Catalytic Serine Labeling in Nonaqueous, Acidic Media

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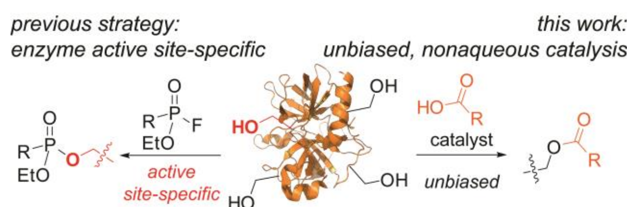
Abstract

Chemoselective modification of alkylalcohols (e.g., serine residues) on proteins has been a daunting challenge especially in aqueous media. Herein, we report chemical modifications of alkylalcohols in protein and cell lysate samples using carboxylic acid-based bioconjugation media. The acidic medium is not only useful to suppress reactivity of other nucleophiles in proteins, but the medium also serves as a potentially biomolecule-compatible solvent. The acidic labeling strategy has a unique selectivity paradigm compared to the common active-serine-targeted method and would act as a new strategy for studying biological roles of serine residues.



Although selective chemical reactions of alkylalcohols are ubiquitous processes in synthetic organic chemistry as well as cellular processes of biological systems, chemical tagging of alkylalcohol-based amino acid residues such as serine remains a formidable challenge in the field of chemical biology. Existing protein bioconjugation methods often are harnessed by inherent high nucleophilicity of amino acid side chains (e.g., lysine, cysteine, and tyrosine),[1] facilitating a wide range of applications including therapeutic/diagnostic use of bioconjugates[2,3] and interrogation of cellular processes through labeling of such target amino acids in living systems.[4] Even if serine is known to undergo post-translational modification most frequently among the 20 canonical amino acids,[5] chemoselective labeling of serine or chemical tools for serine to study its biological diversity is simply lacking.[6] The dearth of the serine labeling methods is likely due to the ubiquity of the functional group (i.e., OH group) and its modest nucleophilicity in aqueous media, and common oxophilic reagents used in organic chemistry for alkylalcohol substrates would be readily quenched by the solvent molecule before reaching the target amino acid. As natural systems take advantage of enzymatic processes for selective serine modification such as phosphorylation even in complex cellular environments,[7] various bioinspired phosphorous-based approaches have been devised to chemically tag serine residues in aqueous media including fluorophosphonates (active-site

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serine modification)[8] and oxathiophospholanes (chemoselective serine modification in peptides and a small protein)[9] although there has not been a general chemoselective modification method applicable to protein and cellular samples. Historically, organic chemists, on the other hand, began utilizing aprotic organic solvents to address the challenges of the alkylalcohol reactions.[10,11] Of course, the majority of aprotic organic solvents are incompatible with proteins because of their denaturation/aggregation,[12] but it is intriguing that serine-targeting protein bioconjugation in nonaqueous media that is compatible with biomolecules has not been pursued to date even though such an approach would address the challenges of serine labeling.

Recognizing the biocompatibility of various carboxylic acid-based molecules and protein stabilization by pH controls, this work demonstrates acid-catalyzed acylation reaction in nonaqueous acid media (Figure 1A,B). Our previous studies demonstrated protein bioconjugation in protein-compatible nonaqueous media such as ionic liquid[13–15] and fluoroalcohol solvents.[16,17] Notably, the urea-forming lysine modification process in ionic liquid did not cause any noticeable effects on native protein abilities such as streptavidin and antibody binding capabilities even by introducing the strong base/nucleophile iminophosphorane (pK_a of its conjugate acid is >20[18]).[13] In aqueous media, extremely acidic and basic conditions are often avoided for studying proteins in part due to the potential hydrolysis of amide backbones, though those extreme pH ranges could be helpful for the prevention of protein precipitation in terms of their isoelectric points (Figure 1C).[19] Because a hydrolysis process would not be a favorable chemical event in nonaqueous media with a substantially low concentration of water compared to aqueous solutions, we hypothesized that extreme pH conditions could be potentially protein-compatible, which has been demonstrated by the iminophosphorane-based bioconjugation in ionic liquid as an extremely basic process. Carboxylic acid-based molecules are often biocompatible and used as common buffer components for biochemical studies (e.g., acetate, glycine, and citrate buffers),[20] and therefore, we examined alcohol-selective chemical reactions in nonaqueous carboxylic acid-based media as an extremely acidic bioconjugation process. In particular, our focus was an acid-catalyzed acylation approach (i.e., Fischer esterification) that would be not only potentially biomolecule-compatible due to the abovementioned reasons, but would the acidic media also suppress the reactivity of many intrinsically basic/nucleophilic amino acid side chains through their protonation.

