NMR Profiling of Histone Deacetylase and Acetyl-transferase Activities in Real Time

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Supporting Information

ABSTRACT: Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are universal regulators of eukaryotic transcriptional activity and emerging therapeutic targets for human diseases. Here we describe the generation of isotope labeled deacetylation and acetylation reporters for simultaneous NMR readouts of multiple deacetylation and acetylation reactions at different histone H4 sites. The site preferences of two prototypic histone deacetylases (Sir2.1 and HDAC8) and two acetyl transferases (HAT1 and p300/CBP) were studied in intramolecular competition assays. We identify a previously ill defined acetylation site, lysine 20 of histone H4, as a preferred target of three of these enzymes. In situ analyses of endogenous deacetylation reactions at H4 sites in HeLa nuclear extracts point to abundant HDAC activities in human cellular environments.

Reversible lysine acetylation is a common post translational protein modification with over 1700 identified substrates in the human proteome.1 Histone proteins and more specifically their N terminal ‘tail’ regions are among the most prominent acetyl transferase targets. Histones package DNA into chromatin, and acetylation of their ‘tail’ regions establishes, in part, the ‘epigenetic chromatin code’ by promoting permissive (acetylated) or repressive (deacetylated) states of gene expression.25 Histone acetylation is catalyzed by a set of enzymes collectively referred to as histone acetyl transferases, or HATs.6 Histone deacetylases, or HDACs, perform the ‘opposite’ reaction and selectively remove acetyl moieties from modified lysine residues.7 Because of their roles as general transcriptional modulators, HATs and HDACs have emerged as promising drug targets for a number of human diseases.8 A detailed knowledge about the enzymatic properties of HATs and HDACs, in terms of site preferences and kinetics, is essential for understanding the complex regulation of transcriptional activity.9

Existing biochemical assays to monitor lysine acetylation in vitro typically employ readout mechanisms like the incorporation or release of radioactive probes, fluorophores, or dyes.10–12 While these methods are widely used to study the catalytic properties of HATs and HDACs, they are generally limited to observing modification reactions at single substrate sites only. In Vivo, reversible lysine acetylation often occurs simultaneously at multiple sites, which emphasizes the need for tools to unveil the mechanistic properties and dynamics behind such complex modification events. Here, we report the generation of peptide based NMR reporters for direct readouts of histone specific HAT and HDAC activities by high resolution NMR measurements. Our approach includes a chemical synthesis scheme for the incorporation of stable, NMR active isotopes at selected lysine positions within histone tails. These NMR reporters then enable time resolved observations of multiple lysine acetylation events in parallel and in a continuous and quantitative manner. We demonstrate the feasibility of our NMR approach by analyzing the catalytic properties of HATs and HDACs at individual lysine residues within the highly sequence degenerate, N terminal tail region of histone H4 (Figure 1a).

Acetylated lysine residues display two characteristic features in 1H/15N correlation experiments.13,14 Backbone amide resonances (1H/15Nε) experience upfield chemical shift changes of 0.1–0.2 ppm, whereas acetylated lysine side chains give rise to additional 1H/15Nε cross peaks that correspond to the newly established side chain amide groups (Figure 1b). Because these 1H/15Nε signals are only present when the distal amino groups of lysine residues are acetylated, they serve as general acetylation indicators.13,14 In fact, most 1H/15Nε signals of acetylated lysines display very similar resonance frequencies (7.95/127 ppm), and indicator signals of multiple acetylated lysine residues often superimpose as one resonance crosspeak. In contrast, backbone amide chemical shift changes more specifically function as ‘acetyl lysine identifiers’ because they unambiguously map the sites of acetylation. Together, both NMR signals report the acetylation states of individual lysine residues in a protein of interest. We have previously shown that acetylation of

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recombinant, uniformly $^{15}$N labeled fragments of cmyc and histone H3 can be directly monitored by time resolved NMR measurements.\textsuperscript{13} However, many HAT substrates contain a debilitating degree of sequence degeneration and, conversely, confounding signal overlap in their backbone amide resonances. One such example is the N terminus of histone H4, for which, in a uniformly labeled form, a sequence specific readout of acetylation events by NMR spectroscopy is not possible. (Supporting Figure S1). The backbone amides ($^1$H/$^{15}$N) of many of the known H4 acetylation sites (Lys5, Lys8, Lys12) resonate at very similar NMR frequencies so that the mapping capacity of NMR spectroscopy is unable to resolve which of the possible acetylation sites does indeed get modified. We reasoned that synthetic NMR reporters, designed in a way to allow for maximum chemical shift dispersion of both the modified and unmodified lysine residues could resolve this problem.

