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COMMUNICATION

Highly-charged protein ions: The strongest organic acids to date

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Abstract: The basicity of highly protonated cytochrome *c* (cyt *c*) and myoglobin (myo) ions were investigated using tandem mass spectrometry, ion-molecule reactions (IMRs), and theoretical calculations as a function of charge state. Surprisingly, highly-charged protein ions (HCPI) can readily protonate non-polar molecules and inert gases, including Ar, O_2, and N_2 in thermal IMRs. The most HCPIs that can be observed are over 130 kJ/mol less basic than the least basic neutral organic molecules known (tetrafluoromethane and methane). Based on theoretical calculations, it is predicted that protonated cyt *c* and myo ions should spontaneously lose a proton to vacuum for charge states in which every ~3rd residue is protonated. In this study, HCPIs are formed where every ~4th residue on average is protonated. These results indicate that protein ions in higher charge states can be formed using a low-pressure ion source to reduce proton-transfer reactions between protein ions and gases from the atmosphere.

Electrospray ionisation (ESI) is renowned for its ability to form intact, gaseous multiply charged protein ions for rapid and sensitive detection by mass spectrometry.[1] However, the mechanism by which protein ions are formed in ESI is controversial and continues to be actively debated. The two primary competing models to explain ion formation are known as the Charge Residue Model (CRM)[2] and the Ion Evaporation Model (IEM).[3] In both models, as neutral molecules evaporate from a charged droplet, the electric field at the surface of the droplet increases, which initiates fission.[4] Such droplet fission events result in the emission of a fine stream of smaller droplets that remove less than 1 % of the mass but more than 30 % of the charge of the precursor droplet.[4-5] In the CRM, sequential droplet evaporation and Coulombic fission events yield a charged droplet that contains a single analyte ion, which evaporates to dryness via the loss of neutral solvent molecules. In the IEM, the electric field on the surface of a highly charged droplet near the moment of ion formation is sufficient to result in the ejection of an analyte ion from the surface of the ionic droplet. The majority of current evidence indicates that fully desolvated protein ions formed from buffered aqueous solutions are formed by the CRM. Charge carriers such as solvated hydronium ions can be lost via ion evaporation during the ESI process.[6] Recently, the chain-ejection model (CEM),[7] which is related to the IEM, was proposed to explain the formation of protein ions from denaturing solutions based on results from molecular dynamics simulations.[7] In the CEM, a denatured, disordered protein chain is ejected from a highly-charged, nanometer-sized ionic droplet. As the protein ion protrudes and is ejected from the droplet, proton transfer to the protein ion can occur. However, the mechanism by which highly-charged protein ions (HCPIs) are formed from denaturing solutions is less well established with evidence supporting both the CRM[8] and CEM/IEM having been reported.[7]

Central to the CRM is the theoretical proton transfer limit to protein ion charging in ESI.[9] As the number of protons on a protein ion increases, the gas-phase basicity (GB) decreases owing primarily to Coulombic repulsion.[9a] In the theoretical proton-transfer limit, protein ions that are less basic than the neutral molecules from the ESI source should not be formed;[9b] that is, protein ions should readily transfer protons to evaporating, neutral solvent molecules and not survive ESI.

The thermochemistry of proton-transfer reactions underpin the rationalisation of many chemical reactions. The investigation of gas-phase basicities (GBs, Eq. 1) of molecules has been a significant undertaking in gas-phase ion chemistry since the late 1960's[10] and values have been determined for over a thousand compounds.

$$M(g) + H^+(g) \rightarrow MH^+(g) \qquad \Delta G^\circ = -GB(M) \quad [1]$$

For organic molecules, these values range from 505 kJ/mol for tetrafluoromethane to more than 1,000 kJ/mol for highly basic molecules such as arginine (GB = 1,007 kJ/mol).

