

Leaky expression of PRMT1 in *Escherichia coli* and redox regulation of its histone methyltransferase activity

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SUMMARY

Protein arginine methyltransferase 1 (PRMT1) is a major regulator of gene expression and genome stability through its ability to methylate histone and non-histone substrates. Because PRMT1 activity is tightly linked to its cellular abundance and biochemical environment, understanding how experimental conditions influence its expression and enzymatic function is critical for accurate biochemical characterization. In this study, we examined recombinant human PRMT1 produced in *Escherichia coli*. We assessed how variation in expression conditions influences PRMT1 production and how the reduction–oxidation (redox) environment modulates its enzymatic activity. We hypothesized that basal expression from a T7 promoter–based pET28a(+) expression vector system would be sufficient to generate functional PRMT1 protein even without isopropyl β -D-1-thiogalactopyranoside (IPTG) induction. We further hypothesized that a reducing biochemical environment would enhance PRMT1 catalytic activity by maintaining key cysteine residues in a reduced state. Consistent with this hypothesis, PRMT1 protein was readily detected and purified even in the absence of strong induction, and its histone methyltransferase activity was selectively sensitive to the nature of the reducing environment. Together, these findings demonstrate that both expression context and the specific redox conditions applied are critical variables governing PRMT1 activity and provide a practical framework for designing and interpreting *in vitro* biochemical assays of PRMT1 and related methyltransferases.

INTRODUCTION

Gene expression in eukaryotic cells is tightly regulated at multiple levels, including chromatin organization and epigenetic modification. DNA is packaged into chromatin through its association with histone proteins, and chromatin accessibility plays a central role in transcriptional regulation (1). Post-translational modifications (PTMs) of histones regulate chromatin structure and gene expression (2). Among these, histone methylation can either activate or repress transcription depending on context (3). Dysregulation of histone methylation is linked to various diseases (4).

In mammalian cells, the protein arginine methyltransferase (PRMT) family comprises nine members (PRMT1–PRMT9), which are classified into type I, type II, and type III enzymes (5). Type I PRMTs produce mono-methylarginine (MMA) and asymmetric di-methylarginine (ADMA), type II PRMTs generate MMA and symmetric di-methylarginine, and type III PRMTs catalyze only MMA formation. PRMT1 is the predominant type I PRMT and catalyzes the transfer of methyl

groups from S-adenosylmethionine (SAM) to the guanidino nitrogen atoms of arginine residues, generating MMA and ADMA, and functions as a homodimer. PRMT1 is responsible for approximately 90% of arginine methyltransferase activity *in vivo* (6, 7).

PRMT1 was initially characterized for its ability to methylate histone H4 at arginine 3 (H4R3me2a), a modification linked to transcriptional activation (8, 9). Beyond histones, PRMT1 also methylates a wide range of non-histone proteins, including transcription factors, RNA-binding proteins, and components involved in genome maintenance. Through these modifications, PRMT1 influences diverse cellular processes such as transcriptional regulation, RNA processing, and DNA damage responses, thereby regulating cell phenotypes (10–16). Together, these findings highlight PRMT1 as a broadly acting regulatory enzyme whose activity must be precisely controlled across multiple cellular contexts.

Dysregulation of PRMT1 has been implicated in disease. PRMT1 is frequently overexpressed in breast cancer and associated with poor prognosis, and PRMT1-dependent methylation events support leukemogenesis by altering transcriptional regulators (16, 17). Broader surveys implicate PRMT1 in cancer progression across multiple tumor types, reinforcing its therapeutic relevance (18, 19).

At the enzymatic level, PRMT1 transfers methyl groups from SAM to arginine side chains on histone and non-histone substrates to generate ADMA marks that modulate chromatin and RNA binding protein, RBP, networks (8,10). *In vitro* methyltransferase assays require careful control of reaction conditions, as PRMT1 activity is sensitive to the redox state of the reaction environment. Oxidative conditions (e.g., hydrogen peroxide, H₂O₂) inhibit catalysis, whereas reducing conditions restore enzymatic activity, making redox control essential for quantitative PRMT1 kinetics (20).

