CRITICAL INSIGHT

Reflections on Charge State Distributions, Protein Structure, and the Mystical Mechanism of Electrospray Ionization

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Abstract

The connection between charge state distributions, protein structure, and mechanistic details of electrospray are discussed in relation to the emerging field of gas phase structural biology. Comparisons are drawn with the established area of enzymatic catalysis in organic solvents, which shares many similar challenges. Charge solvation emerges as a dominant force in both systems that must be dealt with to enable kinetic trapping of native structures in foreign environments. Potential methods for mediating unfavorable charge solvation effects are discussed and, ironically, do not include partial solvation by water. The importance of timescale in relation to the evolution of protein structure during the process of electrospray ionization is discussed. Finally several prospects for future endeavors are highlighted.

Key words: Structural biology, Ion mobility, H/D exchange, Organic

Introduction

Electrospray ionization (ESI) is great. It can be used to gently transfer just about anything, except perhaps small animals, into the gas phase in an ionized state [1, 2]. The full impact that ESI will have on chemistry, biochemistry, biology, medicine, and other areas has not been fully determined, but will certainly be substantial. The utility and importance of ESI cannot, therefore, be reasonably disputed; however, ESI is not completely (or perhaps even well) understood. The basics can be inferred: a voltage difference is used to create charged droplets; these droplets fission, evaporate, and eventually yield ions. Exactly how these events occur, particularly at a molecular level, is not known [3]. Similarly, the influence that the electrospray process has on the molecules being ionized is not fully understood. Why not? There are experimental challenges, i.e., the droplets of interest are very small and exist only transiently in an awkward to access environment. Furthermore, methods for confident molecular level characterization of such droplets may not exist. There are also philosophical barriers—we know everything that we need to in order to make ESI useful, so why invest the time and money? Then there is the issue of source diversity, which is rarely addressed in relation to how ESI works. Electrospray itself can be found in numerous variations, all differing in some way with respect to liquid flow rates, gas flow rates, geometries, voltages, ion optics, materials, etc. In addition there are numerous closely related methods such as nanospray, paperspray, electrosonic spray, and desorption electrospray (DESI), just to mention a few [4, 5]. Do all of these variations operate under similar general principals, or are there important differences? The point is, at the moment, we do not know.

One of the interesting phenomena associated with ESI is that molecules are frequently observed in multiple charge states, especially larger molecules such as proteins [6, 7]. The collection of charge states observed for a particular molecule under a given set of experimental conditions is

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referred to as the charge state distribution. Charge state distributions can be influenced by a variety of factors. For example, the addition of organic solvent and acid to an aqueous protein solution will frequently lead to a dramatic broadening and shift of the charge state distribution to lower m/z (higher charge states). In contrast, addition of buffer (such as ammonium acetate) to an aqueous solution frequently favors lower charge states and narrower distributions. These observations are presumably linked to protein structure, where unfolded proteins are able to accommodate more charge, which leads to higher and broader charge state distributions, while folded proteins typically exhibit lower and narrower charge state distributions.

It is clear from ion mobility experiments that in the gas phase, higher charge states do correspond to more unfolded proteins while lower charge states represent more compact structures [8, 9]. It is therefore often inferred that such structures were present in the original solutions. It is entirely possible that this is the case; however, it is also possible that proteins may unfold during the process of ESI itself. This issue will be discussed further below, but it is sufficient for the moment to say that our ignorance about the mechanistic process of ESI makes it dangerous to assume that structures present in solution are always (or perhaps even can be) directly transferred into the gas phase. This issue is particularly important given the increasing interest in the area of gas phase structural biology, where gas phase methods, including structural characterization *in vacuo*, are employed to examine protein structure. Proponents for this field argue that proteins can be transferred with structural fidelity into the gas phase and meaningfully examined within that environment, while others argue that the gas phase is a wildly unnatural medium that in no way mimics aqueous or cellular conditions and that examination of protein structure in the gas phase is a waste of time.

