

## Simultaneous Detection of Protein Phosphorylation and Acetylation by High-Resolution NMR Spectroscopy

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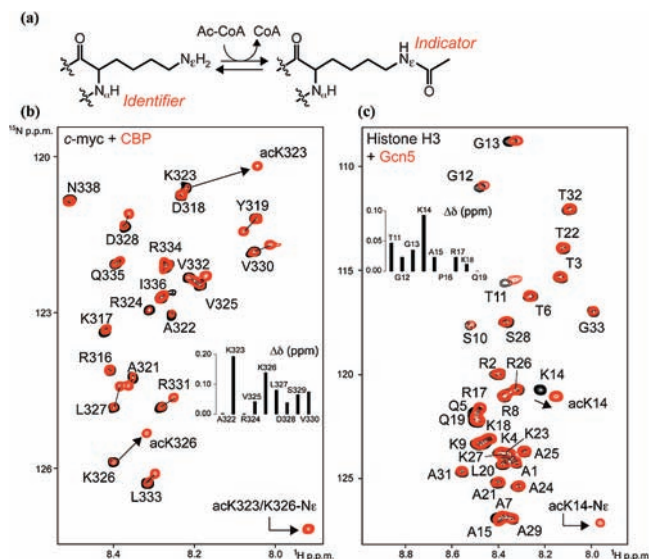
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**Abstract:** Post-translational protein modifications (PTMs) such as phosphorylation and acetylation regulate a large number of eukaryotic signaling processes. In most instances, it is the combination of different PTMs that “encode” the biological outcome of these covalent amendments in a highly dynamic and cell-state-specific manner. Most research tools fail to detect different PTMs in a single experiment and are unable to directly observe dynamic PTM states in complex environments such as cell extracts or intact cells. Here we describe in situ observations of phosphorylation and acetylation reactions by high-resolution liquid-state NMR spectroscopy. We delineate the NMR characteristics of progressive lysine acetylation and provide in vitro examples of joint phosphorylation and acetylation events and how they can be deciphered on a residue-specific basis and in a time-resolved and quantitative manner. Finally, we extend our NMR investigations to cellular phosphorylation and acetylation events in human cell extracts and demonstrate the unique ability of NMR spectroscopy to simultaneously report the establishment of these PTMs by endogenous cellular enzymes.

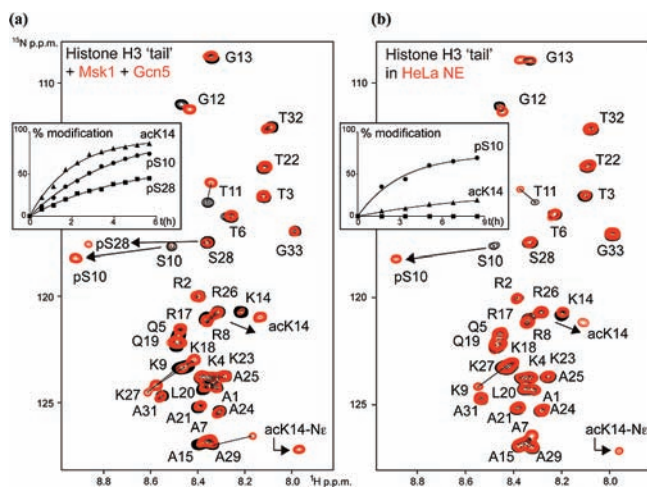
Protein phosphorylation and acetylation constitute abundant post-translational protein modifications (PTMs) that regulate a plethora of biological processes.<sup>1</sup> Differential histone modifications, for example, establish in part the epigenetic “chromatin code” that ultimately determines the transcriptional activities of whole genomes.<sup>2</sup> Similarly, transcription factors such as p53, *c-myc*, or CBP/p300 are regulated by phosphorylation and acetylation at multiple modification sites.<sup>3</sup> Here we employed high-resolution NMR spectroscopy to simultaneously observe protein phosphorylation and acetylation reactions by recombinant kinases and acetyltransferases in vitro and by endogenous cellular enzymes in human cell extracts.