In a first step, we employed solid phase peptide synthesis and classical protocols for orthogonal lysine protection to prepare $^{15}$N\textsubscript{O} and $^{15}$N\textsubscript{e} labeled building blocks (Supporting Scheme S1).\textsuperscript{15} Because NMR signals of Lys5, Lys8, and Lys12 were not well resolved, we incorporated $^{15}$N\textsubscript{O} labels at Lys16 and at one of the overlapped positions, i.e., Lys12. In order to monitor the acetylation state of one additional superimposed site, we incorporated a side chain $^{15}$N\textsubscript{e} label at Lys5. Because N\textsubscript{e} amides of acetylated lysine side chains resonate at very different chemical shift dispersion of both the modified and unmodified lysine residues, although at significantly different rates (Figure 3b). As a result, acetylation of Lys12 turns Lys5 into a reasonably weak, secondary modification event by NMR spectroscopy is not possible. (Supporting Figure S1). The enzyme exclusively modified Lys12 and Lys5 of H4.\textsuperscript{16}A series of time resolved 2D $^1$H/$^{15}$N correlation experiments initially revealed exclusive acetylation of Lys12 (Figure 3a). After completion of Lys12 acetylation, modification of Lys5 ensued in a second reaction step. Thus, our NMR HAT reporter correctly identified the known acetylation sites of HAT1 and additionally provided mechanistic insights into the previously unknown modification behavior of the enzyme. On the basis of our data, we conclude that Lys12 of histone H4 constituted the preferred substrate site for HAT1. The enzyme exclusively modified Lys12 until all substrate molecules were acetylated at this position. Only then did HAT1 modify Lys5. These reaction characteristics are consistent with the notion that the positive charge of Lys12 renders Lys5 an unfavorable substrate site.\textsuperscript{17} As a result, acetylation of Lys12 turns Lys5 into a reasonably weak, secondary modification site. Next, we probed the acetylation behavior of recombinant human p300/CBP. Here, we observed the joint acetylation of all four histone H4 tail lysines in every single reaction (Figure 2a and b).

In a first instance, we reacted the unmodified HAT reporter with recombinant HAT1 from yeast, an acetyl transferase known to modify Lys12 and Lys5 of H4.\textsuperscript{16} A series of time resolved 2D $^1$H/$^{15}$N correlation experiments initially revealed exclusive acetylation of Lys12 (Figure 3a). After completion of Lys12 acetylation, modification of Lys5 ensued in a second reaction step. Thus, our NMR HAT reporter correctly identified the known acetylation sites of HAT1 and additionally provided mechanistic insights into the previously unknown modification behavior of the enzyme. On the basis of our data, we conclude that Lys12 of histone H4 constituted the preferred substrate site for HAT1. The enzyme exclusively modified Lys12 until all substrate molecules were acetylated at this position. Only then did HAT1 modify Lys5. These reaction characteristics are consistent with the notion that the positive charge of Lys12 renders Lys5 an unfavorable substrate site.\textsuperscript{17} As a result, acetylation of Lys12 turns Lys5 into a reasonably weak, secondary modification site. Next, we probed the acetylation behavior of recombinant human p300/CBP. Here, we observed the joint acetylation of all four lysines, although at significantly different rates (Figure 3b). Surprisingly, p300/CBP also acetylated Lys20 in an efficient manner. The implications of this observation will be discussed later in the text.