Nonaqueous Environments

Let us take a brief aside to examine this issue from another angle. Oddly enough, insight into the dilemma of protein structure in the gas phase can be acquired by examination of enzymatic catalysis in organic solvents, which is actually a fairly established field and has been reviewed [10, 11]. Organic solvents (more specifically anhydrous organic solvents—at least to the extent that is possible) are not typically associated with biology or proteins, but are more comparable to the gas phase than water in many respects. Interestingly, even though most proteins are not soluble in organic solvents, they are frequently employed for catalytic transformations in organics despite this shortcoming. In fact, several applications in industry have been used to generate kilograms of product in this fashion [10]. Instinctively, one might assume that proteins would be denatured in organic solvents; however, experimental characterization, though limited, has suggested that this is not the case [12, 13]. Furthermore, the ultimate test for structural fidelity with proteins is generally considered to be functionality,

which can obviously be retained and utilized in organic media. It is, therefore, reasonable to conclude that sufficient core structure is retained in organic solvents to allow for the observed catalysis.

Many of these experiments are carried out by lyophilization of the protein, which is then dispersed in an organic solvent by vigorous and constant agitation. Importantly, the conditions under which the protein is lyophilized can have significant impact on catalytic activity. If the protein is denatured prior to or during lyophilization, no catalysis is observed. This further supports retention of protein structure as being important for successful catalysis. In addition, experiments have shown that the addition of salts or crown ethers to solution prior to lyophilization greatly enhances catalytic activity [14, 15]. There are multiple potential explanations for this observation. For example, it has been suggested that proteins become very rigid in organic solvents due to loss of the dynamic hydrogen bonding environment afforded by water. If this rigidity concept is correct, then salts or crown ethers may restore a degree of flexibility to the enzymes (which is necessary for catalysis). Another (not mutually incompatible) way of looking at this issue relates to charge solvation. It is likely that counterions or crown ethers enhance solvation of charged groups on the protein surface during and after lyophilization, which helps mitigate loss of structure due to Coulombic forces. Interestingly, solvation of the charge groups by retention of water itself is not a viable option. Addition of water, even in small quantities, is not beneficial for catalysis and leads to rapid denaturation [10]. When these observations are considered together, it would appear that proteins retain structure in organic media due to kinetic trapping. In other words, although native structures are likely not the lowest energy states in organic solvents, the barriers to rearrangement to the lowest energy structures are sufficiently large to prevent this from occurring. The addition of water likely lowers the kinetic barriers, enabling transitions between different structural states.

To sum up, there are a few important take-home points. One, proteins can be kinetically trapped in their native state in nonaqueous environments and retain enzymatic activity under certain conditions. The theoretical possibility for gas phase structural biology is, therefore, possible. Two, water actually facilitates protein unfolding in denaturing environments. As a result, many of the water/organic solution combinations frequently employed in ESI are likely to be highly denaturing (ironically because of the combination, not just the organic). Three, lack of charge solvation can interfere with kinetic trapping of native states. The gas phase is even worse at charge solvation than organic solvents, making this a primary obstacle, which will interfere with retention of native or native-like structures.

Timescale

One issue that is often overlooked in relation to protein structure and ESI is time [3, 16]. Proteins are large molecules and require a certain amount of time to undergo structural

transitions. The exact amount of time is dependent on the protein and the particular conditions which are present. Can proteins unfold within the timescale of ESI? For that matter, what is the relevant timescale? In most instruments, the time from droplet generation to naked ions will typically be in the millisecond timeframe, but at what point does the environment within a charged droplet appear to be substantially different to a protein? Immediately? After droplet fission?

Recent experiments by the Williams group offer compelling evidence that protein unfolding can occur within the timescale of ESI [17]. In experiments designed to elucidate the mechanism of supercharging (a phenomenon where the charge state distribution is pushed to very low m/z), proteins were demonstrated to be folded in bulk solution, yet unfolded in the gas phase. To demonstrate this, various amounts of DMSO was added to aqueous solutions that were examined by ESI and circular dichroism (CD). The onset of unfolding was observed at a much smaller percentage of DMSO in the ESI experiments. DMSO has a much higher boiling point than water and only acts as a denaturant at high concentrations [18]. It was postulated that the concentration of DMSO must increase during droplet evaporation in ESI. If this occurred, the bulk solution with a low concentration of DMSO would be non-denaturing (as confirmed by CD) while droplets generated by ESI would become more denaturing, leading to protein unfolding and a shift in charge state distribution.

