Analysis of JmjC Demethylase-Catalysed Demethylation using Geometrically-Constrained Lysine Analogues

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Abstract

The dynamic post-translational modifications of histones play important roles in the regulation of transcription in animals. The demethylation of N^{ε}-methyl lysine residues in the *N*-terminal tail of histone H3 is catalysed by demethylases, of which the largest family is the ferrous iron and 2-oxoglutarate dependent demethylases (JmjC KDMs), which catalyse demethylation via initial hydroxylation of the *N*-methyl groups. We report studies on the conformational requirements of the JmjC KDM substrates using *N*-methylated lysine analogues prepared by metathesis reactions of suitably protected *N*-allylglycine. The results support the proposed requirement for a positively charged N^{ε}-amino group in JmjC KDM catalysis. Demethylation of an *anti*-C-4/C-5 dehydrolysine analogue was observed with both KDM4E and KDM7B, which are predicted, based on crystallographic analyses, to bind the N^{ε}-methylated lysine residue in different conformations during catalysis. This information may be useful in the design of JmjC KDM selective inhibitors.

Introduction

Post-translational modifications to the *N*-terminal tail regions of histone proteins, in particular histone H3, play important roles in the regulation of eukaryotic gene expression.[1] It is proposed that the set of post-translational modifications to histone tails acts as a code that contributes to the regulation of expression.[2] The N^{ϵ}-amino groups of lysine residues on histones are subject to methylations, which may be transcriptionally activating or repressive depending on the context.[3] Control of lysine N^{ϵ}-methylation status, along with those of other post-translational modifications to chromatin associated proteins and nucleic acids is of major therapeutic interest for diseases including cancer.[4-6] An emerging body of evidence implies that many histone modifications, and their associated molecular interactions, are dynamic and complex. A detailed chemical understanding of the kinetics of transcriptional regulation requires knowledge not only of covalent modifications, but also of non-covalent changes during transcription, which are more difficult to measure. N^{ϵ}-Amino groups are subject to methylation, giving mono-, di- and tri- methylation states alongside the non-methylated forms.[7, 8] At least at some sites, lysine methylation is dynamic, with the action of *S*-adenosylmethionine-dependent methyltransferases being 'reversed' by the action of demethylases.[9-11] Demethylation is catalysed by flavin dependent lysine specific demethylases (KDM1s or LSDs), and the larger JmjC family of ferrous iron, oxygen and 2-oxoglutarate (2OG) dependent demethylases (JmjC KDMs).[7, 8, 12, 13]

Catalysis by both the KDM1 and JmjC KDM subfamilies requires oxidation of a methyl C-H bond on the N^{ϵ}-methyl group. KDM1 catalysis is coupled to the reduction of flavin adenine dinucleotide (FAD) and likely proceeds *via* a hydride transfer from the methylated lysine in a process dependent on the unprotonated state / lone pair of

the N^{ϵ}-nitrogen.[14] Hence, the KDM1 enzymes do not act, as far as is known, on the N^{ϵ}-trimethylated state.

JmjC KDM catalysis proceeds via the 'direct' hydroxylation of the substituted N^{ϵ}-methyl group and consequently can occur on all three N^{ϵ}-methylation states (**Figure 1**). Sequential binding of the iron cofactor, 2OG, methylated histone substrate and oxygen occurs; oxidative decarboxylation results in a reactive iron(IV)-oxo intermediate that inserts an oxygen atom into the methyl group C-H bond, thereby reducing the iron(IV) species to iron(II). The resulting hemiaminal intermediate is unstable, fragmenting to form the demethylated product and formaldehyde.[12] The JmjC KDMs are classified into five subfamilies on the basis of sequence / structural homologies, the presence of 'non-catalytic' domains and their substrate selectivity. The JmjC family includes proteins that catalyse protein hydroxylations other than an *N*-methyl group, some of relevance in the regulation of protein biosynthesis. Recent work with histone fragment peptides containing lysine analogues has shown that some JmjC KDMs can catalyse reactions other than demethylation including *N*-dealkylation of groups other than methyl-, and reaction to give stable alcohol products.[15] These studies imply that the role of JmjC KDM catalysis in cells may be more complex than simple N^{ϵ}-methyl group demethylation.

