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A chemical biology route to site-specific authentic protein modifications

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Abstract

Many essential biological processes are controlled by posttranslational protein modifications. The inability to synthetically attain the diversity enabled by these modifications limits functional studies of many proteins. We designed a three-step approach for installing authentic posttranslational modifications in recombinant proteins. We first use the established *O*-phosphoserine (Sep) orthogonal translation system to create a Sep-containing recombinant protein. The Sep residue is then dephosphorylated to dehydroalanine (Dha). Last, conjugate addition of alkyl iodides to Dha, promoted by zinc and copper, enables chemoselective carbon-carbon bond formation. To validate our approach, we produced histone H3, ubiquitin, and green fluorescent protein variants with site-specific modifications, including different methylations of H3K79. The methylated histones stimulate transcription through histone acetylation. This approach offers a powerful tool to engineer diverse designer proteins.

Posttranslational modifications (PTMs) play vital roles in expanding protein functional diversity and critically affect numerous biological processes (1). The availability of proteins with specific modifications at selected residues is essential for experimental strategies to investigate fundamental biological mechanisms. Methods to generate diverse native protein covalent modifications do not exist at present. Genetic code expansion approaches are useful in producing recombinant proteins with specific modifications (2, 3) but rely on the availability of an orthogonal transfer RNA (tRNA) synthetase•tRNA pair for acylation of a

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SUPPLEMENTARY MATERIALS

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Materials and Methods

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specific noncanonical amino acid. Despite much progress, the creation of many important protein modifications (e.g., trimethyllysine) is not yet feasible. Among chemical conjugation approaches (4), Cys-based strategies have been widely applied to generate protein conjugates (5) and mimics of PTMs (6, 7). Yet the final products are PTM analogs whose value for finding unidentified properties of the natural system may be questionable (8). Thus, despite extensive efforts, synthetic approaches for many authentic PTMs are not available, because no carbon-carbon bond-forming reactions have thus far been successfully applied to protein modifications, despite the prevalence of such reactions in organic chemistry (9).

Here we propose a three-step strategy (Fig. 1A) that, in principle, is applicable to generating diverse forms of authentic and selective protein modifications. (i) The site of the intended modification is established by cotranslational *O*-phosphoserine (Sep) incorporation into a recombinant protein by using the Sep orthogonal *Escherichia coli* translation system (10, 11). (ii) Then, the Sep residue of the purified recombinant protein is converted by phosphate removal to dehydroalanine (Dha), which in turn serves as a radicalophile, enabling a bioorthogonal chemical reaction. (iii) Last, PTM moieties are directly coupled to Dha through metal-mediated conjugate additions of alkyl iodides in aqueous solution, facilitating chemoselective carbon-carbon bond formation in proteins (Fig. 1B).

To demonstrate the feasibility and versatility of our strategy, we set out to generate recombinant *Xenopus laevis* histone H3K79 with five different modifications: monomethylation, dimethylation, trimethylation, formylation, and acetylation. Reversible lysine methylation in proteins presents the most complex and dynamic modification (12). Several approaches have attempted lysine methylation (13), but the trimethylated product has proved elusive. Histone H3K79 appears to be dynamically regulated (14) and associated with diverse cellular processes; its exact role has not been fully examined because such authentically modified histones have not been generated.

First, the Sep-containing histone H3Sep79 was made by expressing a *X. laevis* histone H3 mRNA containing a UAG codon at position 79 and a coding sequence for a C-terminal His₆ tag in *E. coli* containing the orthogonal Sep translation system [the engineered SepRS9•tRNA^{Sep} pair and the evolved elongation factor (EF)–Sep21] (11). H3Sep79 was routinely obtained in good yield (~20 mg/liter of culture) (fig. S1). The incorporation of Sep at the intended position (79) was confirmed by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the purified recombinant histone (fig. S2) and its tryptic peptides (Fig. 2A and tables S1 and S2).

Because phosphoamino acids are known to be labile under alkaline conditions (15), we attempted to convert Sep to Dha by mild alkali treatment. To determine the optimal procedure, purified H3Sep79 was incubated with LiOH, Ba(OH)₂, or Sr(OH)₂ under different conditions, followed by neutralization with acetic acid and dialysis against distilled water (fig. S3). The reaction progress was analyzed by MALDI-TOFMS after in-gel trypsin digestion (tables S1 and S2). Near-complete disappearance of the Sep residue and concurrent generation of Dha took 30 min at room temperature in 40mMBa(OH)₂ solution. Mass analysis of whole proteins (fig. S4) and tryptic peptides (Fig. 2A) demonstrated that

these conditions led to highly selective chemical transformation of H3Sep79 into H3Dha79 with no noticeable protein oxidation or other side reactions (16).

