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A Versatile Approach for Site-Specific Lysine Acylation in Proteins

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Abstract: *Using amber suppression in coordination with a mutant pyrrolysyl-tRNA synthetase-tRNA^{Pyl} pair, azidonorleucine is genetically encoded in E. coli. Its genetic incorporation followed by traceless Staudinger ligation with a phosphinothioester allows the convenient synthesis of a protein with a site-specifically installed lysine acylation. By simply changing the phosphinothioester identity, any lysine acylation type could be introduced. Using this approach, we demonstrated that both lysine acetylation and lysine succinylation can be installed selectively in ubiquitin and synthesized histone H3 with succinylation at its K4 position (H3K4su). Using an H3K4su-H4 tetramer as a substrate, we further confirmed that Sirt5 is an active histone desuccinylase. Lysine succinylation is a recently identified post-translational modification. The reported technique makes it possible to explicate regulatory functions of this modification in proteins.*

As the only amino acid with a side chain amine, lysine undergoes a myriad of post-translational acylations (Figure 1 A).[1,2] The most studied lysine acylation is acetylation. It was initially discovered in histones and is widespread in transcription factors and cytosolic proteins.[3] Two other well-known lysine acylations are biotinylation and lipoylation. Both modifications anchor a catalytic cofactor to enzymes.[4] With the advent of sophisticated proteomic techniques, there has been an increased diversity of lysine acylations revealed in histones and non-histone proteins. Three of these novel acylations, malonylation, succinylation, and glutarylation, reverse the charge state of the lysine side chain, which potentiates a unique way of controlling protein functions.[2] So far, more than ten lysine acylation types have been discovered. However, the functions of novel acylations are largely unexplored. One impediment is the difficulty of obtaining proteins with site-specific lysine acylations. The enzymatic synthesis of proteins with novel lysine acylations is not applicable since the enzymes responsible for these modifications are elusive.[5] Alternatively, native chemical ligation/expressed protein ligation (NCL/EPL), a cysteine-based alkylation approach, and a recently developed dehydroalanine-based coupling approach may be applied for the synthesis of proteins with lysine acylations.[6] However, ECL/EPL requires a cysteine for ligation and the structures generated by the latter two methods are slightly different from the natural modifications. Herein, we report the synthesis of proteins with genuine lysine acylations by coupling the amber-suppression-based mutagenesis approach[7] with traceless Staudinger ligation.

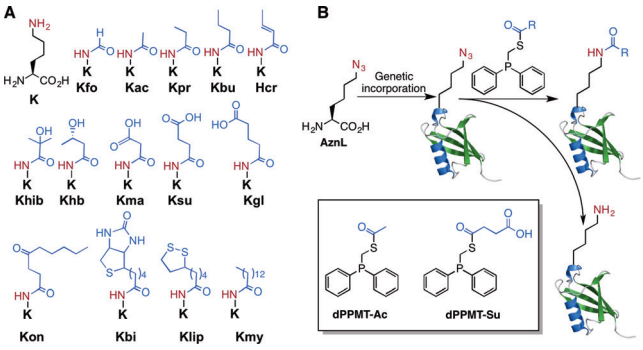


Figure 1. A proposed versatile approach to access proteins with lysine acylations. A) Post-translationally acylated lysines and their abbreviations. Acylations with small proteins such as ubiquitin and ubiquitin-like proteins are not shown. B) Genetic incorporation of AznL followed by traceless Staudinger ligation with a phosphinothioester to install a site-specific lysine acylation in a protein. The reducing nature of the phosphinothioester leads to the partial conversion of AznL to lysine as a side product. Shown in the inset are two initially designed phosphinothioesters.