We decided to test this hypothesis with a very simple experiment. A nondenaturing solution of water and DMSO (90/10) containing myoglobin was reduced in volume by sequential partial lyophilization. Indeed, as shown by CD in Figure 1 the protein unfolded after the volume of the solution was significantly reduced, presumably due to concentration of DMSO. In fact, if it is assumed that only water is lost, then the percent DMSO where denaturation occurs is in good agreement with previous experiments [22, 23]. The point here is that the solution composition in ESI droplets is most likely evolving with time as evaporation and ionization occur. If one were to attempt to evaluate the amount of organic solvent that causes denaturation of a
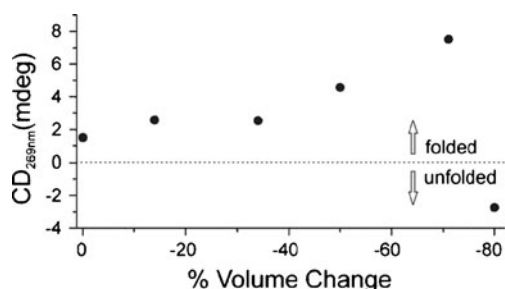
protein using ESI, these considerations would need to be taken into account.

Do proteins always unfold or otherwise undergo structural rearrangement within the timescale of ESI? This is a much more difficult question to answer, as proteins which retain compact structures may not represent native structures. For example, the addition of some acids to aqueous solutions can cause apparent charge state reduction and compaction, though the equilibrium has not been shifted towards the native state by such conditions [19]. However, there are experiments which demonstrate that mildly denaturing conditions may not be sufficient to enable protein unfolding within the timescale of ESI. Liquid desorption electrospray ionization, or liquid DESI, developed by the Chen group, [20] can be used to introduce the analyte of interest independently from the ionization solution as shown in Figure 2a. For example, an aqueous myoglobin solution can be intersected with an ionized plume of water/methanol/acetic acid (50/50/1). When this is done with 2:1 (spray/sample) flow rates, the observed protein charge state distribution is very similar to that obtained by electrospraying the aqueous solution, as shown in Figure 2b. In fact, if anything, the aqueous ESI spectrum (inset in Figure 2b) appears to be slightly more denatured than the liquid DESI spectrum. However, if a mixture containing proportional volumes of the spray and sample solutions is mixed and then directly electrosprayed, significant shifting of the charge state distribution is observed, as shown in Figure 2c. This suggests that the solvent mixture is denaturing in both cases, but that the timescale is too short for the protein to unfold in the liquid DESI configuration. If the proportion of water in the final mixture is reduced by decreasing the flow rate of the sample, protein unfolding can be observed in liquid DESI as well. These results suggest that protein unfolding can take place within the timeframe of ESI, but also that such unfolding is dependent on the severity of denaturing conditions. It should also be mentioned that due to the relative boiling points of water and methanol, in contrast to the DMSO experiments described above, the amount of organic solvent present will be decreasing with time when methanol is the organic component.

