The Major Protein Arginine Methyltransferase in *Trypanosoma brucei* Functions as an Enzyme-Prozyme Complex*

Lucie Kafková†, Erik W. Debler‡, John C. Fisk§, Kanishk Jain¶, Steven G. Clarke‡, and Laurie K. Read*†

From the †Department of Microbiology and Immunology, Witebsky Center for Microbial Pathogenesis and Immunology, and Jacobs School of Medicine and Biomedical Sciences, University at Buffalo, Buffalo, New York 14214, the §Laboratory of Cell Biology, The Rockefeller University, New York, New York 10065, and the ¶Department of Chemistry and Biochemistry and The Molecular Biology Institute, UCLA, Los Angeles, California 90095

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Prozymes are catalytically inactive enzyme paralogs that dramatically stimulate the function of weakly active enzymes through complex formation. The two prozymes described to date reside in the polyamine biosynthesis pathway of the human parasite *Trypanosoma brucei*, an early branching eukaryote that lacks transcriptional regulation and regulates its proteome through posttranscriptional and posttranslational means. Arginine methylation is a common posttranslational modification in eukaryotes catalyzed by protein arginine methyltransferases (PRMTs) that are typically thought to function as homodimers. We demonstrate that a major *T. brucei* PRMT, *Tb*PRMT1, functions as a heterotetrameric enzyme-prozyme pair. The inactive PRMT paralog, *Tb*PRMT1PRO, is essential for catalytic activity of the *Tb*PRMT1**ENZ** subunit. Mutational analysis definitively demonstrates that *Tb*PRMT1**ENZ** is the cofactor-binding subunit and carries all catalytic activity of the complex. Our results are the first demonstration of an obligate heteromeric PRMT, and they suggest that enzyme-prozyme organization is expanded in trypanosomes as a posttranslational means of enzyme regulation.

Trypanosoma brucei, the causative agent of human African trypanosomiasis, poses a severe health risk in Sub-Saharan Africa. An estimated 70 million people are at risk of the infection, and the World Health Organization estimates about 20,000 new cases per year (1). In the search for new treatments, understanding the basic biology of the parasite is a cornerstone of this major transcriptome regulator as well as the composition of DRBD18-containing ribonucleoproteins (11). Our proteome-wide studies revealed that about 15% of the proteome of *T. brucei* insect vector procyclic forms (PFs) harbors arginine methyl marks (9, 10). Thus, arginine methylation is poised to play a crucial role in regulating *T. brucei* biology. In support of this notion, we showed that *Tb*PRMT1-catalyzed arginine methylation of the essential RNA-binding protein DRBD18 acts as a switch that controls the RNA-stabilizing and -degrading activity of this major transcriptome regulator as well as the composition of DRBD18-containing ribonucleoproteins (11).

An enzyme family containing three major types of protein arginine methyltransferases (PRMTs) catalyzes arginine methylation (12). All PRMTs catalyze formation of ω-N°,monomethylarginine (MMA), type I PRMTs catalyze ω-N°,N°-asymmetric dimethylarginine (ADMA), and type II PRMTs create ω-N°,N°-symmetric dimethylarginine (SDMA). Humans possess nine PRMTs (12). *T. brucei* apparently contains just four PRMTs, and we showed that together these enzymes have the capacity to catalyze MMA, ADMA, and SDMA formation (13–

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†To whom correspondence should be addressed: Dept. of Microbiology and Immunology, University at Buffalo, Jacobs School of Medicine and Biomedical Sciences, Buffalo, NY 14214. Tel.: 716-829-3307; E-mail: lread@buffalo.edu.

‡The abbreviations used are: PF, procyclic form; PRMT, protein arginine methyltransferase; Tb, *T. brucei*; MMA, ω-N°-monomethylarginine; ADMA, ω-N°,N°-asymmetric dimethylarginine; SDMA, ω-N°,N°-symmetric dimethylarginine; BF, bloodstream form; LSH, linker-Strap-His; AdoMet, S-adenosyl-l-methionine; MBP, maltose-binding protein; MALS, multangle light scattering; AdoMetDC, AdoMet decarboxylase; MCS, multiple cloning site; qRT-PCR, quantitative RT-PCR; qPCR, quantitative PCR.

