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A clickable glutamine (CliQ) derivative for the traceless reversible modification of peptides and proteins

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Samuel D. Whedon,[a] Marissa K. Parker,[a] Elizabeth L Tyson,[a] Tobias Ritterhoff,[b] Patrick M. M. Shelton,[a] and Champak Chatterjee [a]

The Cu(I)-mediated click reaction of proteins with affinity tags enables their selective isolation from complex mixtures. However, irreversible protein modification limits the interpretation of results from subsequent biophysical and biochemical assays. We report a facile and modular chemical strategy to reversibly modify peptides and proteins with biotin and FLAG affinity tags at a clickable glutamine (CliQ) residue.

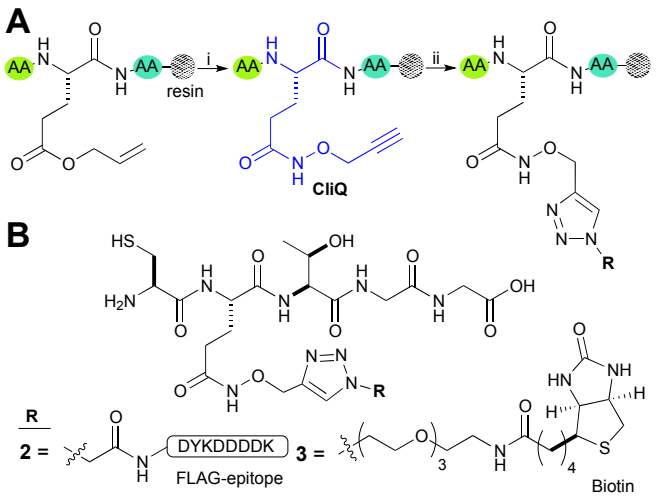
The Cu(I)-mediated Huisgen 1,3-dipolar cycloaddition of azides with alkynes, commonly known as a click reaction, is widely employed for protein bioconjugation.[1] Either azide or alkyne reaction components may be site-specifically introduced in proteins by enzymatic,[2] semisynthetic[3] or molecular biological methods.[4] This enables the introduction of a range of small molecules into proteins, including therapeutic drugs,[5] fluorophores,[6] and affinity tags.[1,7] The introduction of an affinity tag enables specific protein isolation from complex mixtures, and is a mainstay of the widely applied activity-based protein profiling approach.[8] Although irreversibly clicked affinity tags do not interfere with proteomic analysis, they complicate interpretation of the biophysical and biochemical properties of their protein target. This has led to the development of reversible protein tagging strategies that yield wild-type unmodified proteins after tag removal.[9,10-12] Due to their nucleophilic side-chains, most reversible protein tagging strategies have focused on cysteine (Cys) and lysine (Lys) residues.[10,11,13] However, Cys is not an abundant amino acid in proteins,[14] and Lys is often targeted for enzymatic post-translational modification (PTM).[15] This limits the potential sites in a protein that may be site-specifically reversibly modified while maintaining desired PTM states. Furthermore, reversible affinity tags commonly employ a photocleavable *o*-nitrobenzyl linker that requires challenging multi-step synthesis and UV-irradiation for tag removal.[10,12] Photooxidation and photobleaching are two well-known issues associated with the UV irradiation of proteins.[16] Therefore, we sought to develop a chemical strategy for reversibly modifying proteins with affinity tags that is synthetically facile, targets a rarely modified amino acid, and does not require UV irradiation for tag removal.

Glutamine (Gln or Q) is a relatively abundant amino acid in proteins that is seldom targeted by PTMs,[17] which makes it an ideal site for reversible protein derivatization. We envisioned a reducible hydroxamate ester derivative of Gln, which may be easily generated during solid phase peptide synthesis (SPPS) (**Scheme 1A**). The desired side-chain functionalization was achieved by coupling an orthogonally protected Fmoc-Glu γ-allyl ester in place of Gln in the 5-mer peptide H_2N-CQTGG-CO_2H (**1**), corresponding to residues 88-92 of the small ubiquitin-like modifier protein 3 (SUMO-3). Following Pd-mediated allyl deprotection on the solid-phase, the Glu side chain was condensed with O-(2-

