Redox Control of Protein Arginine Methyltransferase 1 (PRMT1) Activity*

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Background: Oxidative stress leads to increased PRMT1 expression and ADMA accumulation.
Results: PRMT1 activity is increased by reductants and decreased by oxidants.
Conclusion: PRMT1 activity is regulated in a redox-sensitive manner.
Significance: The role of PRMT1 activity in the oxidative stress response may be more complex than previously thought.

ABSTRACT
Elevated levels of asymmetric dimethylarginine (ADMA) correlate with risk factors for cardiovascular disease. ADMA is generated by the catabolism of proteins methylated on arginine residues by protein arginine methyltransferases (PRMTs), and is degraded by dimethylarginine dimethylaminohydrolase (DDAH). Reports have shown that DDAH activity is downregulated and PRMT1 protein expression is upregulated under oxidative stress conditions, leading many to conclude that ADMA accumulation occurs via increased synthesis by PRMTs and decreased degradation. However, we now report that the methyltransferase activity of PRMT1, the major PRMT isoform in humans, is impaired under oxidative conditions. Oxidized PRMT1 displays decreased activity, which can be rescued by reduction. This oxidation event involves one or more cysteine residues that become oxidized to sulfenic acid (-SOH). We demonstrate a hydrogen peroxide concentration-dependent inhibition of PRMT1 activity that is readily reversed under physiological H₂O₂ concentrations. Our results challenge the unilateral view that increased PRMT1 expression necessarily results in increased ADMA synthesis, but rather demonstrate that enzymatic activity can be regulated in a redox-sensitive manner.

Endothelial dysfunction plays a major role in cardiovascular disease, the leading cause of death in the United States (1). Several factors have been suggested to contribute to endothelial dysfunction such as decreased activity and/or expression of endothelial nitric oxide synthase (eNOS) and/or increased vascular formation of oxygen-derived free radicals (2,3). Asymmetric dimethylarginine (ADMA) is an endogenously synthesized inhibitor
of NOS that has been gaining increased attention in the cardiovascular field (2,4-9). In the heart, ADMA and other NOS inhibitors cause reduced heart rate and cardiac output (10,11). Interestingly, in addition to decreasing levels of nitric oxide, evidence suggests that ADMA may also uncouple NOS (conditions under which NOS generates superoxide anion), thus increasing oxidative stress and inducing additional endothelial dysfunction (7,12). Furthermore, ADMA was shown to increase endothelial oxidative stress and up-regulate expression of redox-sensitive genes that encode endothelial adhesion molecules (13), increasing propensity for plaque buildup. Taken together, the available data indicates that ADMA levels represent a risk factor for the development of endothelial dysfunction.

ADMA is generated through the degradation of cellular proteins containing asymmetrically dimethylated arginine residues (Fig. 1). Arginine residues in certain proteins can be modified by the addition of one or two methyl groups; this modification is catalyzed by the protein arginine methyltransferase (PRMT) family of enzymes. Nine human PRMT isoforms can be subdivided into three types determined by their final methylation products. Type 1 PRMTs (such as PRMT1) form monomethylarginine (MMA) and/or ADMA and represent the majority of identified PRMTs. Type 2 PRMTs form MMA and/or symmetric dimethylarginine (SDMA), and type 3 PRMTs produce only MMA (14). Each of the methylated arginine products (MMA, ADMA, and SDMA) can induce different biological responses in the cell; however, only MMA and ADMA are inhibitors of NOS activity (9,14). ADMA in the body is metabolized by dimethylarginine dimethylaminohydrolase (DDAH) to citrulline and dimethylamine (15). Thus, the amount of free ADMA at any given time is a reflection of PRMT, proteasome, and DDAH activities.

Evidence has shown that under oxidative stress, a condition linked to a variety of disease states, free ADMA levels are increased. In many instances these studies also showed that PRMT1 RNA or protein expression is increased and DDAH activity is decreased (11,16-20). This has led many to conclude that the increased expression of PRMT1 protein results in increased protein methylation, giving rise to a larger pool of the precursors for free ADMA (6,18,21). While it is clear that elevated ADMA levels are connected to oxidative stress, the assumption that increased levels of PRMT1 protein expression are directly responsible for increased free ADMA production has not been validated. In fact, a recent report showed a significant reduction in the production of ADMA-containing polypeptides in both replicative and H2O2-induced premature senescent cells (22). In order to clarify the role of PRMT1 in free ADMA accumulation under oxidative stress conditions, we set out to investigate if oxidative conditions affect PRMT1 catalytic activity.

Here we report that PRMT1, the major human PRMT isoform, is susceptible to oxidation. Oxidized rat PRMT1, which differs from human PRMT1 at just one residue, is characterized by impaired enzymatic activity that can be rescued by reduction. We demonstrate a reversible, concentration-dependent inhibition of PRMT1 activity by H2O2. Furthermore, we show that this oxidation event involves at least two cysteines which are oxidized to sulfenic acid (-SOH). Our results provide the first direct evidence that PRMT1 enzymatic activity can be regulated in a redox-sensitive manner.

**EXPERIMENTAL PROCEDURES**

**Reagents**— AdoMet was purchased from Sigma as a chloride salt (≥ 80%, from yeast). [3H]AdoMet (83.1 Ci/mmol) was purchased from Perkin Elmer. R3 peptide (acetGGRGGFGGRGGGFGGRGGRG) was synthesized by the Keck Institute (Yale University) and purified to ≥95%. The lyophilized peptide was dissolved in water and its concentration was determined by mass or by UV spectroscopy (ε280 nm=5,690 M⁻¹cm⁻¹). Bulk histones from calf thymus were purchased from Sigma. Histone 4 protein was purchased from New England Biolabs. ZipTip®C4/C18 pipette tips were purchased from Millipore. DCP-Rho1 sulfenic acid probe was purchased from Kerafast and 5IAF thiol-specific probe was purchased from Life Technologies.