Crystallographic analyses of the catalytic domains of some JmjC KDMs complexed with fragments of their histone substrates have revealed different conformations for the lysine side chains, sometimes extended as in the KDM4 enzymes, or sometimes not as in KDM7B. The use of conformationally constrained and differently charged lysine residue analogues has potential for probing charge and conformational requirements in KDM catalysis; however, a hindrance to studies with lysine analogues (and indeed with methylated lysines) has been the lack of efficient

synthetic approaches. We report methods for the synthesis of N^{ε}-methylated lysine analogues conformationally constrained by the presence of an alkene and in which the N^{ε}-amino group is replaced by carbon and oxygen. Four analogues were incorporated into histone fragment peptides and tested for JmjC KDM activity. The results imply that, at least for the JmjC KDM KDM4E, a positively-charged N^{ε}-amino group is required for efficient demethylation. Both KDM4E and KDM7B were observed to catalyse demethylation of a C4-C5-*anti*-dehydrolysine analogue; this is interesting because crystallographic analyses predict that the two enzymes bind the N^{ε}-methylated lysine residue in different conformations about the C4-C5 bond, suggesting a requirement for flexibility in substrate binding during catalysis. The results are of relevance to both mechanistic work and the design of KDM inhibitors.

Results

Synthesis of Methylated Lysines and Analogues *via* Olefin Cross Metathesis

We envisaged that olefin cross metathesis using *N*-Fmoc-allyl-L-glycine (**1**) would be a useful methodology for the synthesis of methylated lysines and analogues (**Scheme 1**). Enantiopure allylglycine is commercially available or can be readily prepared by efficient enzymatic resolution of easily prepared synthetic racemic allylglycine.[16] We initially tested the route by reacting *N*-Boc-allylamine (**2**) with **1** via cross-metathesis using the 'second generation' Hoveyda-Grubbs catalyst; the un-optimised reaction proceeded in reasonable yield (63 %) to give the lysine analogue *N*2-Fmoc-*N*6-Boc-4,5-(*E*)-dehydrohexanoic acid (**3**) in an Fmoc protected form suitable for use in solid phase peptide synthesis. NMR characterisation of **3** (or indeed other analogues) did not afford accurate *J*-coupling values for the alkenyl protons due to peak overlap; however the geometry about the double bond was

assigned as *trans* based on Nuclear Overhauser Effect correlation patterns observed between the alkenyl protons and the protons at C3 and C6 (**Figure S1**). *N*2-Fmoc-*N*7-Boc-4,5-(*E*)-dehydroheptanoic acid (**4**), an analogue of **3** with an additional methylene unit in the side chain, was also synthesised *via* the reaction of **1** with 1-(Boc-amino)-3-butene (**5**, 68 %). Reduction of **4** using H_2/Pd/C afforded the saturated *N*2-Fmoc-*N*7-Boc-heptanoic acid (**6**) in good yield (93 %).

Attention then focused on the synthesis of N^{ϵ}-methylated lysines. *N2*-Fmoc-*N6*-Boc-*N6*-monomethyl-4,5-(*E*)-dehydrohexanoic acid (**7**) was synthesised from **1** *via* cross-metathesis with *N*-Boc-methylallylamine (**8**, 46 %), which was converted to the saturated analogue *N2*-Fmoc-*N6*-Boc-*N6*-monomethyl-hexanoic acid (**9**) via reduction of the 4,5-double bond using H_2/Pd/C (84 %). Attempted 'direct' synthesis of the dimethylated variant *N2*-Fmoc-*N6*-dimethyl-4,5-dehydrohexanoic acid **10** using *N*-dimethylallylamine, or the analogous trimethylated variant *N2*-Fmoc-*N6*-trimethyl-4,5-dehydrohexanoic acid using *N*-trimethylallylamine, did not produce the desired products, at least efficiently, instead resulting in degradation of the metathesis catalyst. The synthesis of **10** was achieved from **3** *via* N^{ϵ}-Boc deprotection and subsequent reductive methylation of the free amine using formaldehyde and sodium cyanoborohydride (97 % over 2 steps).

