Anal. Chem. **2009,** *81,* 9885–9895

Method for the Affinity Purification of Covalently Linked Peptides Following Cyanogen Bromide Cleavage of Proteins

Tujin Shi,[†] Rasanjala Weerasekera,[†,‡] Chen Yan,[‡] William Reginold,[‡] Haydn Ball,[§] Thomas Kislinger,[||] and Gerold Schmitt-Ulms*[,†,‡]

Centre for Research in Neurodegenerative Diseases, University of Toronto, Toronto, Ontario, Canada, Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada, Department of Biochemistry, University of Texas Southwestern Medical School, Dallas, Texas, and Division of Cancer Genomics and Proteomics, Ontario Cancer Institute, Toronto, Ontario, Canada

The low resolution structure of a protein can sometimes be inferred from information about existing disulfide bridges or experimentally introduced chemical crosslinks. Frequently, this task involves enzymatic digestion of a protein followed by mass spectrometry-based identification of covalently linked peptides. To facilitate this task, we developed a method for the enrichment of covalently linked peptides following the chemical cleavage of a protein. The method capitalizes on the availability of homoserine lactone moieties at the C-termini of cyanogen bromide cleavage products which support selective conjugation of affinity tags. The availability of two C-termini within covalently linked peptides allows for the conjugation of two distinct affinity tags and thereby enables subsequent removal of unmodified peptides by tandem affinity chromatography. Here, we demonstrate the stepwise implementation of this method using a polyhistidine tag and a biotin tag for the selective two-step purification of covalently linked cyanogen bromide fragments from increasingly complex protein samples. The method is independent of the nature of the covalent bond, is adaptable to fully denaturing conditions, and requires only low picomole quantities of starting material.

The topology of a protein and the interactions it engages in are important determinants of its biology. Proteins do not act in isolation but interact with other proteins, nucleic acids, and a range of cellular factors to fulfill their diverse cellular roles.[1−4] Whereas tools for the identification of proteins,[5] the characterization of their posttranslational modifications,[6] and the mapping of protein−nucleic acid interactions are increasingly well-developed,[7−13] gaining insights into the folds of proteins and contact sites between proteins remains a challenging undertaking. Despite major advances in speed and application range, high-resolution structure methods based on X-ray crystallography or nuclear magnetic resonance (NMR) analyses continue to be time-consuming and are limited by the need to obtain analytes at high levels of purity and quantity. The quest for a robust methodology that fills this need has arguably become one of the most pressing problems in protein science. For proteins which harbor internal disulfide bridges, a first glimpse into their fold may be obtained by characterizing these linkages. Whenever no disulfide linkages exist in a protein of interest, chemical crosslinking reagents can be employed to experimentally introduce covalent linkages.[14] Alternatively, cysteine residues can be engineered into a protein which subsequently can inform one about the structural fold of a protein.[15] These methods may become particularly useful for studying the topology and interfaces of macromolecular protein complexes that have proven to pose a formidable challenge to the above-mentioned high-resolution methods. The typical methodological progression in these studies is the enzymatic digestion of proteins followed by a mass spectrometry (MS)-based identification of covalently linked peptides. Multiple reports have documented the successful application of this approach to explore the topology of proteins[16−19] or to characterize protein−protein

* To whom correspondence should be addressed. Address: Centre for Research in Neurodegenerative Diseases, University of Toronto, Room 209, Tanz Neuroscience Building, Toronto, Ontario, M5S 3H2, Canada. Tel: 416-946-0066. Fax: 416-978-1878. E-mail: g.schmittulms@utoronto.ca.

[†] Centre for Research in Neurodegenerative Diseases, University of Toronto.

[‡] Department of Laboratory Medicine and Pathobiology, University of Toronto.

[§] University of Texas Southwestern Medical School.

[||] Ontario Cancer Institute.

(1) Gingras, A. C.; Gstaiger, M.; Raught, B.; Aebersold, R. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 645–654.

(2) Aloy, P.; Russel, R. B. *Trends Biochem. Sci.* **2002**, *12*, 633–638.

(3) Pandey, A.; Mann, M. *Nature* **2000**, *405*, 837–846.

(4) Sobott, F.; Robinson, C. V. *Curr. Opin. Struct. Biol.* **2002**, *12*, 729–734.

(5) Lu, B.; Xu, T.; Park, S. K.; Yates, J. R., 3rd *Methods Mol. Biol.* **2009**, *564*, 261–288.

(6) Hoffman, M. D.; Sniatynski, M. J.; Kast, J. *Anal. Chim. Acta* **2008**, *627*, 50–61.

(7) Geyer, H.; Geyer, R.; Pingoud, V. *Nucleic Acids Res.* **2004**, *32*, e132.

(8) Jensen, O. N.; Barofsky, D. F.; Young, M. C.; von Hippel, P. H.; Swenson, S.; Seifried, S. E. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 496–501.

(9) Jensen, O. N.; Kulkarni, S.; Aldrich, J. V.; Barofsky, D. F. *Nucleic Acids Res.* **1996**, *24*, 3866–3872.

(10) Lenz, C.; Kuhn-Holsken, E.; Urlaub, H. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 869–881.

(11) Pingoud, V.; Geyer, H.; Geyer, R.; Kubareva, E.; Bujnicki, J. M.; Pingoud, A. *Mol. BioSyst.* **2005**, *1*, 135–141.

(12) Urlaub, H.; Hartmuth, K.; Luhrmann, R. *Methods* **2002**, *26*, 170–181.

(13) Urlaub, H.; Kuhn-Holsken, E.; Luhrmann, R. *Methods Mol. Biol.* **2008**, *488*, 221–245.

(14) Sinz, A. *Mass Spectrom. Rev.* **2006**, *25*, 663–682.

(15) Sato, C.; Morohashi, Y.; Tomita, T.; Iwatsubo, T. *J. Neurosci.* **2006**, *26*, 12081–12088.

(16) Pearson, K. M.; Pannell, L. K.; Fales, H. M. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 149–159.

10.1021/ac901373q CCC: $40.75 © 2009 American Chemical Society
Published on Web 11/19/2009

interfaces.[20−24] Despite impressive progress in this direction, this type of investigation has so far been of modest practical benefit to mainstream biochemists because the demand for the purity of samples remains relatively high. A reason for the limitation of current protocols is their reliance on the direct detection of crosslinked peptides by mass spectrometry. This conceptual feature translates into a search not unlike the proverbial search for a "needle in a haystack", since informative through-space covalent linkages, hereafter referred to as intercrosslinks, are present in such protein samples at substoichiometric levels, often masked by an overabundance of unmodified, derivatized, or internally crosslinked peptides. Moreover, upon enzymatic digestion, intercrosslinked peptides give rise to relatively large analytes which tend to ionize relatively poorly. Whenever tandem MS spectra are obtained, their interpretation is far from trivial due to the population of these spectra with fragments from two different peptides. In the past 2 years, we developed a protocol which addresses this problem by deriving topology and interface information in an indirect manner following consecutive cleavages of chemically crosslinked material with cyanogen bromide (CNBr) and trypsin.[25] CNBr cleavage has long been established as a useful tool for the characterization of endogenous crosslinks present in collagens.[26−30] We demonstrated that this approach can be adapted for the design of a topology mapping method that does not rely on specialized data mining software for the assignment of crosslinks. Despite these advances, the application range of this method remains limited without a strategy that deals with the challenges posed by the overabundance of uncrosslinked material present in complex protein samples. This problem has also been recognized by others and has led to methods (reviewed in refs 31 and 32) that facilitate the detection of crosslinks by incorporation of isotopic labels in crosslinkers,[16,33,34] proteins,[35] or peptides.[36,37] Even more promising are approaches which selectively remove noninformative peptides from the sample. For this purpose, crosslinkers can be equipped with a functional group such as a biotin moiety which facilitate enrichment following their conjugation to a peptide.[24,38−43] However, the application of more complex crosslinkers may be counterintuitive if the long-term objective is the study of protein complex topologies following in vivo crosslinking of cells and intact tissues. Furthermore, the use of affinity-tagged crosslinking reagents will lead to the concomitant purification of noninformative peptides which are merely derivatized or contain internal covalent linkages. We, therefore, favor a strategy which capitalizes on the fact that intercrosslinked CNBr fragments can be distinguished from all other contaminants by the presence of two peptide chains and, therefore, two N- and C-termini. Here, we report on a novel tandem affinity strategy for the purification of crosslinked peptides based on these concepts. The method employs the presence of homoserine lactone (HSL) moieties at the C-termini of CNBr-cleaved peptides for the attachment of polyhistidine and biotin tags. Using model peptides and proteins, we demonstrate sensitive and selective enrichment of intercrosslinked peptides from samples of increasing complexity.

