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Top-Down ESI-ECD-FT-ICR Mass Spectrometry Localizes Noncovalent Protein-Ligand Binding Sites

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Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry, unprecedented in mass resolution and mass measurement accuracy, combined with electrospray ionization (ESI) for top-down protein sequencing (as coined by McLafferty and co-workers[1]), delivers substantial sequence information from intact proteins, especially when FT-ICR−MS is armed with electron capture dissociation (ECD).[2−4] ECD is valued for top-down protein sequencing to locate post-translational modifications[5,6] because ECD product ions arise from cleavages between most of the protein's amino acids.[7] Following ECD with ion heating (e.g., infrared multiphoton dissociation, IRMPD) increases sequencing yields.[4,8] Moreover, ESI-MS can measure protein−ligand associations.[9−11] Zubarev suggests that ECD can dissociate covalent bonds, yet retain other noncovalent bonds.[12,13] A related technique, electron detachment dissociation (EDD) was shown to preserve noncovalent bonding in negatively charged DNA duplexes.[14] Generally, collisionally activated dissociation (CAD) and IRMPD of noncovalent protein−ligand complexes yields apoprotein, liberated ligand, and little or no binding site information.[11] In contrast, we demonstrate ECD's special ability for revealing ligand binding sites in a noncovalent α-synuclein (AS)−spermine complex.

Pathologically, Parkinson's disease (PD) presents as intracellular inclusions (Lewy bodies) in the dopaminergic neurons of the substantia nigra and several other brain regions. Filamentous AS (140 amino acids, $M_r = 14460$) protein mainly comprises these deposits, and its aggregation is believed to play an important role in PD. AS binding to natural polycations, for example, spermidine, spermine, and basic histone proteins, has a role in its aggregation.[15,16] α-Synuclein is natively unfolded at physiological pH.[17] Moreover, the C-terminal region is very acidic, presenting 5 aspartate and 10 glutamate residues within residues 96−140. A previous NMR study suggests that spermine ($M_r = 202$) binds to the AS C-terminal acidic region with solution binding affinity (K_d) of 0.6 mM.[17] ESI-MS titrations with a quadrupole time-of-flight (QqTOF) instrument estimate binding affinities to ∼0.36 mM for the 1:1 AS−spermine complex (Supporting Information). γ-Synuclein (GS) is 60% similar to AS by amino acid sequence, but it lacks the acid-rich C-terminal domain. No GS−spermine complex was found by ESI-MS, confirming that the C-terminus is important for spermine binding.

Despite a millimolar AS−spermine solution affinity, NMR and this MS study strongly suggest a *specific* rather than nonspecific interaction. A small amount of nonspecific binding is likely present, as a 1:2 protein−ligand complex is observed at higher concentrations. However, recall that numerous specific biological interactions display millimolar binding strengths[18] and that specific weak interactions are exploited in the popular SAR-by-NMR method, which links two weak-binders to produce potent inhibitors.[19] ESI-MS is advantageous when probing important but weak solution interactions, because the electrostatic component of an interaction is enhanced in the gas phase; solution equilibrium constants do not predict gas phase avidity.[20]

Using a linear quadrupole ion trap coupled to FT-ICR (ThermoFinnigan LTQ-FT),[21] free AS protein was subjected to CAD and ECD. In denaturant (50% acetonitrile and 0.1% formic acid), CAD of 14+-charged AS produces b- and y-type products spanning ∼40% of the sequence. ECD of AS produces c- and z-type products to span ∼33% of the sequence, generally from regions not covered by CAD. Combining complementary CAD and ECD yields nearly 65% of the total sequence.

Top-down CAD-MS/MS (QqTOF and FT-ICR) and IRMPD−MS/MS (FT-ICR) of 1:1 AS−spermine ($M_r = 14662$; 5 μM AS and 100 μM spermine, pH 6.8; Supporting Information) releases spermine from the *holo*-protein, but ECD FT-ICR−MS of noncovalent AS−spermine yields information on ligand-bound sites *by retaining spermine*. As expected for a natively unstructured protein, the ESI charge state distribution does not change significantly upon increasing the solution pH (3 to 6.8) or adding spermine ligand (Supporting Information). The average charge states measured by ESI-QqTOF-MS for the *apo*-protein are +15.6 and +15.8 at pH 3 and pH 6.8, respectively. Spermine binding decreases the average charge slightly to +14.7, perhaps consistent with salt-bridge interactions.

