A Cell-Permeable Biscyclooctyne As a Novel Probe for the Identification of Protein Sulfenic Acids

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ABSTRACT: Reactive oxygen species act as important second messengers in cell signaling and homeostasis through the oxidation of protein thiols. However, the dynamic nature of protein oxidation and the lack of sensitivity of existing molecular probes have hindered our understanding of such reactions; therefore, new tools are required to address these challenges. We designed a bifunctional variant of the strained bicyclo[6.1.0]nonyne (BCN-E-BCN) that enables the tagging of intracellular protein sulfenic acids for biorthogonal copper-free click chemistry. In validation studies, BCN-E-BCN binds the sulfenylated form of the actin-severing protein coflin, while mutation of the cognate cysteine residues abrogates its binding. BCN-E-BCN is cell permeable and reacts rapidly with cysteine sulfenic acids in cultured cells. Using different azide-tagged conjugates, we demonstrate that BCN-E-BCN can be used in various applications for the detection of sulfenylated proteins. Remarkably, cycloaddition of an azide-tagged fluorophore to BCN-E-BCN labeled proteins produced in vivo can be visualized by fluorescence microscopy to reveal their localization. These findings demonstrate a novel and multifaceted approach to the detection and trapping of sulfenic acids.

Protein thiol (sulphydryl) groups are important mediators of redox signaling.1 In response to increases in cellular oxidation, thiols act as nucleophiles to reduce reactive oxygen species (ROS) in order to maintain redox homeostasis. In general, thiol oxidation leads to the formation of disulfides and oxidative intermediates, leading to structural changes of the protein thiols.2 In response to ROS, free thiols become oxidized to form sulfenic acids (sulfenylation), unstable moieties that can contribute to the formation of post-translational modifications that may vary depending on microenvironmental conditions and the duration of the ROS generated.3 Sulfenic acids may undergo further oxidation to sulfinic and sulfonic acids, or react with other cysteines or glutathione to form disulfides or thiosulfonates, respectively.4 These structural changes are diverse in nature and may impact protein function, activity, and localization. As such, ROS has significant influence on cell functions, acting as a second messenger to promote a diverse number of signaling events and subsequent responses including proliferation and differentiation,1,5 hypoxia,6 ferroptosis,7 and autophagy.8 Furthermore, there are clear ROS signatures in the etiology of several diseases,9 therefore, identifying the proteins subject to ROS regulation is critical to understanding how oxidative stress can influence these pathways and biological processes. Protein thiol sulfenylation is central to ROS physiology and pathophysiology as it is the first intermediate step in protein oxidation. As such, a number of methods have been developed to exploit the reactivity of the protein thiol group in order to “trap” proteins in their sulfenylated state to characterize the proteins susceptible to ROS modification. Typically, the use of 1,3-dicarbonyl-based probes, such as dimedone, has been employed to label sulfenylated proteins, with over 700 proteins having been identified to date.10,11 Although the use of dimedone and dimedone-like compounds is generally accepted, there are a number of limitations associated with these probes. Dimedone derivatives have slow rates of reaction toward sulfenic acids; in fact, there have been several recent efforts to overcome this problem by balancing the pKa and nucleophilicity of the enolates of beta-functionalized carbonyl compounds.12,13 There is also evidence suggesting that they may react with electrophilic sulfenamides.14 Furthermore, due to the limited range of applications with such probes, there is a lack of spatial and temporal data revealing the subcellular distribution of sulfenylated proteins. As a result, there is a need to develop better tools to overcome the challenges to examining this dynamic and labile redox modification.

Strained cycloalkynes, such as bicyclo[6.1.0]nonyne (BCN), show great promise as sulfenic acid traps as they react with sulfenic acids with rate constants that are several-hundred-fold greater than other sulfenic acid probes.15 Rather than acting similarly to dimedone-based probes that exploit the electrophilicity of sulfenic acids (Supporting Information Figure S1A),12,13 or to probes that use the weak nucleophilicity of this group,16 the...
reaction involves a specific concerted syn addition to the alkyne (Supporting Information Figure S1B). In this study, we describe the development and validation of a novel cell-permeable bifunctional reagent, consisting of two linked BCN moieties (BCN-E-BCN), which makes use of the BCN reactivity for sulfenic acids to enable the detection of sulfenylated proteins.

BCN-E-BCN is comprised of two symmetrical strained cyclooctynes connected by a short ethylenediamine-derived linker, which allows protein sulfenic acids to be selectively tagged with a BCN group for copper-free click chemistry with azide-tagged reagents (Figure 1). The symmetrical design of the bifunctional probe allows for straightforward two-step synthesis from commercially available compounds (Supporting Information Figure S2) and exploits the most reactive and practical functionalities for sulfenic acid detection and click chemistry.

