Chemistry & Biology
Article

Useful Tools for Biomolecule Isolation, Detection, and Identification: Acylhydrazone-Based Cleavable Linkers

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DOI 10.1016/j.chembiol.2009.06.005

SUMMARY

Proteomic searches using affinity-based chromatography (e.g., biotin-[strept]avidin) have been severely hampered by low protein recovery yields, protein destruction and denaturation, and the release of background proteins from the support. These limitations confound protein identification. A new acylhydrazone-based cleavable linker was developed to permit the efficient isolation of proteins with a traceable tag allowing detection and identification under mild conditions. The utility of the acylhydrazone linker was validated in a proteomic search wherein aldehyde dehydrogenase-1 was selectively captured and isolated from the mouse soluble liver proteome without interfering background proteins. The use of acylhydrazone linkers is expected to be generalized, allowing for the selective release of tagged molecules from noncovalent and covalently tagged supports.

INTRODUCTION

In recent years, significant progress has been made in the identification of protein-ligand, protein-drug, and protein-protein interactions (Berggard et al., 2007; Walsh and Chang, 2006). Nonetheless, the complexity of the proteome, the low abundance of many proteins, and the temporal and spatial distribution of proteins make protein target identification difficult. However, new methods have been advanced to identify proteins involved in biochemical and regulatory pathways. Common to many of these is the selective capture of the tagged protein of interest from the biological milieu using affinity-based chromatography (Scriba, 2004). Because the biotin-avidin association is among the tightest noncovalent interactions known (K_A = 10^{14}–10^{15} M^{-1}) (Green, 1990), biotinylated tags and (strept)avidin supports have been extensively used in affinity-based chromatographic strategies (Wilchek and Bayer, 1990). This method's utility has been diminished by the very inefficient release of the intact biotinylated protein from the streptavidin support under mild conditions (Holmberg et al., 2005; Marie et al., 1990), a situation that makes it difficult to identify low-abundance proteins.

Several methods have been advanced to address the difficulty of the biotinylated product release step. They include using either biotin analogs (Hirsch et al., 2002; Zeheb and Orr, 1986) or protein-engineered streptavidin mutants (Howarth et al., 2006; Malmstadt et al., 2003; Morag et al., 1996; Wu and Wong, 2005), structural modifications that weaken the biotin-(strept)avidin interaction. Although these methods improve the release of biotinylated molecules, the reduced association constant for the complex does not allow the application of stringent wash conditions to remove nonspecific protein interactions from the resin and adversely affects the capture efficiency of the biotin-tagged adducts. Another approach has been to use conditions that disrupt the biotin-(strept)avidin complex (Holmberg et al., 2005; Jenne and Famulok, 1999; Tong and Smith, 1992). Unfortunately, several of these conditions (e.g., 1% aqueous SDS/heat) are harsh and generally lead to the release of abundant proteins that nonspecifically bind to the matrix or streptavidin. Furthermore, the recovery yields of the biotinylated materials are still low and the captured molecules are often either destructed or denatured. A third strategy has been to use biotinylated agents that incorporate a cleavable site between the biotin and the bound (bio)molecule. Examples include linkers that contain a proteolytic (Dieterich et al., 2007; Speers and Cravatt, 2005) or a chemically labile (e.g., disulfide (Finn et al., 1985; Marie et al., 1990; Shimkus et al., 1985), acid-sensitive (van der Veken et al., 2005), base-sensitive (Ball and Mascagni, 1997; Kazmierski and McDermed, 1995), nucleophile-sensitive (Lin and Morton, 1991), photolytic (Bai et al., 2004; Thiele and Fahrenholz, 1994), reductive (Verhelst et al., 2007) site.

We looked for an easy-to-prepare, cleavable linker that would, under mild conditions, efficiently release the (bio)molecules tethered to a support and for which destruction and/or denaturation of the (bio)molecules would be minimal. Ideally, the cleavage reaction would allow the incorporation of a traceable tag (i.e., isotopic, radioactive, fluorescent) that permits the detection and identification of the captured (bio)molecule by chromatographic and spectroscopic methods when it is released.

In this report, we describe our test system, the biotin/streptavidin complex in which the protein of interest is attached to the biotin by an acylhydrazone cleavable linker (Figure 1). Acylhydrazones have been used extensively in drug-delivery systems

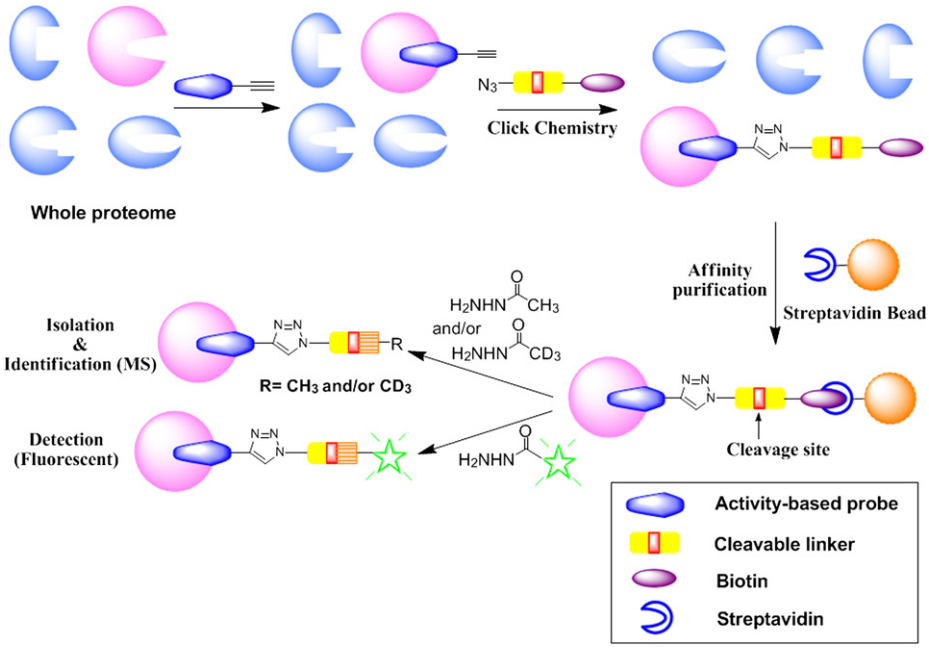

Figure 1. General Strategy of Cleavable Linker for Isolation, Identification and Detection of Target Proteins

(Sawant et al., 2006; Kale and Torchilin, 2007), in monitoring conjugation reactions (Flinn et al., 2004), and as structural units in the construction of dynamic combinatorial libraries (Goral et al., 2001). Studies show that in most instances, acylhydrazones are stable under near-neutral to basic pH conditions (pH ~8–10), but they undergo both hydrolysis (Flinn et al., 2004; Smith et al., 2007) and hydrazone exchange with hydrazides (King et al., 1986) in moderately acidic solutions (pH ~4–5). Herein, we show that biotinylated probes with an imbedded acylhydrazone unit are readily cleaved in high yields at near-neutral pH values with acylhydrazides that contain a traceable tag providing (bio)molecules that can be identified in proteomic searches by conventional analytical techniques. We expect that incorporation of an acylhydrazone unit within a linker will be generalized, allowing for the selective release of tagged molecules from noncovalently or covalently bound supports.

