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Comparative Analysis of Cleavable Azobenzene-Based Affinity Tags for Bioorthogonal Chemical Proteomics

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SUMMARY

The advances in bioorthogonal ligation methods have provided new opportunities for proteomic analysis of newly synthesized proteins, posttranslational modifications, and specific enzyme families using azide/alkyne-functionalized chemical reporters and activity-based probes. Efficient enrichment and elution of azide/alkyne-labeled proteins with selectively cleavable affinity tags are essential for protein identification and quantification applications. Here, we report the synthesis and comparative analysis of $Na_2S_2O_4$-cleavable azobenzene-based affinity tags for bioorthogonal chemical proteomics. We demonstrated that *ortho*-hydroxyl substituent is required for efficient azobenzene-bond cleavage and show that these cleavable affinity tags can be used to identify newly synthesized proteins in bacteria targeted by amino acid chemical reporters as well as their sites of modification on endogenously expressed proteins. The azobenzene-based affinity tags are compatible with in-gel, in-solution, and on-bead enrichment strategies and should afford useful tools for diverse bioorthogonal proteomic applications.

INTRODUCTION

Bioorthogonal chemical proteomics has afforded new opportunities to investigate protein function and regulation (Sletten and Bertozzi, 2009; Cravatt et al., 2008). The advent of bioorthogonal ligation methods such as the Staudinger ligation (Jewett and Bertozzi, 2010; Laughlin and Bertozzi, 2009) and click chemistry cycloaddition reactions (Rostovtsev et al., 2002; Tornoe et al., 2002; Best, 2009; Saxon and Bertozzi, 2000) has enabled the introduction of small molecule probes into cells and animals for imaging and proteomic studies of specific proteins that were previously impossible. For example the administration of azide- or alkyne-functionalized substrates allows metabolic labeling of proteins with bioorthogonal chemical reporters to monitor protein synthesis and/or posttranslational modifications as well as nucleic acid synthesis and lipid metabolism (Dieterich et al., 2007; Baskin et al., 2010; Zhang et al., 2010; Salic and Mitchison, 2008). Alternatively, enzyme/mechanism-based inhibitors (Evans and Cravatt, 2006; Simon and Cravatt, 2010) and natural products (Böttcher et al., 2010) that are modified with azides or alkynes probes can facilitate target identification of small molecules as well as profiling of specific protein families in cells and animals (Cravatt et al., 2008). The pioneering studies by Cravatt et al. (2008) demonstrated that alkyne-modified activity-based probes and click chemistry could be used for in-gel fluorescence profiling of enzyme families as well as characterization of unclassified enzymes using multidimensional protein identification technology (MudPIT) (Simon and Cravatt, 2010), but the robust identification of azide/alkyne-labeled proteins and their subsequent functional analysis is still challenging.

The multiple steps required for bioorthogonal chemical proteomics can significantly limit the identification of proteins and specific amino acids that are targeted by chemical reporters or activity-based probes. In vitro studies suggest that Cu^{I}-catalyzed azide-alkyne cycloaddition (CuAAC) is more efficient for bioorthogonal detection of azide/alkyne-modified proteins in cell lysates (Charron et al., 2009; Speers and Cravatt, 2005), although the Staudinger ligation and Cu-free cycloaddition reactions are preferred with live cells and in animals (Jewett and Bertozzi, 2010; Laughlin and Bertozzi, 2009). One important issue is the enrichment and recovery of azide- or alkyne-modified proteins/peptides after bioorthogonal ligation reactions from complex mixtures for mass spectrometry (MS)-based protein identification. Although functionalized biotinylated affinity tags and streptavidin beads provides an effective approach for enrichment of chemical reporter/probe-labeled proteins/peptides, the high-affinity binding ($\sim K_D$ 10^{-15} M) of biotin to streptavidin makes quantitative elution of captured proteins/peptides from beads challenging and is not ideal for large-scale proteomic studies as well as mapping sites of protein modifications. To address this issue, selectively cleavable affinity tags have been developed for bioorthogonal chemical proteomics. For example, protease- (Speers and Cravatt, 2005; Dieterich et al., 2006), pH- (van der Veken et al., 2005; Fauq et al., 2006), photo- (Petrotchenko et al., 2009), and redox-cleavable- (Shimkus et al., 1985; Gartner et al., 2007; Verhelst et al., 2007; Nessen et al., 2009) linkers have been incorporated into biotinylated affinity tags to facilitate the elution of proteins and peptides from streptavidin beads for protein identification. The compatibility of cleavable linkers with bioorthogonal ligation conditions and MS-based peptide sequencing is also crucial. CuAAC requires reducing agents such as 1 mM

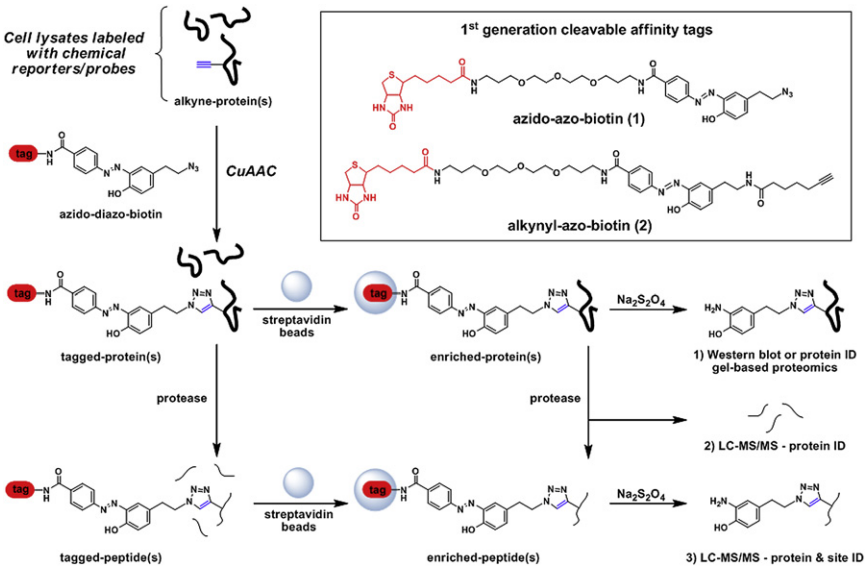

Figure 1. Schematic of Selective Enrichment and Elution of Alkyne/Azide-Labeled Proteins or Peptides Using Azobenzene-Based Cleavable Affinity Tags for Bioorthogonal Proteomics

In this two-step labeling approach, target proteins labeled with alkyne or azide-functionalized chemical reporters/probes can be selectively reacted with CuAAC reagents for detection and identification. For example, alkyne-modified proteins/peptides can be reacted with azido-azo-biotin (**1**) and enriched at the protein level and eluted with $Na_2S_2O_4$ for gel-based proteomics or subjected to on-bead protease digestion and LC-MS/MS analysis. Alternatively, tagged peptides can be enriched and cleaved with $Na_2S_2O_4$ for LC-MS/MS analysis.

See also Figure S1.

tris(2-carboxyethyl)phosphine (TCEP) or ascorbic acid (Wang et al., 2003; Chan et al., 2004), which is not compatible with disulfide linkers commonly used with biotinylated affinity tags (Shimkus et al., 1985; Gartner et al., 2007; Nessen et al., 2009). However, disulfide cleavable linkers can be used with Cu-free cycloadditions for bioorthogonal proteomic studies, but the synthesis of cyclooctyne reagents is more cumbersome (Nessen et al., 2009). Acylhydrazone linkers are compatible with CuAAC, can be cleaved with mildly acidic conditions (pH ~5.8) for elution of proteins from beads, and used to reintroduce detection tags onto recovered proteins but have not been used for large-scale bioorthogonal proteomics or mapping sites of protein modifications yet (Park et al., 2009). Protease-sensitive cleavable linkers provide mild enzymatic conditions for eluting captured proteins from beads and subsequent protein identification (Speers and Cravatt, 2005; Dieterich et al., 2006). Notably, the development of TEV-protease cleavable affinity tags by the Cravatt laboratory has enabled high-content bioorthogonal proteomic studies of activity-based probes and mapping modification sites (Speers and Cravatt, 2005; Weerapana et al., 2007).