Following the pioneer work by Chin et al., several groups have successfully engineered the native pyrrolysine incorporation system for the genetic incorporation of acyl-lysines into proteins, including Kac, Kpr, Kbu, Kcr, and Khib.[8] Although convenient, the genetic acyl-lysine incorporation approach requires that a pyrrolysyl-tRNA synthetase (PylRS) mutant that recognizes a particular acyl-lysine, but not any native amino acid, must be identified. The process of identifying a mutant PylRS is tedious and doesn't always work. So far, our efforts to find PylRS mutants for Kma, Ksu, and Kgl have ended with no success. Given the large sizes of Kon, Kbi, Klip, and Kmy, their direct incorporation at the amber codon is undoubtedly difficult if not impossible. Inspired by the traceless Staudinger ligation reaction developed by Bertozzi, Raines, and others for the conversion of an azide to an acyl

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amide,[9,10] we anticipated that the genetic incorporation of azidonorleucine (AznL, Figure 1B) followed by a reaction with a phosphinothioester would effectively combine to produce a one-size-fits-all approach for the installation of a large variety of lysine acylations in proteins by simply changing the acyl group in the phosphinothioester. For example, using two phosphinothioesters, dPPMT-Ac and dPPMT-Su, will in theory allow the synthesis of proteins with site-specific acetylation and succinylation. Although the intrinsic reductive nature of the phosphinothioester[10,11] will inevitably convert part of AznL to lysine, this side reaction leads to the formation of unacylated proteins that could be resolved from acylated ones using lysine acylation-specific antibodies for immunoaffinity separation. AznL was previously installed at the methionine positions of proteins using the residue-replacement method.[12] Although advantageous in analyzing nascent proteomes in cells, its global substitution of methionines makes the approach undesirable for the purpose of synthesizing proteins with selective lysine acylations. For this reason, we chose the amber-suppression-based mutagenesis approach for coding AznL. The synthesis of AznL followed procedures described in the Supporting Information, Schemes S1 and S2. A bulk amount around 5 g was easily made. To identify AznL-specific PylRS mutants, a PylRS gene library with randomization at five active site residues, Y306, L309, C348, Y384, and W411, was constructed. This library was subjected to a widely adopted double-sieve selection,[13] yielding a highly efficient AznL-specific mutant Y306L/C348I/Y384F that is referred to as AznLRS. For optimal expression, the AznLRS gene was codon-optimized and then, in coordination with $tRNA^{Pyl}$, used in the *E. coli* BL21 cells to drive the expression of full-length superfolder green fluorescent protein (sfGFP) in which an amber codon was introduced at the D134 coding position of its gene. When cells were grown in the LB medium supplemented with 5 mM AznL, full-length sfGFP with an expression level of 20 mg L^{-1} was achieved, which was in marked contrast with the non-detectable full-length sfGFP expression observed in the absence of AznL (Figure 2A). The electrospray ionization mass spectrometry (ESI-MS) analysis of the expressed protein sfGFP-D134AznL displayed one major peak at 27866 Da and one minor peak at 27735 Da,

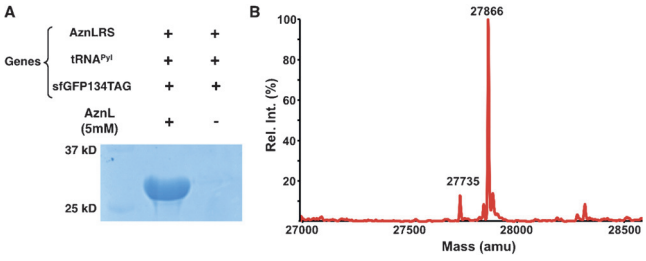


Figure 2. Genetic incorporation of AznL into a model protein sfGFP. A) Site-specific incorporation of AznL into sfGFP at its D134 position to produce sfGFP-D134AznL. Cells were transformed with two plasmids coding genes for AznLRS, $tRNA^{Pyl}$, and sfGFP with an amber mutation at its D134 position and grown in the LB medium with or without 5 mM AnzL. B) Deconvoluted ESI-MS spectrum of purified sfGFP-D134AznL.

which agree well with theoretical molecular weights of full-length sfGFP with AnzL installed at its 134 position (27866 Da) and its N-terminal methionine cleavage product (27835 Da) (Figure 2B). The presence of the azide functionality in sfGFP-D134AznL was further confirmed by its selective labeling with an alkyne-fluorescein dye (Supporting Information, Figure S1). These combined results validated the specificity of AznLRS toward AznL and the selective incorporation of AznL at the amber codon.

