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Bioconjugate Chemistry

Article

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Installation of Trimethyllysine Analogs on Intact Histones *via* Cysteine Alkylation

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ABSTRACT

Site-specific incorporation of posttranslationally-modified amino acids into proteins, including histones, has been a subject of great interest for chemical and biochemical communities. Here, we describe a site-specific incorporation of structurally simplest trimethyllysine analogs into the position 4 of the intact histone H3 protein. An efficient alkylation of cysteine 4 of the recombinantly expressed histone H3 provides a panel of trimethyllysine analogs that differ in charge, charge density, sterics and chain length. We demonstrate that H3 histone that bears trimethyllysine analogs can be further assembled into the octameric histone complex that constitutes the nucleosome. Binding studies showed that H3 histone that possesses trimethyllysine analogs is well recognized by a PHD3 reader domain of human JARID1A. This work provides important (bio)chemical tools for fundamental biomolecular studies aimed at unravelling the molecular basis of the higher order nucleosome and chromatin assemblies.

KEYWORDS

Biomolecular recognition, Cysteine alkylation, Epigenetics, Histone, Posttranslational modifications, Trimethyllysine

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INTRODUCTION

Numerous posttranslational modifications (PTMs) of the nucleosomal histone proteins are centrally important for the regulation of epigenetic processes in humans (Figure 1a).[1] One of the most widespread PTMs is the methylation of lysine residues on histone tails and core histones.[2, 3] Histone lysine methylation is catalyzed by S-adenosylmethionine (SAM)-dependent lysine methyltransferases that install one (Kme), two (Kme2) or three methyl (Kme3) groups on lysine residues (Figure 1b).[4] These methylation marks are specifically recognized by methyllysine binding proteins (known as reader proteins), including plant homeodomain (PHD) zinc fingers, tandem tudor domains and chromodomains.[5] Positively charged Kme3 is recognized by an electron-rich aromatic cage present in the reader domains (Figure 1c).[5] Recent work using histone peptides revealed that tuning the chemical structure of the trimethyllysine enabled a better understanding of the nature of biomolecular recognition by epigenetic readers.[6-9] Among examined trimethyllysine analogs, the cysteine-derived trimethyllysine ($K_c$me3) appears to be recognized by epigenetic reader proteins with similar affinity as the natural trimethyllysine, thus making it the closest mimic known to date (Figure 1d).[10]

Site-specific introduction of unnatural Kme3 analogs into intact histone proteins remains a challenge, and among others contributes to incomplete understanding of the role of lysine methylation on the nucleosome structure and function (Figure 1a). Incorporation of unnatural amino acids into proteins can be achieved by changing the biochemical machinery of cells to express unnatural variants. One such method is the use of auxotrophic bacterial strains, which results in incorporation of an unnatural amino acid in every position it would naturally occur, hence no positional selectivity is possible.[11] A more recent approach uses the amber stop codon (TAG), which does not code for any natural amino acid.[12] This method allows an introduction of an unnatural

amino acid at a specific site, since the amber stop codon can be introduced anywhere using mutagenesis.[13] Major drawbacks of these methods include severe limitations in the diversity of amino acid that can be introduced into proteins. Furthermore, when using auxotrophic strains, the protein expression yield is reduced dramatically and the process of developing an amber codon pair is time-consuming and laborious.[13] To circumvent disadvantages that are encountered with these expression methods, several synthetic and semi-synthetic methods in histone research have been developed recently.[14-16] For instance, native chemical ligation (NCL) enables the preparation of a wide variety of proteins, including histones with installed PTMs.[16] Still, full protein synthesis can be laborious, and is somewhat limited in sequence length, which is why synthetically simpler methods can provide an important alternative.