Our laboratory has focused on azobenzene-functionalized biotinylated tags that are stable to reducing conditions of CuAAC and efficiently cleaved by sodium dithionite ($Na_2S_2O_4$) for bioorthogonal proteomic studies (Figure 1) (Verhelst et al., 2007; Landi et al., 2010; Yang et al., 2010; Yount et al., 2010; Grammel et al., 2010; Rangan et al., 2010). Our first-generation clickable and azobenzene-functionalized biotinylated tag (azido-azo-biotin, **1**) (Figure 1) enabled the CuAAC-based proteomic analysis of acetylated (Yang et al., 2010) and S-palmitoylated proteins (Yount et al., 2010) in mammalian cells as well as lipoproteins (Rangan et al., 2010) and newly synthesized proteins (Grammel et al., 2010) in bacteria using alkyne-functionalized chemical reporters. Additionally, we generated an alkyne- and azobenzene-functionalized biotinylated tag (alkynyl-azo-biotin, **2**) (Figure 1) for proteomic analysis of azide-modified proteins and used this cleavable affinity tag to profile fatty-acylated proteins in mammalian cells (unpublished data). Here, we describe the modular synthesis, scope, and utility of several azobenzene-functionalized biotinylated tags for bioorthogonal chemical proteomics. We demonstrated that clickable and azobenzene-based affinity tags require *ortho*-hydroxy-functionalization of the aromatic ring for efficient $Na_2S_2O_4$ cleavage and elution of proteins from streptavidin beads for bioorthogonal chemical proteomics. Using amino acid reporters of newly synthesized proteins in bacteria, we showed that these clickable azobenzene-based affinity tags allow robust protein identification and mapping of modification sites.

RESULTS

Synthesis and Evaluation of Second-Generation Clickable and Azobenzene-Based Affinity Tags

Given the utility of our first-generation clickable azobenzene-based affinity tags (**1** and **2**) (Figure 1) for bioorthogonal chemical proteomics (Yang et al., 2010; Yount et al., 2010; Grammel et al., 2010; Rangan et al., 2010), we synthesized second-generation clickable azobenzene-based affinity tags using a more efficient and modular synthetic route to generate both azide- and alkyne-functionalized azobenzene affinity tags (Figure 2A). The azobenzene moiety was generated by coupling of azonium salt of 4-aminobenzoic acid with phenol as previously described (Bahulayan et al., 2003). The resulting azobenzene acid was esterified and then alkylated with 2-azdioethyl tosylate or propargyl bromide to give compounds **8** and **10**, respectively. The methyl esters were then converted into their corresponding acyl chlorides (**9** and **11**) and reacted with biotin-PEG-NH_2 (Huang et al., 2006) to yield the affinity tags **3** and **4**, respectively (Figure 2A). Starting from the same intermediate **7**, this new synthetic route allows us to generate both azide- and alkyne-derivatized azobenzene-based affinity tags more efficiently compared to **1** and **2**. The overall yields for **1** and **2** were 33% (Yang et al., 2010) and 8% (unpublished data), respectively, whereas **3** and **4** were obtained with 49% and 35% over three steps.

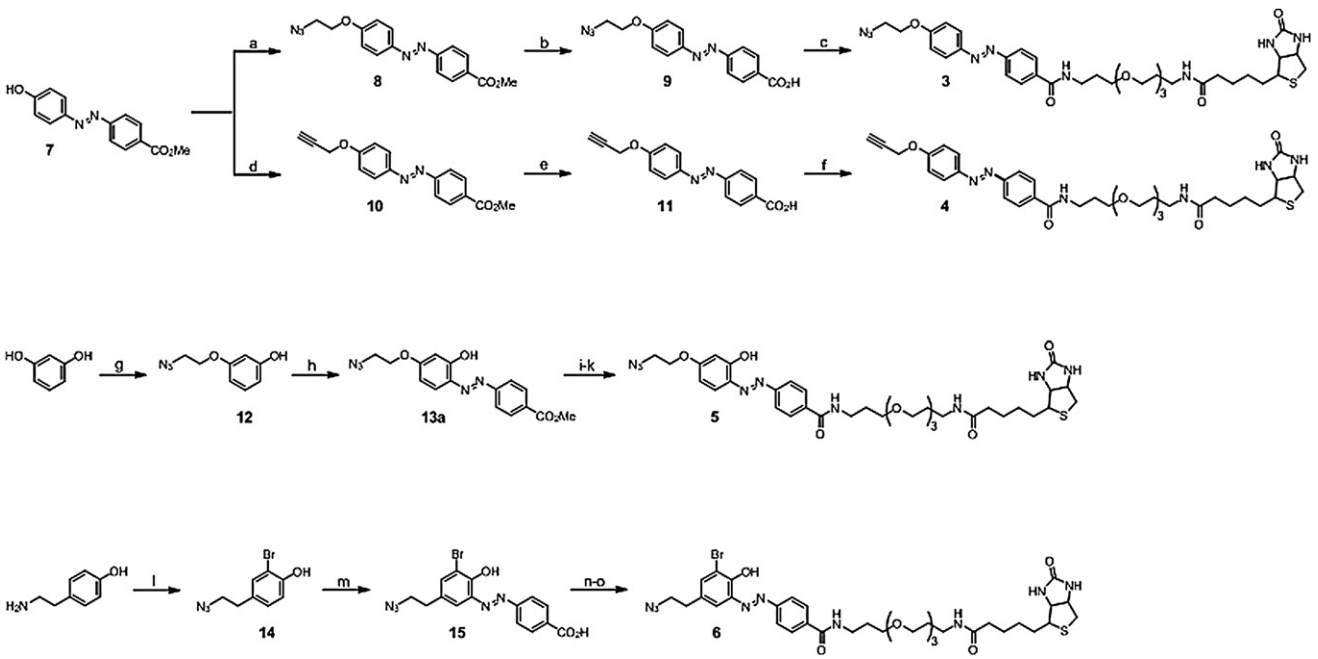

Figure 2. Synthesis of the Second-Generation Azobenzene-Based Cleavable Affinity Tags (3–6)

(A) 2-azidoethyl tosylate, K_2CO_3, DMF, 0°C then rt, 85%; (B) LiOH, THF/H_2O (v/v = 1/1) (pH = 12.0), 90%; (C) i. oxalyl chloride, cat. DMF, CH_2Cl_2; ii. biotin-PEG-NH_2, Et_3N, CH_2Cl_2, 55%; (D) propargyl bromide, K_2CO_3, DMF, rt, 70%; (E) LiOH, THF/H_2O (v/v = 1/1) (pH = 12), 90%; (F) i. oxalyl chloride, cat. DMF, CH_2Cl_2; ii. biotin-PEG-NH_2, Et_3N, CH_2Cl_2, 39%; (G) resorcinol, 2-azidoethyl tosylate, EtOH, $KOH_{(aq)}$, reflux, 60%; (H) i. methyl 4-aminobenzoate, 6 N $HCl_{(aq)}$, $NaNO_2$, 0°C, 15 min; ii. K_2CO_3, THF (pH 8.0), 0°C then rt, 5%; (I) LiOH, THF/H_2O (v/v = 1/1) (pH = 12.0), >95%; (J) *N*-hydroxysuccinimide, DCC, THF, 2.5 hr; (K) biotin-PEG-NH_2, DMF, 4 hr, 65% over 2 steps; (L) Br_2, AcOH, 84%; (M) i. 4-aminobenzoic acid, 6 N $HCl_{(aq)}$, $NaNO_2$, 0°C, 25 min; ii. K_2CO_3, THF (pH 8.0), 0°C then rt, 8%–15%; (N) *N*-hydroxysuccinimide, DCC, THF, 3 h; (O) biotin-PEG-NH_2, DMF, 4 hr, 53% over 2 steps.