After demonstrating the selective incorporation of AznL, we demonstrated its application in the synthesis of proteins with site-specific acylations. Owing to our ready access to a MALDI-TOF-MS instrument that is not optimal for analyzing large proteins such as sfGFP, we switched to work with ubiquitin. A ubiquitin variant Ub-K48AznL with a C-terminal 6×His tag and AznL incorporated at the original K48 position was produced similarly as sfGFP-D134AznL. Expression levels around 10 mg L^{-1} were routinely obtained (Supporting Information, Figure S2). Ub-K48AznL was then reacted with excess dMMPT-Ac. DMMPT-Ac and dMMPT-Su, to be used later, were synthesized according to procedures described in the Supporting Information, Schemes S3 and S4. Owing to the slow kinetics of the traceless Staudinger ligation (a second-order rate constant around 0.001 $M^{-1}s^{-1}$),[14] 5 mM dMMPT-Ac and 37 °C at pH 6.0 were chosen as the reaction conditions. Products from different reaction times were analyzed by SDS-PAGE and then probed by a pan *anti*-Kac antibody in the western blot analysis. Ub-K48ac that was produced using a previously identified AcKRS-$tRNA^{Pyl}$ pair was used as a positive control. The western blot analysis clearly showed improved acetylation when the incubation time increased (Figure 3A). The reaction is deemed close to completion at 48 h since the acetylation level at this reaction time was not significantly higher than at 36 h. In contrast to the intense acetylation detected in the reaction products of Ub-K48AznL, a similar reaction with wild type Ub did not yield a detectable acetylation level. This result indicates a much slower intermolecular S-to-N acyl transfer between dMMPT-Ac and Ub than the traceless Staudinger ligation, assuring the selectivity of using traceless Staudinger ligation to convert AznL in a protein specifically to Kac. The conversion of Ub-K48AznL to its corresponding acetylation product Ub-K48ac was further confirmed with the MALDI-TOF-MS analysis of the 48-h reaction product of Ub-K48AznL. The spectrum displayed two major peaks at 9386 and 9428 Da, representing the corresponding Staudinger reduction product (a wild type 6×His-tagged Ub) and the traceless Staudinger ligation product (Ub-K48ac), whose theoretical molecular weights are 9387 and 9429 Da, respectively (Figure 3B). In the final products, Ub-K48ac is slightly more abundant than wild type Ub. To confirm that the acetylation is at K48, the 48-h reaction product was trypsinized and analyzed by the tandem mass spectrometry (MS/MS) analysis. The K48ac-containing fragment was clearly observed. Its further fragmentation clearly indicated the presence of acetylation at K48 (Figure 3C).

Encouraged by the acetylation results, we applied dPPMT-Su to the conversion of Ub-K48AznL into Ub-K48su that is succinylated at the K48 position. The reactions

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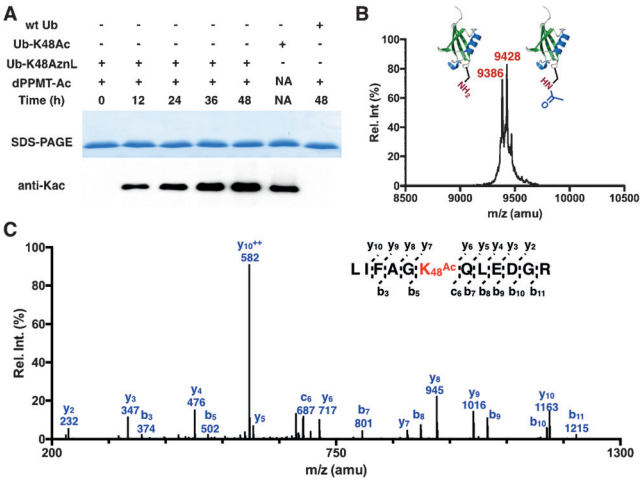


