Protein Structure

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# Hofmeister Salts Recover a Misfolded Multiprotein Complex for Subsequent Structural Measurements in the Gas Phase**

*Linjie Han and Brandon T. Ruotolo**

Proteins are the workhorses of the cell, the functions of which are largely predicated on their structures and dynamics. Detailed knowledge of these attributes has enabled countless breakthroughs in human health and disease.[1] Many aspects, however, of protein structure remain poorly understood, including their high-order interactions.[2] This knowledge gap drives the development of new methods that are capable of protein structure characterization, some of which operate by measuring desolvated protein structure.[3] Although desolvation enables the application of powerful analytical techniques that cannot be used in a solvated environment, and recent results indicate that many features of native protein structure survive in the gas phase,[4] desolvation may also act to obfuscate critical details of protein conformation.[8] Recently, multiple strategies have emerged for observing labile protein structures in the absence of bulk water and have proven useful in stabilizing protein–small-molecule interactions, globular proteins, and their complexes.[5] Herein, we present the first evidence that a misfolded protein complex, which exists both in solution and in the gas phase, can be recovered back to a native-like structure by the addition of salts prior to desorption/ionization. This is the first time that such a solution-phase multiprotein folding equilibrium has been captured by gas-phase measurements.

Our experiments involve the direct addition of salt additives to proteins in solution, mimicking the well-known Hofmeister series,[6] followed by transfer into the gas phase using nano-electropsray ionization (nESI). We then utilize ion mobility-mass spectrometry (IM-MS) to measure the influence of such additives on both the composition and structure of the resulting gas-phase ions. IM separates proteins and complexes based on their collision cross-section (CCS). Such information can be used, along with computational procedures, to deduce the three-dimensional structures of biomolecules.[7] MS can then be used to analyze the composition of ions that elute from the IM separator.[8] While previous measurements have allowed us to rank the ability of bound anions and cations to stabilize proteins in the gas phase, these experiments started from thermodynamically stable proteins that were natively folded prior to nESI and did not reflect protein stabilities in solution.[5b–d] The protein system we have chosen to study here is the lectin concanavalin A (ConA), a 103 kDa homo-tetramer having a dimer-of-dimers arrange-

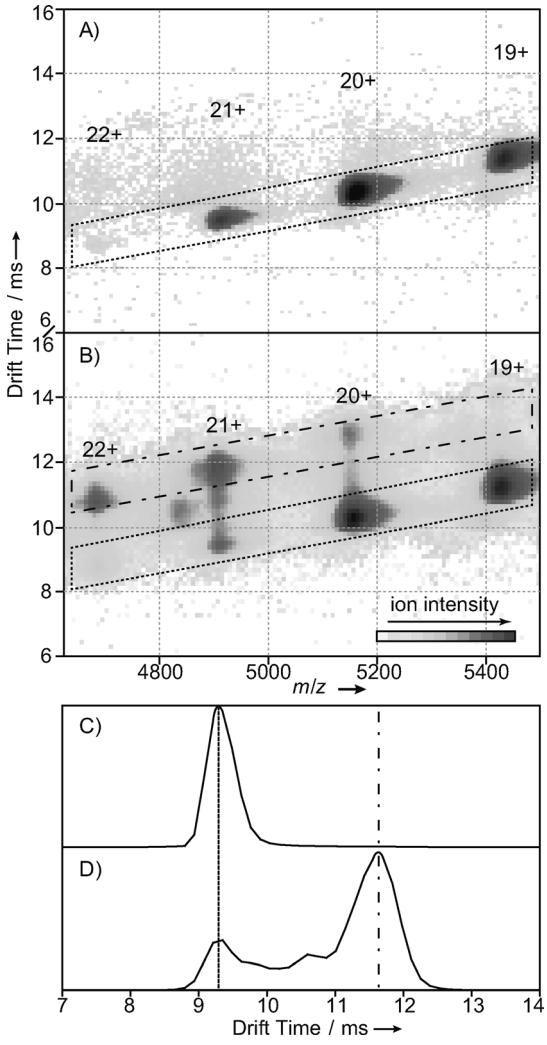


***Figure 1.*** An alternative form of the ConA tetramer is observed by comparing IM-MS data acquired from control (100 mM ammonium acetate) conditions (A) and following multiple freeze–thaw cycles (B). The conversion between ConA (dotted box) and ConA' (dashed-dotted box) can also be achieved by adding small amounts of methanol or acetic acid in solution prior to nESI. Drift-time distributions for $21^+$ charge-state tetramer ions are shown in (C) and (D) for ConA and ConA' respectively (same $m/z$). The centroid CCS of peaks corresponding to ConA and ConA' are highlighted with dotted and dashed-dotted lines, respectively (dashed: 5400, dashed-dotted: 6040 Å$^2$).

