HHS Public Access

Author manuscript

Mol Pharm. Author manuscript; available in PMC 2019 March 05.

Published in final edited form as:

Mol Pharm. 2018 March 05; 15(3): 743–749. doi:10.1021/acs.molpharmaceut.7b00832.

Isotopically-Labeled Iodoacetamide-alkyne Probes for Quantitative Cysteine-Reactivity Profiling

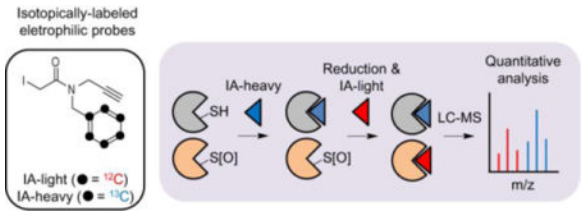
Masahiro Abo, **Chun Li**, and **Eranthie Weerapana**

Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02467, United States

Abstract

Cysteine residues on proteins serve a variety of catalytic and regulatory functions due to the high nucleophilicity and redox activity of the thiol group. Quantitative proteomic platforms for profiling cysteine reactivity can provide valuable information related to the posttranslational modification state and inhibitor occupancy of functional cysteine residues within a complex proteome. Cysteine-reactivity profiling typically monitors changes in the extent of cysteine labeling by cysteine-reactive chemical probes such as iodoacetamide (IA)-alkyne. To enable accurate measurements of cysteine reactivity changes, isotopic labels are introduced into the two proteomes of interest using either isotopically tagged proteomes (SILAC) or cleavable linkers (isoTOP-ABPP) that are installed using copper-catalyzed azide-alkyne cycloaddition (CuAAC). Here we provide an alternative strategy for isotopic tagging of two proteomes for cysteine-reactivity profiling, by developing IA-light and IA-heavy, a pair of isotopically labeled iodoacetamide-alkyne probes. These probes can be utilized for proteome samples that are not amenable to SILAC labeling and are facile to synthesize, especially when compared to the isotopically tagged cleavable linkers. We confirm the quantitative accuracy of IA-light and IA-heavy by assessing cysteine reactivity in a purified thioredoxin protein, as well as globally within a complex proteome where IA-light treatment generates mass-spectrometry identification of 992 cysteine residues. Importantly, these isotopically tagged probes can also be utilized for quantifying the percentage of cysteine modification within a single sample. Preliminary data supports the use of these tags to quantify the stoichiometry of TCEP-susceptible cysteine oxidation events in cell lysates.

Graphical abstract



SUPPORTING INFORMATION

Supporting Information is available free of charge on the ACS Publications website.

^{1}H and ^{13}C NMR spectra of synthesized compounds (PDF); Full lists of mass-spectrometry data (Excel).

Keywords

Cysteine; quantitative proteomics; electrophilic probe; post-translational modification; disulfide

INTRODUCTION

Cysteine is unique among proteinogenic amino acids due to the high nucleophilicity and redox activity of the thiol group.[1] Due to this characteristic chemical reactivity, cysteine participates in diverse protein functions such as nucleophilic and redox catalysis, metal binding, and disulfide-bond formation.[2] Cysteine can also act as regulatory switches through post-translational modifications (PTMs) including oxidation,[3] nitrosation,[4] and glutathionylation.[5] Chemoproteomic platforms, such as isoTOP-ABPP, apply a cysteine-reactive iodoacetamide-alkyne (IA-alkyne) probe, coupled with quantitative mass spectrometry (MS), to monitor cysteine-reactivity changes in two proteomes.[6] IsoTOP-ABPP, and derivatives thereof, has enabled relative quantification of reactivity across hundreds of cysteines in lysates and live cells,[7, 8] and has been applied to monitor PTMs of cysteine including oxidation,[9] nitrosation,[10] modification by endogenous and exogenous electrophiles,[11, 12] and metal chelation.[13]

Although label-free quantitation methods have improved significantly, incorporation of stable isotopes into peptides prior to analysis is still a commonly utilized strategy for quantitative analysis of differences among two or more samples. For cysteine-reactivity profiling studies, isotopic labels are commonly incorporated by two different workflows (Figure 1A). One method is by metabolic incorporation of heavy ^{13}C and ^{15}N isotopes into protein backbones, using established stable isotope labeling of amino acids in cell culture (SILAC) protocols. However, the use of SILAC is not amenable to the study of human tissue samples, and is cost-limited for the study of mice and other higher order organisms. To circumvent the need for SILAC labeling, the isoTOP-ABPP platform utilizes isotopically labeled cleavable biotin-azide tags that are incorporated into IA-alkyne tagged proteins through copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (Figure 1B).[14] In a typical isoTOP-ABPP workflow, two IA-alkyne treated proteome samples are appended to either a light or heavy cleavable biotin-azide tag, subjected to on-bead trypsin digestion and release of probe-labeled peptides for MS analysis. Changes in cysteine reactivity across the two proteomes being analyzed is reflected in the light:heavy ratio for each cysteine-containing peptide upon MS analysis. One technical limitation of isoTOP-ABPP has been the synthesis of isotope-containing cleavable biotin tags that requires costly reagents (L-valine-N-Fmoc ($^{13}C_5$, $^{15}N_1$)), and is low yielding. Here, we sought to develop an alternative to the isotopically tagged cleavable biotin-azide linkers by developing light and heavy analogs of IA-alkyne (IA-light and IA-heavy), which are accessible with greater synthetic ease.

