per 1L LB media) and incubated at 37 °C with constant shaking until OD_{595} values were between 0.6 and 0.8. The cultures were chilled on ice before inducing protein expression with 0.3 mM IPTG followed by overnight incubation at 16 °C with constant shaking. Cells were disrupted in lysis buffer (25 mM Na-HEPES pH 7, 150 mM NaCl, 1 mM $MgSO_4$, 5% glycerol, 5% ethylene glycol, 1 mM PMSF) with a Microfluidics cell disruptor. Cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C, and the supernatant was gently rocked for 1–1.5 h with Ni-NTA His·Bind® Resin (Novagen) that was pre-equilibrated in 25 mM Na-HEPES pH 7, 300 mM NaCl, 30 mM imidazole, and 1 mM PMSF. The resin was thoroughly washed with a 25 mM Na-HEPES pH 7 buffer containing 300 mM NaCl, 70 mM imidazole, 1 mM PMSF. Protein was eluted with a 25 mM Na-HEPES pH 7 buffer containing 300 mM NaCl, 100 mM EDTA, 200 mM imidazole, 10% glycerol, 1 mM PMSF. Protein purity was checked by resolving the eluted proteins on a 12 % polyacrylamide gel using SDS-PAGE. Elution fractions were concentrated in centrifugal filter units (regenerated cellulose 10,000 NMWL, Millipore) for 0.5–1 h by centrifugation (4 °C, 5000 rpm) before dialysis in a 25 mM Na-HEPES pH 7 buffer

containing 300 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT. Purified proteins were concentrated again by centrifugation (4 °C, 5000 rpm, 1–2 h), and protein concentration was determined with the Bio-Rad Protein Assay by following the manufacturer's protocol. All absorbance values were measured with a Shimadzu UV Spectrophotometer (UV-1800). Purified proteins were aliquoted and flash frozen with liquid nitrogen before storing at −80 °C.

Peptide Synthesis

All peptide sequences are based on the histone H4 (H4) N-terminal tail. Ac-H4(1-20) includes N-terminal H4 residues 1-20, Ac-H4(1-21) includes H4 residues 1-21, and Ac-H4(1-22) includes H4 residues 1-22. All peptides were synthesized on an AAPPTec Focus XC synthesizer from Wang resin (Novabiochem) with *N*-α-Fmoc-protected amino acids. Each amino acid was double coupled to the solid phase with 5 eq. of amino acid/HCTU [O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (Novabiochem, Darmstadt, Germany) and 20 eq. of N-methyl-morpholine (NMM). The Fmoc deprotection reactions were performed with 20% (v/v) piperidine in DMF. The N-terminus of each peptide, unless stated otherwise, was acetylated with acetic anhydride. Peptides were cleaved from Wang resin with 2.5% ethane dithiol (EDT), 5% deionized water, 5% thioanisole, 5% phenol, 1% triisopropylsilane, and 81.5% trifluoroacetic acid (TFA). Peptides were precipitated in cold diethyl ether and pelleted by centrifugation (5 min, 2000 rpm). After centrifugation, the crude peptides were dissolved in water for lyophilization. Purification was performed on a Shimadzu reversed-phase high performance liquid chromatography (RP-HPLC) equipped with a Polaris 5 C18-A, 150 × 21.2 mm (Agilent). Peptides were purified with a linear gradient using 0.05% TFA in water and 0.05% TFA in acetonitrile as the two mobile phases. The purified peptides were confirmed and characterized by MALDI, and the peptide purity was checked by analytical HPLC. The stock solutions of Ac-H4(1-21), NH2-H4(1-21), Ac-H4(1-22)R3me, Ac-H4(1-22)R3me2a, and Ac-H4(1-22)R3me2s were prepared based on weight and not calibrated. The concentrations of Ac-H4(1-20) and Ac-H4(1-20)K5 modified peptides were calibrated by NMR.

Determine Peptide Concentrations with NMR

The method is similar to that previously described.[20] A Varian Unity INOVA 500 MHz was used to determine the one-dimensional ^{1}H NMR spectrum at room temperature for Ac-H4(1-20) and the Ac-H4(1-20)K5 modified peptides. D_2O solutions containing 1.8 mM (weight-based) of the Ac-H4(1-20) or a Ac-H4(1-20)K5 modified peptide and 0.2 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) were prepared for each NMR sample. The integration ratio was measured for each peptide as previously described,[20] note (δ = 6.5–8.0). Ac-H4(1-20) and Ac-H4(1-20)K5 modified peptides were dissolved in deionized water to prepare stock solutions. The NMR spectra are available in the supporting information.

Radioactive Methylation Assay (Filter-Binding Assay)

Each methylation reaction was supplied with either ^{14}C-isotope-labeled SAM ([^{14}C]-SAM 56.3 mCi/mmol or [^{14}C]-SAM 58 mCi/mmol, catalog No. NEC363050UC from PerkinElmer, Inc.). The reaction buffer contained 50 mM HEPES (pH 8), 10 mM NaCl, 0.5

mM EDTA, and 0.5 mM DTT. Each peptide was pre-mixed at room temperature with [^{14}C]-SAM before initiating the reaction by adding the PRMT for a final concentration of 0.02 μM with the 15 min reactions or 0.05 μM with the 35 min reactions as indicated in the results. Once initiated, all reactions were incubated at 30 °C. Reactions were quenched by an equal volume of isopropanol, immediately vortexed, pulse spun down, and then loaded onto P81 Whatman filter paper (2.2 cm × 2 cm for each sample, Reaction Biology) to dry for 30 min at room temperature. Afterwards, the filter paper samples (except [^{14}C]-SAM reference samples) were washed three times (20 min/wash) with 50 mM $NaHCO_3$ (pH 9). All filter paper samples were allowed to dry for at least 3 h at room temperature. Each filter paper sample was immersed in 5 mL of scintillation cocktail (Ultima Gold mixture, PerkinElmer) and incubate for 30 min–1 h at room temperature in the dark before liquid scintillation counting on a Beckman Coulter LS 6500 Multi-Purpose Scintillation Counter (5 min/sample). The counts per minute (cpm) measured by liquid scintillation counting was converted to the concentration of methylated products (*P*) based on the known concentration and cpm measured for the [^{14}C]-SAM reference samples (equation 1). The experiments were performed, at minimum, in duplicate. Either KaleidaGraph (Version 4.03) or GraphPad Prism 7 was used to fit the kinetic data with equation 2 (Michaelis-Menten) or equation 3 (Hill) to calculate the steady-state kinetic parameters k_{cat}, $K_{0.5}$, and n (Hill coefficient).