EXPERIMENTAL SECTION

Peptides and Other Reagents. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (Oakville, ON, Canada). The N-terminally acetylated model peptide AcCAPQEGILEDMPVDPDNEAY was synthesized using an automated peptide synthesizer (Applied Biosystems, Foster City, CA), and azurin protein was generously provided by Dr. Yi Lu (University of Illinois, Urbana, IL). The tetrahistidine (His−His−His−His−CO−NH−CH2−CH2−NH2) and biotin (biotin−CO−NH−CH_2−CH_2−NH_2) conjugation reagents were purchased from AnaSpec (San Jose, CA) and Biotium (Hayward, CA), respectively. All other chemicals were from Sigma-Aldrich.

Cyanogen Bromide Cleavage. Fifty micrograms of model peptide or protein was subjected to cyanogen bromide cleavage in a 100 μL reaction volume containing 100 mM CNBr (diluted from a 5 M CNBr stock in ACN) in 86% trifluoroacetic acid (TFA). The incubation occurred in the dark at room temperature for the duration of 16 h. Following CNBr cleavage, all nonpeptide reagents were removed by centrifugal vacuum concentration. Please note that near-complete removal of CNBr and TFA required three cycles of water addition and brief mixing of solvents followed by speed vacuum concentration to a volume of 10 μL.

(17) Schulz, D. M.; Ihling, C.; Clore, G. M.; Sinz, A. *Biochemistry* **2004**, *43*, 4703–4715.
(18) Sinz, A. *J. Mass Spectrom.* **2003**, *38*, 1225–1237.
(19) Young, M. M.; Tang, N.; Hempel, J. C.; Oshiro, C. M.; Taylor, E. W.; Kuntz, I. D.; Gibson, B. W.; Dollinger, G. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5802–5806.
(20) Lanman, J.; Lam, T. T.; Barnes, S.; Sakalian, M.; Emmett, M. R.; Marshall, A. G.; Prevelige, P. E., Jr. *J. Mol. Biol.* **2003**, *325*, 759–772.
(21) Rappsilber, J.; Siniossoglou, S.; Hurt, E. C.; Mann, M. *Anal. Chem.* **2000**, *72*, 267–275.
(22) Schmitt-Ulms, G.; Hansen, K.; Liu, J.; Cowdrey, C.; Yang, J.; DeArmond, S.; Cohen, F. E.; Prusiner, S. B.; Baldwin, M. A. *Nat. Biotechnol.* **2004**, *22*, 724–731.
(23) Tang, X.; Yi, W.; Munske, G. R.; Adhikari, D. P.; Zakharova, N. L.; Bruce, J. E. *J. Proteome Res.* **2007**, *6*, 724–734.
(24) Trester-Zedlitz, M.; Kamada, K.; Burley, S. K.; Fenyo, D.; Chait, B. T.; Muir, T. W. *J. Am. Chem. Soc.* **2003**, *125*, 2416–2425.
(25) Weerasekera, R.; She, Y. M.; Markham, K. A.; Bai, Y.; Opalka, N.; Orlicky, S.; Sicheri, F.; Kislinger, T.; Schmitt-Ulms, G. *Proteomics* **2007**, *7*, 3835–3852.
(26) Eyre, D. R.; Glimcher, M. J. *Biochem. J.* **1973**, *135*, 393–403.
(27) Matsui, Y.; Wu, J. J.; Weis, M. A.; Pietka, T.; Eyre, D. R. *Matrix Biol.* **2003**, *22*, 123–129.
(28) Piez, K. A.; Miller, E. J.; Lane, J. M.; Butler, W. T. *Biochem. Biophys. Res. Commun.* **1969**, *37*, 801–805.
(29) Stanescu, V.; Maroteaux, P.; Stanescu, R. *Ann. Genet.* **1976**, *19*, 119.
(30) Wu, J. J.; Eyre, D. R. *Biochemistry* **1984**, *23*, 1850–1857.
(31) Sinz, A. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 1100–1113.
(32) Weerasekera, R.; Schmitt-Ulms, G. *Biotechnol. Genet. Eng. Rev.* **2006**, *23*, 41–62.
(33) Mueller, D. R.; Schindler, P.; Towbin, H.; Wirth, U.; Voshol, H.; Hoving, S.; Steinmetz, M. O. *Anal. Chem.* **2001**, *73*, 1927–1934.
(34) Petrotchenko, E. V.; Olkhovik, V. K.; Borchers, C. H. *Mol. Cell. Proteomics* **2005**, *4*, 1167–1179.
(35) Taverner, T.; Hall, N. E.; O'Hair, A. J.; Simpson, R. J. *J. Biol. Chem.* **2002**, *277*, 46487–46492.
(36) Back, J. W.; Notenboom, V.; De Koning, L. J.; Muijsers, A. O.; Sixma, T. K.; De Koster, C. G.; De Jong, L. *Anal. Chem.* **2002**, 74.
(37) Huang, B. X.; Kim, H.-Y.; Dass, C. *J. Am. Soc. Mass. Spectrom.* **2004**, *15*, 1237–1247.
(38) Hurst, G. B.; Lankford, T. K.; Kennel, S. J. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 832–839.
(39) Itoh, Y.; Cai, K.; Khorana, G. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4883–4887.
(40) Alley, S. C.; Trakselis, M. A.; Mayer, M. U.; Ishmael, F. T.; Jones, A. D.; Benkovic, S. J. *J. Biol. Chem.* **2001**, *276*, 39340–39349.
(41) Back, J. W.; Sanz, M. A.; De Jong, L.; Koning, L. J.; Nijtmans, L. G. J.; De Koster, C. G.; Grivell, L. A.; Van Der Spek, H.; Muijsers, A. O. *Protein Sci.* **2002**, *11*, 2471–2478.
(42) Bennett, K. L.; Kussmann, M.; Bjork, P.; Godzwon, M.; Mikkelsen, M.; Sorensen, P.; Roepstorff, P. *Protein Sci.* **2000**, *9*, 1503–1518.
(43) Chowdhury, S. M.; Du, X.; Tolic, N.; Wu, S.; Moore, R. J.; Mayer, M. U.; Smith, R. D.; Adkins, J. N. *Anal. Chem.* **2009**.

Conjugation of Affinity Tags to Homoserine Lactone Moiety. CNBr-cleaved peptides or proteins (1−5 μg) were fully converted into their lactonized forms by the addition of 20 μL of 100% TFA and subsequent drying in a speed vacuum concentrator. Affinity tags functionalized with a primary amine group were dissolved in anhydrous dimethyl sulfoxide (DMSO) and added to the dried lactonized pellet. To maximize the conjugation efficiency, multiple parameters of this reaction were optimized (as detailed in the Results and Discussion section). The yield of conjugates was monitored by MALDI-time-of-flight (TOF) mass spectrometry. Following the conjugation of the tetrahistidine tag, C_{18} reversed-phase ZipTip (Millipore, Bedford, MA) cleanup was undertaken to remove nonreacted tetrahistidine reagent. The peptides were eluted from the ZipTip matrix with 50% isopropanol in 0.1% TFA, and the eluent was evaporated to dryness in a speed vacuum concentrator. No cleanup of nonreacted labeling reagent was required following the second conjugation step with the biotin reagent.

