Surveying Ubiquitin Structure by Noncovalent Attachment of Distance Constrained Bis(crown) Ethers

ARTICLE *in* ANALYTICAL CHEMISTRY · AUGUST 2008

Impact Factor: 5.64 · DOI: 10.1021/ac800177s · Source: PubMed

5 AUTHORS, INCLUDING:

Tony Ly
University of Dundee
25 PUBLICATIONS **672** CITATIONS
SEE PROFILE

Ryan R Julian
University of California, Riverside
96 PUBLICATIONS **2,043** CITATIONS
SEE PROFILE

Anal. Chem. **2008,** *80,* 5059–5064

Surveying Ubiquitin Structure by Noncovalent Attachment of Distance Constrained Bis(crown) Ethers

Tony Ly,[†] Zhenjiu Liu,[†] Brian G. Pujanauski,[‡] Richmond Sarpong,[‡] and Ryan R. Julian*,[†]

Department of Chemistry, University of California, Riverside, California 92521, and College of Chemistry, University of California, Berkeley, California 94720

Selective noncovalent adduct protein probing (SNAPP) mass spectrometry was recently developed to study solution-phase conformations of proteins by exploiting the specific affinity between 18-crown-6 ether (18C6) and lysine side chains. To obtain more detailed information about protein tertiary structure, a novel noncovalent cross-linking reagent with two 18C6 molecules bridged by a covalent phenyl linker (called PBC for phenyl bis-crown) was synthesized. PBC introduces a distance constraint into SNAPP experiments where pairs of lysine side chains that are held in proximity by tertiary structure should be the most favored binding sites. Application of this method to ubiquitin reveals that PBC can bind to one lysine in a monodentate fashion or bind to two lysines via a bidentate interaction. Comparison with 18C6 can be used to reveal the mode of binding. For the native state of ubiquitin, bidentate binding of PBC is not observed. The partially denatured A-state, however, contains a single pair of lysines that are both chemically available and spaced by less than ~19 Å (the maximum distance spanning the crown ether binding sites in PBC). Collision-induced dissociation and site-directed mutagenesis reveal that the bidentate PBC attaches to K29 and K33, which is in agreement with previous structural data on the A-state of ubiquitin. PBC is shown to be an effective probe of protein structure in SNAPP experiments, although assigning the specific residues to which PBC is attached can be experimentally challenging.

Protein structure is one of the keys to understanding function or malfunction[1] in biological systems at the molecular level; therefore, methods that enable the rapid screening of protein structure are needed. Mass spectrometry (MS) has several inherent advantages over X-ray[2] or NMR[3] in terms of speed and sensitivity, which has led to the development of several tools[4–14] for examining protein structure. This statement requires clarification because the most relevant environment for studying protein structure is obviously in solution, yet MS is conducted exclusively in the gas phase. Despite this fact, cleverly designed experiments can utilize MS to track structurally dependent mass shifts that occur in solution yet are retained and observed in the gas phase. In other words, the mass spectrometer is used as a detector, not a reaction vessel. One of the most common techniques employs hydrogen/deuterium exchange to probe the chemical availability of backbone amide hydrogens.[5–7] The degree of exchange is largely determined by intramolecular interactions (typically hydrogen bonds)[15] within the protein and can be easily quantified by the resulting mass shift for each exchange. In practical terms, faster exchange is usually observed for more exposed portions of the protein. Quenching of the exchange, followed by enzymatic digestion, and further MS analysis of the fragments can be used to localize fast- or slow-exchanging regions.[6] Three-dimensional structures, such as those obtained by X-ray or NMR, are not revealed and neither are tertiary structural interactions.

This shortcoming can be partially addressed by the use of chemical cross-linking studies.[9–11] In these experiments, reactive amino acid side chains are covalently cross-linked to one another. The cross-linking reagents are chosen such that the maximum distance between two amino acids can be determined.[16] Following enzymatic digestion and analysis by MS, the residues that are cross-linked are revealed because of the mass shift caused by the cross-linking agent. In this manner, proximity relationships between different amino acids can be established and provide experimental constraints on computational models of protein structure.[17] These experiments usually only provide information

* To whom correspondence should be addressed. E-mail: ryan.julian@ucr.edu.
† Department of Chemistry.
‡ College of Chemistry.

