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Gas-phase processes and measurements of macromolecular properties in solution: On the possibility of false positive and false negative signals of protein unfolding

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Abstract

Electrospray ionization mass spectrometry is increasingly applied to study protein behavior in solution, including characterization of their higher order structure, conformational dynamics and interactions with small ligands and other biopolymers. However, actual measurements of fundamental ionic parameters (mass and charge) take place in vacuum, and an array of gas phase processes occurring prior to protein ion detection and characterization may certainly affect them. While most previous studies were concerned primarily with the effect of gas phase processes on mass measurement (e.g., integrity of macromolecular complexes in the absence of solvent, non-specific interactions, etc.), the focus of our attention is the ionic charge. Charge state distributions of protein ions in ESI MS are often used to characterize large-scale dynamic processes in solution (such as protein unfolding). Formation of metastable protein aggregates either in the bulk of solution or in the electrosprayed droplets, their consequent transfer to the gas phase and asymmetric dissociation may give rise to a population of highly charged ions. Presence of such ions in ESI mass spectra usually indicates loss of native structure in solution. Therefore, studies of large-scale conformational dynamics in solution by monitoring protein ion charge state distributions should be carried out at low protein concentrations in order to minimize the occurrence of false positive signals of protein unfolding. The opposite phenomenon, absence of highly protonated ionic species in ESI spectra of unfolded proteins, does not occur even in the case of highly acidic proteins lacking a sufficient number of basic residues.

Keywords: Electrospray ionization; Charge state distribution; Non-covalent complex; Asymmetric dissociation; Proton affinity

1. Introduction

In the past decade electrospray ionization mass spectrometry (ESI MS) has become one of the most popular tools to study macromolecular behavior in solution [1,2]. Arguably, the most popular experimental strategy that utilizes ESI MS to probe protein structure and dynamics is hydrogen/deuterium exchange (HDX) [3,4], although various other methods are becoming increasingly reliant on ESI MS as a method of detection. These include chemical cross-linking, selective chemical labeling, radiolytic foot-printing and covalent trapping of intermediate states [2]. It is important to remember, however, that MS measurements are carried out in the absence of solvent, and such a dramatic change of the environment surely affects macromolecular properties in multiple (and often unexpected) ways [5]. Therefore, prior to applying any MS-based method to probing macromolecular properties in solution, the following questions must be addressed. Are there any processes occurring in the gas phase prior to MS detection that may influence the measured ionic parameters? If so, what effect will such interference have on the measurements of macromolecular properties in solution? Finally, what can be done in order to minimize these effects?

Even though all ions without exception suffer loss or alteration of at least some of their properties upon transition from solution to the gas phase, it does not necessarily invalidates the characterization of their behavior in solution using experimental methods that rely on MS detection. For example, studies of higher order protein structures by chemical cross-linking or selective chemical labeling can be carried out in most cases

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without any regard for protein behavior in the gas phase. This contrasts sharply with the studies of protein dynamics that employ a combination of HDX in solution and protein ion fragmentation in the gas phase [6], where intrinsic mobility of protons within solvent-free polypeptide ions may lead under certain conditions to hydrogen scrambling [7]. Another example highlighting the need for very careful planning of experiments and interpretation of their results is an ESI MS titration applied to measurements of formation constants of non-covalent complexes in solution [8]. What is clear now is the fact that in each case a good understanding of the physical principles governing behavior of macromolecular ions in the gas phase is needed in order to design optimal experimental schemes and avoid data misinterpretation.

Analysis of protein ion charge state distributions in ESI MS is another biophysical method that has been steadily gaining popularity as a technique allowing large scale conformational transitions in solution to be monitored [9,10]. The link between the degree of compactness of polypeptide chains in solution and the extent of multiple charging of corresponding ions in the gas phase was established over a decade ago [11] and this phenomenon was used extensively in the past as a means to monitor protein folding and unfolding events in solution. Loss of native structure (either complete or partial) is almost always easily detectable in ESI MS, as it manifests itself by the appearance of bimodal charge state distributions with clearly distinguishable contributions from both low and high charge density protein ions (Fig. 1). The former correspond to folded (compact) protein molecules, which can accumulate only few charges upon their transition from solution to gas phase due to limited solvent-exposed surface area (SASA). Loss of structure (which in the case of proteins is synonymous with loss of compactness and, therefore, dramatic increase of SASA) allows a significantly higher number of charges to be accumulated. Therefore, changing solvent conditions from near-native to denaturing almost always has a profound effect on the appearance of the ESI mass spectra of proteins, and the appearance of bimodal charge state distributions in ESI mass spectra almost always signals the emergence of non-native state(s) in solution.

In addition to indicating the presence of non-native states in solution, the evolving shape of the high charge (low *m*/*z*) end of the protein ion charge state distribution often hints at transitions among various non-native states. Indeed, not only does a progressive departure from the native conditions lead to continuous elevation of the total abundance of highly charged ions, but also a prominent shift in the intensity distribution can be seen in the low-*m*/*z* part of the spectrum (Fig. 1). Such a shift (towards protein ions carrying more charges and, therefore, representing conformations with less residual structure) clearly indicates that highly unstructured conformers become more favored species in solution as the denaturant concentration becomes sufficiently high. Since the contributions from various non-native states almost always overlap [10], their distinction requires that the ESI MS data be processed using chemometric methods, such as factor analysis [12]. Briefly, a large array of ESI MS data is acquired over a wide range of solution conditions