**Plasmid generation**— The His6-ratPRMT1 plasmid (HisPRMT1) was previously generated (23). To create a construct with a cleavable His6-tag, a DNA segment coding for a tobacco etch virus (TEV) cleavage site was designed with both N- and C-terminal Ndel restriction sites. The Ndel

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Restriction enzyme was used to cut open the vector at an Ndel site between the His$_{6}$-tag and the enzyme coding sequence, and the designed TEV insert ligated using the Quick Ligation™ kit (New England Biolabs) to form the His$_{6}$-TEV-ratPRMT1 plasmid (HisTevPRMT1). The C101S, C342S, C254S, C208S, C101S/C208S variants were generated using the HisTevPRMT1 plasmid (or confirmed HisTevPRMT1 C101S plasmid for C101S/C208S) as a PCR template for site directed mutagenesis using the QuikChange Lightning Kit (Stratagene) and sets of complementary oligonucleotide primers spanning the desired site of mutation. To replace all 11 cysteine residues of PRMT1 with serines and create HisTevPRMT1Acys (or cys-), properly coded DNA with N-terminal Ndel and C-terminal BamHI restriction sites was ordered from GenScript. The PRMT1 sequence in His$_{6}$-TEV-PRMT1 was excised with Ndel/BamHI and replaced with the synthetic PRMT1Acys insert. The cysteine-less with C101 reintroduced (cys-C$^{101}$), cysteine-less with C208 reintroduced (cys-C$^{208}$), and cysteine-less with both C101 and C208 reintroduced (cys-C$^{101}$C$^{208}$) variants were generated using the HisTevPRMT1Acys plasmid as a PCR template for site directed mutagenesis using the QuikChange Lightning Kit (Stratagene) and sets of complementary oligonucleotide primers spanning the desired site of mutation. All plasmids were transformed using the E.coli DH5α cell line. Plasmids were purified (Qiagen Plasmid Mini Kit) and sequenced to confirm the correctness of the open reading frame prior to protein expression.

**Recombinant protein expression and purification**—Full-length His-tagged PRMT1 (residues 1-353) was expressed and purified as described previously (23). Briefly, E. coli BL21 cells were transformed with the above constructs and grown on LB medium / kanamycin agar plates. Selected colonies were grown in LB broth to $A_{600}=0.6$ and induced with 0.5 mM IPTG for 24 hours at 25°C. Cell pellets were resuspended in lysis/wash buffer (50 mM sodium phosphate [pH 7.6], and 20 mM imidazole), sonicated, and centrifuged at 47,000 x g for 20 minutes at 4°C. The clarified supernatant was incubated with Nickel Sepharose 6 Fast Flow resin (Qiagen) rotating for 2 hours at 4°C. The binding reaction was pelleted at 700 x g, the supernatant discarded, and the resin washed 4 times with lysis/wash buffer, 7 times with wash buffer containing 70 mM imidazole, and then eluted in 6 washes with 250 mM imidazole buffer. The elutions were pooled and dialyzed into storage buffer (50 mM sodium phosphate [pH 7.6], and 10% glycerol), concentrated to greater than 1 mg/mL, and beaded in liquid nitrogen for storage at -80°C. To generate cleaved constructs, half of the His$_{6}$-TEV enzymes were put into cleavage buffer (50 mM sodium phosphate, 1 mM DTT, 2 mM EDTA, and 5% glycerol); the TEV enzyme added and allowed to cleave overnight. The cleaved enzyme was dialyzed into storage buffer, re-incubated with nickel resin (removal of TEV and remaining His$_{6}$-tag), and the subsequent supernatant was dialyzed, concentrated, and stored the same as the His$_{6}$-tagged elutions. Enzyme cleavage and purity (>90%) were assessed during and after purification using SDS-page.

**Kinetic assays of PRMT1 constructs**—Conditions of methylation reactions were as published previously (23). Briefly, enzyme activity was assessed at 37°C in assays containing 100 nM PRMT1, 2 μM AdoMet (1 μM [H]$^{3}$AdoMet), 0.38 μM bovine serum albumin, 100 nM AdoHcy nucleosidase (MTAN, purified as described in ref. (25)) and initiated with 200 μM peptide substrate or 2 μM protein substrate. Reactions in the presence of DTT were performed by pre-incubation of the reaction with 1 mM DTT for 10 minutes at 4°C prior to initiation with substrate. At different time points, 5 μL samples were removed from reactions and quenched with 6 μL of 8 M guanidinium hydrochloride. Samples were processed with ZipTip® C4/C18 pipette tips (Millipore) (for protein or peptide substrates, respectively) to separate the unreacted [H]$^{3}$AdoMet and the radiolabeled product (26). Time-dependent incorporation of radiolabel into substrates was quantified using a scintillation counter (Beckman Coulter). Methyltransferase activity for hPRMT3, hPRMT6, and TbPRMT7 were assessed during and after purification using SDS-page. pET21a TbPRMT7 and pET28b human PRMT6 were purified as PRMT1. pET28a human PRMT3 (residues 211-531) was grown and purified as described in Wang et al. (24). Protein concentrations were determined by UV spectroscopy (PRMT1 $\varepsilon_{280}$ mm$^{-1}$cm$^{-1}$ =54,945 M$^{-1}$cm$^{-1}$, PRMT3 $\varepsilon_{280}$ mm$^{-1}$ =37750 M$^{-1}$cm$^{-1}$, PRMT7 $\varepsilon_{280}$ mm$^{-1}$ =37150 M$^{-1}$cm$^{-1}$, PRMT6 $\varepsilon_{280}$ mm$^{-1}$ =59040 M$^{-1}$cm$^{-1}$) and by the Bradford assay with bovine serum albumin as a standard.