See also Figure S4.

We then evaluated the utility of cleavable affinity tags **3** and **4** for capture and enrichment of azide/alkyne-labeled proteins. As an abundant source of azide/alkyne-labeled proteins, 2-amino-octynoic acid (AOA) and azido-norleucine (ANL)-metabolically labeled *S. typhimurium* cell lysates were utilized based on our previous studies with these amino acid chemical reporters (Figure 3) (Grammel et al., 2010). AOA (Link et al., 2006; Tanrikulu et al., 2009) and ANL (Grammel et al., 2010) are methionine (Met) surrogates that can be efficiently utilized by mutant Met tRNA synthetases (MetG) when expressed in bacteria such as *S. typhimurium*, serving as orthogonal chemical reporters of newly synthesized proteins. MetRS-PLL-*S. typhimurium* was labeled with 1 mM Met (negative control), 1 mM AOA, or 1 mM ANL for 3 hr, lysed with 4% sodium dodecyl sulfate (SDS) buffer to give total cell lysates (Grammel et al., 2010). The bacterial cell lysates were then reacted with clickable fluorophores (Charron et al., 2009) via CuAAC to confirm metabolic labeling of bacterial proteins with AOA or ANL, respectively (Figure 3). Indeed, AOA and ANL enable robust labeling of bacterial proteins in MetRS-PLL-*S. typhimurium* as previously described (Figure 3) (Grammel et al., 2010). The clickable and cleavable affinity tags **3** and **4** were then evaluated for CuAAC, streptavidin enrichment, and $Na_2S_2O_4$ elution of AOA- and ANL-labeled proteins using our previously reported conditions (Yang et al., 2010; Yount et al., 2010; Grammel et al., 2010; Rangan et al., 2010). Contrary to azido-azo-biotin **1**, cleavable affinity tags **3** and **4** showed relatively poor protein recovery in $Na_2S_2O_4$ eluants, where most labeled proteins remained bound to the beads that were released after boiling with reducing and denaturing protein-loading buffer (see Figure S1 available online). Comparative analysis of compounds **1** and **3** with *N*-ethyl-6-ethynyl-1,8-naphthalimide (Sawa et al., 2006), a model CuAAC substrate, yielded comparable formation of triazole products (Figure S2), demonstrating that click chemistry reactivity was not responsible for the differences in protein elution yields between the first and second-generation affinity tags.

We analyzed the $Na_2S_2O_4$-cleavage efficiencies of affinity tags **1**, **3**, and **4** by HPLC to determine discrepancies in their reactivity (Figure 4A). As expected, the azobenzene motif of **1** was completely reduced to the corresponding cleaved product after 1 min with 25 mM $Na_2S_2O_4$ in phosphate buffer saline (PBS) at pH 7.4 (Figure 4B). The identical conditions resulted in >95% disappearance of **3** and **4**, emergence of the azobenzene cleaved products, but also the accumulation of intermediates that comprised about 40% of the resulting reaction products (Figures 4C and 4D). These intermediates persisted even after 1 hr (Figures 4C and 4D). MS analysis of these intermediates revealed partially reduced hydrazine products of **3** and **4** (Figures S3B and S3C). Compounds **3** and **4** both afforded two peaks of equal mass by LC-MS analysis, which likely reflect the *cis-trans* isomers of azobenzene moiety, presumably due to the lack of the *ortho*-hydroxyl group on their aromatic rings (Figures 4C and 4D). The slower eluting isomers of both **3** and **4** (isomer 2) appear to react with $Na_2S_2O_4$ more rapidly because the majority of these

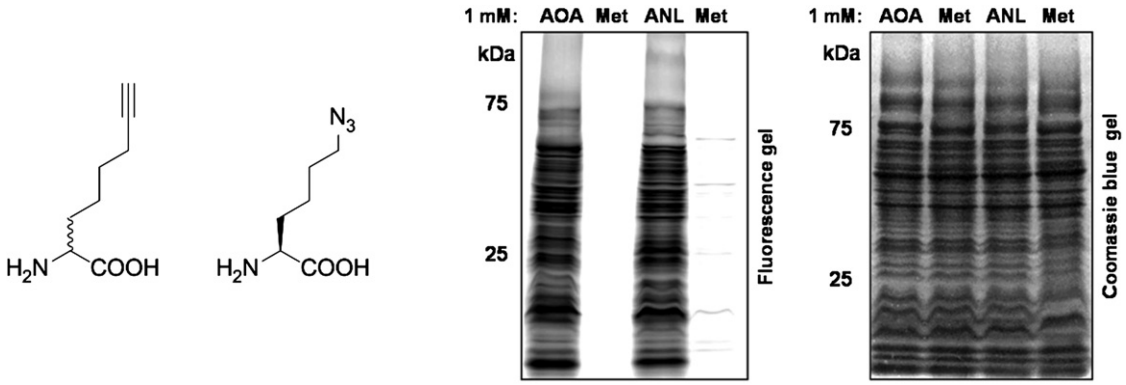

Figure 3. In-Gel Fluorescent Profiling of Met-, AOA-, and ANL-Metabolically Labeled Bacterial Proteomes

MetRS-NLL *S. typhimurium* was labeled with 1 mM Met (negative control), AOA, or ANL for 1 hr. The cell lysates were subjected to CuAAC with azido-rhodamine (Charron et al., 2009) and analyzed by in-gel fluorescence scanning. Coomassie blue gel shows proteins were equally loaded.

compounds are absent after 1 min, whereas the less abundant and faster eluting isomers (isomer 1) still persist (Figures 4C and 4D). After 1 hr, all starting isomers of **3** and **4** were converted into the $Na_2S_2O_4$-cleaved product or partially reduced intermediate. We also examined azobenzene cleavage with 300 mM $Na_2S_2O_4$, which was reported by Hulme and coworkers for a structurally similar azobenzene-functionalized affinity tag (Landi et al., 2010). Although 300 mM $Na_2S_2O_4$ improved the cleavage efficiency of **3** and **4**, 20%–35% of the partially reduced hydrazide products persisted even after 1 hr (Figures 4C and 4D). The different azobenzene reduction efficiencies observed on our first-generation (**1**) and second-generation (**3** and **4**) cleavable affinity tags suggested that the *ortho*-hydroxyl substituent on the aromatic ring of azobenzene might be essential for rapid $Na_2S_2O_4$ reduction.