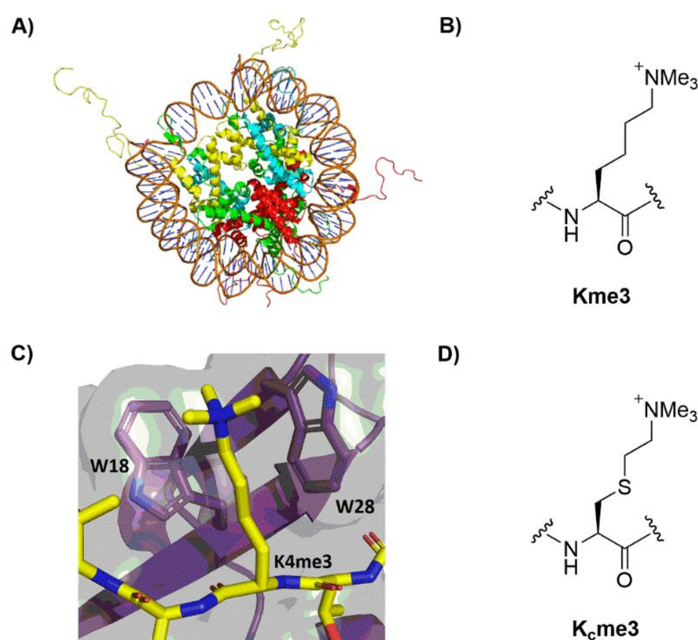

Figure 1. A) Structure of the nucleosome. Green: H2A, cyan: H2B, yellow: H3 and red: H4. (PDB ID: 1KX5); B) Structure of trimethyllysine (Kme3); C) View from the PHD3 domain of JARID1A (purple) (PDB ID: 3GL6) complexed with H3K4me3 (yellow); D) Structure of the cysteine-derived trimethyllysine ($K_c$me3).

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One such method is modification of cysteines to produce unnatural amino acids that strongly mimic the natural amino acids.[17] This can be achieved either by cysteine alkylation[18] (Figure 2a) or the conversion of native cysteine to dehydroalanine.[19] Employing the dehydroalanine method has provided access to a variety of PTMs, which can be installed into intact histones.[20, 21] Among these modifications are methylated and acetylated lysine analogs, and synthetically challenging phosphorylated and glycosylated serine analogs. However, converting cysteine to dehydroalanine causes racemization of the target amino acid.[17] Because epigenetic proteins very poorly (or not at all) accept histones that bear (methylated) D-lysine,[9] it is important to further explore the cysteine alkylation strategy, as L-stereochemistry is retained in this case. This method[18] is especially suitable for studying histones, because only one or two native cysteines exist (C110 and C96 in H3.1, C110 in H3.2, C110 in H3.3) in the four different histones that constitute the nucleosomal octamer (Figure 1a). Histone H3 isoforms that possess C110A and C96S display a biological function.[22, 23] This provides the freedom to position cysteine residues in desired locations in the histone proteins by single amino acid mutagenesis and subsequent expression. Cysteine alkylation variants or methylated lysine analogs (MLA) do not adversely change the residues' association with epigenetic readers, but are somewhat poorer substrates for histone demethylases,[24] when compared to natural lysine or methylated lysine residues.[10, 22, 25] This approach generally is suitable to study the recognition of trimethyllysine-containing histone peptides and intact histones.[10, 22, 25] Building on this precedent,[22] we report an installation of trimethyllysine analogs with smallest controllable perturbations in molecular structure into intact histones (Figure 2b). We show that such modified histones can assemble into the octameric histone assembly and that a human epigenetic reader protein recognizes intact histones that possess the simplest trimethyllysine analogs.