[a] University of Washington, Department of Chemistry, Box 351700, Seattle, WA 98195-1700
[b] University of Washington, Department of Biochemistry, Seattle, WA 98195-1700

propynyl)hydroxylamine to generate the clickable Gln (CliQ) at position 89. Introduction of the alkyne handle enabled divergent functionalization of the peptide by Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC). Importantly, undertaking cycloaddition with protected peptides on the solid-phase both circumvented inadvertent oxidation of the N-terminal Cys,[18] and facilitated removal of the residual copper before peptide purification.[19] Click reactions on the solid-phase peptide with either a side-chain protected azidoacetyl-FLAG epitope peptide or Biotin-PEG_3-azide provided the respective FLAG-labeled (**2**) and biotinylated (**3**) 5-mer peptides that were purified to homogeneity (**Scheme 1B and Figure S1**).

We first tested reduction of the N-O bond in the context of peptides **2** and **3**. *N*-alkylated hydroxamate esters are known to be reducible with metallic Zn,[20] SmI_2,[21] photocatalysis,[22] and some electron-rich aromatic thiols.[23] Aromatic thiols are particularly appealing due to their compatibility with folded proteins in aqueous buffers. However, aromatic thiol-mediated reduction was not observed for either **2** or **3**. It is possible that additional *N*-alkylation may be required to stabilize the amidyl radical product of single electron transfer from aromatic thiols.[23] Gratifyingly, reduction of the N-O bond in **3** to yield the unmodified peptide **1** and biotin-triazole derivative (**4**) proceeded quantitatively with



Scheme 1. Synthesis and application of CliQ. A. Strategy for generating CliQ in peptides attached to the solid-phase. i) (a) $Pd(PPh_3)_4$, $PhSiH_3$; (b) HATU, DIEA, O-2-propynyl hydroxylamine. ii) Cu(I)Cl, Ascorbic acid, Piperidine, DMF, R-N_3. **B.** Affinity tagged SUMO-3(88-93) peptides. R_2= FLAG, R_3= PEG_3-Biotin. AA= amino acid.

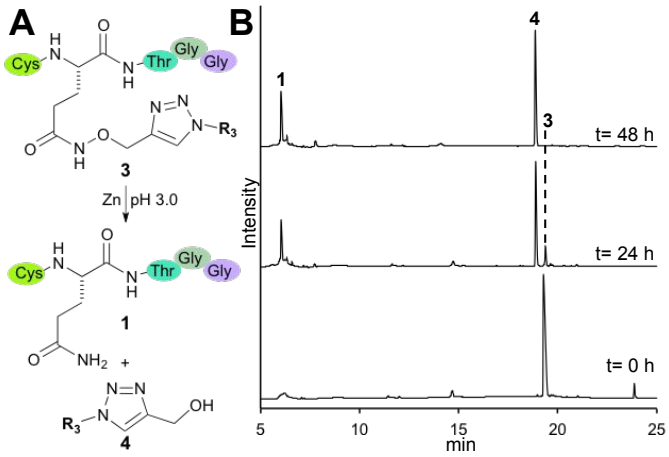


Figure 1. Traceless and reversible peptide-labelling with CliQ. A. Predicted fragments generated from Zn-mediated reduction of the labile $^{\delta}$N-O bond in the biotinylated CQTGG peptide **3**. **B.** Time-course of N-O bond reduction followed by C18 analytical RP-HPLC. R3= PEG_3-Biotin.

activated Zn in both denaturing and non-denaturing buffers ranging in pH from 3.0 to 7.2, and equally efficient cleavage was seen for peptide **2** (**Figures 1 and S2-S3, Table S1**). This demonstrated the utility of CliQ for traceless labelling at Gln in a peptide substrate. CliQ was also incorporated in place of Q19 in an H3(1-28) peptide and reacted with azide-PEG_3-Biotin to generate a biotin-tagged H3(1-28) peptide that could be easily untagged with activated Zn (**Figure S4**). This confirms that interior sites in longer peptides are also accessible for labeling by CliQ.

