CHAPTER THREE

A Method to Site-Specifically Incorporate Methyl-Lysine Analogues into Recombinant Proteins

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Abstract

The site-specific and degree-specific methylation of histone lysine residues is important for the regulation of chromatin. To study the biochemical roles of lysine methylation, several approaches have been developed to reconstitute chromatin fibers *in vitro* with well-defined methylation patterns. Here, we describe the installation of methyl-lysine analogues (MLAs) as a simple and scalable method to introduce mono-, di-, or trimethylation at specific sites of recombinantly expressed histones. In this method, a histone is engineered to harbor a lysine-to-cysteine mutation at the desired site of modification. These mutant histones are treated with halo-ethylamines that react with the cysteine side chain, providing high yields of *N*-methylated aminoethylcysteines,

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analogues of *N*-methylated lysine residues. These MLA histones have been used to construct well-defined chromatin templates to study the direct biochemical consequences of histone lysine methylation in a variety of contexts.

1. INTRODUCTION

1.1. The biochemistry of methylated lysine residues

Genetic studies to identify factors that regulate development have identified numerous proteins implicated in histone lysine methylation as important developmental regulators in higher organisms (Campos & Reinberg, 2009; Kouzarides, 2007). Histones, the most abundant chromatin proteins, function both to compact DNA in the nucleus and also to regulate specific genomic loci. Histones harbor a wide range of posttranslational modifications, including acetylation, phosphorylation, and methylation. For example, at some loci, the spreading of heterochromatin is regulated by the methylation of histone H3 on lysine 9; this methylated residue is bound by the heterochromatin protein 1 (Grewal & Moazed, 2003). Mechanistic understanding of the role of lysine methylation in the regulation of chromatin can be greatly assisted by studying histone methylation in reconstituted chromatin substrates. Therefore, facile methods to introduce site-specific methylation into proteins are important for understanding genomic regulation at the level of chromatin.

1.2. Methods to install specific methylation into proteins

In theory, it would be possible to use existing enzymes to make specifically methylated proteins *in vitro*. Unfortunately, enzymes with the desired activity and specificity are rarely available. Chemical approaches are appealing ways to construct specifically methylated chromatin (Allis & Muir, 2011). One powerful approach is to use synthetic peptides to incorporate modified lysine residues at well-defined locations using native chemical ligation reactions (He et al., 2003; Shogren-Knaak, Fry, & Peterson, 2003). Another powerful approach is to use unnatural amino-acid mutagenesis in *Escherichia coli* to express modified lysine residues (Nguyen, Garcia Alai, Kapadnis, Neumann, & Chin, 2009) but is currently limited to monomethylated lysine residues and has not been extended to di- and trimethylated lysines. (Fig. 3.1)

Here, we describe a chemical approach that was developed to generate large quantities of site-specific methylated histones (Simon et al., 2007). This approach relies on the long-appreciated similarity between the lysine side

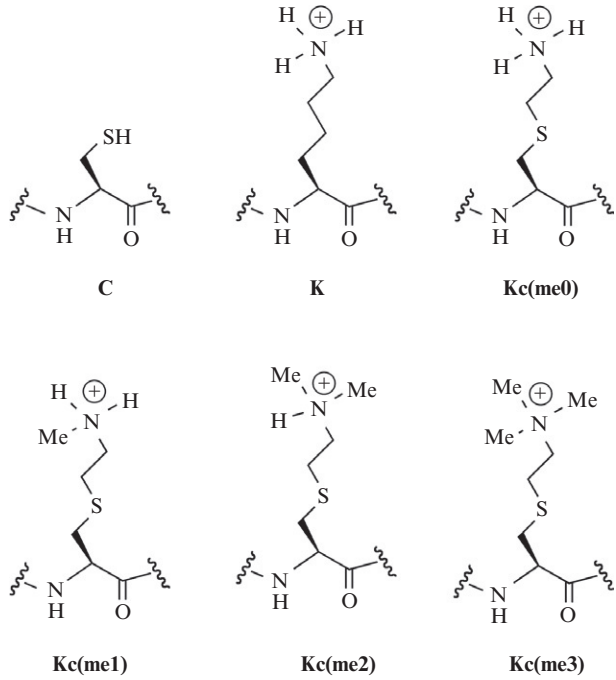

Figure 3.1 Structures of the amino-acid side chains of cysteine (C), lysine (K), and the methyl-lysine analogues for unmethylated lysine, Kc(me0); monomethylated lysine, Kc (me1); dimethylated lysine, Kc(me2); and trimethylated lysine, Kc(me3).

