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RESEARCH ARTICLE

Ion Mobility-Mass Spectrometry Reveals Conformational Changes in Charge Reduced Multiprotein Complexes

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Abstract

Characterizing intact multiprotein complexes in terms of both their mass and size by ion mobility-mass spectrometry is becoming an increasingly important tool for structural biology. Furthermore, the charge states of intact protein complexes can dramatically influence the information content of gas-phase measurements performed. Specifically, protein complex charge state has a demonstrated influence upon the conformation, mass resolution, ion mobility resolution, and dissociation properties of protein assemblies upon collisional activation. Here we present the first comparison of charge-reduced multiprotein complexes generated by solution additives and gas-phase ion-neutral reaction chemistry. While the charge reduction mechanism for both methods is undoubtedly similar, significant gas-phase activation of the complex is required to reduce the charge of the assemblies generated using the solution additive strategy employed here. This activation step can act to unfold intact protein complexes, making the data difficult to correlate with solution-phase structures and topologies. We use ion mobility-mass spectrometry to chart such conformational effects for a range of multi-protein complexes, and demonstrate that approaches to reduce charge based on ion-neutral reaction chemistry in the gas-phase consistently produce protein assemblies having compact, 'native-like' geometries while the same molecules added in solution generate significantly unfolded gas-phase complexes having identical charge states.

Key words: Noncovalent complexes, Gas-phase protein structure, Charge manipulation, Collision cross section

Introduction

Over the last two decades, the interconnected nature of life processes has been revealed through the development of analytical approaches that are able to capture and characterize the complexity of interacting proteins and other biomolecules. Because virtually all cellular processes are interconnected, such multi-component protein assemblies have critical significance in health and medicine. Mass spectrometry (MS) has been a key tool in assessing such complex biological systems [1, 2]. These experiments have been conducted using both "bottom-up" methods, where interacting partners are detected by defining the composition of fractions derived from affinity capture or chemical cross-linking [3, 4], and by "top-down" approaches, where the interaction network is observed intact by MS [5, 6]. Both types of datasets have been instrumental in defining contact diagrams for protein interaction networks, and can often provide limited information on the three-dimensional structure of such assemblies [7, 8].

The incorporation of ion mobility (IM) separation into such MS experiments is an important emerging approach for

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the structural characterization of biomolecules and their higher-order complexes [9–11]. Following on from early results indicating that the topology and quaternary organization of multiprotein complexes can be assessed and related to structures determined using X-ray crystallography and nuclear magnetic resonance spectroscopy [12, 13], the applications of IM-MS to protein assemblies have increased dramatically. Recent examples include studies aimed at assessing binding events within protein cavities [14], refining protein interaction networks [15, 16], characterizing multiprotein complexes bound to small molecules [17], and assessing the relative stability of membrane protein complexes [18]. In all of these reports, IM provided a critical structural assessment of a multiprotein complex system found difficult or impossible to characterize using other structural biology technologies.

In multiple studies, the influence of protein complex ion charge state is often highlighted as a key factor in altering the information content of IM-MS and tandem MS measurements [13, 14]. For example, the collision induced dissociation (CID) process for natively-charged multiprotein complexes produced by nano-electrospray ionization (nESI) often results in an effective charge reduction step, where the stripped protein complexes that have lost a monomeric unit also lose a large portion of the charge originally carried by precursor ions [5, 19]. This effect has been used to great advantage to characterize polydisperse protein complexes for which the primary mass spectrum is difficult to interpret due to spectral overlap [20]. Further, several datasets have highlighted the influence of precursor ion charge state on both the structure and type of product ions produced from multiprotein complex CID. Recent data have highlighted the ability of ion charge state to alter the apparent mechanism of dissociation, where precursor ions of lower charge tend to eject compact (rather than the typically unfolded) monomers [21]. Additional datasets have indicated similar effects for charge amplified protein complex ions in specialized cases [22, 23]. In datasets where extremely low or high charge states are achieved through altering either solvent composition or nESI emitter position, covalent bonds rather than noncovalent protein–protein interactions can be broken to produce sequence informative fragment ions [21, 22].