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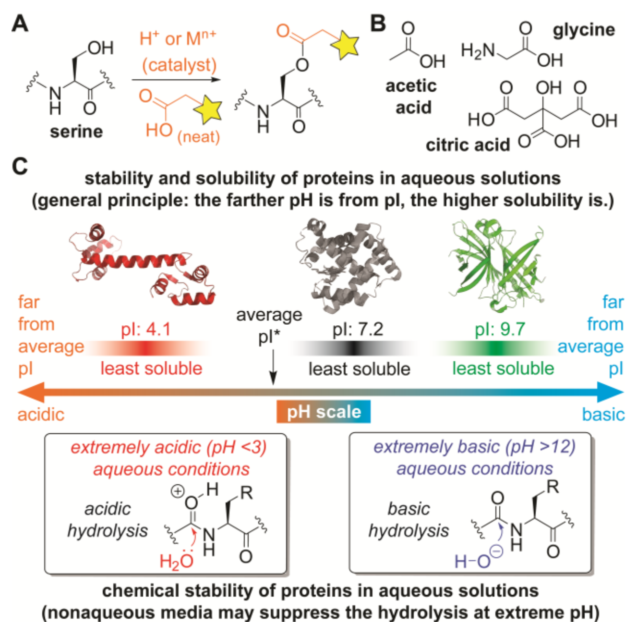

Figure 1. Nonaqueous, acidic media for labeling alkylalcohol of proteins. (A) General reaction scheme of this work using carboxylic acid-based molecule as a bioconjugation medium. (B) Chemical structures of common biological buffers containing carboxylic acid groups. (C) Schematic depiction of protein stability/solubility and their hydrolysis susceptibility in aqueous solution at different pH ranges. *Average isoelectric point (pI) for proteins across kingdoms of life based on literature.[21]