chain and aminoethylcysteine, a lysine analogue that can be made by alkylating cysteine residues (Kenyon & Bruice, 1977). Fortuitously, the core histones are highly conserved and have only a single conserved cysteine that can be mutated to alanine without any known effects. Therefore, cysteine residues can be engineered at any position in the histone, and by choosing the appropriate alkylating reagent, these cysteines can be converted chemically into analogues of mono-, di-, or trimethyl lysine. These methylated histones can be incorporated into histone octamers, nucleosomes, and chromatin to study the direct biochemical effects of specific histone methylation events.

2. PROTOCOL TO INSTALL MLA INTO RECOMBINANT PROTEINS

Here, we present protocols to install analogues of mono-, di-, or trimethyl lysine into recombinant histones. We also present a protocol for installation of the unmethylated lysine, which can be used to control for

potential artifacts caused by the unnatural aminoethylcysteine side chain. These protocols lead to histones that can be incorporated into histone octamers that can in turn be used to reconstitute chromatin. The resulting materials have been demonstrated to be of suitable quality for crystallographic analysis (Lu et al., 2008).

Starting from lyophilized cysteine-containing histones, the methyl-lysine analogue (MLA) reactions can be performed in a single day, with several alkylation reactions run in parallel. The characterization of the MLA histones can be performed either later the same day or on a subsequent day.

Day 1. Reduction of histones, alkylation, desalting, and lyophilization (to be left overnight).

Day 2. Storage of histones and characterization of MLA reactions by mass spectrometry.

2.1. General issues

While these reactions are neither chemically complicated nor labor intensive, there is one issue worth particular attention: it is important to avoid oxidation of the proteins before, during, and after the protocols described here. The thioether of the MLA moiety is similar to methionine, and like methionine, the MLA can oxidize to a sulfoxide in the presence of oxygen. Good biochemical technique generally dictates the maintenance of reducing conditions when handling proteins, and this is particularly critical when working with MLA histones, as side-chain oxidation would occur immediately at the site of interest and therefore is likely to lead to faulty conclusions. Further, it is difficult to characterize the oxidation state of histones once they are incorporated into chromatin substrates; the best practice is to be particularly careful to avoid oxidation of the samples in the first place.

The steps we recommend to avoid oxidation include using fresh solid DTT that has been stored under airtight conditions at $-20\ ^{\circ}C$ and dissolved immediately prior to the reactions. Buffers should be degassed by stirring under vacuum. After the MLAs have been installed into histones, it is important to use them with buffers containing fresh reducing reagents. After the MLAs have been incorporated into chromatin fibers, we recommend storing these reagents with 0.5–1 m*M* Tris(2-carboxyethyl)phosphine (TCEP). As TCEP·HCl can be very acidic, it is convenient to use a premade neutralized solution (e.g., Bond-Breaker TCEP Solution, Pierce, 77720).

2.2. Required starting protein

This protocol requires the previous expression and purification of histones that have been mutated to contain a unique cysteine residue at the desired site of modification. The wild-type core *Xenopus* histones, which are most commonly used species since their expression was optimized (Luger, Rechsteiner, & Richmond, 1999), have a single additional cysteine residue (xH3 C110) that must be mutated to avoid alkylating this site. Therefore, all xH3 MLA histones are constructed in the background of a C110A mutation. If this protocol is used to construct MLAs in proteins other than *Xenopus* histones, it is important to check that there are no other Cys residues present, as they will also be modified. A description of histone expression and purification can be found elsewhere in this series (Luger et al., 1999). We have found that histones purified only over size exclusion chromatography have proven sufficient for most uses without the need for additional ion exchange chromatography.

2.3. Preparation of histones for alkylation

The first steps of this protocol are to make the alkylation buffer, dissolve the histones, and reduce their cysteine residues to ensure they begin the reaction as free thiols available for alkylation.

The buffer for these reactions is strongly denaturing to ensure all residues accessible for alkylation. These alkylations work under less denaturing conditions for accessible sites (e.g., xH3 K4C), but we have found other residues (such as xH3 K79C) requiring strongly denaturing conditions for alkylation. Since histones are generally denatured before assembling histone octamers (Luger et al., 1999), complete denaturation of the histones is acceptable at this stage of handling.

