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Avidin plate assay system for enzymatic characterization of a histone lysine methyltransferase

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Abstract

Modification of proteins by protein methyltransferases has several important biological functions. Here, we study the methylation of histone H3 tail at position Lys9 by the Dim-5 histone lysine methyltransferase, which is involved in epigenetic signaling and gene silencing and which triggers DNA methylation in *Neurospora crassa*. We have developed a new assay to detect protein methylation using a biotinylated synthetic peptide substrate and a radioactively labeled coenzyme. We show that the assay is linear with respect to time and enzyme concentration (under multiple turnover conditions) and that its background is very low. Data points were reproducible within 3%. At least 200 pmol of biotinylated peptide is bound completely to the microplate. We employed the assay system to determine the K_m and k_{cat} values of the Dim-5 enzyme for the methylation of a 20mer peptide to be 7.4 μM and 2.3 min^{-1} , respectively. In addition, we determined the activity of four Dim-5 variants, ranging from full activity to less than 1% of residual activity. The microplate biotin/avidin peptide methylation assay developed here is convenient, very accurate, reproducible, and inexpensive. Because it yields quantitative results, it can be employed for a characterization of the enzymatic properties of histone lysine methyltransferases and other protein methyltransferases. The assay also is well suited for high-throughput applications.

Keywords

Protein methyltransferase; Peptide methylation assay; H3K9 methyltransferase; Avidin-biotin; Dim-5

Methylation of proteins occurs at different amino acids such as Asp, Glu, His, Lys, and Arg [1]. It has a variety of biological functions. First, methylation of glutamate residues by the CheR methyltransferase (MTase)¹ is involved in bacterial chemotaxis (for a review, see [2]). Second, protein aspartyl MTases function as protein repair enzymes to convert isoaspartyl residues formed during protein aging back into the aspartyl configuration (for a review, see [3]). Third, protein arginine methylation is involved in protein–RNA interaction, protein–protein interaction, protein sorting, transcription, and signaling (for reviews, see [4,5]). Fourth, methylation of Arg and Lys residues in the N-terminal tails of histone also is an important epigenetic signal involved in many biological processes (for reviews, see [6,7]).

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¹Abbreviations used: MTase, methyltransferase; HKMT, histone lysine methyltransferase; EIA, enzyme immunoassay; RIA, radioimmunoassay; DTT, dithiothreitol; BSA, bovine serum albumin; TCA, trichloroacetic acid.

Histone tails are methylated by specific MTases, the histone lysine methyltransferases (HKMTs) that are the subject of this work. Known targets for HKMTs are histone H3 positions Lys4, -9, -27, -36, and -79 and histone H4 position Lys20. The Dim-5 enzyme from *Neurospora crassa* is one example of an HKMT with specificity for histone H3 Lys9. The enzyme processively introduces three methyl groups to the target lysine residue [8]. Trimethylated histone H3 Lys9 is a mark for repressed heterochromatin [9] and has been shown to trigger DNA methylation in some species, including *N. crassa* [10–14]. In *N. crassa*, the HP1 protein binds to methylated histone H3 Lys9 and serves as an adaptor between the histone methylation and DNA methylation machinery [15,16]. The structure of Dim-5 is known in the presence and absence of peptide substrate [8,17]. It consists of two domains: SET and pre-SET. The SET domain is conserved among the family of HKMTs and harbors the enzyme's active site. The pre-SET domain contains a triangular Zn₃Cys₉ zinc cluster. Histone MTases are active participants in epigenetic signaling and, thus, are interesting objects for enzymatic investigation. Here, we describe the design and validation of a new peptide methylation assay that is inexpensive, convenient, and robust and that provides quantitative data that are highly accurate and reproducible.

Materials and methods

Dim-5 was expressed and purified as described previously [17]. The design and generation of Dim-5 variants is described in [8]. A synthetic peptide containing a biotin at its N terminus was purchased from IRIS Biotech (Marktredwitz, Germany) in HPLC-purified form and was dissolved in water. Purity of the peptide was greater than 95%, as confirmed by HPLC and mass spectrometric analysis. The length of the peptide was 20 amino acid residues, corresponding to the first 19 amino acids of histone H3 tail plus a methionine (Bt-MARTKQTARKSTGGKAPRKQ), with the target lysine residue underlined.