Synthesis and Evaluation of *ortho*-Hydroxylated Azobenzene-Functionalized Affinity Tag

To determine whether the *ortho*-hydroxyl group of the azobenzene moiety was a key factor for $Na_2S_2O_4$ cleavage, we generated azidoethoxy *ortho*-hydroxyl-azo-biotin affinity tag **5** (Figure 2B). Tag **5** exhibits the same structure as tag **3**, except for an *ortho* hydroxyl group. Compound **5** was synthesized by monoalkylation of resorcinol followed by azo-coupling of **12** with methyl 4-aminobenzoate to afford **13**. Although both the hydroxyl and 2-azidoethoxyl groups of compound **12** can direct azo-coupling to their *ortho* positions to yield three possible regioisomers **13a–c** (Figure S4), after flash column chromatography, we only isolated one product with the correct mass. ^{1}H-NMR analysis of this product excluded the possibility of regioisomer **13c** because it exhibits a different peak-splitting pattern from what we observed (Figure S4). To differentiate regioisomer **13a** from **13b**, compound **13** was subjected to $Na_2S_2O_4$ reduction to yield product **16**, which was then reacted with phenol isothiocyanate followed by cyclization under the treatment with sodium hydroxide and 4-toluenesulfonyl chloride to give compound **17** (Figure S4). Only the 2-aminophenol moiety is capable of forming the compound **17**, confirming that the azo-coupling product was the desired regioisomer **13a** (Figure S4). Compound **13a** was then saponified, converted to *N*-hydroxysuccinimide ester, and coupled with biotin-PEG-NH_2 to give cleavable affinity tag **5**. The cleavage efficiency of tag **5** was evaluated by treatment with 25 mM $Na_2S_2O_4$ (in PBS [pH 7.4]) for 1 min. As shown by HPLC analysis (Figure 4E), the reactivity of **5** toward $Na_2S_2O_4$ is comparable to tag **1**, indicating that the *ortho*-hydroxyl group is essential for efficient azobenzene cleavage.

Comparative Analysis of Protein Enrichment and Elution with Cleavable Affinity Tags

Based on our HPLC analytical results (Figure 4), we reexamined the elution of AOA-labeled proteins, which were clicked to tags **1**, **3**, and **5**, from the streptavidin beads in the gel-based proteomics approach (Figure 1). AOA-labeled *S. typhimurium* cell lysates were reacted with azide-functionalized affinity tags **1**, **3**, and **5** via CuAAC and then precipitated to remove excess "click reagents." Air-dried protein pellets were resuspended in 6 M urea/2 M thiourea/10 mM HEPES (pH 8.0) (Choudhary et al., 2009), instead of previously reported 4% SDS (Yang et al., 2010), because this urea buffer is more compatible with the solubility of membrane proteins (Rabilloud, 1998) and the conditions used for in-solution protein reduction-alkylation and protease digestion (Smejkal et al., 2006). The protein suspensions were then reduced with 1 mM dithiothreitol (DTT), alkylated with 5.5 mM iodoacetamide, and incubated with streptavidin beads. After extensive washing, streptavidin beads-bound **1**- and **5**-tagged proteins were treated with 25 mM $Na_2S_2O_4$ in 1% SDS at pH 7.4. In contrast, beads-bound proteins bearing affinity tag **3** were eluted by 300 mM $Na_2S_2O_4$ in 1% SDS at pH 7.4. Affinity tag **5** exhibited similar protein elution efficiencies as tag **1** (Figure 5). The majority of bound proteins were cleaved from beads within the first 30 min. Notably, SDS was essential for solubilizing proteins and recovering proteins from the beads during $Na_2S_2O_4$ elution (Figures S5A and S5B), even though it is not essential for rapid azobenzene cleavage in vitro (Figure 4). On the other hand, affinity tag **3** still yielded poor protein elution results under the treatment of 300 mM $Na_2S_2O_4$ (Figure 5), which suggests that the *ortho*-hydroxyl group is needed for $Na_2S_2O_4$ elution of proteins from the beads. AOA-metabolically labeled *S. typhimurium* proteins enriched and recovered by using

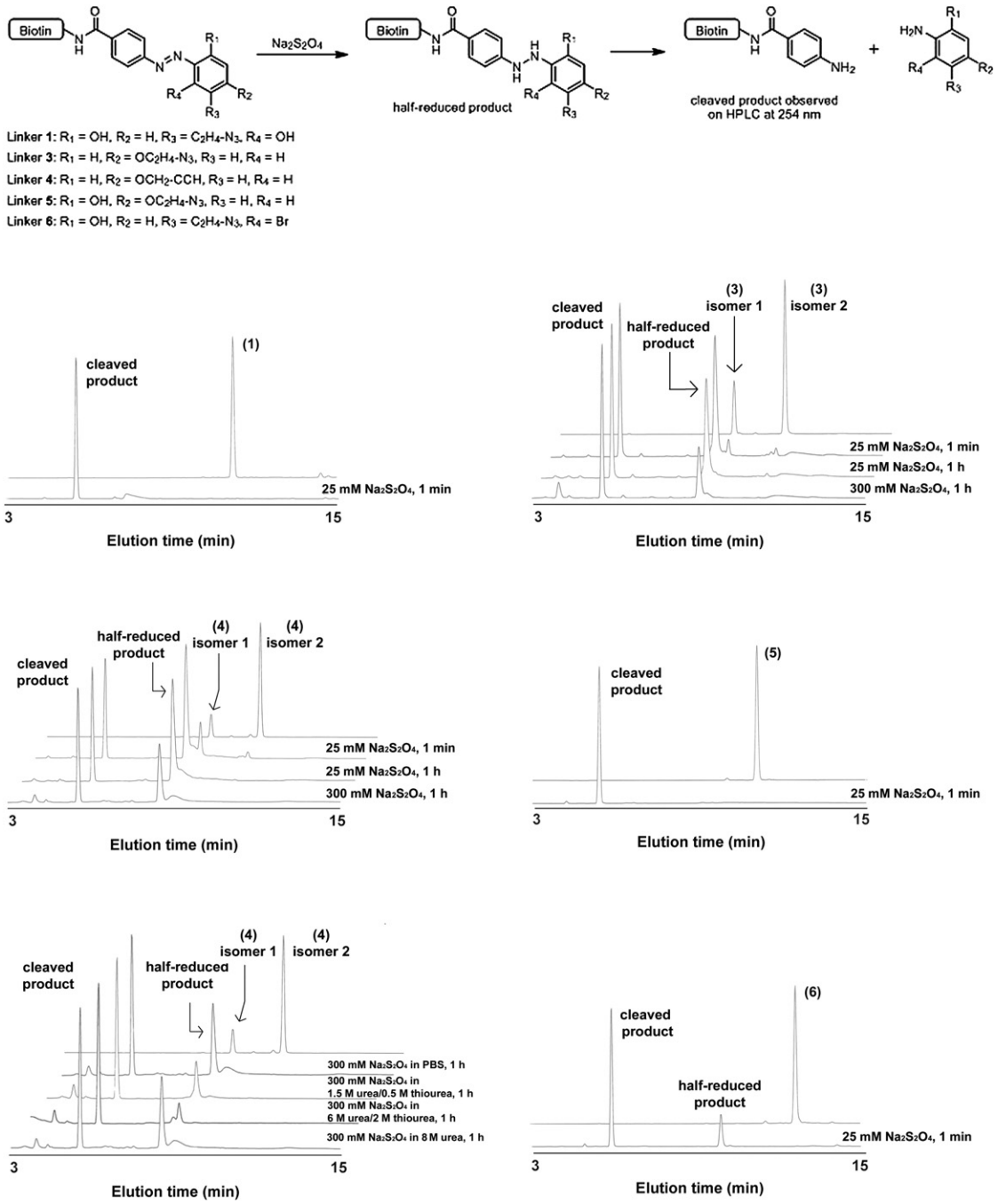

Figure 4. HPLC Analysis of the Azobenzene Reduction Efficiencies for Affinity Tags 1 and 3–6

Each azobenzene-based affinity tag (2 μl from 5 mM stock solution, final concentration = 0.1 mM) was treated with 100 μl of freshly made $Na_2S_2O_4$ (in PBS [pH 7.4]) of the indicated concentrations. At the described time points, the reaction solution was immediately injected into analytical reversed-phase HPLC. HPLC analysis was conducted with H_2O/CH_3CN: 90%/10% to 15%/85% over 20 min.

