A continuous kinetic assay for protein and DNA methyltransferase enzymatic activities

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Abstract

Background: Methyltransferases (MTs) catalyze the S-adenosylmethionine (SAM)-dependent methylation of a wide variety of protein and DNA substrates. Methylation of lysine, arginine or cytosine regulates a variety of biological processes including transcriptional activation and gene silencing. Despite extensive studies of the cellular roles of MTs, their quantitative kinetic characterization remains challenging. In the past decade, several assays have been developed to monitor methyl transfer activity utilizing different approaches including radiolabeling, antibodies or mass-spectrometry analysis. However, each approach suffers from different limitation and no easy continuous assay for detection of MT activity exists.

Results: We have developed a continuous coupled assay for the general detection of MTs activity. In this assay, the formation of S-adenosylhomocysteine (SAH) product is coupled NAD(P)H oxidation through three enzyme reactions including glutamate dehydrogenase leading to absorbance changes at 340 nm. The utility and versatility of this assay is demonstrated for SET7/9 and SETD6 with peptides and full length protein substrates and for M.Hae III with a DNA substrate.

Conclusions: This study shows a simple and robust assay for the continuous monitoring of MT enzymatic activity. This assay can be used for the determination of steady-state kinetic enzymatic parameters (e.g., $k_{cat}$ and $K_M$) for a wide variety of MTs and can be easily adapted for high-throughput detection of MT activity for various applications.

Background

Methylation is a common covalent modification of nucleic acids and proteins. Recently, methylation has emerged as a prominent posttranslational modification of proteins regulating diverse cellular signaling pathways that influence cell survival, growth, and proliferation. Disruption of methylation is thought to fundamentally impact the initiation and progression of many biological processes, leading to altered cellular phenotypes and the development of diseases [1–4]. Methylation of lysine residues in target proteins is performed by protein lysine (K) methyltransferases (PKMTs) [5, 6]. Currently, there are over 60 candidates and known members of this enzyme family, the vast majority of which contain a conserved SET domain that is responsible for the enzymatic activity [5, 6]. Methylation also takes place on genomic DNA on cytosine bases in CpG dinucleotide repeats [7]. These regions of the genome are enriched in transcriptionally repressed chromatin and methylation mediates epigenetic silencing within these domains [8]. Thus, DNA methylation was shown to play a crucial role in the epigenetic control of gene expression [9].

All methyltransferases (MTs) utilize the S-adenosylmethionine (SAM) as a universal methyl donor leading to the generation of S-adenosylhomocysteine (SAH) following methylation. Due to the central biological roles of MTs, it is important to biochemically characterize and quantitatively measure their catalytic activity. Previous efforts for the development of quantitative assays for MTs activity rely on the detection of methylated product or the formation of SAH [10, 11]. Radioactive assays utilizing $^3$H-SAM are considered the most sensitive and reliable but require chromatographic separation and thus tend to be slow and labor intensive [12]. Recently, more advanced and rapid radioactive assays were developed...
to allow the detection of many MTs reactions in parallel [13–16]. More recently, fluorescent [17], antibody-based immunoassays or reading domain-based assays [18–20] for the detection of methylated lysine, arginine and cytosine were developed utilizing enzyme-linked immuno- sorbent assay (ELISA) or fluorescence resonance energy transfer (FRET) [21–23]. These assays based on fluorescence or absorbance are highly sensitive but are not general for the detection of a wide range of MT activities because a specific antibody for each substrate and/or type of modification must be used.

Assays for the detection of SAH [24–26] have the advantage of providing a general detection method for MTs regardless of the protein or DNA acceptor. Previously, the accumulation of SAH product at high concentration was shown to inhibit most MTs activity [27, 28]. The coupled assay can overcome this problem by preventing the accumulation of the SAH following methyl transfer reaction to hypoxanthine formation that is monitored at 265 nm [24]. However, measuring MTs activity at 265 nm is highly problematic due to the high absorbance of proteins at 280 nm and the inability to utilize standard cuvettes or multi-well plates due to their high absorbance at this wavelength. To overcome these limitations, we have coupled the activity of SAHN and ADE with glutamate dehydrogenase that utilizes ammonia and α-ketoglutarate to generate glutamate while oxidizing NADPH. The coupling of this reaction to MTs activity allows monitoring the continuous change in absorbance at 340 nm due to NADPH oxidation (Fig. 1) which linearly correlates with the reduction of the SAM concentration.