The cross-metathesis method was then applied to the synthesis of analogues not containing an N^{ϵ}-amine. 4,5-Unsaturated C7-monomethylated and C7-dimethylated 2-amino-octanoic acid analogues **11** and **12**, which contain a carbon atom in place of the lysyl N^{ϵ}-amine, were synthesised in good yields from **1** using the metathesis procedure and 1,2-dehydro-4-methylpentane (**13**) and 1,2-dehydro-4-dimethylpentane (**14**), respectively (72 % and 74 % respectively). Hydrogenation of **11** and **12** afforded the corresponding 4,5-saturated variants **15** and **16** in near

quantitative yield (>95 %); in the case of **16**, the metathesis route is a significant improvement on reported methods (20 % over 4 steps).[17, 18] Reaction of **1** with allylmethylether (**17**) afforded 4,5-unsaturated 2-amino-6-methoxy-heptanoic acid (**18**, 69 %). Hydrogenation of **18** afforded the methyl ether variant **19** in good yield (84 %).

Biochemical Studies

We then incorporated selected examples of the lysine analogues into histone H3 *N*-terminal sequences in order to test whether JmjC KDMs (i) require a positive charge at the N^{ε}-position, (ii) can catalyse *O*-methyl demethylation (as observed for other 2OG oxygenases),[19] and (iii) to investigate the catalytically productive conformation of the lysine side chain. The Fmoc-protected amino acids **6**, **10**, **16** and **19** were individually incorporated into the human histone H3 *N*-terminal fragment peptides (residues 1-15, sequence ARTKQTAR-lysine analogue-STGGKA) using Fmoc solid phase peptide synthesis (**Figure S2**). In the case of **10**, the alkene was found to be sufficiently stable during the peptide synthesis; ^{1}H NMR analyses of two peptides containing **10** (see peptides **P1** and **P3** below) also revealed ^{2}J-coupling values of 15.5 Hz between the alkenyl protons, supporting a *trans* configuration about the double bond. After purification by high-performance liquid chromatography, the peptides were incubated with recombinant JmjC KDM KDM4E, which can be easily prepared in *Escherichia coli* and catalyses the relatively efficient demethylation of N^{ε}-methylated lysine residues in histone fragments.[15, 20-22]

The peptide containing analogue **10** (peptide **P1**,10 μM) was first incubated with KDM4E (2 μM), 2OG (50 μM), Iron (II) (10 μM) and ascorbate (100 μM) in 50 mM HEPES (pH 7.5) at 37 °C. MALDI-TOF mass spectra were obtained and comparison

with a no-enzyme control showed apparent demethylation of the analogue under standard assay conditions (as evidenced by mass shifts of -14 Da and -28 Da relative to the substrate peptide, **Figures 2A** and **S3**). Demethylation was also observed by ^{1}H NMR analyses, which revealed an increase in 2OG oxidation (to form succinate) upon incubation with the peptide (**Figure 2B**). Notably, succinate production was markedly increased in the NMR experiments in the presence of peptide, although peptide demethylation was relatively inefficient; this finding may indicate that the substrate analogue **10** can adopt binding modes in the KDM4E active site that stimulate 2OG conversion to succinate but are not amenable to efficient demethylation (note: such effects have been observed for KDM4E with other substrates).[15] Overall, the MS and NMR experiments confirm that the unsaturated analogue **10** is accepted as a substrate of KDM4E, albeit a non-optimal one, resulting in demethylation of the N^{ε}-methyl groups.

A 1:1 competition assay was then conducted between peptide **P1** and an analogous 'wild-type' peptide (peptide **P2**), which alongside N^{ε}-dimethyl-L-lysine and the K9 position (*i.e.* the equivalent position as **10** in peptide **P1**), also contained trimethylation at K4, thereby allowing the two peptides to be distinguished during MS analysis. Both peptides were demethylated; however, peptide **P2**, *i.e.* the N^{ε}-dimethyl-L-lysine containing peptide, was the preferred substrate for KDM4E (**Figure 2C**).