$$\mathrm{P}=\frac{\mathrm{total}[14\mathrm{C}]-\mathrm{SAM}\times \mathrm{cpm}_{\mathrm{sample}}}{\mathrm{cpm}_{\mathrm{total\ SAM}}} \quad \text{(Eq. 1)}$$

$$\mathrm{Rate}\left(\mathrm{min}^{-1}\right)=\frac{v}{[E]}=\frac{k_{\mathrm{cat}}[S]}{K_{\mathrm{m}}+[S]} \quad \text{(Eq. 2)}$$

$$\mathrm{Rate}\left(\mathrm{min}^{-1}\right)=\frac{v}{[E]}=\frac{k_{\mathrm{cat}}[S]^{n}}{{K_{0.5}}^{n}+[S]^{n}} \quad \text{(Eq. 3)}$$

RESULTS

Arginine methylation by PRMT1 was reduced with H4K5 acylation

We used a radioactive filter-binding biochemical assay to determine the steady-state activities of PRMTs. In the reaction, Ac-H4(1-20) (with or without K5 modifications, Figure 1) or Ac-H4(1-21) was used as the peptide substrate, and [^{14}C]-SAM was the co-substrate. Unless otherwise specified, all the H4 peptides are N-terminal capped with an acetyl group since the endogenous histone H4 protein is N-terminally acetylated *in vivo*.[39–41] The reaction mixture was loaded on P81 filter paper and washed with sodium bicarbonate buffer (pH 9) to remove unreacted [^{14}C]-SAM. With an internal [^{14}C]-SAM standard of known concentration, the filter paper samples immersed in the scintillation cocktail and the

radioactivity of the product on the filter paper was quantified by a Beckman Coulter scintillation counter.

We performed a single-point methylation assay to identify trends in arginine methylation with all seven Ac-H4(1-20) peptides (Figure 2A and Table 1). Methylation of H4K5 did not affect the rate of arginine methylation by PRMT1 (Figure 2A). Strikingly, we observed that with all the acylation modifications, a 2-fold or greater reduction was observed in the rate of arginine methylation (Figure 2A). Clearly, Lys-5 acylation results in an inhibitory effect on Arg-3 methylation by PRMT1. Hence, to examine this effect more closely, we measured the steady-state kinetic parameters of PRMT1 with the seven peptide substrates. Reaction rates were calculated based on the [^{14}C]-SAM internal standard (equation 1) and plotted against a range of individual H4 peptide substrates for steady-state kinetic quantitation.

We carefully examined the concentration-dependent activity data of PRMT1 with respect to differently modified Ac-H4(1-20) substrates. Interestingly, the majority of the steady-state kinetic data points did not fit the traditional Michaelis-Menten equation (Figure 2B). Instead, the concentration-activity data points fit considerably well with the Hill equation. Hence, the kinetic parameters were determined by applying the Hill equation to the kinetic data (Table 1). Remarkably, the range of Hill coefficients (n) was 1.3 to 5.1, and the highest n value was observed when Ac-H4(1-20) was the substrate (n = 5.1). Interestingly, acylation of Lys-5 appeared to decrease n, particularly if Lys-5 was crotonylated or 2-hydroxyisobutyrylated. Most of the values were above 2, and the case exception was when PRMT1 was provided Ac-H4(1-20)K5$_{cr}$ as the substrate. The high Hill values imply possible positive cooperativity in the enzyme catalysis, which is a phenomenon where the binding of one substrate can enhance the binding of a subsequent substrate molecule to the enzyme.[42] The mechanistic basis for the high cooperativity of PRMT1 is not clear at this stage.

In comparison to the unmodified Ac-H4(1-20) peptide, methylation of H4K5 did not strongly affect k_{cat} or $K_{0.5}$, resulting in a catalytic efficiency comparable to Ac-H4(1-20) (Table 1). In contrast, H4K5 acetylation reduced k_{cat} by nearly 3-fold in comparison to Ac-H4(1-20) while not affecting $K_{0.5}$, and this dropped the $k_{cat}/K_{0.5}$ over 2-fold. The effect from K5 acetylation was consistent with our previous study.[20] Yet, with increasing length and steric bulk of the acyl group, $K_{0.5}$ began to increase while k_{cat} appears to remain low. This indicates that the length and steric hindrance of the acyl modifications predominantly affects binding.

Arginine methylation by PRMT3 was enhanced by H4K5 methylation and reduced by H4K5 acylation

As a comparison to hPRMT1, arginine methylation was monitored with another type I PRMT, hPRMT3. As before, we setup a single-point methylation assay to identify the trends of PRMT3 activity in arginine methylation with all the seven peptides (Figure 3A). Similar to PRMT1, the overall arginine methylation was decreased upon acylation, and this responded to increasing length and steric bulk to the acyl modifications. However, most strikingly there was a robust increase in arginine methylation (over 5-fold) of Ac-H4(1-20)K5$_{me}$ in comparison to Ac-H4(1-20). To examine this more closely, we determined the full kinetic parameters for Ac-H4(1-20) and Ac-H4(1-20)K5$_{me}$. Similar to PRMT1, the

PRMT3 kinetic data fit the Hill equation (Figure 3B). Indeed, there was a 3-fold increase of k_{cat} upon methylation of Lys-5 with a decrease in $K_{0.5}$ that resulted in a catalytic efficiency nearly 4-fold higher than Ac-H4(1-20) (Table 2). Based on these results, Ac-H4(1-20)K5$_{me}$ is a better substrate than Ac-H4(1-20) for PRMT3.