Coating microplates (enzyme immunoassay (EIA)/radioimmunoassay (RIA) high binding, Corning, Corning, NY) with avidin was performed as described previously [18]. Briefly, 1 µg avidin (Sigma, St. Louis, MO, USA) was dissolved in 100 µl of 100 mM NaHCO₃ (pH 9.6), pipetted into each well, and incubated overnight at 4°C (coated plates can be stored for several days at 4°C). The wells were washed five times with 200 µl PBST buffer (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM K₂HPO₄, 0.05% (v/v) Tween 50, pH 7.2) before use.

Methylation reactions were carried out in methylation buffer (50 mM glycine (pH 9.8), 2 mM dithiothreitol (DTT), 25 µg/ml bovine serum albumin (BSA), 10% glycerol) at ambient temperature in the presence of 0.35 µM labeled [methyl-³H]AdoMet (2.93 × 10¹⁵ Bq/mol, NEN Life Sciences, Boston, MA, USA). Aliquots (2 µl) were transferred from the reaction mixture to the wells of an avidin-coated microplate containing 50 µl PBST supplemented with 5 mM unlabeled AdoMet (Sigma) to stop the incorporation of radioactivity. The mixture was incubated for 30 min to allow binding of the biotinylated peptide to the microplate. The wells were washed five times with 200 µl PBST to remove the unreacted Ado-Met and the enzyme. Complete removal of the MTase is important because unreacted AdoMet could bind to the protein and thereby be retained. Subsequently, the bound peptide was eluted by incubation with 50 mM HCl at ambient temperature for 30 min. The released radioactivity was analyzed by liquid scintillation counting of the reaction mixture after adding 2 ml liquid scintillator Rotiszint Ecoplus (Roth, Karlsruhe, Germany). Usually, average values of duplicate measurements of each data point were used. To avoid degradation, labeled and unlabeled AdoMet was stored in small aliquots in 10 mM H₂SO₄ at -20 °C and was thawed only once.

Results and discussion

Analysis of DNA methylation using a microplate assay and biotinylated oligonucleotide substrates was introduced some years ago [18]. Here, we report the adaptation of this assay to detect methylation of peptides by protein MTases (Fig. 1). Our system employs as substrate a synthetic peptide that carries a biotin group on its N-terminal end. Methylation of the peptide was performed in solution in the presence of [methyl-³H]AdoMet, whose methyl group was radioactively labeled. During the methyl transfer reaction, the radioactivity was transferred to the peptide. For separation of the radioactively labeled product and free coenzyme, an avidin-coated microplate was used. After washing, the bound peptide was released and radioactivity was quantified by scintillation counting. To elute the radioactivity, diluted hydrochloric acid was used, disrupting the avidin–biotin interaction. We tested elution of methylated peptides using a range of concentrations of HCl and observed a broad plateau of eluted activity between 10 and 100 mM HCl. Lower amounts of radioactivity were released at lower and higher concentrations, most likely due to incomplete disruption of the biotin–avidin interaction at lower concentrations and to interference of high concentrations of HCl with the liquid scintillation process.

Assay validation

To validate the assay, we investigated the linearity of the radioactive signal with respect to variation of the incubation time and enzyme concentration. Fig. 2A illustrates that linear time courses were obtained for up to 10 min. Decreasing the relative amounts of enzyme to substrate prolonged the linear phase (data not shown). To determine the background of the assay (corresponding to incomplete removal of unreacted [methyl-³H] AdoMet), labeled AdoMet and biotinylated peptide substrate were incubated in the absence of Dim-5 and the sample was processed as described. We detected very low levels of background (Fig. 2A), with repeated experiments yielding background levels of 28 ± 6 cpm. Experiments using Dim-5 enzyme and labeled AdoMet without peptide did not show any detectable activity above background radiation (data not shown).

Under multiple turnover conditions, the rate of an enzyme reaction should be proportional to the concentration of the enzyme in the reaction mixture. To test for this requirement, we performed methylation kinetics using different amounts of Dim-5. Time courses were analyzed by linear regression of the linear phase of the reaction, and the slopes of the regression lines were compared. As shown in Fig. 2B, the slopes obtained were strictly correlated to the concentration of the enzyme in the assay.