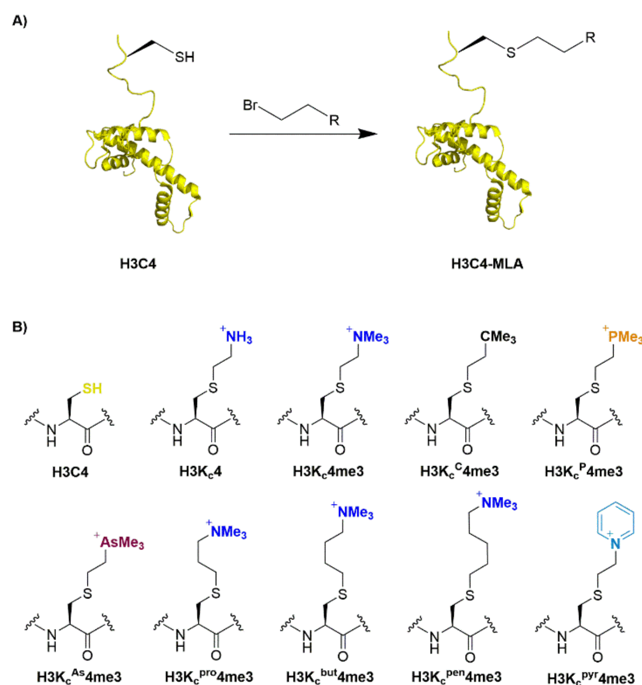

Figure 2. A) Conversion of cysteine 4 on histone 3 (H3C4) into a methyllysine analogs (MLA) by alkylation using bromide alkylation reagents; B) H3C4 and the MLA-bearing histones studied in this work.

RESULTS AND DISCUSSION

Trimethyllysine has interesting chemical properties: it possesses a fixed positive charge, three hydrophobic methyl groups and a side chain comprised of four methylene groups. To investigate the role of individual properties (i.e. charge, charge density, chain length, sterics) on histone biomolecular recognition, we sought to expand the structural and chemical diversity of MLAs on intact histones through cysteine alkylation (Figure 2a-b). An uncharged carba (K_c^{C}me3) analog was designed to investigate the effect of the positive charge on biomolecular recognition. Furthermore, two MLAs were designed to probe the effect of the charge density of the trimethylammonium moiety, namely a phosphonium (K_c^{P}me3) and an arsonium (K_c^{As}me3) analog. Three additional MLAs, propane-trimethyllysine (K_c^{pro}me3), butane-trimethyllysine (K_c^{but}me3) and pentane-

trimethyllysine (K_c^{pen}me3) were synthesized to explore the effect of side chain length. Finally, a pyridine analog (K_c^{pyr}me3) was developed to investigate the effect of sterics on biomolecular recognition. Our objective was to examine whether such intact histones can be assembled into the higher order octameric histone complex and whether intact histone proteins that bear MLAs are recognized by the PHD3 zinc finger domain of human JARID1A (Figure 1c).

We used an H3 construct containing K4C and C110A mutations, hereafter denoted as H3C4, for biomolecular studies on intact histone proteins. The H3C4 protein was successfully expressed in *E. coli* and purified from the pellet fraction and characterized by denaturing ESI-MS (Figure S1).[26] H3C4 was then alkylated using a variety of MLA reagents, thus leading to subtle alterations of the side chain structure when compared with Kme3 (Figure 2b). The alkylation reactions were carried out on intact H3C4 protein at pH 7.8 with the addition of DTT; denaturing ESI-MS experiments were performed on these alkylated products (Figure 3, Figure S1 and Table S1). The mass of unalkylated H3C4 protein corresponds to the expected calculated mass minus 2 Da (Table S2). This shift is possibly caused by deprotonation of the H3 histidines during ESI-MS measurements. A peak with an increased mass of 43 Da was visible as well; this peak was reported previously and likely corresponds to the addition of a carbamoyl group during production of intact histone proteins.[22] A good site-selective conversion of H3C4 to H3K_c4, H3K_c4me3, H3K_c^{As}4me3, H3K_c^{C}4me3, H3K_c^{pro}4me3, H3K_c^{but}4me3 and H3K_c^{pyr}4me3 was observed, while H3K_c^{P}4me3 and H3K_c^{pen}4me3 appear to be also partially over-alkylated. The reagents used for the latter alkylations proved to be more reactive than expected and histones containing multiple adducts could not be prevented despite our optimisation efforts; notably, the monoalkylated products were predominantly present in all cases.