The Interesting Case of Calmodulin

Calmodulin regulates many cellular processes by binding calcium, which elicits a significant rearrangement of the tertiary structure of the protein. Calmodulin has been examined frequently by mass spectrometry, [21, 22] but one experiment is particularly relevant here. [23] Selective noncovalent adduct protein probing (SNAPP) is a mass spectrometry based method that utilizes 18-crown-6 ether (18C6) to examine protein structure. 18C6 forms stable noncovalent adducts with proteins in the gas phase when electrosprayed under gentle conditions. The number of 18C6 adducts that attach to a protein (or SNAPP distribution) is a function of the solution phase structure of the protein. Importantly, 18C6 does not bind to proteins to a significant
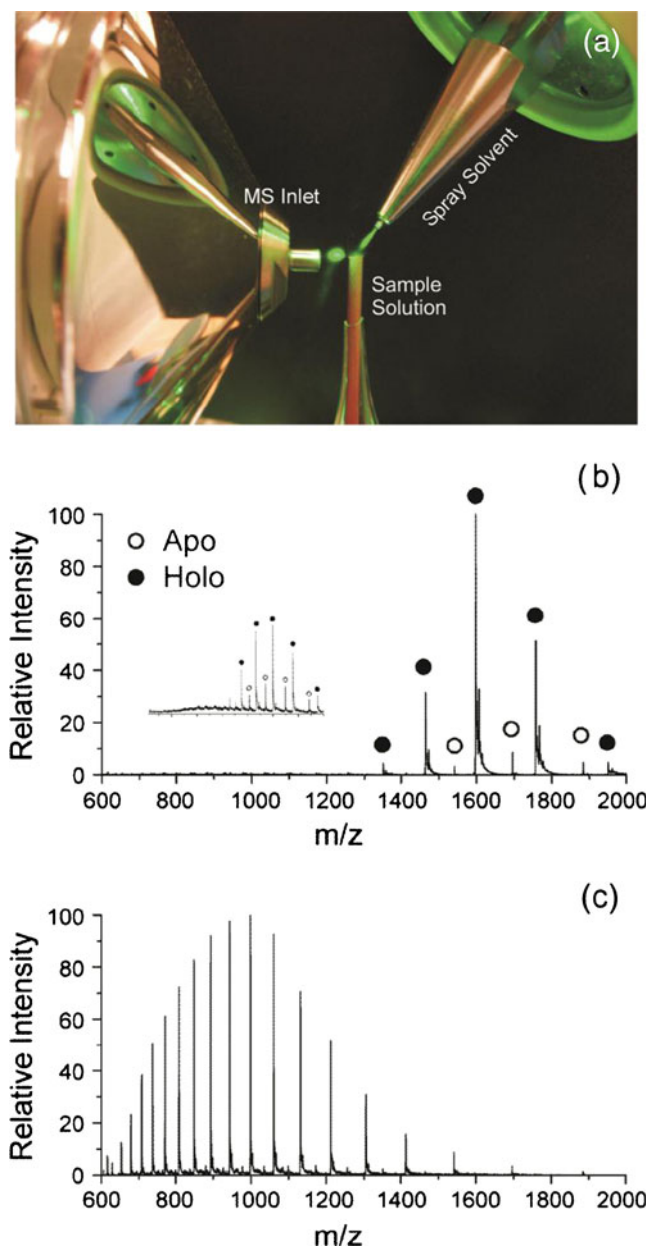

Figure 1. Circular dichroism at 269 nm as a function of reduced volume following sequential lyophilization of 20 μM myoglobin initially in 90/10 water/DMSO. Unfolding occurs when the solution would hit ~50% DMSO (assuming only marginal DMSO loss)

Figure 2. **(a)** Photo of liquid DESI setup. A green laser was used to illuminate the initial and reflected droplet plumes. The sample is infused through the peek tubing in the center, while voltage is only applied to the spray solvent tip. The spray solvent is 50/50/1 water/MeOH/acetic acid and injected at twice the flow rate of the sample. **(b)** Liquid DESI spectrum of myoglobin in water, which is very similar to that obtained by ESI of myoglobin from water (see inset of identical *m/z* range). **(c)** Direct ESI of a solution corresponding to a mixture of the sample and spray solutions in **(b)**, demonstrating that indeed the mixture is denaturing

extent in bulk water, meaning that 18C6 adduct formation occurs sometime after droplet formation during the process of ESI. Calmodulin also undergoes an interesting transition after droplet formation in ESI. Calmodulin has four calcium binding sites with 10^6 M^{-1} association constants or higher,

meaning that calcium is strongly bound in solution. However, when calmodulin is electrosprayed in positive ion mode, the number of calcium ions that remain attached to the protein is much lower than would be predicted by solution phase affinity. Likely, this may occur due to protonation of the acidic residues which bind calcium by the excess positive charges in the ESI droplet, leading to loss of calcium ions during desolvation of the protein.