§K. Lott and L. Read, unpublished results.
T. brucei PRMT Is an Enzyme-Prozyme Pair

In this study, we show that TbpPRMT1 functions as a heterotetrameric complex formed by the enzymatic subunit TbpPRMT1ENZ (previously TbpPRMT1) and the inactive PRMT paralog TbpPRMT1PRO (previously TbpPRMT3). Our results demonstrate a novel PRMT organization and represent the first expansion of the trypanosome prozyme paradigm outside the polyamine synthesis pathway. These findings suggest that allosteric enzyme activation by catalytically inactive paralogs may be a more widespread mechanism for posttranslational regulation in trypanosomes than previously appreciated. Furthermore, our results suggest the presence of novel PRMT regulatory mechanisms that could also function in higher organisms under specific conditions.

**Results**

TbpPRMT1PRO Is Missing Key Catalytic Residues—The T. brucei genome encodes five proteins with high homology to human PRMTs, four of which have been characterized previously (13–16). Pairwise BLAST comparisons with human PRMTs indicated that the remaining putative TbpPRMT (Tbp927.10.3560) has the highest sequence similarity to human PRMT3, and therefore this enzyme has been formerly referred to as TbpPRMT3 (18, 22). In light of the functional results presented in this work, we have renamed Tbp927.10.3560 as TbpPRMT1PRO. Our studies also have led us to rename the former TbpPRMT1 (Tbp927.1.4690) as TbpPRMT1ENZ, and these names will be used hereafter. To begin to understand the function of TbpPRMT1PRO, we first examined its amino acid sequence. In general, type I PRMTs comprise a Rossmann fold that harbors conserved motifs I, post-I, II, and III and the double-E loop as well as a β-barrel domain containing a THW loop (Fig. 1A). The TbpPRMT1PRO amino acid sequence reveals conserved motifs I, post-I, II, and III. However, this protein harbors a Glu to Asp mutation within its double-E loop (Fig. 1B). Although conservative, the analogous mutation in rat PRMT1 was shown previously to decrease in vitro methylation activity to 0.03% compared with wild type enzyme (23). Furthermore, based on phylogenetic analysis, TbpPRMT1PRO clusters with type I PRMTs (24), and as such, it is expected to contain a THW loop. Strikingly, neither the threonine nor the histidine residue is conserved in the region of TbpPRMT1PRO corresponding to the THW loop (Fig. 1B). These observations suggested that TbpPRMT1PRO could be an inactive PRMT paralog.

TbpPRMT1PRO Forms a Complex with TbpPRMT1ENZ—TbpPRMT1ENZ has been described previously as an active enzyme that is responsible for the majority of ADMA formation in vivo (14). Surprisingly, in vitro we observed a narrow substrate specificity and very low activity compared with rat PRMT1 (14). Attempts to detect TbpPRMT1PRO activity have been unsuccessful, reinforcing the idea that it possibly is a catalytically inactive PRMT paralog. While investigating the interplay of TbpPRMTs in T. brucei PF, we noticed that TbpPRMT1ENZ and TbpPRMT1PRO share mutual protein stability dependence (18). Because this phenomenon is commonly associated with proteins that form a complex, we explored the
possibility that *TbPRMT1* ENZ and *TbPRMT1* PRO form a PRMT heteromorphic complex. First, we asked whether the mutual stability dependence of *TbPRMT1* ENZ and *TbPRMT1* PRO is conserved in both culturHar.
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induction of RNAi in PF cells, only the lower band was visibly diminished, leading us to conclude that the upper band is likely nonspecific (data not shown). In contrast to TbPRMT1<sup>ENZ</sup> and TbPRMT1<sup>PRO</sup>, TbPRMT6 (41 kDa) peaked in a fraction corresponding to a dimer. Together, these data indicate that TbPRMT1<sup>ENZ</sup> and TbPRMT1<sup>PRO</sup> are part of the same complex in vivo.

As our in vivo studies did not allow us to determine whether TbPRMT1<sup>ENZ</sup> and TbPRMT1<sup>PRO</sup> interact directly, we used an in vitro approach to answer this question. To this end, we utilized a pETDuet bacterial expression vector that allows for co-expression of two proteins under separate T7 promoters (Fig. 3A). The two TbPRMT1 subunits were first cloned separately to permit expression of a single histidine (His)-tagged PRMT (Fig. 3A, constructs “a” and “c”). For simplicity, these will be referred to as His-ENZ and His-PRO. In separate plasmids, the remaining TbPRMT1 ORF was cloned into the second site, which resulted in two additional constructs containing TbPRMT1<sup>ENZ</sup> with untagged TbPRMT1<sup>PRO</sup> or His-TbPRMT1<sup>PRO</sup> with untagged TbPRMT1<sup>ENZ</sup>. These constructs will be hereafter referred to as His-ENZ/PRO and His-PRO/ENZ (Fig. 3A, constructs “b” and “d”). Each of the four constructs was separately expressed in E. coli, and metal affinity resin was used to purify the His-tagged protein under stringent washing conditions (1 M NaCl). Next, we probed Western blots containing the eluted proteins with α-TbPRMT1<sup>ENZ</sup> and α-TbPRMT1<sup>PRO</sup> antibodies (Fig. 3B). We observed that, in both cases, the untagged subunit was purified together with the His-tagged protein, clearly demonstrating a strong, direct non-ionic interaction between TbPRMT1<sup>ENZ</sup> and TbPRMT1<sup>PRO</sup>.