IMAC Purification. The tetrahistidine-tagged peptides were purified by immobilized metal ion affinity chromatography (IMAC), essentially following a method that had been described before.[44] Briefly, Gelloader pipet tips (Eppendorf, Hamburg, Germany) were packed with 10−15 μL of 50% w/v nickel-nitrilo-triacetic acid (Ni-NTA) agarose (Qiagen, Valencia, CA). Following the conjugation step, the volume of the reaction mixture containing an excess of nonconjugated tag reagent was initially reduced in a speed vacuum concentrator to 2 μL, then diluted with Ni-NTA loading buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH 8.0), and loaded onto a pre-equilibrated Ni-NTA-packed Gelloader tip. Following extensive washing with Ni-NTA loading buffer containing 2 mM imidazole, the slurry was briefly subject to a pre-elution rinse with water, and peptides were eluted with 0.2% TFA in 50% acetonitrile. Following volume reduction by speed vacuum concentration, the pH of the eluate was neutralized by the addition of an excess of phosphate buffered saline (PBS) (20 mM Na_2HPO_4, 150 mM NaCl).

Streptavidin Beads. Streptavidin agarose beads (Pierce, Rockford, IL) were pre-equilibrated in PBS and added to the pH-adjusted eluent from the Ni-NTA purification step (15 μL of 50% slurry per sample). The capture of biotinylated proteins occurred with end-over-end rotation overnight at 4 °C. Subsequently, streptavidin beads were subjected to washes with: 2 × 50 μL of 0.1% BSA in PBS; 2 × 50 μL of 0.1% BSA + 0.1% SDS in 1× PBS; 2 × 50 μL of 0.1% BSA + 1 M NaCl in 1× PBS; 2 × 50 μL of 20% methanol + 100 mM imidazole in 50 mM NH_4HCO_3, and 50 μL of water. The inclusion of BSA and imidazole in the wash buffer served to minimize unspecific interactions of unlabeled and polyhistidine-containing peptides to the affinity matrix during this step. Elution of biotin-tagged peptides proceeded in the presence of 30% acetonitrile in 5% formic acid.

Mass Spectrometry. Analytes were mixed on the matrix-assisted laser desorption/ionization (MALDI) target with an equal volume of 2,5-dihydroxybenzoic acid (DHB) matrix (100 mg/mL in 50% acetonitrile, 0.1% TFA), and droplets were air-dried. Data were acquired on a QStarXL quadrupole time-of-flight (QqTOF) mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with an orthogonal MALDI (oMALDI) source and a nitrogen laser operating at 337 nm with a pulse frequency of 20 Hz. Mass spectra were collected under the control of the operating software Analyst QS (Applied Biosystems/MDS Sciex) by averaging laser shots as indicated in the individual figures.

RESULTS AND DISCUSSION

Design of Affinity Enrichment Method. In designing a method for the purification of covalently linked peptides, key concerns were (i) to ensure tolerance of the method to denaturing conditions, (ii) to minimize sample loss, and (iii) to avoid steps limiting its applicability to a particular type of covalent linkage or protein sample. In contrast to conventional peptides, covalently linked peptides contain two peptide strands and, thus, two N- and C-termini. A method that capitalizes on this discriminating feature for affinity enrichment has conceptually the advantage, compared to methods which rely on crosslinking reagents with built-in enrichment functionalities, that it restricts enrichment to inter-crosslinked peptides, as opposed to internally linked or merely derivatized peptides (Figure 1a). Conveniently, the cleavage of methionine-containing peptides with CNBr generates homoserine lactone (HSL) moieties at the C-termini of peptides, which can conjugate to any reagent harboring a primary amine group (Figure 1b). The intermediate reactivity of the HSL electrophile is ideal in this context, as it translates into a negligible conjugation rate to primary amines present at the N-terminus of peptides or within lysine side chains. However, when reagents which harbor a primary amine are added in excess of millimolar concentrations, the conjugation of HSL moieties proceeds at neutral pH within a few hours with excellent yields (>90%). This bio-orthogonal chemical characteristic of the HSL moiety has been recognized before and has, for example, found application in the highly specific coupling of HSL-containing peptides to amino glass resins used in solid-phase peptide sequencing.[45,46]

Optimization of the Homoserine Lactone Conjugation Reaction. A critical step in this method constitutes the conjugation of HSL moieties to affinity tags harboring primary amine groups through an aminolysis reaction. While reports describing similar reactions can be found in the literature, most of the available reports date back more than two decades[45−47] and objectives pursued here were sufficiently different from earlier applications to warrant reinvestigation of the characteristics of this reaction. A series of small-scale optimization experiments was conducted in which CNBr cleavage products of bovine serum albumin (BSA) or alcohol dehydrogenase (ADH) were conjugated to small compounds harboring primary amines (propylamine, tris, ethanolamine, amino acids, etc.). Parameters explored were (i) the duration of the reaction; (ii) the concentration of the reagent contributing the primary amine; (iii) the reaction temperature; (iv) the pH during the reaction; and (v) the reaction solvent. These experiments revealed that the coupling reaction proceeds to completion within 6 h. However, extended overnight incubations did not cause appreciable side reactions. The optimum molar ratio of primary amine- and HSL-containing reactants depended somewhat on the chemical environment of the primary amine and,

(44) Stensballe, A.; Andersen, S.; Jensen, O. N. *Proteomics* **2001**, *1*, 207–222.

(45) Horn, M. J. *Anal. Biochem.* **1975**, *69*, 583–589.
(46) Horn, M. J.; Laursen, R. A. *FEBS Lett.* **1973**, *36*, 285–288.
(47) Offord, R. E. *Biochem. J.* **1972**, *129*, 499–501.