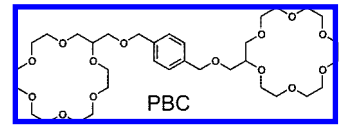
(1) Chiti, F.; Dobson, C. M. *Annu. Rev. Biochem.* **2006**, *75*, 333–366.
(2) Matthews, B. W. *Annu. Rev. Phys. Chem.* **1976**, *27*, 493–523.
(3) Dyson, H. J.; Wright, P. E. *Chem. Rev.* **2004**, *104*, 3607–3622.
(4) Loo, J. A.; Loo, R. R. O.; Udseth, H. R.; Edmonds, C. G.; Smith, R. D. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 101–105.
(5) Katta, V.; Chait, B. T. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 214–217.
(6) Zhang, Z. Q.; Smith, D. L. *Protein Sci.* **1993**, *2*, 522–531.
(7) Wales, T. E.; Engen, J. R. *Mass Spectrom. Rev.* **2006**, *25*, 158–170.
(8) Kaltashov, I. A.; Eyles, S. J. *Mass Spectrom. Rev.* **2002**, *21*, 37–71.
(9) Sinz, A. *Mass. Spectrom. Rev.* **2006**, *25*, 663–682.
(10) 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.
(11) Kruppa, G. H.; Schoeniger, J.; Young, M. M. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 155–162.
(12) Ly, T.; Julian, R. R. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 1209–1215.
(13) Friess, S. D.; Daniel, J. M.; Zenobi, R. *Phys. Chem. Chem. Phys.* **2004**, *6*, 2664–2675.
(14) Friess, S. D.; Daniel, J. M.; Hartmann, R.; Zenobi, R. *Int. J. Mass Spectrom.* **2002**, *219*, 269–281.
(15) Englander, S. W. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 213–238.
(16) Green, N. S.; Reisler, E.; Houk, K. N. *Protein Sci.* **2001**, *10*, 1293–1304.
(17) van Dijk, A. D. J.; Boelens, R.; Bonvin, A. M. J. J. *FEBS J.* **2005**, *272*, 293–312.

10.1021/ac800177s CCC: $40.75
Published on Web 05/23/2008

about the proximity of side chains, and the backbone is not directly probed. Caution should be employed while interpreting the results, because factors other than spatial proximity can influence cross-linking potential including chemical reactivity[18] (which may be a function of intramolecular entanglement) and protein structural dynamics.

A third method for examining protein structure, known as SNAPP (selective noncovalent adduct protein probing) has been developed recently.[12] SNAPP utilizes a selective interaction between 18-crown-6 ether (18C6) and lysine to examine protein structure.[19] 18C6 associates weakly with protonated primary amines (such as the side chain of lysine) in solution via the formation of three hydrogen bonds. The dissociation constant for the 18C6-protonated lysine complex has not been determined and may be influenced by a variety of factors including local sequence or protein structure. Nevertheless, the binding constant should be similar in magnitude to protonated butylamine, for which thermodynamic data are available. Protonated butylamine binds to 18C6 with a dissociation constant of ~110 mM in water.[20] Therefore, attachment of 18C6 to lysine depends on the degree to which the side chain is occupied by intramolecular interactions such as salt bridges or hydrogen bonds.[21] These potentially interfering intramolecular interactions prevent attachment of 18C6 and are inseparably connected to the structure of the protein, making attachment of 18C6 (or lack thereof) a sensitive probe of protein structure.[12] The number of 18C6s that attach to a protein can be easily determined with MS because the 18C6/lysine interaction becomes strong in the gas phase (ΔH ~−150 kJ/mol)[19] and causes an easily detectable mass shift. A typical SNAPP experiment is conducted by electrospraying a solution containing the protein and 18C6 directly into the mass spectrometer. The resulting mass spectrum contains an intensity distribution of protein−18C6 complexes. If the ensemble of conformations is heterogeneous, then the observed 18C6 distributions represent statistical averages of the entire ensemble. Assuming that distinct conformations have different propensities for protonation, then the 18C6 distributions for different structures are resolvable by differences in charge state, and therefore, different dynamic states of the protein can be simultaneously detected. Site-specific information can be obtained by determining which lysine residues are capable of attaching 18C6.[21] The three-dimensional relationship between different lysine side chains, however, is not examined.

The present work extends the utility of SNAPP with the implementation of the bis(crown) probe shown (phenyl bis-crown, PBC). The covalent attachment of two 18C6 moieties[22] introduces an additional distance constraint factor into SNAPP experiments. Molecular modeling suggests that the maximum distance between the centers of the two crown ethers is ~19 Å. Results with ubiquitin demonstrate that PBC is a viable SNAPP reagent. Comparison with SNAPP experiments using 18C6 can be used to easily identify the degree of bidentate binding to a protein. The A-state of ubiquitin contains one abundant PBC adduct, which is attached by a bidentate interaction. Determination of the precise binding sites for PBC is more challenging. Collision-induced dissociation (CID) experiments can be used to narrow down the possible sites and can also be used to confirm the degree of bidentate binding. Site-directed mutagenesis was used to pinpoint the preferred bidentate binding location to residues Lys29 and Lys33. These results suggest that PBC will be useful for SNAPP experiments to probe changes in protein structure under equilibrium conditions in a general manner; however, the further extraction of site-specific information with the noncovalent cross-linking approach may be challenging.