RESULTS

Design and Chemical Properties of Cleavable Linker

Compound **1** was selected and synthesized as our test linker (Figure 2). It contained an acylhydrazone cleavage site, a biotin

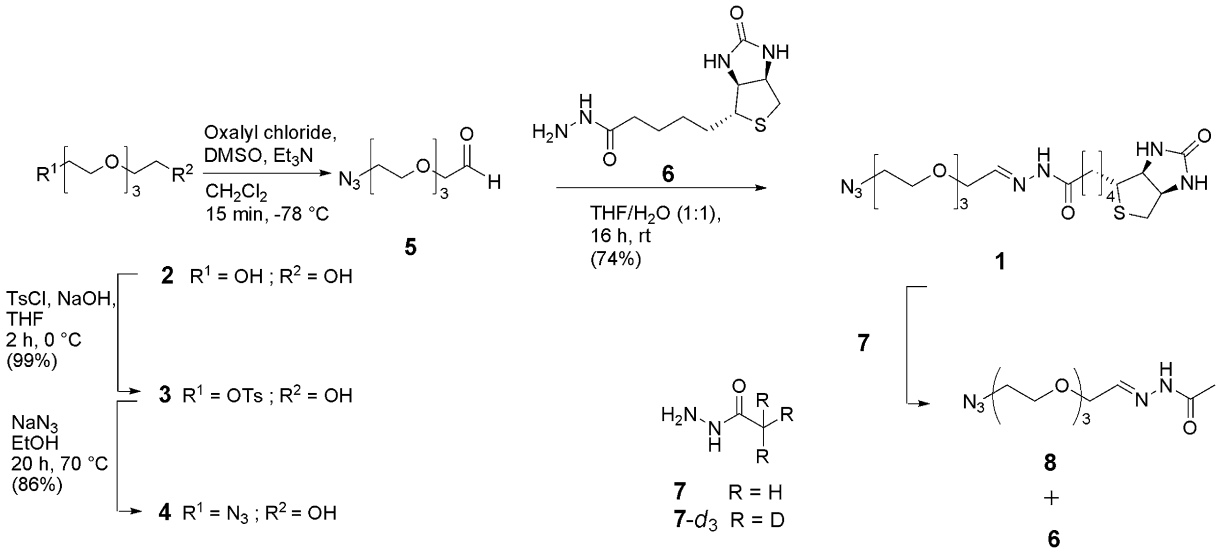

Figure 2. Synthesis of Acylhydrazone Linker 1 and Exchange of Hydrazone Linkage in 1 with Acethydrazide (7)

unit, a terminal azide on **1** to permit capture with an alkyne-modified protein using a copper(I)-mediated cycloaddition reaction ("click chemistry") (Agard et al., 2006; Dieterich et al., 2007; MacKinnon et al., 2007; Rostovtsev et al., 2002; Speers et al., 2003; Tornoe et al., 2002), and a polyethylene glycol linker to increase its water solubility and to minimize adverse steric interactions with the immobilized streptavidin during protein capture (Marie et al., 1990). The success of **1** to capture biomolecules hinged upon its ability to undergo Cu(I)-mediated cycloaddition without disrupting the acylhydrazone linkage, its stability in aqueous solutions, and its ability to undergo cleavage with acylhydrazides. We first showed that the acylhydrazone linkage in **1** was stable to Cu(I)-mediated cycloaddition conditions (see Figure S1 available online), and remained intact at pH 7.4 and above but at pH 5.8 underwent consumption (38%) at 22°C (2 hr) (Table S1). Significantly, **1** efficiently reacted with acethydrazide (**7**) (20–100 Eq) at 22°C to give **8** (Figure 2). For example, at pH 5.2 and 6.2, the reaction was essentially complete within 1 hr at 22°C, using as little as 20 Eq **7** (high-performance liquid chromatography [HPLC] analysis) (Table S2). The ease of this imine exchange was markedly faster than that reported by King and coworkers for a similar transformation (King et al., 1986). Next, we examined the stability of the acylhydrazone linker in the presence of detergents (SDS, NP-40, Triton X-100) because many proteomic protocols employ these agents to facilitate protein solubilization. HPLC analysis showed that when SDS (0.5% and 1%) was included in the buffered solutions (pH 7.4) there was a rapid loss of **1**, whereas similar losses were not observed with the detergents NP-40 (1%) and Triton X-100 (1%) (Table S3).

Use of 1 for Protein Capture, Release, and Detection

To evaluate the utility of **1** for protein capture and release, we prepared bovine serum albumin (BSA) sample **9** (proposed general structure of the photoadduct; the structure of the azide photoadduct has not been determined) (Figure 3A) after photocrosslinking with photoactivable lacosamide probe followed by click chemistry with **1** (Figure S2). The BSA sample **9** was captured by streptavidin-agarose beads and divided into equal aliquots. Each portion was either treated with or without **7** (50, 200 mM) at different pH values (5.8, 7.4) to release the captured proteins. We also tested whether either *p*-anisidine or aniline would facilitate acylhydrazone cleavage because these aryl amines have been reported to catalyze oxime ligation, hydrazone formation, and transimination (acylhydrazone exchange) at moderate pH values (Dirksen et al., 2006a, 2006b). Consistent with our HPLC findings (Tables S1 and S2), we observed increased linker cleavage at pH 5.8 compared with at pH 7.4, upon the addition of **7**, and upon the inclusion of either *p*-anisidine or aniline with **7** (Figure S3). We then examined the effect of 20 mM SDS (0.5%) on the cleavage reaction of **9** in aqueous pH 5.8 solutions at 22°C. In agreement with our HPLC studies (Table S3), we found that 20 mM SDS facilitated linker cleavage, and that inclusion of both SDS and **7** provided slightly enhanced levels of cleavage than with either one alone (Figure S4).

To examine the recovery efficiency of protein capture and release of our method, we quantified the percentage yield of the immobilization, flow-through/wash, and elution steps using model protein **9**. The release efficiency could be readily determined from the ratio of the amount eluted under mild cleavage conditions to that captured by streptavidin prior to cleavage. Significantly, more than 85% of the protein captured by streptavidin-agarose beads was released with **7** using mild conditions (**7** [100 mM], SDS [20 mM] in aqueous 50 mM HEPES [pH 5.8] [50°C, 1 hr]). Under harsh cleavage conditions (SDS loading buffer [2% SDS, 10% glycerol, 1% mercaptoethanol, 0.01% bromophenol blue] [95°C, 5 min]), protein recovery was near-quantitative (see Supplemental Experimental Procedures). These harsh conditions are expected to completely cleave the acyl hydrazone bond.

