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A Caged Electrophilic Probe for Global Analysis of Cysteine Reactivity in Living Cells

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Supporting Information

ABSTRACT: Cysteine residues are subject to diverse modifications, such as oxidation, nitrosation, and lipidation. The resulting loss in cysteine reactivity can be measured using electrophilic chemical probes, which importantly provide the stoichiometry of modification. An iodoacetamide (IA)-based chemical probe has been used to concurrently quantify reactivity changes in hundreds of cysteines within cell lysates. However, the cytotoxicity of the IA group precludes efficient live-cell labeling, which is important for preserving transient cysteine modifications. To overcome this limitation, a caged bromomethyl ketone (BK) electrophile was developed, which shows minimal cytotoxicity and provides spatial and temporal control of electrophile activation through irradiation. The caged-BK probe was utilized to monitor cysteine reactivity changes in A431 cells upon epidermal growth factor (EGF)-stimulated release of cellular reactive oxygen species. Decreased reactivity was observed for cysteines known to form sulfenic acids and redox-active disulfides. Importantly, the caged-BK platform provided the first quantification of intracellular disulfide bond formation upon EGF stimulation. In summary, the caged-BK probe is a powerful tool to identify reactivity changes associated with diverse cysteine modifications, including oxidation, metal chelation, and inhibitor binding, within a physiologically relevant context.

The chemical reactivity of cysteine is unique among amino acids due to the high nucleophilicity and redox sensitivity of the cysteine thiol.[1] Therefore, cysteine residues can perform a variety of specialized functions within proteins, including nucleophilic and redox catalysis, metal binding, and protein regulation.[2] Diverse families of enzymes rely on a functional cysteine for activity, such as thiol oxidoreductases, proteases, and phosphatases.[3] The activity of these and other proteins can be regulated through cysteine post-translational modifications (PTMs) such as oxidation,[4] nitrosation,[5] palmitoylation,[6] prenylation,[7] and Michael additions to oxidized lipids.[8] In particular, oxidative modifications mediated through cellular reactive oxygen species (ROS) are implicated in various physiological and pathological processes such as cell proliferation,[9] differentiation,[10] and migration.[11]

To fully understand the role of cysteine PTMs in regulating protein function *in vivo*, it is critical to develop chemical and proteomic methods to enrich and identify modified cysteine residues within proteomes.[12] One strategy to globally evaluate cysteine modifications is through the use of a highly electrophilic probe that monitors the characteristic loss in nucleophilicity associated with all common PTMs. Specifically, in a method termed isoTOP-ABPP,[13] an iodoacetamide (IA)-alkyne probe is coupled with quantitative mass spectrometry (MS) to concurrently report on reactivity changes in hundreds of cysteines within a proteome. Importantly, isoTOP-ABPP and its derivatives[14] have identified bacterial proteins susceptible to oxidation by hydrogen peroxide,[15] sites of modification by endogenous lipid-derived electrophiles,[16] and cysteine residues that chelate to zinc ions.[17] These previous applications demonstrate the versatility of coupling highly reactive electrophilic probes with quantitative MS to quantify the stoichiometry of diverse cysteine modifications.

Previous applications of the IA-alkyne probe have primarily utilized cell lysates exposed to an oxidant or other cysteine modifying agents *in vitro*. Due to the chemical lability of many cysteine PTMs and the importance of preserving native redox environments during analysis, it would be ideal to perform the initial cysteine labeling step directly in living cells. Although the IA-alkyne probe is cell permeable and enables live-cell cysteine labeling,[18] the high toxicity of this probe prohibits its use at high concentrations to obtain expansive coverage of cellular cysteines. Furthermore, serum proteins in the media serve to inactivate a fraction of the probe prior to internalization, reducing intracellular concentrations. Lastly, there is limited temporal control over cysteine labeling, rendering it a challenge to monitor cellular changes that occur within a short time scale. To overcome these inherent limitations associated with use of the IA-alkyne probe in cells, a cysteine-reactive probe with reduced cytotoxicity and spatial and temporal control of cysteine labeling in living cells was developed.