A further set of charge-related effects observed for multiprotein complex ions center on datasets that demonstrate the apparent reliance of gas-phase structure upon ion charge state. In most cases, protein ions display a distribution of charge states when produced through nESI, and IM and MS measurements indicate that ions having the lowest ionic charge are the most “native-like” [11, 13, 24], in that IM data for ions of low charge states are, in general, the most-correlated to X-ray and nuclear magnetic resonance (NMR) datasets [25–27] and are often the most useful data points when constraining topological models of multiprotein complexes from IM-MS data [16]. For example, in early experiments involving tryptophan-RNA binding attenuation protein (TRAP) 11-mer ions, higher charge states were observed to be more compact relative to ions having lower charge [13]. The charge state dependent nature of protein complex structure has also been observed in other datasets, including a large database of high-precision collision cross-section measurements, in which both increases and decreases in ion size as a function of charge state are observed [28]. Although all of these reports end with similar conclusions regarding the influence of charge on gas-phase protein structure, the mechanistic details that lead to this apparent charge-state dependant behavior have yet to be completely elucidated.

There are multiple methods and protocols available for manipulating the charge state of ions produced by nESI [29–39]. In general, charge manipulation is achieved using three strategies: solution additives, ion-neutral chemistries, and ion–ion chemistries. Methods that utilize solution additives are among the simplest approaches to implement experimentally [31], but have distinct disadvantages for the analysis of multiprotein complexes where the stability of the assembly may be altered significantly through only small changes in solution composition, pH, or ionic strength [40]. Ion–ion chemistries allow for fine control and high efficiency in manipulating the charge states of biomolecules, but require either modified ion sources or specialized ion trap approaches for successful charge reduction or amplification [36, 39]. Approaches centering upon ion-neutral chemistries are inherently less efficient that those described above [30], but combine some of the simplified aspects of solution additive approaches with the fine control of ion–ion approaches for the generation of charge-modulated biomolecular ions. Critically, there is little data currently that describes the relative influence of these different charge modulation strategies on the gas-phase structures of biomolecular ions, with the majority of data focused on monomeric proteins [29, 41–46]. Multiprotein complexes have been charge-modulated using primarily solution additive-type strategies [31], but the influences of such approaches on the quaternary structure of multiprotein complexes in the absence of bulk solvent is poorly understood.

In this report, we compare charge reduction methods based on solution-additives to those based on gas-phase ion-neutral reaction chemistry by assessing and comparing the structures of the ions generated in terms of their collision cross-sections as a function of charge state. We find that while both approaches can achieve similar amounts of charge reduction, ranging from 2.1%–28% effectiveness, the solution based-additive approach studied here requires significant levels of collisional activation in order to shed positive ions and generate charge reduced protein complex ions. Therefore, ions produced by the solution-additive based method produce larger ions having undergone conformational rearrangements and unfolding [47]. Conversely, the gas-phase ion-neutral approach universally produces compact, ‘native-like’ ions. Critically, charge reduction carried out by ion-neutral chemistry in the gas-phase can impart similar charge reduction effectiveness

when compared with analogous solution-phase approaches, without the need to introduce solution additives that may act to disrupt the oligomeric state or structure of ions prior to nESI analysis. Thus, we demonstrate that charging on multiprotein complexes by ESI can be modulated by the gas-phase ion-neutral chemistry methods described herein, and can be used in conjunction with CID to improve the utility of IM-MS as a tool for characterizing the structure of biomolecules.

Experimental

The protein complexes studied here, avidin (chicken egg white), alcohol dehydrogenase (yeast, ADH), and pyruvate kinase (rabbit muscle, PK), were purchased from Sigma-Aldrich (St. Louis, MO, USA) as well as ammonium acetate and the charge reducing bases triethylamine (TEA), 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), and 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU). Protein stock samples prepared to 100 or 50 μM in 100 mM ammonium acetate (pH 6.9) were de-salted in 100 mM ammonium acetate using Micro Bio-Spin 6 columns (Bio-Rad, Hercules, CA, USA) and diluted to a final concentration of 10 μM. Protein samples prepared with solution additives contained charge reducing agents at concentrations of 5–20 mM. For the study of gas-phase ion-neutral reactions, aqueous solutions of the charge reducing agents TEA, DBN, and DBU were prepared to 1.4, 1.7, and 0.7–2.0 M concentrations, respectively. Bases were chosen based on their common use in previous experiments involving multiprotein complexes both in our lab and others [31].