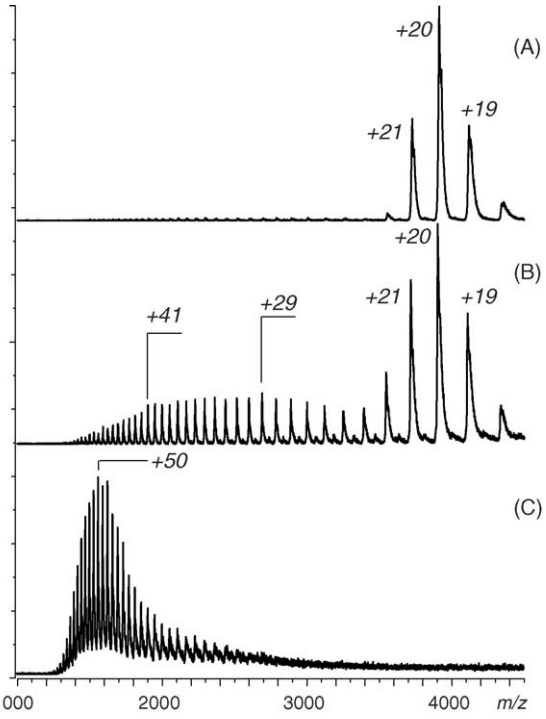

Fig. 1. ESI mass spectra of an 80 kDa protein transferrin acquired on a magnetic sector instrument under near-native (10 mM ammonium acetate, pH 7.0, panel (A)), mildly denaturing (10 mM ammonium acetate, pH adjusted to 5.0, panel (B)) and strongly denaturing (water/methanol/acetic acid, 47:50:3, v:v:v, panel (C)) conditions. Emergence of non-native (partially unfolded) states is evident in (B) as the charge state distribution becomes bimodal. Further unfolding of the protein (population of significantly less compact states) is manifested in (C) by a dramatic increase of the abundance of highly charged protein ions.

to ensure adequate sampling of the protein conformational space. Singular value decomposition analysis of this array yields a number of independent components, which (apart from the noise) are responsible for the observed variations of the charge state distributions. This number is equal to a number of protein conformers whose geometries are different enough to allow at least some distinction to be made as far as their individual contributions to the overall charge state distributions. A set of basis functions is then constructed, each representing a "pure signal" of a certain conformer and the entire data array is fitted using this set, yielding ionic profiles of individual protein states over the range of solution conditions [12]. At least in the case of the natively folded proteins and their complexes, average charge accumulated by ESI-generated ions can be used to estimate their SASA [13]. It remains to be seen whether similar estimates of SASA of non-native (partially unfolded) states can be carried out using the extent of multiple charging of protein ions corresponding to non-native conformations.

As is the case with many other MS-based techniques aiming at characterization of macromolecular properties and behavior in solution, the analysis of protein ion charge state distributions in ESI MS can be also affected by various processes occurring in the gas phase. One has to be mindful of the fact that protein compactness is not the only factor that determines the appearance of charge state distributions in ESI MS. Sometimes changes in solvent composition may trigger certain gas phase processes,

which exert significant influence on the extent of multiple charging of protein ions. For example, apparent charge state reduction is observed when low molecular weight anions are present in the solvent at relatively high concentrations, a situation favoring formation of loose protein-anion complexes in solution [14]. Dissociation of such complexes in the gas phase inevitably leads to a proton transfer from the multiply charged protein ion to the departing anion to avoid enormous enthalpic penalty associated with separation of charges of opposite polarity in vacuum [14]. Clear understanding of these processes makes it possible to select a range of solvent conditions (e.g., pH interval), which is wide enough to allow adequate sampling of the conformational space, without triggering gas phase processes that may compromise the results of the analysis of protein ion charge state distributions.

In the present work we consider asymmetric dissociation of protein complexes and aggregates in the gas-phase, another process that under certain conditions may alter charge state distributions of protein ions in ESI MS and provide a "false positive" signal of protein unfolding in solution. We also briefly consider the possibility of having "false negatives," a situation when a protein molecule does not possess a sufficient number of basic sites to accommodate the requisite amount of protons in the unfolded state.

2. Experimental

All mass spectra were acquired on QStar-XL (MDS Sciex, Toronto, Canada) hybrid quadrupole/time-of-flight and JMS-700 MStation (JEOL, Tokyo, Japan) magnetic sector (double focusing) mass spectrometers equipped with standard ESI sources. Protein solutions were continuously infused into the ESI sources at a flow rate of 3–5 μL/min, and N_2 was used as nebulizing gas in each case.

To acquire spectra on the MStation, the nominal resolution was set at 1000 and the magnet was scanned at a rate of 5 s/decade. All ESI source parameters, i.e., desolvating plate temperature, electrostatic potentials on ion optics elements, etc. were kept constant throughout the measurements to insure constancy in protein ion desorption and transmission conditions for each instrument.

To acquire mass spectra on a QStar-XL instrument, decelerating potentials on the skimmer and the orifice plate, were tuned to provide optimal ion desolvation in the interface region. Stability of non-covalent complexes in the gas phase was enhanced by using collisional cooling (a gas flow-restricting sleeve in the ion guide region Q0) [15]. Trapping and gating parameters (IRD, IRW, RO2, IQ3) in the MS/MS mode were selected to optimize the ionic signal in the low *m*/*z* range (monomeric fragments), except in the case of the sickle cell hemoglobin octamer fragmentation, in which case the parameters were adjusted to improve sensitivity in the high *m*/*z* region.

Cellular retinoic acid binding protein I (CRABP I) was expressed using a plasmid, which was generously provided by Prof. Lila M. Gierasch (University of Massachusetts-Amherst). The apo-form of human Ferritin (HuHf) was generously provided by Prof. N. Dennis Chasteen (University of New Hampshire). All other proteins used in this work were purchased from Sigma–Aldrich Chemical Co., St. Louis, MO and used following purification by ultra-filtration to remove low molecular weight impurities. All chemicals and solvents were of analytical grade or higher.