Our strategy utilizes a protected (caged) electrophile that is unreactive with cellular proteins to enable accumulation in cells at high concentration with low toxicity. Protein labeling is triggered *in situ* using photo-uncaging to unveil a cysteine-reactive electrophile directly in living cells. This *in situ* uncaging strategy will provide spatial and temporal control of cysteine labeling when necessary. An α-bromomethyl ketone (BK) electrophile, which is known to be thiol reactive and has reactivity equivalent to that of IA, was used for this application. BK is an ideal electrophile because a photolabile acetal protecting group provides an effective caging strategy. An alkyne-functionalized BK probe and its caged derivative (caged-BK), utilizing *o*-nitrophenylethylene (NPE) glycol as a

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photolabile protecting group (Figure 1A), were synthesized.[19] The alkyne group was incorporated as a bioorthogonal handle

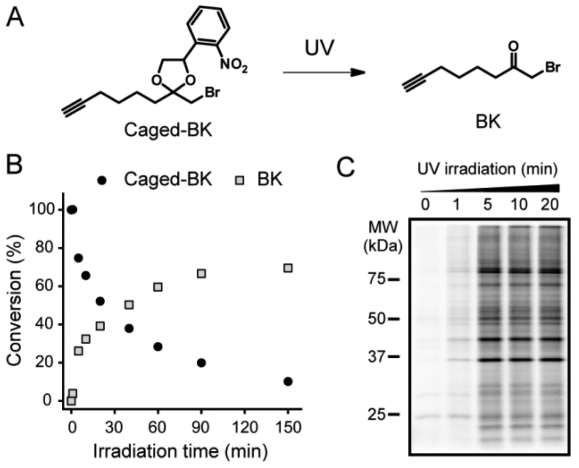

Figure 1. Monitoring the uncaging process of caged-BK. (A) Scheme for the uncaging of caged-BK upon UV irradiation. (B) Uncaging efficiency determined in CD_3OD using NMR. (C) In-gel fluorescence analysis of caged-BK (200 μM) uncaging in live HeLa cells, where BK-adducted proteins were fluorescently tagged using CuAAC prior to SDS-PAGE.

for incorporation of reporter tags using copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC). NPE derivatives are commonly used for caging biomolecules such as neurotransmitters, and they are known to have high uncaging efficiency and compatibility with a variety of living organisms.[20] After synthesis of caged-BK (Supporting Information, Figure S1), the uncaging process upon irradiation with ultraviolet (UV) light (365 nm, 160 W) was monitored by NMR. These solution uncaging experiments indicated that 90% of caged-BK was consumed, affording 70% conversion to BK after irradiation for 150 min in CD_3OD (Figures 1B and S2). To monitor uncaging in a biologically relevant environment, covalent labeling of proteins upon irradiation of caged-BK in HeLa cells or lysates was observed using in-gel fluorescence after CuAAC-based incorporation of a fluorescent rhodamine group. Caged-BK displayed minimal protein labeling without UV irradiation. The extent of labeling reached a maximum after 5 min of irradiation in both cell lysates as well as living cells pretreated with caged-BK (Figures 1C and S4). The conditions for labeling in living cells were optimized (Figure S5−S7) to arrive at the following protocol: (1) treatment of cells with 200 μM caged-BK for 60 min; (2) washing to remove non-internalized probe; (3) irradiation for 5 min on ice; and (4) incubation for 20 min to allow for protein labeling with the newly uncaged probe.