[*] L. Han, Prof. Dr. B. T. Ruotolo
Department of Chemistry, University of Michigan
930 North University Avenue, Ann Arbor, MI 48109-1055 (USA)
E-mail: bruotolo@umich.edu

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ment.[9] The ConA tetramer can reversibly self-assemble to form dimers and tetramers in a manner that depends upon solution pH, temperature, and ionic strength.[10] Along with these properties, IM-MS reveals that the ConA tetramer can generate an alternate quaternary structure, which can be recovered back to a native-like conformation in a salt-dependent manner.

ConA has been long studied for its mitogenic, cell surface, and highly-selective metal-binding ($Mn^{2+}$ and $Ca^{2+}$) properties.[9] While many reports indicate that the protein interfaces within the complex can be disrupted to produce alternative structural forms,[10a,b] the native holo-protein complex exists primarily within a narrow conformational space, as observed across all charge states observed in our IM-MS measurements (Figure 1 A, black dashed box). When the protein is exposed to freeze–thaw cycles or a small amount of denaturant in solution, however, we observe a new set of peaks having longer drift times (Figure 1 B, dashed-dotted box). As all of the ions shown in Figure 1 are measured under identical instrument conditions, we can rule out the possibility that these new signals result from different levels of collisional activation. Furthermore, tandem MS experiments (see below), and the fact that the results shown in Figure 1 A and B were acquired from solutions having identical protein concentrations, confirm that the features observed at longer drift times are a tetrameric form of ConA. Therefore, we assign these new features to an additional conformer of the ConA tetramer which coexists with its native form in solution and is apparently stable on the timescale of our experiments (minutes to hours), having slightly higher average charge (Supporting Information, Figure S1). In Figure 1 C and D, this alternate form of ConA (ConA′) is about 12% larger (CCS = 6040 Å$^2$) than the compact form typically observed (CCS = 5400 Å$^2$).[11]

To probe the origin of ConA′, and further verify our assignment above, we undertook a series of experiments where we aimed to structurally characterize the conformations of sub-assemblies and subunits produced under conditions favoring either ConA or ConA′ (Supporting Information, Figure S2). As noted above, we found that both small amounts of acetic acid (adjusted to a minimum pH of 5.2) and methanol (up to 30% by volume) convert ConA to ConA′. Furthermore, for those conditions that give rise to significant amounts of ConA′ tetramer, we observe a mirrored set of conformational changes in the protein monomers produced upon complex disruption. For example, in a weakly acidic solution, the ConA tetramer is disrupted to produce monomers in a relatively narrow band of structural states; however, increasing the solution methanol content to 10% generates both ConA′ and low charge state monomers, the latter existing within at least three conformational families simultaneously. Furthermore, we note that the dimers produced under any set of solution conditions exhibit a single, relatively narrow CCS distribution. From this data, we conclude that the structural transformations observed within the ConA tetramer are caused by deformations in the monomeric subunits of ConA that do not influence the overall size of ConA dimers. Furthermore, while polar protic solvents (that is, methanol; Supporting Information, Figure S3) can efficiently produce ConA′, polar aprotic solvents (that is, DMSO; Supporting Information, Figure S4) cannot. Collision-induced unfolding (CIU) and dissociation (CID) confirm the stoichiometry of ConA′ as well as its construction from likely unfolded protein subunits, as the misfolded tetramer produces unfolding patterns having ΔCCS values identical to ConA yet ejects monomers that cover a broader range of charge states upon dissociation in the gas phase (Supporting Information, Figure S5).