So in SNAPP experiments with calmodulin, calcium ions are being lost from the protein at the same time that 18C6 is attaching to the protein. Shown in Figure 3 are several SNAPP distributions for calmodulin in the +11 charge state. The apo form obtained from an aqueous solution with no calcium present is shown in blue. The SNAPP distributions in green were obtained from a calcium containing solution and are shown as a function of the number of calcium adducts (zero, one, and two calciums attached). Based on solution phase binding, the two calcium adduct peak (far right) should be ~50× more intense than the naked protein (solid green). Clearly, this is not the case, confirming that significant calcium loss occurred during ESI. The distributions observed in the solid blue and solid green bars are significantly different from each other, despite the fact that both of them correspond to observation of apo-calmodulin. The solid green distribution is much more similar to those observed with calcium ions still attached to the protein. This suggests that even though calcium is removed from the protein during ESI, which will eventually induce a large structural rearrangement, calmodulin does not have time to complete this transition before 18C6 adducts attach. However, the distributions in Figure 3 originating from the calcium solution suggest that this transition may be beginning to occur in the droplet. Comparison of the three distributions in green reveals a consistent shift towards the true apo distribution (blue) as fewer calcium ions remain attached.

The loss of calcium in this example represents a situation where ESI elicits undeniable (due to the mass shift) and
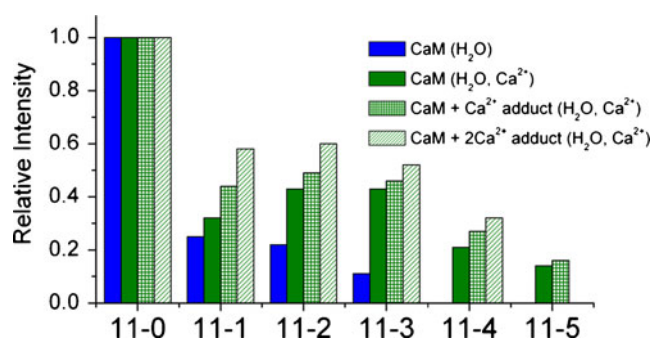

Figure 3. SNAPP distributions for the +11 charge state of calmodulin. Although the protein can lose all four bound calcium ions (solid green), the structure does not have time to revert to the apo form (blue). X-axis labels correspond to (charge state) – (# 18C6s attached). Reprinted from Reference 31 with permission

undesirable (due to the known effect of calcium ions on structure) perturbations related to charge solvation. Interestingly however, structure probing by 18C6 is not significantly affected by loss of calcium, most likely due to the abbreviated timeframe. It is not known from the SNAPP experiment whether the apo-protein remains kinetically trapped in the holo-structure following complete desolvation (although this system has been examined by ion mobility), [24, 25] but it is clear that the residual structure is retained for some amount of time even though the charge landscape of the protein has been significantly altered. Again these results indicate that in terms of gas phase structural biology, it will be important to consider not only how to examine protein structure, but also how quickly the structure is examined.

Future Outlook

Although we may not know everything about ESI that we would like to, it is still possible to use information that we do have to steer future endeavors. If protein structure is denatured in solution, it is unlikely that the process of ESI will favor or allow refolding. Thus, care should be taken to prepare samples in solvents that do not cause the protein to denature, if one desires to obtain information about the protein structure. It should also be taken into account that changes to the solvent composition may occur during ESI, and the effect that these changes might have on the folding state of the protein should be considered. Proteins can be kinetically trapped in their native state in very non-native conditions; however, insufficient charge solvation is a primary driving force which can interfere with or prevent kinetic trapping. It should be mentioned that the importance of charge solvation will scale inversely with the size of the protein. This can be rationalized by the fact that charged groups are almost always present at the surface of a protein. Proteins are almost all roughly globular in shape, meaning that uncharged stabilizing interactions will increase in proportion to volume while charged interactions will scale with surface area. As protein size increases, the volume scales according to $\sim r^3$ while the surface area increases by $\sim r^2$. Thus, for larger proteins, the fractional abundance of charged interactions becomes smaller and, therefore, represents a weaker driving force for structural rearrangement. This rationale assumes that internally stabilizing interactions, i.e., hydrogen bonds, van der Waals interactions, etc., maintain approximately similar relative contributions as protein size increases. Larger proteins should also fold and unfold more slowly, which should also favor kinetic trapping. Regardless of protein size, thermal excitation post (or in the final stages of) desolvation is highly likely to overcome kinetic trapping and should be avoided. Similarly, protein structure should be examined on the shortest timescale possible.

