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A novel protein crosslinking reagent for the determination of moderate resolution protein structures by mass spectrometry (MS3-D)☆

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Abstract—A new approach to the determination of moderate resolution protein structures, termed MS3-D—Mass Spectrometry in 3 Dimensions—has recently been disclosed. The method involves the formation of covalent crosslinks between reactive residues on the protein surface, the determination of the location of those crosslinks in primary sequence space by mass spectrometry, and then the imposition of a distance constraint upon the location of the respective side chains during distance geometry calculations of protein structure. MS3-D is rapid, requires small amounts of protein, and works in native biochemical conditions. Therefore, it offers the potential for determination of the structures of all proteins expressed by an organism in a high throughput manner. However, the methodology is completely dependent upon the production of chemical crosslinks and technical limitations of available crosslinkers have proven problematic in generalization and automation of the method for the determination of the structures of complete proteomes. Presented herein is the design, synthesis, and proofing of a novel modular protein crosslinking reagent designed to enhance hydrophilicity, provide an increased effective signal to noise ratio for MS3-D, and allow the sampling of a wider variety of side chains during the process.

Two approaches have been reported for the determination of moderate resolution protein structures by chemical crosslinking, mass spectrometry, and computation (mass spectrometry in 3 dimensions, MS3-D): the bottom up, in which crosslinked proteins are digested by proteases prior to analysis,[2,3,9] and the top down, in which intact crosslinked protein is used for the analysis.[6] Inherent limitations of existing crosslinking reagents have proven to be a major technical obstacle in high throughput MS3-D. First, the hydrophobicity of crosslinkers with long aliphatic arms is problematic due to hydrophobic collapse. Second, the efficiency of detection of the required singly crosslinked protein or peptides is often low. Finally, available commercial reagents do not allow for formation of crosslinks of the same length with diverse chemistries. We report herein the design, synthesis, and evaluation of a modular affinity-tagged crosslinker, **1**, optimized for use in MS3-D.

Hydrophilic crosslinkers exist with PEG chains, with amino acid cores,[7] and with combinations of PEG and cleavable linkers. However, none were available with bifunctional lysine reactivity, a noncleavable linker arm, and an affinity tag. Studies with analogous crosslinkers of various length, lacking the affinity tag, have revealed that crosslinkers with a sampling range of 5–10 angstroms are optimal for providing distance geometry information about the placement of lysine backbone for threading calculations.[4] Thus, combination of a single amide core and a PEG linker led to designed crosslinker **1**, with a −1.7 ClogP value,[5] and a similar crosslinking distance to disuccynimidylsuberate (DSS) (Fig. 1). The reagent also included an affinity tag (biotin) to facilitate signal enhancement.

Keywords: Protein crosslinking affinity reagent MS3D.
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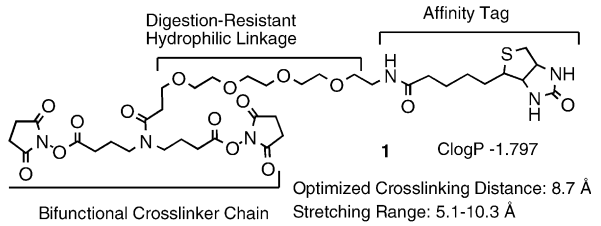

Figure 1. Design of hydrophilic affinity-tagged crosslinking reagent **1**.

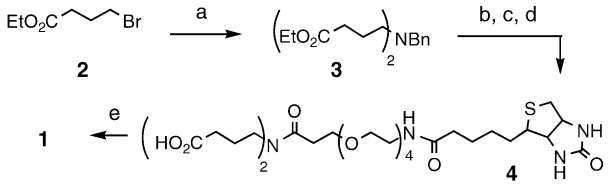

Figure 2. Synthesis of crosslinking reagent **1.** (a) Benzylamine (0.5 equiv), K_2CO_3 (3.0 equiv), DMF, 30–40 °C, 2 d, 70%; (b) H_2, Pd–C, MeOH, HCl, rt, 1.5 h quant; (c) dPEG4-biotin acid (0.8 equiv), HBTU (1.3 equiv), DIPEA (3.0 equiv), DMF, rt, 1 h, quant; (d) MeOH, NaOH (2.5 equiv), H_2O, 45 °C, 7 h, then HCl, 60%; (e) HOSu (2.0 equiv), PL-EDC (3.4 equiv), 4 Å molecular sieves, DMF, $CHCl_3$, rt, 15 h, 86%.