TbPRMT1<sup>ENZ</sup>/TbPRMT1<sup>PRO</sup> Heteromer Is the Functional Unit of the Major Type I PRMT in T. brucei—Having confirmed His-TbPRMT1<sup>ENZ</sup>/TbPRMT1<sup>PRO</sup> heteromer formation in vivo and in vitro, we next wanted to investigate the in vitro activity of the heteromer. Because our previous TbPRMT1<sup>ENZ</sup> characterization showed that TbPRMT1<sup>ENZ</sup> down-regulation almost completely abolishes ADMA formation in T. brucei (14), we expected the heteromer to exhibit activity comparable with the major mammalian type I PRMT, PRMT1. To test the PRMT activities of TbPRMT1<sup>ENZ</sup>, TbPRMT1<sup>PRO</sup>, and the heteromer containing both proteins and compare their activities with mammalian PRMT1, we incubated rat PRMT1, His-ENZ, His-PRO, His-ENZ/PRO, or His-PRO/ENZ with S-adenosyl-L-[methyl-<sup>3</sup>H]methionine ([<sup>3</sup>H]AdoMet) in the absence or presence of an RGG-rich substrate (Fig. 4). Following the reactions, proteins were resolved by SDS-PAGE and Coomassie-stained, and the radiolabeled signal was visualized by fluorography. We observed that under our experimental conditions both heteromers, regardless of which subunit was fused to the N-terminal His tag, exhibited activity, whereas neither subunit alone produced any signal. Furthermore, the His-PRO/ENZ complex showed activity comparable with that of the rat PRMT1. From these data, we conclude that the TbPRMT1<sup>ENZ</sup>/TbPRMT1<sup>PRO</sup> heteromer is the active form of TbPRMT1.

We next wanted to determine the type of methylarginine produced by TbPRMT1. To this end, we performed a methylation reaction containing TbPRMT1 heteromer, MBP-RGG substrate, and [<sup>3</sup>H]AdoMet. The reaction was allowed to proceed for 2 h based on a time course experiment showing that at this time point the reaction was progressing within the linear

FIGURE 3. A, schematic of pETDuet-1 constructs used to express His-TbPRMT1<sup>ENZ</sup> (a), His-TbPRMT1<sup>ENZ</sup>/PRO (b), His-TbPRMT1<sup>PRO</sup> (c), and His-TbPRMT1<sup>PRO</sup>/ENZ (d). B, TbPRMT1<sup>ENZ</sup> and TbPRMT1<sup>PRO</sup> interact directly. Constructs a–d shown in A were expressed in E. coli. His-tagged protein was purified, and the elutions were probed with the indicated α-TbPRMT antibodies. The image is representative of two biological replicates.
range (Fig. 5A). Subsequently, the proteins were TCA-precipitated, hydrolyzed into amino acids, and analyzed on a cation exchange chromatography column together with unlabeled MMA, ADMA, and SDMA standards. Reactions omitting either substrate or enzyme served as controls (Fig. 5B). We observed a significant amount of ADMA and MMA present in the experimental sample, whereas no trace of SDMA was recorded. This led us to conclude that the TbPRMT1 heteromer is a type I PRMT. We also observed a low level of both ADMA and MMA in the experimental sample, whereas no trace of SDMA was recorded. This led us to conclude that the TbPRMT1 heteromer is a type I PRMT.

