## A Direct Method for Site-Specific Protein Acetylation\*\*

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Post-translational modification (PTM) is a fundamental mechanism for modulating protein function. One such PTM with increasingly recognized significance is protein lysine acetylation.<sup>[1]</sup> A reversible biochemical process,<sup>[2]</sup> lysine acetylation was initially discovered in histones.<sup>[3]</sup> Recently it has also been observed in a very large number of other proteins,<sup>[4]</sup> thus suggesting its diverse regulatory functions in the cell.<sup>[5]</sup> There is mounting evidence that aberrant lysine acetylation is implicated in many disease conditions such as cancer and neurological disorders.<sup>[6]</sup> Therefore, the study of lysine acetylation biology is of great importance and will lead to continued therapeutic innovations.<sup>[7,8]</sup>

Although lysine acetylation has long been recognized as a histone epigenetic mark affecting chromatin structure and function,<sup>[2]</sup> the exact effects of most individual protein acetylation events, especially those identified more recently remain to be elucidated. A major difficulty in the study of lysine acetylation biology lies in the limited availability of homogeneous protein samples that contain the acetylated lysine residue(s) of interest. Such materials would be invaluable reagents for discerning the structural and functional effects of a particular Lys acetylation PTM by biophysical and biochemical means.<sup>[9]</sup> Several methods can be used to prepare site-specifically acetylated proteins, such as unnatural aminoacid mutagenesis using the amber stop codon/suppressor tRNA system<sup>[10]</sup> and protein chemical synthesis.<sup>[11]</sup> While the stop codon suppression strategy is a powerful method, it is currently not widely available. And significant technical barriers exist for the adoption of chemical synthesis methods by the large bioscience community. Another method combines unnatural amino acid mutagenesis with chemical modification to introduce an acetyl lysine analogue into a protein; however, the chiral integrity of the modified amino acid is compromised in the process.<sup>[12]</sup> Direct enzymatic Lys acetylation is unrealistic given the often promiscuous as well as inefficient and incomplete nature of such enzymatic reactions. Chemical acetylation of a selected lysine among many Lys residues in a protein is also obviously not feasible. The unique reactivity of the thiol group of cysteine as a soft nucleophile has been exploited extensively for selective protein modification.<sup>[13]</sup> For instance, the classic reaction of aminoethylation of Cys has long been used for converting a cysteine residue to 4-thialysine as a functional equivalent to lysine.<sup>[14a]</sup> This method was also extended recently to the preparation of  $N^{\varepsilon}$ -methyl-lysine analogues by using Nmethylaminoethyl halides as the alkylating agents.<sup>[14b]</sup> The efficiency of this reaction is attributed to the formation of a highly reactive aziridinium intermediate.<sup>[14c]</sup> Unfortunately, this reaction system does not work for N-acetyl-thialysine as seen in the failed attempts by others<sup>[15]</sup> and us to use N-acetylaziridine and N-acetyl-aminoethyl bromide or iodide for cysteine alkylation. More recently it was reported that the use of methylthiocarbonyl-aziridine led to selective Cys alkylation.<sup>[15]</sup> The resultant methylthiocarbonyl-thialysine was shown to mimic  $N^{\varepsilon}$ -acetyl-lysine in certain functions,<sup>[15]</sup> although the methylthio-carbamate moiety is electrosterically rather different from the acetamide in  $N^{\varepsilon}$ -acetyl-lysine. Clearly, analogously to 4-thialysine and N-methyl-thialysine being ideal mimics of lysine and  $N^{\varepsilon}$ -methyl-lysine respectively, N-acetyl-thialysine [sLys(Ac)] would also be an ideal mimic of Lys(Ac), in which the only difference is the isosteric thioether in lieu of the 4-methylene in natural Lys(Ac). As the position of this substitution is rather far away-by 2 carbon atoms-from the acetamide nitrogen, little difference is expected between this Lys(Ac) mimic and native Lys(Ac) in their exhibited physicochemical and biochemical properties. However, since existing methods for cysteine modification are not applicable here, a new method must be discovered to obtain such a Lys(Ac) mimic.