In the following we studied histone deacetylation reactions. Because HDACs play important roles in gene regulation, they represent attractive biomedical targets for therapeutic intervention of aberrant gene activities in a number of human diseases.\textsuperscript{18} To characterize the deacetylation properties of different HDACs, the respective substrate molecules must be available in preacetylated forms. This is another advantage that a chemical synthesis
scheme provides over recombinant protein production: the ability to produce preacetylated versions of H4 reporters in a site-specific manner (Figure 2b). We first analyzed the deacetylation behavior of Sir2.1 from *C. elegans*. A member of class III HDACs, also referred to as sirtuins, this enzyme requires NAD$^+$ as a cofactor. *In situ* NMR recordings of Sir2.1 mediated deacetylation reactions revealed that Lys16 and Lys20 were efficiently processed, whereas there was no detectable deacetylation of Lys5 and Lys12 (Figure 4a). Preferential deacetylation of Lys16 of histone H4 is in agreement with published data for other sirtuins. 19 In contrast, Lys20 deacetylation by sirtuins has not been reported before. Again, deacetylation of this site by an enzyme from *C. elegans* was surprising given the fact that Lys20 acetylation is not known to occur in metazoans and has only been described in plants. 20 Our results demonstrate the unique ability of NMR spectroscopy, in conjunction with the multisite deacetylation reporter, to uncover Sir2.1’s site preferences for acetylated H4 tail lysines in a single run of consecutive NMR experiments. This underscores another important aspect of our NMR-based approach: the characterization of Sir2.1 only required a single sample and, conversely, only a small amount of enzyme. This is especially useful when recombinant HDACs of classes I and II are to be studied, as these enzymes are difficult to produce. 21, 22 We took advantage of this feature to also analyze a commercially available class I deacetylase, the human enzyme HDAC8. With regard to HDAC8’s substrate specificity, there is conflicting evidence as to whether this enzyme deacylates histone H4 sites at all. 23, 24 To address this question, we performed a time resolved deacetylation assay using our HDAC reporter. HDAC8 was less active in deacetylating H4 than Sir2.1, and the enzyme exclusively processed the modified Lys20 site (Figure 4b). Taken together, our results indicate that HDAC8, as well as Sir2.1 and p300/CBP, displays substantial enzymatic activities toward a lysine residue of the histone H4 tail that is not known to represent a *bona fide* acetylation site. 20 This led us to investigate whether acetylated Lys20 also constituted a substrate site for endogenous HDACs in cell free extracts of cultured human cells. We incubated the HDAC reporter in organelle fractioned, nuclear extracts of human HeLa cells and directly followed the site specific deacetylation reactions in the lysate suspension. Progressive deacetylation of all substrate sites, including Lys20, was readily observed in consecutive, time resolved NMR experiments (Figure 4c). This indicates that endogenous cellular enzymes can, in principle, deacetylate the histone H4 Lys20 site when presented in a tail peptide context. Given the notion that Lys20 acetylation/deacetylation has not been observed in metazoan systems before, the question arises as to whether these modifications ever occur in a physiological context. Here, one observation may be of particular importance: *In Vivo*, Lys20 of histone H4 is a virtually constitutive methylation site. 26 Although stable Lys20 methylation may prevent acetylation, cellular deacetylase activities that target Lys20 might ensure that this site is kept available for methylation only. 27 An alternative explanation involves the chromosomal context of histones and their N terminal tail regions as integral parts of nucleosomes. These environmental differences may critically modulate some of the observed modification characteristics and could render Lys20 inaccessible for HAT and HDAC enzymes *In Vivo*. In this regard, it is interesting to note that the sequence context of Lys20 of histone H4 (Arg His Arg Lys) displays a high degree of similarity to Lys382 of the human transcription factor and oncoprotein p53 (Arg His Lys Lys). Moreover, all of the enzymes found to modify Lys20 in our study, p300/CBP, Sir2.1, and HDAC8, are also known to modify Lys382 of p53. 10, 28, 29 This may relate to the fact that Lys20 in our reporter peptides, as well as Lys382 in wild type p53 reside in C terminally ‘unrestrained’ environments that may provide optimal access for the above enzymes.