Ion mobility-mass spectrometry experiments were performed on a quadrupole-ion mobility-time-of-flight mass spectrometer (Q-IM-TOF MS) purchased from Waters (Synapt G2 HDMS; Manchester, UK) [48]. While earlier versions of the Synapt instrument platform utilizing a nano-electrospray ionization (nESI) source were also fitted with a reference emitter that included a nebulizing sheath flow, the reference sprayer provided by the manufacturer for the nESI source on the Synapt G2 does not. Figure 1 shows a schematic diagram of the modified nESI ion source, where the nESI reference sprayer is replaced with a simple nebulizer, which acts to introduce neutral base molecules into the source near the sampling cone, perpendicular to the analyte spray, allowing ion-neutral chemistry to occur before protein ions enter the sampling orifice. The device was designed to introduce base into the source through a stainless steel capillary; the capillary passes through a hollow chamber within the sprayer, which directs the sheath gas flow. The sprayer tip then focuses the sheath gas to pass directly over the capillary tip in order to nebulize the base molecules. The base flow rate ranges from 10–25 mL/hr with the nitrogen sheath gas pressure optimized for continuous nebulized droplet formation ranging from 20–35 psi. A typical nebulizer flow rate used in these studies is 10 mL/h, with a gas pressure of 20 psi.

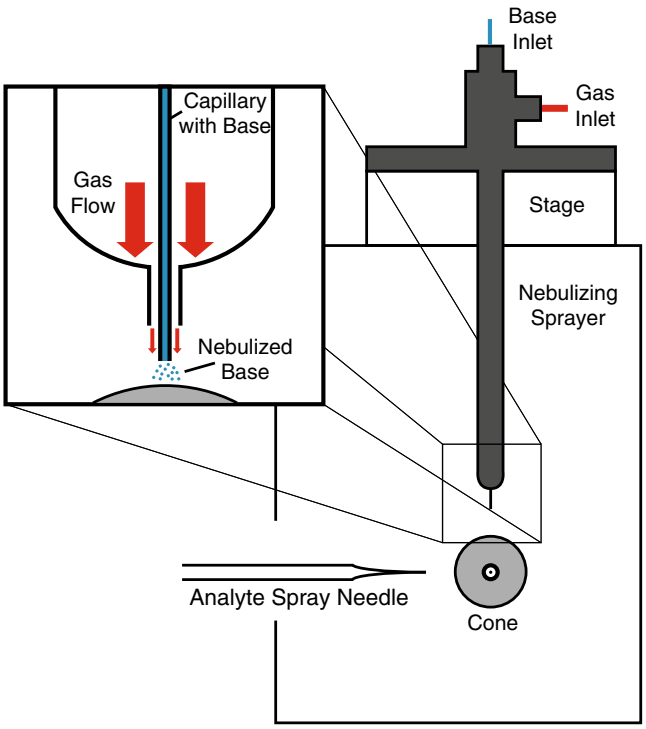

Figure 1. A schematic of the modified nano-electrospray ion source for the Synapt G2 instrument used in these studies. Nebulized base molecules are introduced into the source perpendicular to the analyte spray. Base solution flows through a stainless steel capillary, which is enclosed in a hollow chamber within the sprayer. Sheath gas flows through the hollow chamber and exits out the sprayer tip. The tip is designed to force sheath gas over the end of the capillary, causing the base to nebulize for interaction with electro-sprayed protein complex ions

To generate protein complex ions, an aliquot of sample (~5 μL) was sprayed from the nESI emitter using capillary voltages ranging from 1.8–2.0 kV, with the source operating in positive ion mode and the sample cone operated at 20 V. The trap travelling-wave ion guide was operated with an argon gas pressure of 3.3×10^{-2} mbar and a 50 V trap bias (as much as 150 V was used in solution phase charge reduction experiments). The travelling-wave IM region was operated with a nitrogen gas pressure of 3.5 mbar and employed a series of DC voltage waves (wave heights: 30–35 V, wave velocities: 500–600 m/s) to generate IM separation. The TOF-MS was operated over an *m/z* range of 800–15000 with a pressure of 1.6×10^{-6} mbar. Collision cross-section (CCS) measurements were made using known CCS values of avidin, ADH, and PK, as well as cytochrome *c*, concanavalin A tetramer, and glutamine dehydrogenase (Sigma-Aldrich), as calibrants using the method described previously [8, 28]. All mass spectra were calibrated

