Published in final edited form as:

Chem Commun (Camb). 2016 April 7; 52(31): 5451–5454. doi:10.1039/c6cc01321j.

A Simple Method for Enhancing the Bioorthogonality of Cyclooctyne Reagent

He Tian^a, Thomas P. Sakmar^{a,b}, and Thomas Huber^a

Thomas P. Sakmar: sakmar@rockefeller.edu; Thomas Huber: hubert@rockefeller.edu

^aLaboratory of Chemical Biology and Signal Transduction, The Rockefeller University, 1230 York Ave., New York, NY 10065 USA

^bDepartment of Neurobiology, Care Sciences and Society, Division for Neurogeriatrics, Center for Alzheimer Research, Karolinska Institutet, 141 57 Huddinge, Sweden

Abstract

The cross-reactivity between some cyclooctynes and thiols limits the bioorthogonality of the strain-promoted azide–alkyne cycloaddition reaction. We show that a low concentration of β -mercaptoethanol significantly reduces the undesirable side reaction between bicyclononyne (BCN) and cysteine and while preserving free cysteines. We site-specifically label a genetically-encoded azido group in the visual photoreceptor rhodopsin to demonstrate the utility of the strategy.

Graphical Abstract

The strain-promoted azide–alkyne [3+2] cycloaddition (SpAAC) between azide and cyclooctyne has emerged as an important bioorthogonal reaction for labeling biomolecules.^{1,2} We previously investigated the SpAAC reaction of dibenzocyclooctynes (DIBO) with the azido-containing unnatural amino acid *p*-azido-L-phenylalanine (azF). azF was genetically encoded into G protein-coupled receptors (GPCRs) by amber codon

Correspondence to: Thomas P. Sakmar, sakmar@rockefeller.edu; Thomas Huber, hubert@rockefeller.edu.

Electronic Supplementary Information (ESI) available: Experimental Section; Supporting Figures S1–S8; Supporting Table S1, S2. See DOI: 10.1039/x0xx00000x

suppression.³⁻⁵ We found that azF could be labeled with DIBO with minimal background.⁶ We showed the utility of micelle-enhanced SpAAC for labeling membrane proteins where the hydrophobic DIBO partitions into detergent micelles, resulting in a high local concentration and up to 1000-fold accelerated labeling rates ($k_2 > 10^2 \text{ M}^{-1}\text{s}^{-1}$) for azF situated in the transmembrane region of detergent-solubilized GPCRs.^{7, 8} However, when attempting to label solvent accessible extracellular or intracellular regions of GPCRs slower reaction rates are observed, because the effective concentration of hydrophobic DIBO is lower than the apparent concentration in a homogenous system. Here we suggest a strategy to label azF residues that are water-exposed.

A more hydrophilic version of the DIBO reagents would be desirable, and among the cyclooctynes reagents, (1R,8S,9S)-bicyclo[6.1.0]nonyne (BCN) provides a good balance in terms of reactivity and hydrophilicity.⁹ However, van Geel et al. reported that BCN exhibits non-negligible cross-reactivity with thiols, making it less bioorthogonal than other cyclooctynes, including DIBO.¹⁰ These findings suggest that BCN may be unsatisfactory for site-specific labeling of azido-tagged proteins containing free cysteines. Although van Geel et al. showed that such background labeling could be reduced by alkylating free thiols, for many proteins free cysteines are structurally and functionally critical, and cannot be simply blocked or substituted by other residues.

Van Geel et al. also observed that the addition of non-peptidyl thiols like β -mercaptoethanol (β ME, 10 mM) reduced the conjugation between cysteine residues and BCN, which was attributed to the competition between these cysteines and β ME for reaction with BCN.¹⁰ This explanation suggests that the presence of β ME could interfere with SpAAC since a substantial fraction of BCN would be destroyed.