The pH of the solution is also critical. The deprotonated thiolate of cysteine is substantially more nucleophilic than its protonated form; higher pH favors MLA alkylations. However, proteins have many amines that can be alkylated if the pH of the solution is too high. The reaction conditions have carefully tuned pH to maintain conditions with high Cys reactivity, but very low levels of unwanted alkylation events. Further, as the alkylating reagents are consumed, they generate a molar equivalent of acid. For this reason, the reactions are performed at high concentrations of buffer to minimize acidification of the solution during the course of the reaction.

2.3.1 Making alkylation buffer

Given the importance of pH and the modest solubility of the reagents used for the alkylation buffer, making this buffer is a critical step for successful alkylation reactions.

1. Weigh into a 100 mL beaker 6.46 g of HEPES, 18.98 g of HEPES sodium salt (to give a 1 *M* solution at the desired pH of 7.8), and 149 mg of D/L-methionine (10 m*M* methionine is included as an extra oxygen scavenger to help reduce oxidation to the desired histones).
2. Add distilled water to about 55 mL with stirring.
3. After most of the HEPES has dissolved, add 38.21 g of guanidinium chloride slowly (over a few minutes) while stirring.
4. After the solution has warmed to room temperature (rt), add minimal water to bring all the solids into solution keeping the volume below 100 mL. Transfer the solution to a 100-mL graduated cylinder and adjust with water to 100 mL.
5. Filter the thick solution through a 0.22-μm filter and store at rt.
6. Before use, degas the solution under vacuum for about 30 min either with stirring or using a bath sonicator.

2.3.2 Reducing the proteins prior to alkylation

While there are several reducing agents that are commonly used, we have had the best results using DTT.

1. Start by dissolving the lyophilized proteins in alkylation buffer at approximately 5–10 mg/mL.
2. Mix by gentle inversion until the entire solid has dissolved.
3. Dissolve fresh solid DTT to 1 *M* with water (154 mg/mL).
4. Add 20 μL of DTT solution from step 3 for every milliliter of the histone solution.
5. Incubate this solution for 1 h at 37 °C.

2.4. Installing the appropriate MLA side chain

After the proteins are reduced, they are treated with high concentrations of the appropriate alkylating reagents. Due to differences in the reactivity of each electrophile, the temperature, time, and number of additions have been adjusted to provide maximally robust results for each, as described below.

2.4.1 Alkylation to make analogues of monomethyl lysine, Kc(me1)

The original conditions we reported for installing monomethyl lysine analogues (Simon et al., 2007) required an overnight reaction. Further optimization of this reaction (Simon, 2010) has decreased reaction times and led to

more robust alkylation results. These optimized conditions are described below:

1. Weigh at least 13.0 mg of (2-chloroethyl)-methylammonium chloride (130.02 g/mol) into a 1.7-mL tube and dissolve with the appropriate volume of water to make a 1 *M* solution. After dissolving the solids by vortexing, protect the tube from light. Perform the following steps in a fume hood, as the aziridine intermediate is volatile and toxic. Protect your hands by wearing gloves and do not breathe the vapors while handling the reaction.
2. Add 960 μL of the reduced protein solution from Section 2.3.1 to a fresh microcentrifuge tube.
3. Add 50 μL of the 1 *M* (2-chloroethyl)-methylammonium chloride stock and mix gently by flicking.
4. Incubate the reaction for 2.5 h at rt keeping the reaction protected from light using aluminum foil.
5. To extend the reaction and maintain reducing conditions, add 10 μL of 1 *M* DTT.
6. Flick to mix the solution and allow the reaction to continue for 2.5 h.
7. To consume the remaining alkylating reagent, quench using 50 μL BME (neat) and incubate 30 min at rt.

2.4.2 Alkylation to make analogues of dimethyl lysine, Kc(me2)

1. Weigh at least 14.5 mg of (2-chloroethyl)-dimethylammonium chloride (144.05 g/mol) into a 1.7-mL tube and dissolve with the appropriate volume of water to make a 1 *M* solution. After dissolving the solids by vortexing, protect the tube from light. Perform the following steps in a fume hood.
2. Add 950 μL of the reduced protein solution from Section 2.3.1 to a fresh microcentrifuge tube.
3. Add 50 μL of the (2-chloroethyl)-dimethylammonium chloride stock and mix gently by flicking.
4. Incubate the reaction for 2 h at rt keeping the reaction protected from light using aluminum foil.
5. To extend the reaction and maintain reducing conditions, add 10 μL of 1 *M* DTT.
6. Allow the reduction to proceed for 30 min and then add an additional 50 μL of the (2-chloroethyl)-dimethylammonium chloride stock.
7. Flick to mix the solution and allow the reaction to continue for 2 h.
8. To consume the remaining alkylating reagent, quench using 50 μL BME (neat) and incubate 30 min at rt.