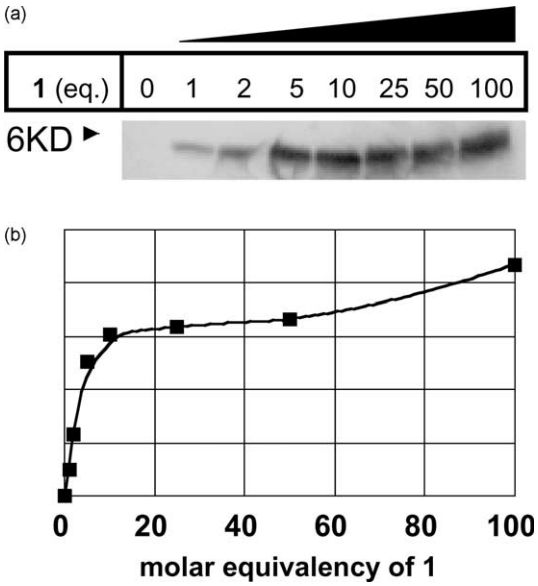

Figure 3. Western blot analysis of ubiquitin-**1** conjugates confirms biotinylation of ubiquitin and binding to streptavidin. Ubiquitin (1 μg/lane) was reacted with varied molar equivalents of **1**, purified by SDS-PAGE on an 18% Tris–Glycine gel, electroblotted to PVDF membrane, and incubated with Streptavidin–AP conjugate to detect the crosslinking. Panel A shows the blot and molar equivalency of **1**. Panel B shows the quantitative measurement of the relative amounts of the biotin conjugation.

The synthetic route to **1** (Fig. 2) proceeds smoothly and allows for large-scale preparations. The crosslinking subunit is assembled convergently, allowing for later variation of the pendant crosslinkers. In this case, the bis-succinimidyl ester is formed using solid-supported carbodiimide[1] to avoid aqueous work up and chromatographic purification. Crosslinker **1** is water soluble, easily handled, and stable in DMSO solution at −80 °C for more than 2 months.

Crosslinking reagent **1** efficiently crosslinks the pendant lysine sidechains of proteins. Ubiquitin was allowed to react with increasing amounts of **1**, purified by SDS-PAGE, and detected by streptavidin–AP conjugate blotting. The only observed species corresponded to the molecular weight of ubiquitin (Fig. 3A). Saturation of biotin conjugation was observed slightly above 5-molar equivalents of **1** (Fig. 3B), corresponding to the six lysines in the protein. Overall, reactivity is comparable to DSS. Thus, **1** efficiently crosslinks ubiquitin at equimolar concentrations, single crosslinks can be titrated by using lower concentrations of **1**, and large excess of **1** does not cause side reaction with other residues.

Protein conjugates of **1** can be purified by avidin affinity chromatography to remove unconjugated protein (due to lack of affinity for avidin) *and* multiply labeled protein (due to high avidity for avidin). Ubiquitin was allowed to react with **1** and excess crosslinking reagent removed by centrifugal size exclusion filtration. The protein sample was then incubated with streptavidin agarose. The agarose was subsequently washed and the bound ubiquitin was eluted from the agarose using acidic acetonitrile. The eluted sample was analyzed by ESI-QTOF MS. The relative concentrations of doubly modified and unmodified ubiquitin were strongly reduced and desired mono- modified ubiquitin strongly enhanced in the eluted solution (Fig. 4). Thus, the species needed for generation of constraints for MS3-D are essentially the exclusive product of the use of this crosslinking reagent and procedure.

This study clearly demonstrates the utility of a chemical protein crosslinker designed to overcome the difficulties with hydrophobicity, lack of selectivity, and lack of sensitivity observed when commercially available crosslinkers are used in the MS3-D process. Crosslinker **1** is stable and water soluble, has good reactivity, and is both selective (suppresses uncrosslinked and multiply-labeled species relative to singly labeled) and sensitive (enhances crosslinked species relative to unreacted)

Compound **1** also provides a chemically flexible platform for the production of a wide range of crosslinkers. Studies using tetra O^{18}-labeled **1** to apply isotope driven MS^n methodology,[8] cysteine reactive and photoreactive versions of **1** to allow sampling of a wider range of side chain placements, and multiple other protein systems are underway and will be reported in due course.

Supporting information available

Synthetic procedures, SDS-PAGE/Western blotting protocols, mass spectrometry protocols and data which can be found with the online version of this paper.