Recombinant PRMT1 is typically produced in *Escherichia coli* (*E. coli*) using T7 promoter-based expression systems; however, a well-recognized limitation of these systems is “leaky expression,” in which basal transcription occurs even in the absence of inducer (21). In BL21(DE3) cells, expression of T7 RNA polymerase is controlled by the lac promoter and repressed by LacI; however, this repression is not absolute. As a result, low levels of T7 RNA polymerase can be produced even without inducer, which in turn drives basal transcription from the T7 promoter and leads to detectable PRMT1 expression under uninduced conditions (22). Although the pET28a(+) vector incorporates a T7 promoter and lac operator to facilitate controlled expression and purification, these regulatory features do not fully prevent uninduced transcription (21, 23). In parallel, PRMT1 catalytic function depends on maintaining key cysteine residues in a

reduced state, which is typically achieved *in vitro* by including reducing agents such as dithiothreitol (DTT), whereas oxidation disrupts proper enzymatic conformation (20). Although recombinant PRMT1 expressed in *E. coli* is devoid of post-translational modifications (PTMs), it has been widely used as an active enzyme source for *in vitro* methyltransferase assays, enabling controlled biochemical characterization. Moreover, since cellular redox states vary across physiological and disease contexts, defining redox-dependent effects on PRMT1 activity *in vitro* provides a baseline for interpreting how PRMT1 function may be modulated in human cells (20). Notably, PRMT1 expression levels do not always directly reflect enzymatic activity, because oxidative modifications can transiently suppress catalysis even when protein abundance is unchanged (20).

Based on these considerations, we hypothesized that both the production and catalytic activity of PRMT1 are influenced by experimental expression conditions and the redox environment. While basal expression from T7-based systems and redox sensitivity of PRMT1 have been previously reported, we sought to systematically evaluate the extent of leaky expression across induction conditions and to directly compare the effects of distinct reducing agents (DTT versus β -mercaptoethanol) on PRMT1 catalytic activity.

Specifically, we predicted that basal expression from a T7-based system would be sufficient to generate functional PRMT1 protein, even in the absence of strong chemical induction. In T7 promoter-based expression systems, isopropyl β -D-1-thiogalactopyranoside (IPTG) is commonly used as an inducer to relieve LacI-mediated repression and stimulate transcription; however, incomplete repression can result in basal expression even without IPTG. We therefore examined PRMT1 expression under different IPTG induction conditions to assess the extent of this leaky expression. In

addition to expression level, we further hypothesized that variation in the redox state of the biochemical environment would significantly modulate PRMT1 catalytic activity. To test this, we assessed PRMT1 activity under different reducing conditions using two commonly employed reducing agents, DTT and β -mercaptoethanol (BME), which differ in their chemical properties and effective reducing strength. Because reducing agents can alter protein function by maintaining cysteine residues in a reduced state, comparing DTT and BME provided a controlled approach to examine how redox conditions influence PRMT1 histone methyltransferase activity *in vitro*. Here, we report that PRMT1 undergoes leaky expression from the pET28a(+) vector in *E. coli* BL21(DE3) cells, yielding sufficient protein for nickel-nitrilotriacetic acid (Ni-NTA) affinity purification in the absence of IPTG induction. We further demonstrate that PRMT1 histone methyltransferase activity toward histone H4R3 is enhanced by DTT in a concentration-dependent manner, reaching a plateau at ≥ 1.0 mM DTT, whereas BME fails to potentiate activity even at substantially higher molar concentrations.

RESULTS

To assess whether PRMT1 is expressed in the absence of chemical induction, we analyzed BL21(DE3) cells harboring His-tagged PRMT1 cloned into the T7 promoter-based pET28a(+) expression vector, across varying IPTG concentrations and growth temperatures and detected PRMT1 expression even without IPTG (**Figure 1A**). Whole-cell lysates were analyzed by sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis followed by Coomassie staining to assess PRMT1 expression (**Figure 1B**). A discrete band at the expected size for His-PRMT1 (~42 kDa) was observed under all tested conditions, including the 0 mM IPTG lanes, indicating basal (leaky) T7-driven