Because SDS can affect protein structure, function, and mass spectrometric identification, we identified conditions for the protein recovery step that excluded SDS as well as those that contained 20 mM SDS (0.5%) (Figure S5). We found that **7**-mediated cleavage of streptavidin bound **9** led to high protein recovery rates after 4 hr in pH 5.8 and pH 7.4 (37°C) solutions without 20 mM SDS. Specifically, we observed that at pH 5.8, inclusion of *p*-anisidine in the reaction solution led to efficient cleavage (~97%) at 37°C after 4 hr. Correspondingly, high protein recovery rates (~93% to ~98%) were also obtained at 50°C after 1 hr in acidic solutions (pH 4.2 and 5.8) upon the inclusion of 20 mM SDS with **7**. Reducing the temperature from 50°C

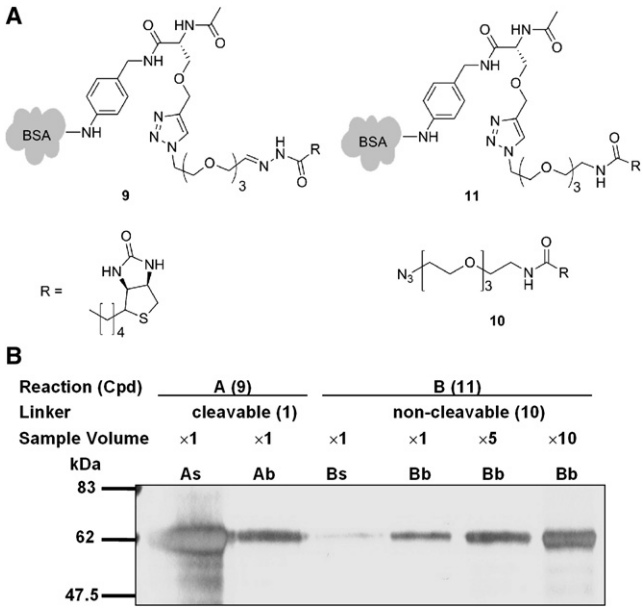

Figure 3. Comparison of the Efficiency of Protein Release from BSA Adducts (9 and 11) Modified with Either Cleavable Linker 1 or Non-cleavable Linker 10

(A) Chemical structure of noncleavable linker **10** and two different BSA samples (**9** and **11**) modified with either cleavable linker **1** or noncleavable linker **10**.

(B) All reactions were run in aqueous 50 mM HEPES (pH 5.8) using optimized condition (**7** [10 mM] and SDS [20 mM] at 50°C [1 hr]) (Figure S5B). Lanes marked "s" correspond to the supernatant removed from the bead mixture after the initial treatment, whereas the lanes marked "b" refer to the sample obtained after the beads were washed and then treated with loading buffer and heated at 95°C for either 5 min (Ab) or 15 min (lane Bb). For reaction B, 5- to 10-fold sample volumes from the recovered Bb were loaded to facilitate comparison. The proteins were visualized by silver staining.

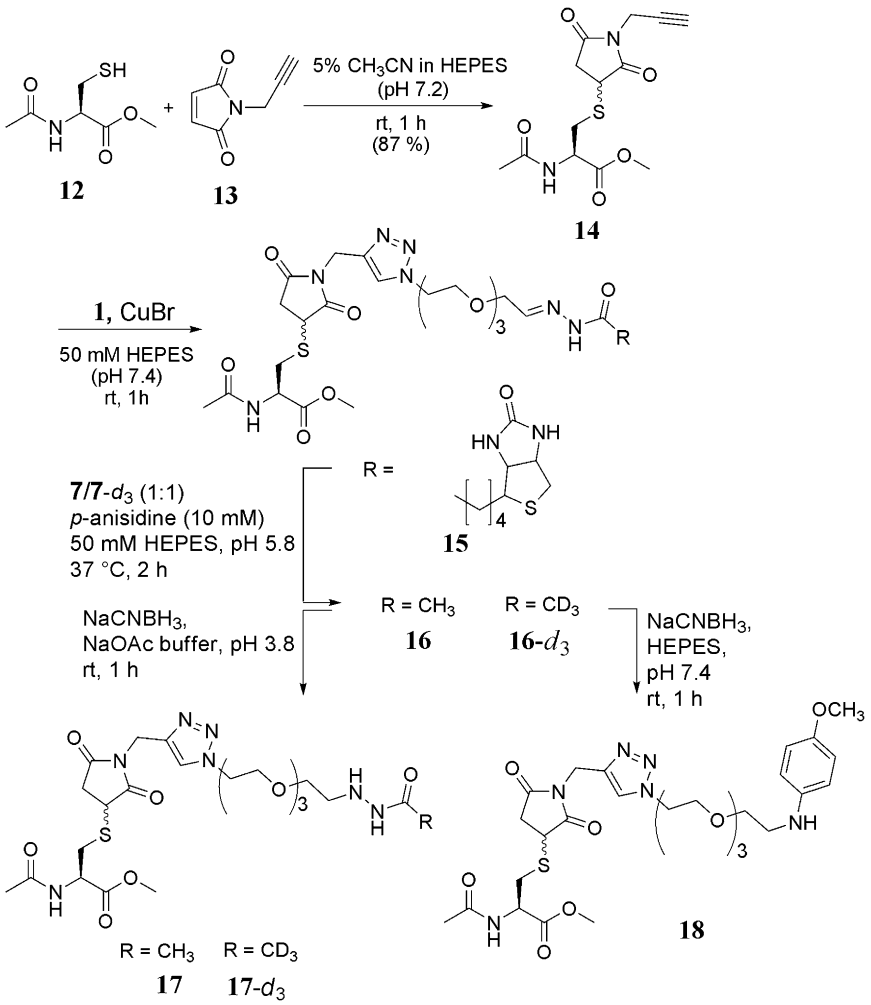

Figure 4. Use of 1 for Adduct Capture and Validation of the Release and Reduction Steps

(~93%) to 37°C (~72%) to 22°C (~48%) for the pH 5.8 reactions led to progressively lower cleavage yields.

To investigate the efficiency of cleavable linker **1** for protein capture and release compared with a noncleavable linker, we synthesized linker **10** (Sun et al., 2006) (Figures 3A and S6). We compared streptavidin-bound BSA **9** containing **1** with streptavidin-bound BSA **11** containing **10** (Figure 3A). Noncleavable linker **10** cannot undergo acylhydrazone cleavage, so protein release can only occur by disruption of the biotin-(strept)avidin complex (Holmberg et al., 2005; Jenne and Famulok, 1999; Tong and Smith, 1992). Both streptavidin-bound BSA **9** and **11** were treated for 1 hr at pH 5.8 using **7** (100 mM) and SDS (20 mM) at 50°C (Figure 3B). The extent of modified BSA recovery was determined by analyzing the supernatant and bead samples. Inspection of Figure 3B showed that very low levels of protein recovery were obtained from the initial supernatant from the BSA sample **11** modified with the noncleavable linker **10** (Figure 3B, lane Bs). Correspondingly, we observed high protein recovery from the supernatant from BSA **9** containing the cleavable linker **1** (Figure 3B, lane As). Consistent with reports in the literature (Holmberg et al., 2005; Tong and Smith, 1992), we observed low levels of protein recovery from the streptavidin-bound BSA **11** containing the noncleavable linker **10** after the bead was treated with SDS-loading buffer at 95°C for 15 min (Figure 3B, lane Bb). We estimated that the relative efficiencies of protein recovery from the cleavable linker BSA adduct **9** under moderate conditions (pH 5.8, **7** [100 mM], SDS [20 mM], 50°C, 1 hr) compared with the noncleavable linker BSA adduct **11** after heating (95°C) with SDS-loading buffer for 15 min to be ~20:1, based on comparison of the silver-stained protein lanes in Figure 3B for As and Bb.