Upon optimizing caged-BK labeling using in-gel fluorescence, MS-based proteomics was applied to identify BK-labeled cysteine residues. In order to ensure that the ketone that is generated after protein labeling with BK does not interfere with downstream steps involved in the MS workflow, an additional reduction step, using sodium borohydride to reduce the ketone group, was incorporated.[21] Conditions for this reduction step were optimized by chemical synthesis of a BK-cysteine adduct (Figure S8), followed by evaluation of reduction efficiency by LC-MS upon treatment with sodium borohydride (Figure S9). This reduction step was confirmed to be compatible with the subsequent CuAAC step (Figure S10), and these optimized reducing conditions were applied to HeLa cell lysates treated with BK (100 μM). After reduction, probe-labeled proteins were appended using CuAAC to the Azo tag,[14] which is comprised of an azide coupling partner for CuAAC, a biotin group for enrichment and an azobenzene cleavable unit for selective release of labeled peptides after enrichment. BK-labeled proteins were enriched on streptavidin beads, subjected to an on-bead trypsin digestion and subsequent treatment with sodium dithionite to release probe-labeled peptides. Analysis of these peptide mixtures by LC/LC-MS/MS resulted in identification of 1174 cysteines (Figure 2A, Table S1). This

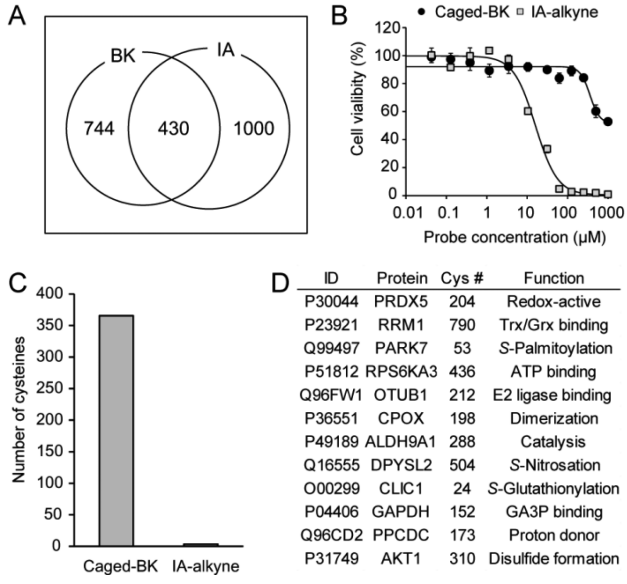

Figure 2. MS experiments with IA-alkyne, BK, and caged-BK. (A) Venn diagram demonstrating overlap between cysteines detected from IA-alkyne (100 μM) or BK (100 μM)-labeled cell lysates. (B) Cell viability assays with caged-BK or IA-alkyne. (C) Cysteines identified from MS analysis of live HeLa cells labeled with caged-BK (200 μM) or IA-alkyne (3 μM). (D) Examples of functional cysteines labeled by caged-BK in living cells.

number was comparable to that detected with 100 μM IA-alkyne probe (1430 cysteines, Table S2). Considering the structural and reactivity differences between the IA and BK probes, there was significant overlap between the identified cysteines (430; 37%). A previous comparison between cysteine-reactive maleimide and IA electrophiles showed a similar overlap,[22] suggesting that diverse thiol-reactive electrophiles can target different subsets of cysteines. Regardless, the BK electrophile enables identification of hundreds of cysteine residues within cell lysates, rendering it an ideal probe for global analysis of cysteine reactivity.

To determine the cytotoxicity of caged-BK relative to the IA-alkyne probe, cell viability assays were performed using HeLa cells treated with either probe for 1 h (Figures 2B and S3). Caged-BK had no measurable cytotoxicity up to 250 μM, whereas IA-alkyne showed a decrease in cell viability above 3 μM, with an LC_{50} of 16 μM. MS experiments were performed to identify cysteines labeled by caged-BK and IA-alkyne upon treatment of live cells at non-cytotoxic concentrations. Cells were treated with caged-BK at 200 μM, whereas labeling with IA-alkyne was performed at 3 μM, since concentrations above that resulted in a measurable loss in cell viability. We identified 366 cysteines with caged-BK (200 μM), while IA treatment (3