We also determined the full kinetic parameters for Ac-H4(1-20)K5$_{ac}$ as a representative acylated peptide to compare with methylated and unmethylated Ac-H4(1-20) (Table 2). This was to tease apart whether or not the decreased arginine methylation by PRMT3 was due to reduced k_{cat} and/or $K_{0.5}$. We suspected the positive charge on Lys-5 may be important for PRMT3 catalysis as it appeared to be with PRMT1. While k_{cat} was reduced slightly upon acetylation in comparison to Ac-H4(1-20), $K_{0.5}$ increased 2-fold (Figure 3B and Table 2). Hence, it is not as clear as with PRMT1 that the positive charge on Lys-5 alone appears to be important for PRMT3 catalysis.

Acylation negatively affected arginine methylation by PRMT8

PRMT8 shares the highest structural homology with PRMT1.[43] We were interested in determining how methylation of Arg-3 by PRMT8 was affected by Lys-5 modifications. A single-point methylation assay was setup to snapshot any general trends in the arginine methylation upon H4K5 methylation or acylation. Upon H4K5 methylation, there was a marginal increase in arginine methylation (0.19 min^{-1} ± 0.017 Ac-H4(1-20) vs. 0.23 min^{-1} ± 0.0068 Ac-H4(1-20)K5$_{me}$) (Figure 4A). In contrast, all the types of acylations led to a dramatic decrease (2-fold or greater) in arginine methylation in comparison to unmodified Ac-H4(1-20) substrate, and crotonylation gave the strongest inhibitory effect on PRMT8 activity. Similar as before, we pursued a closer examination of the complete kinetics of methylated versus acetylated Ac-H4(1-20) (Figure 4B). While k_{cat} was not affected by methylation of Lys-5, the $K_{0.5}$ decreased over 2-fold resulting in the higher catalytic efficiency in comparison unmodified Ac-H4(1-20) (Table 2). In contrast, acetylation of Lys-5 reduces k_{cat} and dramatically increases $K_{0.5}$ over 3-fold in comparison to Ac-H4(1-20) and over 10-fold in comparison to Ac-H4(1-20)K5$_{me}$. Hence, similar to PRMT3, Ac-H4(1-20)K5$_{me}$ is the best substrate for PRMT8. Also, acylation of Lys-5 negatively impacts arginine methylation by PRMT8, which is comparable to what we observed with PRMT1 and PRMT3.

Acetylation of H4K5 promoted arginine methylation by PRMT5

To compare our observations with the members of the type I PRMTs, we investigated how the modifications of H4K5 affected arginine methylation by the major type II enzyme, PRMT5. As shown in Figure 5, methylation of H4K5 does not affect arginine methylation in comparison unmodified Ac-H4(1-20). Moreover, contrary to the type I PRMTs in this study, there was an increase arginine methylation upon acetylation of Lys-5 in comparison to Ac-H4(1-20), which is consistent with our previous study.[20] Nevertheless, there was a decline in arginine methylation thereafter as the alkyl chain increased with propionylation and butyrylation. Upon introduction of a *trans* double bond, crotonylated Lys-5, led to a slight increase in arginine methylation in comparison to unmodified Ac-H4(1-20). However, 2-hydroxyisobutyrylation of Lys-5 decreased arginine methylation by PRMT5-MEP50.

Acetylation of the H4 N-terminal amino group reduced arginine methylation by PRMT3, but not PRMT1, PRMT8, or PRMT5-MEP50

Since Arg-3 is very close to the H4 N-terminus, we wondered whether or not the naturally occurring acetylation of the N-terminal amino group of H4 affects arginine methylation by PRMTs. A single-point radioactive methylation assay was used to observe arginine methylation of NH_2-H4(1-21) or Ac-H4(1-21) by PRMT1, 3, 8, and PRMT5-MEP50. Interestingly, acetylation of the H4 peptide N-terminus did not strongly affect arginine methylation by PRMT1, 8, or PRMT5-MEP50. There was a two-fold reduction observed ($0.12\ min^{-1} \pm 0.0029$ with NH_2-H4(1-21) vs. $0.063\ min^{-1} \pm 0.0028$ with Ac-H4(1-21)) in the presence of PRMT3 (Figure 6). Hence, despite masking the positive charge on the N-terminal alpha amino group, N-terminal acetylation did not strongly affect arginine methylation by most of the PRMTs examined.

DISCUSSION

The nuclear core histones are rich in a variety of PTMs, especially at the N-terminal tail region. The histone H4 N-terminal modifications include methylation, acetylation, propionylation, crotonylation, butyrylation, 2-hydroxyisobutyrylation, citrullination, succinylation, formylation, and phosphorylation.[2] MS-based proteomic studies infer that these various modifications can give rise to diverse PTM combinations and opportunities of histone code control.[41, 44] Histone H4 Arg-3 (H4R3) methylation is a major epigenetic mark, and H4R3 is a shared substrate for several PRMT enzymes including PRMT1, 3, 5, 6, and 8. [18, 21, 22, 45–48] In our experiment, when ADMA or SDMA is present on H4R3, the activities of PRMT1, 3, 5, and 8 were largely abolished (Supplemental Figure S2), which is consistent with previous observations.[16, 21] We and others have previously shown that different acetylation combinations on H4K5, 8, 12, and 16 inhibited the H4R3 methylation activity of PRMT1, which is consistent with cellular observations by Wang and coworkers,[19] and also that $H4K5_{ac}$ increased PRMT5 activity.[20] Our recent work demonstrated that the steady-state kinetic data of PRMT1 fit well with the Hill equation, instead of the classic Michaelis-Menten equation.[49] This has been consistent with this study. As a matter of fact, the Hill equation appeared to be suitable for fitting all the kinetic results of PRMT1, 3, and 8. These findings suggest that various levels of cooperativity exist among type I PRMTs. Future investigation is necessary to illuminate the molecular basis of the cooperativity.