3. Results and discussion

3.1. Protein aggregation and charge state distribution of monomeric ions in ESI MS

The soft nature of the ESI process allows weak non-covalent interactions among various macromolecules in solution to be preserved in many cases following transition to the gas phase, thus enabling direct observation of specific assemblies of proteins and other biopolymers [16,17]. In addition to forming highly specific assemblies, many proteins may also form non-specific complexes and even aggregates in solution. Although some proteins are infamous for their propensity to aggregate under certain conditions both in vivo and in vitro (such as a variety of amyloid-forming proteins), many others also can aggregate in solution, given high enough concentration. While the molecular mechanisms leading to amyloid formation remain an area of extensive research, a consensus is already emerging that (partially) unfolded states play an important role in protein aggregation [18]. The ability of ESI MS to provide information on composition of protein aggregates (if they survive the transition to the gas phase) [19], as well as conformational dynamics of individual proteins [20,21], gives rise to an expectation that it may become an extremely powerful experimental tool to study mechanisms of protein aggregation in solution.

One of the proteins that aggregates readily in solution, although its amyloid-forming properties in vivo have not been documented, is cellular retinoic acid binding protein I, CRABP I [22]. The high β-sheet content of CRABP I is likely the most important factor making it aggregation-prone in solution, particularly at elevated concentrations. The endogenous concentration of CRABP I in tissues expressing this protein (e.g., limb buds, neuronal tissues, etc.) is estimated to be in the range 1–10 μM, a level that corresponds to ca. 10^7 to 10^8 protein molecules per cell [22]. In the low mM concentration range formation of dimers and higher oligomers becomes detectable by high-field NMR (translational self-diffusion coefficient measurements), and the presence of small amount of dimers or other oligomers cannot be ruled out at lower concentrations [23].

The ESI mass spectrum of a 2 μM aqueous solution of apo-CRABP I contains only ion peaks corresponding to the monomeric state of the protein (Fig. 2, top). The charge state distribution is narrow, and the average number of charges accommodated by the protein ions is low, indicating that the protein is compactly folded in solution. A noticeable change in the spectrum occurs when the protein concentration is increased 10-fold to 20 μM (Fig. 2, middle). A new set of ion peaks appears in the *m*/*z* range 2300–2800. The mass of this ionic species exceeds that of CRABP I by a factor of 2, and the average charge is +12, suggesting that it corresponds to a protein dimer. Further increase of the protein concentration leads to broadening of the

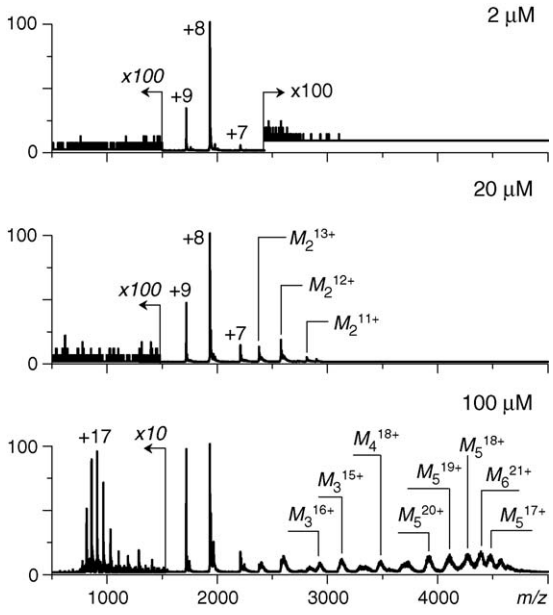

Fig. 2. ESI mass spectra of CRABP I acquired on a magnetic sector mass spectrometer under mild desolvation conditions in the ESI interface. Protein concentration in solution increases from 2 μM (top) to 100 μM (bottom). All spectra are acquired under conditions precluding detector saturation.

dimer ion peaks, as well as to the appearance of new ionic species, whose masses correspond to a CRABP I trimer (*m*/*z* 2800–3300), tetramer (*m*/*z* 3300–3800) and higher oligomers (penta- and hexameric protein species at *m*/*z* 3800–4700, Fig. 2, bottom).

In addition to prominent signal corresponding to oligomeric ions, elevated protein concentration gives rise to monomeric protein ions with a high number of charges (a distribution centered around *m*/*z* 900), a feature that is notably absent in the spectra acquired at low protein concentration. Although it may be tempting to assign the highly charged protein ions to partially unstructured states of CRABP I that serve as obligatory precursors of protein aggregation, several factors make such conclusions highly suspicious. First of all, the extent of multiple charging of these ions is very high (average charge between +17 and +18), which significantly exceeds the average charge accumulated by partially unstructured CRABP I ions upon acid-induced unfolding (Fig. 3E). In fact, +18 is the average charge corresponding to the conformation which was previous assigned as fully unstructured state of CRABP I [10]. More important, however, is the fact that although the ESI mass spectra of concentrated CRABP I acquired on a quadrupole-TOF mass spectrometer (QStar) also feature bimodal charge state distributions of monomeric protein ions, the average charge corresponding to the unfolded monomers is now significantly lower (Fig. 3B). Although some variations in the average charge states of ionic signals corresponding to the same protein conformation are indeed expected when the data obtained with two different mass analyzers are compared, the observed discrepancy is too high to be accounted for by different transmission characteristics of the magnetic sector and hybrid quadrupole-TOF analyzers. This becomes particularly evident when one compares the average charges of natively folded proteins in the ESI mass spectra acquired with these two instruments (Fig. 3C and D). Furthermore, there are no dramatic differences in the charge state distributions of CRABP I ions in the ESI mass spectra acquired with the two mass analyzers under mildly denaturing (acidic) conditions when the protein concentration is relatively low (compare Fig. 3E and F).