Structural Validation of the Capture, Release, and Reduction Steps and Detection of Protein Using Traceable Tag

An important structural feature in the acylhydrazide-mediated cleavage of linker **1** is the formation of a new acylhydrazone (e.g., **8**). We anticipated that incorporating a traceable acylhydrazone tag (i.e., isotopic, radioactive, fluorescent) in the captured (bio)molecule would facilitate its detection and identification. The key chemical steps of the acylhydrazone-based cleavable linker methodology were validated by HPLC and mass spectrometry beginning with *N*-acetyl-L-cysteine methyl ester (**12**) (Figure 4). Reacting **12** with *N*-(propyl-2-ynyl)maleimide (**13**) gave **14** as a mixture of diastereomers. Compound **14** was then treated with **1** using CuBr-mediated cycloaddition conditions to provide **15**. The ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and mass spectral data were in agreement with the proposed adduct (Figure S7A). Treatment of **15** with an ~1:1 mixture of **7** and **7**-d_3 (*p*-anisidine [10 mM], pH 5.7, 37°C, 2 hr) gave the corresponding exchanged acetylhydrazone **16** and **16**-d_3 as the major product (HPLC t_R 12.8 min, Figure S7-B1). Mass spectral analysis of the HPLC-purified 12.8 min product showed a diagnostic "doublet" at *m/z* 608.2 and 611.2 for **16** and **16**-d_3 [M + Na]$^+$, respectively (Figure S7-B2). Correspondingly, sequential conversion of **15** to imine **16** and **16**-d_3 (pH 5.8) followed by $NaCNBH_3$ reduction (pH 3.8) led to the appearance of a new peak in the HPLC (Figure S7-C1). Isolation of the new product by HPLC and mass spectral analysis gave a doublet pattern at *m/z* 610.2 and 613.2 consistent with the reduced acethydrazides **17** and **17**-d_3 [M+Na]$^+$, respectively (Figures 4 and S7-C2). The facility of this reduction is important because it provides a chemically stable product not readily susceptible to acid hydrolysis and which is suitable for mass spectral analysis. When the $NaCNBH_3$ reduction was conducted at higher pH values (pH ~7.4) **17** and **17**-d_3 were not observed but rather we isolated the anisidine

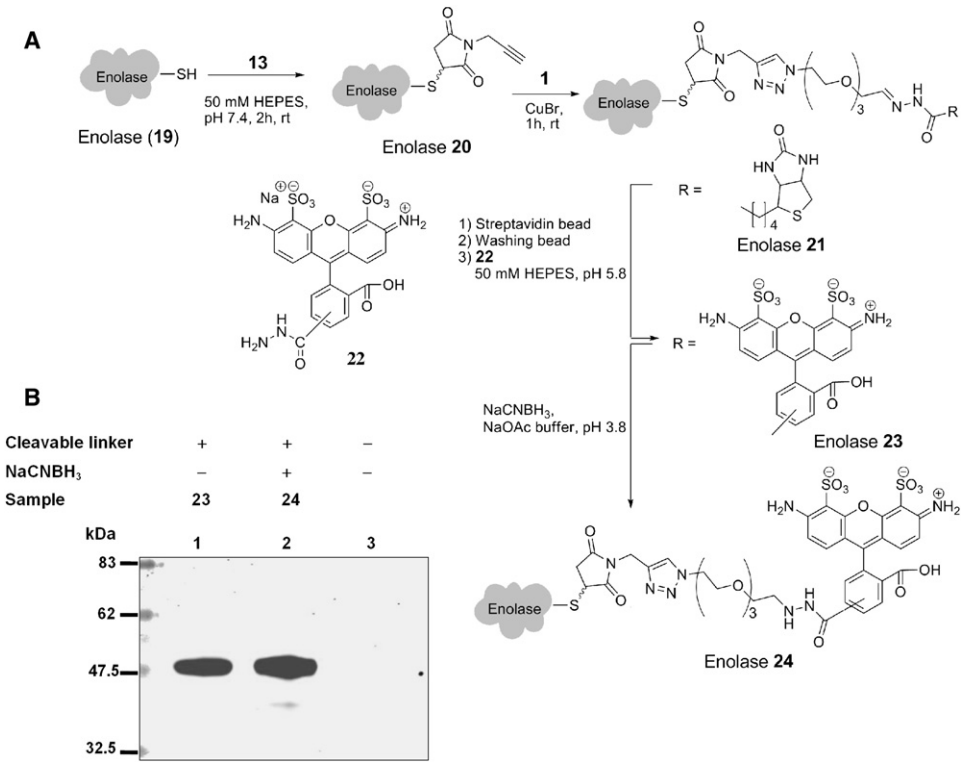

Figure 5. Use of 1 for Enolase Capture, Release, and Detection

(A) Scheme of enolase capture, release, and detection. The single cysteine unit in enolase (**19**) was treated with **13** to give approximately 30% of alkyne functionalized enolase **20** based on mass spectrometry (data not shown) and then converted to enolase **21** under Cu(I)-mediated cycloaddition condition.

(B) Use of fluorescein hydrazide **22** for detection and isolation of streptavidin-bound modified enolase **21.** Lane 1, **23** (the enolase recovered after incubation of enolase **21** with streptavidin beads in aqueous 50 mM HEPES (pH 5.8), followed by washing of the beads, and then treatment of the beads with **22** (1.5 mM) using an optimized condition (*p*-anisidine [10 mM], 37°C, 4 hr); lane 2, **24** (reduction of **23** with $NaCNBH_3$ at pH 3.8); lane 3, control (all reactions were the same as **23** except the cleavable linker was excluded in the cycloaddition step). The proteins were visualized by fluorescent detection by excitation at 488 nm and detection at 520 nm.

derivative **18** (*m/z* 637.3 $[M+H]^+$) (Figures 4 and S7-D2). This finding suggested that at near-neutral pH values, **16** and **16**-d_3 underwent transimination with anisidine and then reduction to give **18** as the major product. These results demonstrated that the acylhydrazide-mediated cleavage of the linker provided a traceable tag in the cleaved product that can be monitored by mass spectrometry.

Similarly, we showed that fluorescein hydrazide treatment of enolase sample **21** modified with cleavable linker **1** (Figure 5A) led to the appearance of a strong fluorescent band corresponding to the expected enolase adducts both before and after $NaCNBH_3$ (pH 3.8) treatment (Figure 5B). Because only fluorescently labeled proteins are released, this methodology can significantly increase both the detection sensitivity and purification efficiency of the proteins of interest in proteomic analyses.

Use of Cleavable Linker 1 in Proteomic Target Searches: Capture and Identification

Next, we tested whether cleavable linker **1** can be applied to capture proteins of interest from a natural proteome. The Cravatt laboratory showed that alkyne phenyl sulfonate ester **25** (Speers and Cravatt, 2004; Weerapana et al., 2008) (Figure 6A) selectively reacted with aldehyde dehydrogenase 1 (ALDH-1) in the mouse soluble liver lysate. We asked whether the use of **25** and cleavable linker **1** would permit efficient capture and removal of ALDH-1 from the mouse soluble liver proteome using streptavidin beads, and whether the release of the captured ALDH-1 from the streptavidin support with **7** would proceed with minimal release of nonspecific ''background'' proteins that bound to the streptavidin resin (Fonovic et al., 2007).