For positive ions, GBs are lower than the corresponding neutral molecules owing to the Coulombic repulsion between the proton and cationic molecule (Eq. 2).[9]

$$MH^+(g) + H^+(g) \rightarrow MH_2^{2+}(g) \qquad \Delta G^\dagger = -GB^{app}(MH^+) \quad [2]$$

For multiply protonated ions, the apparent GB (GB^{app}; includes the repulsive Coulomb barrier to proton transfer) of a given charge state (CS) can be measured using the bracketing method.[9, 11] The rates of proton transfer between an ion with an unknown GB^{app} value and neutral molecules with known GB values are measured. By use of a "ladder" of neutral molecules with well-established GBs, unknown GB^{app} values can be determined to within 15 kJ/mol.[9, 11-12]

Since the gas-phase proton-transfer reactivity of protein ions was first reported,[13] GB^{app} values have been measured for protonated molecules,[14] peptides,[15] cyt *c*,[9a] and ubiquitin.[16] As charge increases, the GB^{app} of the protein ion decreases[9] because fewer basic sites are available for protonation and Coulombic repulsion between charge sites increases with CS.[9, 17] For example, the GB^{app} values of protonated cyt *c* decrease from 980 kJ/mol to 799 kJ/mol as the CS increased from 3+ to 15+.[9a] For the most highly charged cyt *c* ions that were investigated, the GB^{app} values are nearly the same as the GB values of most basic components of common ESI solutions (*ca.* 800 kJ/mol).[9]

Tandem mass spectrometry experiments benefit from the use of highly charged protein ions since they tend to fragment more extensively than those in lower charge states, resulting in more sequence ions to yield greater sequence information. Ion detection efficiencies increase proportionally to charge state using high performance mass analysers that detect ions based on image current detection (FTICR and orbitrap mass spectrometers). In electron capture and transfer based ion activation methods, the efficiency,[18] extent of energy deposition,[19] number of fragment ions,[18] and sequence coverage[18-19] can increase significantly as the protein ion charge

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states increase. Thus, determining the mechanisms of ion formation and factors that limit ion charging is of fundamental importance to many types of mass spectrometry applications.

We have discovered that protein ions can be formed in higher charge states in ESI[20] than other known approaches[8, 21] by doping butylene carbonate into the ESI solutions. It was hypothesised that such ions should be highly reactive with respect to proton transfer reactivity. Here, we use gas-phase ion chemistry techniques and theoretical calculations to demonstrate that multiply protonated protein ions in very high charge states are significantly less basic than atmospheric gases (N_2, O_2, and Ar) and all organic neutral and ionic molecules for which GB values are reported.

To measure GB^{app} values using the bracketing method,[9, 11] IMRs were performed on an ESI-equipped linear quadrupole ion trap mass spectrometer (LTQ-MS; Thermo Scientific) that was modified to perform IMRs[22] at thermal ion effective temperatures.[23] Full experimental details are in the Supplementary Information.

ESI of aqueous solutions containing 5 µM cyt *c*, 1 % acetic acid and 5 % of the "supercharging" agent 1,2-butylene carbonate resulted in the formation of protonated cyt *c* with an average CS of 21.9 ± 0.2 (**Figure 1A**), which is nearly the same as observed previously.[20b] Isolation and storage of [cyt *c*, 23H]$^{23+}$ in the presence of 1 % Ar in He (~1 mTorr) resulted in the formation of an ion corresponding to [cyt *c,* 22H]$^{22+}$ with an abundance that is ~8 % of [cyt *c*, 23H]$^{23+}$ (**Figure 1B**). In 3 % Ar, the abundance of the ion corresponding to [cyt *c*, 22H]$^{22+}$ increases to ~75 % of the precursor ion, consistent with the loss of a proton. In addition, an ion corresponding to [cyt *c*, 21H]$^{21+}$ is formed in relatively low abundance (~13 % of precursor ion). Storing [cyt *c*, 23H]$^{23+}$ in 100 % He without Ar results in essentially no reaction (**Figure 1A**). As an additional control, the gas line for introducing 100 % He, 1:99 % and 3:97 % Ar:He, and 1:99 % N_2:He was modified to pass the gases through a cyrotrap (N_2(l); –196 °C) prior to introduction to the ion trap. The use of the cryotrap had no measureable effect on the rates of proton transfer for [cyt *c*, 23H]$^{23+}$ using He, and mixtures of Ar:He and N_2:He (**Figure S1**). Overall, these data indicate that a proton can be transferred from [cyt *c,* 23H]$^{23+}$ to Ar (Eq. 3).