2.4.3 Alkylation to make analogues of trimethyl lysine, Kc(me3)

Unlike the reagents required for making mono- and di-MLAs, the trimethylated bromoethylamine cannot form an aziridine intermediate and requires higher temperatures for the reaction. Further, its solubility is limited at rt, so the heated reaction also serves to help dissolve this reagent.

1. Into a fresh 1.7-mL microcentrifuge tube, weigh 100 mg of (2-bromoethyl) trimethyl ammonium bromide.
2. Add 1 mL of the reduced protein solution from Section 2.3.1.
3. Flick to mix and transfer the mixture to a 50 °C heat block.
4. Flick to mix approximately every 30 min until the entire solid has dissolved. Alternatively, use a thermomixer and shake at 1000 rpm at 50 °C until the entire solid has dissolved.
5. From the beginning of step 2, allow the reaction to proceed for 2.5 h.
6. To extend the reaction and maintain reducing conditions, add 10 μL of 1 *M* DTT.
7. Flick to mix the solution and allow the reaction to continue for an additional 2.5 h.
8. To consume the remaining alkylating reagent, quench using 50 μL BME (neat) and incubate 30 min at rt.

2.4.4 Alkylation to make analogues of unmethylated lysine, Kc(me0)

To ensure that effects observed with the MLA histones are due to methylation, and not differences between the natural lysine side chain and the sulfur-containing side chain, a helpful control is to use an unmethylated MLA.

1. Weigh at least 10.3 mg of (2-bromoethyl)-ammonium bromide (204.89 g/mol) into a 1.7-mL tube and dissolve with the appropriate volume of water to make a 1 *M* solution.
2. After dissolving the solids by vortexing, protect the tube from light. Perform the following steps in a fume hood, as the aziridine intermediate is volatile and toxic. Protect your hands by wearing gloves and do not breathe the vapors while handling the reaction.
3. Add 960 μL of the reduced protein solution from Section 2.3.1 to a fresh microcentrifuge tube.
4. Add 50 μL of the 1 *M* (2-bromoethyl)-ammonium bromide stock and mix gently by flicking.
5. Incubate the reaction for 2.5 h at rt keeping the reaction protected from light using aluminum foil.

6. To extend the reaction and maintain reducing conditions, add 10 μL of 1 *M* DTT. Flick to mix the solution and allow the reaction to continue for 2.5 h.
7. To consume the remaining alkylating reagent, quench using 50 μL BME (neat) and incubate 30 min at rt.

2.5. Purification of MLA proteins

To isolate the MLA proteins from the reaction mixture, a standard PD-10 desalting column is used. Gravity flow allows several to be run in parallel.

1. Before purification, each column is equilibrated with 25 mL of water that is supplemented with 2 m*M* β-mercaptoethanol (BME).
2. Apply the ~1 mL alkylation reaction to the column.
3. Rinse the samples into the column using an additional 1.5 mL of water with 2 m*M* BME. Note that it is important that the total volume loaded in steps 2 and 3 is 2.5 mL. The eluant at this step may be discarded.
4. Into a fresh 15-mL conical tube, elute the MLA proteins using 3.2 mL of water with 2 m*M* BME.
5. Save 10 μL of eluted proteins and dilute into 90 μL of water/BME for analysis by mass spectrometry.
6. Measure the OD_{276} of the solutions to determine the concentrations of proteins. For histones, use the following values: xenopus histone H3: 15,273 g/mol, 4040 M^{-1}/cm, and histone H4: 11,236 g/mol, 5040 M^{-1}/cm (Luger et al., 1999).
7. Split each protein solution into the desired aliquots. Flash freeze in liquid nitrogen and lyophilize to dryness.
8. Store protein pellets at −80 °C.