In addition to providing an alternative strategy for stable isotope incorporation for typical reactive cysteine-profiling studies, the IA-light and IA-heavy probes have potential utility for differential labeling experiments, whereby cysteine reactivity changes can be analyzed within a single proteome sample. For example, a method termed OxICAT performs sequential labeling with a pair of isotopically labeled electrophiles, or ICAT reagents.[15, 16]

ICAT reagents consist of an iodoacetamide (IAM) electrophile, a biotin group, and a linker containing either hydrogen (light) or deuterium (heavy) atoms providing a ~9 Da mass difference between the light and heavy variants. In OxICAT, a proteome sample is labeled with the light ICAT reagent to cap reduced cysteines under denaturing conditions. After reduction with a strong reductant, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), the newly reduced cysteines are labeled with the heavy ICAT reagent. MS analysis provides heavy-to-light ratios which reveal the stoichiometry of cysteine oxidation within a single proteome sample. The IA-light and IA-heavy probes described herein, can provide a cheaper alternative to the light and heavy ICAT linkers utilized in typical OxICAT studies. Additionally, the small size of the IA-light and IA-heavy tags, relative to the ICAT linkers, make it feasible to perform the initial cysteine labeling step on fully folded proteins, thereby maintaining secondary structural elements that influence cysteine reactivity. Thereby, cysteine oxidation of highly reactive and functional cysteines can be selectively evaluated. We demonstrate the feasibility of this workflow in preliminary experiments that explore the stoichiometry of endogenous oxidation of proteins within a cell lysate. These experiments follow a similar workflow to OxICAT, in that a single proteome is treated with IA-heavy, prior to reduction by TCEP and addition of IA-light. Highly oxidized, partially oxidized, and highly reduced cysteines are differentiated from a single cell lysate sample. In summary, IA-light and IA-heavy provide cheaper alternatives that can be utilized for both isoTOP-ABPP and OxICAT workflows.

EXPERIMENTAL SECTION

Synthesis of intermediate 1

To benzaldehyde (530 mg, 5.00 mmol) in 10 mL of dichloromethane were added propargylamine (386 mg, 7.00 mmol) and magnesium sulfate (1.81 g, 15.0 mmol), and the mixture was stirred at 25 °C overnight. The mixture was filtered to remove magnesium sulfate, and the filtrate was concentrated under vacuum. The residual oil was suspended in 10 mL methanol, to which sodium borohydride (378 mg, 10.0 mmol) was added. The mixture was stirred at 0 °C for 30 minutes. After quenching the reaction by addition of 10 mL of 2 M aqueous HCl, methanol was evaporated to afford a dark yellow viscous solution. The solution was diluted with water and washed with dichloromethane. The aqueous layer was neutralized with 5 mL of 5 M NaOH, and extracted with dichloromethane (15 mL × 7). The combined organic layer was washed with 10 mL of brine, dried with sodium sulfate, and concentrated, affording 168 mg of N-benzylprop-2-yn-1-amine as a pale yellow oil (1.15 mmol, 23 % for two steps). The yellow oil was dissolved in 11.5 mL of dichloromethane and cooled to −10 °C. Chloroacetyl chloride (156 mg, 1.39 mmol) and triethylamine (234 mg, 2.31 mmol) were added to the solution, and the mixture was stirred at −10 °C for 20 minutes. The reaction was quenched by 10 mL of saturated sodium bicarbonate solution. The organic layer was isolated, washed with 2M HCl and brine, dried with sodium sulfate, and concentrated to give a pale yellow oil. The oil was purified by column chromatography (15 % ethyl acetate in hexane), affording 138 mg of **1** (0.624 mmol, 54 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36-7.24 (m, 5H), [4.72 (s), 4.70 (s), (2H)], [4.22 (s), 4.21 (s), (2H)], [4.12 (s), 4.01 (s), (2H)], [2.34 (s), 2.24 (s), (1H)]. ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.5 (s), (135.9, 135.2), 129.1-126.8 (m), (77.9, 77.6), (73.5, 72.7), (50.5, 48.7), 41.2 (s), (36.5, 34.8).

Due to the presence of rotamers around the amide bond, some ^{1}H and ^{13}C signals are split into two peaks, which are represented within parentheses. HRMS (ESI+) Calcd for [M+H], 222.0686; Found, 222.0691 (0.5 mmu).

Synthesis of intermediate 2

Intermediate **2** was prepared by following the procedure for intermediate **1** using 500 mg (4.46 mmol) of heavy benzaldehyde (ring-$^{13}C_6$) instead of light benzaldehyde, affording 385 mg of **2** (1.70 mmol, 38 % for three steps). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.50-7.12 (m, 5H), 4.72 (d, 2H, J = 10.8 Hz), 4.23 (s, 2H), [4.12 (s), 4.01 (s), (2H)], [2.33 (s), 2.24 (s), (1H)]. ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.2, 138.9 -137.5 (m), 132.2-129.1 (m), (80.6, 80.2), (76.1, 75.3), (53.3, 53.0), (51.5, 51.2), 43.9, (39.1, 37.5). HRMS (ESI+) Calcd for [M +H], 228.0887; Found, 228.0889 (0.1 mmu).

Synthesis of IA-light

To the solution of intermediate **1** (138 mg, 0.624 mmol) in 3 mL acetone was added sodium iodide (468 mg, 3.12 mmol), and the mixture was stirred at 25 °C for 30 minutes. After filtration, the filtrate was concentrated under vacuum, and suspended in dichloromethane. The organic layer was washed with water (15 mL × 5), dried with sodium sulfate, and concentrated to obtain a pale yellow oil. The oil was purified by column chromatography (15 % ethyl acetate in hexane), affording 100 mg of IA-light (0.319 mmol, 51 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38-7.23 (m, 5H), 4.67 (s, 2H), [4.19 (d), 3.94 (d), (2H)], [3.87 (s), 3.74 (s), (2H)], [2.32 (t), 2.23 (t), (1H)]. ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.0 (d), (136.1, 135.3), 129.0-126.7 (m), (77.8, 77.6), (73.4, 72.7), (51.7, 48.6), (37.4, 35.1), (-3.6, -3.7). HRMS (ESI+) Calcd for [M+H], 314.0042; Found, 314.0032 (-1.0 mmu).