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μM) detected only four cysteines (Figure 2C). In two replicate experiments using caged-BK, 435 and 296 cysteines were detected, and 160 of these cysteines were detected in both samples (Figure S11). Detected proteins were distributed across the cytosol, nucleus, mitochondria, and other organelles, and the cellular distributions were similar between lysate and live-cell labeling (Figure S12). These data indicate that caged-BK is equally distributed within the cell and is not preferentially localized in a particular organelle. Cysteines labeled by caged-BK include functional cysteines involved in redox regulation, catalysis, protein and small-molecule recognition, and sites of PTMs (Figures 2D and S13, Table S3). Therefore, proteomic studies with caged-BK provide access to functionally important cysteine residues within the cell. Lastly, to confirm that BK selectively labels cysteine and not lysine within proteomes, BK labeling in lysates was shown to be completely competed by pretreatment with IA, a known cysteine-specific modifying agent (Figure S14). Furthermore, analysis of the proteomic data upon specifying the mass of the probe on lysine identified a negligible number of peptides relative to those labeled on cysteine (Figure S15).

Lastly, we sought to apply the caged-BK probe to characterize oxidative modifications on cysteine under physiological stimulation of ROS in living cells. A431 epidermoid carcinoma cells overexpress the epidermal growth factor receptor (EGFR) and produce ROS under EGF stimulation.[23] ROS production is implicated in EGF-mediated signal transduction by oxidizing functional cysteines on enzymes such as EGFR and phosphatases.[24] Previous studies have evaluated sulfenic acid formation upon EGF stimulation,[24,25] but other reversible oxidative modifications, in particular disulfide bond formation, have not been explored. Our platform enables concurrent monitoring of all oxidative cysteine PTMs while providing the stoichiometry of modification and will therefore complement previous sulfenic acid specific studies. To identify cysteine residues subject to oxidation upon EGF stimulation of A431 cells, we utilized isotopically tagged variants of the Azo tags[14] for quantitative proteomics. We performed two comparisons using our established light and heavy Azo tag system (Figure 3A): (1) vehicle-treated (light) versus vehicle-treated (heavy) (control sample); and (2) EGF-treated (light) versus vehicle-treated (heavy) (experimental sample). For each identified cysteine, a heavy:light ratio ($R_{H/L}$) provides a measure of the relative amount of cysteine labeling in each of the two samples being compared. $R_{H/L} \approx 1$ indicates no change in cysteine reactivity, whereas $R_{H/L} > 1$ is indicative of a decrease in cysteine reactivity in the EGF-stimulated sample. The experimental and the control samples gave 346 and 300 valid $R_{H/L}$ values, respectively (Table S4). As expected, the majority of cysteines (85%) in the control sample were distributed within the 0.8−1.2 ratio window, indicating no significant change in cysteine reactivity across the two samples. In comparison, EGF stimulation increased the number of cysteines found in the high $R_{H/L}$ range ($R_{H/L}$ = 1.2−2.0) (increase from 8.7% in control sample to 25% in experimental sample) (Figure 3B), signifying decreased reactivity of these cysteines upon EGF stimulation. Given that EGF stimulation is known to increase ROS levels in cells, the observed decrease in cysteine reactivity is indicative of the formation of various oxidized cysteine adducts including sulfenic/sulfinic/sulfonic acids and disulfides. The UniProt database was mined to determine if the cysteines with decreased reactivity were known functional cysteines. Within the subset of cysteines with $R_{H/L}$ > 1.2 in the EGF-stimulated sample, a significant enrichment was observed for cysteines known to form redox-active disulfides (Figures 3C and S16). In contrast, there was no observed bias in the functional annotation of cysteines in the control sample (Figure 3D). These data were compared to a recently published study on the proteomic analysis of *S*-sulfenylation in EGF-stimulated A431 cells.[25] Within the subset of cysteines that were identified to be oxidized were highly *S*-sulfenylated cysteines in proteins such as HSPD1, GSTP1, and REEP5 (Table S4). Therefore, our caged-BK strategy allows for monitoring of multiple oxidative cysteine modifications concurrently within living cells, and provides the first look at redox-active disulfide bond formation upon EGF stimulation. It is important to note that the peroxide burst generated upon EGF stimulation is maximal ∼5−15 min after addition of EGF.[24] This short time frame renders the temporal control provided by the caged-BK probe essential to this analysis. Use of an uncaged and reactive electrophile would require a longer time period for internalization and equilibration in cells after EGF stimulation, at which point the maximal oxidative effects have diminished.

