Site-Specific Dual Functionalization of Cysteine Residue in Peptides and Proteins with 2-Azidoacylates

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Supporting Information

ABSTRACT: Herein, we report use of 2-azidoacylates to perform site-specific dual functionalization of the cysteine residue of peptides and bovine serum albumin (BSA), a native protein containing one free cysteine residue. The sulfhydryl group of the cysteine residue could be conjugated with 2-azidoacylates bearing various functionalities, such as fluorescent dyes under physiological aqueous buffer conditions, to afford peptide and protein conjugates anchoring an azide moiety. Successive azide–alkyne cycloaddition enables installation of the second functionality, thus affording dual-functionalized peptide- and protein-based materials.

Chemical conjugation of proteins is paramount for various kinds of biological applications such as the probing of protein dynamics in vitro or in vivo, investigation of protein structures and their functions, enhancement of protein stability in biological systems and resulting therapeutic efficacy, development of artificial enzymes or protein-based materials, and construction of protein–drug conjugates. Because proteins consist of various amino acid residues having different functional groups, protein modification should be carried out selectively at the defined site and should be able to be performed under physiologically relevant conditions. Among about 20 different amino acids involved in the protein composition, cysteine (Cys) is one of the most convenient targets for bioconjugation because of higher nucleophilicity of the sulfhydryl group. In addition, lower natural abundance of the cysteine residue might be useful to avoid undesired multiscite modification. Therefore, various methods for the Cys modification have been developed, such as alkylation through nucleophilic substitution with α-halo carboxylic reagents and 1-(arylsulfonyl)bicyclo[1.1.0]butanes (strain-release reagents), disulfide exchange, nucleophilic aromatic substitution (SNAr) of perfluorobenzene derivatives, thiol-yne or yne reactions, and conjugate addition onto the maleimide derivatives. Among them, the maleimide derivatives are the most commonly used chemical linker because of their excellent kinetic property in spite of the instability of the resulting conjugates due to the retro-Michael reaction.

Ability in multifunctionalization for chemical modification of proteins has been in high demand because advanced peptide probes and protein-based therapeutics frequently need multiple functions on the basis of the conjugation through photo- and fluorophor dyes or targeting moieties such as biotin and drug molecules. However, current methods for the multifunctionalization of proteins mainly depend on genetic engineering to prepare artificial proteins having different bioorthogonal handles and subsequent multistep bioorthogonal reactions under the precisely controlled conditions. However, development of multifunctionalization of native proteins is still amateur due to lacking of the versatile linkers. To develop the linker capable of multifunctionalizing native proteins, we became interested in design and use of 2-azidoacylate derivatives as the Michael acceptor for cysteine-selective conjugation, which can simultaneously install an azide group at the same position of the target proteins for the continuous second functionalization by azide–alkyne cycloaddition. At the outset of the project, we prepared four types of 2-azidoacylates having an ethyl group (1a), a hydrophilic short polyethylene glycol (short-PEG) moiety (1b), and hydrophobic parts (dodecyl 1c and fluorescent dicyanamide 1d). The optimization of the reaction conditions of the first conjugation using ethyl 2-azidoacylate (1a) and cysteine methyl ester (2) revealed that the conjugation proceeds smoothly and selectively in sodium phosphate buffer (50 mM) at pH 7.4, affording α-azidoester-cysteine conjugate.
Scheme 1. Site-Specific Dual Functionalization of Proteins with 2-Azidoacrylates

3a in 77% isolated yield within 0.5 h. Similarly, the reaction with short-PEG derivative 1b proceeded well, giving 3b in 76% isolated yield. However, these with hydrophobic 1c and 1d should be conducted in the cosolvent system of aqueous buffer and THF to afford conjugates 3c and 3d, respectively, in good yields, albeit with longer reaction times. The reactions of tripeptide, glutathione (4) with 2-azidoacrylates 1a–d could also show chemoselectivity with the sulphydryl group to deliver the corresponding conjugates 5a–d, respectively, in good yields (Scheme 2C). Furthermore, the installed azide group in 5b smoothly reacted with fluoresein-linked dibenzoyletoctyne (DBCO) 6 through strain-promoted alkyn–azide cycloaddition (SPAAC) to afford dual-functionalized glutathione 7 (Scheme 2D).