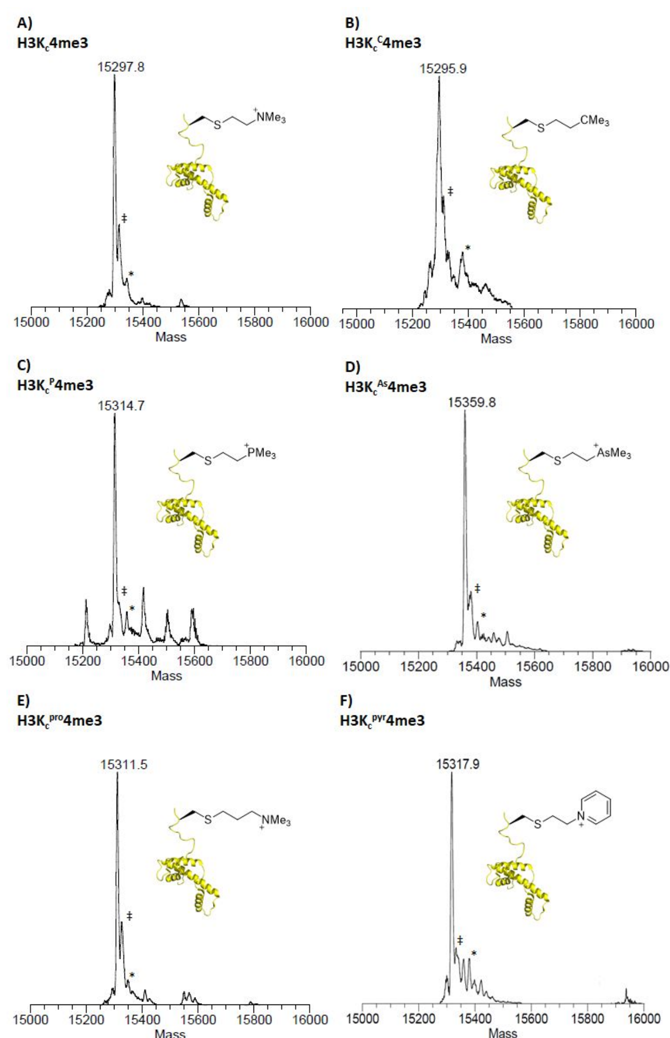

Figure 3. Deconvoluted ESI-MS spectra of trimethyllysine-possessing histone proteins. A) $H3K_c4me3$; B) $H3K_c{}^{C}4me3$; C) $H3K_c{}^{P}4me3$; D) $H3K_c{}^{As}4me3$; E) $H3K_c{}^{pro}4me3$; F) $H3K_c{}^{pyr}4me3$. Peaks indicated with a double dagger correspond to a mass of +16 Da (ammonium adduct) and peaks indicated with an asterisk correspond to a mass of +43 Da (carbamyl adduct).

Next, the alkylated histones were assessed by SDS-PAGE and western blotting (Figure 4). These results showed that the alkylated histones have been obtained in high purity (Figure 4a) and still contained epitopes for recognition by antibodies directed