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were performed as above, except 1 mM bulk histones (Sigma), and 1 mM Histone 4 protein (NEB) were used as substrates for hPRMT6 and TbPRMT7, respectively.

Conditions for testing the effect of hydrogen peroxide on enzymatic activity were published previously (27) and were modified as follows. PRMT1 was pre-reduced in 1 mM DTT for 2 hours on ice then rapidly desalted on a 7 kDa Zeba™ Spin desalting column (Thermo Scientific). PRMT1 (2 µM final concentration) was oxidized with 0, 0.4 µM, 4 µM, 40 µM, 200 µM, 400 µM, or 800 µM H₂O₂ for 10 minutes at 37°C and then incubated with catalase (300 U/ml) for 1 minute at 37°C. The mixture was then kept on ice and used for kinetic assays as above. Methyltransferase activity was unaffected by the addition of catalase (data not shown). In all cases, data reported is the average of at least three independent measurements.

Size exclusion chromatography— Gel filtration chromatography was performed on a Superdex™ 200 10/300GL column (GE Healthcare) in 50 mM sodium phosphate pH 7.6, 150 mM NaCl, and 5% glycerol at 0.4 ml/min flow rate. Freshly purified PRMT1 variants were allowed to equilibrate overnight into gel filtration buffer, with or without the addition of 1 mM DTT, 2 mM EDTA prior to examination. All constructs were analyzed by loading 300 µL of enzyme at a concentration of 10-20 µM and run at 0.4 mL/min for 75 minutes.

In vitro DCP-Rho1 labeling of sulfenic acid in recombinant PRMT1— DCP-Rho1 labeling of purified PRMT1 was modified from the method described in Poole et al. (28). Briefly, 2.5 µM recombinantly expressed, air oxidized WT PRMT1, cysteine-less PRMT1, cysteine-lessC₁₀¹, cysteine-lessC₂₀⁸, or cysteine-lessC₁₀¹C₂₀⁸ enzymes were incubated with 1 mM DTT or with buffer in the presence of 6 M urea for 1 hour at 22°C prior to addition of 2.5 mM SIAF for 1 hour at 22°C in a final reaction volume of 25 µl. The labeling reaction was quenched by addition of 4X SDS loading dye and boiling for 5 minutes. Labeled proteins were separated from unreacted label by 12% SDS-PAGE, and band quantification was performed using the Image Lab software from BioRad and is reported as the average of three independent measurements.

LC-MS/MS analysis of sulfenic acid content in recombinant PRMT1— 120 µg WT PRMT1 was treated with 5 mM dimedone in the presence of 6 M urea for 2 hours at 22°C. The sample was then buffer exchanged twice into 50 mM ammonium bicarbonate to remove excess dimedone and all urea. After reduction and alkylation, the labeled sample was precipitated by stepwise addition of 6 volumes of cold acetone with continuous vortexing and then incubated overnight at -20°C. After centrifugation at 20,000 g for 30 minutes at 4°C, the supernatant was removed, and the pellet was allowed to air-dry. Two consecutive digestions steps were employed for the on-pellet-digestion. In step 1 (digestion-aided pellet dissolution), Tris buffer (50 mM, pH 8.5) containing trypsin at an enzyme/substrate ratio of 1:40 (w/w) was added and incubated at 37°C for 12 hours to achieve a complete digestion. The digestion was terminated by adding 1 % (v:v) formic acid and centrifuged at 20,000 g for 30 min at 4°C; the supernatant was used for LC-MS/MS analysis.
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coupled to an ultra-high pressure Eksigent ekspert NanoLC 425 system (Dublin, CA) with a autosampler of Eksigent NLC 400 (Made in the Netherlands). A nano-LC/nanospray setup was used to obtain a comprehensive separation of the complex peptide mixture and sensitive detection. Mobile phases A and B were 0.1% formic acid in 1% acetonitrile and 0.1% formic acid in 88% acetonitrile, respectively. Samples were loaded onto a large ID trap (300 µm ID × 5 mm, packed with Zorbax 5 µm C18 material) with 1% B at a flow rate of 10 µL/min for 3 min. A series of nanoflow gradients was used to back-flush the trapped samples onto the nano-LC column (75 µm ID × 100 cm, packed with Pepmap 3-µm C18 material). The column was heated at 52 °C to improve both chromatographic resolution and reproducibility. Gradient profile was as follows: i) a linear increase from 3 to 8% B over 5 min; ii) an increase from 8 to 27% B over 117 min; iii) an increase from 27 to 45% B over 10 min; iv) an increase from 45 to 98% B over 20 min; and v) isocratic at 98% B for 20 min.

The mass spectrometry was operating under data-dependent product ion mode. A 3 second scan cycle included an MS1 scan (Orbitrap) followed by MS2 scans (dual-cell ion trap) by alternating CID and ETD activation, was programed. The parameters used for MS and MS/MS data acquisition under the CID mode were: top speed mode with 3s cycle time; Orbitrap: scan range (m/z) = 400-1600; resolution = 120 K; AGC target = 5 × 10^5; maximum injection time (ms) = 50; Filter: precursor selection range = 400-1500; include charge state = 2-7; dynamic exclude after n times = 1, duration time 60 s; Decision: data dependent mode: top speed, precursor priority = most intense; for CID, isolation mode = quadrupole; isolation window = 1.6; collision energy (%) = 30; detection type: Ion trap; iontrap scan rate: Rapid; AGC target = 1 × 10^5; maximum injection time (ms) = 50; microscan = 1; and for ddMS2 (ETD), ETD reaction time (ms) 200; ETD reagent target 1.0 x 10^6; Maximum ETD reagent injection time (ms): 200; AGC target = 1 x 10^6; maximum injection time (ms) = 50; microscan = 1.