Figure 3. Acetylation assay with the HAT reporter. (a) Time resolved acetylation reaction of the HAT reporter in the presence of HAT1. (b) p300/CBP catalyzed acetylation of the HAT reporter. All reactions were performed with a HAT reporter concentration of 200 μM at 295 K.
In summary, we have reported a versatile set of tools for monitoring histone H4 acetylation and deacetylation reactions by NMR spectroscopy. In contrast to conventional assays, our approach enables direct in situ studies of multiple modification reactions in parallel and in a time resolved and quantitative fashion. We delineated the acetylation/deacetylation behaviors of well known HATs (HAT1, p300/CBP) and HDACs (Sir2.1, HDAC8) and identified Lys20 of histone H4, a hitherto ill defined acetylation site, as being efficiently modified by these enzymes in vitro, as well as by endogenous HDACs in nuclear extracts of human HeLa cells.

**METHODS**

**Synthesis of $^{15}$N-Labeled Building Blocks and Recombinant Production of the Homogeneously Labeled H4 Tail.** Detailed information is provided in the Supporting Information.

**Solid-Phase Peptide Synthesis.** NMR reporters were synthesized on a 15 µmol scale using standard Fmoc based solid phase chemistry on an Intavis Resep XL synthesizer. TentaGel R RAM resin (cap. 0.19 mmol/g) was used as solid support, and amino acid side chains were protected as follows: Arg(Pbf), Asn(Trt), Asp(OtBu), His(Trt), Lys(Boc), and Ser(tBu). Coupling reactions were achieved by using 2 (1H benzotriazole 1 yl) 1,1,3,3 tetramethyluroniumhexafluorophosphate (HBTU) as the activation agent and N methylmorpholine (NMM) in DMF/NMP as base. Each successive amino acid was doubly coupled in 5 fold molar excess. Isotope labeled derivatives were synthesized as described above and coupled manually using 2 (7 aza 1H benzotriazole 1 yl) 1,1,3,3 tetramethyluronium hexafluorophosphate (HATU) and NMM in DMF. These couplings were performed once, in 3 molar excess for 1 h with a mixture of Fmoc amino acids (45 µmol), HATU (40 µmol), and NMM (0.6 mmol) in DMF, followed by a capping step using Ac2O and N,N diisopropylethylamine (DIPEA) in DMF. Removal of the Fmoc group was carried out with 20% piperidine in DMF. Peptides were N terminally capped with Ac$_2$O and DIPEA in DMF, cleaved off the resin with complete removal of the side chain protection groups by a cleavage cocktail that contained TFA/phenol/triisopropylsilane/H$_2$O (85:5:5:5) for 4 h. Cleaved products were precipitated in cold diethyl ether, centrifuged, washed with diethyl ether, dissolved in H$_2$O, and lyophilized. Crude peptides were HPLC purified on a semipreparative polymer column to approximately 95% purity.

In addition to the 25 amino acids of the H4 tail, we intended to add a C terminal amidated residue to increase the reporter stability in extracts. Initial use of the native Val residue in this position frequently resulted in incomplete downstream coupling and was therefore replaced by Ala.

**Figure 4.** Deacetylation assays based on the HDAC reporter. (a) Sir2.1 and (b) HDAC8 catalyzed deacetylation reactions of the HDAC reporter. (c) Deacetylation of the HDAC reporter catalyzed by endogenous HDACs in a HeLa nuclear extract. All reactions were performed with a HDAC reporter concentration of 200 µM at 295 K.
Recombinant Protein Production. C. elegans Sir2.1 and yeast HAT1 were expressed in E. Coli BL21 (DE3) express cells (Lucigen) as a His tag fusion protein employing the pET expression system (Novagen). At an OD600 of 0.50, protein expression was induced for 12 h at 18 °C with 0.2 mM IPTG. Cells were lysed by sonication, and the proteins were purified by conventional NINTA chromatography and gel filtration (Hi load 16/60 Superdex 75, GE Healthcare). Pure Sir2.1 and HAT1 were stored in 10% glycerol (v/v) at 80 °C. The HAT domain of human p300/CBP (aa 1097–1757) was expressed as a GST fusion protein and purified via Glutathion Sepharose (GE Healthcare) and gel filtration (Hi load Superdex 75, GE Healthcare).

