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Quantitative and structural assessment of histone methyllysine analog engagement by cognate binding proteins reveals affinity decrements relative to native counterparts

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Abstract

Methyllysine analogs (MLAs), furnished by aminoethylation of engineered cysteine residues, are widely used surrogates of histone methyllysine and are considered to be effective proxies for studying these epigenetic marks in vitro. Here we report the first structure of a trimethyllysine MLA histone in complex with a protein binding partner, quantify the thermodynamic distinctions between MLAs and their native methyllysine counterparts, and demonstrate that these differences can compromise qualitative interpretations of binding at the nucleosome-level. Quantitative measurements with two methyllysine-binding protein modules reveal substantial affinity losses for the MLA peptides versus the corresponding native methyllysine species in both cases, although the thermodynamic underpinnings are distinct. MLA and methyllysine adopt distinct conformational geometries when in complex the BPTF PHD finger, a well-established H3K4me3

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ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website. Experimental details; supporting text; characterization of purified proteins and peptides; and additional structural analyses (PDF).

Author Contributions

ZC and AJR performed all of the experiments and analyses presented. RQN and AJR devised the idea to quantitatively compare MLA binding, RQN cloned CBX5 and performed preliminary binding studies by FP. BU performed top-down MS on MLA histones. ZC and AJR wrote the manuscript with input from RQN.

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binding partner. In this case, a ~13-fold K_d difference at the peptide-level translates to nucleosomal affinities for MLA analogs that fall outside of the detectable range in a pull-down format, whereas the methyllysine species installed by native chemical ligation demonstrates robust binding. Thus, despite their facile production and commercial availability, there is a significant caveat of potentially altered binding affinity when MLAs are used in place of native methyllysine residues.

The histone proteins that package the genome into nucleosomal repeating units are subject to diverse post-translational modifications (PTMs) that play important roles in all aspects of genome management.^{1,2} Of these histone PTMs, mono, di, and trimethylation of ϵ -amine of lysine residues have been most extensively studied, and function almost exclusively by recruiting binding partners that recognize the mark in its flanking amino acid context.^{1–3} Mechanistic study of these binding events and their consequences for transcription, chromatin structure and other chromatin modification pathways, has critically relied upon *in vitro* reconstituted nucleosomes incorporating specifically methylated histones.^{4–14}

High purity histones bearing site-and-degree specific methylation marks can be prepared by native chemical ligation^{4,6,15,16} and for the lower methylation forms, genetically encoded unnatural amino acid mutagenesis.^{17,18} Yet both of these approaches require chemical and biochemical sophistication and a good deal of labor. To enable broader application of nucleosomal methyllysine experiments, Simon and colleagues developed an expedient protocol whereby methyllysines on histones are mimicked by aminoethylating cysteines installed in their stead to furnish various ϵ -methylated γ -thialysine adducts.¹⁹ These isosteric mimics, called methyl lysine analogs (MLAs) (Figure 1A, S1A), have been shown to interact with effector proteins and act as substrates for histone lysine methyltransferases in a qualitative manner.^{7,11,13,14,19–22} Due to their ease of preparation in scale, MLAs have become widely used in the study of histone methyllysines.

However, MLAs and methyllysines are not likely to be identical from the perspective of binding proteins or enzymes due to distinct chemical properties. Biologic thioethers have high conformational entropy and lower bond rotational barriers, and the polarizability and inductive electronic effects of this functional group are distinct.^{23,24} A comparison of ultra-high resolution small molecule crystal structures reveals a ~12° more acute bond angle for acyclic thioethers versus the expanded tetrahedral geometry of their corresponding aliphatic moieties (Figure S1B–C). Moreover, the average C-S bond length in the same set of compounds is ~20% longer than the corresponding alkane. To some extent the smaller bond angle compensates for the longer bond length, yet there is still a 0.3Å expansion of the distance between geminal methylene units in thioethers relative to the aliphatic moieties (Figure S1C).

Quantitative study of how these chemical perturbations of MLAs impact the interaction with cognate binding proteins has been pioneered by the Fischle group, revealing K_d decrements ranging from negligible to 8-fold for five different methyllysine binding proteins.^{10,25} Yet the structural and thermodynamic underpinnings of these effects remain unclear, and there have been no clear examples reported where MLA substitution compromises the interpretation of a nucleosome-level experiment. To address these mechanistic questions, we

compared the native and MLA forms of H3K4me3 and H3K9me3 with respective binding proteins by isothermal titration calorimetry (ITC), solved an MLA complex structure to elucidate binding differences, and examined whether binding deficiencies of MLAs can give rise to incorrect qualitative experimental interpretations at the nucleosome-level.

