Dual-Functional-Tag-Facilitated Protein Labeling and Immobilization

Xinyi Zhang
University of Kentucky, xinyi.zhang@uky.edu

Wei Lu
University of Kentucky

Kevin Kwan
University of Kentucky, kevin.kwan@uky.edu

Dibakar Bhattacharyya
University of Kentucky, db@uky.edu

Yinan Wei
University of Kentucky, yinan.wei@uky.edu

Click here to let us know how access to this document benefits you.

Follow this and additional works at: https://uknowledge.uky.edu/chemistry_facpub

Part of the Amino Acids, Peptides, and Proteins Commons, Cell and Developmental Biology Commons, Chemistry Commons, and the Molecular Biology Commons

Repository Citation
Zhang, Xinyi; Lu, Wei; Kwan, Kevin; Bhattacharyya, Dibakar; and Wei, Yinan, "Dual-Functional-Tag-Facilitated Protein Labeling and Immobilization" (2017). Chemistry Faculty Publications. 96.
https://uknowledge.uky.edu/chemistry_facpub/96

This Article is brought to you for free and open access by the Chemistry at UKnowledge. It has been accepted for inclusion in Chemistry Faculty Publications by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
Dual-Functional-Tag-Facilitated Protein Labeling and Immobilization

Notes/Citation Information
Published in ACS Omega, v. 2, issue 2, p. S22-S28.

Copyright © 2017 American Chemical Society

This is an open access article published under an ACS AuthorChoice License, which permits copying and redistribution of the article or any adaptations for non-commercial purposes.

Digital Object Identifier (DOI)
https://doi.org/10.1021/acsomega.6b00512

This article is available at UKnowledge: https://uknowledge.uky.edu/chemistry_facpub/96
Dual-Functional-Tag-Facilitated Protein Labeling and Immobilization

Xinyi Zhang,† Wei Lu,† Kevin Kwan,† Dibakar Bhattacharyya,‡ and Yinan Wei*†‡

†Department of Chemistry and ‡Department of Chemical and Materials Engineering, University of Kentucky, Lexington, Kentucky 40506, United States

ABSTRACT: An important strategy in the construction of biomimetic membranes and devices is to use natural proteins as the functional components for incorporation in a polymeric or nanocomposite matrix. Toward this goal, an important step is to immobilize proteins with high efficiency and precision without disrupting the protein function. Here, we developed a dual-functional tag containing histidine and the non-natural amino acid azidohomoalanine (AHA). AHA is metabolically incorporated into the protein, taking advantage of the Met-tRNA and Met-tRNA synthetase. Histidine in the tag can facilitate metal-affinity purification, whereas AHA can react with an alkyne-functionalized probe or surface via well-established click chemistry. We tested the performance of the tag using two model proteins, green fluorescence protein and an enzyme pyrophosphatase. We found that the addition of the tag and the incorporation of AHA did not significantly impair the properties of these proteins, and the histidine–AHA tag can facilitate protein purification, immobilization, and labeling.

INTRODUCTION

Proteins are functional materials created by nature to execute diverse activities in living organisms. The superb selectivity, specificity, and performance of proteins have made them highly desirable components in the creation of biomimetic materials. A major obstacle in the utilization of proteins in biotechnology is the difficulty with site-specific immobilization of proteins without compromising their functions. Introduction of a peptide tag has been a popular method to facilitate protein immobilization or detection. Such peptide tags include the GST tag,1,2 strep tag,3–5 HA tag,6,7 flag tag,8–10 arg tag,11,12 c-myc tag,13,14 and His-tag.15,16 Proteins bearing these tags can be captured or detected via interactions with their corresponding binding-partner modules or antibodies. Peptide tags are usually directly encoded into the gene of the target protein and thus have the advantage of being convenient and highly specific. Although they are very useful in the purification and detection of the tagged proteins, the application of most peptide tags in protein immobilization and modification is limited by the noncovalent nature of the interaction, which suffers from drawbacks including a high off-rate and low mechanical resilience. Several systems have been developed to promote the formation of covalent bonds between a residue in the peptide tag and its catcher module, such as split inteins,17 the SpyTag/SpyCatcher,18–20 cysteine and α-chloroacetyl interaction facilitated by the coil–coil interaction,21 the His-tag and nitrotriacetate-based arylazide photoreactive label via click chemistry,22,23 and the use of the substrate peptide of a ligase or transferase, such as the LAP tag,24 the Q tag,25 the sortagging motif,26 the formyl glycine tag,27 and the peptides A1 and S6.28