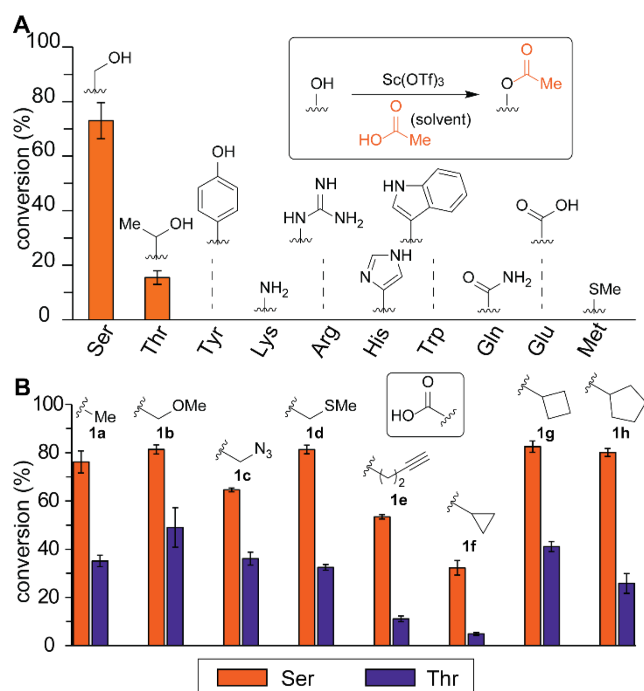

Figure 2. Amino acid screening and carboxylic screening of the acid-catalyzed acylation reaction. Typical reaction conditions: Amino acid (5 mM) and catalytic amount of scandium (III) triflate (1.25 mM) in acetic acid at 50 °C for 24 h. Error bars represent standard deviation (n = 3). (A) Schematic illustration of the acylation reaction and a bar graph showing the reaction conversion of liquid-chromatography (LC)-based analysis of modification of different N-protected (F-moc) amino acids. (B) Bar graph showing the reaction conversion of LC-based analysis of modification of N-protected serine and N-protected threonine with different carboxylic acids.

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Catalytic acylation processes in an acidic medium enabled chemoselective labeling toward the alkyl hydroxyl groups over other nucleophilic side chains at an amino-acid level (Figures 2A, S1–S4). To test our hypothesis, *N*-protected canonical amino acids were subjected to the acylation processes with acetic acid as a solvent and a catalytic amount of scandium triflate. The acylation products were observed only for serine and threonine in liquid-chromatography mass spectrometry (LC-MS) analysis with a higher selectivity toward serine over threonine (Figure S5). In contrast, other nucleophilic amino acids including tyrosine, lysine, arginine, histidine, tryptophan, glutamine, glutamic acid, and methionine did not show meaningful product formation (Figures S6–S8), indicative of the high chemoselectivity through suppression of the reactivities of those typical nucleophiles. Peptide-substrate scope investigation and tandem-mass spectrometry (MS/MS) also suggested chemoselective modification of alkyl hydroxyl groups and suggested more efficient modification of serine compared to threonine (Table S1, Figures S9–S26).

Screening of a series of carboxylic acid labeling reagents implied that reactivity and selectivity control of the bioconjugation is possible through electronic and steric alterations (Figures 2B, S27–S34). We have tested carboxylic acid compounds to assess both the electronic and steric effects on reactivity and selectivity. While some carboxylic acids are solids under ambient conditions such as pentynoic acid (**1e**), we discovered that the labeling process proceeds efficiently in a 4–5 M solution of the acid in a nonaqueous solvent such as acetonitrile and ionic liquid (Figures S35, S61–62). Whereas overall modification efficiency varied by a type of carboxylic acid under the tested conditions, we did not observe clear trends of the selectivity changes toward serine over threonine through the introduction of heteroatoms at the α-position as well as cyclic structures for electronic and steric perturbations. The acylation product formation was also observed by the acids with bioorthogonal handles such as azide and terminal alkyne groups, although the reaction with azidoacetic acid showed an unidentified byproduct in the LC-MS analysis (Figure S29). The feasibility of the acylation process with 4-pentynoic acid (**1e**) and subsequent copper-catalyzed azide-alkyne cycloaddition reaction of the acylated group was confirmed as well (Figures S36–S44). It is noteworthy that typical reactive acylating reagents (i.e., acyl chlorides and acid anhydrides) were reported to modify even tyrosine residues in addition to serine,[22,23] underscoring the importance of the acidic catalysis.