externally using a solution of cesium iodide (100 mg/mL) and were processed on Masslynx 4.1 software (Waters). Charge reduction effectiveness was determined using:

$$Eff(\%) = \frac{q_{avg,Control} - q_{avg,CR}}{q_{avg,Control}} \quad (1)$$

where $q_{avg,Control}$ and $q_{avg,CR}$ are the average charge state distributions (q_{avg}) for control and charge reduced (CR) protein ions, and are calculated using:

$$q_{avg} = \frac{\sum_i q_i \times w_i}{\sum_i w_i} \quad (2)$$

where q_i is the charge on the i^{th} charge state and w_i is the signal intensity for the i^{th} charge state.

Results and Discussion

Initial experiments in our laboratory were aimed at characterizing many different methods of charge reduction in terms of multiple figures of merit, some of which are unique to the IM-MS experiment. For example, in order to successfully build model structures of multiprotein complexes, CCS measurements of proteins in their compact state are usually preferred over measurements of unfolded forms because compact states are more-facilely related to solution-phase architectures [49]. Although limited measurements have been performed to assess the influence of charge reducing ion-neutral chemistries on the structures of small monomeric proteins [38], no data is currently available that rigorously assesses the influence of such chemistries on the gas-phase quaternary structure of proteins or in comparison with the solution additive approaches for charge reducing ions produced by ESI. In addition to monitoring the gas-phase structure of the ions produced using various charge reduction protocols, we have evaluated charge reduction strategies in terms of two other figures of merit. We define charge reduction 'efficiency' as the amount of charge reducing agent required to observe a given level of charge reduction. Further, we define charge reduction 'effectiveness' as the ultimate extent of charge reduction observed relative to control datasets (see Experimental section for details).

Figure 2a shows charge reduction effectiveness and efficiency data for the avidin tetramer (64 kDa) acquired using our two charge reduction conditions. The plots presented show trends in the average protein charge state distribution (q_{avg}) recorded under optimum instrument conditions for charge reduction (i.e., optimized acceleration voltages, see the Experimental section) versus DBU solution concentration for gas-phase ion-neutral charge reduction ranging from 0.7–2.0 M (blue) and DBU solution additive concentration ranging from 10–20 mM (green). The data shows that the concentration of charge reducing agent, used either as a solution additive or nebulized into the source for ion-neutral chemistry, is inversely correlated with the

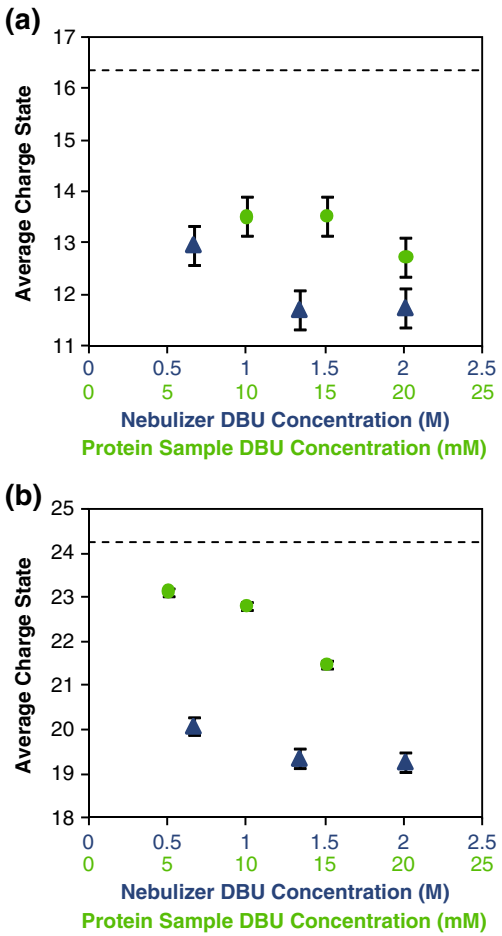

Figure 2. Plots of average avidin **(a)** and ADH **(b)** tetramer charge state against the concentration of DBU used in nebulized base solutions (▲) and as an additive in protein samples (●). Error bars shown for each measurement represent standard deviation calculations from average charge state measurements spanning at least three replicates. The dashed-line represents the average charge state recorded for protein complex ions in control experiments (no solution additives or nebulized base). In all cases, solution-phase additives exhibit higher charge reduction efficiencies (charge reduction per-unit of base concentration) and the use of nebulized base exhibits greater charge reduction effectiveness (ultimate charge reduction amount achieved)