Accordingly, mouse soluble liver lysate (pH 8.0) was incubated with **25** (5, 25 μM) at room temperature (1 hr) and then treated with rhodamine azide (RhN_3, **26**), TCEP, TBTA, and $CuSO_4$. The lysate was separated by SDS-PAGE and the labeled proteins visualized by in-gel fluorescence (Figure 6B). In agreement with earlier reports (Speers and Cravatt, 2004), only a single protein band was observed at ~50 kDa when 5 μM **25** was used (Figure 3A, lane 1). Increasing the concentration of **25** to 25 μM led to increased intensity of this band and the appearance of lower molecular weight bands (Figure 6B, lane 3). The in-gel detection of the ~50 kDa band was consistent with the selective tagging of ALDH-1 by activity probe **25** as detailed in the literature (Speers and Cravatt, 2004).

The experiment was repeated with **25** (25 μM) substituting either cleavable linker **1** or noncleavable linker **10** for **26**. The labeled complexes were captured by adding streptavidin-agarose beads.

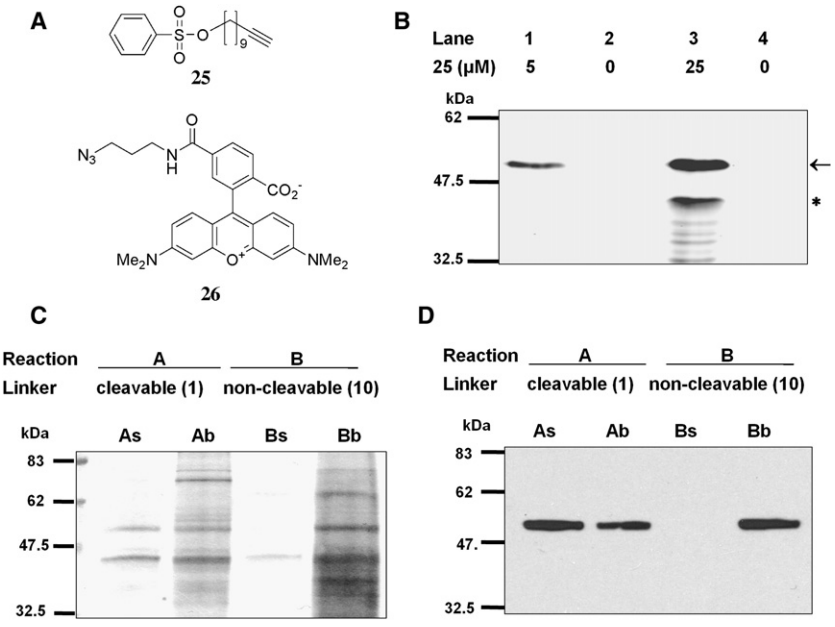

Figure 6. Proteomic Target Search in Mouse Liver Proteome with the Cleavable Linker 1 Using 25 and Comparison with Noncleavable Linker 10

(A) Chemical structures of **25** and **26**.

(B) After labeling with **25** (5 μM or 25 μM) followed by Cu(I)-mediated cycloaddition with **26**, the signal was detected by in-gel fluorescence scanning. Using 5 μM **25**, ALDH-1 (arrow) was selectively labeled. At 25 μM **25**, ALDH-1 (arrow) and an abundant protein(s) (asterisk) in liver lysate were labeled.

(C) After labeling with **25** (25 μM), probe-labeled proteins were captured by streptavidin beads after Cu(I)-mediated cycloaddition to either cleavable linker **1** or noncleavable linker **10**. The beads were washed and treated using a mild cleavage condition (**7** [100 mM], *p*-anisidine [10 mM] in aqueous 50 mM HEPES [pH 5.8] [37°C, 4 hr]). Lanes marked "s" correspond to the supernatant removed from the bead mixture after the initial treatment, whereas the lanes marked "b" refer to the sample obtained after treatment of the remaining beads with loading buffer (95°C, 5 min). The proteins were visualized by silver staining.

(D) Comparison of the release efficiency of **25**-labeled target protein (ALDH-1) after Cu(I)-mediated cycloaddition to either cleavable linker **1** or noncleavable linker **10** by western blot using anti-ALDH-1 antibody.

After extensive wash with buffer, the recovered beads from both the biotinylated cleavable **1** and the noncleavable **10** probes were treated with **7** (100 mM) and *p*-anisidine (10 mM), pH 5.8 at 37°C (4 hr), conditions designed to release proteins from cleavable linker **1**. The supernatants from these reactions were recovered. The remaining beads were treated with SDS-loading buffer, heated at 95°C (5 min), and the supernatants recovered. The recovered protein samples were loaded on SDS-PAGE and the resolved bands visualized by silver staining (Figure 6C) and analyzed by western blot (Figure 6D). The silver-stained gel lane for the **7**-treated supernatant from cleavable linker **1** was remarkably clean, displaying only two prominent bands at ~50 kDa and ~44 kDa (Figure 6C, lane As). This result is consistent with that shown in Figure 6B. Based on the result from Coomassie blue staining of the untreated whole lysate, we suspect that the 44 kDa band corresponds to abundant protein(s) (data not shown). Significantly, the lane from the supernatant recovered from the remaining beads treated with loading buffer (95°C, 5 min) showed these two bands along with significant amounts of other bands (Figure 6C, lane Ab), indicating that many background proteins are indeed nonspecifically captured by the streptavidin-agarose beads. Correspondingly, the **7**-treated supernatant from noncleavable linker **10** displayed only a weak band at ~44 kDa and no band at ~50 kDa (Figure 6C, lane Bs), whereas the remaining beads lane showed significant amounts of the ~44 kDa and ~50 kDa bands corresponding to an abundant protein(s) and ALDH-1, respectively, and many other bands attributed to background proteins (Figure 6C, lane Bb). To confirm that the selectively captured 50 kDa protein by the cleavable linker approach is indeed ALDH-1, we performed western blot analysis using an ALDH-1 antibody (Figure 6D). We observed a prominent band consistent for ALDH-1 in the supernatant recovered from cleavable linker **1** upon treatment with **7** but not in the corresponding supernatant from noncleavable linker **10** (Figure 6D, lane As versus lane Bs). Analysis of the SDS-loading buffer-treated beads from both reactions showed the presence of ALDH-1 (Figure 6D, lanes Ab and Bb). The silver-stained and western blot gels documented that the supernatant recovered from the cleavable linker **1** after **7**-mediated cleavage was highly enriched with target protein ALDH-1 and was devoid of most background proteins present in the lysate. Correspondingly, ALDH-1 was not detected in the supernatant from the noncleavable linker **10** (Figures 6C and 6D, lane Bs), but it was observed after harshly heating (95°C, 5 min) the recovered streptavidin resin with SDS-loading buffer (Figures 6C and 6D, lane Bb), a condition that results in the release of many background proteins.