To ascertain if the changes in gas-phase protein complex structure observed in our IM-MS data are mirrored in solution, we conducted circular dichroism (CD) and differential scanning calorimetry (DSC) experiments on ConA/ConA′ containing solutions designed to mimic those used for our gas-phase measurements (Figure 2). Control samples prepared in ammonium acetate buffer display a bimodal DSC profile, with melt temperatures ($T_m$) corresponding well with known values for the intact tetramer and dimer (85.3 and 78.4 °C respectively),[12] and a CD spectrum with a strong band at 223 nm, characteristic of β-sheet protein structure. Preparation of ConA in solutions containing methanol and acid reveals shifts in the CD spectra toward higher wavelengths and reduced intensities in a manner dependent upon the organic/acid solution content, as well as dramatic decreases in the $T_m$ values recorded from DSC, all in a manner highly-correlated with IM-MS results. Critically,

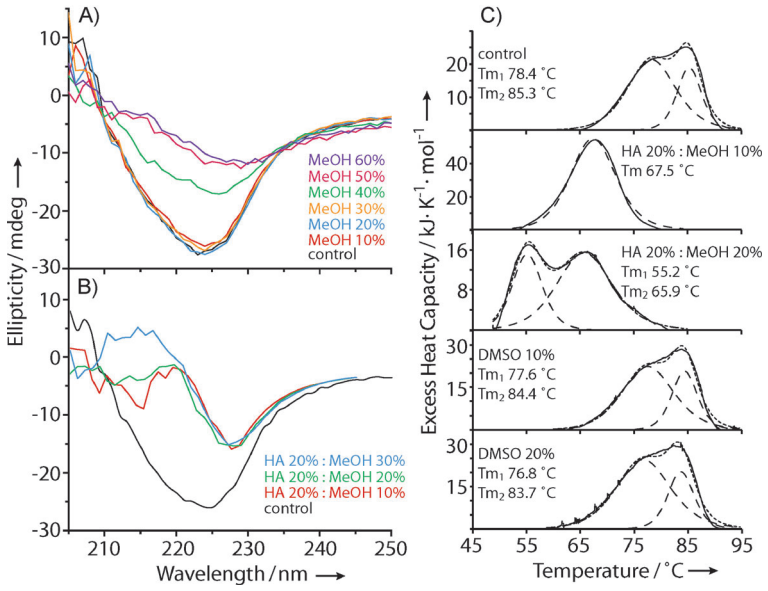


***Figure 2.*** Effect of disrupting agents on ConA structure and stability in bulk solution. A) Far-UV CD spectra of 5 μM ConA as a function of methanol concentration (0–60%, v/v) in 100 mM ammonium acetate buffer, pH 7. B) Far-UV CD spectra of 5 μM ConA as a function of methanol concentration (0–30%, v/v) in 100 mM ammonium acetate buffer, pH 5.2. C) DSC scans of ca. 60 μM ConA (top, 100 mM ammonium acetate) and four additional aqueous solvent compositions prepared using varying amounts of methanol or dimethyl sulfoxide (DMSO) (as marked). The experimental data and fits are indicated by solid and dashed lines, respectively.

the addition of DMSO caused no measurable change in ConA stability in solution, mirroring exactly our gas-phase results.

Given the verification of our assignment of the IM peaks observed in Figure 1, as well as their origins in solution, we then attempted to recover the misfolded ConA′ structure by adding small amounts of anions and cations in solution prior to nESI. Our results indicate a differential stabilization and refolding effect for cations and anions on ConA/ConA′ tetramers (Figure 3). Our data show that multiply charged cations and anions have a greater ability to convert ConA' into CCS values that agree well with ConA data when compared to those that are singly charged in our experiments, which involve adding 2 mM of either acetate-based cations or ammonium-based anions to 5 μM solutions of ConA′ in 100 mM ammonium acetate. As expected, we observe that MS peaks broaden considerably when involatile salts are added prior to nESI, owing to the non-specific adduction of

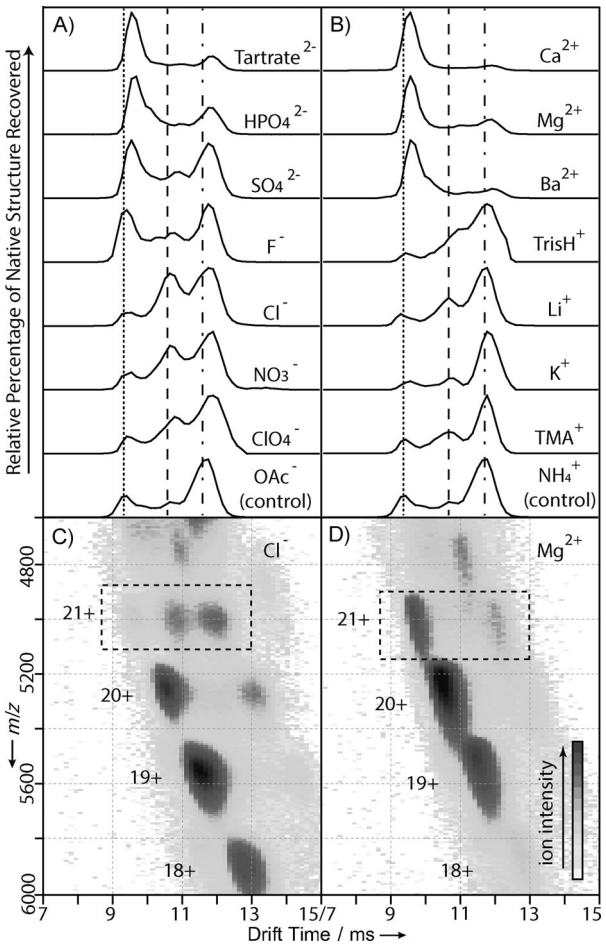


***Figure 3.*** Drift-time distributions measured for $21^+$ charge state of ConA′ tetramer in 100 mM ammonium acetate solutions (control, following freeze–thaw) are treated by increasing the concentration of Hofmeister anions (A) and cations (B) to 2 mM prior to nESI, and are ordered according to the relative amount of ConA recovered. Lines mark the peaks corresponding to ConA (dashed) and ConA' (dashed-dotted) together with a partially misfolded intermediate (dashed). The drift time versus *m/z* contour plots obtained for ConA′ after the addition of $Cl^-$ and $Mg^{2+}$ are shown in (C) and (D), respectively, with the $21^+$ charge state of the tetrameric species highlighted (dashed box).

many anions or cations to the surface of the protein. Conversion between ConA′ and ConA is often incomplete in our dataset, producing significant populations of a new intermediate at 5770 $Å^2$ and slightly swelled (ca. 1%) final sizes. Both anions and cations convert ConA' into ConA in a concentration-dependent manner (Supporting Information, Figure S6), and recovered ConA ions produce CIU fingerprints that are nearly identical to control data (Supporting Information, Figure S7).

We undertook further DSC and CD measurements on ConA' samples treated with selected anions and cations to verify their action on protein structure in solution. These data confirm that anions stabilize ConA in solution according to the Hofmeister series for biopolymers (Figure 4).[6] Conversely, CD and DSC measurements show that cations can act to stabilize ConA according to a reversed Hofmeister series, which is most likely due to its relatively low pI (5.43).[13] Overall, our IM-MS data agrees well with our CD and DSC findings. We also note a strong correlation between the cation-related results shown in Figure 3, and our previous data aimed exclusively at gas-phase protein complex stability.[5d] Binding stoichiometries estimated from MS data (Supporting Information, Figure S8) and the known selectivity of the ConA metal binding sites rule out the influence of specific cation–protein interactions in our dataset.[14]

The implications of the data presented herein are broad, and encompass many long-standing issues in gas-phase protein structure. The observation of ConA′, which had eluded detailed structural characterization until this report, illustrates the power of the IM-MS approach for detecting such small, environment-dependent shifts in protein quaternary structure. Our model for the origin of ConA′ invokes a loosened dimer–dimer interface for the tetramer,[10] misfolded-yet-compact dimers, and monomers that unravel upon their release from higher-order complexes (Supporting Information, Figure S9). Furthermore, it is possible that desolvation may exaggerate the structural differences between ConA and ConA′, leading to CCS values that, while predicated on clear structure changes in solution, result from conformers that are unique to the gas phase. Also, through the addition of specific anions and cations in solution we demonstrate that the differential recovery of ConA′ to ConA can be followed in the gas phase by IM-MS. Our anion data agrees well with Hofmeister's original rank order, as well as recent biopolymer stability measurements in solution.[15] Conversely, while agreeing well with our DSC and CD data, the relative abilities of cations to stabilize the ConA′/ConA transition agrees well with a reversed Hofmeister series, potentially owing to the negative charge of the protein at pH 7.[13] Furthermore, our data are some of the strongest evidence to date suggesting a clear memory effect linking gas-phase protein structures to their analogues in solution.[16] Recent evidence supporting a detailed solution memory for gas-phase biomolecules has focused on peptides,[16e] small proteins,[16b,f] and local interactions within larger biomolecules.[16d] The results shown herein expand the scope of such evidence dramatically to include the global architecture of large multiprotein complexes, their misfolded analogues, and Hofmeister stabilization.

  

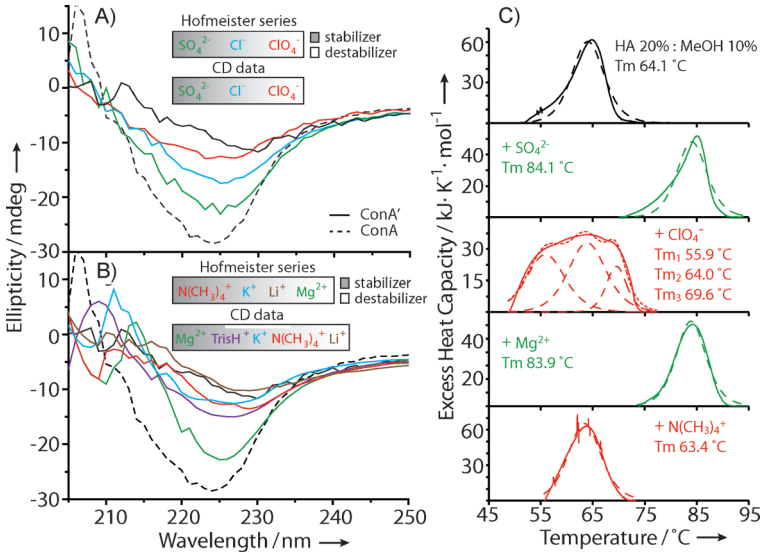


***Figure 4.*** Recovery of the ConA′ structure upon addition of specific salts in bulk solution. A),B) Far-UV CD spectra of 5 μM ConA′ generated from 45% (v/v) methanol (black solid line) in the presence of 1 M ammonium-based anions (sulfate, chloride, and perchlorate) and 1 M acetate-based cations (tetramethylammonium, magnesium, potassium, lithium, and tris), respectively. The CD spectrum corresponding to native ConA (black dashed line) is also included for comparison. C) DSC scans for 60 μM ConA′ generated from 20%:10% (v/v) acetic acid/methanol (top) with 1 M added ammonium sulfate, ammonium perchlorate, magnesium acetate, and tetramethylammonium acetate (bottom). The experimental data and fit data are indicated by the solid and dashed lines, respectively.

## *Experimental Section*

A full description of the methods (detailed materials and methods along with supporting IM-MS disruption experiments, CIU, CID, concentration-dependent salt addition, and detailed MS measurements) is given in Supporting Information. Briefly, IM-MS data were collected using a quadrupole-ion mobility-time-of-flight mass spectrometry (Q-IM-ToF MS) instrument (Synapt G2 HDMS, Waters, Milford MA, USA) as described previously.[5c,d, 8b, 11] Concanavalin A tetramer (ConA, jack bean) was purchased from Sigma (St. Louis, MO, USA) and first buffer exchanged into 100 mM ammonium acetate at pH 7 using Micro Bio-Spin 6 columns (Bio-Rad, Hercules, CA) and prepared to a final concentration of 5 μM prior to the addition of co-solvents and salts (2 mM final concentration). The CD spectra were measured with an Aviv model 202 CD spectrometer (Aviv Instruments, Lakewood, USA). A 1 mm-path-length quartz cuvette was used for scanning between 205 and 250 nm. The concentration of ConA tetramer was 5 μM. DSC experiments were performed on Nano DSC (TA Instruments, New Castle, USA). The concentration of ConA tetramer was 6.0 mg mL$^{-1}$, which is equivalent to about 60 μM. The measurements were performed at temperatures from 45 to 95 °C at a scan rate of 2 °C min$^{-1}$.

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