To resolve the question, we evaluated the thiol-yne reaction between β ME and BCN by LC-MS. Reactivity towards thiols is known for the unsubstituted cyclooctyne¹¹ and some substituted cyclooctynes, such as BCN, DIBO, DIBAC,¹⁰ and BARAC.¹² Here we explored the reaction between N-(1R,8S,9S)-bicyclo[6.1.0]non-4-yn-9-ylmethyloxycarbonyl-1,8-diamino-3,6-dioxaoctane (BCN-POE3-NH₂, 100 μ M) and β ME (10 mM) at 25°C, which resulted in some formation of an adduct product (Figure S1). After 24 h reaction, $8 \pm 1\%$ BCN was converted to the adduct product ($k_2 = 10^{-4} \text{ M}^{-1}\text{s}^{-1}$). The percentage yield was estimated from the integrated area of the MS peaks assuming that the ionization efficiencies for BCN and BCN- β ME are similar.

Therefore, β ME only modestly reduced the effective concentration of BCN. In comparison, van Geel et al. showed a marked decrease of BCN labeling cysteines in presence of 10 mM β ME after only 1 h reaction. The experiment by van Geel et al. was carried out at 4 °C and theoretically the reaction between BCN and β ME should have occurred more slowly. Therefore, the marked decrease of cysteine labeling cannot be explained by the competition between peptidylcysteines and β ME for BCN.

We argue that there might be several alternate explanations for the effect of β ME. First, β ME might prevent the background labeling through its radical scavenging capability since thiol-yne addition is mediated by sulfanyl radicals¹¹. Second, β ME might remove post-

translational modification of cysteines that might be responsible for the background reactivity with BCN. Examples of such post-translational modifications of cysteines are sulfenic acid and persulfide derivatives, which are generated by action of reactive oxygen species. Under reducing conditions (e.g. β ME) these cysteine oxidation products can be converted back to thiols.^{13, 14} Sulfenic acid and persulfides have been reported to be even more reactive with BCN than thiols.^{13, 15} While it would be interesting to differentiate between these potential mechanisms, such experiments would be very difficult using membrane proteins and are beyond the scope of this work. Instead, we focus on the practical implications of the β ME effect in a bioorthogonal labeling reaction.

To test the possibility of exploiting β ME to improve the bioorthogonality of SpAAC, we chose the visual photoreceptor rhodopsin (Rho), a prototypical class A GPCR, as the model system (Figure 1B). Rho possesses a disulfide bond between C110 and C187 that is essential for stabilizing the overall folding of the receptor and the binding with its native ligand 11-*cis*-retinal (11CR).¹⁶ Rho bound with 11CR exhibits a characteristic 500-nm absorbance band. Upon photoactivation, 11CR isomerizes to all-*trans*-retinal, causing the 500-nm absorbance peak to shift to 380 nm. This feature can be used to evaluate the functional integrity of Rho and quantify its concentration. Among the ten cysteines of dark-state Rho, at least two cysteines (C140 and C316) are exposed and easily subjected to modification.¹⁷

We first reacted wild-type (wt) Rho with BCN-DY549 in the presence and absence of β ME (Figure 2A,B and Figure S2). BCN-DY549 is a BCN group linked by a polyethylene glycol chain to the Dyomics fluorophore DY549P1 (Figure S6B). Rho was solubilized in *n*-dodecyl- β -D-maltoside (DM) micelles, immobilized to 1D4-sepharose immunoaffinity beads, and then subjected to the labeling β ME reduced the non-specific labeling from 10% to 1.5%. The in-gel fluorescence image also revealed a stronger fluorescent band for the sample without β ME. Therefore, β ME did reduce the cross-reactivity between BCN and the cysteines in Rho. The photosensitive nature of Rho necessitated the labeling experiments to be performed in the dark. Thus, the background labeling with wt Rho should have resulted from light-independent mechanisms, which argues against a role of the radical, photo-initiated thiolyne addition reaction¹¹ in the background labeling of proteins by BCN.

Initially, we were concerned that the 1D4 monoclonal antibody used for the immunoaffinity purification is sensitive to β ME. We found that the presence of β ME up to 100 mM did not severely interfere with the immunopurification. The SDS-PAGE analysis of the eluted GPCR, however, revealed additional bands in the 100 mM β ME-treated sample, due to the denaturation of antibody (Figure S3B). Such impurities may be removed by an additional cleanup step, if necessitated by the downstream application.