CID and ETD activation spectra were processed using Peaks 7. Briefly, raw files were searched against the sequence of PRMT1, with the precursor mass tolerance of 20 ppm and peptide fragment mass tolerance of 1 Da. And the static side chain modifications of carbamidomethyl (57.021), and dynamic side chain modifications of Oxidation (15.995) and dimedone (138.068) controlled the protein FDR as 0.1%.

RESULTS

PRMT1 activity is reversibly inhibited by oxidation—The elevated levels of free ADMA that are observed under oxidative stress conditions (7) could arise from a few distinct mechanisms. Both proteosomal degradation and methyltransferase activities could affect the rate of free ADMA formation. Increased synthesis of ADMA-containing proteins (the precursors to free ADMA) could occur by increasing the expression of PRMT proteins while maintaining normal enzyme activity, or by increasing the methyltransferase activity of the current pool of PRMT proteins. Since PRMT1 is responsible for ~85% of arginine methylation in cells and is the primary source of ADMA (31), altered expression and/or activity of this isoform would be expected to contribute greatly to altering levels of ADMA. In order to determine if oxidative conditions induce any changes in PRMT1 activity, we treated fully reduced recombinant PRMT1 with hydrogen peroxide, a common cellular oxidant, and measured the resulting methyltransferase activity.

Recombinantly expressed PRMT1 was pre-reduced with dithiothreitol (DTT), and rapidly desalted to remove residual DTT. The reduced PRMT1 was then incubated with various concentrations of H_2O_2 (0.4—800 µM final concentration) followed by the addition of catalase to remove any remaining H_2O_2. Surprisingly, when the peroxide-treated PRMT1 was assayed, methyltransferase activity was found to be significantly inhibited by peroxide in a dose-dependent manner (Fig. 2A). At concentrations of hydrogen peroxide greater than 4 µM, PRMT1 activity was significantly impaired and could not be fully recovered (data not shown). However, at the physiologically relevant concentration of 0.4 µM (physiological levels in mammals range from 0.001 µM to 0.7 µM)(32), hydrogen peroxide was also able to inhibit PRMT1 activity, and subsequent reduction with DTT resulted in full recovery of activity (Fig. 2B). These experiments provide the first evidence that PRMT1 enzymatic activity is susceptible to reversible oxidative...
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Redox control of PRMT1 activity increases after reduction—In order to better understand the sensitivity of PRMT1 to oxidation, we purified recombinant PRMT1 in the absence of reductant and measured methyltransferase activity in the absence and presence of a reductant. Pre-incubation with DTT increased methyltransferase activity by greater than 10-fold with the R3 peptide substrate (Fig. 3A). We examined the duration of this effect by rapidly desalting PRMT1 following the DTT pre-incubation step. The activity of the desalted PRMT1 was increased only by 2-fold using the R3 peptide (Fig. 3A), indicating that the effect of DTT is transient and that PRMT1 oxidation occurs quickly in the absence of a reductant. We further studied the relationship between enzymatic activity and the concentration of DTT by comparing PRMT1 activity with varied concentrations of DTT ranging from 0.1 mM to 10 mM (Fig. 3B). Our results indicate that the effect of DTT is concentration-dependent, achieving a maximal rate enhancement at 1.5-2 mM DTT (Fig. 3B). During the course of this study, more than 50 human and rat PRMT1 proteins were purified in the absence of reductant and showed anywhere from a 1.8-fold to 70-fold increase in methyltransferase activity when DTT was included in the reaction or the enzyme was pre-incubated with DTT (data not shown). We note that in many cases, our purification protocol can be accomplished in as few as ~8 hours. These results demonstrate that PRMT1 is sensitive to oxidation by not only hydrogen peroxide, but also cellular conditions and/or the mild conditions used for purification.

Methylation of the sulfhydryl groups in DTT was previously observed with small molecule plant O-methyltransferases, where it acted as an intermediate acceptor molecule (33). Even though DTT methylation was not detected in our control reactions lacking peptide substrate (control Fig. 3A), we replaced DTT in our assay with an alternative thiol-free reductant, Tris(2-carboxyethyl)phosphine (TCEP). When 1 mM TCEP was used to reduce PRMT1, the rate enhancement with the R3 peptide was identical to that observed using 1 mM DTT (Fig. 3A). We conclude that the enhancement of HisPRMT1 methyltransferase activity is due to the reducing power of the DTT or TCEP additives.

Several different affinity tags are used for PRMT1 purification and variable methylation rates have been observed (34-36). We questioned whether our His$_6$-tag was influencing the observed rate changes upon reduction. To address this concern, we expressed a tag-less version of rat PRMT1. However, this version of PRMT1 was unstable and lost activity rapidly (data not shown). As an alternative strategy, we created a His$_6$-tagged, tobacco etch virus (TEV) cleavable, rat PRMT1 construct (HisTevPRMT1) which allowed us to cleave the tag after an initial purification step and then use the tag-free version (tag-freePRMT1) for kinetic assays. Both tagged and tag-free versions of PRMT1 exhibited rate enhancement upon reduction, although the tag-free version was markedly more active than the tagged version even without DTT in the assay (Fig. 4). This observation is easily explained when the method for acquiring tag-free protein is taken into consideration. The protocol for TEV cleavage includes an overnight dialysis step in a buffer containing 1 mM DTT and 2 mM EDTA and therefore the resulting tag-free enzyme used in the activity assays is already somewhat pre-reduced. To confirm this theory, we again purified the cleavable (HisTevPRMT1) enzyme by nickel chromatography. Purified enzyme was divided into two separate dialysis bags. TEV was added to one, and the other was left uncleaved; however, both tagged and cleaved samples were dialyzed overnight in the same 1 mM DTT, 2 mM EDTA containing buffer. Methyltransferase activity of these newly purified tagged and tag-free PRMT1 enzymes revealed no significant rate enhancement upon addition of DTT (Fig. 4). The addition of only EDTA had no significant effect on methyltransferase activity (Fig. 4). These results confirm that it is not the tag, but rather the reducing treatment of the tag-free enzyme which enhanced the enzymatic activity of PRMT1.