Synthesis of IA-heavy

IA-heavy was synthesized from **2** by following the procedure for IA-light. 385 mg (1.70 mmol) of **2** was used to afford 333 mg of IA-heavy (1.04 mmol, 61 %). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.40-7.12 (m, 5H), 4.69 (s, 2H), [4.22 (d), 3.97 (d), (2H)], [3.89 (s), 3.76 (s), (2H)], [2.33 (t), 2.25 (t), (1H)]. ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.7 (d), 139.1-137.6 (m), 132.2-128.9 (m), (80.5, 80.2), (76.0, 75.3), (54.3 (d), 51.3 (d)), (40.1, 37.8), (−1.1, −1.2). HRMS (ESI+) Calcd for [M+H], 320.0243; Found, 320.0240 (−0.3 mmu).

Labeling of recombinant thioredoxin

Recombinant purified human thioredoxin (30 μg, 12.5 μM) was treated with 100 μM IA-light or IA-heavy at 25 °C for an hour. Labeled proteins were precipitated with 10 % trichloroacetic acid at −80 °C overnight. Upon centrifugation at 16,900 g for 10 min, the pellet was suspended in acetone at 4 °C and centrifuged at 2,150 g for 10 min. The pellet was suspended in 30 μL of 8 M urea in DPBS, and ammonium bicarbonate (100 mM, 70 μL) was added. The solution was treated with 15 mM DTT at 65 °C for 15 min, then 12.5 mM iodoacetamide at 25 °C for 30 min. The sample was diluted with 120 μL of DPBS, mixed with a vortex mixer, and treated with trypsin (2 μg) and 1.0 mM calcium chloride at 37 °C overnight. The peptide sample was analyzed by LC-MS/MS.

Fluorescence gel imaging

The soluble fraction of HeLa cell lysates (2.0 mg/ml) were treated with probes (IA-alkyne, IA-light, or IA-heavy) at 25 °C for 1 h. The labeled lysates underwent Copper(I)-catalyzed Alkyne-Azide Cycloaddition (CuAAC) with a fluorescent reporter, rhodamine-azide, as previously reported.[17] Briefly, the lysates were treated with rhodamine-azide (25 μM), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1.0 mM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA ligand) (100 μM), and copper(II) sulfate (1.0 mM) at 25 °C for 1 h. The fluorescently tagged lysates were analyzed by SDS-PAGE, and visualized by a Bio-Rad ChemiDoc MP System.

Proteome-labeling and analysis by LC/LC-MS/MS

Proteome samples for MS analysis were prepared according to previously reported procedures with modifications.[18] Soluble HeLa cell lysates (2.0 mg/ml, 2.0 mL) were treated with probes (IA alkyne or IA-light, 100 μM) at 25 °C for 1 h. The labeled lysates were treated with diazo biotin-azide (100 μM) (Click Chemistry Tools), TCEP (1.0 mM), TBTA ligand (100 μM), and copper(II) sulfate (1.0 mM) at 25 °C for 1 h. The precipitated proteins were collected by centrifugation (6,500 g, 4 min), washed with cold methanol twice, and solubilized in DPBS containing 1.2 % SDS with sonication and heating (80 °C, 5 min). The probe-labeled proteome samples were diluted with 5 mL of DPBS, and incubated with 100 μL of streptavidin-agarose beads (Thermo Scientific) overnight at 4 °C. The beads were washed by 0.20 % SDS/DPBS (× 1), DPBS (× 3), and water (× 3), and suspended in 500 μL of 6 M urea/DPBS. The suspension was treated with 10 mM DTT at 65 °C for 15 min, then 20 mM iodoacetamide was added to the suspension which was incubated at 37 °C for 30 min. The beads were pelleted by centrifugation (1400 × g, 2 min) and resuspended in 200 μL of 2 M urea/DPBS containing 1.0 mM calcium chloride and trypsin (2.0 μg). The digestion was allowed to proceed overnight at 37 °C. The beads were washed with DPBS (× 3) and water (× 3). The cleavage of azobenzene was carried out by incubation with sodium dithionite (25 mM × 2 and 50 mM × 1) at 25 °C for an hour respectively. The beads were then washed twice with 75 μL of water. To the combined peptide solutions (~350 μL) were added formic acid (17.5 μL), which was analyzed by LC/LC-MS/MS.[18]

Differential labeling

Soluble fractions of HeLa cell lysate (2.0 mL of 4.0 mg protein/ml) were incubated at 37 °C for an hour in the presence or absence of TCEP (1.0 mM). The lysates were labeled with 100 μM IA-heavy at 37 °C for an hour, and IA-heavy was removed by size exclusion chromatography (PD-10 desalting columns, GE Healthcare Life Sciences). The filtrates were treated with 1.0 mM TCEP at 37 °C for an hour, and incubated with 100 μM IA-light at 37 °C for an hour. Diluted lysates (2.0 mL of 1.8 mg protein/ml) underwent CuAAC with diazo biotin-azide, trypsin digestion, and LC/LC-MS/MS as described in the “proteome-labeling and analysis by LC/LC-MS/MS” section.