In searching for ways of introducing an sLys(Ac) residue into proteins, we came across a radical reaction known as thiol-ene addition,<sup>[16]</sup> which might serve our needs. A classic reaction discovered over a century ago,<sup>[17]</sup> radical thiol-ene reaction gives an anti-Markovnikov addition thioether product.<sup>[16a]</sup> Over the years this reaction has found extensive use in polymer chemistry.<sup>[16]</sup> More recently, it has also emerged as a useful click reaction for bioorganic functionalization.<sup>[18,19]</sup> We realized that thiol-ene coupling between the cysteine thiol and N-vinyl-acetamide (NVA) would directly generate the desired acetyl-thialysine (Scheme 1). We first used a small organic thiol compound, benzyl mercaptan (BzSH), as the substrate and examined the alkylation reaction under different conditions. We found that the free radical reaction proceeded well in acetate buffer at pH 4 and in the presence of VA-044 as the initiator under UV irradiation at 365 nm. At a 1:1 ratio of NVA to BzSH and at low concentrations of the two reactants (at 5 or 10 mM), a 30 min reaction gave over 70% conversion of BzSH with the expected thiol acetamidoethylation product. The reaction at 10 mм also produced a

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**Scheme 1.** Radical-mediated addition of Cys thiol to N-vinyl-acetamide (NVA) generates sLys(Ac), a close mimic of native Lys(Ac).

minor side product, which was due to telomerization,<sup>[16a]</sup> that is, polymerization (in this case dimerization only) of NVA on the thiol. When NVA was used in 5-, 10- or even 20-fold excess with respect to BzSH, the reaction was complete within 30 min but more telomerization products were formed (see the Supporting Information). However, even under these conditions, the desired product still formed in a predominant amount. These encouraging results led us to further test this reaction on synthetic peptides. We reasoned that the inclusion of a second thiol compound in the reaction mixture might help to suppress the side reaction of radical telomerization. After numerous trials we found that glutathione (reduced) could perfectly serve this purpose and that a reaction mixture of 15 mм glutathione, 50 mм NVA, and 5 mм VA-044 in 0.2м acetate buffer (pH 4) or 0.2 M phosphate buffer (pH 6 or 7) was well suited for the alkylation of peptide and protein substrates. Using these conditions, the reaction on the synthetic peptides was basically completed in 30 min and no significant side reactions were found when extending the reaction time to 1 h. For instance, when each of the peptides 1-4 (Table 1) was treated with this mixture in acetate buffer (pH 4, final peptide concentration = 5 mM), the desired alkylating product was obtained in near quantitative yield based on HPLC and MS analysis. Under these conditions, no or little telomerization products were detected. Therefore, glutathione together with the peptide substrate participated in the critical radical chain transfer step-which is known as

Table 1: Peptide and protein substrates alkylated with NVA.[a]

N	Substrate	Yield <sup>[b]</sup> [%]
1	Ac-FQPK <b>C</b> G	> 95
2	VG <b>C</b> AEKSL	> 95
3	WA <b>C</b> YKSL	95
4	Biotin-GKGGA <b>C</b> RHRKVLRDN	> 95
5	Biotin-G <b>C</b> GG <b>C</b> GLG <b>C</b> GGA <b>C</b> R	95
6	Ubiquitin K48C	> 95
7	Histone H4 K16C	90
8	Histone H3 K27C	90

[a] Reaction conditions: Peptide or protein substrate (5 mm for peptides 1–4, 1.25 mm for peptide 5, 0.5 mm for ubiquitin and 1 mm for H3 and H4) was treated in a reaction mixture containing 50 mm NVA, 5 mm VA-044 and 15 mm glutathione in 0.2 m acetate buffer at pH 4 (for H3 and H4 the mixture also contained  $6 \times$  Gdn·HCl) under UV (365 nm) irradiation.[b] Yields based on HPLC and MALDI-MS analysis (reaction time: 1 h for 1, 2, 4, and 5, 30 min for 3 and 2 h for the proteins 6–8). See the Supporting Information for experimental details. Peptide 1, ubiquitin (entry 6), and H4 (entry 7) were also reacted at pH 6 or 7 with similar results obtained (see the Supporting Information).