PRMT8 shares high protein sequence identity (80%) with PRMT1.[43] PRMT1 and 8 can exist as homo-oligomers in solution,[43, 50] and oligomer formation is necessary for optimal PRMT1 activity.[51, 52] PRMT1 can be found in the nucleus and cytoplasm,[53] whereas PRMT8 is N-terminally myristoylated and consequently localized to the plasma membrane. [47] While PRMT1 is widely expressed in various tissues,[54] expression of PRMT8 is mainly restricted to brain tissue.[47] In this study, we observed that H4K5 methylation did not appreciably affect k_{cat} of PRMT8 and PRMT1 while H4K5 acetylation led to a lower k_{cat} in comparison to unmodified peptide for both PRMTs. In general, acylation of H4K5 was generally inhibitory towards PRMT1 and 8 activity (Figure 7). It appears that maintaining a positive charge is beneficial for PRMT catalysis, particularly for PRMT1; nonetheless, we cannot exclude the possibility of steric impact. We would expect that if the charge on Lys-5

is important, then replacing the methyl group with an ethyl, propyl, butyl, or possibly 2-methyl-2-butanol (*i.e.* mimic the butyrylation without the carbonyl) would lead to a reduction in $K_{0.5}$ but have little influence on k_{cat} in comparison to the unmodified peptide substrate. Also in this study, we observed high cooperativity with the highest n value (n = 5.1) observed with Ac-H4(1-20) as the substrate for PRMT1 (Table 1). This is above what would be expected for PRMT1 if it was a dimer during the reaction. While the scope of the study was not to address the cooperativity, we hypothesize the cooperativity may be due to higher-order oligomerization in solution, which has been observed for PRMT1 and 8.[43, 50] Detailed studies will be warranted to investigate any kinetic or thermodynamic cooperativity of PRMT catalysis in the future.

Contrary to the type I PRMTs studied herein, H4K5 acetylation appears to increase arginine methylation by PRMT5-MEP50 in comparison to the unacetylated Ac-H4(1-20) substrate. The observation that H4K5 acetylation led to increased arginine methylation by PRMT5-MEP50 was consistent with the observations of Feng and others.[20, 55] Interestingly, increasing the length of the acyl modification had increasingly, though marginal, negative effects on arginine methylation (from acetylation, propionylation, to butyrylation) (Figure 5). Yet, despite introducing steric strain (crotonylation) and increasing the bulk (2-hydroxyisobutyrylation), we did not observe the same 2-fold or more reductions in PRMT5 activity that were apparent with the type I PRMTs. These subtle differences in the regulation of type I and type II PRMT activities may potentially influence whether ADMA or SDMA is the predominant mark on H4R3.

The N-terminal acetylation of proteins is evolutionarily conserved from *Saccharomyces cerevisiae* to *Homo sapiens*, and it is a highly prevalent modification (84% in humans).[56] Previous studies on the crosstalk between N-terminal acetylation and H4R3 methylation have found that N-terminal acetylation negatively affects H4R3 methylation.[1, 28] Indeed, there was a 2-fold decrease in PRMT3 activity upon N-terminal acetylation. However, we did not observe that the N-terminal acetylation had an inhibitory effect on the activities of PRMT1, 5, or 8 (Figure 6). Since PRMT1 is the predominant type I enzyme for catalyzing arginine methylation, [12] it does not seem likely that our results can explain what others have observed. However, the previous studies used antibodies to detect the methylation state of H4R3, and it is not clear if those antibodies are efficacious enough to detect H4R3me in the context of other *cis* histone modifications, such as phosphorylated H4S1. Utley and colleagues performed a dot blot with anti-H4R3me and observed that their antibody can miss the detection of H4R3me in the presence of phosphorylated H4S1.[57] Hence, the relationship between the N-terminal acetylation and H4R3 methylation, especially in the cellular context, remains open for careful inspection.

While many of the natural modifications found on H4K5 are predominantly a form of acylation,[2] methylation of Lys-5 is unique in that it increases local hydrophobicity and allows the retention of the positive charge. SET5 catalyzes lysine methylation on H4K5, K8, and K12 in the yeast trimethylated H4K5, while not as common as monomethylated H4K5, have been identified in two recent proteomic studies.[41, 45] Although we determined the effects of monomethylated lysine on arginine methylation by PRMTs, it would be interesting to know if introducing di- and trimethylation on Ac-H4(1-20)K5 peptide increases catalytic

efficiency in comparison to Ac-H4(1-20), and whether substituting Lys-5 for norleucine to eliminate the charge would abolish catalytic efficiency of PRMT3. In addition, examining how H4R3 methylation affects lysine methylation by SMYD3 or SET5 would be an interesting topic to follow. Nonetheless, it was striking to observe Ac-H4(1-20)K5$_{me}$ perform much better as a substrate than Ac-H4(1-20) for PRMT3 (Figure 7). Plus, retaining the positive charge on Lys-5 appeared to be important, though we expect that in the future we can test the hypothesis that retaining the charge on Lys-5 is important for PRMT3 catalysis. We are also mindful that the PRMT3 in this study is truncated (residues 211-531, PDB ID: 2FYT), and it would be desirable to investigate whether endogenous full-length PRMT3 prefers to methylate nucleosomal H4K5$_{me}$ to unmethylated H4 *in vivo*.