(A) Schematic of reduction of azobenzene-based affinity tags by $Na_2S_2O_4$.

(B) Azido-azo-biotin (**1**).

(C) Azidoethoxy-azo-biotin (**3**).

(D) Alkynylmethoxy-azo-biotin (**4**).

(E) *ortho*-hydroxyl-azidoethoxy-azo-biotin (**5**).

(F) Treatment of tag **4** with 300 mM $Na_2S_2O_4$ solution of various urea/thiourea concentrations. The cleavage efficiencies enhanced when the amounts of thiourea increased in the cleavage solution. The cleavage efficiency difference between 6 M urea/2 M thiourea and 8 M urea indicates that thiourea plays the major role in cleavage efficiency enhancement.

(G) Bromo-azido-azo-biotin (**6**).

See also Figure S3.

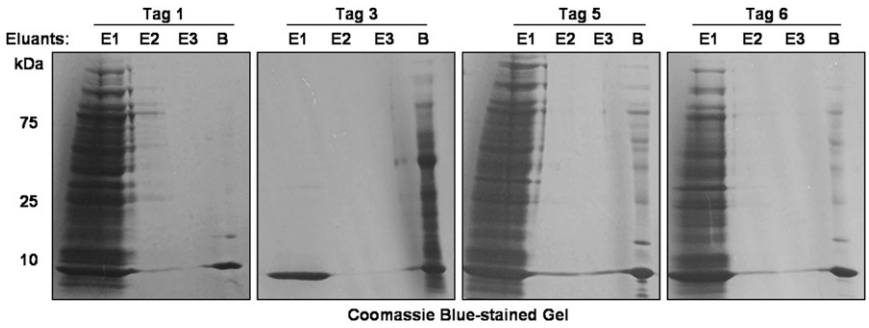

Figure 5. SDS-PAGE Analysis of $Na_2S_2O_4$-Protein Elution Efficiencies of Compounds 1, 3, 5, and 6-Tagged Proteins

Coomassie blue gel images show the elution profiles of metabolically labeled proteins that were reacted with either tag **1**, **3**, **5**, or **6** via CuAAC. Biotinylated proteins were enriched with streptavidin beads from 1.5 mg total cell lysates and then eluted with 25 mM $Na_2S_2O_4$ (in 1% SDS [pH 7.4], for tags **1**, **5**, and **6**) and 300 mM $Na_2S_2O_4$ (in 1% SDS [pH 7.4], for tag **3**). E1, the first elution fraction; E2, the second elution fraction; E3, the third elution fraction; B, the eluant from boiling the streptavidin beads in 4% SDS buffer/10% β-mercaptoethanol/1 × LDS for 10 min. Each elution fraction represents 30 min $Na_2S_2O_4$ treatment. The beads were washed twice between each elution using the cleavage buffer containing no $Na_2S_2O_4$.

See also Figure S5.

cleavable affinity tag **1** were then identified by in-gel proteomic protocol (see Experimental Procedures; Figure S5D) and revealed 456 high-confident protein hits in which each protein contains at least 2-fold of unique peptides over control samples (Table S1). Unfortunately, the sites of AOA-modification within the recovered proteins were not evident by MS/MS analysis. Nonetheless, in comparison to our previous results with cleavable affinity tag **1** (Grammel et al., 2010), a similar number and profile of AOA-labeled *S. typhimurium* proteins were identified here using modified protein-solubilization conditions (Figure 6A; Table S1).

Analysis of Protein Modification Sites of Chemical Reporter with Cleavable Affinity Tags

To characterize the protein modification sites of alkyne- or azide-chemical reporters, we adapted the in-solution digestion approach to our azobenzene-based affinity tags for modified peptide enrichment and elution (Figure 1). For these studies, 10 mg of Met- and AOA-labeled bacterial lysates were reacted with cleavable affinity tag **1** via CuAAC, subjected to protein reduction-alkylation in 6 M urea/2 M thiourea/10 mM HEPES (pH 8.0), and then sequentially digested with endoproteinase Lys-C and trypsin. The resulting digested peptide mixtures were then incubated with streptavidin beads for affinity capture. Because azobenzene-based cleavable tags generally exhibit bright-yellow color, proteins and peptides that are successfully tagged and captured on the streptavidin beads are readily apparent by yellow beads that, after treatment with $Na_2S_2O_4$, become translucent again. After the yellow-colored beads were extensively washed and reacted with 25 mM $Na_2S_2O_4$ (in PBS [pH 7.4]), the peptide eluants were desalted by C8-reversed phase column, lyophilized to dryness, and analyzed by LC-MS/MS using the LTQ-Orbitrap. The search for recovered peptides that contained the AOA-labeled, CuAAC triazole, and $Na_2S_2O_4$ cleaved adduct (M*) of 315 Da revealed 185 unique peptides containing modified amino acid residue in place of Met (Figure 7A; Table S2), which comprised a total of 73 bacterial proteins (Figure 6B; Table S2). We also evaluated alkynyl affinity tag **4** using this protocol with 10 mg of Met- and ANL-labeled bacterial lysates. Surprisingly, 128 unique peptides containing the modified residue in place of Met (Figure 7B; Table S3), which were accounted for a total of 65 MS-identified proteins, were recovered using compound **4** (Figure 6B; Table S4). Because $Na_2S_2O_4$ elution of these AOA-modified peptides was

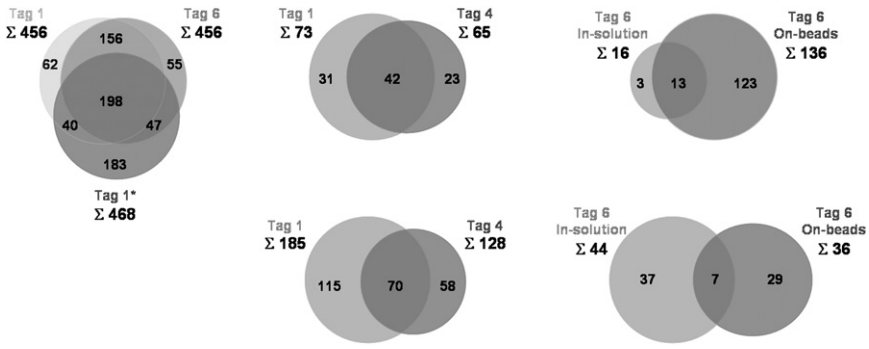

Figure 6. Overview of the Proteomic Analysis of AOA/ANL-Labeled *S. typhimurium* Proteins Using Azobenzene-Based Cleavable Affinity Tags

(A) Comparison of tag **1** and tag **6** in the numbers of their MS-identified AOA-labeled proteins via in-gel digestion approach and the comparison of these data to the previously published data (tag **1***) (Grammel et al., 2010). Starting with 4 mg of 1 mM Met/AOA-metabolically labeled cell lysates, the in-gel digestion approach gave total 456 protein hits for either tag **1** or tag **6**.

(B) Comparison of tag **1** and tag **4** in the numbers of their MS-identified AOA- and ANL-metabolically labeled proteins and unique modified peptides via in-solution digestion approach. Starting with 10 mg of 1 mM Met/AOA-metabolically labeled cell lysates, the in-solution digestion approach using tag **1** yields total 185 unique modified peptides accounted for 73 proteins. Starting with 10 mg of 1 mM Met/ANL-metabolically labeled cell lysates, the in-solution digestion approach using tag **4** yields total 128 unique modified peptides accounted for 65 proteins.