One possibility that may explain the emergence of highly charged protein ions in ESI mass spectra acquired at elevated protein concentration without invoking the notion of unfolding is the asymmetric dissociation of non-covalently bound oligomers in the gas phase [24]. For example, a monomeric ion carrying 17 charges (Fig. 2, bottom) can be a product of an asymmetric gas phase dissociation of a weakly bound hexamer carrying 21 charges (the complementary fragment ion, a pentamer carrying the 4 remaining charges will not be observed in the mass spectra presented in Fig. 2, as its *m*/*z* falls well above 5000). The difference between the charge density of monomeric fragment ions observed with the two types of mass analyzers would indeed be expected in this case, as there is a significant difference in the dissociation time frames. Metastable oligomeric ions that dissociate on a millisecond time scale giving rise to highly charged monomers in a quadrupole mass analyzer, would have ample time to reach the detector without undergoing dissociation when analyzed on a magnetic sector mass spectrometer.

In order to verify the gas phase origin of highly charged monomeric ions of CRABP I, fragmentation of mass-selected oligomeric ions was carried using collision-activated dissociation (CAD) in a hybrid quadrupole-TOF mass spectrometer. An ion peak corresponding to a pentamer carrying 20 charges (M_5^{20+}) is very prominent in the mass spectrum of CRABP I acquired at elevated protein concentration and does not overlap with peaks corresponding to other oligomers. Mass-selection of this ionic species followed by CAD gives rise to a typical asymmetric fragmentation pattern (Fig. 4, top). Interestingly, the shape of the distribution of monomeric fragments is remarkably close to that of the highly charged CRABP I ions seen in the original spectrum of this protein (Fig. 4, bottom). It follows then that the metastable M_5^{20+} ions are among the major contributors to the ionic signal of the monomeric protein ions at charge states +9 through +12. Other metastable oligomeric ions are also likely to contribute to the population of highly charged monomers in the mass spectrum, although extensive overlap of ionic signals corresponding to most oligomers makes it difficult to assess their individual contributions.

In addition to the emergence of highly charged protein ion peaks in ESI mass spectra of CRABP I, another change in the charge state distributions of monomeric ions become apparent at elevated protein concentrations (Fig. 2, top to bottom). As soon as the dimeric species peaks become prominent in the spectrum, the abundance of the charge state +9 begins to increase. This leads to an apparent shift (increase) of the average charge corresponding to the natively folded protein and it may be tempting to interpret this phenomenon as a small-scale conformational change that leads to a modest increase of SASA. Once again, a suspicion arises when intensity distribution of the low-charge

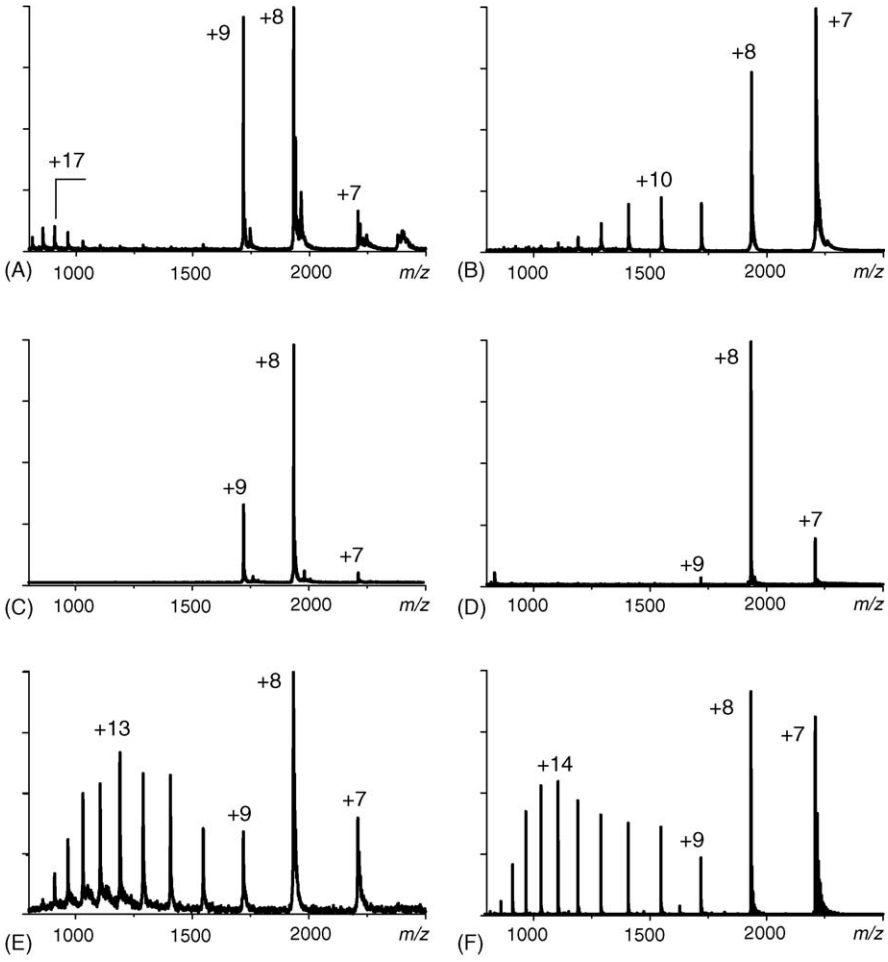

Fig. 3. Charge state distributions of CRABP I ions in ESI mass spectra acquired with magnetic sector (left-hand panels) and hybrid quadrupole-TOF (right-hand panels) mass spectrometers using mild conditions in the ESI interface region. Top panels: protein concentration 100 μM, solution pH 7.0; middle panels: protein concentration 2 μM, solution pH 7.0; bottom panels: protein concentration 2 μM, solution pH 3.5.

density monomers is monitored on a hybrid quadrupole-TOF mass spectrometer. In this case a change in the average charge of ions representing the native state is also evident, although the direction of this shift is opposite to what is observed with a magnetic sector mass spectrometer. Since the onset of these shifts in both cases appears to correlate with the emergence of the dimeric ions, it seems reasonable to assume that it is the dissociation of metastable dimers that leads to the observed alteration of the monomeric signal in the higher *m/z* region.