$$[\text{cyt } c,\ 23\text{H}]^{23+}(\text{g}) + \text{Ar(g)} \rightarrow [\text{cyt } c,\ 22\text{H}]^{22+}(\text{g}) + \text{ArH}^{+}(\text{g}) \qquad [3]$$

Kinetic plots for the depletion of [cyt *c*, 23H]$^{23+}$ to form [cyt *c*, *z*H]$^{z+}$ (*z* = 22 to 19) by the loss of up to 4 protons using 1 % Ar, 1 % O_2, 1 % N_2, and 3 % Ar (total pressure, P_T is ~1 mTorr) are shown in **Figure 1C**. The R^2 and absolute values of the *y*-axis intercepts are greater than 0.99 and less than 0.02, respectively. Thus, the kinetic plots of the linear regression best fit lines in **Figure 1C** are consistent with pseudo-first order kinetics for the depletion of the precursor ion as a function of reaction time under these conditions. Isolation and storage of [cyt *c*, 23H]$^{23+}$ for 30 s in 3:97 % Ar:He results in ~70 % depletion of the precursor ion. For 1 % Ar, O_2 and N_2, the reaction rates (0.6 x 10^{11}, 0.8 x 10^{11} and 6.1 x 10^{11} cm^3 mol^{-1} s^{-1}, respectively; **Table S1**) increased as the GB value of the neutral base increased (the GB of Ar, O_2 and N_2 is 346, 396, and 464 kJ/mol, respectively). For the two least basic gases (Ar and O_2), the measured reaction rates are sufficiently low (< 1.0 x 10^{11} cm^3 mol^{-1} s^{-1}) that the GB^{app} of [cyt *c*, 23H]$^{23+}$ is assigned as being higher than the GB of these two gases. For N_2 the rate of the proton-transfer reaction is significantly higher. Thus, the GB^{app} value of [cyt *c*, 23H]$^{23+}$ is assigned to the average of the GB values of O_2 and N_2 (431 kJ/mol). This approach is consistent with that used previously to measure GB^{app} values using the bracketing method. [9, 11]

In ion-molecule reactions, [myo, 30H]$^{30+}$ can also transfer a proton to Ar, N_2 and O_2, which is consistent with the data for cyt *c* ions (**Figure S2**). For example, isolation and storage of [myo, 30H]$^{30+}$ in 1 % Ar, O_2 and N_2 resulted in depletion of [myo, 30H]$^{30+}$