2.6. Characterization of MLA histones

To characterize the products of the MLA reactions, analyze the resulting proteins by mass spectrometry. Given the high concentrations of proteins used, and the high expected purity of mixture, this analysis does not require a highly sensitive instrument. It is important, however, to achieve a 1- to 2-Da resolution when analyzing the full-length proteins. We use an Agilent 6520 QTOF equipped with an electrospray ionization source.

2.7. Results and troubleshooting

Compare the starting proteins using the MLA proteins. Refer to Table 3.1 for desired mass differences for each MLA reaction and potential contaminants. Generally, the reaction proceeds to completion and the desired

Table 3.1 Commonly observed mass differences in MLA reactions

Delta amu	Identity	Notes
0	Unreacted starting material	If a peak of unreacted starting material is abundant in product (>5% peak height of desired mass), either the reaction had insufficient alkylating reagent, the pH was too low, the reaction time too short, or the insufficient reduction led to disulfides blocking the alkylation.
44	Kc(me0)	A single +44 peak is desired. Additional +44 peaks (i.e., +88, +132, etc.) are indicative of overreaction.
58	Kc(me1)	A single +58 peak is desired. Additional +58 peaks (i.e., +116, etc.) are indicative of overreaction.
72	Kc(me2)	A single +72 peak is desired. Additional +72 peaks (i.e., +144, etc.) are indicative of overreaction.
86	Kc(me3)	A single +86 peak is desired. Additional +86 peaks (i.e., +172, etc.) are indicative of overreaction.
16	Oxidation	Insufficient reducing reagent during reaction.
23	Sodium adduct	Insufficient desalting but not indicative of any problems with the MLA chemistry.
42	Unknown	Common artifact in ESI mass spectrometry. When it appears, it is present in both the starting material and product and therefore is unrelated to the MLA chemistry.

product is the dominant peak, with <5% of any contaminant. If the reaction went poorly, there are three common problems: (1) underreaction, (2) overreaction, and (3) oxidation. These are described below:

1. Underreaction is evident from a strong peak of unreacted histone starting material after the reaction. This generally happens when the pH is too low, there is insufficient alkylating reagent, or the side chains were blocked by disulfides due to insufficient reduction.
2. Overreaction is clear when there are peaks corresponding to multiple additions of the desired modification. This is most likely to occur if

the pH is too high, the temperature is too high, or too much alkylating reagent is used.

3. Oxidation is observed when there is insufficient reducing reagent or when the buffer is not degassed or is too old (the free methionine in the buffer that helps prevent protein oxidation can become oxidized over time). Finally, other peaks found in the product spectra are sometimes also found in the starting material spectra, demonstrating that the problem is insufficiently pure starting material.

2.8. Limitations uses and extensions of the MLA approach

Using MLAs provides rapid access to large quantities of site-specifically methylated proteins. However, the analogue is not equivalent to the natural lysine side chain. In cases where the MLAs have been compared to their natural counterparts in the context of peptides, the specificities of the interactions have always been preserved (Jia et al., 2009; Simon et al., 2007). Nonetheless, quantitative differences in the affinities of methyl–histone binding domains and enzymatic activities have been observed when comparing the MLAs with natural lysine methylation (Krishnan, Collazo, Ortiz-Tello, & Trievel, 2012; Seeliger et al., 2012). Further, MLA histones have primarily been used to study only a single site at a time in a histone. While it is possible to alkylate two cysteine residues in a single histone, the two modifications must be of the same degree of methylation (e.g., this approach allows simultaneous installation of Kc4me3 and Kc27me3 MLAs, but not Kc4me1 and Kc27me3). While this method focuses on the installation of lysine methylation, this approach has also been extended to install analogues of lysine acetylation (Huang et al., 2010; Li et al., 2011).

Despite their limitations, MLA histones have been useful in a wide range of applications. Examples include their use for studying the effect of methylation on chromatin fibers (Lu et al., 2008), on the binding mode of a heterochromatic proteins (Canzio et al., 2011), on the binding of factors through *in vitro* replication (Francis, Follmer, Simon, Aghia, & Butler, 2009), on the recruitment of factors during *in vitro* transcription (Lin et al., 2011), and on the regulation of other enzymatic activities on nucleosomes (Hung et al., 2009; Li et al., 2009; Margueron et al., 2009; Schmitges et al., 2011; Yuan et al., 2011). In summary, MLA histones provide a useful tool to help understand the direct biochemical effects of histone lysine methylation and how these effects contribute to a cell's regulation of its genome.

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