average protein charge state recorded for the avidin tetramer. In addition, while the solution additive approach exhibits substantially higher charge reduction efficiencies (charge reduction per-unit concentration of base utilized), ion-neutral charge reduction is more effective, evidenced by the lower ultimate protein charge states achieved using this approach (Figure 2a). Base concentrations added to protein complex samples in solution above those shown in Figure 2 often result in signal suppression and protein complex disruption (see Supplemental Information). Similar trends are observed for the ADH tetramer (144 kDa), and are shown in Figure 2b over the concentration ranges 0.7–2.0 M and 5–15 mM for in source gas-phase charge reduction (blue) and solution

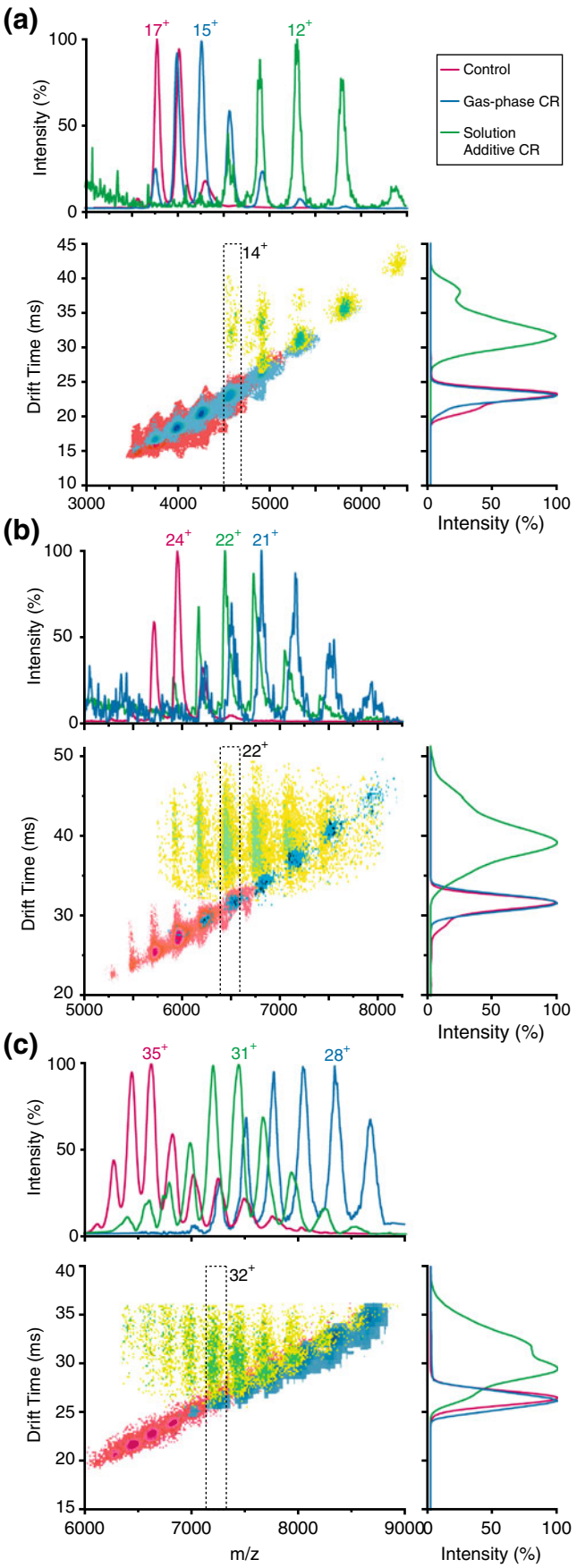

Figure 3. Mass spectra (top), drift time verses *m/z* contour plots (bottom), and IM drift time spectra (right) of **(a)** avidin, **(b)** alcohol dehydrogenase, and **(c)** pyruvate kinase. Drift time spectra shown represent the 14^+, 22^+, and 32^+ ions for avidin, ADH, and PK, respectively. Red spectra and contour plots correspond to control conditions, blue spectra and contour plots correspond to gas-phase charge reduction conditions with DBU, and green spectra and contour plots correspond to solution additive charge reduction conditions with DBU. We have acquired similar datasets where DBU is replaced with either DBN or TEA bases. Increased IM drift times observed on the contour plots and drift time spectra corresponding to solution-phase additive datasets indicate protein complex unfolding relative to control and gas-phase charge reduction data