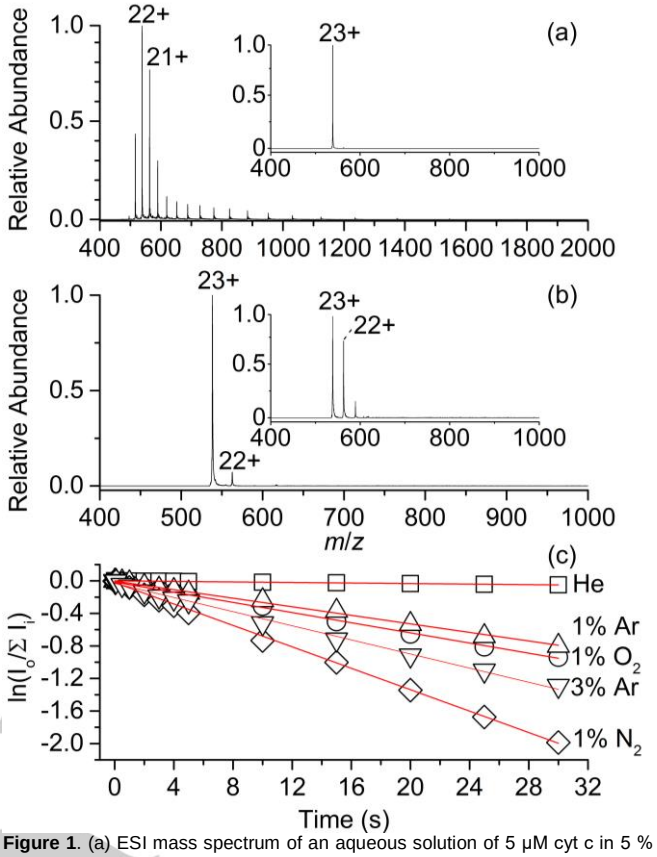

Figure 1. (a) ESI mass spectrum of an aqueous solution of 5 µM cyt c in 5 % 1,2-butylene carbonate with 1 % acetic acid; inset is a mass spectrum of isolated [cyt *c*, 23H]$^{23+}$ stored for 1 s in 1 % He, (b) Isolated [cyt *c*, 23H]$^{23+}$ stored for 1 s in 1 % Ar; inset is a mass spectrum of [cyt *c*, 23H]$^{23+}$ stored for 1 s reaction in 3 % Ar. (c) Kinetic plots for the depletion of [cyt *c*, 23H]$^{23+}$ in ion-molecule reactions with He, Ar, O_2 and N_2 for a reaction time of up to 30 s. I_0 and I_i correspond to the initial precursor ion abundance (*t* = 0 s) and product ion abundances at each reaction time (*t* = *i*).

(**Figure S2**) with pseudo first-order kinetic reaction rates of 0.6 x 10^{11}, 1.8 x $10^{11}$and 5.1 x 10^{11} cm^3 mol^{-1} s^{-1}, respectively (**Table S2)**. Based on these data, the GB^{app} value of [myo, 30H]$^{30+}$ was assigned to the average of the GB values of N_2 and O_2 (403 kJ/mol). These reactions based on this data can be summarized by Eq. 4,

$$[\text{M, } (z{+}1)\text{H}]^{(z+1)+}(\text{g}) + \text{A} \rightarrow [\text{M, } z\text{H}]^{z+}(\text{g}) + \text{AH}^{+}(\text{g}) \qquad [4]$$

where M is a protein ion with a sufficient number of protons (z) that a proton can be transferred to A (Ar, N_2 and O_2).

GB^{app} values [cyt *c*, *z*H]$^{z+}$ and [myo, *z*H]$^{z+}$ were measured as a function of CS (**Figure 2** and **Table S1 and S2**). For low CSs, the basicity of the protein ions decrease monotonically by a relatively minor amount compared to those at higher CSs. For example, the GB^{app} values of [cyt *c*, *z*H]$^{z+}$ decrease by 117 kJ/mol from *z* = 3 to 14+ compared to a decrease of over 519 kJ/mol from 14+ to 24+! Thus, [cyt *c*, *z*H]$^{z+}$ (*z* ≤ 14+) is categorized as having a minor dependence on charge (MDC) and [cyt *c*, *z*H]$^{z+}$ (*z* > 14+) is categorized has having a significant dependence on charge (SDC). For [myo, *z*H]$^{z+}$, the difference in GB^{app} values between the 26+ and 18+ CS is 100 kJ/mol, whereas that between the 33+ and 26+ is more than 290 kJ/mol. Thus, for myo, the transition between MDC and SDC is the
~ 26+ CS (**Figure 2)**.