Figure 3. Synthesis of Ub-K48ac. A) Using dPPMT-Ac to convert Ub-K48AznL to Ub-K48ac. Reaction conditions: 20 μM Ub-K48AznL samples were incubated with 5 mM dPPMT-Ac in a PBS-buffered H_2O/DMSO (1:1) solution (pH 6.0) at 37 °C for several hours. After reactions, the samples were analyzed by SDS-PAGE and probed by a pan-*anti*-Kac antibody by western blot. A control reaction with wild type (wt) Ub was carried out and analyzed similarly. Ub-K48ac was provided as a positive control. B) MALDI-TOF-MS spectrum of the reaction product of Ub-K48AznL after its 48-h incubation with dMMPT-Ac. C) Tandem MS analysis of the trypsinized Kac-containing fragment of the 48-h reaction product of Ub-K48AznL.

were set up almost identically as for the synthesis of Ub-K48ac. Products at different reaction times were analyzed by SDS-PAGE and probed by a pan *anti*-Ksu antibody. Although the succinylation on Ub-K48AznL was clearly detected, the control reaction with wild type Ub also exhibited a high level of lysine succinylation (Figure 4A), indicating non-selective succinylation of seven Ub lysines by dPPMT-Su. Since dPPMT-Su has a carboxylate that potentially triggers an intramolecular S-to-O acyl transfer reaction with the thioester to form more reactive succinic anhydride for succinylating Ub lysines, this result was not a total surprise (Figure 4B). Indeed, we observed that dPPMT-Su in an oil form was completely eliminated to succinic anhydride after storage at −20 °C for several weeks. To avoid the formation of succinic anhydride, we conceived dPPMT-NB-Su (Figure 4C). In dPPMT-NB-Su, a photocleavable nitrobenzyl group[15] shields the carboxylate from the intramolecular S-to-O acyl transfer reaction and also allows its easy recovery by UV photolysis.

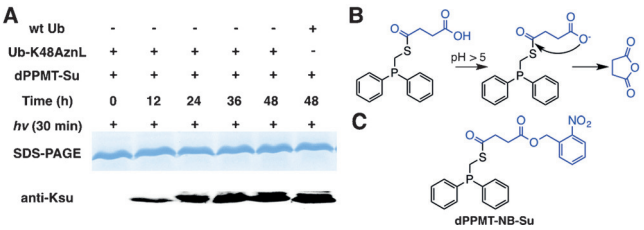


Figure 4. Nonselective succinylation by dPPMT-Su. A) Ub-K48AznL reactions with dPPMT-Su. Reactions conditions were similar to the synthesis of Ub-K48ac. B) An intramolecular S-to-O acyl tranfer reaction of dMMPT-Su. C) Structure of dPPMT-NB-Su.

dMMPT-NB-Su was synthesized according to procedures in the Supporting Information, Scheme S5. The use of dMMPT-NB-Su to trigger the conversion of Ub-K48AznL to Ub-K48su followed a protocol similar to the synthesis of Ub-K48ac except that there was an additional step of 365 nm UV treatment for 30 min before the reaction samples were analyzed by SDS-PAGE and probed by the pan *anti*-Ksu antibody. As shown in Figure 5A, the *anti*-Ksu-probed