additive charge reduction (green), respectively. Again, the solution additive approach is a substantially more efficient process with respect to the base utilized in the experiment, routinely producing significant charge reduction using four orders of magnitude less base than equivalent ion-neutral reaction chemistry approaches. Conversely, ultimate charge reduction effectiveness values for both avidin and ADH datasets, respectively, were calculated to be 22% and 11% for the solution additive protocol and 28% and 20% for the ion-neutral reaction chemistry approach. Similar trends are observed for protein complex charge reduction when other basic molecules are used (e.g., TEA and DBN, data not shown). Thus, in general, while adding base molecules directly into protein complex samples is a more efficient means of charge reduction, nebulized base used for ion-neutral charge reduction following nESI is a more effective charge reduction method under the conditions used here.

The metrics of charge reduction efficiency and effectiveness are critical measures that allow for the analytical comparison of charge reduction protocols in general. However, the influence of such approaches on the gas-phase structure and topology of protein complexes measured by IM-MS is arguably a more important criterion for assessing the applicability of such methods in structural biology. Figure 3a shows data for the avidin tetramer (64 kDa) acquired using three experimental conditions. The red contour plot displays a control dataset, with no base added either in solution or in gas-phase. As the avidin tetramer is ionized and transferred into the gas phase with minimal activation, charge states corresponding to avidin tetramer center around 17^+ with drift times that are consistent with compact 'native-like' tetramers. In contrast, avidin tetramer ions produced from solutions with added DBU (20 mM) exhibit extensive charge reduction (green in Figure 2a). As described in previous reports, DBU (and other similar bases) adheres to the surface of protein complexes in solution and subsequent nESI generates clustered ion-neutral complexes. These ion-neutral clusters further desolvate such that the small basic molecules carry charge away from the protein complex ions, thus resulting in charge reduction [31]. In our experiments with DBU and similar bases, a small amount of

accelerating voltage is necessary to reduce charges during desolvation, presumably by promoting the dissociation of basic molecules from the complex through energetic ion-neutral collisions. For the data shown in Figure 3a, the ions were accelerated at 150 V in the ion trap prior to the IM separation region of the instrument to produce maximum charge reduction, and similar results are achieved by applying activation voltage in the skimmer-cone region of the instrument. While the activation voltage used is insufficient to cause the protein complex to dissociate, IM data reveals that the protein complex ions generated by adding base in solution undergo substantial unfolding under these conditions. In contrast, if the same basic molecules are nebulized into the ion source region of the instrument, charge reduction is observed in the absence of energetic collisions and the ions remain compact (blue dataset, Figure 3a). Drift time spectra for each of the three ionization conditions are presented for the 14^+ ion of avidin tetramer. Control (red) and gas-phase charge reduced (blue) datasets correlate well, while solution additive charge reduction data (green) is shifted to longer drift times. Furthermore, drift time features are substantially broadened in solution additive data when compared with control datasets, and IM resolution is observed to decrease by approximately a factor of 2.

Similar results to these are observed for other multi-protein complex ions. Figure 3b shows data for ADH tetramer ions, where substantial unfolding is observed for the more highly charged region of the charge state distribution produced from solutions with added DBU, and the ions exposed to nebulized DBU remain compact (drift time spectra shown for 22^+ ions). While the conclusions reached for this dataset are similar to those for avidin, some differences are apparent. For example, ADH tetramer ions charge reduced using solution additives exhibit a larger degree of gas-phase unfolding than observed in avidin datasets. In addition, the charge reduction effectiveness for ADH tetramer ions is observed to be substantially higher under the conditions used in Figure 3b than that observed for avidin datasets under similar conditions (27% for ADH versus 8% for avidin in Figure 3a). Similar observations are made in the case of PK (232 kDa) tetramer ions. The data shown in Figure 3c suggest that significant conformational changes occur for most of the PK tetramer charge states produced using the charge reduction method based on solution-additives (green). Similar to avidin and ADH, compact and 'native-like' PK IM data (drift time spectra show 32^+ ion) is observed for complexes exposed to nebulized base after ionization. In some cases, activating ions to produce charge reduction following the addition of base in solution produces ion populations that are compacted relative to both control (red) and gas-phase charge reduced (blue) datasets. Such compaction effects have been observed for other protein complexes upon activation [13, 50], and are consistent with collision-induced remodeling of the complex prior to gas-phase unfolding. We also observe a larger mass increase for ions having undergone charge reduction using the ion-neutral chemistry approach compared with the solution-additive approach described here. This observation is consistent with the lower levels of ion activation that are possible when base is nebulized into the source rather than added in solution (see the Experimental section above for notes on instrument conditions for the two experimental protocols used here) [51].