Highly charged protein ions should be more elongated than those with fewer charges because extended conformations can accommodate more charges than compact structures.[17, 24] The transition between the MDC and SDC regimes in the GB^{app} *vs.* CS data (**Figure 2**) may arise from a structural change and as a result

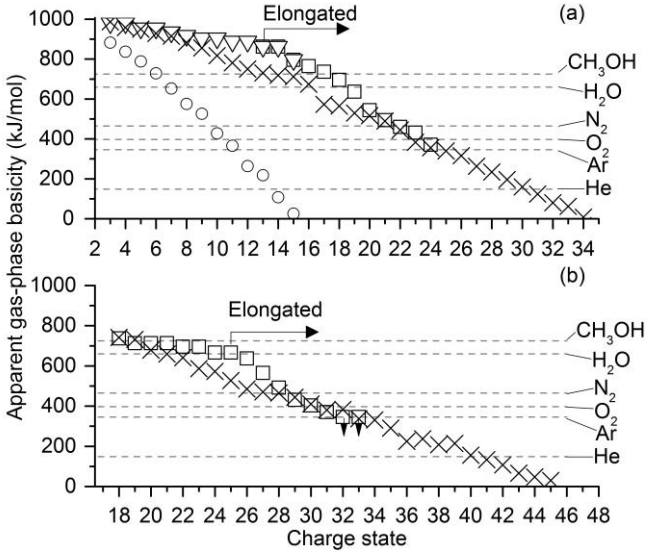

Figure 2. Measured GB^{app} values (squares) *vs.* charge state for protonated (a) cyt *c* and (b) myo. Triangles correspond to GB^{app} values for $[cyt\ c, zH]^{z+}$ (z = 3 to 15) which were measured by Williams and co-workers.[9a] Crosses and circles correspond to calculated GB^{app} values for elongated and globular protein ions, respectively. Based on literature, protein ions adopt more elongated conformations for $z \geq 13+$ and $z \geq 25+$ charge states of cyt *c* and myo, respectively.[17, 25] For $[myo, zH]^{z+}$ (z = 32 and 33+) GB^{app} are less than the GB of Ar, (downward arrows). Horizontal dashed lines are GB values of atmospheric gases and ESI solvents.

of complete protonation of basic residues (*i.e.*, Arg, Lys, and His), which are more basic than other protonation sites by at least ~50 kJ/mol (**Table S4**). For example, cyt *c* and myo have a total of 16 and 23 sites that are considered basic, which are comparable to the number of charges corresponding to the transition between MDC and SDC (14+ and 26+).

The predicted protonation frequency of cyt *c* was calculated using the methods of Williams and co-workers[9] and plotted *vs.* the CS and amino acid residue number (**Figure S3**). As the CS increases to more than 12 (cyt *c*) and 23 (myo) protons, less basic residue sites are predicted to be protonated (**Figure S3**). This data was used to define the starting structures for molecular dynamic (MD) simulations.

MD calculations were performed starting with fully elongated cyt *c* where the proton configuration was taken from the minimum energy structure identified in GB^{app} simulations (8 to 23+). The average collisional cross sections (CCS) of equilibrated trajectories were calculated as a function of CS (**Figure 3A**), and are nearly the same as literature values[17, 26] from ion-mobility measurements (8 to 23+ of protonated cyt *c*). Thus this theoretical approach provides reasonable accuracy for limited computational cost. For the CSs in the MDC regime (≤ 14+), the extent of elongation in the protein ion conformations are more significant than in the SDC regime. For example, representative models of protein ion structures obtained from the MD simulations of the 8+ and 14+ are shown in **Figure 3**. The average length increases from a value of 140 Å for the 8+ to a value of 251 Å for the 14+; *i.e.*, the protein ion elongates by over ~126 %. In contrast, the average calculated length of the 20+ CS is 310 Å, which is ~24 % longer than the calculated length of the 14+ CS. These data indicate that there is a significant decrease in the extent of elongation of the protein ion with increasing CS at the MDC to SDC transition, which likely contributes to a significant increase in the acidity of the protein ions at higher CSs (SDC regime) because protein ion elongation can reduce Coulombic repulsion between charge sites.