(C) Comparison of on-beads digestion approach and in-solution digestion approach in the numbers of their MS-identified AOA-metabolically labeled proteins and unique modified peptides using **6** as the affinity tag. Starting with 2 mg of 1 mM Met/AOA-metabolically labeled cell lysates, the in-solution digestion approach yields total 44 unique modified peptides accounted for 16 proteins, whereas the on-beads digestion approach yields total 136 proteins as well as 36 unique modified peptides.

See also Figure S5D and Tables S1–S5.

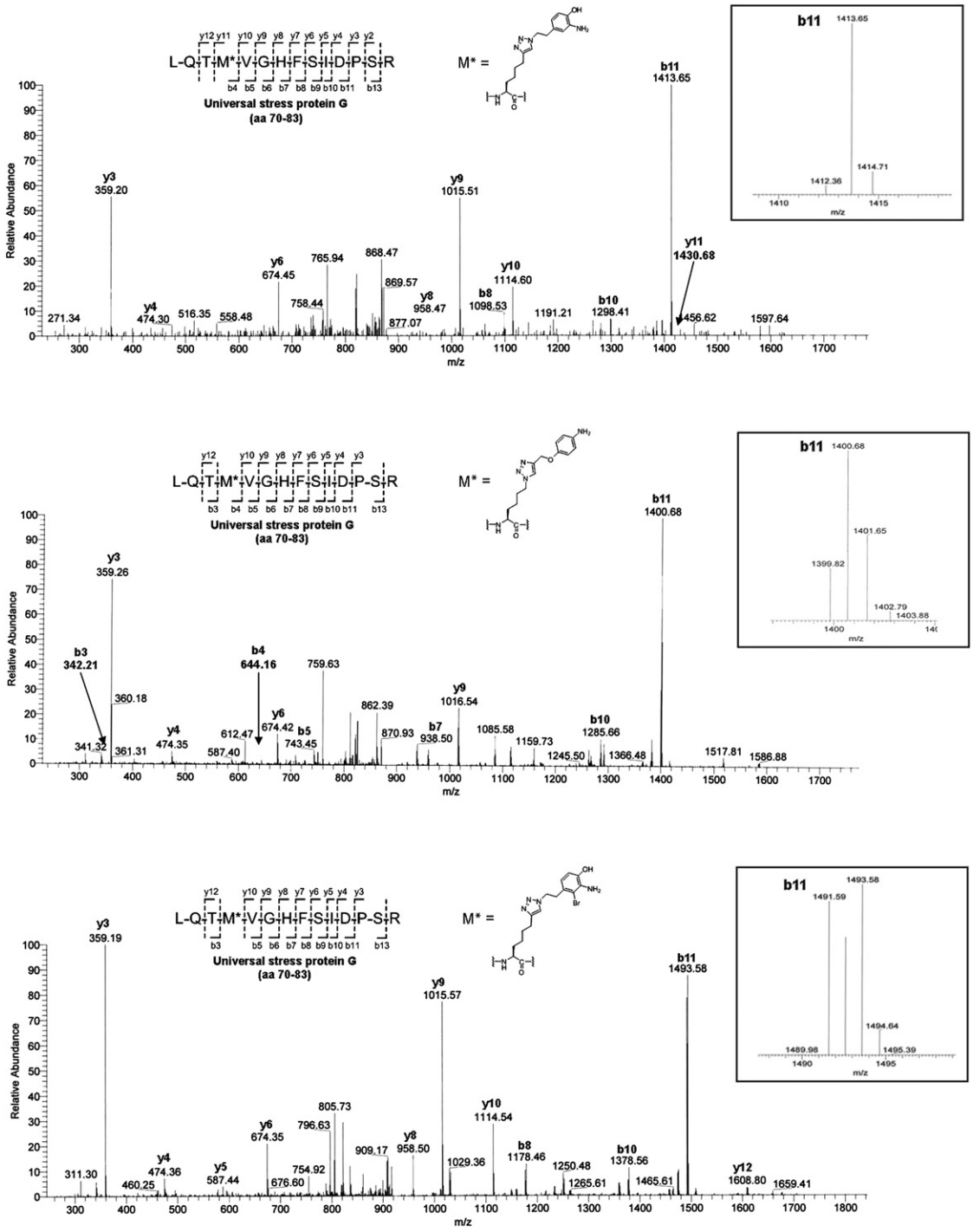

Figure 7. Selected MS/MS Spectra of the Modified Universal Stress Protein G Peptides (aa 70–83)

AOA/ANL-metabolically modified proteins were clicked to tag **1**, **4**, or **6** via CuAAC and then processed via in-solution proteomic strategy. Selected MS/MS spectra were derived from the results of MS analysis of those streptavidin-enriched and $Na_2S_2O_4$-eluted modified peptides. (A) MS/MS spectrum of the modified peptide LQTM*VGHFSIDPSR (M* = AOA + tag **1**/CuAAC/$Na_2S_2O_4$-cleavage adduct, the molecular weight of M* = 315). (B) MS/MS spectrum of the modified peptide LQTM*VGHFSIDPSR (M* = ANL + tag **4**/CuAAC/$Na_2S_2O_4$-cleavage adduct, the molecular weight of M* = 301). (C) MS/MS spectrum of the modified peptide LQTM*VGHFSIDPSR (M* = AOA + tag **6**/CuAAC/$Na_2S_2O_4$-cleavage adduct, the molecular weight of M* = 393).
See also Tables S2–S4.

conducted in PBS, this result led us to analyze whether the azobenzene cleavage efficiencies could be affected by detergents, such as SDS, that were used in the on-bead protein elution experiments by HPLC. Given the incompatibility of the detergents with HPLC, we thus tested the effects of chaotropic agents on the azobenzene cleavage instead. Compound **1** showed slightly decreased $Na_2S_2O_4$ reactivity when 6 M urea/2 M thiourea was included in the reaction buffer (Figures S6A and S6B). However, the azobenzene reduction yields for both compounds **3** and **4** were improved to ~90% when 2 M thiourea was added to 300 mM $Na_2S_2O_4$ (Figures 4F; Figure S6C). These unexpected results led us to revisit the on-bead protein elution of compound **3**-tagged proteins using 300 mM $Na_2S_2O_4$ (in 6 M urea/2 M thiourea/10 mM HEPES [pH 8.0]). Despite the improved cleavage efficiency observed on HPLC (Figure S6A), compound **3** still yielded poor protein recovery even in the presence of 2 M thiourea (Figure S5C). Overall, the azobenzene cleavable affinity tags carrying *ortho*-hydroxyl groups (**1** and **5**) would allow the most efficient protein identification and mapping of chemical reporter modification sites.