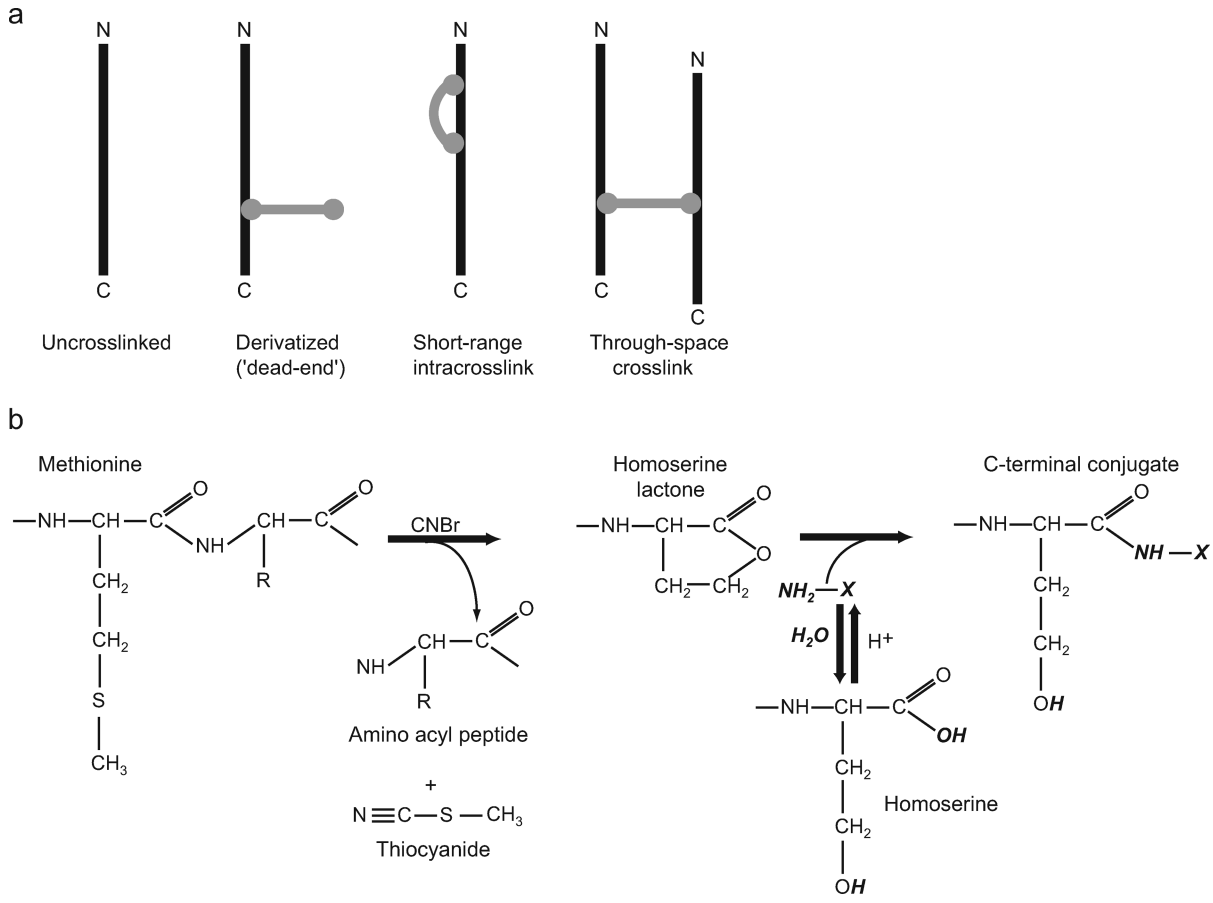

Figure 1. Conjugation of primary amine-containing affinity tags to HSL moieties present at C-termini of CNBr cleavage products. (a) Expected CNBr cleavage products following chemical crosslinking. (b) CNBr cleavage of methionine-containing peptides generates HSL moieties under highly acidic conditions. At neutral pH, the HSL moiety undergoes a slow reversible ring-opening reaction with incorporation of a water molecule to form homoserine. However, in the presence of an excess of reagents harboring primary amines, the homoserine lactone electrophile will form an irreversible bond with this reagent, a feature that can be exploited for the attachment of an affinity tag.

therefore, fell within a range of molar ratios from 1000:1 to 10 000:1 (for peptide-HSL levels of 200 pmol). With propylamine as the coupling reagent, the reaction reached completion at a molar ratio of 3200:1 (Figure 2). The optimum reaction temperature was determined to vary between 45 and 55 °C. Previous reports had already established the importance of a near-neutral pH for efficient conjugation.[47,48] Experiments conducted for this project confirmed that at basic pH, the HSL moiety is removed from the intended reaction by converting to the free acid form of homoserine (HS) (Figure 1b). In an acidic environment, the reactivity of the primary amine-containing molecules is dramatically decreased due to its protonation. Anhydrous solvents (e.g., DMSO, dimethyl formamide (DMF)) are, therefore, preferred over water-based solvents which promote isomerization of HSL into HS and thereby lead to decreases in reaction yield.[49] As expected, the primary amine-containing reactant had to be offered in freebase form. Whenever a freebase form was not commercially available, reaction yields could be improved by increasing basicity through addition of ammonium hydroxide.

Tandem Affinity Purification of Covalently Linked Peptides. Two strategies can be pursued for the enrichment of crosslinked peptides exploiting the C-terminal HSL moiety: (1) a reagent harboring a bio-orthogonal functionality for affinity capture could be attached to all C-termini. The successful implementation of this approach requires the affinity capture step to cleanly separate intercrosslinked CNBr fragments harboring two of these functionalities from singly tagged peptides. Possible examples of such tags represent polyhistidine peptides in conjunction with immobilized metal ion affinity chromatography (IMAC)[50] or perfluorinated compounds that can be separated on solid-phase matrices.[51] (2) Alternatively, HSL moieties at the C-termini of CNBr fragments could be conjugated to either one of two different affinity tags. The presence of two different affinity tags at the two C-termini of through-space crosslinked peptides would then enable their tandem affinity purification on orthogonal affinity matrixes. Because unmodified, derivatized, and internally crosslinked CNBr fragments would at most carry one of the two alternative affinity tags, they would be removed from the sample during one of the two affinity purification steps. Subsequent data presented in this manuscript were generated on the basis of this second strategy and demonstrate the consecutive use of polyhistidine and biotin affinity tags for the tandem affinity purification of intercrosslinked

(48) Kuliopulos, A.; Nelson, N. P.; Yamada, M.; Walsh, C. T.; Furie, B.; Furie, B. C.; Roth, D. A. *J. Biol. Chem.* **1994**, *269*, 21364–21370.
(49) Gross, E.; Witkop, B. *J. Biol. Chem.* **1962**, *237*, 1856–1860.
(50) Hochuli, E.; Bannwarth, W.; Doebeli, H.; Gentz, R.; Stueber, D. *Nat. Biotechnol.* **1988**, *6*, 1321–1325.
(51) Brittain, S. M.; Ficarro, S. B.; Brock, A.; Peters, E. C. *Nat. Biotechnol.* **2005**, *23*, 463–468.