To establish the continuous coupled assay for MTs activity, we initially utilized the PKMTs SET7/9 as a model enzyme. Previous works have shown that SET7/9 exhibits broad substrate specificity catalyzing methyl transfer to a variety of histone and non-histone proteins including H3, TAF10, TAT, RelA, p53 and FoxO3 [5, 32]. Methylation of these proteins by SET7/9 was shown to regulate protein–protein and protein–DNA interactions thus modulating the target protein activity [5, 33]. We initially utilized our coupled assay to monitor SET7/9 activity with a peptide substrate derived from the HIV trans-activator TAT protein. SET7/9 was previously shown to monomethylate TAT protein at K51, thus, a peptide was designed to include the K51 acceptor residue (see peptide sequence below), the rate of NADPH oxidation reflects the rate of methylation. We have shown that this assay is highly versatile allowing the quantitative detection of MTs activity toward peptide or protein substrates and for the detection of DNA MTs activity. This coupled assay permits the Michaelis–Menten (MM) analysis of MTs catalytic activity for mechanistic studies and is readily adaptable for high-throughput screening for the discovery of novel MTs inhibitors.

Results

Development of a coupled continuous assay for MTs

To establish a continuous assay for MTs activity that monitors SAH formation, the transfer of a methyl group to peptide, protein or DNA must be coupled to additional reactions that lead to measurable change in absorbance/fluorescence. Previous work has utilized SAHN and ADE for the conversion of SAH to hypoxanthine and ammonia [24]. These two coupled reactions were shown to be non-rate limiting enabling the efficient coupling of methyl transfer reaction to hypoxanthine formation that is monitored at 265 nm [24]. However, measuring MTs activity at 265 nm is highly problematic due to the high absorbance of proteins at 280 nm and the inability to utilize standard cuvettes or multi-well plates due to their high absorbance at this wavelength. To overcome these limitations, we have coupled the activity of SAHN and ADE with glutamate dehydrogenase that utilizes ammonia and α-ketoglutarate to generate glutamate while oxidizing NADPH. The coupling of this reaction to MTs activity allows monitoring the continuous change in absorbance at 340 nm due to NADPH oxidation (Fig. 1) which linearly correlates with the reduction of the SAM concentration.

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very low activity was observed in accordance with previous report [34] (Fig. 2a). To further verify that SET7/9 is the rate-determining step in our kinetic measurements, we examined the catalytic rate of each step in the coupled reaction independently by monitoring NADPH oxidation. We found that under our reaction mixture (see “Methods” for details) the three coupling enzymes are significantly faster than SET7/9 activity (Fig. 2b). We have also shown that increasing in SET7/9 enzyme concentration is directly proportional to the increase in catalytic activity (Fig. 2c). To further verify that the MT reaction is the rate-determining step, we performed the
reaction with doubled amount of each of the coupling enzymes (ADE, SAHN and glutamate dehydrogenase) and observed no increase in methylation rate (Additional file 1: Figure S2). Next, we examined the sensitivity of our assay by determining NADPH oxidation at limited SAH concentrations. Using this assay, we were able to detect a concentration of 170 nM of SAH highlighting that a transfer of 170 nM of methyl group to a peptide/protein substrate is detectable in our system (Additional file 1: Figure S3). Overall, these controls ensure that the change in absorbance at 340 nm reflects the true measurement of SET7/9 methylation activity and provides a wide dynamic range for activity measurements at different conditions (e.g., substrate concentrations see below).