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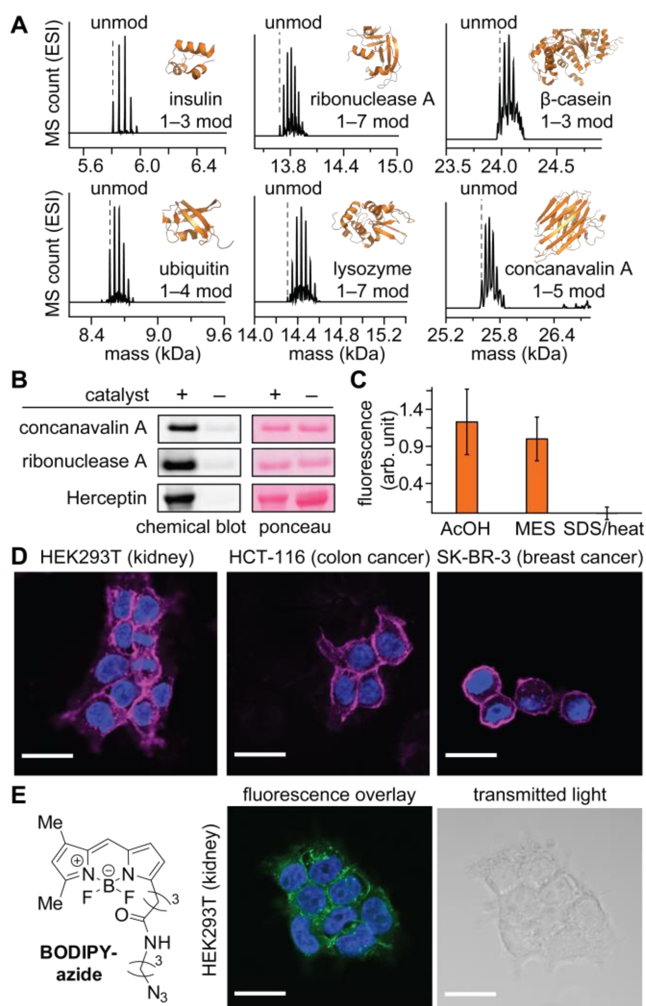

Figure 3. Acid-catalyzed acylation of proteins and effects of the carboxylic acid media/acid-catalyzed acylation process on the activity of proteins. (A) Electrospray ionization mass spectrometry (ESI-MS) analysis of the modification of various proteins with acetic acid in the presence of trifluoroacetic acid (TFA) catalyst. Typical modification conditions: protein (20–100 µM) and TFA (1–12% v/v, 0.13–1.6 M) in acetic acid at 37 °C or 50 °C for 24 h. (B) Chemical blot analysis (detection of an alkyne tag of a protein on a blot membrane with fluorogenic coumarin azide)[24] of the modification of different proteins treated with an alkyne-tagged acid **1e**. Catalyst: TFA. (C) Fluorescence-based activity assay of lysozyme treated with different conditions. (D) Confocal microscopy analysis of three different cell lines (HEK 293T, HCT-116, and SK-BR-3) stained with wheat germ agglutinin (WGA)–fluorophore conjugate (WGA CF®633, magenta) treated in acetic acid. Nuclear stain was performed with DAPI (blue). Scale bars: 20 µm. Each fluorescence channel with positive control experiments (using untreated WGA) is shown in Figure S69–S71. (E) Chemical structure of BODIPY-azide and confocal microscopy analysis of HEK293T cells stained with WGA modified with an alkyne-tagged acid **1e** followed by BODIPY-azide. Cells were visualized by WGA-BODIPY conjugate (green) and nuclear stain (DAPI, blue). Scale bars: 20 µm.

The acid-catalyzed acylation processes were able to modify a range of polypeptide substrates, enabling the installation of a bioorthogonal handle for imaging purposes (Figure 3). Trifluoroacetic acid (TFA) was chosen as a catalyst based on the results of catalyst screenings with peptide and protein as substrates (Table S2–S3, Figures S45–S57) in addition to its compatibility with LC-MS analysis. The acylation products were observed for polypeptides such as insulin (the number of serine residues: 3), ubiquitin (3), ribonuclease A (15), lysozyme (10), β-casein (16), and concanavalin A (31) as shown in Figure 3A and Figures S58–S60. We previously demonstrated that ionic liquids can be conducive for retaining protein structure and activity,[13,25] and we employed imidazolium-based ionic liquid (ethyl-