Deacetylation/Acetylation Assays. Sir2.1 mediated deacetylation reactions were performed in 50 mM sodium phosphate, 25 μM ZnCl₂ at pH 7.5 in the presence of 1 mM NAD⁺, protease inhibitors (Complete, Roche), and 10% D₂O (v/v). Individual HDAC reporter concentrations were 200 μM, and the total reaction volume was 500 μL for all NMR measurements. Acetylation levels (%) were calculated from measured maximum peak intensities at the individual time points. (Alternative integration of the respective peak areas yielded identical results). The starting signal intensities were referenced to an acetylation level of 100%. Deacetylation profiles were measured with 2 μM recombinant Sir2.1 and HDAC reporter at 200 μM. HDAC8 deacetylation reactions were performed in 50 mM sodium phosphate, 140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 25 μM ZnCl₂, at pH 7.5, with protease inhibitors (Complete, Roche) and in 10% D₂O. Fifteen Units (U) of commercial HDAC8 (Enzo Life Sciences) were incubated with 200 μM HDAC reporter at 295 K. Deacetylation reactions in HeLa nuclear extracts (5 mg mL⁻¹ total protein concentration) were performed in 20 mM sodium phosphate, 100 mM KCl, 2 mM MgCl₂, 10% glycerol (v/v), 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF at pH 6.9, with 10% D₂O. All acetylation levels were calculated from NMR signal intensities at maximum peak heights, taking peak splitting of the acLys16 signal into account. All progress curves were fitted to a first order rate equation \( A(t) = A_0 \exp(-kt) \) assuming saturation of the NAD⁺ co substrate for the Sir2.1 assays in GraphPad Prism 5.0. Acetylation assays were performed in 20 mM sodium phosphate, 100 mM NaCl, 0.5 mM acetyl CoA (Sigma), and Complete Pls (Roche) at pH 6.9, 295 K with 2 μM CBP or 0.5 μM HAT1. HAT reporter was used at a final concentration of 200 μM. Acetylation levels (%) were calculated from measured maximum peak intensities at the individual time points. The final intensities of the acetylated \(^{1}H/^{13}N\) (Lys20, p300/CBP acetylation) and \(^{1}H/^{13}N\) (Lys5, p300/CBP acetylation) signals were referenced to an acetylation level of 100% assuming quantitative modification. Reaction profiles were fitted to a first order rate equation \( A(t) = A_0 \left(1 - \exp(-kt)\right) \) in GraphPad Prism 5.0.

NMR Spectroscopy. All experiments were performed on Bruker Avance spectrometers operating at 600 or 750 MHz, equipped with cryogenically cooled triple resonance \(^{1}H/^{13}C/^{15}N\) probes. Natural abundance proton – nitrogen correlation spectra were recorded at 2 mM peptide concentrations, in 20 mM sodium phosphate, 100 mM NaCl at pH 6.8, 10% D₂O (v/v), with 64 transients and 1024 \(^{1}H\) × 256 \(^{15}N\) complex points (acquisition time ~6 h), using a standard HSQC pulse sequence. For the backbone assignment of the uniformly \(^{13}C/^{15}N\) labeled recombinant H4 tail triple resonance HNCA/CB experiment was recorded at 278 K with 8 transients and 1024 \(^{1}H\) × 100 \(^{13}C\) × 80 \(^{15}N\) complex points. Acetylation and deacetylation reactions were recorded with SOFAST HMQC pulse sequences. Individual 2D \(^{13}N\) edited SOFAST HMQC experiments for HAT and HDAC reporter measurements were executed with 32 transients and 1024 \(^{1}H\) × 32 \(^{15}N\) complex points (acquisition time, 251 s). For maximum chemical shift dispersion, the nitrogen carrier frequency and sweep width (SW) were set to 122.5 and 5.5 ppm, respectively. The Ne signal was folded into the spectrum at ~121.5 ppm \(^{15}N\) with a negative signal. The spectra were analyzed with iNMR 3.3.9.