Analyses were then carried out using KDM7B, which also catalyses demethylation of N^{ε}-dimethyl-L-lysine at position 9 of histone H3, but which belongs to a different subfamily of JmjC KDMs.[23] Crystallographic analyses of KDM7B in complex with its substrate (histone fragment incorporating N^{ε}-dimethyllysine at position 9, PDB ID: 3KV4)[24] have predicted that the N^{ε}-methylated lysine residue adopts a more '*syn*-

type' conformation about its C4-C5 bond in the active site. This conformation is different to the crystallographically observed '*anti*-type' conformation adopted by the N^{ϵ}-methylated lysine residue when bound to KDM4 enzymes (for structural comparison of substrate binding to KDM4 and KDM7 subfamilies, see **Figure 3**). In order to test whether analogue **10** may be a substrate for KDM7B, a KDM7B substrate peptide trimethylated at position 4 (to promote binding within the PHD domain of KDM7B)[24] and containing analogue **10** at position 9 (peptide **P3**, sequence ART-K(Me_3)-QTAR-**10**-STGGKA) was synthesised. Interestingly, KDM7B catalysed demethylation of peptide **P3** was observed after two hours at 37 °C (**Figure 4A**). In a 1:1 competition assay with an analogous peptide containing N^{ϵ}-dimethyl-L-lysine at position 9 (peptide **P4**, sequence ART-K(Me_3)-QTAR-K(Me_2)-STGGK), the natural sequence was more efficiently demethylated, although demethylation of both peptides was observed (**Figure 4B**).

Kinetic parameters for the demethylation of peptides containing analogue **10** were determined by analysing formaldehyde production using NAD^+ activated formaldehyde dehydrogenase oxidation (**Figure S4**).[25] Although the absolute values should be treated with caution due to the occurrence of demethylation uncoupled 2OG turnover and trace impurities within the peptide batches, the results are consistent with the peptides containing **10** being relatively efficient substrates of both KDM4E (k_{cat}/K_M for peptide **P1** = 0.00177 μM^{-1} s^{-1}, k_{cat}/K_M for peptide with sequence ARTKQTAR-K(Me_2)-STGGKA (peptide **P5**) = 0.00215 μM^{-1} s^{-1}), and KDM7B (k_{cat}/K_M for peptide **P3** = 0.00059 μM^{-1} s^{-1}, k_{cat}/K_M for peptide **P4** = 0.00020 μM^{-1} s^{-1}). Overall, the combined kinetic and competition experiments reveal that analogue **10**, when incorporated into histone peptide fragments, is a relatively

efficient substrate for KDM4E and KDM7B, displaying comparable, albeit slightly weaker, activity than the corresponding N^{ε}-dimethyl-L-lysine-containing peptides.

Experiments were undertaken to determine whether lysine analogues without a positively-charged N^{ε}-amino group may be accepted as substrates by KDM4E. Peptides containing analogues **6**, **16** and **19** (sequence ARTKQTAR-lysine analogue-STGGKA) were tested for reaction with KDM4E; however, no reactions were observed either during MS or NMR experiments. It is therefore likely that the position and charge of the ε-amine is important for KDM reaction.

Conclusions

Olefin cross metathesis is an efficient method for synthesising 4,5-unsaturated L-lysine analogues for incorporation into peptide synthesis. The metathesis method was successfully applied to synthesise lysine analogues containing 4,5-*trans*-double bonds and saturated analogues (via hydrogenation) that do not contain an N^{ε}-amine. Studies employing NMR and MS assays with KDM4E and KDM7B, which catalyse demethylation of an N^{ε}-dimethyl-L-lysine residue at position 9 of histone H3, reveal that peptides containing appropriately positioned dimethylated analogue **10**, which contains a 4,5-*trans*-double bond, are accepted as a substrate by both enzymes. Kinetic assays monitoring formaldehyde production imply that the peptides containing analogue **10** are of similar efficiency to the natural N^{ε}-methylated lysine substrates, but competition assays imply the analogues are less well accepted; the substrate analogues also cause increased decoupling of 2OG turnover and demethylation.[26]

Analyses of X-ray crystal structures of KDM4 enzymes complexed with histone substrate peptides suggest that the methylated lysine residue adopts an *anti*-

conformation about the 4,5-bond during catalysis (**Figure 3**).[27] It is therefore plausible that analogue **10**, which contains the conformationally rigid 4,5-*trans*-double bond, may be accommodated in the active site of KDM4E in a similar geometry to the natural substrate, reflected in their similar demethylation efficiencies. However, crystallographic studies on KDM7B indicate that N^{ε}-dimethyl-L-lysine likely adopts a more '*syn*-type' conformation about its 4,5-bond in the active site (**Figure 3**),[24] suggesting that analogue **10** may bind in a different geometry to the natural substrate in KDM7B, and/or that there is some conformational flexibility in the KDM7B active site during catalysis. These observations will be useful for characterising the mechanism of KDM7B-catalysed demethylation, and will also be of relevance to inhibitor development studies.