CCS measurements for the ions observed in Figure 3 are generated using calibration procedures that involve a set of known ions having a broad range of ion mobility and CCS values, and are plotted as a function of charge state in Figure 4 [28]. Protein ions produced from gas-phase charge

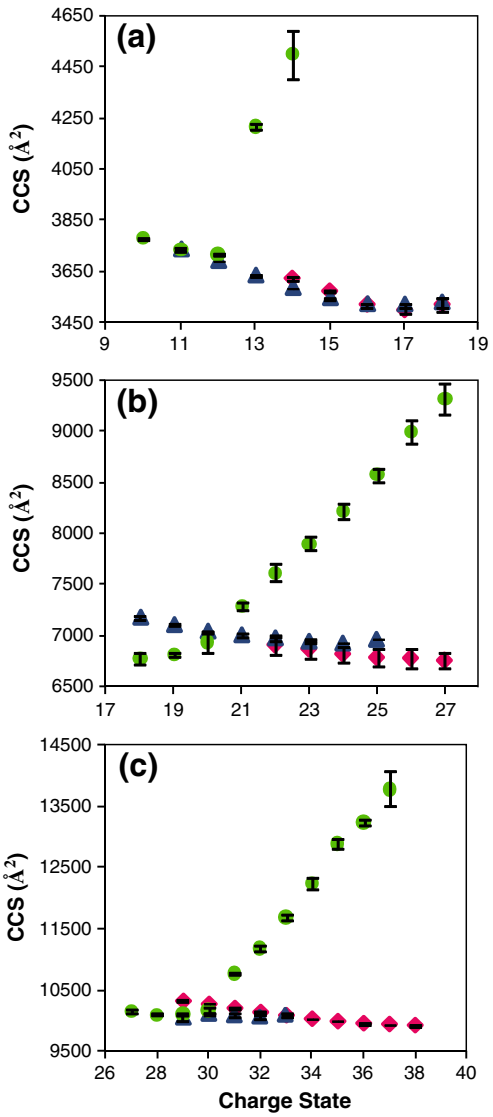

Figure 4. Collision cross-section (CCS) versus charge state plots for **(a)** avidin, **(b)** alcohol dehydrogenase, and **(c)** pyruvate kinase, where (◆) indicate control conditions, (▲) indicate gas-phase charge reduction conditions with DBU, and (●) indicate solution additive charge reduction conditions with DBU. Significant increases in CCS are observed for solution additive datasets compared with both control and gas-phase charge reduction data

reduction generate CCS values similar to control in cases where charge states overlap (blue triangles and red diamonds respectively, Figure 4). A small exception to this general observation is apparent in our PK dataset, where CCS values for ions generated using gas-phase charge reduction are 1%–3% smaller than equivalent ions generated from our control protocol. In contrast, for those ions produced by solution-phase additive approaches, large increases in CCS are observed in most cases when compared with ions produced using either the control or gas-phase charge reduction protocol (green circles). For example, the 33^+ charge state of PK, has a measured CCS from our control dataset of 10084 Å^2. The same ion generated when DBU is nebulized into the ion source has a nearly identical CCS (10094 Å^2, 0.1% different), while the 33^+ ion measured after charge reduction from DBU added in solution is 16% larger than control (11684 Å^2). This increase in CCS across most charge states further indicates protein unfolding upon activation, and confirms our analysis of the data shown in Figure 3. Decreases in CCS of ions following charge reduction via solution additives are also observed in our dataset. Figure 4b shows data for the ADH tetramer, in which CCS decreases ~6% for the 18^+ ions produced with solution additives relative to their counterparts produced by gas-phase ion-neutral chemistry. Such decreases can also be a sign of collision induced remodeling of the protein complex topology [13, 50].