Finally, it is interesting to consider the potential metabolic impact on the diversity of the histone PTMs described in this study. While SAM is the metabolite used for methylation and acetyl-CoA for acetylation, specific acyl-CoAs are the expected source for propionylation, butyrylation, crotonylation, and 2-hydroxyisobutyrylation.[33, 58] Since some HAT enzymes (e.g. p300 and CBP) can accommodate cosubstrates other than acetyl-CoA,[31, 34] the incorporation of diverse acyl-CoAs has been suggested to arise with changes in the acyl-CoA competition for HAT enzymes.[35] This may occur by increasing the concentration of the less prevalent acyl-CoAs by conversion of short-chain fatty acids (SCFAs) and/or reducing the concentration of acetyl-CoA.[35] Moreover, the gut microbiota in the colon can produce SCFAs (proprionate, butyrate, and acetate) from fermentation of proteins and carbohydrates, and the human colon absorbs these SCFAs [59–61]. It may be that the metabolic diversity of the gut microbiota affects the concentrations of SCFAs within the host, and thereby affects the production of acyl-CoAs from SCFAs, leading to changes in the prevalence and diversity of the acylations installed on the core histones [62]. While we have mentioned an enzyme-dependent acylation mechanism, it has also been proposed that the concentration of acyl-CoAs and the alkaline (pH 8) environment of the mitochondria can support non-enzymatic installment of acyl modifications.[36] Certainly, understanding this interface of metabolic and epigenetic biology has prospects for identifying opportunities for therapeutic intervention to promote human health.

CONCLUSION

In this study, we have determined how the histone H4 N-terminal acetylation and various naturally occurring H4K5 PTMs affect Arg-3 methylation by PRMT1, 3, 8, and 5. Based on our findings, PRMT1, 3, and 8 do not appear to consistently obey classical Michaelis-Menten kinetics. Surprisingly, methylation of H4K5 led to improved substrate recognition by PRMT3 and PRMT8, but not PRMT1. Also, acylation of H4K5 predominantly inhibited arginine methylation by the type I PRMTs examined. Specifically, the observation that H4K5 acetylation reduced arginine methylation by PRMT1, yet increases PRMT5 activity, is consistent with previous observations.[20, 55] Finally, the N-terminal acetylation has an inhibitory effect on arginine methylation by PRMT3. These findings provide new mechanistic insights into the understanding of how local PTMs finely tune arginine methylation by different PRMTs, and help unravel the complex interplays in histone codes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

ADMA	ω-N^G,N^G-asymmetric dimethylarginine
CARM1	co-activator-associated arginine methyltransferase 1 (PRMT4)
CBP	CREB-binding protein
MMA	ω-N^G-monomethylarginine
MEP50	methylosome protein 50
p300	histone acetyltransferase p300
PTM	post-translational modification
PRMT	protein arginine methyltransferase
RPS2	40S ribosomal protein S2
SAM	*S*-adenosylmethionine
SCFA	short-chain fatty acid
SDMA	ω-N^G,N'^G-symmetric dimethylarginine
SET5	Su(var) 3–9, Enhancer of Zeste and Trithorax protein 5
SMYD3	SET and MYND domain-containing protein 3

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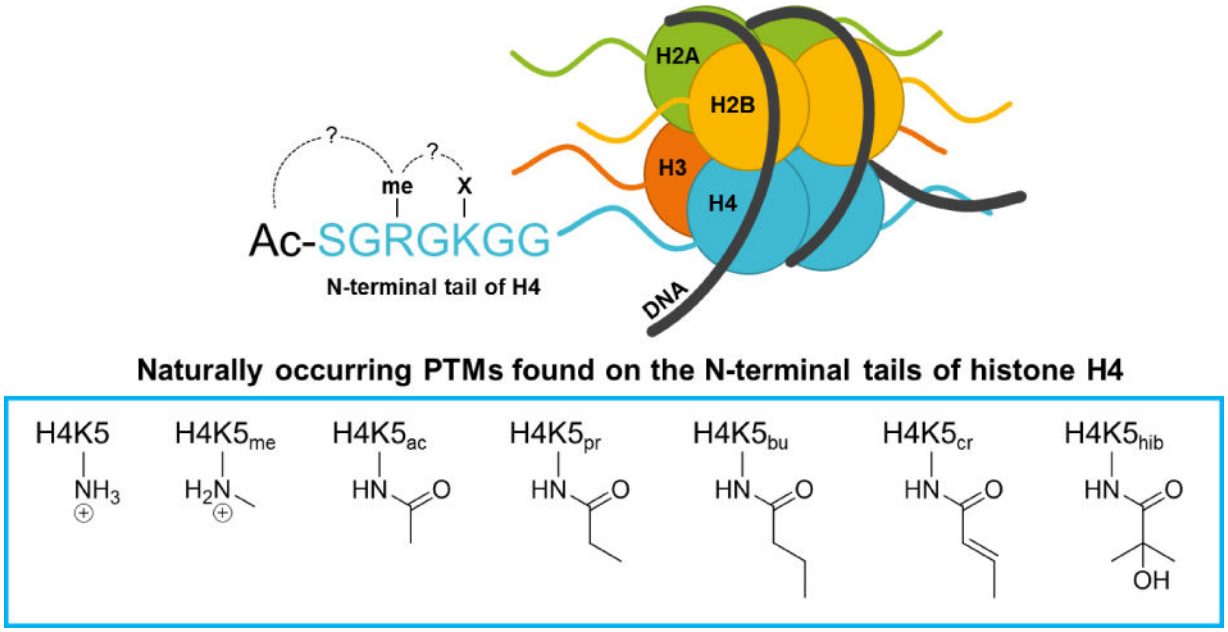

Figure 1.

Elucidating the crosstalk between post-translational modifications (PTMs) on histone H4 Lys-5 (H4K5) and H4 N-terminal acetylation on arginine methylation by PRMTs. Peptides derived from the histone H4 N-terminal tail were synthesized with one of the six known H4K5 PTMs and used as substrates for type I and type II PRMTs.