Synthesis and Evaluation of Clickable and Cleavable Brominated Azobenzene Affinity Tag

Given the utility of *ortho*-hydroxylated azobenzene affinity tags for bioorthogonal chemical proteomic studies, we also synthesized an isotopically encoded cleavable affinity tag **6** bearing a bromine atom. Naturally occurring bromine has two major isotopes of similar abundance that can facilitate protein identification and analysis of chemical reporter modification sites by MS. The brominated azobenzene-functionalized affinity tag **6** was synthesized as described for compound **1**, but starting with 2-bromo-4-(2-azidoethyl)-phenol **14** (Figure 3C). The cleavage efficiency of **6** in the presence of 25 mM $Na_2S_2O_4$ in PBS (pH 7.4) was then evaluated by HPLC. Within 1 min, the majority of **6** were reduced to the corresponding cleaved products, whereas ~10% remained partially reduced. (Figure 4G). Although bromine can serve as a deactivating substituent on the benzene ring that might contribute to the observed slightly slower $Na_2S_2O_4$-reduction efficiency of **6** on HPLC (Figure 4G), cleavable affinity tag **6** exhibited similar performance as tag **1** in CuAAC, streptavidin enrichment, and $Na_2S_2O_4$ elution of AOA-labeled proteins (Figures 5; Figure S5D). In addition, gel-based bioorthogonal chemical proteomics using affinity tag **6** gave total 456 MS-identified AOA-labeled *S. typhimurium* proteins with high confidence, 77% of which overlap with those proteins recovered by using affinity tag **1** (Figure 7A; Table S1).

We also carried out site of modification studies with cleavable affinity tag **6**. AOA-labeled *S. typhimurium* lysates (2 mg) were reacted with **6** via CuAAC, subjected to the in-solution protease digestion and peptide enrichment protocol. LC-MS/MS analysis of those recovered peptides identified 44 unique peptides accounted for 16 proteins which contain the modified residue in place of Met (Table S4; Figure 6C). As shown in Figure 7C, any peptide ion fragment that bears modified residue (y12, b5-11, and b12) shows a characteristic isotopic pattern of bromine. In addition to gel-based and in-solution proteomic approaches (Figure 1), we also evaluated cleavable affinity tag **6** using on-beads digestion approach. AOA-labeled *S. typhimurium* lysates (2 mg) were subjected to CuAAC with **6**, followed by protein reduction, alkylation, and enrichment on the streptavidin beads. After extensive washing, the streptavidin beads-bound proteins were sequentially treated with endoproteinase Lys-C and trypsin (Experimental Procedures). The protease-digested peptides were then collected, extracted by C8 columns, and concentrated for LC-MS/MS analysis. The remaining bound peptides on streptavidin beads were treated with 25 mM $Na_2S_2O_4$ in PBS (pH 7.4) to elute azobenzene-linked AOA-labeled peptides for LC-MS/MS analysis. Using this protocol, we identified 136 *S. typhimurium* proteins together with 36 unique peptides containing the modified residue in place of Met (Table S5; Figure 6C). All the proteins that were identified in $Na_2S_2O_4$ eluants were also presented in protease-digested peptide fractions (Table S5B). These results demonstrated that isotopically encoded cleavable affinity tag **6** allows robust protein identification and mapping of chemical reporter modification sites using different platforms for proteomics.

DISCUSSION

Azobenzene-functionalized probes have been utilized for diverse chemical biology applications, ranging from photoinduced protein regulation (Banghart et al., 2004; Sadovski et al., 2009; Schierling et al., 2010; Fortin et al., 2008), specific recovery of proteins targeted by activity-based probes (Fonović et al., 2007), chemical reporters (Yang et al., 2010; Yount et al., 2010; Grammel et al., 2010; Rangan et al., 2010), as well as non-covalent small molecule ligands (Landi et al., 2010; Budin et al., 2010). Here, we demonstrate that the clickable azobenzene-based affinity tags enable robust proteomic analysis and site mapping of proteins targeted by chemical reporters. These biotinylated azobenzene-based affinity tags are compatible with CuAAC conditions, allow the robust capture of protein/peptides using streptavidin matrices, and enable efficient elution of selectively recovered proteins/peptides for proteomics. We show here that the reactivity of azobenzene toward $Na_2S_2O_4$ was governed by the substituents on the aromatic ring. A strong electron-donating group, like -OH on *ortho* position, accelerates the $Na_2S_2O_4$ cleavage, whereas the presence of the weak electron-withdrawing group, like bromine on *ortho* position, slightly impedes this cleavage efficiency. These results are consistent with recent structure-reactivity studies of azobenzene derivatives by Wagner and coworkers (Leriche et al., 2010). Nevertheless, it is worth mentioning that the *ortho*-hydroxyl group may not impose its effect on azobenzene-bond reactivity toward $Na_2S_2O_4$ merely through mesomeric effect. As indicated in our studies and Wagner and coworkers (Leriche et al., 2010), *para*-alkoxylated azobenzene (**3** and **4** in this study) did not possess better cleavage efficiencies, suggesting that the *ortho*-hydroxyl group might accelerate the azobenzene-bond cleavage through other mechanisms such as intramolecular hydrogen bonding with nitrogen atom of azobenzene (Kuvshinova et al., 2006). Further detailed investigation on $Na_2S_2O_4$-mediated azobenzene cleavage mechanism and structure-and-activity studies would help to reveal the roles of the *ortho*-hydroxyl group in this reaction.

Using bacterial cell lysates that were metabolically labeled with amino acid chemical reporters (AOA or ANL), we

demonstrated that these cleavable affinity tags (**1**, and **3–6**) could be used for proteomic profiling of the newly synthesized proteins in *S. typhimurium*. These cleavable affinity tags are compatible with diverse platforms for proteomics that also allows mapping sites of protein modifications (Figure 1). The advantage of on-bead protease digestion is that it allows modified peptides to be analyzed separately from non-modified peptides, thus improving protein coverage and detection sensitivity during MS analysis. Notably, the triazole adducts from click chemistry/$Na_2S_2O_4$ cleavage were relatively small and not readily fragmented during MS/MS experiments, which were useful for mapping sites of AOA/ANL-modification (Figure 7). Our MS/MS analysis of selectively enriched peptides revealed that AOA/ANL substituted for Met residues in all peptide sequences (Figure 7). These results confirm that both AOA and ANL are indeed Met surrogates and can be efficiently used by mutant $MetG_{PLL}$ overexpressed in *S. typhimurium* (Grammel et al., 2010). Although *ortho*-hydroxylated-azobenzene cleavable affinity tags (**1**, **2**, **5**, and **6**) are effective for bioorthogonal chemical proteomic applications with alkyne-modified proteins/peptides, the *ortho*-hydroxylated derivative of tag **4** should be useful for proteomic studies with azide-modified substrates. In addition the characteristic isotopic pattern of brominated cleavable affinity tag **6** should also aid in distinguishing the modification-bearing peptide ions from other ion peaks. These reagents provide useful tools to circumvent the high-affinity binding of biotin to streptavidin reagents for protein enrichment that are complementary to other enzymatic or chemical cleavage methods (Speers and Cravatt, 2005; Dieterich et al., 2006; Nessen et al., 2009; Park et al., 2009; Dirksen et al., 2010).

SIGNIFICANCE

Chemical probes are affording new opportunities to investigate protein and enzyme regulation. Central to these new technologies is the development of bioorthogonal ligation methods for exploring small molecule-protein interactions using specific probes of enzymes or reporters of protein modifications (Sletten and Bertozzi, 2009; Simon and Cravatt, 2010). The identification of the proteins that are targeted by small molecule probes/reporters is essential for these functional studies. We present here the synthesis and characterization of selectively cleavable affinity tags for versatile and robust bioorthogonal chemical proteomic studies. The azide/alkyne-functionalized *ortho*-hydroxylated-azobenzene cleavable affinity tags enabled selective enrichment and efficient elution of alkyne- and azide-labeled proteins/peptides from complex mixtures and allowed protein identification and modification-site mapping using diverse proteomic platforms. The advances presented here should facilitate bioorthogonal chemical proteomics studies in diverse biological settings.

EXPERIMENTAL PROCEDURES

Detailed characterization of compound **3–17**, the metabolic-labeling procedure, and the mass spectrometric analysis method are in Supplemental Experimental Procedures.