In order to verify the link between dissociation of oligomer ions in the gas phase and the observed changes in charge state distributions of monomeric ions in ESI mass spectra, a prominent dimeric ion M_2^{11+} was mass-selected and subjected to CAD in a collision cell of a hybrid quadrupole-TOF mass analyzer. Fragmentation of this dimer also involves an asymmetric charge partitioning, consistent with the earlier observations of homodimer dissociation [25]. Importantly, the major higher charged fragment monomer carries seven charges (Fig. 4C), hinting at the gas phase origin of the decrease of the average charge of ions corresponding to the compact conformations of CRABP I observed with a quadrupole-TOF MS at elevated protein concentration. Once again, asymmetric dissociation of a non-covalent complex in the gas phase leads to a false positive detection of a conformational transition in solution.

3.2. On the possible mechanisms of charge partitioning upon dissociation of non-covalent complexes in the gas phase

Asymmetric partitioning of both mass and charge between the complementary fragments is an intriguing aspect of dissociation of protein complexes in the gas phase [25–28]. Typically, a monomeric fragment accommodates a disproportionately large fraction of the total charge, while the rest of the complex remains intact. The asymmetric charge partitioning has been linked to conformational changes within the monomeric fragment in the gas phase [25,29] and assumed mobility of surface protons was proposed to be a major factor driving monomer unfolding. However, charge enrichment of a single subunit appears to be thermodynamically unfavorable [28,30] and the exact mechanism leading to such a selective charge-assisted monomer unfolding remains unknown.

Several authors have suggested in the past that dissociation of non-covalent assemblies of macromolecular ions in the gas

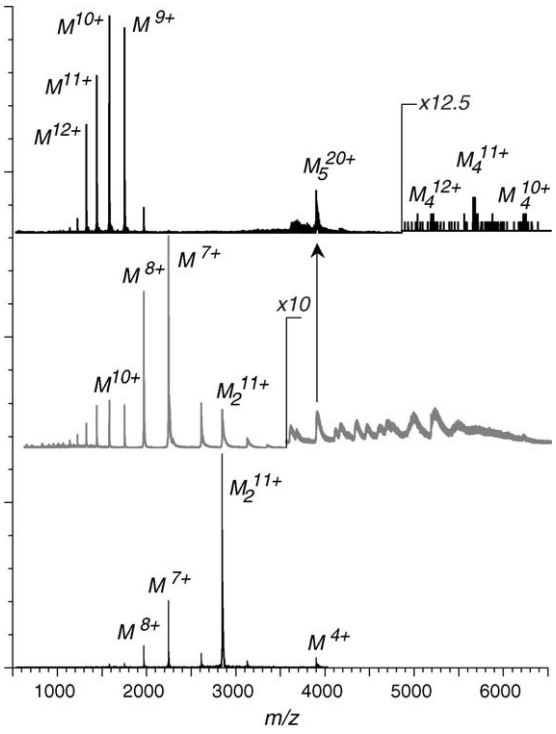

Fig. 4. CAD mass spectra of CRABP I pentamer (top) and dimer (bottom) acquired with a hybrid quadrupole-TOF mass spectrometer. The middle trace represents a mass spectrum of concentrated CRABP I solution (50 μM) acquired under mild conditions in the ESI interface region.

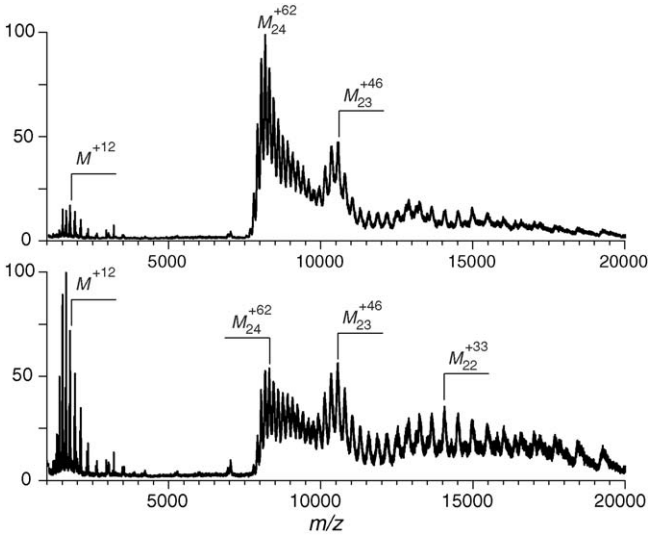

Fig. 5. Mass spectra of apo-form of human ferritin acquired with a hybrid quadrupole-TOF mass spectrometer under mild desolvation conditions (top, de-clustering potential DP = 200 V, focusing potential FP = 265 V) and using collisional activation in the ESI interface region (bottom, de-clustering potential DP = 280 V, focusing potential FP = 300 V). The protein was dissolved in 10 mM ammonium acetate to a concentration of 8 μM.

phase can be qualitatively described within the framework of a charged droplet model, whose various modifications (e.g., deformed jellium model) have been very successful as a means to describe dissociation of charged metal clusters [31]. However, systematic theoretical consideration of the macromolecular complex dissociation using a discretely charged ellipsoid model (DCEM) produced somewhat ambiguous results [30]. While the asymmetric charge partitioning was clearly linked to changes in the fractional surfaces of the fragments, DCEM produced only static information and failed to explain the selectivity of subunit unfolding in the gas phase [30].