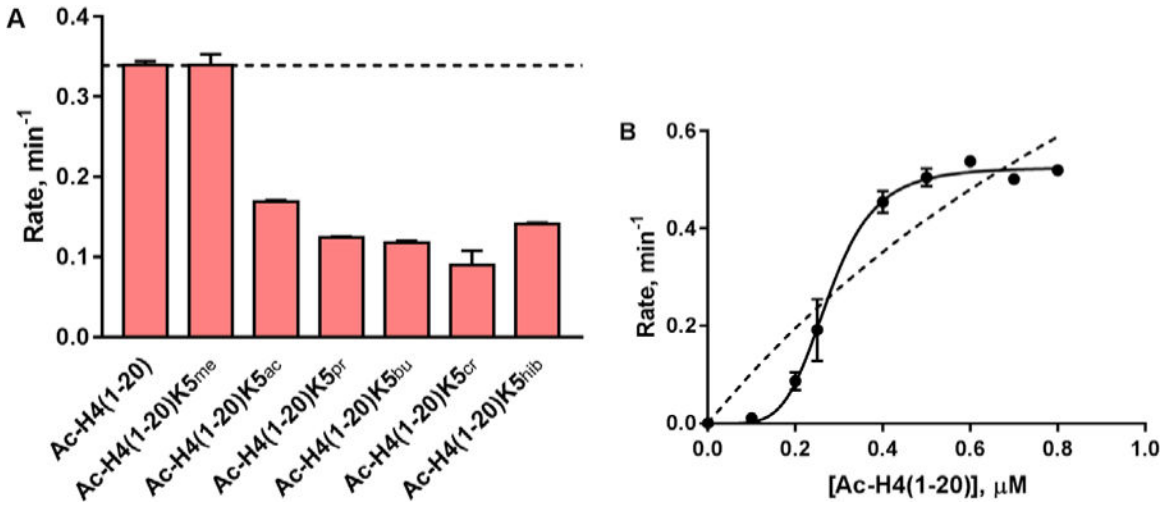

Figure 2.

H4 arginine methylation catalyzed by PRMT1. A) Single-point radioactive methylation assay performed with 0.05 μM PRMT1, 5 μM [^{14}C]-SAM, and 10 μM of peptide substrate at 30 °C over a period of 35 min. B) Arginine methylation of Ac-H4(1-20) by PRMT1 (0.02 μM) was monitored over a course of 15 min at 30 °C in the presence of 15 μM [^{14}C]-SAM and increasing concentration of peptide substrate (0.1–0.8 μM). The dashed line indicates the Michaelis-Menten curve fit while the solid line is the Hill curve fit. Error bars represent standard deviation. For some data points, the associated error value is about the same size or smaller than the size of symbol and difficult to display. Although not shown, we observed substrate inhibition above 1 μM.

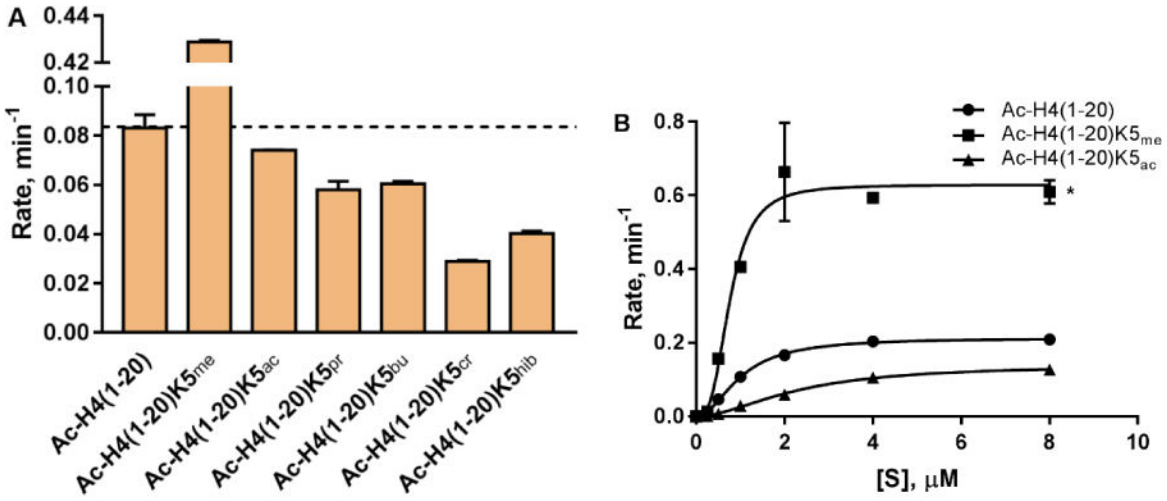

Figure 3.

PRMT3 catalyzed arginine methylation of Ac-H4(1-20) with K5 modifications. A) Single-point radioactive methylation assay performed with 0.05 μM PRMT3, 5 μM [^{14}C]-SAM, and 10 μM of peptide substrate at 30 °C over a period of 35 min. B) PRMT3 kinetics with Ac-H4(1-20), Ac-H4(1-20)K5$_{me}$, or Ac-H4(1-20)K5$_{ac}$ as the substrate. Reactions were held for 35 min at 30 °C with 0.05 μM PRMT3, 15 μM [^{14}C]-SAM, and increasing concentration of peptide substrate (0.25–8 μM). Error bars represent standard deviation. *Although not shown, there appeared to be substrate inhibition above 8 μM for Ac-H4(1-20)K5$_{me}$.