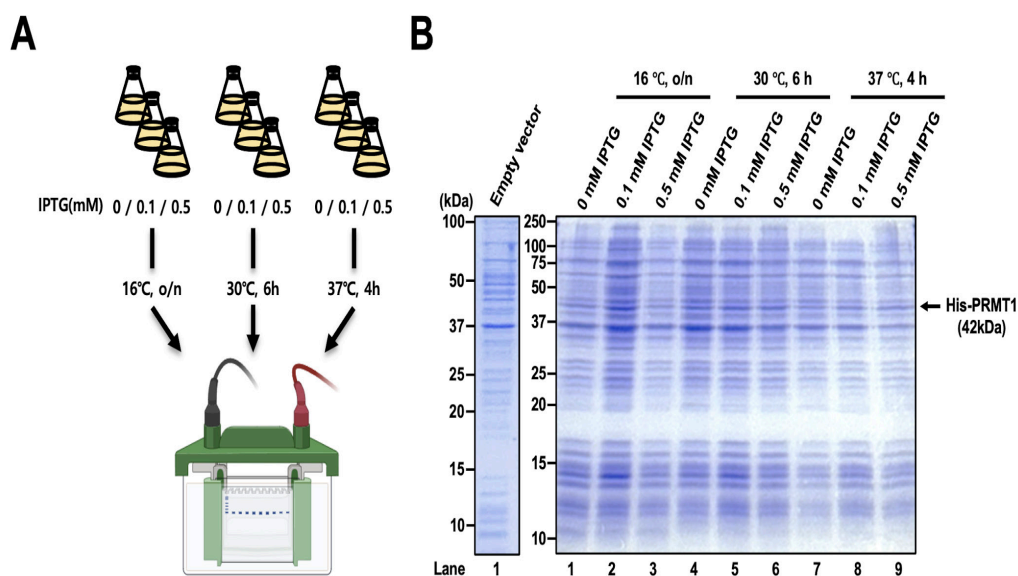


Figure 1. Induction test for His-PRMT1/pET28a(+) plasmid transformed into *E. coli* BL21(DE3) cells.

(A) Schematic representation of the induction conditions tested for His-PRMT1 expression. (B) Coomassie-stained SDS-PAGE of whole-cell lysates. Protein bands are resolved by molecular weight (kDa) along the vertical axis. Lanes 1–3 correspond to 16 °C for overnight (o/n) at 0, 0.1, and 0.5 mM IPTG, respectively; lanes 4–6 correspond to 30 °C for 6 h; and lanes 7–9 correspond to 37 °C for 4 h. An empty vector control is included to indicate background protein expression in the absence of PRMT1. The arrow indicates the position of His-PRMT1.

expression of PRMT1 in BL21(DE3) cells (**Figure 1B**). This pattern was consistent across IPTG concentrations ranging from 0 to 0.5 mM and growth temperatures from 16 °C to 37 °C. In contrast, no enhanced band was detected at this molecular weight in cells transformed with the empty vector control, confirming that the observed ~42 kDa band reflects specific His-PRMT1 expression (**Figure 1B**). Qualitative inspection revealed only modest variation of band intensity across inducer concentrations and temperatures. No condition produced a marked enhancement over the non-induced controls at the whole-cell level, and the overall pattern was consistent with incomplete repression of the T7 transcription system in BL21(DE3) cells. Collectively, the analysis across the tested induction conditions demonstrated that His-PRMT1 is detectably expressed in the absence of IPTG, and that increasing IPTG to 0.1–0.5 mM conferred no substantial advantage in apparent total expression under the conditions tested (**Figure 1A, B**). On this basis, and following confirmation that comparable amounts of His-PRMT1 could be recovered after purification across the tested induction conditions, subsequent *in vitro* assays were performed using protein prepared at 37 °C for 4 h with 0.5 mM IPTG, as a time-efficient and commonly used induction condition.

To assess whether basal expression of His-tagged PRMT1 provides sufficient starting material for downstream purification, we performed Ni-NTA affinity purification. Coomassie-stained SDS-PAGE analysis showed that His-PRMT1 was recovered at comparable levels across all induction conditions (IPTG concentration from 0 to 0.5 mM and growth temperature 16 °C to 37 °C), indicating that IPTG-independent basal expression from the T7 promoter in BL21(DE3) cells yields enough soluble protein to support efficient affinity purification (**Figure 2A**). These results demonstrate that, under the conditions tested, strong chemical induction is not required to obtain recoverable His-PRMT1 for subsequent biochemical analyses. Wild-type (WT) His-PRMT1 was analyzed across all induction conditions to evaluate expression and purification efficiency, whereas

the catalytic-dead mutant His-PRMT1(G98R) was prepared only under the selected condition (37 °C, 4 h, 0.5 mM IPTG) (**Figure 2B**). Based on these optimization experiments, WT His-PRMT1 was readily purified under all conditions tested, and the same protocol was subsequently applied to purify His-PRMT1(G98R) to near homogeneity (**Figure 2B**). The G98R substitution has been previously reported to abolish PRMT1 catalytic activity (24), and both proteins were used in subsequent assays.

To examine the enzymatic activity of purified His-tagged PRMT1, *in vitro* histone methyltransferase (HMT) assays were performed using recombinant histone octamers reconstituted with His-tagged histone H3 as substrate. Immunoblotting with an antibody specific for H4R3me2a revealed that no methylation signal was detected in the absence of PRMT1 (**Figure 3**). Upon increasing the amount of PRMT1 protein, the H4R3me2a signal progressively increased and reached a plateau at the reaction endpoint, with no appreciable difference observed between the highest protein concentrations tested (**Figure 3**). In contrast, the catalytic-dead mutant PRMT1(G98R) failed to produce any detectable H4R3 methylation (**Figure 3**). Coomassie-stained SDS-PAGE confirmed equivalent protein loading across reactions (**Figure 3A**). These findings demonstrate that PRMT1 methylation activity toward histone substrates is dose-dependent, reaches a plateau in final methylation levels under the assay conditions, and is abolished by the G98R mutation.