RESULTS AND DISCUSSION

Design of isotopically tagged cysteine-reactive probes

We designed isotopically tagged cysteine-reactive probes to contain an iodoacetamide electrophile for covalent modification of cysteine thiols, an alkyne bioorthogonal handle for CuAAC-mediated conjugation to reporter tags, and a benzyl group to provide the light and heavy isotope labels (Figure 1B; IA-light and IA-heavy). Since we ideally required the incorporation of 6 heavy atoms to provide the optimal mass difference between the light and heavy probes, we utilized benzaldehyde bearing six ^{13}C atoms on the benzene ring (purchased from Cambridge Isotope Laboratories, Inc.). Since the incorporation of deuterium is known to result in small changes in chromatographic elution relative to the hydrogen-labeled counterpart, we chose to utilize ^{13}C-labeled reagents. Importantly, the ^{13}C-benzaldehyde is a more affordable source of heavy isotopes ($1,100 for 500 mg), especially when compared to the L-valine-N-Fmoc ($^{13}C_5$, $^{15}N_1$) ($906 for 250 mg) used in isoTOP-ABPP cleavable biotin-azide linkers. Furthermore, the isoTOP-ABPP linkers utilize solid-phase synthesis methods that require two or more equivalents of the L-valine-N-Fmoc ($^{13}C_5$, $^{15}N_1$) reagent, resulting in reduced conversion relative to the expensive isotopically tagged reagent. In contrast, the solution-phase synthesis of IA-light and IA-heavy results in higher overall conversion of the heavy reagent.

Synthesis of IA-light and IA-heavy

IA-light and IA-heavy probes were synthesized by treating ^{12}C (light) or ^{13}C (heavy) benzaldehyde with propargyl amine and sodium sulfate to generate the corresponding imines (Figure 1C). Subsequent reduction with sodium borohydride afforded light and heavy *N*-propargylbenzylamines, which were conjugated to chloroacetyl chloride to provide intermediates **1** and **2**. Intermediates **1** and **2** were treated with sodium iodide to generate the iodoacetamide through the Finkelstein reaction, affording IA-light and IA-heavy, respectively. The synthetic procedure requires only 4 steps and two flash chromatography separations, and provides a high overall yield for IA-heavy (23 %) from the expensive ^{13}C-benzaldehyde starting material. The purity and identity of the IA-light and IA-heavy, and the presence of the ^{13}C labels were confirmed by NMR and high resolution MS. Aromatic peaks of the IA-heavy ^{1}H NMR spectrum showed complex coupling with ^{13}C, and the ^{13}C spectra contained large aromatic peaks, signifying the presence of enriched ^{13}C. A mixture of IA-light and IA-heavy (1:1) was analyzed by MS, and clearly showed the presence of two m/z peaks with equivalent intensity with a mass difference of 6 Da (Figure 1D).

IA-light and IA-heavy labeling of purified proteins and cell lysates

Upon synthesis and characterization of IA-light and IA-heavy, we evaluated these probes for labeling of purified recombinant thioredoxin protein. Purified thioredoxin was treated with either IA-light or IA-heavy, and the combined samples were digested with trypsin and analyzed by LC-MS/MS. The C32-containing thioredoxin peptide labeled with IA-light and IA-heavy were identified by LC-MS/MS, with equivalent ion intensities (Figure 2A). Importantly, these studies validated the equivalent cysteine reactivity of IA-light and IA-heavy, and the stability of the resulting covalent adduct in MS/MS fragmentation analyses.

We next evaluated IA-light and IA-heavy labeling in cell lysates by in-gel fluorescence. Soluble fractions of HeLa cell lysates (2.0 mg/ml) were treated with IA-alkyne, IA-light, or IA-heavy. Labeled proteome samples were conjugated with a fluorescent rhodamine-azide tag through CuAAC, and analyzed by SDS-PAGE (Figure 2B). In-gel fluorescence imaging revealed that IA-light and IA-heavy have equivalent fluorescent intensity, again signifying equivalent cysteine reactivity. Interestingly, IA-light and IA-heavy demonstrated a higher intensity of proteome labeling compared to IA-alkyne, indicating enhanced cysteine reactivity of these probes, likely due to the added bulk of the probe resulting in greater binding interactions with protein regions proximal to reactive cysteines within the proteome.

Mass-spectrometry analysis of isotopically tagged IA probes

To identify the cysteine targets of the new IA probes, we performed MS analysis with IA-light treated proteomes, and compared the identified cysteines to data obtained with the IA-alkyne probe. HeLa cell lysates (2.0 mg/ml) were treated with IA-alkyne or IA-light (100 μM), and the labeled proteome samples underwent CuAAC with a commercially available cleavable diazo biotin-azide (Click Chemistry Tools) linker. Biotinylated proteins were enriched by a streptavidin-agarose resin, and digested by trypsin. Upon trypsin digestion, the diazo linker was cleaved by sodium dithionite to release IA-labeled peptides, which were analyzed by LC/LC-MS/MS. Two replicates were performed for the IA-light probe and were compared to one replicate obtained for IA-alkyne. IA-light treatment resulted in the identification of an average of 992 cysteine residues from the two independent runs, while the IA-alkyne analysis identified 837 cysteine residues (Figure 2C, Supporting table S1, S2). Comparison of the unique cysteine residues identified across the two IA-light replicates (1369 cysteines; Supporting table S1) to those unique cysteines identified in the IA-light sample (839 cysteines; Supporting table S2) showed substantial overlap (67.5 % of cysteines identified by IA-alkyne were identified by IA-light; Figure 2D). The high overlap indicates that IA-light can react with a similar subset of cysteines to IA-alkyne, as the observed 67.5% overlap is typical of replicates in standard cysteine-reactivity profiling studies.[6] Cysteine residues identified by IA-light included functionally important cysteines which are involved in a variety of protein functions such as catalysis, nucleotide and metal binding, and sites of PTMs (Figure 2E). These comparison data show that the cysteine targets do not significantly differ between IA-light and IA-alkyne, despite the structural changes induced by incorporation of the aromatic ring. Therefore, IA-light and heavy provide appropriate surrogates for IA-alkyne in isoTOP-ABPP experiments, with the added advantage that commercially available cleavable linkers can now be utilized for quantitative proteomics experiments, without the need to synthesize light and heavy cleavable linkers in house.