Reduction alters the oligomeric state of PRMT1—It has been shown that PRMT1 forms at least a homo dimer in order to be catalytically active (36-38). Feng et al. introduced the idea that changing the oligomeric state of PRMT1 affects its enzymatic activity (34). Since reduction of PRMT1 results in increased activity, we wondered if reduction had an effect on the oligomeric state
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of the enzyme. Size exclusion chromatography experiments in the presence or absence of DTT were carried out to determine the oligomeric state of PRMT1 under different redox environments (Fig. 5). Unreduced PRMT1 migrates over a broad range of oligomeric states, with the majority of the protein existing in oligomers that migrate at molecular weights above 660 kDa. As a reference, PRMT1 is thought to be active as an 80 kDa dimer (although a dimer form has not been observed on size exclusion chromatography) (36,39). Overnight incubation with 1 mM DTT and 2 mM EDTA results in a shift towards a homogeneous oligomeric state migrating between 200 and 450 kDa (Fig. 5). Analytical ultracentrifugation, a more sensitive technique for determining molecular weights, showed the same shift towards a smaller oligomeric species upon reduction as was observed using size exclusion chromatography (data not shown). In conclusion, oxidized PRMT1 forms a large molecular weight functional aggregate, while reduction of PRMT1 causes a shift towards a smaller, more uniform, oligomeric state that correlated with an increase in enzymatic activity.

The oxidation-dependent effects on PRMT1 activity require a cysteine(s) residue—One of the most common mechanisms for oxidative damage is the oxidation of cysteine residues (40-43). We took a broad approach to evaluate whether any cysteine residues were responsible for the effects of oxidation/reduction by creating a PRMT1 variant where all eleven rat PRMT1 cysteine residues were mutated to serine (HisTevPRMT1Δcys or cys-). Interestingly, the cysteine-less PRMT1 showed no enhancement in methyltransferase activity upon pre-incubation with DTT (Fig. 6). In addition to activity measurements, the oligomeric state of the cysteine-less PRMT1 variant was assessed by size-exclusion chromatography (Fig. 5). The cysteine-less PRMT1 enzyme exhibited a migration pattern similar to that of the maximally active, reduced PRMT1, regardless of its treatment (data not shown). We conclude from these observations that indeed one (or more) cysteine(s) are responsible for the redox switching of PRMT1 activity.

C101, C342, C254, and C208 are not individually responsible for reductive effects—We examined the rat PRMT1 crystal structures (36) for cysteine residues capable of undergoing oxidation and found that out of the eleven cysteines present in rat PRMT1, cysteines 101, 208, 232, 254, and 342, are all solvent accessible (Fig. 8). Cysteines 9 and 15 are not visible in the crystal structure and were not considered in this study since a human PRMT1 variant lacking the first 27 amino acids exhibits enhanced activity after reduction (data not shown). A recent quantitative reactivity profiling study identified C101 as hyper-reactive with 4-hydroxyl-2-nonenal (HNE) (44), indicating that C101 may be susceptible to oxidation. Cysteine 101 is situated on the far edge of the AdoMet binding pocket and directly interacts with the adenine ring structure of AdoMet (see Fig. 8), and thus, seemed like an excellent target to control enzymatic activity depending on its redox state. Mutation of C101 to serine results in a construct mimicking wild type methyltransferase activity, including the response to DTT (Fig. 6). Closer evaluation of our rat PRMT1 M48L crystal structure (23) showed additional electron density around cysteine 342, suggesting possible oxidation of the thiolate moiety to a reducible sulfenic acid moiety. Although this residue is relatively removed from the active site, it has been suggested that residues distant from the active site can regulate PRMT substrate specificity (45). However, the C342S variant also mimicked wild type enzymatic activity, including the DTT enhancement (Fig. 6). The PRMT1 crystal structure (36) also shows that C254 forms an intermolecular disulfide bond with another PRMT1 dimer, and was therefore also mutated to determine if reduction of this disulfide caused the increase in activity and the corresponding shift towards a smaller oligomeric state. However, as we saw with the other two individual cysteine variants, C254S activity was still enhanced upon reduction with DTT (Fig. 6). The C254S PRMT1 variant also behaved as WT PRMT1 when run on size exclusion chromatography (data not shown)(36).

As previously mentioned, it is believed that PRMT1 dimerization is necessary for catalytic activity. In fact, all currently available Type 1 and Type 2 PRMT structures reveal a conserved mode of dimerization between catalytic subunits (36-38,46,47). Each subunit contains a dimerization helix-turn-helix dimerization arm (blue in Fig. 8) that extends from the C-terminal β-barrel (dark gray in Fig. 8) and rests upon the N-terminal...
AdoMet binding domain (light gray in Fig. 8) of the other subunit; coming together to form an active dimer with a central cavity and two opposing active sites (Fig. 8).