With KDM4E, no reaction was observed with either the uncharged carba-analogue of N^{ε}-trimethyl-L-lysine (**16**) or the methoxy-containing variant **19**, revealing the importance of a charged ε-amino group for efficient catalysis by KDM4E. These findings support previous studies with KDM4E, where H3 fragment peptides containing uncharged acetyl-, formyl- and crotonyl-L-lysine residues at position 9 were found not to be substrates.[15] However, *O*-demethylation by 2OG oxygenases occur during morphine biosynthesis[19]. Thus, a positively charged group is not an absolute requirement for 2OG oxygenase catalysed demethylation. Another 2OG oxygenase, γ-butyrobetaine hydroxylase, can catalyse hydroxylation, albeit at a much reduced rate, of a carba-analogue of γ-butyrobetaine containing a *tert*-butyl group in place of a trimethylamino group (note: although the trimethylamino group of γ-butyrobetaine binds in a hydrophobic cage, related but different to that in the JmjC KDMs, it does not (normally) catalyse demethylation).[28] The observation that KDM4E, and by implication other JmjC KDMs, requires a charged group for

demethylation/hydroxylation is of interest both with respect to inhibition work and mechanistic analyses.

Figures

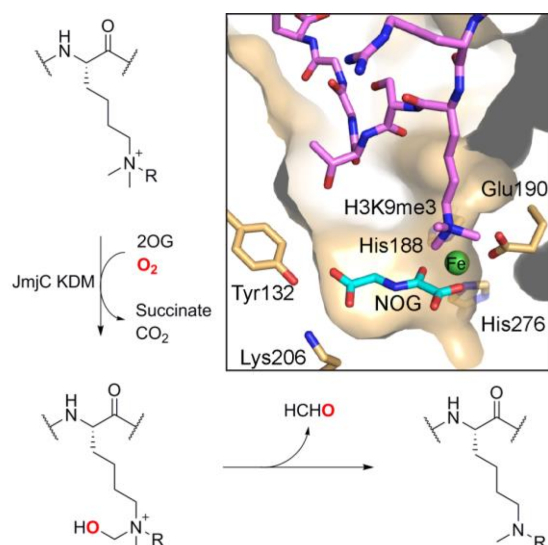

Figure 1. Histone N^{ε}-Methyl Lysyl Demethylation Catalysed by JmjC KDMs. The methylated lysine residue is hydroxylated on its N^{ε}-methyl group via reaction with an enzyme-bound Fe(IV)-oxo intermediate, which is formed via oxidative decarboxylation of 2-oxoglutarate (2OG). The resulting hemiaminal is unstable, fragmenting to form the demethylated product and formaldehyde (HCHO). R = CH_3 or H. The inset shows a view from an X-ray crystal structure of the JmjC KDM KDM4A in complex with *N*-oxalylglycine (NOG, a 2OG mimetic) and a histone H3 fragment peptide trimethylated at lysine 9 (PDB ID: 2OQ6).[27]

Hoveyda-Grubbs II

CH_2Cl_2

H_2, Pd/C

1

X = NHBoc: **2**
CH_2NHBoc: **5**
$N(CH_3)Boc$: **8**
$C(CH_3)_2$: **13**
$C(CH_3)_3$: **14**
OMe: **17**

X = NHBoc: **3** (63 %)
CH_2NHBoc: **4** (68 %)
$N(CH_3)Boc$: **7** (46 %)
$C(CH_3)_2$: **11** (72 %)
$C(CH_3)_3$: **12** (74 %)
OMe: **18** (69 %)

X = CH_2NHBoc: **6** (93 %)
$N(CH_3)Boc$: **9** (84 %)
$C(CH_3)_2$: **15** (96 %)
$C(CH_3)_3$: **16** (97 %)
OMe: **19** (84 %)

HCOOH

HCHO, $NaCNBH_3$

MeOH

3

10 (97 %)

Scheme 1. Synthesis of methylated lysines and analogues by cross-metathesis. N^{α}-Fmoc protected 4,5-unsaturated analogues were prepared from N^{α}-Fmoc-allylglycine **1** using the ‘second generation’ Hoveyda-Grubbs catalyst (upper scheme). The dimethylated analogue **10** was prepared from the unmethylated variant **3** via Boc deprotection followed by reductive methylation (lower scheme).