In order to establish the utility of the CliQ handle in proteins, peptides **2** and **3** were applied to expressed protein ligation (EPL)[24] with a C-terminally truncated cysteineless SUMO-3(2-87) α-thioester (**5**) (**Figure 2A and Figure S5**). Successful EPL afforded the full- length SUMO-3(Q88C) mutant with Q89 conjugated with the FLAG-tag (**6**) or with PEG_3-biotin (**7**) (**Figure S6**). The non-reactivity of CliQ with aromatic thiols facilitated the inclusion of 50 mM 2-mercaptophenylacetic acid (MPAA) to increase ligation rates.[25] The EPL products were reduced with Tris(2-carboxyethyl)phosphine, acidified to pH ~3 and MPAA was extracted with diethyl ether. In order to test the compatibility of CliQ with commonly used protein alkylating agents, Cys88 was alkylated with 2-iodoacetamide to generate *S*-carboxyamidomethyl Cys,[26] which is an analogue of Gln88 in wild-type SUMO-3 (**Figure S7**). Initial attempts

to purify products away from the non-ligated and hydrolysed SUMO-3(2-87)-CO_2H truncant by reverse-phase HPLC yielded 12% of **6** and were entirely unsuccessful for **7** (**Figure S8**).

Indeed, the co-elution of ligation products with truncated hydrolysis products is a known problem in EPL that significantly reduces final yields.[10,27] Nevertheless, the presence of affinity tags in **6** and **7** enabled their selective binding and elution from immobilized FLAG-antibody and Streptavidin, respectively (**Figure 2B-C and S9**). This doubled the isolated yield of **6** to 24% and led to a 73% isolated yield for pure **7**. Finally, Zn-mediated selective reduction of the N-O bond in **6** and **7** yielded full-length SUMO-3 in 56% and 73% yields, respectively (**Figure S10**).

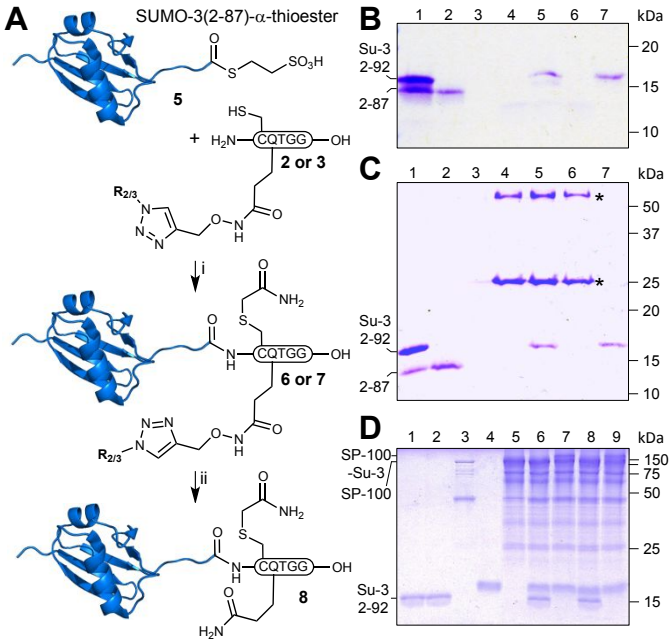


Figure 2. Semisynthesis and CliQ-mediated purification of full-length SUMO-3. A. Expressed protein ligation of **5** and **2** or **3**. i) (1) 6 M Gn-HCl, 150 mM NaCl, 100 mM NaP_i, 50 mM MPAA, 10 mM EDTA, 10 mM TCEP, pH 7.5. (2) 100 mM TCEP, 200 mM iodoacetamide, pH 8. (3) 500 mM DTT, pH 8. ii) 6 M Gn-HCl, pH 3.0, activated Zn. **B.** Coomassie-stained 15% SDS-PAGE of SUMO-3 ligation and streptavidin purification steps. Lanes: 1) Ligation reaction products, 2) Streptavidin resin flow-through, 3) Column wash, 4) Pre-bound resin, 5) Product-bound resin, 6) Resin post-elution, 7) Product eluted with 9:1 (v/v) DMF:H_2O. **C**. Coomassie-stained 15% SDS-PAGE of SUMO-3 ligation and anti-FLAG resin purification steps. Lanes: 1) Ligation reaction products, 2) resin flow-through, 3) Column wash, 4) Pre-bound resin, 5) Product-bound resin, 6) Resin post-elution, 7) Product eluted with 3xFLAG-peptide. **D.** Coomassie-stained 15% SDS-PAGE of in vitro sumoylation. Lanes: 1) SUMO-3(2-92), 2) **8**, 3) SUMO E1 enzymes Sae1/Uba2, 4) SUMO E2 ligase Ubc9, 5) SUMO substrate SP-100, 6) SUMO-3(2-92)+E1+E2+SP-100, no ATP, 7) SUMO-3(2-92)+E1+E2+SP-100, 5 mM ATP, 8) **8**+E1+E2+SP-100, no ATP, 9) **8**+E1+E2+SP-100, 5 mM ATP. R_2= FLAG, R_3= PEG_3-Biotin. Asterisks indicate the heavy and light chains of anti-FLAG antibody.