Differential labeling experiments to quantify the extent of cysteine oxidation

We next sought to apply IA-light and IA-heavy to evaluate the potential of these probes for differential labeling experiments, akin to OxICAT. In this experiment, we monitored cysteine-reactivity changes induced by TCEP, which is a strong reductant of disulfide bonds. Instead of comparing two proteome samples (+/−TCEP) that is typical of standard isoTOP-ABPP workflows, the IA-light and IA-heavy probes allow for assessment of the degree of cysteine oxidation within a single sample. To achieve this, soluble lysates from HeLa cells were treated with 100 μM IA-heavy under native conditions to label reduced and reactive

cysteine residues (Figure 3A). Upon removing IA-heavy by a PD-10 desalting column, oxidized cysteines were reduced by TCEP and exposed to 100 μM IA-light treatment. The labeled proteome sample then underwent the standard sample preparation procedure for MS, including CuAAC with the diazo biotin-azide tag, on-bead trypsin digestion, and cleavage from beads by sodium dithionite. The labeled peptide samples were analyzed by LC/LC-MS/MS to obtain heavy-to-light ratios ($R_{H/L}$) as a measure of the degree of oxidation of each cysteine within the cell lysate sample tested. A low $R_{H/L}$ indicates that the majority of that cysteine exists in the oxidized state, since the fraction that reacted prior to TCEP treatment (heavy label) is less than the fraction labeled after TCEP treatment (light label). In contrast, high $R_{H/L}$ values are indicative of cysteines that exist predominantly in the reduced form (heavy label) with minimal new thiol exposure after TCEP treatment. We identified 700 cysteine residues with valid $R_{H/L}$ (Supporting table S3) including several annotated disulfide-forming cysteines (Supporting table S3). We categorized the identified cysteines into three groups; low ($R_{H/L} < 0.67$), moderate ($0.67 < R_{H/L} < 1.5$) and high ($R_{H/L} > 1.5$) $R_{H/L}$. Cysteines in the low $R_{H/L}$ group exist predominantly in the oxidized state in the cell lysate. As confirmation of our data, cysteines annotated to form redox-active disulfides were found more frequently (15 %) than the groups of moderate and high $R_{H/L}$ (4.7 % and 3.5 % respectively). Known disulfide-forming cysteines such as Cys85 of PDIA3 and Cys70 of CD59 showed low $R_{H/L}$ ratios (0.03 and 0.11 respectively) (Figure 3B). The moderate group included Cys192 of GSTO1 ($R_{H/L} = 1$) and Cys100 of PRDX5 ($R_{H/L} = 1.06$), indicating these cysteines exist as a mixture of reduced and oxidized species within the cell lysates we analyzed. Cysteines with high $R_{H/L}$ ratios such as Cys196 of ACAT1 ($R_{H/L} = 19.32$) and Cys96 of RPL4 ($R_{H/L} = 19.49$) exist predominantly in the reduced form in the cell lysates and are minimally affected by the TCEP treatment.

To confirm that cysteines with low $R_{H/L}$ represent highly oxidized cysteine residues, we performed a targeted analysis of Cys43 of TXNDC17, which was identified in our MS studies with $R_{H/L}$ of 0.1. This low $R_{H/L}$ ratio indicates that only a small fraction of Cys43 exists in the free thiol form in these cell lysates resulting in low labeling by IA-heavy. To confirm that this low IA-heavy labeling was due to oxidation of the cysteine, we preincubated the lysates with TCEP (1 mM) prior to treatment with IA-heavy and continued with the differential labeling protocol as described previously. Pretreatment with TCEP resulted in a $R_{H/L}$ ratio of TXDC17 (Cys43) of 20 (Figure 3C), which is the value that our quantification program uses to indicate singleton peptides (signal detectable only in heavy sample). This large increase in the fraction of Cys43 that was labeled by IA-heavy, confirms that the decreased labeling observed previously was due to oxidation (Figure 3C). In summary, this differential labeling study illustrates the potential utility of the IA-light and IA-heavy probes in differential labeling experiments, akin to the use of ICAT reagents for oxICAT experiments.

CONCLUSIONS

We developed a pair of isotopically tagged iodoacetamide-alkyne probes, IA-light and IA-heavy. These probes can be prepared through a short and effective synthesis, using heavy benzaldehyde as an affordable starting material. These new probes provide an alternative strategy for incorporating light and heavy isotopes into samples for quantitative cysteine-

reactivity profiling, by complementing SILAC and isoTOP-ABPP strategies that have been utilized previously. Importantly, the use of IA-light and IA-heavy circumvents the need to synthesize light and heavy cleavable biotin-azide linkers, enabling the utilization of a variety of commercially available linkers. In addition, IA-light and IA-heavy maintain labeling of reactive and functional cysteines observed with IA-alkyne labeling. Lastly, IA-light and IA-heavy have the potential to be applied to differential labeling experiments that can monitor the stoichiometry of oxidative cysteine PTMs, therefore expanding on the types of experiments amenable to isoTOP-ABPP analysis. Taken together, the IA-light and IA-heavy probes will complement chemoproteomic tools currently available for reactive-cysteine profiling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant 1R01GM117004 to E. W. We thank Tyler Bechtel for providing the purified thioredoxin protein and Aaron Maurais for assistance with MS data analysis.