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methylimidazolium tetrafluoroborate) as a solvent for labeling with a solid alkyne-tagged carboxylic acid **1e**. Using a blot membrane-based alkyne detection method,[24] positive fluorescence responses were observed for modification of model proteins concanavalin A, ribonuclease A, and Herceptin, suggesting the successful installation of the alkyne tag through the acylation process (Figures 3B, S61). The modification of concanavalin A and ribonuclease A was also confirmed by mass spectrometry (Figure S62). While the trypsin digestion and subsequent tandem mass spectrometry analysis of modified protein showed some adduct formation on lysine and tyrosine residues to a certain extent (Figures S63–S93), peaks of modified serine peptide fragments were substantially high in the mass count (Table S4), which also supports our hypothesis about the alkylalcohol selectivity of the method. Although the intact MS analysis did not indicate the adduct formation of TFA catalyst to model protein and peptide substrates, it should be noted that some TFA adducts were observed in the proteomics study to a lesser extent.

The compatibility of the acidic bioconjugation conditions with protein substrates was demonstrated through enzyme activity assays and immunofluorescence experiments (Figure 3C–E). To estimate the effects of an acidic nonaqueous medium on the activity of an enzyme, lysozyme (a glycosyl hydrolase) was incubated in acetic acid at 37 °C for 24 hours and then subjected to a fluorescence-based enzyme-activity assay.[26,27] The acid-treated lysozyme showed a comparable level of fluorescence intensity as the positive control sample treated in an aqueous buffer (Figure 3C), and thus the retained enzymatic activity in aqueous buffer after the acid treatment was indicated. We also tested the binding activity of a commercial fluorophore conjugate of a glycol-protein binding protein, wheat germ agglutinin (WGA CF®633) to cancer cell lines after incubation in acetic acid without a catalyst (i.e., non-acetylating conditions), and the confocal microscope imaging demonstrated similar fluorescence intensity and staining pattern of the acid-treated sample to those of the positive control protein treated in an aqueous buffer (Figures 3D, S96–S98). Encouraged by those results of the retention of protein activities in acidic media without the acylation process, we then evaluated the binding activity of modified WGA after the catalytic bioconjugation in the acidic medium as well as subsequent fluorophore attachment through the copper-catalyzed azide-alkyne cycloaddition reaction. The fluorescently labeled protein was characterized by gel fluorescence (Figure S99). The localization patterns of the HEK293T cells stained with the prepared fluorophore conjugate proved virtually identical to those by the commercial staining dye (Figures 3E, S100), and the acidic modification and alkylalcohol tagging processes probably did not have substantial negative impacts on the protein activity.

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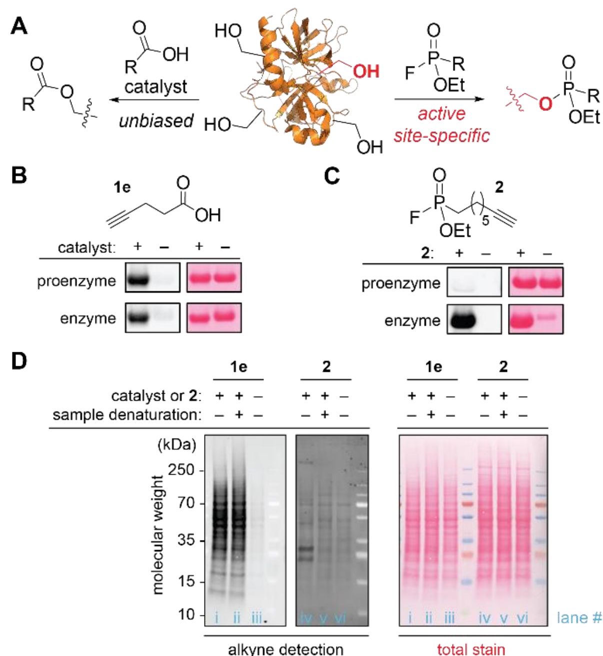