CuAAC of Cleavable Affinity Tags with Met, AOA, or ANL-Labeled Cell Lysates

Met, AOA, or ANL-labeled cell lysates (1–10 mg) were diluted into 4% SDS buffer (4% SDS, 150 mM NaCl, and 50 mM triethanolamine [pH 7.4]) to give 1mg/ml final protein concentration. Protein mixture was subjected to Cu(I)-catalyzed cycloaddition reaction by adding premixed "click chemistry cocktail" (100 μM cleavable biotin linker, 1 mM TCEP, 100 μM (Tris[(1-benzyl-1H-1, 2, 3-triazole-4-yl)methyl] amine [TBTA]; Chan et al., {2004}; and 1 mM $CuSO_4$). The reaction was allowed to sit at room temperature for 1.5 hr. Proteins were then precipitated by adding 10 volumes of chilled MeOH, followed by overnight precipitation at −20°C. Precipitated proteins were pelleted by centrifugation (5200 × g, 30 min, 4°C), washed trice with chilled MeOH, and air dried.

In-Gel Trypsin Digestion of AOA-Labeled Proteins for Mass Spectrometric Analysis

Air-dried protein pellets were resuspended in 6 M urea/2 M thiourea/10 mM HEPES (pH 8.0) (Choudhary et al., 2009). Proteins were reduced with 1 mM DTT (100 mM stock) for 40 min and then alkylated with 5.5 mM iodoacetamide (550 mM stock) in the dark. Prewashed streptavidin beads were added and incubated with protein solution at room temperature for 1.5 hr on end-over-end rotator. The beads were sequentially washed trice with 6 M urea/2 M thiourea/10 mM HEPES (pH 8.0), GIBCO's PBS, and 1% SDS (in 1 × GIBCO's PBS). Centrifugation of the beads between washing steps was carried out (2000 × g, 3 min). Bound proteins were cleaved from the beads by treating with the elution buffer (1% SDS, 25 mM $Na_2S_2O_4$, 1 × GIBCO's PBS) for 30 min trice. Collect and combine the eluants. Removal of the majority of $Na_2S_2O_4$ from the eluants was achieved by applying the eluants onto the Microcon centrifugal filter device (3 kDa NMWL, Millipore) and exchanging the buffer with PBS (the majority of SDS will still retain on the top of the membrane). The concentrated protein mixture was then dried in SpeedVac. Resolubilize the dried protein pellet with 1 × LDS/5% β-mercaptoethanol. Resuspended protein mixtures were boiled at 95°C for 5 min and then loaded onto SDS-PAGE gel (4%–20% Tris-HCl Criterion precast gel, Bio-Rad Laboratories). The profiles of AOA-metabolically labeled proteins together with their negative controls were visualized by Coomassie blue staining. Each lane was sliced into eight fractions and then each excised gel slice was further cut into more pieces. Gel pieces were washed with 50 mM ammonium bicarbonate (ABC) twice, destained with 50 mM ABC/acetonitrile (50/50) twice, and then dehydrated in 100% acetonitrile. After removing acetonitrile in SpeedVac, gel pieces were rehydrated with trypsin solution (2 μg of trypsin for each vial/gel slice) and incubated in 37°C water bath for 18 hr. The eluted trypsin-digested peptides were then collected and dried in SpeedVac. Resolubilize the dried peptides in H_2O (with 0.1% TFA), and submit the samples to nano-HPLC/MS/MS analysis (Thermo LTQ-Orbitrap in the Proteomic Resource Center at Rockefeller University).

For comparative SDS-PAGE analysis of different affinity tags (Figure 5), after each $Na_2S_2O_4$ elution, the beads were washed twice with the elution buffer (without sodium dithionite). The eluant from each cleavage was filtered through Pierce centrifuge column to remove any remaining beads, buffer exchanged on the Microcon centrifugal filter device to reduce the amount of $Na_2S_2O_4$, and then lyophilized to dryness in SpeedVac. After resolubilizing the dried protein mixture in 1 × LDS/5% β-mercaptoethanol, the protein mixtures were boiled at 95°C for 5 min and then loaded onto SDS-PAGE gel. On the other hand, after three times of $Na_2S_2O_4$ elution, the beads were boiled in 4% SDS buffer/10% β-mercaptoethanol/1 × LDS for 10 min to elute the remaining un-cleaved proteins.

In-Solution Protease Digestion of AOA/ANL-Labeled Proteins for MS Analysis

Air-dried pellets were resuspended in 6 M urea/2 M thiourea/10 mM HEPES (pH 8.0) (Choudhary et al., 2009). Proteins were reduced with 1 mM DTT (100 mM stock) for 40 min and then alkylated with 5.5 mM iodoacetamide (550 mM stock) for 30 min in the dark. Reduce urea concentration to 4.5 M urea/1.5 thiourea by adding one-third volume of 10 mM HPEPS (pH 8.0). Lysyl endopeptidase (Lys-C, w/w = 1/100) was added, and protein digestion was carried out at room temperature for 4 hr. Urea concentration was further reduced by adding 3 volumes of 10 mM HEPES (pH 8.0) to allow trypsin

digestion (w/w = 1/100) being carried out at room temperature for 18 hr. The resulting protease-digested peptides were then incubated with prewashed streptavidin beads at room temperature for 1.5 hr on end-over-end rotator. The beads were sequentially washed trice with 1.5 M urea/10 mM HEPES (pH 8.0) and 10 mM HEPES (pH 8.0). Bound peptides were cleaved from the beads by treating with freshly made elution buffer (25 mM sodium dithionite, 1 × GIBCO's PBS) for 1 hr. Repeat this cleavage step by treating the beads with freshly made elution buffer for 10 min twice. Collect and combine the eluants. The cleaved peptides were cleaned up by using C8 cartridge (Waters) and eluted with 70% CH_3CN/20% H_2O/0.1% TFA. Eluted peptides were dried in SpeedVac and then resolubilized in H_2O (with 0.1% TFA) for nano-HPLC/MS/MS analysis.

On-Bead Protease Digestion of AOA-Labeled Proteins for MS Analysis

Air-dried pellets were resuspended in 6 M urea/2 M thiourea/10 mM HEPES (pH 8.0) (Choudhary et al., 2009). Proteins were reduced with 1 mM DTT (100 mM stock) for 40 min and then alkylated with 5.5 mM iodoacetamide (550 mM stock) in the dark for 30 min. Prewashed streptavidin beads were added and incubated with protein mixtures at room temperature for 1.5 hr on end-over-end rotator. The beads were sequentially washed trice with 6 M urea/2 M thiourea/10 mM HEPES (pH 8.0), 0.2% SDS (in PBS), and PBS. The beads were then resuspended in 4.5 M urea/1.5 M thiourea/10 mM HEPES (pH 8.0) and incubated with Lys-C (w/w = 1/100) at room temperature for 4 hr. Urea concentration was further diluted by adding 3 volumes of 10 mM HEPES (pH 8.0) to enable trypsin digestion (w/w = 1/100) being carried out at room temperature for 18 hr. The beads were then spun down at 2000 × g for 1 min. Supernatant (tryptic solution) was collected, and the beads were subjected to $Na_2S_2O_4$ cleavage. Both the protease-digestion fraction and eluant collected from $Na_2S_2O_4$ cleavage were cleaned up using C8 cartridge, eluted by 70% CH_3CN/20% H_2O/0.1% TFA, and dried in SpeedVac for mass spectrometric analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and five tables and can be found with this article online at doi:10.1016/j.chembiol.2010.09.012.

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