the rate-limiting step of this thiol-ene coupling reaction<sup>[16a]</sup> to effectively quench the carbon free radical intermediate formed at the addition step of the thiyl radical to the NVA ethylene double bond and to prevent it from reacting with another molecule of NVA. As expected, glutathione was also alkylated by NVA in the reaction. In fact, when peptide 4 (5 mm) was alkylated deliberately in the absence of glutathione under otherwise identical conditions, products in which the Cys residue was alkylated by di-, tri-, and tetrameric NVAs were observed in addition to the desired product (see the Supporting Information). No reaction occurred on a control peptide with no Cys residue (Ac-FQPKSG); equally, no reaction occurred in the absence of the initiator or without UV irradiation. A weakly acidic pH and the presence of glutathione also prevented oxidative disulfide formation of the thiol in the substrates. Use of the reducing agent tris(2carboxyethyl)phosphine (TCEP) was detrimental to the reaction as it led to desulfurization of the peptide substrate. A noteworthy example to show the high efficiency of this reaction is the alkylation of peptide 5, which contains four Cys residues. Remarkably, when this peptide (1.25 mm) was treated in the reaction mixture for 1 h, a very clean tetraalkylating reaction gave the desired product in 95% yield (Figure 1, top).

This reaction was also highly effective on protein substrates. Firstly, a ubiquitin mutant that contains a Cys at position 48 was prepared and subjected to this modification. The protein (0.5 mM) was used in its native folded state for



**Figure 1.** Top: HPLC and MS analyses of the alkylation reaction on peptide 5. A) peptide 5 (peak a) before alkyation. B) after alkylation. Peak b is the alkylation product; peak c appears because of the oxidation of one of the thioether linkages in the alkylated product. C) MALDI-MS spectrum of the isolated product corresponding to peak b in (B)  $(m/z [M+H]^+$  found: 1735.9, calcd: 1735.7). Bottom: HPLC and MS analyses of histone H4 alkylation. D) HPLC profile of the H4 alkyation reaction at pH 4. Peak a' is the alkylation product; peak b' presumably represents product from oxidation of peak a'. E) ESI-MS of the desired alkylation product with the insert showing the deconvoluted mass (MW found: 11295.5, MW calcd: 11296.2).

alkylation in the same reaction mixture at pH 4 or 7 (see the Supporting Information). MS analysis clearly showed an almost quantitative conversion of the Cys residue to sLys(Ac) in the course of 2 h with the expected +85 Da MW for the alkylated product. The protein remained soluble during the reaction, hence suggesting that no denaturing was occurring and that the presence of 50 mM NVA and 5 mM VA-044 did not affect the structure of the folded protein. It is worth noting that it would be difficult to use a semisynthetic method to prepare such a modified protein, because the modification site is in the middle of the sequence. Two other proteins, histone H4K16C and H3K27C, were also modified with excellent results. In these cases, 6M Gdn·HCl was included in the alkylation reaction mixture. Interestingly, for the alkylation of H4K16C at pH4 or 7, in addition to the desired product, a side product was formed in significant amount (see the Supporting Information). The side product was more hydrophilic with a molecular weight (MW) that was 16 Da higher than that of the expected acetyl-thialysine product. This side product appeared to result from oxidation of the thioether linkage to sulfoxide and the inclusion of dimethylsulfide in the reaction mixture minimized the formation of this side product to about 5% (Figure 1, bottom and the Supporting Information). No alkylation was detected when wild-type H4 was subjected to the same treatment. From the above results we can see that this free radical thiol reaction can tolerate various reaction conditions, for example, native or denatured buffers, to modify a protein.