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3 against intact histone H3 (Figure 4b). When using antibodies specifically directed at
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5 H3K4me3, it was confirmed that these antibodies are indeed specific for $H3K_c4me3$,
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7 and that the presence of the thia-group does not abrogate binding, in line with previous
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9 study;[22] it is worth noting that the anti-H3K4me3 antibodies did not recognize $H3K_c4$
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11 (Figure 4c). Interestingly, replacement of the N^{ε} in the trimethyllysine side chain by
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13 either phosphorus ($H3K_c^{P}me3$) or arsenic ($H3K_c^{As}me3$), or elongation of the side chain
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15 by an additional methylene group ($H3K_c^{pro}me3$) did not visibly affect the recognition by
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17 the anti-H3K4me3 antibodies (Figure 4c). This result indicates that small changes in
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19 charge density or chain length do not affect the ability of antibodies to recognize the
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21 specific Kme3 epitope. Further increases in the chain length resulted in weaker antibody
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23 recognition, although full abrogation of binding was not observed (Figure 4c).
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25 Replacement of the positively charged trimethylammonium group by the neutral carba
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27 analog, on the other hand, completely interfered with recognition of the epitope,
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29 implying that anti-H3K4me3 antibodies recognize epitopes *via* cation–π interactions
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31 (Figure 4c). The installation of the bulky pyridine group did not fully block the
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33 recognition by the antibodies beyond the limits of detection (Figure 4c).
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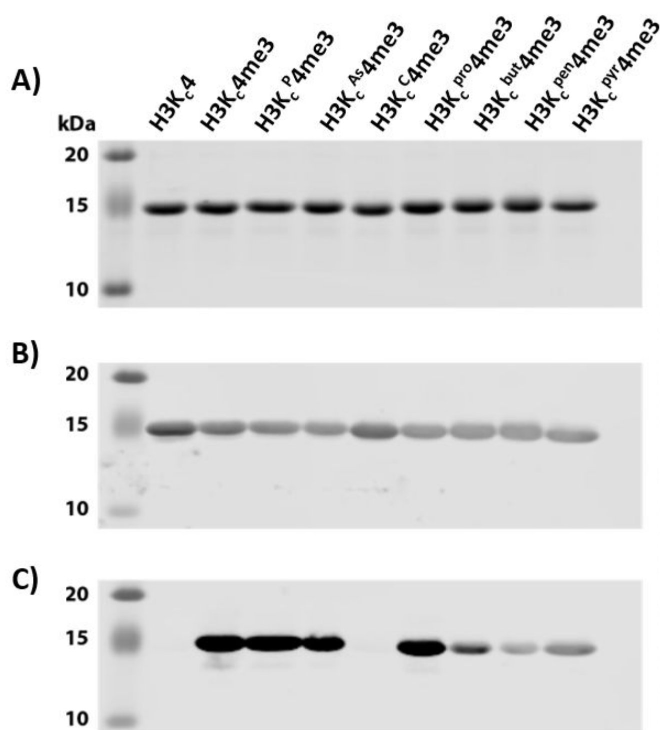

Figure 4. A) SDS-PAGE of purified intact histones that bear Kme3 analogs; B) Western blot of the purified histone alkylation products using antibodies directed against H3; C) Western blot of purified histone alkylation products using antibodies specifically directed against H3K4me3.

To further assess the effects of subtle modifications on the $K_c$me3 side chain, selected alkylated histones H3 in the presence of recombinant H2A, H2B and H4 (Figure S2) were used for the construction of histone octamers, according to reported procedure.[27] SDS-PAGE analysis (Figure 5a) and size exclusion chromatograms (Figure S3) showed that the alkylated H3C4 histones can be assembled into complete histone octamers regardless of the nature of the MLA installed. Further analysis by western blotting and dot blotting verified that anti-H3K4me3 antibodies can readily distinguish between the individual histones without displaying any cross-reactivity under both denatured and

native assembly conditions (Figure 5b-c and Figure S4). This observation indicates that the MLA-containing H3 proteins can be readily assembled into more complex nucleosome-like structures.