Incorporation of non-natural amino acids is another useful technique used in the site-specific modification of proteins. Examples include the azido- or alkyne-containing residues via click chemistry,29 norbornene-containing residues that react with tetrazine-based probes,30 and p-azido-l-phenyalanine via photocatalytic reaction.31 Non-natural amino acids are usually introduced into the protein of interest through the introduction of a dedicated orthogonal pair of tRNA and tRNA synthetase that translate a stop codon (typically TAG) into the specific non-natural amino acid. Recently, this method has been coupled with in situ biosynthesis to incorporate a sulfur-containing noncanonical amino acid, S-allyl-l-cysteine, into Escherichia coli proteins.32 Subsequently, the non-natural amino acid can introduce unique chemistry to facilitate site-specific modification. Alternatively, if the structure of a non-natural amino acid is very similar to that of a natural amino acid, it can be incorporated metabolically using the corresponding auxotrophic strain. In this case, no additional cellular
machineries need to be introduced. For example, azidohomoalanine (AHA) can be incorporated into the sequence of proteins by Met-tRNA and tRNA synthetase (Figure 1A). This method has been used by several groups to label and modify proteins. However, labeling of the incorporated AHAs often suffers from poor efficiency because methionine residues usually form part of the hydrophobic core of a protein and thus have limited accessibility to reactions. Although all proteins contain methionine (and thus the ATG codon for AHA incorporation) in their amino acid sequences, only a small percentage of these residues seemed to be accessible for labeling. One potential solution is to denature and unfold the target protein before labeling to improve the accessibility of the AHA residues. This approach is very useful in the detection of a target protein but not suitable for applications that demand active proteins. Otherwise, the residue can be introduced at the surface of the protein via site-directed mutagenesis. Here, we take advantage of the convenience of metabolic incorporation and address the issue of limited accessibility through the genetic introduction of a tag containing the ATG codon. To avoid the formation of a hydrophobic patch that may potentially affect protein folding, we inserted polar residues (histidine or serine) between neighboring Met/AHA residues. The tag has two functions: histidine in the tag can facilitate protein purification via the conventional metal-affinity chromatography, whereas methionine (or rather, its replacement by AHA) can be used in covalent labeling. The performance of the tags was evaluated using two model proteins, superfolder green fluorescent protein (sfGFP) and inorganic pyrophosphatase from Staphylococcus aureus (PpaC). sfGFP was chosen owing to its intrinsic fluorescence, whereas PpaC was chosen because its enzymatic activity can be conveniently measured using a colorimetric assay. Using these two model proteins, we demonstrated that the incorporation of the tag did not compromise the function of proteins, and the tag could facilitate both protein purification and modification. We expect the dual-functional His–AHA tag to be useful in general for various biotechnological applications.

### RESULTS AND DISCUSSION

#### Incorporation of AHA into the Target Proteins

Expression of AHA-containing proteins was performed using a methionine auxotrophic strain, DL41(DE3). DL41(DE3) could not grow in the absence of methionine (Figure 2A). The replacement of Met by AHA slowed down the growth of the strain and increased the doubling time at the exponential growth phase from 1 to 2 h, but the two cultures grew to similar densities at saturation. This result indicates that AHA can be used effectively as a replacement of methionine in the synthesis of proteins and supports cell growth. To further confirm that AHA was incorporated to replace methionine, we submitted AHA-containing PpaC-H6G3M4 (Table 1) for mass spectrometry peptide fingerprinting analysis. There are 13 methionine residues in the protein, including four in the tag. Among them, the peptide containing AHA-substituted Met1, Met125, or M142 was not detected. Peptides containing AHA replacement of all 10 other methionine residues were identified, indicating that these residues were at least partially replaced by AHA.

#### Incorporation of a Dual-Functional Tag at the C-Terminus of sfGFP

The intrinsic fluorescence of sfGFP makes it a popular model protein in studies involving protein modification, including ones using the azido-alkyne-based click chemistry. The polyhistidine tag has been used extensively in the purification of sfGFP. Here, we have tested two different tag designs, with the addition of two or four methionine residues (Figure 1 and Table 1). To avoid the potential creation of a hydrophobic patch, we juxtaposed methionine with histidine. For protein expression, E. coli strain DL41(DE3) containing the plasmid sfGFP-H6, sfGFP-H7M2, or sfGFP-H6M4 was grown in the M9 medium supplemented with 20 essential amino acids, as described in the Materials and Methods. As shown in Figure 1C, the different tags did not affect the expression level of the protein. The two proteins...
containing methionine residues in the tag can be purified similarly as that of the His-tagged sfGFP, with similar yields and purity (data not shown).