DISCUSSION

We selected **1** as our prototype acylhydrazone linker because of its structural properties and its expected solubility in water. The acylhydrazone moiety in **1** is the cleavage site, and it provided the necessary balance between stability and reactivity. We found that the acylhydrazone unit underwent cleavage under mildly acidic conditions (pH 5.8), but remained intact under near-neutral (pH 7.4) and basic (pH 10) conditions (Table S1). This finding is in general agreement with earlier reports (Goral et al., 2001; King et al., 1986). The observed pH dependency for **1** hydrolysis led us to use moderately basic (pH 7.4–8.5) conditions for storage, handling, and chemical transformations where cleavage of the linker was not desired. We identified additives that promoted cleavage of the acylhydrazone group. Using the findings of Dirksen et al. (2006a, 2006b), we confirmed that both aniline and *p*-anisidine facilitated acylhydrazide exchange

(pH 5.8, 7.4) (Figure S3). A surprising result was that the anionic detergent SDS fostered acylhydrazone cleavage (Figure S4 and Table S3). Correspondingly, use of nonanionic detergents, NP-40 and Triton X-100, in place of SDS did not promote cleavage. The chemical factors responsible for the SDS-mediated cleavage have not been determined. Specifically, we do not know whether SDS serves as a specific or general base in the hydrolysis step, or whether SDS-mediated acylhydrazone loss is due to direct attack of the hydrazone linkage by SDS, or whether SDS-mediated loss is due, in part, to the formation of micelles that facilitates hydrazone modification (Camilo and Pilli, 2004; Gogoi et al., 2005; Wang et al., 2005). Most important for our study was our demonstration that **7** induced hydrazone exchange in **1**. Acethydrazide exchange of acylhydrazones has been described by King et al. (1986). The reaction was reported to proceed to near completion within 2 days at pH 4.7 and 5.2 (25°C), but at pH 7.0 substantial amounts of unreacted starting material (~40%) remained after 8 days. We found that with **1** the reaction proceeded to near completion at pH 5.2 and 6.2 within 1 hr at 22°C, and that high yields of exchange were observed at pH 7.4 after 7 hr at 22°C (Table S2). Similarly efficient cleavage for the acylhydrazone group was observed in the BSA-modified product **9** bound to the streptavidin support, and the reaction was catalyzed by the additive *p*-anisidine, which permitted efficient cleavage at pH 7.4 within 2 hr (Figure S3). The facility with which **7** cleaves **1** at near-neutral pH values compared with the earlier findings (King et al., 1986) suggests that structural changes surrounding the acylhydrazone linkage affect cleavage rates.

To test the utility of **7**-mediated cleavage of acylhydrazone linkers for protein capture and isolation, we used two protein-based model systems. Because linker **1** contained a terminal azide unit, we installed an alkyne moiety (moieties) within the test proteins to permit Cu(I)-mediated cycloaddition reaction. Accordingly, BSA was randomly modified with the photoaffinity lacosamide probe to give an alkyne functionalized BSA (Figure S2). Correspondingly, the single cysteine residue in *S. cerevisiae* enolase (**19**) (Chin et al., 1981; Holland et al., 1981) permitted the Michael addition to *N*-(prop-2-ynyl)maleimide (**13**) to give alkyne functionalized enolase **20** (Figure 5). Together these modified proteins permitted us to test the utility of acylhydrazone linker **1**. The biotinylated BSA products **9** and **11** obtained after Cu(I)-mediated cycloaddition with **1** and **10**, respectively, were captured with streptavidin beads. Utilizing the information learned in our model studies with **1**, we identified a series of conditions that led to efficient cleavage of the acylhydrazone linker in BSA **9,** allowing protein release from the streptavidin support. Using 100 mM **7** and 20 mM SDS, we observed efficient cleavage and release of modified BSA within 1 hr at 50°C in pH 5.8 solutions (Figure S5). High yields of modified BSA release were also observed within 4 hr in both pH 5.8 (22°C) and pH 7.4 (37°C) solutions containing **7** (100 mM) and *p*-anisidine (10 mM). Excluding SDS in the cleavage protocol might facilitate subsequent mass spectrometric detection of the recovered protein and permit us to identify multiprotein complexes that maintain their bioactive conformations. Finally, we expect that the acylhydrazone group generated after linker cleavage can serve as a convenient aldehyde-protecting group that can be removed with mild acid, thereby permitting further protein tagging and modification. Correspondingly, **7**-mediated cleavage of **11** led to the release of very low levels of BSA (Figure 3B). When we compared the relative amounts of BSA recovered from streptavidin-bound BSA **9** (containing cleavable linker **1**) with streptavidin-bound BSA **11** (containing noncleavable linker **10**), we observed a ~20-fold increase in BSA recovery with **9**. This increased amount is significant because BSA release from **9** proceeded under mild conditions whereas harsh conditions were required for **11**. Using the modified BSA protein **9**, we showed that **7**-mediated acyl hydrazone cleavage under mild conditions led to the release of more than 85% of the protein captured by the streptavidin-agarose beads (see Supplemental Experimental Procedures).

The cleavage step was validated by treatment of the modified *N*-acetyl-L-cysteine methyl ester adduct **15** with **7** and **7**-d_3 (Figure 4). Mass spectrometric analysis of both the cleaved acetylhydrazone adducts **16** and **16**-d_3, and the corresponding $NaCNBH_3$-reduced adducts **17** and **17**-d_3 showed a characteristic mass spectrometric signature pattern ([M] + [M+3]) for the desired products (Figure S7). Our finding that $NaCNBH_3$ reduction of the reaction mixture containing **16**, **7**, and **7**-d_3 at pH ~7.4 provided *p*-anisidine adduct **18** indicated that employment of a mixture of nondeuterated and deuterated aniline derivatives in the cleavage step can similarly provide a diagnostic mass spectral pattern useful for protein identification.

An important component in the design of the acylhydrazone linker was incorporating a traceable tag upon protein release from the support. We further documented this design feature using fluorescent hydrazide **22** for acylhydrazone linker cleavage in streptavidin-bound enolase **21** (Figure 5). With **22** we showed that acylhydrazone exchange of streptavidin-bound enolase **21** led to a protein band in the gel that was readily visualized on fluorescent imaging. Because only the tagged proteins were fluorescently labeled and released, this approach could significantly increase both the detection sensitivity and purification efficiency of the proteins of interest in proteomic analyses. Finally, we recognized that the acylhydrazone group in the protein-released product can undergo reduction with $NaCNBH_3$ to the corresponding acylhydrazide, a chemically stable product not readily susceptible to acid hydrolysis. Thus, we reduced the hydrazone group in enolase **23** with $NaCNBH_3$ in acid to give **24**. This reduction step provides a convenient opportunity to incorporate a radioactive tag in the protein through the use of [^{3}H]-$NaCNBH_3$, which can aid protein detection and identification studies.

The utility of cleavable linker **1** was tested in a proteomic search. Cravatt and coworkers have elegantly demonstrated that ALDH-1 was selectively labeled in the mouse soluble liver lysate by phenyl sulfonate ester **25** at pH 8.0. The specificity of this activity probe for ALDH-1 provided a stringent test for our linker. We asked if linker **1** selectively reacted with **25**-modified ALDH-1 by Cu(I)-mediated cycloaddition and whether, upon streptavidin bead capture and treatment of the resin with **7** under mild conditions, the supernatant would be enriched in ALDH-1 and be devoid of background proteins. Using cleavable linker **1**, we demonstrated that the supernatant recovered after treating the streptavidin beads with **7** under mild conditions was enriched in ALDH-1 and was remarkably clean of background proteins (Figure 6C). The silver-stained gel for this sample showed only two prominent protein bands, the first at ~50 kDa corresponding to ALDH-1 and the second at ~44 kDa, which we suspect is an

abundant protein(s) in the lysate that was modified by the 25 μM **25**. The absence of background proteins in Figure 6C, lane As, is important because proteomic target identification studies have been hampered by the presence of such proteins in samples containing the target(s) (Fonovic et al., 2007). As anticipated, ALDH-1 was not detected in the supernatant after treatment of the beads with **7** when the noncleavable linker **10** was employed in the Cu(I)-mediated cycloaddition reaction, but it was observed after harshly heating (95°C, 5 min) the recovered streptavidin resin with loading buffer containing 2% SDS and 1% mercaptoethanol, a condition that results in the release of many background proteins that nonspecifically bound to streptavidin resin. The recovered yield for ALDH-1 in the supernatant was somewhat lower than that observed for cleaved BSA **9** under similar conditions (Figures 6 and S5-A), and might reflect the different nonspecific matrix binding properties of these two proteins.