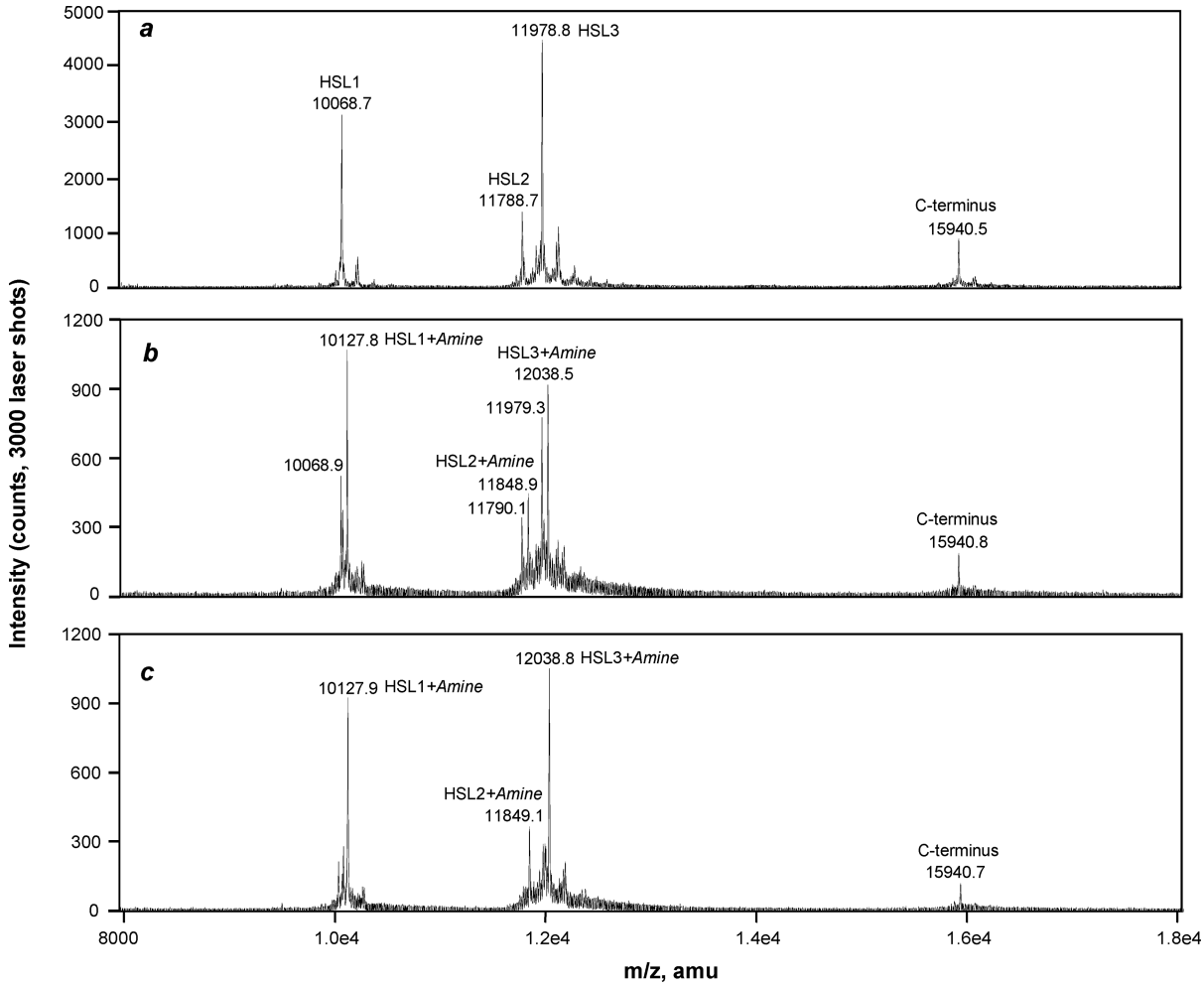

Figure 2. Concentration series documenting conjugation of primary amine-containing reagents to HSL-moieties present at C-termini of CNBr cleavage products. MALDI-TOF mass spectra of (a) CNBr cleavage products of 10 pmol BSA, (b) conjugation of CNBr fragments from 2 pmol bovine serum albumn (BSA) with 100 mM propylamine, and (c) conjugation of CNBr fragments from 2 pmol bovine serum albumn (BSA) with 500 mM propylamine. CNBr fragments of BSA/HSL1, amino acids 25−111; HSL2, amino acids 112−208; HSL3, amino acids 470−571; C-terminus, amino acids 470−607; amine, propylamine (MW: 59 Da). The C-terminal fragment is refractory to the conjugation reaction because it does not carry an HSL moiety.

peptides (Figure 3). More specifically, the method employs a tetrahistidine tag for a first separation on nickel-nitrilo-triacetic acid (Ni-NTA)-derivatized IMAC matrixes and a biotin tag for the second affinity purification step based on streptavidin agarose. CNBr cleavage products equipped with none or only one of the two affinity tags were removed with the unbound material during the extensive washing steps that preceded selective elution from Ni-NTA and streptavidin matrices. Due to the nature of the selected affinity tags, this method should be compatible with fully denaturing conditions such as the presence of 8 M urea. A similar two-step procedure has recently been proposed for the elegant tandem affinity purification of intact proteins engineered to contain a 6-histidine tag and a bacterial signal sequence which mediated in vivo biotinylation.[52]

Application of Purification Method to Disulfide-Linked Model Peptide. To test the purification method on a sample with low complexity, a model peptide was synthesized which contained a cysteine residue, allowing for a readily formed intermolecular disulfide linkage, and a methionine residue to support the formation of C-terminal HSL moieties upon chemical cleavage with CNBr (AcCAPQEGILEDMPVDPDNEAY, theoretical mass: 2396.05 Da). A quantity of 100 pmol of this peptide was subjected to CNBr cleavage (Figure 4a) followed by conjugation of tetrahistidine (MW: 622 Da) and biotin (MW: 286 Da) tags to C-terminal HSL moieties. The conjugation efficiency of the two affinity tags was initially tested by employing the above-mentioned reaction conditions and including both tagging reagents at a 1:1 molar ratio. However, because the coupling of the biotin compound proceeded at a higher rate than the attachment of the tetrahistidine conjugate under those conditions, probably a consequence of a difficulty to obtain the tetrahistidine reagent in its freebase form, subsequent experiments were based on consecutive conjugations of, first, the tetrahistidine compound and, second, the biotin reagent. Please note that the theoretical best outcome is not a quantitative conversion of intercrosslinked peptides to conjugates containing both tags. Due to the fact that the conjugation also generates molecules with two identical biotin tags (25%) or two tetrahistidine tags (25%), the highest yield of the desired end product would be 50% if the two conjugation reagents were offered concomitantly and the reaction proceeded at equal rates for both reagents to

(52) Tagwerker, C.; Flick, K.; Cui, M.; Guerrero, C.; Dou, Y.; Auer, B.; Baldi, P.; Huang, L.; Kaiser, P. *Mol. Cell. Proteomics* **2006**, *5*, 737–748.

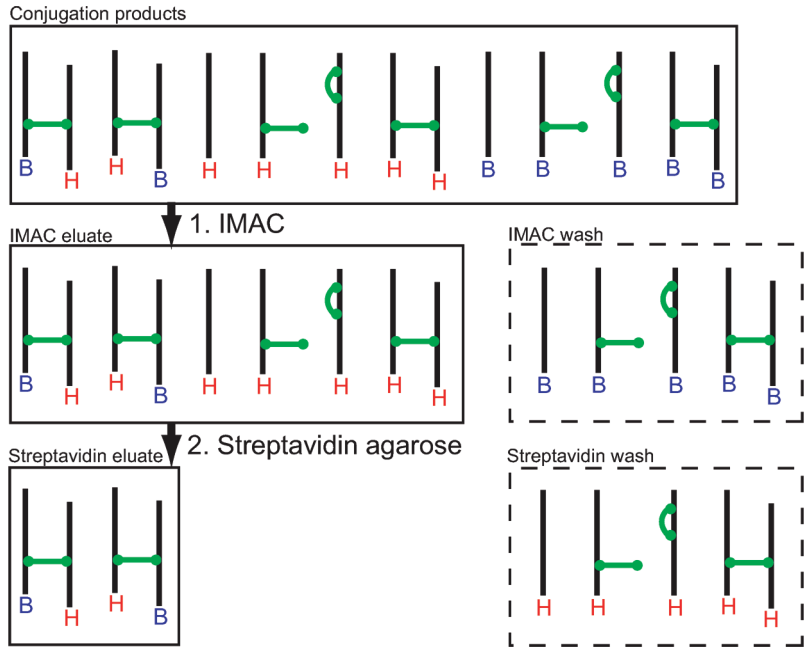

Figure 3. Schematic depiction of method for tandem affinity purification of through-space covalently linked peptides. Following conjugation of affinity tags to C-terminal HSL moieties present at the C-termini of CNBr cleavage products, conjugates are consecutively captured on IMAC and streptavidin agarose matrixes. Unmodified, derivatized, and internally crosslinked peptides, as well as through-space crosslinked peptides which carry only one type of affinity tag, are removed from the sample during washing steps. H, polyhistidine tag; B, biotin tag.

completion. An improved yield of about 65% desired end product was achieved using the following procedure: Initially, the tetrahistidine reagent was added to CNBr cleavage products on its own. Conveniently, the overnight incubation of this reaction mix proceeded reproducibly to 60−70% yields, a level of conjugation which ensured that almost all of the original intercrosslinked substrate molecules had been conjugated to at least one tetrahistidine moiety (Figure 4b). Subsequently, the nonreacted tetrahistidine-tagging reagent was removed by reversed-phase C_{18} cleanup. The second conjugation to the biotin-tagging reagent was then allowed to proceed to completion (Figure 4c).

The affinity-tagged peptides were loaded onto self-packed Ni-NTA gel loading tips, subjected to extensive washing to remove nonreacted biotin-tagging reagent, and eluted in batch format in the presence of imidazole. Subsequently, biotinylated peptides in the Ni-NTA eluate fraction were captured on streptavidin agarose. The near-covalent affinity constant of the biotin−streptavidin interaction ($K_d = 10^{-15}$) enabled stringent washing conditions, which ensured effective removal of nonbiotinylated peptides. The detachment of biotin from streptavidin requires harsh conditions of pH and solvents. Thus, for this step, a protocol was adapted which employs a combination of low pH and inclusion of acetonitrile to dissociate the biotin−streptavidin bond.[53−55] As expected, the MALDI-MS spectrum of analytes in the Ni-NTA eluate is quite similar to that of the initial CNBr fragment mixture since each conjugate contains at least one tetrahistidine (Figure 4d). The analysis of eluate fractions corroborated the choice of the biotin−streptavidin bond for the second purification step as it demonstrated a highly selective purification of disulfide-linked peptides carrying both a tetrahistidine and a biotin tag (Figure 4e).