**Measurements of SET7/9 and SETD6 activity with peptide and protein substrates**

To examine whether our coupled kinetic assay can be utilized for the MM kinetic analysis of SET7/9 activity, we have utilized a peptide derived from the FoxO3 protein. Previous work has shown that SET7/9 methylates FoxO3 at K271 modulating its transcriptional activity and stability [35]. Thus, we have designed a 15 amino acid peptide substrate derived from FoxO3 containing K271 to quantitatively measure SET7/9 catalytic activity (see peptide sequence in the “Methods” section). Using the coupled assay, we measured the initial rates of SET7/9 activity (5 μM) at different peptide substrate concentrations ranging from 0 to 700 μM (Fig. 3a). As expected, we observed a gradual increase in initial rates that is correlated with the increase in FoxO3 peptide concentration. Fitting the initial rate data to the MM equation allowed us to derive kinetic parameters for the activity of SET7/9 with the FoxO3 peptide (Fig. 3b). We found that the $K_M$ and $k_{cat}$ parameters for this activity are $165.4 \pm 20.2 \mu M$ and $32 \pm 0.023 \text{ min}^{-1}$, respectively. The values are in excellent correlation with previous analysis of SET7/9 activity with H3K4 and DNMT1K142 utilizing radioactive MT assay [36]. The previously measured $K_M$ and $k_{cat}$ for H3K4 and DNMT1K142 are 143 and 134 μM and 48 and 42 min$^{-1}$, respectively [36]. This correlation provides strong support for our continuous assay and confirms that the FoxO3 peptide is a substrate for SET7/9 [35].
To further examine whether the coupled assay can monitor MTs activity with full length protein substrates, we examined the activity of SETD6 with RelA protein (residues 1–431 [37, 38]). Previously, SETD6 was shown to methylate RelA on K310 leading to a dramatic modulation of NFκB transcriptional activity [38]. To measure SETD6 activity with RelA protein, we utilized RelA concentrations of up to 3 μM (Fig. 4a). Analysis of SETD6 activity at these RelA concentrations shows a linear increase in reaction rate with a slope of $k_{\text{cat}}/K_M$ of $3.2 \times 10^5 \text{s}^{-1} \text{M}^{-1}$ (Fig. 4b). These results demonstrate that our assay can be utilized to examine MTs activity with full length proteins as substrates, paving the way for additional studies to examine MTs activity with natural protein substrates. To further validate that the coupling enzymes utilized in the MTs kinetic assay do not act as substrates for methylation, we utilized radioactive
[3H]-SAM as part of the SETD6 RelA coupled assay. The utilization of [3H]-SAM in the assay allows monitoring methylation of each protein in the reaction mixture using SDS-PAGE followed by autoradiography. We found that while RelA serves as a substrate for SETD6 [38], the SAH and ADE coupling enzymes do not and thus no significant background is observed in the absence of substrate (Figs. 1, 4c).

**Measurements of M.HaeIII methyl transfer activity with DNA substrate**

To further examine the versatility of the assay for the general monitoring of MTs activity, we examined the methylation activity of M.HaeIII from *Haemophilus aegyptius* with DNA substrate. M.HaeIII belongs to a large family of bacterial DNA MTs that catalyses cytosine C5 DNA methylation [39]. M.HaeIII methylates the internal cytosine of the canonical sequence GGCC and is utilized in the restriction-modification bacterial defense system against phage infection. To examine M.HaeIII activity using our coupled assay, we first prepared DNA substrate by PCR amplification of 1.6 kb DNA fragment from pGex plasmid containing six predicted methylation sites for M.HaeIII (Fig. 5a). We utilized the coupled assay to monitor M.HaeIII activity with different DNA concentrations and measured the initial reaction rates. We observed an increase in the initial rate of M.HaeIII catalysed DNA methylation at increased DNA substrate concentrations, demonstrating our ability to measure the kinetics of MTs with DNA substrates (Fig. 5b). To verify that M.HaeIII methylates DNA under the coupled assay conditions, we performed NotI digestion analysis. Our DNA substrate contains one NotI cleavage site (GGGCGCGC) that is located 1 kb from the 5′ of the DNA. Methylation of this site will prevent NotI cleavage leading to intact 1.6 kb substrate even in the presence of NotI. Indeed, we found that methylation of the DNA substrate by M.HaeIII in the presence of all coupled assay components prevents NotI cleavage leading to the presence of undigested 1.6 kb fragment (Fig. 5c). These results highlight the utility of the coupled assay to efficiently monitor MTs activity with DNA substrates paving the way for quantitative analysis of many DNA methylation enzymes and sites.