To obtain the insight into the kinetic property of the present conjugation with 2-azidoacrylate 1b, the consumption of 1b in the reaction with glutathione 4 under dilute reaction conditions ([1b] = 1.0 mM) was monitored at different time points using high-performance liquid chromatography (HPLC) (see the Supporting Information). The reaction was reached to plateau at about 60% conversion within 2 h, and no side-product was observed in the HPLC analysis. The consumption of 1b was in a well-fitted second-order manner, and its rate constant of 1b against 4 was estimated to be 0.509 ± 0.03 M⁻¹ s⁻¹, which is the similar range to that of strain-promoted alkyn–azide cycloaddition (SPAAC) commonly used as the bioorthogonal reaction.¹⁴ᵇ,c

Chemoselectivity of the present conjugation with 2-azidoacrylates 1 toward the sulphydryl group of the cysteine residue could be further proved by the reactions of 1b with three Cys-containing peptides at the N terminus (for 8a), internal peptide chain (for 8b) or the C terminus (for 8c). The conjugation of peptides 8a–c with 1 equiv of 2-azidoacrylate 1b was completed within 1.5 h (in which [8a–c] = 10 mM was selectivity found at the sulphydryl group, Scheme 3), which was

Scheme 2. Reactions of 2-Azidoacrylates 1 with Cysteine (2) or Glutathione (4)

A 2-Azidoacrylates evaluated

B Reactions of 2-azidoacylates 1 and cysteine methyl ester (2)

C Reactions of 2-azidoacrylates 1 and glutathione (4)

D Dual-functionalization of cysteine residue

¹⁵ mM phosphate buffer (pH 7.4) was used. The reaction was conducted in buffer/tetrahydrofuran (THF) (1:1). The reaction was conducted in buffer/THF (2:1). H NMR yields based on tert-butanol as the internal standard. Chemical conversion was determined by high-performance liquid chromatography. One of the regioisomers is shown.
confirmed by tandem mass spectrometry (MS/MS) analyses of the resulting conjugates 9a–c (see the Supporting Information). In addition, any mis-labeled peptides were not observed even when 10 equiv of 1b were mixed with 9a–c for 24 h. These studies clearly indicated that surroundings around the sulphhydryl group of the Cys residue in terms of the charge, hydrophobic, and hydrophilic properties do not significantly affect efficiency of the conjugation.

We next investigated capability of 2-azidoacrylate 1b in modification of the native protein, bovine serum albumin (BSA) (10), which contains one free cysteine residue in total 583 amino acid residues (MW = 66.5 kDa) (Scheme 4A). To optimize the reaction conditions, the remaining quantity of the free sulphhydryl group in BSA (10) were traced at different reaction time points by the Ellman test.32 Differently from the conjugation of short peptides, the conjugation reaction of BSA (10) under highly diluted reaction conditions required excess amounts (>10 equiv) of 2-azidoacrylate 1b and longer reaction time (>12 h) to achieve sufficient conversion of the conjugation (>85%). We then explored use of the resulting azide moiety in the conjugated-BSA 11 for further functionalization. Namely, successive treatment of 11 with fluorescein-linked DBCO (DBCO–FL) 6 was examined to induce strain-promoted alkyne–azide cycloaddition (SPAAC). This second functionalization took 12 h to attain reasonable conversion (see the Supporting Information) to afford BSA–PEG–FL 12. To confirm the site-specific dual functionalization, the control samples that emitted the incorporation process of either 1b or 6 were prepared and compared with dual-labeled BSA–PEG–FL 12 in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Scheme 4B). As shown in Scheme 4B, BSA–PEG–FL 12 in lane 4 showed both protein-staining and fluorescein-emission bands, whereas lane 3, which lacked 2-azidoacrylate 1b, showed very weak emission signal probably originated from the thiol-yne reaction of BSA (10) with DBCO–FL 6.33 These results indicate that dual functionalization of BSA (10) was accomplished. Furthermore, the MS spectrum of fragments of BSA–PEG–FL 12 digested by trypsin reveals 1b and 6 are site-specifically installed at Cys58 in BSA (10) (see the Supporting Information).