We next tested whether reducing conditions mediated by DTT would enhance PRMT1 enzymatic activity, as has been suggested in prior studies (20, 25). In this assay, 30 ng of PRMT1 was incubated with recombinant histone octamer substrate, while the concentration of DTT was varied across reactions (0.05, 0.1, 0.3, 0.5, 0.7, 1.0, 2.0, and 3.0 mM). Immunoblotting for H4R3me2a revealed a baseline methylation signal in the absence of DTT, attributable to PRMT1 alone (**Figure 4**). Upon increasing the DTT concentration, the H4R3me2a signal progressively increased

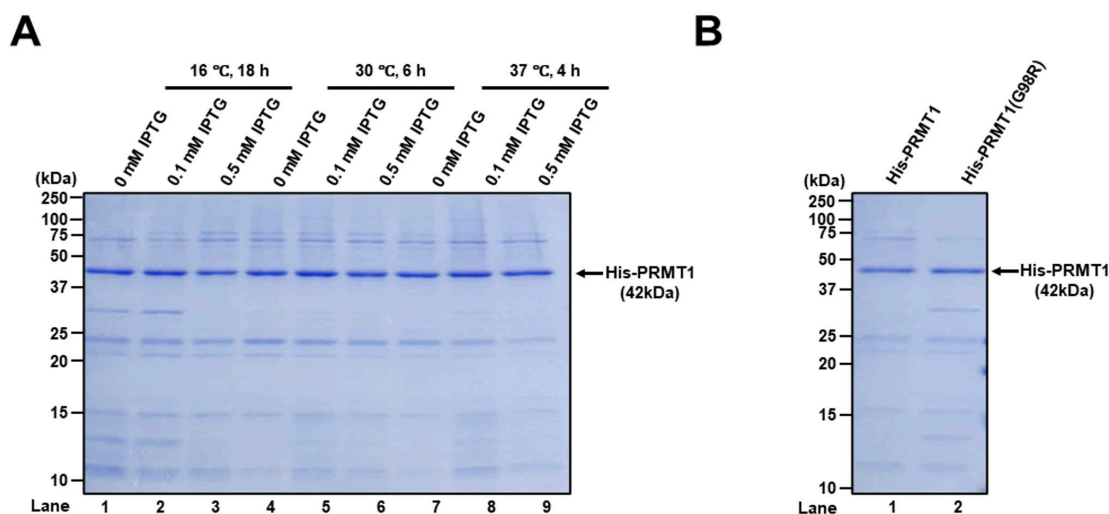


Figure 2. Ni-NTA purification for His-PRMT1 and His-PRMT1(G98R). (A) Coomassie Blue staining of His-PRMT1 proteins, expressed and purified via the *E. coli* expression system under the indicated induction conditions. (B) Coomassie Blue staining of wild-type (WT) and G98R mutant His-PRMT1 proteins purified under the 37 °C for 4 h induction condition.