MATERIALS AND METHODS

Unless otherwise noted, all commercial reagents and solvents were used as received. Bovine ubiquitin, oligonucleotide primers, high-performance liquid chromatography (HPLC)-grade methanol, and HPLC-grade acetonitrile were all purchased from Sigma-Aldrich (St, Louis, MO). Water was purified to 18.2-MΩ resistivity using a Millipore Direct-Q (Millipore, Billerica, MA). *Escherichia coli* BL21 (DE3) cells were purchased from Novagen Inc. (Madison, WI). T4 DNA ligase, pGEM T-vector, *Bam*H1 and *Nde*I were purchased from New England Biolabs (Beverly, MA); Wizard SV Gel and PCR Clean-up System and Plasmid Miniprep Kit were purchased from Promega (Madison, WI). Gene Jet Plasmid Miniprep Kit was purchased from Fermentas Life Science (Glen Bernie, MA).

All air- or moisture-sensitive reactions were conducted in oven-dried glassware under an atmosphere of nitrogen using dry, deoxygenated solvents. Tetrahydrofuran (THF) and diethyl ether were distilled under nitrogen from sodium benzophenone ketyl. 18-Crown-6-methanol was purchased from Alfa Aesar and α,α′-dibromo-*p*-xylene was purchased from Aldrich; both were used without further purification. ^{1}H NMR spectra were recorded on a Bruker AV-500 (at 500 MHz) in chloroform-*d* at 23 °C. Chemical shifts were referenced to the residual chloroform-H peak, which was set at 7.26 ppm for ^{1}H. Data for ^{1}H NMR are reported as follows: chemical shifts (δ ppm), multiplicity, (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublet, dt = doublet of triplet, m = multiplet, br = broad resonance), coupling constants (Hz), and integration. IR spectra were recorded on a Nicolet MAGNA-IR 850 spectrometer and are reported in frequency of absorption (cm^{-1}). All mass spectra were obtained using an LTQ linear ion trap mass spectrometer (Thermo Fisher, Waltham, MA) with a standard electrospray ionization source.

Organic Synthesis. To a stirred solution of 18-crown-6-methanol (28.3 mg, 0.096 mmol), α,α′-dibromo-*p*-xylene (12.7 mg, 0.048 mmol) in THF (500 μL) was added NaH (4.3 mg, 60% dispersion in mineral oil, 0.106 mmol) with stirring. Stirring was continued at room temperature for 24 h. Diethyl ether (2 mL) was added, the mixture was filtered through celite, and the solvent was removed by evaporation under reduced pressure to yield 41 mg of crude product (product + NaCl). The crude product was

(18) Swaim, C. L.; Smith, J. B.; Smith, D. L. *J. Am. Soc. Mass. Spectrom.* **2004**, *15*, 736–749.
(19) Julian, R. R.; Beauchamp, J. L. *Int. J. Mass Spectrom.* **2001**, *210*, 613–623.
(20) Izatt, R. M.; Terry, R. E.; Haymore, B. L.; Hansen, L. D.; Dalley, N. K.; Avondet, A. G.; Christensen, J. J. *J. Am. Chem. Soc.* **1976**, *98*, 7620–7626.
(21) Liu, Z.; Cheng, S.; Gallie, D. R.; Julian, R. R. *Anal. Chem.* I
n press (DOI: 10.1021/ac800176u).
(22) Julian, R. R.; May, J. A.; Stoltz, B. M.; Beauchamp, J. L. *Angew. Chem., Int. Ed.* **2003**, *42*, 1012–1015.