Having determined that the TbPRMT1 heteromer is a functional PRMT, we were intrigued by the possibility that this heteromer could be larger than the canonical PRMT dimer. The glycerol gradient sedimentation of native TbPRMT1 complex suggested this enzyme could function as a tetramer (Fig. 2C)."
We observed a single radioactive band whose signal was fully outcompeted by a 50-fold excess of unlabeled AdoMet but not by excess dATP, thereby demonstrating the specificity of the cross-linking (Fig. 7A). We then performed the same assay using the heteromers in which one of the subunits carried an inactivating mutation (Fig. 7B). We observed that both double-E loop mutants, His-(D180Q)PRO/ENZ and His-PRO/ (E135Q)ENZ, were capable of AdoMet binding, which was in accord with mutational studies on mammalian PRMT1 showing that, although this residue in the double-E loop is crucial for catalysis, it contributes very little to AdoMet binding (23). The retained ability to bind AdoMet also reassured us that the introduced mutations do not distort protein folding. We next analyzed AdoMet binding in heteromers with motif I mutations in each of the subunits. Remarkably, although AdoMet binding of TbPRMT1 was abolished when motif I of the TbPRMT1PRO subunit was mutated (Fig. 7B, His-PRMT1PRO/(G63R)ENZ), the heteromer harboring mutation of motif I in the TbPRMT1PRO subunit was perfectly capable of AdoMet binding (Fig. 7B, His-PRMT1ENZ/(G106R)PRO/ENZ). This led us to conclude that TbPRMT1ENZ is the sole AdoMet-binding subunit of the complex.

To investigate the contribution of the TbPRMT1ENZ and TbPRMT1PRO subunits to the catalytic activity of the heteromeric TbPRMT1 complex, we next performed a gel-based in vitro methylation assay. TbPRMT1, [3H]AdoMet, and substrate were mixed and incubated for 18 h at 22 °C, and methylated products were visualized by fluorography (Fig. 7C). Heteromers containing mutations in either double-E loop (His-(D180Q)PRO/ENZ) or motif I (His-(G106R)PRO/ENZ) of TbPRMT1PRO exhibited activity comparable with wild type complex. In stark contrast, complexes that carried mutations in either the double-E loop or motif I of TbPRMT1ENZ were catalytically dead (Fig. 7C, His-PRMT1PRO/(G63R)ENZ and His-PRMT1PRO/(E135Q)ENZ). Therefore, we conclude that TbPRMT1ENZ is the catalytic subunit of the TbPRMT1 heterotetramer.
Discussion

In this report, we describe a novel mode of PRMT organization and function. *TbPRMT1*, the major type I PRMT in the early branching eukaryote *T. brucei* (14), is a functional hetero-tetramer comprising two subunits. The previously reported *TbPRMT1* protein (14, 22) (here renamed *TbPRMT1\textsuperscript{ENZ}* ) bears a striking sequence identity to human PRMT1. By itself, *TbPRMT1\textsuperscript{ENZ}* exhibits no detectable activity under the conditions tested here despite retaining all critical PRMT motifs and displaying over 50% amino acid identity with human PRMT1. Nevertheless, *TbPRMT1* constitutes the catalytically active subunit of the tetramer. The second subunit of the tetramer previously referred to as *TbPRMT3* (18, 22) is renamed here as *TbPRMT1*\textsuperscript{PRO}. *TbPRMT1*\textsuperscript{PRO} is catalytically inactive but is essential for the allosteric activation of *TbPRMT1*\textsuperscript{ENZ}. This unique PRMT organization represents the first reported obligate heteromeric PRMT in any organism.

Two other *T. brucei* enzymes, deoxyhypusine synthase and AdoMetDC, both within the polyamine synthesis pathway, have been shown to function in a similar manner (19, 21, 32, 33, 39). In both cases, a catalytically inactive enzyme paralog termed prozyme dramatically stimulates the function of the true enzyme. This mode of organization and activation was coined the “prozyme paradigm” by Phillips and co-workers (21), thus leading to the *TbPRMT1* naming convention described above. Inactive enzyme paralogs, called pseudoenzymes, are common in eukaryotes. By some estimates, 10% of human enzyme domains are predicted to be catalytically inactive, and this estimate is even higher for worms and flies at 15% (40). The importance of these proteins is becoming increasingly apparent as pseudoenzymes are assigned biological functions. For example, iRhoms, inactive enzymes resembling rhomboid proteases, act in the endoplasmic reticulum to promote degradation of specific proteins or maturation of others (41). In another example, ornithine decarboxylase homologs regulate activity of ornithine decarboxylases by countering their inhibitory antienzymes (42). Nevertheless, the degree of activation observed in trypanosome enzyme-prozyme pairs is exceptional. It has been postulated that the dramatic control of enzyme activities via the enzyme-prozyme mechanism was expanded in trypanosomes in response to their lack of transcriptional control and the almost exclusive reliance on posttranscriptional and posttranslational mechanisms of gene regulation (43). The present study is the first report of an enzyme-prozyme complex in trypanosomes outside the polyamine synthesis pathway. Our data suggest that this type of control mechanism may indeed be amplified in these organisms and that additional examples of enzyme-prozyme pairs likely await discovery in trypanosomes.