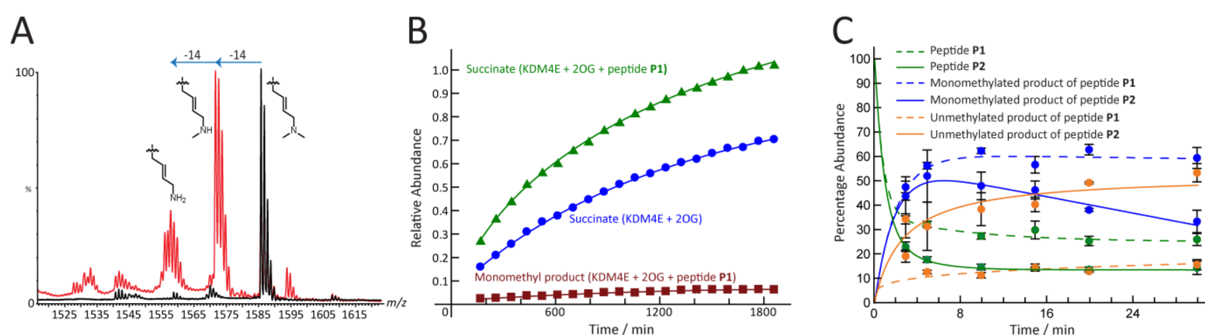

Figure 2. A histone fragment peptide containing *N*$^{\varepsilon}$-dimethyl-4,5-dehydrolysine (10) at position 9 is a KDM4E substrate. (A) MALDI-TOF mass spectra showing KDM4E-catalysed demethylation of an H3 fragment peptide with **10** at position 9 (peptide **P1**, sequence ARTKQTAR-**10**-STGGKA), after one hour at 37 °C. The spectrum of a no enzyme control is in black. (B) KDM4E-catalysed succinate production in the presence (green) and absence (blue) of peptide **P1**. Succinate production is stimulated in the presence of peptide, though most succinate production is uncoupled with respect to peptide demethylation (red). Reactions were monitored by ^{1}H NMR (700 MHz). Abundances are relative to the maximum succinate production observed. (C) KDM4E-catalysed demethylation of a H3 fragment peptide containing *N*$^{\varepsilon}$-dimethyl-L-lysine (peptide **P2**, sequence ART-K(Me_3)-QTAR-K(Me_2)-STGGKA) and peptide **P1** (competition experiment, 1:1 mixture). Demethylation of peptide **P2** (containing *N*$^{\varepsilon}$-dimethyl-L-lysine) is more rapid than that of peptide **P1** (containing analogue **10**). Proportions of starting materials and products were calculated from MALDI-TOF MS ion abundance of the starting materials versus the monomethyled species. Note: peptide **P2**, which is also trimethylated at position 4, was used in order to distinguish between it and peptide **P1** (and their demethylated products) by MS.