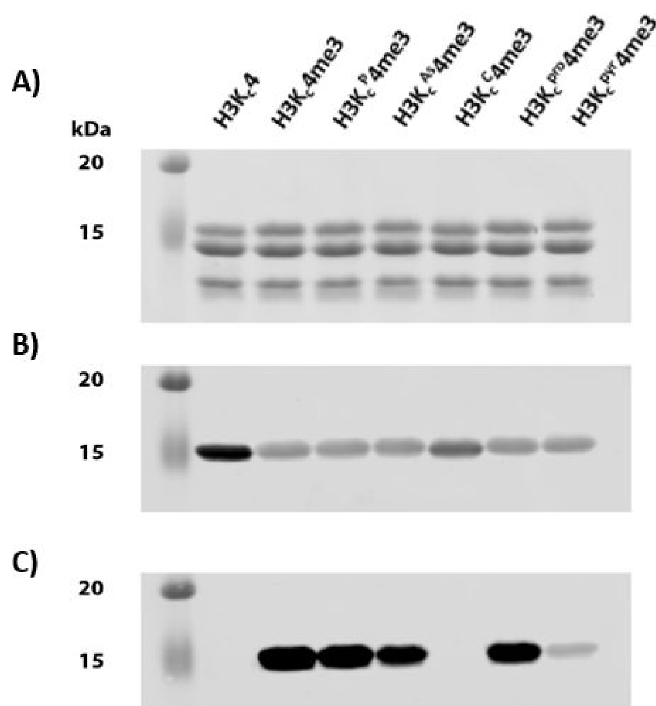

Figure 5. A) SDS-PAGE of assembled histone octamers containing various H3 MLA's; B) Western blot analysis of histone octamers using an antibody directed against H3; C) Western blot analysis of histone octamers using an antibody directed against H3K4me3.

After characterization of the alkylated histone proteins, the variants were used to study recognition by the biomedically important PHD3 domain of human JARID1A.[28] We found that all MLAs were able to function as binding partners for JARID1A with dissociation constants in the range of 0.13–0.6 μM and Gibbs free energies in the range of -8.5 to -9.5 kcal mol^{-1} (Table 1). The $H3K_c4$ variant also bound to JARID1A with reasonable binding affinity, albeit with less favourable enthalpy and more favourable entropy than $H3K_c4me3$. ITC data demonstrated that the association between JARID1A

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and $H3K_c4me3$ is enthalpy driven, while entropy is slightly unfavourable, as observed previously with histone H3 peptides (Figure S5).[7, 29] Results with positively charged $H3K_c^P4me3$ and $H3K_c^{As}4me3$ are similar to those for $H3K_c4me3$ binding, with an enthalpy driven association. The binding affinities for $H3K_c4me3$, $H3K_c^P4me3$ and $H3K_c^{As}me3$ are similar, but small differences in $\Delta H°$ and $-T\Delta S°$ were observed. When examining the binding of $H3K_c^C4me3$, a clear enthalpy-entropy compensation mechanism was observed; $\Delta\Delta H°$ of +2.9 kcal mol^{-1} and $-T\Delta\Delta S°$ of -2.6 kcal mol^{-1} relative to $H3K_c4me3$ was observed. This result is in line with that obtained in our previous work on histone peptides containing a neutral carba variant of trimethyllysine.[7] $H3K_c^{pro}4me3$, $H3K_c^{but}4me3$ and $H3K_c^{pen}4me3$ bind to JARID1A with comparable binding affinities, and slightly weaker than $H3K_c4me3$. These results are in line with recent work on recognition of trimethylhomolysine by JARID1A and other reader proteins.[8] Binding of $H3K_c^{pyr}4me3$ resulted in a marked but overall modest increase in $\Delta G°$ of 1.0 kcal mol^{-1} when compared to $H3K_c4me3$; a relatively large unfavourable $-T\Delta S°$ of +2.6 kcal mol^{-1} was observed in the case of $H3K_c^{pyr}4me3$-JARID1A binding.

Table 1. Thermodynamic data for binding of alkylated intact histones H3 to the PHD3 domain of human JARID1A.