Cellular protein phosphorylation and acetylation are usually detected by generic radionucleotide incorporation assays that employ either [<sup>32</sup>P]ATP or [<sup>3</sup>H/<sup>14</sup>C]acetyl coenzyme A (Ac-CoA).<sup>4</sup> Because these assays do not map the respective PTM sites, mass spectrometry (MS) has become the preferred tool to experimentally verify the presence of PTMs as well as to identify the individually modified protein residues.<sup>5</sup> However, MS analyses cannot be directly performed on complex mixtures such as cell extracts, and they require sample processing and are therefore inherently disruptive. Kinetic analyses of cellular modification events necessitate multiplexed approaches and individual measurements of different reaction time points. In contrast, high-resolution solution-state NMR spectroscopy can be employed to study PTM reactions as they proceed inside an NMR sample tube while NMR recordings are in progress, as well as in complex environments such as cell extracts or intact cells.<sup>6</sup> NMR spectroscopy can thereby provide time-resolved in situ “snapshots” of dynamic modification states at atomic resolution for all residues of a protein simultaneously.



**Figure 1.** NMR characteristics of lysine acetylation. (a) Acetylation yields an NMR-observable side-chain <sup>1</sup>H/<sup>15</sup>N $\epsilon$  correlation signal (*Indicator*). Backbone amide (<sup>1</sup>H/<sup>15</sup>N $\alpha$ ) chemical shift changes (*Identifier*) map acetylation sites on a residue-specific basis. (b) Overlay of 2D <sup>1</sup>H/<sup>15</sup>N correlation spectra of <sup>15</sup>N-labeled *c-myc* (black) upon CBP acetylation (red). Backbone amide chemical shift changes ( $\Delta\delta$ ) unambiguously identify the modified lysine residues (inset). (c) Overlay of 2D <sup>1</sup>H/<sup>15</sup>N correlation spectra of <sup>15</sup>N-labeled histone H3 (black) upon Gcn5 acetylation (red). Backbone amide chemical shift changes unambiguously identify the modified lysine residues (inset). The *indicator* signals of acetylated *c-myc* and H3 resonate at  $\sim$ 7.9/127.5 ppm (<sup>1</sup>H/<sup>15</sup>N).

While protein phosphorylation typically results in large downfield chemical shift changes ( $\Delta\delta$ ) of backbone amide (<sup>1</sup>H/<sup>15</sup>N $\alpha$ ) resonances of modified protein residues,<sup>6</sup> we found that lysine acetylation leads to comparatively smaller, upfield <sup>1</sup>H/<sup>15</sup>N $\alpha$  displacements ( $\Delta\delta < 0.3$  ppm). Additionally, the side-chain amino <sup>1</sup>H/<sup>15</sup>N $\epsilon$  resonances of lysine residues, which are not usually detected in their unmodified forms because of fast chemical exchange with bulk-solvent protons,<sup>7</sup> give rise to observable resonance signals that correspond to the newly established N $\epsilon$  amide groups (Figure 1a). The NMR characteristics of lysine acetylation are exemplified by the in vitro acetylation reaction of uniformly <sup>15</sup>N-labeled *c-myc* [only the regulatory domain of this transcription factor, amino acids (aa) 314–339, was employed] by the acetyltransferase domain of recombinant CBP (Figure 1b). Two lysine residues, K323 and K326, were jointly acetylated with no apparent preference for one site over the other. K326 constitutes a known substrate site for CBP/p300,<sup>8</sup> whereas K323 is a predicted<sup>9</sup> but previously uncharacterized modification site. Similarly, in vitro acetylation of K14 of the N-terminal histone H3 tail region (aa 1–33) was accomplished by recombinant Gcn5, the acetyltransferase known to specifically acetylate this lysine residue<sup>10</sup> (Figure 1c). Acetylation of all three



**Figure 2.** Simultaneous NMR detection of protein phosphorylation and acetylation. (a) Overlay of 2D  $^1\text{H}/^{15}\text{N}$  correlation spectra of  $^{15}\text{N}$ -labeled histone H3 (black) in a joint modification reaction with Msk1 and Gcn5 (red).  $^1\text{H}/^{15}\text{N}\alpha$  peak intensities of the “unmodified” substrate residues at different time points yield the respective reaction profiles (inset). (b) Overlay of 2D  $^1\text{H}/^{15}\text{N}$  correlation spectra of  $^{15}\text{N}$ -labeled histone H3 (black) in HeLa nuclear extracts (red); the inset shows the corresponding reaction profiles. Phosphorylation of S10 and partial acetylation of K14 by endogenous enzymes was detected.

sites was independently confirmed by MS (data not shown). The NMR characteristics of histone H3 acetylation were additionally verified by synthetically modified H3 peptides (Figure S1 in the Supporting Information).