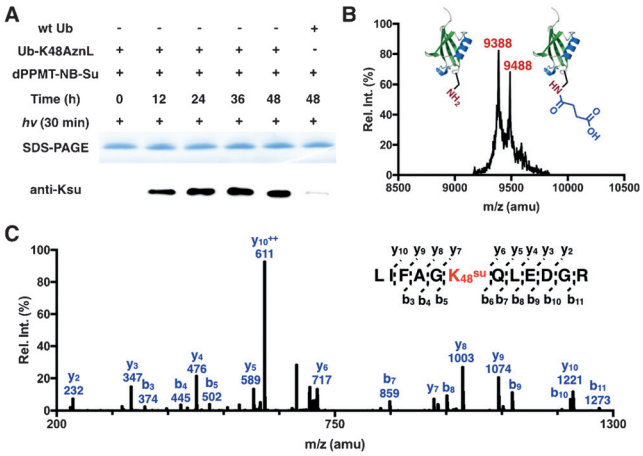


Figure 5. Synthesis of Ub-K48su. A) Using dPPMT-NB-Su to convert Ub-K48AznL to Ub-K48su. Reaction conditions were similar to the synthesis of Ub-K48ac except for an additional 365 nm UV treatment step. B) MALDI-TOF-MS of the 48 h reaction and UV-treated product of Ub-K48AznL. C) Tandem MS spectrum of the trypsinized Ksu-containing fragment of the 48 h reaction and UV-treated product of Ub-K48AznL.

succinylation on Ub-K48AznL was time dependent and appeared to reach completion at 48 h. Although the control reaction with wild type Ub did show detectable lysine succinylation, its level is minimal. Since we did not observe a similar side reaction with dMMPT-Ac, it is possible that the electron-withdrawing inductive effect of the ester in dMMPT-NB-Su activates its thioester for more favorable S-to-N acyl transfer with Ub lysines than dMMPT-Ac. To alternatively confirm the formation of Ub-K48su, the 48-h incubation and UV treated product of Ub-K48AznL was analyzed by MALDI-TOF-MS. The spectrum exhibited two major peaks, one at 9388 Da and the other at 9488 Da, representing the corresponding wild type Ub and Ub-K48su, whose theoretic molecular weights are 9387 and 9487 Da, respectively (Figure 5B). Wild type Ub is a little more abundant than Ub-K48su. To confirm the succinylation at the K48 position, the final product was further trypsinized and analyzed by tandem MS analysis. The K48su-containing fragment was detected. Its fragmentation clearly showed succinylation at K48 (Figure 5C).

Similarly as lysine acetylation, lysine succinylation has been discovered in histones.[16] Since lysine succinylation reverses the charge state of lysine, its occurrence in histones is expected to substantially impact interactions between the histone octamer and its associated DNA. Sirt5 is a NAD-dependent protein deacylase that has been suspected to

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remove succinylation from histones and regulate chromatin functions.[17] To demonstrate Sirt5 is an active histone desuccinylase, we decided to use the above-demonstrated approach to synthesize histone H3 with succinylation at K4 (H3K4su) and its tetramer with H4 as a substrate of Sirt5 for its activity test. H3 with AznL incorporated at K4 (H3K4AznL) was synthesized similarly as Ub-K48AznL. Its reaction with dMMPT-NB-Su was carried out similarly as for the synthesis of Ub-K48su. As shown in Figure 6A,