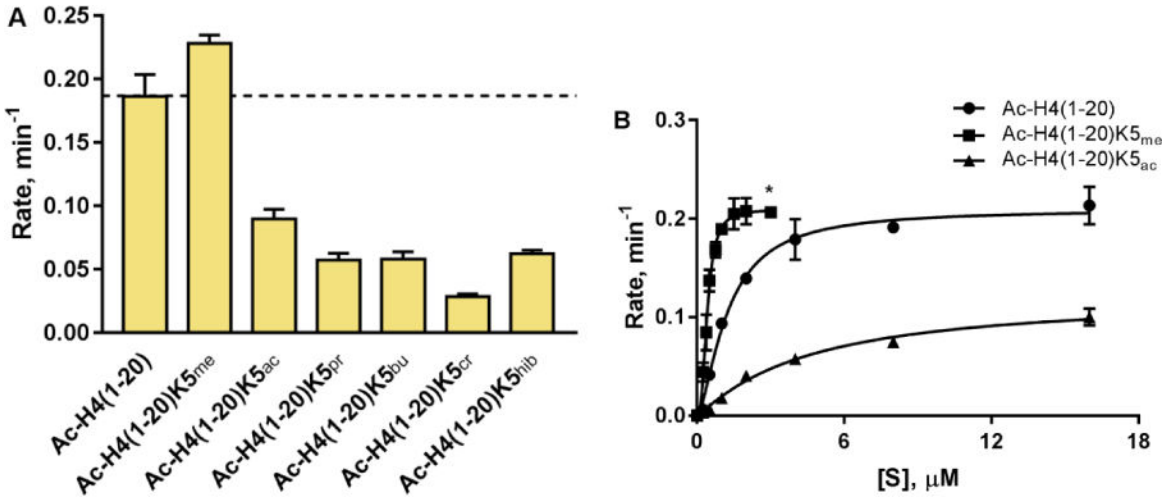

Figure 4.

PRMT8 catalyzed arginine methylation of Ac-H4(1-20) with K5 modifications. A) Single-point radioactive methylation assay performed with 0.05 μM PRMT8, 5 μM [^{14}C]-SAM, and 10 μM of peptide substrate at 30 °C over a period of 35 min. B) PRMT8 kinetics with Ac-H4(1-20), Ac-H4(1-20)K5$_{me}$, or Ac-H4(1-20)K5$_{ac}$ as the substrate. Reactions were held for 35 min at 30 °C with 0.05 μM PRMT8, 15 μM [^{14}C]-SAM, and increasing concentration of peptide substrate (0.25–16 μM). Error bars represent standard deviation. *Although not shown, there appeared to be substrate inhibition with Ac-H4(1-20)K5$_{me}$ above 4 μM.

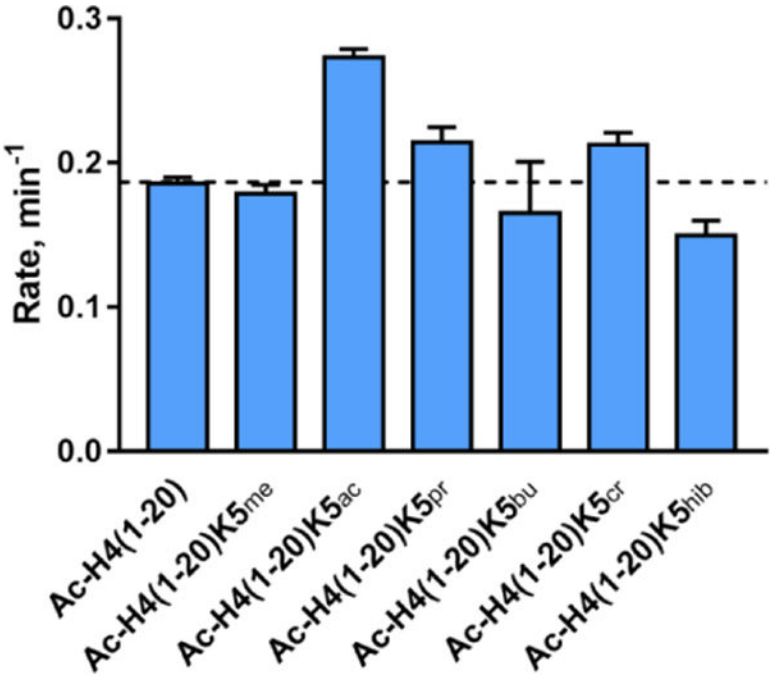

Figure 5.

PRMT5-MEP50 catalyzed arginine methylation of Ac-H4(1-20) with K5 modifications. Single-point radioactive methylation assay performed with 0.05 μM PRMT5-MEP50, 5 μM [^{14}C]-SAM, and 10 μM of peptide substrate at 30 °C over a period of 35 min. Error bars represent standard deviation.

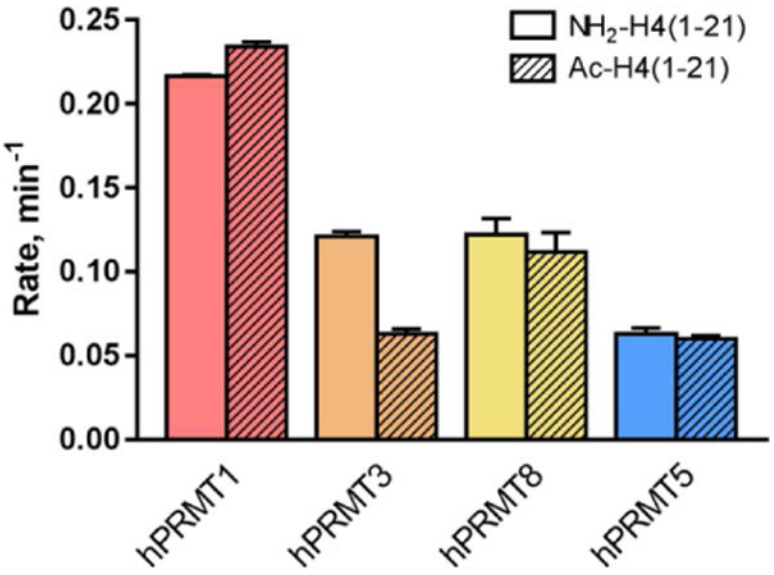

Figure 6.