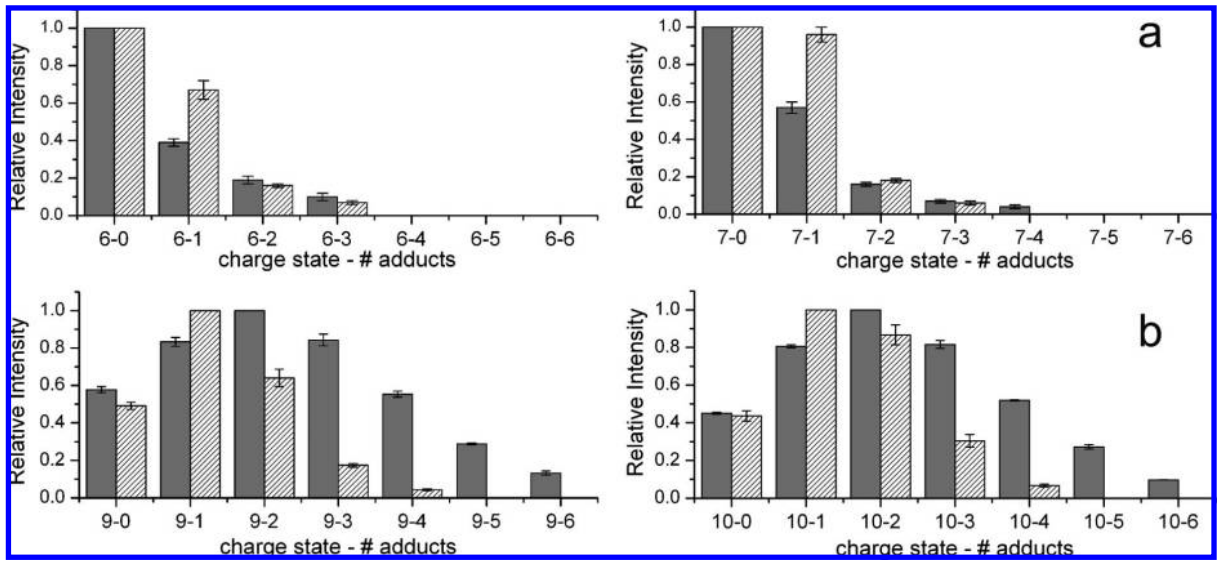


Figure 1. Comparisons of 18C6 (solid) and PBC (hatched) distributions for ubiquitin sampled from native conditions (a) and A-state conditions (b). The average numbers of adducts observed (18C6/PBC) by charge state are (+6) 0.64/0.63, (+7) 0.69/0.68, (+9) 2.34/1.27, and (+10) 2.23/1.46. The difference between the average number of 18C6 and PBC suggests a bidentate interaction is present in the A-state.

resuspended in water and purified using reversed-phase HPLC on a 150 mm × 30 mm, 10-μm particle size C18 Gemini column (Phenomenex, Torrance, CA). The elution gradient was 0−90% acetonitrile over 30 min, using water and acetonitrile as cosolvents. The observed retention time for PBC was ~13.9 min (~42% acetonitrile). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31 (s, 4H), 4.55 (s, 4H), 3.6−3.9 (m, 50H). FT-IR (thin film) 2923.1, 2853.4, 1923.0, 1437.9, 1228.4. MS (m/z): $[M + Na]^+$ calcd for $[C_{34}H_{59}O_{14}Na]^+$, 713.37; found, 713.48.

Selective Noncovalent Adduct Protein Probing Mass Spectrometry. Solutions were prepared either with 50/50 water/methanol + 1% acetic acid to generate the A-state or with water alone to sample the native structure. To ensure stoichiometric excess of adduct, solutions were prepared such that the number of crown moieties was twice the number of possible binding sites. For 18C6, the concentrations used were 10 μM protein/160 μM 18C6. For PBC, which contains two crowns, the molar ratio was 10 μM protein/80 μM 18C6. Ions were generated by direct infusion into the electrospray inlet of the LTQ linear ion trap mass spectrometer. Source and ion lens parameters (electrospray voltage, ion-transfer capillary temperature, tube lens voltage) were optimized for gentle ionization and maximal adduct intensities and are similar to parameters used previously.[12] Once determined, these instrument parameters remained constant for a given analyte. Ions of interest were then isolated in the trap and subjected to a resonance excitation rf voltage to produce fragmentation after many collisions with He buffer gas. Multiple stages of CID (MS^n) can be achieved in the trap by reisolating and exciting subsequent dissociation products. MS^n experiments were used to locate the noncovalent attachment of PBC.

Semiempirical calculations (PM3)[23] were performed as implemented in Gaussian 03 version 6.1 revision D.01. To determine the maximum binding distance for PBC, a ternary complex of PBC and two ammonium adducts were minimized in the gas phase. Coulombic repulsion between the two ammoniums should provide the driving force to maximize separation between the two crown ether centers without significant distortion of PBC. The *Arabidopsis* mutants were prepared as described in detail elsewhere.[21]

RESULTS AND DISCUSSION

Ubiquitin is an important signaling protein that has been well characterized by a variety of mass spectrometric and other techniques.[24] The results for SNAPP experiments with ubiquitin using PBC and 18C6 as the probe reagents are shown in Figure 1. The structure of ubiquitin is quite stable[25] but can be denatured in the presence of acid and organic cosolvent to yield the "A-state".[25,26] In Figure 1a, the natively folded structure is represented in the +6 and +7 charge states. The A-state structure is partially denatured and is observed in higher charges states, such as +9 and +10 shown in Figure 1b.[12,27] Comparison of the SNAPP distributions for the two structures reveals that both PBC and 18C6 attachment are structure dependent. This conclusion is drawn from the fact that both the total number of crowns attaching and the most abundant number of adducts shift to a higher number of adducts for the A-state with PBC and 18C6. Therefore, both reagents can be used for SNAPP experiments to evaluate changes in protein structure, but is there additional information provided by the distance constraint component of PBC?