To examine the potential effect of the tag and the replacement of methionine by AHA on the protein structure, we measured the fluorescence spectra of the three proteins, sfGFP-H6, sfGFP-H7M2, and sfGFP-H6M4, containing methionine or AHA. After expression and purification, the fluorescence spectra of the protein samples were collected (Figure 2B). Six protein samples were examined, including the three different constructs of sfGFP expressed in the presence of either methionine or AHA. The wavelength of the emission peak was not affected by the addition of the tags or the incorporation of AHA. For each construct, the replacement of methionine by AHA did not affect the fluorescence intensity. The fluorescence intensity of sfGFP-H6M4 was approximately 10% lower than that of sfGFP-H6 and sfGFP-H7M2.

Then, we examined the effect of the Met-containing tag in promoting the efficiency of a click-chemistry reaction. Purified sfGFP bearing H6, H7M2, or H6M4 tag was subjected to labeling using biotin alkyn followed by detection using antibiotin western blot (WB). As shown in Figure 3A, the level of labeling of sfGFP-H6 was significantly better than that of sfGFP-H7M2, which was also better than that of sfGFP-H6M4. The relative levels of labeling of H6M4 and H7M2 were approximately 10- and 3-fold, respectively, as the level of sfGFP-H6. There are three intrinsic methionine residues in sfGFP, as highlighted in Figure 1B. The side chain of these methionine residues is likely involved in hydrophobic interactions in the native structure of sfGFP. Therefore, when AHA replaces these methionine residues, its side chains likely have limited accessibility for the reaction.

To demonstrate the usefulness of the tag in facilitating immobilization, we expressed sfGFP-H6M4 in the presence of AHA or methionine and incubated the alkyn agarose resin with purified proteins. After washing, images of the modified resin under normal white light or blue light (Figure 3B,C) were taken. It is clear that the reaction with the alkyn agarose resin depends on the presence of AHA in the protein. The immobilization of AHA-containing sfGFP-H6M4 is highly specific. AHA-containing sfGFP-H6M4 readily attached to the alkyn beads, whereas no binding could be detected for sfGFP-H6M4 expressed in the absence of AHA.

**Addition of the GS3M4 Tag to PpaC.** We used PpaC as a model enzyme to further examine the performance of the methionine-containing tag. Here, we added an octapeptide “GMSMSM” after the His-tag at the C-terminus of PpaC (Table 1). PpaC is a good model enzyme because the protein can be expressed and purified at high yields, and its catalytic activity can be measured using a convenient colorimetric assay. We have previously determined the crystal structure of PpaC-H6. It exists as a dimer, and each subunit contains nine intrinsic methionine residues (Figure 4A).

To examine the effect of the tag and the incorporation of AHA on the catalytic activity of PpaC, we first compared the catalytic activity of PpaC-H6 and PpaC-H6GS3M4 expressed in *DLA1(DE3)* grown in the presence of methionine (Figure 4B, black and red). The fit to the Michaelis–Menten equation yielded $K_{M}$ and $k_{cat}$ of $44 \pm 9 \mu$M and $887 \pm 97 \mu$mol$^{-1}$$\cdot$mg$^{-1}$, respectively, for PpaC-H6GS3M4, which are not significantly different from the $K_{M}$ and $k_{cat}$ of PpaC-H6 ($52 \pm 15 \mu$M and $897 \pm 98 \mu$mol$^{-1}$$\cdot$mg$^{-1}$, respectively). Next, we compared $K_{M}$ and $k_{cat}$ of PpaC-H6GS3M4 expressed with AHA (Figure 4B, red and blue). $K_{M}$ and $k_{cat}$ of the protein expressed from cells grown in the presence of AHA are $43 \pm 11 \mu$M and $1050 \pm 101 \mu$mol$^{-1}$$\cdot$mg$^{-1}$, respectively. Differences in $K_{M}$ and $k_{cat}$ between the AHA-containing PpaC and non-AHA-containing PpaC are not statistically significant.