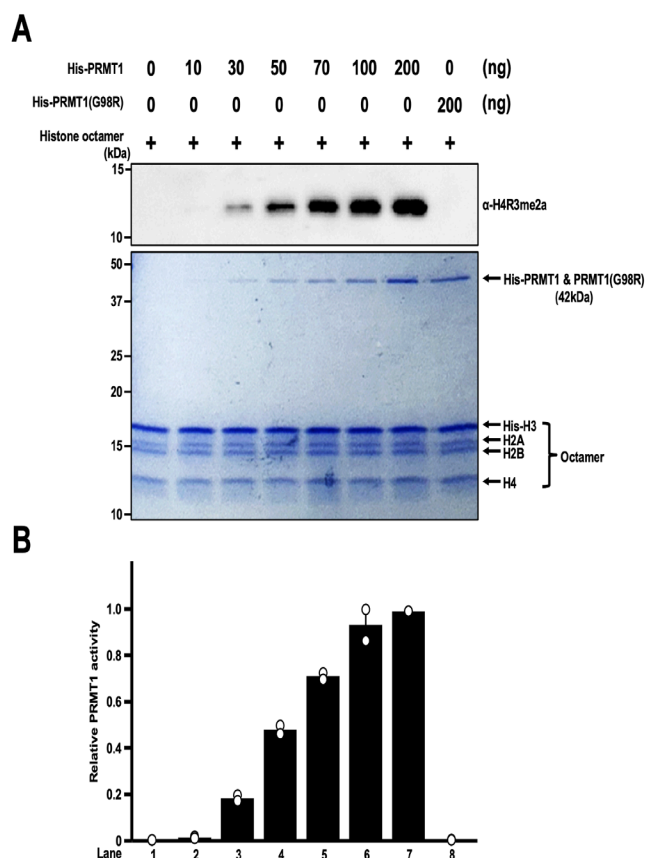


Figure 3. *In vitro* HMT assay assessing PRMT1 methyltransferase activity at different enzyme concentrations. (A) Dose-dependent H4R3 methylation activity of PRMT1 compared to the catalytic-dead mutant PRMT1(G98R). Purified PRMT1 and PRMT1(G98R) were subjected to an *in vitro* methylation assay using recombinant histone octamer as a substrate. Methylation levels were monitored by immunoblotting with the H4R3me2a antibody (top). Protein amounts loaded in the assay were visualized by Coomassie-stained SDS-PAGE (bottom). (B) Bar graph showing mean $\pm$ standard deviation relative H4R3me2a signal intensity ($n = 2$). Relative methylation levels were quantitated by ImageJ software and normalized to the lane containing the highest amount of PRMT1, which was set to 1 (lane 7).

(Figure 4). Notably, methylation reached a plateau at DTT concentrations of 1.0 mM and above, with no substantial further increase observed at higher concentrations (Figure 4). The Coomassie-stained SDS-PAGE, confirmed equal protein input in each reaction. These results demonstrate that PRMT1 methyltransferase activity is potentiated by DTT in a dose-dependent manner and reaches maximal activity under reducing conditions at 1.0 mM DTT (Figure 4).

Consistent with previous studies, PRMT1 enzymatic activity was markedly enhanced under reducing conditions, as DTT reversed the inhibition caused by cysteine oxidation (20). Structural considerations suggest that redox perturbations at cysteine residues may interfere with proper active-site alignment and dimer stability, and DTT may help restores these conformations to enable efficient catalysis. Similarly, we questioned whether BME, as a thiol-based reductant, might exert a comparable effect on PRMT1 by reducing oxidized cysteines, although its reducing capacity is

generally considered weaker and less stable than that of DTT (26). To examine this, *in vitro* HMT assays were performed under the same conditions as in Figure 4, except that DTT was replaced with BME at higher concentrations ranging from 0 to 1.0 M due to its weaker reducing potential. Contrary to expectations, immunoblotting for H4R3me2a showed no appreciable difference between reactions without BME and those with increasing concentrations of BME (Figure 5). These findings suggest that, although both DTT and BME are thiol (-SH) reductants capable of converting oxidized cysteine residues (-SOH, -S-S-) back to their sulfhydryl form, the reducing power of BME is insufficient to enhance PRMT1 enzymatic activity. Alternatively, the results raise the possibility that DTT may promote PRMT1 activity through an additional mechanism beyond simple thiol reduction.

DISCUSSION

Recombinant expression of PRMT1 in *E. coli* BL21(DE3) cells using the T7 promoter-based pET28a(+) expression vector revealed that basal transcription from the T7 promoter was sufficient to produce detectable and purifiable protein, independent of IPTG induction. This phenomenon, previously reported for other T7-based systems, arises from incomplete LacI repression and underscores the challenge of eliminating background transcription in pET-based constructs (27, 28). While our observations were made using the pET28a(+) vector, it remains possible that such leaky expression is specific to this construct. Comparative analyses employing alternative *E. coli* expression vectors will therefore be necessary to determine whether this behavior is broadly applicable or restricted to pET28a(+). Leaky expression is not inherently problematic, but its significance depends on the target protein and experimental goal (27). For toxic or aggregation-prone proteins, basal expression can impair cell growth, reduce plasmid stability, or decrease final yield (28). However, for PRMT1 under the conditions tested here, leaky expression produced soluble, purifiable enzyme and did not interfere with downstream activity assays. Therefore, in this study, leaky expression was not a major limitation but an important feature to consider when interpreting expression results from the pET28a(+)/BL21(DE3) system. Although PRMT1 was detectable without IPTG, we used 0.5 mM IPTG at 37 °C for 4 h for preparative expression because this condition was practical, time-efficient, and provided consistent recovery of both WT PRMT1 and PRMT1(G98R) for comparative biochemical assays.

Functional characterization of recombinant PRMT1 using histone octamers confirmed a concentration-dependent increase in H4R3me2a levels, which reached a plateau at higher enzyme concentrations (Figure 3). These results are consistent with the catalytic properties of PRMT1 described in earlier biochemical studies (6, 9). Importantly, the absence of detectable activity for PRMT1(G98R) validated the assay system and established a benchmark for evaluating redox-dependent modulation of enzymatic activity.