One thing that is often overlooked when considering dissociation of macromolecular complexes is the near-critical number of charges (i.e., close to the Rayleigh instability limit for a liquid droplet of the same size) accumulated by such assemblies generated by ESI [32]. As has been demonstrated in the previous section, some non-covalent complexes produced by ESI are metastable and dissociate even on the μs timescale without additional activation in either ESI interface or collisional cell. Perhaps an even more striking example is presented by the gas phase dissociation of ferritin, a non-covalent assembly of 24 identical subunits (21 kDa) each forming a hollow sphere in solution. Even the combination of most gentle conditions in the ESI interface and collisional cooling in the ion guide region of the quadrupole-TOF mass spectrometer [15] results in limited dissociation of multiply charged ferritin assemblies in the gas phase, which is clearly asymmetric (Fig. 5A). Collisional activation of these non-covalent complexes in the ESI interface region obviously increases the extent of fragmentation, but does not change the mechanism, as the distributions of both highly charged monomers and the complementary M_{23}^{z+} fragments do not appear to be altered (Fig. 5B).

Since collisional activation of both specific non-covalent complexes and protein aggregates facilitates their dissociation without altering fragment ion charge state distributions, it is plausible that such activation simply provides the energy needed to overcome a kinetic barrier without altering the dissociation mechanism. If dissociation of the ESI-generated non-covalent complexes is indeed driven by electrostatic repulsion, it should resemble Coulombic explosion of a liquid droplet charged to the Rayleigh limit [33]. The commonly observed asymmetric dissociation in this case would correspond to a fine fission mode, which is initiated in critically charged droplets by formation of a Taylor cone through which ejection of a small mass of liquid carrying a disproportionately large fraction of the total charge occurs. It seems plausible that fluctuations of charge density on the surface of a globular non-covalent assembly in the gas phase would lead to formation of a local instability similar to the Taylor cone, followed by ejection of highly charged matter. Since the ejection of matter in the fine fission mode occurs through a fine jet, the departing monomer will necessarily unravel in the process of (but not prior to) its ejection (Fig. 7A). Since the jet is formed by a single subunit, it will not disintegrate, unless the departing monomer contains non-covalently bound ligands and co-factors. This is illustrated in Fig. 6C, where asymmetric dissociation of a globular tetrameric complex (human sickle cell hemoglobin HbS) results in ejection of a highly charged monomer (either α or β), whose heme group does not necessarily follow the chain.

A very important question that must be addressed here relates to the nature of the surface charge oscillations leading to formation of a local instability. We note that the non-covalently bound

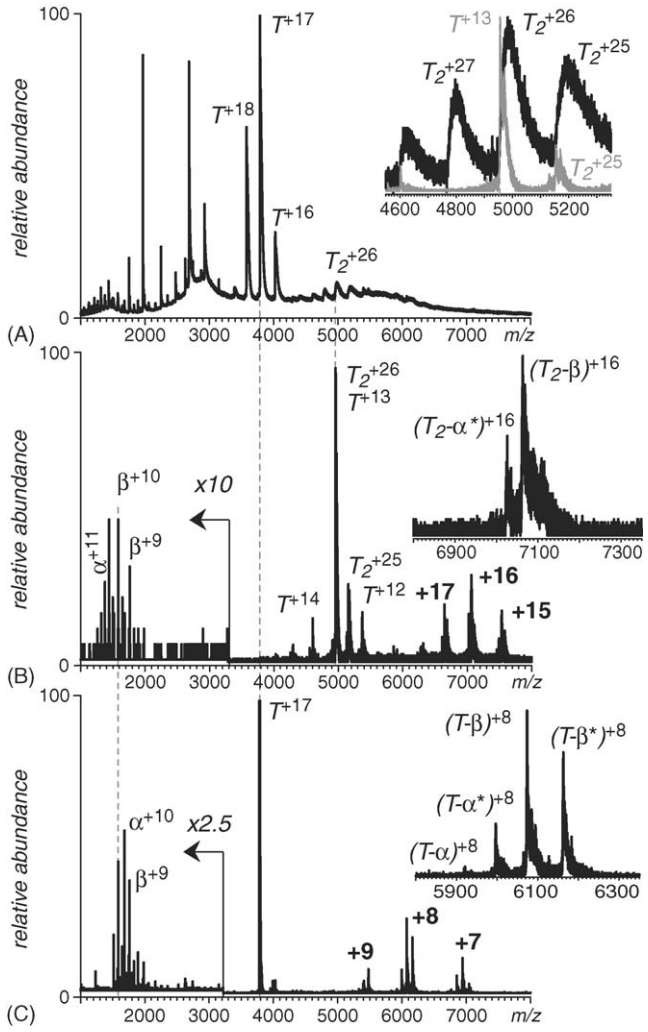

Fig. 6. ESI mass spectra of sickle cell human hemoglobin HbS acquired with a hybrid quadrupole-TOF mass spectrometer. Panel (A) shows an HbS spectrum of acquired under gentle conditions. CAD spectra of HbS octamer and tetramer are shown in panels (B) and (C), respectively. Ionic species labeled in the spectra are tetramers (T), octamers (TT) and their fragments corresponding to losses of α- or β-globins (asterisks denote a heme group). Inset on panel (A) shows overlaid traces of tetrameric signals in the MS1 spectrum of HbS (black) and the CAD spectrum of T_2^{26+} species (gray). Insets on panels (B) and (C) show detailed views of the heptameric (B) and trimeric (C) regions of the corresponding mass spectra.