To compare the level of labeling of PpaC-H6 and PpaC-H6GS3M4, we expressed the two proteins in the presence of AHA as described. Although each PpaC subunit contains nine intrinsic methionine residues, the level of labeling of PpaC-H6 was much lower than the level of labeling of PpaC-H6GS3M4 (Figure 4C). Although similar amounts of proteins were used in the experiment, the band intensity of PpaC-H6GS3M4 in the anti-biotin WB was approximately four times the band intensity of PpaC-H6. In spite of the abundance of the intrinsic

<table>
<thead>
<tr>
<th>Table 1. Primer and Tag Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>construct</td>
</tr>
<tr>
<td>sfGFP-H7M2</td>
</tr>
<tr>
<td>sfGFP-H6M4</td>
</tr>
<tr>
<td>PpaC-H6GS3M4</td>
</tr>
</tbody>
</table>

| Figure 3. | (A) Coomassie blue (CB) stain and anti-biotin WB analysis of three AHA-containing sfGFP constructs reacted with biotin alkyn. (B) Alkyne agarose resin incubated with sfGFP-H6M4 expressed with methionine and then imaged using normal white light (left) or blue light (right). (C) Alkyne agarose resin incubated with sfGFP-H6M4 expressed with AHA and then imaged using normal white light (left) or blue light (right). |
methionine, the addition of an ATG-encoding tag greatly improved the efficiency of modification.

Finally, we examined the usefulness of the AHA-containing tag in the immobilization of PpaC. PpaC-H6GS3M4 expressed in the presence of methionine or AHA was incubated with alkyne agarose resin for immobilization as described in Materials and Methods. After immobilization, the resin was washed with the corresponding lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 200 mM NaCl, pH 7.5 for PpaC or 20 mM phosphate, 200 mM NaCl, pH 7.5 for sfGFP). The protease inhibitor phenylmethylsulfonyl fluoride was added freshly to a final concentration of 0.5 mM. The cells were lysed through sonication and then centrifuged at 10,000 rpm for 10 min. Cell pellets were stored at −80 °C. Control proteins with normal methionine in the tag were expressed the same as described except for replacing 50 μg/mL of AHA with 40 μg/mL of methionine in the culture medium.

**Protein Purification.** For purification, the cell pellet was resuspended in 30 mL of lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 200 mM NaCl, pH 7.5 for PpaC or 20 mM phosphate, 200 mM NaCl, pH 7.5 for sfGFP). The protease inhibitor phenylmethylsulfonyl fluoride was added freshly to a final concentration of 0.5 mM. The cells were lysed through sonication and then centrifuged at 10,000 rpm, 4 °C for 20 min. The supernatant was collected and incubated with Ni-NTA agarose beads (Qiagen) for 40 min at 4 °C with shaking. The resin was then loaded into an empty column, drained, and washed with the corresponding lysis buffer supplemented with 40 mM imidazole. Finally, proteins were eluted using the corresponding lysis buffer supplemented with 500 mM imidazole. After purification, imidazole in the samples was removed by dialysis against the lysis buffer.

**Bacteria Growth Curve.** DL41(DE3) strain was cultured overnight in M9 medium supplemented with 20 essential amino acids. The next morning, the overnight culture was used to inoculate three cultures of M9 medium supplemented with 19 essential amino acids (each at 40 μg/mL, no methionine) plus AHA (50 μg/mL), with 19 amino acids (each at 40 μg/mL, no methionine), or with all 20 essential amino acids (each at 40 μg/mL). Cell growth was measured by monitoring the absorbance at 600 nm. The cells were then grown at 37 °C with shaking at 250 rpm until its absorbance at 600 nm (OD600) reached 0.8, and then the cells were induced with 1 mM isopropyl-β-thiogalactopyranoside (IPTG). Over night-exposure, the cells were harvested by centrifugation at 8000 rpm for 10 min. Cell pellets were stored at −80 °C. Control proteins with normal methionine in the tag were expressed the same as described except for replacing 50 μg/mL of AHA with 40 μg/mL of methionine in the culture medium.

**Materials and Methods.**

**Plasmid Construction.** Plasmids pET22-sfGFP and pET22-PpaC were created as described in earlier studies. C-terminal tags were introduced via the fast cloning method using pET22-sfGFP or pET22-PpaC as the template, and primers are listed in Table 1. All coding sequences were confirmed through DNA sequencing.

**AHA Incorporation into Target Protein.** For protein expression, the corresponding plasmid was transformed into E. coli strain DL41(DE3). Single colonies were first cultured overnight at 37 °C in M9 medium containing ampicillin (100 mg/L), and then the Met-starved overnight culture was used to inoculate 30 mL of fresh M9 medium containing 19 essential amino acids (each at 40 μg/mL without methionine) and 50 μg/mL of AHA with 10-fold dilution. The cells were grown at 37 °C with shaking at 250 rpm until its absorbance at 600 nm (OD600) reached 0.8, and then the cells were induced with 1 mM isopropyl-β-thiogalactopyranoside (IPTG). After overnight-exposure, the cells were harvested by centrifugation at 8000 rpm for 10 min. Cell pellets were stored at −80 °C. Control proteins with normal methionine in the tag were expressed the same as described except for replacing 50 μg/mL of AHA with 40 μg/mL of methionine in the culture medium.
absorbance of the cell cultures at 600 nm (OD600) at the indicated time.