References

1. Poole LB. The basics of thiols and cysteines in redox biology and chemistry. Free Radic Biol Med. 2015; 80:148–157. [PubMed: 25433365]
2. Pace NJ, Weerapana E. Diverse functional roles of reactive cysteines. ACS Chem Biol. 2013; 8:283–296. [PubMed: 23163700]
3. Paulsen CE, Carroll KS. Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. Chem Rev. 2013; 113:4633–4679. [PubMed: 23514336]
4. Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. Protein S-nitrosylation: purview and parameters. Nat Rev Mol Cell Biol. 2005; 6:150–166. [PubMed: 15688001]
5. Grek CL, Zhang J, Manevich Y, Townsend DM, Tew KD. Causes and consequences of cysteine S-glutathionylation. J Biol Chem. 2013; 288:26497–26504. [PubMed: 23861399]
6. Weerapana E, Wang C, Simon GM, Richter F, Khare S, Dillon MB, Bachovchin DA, Mowen K, Baker D, Cravatt BF. Quantitative reactivity profiling predicts functional cysteines in proteomes. Nature. 2010; 468:790–795. [PubMed: 21085121]
7. Abo M, Weerapana E. A Caged Electrophilic Probe for Global Analysis of Cysteine Reactivity in Living Cells. J Am Chem Soc. 2015; 137:7087–7090. [PubMed: 26020833]
8. Abo M, Bak DW, Weerapana E. Optimization of Caged Electrophiles for Improved Monitoring of Cysteine Reactivity in Living Cells. Chembiochem. 2017; 18:81–84. [PubMed: 27813293]
9. Deng X, Weerapana E, Ulanovskaya O, Sun F, Liang H, Ji Q, Ye Y, Fu Y, Zhou L, Li J, Zhang H, Wang C, Alvarez S, Hicks LM, Lan L, Wu M, Cravatt BF, He C. Proteome-wide quantification characterization of oxidation-sensitive cysteines in pathogenic bacteria. Cell Host Microbe. 2013; 13:358–370. [PubMed: 23498960]
10. Zhou Y, Wynia-Smith SL, Couvertier SM, Kalous KS, Marletta MA, Smith BC, Weerapana E. Chemoproteomic Strategy to Quantitatively Monitor Transnitrosation Uncovers Functionally Relevant S-Nitrosation Sites on Cathepsin D HADH2. Cell Chem Biol. 2016; 23:727–737. [PubMed: 27291402]
11. Wang C, Weerapana E, Blewett MM, Cravatt BF. A chemoproteomic platform to quantitatively map targets of lipid-derived electrophiles. Nat Methods. 2014; 11:79–85. [PubMed: 24292485]
12. Backus KM, Correia BE, Lum KM, Forli S, Horning BD, Gonzalez-Paez GE, Chatterjee S, Lanning BR, Teijaro JR, Olson AJ, Wolan DW, Cravatt BF. Proteome-wide covalent ligand discovery in native biological systems. Nature. 2016; 534:570–574. [PubMed: 27309814]

13. Pace NJ, Weerapana E. A competitive chemical-proteomic platform to identify zinc-binding cysteines. ACS Chem Biol. 2014; 9:258–265. [PubMed: 24111988]
14. Qian Y, Martell J, Pace NJ, Ballard TE, Johnson DS, Weerapana E. An isotopically tagged azobenzene-based cleavable linker for quantitative proteomics. Chembiochem. 2013; 14:1410–1414. [PubMed: 23861326]
15. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol. 1999; 17:994–999. [PubMed: 10504701]
16. Leichert LI, Gehrke F, Gudiseva HV, Blackwell T, Ilbert M, Walker AK, Strahler JR, Andrews PC, Jakob U. Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. Proc Natl Acad Sci USA. 2008; 105:8197–8202. [PubMed: 18287020]
17. Speers AE, Cravatt BF. Profiling enzyme activities in vivo using click chemistry methods. Chem Biol. 2004; 11:535–546. [PubMed: 15123248]
18. Weerapana E, Speers AE, Cravatt BF. Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)–a general method for mapping sites of probe modification in proteomes. Nat Protoc. 2007; 2:1414–1425. [PubMed: 17545978]