Figure 4. Comparison of the acid-catalyzed acylation bioconjugation with the active-site serine modification by fluorophoshonate. (A) Schematic illustration of the difference between the acid-catalyzed acylation (unbiased) and modification with fluorophosphonate (active site-specific). (B) Chemical blot analysis (detection of an alkyne tag in proteins on a blot membrane)[24] of the modification of α-chymotrypsinogen A (proenzyme) and chymotrypsin (enzyme) treated with an alkyne-tagged acid **1e**. (C) Chemical blot analysis of the modification of α-chymotrypsinogen A and chymotrypsin treated with fluorophosphonate alkyne **2**. (D) Chemical blot analysis of the modification of HEK293T cell lysate treated with either an alkyne-tagged acid **1e** (lane i–iii) or fluorophosphonate **2** (lane iv–vi). Sample denaturation: 80 °C for 5 min before the labeling process (lane ii and v). Catalyst: trifluoroacetic acid (TFA).

The bioconjugation in the acidic media can offer general alkylalcohol labeling without obvious preferences to active serines in enzymatic pockets (Figure 4). The commonly used alcohol-targeting protein labeling reagents, fluorophosphonates, are known to selectively modify the serine residues of the active site of serine proteases.[8] As the fluorophosphonate-based labeling is limited to enzyme active sites, we envisioned that the acylation catalysis may be able to act as a complementary alkylalcohol-targeting method. Incubation of a model serine protease (α-chymotrypsin) and corresponding proenzyme (α-chymotrypsinogen A) with fluorophosphonates **2** showed a sole modification toward chymotrypsin whereas the proenzyme remained intact.[28] On the other hand, the acid-catalyzed acylation showed adduct formations in both substrates, indicating the independent nature of the labeling efficiency from the active site status (Figures 4A–C, S102–S104).[29] Finally, the unique selectivity profile of the acidic catalysis was also demonstrated at a cell lysate level (Figure 4D). First, the acylation processes in acidic media did not show any noticeable changes to the band shapes and patterns of HEK293T cell lysates in the total stain image from those of the samples in aqueous solutions. Compared to the cell lysate modified by **2**, numerous bands showed fluorescence signals for the acylation samples in the presence of the catalyst in the blot analysis, suggesting modification on many types of proteins beyond specific serine proteases. The fluorescence signals with proteins treated with fluorophoshonate **2** were decreased for heat-denatured cell lysates through the loss of the protease activity as reported in the literature.[8,30,31] By contrast, the acid-catalyzed acylation did not show an apparent difference by the heat denaturation, further confirming that the acid-catalyzed protein modification is independent of the serine protease activities. The band pattern of cell lysate of HEK293T modified with fluorophosphonate alkyne **2** was

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similar to the reported gel-based activity protein profiling results with fluorophosphonate-fluorophore conjugates (Figure S105).[32,33] These results indicate that the acid-catalyzed acylation method would serve as a complementary serine-targeting method to the enzyme-specific labeling with fluorophosphonates.

Though a variety of chemical reactions of serine in natural systems has been known for decades, the challenges to achieve chemical modification of serines have been the longstanding issue of chemists, and the present work demonstrated the catalytic bioconjugation in biomolecule-compatible, nonaqueous acidic media. With the success of cell lysate labeling described above, the acylation method would be applicable to the profiling of the activity of serine residues that are not located in enzyme active sites.[34,35] In contrast to the natural system facilitating reactions of a mildly reactive solvent molecule (i.e., water) through actions of enzymes as catalysts, the nonaqueous strategy promoted the acylation reaction with a solvent molecule through acid catalysis by reaching the extreme pH scale that would not be accessible in aqueous media. As acid catalysis is a common synthetic organic chemistry method,[36] the acidic nonaqueous approach can be extended to a variety of other chemical transformations, which would expand the repertoire of the catalytic bioconjugation strategies.

Notes

The authors declare no competing financial interest.

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