Careful comparison of the 18C6 and PBC distributions reveals that some of the PBC adducts are attached in a bidentate fashion. If two lysines are within ~19 Å (the maximum separation as determined by modeling the distance between two ammonium ions attached to PBC at the PM3 semiempirical level), then one PBC can attach to both residues. If two lysine residues are not within this distance, then two PBC adducts can attach, each in a

(23) Stewart, J. J. P. *J. Comput. Chem.* **1989**, *10*, 209–220.
(24) Hershko, A.; Ciechanover, A. *Annu. Rev. Biochem.* **1998**, *67*, 425–479.
(25) Brutscher, B.; Bruschweiler, R.; Ernst, R. R. *Biochemistry* **1997**, *36*, 13043–13053.
(26) Hoerner, J. K.; Xiao, H.; Kaltashov, I. A. *Biochemistry* **2005**, *44*, 11286–11294.
(27) Pan, J. X.; Wilson, D. J.; Konermann, L. *Biochemistry* **2005**, *44*, 8627–8633.

monodentate fashion. A single bidentate attachment is expected to be more favorable than two monodentate attachments, due to the lower entropic costs of forming the bidentate crown−lysine complex. Additionally, monodentate attachment of PBC may be somewhat less favorable than attachment of 18C6 due to scavenging interactions between the unoccupied crown of PBC with free ions in solution. The unoccupied crown of PBC acts as a receptor for free ions, effectively decreasing binding at lysine for the other crown due to Coulombic repulsion. These scavenging interactions may become more common in the late stages of ionization, i.e., evaporating droplets, where concentrations of free ions are higher than in bulk solution. In contrast, 18C6 can only interact with a single lysine; therefore, comparison of the two adduct distributions should reveal information about the degree of bidentate binding because each bidentate PBC will be replaced by two 18C6s while each monodentate PBC will be matched by a single 18C6. It is not anticipated that there will be an exact quantitative correlation; however, the net result should be a reduction in the number of PBC adducts by approximately one relative to 18C6 for each bidentate PBC.

Returning to the data in Figure 1, the average number of adducts can be calculated for each charge state to determine the extent of bidentate attachment for PBC. The average numbers by charge state for 18C6/PBC are 6+, 0.64/0.63; 7+, 0.69/0.68; 9+, 2.34/1.27; and 10+, 2.23/1.46. For the folded structure, there is almost no change in the number of adducts that attach between PBC and 18C6 (although the shape of the distributions do vary). This suggests that there are no lysine−lysine pairs that are available to bind 18C6 and held within ~19 Å for the native-state structure. Chemical cross-linking studies have found lysine−lysine pairs within this distance.[11,28] There are several experimental differences that may account for this discrepancy. First, noncovalent attachment of 18C6 may be more sensitive to interfering intramolecular interactions than covalent reactions.[29] Second, SNAPP experiments sample equilibrium conditions, whereas covalent cross-linking is not reversible and may trap transient states or exaggerate the importance of minor conformations. In either case, a more dramatic shift occurs in the A-state, suggesting that this structure exhibits a significantly higher degree of bidentate binding. The difference between the average number of 18C6 and PBC adducts in the A-state suggests that one PBC is probably interacting with ubiquitin via bidentate binding. More detailed information, such as the specific binding site for the bidentate PBC, cannot be extracted from the SNAPP distribution alone and requires further experiments.

CID experiments were performed to evaluate potential binding sites. Fortuitously, protonated ubiquitin ions that carry a high net charge (≥+10 charges) have a facile cleavage point at Pro19 (which has been identified in previous work)[30] that facilitates fragmentation of the protein. The results for fragmenting $[Ubi + PBC + 11H]^{11+}$ are shown in Figure 2a. The base peak results from simple loss of the PBC adduct, as would be expected given the noncovalent attachment. Indeed, CID of noncovalent complexes of PBC and ubiquitin at lower charge states results in loss of PBC as the exclusive dissociation pathway. However, for the +11 charge state, several fragments are produced in substantial abundance with PBC still attached. In the comparable experiment with 18C6, no fragments with 18C6 still attached are detected, suggesting that bidentate attachment is required for PBC to be retained following fragmentation of the protein. Following reisolation and fragmentation of the $[y_{58} + PBC + 9H]^{9+}$ fragment, loss of PBC again yields the base peak as shown in Figure 2b. However, there is a small amount of a secondary fragment ($y_{58}-y_{24}$) produced with PBC still attached. In Figure 2c, an MS^4 experiment is conducted to verify the assignment of this peak. Indeed, loss of PBC from this fragment yields the base peak, confirming that PBC was attached to the precursor. These results suggest that the possible locations for bidentate binding are most likely between residues 20−52. Unfortunately, there are four lysine residues in this region of the protein, and further experiments