In contrast with the peptide substrate, a denaturing agent was required for N-O bond reduction in **6** and **7**. Although the use of denaturants may limit applications of CliQ to proteins that can be effectively refolded, these currently include many therapeutically relevant proteins such as tau,[10] α-synuclein,[28] histones[29] and members of the ubiquitin protein family.[30] Importantly Cys S-alkylation is compatible with CliQ and expands its utility to proteins lacking a native Cys. However, S-alkylation is not a pre-requisite for N-O bond cleavage, and the unalkylated SUMO-3(Q88C) mutant was an equally efficient substrate for reduction, indicating full compatibility with protein thiols (**Figure S11**).

Further highlighting the utility of CliQ, the circular dichroism spectrum of S-alkylated SUMO-3 (**8**) after purification and tag removal matched that of the native folded form (**Figure S12**). **8** was also a competent substrate for SUMO ligase enzymes and was efficiently conjugated with the tumour suppressor protein SP-100, which is a known target for sumoylation in humans (**Figure 2D and S13**).[31] Thus, our results demonstrate the utility of CliQ for reversible peptide and protein labelling by two different affinity tags. Although the stronger biotin-streptavidin association led to higher recovery of ligation products, the versatility of the modular CliQ approach in principle permits the attachment of virtually any affinity tag compatible with click chemistry.

In conclusion, we have developed a site-specific, synthetically facile and modular strategy for reversible protein modification at glutamine residues. The CliQ handle is readily incorporated into peptides on the solid phase, and facilitates the isolation of EPL products that are not purifiable by RP-HPLC. The easy reversibility of CliQ to attain a native Gln enables the recovery of wild-type proteins, which are suitable for biophysical and biochemical studies. Although both biotin and FLAG-affinity resins are relatively expensive, reusability of the

antibody-based anti-FLAG-resin and facile assembly of CliQ from commercially available starting materials render our methodology viable for the purification of semisynthetic products in sufficient quantities for biophysical and biochemical assays. We envision that the modularity and ease of CliQ synthesis will enable its extension to Cu-free click reactions.[32] Future work will explore molecular biological strategies for incorporating CliQ in cellular proteins, and will seek to identify milder methods to cleave the biorthogonal N-O bond.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

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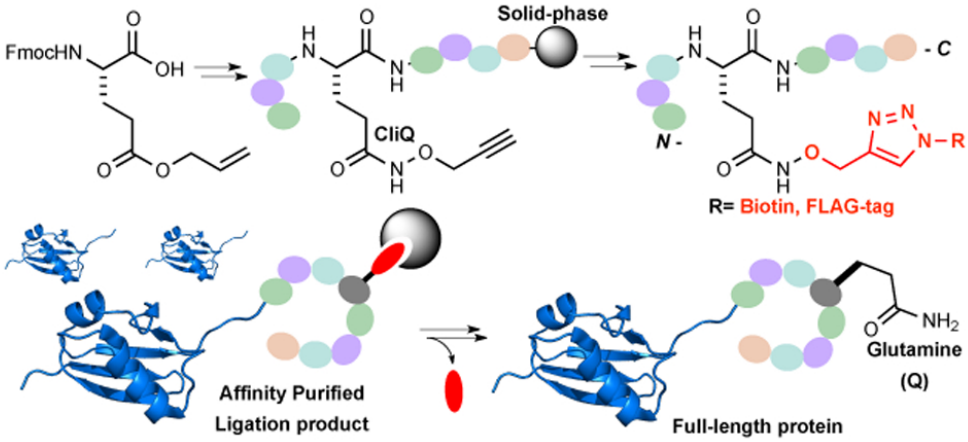

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