**Discussion**

In the past decade, MTs have attracted significant attention due to their roles in regulating central biological processes and their association with diseases [1–4]. Despite extensive investigations of the cellular roles of MTs, much less is known about their biochemical functions, substrate recognition and catalytic mechanism. The establishment of biochemical assay to readily monitor MTs activity will greatly facilitate such mechanistic research. Previously, several groups have attempted to establish biochemical assays for monitoring MTs activity; however, each approach suffers for different draw backs (see “Background” section).
Our assay is based on monitoring SAH formation by three-enzyme coupled reaction leading to the formation of ammonia and the oxidation of NADPH. Utilization of the different enzymes raises the concern that the enzyme used for the coupled reactions can serve as substrates for MTs. Utilizing a radioactive assay, we have shown that SETD6 does not methylate any of the coupling enzymes (Fig. 4c). Previously, the coupling of ammonia formation with α-ketoglutarate activity and NADPH oxidation was utilized for the establishment of Sirt1 deacetylase activity [40, 41]. In addition, the coupling of enzymatic transfer reactions to NADPH oxidation was extensively utilized for the detection of a wide range of glycosyltransferases for the quantitative determination of their catalytic activities [42]. To monitor glycosyltransferase activity, dinucleotides (e.g., UDP, or GDP) generated following sugar transfer activity are coupled to NADH oxidation by pyruvate kinase and lactate dehydrogenase [42, 43]. Thus, coupling different enzymatic reactions to glutamate dehydrogenase activity, enabling monitoring these reactions at 340 nm, is a common tool for enzymatic activity determination.

Our coupled assay allows the continuous monitoring of MTs activity (Fig. 1). We believe that this assay can be used as a fast, convenient and inexpensive approach for the detailed biochemical characterization of a wide variety of MTs. Since the assay can be performed in a multi-well plate format, it can be readily adapted for high-throughput screening of MTs activity. Such screening can be highly useful in the search for new MTs inhibitors or activators. Since the coupling of three enzymes is required to monitor MTs activity, appropriate controls must be taken to ensure the specificity of the new inhibitors to the target methyltransferase.

Conclusion
In conclusion, we have developed a rapid, continuous three-enzyme coupled UV absorption assay for the characterization of enzymes that use SAM to catalyze methyl transfer reactions. We have shown the versatility and robustness of this assay in monitoring the activity of two different protein methyl transferases SET7/9 and SETD6 utilizing peptide and protein substrates, respectively. In addition, we showed that the coupled assay can be utilized for monitoring DNA methylation kinetics using M.HaeIII as a model enzyme.

Methods
Molecular biology
SET7/9 gene was cloned into pET-Duet plasmid for E. coli expression and purification. The SET7/9 gene was PCR amplified from pGex-6p1 plasmid containing the gene as a template. The amplified DNA fragment was then cleaved by SpeI and XhoI restriction enzymes and ligated into a pET-Duet plasmid containing a maltose binding domain (MBP) tag. The adenosine deaminase (ADE, E.C. 3.5.4.4) and S-adenosyl-l-homocysteine nucleosidase (SAHN, E.C. 3.2.2.9) genes were amplified from the genomic DNA of an XL1 blue E. Coli strain using PCR. The amplified DNA fragments were then cleaved using NheI and HindIII restriction enzymes and ligated into a pET28a plasmid containing a Hisx6 tag at the N-terminal of the protein.

Protein expression and purification
SET7/9 enzyme was expressed from a pET-Duet plasmid containing the SET7/9 gene fused to a MBP tag in E. Coli BL21 (DE3). Expression was induced using 0.5 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) for 6 h at 30 °C. Following inductions, cells were centrifuged and resuspended at a buffer containing 25 mM Tris–HCL (pH 7.5), 200 mM NaCl and 1 mM DTT. The cells were lysed using a French press and the resulting cell extract was centrifuged at 13,000g for 1 h. The SET7/9 protein was then purified from the clear lysate using amylose beads (Amersham) according to standard procedures. The MBP tag was then cleaved using TEV protease for 4 h at 4 °C and purified using gel-filtration chromatography to obtain a monomeric SET7/9 protein. GST-SETD6 was expressed and purified as previously described [38].