GB^{app} values were calculated[9] for the extreme case in which the protein ions are represented as fully-elongated line segments (**Figure 2**). The calculated GB^{app} values decrease steadily and

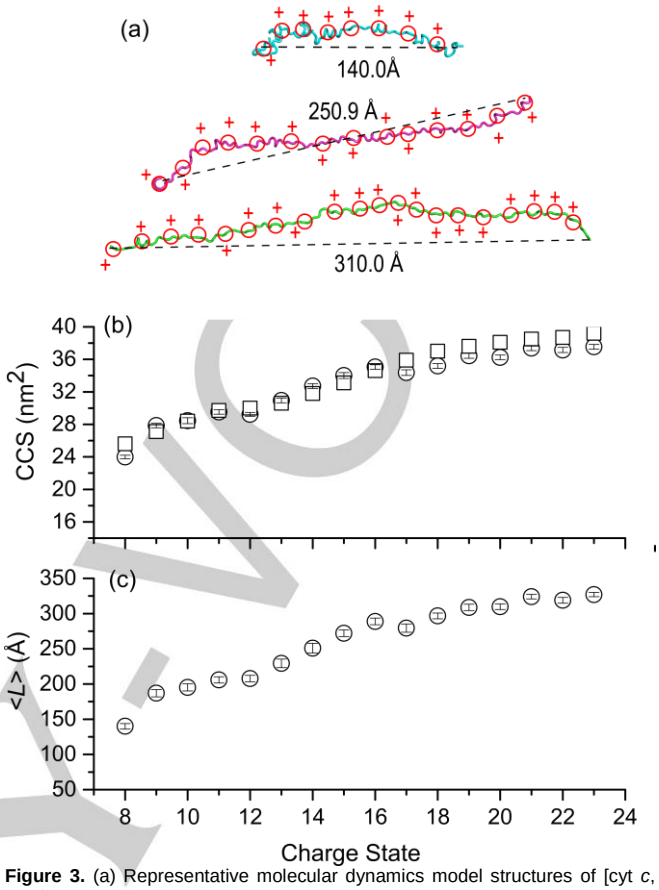

Figure 3. (a) Representative molecular dynamics model structures of $[cyt\ c, zH]^{z+}$, z = 8, 14 and 20, where red circles indicate charge sites; (b) measured (squares) and theoretical (circles) collision cross-sections (CCS) *vs.* charge state for protonated cyt *c*; and (c) the average length (<*L*>) of $[cyt\ c, zH]^{z+}$ *vs.* charge state. Experimental CCS values for $[cyt\ c, zH]^{z+}$ (z = 8 to 20+) were measured by Clemmer and co-workers,[17] and CCS values for $[cyt\ c, zH]^{z+}$ (z = 20 to 23+) were measured by Williams and co-workers.[26]

monotonically as the CSs increase (**Figure 2**). Because the calculations do not account for any structural transitions, these results are consistent with a change of slope in the experimentally measured GB^{app} *vs.* CS (MDC to SDC transition) originating from a structural change in the protein ions. Because the calculated GB^{app} values of the most highly charged protein ions are within 5 % of the experimental values using a dielectric constant of one, these data suggest that structures of the most highly charged protein ions are highly extended and any dielectric screening between charge sites is negligible. Moreover, the total calculated electrostatic Coulomb energy of protonated cyt *c* increases from 10.9 to 67.4 eV for the 10+ to 24+ CSs (**Table S3**), which is over 180 % higher than the highest electrostatic repulsion for an ion that has been reported.[9, 12]

Given that HCPIs can protonate O_2 and N_2 readily at reduced pressures (~1 mTorr), it was hypothesised that protein ion charging in ESI can be limited by proton-transfer reactions with O_2 and N_2 in the atmospheric pressure ion source. ESI was performed using a "shroud" surrounding the ion source that allowed the gas composition in the region of ion formation to be controlled (see Supplementary Information). ESI mass spectra of protonated cyt *c* and myo ions formed from solutions of 94/5/1 water/butylene carbonate/acetic acid using a positive pressure of atmospheric gas are shown in **Figures S3 and S4**. For $[cyt\ c, zH]^{z+}$, the average CS formed was 23.2+ using He, which is higher than that obtained using Ar (21.9+), without the shroud gas (20.9+) and N_2 (20.4+). For myo, the average CS obtained using Ar (29.8+) increased by an average of 4.8 protons compared to the use of N_2 (**Figure S4**). The atmosphere is composed of ~78 %

N_2 (GB = 464 kJ/mol) and the balance is mostly O_2 (GB = 397 kJ/mol). Given that He and Ar have respective GBs of 149 and 346 kJ/mol and the extent of protein ion charging correlates with the basicity of the shroud gases (**Figures S3 and S4**), these data indicate that the extent of protein ion charging can be limited by proton-transfer reactions with atmospheric gases under these conditions.