	K_d (μM)	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$-T\Delta S°$ (kcal mol^{-1})
$H3K_c4$	0.6*	-8.5	-8.2	-0.4
$H3K_c4me3$	0.13	-9.5 ± 0.5	-10.9 ± 0.2	1.4 ± 0.3
$H3K_c^P4me3$	0.23	-9.1 ± 0.2	-9.4 ± 0.1	0.3 ± 0.1
$H3K_c^{As}4me3$	0.29	-8.9 ± 0.1	-10.1 ± 0.6	1.2 ± 0.5
$H3K_c^C4me3$	0.21	-9.1 ± 0.1	-7.9 ± 0.5	-1.2 ± 0.6
$H3K_c^{pro}4me3$	0.29	-8.9 ± 0.3	-11.1 ± 2.6	2.2 ± 2.9
$H3K_c^{but}4me3$	0.30	-8.9 ± 0.1	-10.5 ± 1.2	1.6 ± 1.3
$H3K_c^{pen}4me3$	0.27	-9.0 ± 0.1	-11.0 ± 1.0	2.0 ± 1.1
$H3K_c^{pyr}4me3$	0.60	-8.5 ± 0.1	-11.1 ± 0.7	2.6 ± 0.7

Data was obtained in duplicate. *Single measurement

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CONCLUSION

We have expanded the molecular toolbox for the incorporation of trimethyllysine analogs on intact histones by site-specific cysteine alkylation. Simplest trimethyllysine analogs that differ from trimethyllysine in charge, charge density, chain length and steric properties were successfully incorporated into histone H3 and in the octameric histone assembly. Such trimethyllysine analogs can be used to investigate the function of histones that possess the trimethyllysine moiety without causing major structural and chemical perturbations. We believe that these trimethyllysine analogs can be used to probe the epigenetic function of trimethyllysine in a sequence-specific context and that they provide essential molecular tools for fundamental physical-organic chemistry studies aimed at dissecting the chemical basis of the higher order nucleosome and chromatin assemblies at the unprecedented level of molecular detail.

MATERIALS AND METHODS

Preparation of full-length histone proteins

Full length histone proteins (*X. laevis*) were prepared as described previously.[22, 26] The H3 construct containing a C110A mutation was adjusted with a K4C mutation using PCR, which was subsequently verified using sequence analysis. The obtained histone constructs were transformed into competent *E. coli.* Rosetta BL21 DE3 PlysS cells and used for expression in TB medium. H2A and H2B were expressed by inducing the cultured cells at OD_{600} = 0.6 with 0.1 mM IPTG whereas H3 was induced with 0.4 mM and H4 with 1 mM IPTG. The cells were then cultured for 3 hours at 37°C after which the cells were harvested and stored at -80°C until further use.

The expressed histone proteins were then purified from the pellet fraction by soaking the pellet in DMSO for 30 minutes followed by a 1-hour extraction with 6 M guanidinium HCl, 20 mM NaAc pH 5.2 and 1 mM DTT. The extracted protein was further purified by gel filtration using a HiPrep Sephacryl S-200 HR column. H2A, H2B and H4 protein fractions were eluted in 7 M deionized urea, 20 mM NaAc pH 5.2, 200 mM NaCl and 2 mM 2-mercaptoethanol at 3 ml min^{-1} at room temperature. H3 was purified using 6 M guanidinium HCl, 20 mM NaAc pH 5.2, 1 mM DTT as eluent, in order to more effectively separate the H3 protein from DNA contaminants. Fractions containing the histone proteins were collected, dialyzed against 1 mM 2-mercaptoethanol and subsequently lyophilized for prolonged storage at -20°C.

The PHD3 domain of JARID1A was expressed and purified as described by Pieters et al.[29]