Another potential concern is whether β ME itself could cause any undesirable modification to Rho.¹⁸ To address this question, we incubated wt Rho with various concentrations of β ME (0.1~100 mM) (Figure S3). We found that the 500-nm peaks and the ability of photoactivation were preserved through β ME treatment, indicating the correct folding of Rho. We also asked whether β ME could form mixed disulfides with the protein cysteines and thereby reduce the reaction between BCN and those cysteines. Analyzing such modification on a GPCR by MS would be non-trivial. Instead, we used a cysteine-reactive

reagent, fluorescein-5-maleimide (FL-5-ML), to quantify the amount of free cysteines on Rho with and without prior β ME treatment (Figure S4). β ME treatment caused the resulting FL/Rho ratio to increase from 1.01 ± 0.11 to 1.28 ± 0.08 . The in-gel fluorescence image revealed that the β ME-treated samples contained FL-labeled antibody light chain dissociated from the matrix (Figure S4B), which contributed to the FL absorption peak and results in overestimation of the FL/Rho labeling ratio of the β ME-treated samples. Overall, the concentration of β ME (10~14 mM) in the labeling experiments is not sufficient to give stable modification of Rho.

We then assessed whether the presence of β ME would be useful for labeling azide-tagged Rho (Figure 2C,D and Figure S5). For benchmarking the utility of the hydrophilic BCN, we chose a site that was difficult to label with hydrophobic DIBO reagents due to partitioning of the reagent into the hydrophobic micelles. We previously found that for Y102azF (Figure 1B), a solvent exposed extracellular site in Rho, the reaction rate was too slow to give stoichiometrically labeled receptor ($k_2 \sim 0.2 \text{ M}^{-1} \text{ s}^{-1}$).⁶

Since detergents or lipids are indispensable for reconstituting membrane proteins, it is advantageous to utilize more hydrophilic cyclooctynes like BCN ($\log P = 1.2$)¹ as compared to DIBO ($\log P = 4.4$)¹ to label the exposed protein surfaces. We measured the partition coefficient of BCN-DY549 between water and DM micelle, and found it about two orders of magnitude smaller than that of DIBO-Alexa488 (Figure S6).⁷ The lower partition coefficient of BCN-DY549 would result in 2.8-fold increase in the effective concentration relative to that of DIBO-Alexa488 under the condition used for labeling (Figure S6D). We found that in the presence of β ME (14 mM), the resulting labeling stoichiometries with 50 μM BCN-DY549 after 18 h reaction was 0.79, corresponding to a k_2 of $0.48 \text{ M}^{-1} \text{ s}^{-1}$ (Figure S5). The k_2 for the SpAAC between two small molecules (BCN-OH and benzyl azide) was reported to be $0.14 \text{ M}^{-1} \text{ s}^{-1}$ in $\text{CD}_3\text{CN}/\text{D}_2\text{O}$ (3:1) and $0.29 \text{ M}^{-1} \text{ s}^{-1}$ in $\text{CD}_3\text{CN}/\text{D}_2\text{O}$ (1:2), indicating that reaction rate increases with the polarity of the solvent.⁹ Here the reaction rate between an azido-tagged membrane protein and a small-molecule labeling reagent is only slightly higher than that between two small molecules. Labeling with 100 μM BCN-DY549 for 18 h reaction gave a dye/protein ratio of 1.02 (Figure 2B). The specific labeling with azF-Rho is 30~40-fold greater than the non-specific labeling with wt Rho. Since BCN and DIBO have been reported to be similarly reactive towards azide,¹ the higher efficiency of BCN in modifying the hydrophilic region of Rho can be at least partially attributed to its weaker partitioning into the micelles.

Since β ME also forms adduct product with BCN through thiol-yne reaction, we asked how much BCN would be sacrificed by the use of β ME. We have shown that the thiol-yne addition between β ME and BCN has a k_2 of $10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ (Figure S1), at least three orders of magnitude slower than the SpAAC between BCN and azF-Rho. At the end of 18-h reaction, β ME (14 mM) would cause approximately 9% decrease in the effective concentration of BCN, which should not significantly compromise the efficiency of SpAAC.