Application of the Method to Azurin Protein. We next applied the method to azurin, a copper-binding protein named after the characteristic blue color it acquires in its metal-bound state. Azurin was selected for its relatively small size of around 14 kDa, the presence of only one internal disulfide bond, and the existence of multiple methionine residues in its sequence, which suggested that it may provide a suitable CNBr cleavage substrate. CNBr cleavage of azurin was expected to generate multiple small fragments, a disulfide-linked fragment of 4756.29 Da with two HSL moieties and an internal fragment of 4922.51 Da containing only one HSL at its C-terminus (Table 1). In the native azurin protein, the copper ion is coordinated by two imidazole moieties within histidines at amino acid positions 46 and 117, one cysteine thiolate (Cys112), and two weaker axial ligands, the sulfur of methionine (Met121) and the carbonyl of glycine (Gly45).[56] During the CNBr cleavage which requires highly acidic conditions, the copper ion dissociated from its binding site, a process which could be monitored by the loss of the blue color of the protein solution. MALDI-MS analysis of this sample confirmed the presence of the two above-mentioned cleavage fragments which were observed at 4756.60 and 4922.59 Da (Figure 5a and supplemental Table 1 in the Supporting Information). To minimize side reactions that may occur between disulfide bonds and free copper,[57,58] a chelation step was included for this sample prior to the conjugation

(53) Girault, S.; Chassaing, G.; Blais, J. C.; Brunot, A.; Bolbach, G. *Anal. Chem.* **1996**, *68*, 2122–2126.
(54) Rosli, C.; Rybak, J. N.; Neri, D.; Elia, G. *Methods Mol. Biol.* **2008**, *418*, 89–100.
(55) Schriemer, D. C.; Li, L. *Anal. Chem.* **1996**, *68*, 3382–3387.
(56) Garner, D. K.; Vaughan, M. D.; Hwang, H. J.; Savelieff, M. G.; Berry, S. M.; Honek, J. F.; Lu, Y. *J. Am. Chem. Soc.* **2006**, *128*, 15608–15617.
(57) Gunawardena, H. P.; O'Hair, R. A.; McLuckey, S. A. *J. Proteome Res.* **2006**, *5*, 2087–2092.
(58) Lioe, H.; Duan, M.; O'Hair, R. A. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2727–2733.

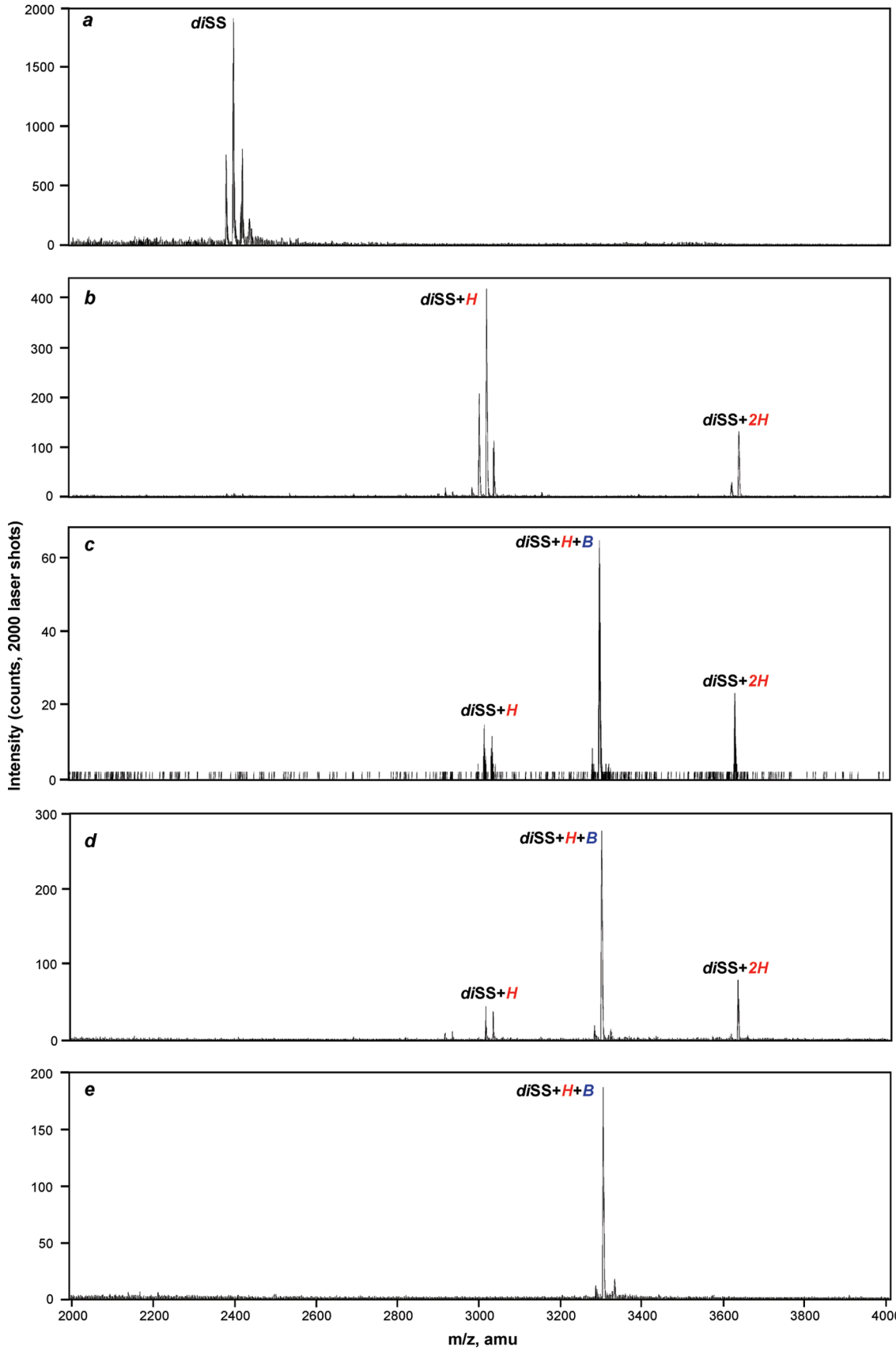

Figure 4. Successful application of method to through-space covalently linked model peptide. MALDI-TOF mass spectra of (a) CNBr cleavage product of 100 pmol disulfide-bridged model peptide; (b, c) products of conjugation of 20 pmol CNBr-cleaved disulfide model peptide with tetrahistidine (b) and biotin (c) tags; (d, e) eluate fractions from the tandem affinity purification on Ni-NTA agarose (d) and streptavidin agarose (e) matrixes. diSS, disulfide-linked CNBr fragments AcCAPQEGILEDh carrying a C-terminal HSL (h) moiety; H, tetrahistidine ethylenediamine (MW: 622 Da); B, biotin ethylenediamine (MW: 286 Da).