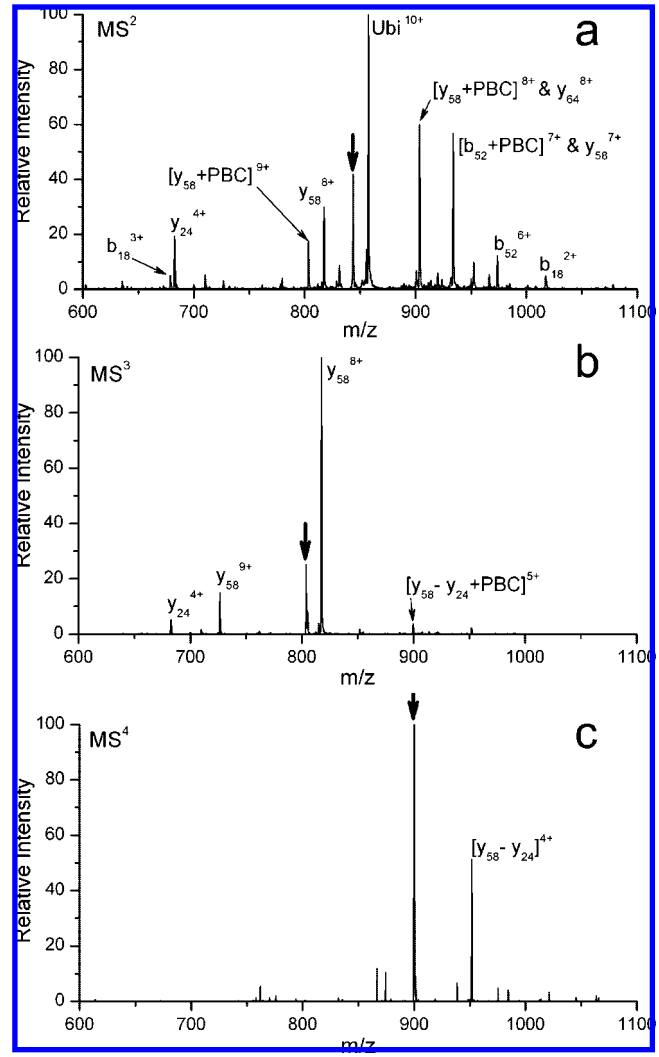


Figure 2. CID spectra of (a) $[ubiquitin + PBC + 11H]^{11+}$, (b) PBC-retaining fragment product $[y_{58} + 9H + PBC]^{9+}$, and (c) $[y_{58}-y_{27} + PBC + 5H]^{5+}$. Observation of $[y_{58}-y_{27} + PBC + 5H]^{5+}$ localizes the bidentate attachment to residues 20−52. However, more specific information could not be obtained by CID alone, due to competitive noncovalent loss of PBC from $[y_{58}-y_{27} + PBC + 5H]^{5+}$. Bold down arrows indicate peaks being subjected to collisional excitation.

(28) Novak, P.; Young, M. M.; Schoeniger, J. S.; Kruppa, G. H. *Eur. J. Mass Spectrom.* **2003**, *9*, 623–631.
(29) For example, compare ref 21 and Novak, P.; Kruppa, G. H.; Young, M. M.; Schoeniger, J. *J. Mass Spectrom* **2004**, *39*, 322–328.
(30) Reid, G. E.; Wu, J.; Chrisman, P. A.; Wells, J. M.; McLuckey, S. A. *Anal. Chem.* **2001**, *73*, 3274–3281.