A potential approach to reduce structural rearrangement due to insufficient charge solvation would be to provide partial solvation, similar to what is achieved by adding salts and crown ethers to the organic catalysis experiments. One possibility would be to examine proteins that are still partially solvated by water molecules; however, since water enables denaturation of proteins in organic solvents, this may have the opposite of the desired effect. Ironically, partially hydrated proteins may be less representative of solution phase structures than fully desolvated proteins because sustained partial hydration would likely facilitate structural rearrangement. Buffers may provide a better solution. The addition of buffers such as ammonium acetate likely reduces Coulombic effects up until the point that the buffer is lost in the gas phase (assuming that it is lost). Perhaps this is sufficient to provide for kinetic trapping for some proteins. Interestingly, crown ethers (particularly 18C6, which was found to be most effective in organic catalysis) have already found utility in variety of mass spectrometry experiments, but the potential of crowns as partial solvation agents has not been fully explored. These experiments are currently underway in several labs, so we may have more information on this exciting idea shortly. Finally, if the organic catalysis work is correct, perhaps the best hope for retention of protein structure is to electrospray proteins directly out of organic solvents. Of course, that will require clever solutions to the problems of insolubility, among other things.

Acknowledgments

References

1. Tito, M.A., Tars, K., Valegard, K., Hajdu, J., Robinson, C.V.: Electrospray time-of-flight mass spectrometry of the intact MS2 virus capsid. *J. Am. Chem. Soc.* **122**(14), 3550–3551 (2000)
2. Cheng, X.H., Camp, D.G., Wu, Q.Y., Bakhtiar, R., Springer, D.L., Morris, B.J., Bruce, J.E., Anderson, G.A., Edmonds, C.G., Smith, R.D.: Molecular weight determination of plasmid DNA using electrospray ionization mass spectrometry. *Nucleic Acids Research* **24**(11), 2183–2189 (1996)
3. The charge residue versus ion evaporation debate will not be treated here, as molecular level experimental observations are lacking for either. For a recent review: Kebarle, P.; Verkerk, U. H. Electrospray: from ions in solution to ions in the gas phase, what we know now. *Mass Spectrom. Rev.* **2009**, *28*(6), 898–917.
4. Gatlin, C.L., Kleemann, G.R., Hays, L.G., Link, A.J., Yates, J.R.: Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography micro-spray and nanospray mass spectrometry. *Anal. Biochem.* **263**(1), 93–101 (1998)
5. Takats, Z., Wiseman, J.M., Gologan, B., Cooks, R.G.: Mass spectrometry sampling under ambient conditions with desorption electrospray ionization. *Science* **306**(5695), 471–473 (2004)
6. Dobo, A., Kaltashov, I.A.: Detection of multiple protein conformational ensembles in solution via deconvolution of charge-state distributions in ESI MS. *Anal. Chem.* **73**(20), 4763–4773 (2001)
7. Pan, J.X., Wilson, D.J., Konermann, L.: Pulsed hydrogen exchange and electrospray charge-state distribution as complementary probes of protein structure in kinetic experiments: Implications for ubiquitin folding. *Biochemistry* **44**(24), 8627–8633 (2005)
8. Clemmer, D.E., Jarrold, M.F.: Ion mobility measurements and their applications to clusters and biomolecules. *Journal of Mass Spectrometry* **32**(6), 577–592 (1997)

9. Bernstein, S.L., Liu, D.F., Wyttenbach, T., Bowers, M.T., Lee, J.C., Gray, H.B., Winkler, J.R.: alpha-synuclein: Stable compact and extended monomeric structures and pH dependence of dimer formation. *J. Am. Soc. Mass Spectrom.* **15**(10), 1435–1443 (2004)