Alkylation of full-length histone H3

The K_c, $K_c$me3, K_c^{P}me3, K_c^{As}me3, K_c^{pyr}me3, K_c^{pro}me3, K_c^{but}me3 and K_c^{pen}me3 groups were deposited according to our own optimized conditions as described in Table S1 based on the protocols described by Simon et al.[22, 26] Briefly; 10 mg of purified and lyophilized histone 3 was dissolved in 980 µl alkylation buffer (4 M GuHCl, 1 M Hepes pH 7.8 and 10 mM D/L-methionine) and allowed to incubate for 1h at 37°C under reducing conditions by adding 20 µl 1 M DTT. The desired alkylation reagent was directly dissolved into the reaction mixture and allowed to react at the specified temperatures described in table 2. After 2.5 h reaction time, 10 µl 1 M DTT was added to the reaction mixture and the reaction was allowed to proceed for another 2.5 h. The reaction was quenched by incubating the reaction mixture with 50 µl 2-mercaptoethanol for 30 minutes at RT after which the alkylated histones were desalted using a PD-10 column (#17-0851-01, GE Healthcare) and subsequently buffer exchanged into 1 mM 2-mercaptoethanol using a centrifugal filter unit. The alkylated histone proteins were then lyophilized and stored at -20°C.

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For installing $K_c{}^{C}me3$ a separate protocol was devised: 10 mg of purified and lyophilized histone 3 was dissolved in 980 µl DMSO and reduced for 1 h at 37°C by adding 20 µl 1M DTT in DMSO. 1 ml reactions were performed in 1.5 ml conical tubes by pipetting the reagent directly into the reaction mixture and incubating at 50°C. After 2.5 hrs. 10 µl 1M DTT in DMSO was added and the reaction was allowed to proceed for another 2.5 hrs. After a total of 5 h reaction time, the reaction was quenched by incubating the reaction mixture with 50 µl 2-mercaptoethanol for 30 minutes at RT. After quenching, the mixture was diluted with ddH_2O and buffer exchanged into 1 mM 2-mercaptoethanol using a centrifugal filter unit. The hydrophobic reagent was removed from solution by ether extraction and the protein was subsequently lyophilized and stored at -20°C. All histone proteins were analyzed by denaturing mass spectrometry (Ultima Q-TOF).

SDS-PAGE and western blotting

SDS-PAGE analysis was performed on the histone proteins and assembled histone octamers using a 15% PAA gel (Figure S2). PAGE was run for 30 minutes at 100 V and subsequently run for 1 hour at 150 V. Gels were stained with coomassie brilliant blue for protein visualization. Western blotting was performed according to standard procedures. Briefly an SDS-PAGE gel was prepared as described above, after which it was blotted onto a nitrocellulose membrane for 1 hour at 250 mA. The histone proteins were visualized using antibodies directed against H3 and H3K4me3 respectively (Abcam, Ab1791 and Novus Biologicals, NB21-1023).

Histone octamer assembly

Histone octamers were assembled as described by Luger et al.[27] Upon expression and purification of full length histones (Figure S2) the lyophilized histones were re-dissolved and

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allowed to unfold for maximally 3 hours at a concentration of 2 mg ml^{-1} in freshly prepared 6 M guanidinium chloride, 20 mM Tris pH 7.5 and 5 mM DTT solution. The histone proteins were mixed in exact equimolar amounts and diluted to a final total protein concentration of 1 mg ml^{-1}. The histone proteins were then dialyzed 3 times at least 6 hours against 2 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA and 5 mM 2-mercaptoethanol at 4 degrees using a 10 kD MWCO dialysis membrane. The assembled octamers were concentrated and subsequently purified by size exclusion chromatography on a superdex 200 HR column using 2 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA and 5 mM 2-mercaptoethanol as eluent (Figure S3). The obtained octamers were stored at 4°C.

ITC measurements

ITC measurements were conducted with recombinant JARID1A PHD3 reader domain and full length histones at a temperature of 298 K (Figure S5). Buffers identical to the buffers used for reader protein purification were used for ITC experiments. Typically, 30 μM reader protein was titrated with 300 μM histone protein. Each ITC titration consisted of 19 injections and experiments were repeated 3 to 5 times. The ITC experiments were performed on a fully automated Microcal Auto-iTC200 (Malvern) and curve fitting was performed using Origin 6.0 (Microcal Inc., USA) using a one-site model.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Synthesis, protein modification, mass spectrometry, isothermal titration calorimetry.

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Notes

The authors declare no competing financial interest.

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TOC IMAGE