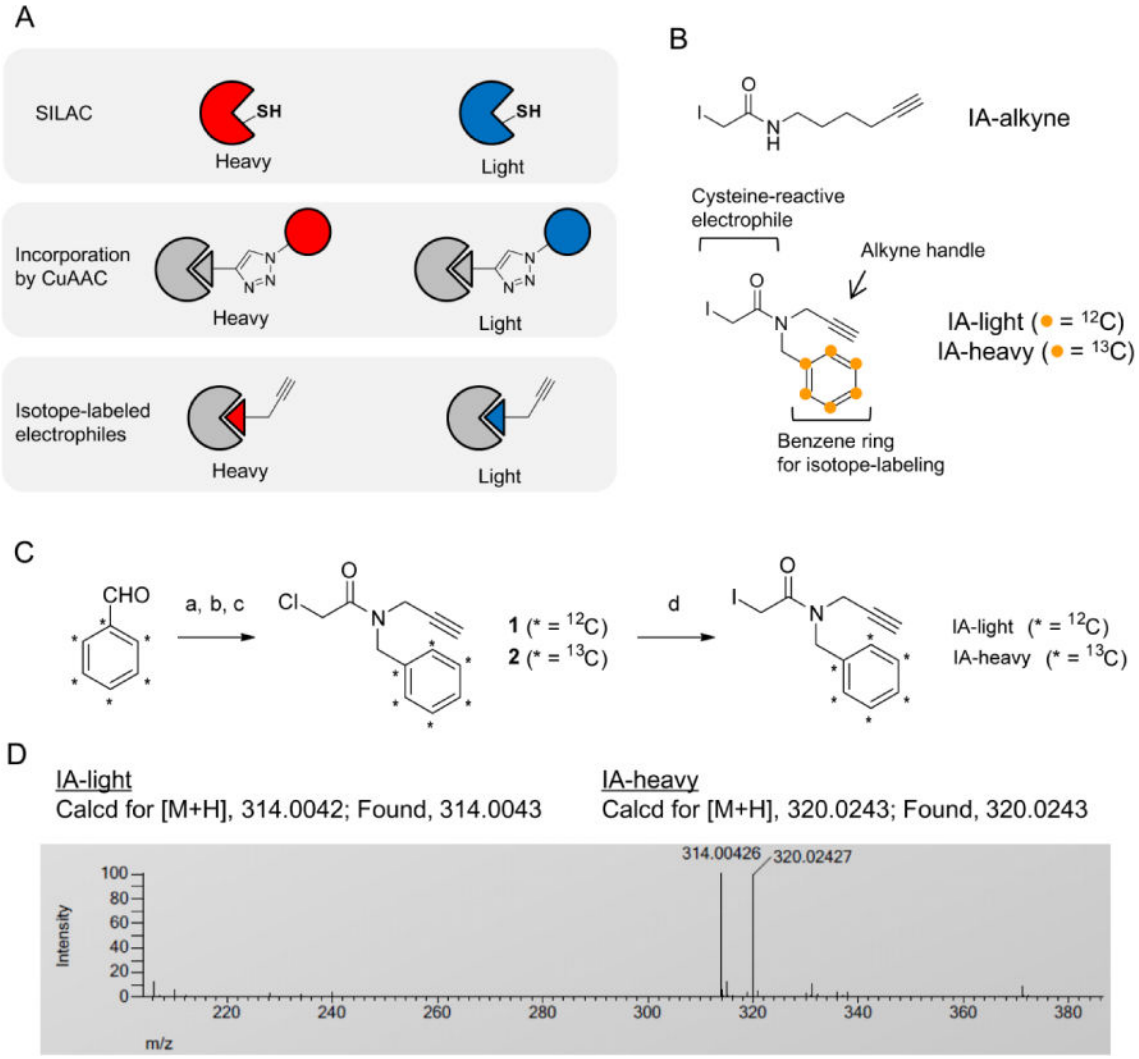
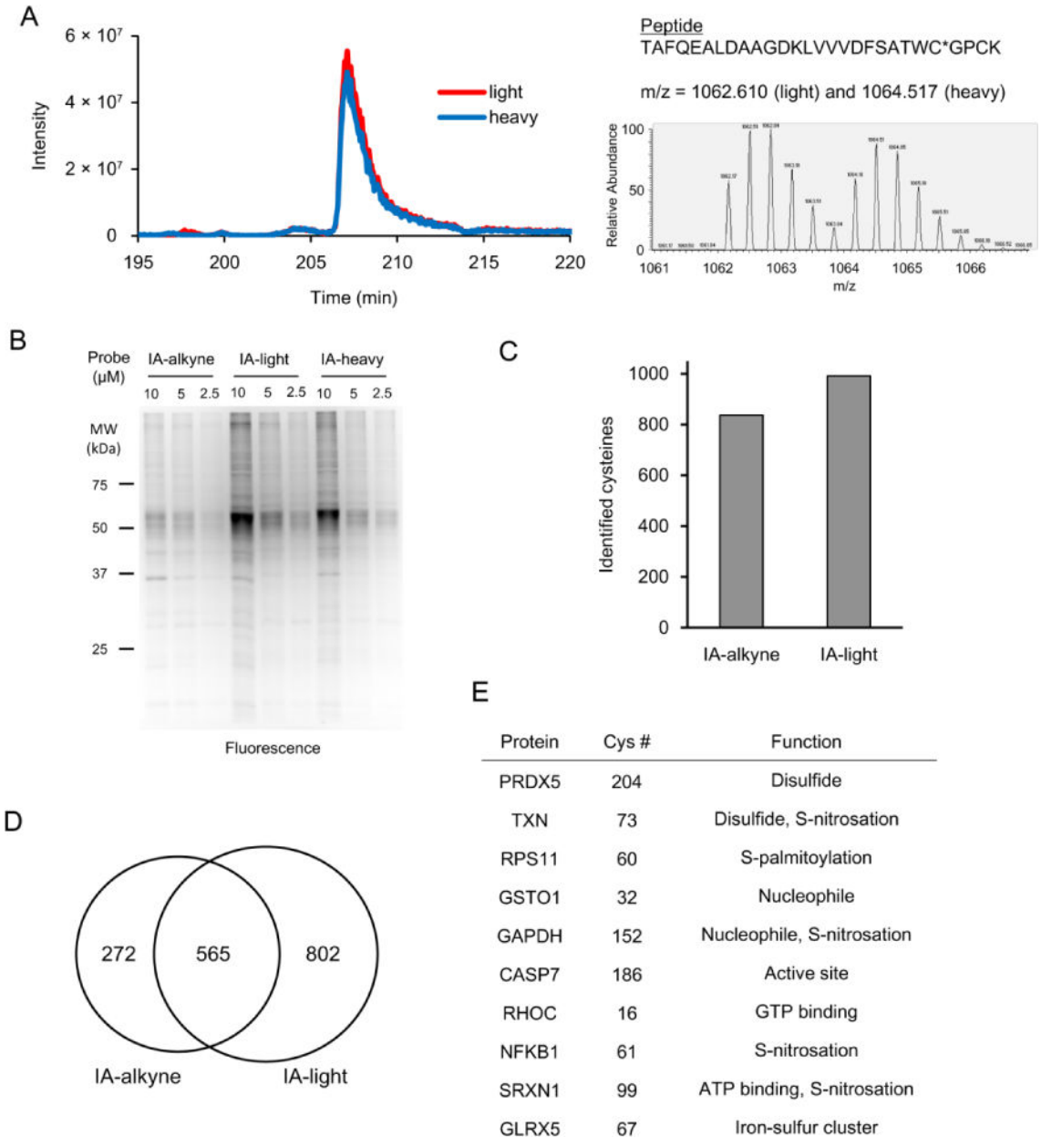


