Ethynylbenziodoxolone reactivity in cysteine bioconjugation

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Selective labeling of cysteine is crucial to enable the study of the many biological processes in which cysteine plays a key part in the structure and activity of proteins. In this issue of *Chem*, Waser, Fierz and coworkers describe an efficient, chemoselective cysteine bioconjugation method to introduce vinylbenziodoxolone into biomolecules.

Chemical biologists need tools to precisely engineer proteins so that they can understand and manipulate their structure and function.[1] The demand for ever better diagnostics and therapeutics has driven the development of new chemical methods for single-site attachment of biophysical probes or cytotoxic drugs onto proteins.[2,3] Initial efforts to selectively label engineered proteins with an non-canonical amino acid or peptide fragment revealed the advantages of using precisely modified proteins for biological studies.[4,5] Although these methods require prior sequence manipulation, successful site-selective labeling of engineered proteins has encouraged scientists to develop chemical methods for native protein modification. In recent years, there have been numerous studies on site-selective labeling of native proteins.[6,7]

The challenges for site-selective labeling of engineered proteins of reactivity and selectivity can be circumvented through the use of chemoselective transformations — often an orthogonal reaction.[4] However, for native proteins, selective single-site labeling is difficult because of the wide reactivity profile resulting from the presence of multiple nucleophilic side-chain amino acids.[6,7] Ideally, bioconjugation methods should be fast at low protein concentrations, and work at physiological pH and ambient temperature. Such reactions should also be able to differentiate one functionality from several nucleophilic side-chain residues (chemoselective) and one residue among multiple copies (site-specific). Most approaches in native protein modification rely on chemoselective transformations that target the low to moderate-frequency residues and the N-terminus α-amine. Cysteine residues of both native and engineered proteins are ideal targets because they are highly nucleophilic, have relatively low abundance, are easy to incorporate into a protein's sequence, and they react well with a number of readily available chemoselective reagents.[6,7,8]

α-Halocarbonyl and maleimide derivatives are popular electrophilic reagents for labeling cysteine residues. Despite their use in bioconjugation reactions, iodoacetamide derivatives lack chemoselectivity in the presence of other nucleophilic amino acids when these reagents are used at high concentrations. The short reaction times and high chemoselectivity in reactions between maleimide derivatives with cysteine makes them appealing for use in the construction of conjugates for advanced diagnostics and therapeutics.[2,3] However, conjugates formed through maleimide bioconjugation undergo thiol-exchange reactions in the presence of external thiols, which leads to loss of label. Reversibility is a serious problem in applications that involve antibody-drug conjugates or fluorescence imaging studies because it leads to off-target toxicity and non-specific imaging, respectively. Recent approaches for cysteine bioconjugation have sought to control reactivity, provide excellent chemoselectivity, be metal-free, and yield sufficiently stable conjugates.[6,7]

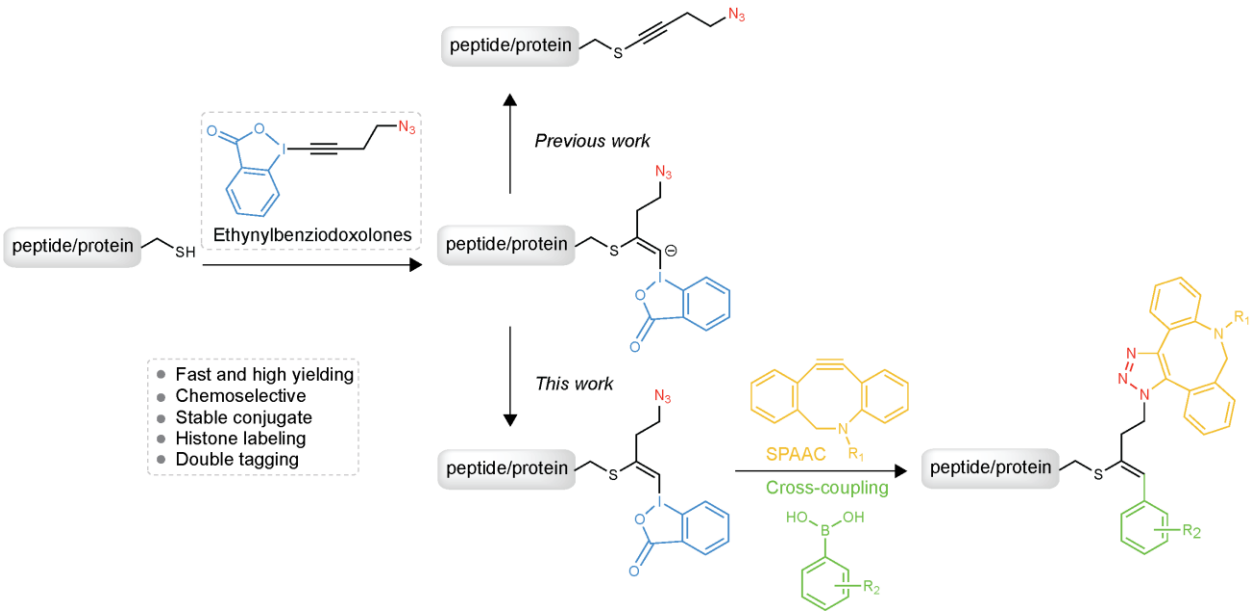

Figure 1. Bioconjugation of cysteine with EBX.