Systematic titration of DTT revealed a dose-dependent enhancement of PRMT1 activity, with a progressive increase in methylation levels until a plateau was reached at 1.0 mM DTT (Figure 4). This observation agrees with prior reports showing that PRMT1 is highly sensitive to cysteine oxidation, and that reducing agents restore catalytic activity by maintaining active-site cysteines in a reduced conformation (20, 25). In

contrast, when we substituted DTT with β -mercaptoethanol at over 300-fold higher concentrations, we did not observe a measurable increase in PRMT1 activity. One explanation for this difference lies in the distinct reducing capacities of the two reagents: DTT has a more favorable redox potential and can more efficiently reduce disulfide bonds, whereas BME is a weaker reductant, despite its thiol chemistry, and may be less effective at maintaining critical cysteine residues in a reduced state under the assay conditions. However, the observed disparity between DTT and BME is not necessarily attributable solely to differences in redox potential. Efficient reduction of catalytic cysteine residues may require not only sufficient reducing power but also appropriate access to, and stabilization within, the enzyme's active site. In this context, the smaller size and higher diffusivity of BME may limit its ability to remain positioned long enough to reduce the relevant cysteine residues effectively. Conversely, the larger and more rigid structure of DTT may allow more sustained interactions with the protein, thereby facilitating reduction before diffusing away. These considerations raise the possibility that steric or binding-related factors, in addition to intrinsic chemical properties, contribute to the differential effects of these reducing agents on PRMT1 activity. Dissecting the relative contributions of redox potential, molecular size, and active-site accessibility will require further mechanistic studies, potentially involving structural analyses or kinetic measurements under defined redox conditions. In interpreting these activity measurements, it should also be noted that protein amounts used in the assays were estimated based on Coomassie-stained SDS-PAGE band intensity rather than by an independent chemical quantification method. Accordingly, while relative changes in PRMT1 activity across conditions could be robustly compared, precise calculation of specific enzymatic activity was not possible. In future studies, specific enzymatic activity could be determined by independently quantifying PRMT1 concentration and measuring product formation within the linear range of the reaction, enabling normalization to enzyme amount and reaction time.

Although histone H4R3 was used as a prototypical substrate in this study, PRMT1 is known to target a wide range of non-histone proteins, including transcription factors, RNA-binding proteins, and components of the DNA damage response (5, 29, 30). Notably, the recombinant histone octamers used here were expressed in *E. coli* and therefore lack pre-existing post-translational modifications (PTMs) such as methylation or acetylation that are commonly present on histones in mammalian cells. The absence of these native PTMs may influence PRMT1–substrate interactions and catalytic efficiency, and thus the redox-dependent effects observed in this study should be interpreted in the context of a simplified, unmodified chromatin substrate. Each substrate displays distinct methylation sites and context-dependent efficiencies, implying that the magnitude of redox-dependent regulation may vary considerably depending on the substrate. Nevertheless, our data establish quantitative relationships between PRMT1 abundance, cofactor conditions, and catalytic output, thereby providing a reference framework for future analyses of PRMT1 activity on diverse targets.

In addition to DTT and BME, a variety of reductants are widely used in biochemical assays, including Tris(2-carboxyethyl) phosphine glutathione, and thioredoxin-