Figure 1.

Design and synthesis of isotopically-labeled iodoacetamide probes. (A) Potential workflows for installing isotopic labels for quantitative cysteine-reactivity profiling. (B) Structures of IA-alkyne, IA-light, and IA-heavy. (C) Synthetic route to IA-light and IA-heavy; (a) propargylamine, magnesium sulfate, dichloromethane; (b) sodium borohydride, methanol; (c) chloroacetyl chloride, triethylamine, dichloromethane; (d) sodium iodide, acetone. (D) Mass spectrometry (DART+) of an equimolar mixture of IA-light and IA-heavy.



Protein	Cys #	Function
PRDX5	204	Disulfide
TXN	73	Disulfide, S-nitrosation
RPS11	60	S-palmitoylation
GSTO1	32	Nucleophile
GAPDH	152	Nucleophile, S-nitrosation
CASP7	186	Active site
RHOC	16	GTP binding
NFKB1	61	S-nitrosation
SRXN1	99	ATP binding, S-nitrosation
GLRX5	67	Iron-sulfur cluster

Figure 2.

Evaluation of IA-light and IA-heavy with recombinant human thioredoxin protein and HeLa cell lysates. (A) Chromatogram and isotopic distribution of the Cys-32 containing peptide of thioredoxin labeled by IA-light and IA-heavy. (B) In-gel fluorescence image of HeLa cell lysates labeled with IA-alkyne, IA-light, and IA-heavy. (C) Numbers of cysteines identified by IA-alkyne and IA-light. (D) Venn diagram showing overlap of cysteines identified by IA-alkyne and IA-light. (E) Examples of functional cysteines identified by IA-light.

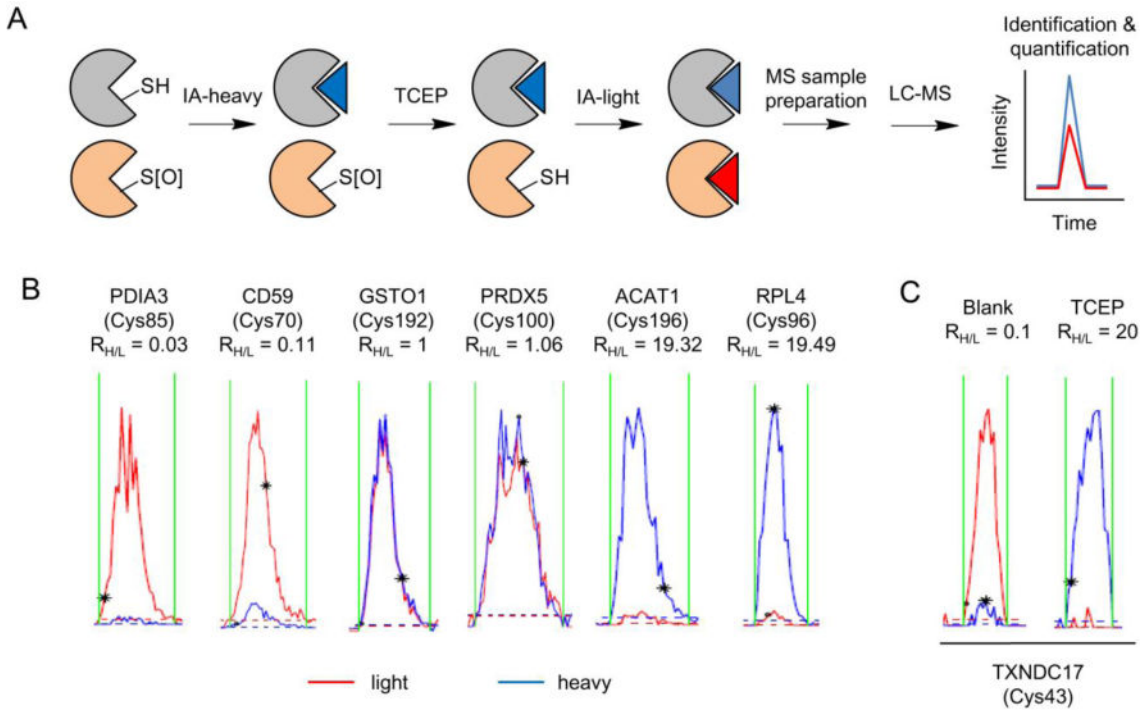


Figure 3.

Differential labeling with IA-light and IA-heavy. (A) Experimental scheme of differential labeling. (B) Chromatograms of select identified peptides with low, moderate and high $R_{H/L}$ values. (C) Chromatograms for the Cys-43 containing peptide from TXNDC17 in the presence or absence of pretreatment with TCEP.