To approximate the maximum number of protons a protein ion can accommodate without the spontaneous loss of a proton when stored in vacuum, GB^{app} values were calculated for protonated cyt *c* and myo ions to determine the highest CS that have positive GB^{app} values. The ~34 and ~45+ CS of protonated cyt *c* and myo should be the highest CS that can be isolated in vacuum without spontaneous proton ejection (**Figure 2**). In contrast, when calculations are conducted starting from a globular structure, the charge state limits (15+ and 17+) are lower owing to the distance between charge states being much less for compact than elongated protein ions (**Figure 2**). Thus, the predicted upper maximum limit to the extent of charging corresponds to every ~3rd amino acid residue being protonated on average compared to every ~4th residue of the protein ions that can currently be formed and detected.

Highly charged cyt *c* and myo ions are the most acidic organic species to be isolated and detected. For example, such ions are over 130 kJ/mol less basic than the least basic known organic molecules, including CF_4 (GB of 504 kJ/mol)[10a, 27] and CH_4 (GB of 521 kJ/mol).[10a, 28] The extent of protein charging in ESI can be limited by proton transfer reactions with atmospheric gases. Remarkably, HCPIs have GB values that are more than 375 kJ/mol less basic than the components of the ESI solutions, which indicates that the proton transfer limit in ESI is more than 100 % lower (in terms of GB values) than the theoretical proton transfer limit.[9b] Departing neutral solvent molecules should readily deprotonate such highly acidic protein ions near the moment of ion formation. Thus, HCPIs should not survive the ESI process based on the CRM. In the CEM, protein ions are ejected from highly charged ionic droplets. In a competition for charge between the protein ion and highly charged ionic droplets during protein chain ejection, if the electric field on the droplet lowers the "basicity" of the droplet to a sufficient extent, then it is feasible that protein ions that are significantly less basic than the individual neutral solvent molecules can be formed. Overall, these results are more consistent with the CEM than the CRM for the formation of highly charged protein ions under these conditions. Given that such protein ion "superacids" have very high Coulombic repulsion, minimal dielectric screening between adjacent charge sites, and are in highly elongated conformations, they should fragment readily in ion-electron recombination experiments. It is anticipated that ESI in an inert, reduced pressure atmosphere will be useful for increasing ion charging even further to maximize the performance of many tandem mass spectrometry experiments.

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Keywords: Gas-phase basicity • Electrospray ionization • Ion-molecule reactions • Mass spectrometry • Proteins

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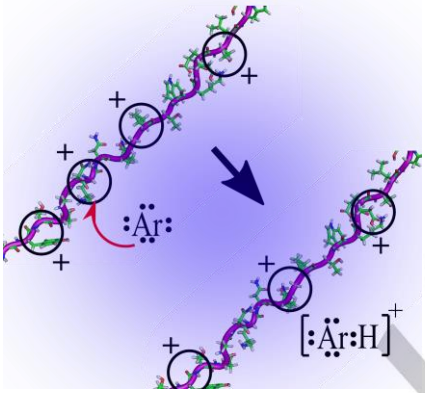
Entry for the Table of Contents

COMMUNICATION

Protein ions in extremely high charge states are sufficiently acidic that noble gases can be protonated in ion-molecule reactions. Given that the protein ions are more than ~375 kJ/mol less basic than the least volatile components of the ESI solutions, these results are not consistent with the charge residue model for the formation of highly charged protein ions in electrospray ionization. The chain ejection model is more likely.



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