We first assessed the binding affinity by Isothermal Titration Calorimetry (ITC) of both native and MLA forms of H3K4me3 with one of its well-established cognate effector proteins^{26,27}: the PHD finger of human BPTF protein. We find the dissociation constant for binding to the BPTF PHD to the H3K4me3 peptide MLA to be ~13-fold weaker than that of the native H3K4me3 counterpart (Figure 1B and 1C). As a negative control, unmodified H3 tail peptide was titrated into the PHD finger and the binding observed was so weak that it precluded accurate K_d determination (Figure 1B and S2D). The binding deficit for the MLA manifests as an enthalpic effect with some entropy compensation. The enthalpic decrement is consistent with the H3K4me3-binding mode energetically counterbalanced by the introduction of strain or slight van der Waals repulsion within the MLA or pocket.

To understand the molecular underpinnings of this differential recognition, we crystallized the H3K4me3 peptide in complex with the BPTF PHD-finger and adjacent bromodomain (Figure 2A, S5A). To our knowledge, this is the first X-ray crystal structure of a trimethyllysine MLA co-crystallized with an effector protein. Despite ~13-fold lower affinity, the binding mode observed in our MLA complex structure is superficially similar to the BPTF the structure in complex with native H3K4me3 peptides at high resolution,²⁷ with the PHD finger domain displaying no significant deviations (Figure S5B). Given this similarity, we took pains during the structural refinement to properly model the geometry of the thioether. Specifically, the MLA bond lengths and angles were constrained to be within the standard deviation of both the mean and median parameters for acyclic thioethers from ultra-high resolution small molecule crystal structures in the Cambridge Structure Database (Figure S1B–C), and other geometry of the side chain forced to be consistent with that found in trimethyllysine. Nevertheless, there are differences in the H3 peptide conformation, localized to the MLA side chain largely as a consequence of expanded C-S bond lengths that are partially offset by a more compressed bond angle (Figure 2B–D). Compared to native methyllysine, the quaternary amine of the MLA projects slightly further into the pocket defined by the aromatic cage, the α -carbon of the MLA displays a slight distal shift, and the slightly compressed bond geometry about the thioether appear to reflect the spatial limits imposed by PHD binding cleft (Figure 2B–D). Given that the enthalpy term of the binding free energy bears the brunt of the differences in binding, and that the PHD-finger itself displays no structural alterations, we infer that strain and steric impingement – hints of which are seen in the MLA conformation – account for the bulk of this effect.

Do these subtle chemical and structural perturbations of MLAs undermine their fidelity as mimics for methyllysines on histones or in nucleosomal contexts? We next sought to examine multivalent readout of doubly modified nucleosomes by the BPTF PHD-bromodomain module that we had previously observed with histones made by chemical ligation^{8,28}, upon substitution with MLA H3K4me3 histone. In addition to the BPTF PHD finger binding H3K4me3,^{26,27} the adjacent bromodomain can bind H4K16ac acetyl marks adding apparent binding affinity for nucleosomes bearing both marks.^{8,28} Under identical

conditions, nucleosomes containing native H3K4me3 generated by chemical ligation^{4,16} are robustly captured in GST pull-downs, while nucleosomes bearing H3K_C4me3 are not discernably enriched (Figure 3B). Therefore, the substantial loss of binding affinity measured at the peptide level can manifest as a false negative interpretation of binding at the nucleosome-level in this experimental format.

To expand upon the generality of these conclusions, we performed another case study with the chromodomain of CBX5, which engages its cognate H3K9me3 ~6-fold more tightly than H3K_C9me3 (Figure 4). While the more unfavorable entropy dominates the overall affinity erosion, the enthalpy term is more favorable for the MLA relative to the H3K9me3 (Figure 4C). This could be due to the thioether's greater propensity²³ for adopting the partially eclipsed dihedral angle in the H3K9me3 structure²⁹ (Figure S5C–D). The aromatic-cage distal portion of the trimethyllysine-binding pocket in CBX5 is less constrained than the BPTF PHD (Figure S5E), such that it could accommodate the expanded MLA side chain by peptide backbone displacement. A similar conformation to this structure²³ would position the sulfur such that it could exploit its superior capacity for attractive van der Waals contacts with the hydrophobic pocket. In BPTF-PHD complexes, both MLA and trimethyllysine side chains are in fully extended “anti” conformations throughout (Figure S5F–G), displacement of the backbone is constrained by more restrictive binding groove sterics and the thioether sulfur projects into solvent (Figure 2B) so the mechanisms that may account for the favorable CBX5-H3K_C9me3 enthalpy would not be anticipated. Another non-mutually exclusive possibility could account for more favorable enthalpy in MLA binding CBX5: increased inductive electronic withdrawal of the thioether, evidenced by >10-fold higher ϵ -ammonium acidity for γ -thialysine relative to lysine,³⁰ may potentiate cation- π interactions.³¹ However such effects would be anticipated to be similar for the BPTF aromatic cage, unless offset by strain or sterics clashes during pocket engagement. While it is clearly the entropy term that drives the weaker binding for the MLA for CBX5, it is not obvious what entropy manifestation could be operant in the case of CBX5 binding that would not also be apparent in MLA engagement of BPTF.