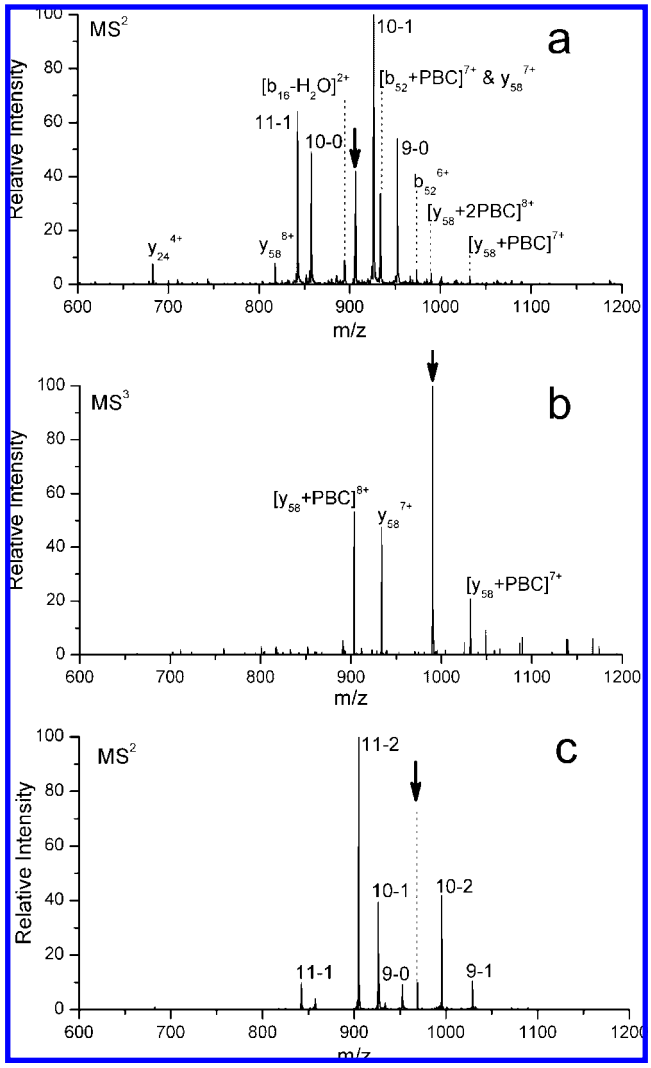


Figure 3. (a) CID of [ubiquitin + 11H + 2PBC]$^{11+}$ producing [y_{58} + 8H + 2PBC]$^{8+}$ in low abundance, suggesting that the second bidentate attachment is more weakly bound than the first. (b) Confirmation of the assignment is provided by CID of the PBC-retaining product [y_{58} + 8H + 2PBC]$^{8+}$, which results in loss of noncovalently bound PBC without backbone fragmentation. (c) CID of [ubiquitin + 11H + 3PBC]$^{11+}$ and other higher order ubiquitin−PBC complexes (data not shown) exclusively produces noncovalent losses of PBC and [PBC + H]$^{+}$. Bold down arrows indicate peaks being subjected to collisional excitation.

(which are described below) are necessary to pinpoint the location. Fragmentation of higher charge states produced similar product ions.

A comparison of the adduct distributions and the CID experiments discussed above reveals that there is a single lysine−lysine pair bridged by a bidentate PBC. To confirm that there is only one bidentate PBC attachment, we performed CID experiments on complexes where the number of PBC molecules attached is greater than one, as shown in Figure 3. Figure 3a shows the MS^2 of the ternary complex of ubiquitin and two PBC molecules, which yielded primarily loss of one or both PBC adducts. Nevertheless, a very small amount of the y_{58} fragment with two PBC adducts attached was produced. This assignment was confirmed with MS,[3] which generated several peaks corresponding to the loss of PBC

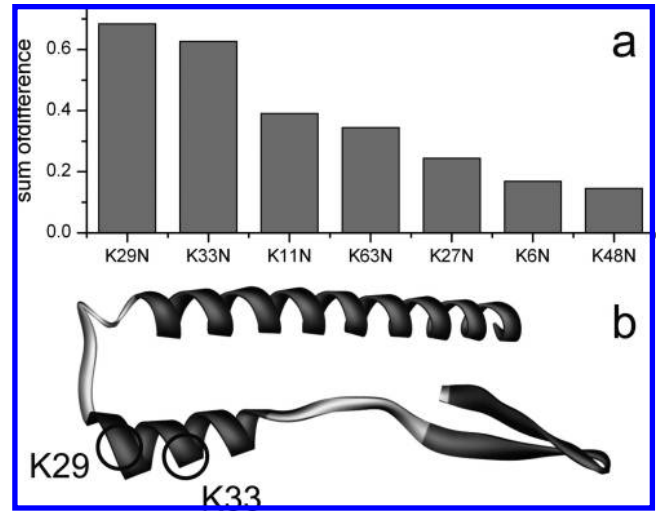


Figure 4. (a) Data from seven single residue mutants of *Arabidopsis* ubiquitin. Differences in PBC distributions for the 10+ charge state between wild-type and each mutant were summed. Mutations that affected the PBC distribution most, K29N and K33N, are the putative sites for PBC bidentate attachment. (b) Ribbon cartoon of the A-state of ubiquitin depicting a loose tertiary structure composed of two α helices and a β sheet. Random coil and highly flexible regions are shaded light gray, while distinct secondary structures are shaded dark gray. A single PBC was to found bridge K29 and K33 (circled), which are on the same α helix.

as shown in Figure 3b. No additional fragmentation with retention of both adducts was observed. It is known that the A-state of ubiquitin is dynamic in nature, and it is possible that the second bidentate PBC attaches to one of the less abundant conformations or only attaches to the protein transiently due to a less favorable interaction. In either case, the amount of the second bidentate adduct appears to be minimal from these results and analysis of Figure 1. The PBC triple adduct was subjected to CID as shown in Figure 3c. In this case, only loss of PBC is observed, confirming that some of the PBC adducts are most likely attached to the protein by a monodentate interaction. Furthermore, this result demonstrates that it is not possible for any arbitrary PBC adduct to adopt a bidentate state in the gas phase during the CID process.