Zhang, X. et al. reported that removal of the helix-turn-helix dimerization arm of PRMT1 not only eliminated homodimerization, but also AdoMet binding and methyltransferase activity. It was thus suggested that the dimer interaction is required to engage the residues on the other side of the structural elements to interact with AdoMet properly (36). In the analysis of another type 1 PRMT structure, Cheng, Y. et al. computed the atomic position fluctuations (APFs) of the monomer and dimer to determine the impact of dimerization on the motion of atPRMT10, also a type 1 PRMT. They found two regions to have lower APFs in dimeric form than in monomeric form: the αγ-loop-αZ (40-68 in atPRMT10) (orange in Fig. 8) and the dimerization arm (187-235 in atPRMT10) (blue in Fig. 8), suggesting that these regions are stabilized upon dimer formation. The αγ-loop-αZ region is directly involved in AdoMet binding and the formation of the substrate binding groove I (36). It was also suggested that stabilization of this region by dimerization likely improves the binding of AdoMet and substrate proteins (37). Additionally, Higashimoto et al. demonstrated that in PRMT4 (CARM1), phosphorylation of S229, located on the outer face of the AdoMet binding domain, compromised dimerization, negatively regulated methyltransferase activity, and lowered AdoMet binding (48). The presence of a charged functional group adjacent to the hydrophobic dimerization arm binding surface likely destabilizes dimerization interactions. Cysteine 208 of PRMT1 is located on the PRMT1 dimer interface (Fig. 8). Given the importance of dimerization for PRMT activity and the predicted susceptibility to oxidation, this residue was also deemed likely to be responsible for the effect of oxidation in impairing methyltransferase activity. Additionally, use of the Cysteine Oxidation Prediction Algorithm (COPA) which uses distance to the nearest cysteine sulfur, solvent accessibility, and pKa as parameters for thiol oxidation susceptibility, suggested Cysteine 208 is susceptible to oxidation by exposure (49). However, measurement of the enzymatic activity of the individual C208S variant revealed this construct was also enhanced by DTT (Fig. 6).

If PRMT1 dimerization is indeed stabilizing substrate binding, we rationalized that oxidation of C208 in the dimerization arm in conjunction with C101 in the AdoMet binding pocket could be responsible for the diminished methyltransferase activity observed under oxidizing conditions. In order to explore this hypothesis, a C101S/C208S variant of PRMT1 was created, and methyltransferase activity was measured in the presence and absence of DTT. Remarkably, methyltransferase activity of the double variant was the same under both oxidizing and reducing conditions (Fig. 6). We note that the activity of the double variant (C101S/C208S) is lower than the activity of reduced wild type enzyme, suggesting that these residues may have an additional role in PRMT1 activity. Surprisingly, when both C101 and C208 are unavailable for oxidation, the protein does not completely shift to a smaller oligomeric state as the cysteine-less construct (data not shown). In conclusion, making C101 and C208 unavailable for oxidation resulted in abolished redox control over PRMT1 catalytic activity.

**Sulfenic acid formation at C101 and C208 in vitro**—In order to further analyze the oxidation events occurring at C101 and C208, we used the sulfenic acid-specific probe DCP-Rho1, which contains a rhodamine attached to the functional core of dimedone. To detect sulfenic acids, air oxidized wild type PRMT1 (WT), cysteine-less with C101 reintroduced (cys-C101), cysteine-less with C208 reintroduced (cys-C208), and cysteine-less with both C101 and C208 reintroduced (cys-C101C208) were incubated with DCP-Rho1 or DMSO as a negative control in the presence or absence of reductant under denaturing conditions. Cysteine-less PRMT1 (cys-)) was also subjected to labeling as a negative control. Analysis of the fluorescent label incorporation (Fig. 7A and B) clearly shows the presence of sulfenic acid at cysteine 101 and 208 of PRMT1. Interestingly, while both cysteine 101 and 208 are necessary for oxidative impairment of C101, we consistently observed more sulfenic acid formation at C208, suggesting either that C101 is less susceptible to oxidation than C208, or that the sulfenic acid form of C101 is transient and can be further oxidized to sulfonic or sulfonyl acid. Free thiol content analysis using the 5IAF probe (Fig. 7C and D) and
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performed in parallel with the DCP-Rho1 labeling, shows similar free thiol content for cys-C^{101} and cys-C^{208}. When analyzed together, these results are consistent with a higher propensity for irreversible oxidation at C101. In addition to the fluorescent probes, WT PRMT1 treated with sulfenic-acid-specific dimedone was subjected to LC-MS/MS analysis to further confirm sulfenic acid formation. Using this method, dimedone incorporation was observed at both C101 and C208 (Figure 7E and F), confirming the fluorescent probe results. In conclusion, the reversible activity impairment observed under oxidative conditions is consistent with sulfenic acid formation at C101 and C208 of PRMT1.

DISCUSSION

Elevated ADMA concentrations have been proposed to predict cardiovascular mortality in patients with chronic renal insufficiency (50) and acute coronary events (8). Additionally, ADMA is not only a marker, but also a mediator of oxidative stress within vascular tissue (7). Given that PRMT1 is the primary source of ADMA in eukaryotes, it is of the utmost importance to understand how PRMT1 activity and ADMA synthesis are regulated under oxidative stress conditions. Here, we have shown that PRMT1 activity is decreased in vitro under physiologically relevant oxidative conditions, an effect which is readily reversed upon reduction and is associated with cysteine oxidation to sulfenic acid. Our results complicate the view that the increased PRMT1 protein expression observed under oxidative stress conditions is responsible for increased ADMA levels, by supporting a model in which PRMT1 activity can be regulated in a redox-sensitive manner. Our results complicate the view that the increased PRMT1 protein expression observed under oxidative stress conditions is responsible for increased ADMA levels, by supporting a model in which PRMT1 activity can be regulated in a redox-sensitive manner. While it has long been known that DDAH breakdown of ADMA is under redox control, we provide the first evidence that the activity of enzymes involved in the formation of ADMA precursors are regulated in a redox-sensitive fashion.