Our studies demonstrated that incorporating an acylhydrazone group within the biotin-containing linker permitted efficient linker cleavage from the streptavidin support. Some advantages associated with this method include: (1) the ease of linker synthesis; (2) stability of the linker to bio-orthogonal coupling transformations (e.g., Cu(I)-mediated cycloaddition reactions); (3) linker stability at near neutral and moderately basic pH values; (4) chemoselective cleavage of the linker with acylhydrazides with minimal release of background proteins nonspecifically bound to (streptavidin) supports; (5) high recovery yields of the cleaved proteins; and (6) ability to incorporate a traceable tag (e.g., isotopic, fluorescent) in the captured protein to facilitate detection and identification. Finally, the mild conditions needed for acylhydrazone cleavage offer the possibility that nondenatured proteins or protein complexes can be captured and then released. Countering these advantages is the need to avoid moderately acidic pH values during protein capture to prevent premature linker cleavage and the recognition that linker cleavage leads to a permanent structural modification in the protein.

The acylhydrazone linker compares favorably with previously introduced cleavable linkers. Like **1**, each linker has advantages and disadvantages to consider prior to selection and use. For instance, photolytic-based cleavable linkers typically undergo efficient release, but upon cleavage, they release reactive carbonyl compounds (aldehydes, ketones) that can react with the protein (Bai et al., 2004; Thiele and Fahrenholz, 1994). Acid-sensitive linkers that require trifluoroacetic acid (TFA) can lead to nonspecific cleavage, and TFA use requires its removal prior to mass spectrometry analysis (van der Veken et al., 2005). Linkers that rely on proteolytic enzymes (e.g., trypsin) provide a selective cleavage method but require that a designed peptide sequence be installed within the linker that is efficiently cleaved (Dieterich et al., 2007; Fonovic et al., 2007; Speers and Cravatt, 2005). The use of proteolytic enzymes for cleavage may prevent the isolation of the intact protein. Proteomic experiments utilizing disulfide linkers require the use of buffers that are devoid of reducing reagents (Finn et al., 1985; Fonovic et al., 2007; Marie et al., 1990; Shimkus et al., 1985). Moreover, these disulfide linkers can be cleaved under intracellular conditions. This concern for premature cleavage of the linker is not an issue for the diazobenzene linker advanced by Bogyo and coworkers and where $Na_2S_2O_4$ serves as the chemoselective reductant (Fonovic et al., 2007; Verhelst et al., 2007).

Our procedure compliments the widely used method that couples on-bead digestion and mass spectrometry (Chrestensen et al., 2004). This on-bead method benefits from its simplicity and ease of use. Unlike the on-bead method, our procedure allows the chemoselective release of the protein of interest, the incorporation of an isotopic tag that facilitates mass spectrometric identification, the isolation of the intact protein, and the opportunity to identify the site of protein adduction.

SIGNIFICANCE

In this study, we showed that the use of 1 permitted the rapid, selective release of proteins from streptavidin supports with acylhydrazides under mild conditions at near-neutral pH values. Protein release proceeded with significantly reduced levels of background proteins. The lack of appreciable amounts of background proteins is important because proteomic target identification studies have been hampered by the presence of such proteins in samples containing the target(s). In addition, the acylhydrazone group permitted the incorporation of a traceable tag (i.e., isotopic, fluorescent) in the released protein. The use of tailored acylhydrazides that contain a traceable tag provides opportunities to detect and identify biomolecules. We anticipate that the acylhydrazone group will serve as a useful, cleavable structural entity for the release of tethered molecules from supports other than (strept)avidin (e.g., noncovalent complexes and systems/resins) that employ a covalent linkage for (bio)-molecule attachment to the support.

EXPERIMENTAL PROCEDURES

Synthesis of Compounds 3, 4, 7-d_3, 8, 10, 13–15, 16, 16-d_3, 17, and 17-d_3

See Supplemental Data.

Preparation of *N′*-(2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy) acetaldehyde Biotinylhydrazone (1)

To an anhydrous CH_2Cl_2 solution (2 ml) of oxalyl chloride (0.10 ml, 1.10 mmol) maintained at −78°C under Ar was slowly added freshly distilled DMSO (0.19 ml, 3.29 mmol). The mixture was stirred (30 min), and then dry **4** (0.20 g, 0.91 mmol) was added via syringe. The mixture was stirred for an additional 30 min at −78°C, and then Et_3N (0.78 ml, 6.57 mmol) was added (10 min) and the mixture was stirred (15 min) at this temperature and warmed to ambient temperature. The reaction solution was diluted with CH_2Cl_2 (10 ml) and washed with H_2O (2 × 10 ml). The organic layer was dried (Na_2SO_4), concentrated, and the crude aldehyde **5** (0.19 g, 0.88 mmol) was dissolved in CH_2Cl_2 (1 ml) and added to a solution of **6** (0.15 g, 0.58 mmol) in THF/H_2O (1:1, 5 ml). The reaction mixture was allowed to stir at room temperature (16 hr), the solvent was evaporated in vacuo, and then the crude product purified by column chromatography (SiO_2; 1/9 MeOH/$CHCl_3$) to yield 0.20 g (74%) of **1** as a white solid: mp 134°C–135°C; R_f = 0.35 (1/9 MeOH/$CHCl_3$); IR (nujol) 2924, 2101, 1667, 1557, 1459 cm^{-1}; ^1H NMR (CD_3OD) (minor conformer in parenthesis [a mixture of conformers similar to other hydrazones (Syakaev et al., 2006)]) δ 1.45–1.52 (m, C(6)**H$_2$**), 1.58–1.79 (m, C(7)**H$_2$,** C(8)**H$_2$**), 2.27 (t, *J* = 7.4 Hz, C(9)**H$_2$** (2.65 (t, *J* = 7.5 Hz, C(9)**H$_2$**))), 2.71 (d, *J* = 12.6 Hz, C(5)**H**H′), 2.93 (dd, *J* = 4.7, 12.6 Hz, C(5)H**H**′), 3.18–3.25 (m, C(2)**H**), 3.37 (t, *J* = 4.8 Hz, N_3C**H$_2$**), 3.65–3.69 (m, 2 O**CH_2CH_2**O, N_3CH_2**CH_2**), 4.18 (d, *J* = 5.3 Hz, O**CH_2**CHN (4.15 (d, *J* = 5.3 Hz, O**CH_2**CHN))), 4.31 (dd, *J* = 4.2, 7.6 Hz, C(3)**H**), 4.49 (dd, *J* = 4.7, 7.6 Hz, C(4)**H**), 7.49 (t, *J* = 5.3 Hz, OCH_2**C**H**N** (7.33 (t, *J* = 5.3 Hz, OCH_2**C**H**N**))); ^{13}C NMR (CD_3OD) (minor conformer in parenthesis) δ 26.0 (26.6) (**C**(6)), 29.6 (**C**(7)), 29.9 (30.0) (**C**(8)), 33.2 (35.2) (**C**(9)), 41.2 (**C**(5)), 51.9 (N_3**C**H_2), 57.1 (57.2) (**C**(2)), 61.8 (**C**(3)), 63.5 (**C**(4)), 71.2, 71.3, 71.4,

71.5, 71.7, 71.8 (3 **C**H_2O**C**H_2), 145.6 (149.3) (OCH_2**C**HN), 166.3 (**C**(2′)), 172.7 (177.9) (**C**(10)); HRMS (ESI) 458.2182 [M + H^+] (calcd. for $C_{18}H_{32}N_7O_5S$ 458.2186); Anal. ($C_{18}H_{31}N_7O_5S$, 0.25 H_2O) calcd.: C, 46.79%; H, 6.87%; N, 21.22%; S, 6.94%. Found: C, 46.67%; H, 6.82%; N, 20.88%; S, 6.88%.