Computing ΔG for the binding comparison for CBX5 and the two peptides yields a value of 4.7 ± 0.5 kJ/mol. Although more modest than a computational prediction of this binding difference (7.3 ± 1.1 kJ/mol),²⁵ our experimentally derived value is virtually identical to that reported for the related Cbx1 protein with peptides (FP 4.6 ± 1.1 kJ/mol; ITC 3 ± 1 kJ/mol) and nucleosomes (3 ± 2 kJ/mol).^{10,25} Interestingly, Seeliger et al. did not discern MLA binding deficits for the PHD-finger ING1 PHD finger, which is structurally similar to the BPTF-PHD.²⁵ Their work coupled with our current data strongly suggest that MLA binding energetics are idiosyncratic, and may not be readily predicted.

Regardless of the precise mechanistic and energetic details, in both cases examined here, the effector proteins engage the MLAs less tightly than native methyllysines, suggesting that that other effector proteins evolved to recognize native methyllysines may also not accommodate the methylene-to-sulfur substitution of MLAs. Indeed, binding affinity losses with MLAs and histone methyllysine binding proteins ranging from 5–8 fold have been observed for three other cases.^{10,25} When subject to quantitative analysis, MLAs do not behave identically to their native counterparts in biochemical assays and therefore

conclusions should be drawn with caution when MLAs are used in the study of histone methyllysines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MLA	methyllysine analog
NCL	Native chemical ligation
H3K4me3	histone 3 trimethylated on lysine 4
H3K9me3	histone 3 trimethylated on lysine 9

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Figure 1.

Quantitative comparison of binding partner engagement of methyllysine analogs (MLA)¹⁹ versus native counterparts. (A) The chemical structures of trimethyllysine (Kme3) and the MLA afforded by cysteine alkylation (K_Cme3) overlaid on a schematic representation of the Histone 3 positions examined here. (B,C) A quantitative comparison of binding affinities of the H3K4me3 to its the MLA counterpart, H3K_C4me3, and unmodified H3 peptide for the second BPTF PHD-finger by ITC. (D) Thermodynamic parameters derived from the ITC experiment.

Figure 2.

Comparative structural analysis of the BPTF PHD-finger bound to H3K_C4me₃. (A) Surface and ribbon representation of the BPTF PHD finger (gray, with aromatic cage residues rendered in purple, H3R2 hydrogen bonding side chains in orange) and Zn-coordinating residues in red), H3K_C4me₃ depicted in blue. (B) The methyllysine interface showing the spatial overlap of the MLA peptide ε-trimethyl-γ-thialysine (blue) with trimethyllysine residue from the prior structure²⁷ superimposed (cyan, PDB:2F6J). (C,D) The geometric parameters of Cβ-Cγ-Cδ and corresponding thioether from each structure. Lower panels depict the average bond lengths and angles from ultra-high resolution structures of acyclic aliphatic compounds and thioethers from the CSD (Fig S1B).

Figure 3.

Pull-downs reveal important mimicry deficits for nucleosomal MLAs engaging the BPTF protein that could lead to the false-negative binding inferences. (A) Schematic of nucleosome pull-down with the GST-tagged BPTF PHD-bromodomain module. (B) Unmodified (un, red), H4K16ac (ac, lavender), H3K4me3 (me, blue) and H3K4me3+H4K16ac (dual, orange) radiolabeled nucleosomes retained after substantial washing of glutathione-resin bearing either GST-BPTF PHD-bromodomain (PB) or GST alone.⁸ Modifications made by native chemical ligation (NCL, top panel) are compared to

the H3K_C4me3 methyllysine analogue paired with NCL produced H4K16ac (lower panel, as indicated) under identical conditions.

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Figure 4.

Measurement of MLA binding the CBX5 chromodomain by ITC. (A) Double background subtracted thermograms, and (B) Enthalpy of binding of trimethylated H3 lysine 9 peptide (green, 0.764mM H3K9me3 titrated into 0.0784mM CBX5) and the MLA counterpart (blue, 4.68mM H3K_c9me3 titrated into 0.41mM CBX5). (C) Thermodynamic parameters derived from the ITC experiment.