Binding capacity of the microplate

For quantitative analyses, it is important that the peptide is completely bound to the microplate. Therefore, we determined the capacity of the microplate for our peptide. To this end, peptide was methylated at high concentration (45 μ M), and different volumes of methylated peptide were applied to the plate, washed, and eluted. As shown in Fig. 2C, a linear relation between peptide concentration and radioactivity was observed if less than 100 pmol of peptide was loaded onto the plate. In our experiments, typically 1–5 pmol per well was used, thereby ensuring a complete binding of the peptide to the plate.

Accuracy of the assay

To estimate the accuracy of the assay, the deviations of two individual measurements for each time point were calculated. In several experiments, this deviation was found to be within 3–10%. For subsequent analyses, the averages of the two duplicate points were used. These numbers were highly accurate, as shown in Figs. 2A and B. In both cases, the average deviation of the values of data points in the linear part of the curve from the linear regression line was

3%. In additional experiments, similar degrees of fluctuations were observed (data not shown), demonstrating that the assay is highly accurate and reproducible.

Michaelis–Menten analysis of kinetic data

To determine the K_m and k_{cat} values of Dim-5 for the substrate peptide, methylation kinetics were determined at different concentrations of peptide ranging from 0.2 to 6 μM . The initial points of time courses of the reactions were analyzed by linear regression, and the initial slopes were fitted to the Michaelis–Menten model (Fig. 3), yielding a K_m value of 7.4 μM and a k_{cat} value of 2.3 min^{-1} . This K_m value is similar to K_d values reported for the Set7/9 MTase interacting with a substrate peptide [19].

Analysis of the activity of Dim-5 variants

We purified and analyzed the following variants of Dim-5: Y178F, Y178V, F281Y, and F281W. All mutants showed catalytic activity, although the methylation rates observed with the Y178F variants were low (Fig. 4). The activities determined here generally agree with results reported previously using full histone H3 as substrate [8]. The slightly higher activities of the Y178V and F281W observed here could be due to the change in substrate or different assay conditions.

Conclusions

Different assays are used to analyze the enzymatic activities of protein MTases on peptide substrates in vitro, including assays using labeled coenzyme and assays based on direct detection of the modification in the product, for example, by mass spectrometry, peptide sequencing, or antibody detection [8,17]. Labeled coenzyme can be separated from methylated product by filtration or precipitation techniques [19,20]. However, all of these assays have certain disadvantages. First, radioactive filter assays using trichloroacetic acid (TCA) precipitation have a relatively high background and low sensitivity. Second, antibody-based assays are not very quantitative. Third, mass spectrometric assays also have problems in quantitation. Here we have established an alternative in vitro assay system that allows studying methylation of peptides. The assay is sensitive, convenient, inexpensive, and suitable for high throughput and automation. It provides quantitative data that are highly reproducible and accurate. We applied this assay to determine the Michaelis–Menten parameters of Dim-5 and to characterize the activities of four Dim-5 variants.

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Fig. 1.
Schematic drawing of the principal steps of the peptide methylation assay developed here.

Fig. 2.

Validation of peptide methylation assay. (A) Time course of methylation of substrate peptide (2.25 μ M) by Dim-5 (4 nM) (diamonds), demonstrating that the kinetics are linear with respect to time for at least 10 min. The straight line shows a linear regression of the first five data points. The gray squares show results of an identical experiment carried out without adding Dim-5. (B) Methylation of substrate peptide (2.25 μ M) by different concentrations of Dim-5, demonstrating that the assay is linear with respect to enzyme concentration. The line shows a linear regression of the first five data points. (C) Binding capacity of avidin plate. Different amounts of substrate peptide were methylated using large excess of Dim-5 (1.25 μ M) for 30 min, and the methylated peptide was applied to the avidin microplate. The results show that each well binds to at least 200 pmol peptide completely. The line shows a linear regression of the first six data points.

Fig. 3. Michaelis–Menten analysis of Dim-5. Methylation kinetics were performed using 4.1 nM Dim-5 at different concentrations (conc.) of peptide. The individual time courses were analyzed by linear regression, and the initial slopes were fitted to the Michaelis–Menten model.

Fig. 4. Catalytic activities of some Dim-5 variants determined using the microplate assay. Kinetics were determined using 4 nM enzyme (400 nM in the case of Y178F) and 4.5 μ M peptide.