Treatment of Enolase (19) with Maleimide 13 to Afford Enolase 20

To a 200 μM solution (1 ml) of *S. cerevisiae* enolase (**19**) (Sigma, E6126) in aqueous 50 mM HEPES (pH 7.4) was added a 200 mM solution (0.1 ml) of **13** (2.7 mg, 20.0 μmol) in 5% CH_3CN/aqueous 50 mM HEPES (pH 7.4). The solution was incubated at room temperature (2 hr) and then the reaction solution was diluted with aqueous 50 mM HEPES buffer (pH 7.4) to 5 ml and passed through NAP-5 columns pre-equilibrated with 50 mM HEPES buffer (pH 7.4). The eluents (~10 ml) were combined and stored at 4°C.

Cycloaddition Reaction between Enolase 20 and Cleavable Linker 1 to Afford Enolase 21 (Method A)

To an aqueous 50 mM HEPES solution containing **20** obtained after passage through the NAP-5 column (1 ml), was sequentially added a 20 mM solution (50 μl) of **1** (0.44 mg, 1.0 μmol) in 5% CH_3CN/aqueous 50 mM HEPES (pH 7.4), a 20 mM solution (50 μl) of biotin hydrazide (**6**) (0.26 mg, 1.0 μmol) in 5% CH_3CN/aqueous 50 mM HEPES (pH 7.4), and CuBr (0.2 mg, 1.4 μmol). The reaction mixture was rotated using Roto-shake (8 rpm, Scientific Industries Inc., model SI-1100, Bohemia, NY) at room temperature (1 hr), then divided in two equal portions and passed through separate NAP-5 columns pre-equilibrated with HEPES buffer (pH 7.4) to give an aqueous solution of enolase **21**. The eluents were combined (~2 ml) and stored at 4°C.

Use of Fluorescent Hydrazide 22 for Detection and Isolation of Streptavidin-Bound Modified Enolase 21 and Reduction of Imine 23 to 24 (Method B)

An aliquot of enolase **21** (200 μl) in aqueous 50 mM HEPES buffer (pH 7.4) was added to an immobilized streptavidin slurry (1 ml) (High Capacity Streptavidin Agarose Resin, Pierce, Rockford, IL) and rotated using a shaker (15 rpm) at 4°C (90 min). The streptavidin beads were washed with aqueous 15 mM HEPES buffer (pH 7.4) (10 × 0.8 ml). The beads were centrifuged (1000 rpm, 1 min), and the supernatant removed. An aqueous 50 mM HEPES solution (100 μl, pH 5.8) was added and the beads treated with fluorescent hydrazide **22** (Alexa Fluor hydrazide, A10436, Molecular Probes, 85.5 μg, 0.15 μmol; final concentration 1.5 mM) and *p*-anisidine (123.0 μg, 1 μmol; final concentration 10 mM). The reaction mixture was gently shaken at 37°C (4 hr) and the supernatant (100 μl) containing **23** was collected.

The supernatant (50 μl) was treated with a 10 mM solution of $NaCNBH_3$ (50 μl) in 50 mM NaOAc buffer (pH 3.8) and rotated using Roto-shake (8 rpm) at room temperature (2 hr) to give a mixture containing **24**. Both the supernatant containing **23** and the reaction mixture containing **24** were loaded on a 10% SDS-PAGE gel. Labeled proteins were visualized using a typhoon 9400 scanner (Amersham Bioscience) with excitation at 488 nm and detection at 520 nm.

Proteome Sample Preparation, Probe Labeling, Cycloaddition Reaction, and In-Gel Fluorescence Scanning

Compound **25** and azide-rhodamine reporter tag (RhN_3, **26**) were prepared according to previous reports (Speers and Cravatt, 2004). Mouse liver lysates (43 μl 2.0 mg/ml protein in PB [pH 8.0]), prepared as described in the Supplemental Data, were treated with 5 μM or 25 μM **25** at room temperature (1 hr). To incubated lysates with **25** were sequentially added **26** (100 μM), TCEP (1 mM), TBTA (100 μM), and $CuSO_4$ (1 mM). Samples were vortexed and allowed to react at room temperature (1 hr). Proteins were separated by SDS-PAGE after addition of 4 × SDS-PAGE loading buffer and visualized in-gel using a typhoon 9400 scanner (Amersham Bioscience) with excitation at 555 nm and detection at 580 nm.

Capture and Release of Probe-Labeled Proteins from Mouse Liver Lysate

Mouse soluble liver lysate (400 μl) was treated with **25** (3.08 μg, 10 nmol, 25 μM) at room temperature (1 hr) and passed through a NAP-5 column to exchange to an aqueous 50 mM HEPES buffer (pH 7.4) and then divided into two 250 μl aliquots. Using method A, to each aliquot was added a 5 mM solution (10 μl) of either **1** or **10** (22 μg, 50 nmol) in 5% CH_3CN/aqueous 50 mM HEPES (pH 7.4), a 5 mM solution (10 μl) of biotin hydrazide (**6**) (13 μg, 50 nmol) in CH_3CN/aqueous 50 mM HEPES (pH 7.4), and CuBr (0.2 mg, 1.4 μmol). Using method B, immobilized streptavidin slurry (300 μl) was added and the beads were treated with **7** (0.78 mg, 10 μmol) and *p*-anisidine [final concentration 10 mM] at 37°C (4 hr). The supernatants were collected and then the remaining streptavidin beads were boiled (5 min) with SDS-PAGE loading buffer (2% SDS, 10% glycerol, 1% mercaptoethanol, 0.01% bromophenol blue). The samples were loaded on two SDS-PAGE (10%) gels and the proteins visualized by silver staining and ALDH-1 detected by western blot using anti-ALDH-1 antibody (ab23375, Abcam) as detailed in Supplemental Data.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, eight figures, and three tables and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00184-7.

ACKNOWLEDGMENTS

We thank Steven Cotten (University of North Carolina-Chapel Hill) for helpful discussions and advice. The project described was supported by grant 5R01NS054112 (to H.K., R.L.) from the National Institute of Neurological Disorders and Stroke, and by a Korean Research Foundation Grant funded by the Korean Government (MOEHRD, grant KRF-2006-352-C00042, to K.D.P.).

Received: March 26, 2009
Revised: May 29, 2009
Accepted: June 5, 2009
Published: July 30, 2009

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