To demonstrate the versatile capability of the present dual functionalization strategy with 2-azidoacrylates, we further prepared fluorescein-linked 2-azidoacrylate 1e and use it for conjugation reactions of BSA (10) (Scheme 5). By following the procedure of dual functionalization (Scheme 5), BSA (10) (in 0.1 M) was treated with 2-azidoacrylate 1e (10 equiv) to form the first conjugate, 43, which was followed by SPAAC with cyanine dye-linked dibenzocyclooctyne (DBCO–Cy5) 14 to afford dual-color-labeled BSA 15. The SDS-PAGE analysis of 15 in Scheme 5 clearly indicated that BSA was modified with both fluorescein and Cy5 at the single site. Thus, the present dual color-labeling of native proteins would be useful for design of advanced protein-based chemical probes, such as ratiometric fluorescent probes and fluorescence resonance energy transfer (FRET)-based probes.34

Furthermore, BSA–FL 13 was applied for biotinylation using DBCO-biotin 16 to prepare dual-functionalized BSA–FL–biotin 17 (Scheme 6A). In SDS-PAGE analysis (Scheme 6B), BSA–FL–biotin 17 exhibited green fluorescence originated from fluorescein. To confirm the successful incorporation of biotin, enzyme-linked immunosorbent assay (ELISA) of BSA–FL–biotin 17, and its control samples were conducted by using streptavidin-coated beads, anti-BSA antibody and horseradish peroxidase (HRP)-conjugated secondary antibody (Scheme 6C). Only sample 4, containing BSA–FL–biotin 17, exhibited significant HRP activity, suggesting that biotin moiety is incorporated into BSA through the dual functionalization. Because biotin itself does not bear fluorescence property, concise incorporation of another reporter moiety such as
Scheme 5. Dual-Color Labeling of BSA (10) with 1e and DBCO–Cy5 14

In summary, we have developed site-specific dual functionalization of the cysteine residue of peptides and bovine serum albumin, a native protein containing one free cysteine residue with 2-azidoacrylates linked with various hydrophilic and hydrophobic functionalities. The 2-azidoacrylates could react with the sulphydryl group of the cysteine residues in a chemoselective manner over other amino acid residues to give the α-azidoester conjugate, which was further functionalized through alkyne–azide cycloaddition, thus enabling facile dual functionalization at the single site of the cysteine residue. We anticipate that the concept for concise dual functionalization of proteins will be useful to develop advanced protein-based chemical probes or therapeutics. The future work will be directed toward development of the site-specific conjugation of the targeted Cys residue(s) in various native proteins having multiple Cys residues using functionalized 2-azidoacrylates of rational design.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00024.

General details, safety issues, synthesis and conjugation details, a kinetic study in modification, dual functionalization and dual-color labeling details, and 1H and 13C NMR spectra. (PDF)

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
Cys, cysteine; SαAr, nuclophilic aromatic substitution; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; PEG, polyethylene glycol; DBCO, dibenzocyclooctyne; SPAAC, strain-promoted alkyne–azide cycloaddition; HPLC, high-performance liquid chromatography; Gly, glycine; Lys, lysine; Ser, serine; Arg, arginine; Phe, phenylalanine; MS/MS, tandem mass spectrometry; BSA, bovine serum albumin; FL, fluorescein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase