

# Protons Are Fast and Smart; Proteins Are Slow and Dumb: On the Relationship of Electrospray Ionization Charge States and Conformations

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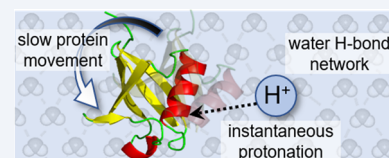


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**ABSTRACT:** We present simple considerations of how differences in time scales of motions of protons, the lightest and fastest chemical moiety, and the much longer time scales associated with the dynamics of proteins, among the heaviest and slowest analytes, may allow many protein conformations from solution to be kinetically trapped during the process of electrospraying protein solutions into the gas phase. In solution, the quantum nature of protons leads them to change locations by tunneling, an instantaneous process; moreover, the Grotthuss mechanism suggests that these small particles can respond nearly instantaneously to the dynamic motions of proteins that occur on much longer time scales. A conformational change is accompanied by favorable or unfavorable variations in the free energy of the system, providing the impetus for solvent  $\leftrightarrow$  protein proton exchange. Thus, as thermal distributions of protein conformations interconvert, protonation states rapidly respond, as specific acidic and basic sites are exposed or protected. In the vacuum of the mass spectrometer, protons become immobilized in locations that are specific to the protein conformations from which they were incorporated. In this way, conformational states from solution are preserved upon electrospraying them into the gas phase. These ideas are consistent with the exquisite sensitivity of electrospray mass spectra to small changes of the local environment that alter protein structure in solution. We might remember this approximation for the protonation of proteins in solution with the colloquial expression—*protons are fast and smart; proteins are slow and dumb*.



## INTRODUCTION

**Fast Migration Rates of Protons in Solution.** The Grotthuss mechanism (Scheme 1) predicts that protons can be transferred through long distances in water with extraordinary speed.<sup>1</sup> Unlike all other ions, a proton lacks electronic structure; as the lightest chemical moiety, these particles behave quantum mechanically and can change position by tunneling.<sup>2</sup> In solution, protons are conducted nearly instantaneously in a manner that can be thought of in analogy with the conduction of electrons through a wire. The mechanism in Scheme 1 has a proton migration rate that is limited by the vibrational frequency of the conducting water molecules. Perhaps the most remarkable aspect of this mechanism lies not in the profound consequences of such high velocity migration (estimated to be  $>800 \text{ m} \cdot \text{s}^{-1}$ ; nearly  $1 \mu\text{m}$  in a ns)<sup>3</sup> but rather that Grotthuss proposed it in 1806 using OH as the molecular formula of water instead of  $\text{H}_2\text{O}$ , as the correct stoichiometry was not determined by Avogadro until 1811.<sup>4</sup>

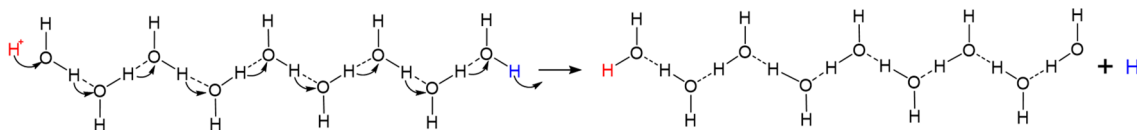
**Structural Changes in Large Molecules Such As Proteins Are Relatively Slow.** One consequence of high-speed proton motion is that rates of proton-transfer reactions in large flexible molecules such as proteins will be limited by the availability of acidic and basic sites rather than the rate of transferring a proton from solution to these sites. Consider the

dynamics of an ensemble of protein conformational states in solution. Interconversion of different structures involves rearrangements of the positions of a plethora of atoms and molecules involved in intramolecular, solvent–protein, and solvent–solvent interactions that frame each structure-coupled processes that require substantial time. For example, secondary structures such as  $\alpha$ -helices are often formed on nanosecond to microsecond time scales, whereas tertiary structures often require microsecond to second and quaternary structures even longer.<sup>5–9</sup> As these structures are being established, new basic or acidic sites may become solvent exposed many times as conformations fluctuate. Because of the differences in mechanisms of motions (and time scales) of conformational changes and protons, any changes in the solvent accessibility of such sites due to conformer fluctuation would rapidly accommodate changes in protonation state. That is, during a large conformational change, protons may flicker on and off

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**Scheme 1. Description of  $\text{H}^+$  Transfer through  $\text{H}_2\text{O}$  Wire Predicted by Grotthuss in 1806 (albeit for OH instead of  $\text{H}_2\text{O}$ )<sup>a</sup>**


<sup>a</sup>See ref 1 for details.

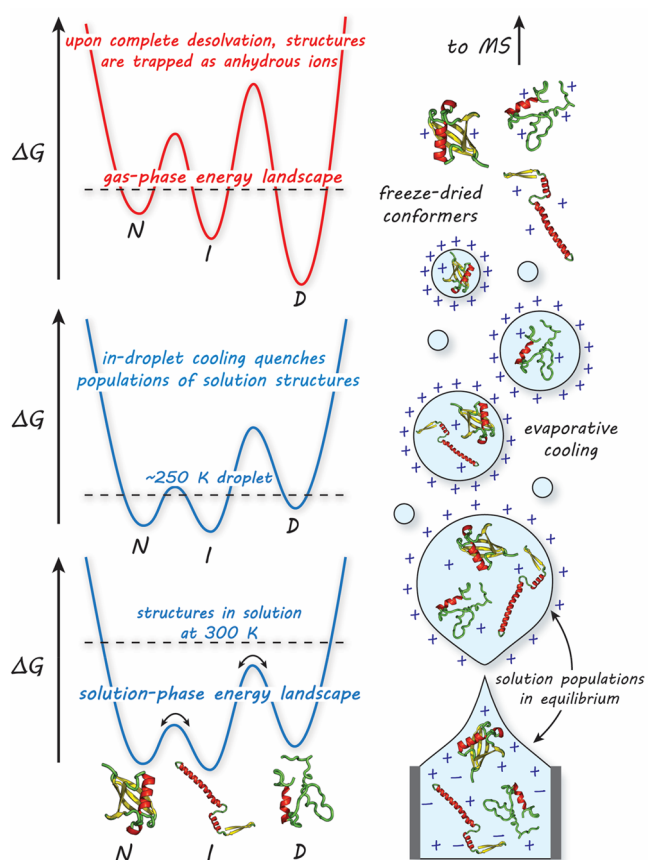
specific sites many times as a new solution structure is established. Crudely speaking, we might say that *protons are fast and smart; proteins are slow and dumb*.<sup>10</sup>

**Proposed Electrospray Mechanisms.** The differences in proton migration and protein conformation time scales raise interesting possibilities for capturing information about solution structure by nanoelectrospray ionization (n-ESI) paired with mass spectrometry (MS).<sup>11</sup> The mechanisms of ESI have been debated extensively, and efforts toward characterizing these processes are still an active area of research. There are three predominant mechanisms that account for experimental ESI observations: the ion evaporation, charge residue, and chain ejection models, all of which have been described in detail elsewhere.<sup>12–16</sup> In general, electrified analyte solutions are sprayed from a small tip (typically nanometer to micrometer) to form a Taylor cone that emits a fine mist of charged droplets containing analyte molecules. In the case of positive-mode n-ESI, each droplet carries positive charges due to the presence of excess ions including  $\text{H}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ , etc. Once emitted from the Taylor cone, the charged droplets undergo rapid solvent evaporation and shrinkage that ultimately increases their charge density. As the droplets reach the Raleigh limit,<sup>21</sup> which depends on the solution properties, the droplets can no longer sustain increased Coulomb repulsion. Jet fissioning occurs to produce smaller droplets, and repeated evaporation and fission events yield highly charged, nm-radii droplets that enter the MS instrument.<sup>17–19</sup>

As the droplet shrinks, its surface-to-volume ratio increases, as does its internal strain energy;<sup>22</sup> because of this, we anticipate that small droplets will be less stable and evaporate faster than large droplets. Therefore, the quenching process should speed up as the protein approaches the anhydrous state. There are several factors that influence the droplet size and time scale of solvent evaporation, including emitter size, flow rate, distance from the MS inlet, and solvent. We note that in n-ESI experiments, where emitter sizes are typically  $>5\ \mu\text{m}$ , droplet effects such as pH changes, recondensation events due to poor desolvation, and salt adduction are diminished.<sup>23,24</sup> This is further supported by ion mobility spectrometry (IMS) cross-section measurements made with n-ESI that agree with theoretical measurements of compact, dehydrated structures.<sup>25–27</sup> In n-ESI, it is estimated that protein ions are evaporated and quenched (i.e., kinetically trapped as gaseous ions) on the order of 1–100  $\mu\text{s}$ .<sup>28–30</sup> This suggests that under some conditions the key step of establishing the charge state by n-ESI is much faster than protein conformational changes.

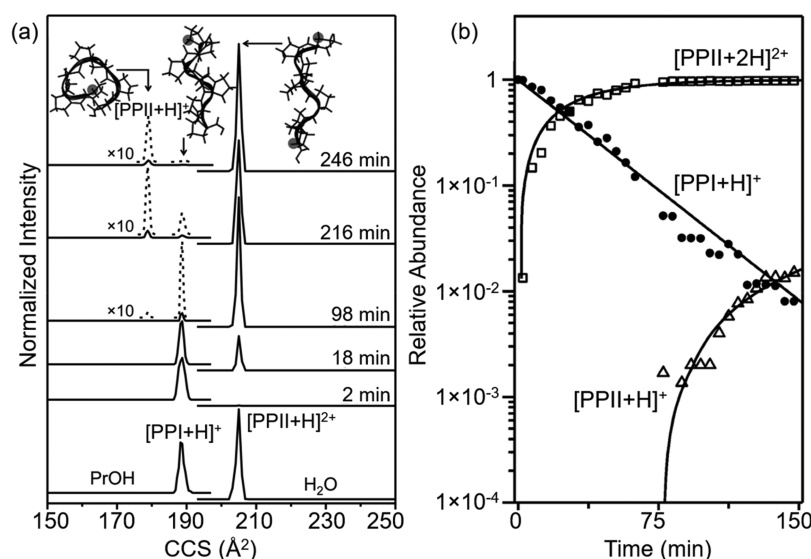
**Are Unique Solution Conformations Trapped as Specific Protonation States during the Process of Electrospray Ionization?** There are many reports stating that ESI appears to capture solution-like conformations in the gas phase.<sup>26,31–37</sup> The evaporative-cooling phenomenon associated with droplet shrinkage appears to “freeze out” a

number of structures that are in equilibrium in solution, as illustrated in Figure 1.<sup>36,38–41</sup>



**Figure 1.** Hypothetical drawing of the process of nanoelectrospraying proteins into the gas phase (right) and its implications on the protein free-energy landscape (left). In solution, rapid interconversion of protein structures [native (N), intermediate (I), denatured (D)] is mediated by solvent. During early states of n-ESI, the droplet initiates with solution populations in equilibrium with rapidly exchanging protons (bottom). As the droplet dries, the solution equilibrium is quenched due to evaporative cooling (middle). Just before entering the MS instrument, protein structures are kinetically trapped on a new, gas-phase free energy landscape associated with the solvent-free ion (top).

Protein structures in solution resemble rugged free-energy landscapes where rapid interconversion of structures is mediated by solvent. During n-ESI, the droplets containing protein solutions undergo rapid cooling to below room temperature, resulting from desolvation. The temperature drop over this short time scale is likely to be substantial;<sup>39,42–44</sup> however, it is difficult to estimate an exact temperature due to various factors including solvent behavior, ambient collisions that warm the droplet, and instrumental conditions. Nonetheless, within the lower temperature droplet



**Figure 2.** (a) Collision cross section (CCS) distributions for  $\text{PPI}_{\text{PrOH}} \rightarrow \text{PPII}_{\text{aq}}$  transition for Pro7. Bottom CCS distributions obtained by integrating the ions with different  $m/z$  values show the formation of the PPI helix in propanol and the formation of the PPII helix in water. Kinetic studies from 2 to 246 min at 296 K in 40:59.5:0.5 1-propanol/ $\text{H}_2\text{O}$ /HOAc (v/v/v) show the transition from PPI to PPII. Structures derived from molecular dynamic simulations are shown for each CCS peak. (b) Relative abundance of different charge states and conformers as a function of transition time. See text and ref 27 for details. Adapted ref 27. Copyright 2015 American Chemical Society.

environment, the energy barriers between structures on the free-energy landscape become more difficult and take longer to overcome, resulting in the “freezing out” of protein structures in the absence of a lubricating solvent. Changes to the free energy of the system during n-ESI are likely to be small; recent thermochemical results ( $\Delta G$ ,  $\Delta H$ ,  $\Delta S$ , and  $\Delta C_p$ ) derived from solution temperature-dependent IMS data agree with literature values provided from experiments done directly in solution with calorimetry.<sup>45</sup>

As distinct solution conformations emerge into the gas phase as desolvated ions, different numbers of protons (and other excess ions) are retained according to the types of residues exposed, leading to the observed distribution of protonation states in ESI-MS (e.g.,  $[\text{M} + n\text{H}]^{n+}$ ,  $[\text{M} + (n+1)\text{H}]^{n+1}$ ,  $[\text{M} + (n+2)\text{H}]^{(n+2)+}$ , etc.).<sup>46–49</sup> The outcome is that individual charge states may be specific to protein structure in a manner that allows access to even subtle differences in solution conformations. Below, we revisit prior published results<sup>27,45,50</sup> and find them to be consistent with the notions presented here. It is likely that this analogy has not been drawn previously because little is known about the structures of non-native protein conformations and the interconversions among them. Equilibrium ensembles and folding intermediates<sup>51–54</sup> often have short lifetimes, and their fleeting existence leaves them difficult to characterize.<sup>55</sup> Because of this, we begin by considering a simple system with large energetic barriers between well-characterized structures such that structural transitions are slow and obvious. Such a situation is often found for peptide bonds involving a proline residue, which can rotate between *cis*  $\leftrightarrow$  *trans* forms, inducing large structural changes.<sup>56,57</sup> Folding studies reveal that these transitions are often rate limiting.<sup>58</sup>

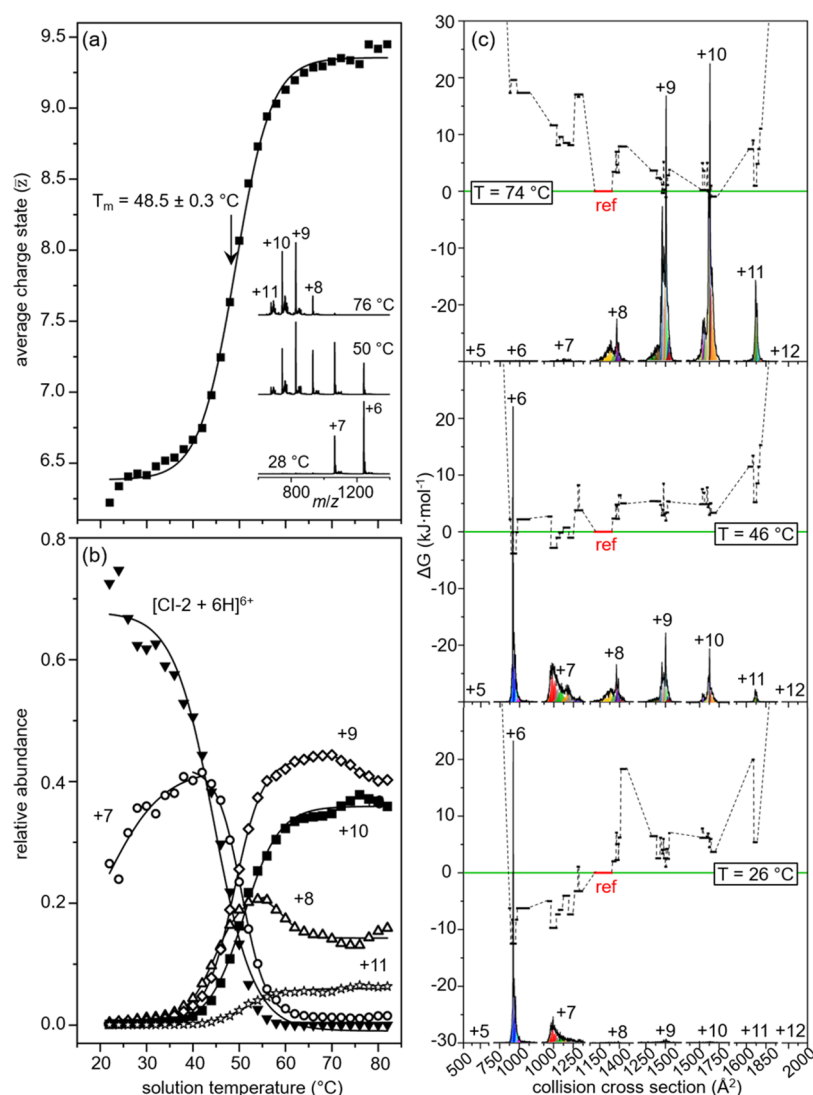
## EXAMPLES

**Charge-State Populations Controlled by a Conformational Change: The PPI to PPII Helix.** The simple model peptide polypyrroline-7 (Pro7) provides an excellent starting point for understanding conformational regulation of proton transfer.<sup>27</sup> When dissolved in a nonpolar solvent such as 1-

propanol, Pro-7 favors the right-handed PPI helical structure in which each of the six peptide bonds adopts a *cis* configuration. In aqueous solutions, the all-*trans* PPII left-handed helix is observed. The PPI helix possesses a macrodipole in the direction of the N-terminal residue that is stabilized upon protonation. As shown in Figure 2a, when PPI is electrosprayed from a 1-propanol solution, a single peak in the mass spectrum, having a size (collision cross section) consistent with the singly protonated ion  $[\text{PPI} + \text{H}]^+$ , is observed. Upon dilution of the Pro7 PPI helix into a more aqueous environment (e.g., 2.0 min after dilution as shown in Figure 2a), the singly protonated PPI helical species persists as the only ion formed upon electrospray. At longer times, doubly protonated PPII is formed by the reaction:  $\text{PPI}^+_{\text{PrOH}} + \text{H}_3\text{O}^+ \rightarrow \text{PPII}^{2+}_{\text{PrOH/aq}} + \text{H}_2\text{O}$ .

The long time scale associated with the formation of the  $\text{PPII}^{2+}$  ion is remarkable as it is the slowest proton-transfer reaction ever reported due to the slow interconversion of structures. The kinetics associated with this reaction are shown in Figure 2b. The structure of PPI must not be able to instantaneously accommodate an additional charge. Instead, transfer of the proton from solution to form the doubly protonated state is regulated by a slow structural transition, in this case one in which every peptide bond flips to a *trans* configuration, leading to the PPII helix. Ultimately, the final location of the proton depends upon the structure of Pro7.

**Water Stabilizes Charge and Conformation.** If an understanding of how many different conformations contribute to the charge state distribution in an electrospray mass spectrum is inhibited by the fleeting nature of “non-native” states, then one might imagine that appreciating the role of solvent will be even more challenging. The competition for protons is defined not only by the accessible basic sites on each conformation but also the willingness of the solvent to give them up. That is, it is the interactions of the system, consisting of the molecule and the solvent, that lead to the protonation state. Of the many examples of this, consider the key role of hydration illustrated by recent studies of 4-aminobenzoic acid



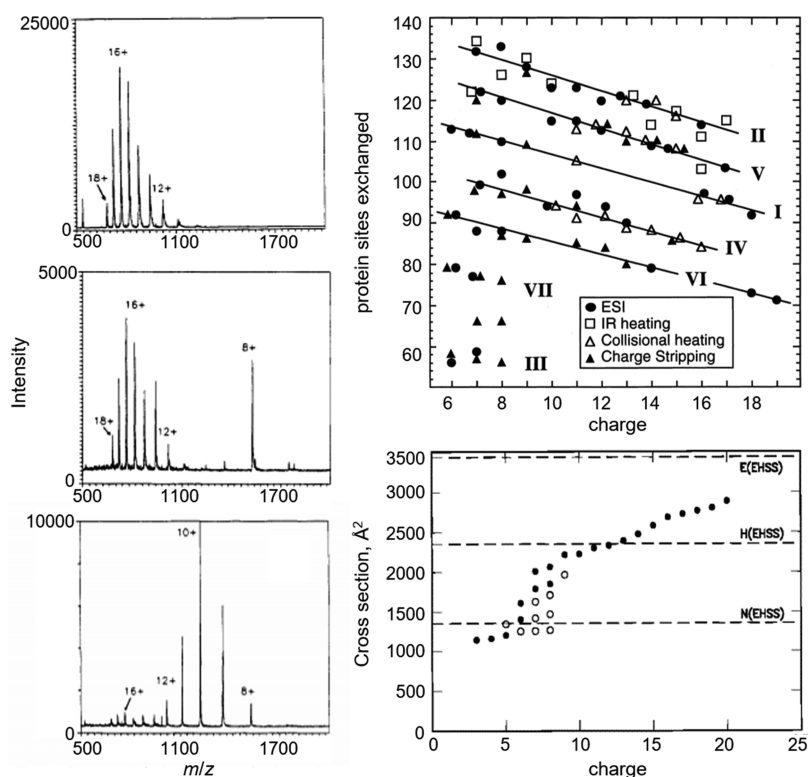
**Figure 3.** Variable-temperature ESI-IMS-MS studies of CI-2 in water at pH 2.6. (a) Weighted average charge state as a function of solution temperature for CI-2 with a  $T_m = 48.5 \pm 0.3$  °C; inset shows the mass spectra at various solution temperatures. (b) Relative abundance as a function of solution temperature for each of the six individual charge states of CI-2. (c) Free-energy landscape depicting  $\Delta G$  as a function of cross section (for each charge state) showing 41 unique solution structures. Relative abundance of CCS distributions for each charge state are shown at the bottom of each landscape. Each black line represents the free energy of a structure populated in solution, arbitrarily referenced to product conformation 1 ( $P_1$ ) shown as a red line at  $\Delta G = 0$  kJ·mol<sup>-1</sup>. At low solution temperature (26 °C, bottom), structures present within the +6 and +7 distributions are favored with low  $\Delta G$  values; at the highest temperatures (top), denatured structures become the favored signals while the folded structures in the +6 and +7 charge states become unfavorable. See text and ref 45 for details. Adapted from ref 45. Copyright 2020, American Chemical Society.

(4-ABA).<sup>50</sup> The protonation of the amine group of 4-ABAH<sup>+</sup> is favored in protic solvents (N-protomer), whereas protonation of the carboxylic acid group is favored in the gas phase and in aprotic solvents (O-protomer). A series of hydrated 4-ABAH<sup>+</sup> ions are observed upon electrospraying the analyte into a cryogenic IMS-MS with a range of  $n = 0$  to  $\sim 50$  water molecules [4-ABAH<sup>+</sup>(H<sub>2</sub>O)<sub>*n*</sub>]. The proton transfer of hydrated 4-ABAH<sup>+</sup>(H<sub>2</sub>O)<sub>*n*</sub> from the N- to O-protomer occurs at  $n = 6$ , which is mediated by a stable water bridge between the polar groups. The head-to-tail arrangement of this water bridge strongly implies proton hopping between water molecules, i.e., the Grotthuss mechanism. In the presence of acetonitrile, the proton migration is inhibited owing to favorable solvent interactions with the N-protomer. We note that there are results that do not appear to be consistent with a Grotthuss-type mechanism. For example, in nonaqueous solvents, this

mechanism would not work well. But it does appear that such a mechanism is reasonable under aqueous conditions.<sup>59</sup> Similar to this example, several recent studies have found evidence for specific conformations of isotopomers.<sup>60,61</sup> It is important to note that during the final stages of the dehydration process, transporting protons away from their solution phase locations can fundamentally alter the structural integrity of polar molecules.

**Evidence That Specific Non-native Conformations Formed upon Denaturation Favor Specific Protonation States.** The configurational regulation of protons in the small Pro7 and 4-amino benzoic acid systems is also apparent in larger systems. Consider, for example, protein denaturation, which can be induced by many types of perturbations, including introduction of chaotropes,<sup>62</sup> variation of pH,<sup>63</sup> and thermal heating.<sup>64</sup> The ESI charge state distribution is extremely sensitive to the structure of proteins in solution. A





**Figure 4.** Classic examples of changes in charge state and conformation of cytochrome *c* measured by MS depicted from ref 46 and adapted from refs 76 (copyright 1998 American Chemical Society), and 77 (copyright 1997 American Chemical Society). Left: changes in MS charge-state distribution of cytochrome *c* at different solvent pH: 2.6 (bottom), 3.0 (middle), 5.2 (top). Top right: isotopic  $^1\text{H}/^2\text{H}$  exchange profiles of different charge states of cytochrome *c* in the gas phase formed by ESI (circles), IR laser heating (squares), collisional heating (open triangles), and charge stripping (closed triangles). Bottom right: cross sections of cytochrome *c* populated in each charge state at high (filled circles) and low (open circles) injection energies measured by IMS-MS.

narrow distribution of low charge states is often observed for “native” electrospray; a broader distribution of  $m/z$  peaks corresponding to higher charge states is observed from denatured solutions. But, do specific solution conformations contribute uniquely to specific charge states in these larger systems?