For all protein complex ions generated in control and ion-neutral charge reduction datasets, the trend observed in CCS as a function of charge state is near-linear, having a negative slope in Figure 4. In addition, the observed slope is shallow, with the range of CCS values spanning less than 10% for all protein complex ions produced by these two methodologies. As discussed above, the apparent compaction observed for protein complex ions as charge state is increased is similar to previous datasets [13, 19, 50], and is likely due to the enhanced kinetic energies, and thus internal energies upon collisional activation, of the higher charge state ions observed. Other plausible explanations for the slight decrease in CCS observed center on the calibration function used to convert traveling-wave IM drift times to CCS values using ions of known CCS [49]. While the possibility of calibration errors cannot be completely eliminated from our analysis, our dataset utilizes a recently developed database of calibrant values that span a large range of CCS and ion mobility values in order to minimize errors in the process [28] and makes significant errors in calibration unlikely.

Again, in contrast to datasets collected from ions produced from control samples or ion-neutral chemistry-based charge reduction, ions generated from solutions with basic additives result in significantly larger CCS values relative to control values and varying trends as a function of charge state. We have also collected data on concanavalin A tetramers (from jack bean, 103 kDa, see Supplemental Information), where the trends in CCS as a function of charge state are parabolic in nature, with the intermediate charge states exhibiting minimum CCS values. It is possible that trends in CCS for protein complex ions as a function of charge state could be used to recover structural information from the assembly or as a means of protein complex identification, and these are active areas of research in our laboratory. Note also that in some cases, the addition of base in solution acts as a disrupting agent similar to the addition of organic solvent or salts, resulting in the formation of protein dimers and monomers in solution. This is the case with concanavalin A tetramers (Supplemental Information, Figure S1) where solution additives cause the dissociation of tetramer into dimers while gas-phase charge reduction approaches are able to retain the tetramer and reduce its charge. This further makes the case, in general, for gas-phase approaches to charge reduction, especially for more fragile multiprotein complexes.

Conclusions

In this report, we demonstrate that the addition of strong bases (TEA, DBN, DBU) in solution, while leading to charge reduced populations of electrosprayed protein complexes, can also result in unfolded protein conformers in the gas phase through the activation necessary to dissociate proton-bound base molecules from the surface of the complex. It is important to note that while operative for the base molecules studied here, gas-phase unfolding has not been observed in other cases where small molecules have been added in solution in order to charge reduce multiprotein complexes. For example, imidizole has been used to reduce the charge state of the GroEL tetradecamer and IM-MS data indicate a compact configuration for the charge reduced species generated [14]. Similarly, crown ether compounds and triethylammonium acetate buffer have recently been used to alter the charge state of transthyretin tetramers in order to study their collision induced dissociation properties [21]. Here again, IM-MS data confirm compact conformations for the charge reduced tetramers prior to activation.

The critical differences associated with the base-protein complexes formed in our experiments likely relates to the stronger proton-bound interactions formed between highly-basic molecules and protein surfaces. These stronger interactions require increased energy to break, generated here in the form of activating collisions with inert neutrals. In addition to dissociating the protein-bound base molecules from the complex, the assembly unfolds in our experiments as well. This result is in stark contrast to those where the base is nebulized in the ion source and allowed to interact with the protein in the gas-phase through ion-neutral reaction chemistry. In these cases, base likely interacts with proteins in fewer numbers, and in a more discrete fashion, than the action of the same base molecules in solution. This would likely lead to fewer base molecules bound to the surface of the protein at any given time and, thus, the complexes created would require less thermal energy to dissociate and generate charge reduced species. Moreover,

while the gas-phase ion-neutral chemistry method described lacks efficiency compared with solution additive based approaches, it is a more effective charge reduction method under the conditions studied here. In summary, our results suggest that gas-phase ion-neutral chemistry approaches offer an alternative method to reduce charges for protein complexes that is accessible to a larger number of basic molecules than equivalent approaches in solution, while having an enhanced ability to preserve 'native-like' protein complex structures in the gas phase.

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