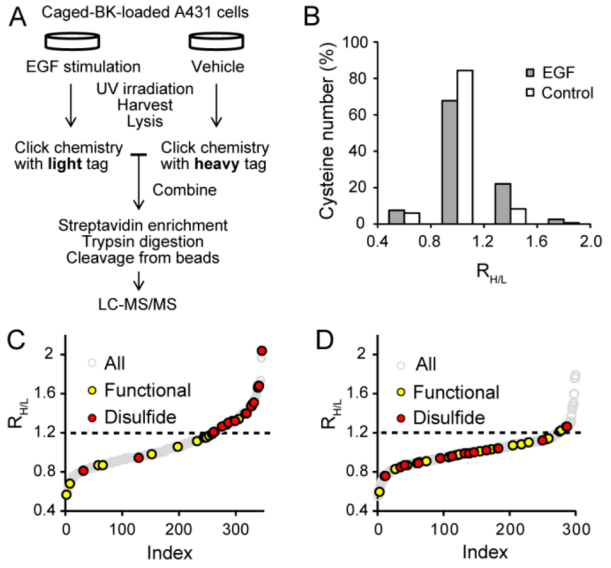

Figure 3. Quantitative MS analysis of cysteine reactivity changes upon EGF stimulation of A431 cells. (A) Workflow for caged-BK labeling, EGF activation, irradiation, and subsequent MS analysis. (B) Number of cysteine residues identified in each $R_{H/L}$ range. (C,D) $R_{H/L}$ obtained from EGF-stimulated (C) and control (D) samples. Functional cysteines are represented as yellow (all functional cysteines) or red (redox-active disulfides).

In summary, a caged cysteine-reactive probe was developed that demonstrates low cytotoxicity in cells, enabling internalization at higher concentrations than permissible by the widely utilized IA-alkyne probe. The caged-BK probe can be uncaged upon ∼5 min of UV irradiation in cells to generate a thiol-specific electrophile that covalently modifies proteins in a variety of cellular subcompartments. Importantly, this probe provides temporal control over electrophile activation. Furthermore, spatial control can also be obtained by targeted irradiation of a specific region of a cellular or tissue sample. Hundreds of cysteines in living cells are labeled by caged-BK, including critical catalytic and regulatory residues. Quantitative MS experiments were used to monitor the loss of cysteine reactivity induced by EGF stimulation of A431 cells. Several annotated sites of cysteine oxidation, including sulfenic acids

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and redox-active disulfides, were identified and serve to illustrate the utility of this method to monitor the stoichiometry of diverse cysteine modifications directly within living cells. The versatility of this platform enables quantification of cysteine modification in living cells upon treatment with various oxidative insults, cysteine-reactive small molecules, or thiophilic metal ions. Importantly, the preservation of native cellular environments during the cysteine-trapping step minimizes disruptions to PTMs and protein−protein interactions caused during cell lysis.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis and characterization of BK and caged-BK; NMR spectra for monitoring the uncaging of caged-BK; cell viability assays with prolonged exposure to probes; labeling of cysteines in cell lysates with BK, caged-BK, or IA-alkyne; optimization of labeling conditions with caged-BK in living cells; development of a procedure for BK-labeled MS samples; detailed procedures of proteomic analysis; cellular localizations of BK-labeled proteins; MS data tables. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b04350.

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Notes

The authors declare no competing financial interest.

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