The impact of H4(1-21) N-terminal acetylation on arginine methylation by various PRMTs. Single-point radioactive methylation assay performed with 0.05 μM PRMT, 5 μM [^{14}C]-SAM, and 10 μM of peptide substrate at 30 °C over a period of 35 min. From left to right, PRMT1 is shown in red color, PRMT3 is in orange, PRMT8 is in yellow, and PRMT5 is in blue.

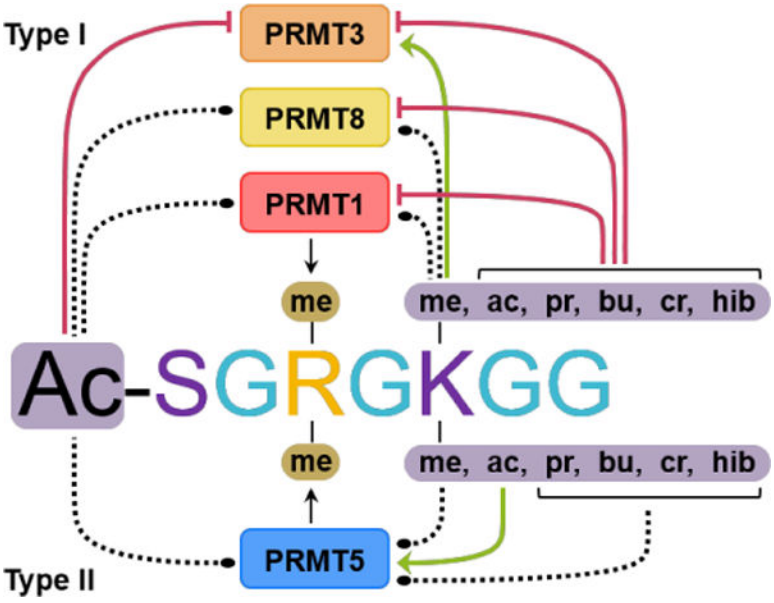

Figure 7.

A model to summarize the impact of histone H4K5 and α-amino PTMs on PRMT1, 3, 5, and 8 activity. Green solid line with arrow = activating, red solid line with blunt end = inhibiting, and black dotted line with oval end = marginal or no effect. Ac, acetylation; me, methylation; pr, propionylation; bu, butyrylation; cr, crotonylation; hib, 2-hydroxyisobutyrylation.

Table 1

Summary of kinetic parameters for arginine methylation by PRMT1.

Substrate	Sequence	Equation Fitted	R^2	k_{cat} (min^{-1})	$K_{0.5}$ (μM)	*n*	$k_{cat}/K_{0.5}$ (min^{-1} $μM^{-1}$)
Ac-H4(1-20)	Ac-SGRGKGGKGLGKGGAKRHRK	Hill	0.99775	0.53 ± 0.0085	0.28 ± 0.0059	5.1 ± 0.45	1.9
Ac-H4(1-20)$K5_{me}$	Ac-SGRGK_{me}GGKGLGKGGAKRHRK	Hill	0.99792	0.51 ± 0.013	0.28 ± 0.0078	4.9 ± 0.56	1.8
Ac-H4(1-20)$K5_{ac}$	Ac-SGRGK_{ac}GGKGLGKGGAKRHRK	Hill	0.99833	0.18 ± 0.0023	0.28 ± 0.0050	4.0 ± 0.31	0.64
Ac-H4(1-20)$K5_{pr}$	Ac-SGRGK_{pr}GGKGLGKGGAKRHRK	Hill	0.99014	0.20 ± 0.0048	0.35 ± 0.011	4.3 ± 0.55	0.57
Ac-H4(1-20)$K5_{bu}$	Ac-SGRGK_{bu}GGKGLGKGGAKRHRK	Hill	0.99221	0.11 ± 0.0032	0.46 ± 0.015	2.9 ± 0.32	0.24
Ac-H4(1-20)$K5_{cr}$	Ac-SGRGK_{cr}GGKGLGKGGAKRHRK	Hill	0.98264	0.17 ± 0.010	0.40 ± 0.066	1.3 ± 0.34	0.43
Ac-H4(1-20)$K5_{hib}$	Ac-SGRGK_{hib}GGKGLGKGGAKRHRK	Hill	0.99567	0.13 ± 0.0030	0.50 ± 0.026	2.4 ± 0.28	0.26

15 min reactions with 0.02 μM [E] and 15 μM [^{14}C]-SAM

n = Hill Coefficient

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Table 2

Comparison of PRMT3 and PRMT8 kinetic parameters for arginine methylation.

Enzyme	Substrate	k_{cat} (min^{-1})	$K_{0.5}$ (μM)	n	$k_{cat}/K_{0.5}$ (min^{-1} $μM^{-1}$)
PRMT3	Ac-H4(1-20)	0.21 ± 0.0050	1.0 ± 0.049	2.0 ± 0.16	0.21
	Ac-H4(1-20)$K5_{me}$	0.63 ± 0.029	0.76 ± 0.070	3.0 ± 0.68	0.83
	Ac-H4(1-20)$K5_{ac}$	0.14 ± 0.0056	2.3 ± 0.15	1.8 ± 0.14	0.061
PRMT8	Ac-H4(1-20)	0.21 ± 0.0070	1.2 ± 0.10	1.6 ± 0.17	0.18
	Ac-H4(1-20)$K5_{me}$	0.21 ± 0.0032	0.42 ± 0.010	2.7 ± 0.18	0.50
	Ac-H4(1-20)$K5_{ac}$	0.12 ± 0.015	4.5 ± 1.2	1.1 ± 0.16	0.027

35 min reactions with 0.05 μM [E] and 15 μM [^{14}C]-SAM