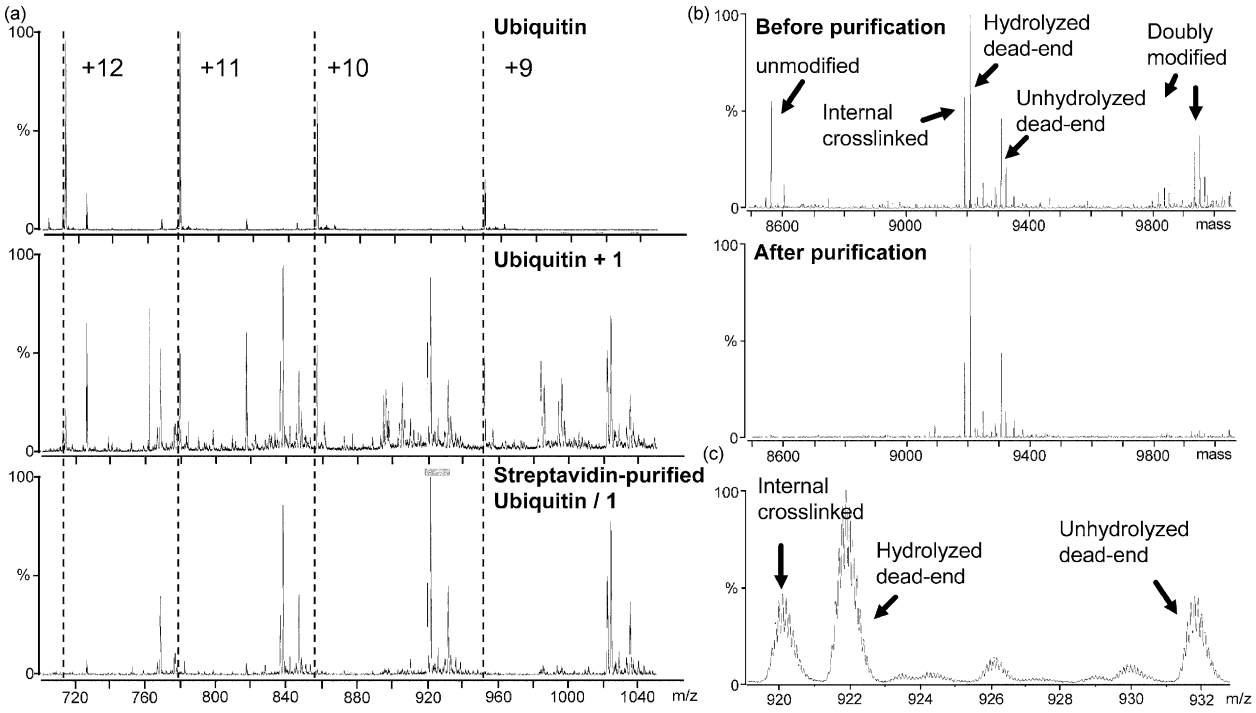

Figure 4. Ubiquitin-**1** conjugates purified using streptavidin-agarose. (a) The masses of control and modified ubiquitin were measured by QTOF-MS. Upper panel, raw data from a portion of a control ubiquitin spectrum showing the +9 to +12 charge states. Middle panel, the same *m*/*z* range following reaction of ubiquitin with 5-molar equivalents of **1**. Singly- and doubly-modified, as well as unmodified, ubiquitin can be seen, as summarized in (b). Lower panel, ubiquitin reacted with **1** eluted from streptavidin using 70% acetonitrile/5% TFA; (b) De-convolution (MaxEnt1 software, Micromass) of masses in **1**-modified spectra before (upper panel) and after (lower panel) streptavidin purification. The internally crosslinked, singly modified and hydrolyzed, and singly modified and unhydrolyzed species are labeled in the upper panel. Note the absence of unmodified and doubly modified protein following streptavidin purification in the lower panel; (c) Expansion of the +9 charge region indicated by a bar in the lower panel of (a).

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References and notes

1. Adamczyk, M.; Fishpaugh, J. R.; Mattingly, P. G. *Tetrahedron Lett*. **1995**, *36*, 8345.
2. Back, J. W.; Sanz, M. A.; De Jong, L.; De Koning, L. J.; Nijtmans, L. G.; De Koster, C. G.; Grivell, L. A.; Van Der Spek, H.; Muijsers, A. O. *Protein Sci*. **2002**, *11*, 2471.
3. Bennett, K. L.; Matthiesen, T.; Roepstorff, P. *Meth. Mol. Biol*. **2000**, *146*, 113.
4. Collins, C. J.; Schilling, B.; Young, M.; Dollinger, G.; Guy, R. K. *Bioorg. Med. Chem. Lett*. **2003**, *13*, 4023.
5. Daylight Chem Info, I.
6. Kruppa, G.; Schoeniger, J. S.; Young, M. M. *Rapid Commun. Mass Spectrom*. **2003**, *17*, 155.
7. Trester-Zedlitz, M.; Kamada, K.; Burley, S. K.; Fenyoe, D.; Chait, B. T.; Muir, T. W. *J. Am. Chem. Soc*. **2003**, *125*, 2416.
8. Turecek, F. *J. Mass Spectrom*. **2002**, *37*, 1.
9. Young, M. M.; Tang, N.; Hempel, J. C.; Oshiro, C. M.; Taylor, E. W.; Kuntz, I. D.; Gibson, B. W.; Dollinger, G. *Proc. Nat. Acad. of Sci. U.S.A*. **2000**, *97*, 5802.