Table 1. MALDI MS Masses of CNBr Fragments of Bovine Serum Albumin, Disulfide-Linked Synthetic Peptide, and Azurin

Analyte	Amino acids	Peptide sequence[b]	$[MH]^+$ observed	$[MH]^+$ calculated
BSA[a]	572-607	(h)ENFVAFVDKCCAADDKEACFAVEGPKLVVSTQTALA(-)	3960.88	3960.85
	25-111	VFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVNELTEFAKT CVADESHAGCEKSLHTLFGDELCKVASLRETYGDh	10068.73[c]	10068.34[c]
	112-208	(h)ADCCEKQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYE IARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETh	11788.70[c]	11780.33[c]
	470-571	(h)PCTEDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVP KAFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVh	11977.21[c]	11976.96[c]
	470-607	(h)PCTEDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVP KAFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFV DKCCAADDKEACFAVEGPKLVVSTQTALA(-)	15941.32[c]	15944.52[c]
Disulfide-linked synthetic peptide	1-11 1-11	AcCAPQEGILEDh AcCAPQEGILEDh	2396.07	2396.05
Azurin	45-56	(h)GHNWVLSTAADh	1253.57	1253.59
	1-13 14-44	(-)AECSVDIQGNDQh (h)QFNTNAITVDKSCKQFTVNLSHPGNLPKNVh	4756.60	4756.29
	65-109	(h)ASGLDKDYLKPDDSRVIAHTKLIGSGEKDSVTFDVSKLKEGEQYh	4922.59	4922.51
	110-121	(h)FFCTFPGHSALh	1309.54	1309.60

[a] Reduced with dithiothreitol and subsequently alkylated with iodoacetamide. [b] Homoserine lactone moieties are depicted as "h", and C-termini of parent proteins are shown as "—". [c] Average isotopic mass.

reaction.[59] The downstream affinity tag incorporation followed procedures described above for the synthetic peptide and led to a relatively complex MALDI-TOF spectrum populated by multiple conjugation reaction products containing no tag, a single biotin or tetrahistidine tag, or a combination of these tags (Figure 5b−c). The purification of this sample on a Ni-NTA-packed gel loading tip, however, led to the expected enrichment of tetrahistidine-containing peptides (Figure 5d). Finally, the streptavidin-capture step allowed efficient removal of unbound peptides and enabled the exclusive purification of intercrosslinked azurin peptides which had been conjugated to both one tetrahistidine and one biotin tag (Figure 5e). The tandem affinity purification method, thus, enabled rapid enrichment of intercrosslinked peptides from an excess of noninformative peptides. This experiment further documented a strong increase in the sensitivity with which the informative species could be detected once an excess of signal-quenching analytes had been removed from the sample. The experiment was replicated more than five times with virtually identical outcomes.

Selective Purification of Covalently Linked Peptide from Large Excess of Bacterial Peptides. To assess the selectivity and robustness of the purification protocol, we next spiked 16 pmol of the CNBr- cleaved and affinity-conjugated peptides into a complex peptide mixture obtained after CNBr cleavage, reduction, and alkylation of proteins present in a crude bacterial cell extract (Figure 6a). The subsequent purification of this material on the IMAC matrix led to the copurification of a small number of peptides from the bacterial extract, thereby emphasizing the need for a tandem affinity purification methodology (Figure 6b). This observation was expected as a small number of bacterial proteins are known to harbor histidine-rich sequence motifs which can mediate binding to IMAC matrixes under the separation conditions employed. The subsequent streptavidin-based purification step achieved the desired purity of the material, confirming the excellent selectivity of the tandem purification steps employed (Figure 6c).

Efforts to minimize protein consumption during sample handling steps are ongoing. So far, a minimum amount of 10 pmol of azurin protein sample was found to be sufficient in experiments which aimed for a minimum 3:1 signal-to-noise ratio with which intercrosslinked peptides had to be detectable following their tandem affinity purification (not shown). Factors that contribute to sample losses are (i) the yield of doubly affinity-tagged crosslinked peptides, (ii) the less than quantitative recovery from affinity matrixes, and (iii) unspecific binding of analytes.

As demonstrated in this work, the affinity-enrichment of intercrosslinked CNBr fragments can be followed by their direct mass spectrometry-based analysis. Alternatively, more complex mixtures of intercrosslinked CNBr fragment can be separated by two-dimensional gel separation. The identification of these fragments can then follow their in-gel trypsinization. Given a statistical representation of methionines of approximately 1.8% of all amino acids in vertebrate genomes, the average length of CNBr fragments is expected to be just over 50 amino acids. The tryptic digestion of intercrosslinked CNBr fragments, therefore, can give rise to multiple short peptides which can support the identification of these CNBr fragments.[25]

The assignment of crosslinked CNBr fragments following their enrichment may also be realized by accurate mass determination on a high resolution mass spectrometer. For this alternative assignment method to work, an inventory list of proteins present

(59) Gabellieri, E.; Strambini, G. B. *Biophys. J.* **2006**, *90*, 3239–3245.

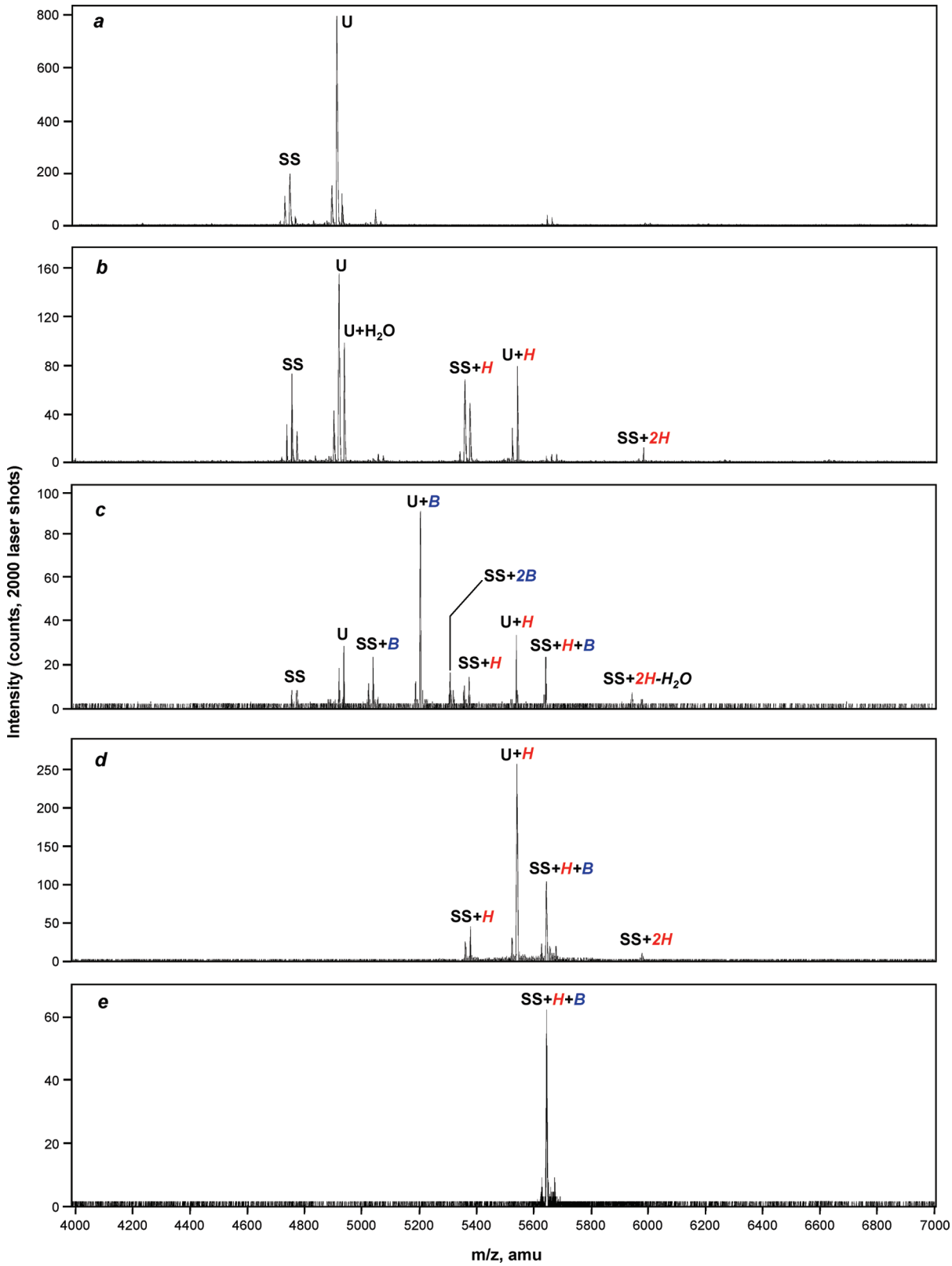

Figure 5. Successful application of method to through-space covalently linked CNBr cleavage product of azurin by tandem affinity purification. MALDI-TOF mass spectra of (a) CNBr cleavage products of 40 pmol azurin; (b, c) products of conjugation of 16 pmol CNBr-cleaved azurin with tetrahistidine (b) and biotin (c) tags; (d, e) eluate fractions from the tandem affinity purification on Ni-NTA agarose (d) and streptavidin agarose (e) matrixes. CNBr fragments of azurin: SS, disulfide-linked peptides containing amino acids 1−13 and 14−44 with two HSL moieties at C-termini; U, amino acids 65−109, noncrosslinked peptide with one HSL moiety; H, tetrahistidine ethylenediamine (MW: 622 Da); B, biotin ethylenediamine (MW: 286 Da).