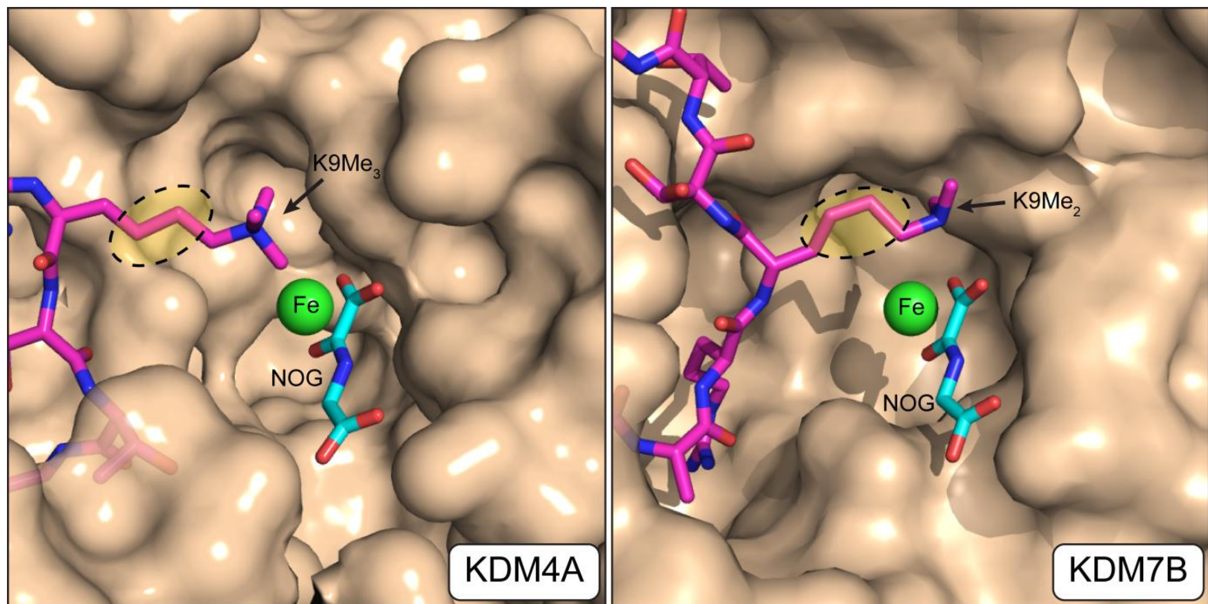

Figure 3. Comparison of N^{ε}-methyllysine substrate binding in the active sites of KDM4A and KDM7B. (Left) View from an X-ray crystal structure of a histone 3 fragment peptide trimethylated at K9 in the active site of KDM4A, a close homologue of KDM4E (PDB ID: 2OQ6).[27] The peptide backbone binds in a cleft along the enzyme surface, with the N^{ε}-trimethyl-L-lysine side-chain protruding into the active site. The lysyl side-chain is observed to adopt an *anti*-conformation about its 4,5 bond (C3-C4-C5-C6 dihedral angle = -174°). (Right) View from an X-ray crystal structure of a histone 3 fragment peptide dimethylated at K9 complexed in the KDM7B active site (PDB ID: 3KV4).[24] In this structure, the methylated lysine residue protrudes more deeply into the active site; note that the lysine side-chain does not adopt an *anti*-conformation about the 4,5-bond (C3-C4-C5-C6 dihedral angle = -62°).

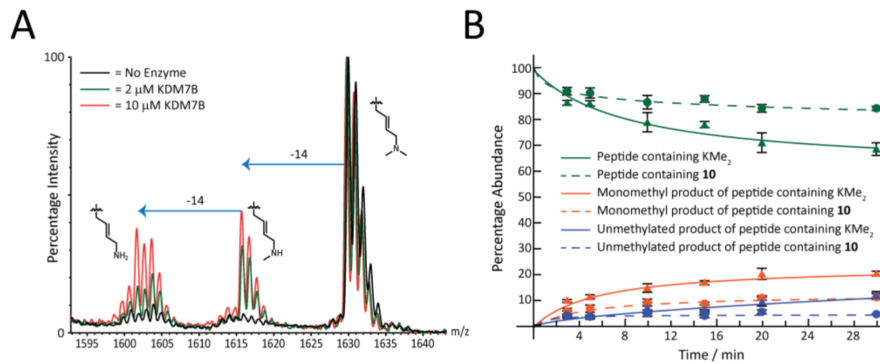

Figure 4. A histone fragment peptide containing N^{ε}-dimethyl-4,5-(*E*)-dehydrolysine (10) at position 9 is a KDM7B substrate. (A) MALDI-TOF mass spectra showing demethylation of an H3 fragment peptide containing N^{ε}-trimethyl-L-lysine at position 4 and analogue **10** at position 9 (peptide **P3**, sequence ART-K(Me3)-QTAR-**10**-STGGKA). Demethylation is observed. (B) KDM7B-catalysed demethylation of an H3 fragment peptide containing N^{ε}-dimethyl-L-lysine (peptide **P4**, sequence ART-K(Me_3)-QTAR-K(Me_2)-STGGK) and peptide **P3** (competition experiment, 1:1 mixture). Demethylation of the peptide **P4** is preferred. Note: peptide **P4** contains 14 residues in order to distinguish between it and peptide **P3** (and their demethylated products) by MS.

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