For the 14+ AS−spermine complex (*m*/*z* 1048), nondissociative charge reduction to 13+, 12+, and 11+ is predominant (Figure 1, top). No products for the free ligand were observed. ECD efficiencies for dissociating precursor ions can range from 5 to 30% or lower because of concomitant charge reduction, highly dependent on precursor charge and the protein.[13,22] The most abundant fragments for AS−spermine were only 3−4% relative abundance. However, c- and z-products that retained ligand [e.g., $(z_{36} + \text{spermine})^{3+}$] localized near the C-terminus were observed for 14+ holo-protein, and not for 14+ apoprotein (*m*/*z* 1034). Several y-type ions retained spermine [e.g., $(y_{82} + \text{spermine})^{7+}$] (Figure 1, bottom). Generation of y-products by ECD has been observed previously.[23,24] Product ion yields are enhanced slightly by IR laser-heating after ECD, consistent with hydrogen bond retention upon ECD, followed by dissociation upon heating.[8,25] In total, for 14+-charged AS−spermine, 82 unique product ions with an average mass accuracy ±3 ppm were measured, representing 54 cleavages out of 139 possible amino acid pairs. Remarkably, nearly 50% of the products retain spermine (28, 2, and 12 spermine-bound z-, c-, and y-type ions, respectively; 40 c-ions without spermine) (Figure 2). In no instance was a pair of equivalent product ions observed reflecting both spermine-bound and unbound status; for example, a z_{36}^{3+} associated with the $(z_{36} + \text{spermine})^{3+}$ product was not found. Thus, *all* C-terminal-containing z-/y-products are spermine-bound, while

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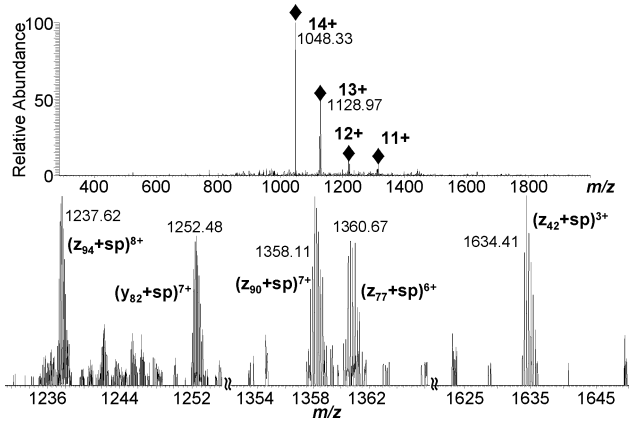

Figure 1. ECD mass spectrum of the 14+ 1:1 α-synuclein−spermine complex (*m*/*z* 1048; top), with expanded regions shown (bottom).

Figure 2. ECD generated products from the 14+-charged 1:1 α-synuclein−spermine complex. Product ions that retain spermine binding are indicated by the extra line underneath the fragments (e.g., ⌊).

nearly all N-terminal c-products do not include spermine. Furthermore, many of the 46 products (36, 9, and 1 c-, z-, and y-ions, respectively) found by ECD of 14+ apoprotein match observed amino acid scissions for c-ions generated from holo-protein.

For 14+ AS−spermine, ligand binds between residues Gly106 and Pro138 based on $(y_{35} + \text{spermine})^{3+}$ and $(c_{138} + \text{spermine})^{12+}$ products. Similar profiles were obtained by ECD of the 15+ complex, as $(z_{36} + \text{spermine})^{3+}$ and $(c_{139} + \text{spermine})^{13+}$ products were measured. The earlier NMR titration of ^{15}N-enriched AS with spermine, monitoring backbone chemical shifts in ^{1}H-^{15}N HSCQ spectra, measured chemical shift changes in residues located in the Gln109-Ala140 C-terminal region; residues Ala124, Glu126, Met127, Ser129, and Asp135 showed the largest displacements in their amide resonances upon interaction with spermine.[17] Thus, the ECD-MS data localizing polycationic spermine binding to the acidic C-terminal portion of AS are consistent with the NMR study.

Also consistent with a recent NMR study indicating that spermine binding *induces* an AS conformational extension,[26] ECD generated >20% more interresidue cleavages for the holo-protein compared to the apo-form. Although AS is presumed natively unfolded, paramagnetic relaxation enhancement and NMR dipolar coupling showed that the negatively charged C-terminal domain interacts with the positively charged N-terminus.[26] Spermine binding to the C-terminal domain shields electrostatic interactions with the N-terminus and reduces the overall compactness of the protein, thus promoting aggregation.[27] ECD of the 14+-holo yielded 54 interresidue cleavages versus 42 found for the apo-form, and 20 cleavages are unique to the holo-form. A more open structured protein should be more amenable to gas-phase dissociation processes, such as ECD.

We have used a combination of ESI-MS and ECD to probe binding of a small molecule ligand to Parkinson's disease related α-synuclein. Covalent bonds along the peptide/protein backbone can be broken by ECD, yet noncovalent bonds may be unaffected, as suggested by the proposed mechanism.[8,12,13] Spermine binding is localized to the C-terminus of α-synuclein, not only in solution, but also in the gas-phase complex, and is clearly specific, even in a natively unstructured protein. Solution-phase structure is sufficiently preserved in the gas-phase such that the positional information of intermolecular interactions is not alterred.[28] We demonstrate that ESI-ECD-MS/MS of protein noncovalent complexes reveals contacts between protein and ligand and potentially for protein−protein interfaces.