To validate the specificity of this probe, we incubated BCN-E-BCN with coflin, a filamentous actin (F-actin) severing protein that has been shown to be oxidized by hydrogen peroxide (H₂O₂). Recombinant human coflin was first incubated with 100 μM BCN-E-BCN, followed by a copper-free click reaction with azide-PEG3-biotin. BCN-E-BCN tagging was characterized by Western blotting using fluorescently labeled streptavidin to detect consequent protein-biotin conjugates formed by this reaction. BCN-E-BCN tagged coflin in vitro, which could be increased above spontaneously oxidized basal levels with increasing H₂O₂ concentrations (Figure 2A). We previously determined that coflin oxidation by H₂O₂ occurs at cysteines 139 and 147, resulting in reduced binding and severing of F-actin by coflin. Mutation of these cysteines to alanine residues (C139/147A) decreased H₂O₂-induced BCN-E-BCN labeling in vitro (Figure 2B), indicating that BCN-E-BCN tags coflin at these cysteine sulfenic acids. Mass spectrometry analysis of recombinant coflin incubated with BCN-E-BCN verified that cysteine 139 was a BCN-modified residue (Figure 2C). In addition, we did not detect that BCN-E-BCN labeled the free thiol, sulfinic, or sulfonic forms of coflin by mass spectrometry.

MDA MB 231 cells are triple negative human breast cancer cells that have been shown to have high basal levels of sulfenic...
To test the reactivity of BCN-E-BCN in a whole cell system, we incubated cultured cells with varying concentrations of BCN-E-BCN for 30 min at 37 °C, followed by cell lysis and conjugation with azide-PEG3-biotin for detection. BCN-E-BCN was determined to be cell permeable and labeled sulfenic acids in a concentration-dependent manner (Figure 3A), with efficient labeling at concentrations lower than typically used for dimedone based probes.11 When compared to a commercially available biotinylated form of BCN (BCN-biotin), BCN-E-BCN demonstrated significantly greater efficacy in labeling sulfonylated proteins (Figure 3B). Treatment with 50 μM H2O2 induced a modest 50% increase in BCN-E-BCN labeling, as calculated by quantifying fluorophore-conjugated band intensities using an Odyssey CLx Imaging System (Figure 3C). To attenuate this increase and demonstrate that the labeling responds to changes in redox state, we incubated the reaction in the presence of the antioxidant N-acetylcysteine (NAC; Figure 3C), which resulted in decreased BCN-E-BCN labeling. Furthermore, treatment with high H2O2 concentrations decreased BCN-E-BCN labeling and correlated with increased peroxiredoxin trioxide (Prx-O3) formation (Figure 3D), suggesting that proteins were oxidized beyond the single oxygen-containing sulfenic acid and were forming BCN nonreactive sulfinic and sulfonic acids at these concentrations. Collectively, these data are consistent with BCN-E-BCN being specific for the sulfenic acid form of thiol oxidation.

We next examined BCN-E-BCN tagging of a previously characterized sulfonylated candidate protein by immunoprecipitation from whole cell lysates. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a key regulator of the glycolytic pathway and...
is susceptible to thiol oxidation.\textsuperscript{20–22} HEK 293 human embryonic kidney cell lysates were oxidized with 0.5 mM H\textsubscript{2}O\textsubscript{2} before labeling with 250 μM BCN-E-BCN. GAPDH was immunoprecipitated and BCN-E-BCN labeling determined by Western blotting. As shown in Figure 3E, BCN-E-BCN labeled GAPDH in HEK293 cell lysates, demonstrating the utility of this compound in identifying sulfenylated proteins from complex lysates.

To utilize the flexibility of the cyclooctyne ring in click chemistry reactions, we employed an azide-tagged fluorophore to label BCN-E-BCN-protein conjugates in fixed cells in order to visualize their subcellular localization. MDA MB 231 cells were fixed with 4% paraformaldehyde, tagged with 100 μM BCN-E-BCN followed by Alexa Fluor 488 azide and analyzed by fluorescence microscopy. We were able to visualize the spatial distribution of BCN-E-BCN tagged proteins, with the majority being predominantly cytoplasmic, with some mitochondrial, plasma membrane and weak nuclear signals detected (Figure 4).

It has been suggested that free thiols may undergo thiol-yne addition with cyclooctyne groups, such that pretreating proteins with iodoacetamide (IAM) would be expected to reduce non-specific binding.\textsuperscript{23} However, we found that IAM pretreatment of MDA MB 231 cells did not markedly affect BCN-E-BCN labeling (Figure 4), consistent with the sulfenic acid specificity of the reaction and in line with previous findings.\textsuperscript{15} It should be noted that BCN should not be regarded as an electrophilic reagent for sulfenic acids but reacts via a concerted syn addition of the sulfenic acid to the alkyne: the C−S bond and the C−H bond are formed in the same step via a cyclic transition state, analogous...
to a pericyclic reaction (Supporting Information Figure S1B). This type of reactivity is not possible for protein thiols or sulfenamides, and our results indicate that electrophilic reaction with thiolates or thyl radicals is unlikely to contribute to the observed BCN-tagging of proteins under physiological conditions. Protein sulfenylation has received much recent interest because of its dynamic and complex role in ROS mediated cell signaling. However, a major limitation is a lack of convenient tools to measure this post-translational modification. Existing reagents are compromised by low reaction rates for sulfenic acids or have limited utility in biochemical and cell biological applications. Here, we now report the development and characterization of a novel bifunctional cyclooctane compound, BCN-E-BCN, which can rapidly label sulfenylated proteins in cultured cells and be used in a number of diverse applications. We demonstrate that BCN-E-BCN is cell permeable, has high reactivity compared to other BCN derivatives, and can facilitate a range of experimental approaches via copper-free click chemistry reactions that allow for labeling of BCN-tagged proteins with a variety of readily detectable moieties.

■ ASSOCIATED CONTENT

* Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00742.
Methods and Figures S1 and S2 (PDF)

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Notes
The authors declare no competing financial interest.

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