Removal of two cysteine residues implicated in PRMT1 dimerization and cofactor binding eliminates the redox-dependent control over PRMT1 methyltransferase activity. Dimerization is strictly conserved in all known PRMTs and seems necessary for methyltransferase activity (36-38). One of the pathways proposed for signal communication between the dimer interface and the catalytic center uses the mainly hydrophobic dimer interactions between the αγ-loop-αZ (orange in Fig. 8) of one monomer and the helix-turn-helix dimerization arm of another (blue in Fig.8) (36,37,46). It has been proposed that this dimer interaction is required to engage residues into proper conformation for interaction with the AdoMet cofactor (36). It is feasible that oxidation of residues involved in this cooperative effort could disrupt PRMT1 methyltransferase activity by affecting AdoMet binding. Interestingly, cysteine 208 is conserved in PRMT3 and PRMT6, while both cysteine 101 and 208 are conserved in PRMT8. Remarkably, the type III PRMT7 from Trypanosoma brucei, which contains only three cysteine residues that do not align with any cysteines in PRMT1, but does contain a cysteine at a different location in its dimerization arm, also shows increased activity upon reduction. Activity measurements using human PRMT3, human PRMT6, and TbPRMT7 demonstrate that these type 1 and type 3 PRMTs are also under redox control (Fig. 9). Ongoing studies will help provide insight as to how these residues affect AdoMet interactions and PRMT methyltransferase activity.

While the active form of PRMT1 is expected to be a dimer, size exclusion chromatography analysis, as well as dynamic light scattering, have shown the enzyme typically migrates as a high molecular weight oligomer (34,36,38,51,52). Feng et al. suggested that PRMT1 multi-oligomerization (i.e., greater than a dimer) is concentration dependent in the range of 0-0.5 µM and that the final PRMT1 multi-oligomeric complex is the most active form (34). It is important to note that the Feng study was done with fully reduced PRMT1 and at significantly lower enzyme concentrations than those used in this study. Our results show that oxidized PRMT1, which displays less activity, shifts from a large oligomeric species to a smaller, but still large oligomeric state under reducing conditions. While our results seem to be at odds with the Feng study, they only serve to highlight the high degree of complexity that exists in relating PRMT1 oligomerization and activity. Interestingly, while cysteine-less PRMT1 remains a smaller oligomer under both reducing and oxidizing conditions, the C101S/C208S PRMT1 variant presents as a mix of both large and smaller oligomers under reducing conditions (data not shown). It is difficult to assess what may be
causing PRMT1 to form such large oligomeric aggregates, and the link between PRMT1 cysteine oxidation and protein oligomerization remains to be determined. While our results add to the larger story, much remains to be discovered on the impact of oligomeric state on PRMT1 activity.

It is also important to point out that much of the work in the PRMT field is carried out exclusively under reducing conditions. Our work emphasizes the importance of PRMT1 as a redox-sensitive enzyme and the need for careful control of its redox environment. Since PRMT1 activity is under redox control, it is possible that experiment conditions might inadvertently alter activity and/or interaction partners. In the future, it will be highly important to take into account the redox environment of experiments before reaching conclusions. Additionally, while the different affinity tags that were used in this study did not change the increase in activity observed upon PRMT1 reduction, the overall methyltransferase activity was generally lower for HisTevPRMT1 constructs than for HisPRMT1 constructs. Since the tags are located on the N-terminal end of the enzyme, this observation may hint at the importance of the N-terminal PRMT1 sequence in controlling enzymatic activity.

In addition to providing ADMA precursor pools, growing evidence supports the involvement of PRMT1 in the cellular oxidative stress response as a modifier of signal transduction. PRMT1 has recently been reported to be involved in the transcriptional regulation of the human ferritin gene, up-regulation of which is an important cellular defense response to oxidative stress (53). It was also recently reported that PRMT1 methylation is involved in the transcriptional repression of HIFα (54), a protein needed to activate the cellular adaptive response to hypoxia, another condition linked to high levels oxidative stress. Finally, we note that oxidative stress in HEK293 cells was reported to enhance PRMT1-mediated methylation of FOXO1 in the nucleus (55). The observed increase in FOXO1 methylation seems contradictory to our results and highlights the importance of further investigating the effect of oxidation in vivo on PRMT1 substrate specificity, and/or cellular localization. In future studies, it will also be interesting to determine how redox regulation affects the role of PRMT1 in signal transduction.

In summary, this work has demonstrated that PRMT1 is a redox-responsive enzyme. Oxidation at two cysteine residues potentially destabilizes dimerization leading to diminished methyltransferase activity, while reduction readily reverses the effects of oxidation under physiologically relevant conditions, consistent with sulfenic acid formation observed at both cysteine residues. Our results provide the first direct evidence that PRMT1 enzymatic activity can be regulated in a redox-sensitive manner and raise the concern that the current paradigm used to explain free ADMA accumulation in vivo may be incomplete.
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FOOTNOTES

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The abbreviations used are: ADMA, asymmetric dimethylarginine; PRMT, protein arginine methyltransferase; DDAH, dimethylarginine dimethylaminohydrolase; H\textsubscript{2}O\textsubscript{2}, hydrogen peroxide; MMA, monomethylarginine; SDMA, symmetric dimethylarginine; NOS, nitric oxide synthase; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine; TEV, tobacco etch virus; EDTA, ethylenediaminetetraacetic acid; DCP-Rho1, rhodamine B [4-[3-(2,4-dioxocyclohexyl)propyl]carbamate]piperazine amide; 5IAF, 5-iodoacetamidofluorescein; HNE, 4-hydroxyl-2-nonenal; PDTC, pyrrolidine dithiocarbamate; AdoMet, S-adenosyl methionine; AdoHcy, S-adenosyl homocysteine; APF, atomic position fluctuations; CARM1, coactivator-associated arginine methyltransferase 1; HIFα, hypoxia-inducible factor α; FOXO, forkhead box O transcription factor
FIGURE LEGENDS

FIGURE 1. ADMA formation and degradation. Protein arginine methyltransferases (PRMTs) transfer a methyl group from donor AdoMet to arginine residues in substrate proteins. Type 1 PRMTs can transfer one or two methyl groups to the same terminal guanidino nitrogen producing monomethyl arginine (MMA) or asymmetric dimethylarginine (ADMA), respectively. Upon degradation of the methylated proteins, free MMA and ADMA inhibit NO synthesis by acting as competitive inhibitors of nitric oxide synthase. Free ADMA is catabolized by dimethylarginine dimethylaminohydrolase (DDAH) to citrulline and dimethylamine, or excreted in urine.

FIGURE 2. PRMT1 activity is (A) inhibited by H$_2$O$_2$ in a concentration dependent manner and (B) activity lost can be recovered by reduction. In (A), reduced PRMT1 was incubated with 0 (◊), 40 (□), 200 (○), 400 (×), or 800 (△) µM H$_2$O$_2$ for 10 minutes at 37° C, followed by the addition of catalase. Methyltransferase activity of the treated PRMT1 was measured with 200 µM R3 peptide as a substrate as described in Experimental Procedures. In (B), reduced PRMT1 was treated with a physiologically-relevant concentration of hydrogen peroxide or phosphate-buffered saline (PBS) and subsequently treated with 1mM DTT or PBS prior to methyltransferase assays.

FIGURE 3. The effect of reducing agents on PRMT1 enzymatic activity. In (A) PRMT1 methyltransferase activity was measured without substrate (△), or with 200 µM R3 peptide in the absence (□) and presence of DTT (○) or TCEP (×), or after desalting following a 10 minute pre-incubation with DTT (◊). In (B), methyltransferase activity was measured as a function of DTT concentration.

FIGURE 4. The enhancing effect of DTT on PRMT1 methyltransferase activity is independent of the His$_6$-tag. Enzymatic activity of HisTevPRMT1 (○), cleaved PRMT1 (△), and dialyzed HisTevPRMT1 (□) measured with 200 µM R3 peptide in the absence (open) and presence (closed symbols) of DTT respectively, as well as HisTevPRMT1 treated with EDTA only as a control (◊).

FIGURE 5. Oligomeric state of PRMT1 proteins assessed by size exclusion chromatography. PRMT1 without DTT treatment (top) or incubated overnight with 1 mM DTT and 2 mM EDTA (middle), compared to Cysteine-less PRMT1 which migrates at the same position regardless of treatment with DTT or EDTA. Reduction of PRMT1 or removal of all cysteine residues results in a shift towards a smaller oligomeric state.

FIGURE 6. Methyltransferase activity of PRMT1 cysteine variants in the absence or presence of DTT. WT PRMT1, Cysteine-less PRMT1, C101S PRMT1, C342S PRMT1, C254S PRMT1, and C101S/C208S PRMT1 methylated 200 µM R3 peptide in the absence (light gray/purple) or presence (dark gray/purple) of DTT. Results shown correspond to the average of at least two biological replicates. Removing all cysteine residues from PRMT1, or making C101 and C208 unavailable for oxidation resulted in abolished redox control over PRMT1 catalytic activity.

FIGURE 7. Sulfenic acid detection and free thiol content in PRMT1. In (A), (B), (C), and (D) Air oxidized wild type PRMT1 (WT), cysteine-less (cys-), cysteine-less with C101 reintroduced (cys-C$^{101}$), cysteine-less with C208 reintroduced (cys-C$^{208}$), and cysteine-less with both C101 and C208 reintroduced (cys-C$^{101}$C$^{208}$) were denatured in 6M urea and incubated with 1 mM DTT or buffer prior to addition of 10 µM DCP-Rho1 or 2.5 mM 5IAF. Labeled samples were resolved by SDS-PAGE. (A) shows a representative image of the rhodamine fluorescence signal and the corresponding coomassie bands. (B) graphical representation of triplicate gel analysis. Normalized percent DCP-Rho1 fluorescence represents the percentage of fluorescent signal divided by the amount of protein observed in the coomassie bands which is interpreted as the relative amount of sulfenic acid present. (C) shows a representative image of
the 5IAF fluorescence signal and the corresponding commassie bands. (D) graphical representation of triplicate 5IAF gel analysis, interpreted as relative amount of free thiols present. (E) and (F) show a representative MS/MS fragmentation spectra for peptides containing dimeredone-modified sulfenic acids in PRMT1. In (E), the CID fragments of peptide IECSSISDYAVK labeled with dimeredone at C101. (F) ETD fragments of peptide MCSIKDVAIK labeled with dimeredone at C208. The mass shift by the modification is 138.068 (exact number), as denoted in peptide sequence.

FIGURE 8. Cysteine residues in rPRMT1. PRMT1 dimer (1OR8) colored as described in text (AdoMet binding domain in light gray, β-barrel domain in dark gray, dimerization arm in blue, αγ-loop–αZ in orange, AdoHcy in green, and cysteine residues in red. Residues 26-39 were modeled based on the position of this helix in the PRMT3 structure (1F3L). (A) PRMT1 dimer (B) Surface representation showing close active site interactions between AdoHcy, C101 and F36. (C) Top view of dimerization arm in one monomer interacting with AdoMet binding site in other monomer. (D) Back view showing packing of C208 in one monomer with alpha helix in AdoMet binding domain of other monomer.

FIGURE 9. Redox control is conserved among PRMT family members. Human PRMT3 (residues 211-531), human PRMT6, and TbPRMT7 activities were tested with R3 peptide, bulk histones, or Histone 4 respectively in the absence or presence of DTT. The average of three activity measurements are shown as relative percent activity for each isoform with its corresponding substrate.
FIGURE 2

A

Methyl groups transferred (µM)

Time (min)

B

Rate (µM CH/min)

PRMT1

- + + 0.4 µM H₂O₂
- + + 1 mM DTT
+ - +
FIGURE 4

Redox control of PRMT1 activity
FIGURE 5
FIGURE 6

![Graph showing the redox control of PRMT1 activity. The x-axis represents different conditions, and the y-axis shows the rate (µM/min). The graph includes bars for each condition with error bars indicating variability.](http://www.jbc.org/Downloaded from)
Redox control of protein arginine methyltransferase 1 (PRMT1) activity
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