The RelA protein (residues 1–431) was expressed using a pGEX-6p1 plasmid containing the RelA gene fused to a GST tag in E. coli BL21 (DE3). Expression was induced with 0.5 mM IPTG for 16 h at 20 °C. Following inductions, cells were centrifuged and resuspended in buffer containing 25 mM Tris–HCL (pH 7.5), 200 mM NaCl and 1 mM DTT. The cells were lysed using a French press and the cell extract was centrifuged at 13,000g for 1 h. The RelA protein was then purified using glutathione beads (Amersham) according to standard procedures.

The ADE enzyme was expressed from pET28a plasmid containing the ADE gene fused to a 6xHis tag at the N-terminal in E.coli BL21 (DE3). Expression was induced with 0.5 mM IPTG for 16 h at 20 °C. To replace the Fe2+ metal at the ADE active site with Mn2+, 50 μM of 2,2′-dipyridyl and 1.0 mM MnCl2 were added at time of the induction [44]. Following induction, cells were centrifuged and resuspended at a buffer containing 25 mM Tris–HCL (pH 7.5), 200 mM NaCl and 1 mM DTT. The cells were lysed using French press and the cell extract was centrifuged at 13,000g for 1 h. The ADE protein was then purified using nickel-NTA beads using standard procedures. Following purification, a dialysis against the original buffer was performed to remove the imidazole.
The SAHN protein was purified using the same purification protocol without the metal replacement procedure performed for the ADE purification. For DNA methylation assays, commercial M.HaeIII enzyme was purchased from NEB.

**Methylation assays**

The continuous coupled methylation assay was carried out with clear flat bottom 96 well plates, containing 4.5 µM SAHN, 3 µM ADE, 2.62 units of glutamate dehydrogenase (Ammonia detection kit, Sigma), 300 µM SAM and a varying concentration of methyltransferase enzyme and methyl acceptor. A concentration of 300 µM SAM was used to ensure saturation of the methyl donor. A final volume of 250 µl was reached in the well using the ammonia assay kit buffer (Sigma). The assay was performed at 30 °C, and the reaction was monitored at 340 nm using Tecan Infinite M200 plate reader. Kinetic parameters were derived by fitting to Michaelis–Menten $V_o = \frac{k_{cat}[E][S]}{[S] + K_M}$ model. The TAT and FoxO3 peptide substrate sequences are AALQTA (residues 264–277), respectively (methyl donor). The detection of MTs using short peptide as a substrate a. The detection of MTs activity is dependent on all assay components. The reaction cannot be monitored in the absence of glutamate dehydrogenase (orange) or adenine deaminase (ADE, grey). Activity is measured only when all reaction components are present including the peptide substrate (TAT peptide as an example, blue). Figure S2. Methyltransferase activity is the rate-determining step of NADPH oxidation. Doubling the concentration of each component in the total methyltransferase reaction does not lead to increase in methylation rate (A) SET7/9 (5 µM) activity with TAT peptide (500 µM) at 3 µM (blue) or 6 µM (orange) of adenine deaminase (ADE), 2.62 units (blue) or 5.24 units (orange) of glutamate dehydrogenase and 4.5 µM (blue) or 9 µM (orange) of SAH nucleosidase. (B) ADE activity with 3 µM (blue) or 6 µM (orange) of the enzyme at 150 µM concentration of adenine (C) Glutamate dehydrogenase activity with 2.62 units (blue) or 5.24 units (orange) of the enzyme at 30 µM NH₄⁺ concentration. (D) SAH nucleosidase activity 4.5 µM (blue) or 9 µM (orange) with 100 µM SAH concentration. Figure S3. Monitoring of SAHN activity at limiting SAH (Sigma) concentrations using the coupled assay. Activity was detected using SAHN 5 µM, ADE 3.5 µM and 2.62 units of glutamate dehydrogenase in the presence of 300 µM SAM by monitoring changes at 340 nm. Figure S4. Raw measurement of MT activity coupled with NADPH oxidation. A. The rate of absorbance decrease at 340 nm reflects the rate of SET7/9 activity with the TAT peptide (see also Figure S1 and Figure 2 main paper). B. Absolute absorbance values are transformed to change in absorbance at 340 nm and the values of 1-absorbance change are shown to highlight the rate of methylation. Figure S5. SET7/9 activity with different FoxO3 peptide concentrations in a defined reaction conditions including Tris 25 mM pH 7.5, bovine serum albumin (BSA) 0.5 % (v/v), 300 mM SAM, α-ketoglutarate 5 mM, NADPH 0.5 mM, SAH 5 µM, ADE 3.5 µM and 2.62 units of glutamate dehydrogenase. Activity increases at increased peptide concentration.