In order to further pinpoint the primary bidentate binding site, experiments were performed with a series of ubiquitin mutants from *Arabidopsis thaliana*. *Arabidopsis* ubiquitin yields results nearly identical to bovine ubiquitin for every structural probing technique that has been applied to both proteins.[21] Furthermore, the overall backbone structure is highly conserved within the ubiquitin family.[31] In fact, the ubiquitin-related protein, RUB1, has 29 amino acid substitutions (as compared to bovine), yet yields a structure with an essentially identical backbone structure.[32] The primary sequences of ubiquitin derived from bovine and *Arabidopsis* differ by only three residues (S19P, D24E, A57S), indicating that results from both proteins should be highly comparable. In the *Arabidopsis* mutants, each lysine is mutated to asparagine one at a time. This enables the contribution of each lysine as a binding site for 18C6 or PBC to be evaluated independently. The results are summarized in Figure 4a, where the magnitude of the peak

(31) Walters, K. J.; Goh, A. M.; Wang, Q. H.; Wagner, G.; Howley, P. M. *Biochim. Biophys. Acta* **2004**, *1695*, 73–87.
(32) Rao-Naik, C.; de la Cruz, W.; Laplaza, J. M.; Tan, S.; Callis, J.; Fisher, A. J. *J. Biol. Chem.* **1998**, *273*, 34976–34982.

is proportional to the degree of binding at a particular lysine. The results suggest that K29 and K33 are the most likely sites for PBC binding. Since bidentate binding is predicted to be strongly preferred over monodentate binding, these sites are also the most likely pair for the bidentate PBC. This conclusion is in good agreement with the results obtained by CID in Figure 3. Furthermore, predictions based on NMR data for the A-state structure identified organized secondary structural regions, but an absence of stable tertiary structure.[25] The cartoon structure shown in Figure 4b represents the secondary structural elements that were predicted by NMR with an absence of any tertiary structure.[25] Lys29 and Lys33 are the same side of the central α-helix. The predicted distance between these residues is in the 10−15-Å range, depending on the relative orientation of the side chains. Therefore, a sustainable bidentate interaction is possible between these residues in the absence of tertiary structure. The absence of a strong bidentate PBC bridging any two of the secondary structure elements in Figure 4b agrees with the NMR prediction of a highly dynamic structure lacking stable tertiary features. It is interesting to note that Lys29 and Lys33 are on the same helix in the folded structure as well; however, results with 18C6 indicate that these residues are intramolecularly entangled. Analysis of the crystal structure suggests that the sequence remote residues 52 and 14 are the interaction sites. This information also suggests that the A-state lacks any stable tertiary structure.

CONCLUSIONS

We have studied whether noncovalent attachment of a novel bis(crown) ether molecule is specific toward lysine pairs within a distance imposed by a semirigid linker. This approach is similar to chemical cross-linking in that it potentially provides distance constraints between residues that are spatially close, information which is useful in constructing theoretical models. However, by using noncovalent interactions, this approach avoids kinetic traps that can potentially occur during covalent modification and provides complementary information. Our SNAPP data for the A-state of ubiquitin suggest a single lysine−lysine pair within ~19 Å. Data from CID experiments and *Arabidopsis* ubiquitin mutants confirm the bidentate attachment and localize the pair to K29 and K33, in agreement with the NMR studies of the A-state.[25] These results also demonstrate that some noncovalent interactions in solution are strong enough in the gas phase to be competitive with protein backbone fragmentation using conventional CID. However, CID experiments may only provide a rough estimate of where the noncovalent adduct is attached and are limited by the number of facile cleavage sites on a protein. Nevertheless, PBC is shown to be a useful SNAPP reagent that can rapidly and easily probe protein structure. The noncovalent approach for probing distance constraints may find utility in systems where dynamics are important, or where the required pH may prove problematic for traditional covalent cross-linking studies. Both issues are likely important for studying the A-state of ubiquitin.

ACKNOWLEDGMENT

T.L., Z.L., and R.R.J. acknowledge funding from the University of California, Riverside, and the American Society for Mass Spectrometry Research Award. R.S. and B.G.P. acknowledge support from the University of California, Berkeley, and the Roche Predoctoral Fellowship (B.G.P.).

Received for review January 24, 2008. Accepted April 22, 2008.

AC800177S