in a given sample will have to be generated beforehand to narrow the computational protein search space. Fourier transform mass spectrometers and orbitrap instruments perform well in the intermediate mass range required for the direct detection of

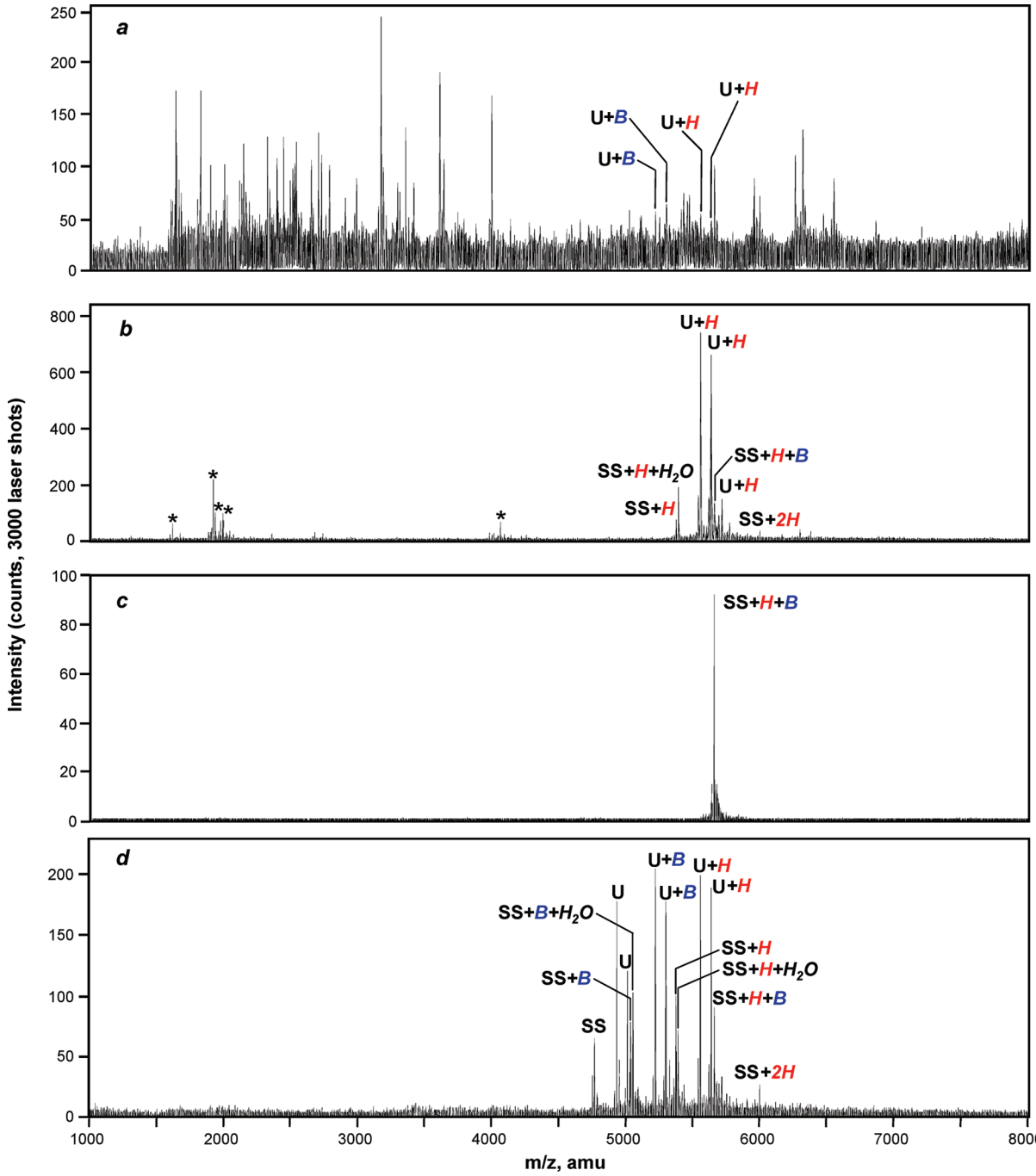

Figure 6. Selective purification of through-space covalently linked CNBr cleavage product of azurin from an excess of CNBr cleavage products of bacterial cell lysis by tandem affinity purification. MALDI-TOF mass spectra of (a) 16 pmol of affinity-tagged CNBr cleavage products of azurin spiked into CNBr digest of a bacterial cell lysate (without detergents), (b) Ni-NTA agarose eluate, (c) streptavidin agarose eluate, and (d) affinity-tagged CNBr cleavage products of 16 pmol azurin used for spiking of bacterial extract. Please note that a comparison of relative intensities of signals seen in panels c and d only allows an approximation of recovery yields because of ion suppression effects and related phenomena. CNBr fragments of azurin: SS, disulfide-linked peptides containing amino acids 1−13 and 14−44 with two HSL moieties at C-termini; U, amino acids 65−109, noncrosslinked peptide with one HSL moiety; phosphorylated U, amino acids 65−109 with one phosphorylation modification; H, tetrahistidine enthylenediamine (MW: 622 Da); B, biotin enthylenediamine (MW: 286 Da).

crosslinked CNBr fragments and the larger size of these fragments (compared to peptides present in tryptic digests) is expected to add discriminatory power for the computational assignment of CNBr fragment identities.

It should be noted that this method is conceptually fully compatible with its application to biological samples in vivo. However, it is anticipated that a move to in vivo crosslinking would have to be paralleled by an increase in the amount of analyte. This need arises from the observation that only substoichiometric levels of crosslinking can be introduced because excessive crosslinking may interfere with the extraction and subsequent fragmentation of proteins. Furthermore, given the wide range of levels of protein expression in biological source materials, work with in vivo crosslinked protein samples may require additional prefractionation steps to accommodate the increase in complexity of CNBr digests. Additional efforts, beyond the scope of this

manuscript, are required to reach the long-term objective to generate a robust method for the sensitive mapping of interfaces/topologies in cells and tissues. Currently, relatively little is known about the true complexity of samples derived from in vivo crosslinked cells and tissues. Whereas multiple reports exploit in vivo crosslinking to stabilize protein complexes prior to their affinity capture, the extent to which individual cellular proteins (and the complexes they are part of) are crosslinked in such biological material has remained poorly understood.

CONCLUSIONS

This work described a novel method for the effective purification of intercrosslinked peptides. The method capitalizes on the presence of C-terminal homoserine lactone moieties in CNBr-cleaved proteins for the bio-orthogonal tagging and purification of intercrosslinked peptides. Unlike alternative methodologies, which have been described in the literature and employ crosslinkers with built-in enrichment functionalities, this strategy is not conceptually limited in its application to a specific type of covalent linkage. It, thereby, avoids the unintended copurification of derivatized or internally linked peptides. Our data document excellent selectivity for exclusively intercrosslinked peptides with this method. Future method development efforts will be needed to extend the utility of this approach to low quantity biological samples and ultimately the systematic and proteome-wide analysis of material obtained following in vivo crosslinking of cells or tissues.

ACKNOWLEDGMENT

We thank Sepehr Ehsani for his critical comments and suggestions for improving the manuscript. Work on this project was funded through support from the Canadian Institutes of Health Research (MOP-74734-GSU). G.S.-U. received generous support from the W. Garfield Weston Foundation.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review June 24, 2009. Accepted October 26, 2009.

AC901373Q