**Radioactive in vitro methylation assay**

Assays were performed as previously described [38]. Briefly, recombinant proteins were incubated with recombinant SETD6, and 2 mCi ³H-SAM (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) in methylation buffer [50 mM Tris–HCL (pH 8.0), 10 % glycerol, 20 mM KCl, 5 mM MgCl₂ and 1 mM PMSF] at 30 °C overnight. The reaction mixture was resolved by SDS-PAGE, followed by either autoradiography or Coomassie blue stain.

**Additional file**

**Additional file 1: Figure S1.** Establishment of the continuous coupled assay for MTs using short peptide as a substrate a. The detection of MTs activity is dependent on all assay components. The reaction cannot be monitored in the absence of glutamate dehydrogenase (orange) or adenine deaminase (ADE, grey). Activity is measured only when all reaction components are present including the peptide substrate (TAT peptide as an example, blue). Figure S2. Methyltransferase activity is the rate-determining step of NADPH oxidation. Doubling the concentration of each component in the total methyltransferase reaction does not lead to increase in methylation rate (A) SET7/9 (5 µM) activity with TAT peptide (500 µM) at 3 µM (blue) or 6 µM (orange) of adenine deaminase (ADE), 2.62 units (blue) or 5.24 units (orange) of glutamate dehydrogenase and 4.5 µM (blue) or 9 µM (orange) of SAH nucleosidase. (B) ADE activity with 3 µM (blue) or 6 µM (orange) of the enzyme at 150 µM concentration of adenine (C) Glutamate dehydrogenase activity with 2.62 units (blue) or 5.24 units (orange) of the enzyme at 30 µM NH₄⁺ concentration. (D) SAH nucleosidase activity 4.5 µM (blue) or 9 µM (orange) with 100 µM SAH concentration. Figure S3. Monitoring of SAHN activity at limiting SAH (Sigma) concentrations using the coupled assay. Activity was detected using SAHN 5 µM, ADE 3.5 µM and 2.62 units of glutamate dehydrogenase in the presence of 300 µM SAM by monitoring changes at 340 nm. Figure S4. Raw measurement of MT activity coupled with NADPH oxidation. A. The rate of absorbance decrease at 340 nm reflects the rate of SET7/9 activity with the TAT peptide (see also Figure S1 and Figure 2 main paper). B. Absolute absorbance values are transformed to change in absorbance at 340 nm and the values of 1-absorbance change are shown to highlight the rate of methylation. Figure S5. SET7/9 activity with different FoxO3 peptide concentrations in a defined reaction conditions including Tris 25 mM pH 7.5, bovine serum albumin (BSA) 0.5 % (v/v), 300 mM SAM, α-ketoglutarate 5 mM, NADPH 0.5 mM, SAH 5 µM, ADE 3.5 µM and 2.62 units of glutamate dehydrogenase. Activity increases at increased peptide concentration.

**Abbreviations**

MT: methyltransferase; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; ADE: adenine deaminase; GST: glutathione S-transferase; MBP: maltose binding domain.

**Authors’ contributions**

SD, DL and AA conceived and designed the experiments. SD performed the experiments. ZV performed the radioactive in vitro methylation assay. All authors read and approved the final manuscript.

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**Competing interests**

A provisional patent application covering the use of the method is in preparation by the National Institute for Biotechnology in the Negev at Ben-Gurion University.

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