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Supporting Information Available: Experimental procedures, complete ref 19, mass spectra of AS and AS−spermine complex, and tables of product ions. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Kelleher, N. L.; Lin, H. Y.; Valaskovic, G. A.; Aaserud, D. J.; Fridriksson, E. K.; McLafferty, F. W. *J. Am. Chem. Soc.* **1999**, *121*, 806−812.
(2) Zubarev, R. A.; Kelleher, N. L.; McLafferty, F. W. *J. Am. Chem. Soc.* **1998**, *120*, 3265−3266.
(3) Horn, D. M.; Zubarev, R. A.; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 10313−10317.
(4) Ge, Y.; Lawhorn, B. G.; ElNaggar, M.; Strauss, E.; Park, J. H.; Begley, T. P.; McLafferty, F. W. *J. Am. Chem. Soc.* **2002**, *124*, 672−678.
(5) Kelleher, N. L.; Zubarev, R. A.; Bush, K.; Furie, B.; Furie, B. C.; McLafferty, F. W.; Walsh, C. T. *Anal. Chem.* **1999**, *71*, 4250−4253.
(6) Shi, S. D.-H.; Hemling, M. E.; Carr, S. A.; Horn, D. M.; Lindh, I.; McLafferty, F. W. *Anal. Chem.* **2001**, *73*, 19−22.
(7) McLafferty, F. W.; Horn, D. M.; Breuker, K.; Ge, Y.; Lewis, M. A.; Cerda, B.; Zubarev, R. A.; Carpenter, B. K. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 245−249.
(8) Breuker, K.; Oh, H.; Lin, C.; Carpenter, B. K.; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 14011−14016.
(9) Loo, J. A. *Mass Spectrom. Rev.* **1997**, *16*, 1−23.
(10) Loo, J. A. *Int. J. Mass Spectrom.* **2000**, *200*, 175−186.
(11) Heck, A. J. R.; van den Heuvel, R. H. H. *Mass Spectrom. Rev.* **2004**, *23*, 368−389.
(12) Haselmann, K. F.; Jorgensen, T. J. D.; Budnik, B. A.; Jensen, F.; Zubarev, R. A. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 2260−2265.
(13) Zubarev, R. A. *Curr. Opin. Biotechnol.* **2004**, *15*, 12−16.
(14) Yang, J.; Mo, J.; Adamson, J. T.; Hakansson, K. *Anal. Chem.* **2005**, *77*, 1876−1882.
(15) Goers, J.; Uversky, V. N.; Fink, A. L. *Protein Sci.* **2003**, *12*, 702−707.
(16) Antony, T.; Hoyer, W.; Cherny, D.; Heim, G.; Jovin, T. M.; Subramaniam, V. *J. Biol. Chem.* **2003**, *278*, 3235−3240.
(17) Fernández, C. O.; Hoyer, W.; Zweckstetter, M.; Jares-Erijman, E. A.; Subramaniam, V.; Griesinger, C.; Jovin, T. M. *EMBO J.* **2004**, *23*, 2039−2046.
(18) Houk, K. N.; Leach, A. G.; Kim, S. P.; Zhang, X. *Angew. Chem., Int. Ed.* **2003**, *42*, 4872−4897.
(19) Hajduk, P. J.; et al. *J. Am. Chem. Soc.* **1997**, *119*, 5818−5827.
(20) Goodlett, D. R.; Ogorzalek Loo, R. R.; Loo, J. A.; Wahl, J. H.; Udseth, H. R.; Smith, R. D. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 614−622.
(21) Syka, J. E. P.; Marto, J. A.; Bai, D. L.; Horning, S.; Senko, M. W.; Schwartz, J. C.; Ueberheide, B.; Garcia, B.; Busby, S.; Muratore, T.; Shabanowitz, J.; Hunt, D. F. *J. Proteome Res.* **2004**, *3*, 621− 626.
(22) Cooper, H. J.; Hakansson, K.; Marshall, A. G. *Mass Spectrom. Rev.* **2005**, *24*, 201−222.
(23) Cooper, H. J. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1932−1940.
(24) Zubarev, R. A.; Kruger, N. A.; Fridriksson, E. K.; Lewis, M. A.; Horn, D. M.; Carpenter, B. K.; McLafferty, F. W. *J. Am. Chem. Soc.* **1999**, *121*, 2857−2862.
(25) Oh, H.; Breuker, K.; Sze, S. K.; Ge, Y.; Carpenter, B. K.; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15863−15868.
(26) Bertoncini, C. W.; Jung, Y.-S.; Fernandez, C. O.; Hoyer, W.; Griesinger, C.; Jovin, T. M.; Zweckstetter, M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 1430−1435.
(27) Hoyer, W.; Cherny, D.; Subramaniam, V.; Jovin, T. M. *Biochemistry* **2004**, *43*, 16233−16242.
(28) Loo, J. A.; He, J.-X.; Cody, W. L. *J. Am. Chem. Soc.* **1998**, *120*, 4542−4543.

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