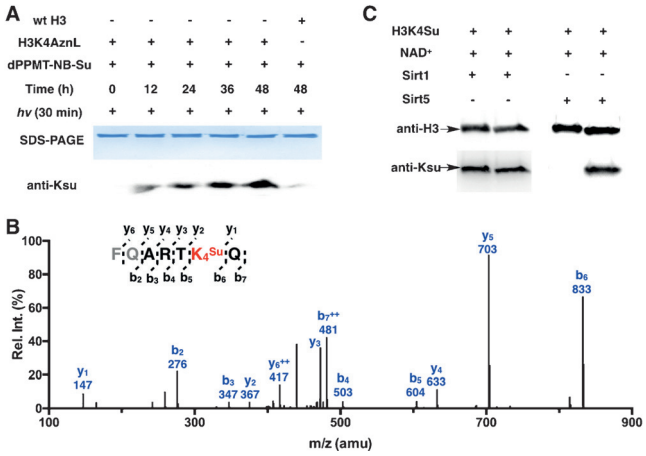


Figure 6. Synthesis of H3K4su and its application as a probe of Sirt5 desuccinylation activity. A) Using dPPMT-NB-Su to convert H3K4AznL into H3K4su. Reaction conditions and analysis were as same as for the synthesis of Ub-K48su. B) Tandem MS analysis of the trypsinized Ksu-containing fragment of the 48-h reaction and UV-treated product of H3K4AznL. C) Desuccinylation of the H3K4su-H4 tetramer by Sirt1 and Sirt5. 180 μM H3K4su-H3 tetramer was incubated with or without 0.5 μM Sirt1/Sirt5 in the presence of 10 mM NAD^+ for 4 h. After reactions, proteins were analyzed by western blot analysis using *anti*-H3 and pan *anti*-Ksu antibodies.

H3K4AznL was successfully converted to H3K4su, which was detected at high levels by the pan *anti*-Ksu antibody. The control reaction with wild type H3 led to a very low level of lysine succinylation. To confirm K4 succinylation, the 48-h incubation and UV-treated product of H3K4AznL was trypsinized and analyzed by the MS/MS. The tandem MS spectrum of the Ksu-containing fragment clearly showed succinylation at K4 (Figure 6B). After confirming the succinylation site, we directly used this 48-h incubation and UV-treated product of H3K4AznL to assemble an H3K4su-H4 tetramer that was subsequently used as a substrate to test Sirt5 activity. As shown in Figure 6C, when Sirt5 and its cofactor NAD^+ were provided to the H3K4su-H4 solution, the succinylation was actively removed from H3. On the contrary, a control reaction with Sirt1 showed no succinylation removal from H3. These combined results unequivocally approve show that Sirt5 but not Sirt1 is a histone desuccinylase.

In summary, a versatile approach has been developed for the site-specific installation of lysine acylations in proteins. We demonstrated that this approach can be readily applied for the synthesis of proteins with site-selective lysine acetylation and succinylation. Succinylation is a recently discovered novel lysine acylation. Its proteomes have been profiled in *E. coli*, *Saccharomyces cerevisiae*, *Toxoplasma gondii*, *Vibrio parahemolyticus*, *Mycobacterium tuberculosis*, human cells, and mouse liver tissues.[18] Many proteins in these organisms that undergo lysine succinylation are metabolic enzymes. Their catalytic alteration by lysine succinylation is anticipated. Xie et al. recently reported lysine succinylation on histone proteins.[16] One key feature of lysine succinylation of histones is that it mainly takes place on lysines in the nucleosome core region. The majority of these lysines involve direct charge–charge interactions with DNA. Their succinylation not only removes these charge–charge interactions but also creates repulsion interactions with DNA that loosen the nucleosome structure. Whether or not cells use this strategy to unwrap DNA and Sirt5 for its reversal is an interesting aspect to explore. With our current method, many questions related to lysine succinylation can be addressed. Since the method potentiates the synthesis of proteins with any lysine acylation type, it provides a myriad of opportunities to understand the functional roles of other novel posttranslational lysine acylations such as malonylation, glutarylation, and myristoylation. Although not explored in the current study, the method reported here could be further improved by using water-soluble phosphinothioesters at high concentrations to accelerate the traceless Staudinger ligation reaction.[11] One may also consider the addition of substituents to phenyl groups of phosphinothioesters to retard the hydrolysis of the P-N ylide to reduce the side Staudinger reduction product.[19]

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Conflict of interest

The authors declare no conflict of interest.

Keywords: amber suppression · azidonorleucine · lysine acylation · protein modification · traceless Staudinger ligation

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