Another concern was the stability of azF in presence of reducing several reducing reagents (10 mM β ME, DTT, GSH; Figure S7). The LC-MS results showed that after 48 h less than 2% of azide was reduced to amine by β ME, and only 0.05% by GSH. DTT caused the most

reduction due to its strong reduction capability. Note that the cytoplasmic concentration of GSH is typically below 10 mM in mammalian cytosol.²⁰ These data suggest that azF is sufficiently stable in mammalian cells. By contrast, azido unnatural amino acids could be substantially degraded in the more reducing environment of *E. coli*.²¹

Finally, we tested whether β ME could reduce the background labeling of cell lysates by BCN. While blocking the reactive thiol with alkylation reagents reduced background labeling, the addition of β ME in fact enhanced it (Figure S8). At this point, it is not clear why β ME increases the background labeling in cell extracts, but reduces it in the case of purified proteins. A plausible explanation would be that the disulfide bonds in some proteins are more susceptible to the reducing ability of β ME. By comparison, we observed that the disulfide bond between C110 and C187 of rhodopsin was generally resistant to β ME. However, we do not have any direct evidence supporting this hypothesis, and we do not rule out more complicated mechanisms. Thus, a more precise understanding on how β ME could reduce the background labeling for purified proteins would be important for utilizing this approach for live cell labeling.

In summary, we showed that using a low concentration of β ME enhances the bioorthogonality of the hydrophilic cyclooctyne BCN for labeling reactions, such as SpAAC. This method is simple to implement, did not appear to compromise the kinetics of SpAAC, and keeps the free cysteines in the protein of interest intact for subsequent applications. While the proof-of-concept was demonstrated using a GPCR, its utility may extend beyond protein labeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the generous support from the Crowley Family Fund, the Danica Foundation, and the NIH R01 EY012049 (T.P.S. and T.H.), as well as the Tri-Institutional Training Program in Chemical Biology for supporting H.T. We thank the Rockefeller University Proteomic Center for the mass spectroscopy analysis. This work was also supported by an International Research Alliance with Prof. Thue W. Schwartz at The Novo Nordisk Foundation Center for Basic Metabolic Research (<http://www.metabol.ku.dk>) through an unconditional grant from the Novo Nordisk Foundation to the University of Copenhagen.

ABBREVIATIONS

11CR	11- <i>cis</i> -retinal
βME	β -mercaptoethanol
azF	<i>p</i> -azido-L-phenylalanine
BCN	(1R,8S,9S)-bicyclo[6.1.0]non-4-yn
BCN-POE3-NH₂	<i>N</i> -BCN-9-ylmethyloxycarbonyl-1,8-diamino-3,6-dioxaoctane
DIBO	dibenzocyclooctyne
DM	<i>n</i> -dodecyl- β -D-maltoside

FL-5-ML	fluorescein-5-maleimide
GPCR	G protein-coupled receptor
Rho	rhodopsin
SpAAC	strain-promoted [3+2] azide–alkyne cycloaddition
wt	wild-type

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Figure 1. Using β -mercaptoethanol (β ME) to reduce the cross-reactivity between bicyclononyne (BCN) and cysteine

A) BCN can react with azide through strain-promoted azide-alkyne [3+2] cycloaddition (SpAAC), or with thiol through thiol-alkyne addition. The presence of β ME reduces the unwanted reaction with cysteine. **B)** The structure of the visual photoreceptor rhodopsin (PDB: 1U19). Highlighted residues and prosthetic group: Y102azF (red); free cysteines (green); cysteines that form a disulfide bond (C110–C187, cyan); 11-*cis*-retinal (orange).

Figure 2. Labeling of Y102azF-Rho by BCN-DY549

Labeling was performed at 25 °C for 18 h. **A**) The non-specific reaction between wt Rho and BCN-DY549 (50 μ M) in the presence and absence of β ME (14 mM). The DY549/Rho ratios are 0.015 and 0.095, respectively. **B**) The corresponding in-gel fluorescence image for **A**). **C**) Labeling of Y102azF and wt Rho with BCN-DY549 (100 μ M) in the presence β ME (14 mM). The DY549/Rho ratios are 1.02 and 0.04, respectively. **D**) The corresponding in-gel fluorescence image for **C**). Note that **B** was detected with higher sensitivity compared with **D**.