**Protein Biotinylation via Click Chemistry.** The reactivity of AHA residues in the structure of sfGFP and PpaC was examined through their reaction with PEG4 carboxamide-propargyl biotin (biotin alkyne). To initiate the reaction, biotin alkyne (50 μM), tris[1-(benzyl-1H-1,2,3-triazol-4-yl) methyl]amine (TBTA, 600 μM), and CuBr (600 μM) were added into the purified protein sample in the HEPES buffer. The reaction mixture was incubated at room temperature with shaking for 5 min for sfGFP constructs and 1 h for PpaC constructs and then analyzed using anti-biotin WB.

**Protein Immobilization to Alkyne Agarose Resin.** Alkyne agarose resins were purchased from Jena Bioscience. The alkyne agarose beads were first washed with 10 bed volume of HEPES buffer (20 mM HEPES, 200 mM NaCl, pH 7.5) three times and then resuspended in 2 bed volume of HEPES buffer containing the indicated protein. TBTA was added to a final concentration of 600 μM followed by mixing using a pipette tip. CuBr was then added to a final concentration of 200 μM. The reaction mixture was mixed thoroughly using the pipette tip and incubated at room temperature for 2 h. Finally, the beads were washed using 10 bed volume of HEPES buffer via centrifugation three times.

**Gel Electrophoresis and WB.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was preformed using 20% tris–glycine gel. For WB, proteins were transferred to a polyvinylidene fluoride membrane after SDS-PAGE and detected using a monoclonal antibiotic antibody—alkaline phosphatase conjugate.

**Fluorescence Spectroscopy.** Fluorescence emission spectra were recorded using a PerkinElmer LS-55 fluorescence spectrometer (PerkinElmer, Waltham, MA) at 20 °C at an excitation wavelength of 485 nm. Fluorescent images were taken using a fluorescence microscope (Nikon, Melvyl, NY).

**PpaC Activity Assay.** The PPase activity was measured using Mn pyrophosphate as the substrate as described.\(^{48}\) Stock solutions of MnCl\(_2\) and sodium pyrophosphate were mixed at a 1:1 molar ratio at 0.5 mM right before the analysis (mixing an equal volume of 1.0 mM MnCl\(_2\) and 1.0 mM sodium pyrophosphate) and diluted to the indicated substrate concentration for the activity measurement. At neutral pH, pyrophosphate is not fully deprotonated. The major species is MnH\(_2\)PPi (MnPPi), which was considered to be the substrate for PpaC.\(^{50}\) A stock solution of malachite green (0.12%, w/v) was made by dissolving the dye in 3 M sulfuric acid. A working solution was always prepared fresh by adding one volume of 7.5% (w/v) ammonium molybdate into four volumes of the malachite green stock solution followed by the addition of Tween 20, to a final concentration of 0.2% (v/v). This solution is used to both terminate the enzyme reaction and initiate the colorimetric reaction to determine the concentration of phosphates. For activity measurements, PpaC was added into a freshly prepared reaction mixture containing the indicated concentration of MnPPi, in a reaction buffer (25 mM Tris-Cl, 50 mM NaCl, pH 7.0) at room temperature for 5 min. To terminate the enzymatic reaction and determine the phosphate concentration of a sample, one volume of the working solution was mixed with four volumes of the enzymatic reaction mixture to be analyzed. For immobilized PpaC, the reaction mixture was subjected to a quick centrifugation, and the supernatant was collected for analysis. The mixture was incubated for 5 min for the color to develop, and the absorbance at 630 nm was measured. We have experimented with the conditions and confirmed that under these conditions, the product phosphate accumulation over time was linear. Therefore, the absorbance at 630 nm, after correction for background, directly correlates with the rate of hydrolysis. Michaelis–Menten constant \(K_M\) and specific enzymatic activity were determined through fitting the measured values using SigmaPlot.

**ACKNOWLEDGMENTS**

We would like to thank Drs. Jing Chen and Haining Zhu for their help with the mass spectroscopy peptide fingerprinting analysis. This work was supported by the Kentucky NSF EPSCoR RII Award 1355438 (to D.B. and Y.W.) and the Igniting Research Collaboration Award from the University of Kentucky (to Y.W.).

**REFERENCES**

(3) Schmidt, T. G. M.; Skerra, A. The random peptide library-assisted engineering of a C-terminal affinity peptide, useful for the detection and purification of a functional Ig Fv fragment. Protein Eng. 1993, 6, 109–122.