10. Doukyu, N., Ogino, H.: Organic solvent-tolerant enzymes. *Biochem. Eng. J.* **48**(3), 270–282 (2010)

11. Klibanov, A.M.: Improving enzymes by using them in organic solvents. *Nature* **409**(6817), 241–246 (2001)

12. Santos, A.M., Vidal, M., Pacheco, Y., Frontera, J., Baez, C., Ornellas, O., Barletta, G., Griebenow, K.: Effect of crown ethers on structure, stability, activity, and enantioselectivity of subtilisin Carlsberg in organic solvents. *Biotechnol. Bioeng.* **74**(4), 295–308 (2001)

13. Griebenow, K., Klibanov, A.M.: On protein denaturation in aqueous-organic mixtures but not in pure organic solvents. *J. Am. Chem. Soc.* **118**(47), 11695–11700 (1996)

14. Morgan, J.A., Clark, D.S.: Salt-activation of nonhydrolase enzymes for use in organic solvents. *Biotechnology and Bioengineering* **85**(4), 456–459 (2004)

15. van Unen, D.J., Engbersen, J.F.J., Reinhoudt, D.N.: Why do crown ethers activate enzymes in organic solvents? *Biotechnol. Bioeng.* **77**(3), 248–255 (2002)

16. Breuker, K., McLafferty, F.W.: Stepwise evolution of protein native structure with electrospray into the gas phase, $10(−12)$ to $10(2)$ S. *Proc. Nat. Acad. Sci.* **105**(47), 18145–18152 (2008)

17. Sterling, H.J., Prell, J.S., Cassou, C.A., Williams, E.R.: Protein conformation and supercharging with DMSO from aqueous solution. *J. Am. Soc. Mass Spectrom.* **22**(7), 1178–1186 (2011)

18. Voets, I.K., Cruz, W.A., Moitzi, C., Lindner, P., Areas, E.P.G., Schurtenberger, P.: DMSO-induced denaturation of hen egg white lysozyme. *J. Phys. Chem. B* **114**(36), 11875–11883 (2010)

19. Samalikova, M., Matecko, I., Muller, N., Grandori, R.: Interpreting conformational effects in protein nano-ESI-MS spectra. *Anal. Bioanal. Chem.* **378**(4), 1112–1123 (2004)

20. Miao, Z.X., Wu, S.Y., Chen, H.: The study of protein conformation in solution via direct sampling by desorption electrospray ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **21**(10), 1730–1736 (2010)

21. Hu, P.F., Ye, Q.Z., Loo, J.A.: Calcium stoichiometry determination for calcium-binding proteins by electrospray-ionization mass-spectrometry. *Anal. Chem.* **66**(23), 4190–4194 (1994)

22. Schulz, D.M., Ihling, C., Clore, G.M., Sinz, A.: Mapping the topology and determination of a low-resolution three-dimensional structure of the calmodulin-melittin complex by chemical cross-linking and high-resolution FTICRMS: Direct demonstration of multiple binding modes. *Biochemistry* **43**(16), 4703–4715 (2004)

23. Ly, T., Julian, R.R.: Protein-metal interactions of calmodulin and →-synuclein monitored by selective noncovalent adduct protein probing mass spectrometry. *J. Am. Soc. Mass Spectrom* **19**(11), 1663–1672 (2008)

24. Faull, P.A., Korkeila, K.E., Kalapothakis, J.M., Gray, A., McCullough, B.J., Barran, P.E.: Gas-phase metalloprotein complexes interrogated by ion mobility-mass spectrometry. *Int. J. Mass Spectrom.* **283**(1/3), 140–148 (2009)

25. Wyttenbach, T., Grabenauer, M., Thalassinos, K., Scrivens, J.H., Bowers, M.T.: The effect of calcium ions and peptide ligands on the relative stabilities of the calmodulin dumbbell and compact structures. *J. Phys. Chem. B* **114**(1), 437–447 (2010)