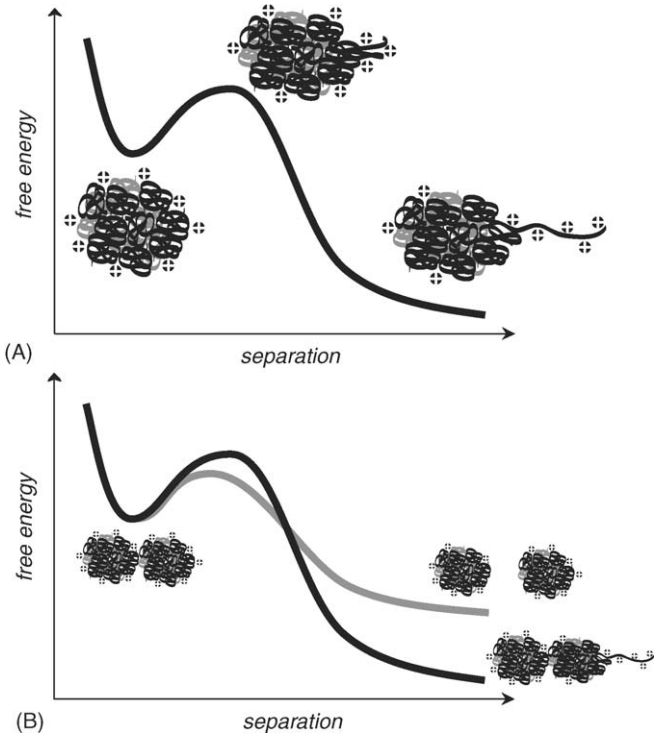

Fig. 7. Hypothetical energy surfaces for gas phase dissociation of a globular (A) and non-globular (B) protein ion complexes.

ionic species are highly solvated in the ESI mass spectrum of HbS (Fig. 6A), a feature which is notably absent from the spectra of dissociation products. It is this solvation shell (composed mostly of the ionic and polar solvent components) that may shuttle the charges on the surface of the complex, eventually leading to formation of a local instability.

Finally, it must be mentioned that disintegration of charged liquid droplets occurs under some circumstances in the so-called rough fission mode [33], which leads to generation of a few (often two) progeny droplets with similar masses. Had the rough fission of a non-covalent complex occurred in the gas phase, it would have generated two fragments of similar size and charge. This situation clearly does not occur upon dissociation of HbS tetramer, which is consistent with numerous earlier reports on dissociation of globular complexes generated by ESI under near-native conditions [17]. Since rough fission of electrically charged droplets proceeds via a saddle shape deformation [34], it may be possible to bias the dissociation towards the rough fission mode by activating a complex whose shape already has a saddle-type deformation, such as sickle cell hemoglobin octamer. Polymerization of human sickle cell hemoglobin in solution occurs via dimerization of tetrameric $(\alpha^*\beta^*)_2$ species, where the interface between the two tetramers is minimal, giving rise to a symmetric two-sphere configuration with a saddle point in the middle [35]. Dissociation of HbS octamer is qualitatively very different from that of HbS tetramer, as it proceeds through two competing channels. Appearance of tetrameric fragments in the spectrum (Fig. 6B) provides a clear indication that symmetric dissociation does occurs in this case, giving rise to two complementary fragment ions whose charges and masses are close to each other (e.g., T^{+12} and T^{+14}). It is also very likely that an abundant signal corresponding to a T^{+13} fragment ions is present of the spectrum as well, but it overlaps with a precursor ion peak. At the same time, the asymmetric dissociation channel is also present in this case, as evidenced by the appearance of abundant heptameric fragments and complementary monomers. This observation is quite remarkable, as it suggests that fine fission competes effectively with rough fission, despite the bias introduced by the shape of the complex. It seems reasonable to assume that such a deformation lowers the energy barrier on the symmetric dissociation (rough fission) pathway, thus making it a viable fragmentation pathway (Fig. 7B). Furthermore, it is rather intriguing that the extent of multiple charging of monomeric fragments does not appear to depend on their origin (tetramer carrying 17 charges or

octamer carrying 26 charges). It seems plausible that the charge of monomeric fragments reflects the value of a critical charge required to trigger local instability followed by a fine fission of the complex.

The fission model of complex ion dissociation presented in this work accounts for all features of asymmetric dissociation reported earlier [25,27]. It provides a dynamic picture of the dissociation process as a fission event resulting from accumulation of a critical charge locally, which leads to ejection of a monomeric thread akin to a fine jet formed at the tip of the Taylor cone on a critically charged droplet. A competing symmetric dissociation channel (rough fission) can be introduced by deforming the complex shape, however, fine fission still remains the most effective dissociation pathway.

3.3. Unfolding and the extent of multiple charging of proteins deficient in basic sites

The examples considered in the previous sections clearly suggest that the emergence of highly charged protein ion peaks in ESI mass spectra do not always signal loss of structure in solution. Ignoring gas phase dissociation processes may give false positives when protein conformational changes in solution are monitored based on the appearance of charge state distributions in ESI mass spectra. It is not inconceivable that the analysis of protein ion charge state distributions may also give false negatives when used as a means of detecting protein unfolding in solution. For example, it is often argued that the extent of multiple charging (protonation) of an unstructured polypeptide chain should be limited by the number of basic residues in its sequence [36]. Specifically, proton affinity of the solvent molecules is argued to provide a "cut-off" level for amino acid residues that can be protonated in the gas phase [37]. Since most proteins possess a high number of basic sites (arginine, lysine and histidine), the requisite number of positive charges can almost always be distributed among the available basic sites in the unstructured polypeptide chain. However, there are several examples of highly acidic proteins, which contain very few basic sites. A classic example of such a system is pepsin, a 33 kDa protein containing 41 acidic and only 4 basic residues (the protein N-terminal amino group can be counted as the fifth basic site). Therefore, one may argue that there are simply not enough basic sites in this protein to afford adequate protonation of the unfolded polypeptide chain in the gas phase.