The generated sLys(Ac) was shown to be a good functional mimic of the natural Lys(Ac). Firstly, histone protein H4 K<sub>s</sub>16Ac was recognized by a specific anti-H4K16Ac antibody, whereas the unmodified H4K16C was not recognized by the same antibody (Figure 2A). Next, an enzymatic test was conducted to investigate whether the acetyl-lysine mimic could be recognized by a histone deacetylase and be substrate for deacetylation. SIRT2, a class III NAD-dependent deacetylase, was used for the deacetylation reaction of the alkylated peptide 1 and its native counterpart Ac-FOPKK(Ac)G. Using an HPLC assay (Figure 2B), we showed that the sLys(Ac) residue in the alkylated peptide 1 was susceptible to enzymatic deacetylation, albeit to a lesser degree than its native counterpart (see the Supporting Information), which might be due to the fact that the side chain of sLys is slightly longer than that of native Lys. The recently reported methylthiocarbamate modification, on the other hand, was resistant to enzymatic deacetylation.<sup>[15]</sup>

Acetylation of Lys16 in histone H4 is known to inhibit the folding of nucleosome arrays and hence the formation of the compact 30 nm chromatin fibre.<sup>[9,20]</sup> H4 K<sub>s</sub>16Ac and three other control H4 proteins (H4 K16Ac, H4 K<sub>s</sub>16, and H4-WT) were incorporated respectively, together with H3, H2A, and H2B into histone octamers. H4 K16Ac was prepared using a semisynthetic approach<sup>[20]</sup> and H4 K<sub>s</sub>16 was synthesized from alkylating H4 C16 with 2-bromoethylamine. The four different octamers were then individually combined with the 12-177-601 DNA to assemble into the 12-nucleosome arrays. Using analytical ultracentrifugation (AUC), we demonstrated that the K<sub>s</sub>16Ac in abolishing Mg<sup>2+</sup>-induced folding of the



**Figure 2.** A) Western blots with anti-H4 K16Ac Ab on the H4 proteins: H4 K16C and H4 K<sub>5</sub>16Ac; B) deacetylation assay of Ac-FQPKKs(Ac)G by SIRT2 after 16 h; C) effects of H4 K16 acetylation on nucleosome array folding as seen from sedimentation distributions of the nucleosome arrays before (no Mg<sup>2+</sup>, open symbols) and after Mg<sup>2+</sup>-induced folding (with 1.0 mM MgCl<sub>2</sub>, solid symbols). See the Supporting Information for details.

reconstituted nucleosome array (Figure 2 C). The AUC data clearly showed that, in the presence of 1 mM MgCl<sub>2</sub>, the nucleosome array containing wild-type H4 or its equivalent H4 K<sub>s</sub>16 folded into a significantly more compact state (sedimentation coefficient  $S_{20^{\circ}C,w} = 52-53S$ ) than did the K16Ac and K<sub>s</sub>16Ac arrays ( $S_{20^{\circ}C,w} = 44-45S$ ). Remarkably, these results prove not only the functional equivalency between sLys(Ac) and Lys(Ac) but also that between sLys and Lys.

To summarize, a previously unexplored thiol-ene radical addition reaction involving the commercially available NVA is well suited for the S-acetamidoethylation of cysteine residues in synthetic peptides and recombinant proteins. The resultant N-acetyl-thialysine differs from natural acetyllysine only isosterically at the  $\gamma$  position of the amino acid structure and is functionally equivalent or similar to the latter. Although a limitation of the method is that the protein substrates should not contain other cysteines, it nevertheless has many potential applications, such as the histone epigenetic study-an intense research area at present. The reaction system is robust and gives near quantitative yields of sitespecifically acetylated proteins that can be purified in a simple chromatography or dialysis step. The ease of implementation of this method also makes it easily adoptable for researchers from the bioscience research community. As such, this radical reaction approach provides a convenient enabling tool for the study of lysine acetylation biology and will help to advance research in this important field.

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