Dha-mediated conjugation approaches have been successfully applied to the generation of thiol-linked PTM analogs or mimics (17, 18). To produce authentic PTMs from Dha, we needed a new coupling scheme enabling chemoselective carbon-carbon bond formation in proteins (Fig. 1B). We were encouraged by reports of water-based organic reactions in which an alkene group functions as a radical acceptor (19). Knowing that Dha could function as a radical acceptor, we chose alkyl radicals that can be generated from alkyl iodides by transition metals (19). Inspired by recent metal-mediated conjugate addition reactions (20), we reasoned that organozinc species would form from the precursor halides and Zn metal. Subsequently, zinc-to-copper transmetalation would occur, generating organo copper reagents, which would lead to the formation of alkyl radical species. Last, conjugate coupling of the alkyl radical with the Dha residue of a protein would take place (Fig. 1B). To demonstrate the proposed coupling scheme, we tested first the addition of trimethyl iodide (3-iodo-*N,N,N*-trimethylpropan-1-amine, **3**; fig. S5) to H3Dha79 to generate the expected product H3K79me3. The reaction products were analyzed by Western blotting using an antibody against H3K79me3. Our initial attempts to directly use water-based organic reactions were unsuccessful. After testing a large array of conditions (described in the supplementary materials, figs. S6 to S16)—including different buffers and pH ranges, surfactants for protein stabilization, essential reagents for the metal-mediated coupling reaction (Zn metals and copper salts), and possible auxiliary reagents [e.g., tetramethylethylenediamine (TMEDA)]—we arrived at conditions that reproducibly led to the formation of carbon-carbon bonds in high yield. The deduced optimal conditions were H3Dha79 (10 μ M), alkyl iodide (30 mM), Zn powder (0.4 mg), Cu(OAc)₂ (1 mM), Triton X-100 (2.0 weight %), and TMEDA (10 mM) in sodium acetate (pH 4.5, 0.5 M). Reactions (20 to 50 μ l) were incubated at room temperature.

With optimized reaction conditions in hand, we attempted to synthesize H3K79me1, H3K79me2, and H3K79me3 by incubating H3Dha79 with three different methyl iodides: monomethyl iodide (3-iodo-*N*-methylpropan-1-amine, **1**), dimethyl iodide (3-iodo-*N,N*-dimethylpropan-1-amine, **2**), or trimethyl iodide (Fig. 2C and fig. S5). MALDI-TOF MS analysis revealed the disappearance of Dha and the concomitant generation of methylated lysine residues (Fig. 2A). The coupling reactions were highly selective and efficient (normally >80%), as demonstrated by mass analyses of tryptic peptides (Fig. 2A and table S2) and whole proteins (fig. S17), and had a good recovery yield (between 50 and 70%). Selective and differential lysine methylations were also demonstrated by Western blot analysis using antibodies against H3K79me1, H3K79me2, and H3K79me3 (Fig. 2B). To determine whether other PTM moieties could be coupled onto H3Dha79, we synthesized formyl iodide [*N*-(3-iodopropyl)formamide, **4**] and acetyl iodide [*N*-(3-iodopropyl)-acetamide, **5**; fig. S5] and used them for producing histones H3K79^N-formyl and H3K79^N-acetyl, respectively, as verified by MALDI-TOF MS analysis (fig. S18).

To demonstrate whether our three-step synthesis can be used for modification of other proteins, we set out to generate ubiquitin variants with site-specific modifications. Eight ubiquitin variants with various lysine modifications (acetylation and differential

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definitive analysis of the epimeric ratio will require high-resolution crystallography of the protein. However, the steric context of the local protein conformation may substantively affect the final diastereomeric ratio of the products. The fact that our synthetic proteins H3K79me1, H3K79me2, and H3K79me3 were well recognized by antibodies, and that these modified histones could assemble into octamers with biological in vitro activity, underscores the utility of our approach.

We anticipate that with well-tailored alkyl iodides [e.g., (25)], our approach can be extended to produce designer proteins with diverse forms of chemical modifications (e.g., glycosylated amino acids and phosphotyrosine). Such efforts will markedly expand the available chemical diversity in proteins and facilitate the study of many PTM-mediated biological processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Approach for protein chemical modifications

(A) Schematic representation of the three-step synthesis of proteins with authentic PTMs.

(B) Our coupling scheme enables chemoselective carbon-carbon bond formation in proteins.

R, PTM moiety.

Fig. 2. Synthesis of proteins with selective differential methylations

(A) MALDI-TOF MS analysis of proteins after trypsin digestion. Formation of H3Sep79 is evidenced by the Sep-containing peptide (Sep79, blue). Synthesis of H3Dha79 generates a new Dha-containing peptide (Dha79, green) but eliminates the Sep-containing peptide. Coupling of the methyl iodides onto H3Dha79 produces new methylated lysine-containing peptides (K79me1, K79me2, and K79me3, red). m/z , mass/charge ratio; $[M+H]^+$, monoisotopic mass; a.u., arbitrary units. (B) Western blot analysis of the modified proteins, using antibodies (α) against H3K79me1, H3K79me2, and H3K79me3. CB, Coomassie blue. (C) Chemical structures of lysine and differentially methylated lysine residues.

Fig. 3. Effects of H3K79 methylation on chromatin transcription

(A) Schematic of the standard in vitro transcription assay. AcCoA, acetyl coenzyme A; NTP, nucleoside triphosphate. (B) Effect of methylated H3K79 on chromatin transcription (*x* axis, lane number; *y* axis, relative transcription activity). (C) Effect of methylated H3K79 on p300-mediated chromatin acetylation. Histone acetylation status was monitored by fluorography. (D) Schematic representation of transcriptional activation by H3K79 methylation. Pol, polymerase; GTF, general transcription factor; TBP, TATA-binding protein.