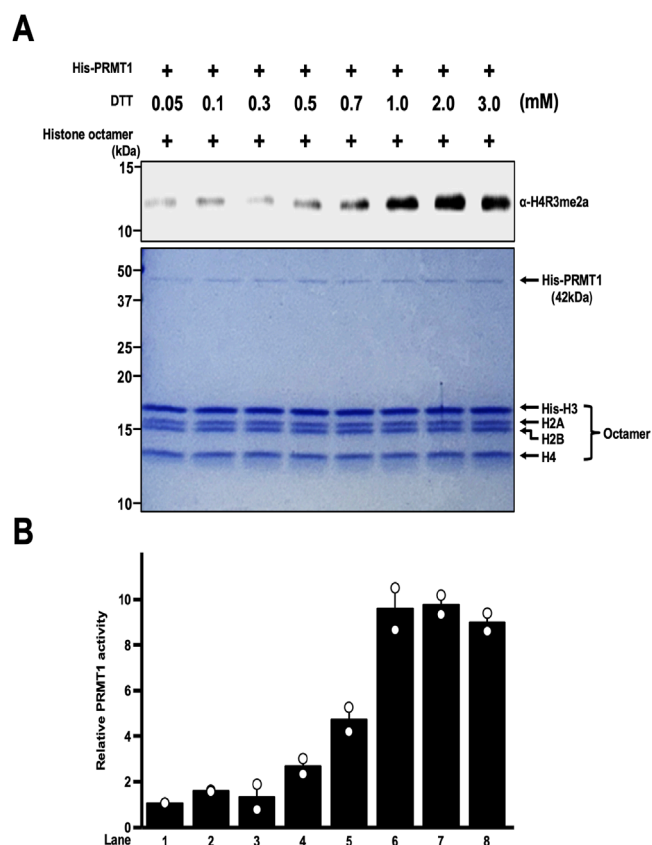


Figure 4. In vitro HMT assay assessing PRMT1 activity under increasing DTT concentrations. (A) Purified PRMT1 and recombinant histone octamer substrate were subjected to in vitro methylation assays with increasing concentrations of DTT. H4R3me2a levels were monitored by immunoblotting (top), and the amounts of proteins used in each reaction were confirmed by Coomassie-stained SDS-PAGE (bottom). (B) Bar graph showing mean $\pm$ standard deviation relative H4R3me2a levels ($n = 2$). Relative methylation levels were quantitated by ImageJ software and the lane containing the lowest DTT concentration was normalized to 1.

based systems (31, 32). Their distinct redox potentials and chemical stability may differentially affect PRMT1 activity, and comparative studies will be necessary to identify optimal conditions for each experimental context. Extending such analyses to other PRMTs will also be important, as type I, II, and III enzymes exhibit distinct catalytic outputs (5, 29). Systematic biochemical comparisons across the PRMT family would clarify whether the redox sensitivity observed for PRMT1 is a general property or an enzyme-specific characteristic. Future work will be required to assess the impact of alternative reducing agents, to investigate the contribution of DTT-specific mechanisms, and to evaluate PRMT1 activity across a broader spectrum of histone and non-histone substrates.

In conclusion, our study demonstrates that leaky expression from pET28a(+) in *E. coli* is sufficient for purification of catalytically active PRMT1, and that enzymatic activity is increased in the presence of DTT in a concentration-dependent manner, but not by BME. These findings highlight the importance of redox control in PRMT1 catalysis and suggest that both chemical and structural features of reducing agents may influence enzyme function.

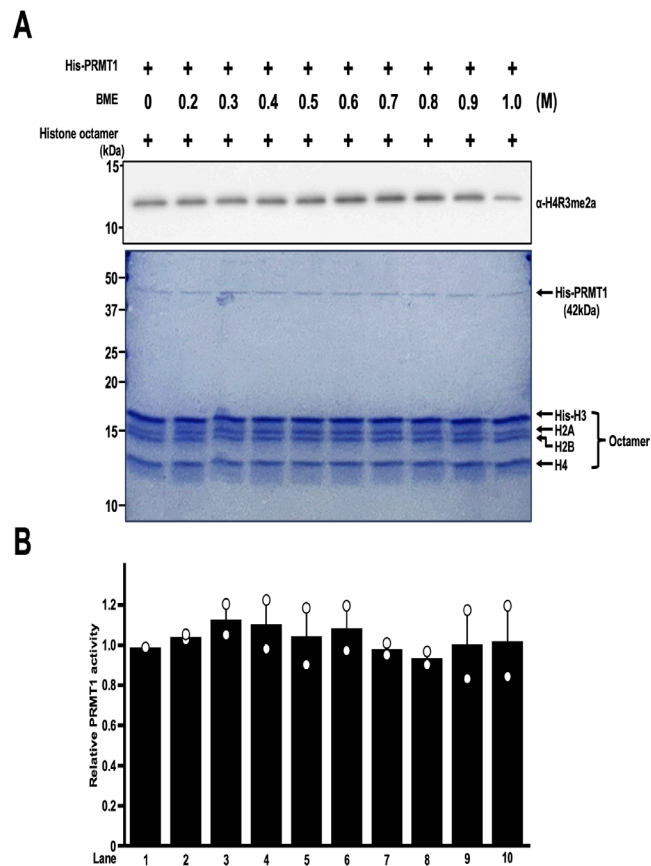


Figure 5. *In vitro* HMT assay assessing PRMT1 activity under increasing β -mercaptoethanol concentrations. (A) As previously described, purified PRMT1 and recombinant histone octamer substrate were analyzed in an *in vitro* methylation assay under varying BME concentrations. H4R3me2a signals were detected by immunoblotting (top), and total protein inputs were verified by Coomassie staining of SDS-PAGE gels (bottom). (B) Bar graph showing mean $\pm$ standard deviation relative H4R3me2a fold change ($n = 2$). Relative methylation levels were quantified using ImageJ software, and protein band intensities were normalized by setting the lane without BME (lane 1) to 1.