Our previous studies indicated that at neutral and mildly acidic pH an average positive charge displayed by pepsin ions in ESI mass spectra is 10.7 [13]. This number is very close to that calculated on the basis of native pepsin SASA, and is twice as high as the number of available basic sites. However, instead of simple protonation, the multiple charging appears to proceed in this case via formation of ammonium adducts, which are only marginally stable in the gas phase. Facile dissociation of such protein–NH_4^+ complexes inevitably leads to charge reduction of the protein ions [13], a process driven by the relatively high proton affinity of ammonia.

Our initial expectation was that even if unfolded pepsin can accumulate some extra charges (due to the increase of SASA), these would also be in the form of NH_4^+ or other cations, and the resulting adducts would be prone to facile dissociation in the gas phase (and, therefore, undergo apparent charge state reduction). This, of course, would make the detection of unfolded pepsin difficult and may actually give rise to false negatives if the highly charged pepsin ions are to be used as indicators of protein unfolding in solution. In order to investigate the extent of multiple charging of pepsin under denaturing conditions, a series of ESI spectra were collected over a wide range of pH both in the absence and in the presence of alcohol. Since the physiological environment of pepsin (stomach) is highly acidic, this protein remains compact at strongly and mildly acidic pH [38]; unfolding occurs only in basic environment [39]. Not surprisingly, charge state distributions of pepsin ions in ESI spectra acquired within pH range 2–6 are relatively narrow and display the average number of charges close to that calculated based on the SASA of the native conformation (Fig. 8, bottom trace), consistent with our earlier observations [13]. The increase of solution pH above 7 and/or addition of alcohol result in a dramatic change in the appearance of the charge state distribution (Fig. 8, top trace). Its bimodal character is now evident, and, quite surprisingly, the highly charged pepsin ions can accommodate as many as 38 charges. Furthermore, accurate measurements of masses of these highly charged ions indicate that they are multiply protonated species, not adducts of larger cations. Average molecular weight of pepsin calculated based on measured *m/z* values for charge states +26 through +35 is 34,570 ± 11 Da, which is within the experimental error range of the average mass of the protein calculated based on its sequence (34,581 Da). Should the extensive adduct formation occur, the measured mass would exceed the calculated one by a multiple of the cation mass less one (e.g., 16 for NH_4^+ adducts, 22 for Na^+, etc.).

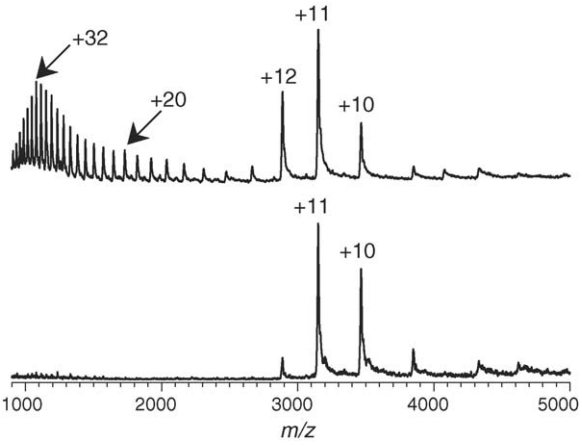

Fig. 8. ESI mass spectra of pepsin acquired with a magnetic sector instrument at pH 10, 50% methanol by volume (top) and at pH 6 in the absence of alcohol (bottom). The pH of a protein solution (10 μM pepsin in 10 mM $CH_3CO_2NH_4$) was adjusted to a desired level with either CH_3CO_2H or NH_4OH.

It is quite remarkable that so many protons can be accommodated by a polypeptide chain, which contains only five basic sites. This is probably a consequence of the greater conformational flexibility of the polypeptide chain in the unfolded state.

Perhaps, such flexibility allows more than one functional group to participate in binding a single proton in a fashion similar to that observed in solvation of larger cations by polymer chains in the gas phase [40]. Such collective proton binding (or "sharing") would certainly increase the apparent proton affinity of any particular binding site and, therefore, lift the restrictions on the extent of protonation imposed by thermodynamic considerations [37,36]. This, of course, would allow the requisite amount of charges to be accumulated by unfolded proteins even if they are deficient in basic sites.

4. Conclusions

Gas phase behavior or protein ions must be taken into account if their characteristics are used to evaluate protein properties in solution. While the emergence of bimodal charge state distributions in ESI MS is often taken as a manifestation of protein unfolding in solution, dissociation of protein complexes and aggregates in the gas phase can give rise to similar distributions as well. Therefore, care must be taken in order to interpret the ESI MS data correctly and avoid false positive signals of protein unfolding. At the same time, unfolding (or, more specifically, loss of compactness) of polypeptide chains in solution necessarily leads to emergence of highly charged protein ions in ESI mass spectra, even if the polypeptide chains lack a sufficient number of basic sites. The surprising ability of highly acidic proteins to accommodate a requisite number of positive charges, which may significantly exceed the number of basic sites, effectively rules out false negatives when charge state distributions are relied upon as indicators of unfolding in solution.

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