Most of the acetylation reactions that we and others<sup>11</sup> have investigated to date have displayed  $^1\text{H}/^{15}\text{N}\epsilon$  acetyl signals at very similar resonance frequencies ( $\sim 7.9/127.5$  ppm). This is most probably due to the highly solvent-exposed and degenerate chemical environment that these acetylated lysine residues experience in different proteins and peptides. We therefore refer to  $^1\text{H}/^{15}\text{N}\epsilon$  signals as generic *acetylation indicators*. Changes in the backbone amide chemical shifts more specifically map the sites of acetylation and are hence the residue-specific *acetyllysine identifiers*. Thus, NMR detection of lysine acetylation by  $^1\text{H}/^{15}\text{N}$  correlation experiments relies on two interdependent readout parameters: the *indicator* signal irrevocably reports the presence of an acetylated lysine residue in a protein of interest, and the *identifier* chemical shift change maps the respective acetylation site.

To determine whether NMR spectroscopy can simultaneously report residue-specific phosphorylation and acetylation events *in vitro*, we reacted  $^{15}\text{N}$ -labeled histone H3 (aa 1–33) with equal amounts of Msk1, the protein kinase known to phosphorylate S10 and S28 of histone H3<sup>12</sup> and Gcn5 (Figure 2a). Progressive phosphorylation of S10 and S28 and acetylation of K14 were simultaneously detected in a series of time-resolved 2D  $^1\text{H}/^{15}\text{N}$  correlation experiments. The dynamic changes in signal intensities of the “unmodified” backbone amide resonances directly revealed the respective modification kinetics of the three PTM sites. On the basis of these experimentally obtained reaction profiles, we concluded that acetylation of K14 and phosphorylation of S10 and S28 proceeded in parallel but with different turnover rates. Acetylation of K14 was accomplished most efficiently, while phosphorylation of S10 proceeded notably “faster” than the modification of S28 (Figure 2a). Overall, these modification characteristics remained unchanged when we probed Gcn5-mediated

acetylation and Msk1-dependent phosphorylation in individual reactions, although modification of S10 and K14 occurred somewhat “faster” (data not shown). In this case, it is likely that the absence of steric exclusion under monotypic reaction conditions enables higher turnover rates of nearby substrate sites.

In a next step, we performed *in situ* NMR measurements of endogenous modification events in nuclear extracts (NE) from cultured human HeLa cells (Figure 2b). S10 of the  $^{15}\text{N}$ -labeled histone H3 tail was efficiently phosphorylated by cellular kinases, whereas no modification of Ser28 was detected. While S10 phosphorylation proceeded “slower” than in the corresponding *in vitro* reactions, similar modification levels were achieved at the respective reaction end points. This may indicate that kinases other than Msk1 execute S10 phosphorylation in HeLa NEs. Indeed, a number of enzymes have been implicated in S10 phosphorylation under different cellular conditions.<sup>12</sup> Surprisingly, none of the other known H3 phosphorylation sites (T3, T6, and T11) were modified.<sup>12</sup> Acetylation of K14 by cellular lysine acetyltransferases (KATs) was clearly detected but markedly reduced in comparison with the *in vitro* reactions with recombinant Gcn5. Whether this suggests that K14-specific KATs are less abundant in HeLa NEs or that their activities are more tightly regulated under those conditions remains to be determined. None of the other known H3-tail modification sites, K9 and K18, were acetylated to any visible extent.

In summary, our results exemplify some of the advantages that NMR detection of protein phosphorylation and acetylation provides over other analytical tools, including its unique ability to jointly detect these PTMs in a nondisruptive, instantaneous, and continuous fashion; its suitability for residue-resolved *in situ* observations in complex reaction mixtures, including cell-free extracts; its straightforward *de novo* PTM site-mapping capabilities without the need for destructive protein fragmentation or modification- and site-specific antibodies; and its potential for immediate retrieval of reaction profiles of different modification events in parallel in a time-resolved and quantitative manner.

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**Supporting Information Available:** Detailed experimental procedures and NMR data on synthetically acetylated H3 peptides. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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