MATERIALS AND METHODS

Plasmids

N-terminal His-tagged constructs His-PRMT1 and His-PRMT1(G98R), encoding human WT PRMT1 and the catalytic-dead mutant PRMT1(G98R) in pET28a(+) vector (Novagen) were derived from constructs previously described in previous work (16).

Induction Test

For protein expression tests, BL21(DE3) cells transformed with His-PRMT1/pET28a(+) were grown in LB (100 ml) at 37°C to an OD₆₀₀ of 0.6. Cultures were induced with IPTG (Roche) to final concentrations of 0, 0.1, or 0.5 mM, and then incubated under one of the following conditions: 16°C for overnight, 30°C for 6 h, or 37°C for 4 h. Cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl pH 7.9, 300 mM KCl, 20% glycerol, 0.1 mM PMSF, and 0.1% NP-40), and lysed by incubation at 4 °C for 30 min with gentle shaking. The expression test was performed using the

same base buffer composition as that used for subsequent Ni-NTA purification. Whole-cell lysates were then analyzed by SDS-PAGE followed by Coomassie staining to assess protein expression.

SDS-PAGE and Coomassie Staining

Proteins were resolved by SDS-PAGE using gels composed of either 10% or 15% resolving gel with a 4% stacking gel, depending on the molecular weight range of interest. Protein samples were mixed with 5X loading dye (LPS Solution), heated at 100 °C for 5 min, and 10 μ L of each sample was loaded per lane. Electrophoresis was performed using standard running buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. Following electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (CELLPICK) overnight at room temperature and subsequently destained using destaining buffer (40% methanol, 10% acetic acid) until protein bands were clearly visible.

Protein Purification

For purification of His-PRMT1, the plasmid-induced cell pellets were resuspended and bound to Ni-NTA agarose (Qiagen) in 20 mM Tris-HCl (pH 7.9), 300 mM KCl, 20% glycerol, 0.1 mM PMSF, 1 mM DTT, and 0.1% NP-40 without EDTA, supplemented with 5 mM imidazole during the binding step. Resin was washed extensively with the same buffer containing 30 mM imidazole, and His-tagged proteins were eluted with 400 mM imidazole. Eluted fractions were analyzed by SDS-PAGE/Coomassie and either used directly or further buffer-exchanged as indicated in subsequent assays. The concentrations of purified proteins were estimated by visual comparison of band intensities to known amounts of bovine serum albumin (BSA) standards run in parallel on the same Coomassie-stained SDS-PAGE gel, without formal densitometric quantification.

In Vitro Protein Methylation Assays

For histone methylation assays, reactions were performed in a total volume of 20 μ L containing 400 ng recombinant histone octamers (His-H3) and 200 μ M S-adenosylmethionine (SAM, NEB) in reaction buffer (20 mM Tris-HCl [pH 7.9], 150 mM NaCl, and 15% glycerol). To assess PRMT1 dose-dependent activity, 10–200 ng of His-PRMT1 was added to each reaction, and 200 ng of His-PRMT1(G98R) was used as a catalytic-dead control. To evaluate the effect of reducing conditions, reactions were supplemented with either DTT (0–3 mM, Thermo) or β -mercaptoethanol (0–1000 mM, Sigma), as indicated. Reactions were incubated at 30°C for 0.5 h. Proteins were resolved by SDS-PAGE and subjected to immunoblotting with an anti-H4R3me2a (Active Motif, 39705) at a dilution of 1:5000.

Western Blotting

After SDS-PAGE, proteins were transferred to nitrocellulose membranes (0.45 μ m pore size; Bio-Rad, 1620115) using a wet-transfer system (Bio-Rad) in transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Transfers were carried out at approximately 100 V for ~2 h with constant current conditions. Membranes were blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween-20 (0.05% TBS-T; CELLPICK) at room temperature for 30 min, followed by incubation with anti-H4R3me2a primary

antibody (Active Motif, 39705) at 4 °C overnight at a dilution of 1:5000. Membranes were washed three times for 10 min each with 0.05% TBS-T and then incubated with HRP-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Invitrogen, 31460) at room temperature for 1 h at a dilution of 1:3000. After three additional washes with 0.05% TBS-T, signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500) and visualized with a ChemiDoc™ Imaging System (Bio-Rad, #12003153).

Quantification and Statistical Analysis

All Western blot experiments were performed in two independent biological experiments, and representative blots are shown. Band intensities corresponding to H4R3me2a signals were quantified using ImageJ software, where signal intensity was measured using the “Plot Lanes” function and calculated as the area under each peak. For each experiment, band intensities were normalized by setting the intensity of a specified reference lane to 1, as indicated in the corresponding figure legends. Quantified values are presented as mean ± standard deviation, with error bars representing SD from independent experiments, as described for each figure.

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