In this issue of Chem[9], Waser, Fierz and colleagues report a fast, efficient, and highly chemoselective approach to give stable cysteine conjugates with ethynylbenziodoxolone (EBX; Figure 1). The resulting cysteine conjugate has a vinylbenziodoxolone (VBX) hypervalent iodine bond and an azide group with orthogonal reactivity. These reactive handles mean that a tag of interest can be included at a late stage through either strain-promoted cycloaddition reaction with the azide or by Suzuki–Miyaura cross-coupling reaction with the vinyl hypervalent iodine bond. In this work, the authors used glutathione — a thiol-containing tripeptide — as a model substrate to optimize the conditions for bioconjugation. Unexpectedly, EBX gave cysteine adduct VBX instead of the expected alkynyl sulfide.[10] This transformation hinges on protonation, rather than α-elimination, followed by S-1,2 shift of the vinylic carbanion intermediate generated from the cysteine biomolecule and EBX. Importantly, solvent polarity is key to being able to switch between a protonation versus elimination step. The cysteine-EBX conjugate is stable at room temperature, at 100 °C in water, in acidic buffer, and in basic buffer with minimal degradation. The cysteine-EBX conjugate is also resistant against the thiol-exchange reactions in the presence of external thiol nucleophiles, such as tiopronin.

The reaction works with EBX derivatives that bear a variety of functional groups (azide, alkyne, halide, alcohol, and less efficient with lipophilic groups) and small-molecule thiols (thiophenol, benzyl mercaptan, cysteine, tiopronin, 6-thioguanine, and thio-β-glucose). EBX exhibits excellent reactivity and high chemoselectivity towards cysteine in various natural tetrapeptides and larger peptides (L55-H63 fragment of human serum albumin). However, caution needs to be considered when cysteine is present at the C-terminus as well as next to arginine because of potential oxidation and side products, respectively. Also, the method was employed to modify cysteine residues in proteins. Initially, ubiquitin that had an N-terminal His-tag followed by a cysteine residue was chosen as a model to test the efficiency of the bioconjugation reaction. Two equivalents of EBX resulted in complete conversion to the corresponding ubiquitin-EBX conjugate, whereas no labeling occurred with native ubiquitin (lacks a free cysteine), which demonstrates the high chemoselectivity of EBX toward cysteine residues. Subsequently, the orthogonality of hypervalent iodine reactivity relative to the azide was tested by means of a copper-free strain-promoted azide-alkyne

cycloaddition (SPAAC) reaction. This effect was demonstrated on glutathione and ubiquitin by using dibenzocyclooctynes with polar groups (acid), affinity tags (biotin), and fluorophores (cyanine dyes). Interestingly, the cycloaddition reaction was completely orthogonal to the hypervalent iodine reactivity, which opens the possibility to attach yet another tag of interest.

The bioconjugation conditions can label cysteine residues that result from disulfide reduction in bioactive peptides, such as oxytocin and somatostatin. Importantly, the resulting conjugate was stable in the presence of a reducing agent. However, the structure of the cyclic peptide was not conserved after labeling, which can affect its affinity and function-based applications. Next, the versatility of the method was demonstrated by labeling a more complex histone octamer. The mutation of glutamate with cysteine in H4 (E63C) followed by assembly with other histones, H2A, H2B, and H3 (C110A) gave the histone octamer complex. The cysteine residues were then labeled with EBX and Cy5-DBCO was installed through an azide-cyclooctyne cycloaddition reaction to give the fluorescently labeled histone octamer complex. The native structure of the octamer complex was conserved after labeling, which was demonstrated by continued nucleosome formation. The formation of the fluorescently labeled nucleosome was confirmed by native gel electrophoresis and fluorescent microscopy analysis. The efficiency of this method for site-specific labeling of proteins or more complex systems has yet to be demonstrated, such as with multiple cysteine residues present in a protein, therapeutic antibodies or in the presence of other cysteine-containing proteins.

The stability of hypervalent bond during the azide-cyclooctyne cycloaddition led the authors to investigate the dual functionalization of the cysteine-EBX conjugate. To functionalize the hypervalent bond, a Suzuki-Miyaura cross-coupling reaction was used to give glutathione-EBX and ubiquitin-EBX conjugates without degradation of the azide group. Synthesis of a doubly functionalized Cys-labeled ubiquitin was explored because of the compatibility of the azide and VBX hypervalent iodine groups together by means of a one-pot SPAAC-Suzuki-Miyaura reaction. The efficiency the cross-coupling is moderate, but it opens avenues for further functionalization of VBX. The authors went on to showcase an application of their method to fluorescently label receptors on living cells. The authors functionalized a high-affinity neuropeptide against neurokinin-1 receptor that had an additional N-terminal cysteine residue with EBX and then tagged it with Cy5-DBCO. The resulting conjugate readily labeled the human embryonic kidney cell line expressing green fluorescent protein-tagged NK1 receptor at the cell surface and showed colocalization with the GFP-tagged NK1 receptor. Finally, the doubly orthogonal functionalization strategy was applied to improve the photostability of the fluorophore through attachment of a fluorescent stain (TSQ) close to the Cy5 dye.

In summary, this article[9] details an efficient protocol to incorporate VBX hypervalent iodine derivatives into peptides and proteins through cysteine bioconjugation. VBX and azide are orthogonal in reactivity, which enables dual tagging at one site. The high chemoselectivity of cysteine bioconjugation proceeds smoothly to give stable conjugates. By understanding of factors that affect the reaction of EBX with cysteine and single site-selective labeling of cysteine among multiple copies, applications for cysteine labeling in chemical biology and medicinal chemistry can be improved.

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