Novel means of enzyme regulation stemming from the enzyme-prozyme mechanism have been reported. For example, the AdoMetDC prozyme is present in limiting amounts, and it is rapidly up-regulated when AdoMetDC activity is chemically inhibited, allowing increased flux through the pathway (33). This regulation takes place at the level of prozyme translation, apparently triggered by decarboxylated AdoMet (44). *TbPRMT1* is unlikely to be controlled by an analogous mechanism because the stabilities of *TbPRMT1*\textsuperscript{PRO} and *TbPRMT1*\textsuperscript{ENZ} proteins are mutually exclusive, similar to deoxyhypusine synthase and its prozyme (19). Intriguingly, however, *TbPRMT1*\textsuperscript{PRO}, but not *TbPRMT1*\textsuperscript{ENZ}, is reportedly phosphorylated (8, 45), bound to mRNA (46), and trafficked to stress granules in starved trypanosomes (47). This suggests that *TbPRMT1*\textsuperscript{PRO} could be, in certain situations, operating independently of *TbPRMT1*\textsuperscript{ENZ}, possibly as a homodimer (Fig. 6). An interesting observation made during our work was that, although *TbPRMT1*\textsuperscript{PRO} can be readily purified from *E. coli* in the absence of the enzyme, *TbPRMT1*\textsuperscript{ENZ} is notably unstable by itself and aggregates. This led us to postulate that *TbPRMT1*\textsuperscript{PRO} may act as a chaperone toward *TbPRMT1*\textsuperscript{ENZ}, and the mutual protein stability effect we observed could reflect different protein degradation mechanisms for the two subunits. In that case, sequestration of *TbPRMT1*\textsuperscript{PRO} into stress granules would efficiently abolish *TbPRMT1* activity for the duration of the stress and allow for a quick recovery once the cells enter a less hostile environment. Further research will focus on mechanisms of *TbPRMT1* regulation, allowing us to conclusively determine the advantages *T. brucei* gains from utilizing a heteromeric PRMT1.

The discovery of a heterotetrameric, prozyme-activated PRMT may also have relevance to methyltransferase organization and regulation in higher eukaryotes. The most striking parallel with the prozyme paradigm emerged from the work on the human RNA methyltransferase complex METTL3-METTL14. In this complex, METTL3 constitutes the catalytic core and binds AdoMet but requires allosteric activation and stabilization by METTL14 (48, 49). Although METTL14 has been reported to exhibit weak *in vivo* methylation activity (50), the phylogenetic analysis suggests that the METTL14 catalytic core has lost its function (51). In another example, mammalian PRMT7 and PRMT9 both harbor two catalytic modules in tandem, forming a pseudodimer. The data suggest that in both cases only the N-terminal PRMT module contains conserved residues and binds AdoMet, although the inactive module is necessary for the enzyme activity (52–55), which is somewhat reminiscent of activation of *TbPRMT1*\textsuperscript{ENZ} by *TbPRMT1*\textsuperscript{PRO}. In regard to possible PRMT multimerization, we show here that *TbPRMT1* forms a tetramer *in vitro* and sediments at a size corresponding to a tetramer in wild type cell lysate separated on a glycerol gradient. Based primarily on crystallographic studies, types I and III PRMTs are considered to function predominantly as homodimers with the exception of yeast PRMT1 (HMT1) (23, 27, 36, 37, 56, 57). However, two recent structural studies of human PRMT8 revealed a tetrameric enzyme bound to a single molecule of AdoMet per canonical dimer (31) or a possible octameric helical assembly (38). Furthermore, larger oligomers of type I PRMTs are often observed by both size exclusion chromatography and glycerol gradient fractionation, and some evidence suggests that the oligomerization may be necessary for PRMT activity (23, 28, 29, 38, 58, 59). Together, these findings imply that PRMT oligomerization may be more common than previously thought. Moreover, not only have PRMTs been shown to homooligomerize, but some studies support the possibility that PRMTs in mammals and plants have the ability to form heteromers. For example, mammalian PRMT2 can interact with PRMT1 both *in vivo* and *in vitro,*...
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Experimental Procedures

T. brucei Cell Culture and Generation of Cell Lines and Antibodies—TbPRMT1\textsuperscript{ENZ} and TbPRMT1\textsuperscript{PRO} doxycycline-inducible RNAi cell lines were generated as described (18). Briefly, plasmids were linearized using the NotI restriction site and electroporated into a BF single marker T. brucei strain (66) with the Amaxa Nucleofactor\textsuperscript{TM} system. Cells were cultured in HMI-11 medium supplemented with 10% FBS (67). Transformants were selected with 2.5 μg/ml phleomycin, and clones were obtained by limiting dilution. RNAi was induced for 4 days with 2.5 μg/ml doxycycline.

pLEW100-Myc-BirA\textsuperscript{a} vector (68) was a kind gift from Graham Warren (Max F. Perutz Laboratories, University of Vienna and Medical University of Vienna, Vienna, Austria). The complete open reading frame for TbPRMT1\textsuperscript{ENZ} was amplified using TbPRMT1\textsuperscript{ENZ} 5' AfII and TbPRMT1\textsuperscript{ENZ} BamHI 3' STOP primers (primers listed in Table 1) and cloned into the AflII and HindIII sites of pLEW100 (66) digested with HindIII/BamHI. 15 pmol of each primer used in the reaction described below was phosphorylated with T4 polynucleotide kinase (ThermoFisher) and ATP in manufacturer supplied buffer A for 30 min at 37 °C followed by inactivation for 10 min at 75 °C. Primer pairs were pooled and incubated at 94 °C for 1 min, 50 °C for 1 min, and 24 °C for 1 min to facilitate annealing. Primers 4A, 4B, 5A, 5B, 6A, and 6B were then pooled; the primer mixture (0.75 pmol of each primer) was ligated with 200 ng of restricted pLEW100 plasmid with T4 DNA ligase; and ligated plasmids were transformed into DH5-α E. coli. Linker was then added to the resulting plasmid by

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**List of primers**

| Primer name | Primer sequence |
|-------------|-----------------|
| PRMT1ENZ 5' | AGCTTCGAATTCGTCAGGATCCATGTTGAAGCC |
| PRMT1ENZ 3' | CCACACTGATCCGCTGGAGTCATCAAGTTGA |
| PRMT1PRO 5' | ATGGAATTCGTCAGGATCCATGTTGAAGCC |
| PRMT1PRO 3' | CCACACTGATCCGCTGGAGTCATCAAGTTGA |
| PRMT1ENZ BamHI 3 | AACACTACTTCTCGACACACAC |
| PRMT1ENZ 5 | GTACATATGATGACGGTGGACGCAAATG |
| PRMT1ENZ 3 | GTACTCGAGCTACCGCAGCCAAAATCCT |
| PRMT1PRO 5 | GTAAAGCTTTCAATACCTTTGGTAGTTGTACGTG |
| PRMT1PRO 3 | CCTGATGTTGGTTTCAGGACGGGAATCCTTTC |
| PRMT1ENZ 5 | GTAAAGCTTTCAATACCTTTGGTAGTTGTACGTG |
| PRMT1PRO 3 | CCTGATGTTGGTTTCAGGACGGGAATCCTTTC |
| PRMT1ENZ 5 | GTAAAGCTTTCAATACCTTTGGTAGTTGTACGTG |
| PRMT1PRO 3 | CCTGATGTTGGTTTCAGGACGGGAATCCTTTC |
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| PRMT1PRO 3 | CCTGATGTTGGTTTCAGGACGGGAATCCTTTC |
| PRMT1ENZ 5 | GTAAAGCTTTCAATACCTTTGGTAGTTGTACGTG |
| PRMT1PRO 3 | CCTGATGTTGGTTTCAGGACGGGAATCCTTTC |
Co-immunoprecipitation of PRMT1 Isoforms—The immunoprecipitation of TbPRMT1 

\[ \text{TbPRMT1}^{\text{ENZ}} \] with the tagged \( \text{TbPRMT1}^{\text{PRO}} \)-LSH was performed following a 4-day induction of \( \text{TbPRMT1}^{\text{PRO}} \)-LSH using 2.5 \( \mu \text{g/ml} \) tetracycline. Cells were pelleted and resuspended in 6.5 ml of PBS with 0.1\% Nonidet P-40, an EDTA-free protease inhibitor tablet (Roche Applied Science; one tablet/50 ml of lysis buffer), 50 \( \mu \text{g/ml} \) DNase I, and 1 \( \text{mm} \) CaCl₂. Cells were lysed for 20 min at 4 °C followed by centrifugation at 14,400 \( \times g \) for 15 min. The recovered supernatant was bound to TALON\textsuperscript{®} cobalt beads (Clontech) for 1 h at 4 °C. The flow-through was collected followed by washing with 50 ml of PBS. Bound \( \text{TbPRMT1}^{\text{PRO}} \) was then eluted with 150 \text{mM} imidazole. Eluted fractions were analyzed by Western blotting.

The reciprocal immunoprecipitation was performed using PF cells expressing Myc-BirA\textsuperscript* for expression followed the same protocol as described previously (14). Briefly, 1.2 \( \mu \text{m} \) total PRMT was mixed with 0.2 or 2 \( \mu \text{M} \) substrate and 0.7 \( \mu \text{M} \) \( [3\text{H}] \) AdoMet (55–85 Ci (2.03–3.15 TBq)/mmol; PerkinElmer Life Sciences) in PBS (pH 7.4) containing 1 \( \text{mM} \) PMSF in a final volume of 50 \( \mu \text{l} \). Reactions were carried out for \( \sim 18 \text{ h} \) at 22 °C. After separation by SDS-PAGE, the gel was incubated with EN\textsuperscript{3}HANCE solution (PerkinElmer Life Sciences) for 1 h, dried, and exposed to film at \( \sim 80 \text{ °C} \) overnight. The samples shown in Fig. 7C also contained 2 \( \mu \text{M} \) DTT, which led to substantial reduction of necessary exposure time from \( \sim 18 \text{ to } 1 \text{ h} \).

**Time Course of His-PRO/ENZ Complex Activity**—Under our experimental conditions, we showed that product formation from the His-PRO/ENZ complex was linearly dependent upon time for at least the 2-hour reaction time used for chromatographic analysis in Fig. 5B. Five PBS-based reactions containing 60 pmol of His-PRO/ENZ, 10 pmol of MBP-RRG substrate, and 1 \( \text{mM} \) PMSF were initiated by addition of 2 \( μ \text{Ci} \) of \( [3\text{H}] \) AdoMet (55–85 Ci (2.03–3.15 TBq)/mmol) during lysis and 15 \( \text{mM} \) imidazole throughout. Rat GST-PRMT1 and MBP-RRG substrate were expressed and purified as described previously (14, 70).

The protein used for light scattering was expressed from a pETDuet-1 vector with \( \text{TbPRMT1}^{\text{PRO}} \) in MCS1 (restriction sites Ncol and NotI) and \( \text{TbPRMT1}^{\text{ENZ}} \) in MCS2 (Ndel and Xhol). The vector contained a PreScission protease (GE Healthcare) cleavage site for His tag removal from \( \text{TbPRMT1}^{\text{PRO}} \). Protein expression followed the same protocol as described previously for \( \text{TbPRMT7}^{\text{PRO}} \) (36). Briefly, protein purification encompassed affinity chromatography on a nickel-nitrilotriacetic acid column (Qiagen), ion exchange chromatography on a HiTrap Heparin HP 5-ml column (GE Healthcare), and gel filtration on a HiLoad Superdex 200 16/60 column (GE Healthcare).

**In Vitro Methylation Assays and Methyalted Species Identification—In vitro** methylation assays were performed as described previously (14). Briefly, 1.2 \( \mu \text{m} \) total PRMT was mixed with 0.2 or 2 \( \mu \text{M} \) substrate and 0.7 \( \mu \text{M} \) \( [3\text{H}] \) AdoMet (55–85 Ci (2.03–3.15 TBq)/mmol; PerkinElmer Life Sciences) and then bound to 100 \( \mu \text{l} \) anti-Myc Sepharose beads (ICL, Inc.) for 3 h at 4 °C. The flow-through was collected, and the beads were rinsed with 20 ml of PBS with 0.1\% Tween 20. Bound \( \text{TbPRMT1}^{\text{PRO}} \) was then incubated with EN3HANCE solution for 1 h, dried, and exposed to film at \( \sim 80 \text{ °C} \) overnight. The samples shown in Fig. 5C also contained 2 \( \mu \text{M} \) DTT, which led to substantial reduction of necessary exposure time from \( \sim 18 \text{ to } 1 \text{ h} \).
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were analyzed by cation exchange chromatography in the presence of unlabeled ADMA, SDMA, and MMA standards.

AdoMet-PRMT UV Cross-linking—To cross-link PRMTs to [3H]AdoMet, 1.6 μM total PRMT was mixed with 1.4 μM [3H]-AdoMet (78 Ci/mmol) in 50 mM phosphate buffer (pH 7.4) in the presence of 5 mM DTT in a final volume of 50 μl and incubated at 4°C for ~18 h. Samples were then UV cross-linked for 10 min on ice 1 cm from the UV lamp using a UV sterilization solution for 1 h, dried, and exposed to film at ~80°C for 1 week.

To determine the specificity of UV cross-linking assay to AdoMet, 1.6 μM total PRMT was mixed with 1.4 μM [3H]-AdoMet in 50 mM phosphate buffer (pH 7.4) in the presence of 5 mM DTT and incubated at 22°C for ~10 min (50-μl reaction volume). Following initial incubation, a 50× excess of dATP or unlabeled AdoMet was added to control reactions. Samples were then incubated at 4°C for ~18 h. Samples were UV cross-linked for 10 min on ice 1 cm from the UV lamp using a UV Sterilinker 2400 (Stratagene). After separation of proteins by SDS-PAGE, the gel was Coomassie-stained, incubated with EN3HANCE reagent, dried, and exposed to film at ~80°C for 1 week.

Multiangle Light Scattering—Untagged TbPRMT1<sup>ENZ</sup>/PRO and TbPRMT1<sup>ENZ</sup>/PRO were characterized by multiangle light scattering following size exclusion chromatography. The identity of the proteins used for light scattering analysis was confirmed by mass spectrometry analysis. Protein at 50 μM (TbPRMT1<sup>ENZ</sup>/PRO) and 130 μM (TbPRMT1<sup>PRO</sup>/PRO) was injected onto a Superdex 200 10/300 GL size exclusion chromatography column equilibrated in a buffer containing 20 mM HEPES (pH 7.5), 200 mM NaCl, and 0.5 mM tris(2-carboxyethyl)phosphine. The chromatography system was connected in series with an 18-angle light scattering detector (DAWN HELEOS) and refractive index detector (OptilabEX) (Wyatt Technology). Data were collected every second at a flow rate of 0.15 ml/min at 25°C. Data analysis was carried out using the program ASTRA, yielding the molar mass and mass distribution (polydispersity) of the sample.

Quantitative RT-PCR—TbPRMT1<sup>ENZ</sup> and TbPRMT1<sup>PRO</sup> RNAi cell lines were grown for 4 days in the absence or presence of 2.5 (replicate 1) or 4 μg/ml (replicate 2) doxycycline. 250 ml of each cell culture was then harvested and resuspended in 1 ml of TRIZol reagent (Ambion). RNA was isolated according to the manufacturer’s instructions and subsequently re-extracted by an acidic phenol RNA purification procedure. 10 μg of RNA sample was DNase-treated using an Ambion DNA-free kit (Invitrogen) and re-extracted with acidic phenol. cDNA was synthesized using random hexamer primers with an iScript Select cDNA Synthesis kit (Bio-Rad) according to the manufacturer’s instructions. Levels of each PRMT mRNA were then assayed by qRT-PCR using qPCR1 TbPRMT1<sup>ENZ</sup> and qPCR1 TbPRMT1<sup>PRO</sup> primer pairs in the first replicate. Second replicate qPCR used primer pairs qPCR2 TbPRMT1<sup>ENZ</sup> and qPCR2 TbPRMT1<sup>PRO</sup>. Between samples, mRNA levels were normalized to the levels of β-tubulin mRNA.

Glycerol Gradient Sedimentation—5–20% glycerol gradients were prepared as follows. 5.5 ml of buffer A (20 mM HEPES (pH 7.9), 10 mM MgCl₂, 50 mM KCl, 1 mM EDTA, and 20% glycerol) was poured into an ultracentrifugation tube and frozen at ~80°C. 5.5 ml of buffer B (20 mM HEPES (pH 7.9), 10 mM MgCl₂, 50 mM KCl, 1 mM EDTA, and 5% glycerol) was layered on top of the frozen buffer A. The tubes then underwent four freeze/thaw cycles at ~80°C to create a linear glycerol gradient. 1 × 10<sup>7</sup> wild type 29-13 procyclic form T. bрюсеи cells per sample were harvested. The cell pellet was resuspended in 0.5 ml of lysis buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, an EDTA-free protease inhibitor tablet (one tablet/50 ml of lysis buffer), 50 μg/ml DNase I, and 1 mM CaCl₂). Cells were lysed for 20 min at 4°C by addition of Triton X-100 to a final concentration of 1% (v/v). Lysates were cleared by centrifugation for 15 min at 15,000 × g. Clear lysate was loaded on top of a 5–20% gradient and centrifuged at 32,000 rpm for 16 h in an SW41 Beckman rotor. Size standards consisting of proteins with known sedimentation coefficients were run on a parallel gradient (cytochrome c (1.9S), BSA (4.3S), yeast alcohol dehydrogenase (7.4S), catalase (11.3S), and thyroglobulin (19S)). 0.5-ml fractions were collected and probed with α-TbPRMT antibodies. Size standard fraction contents were visualized by Coomassie staining.

Author Contributions—L. K. R., L. K., S. G. C., and E. W. D. conceived the project. L. K., E. W. D., J. C. F., and K. J. carried out the experiments. All authors contributed to interpretation of the data. L. K. and L. K. R. wrote the manuscript, and S. G. C. and E. W. D. provided valuable support with the writing.

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