An example of this is found upon analyzing the 64 amino acid protein chymotrypsin inhibitor 2 (CI-2). The solution transition from native to denatured states of CI-2 has been studied extensively by traditional calorimetric techniques.<sup>65–67</sup> Protein denaturation is generally viewed as a cooperative two-state process in which loss of the native structure gives rise to the denatured state—a distribution of random, amorphous forms.<sup>65</sup> However, it is postulated that different types of intermediate structures should be populated near the phase transition between the native and denatured forms.<sup>68</sup> Figure 3a shows the analysis of CI-2 with variable-temperature ESI to measure the cooperative two-state transition by means of an average charge state derived from temperature-dependent MS data. Fitting these data to a two-state sigmoidal transition yields a melting temperature ( $T_m$ ) which provides an ensemble signature of stability for the native fold.  $T_m$  values measured by this analysis have been in agreement with values measured with traditional techniques.<sup>26,69,70</sup>

This interpretation does not preclude a more complex process involving many structures that are hidden within each charge state. Figure 3b shows that each charge state varies uniquely with solution temperature, requiring that multiple solution structures are contributing differently to each of the

charge states within the MS distribution. Inspection of the mobilities within each charge state shows multiple peaks that behave differently with solution temperature. A Gaussian model of the temperature-dependent cross section distributions is used to elucidate 41 unique structures behaving differently with solution temperature. Figure 3c shows an example of the conformational free energy landscape of CI-2 consisting of 41 structures at various solution temperatures. Because of the broad dynamic range of IMS-MS techniques, the 41 structures can be seen across many temperature ranges even if they are in very low abundance. We might think of this as a transition that is taking place similar to a more flexible, membrane-like surface; as the temperature changes, the free energy surface evolves to favor some new species (unfolded structures with high collision cross sections) and disfavor others (folded structures with low collision cross sections). In this way, a conformation that we would consider energetically unfavored may be preferred at elevated temperatures. Of course, depending upon the nature of the system, new non-native structures may be formed by lowering the temperature as well (i.e., cold denaturation).

**Are These Ideas Consistent with Other Protein Mass Spectrometry Data?** Many clues about the relationship of conformations and charge-state distribution come from early electrospray studies. Since Chowdhury, Katta, and Chait’s classic 1990 paper describing how the populations of the +8 to +18 charge states of cytochrome *c* change upon varying the pH of aqueous solutions (as shown in Figure 4),<sup>46</sup> many reports of variations in protonation states with changing solution

environment have been made.<sup>71</sup> Implicit in the interpretation of these results is the impression that conformation states are distinct from one another, but each is assumed to give rise to multiple charge states in the electrospray distribution. This behavior is common for many proteins and changes in charge states can be induced by other means of protein denaturation, e.g., variation in solvent composition<sup>72</sup> or solvent temperature.<sup>73–75</sup> The results described above suggest that far more detail about many conformations is likely to be accessible from the MS distribution.

The idea that protons are far more mobile than conformational states would suggest that composites of multiple distinct species are hidden within a charge state distribution. In their 1993 paper, McLafferty and co-workers rigorously tested this hypothesis by reacting gaseous cytochrome *c* ions with deuterated solvents and measuring the isotopic <sup>1</sup>H/<sup>2</sup>H exchange profiles of different charge states as shown in Figure 4.<sup>78</sup> They interpreted the varying exchange levels for different charge states as evidence for families of different gas-phase conformations, that in some cases appeared to be correlated to unique structures in solution. Subsequent studies showed that unique conformations could be resolved based on differences in their mobilities through a buffer gas on millisecond time scales (Figure 4).<sup>25,77</sup> The first remarkable aspect of these results is that each requires multiple, well-defined conformations that can coexist in the absence of solvent. Additionally, these measurements reveal that unlike the rapid interconversion of conformations in solution, when solvent is removed, specific structures can be stable for long time periods (at least milliseconds in the case of mobility studies to many minutes, or even hours, as revealed by <sup>1</sup>H/<sup>2</sup>H exchange).<sup>79</sup> The large differences imply that the solution acts as a lubricant, allowing structures that are long-lived in the gas-phase to be highly flexural in solution. Several years later, Breuker and McLafferty discussed the time scales of the formation of specific types of intramolecular interactions in the gas phase.<sup>29</sup> Their estimates suggest that gentle instrument conditions can preserve solution structures into the gas phase and the ESI dehydration process may even stabilize aspects of the solution fold.

**Mobile Protons in the Gas Phase.** In the absence of a conducting medium (in nonactivating, gas-phase conditions), protons are solvated by intramolecular interactions with the peptide backbone groups and side chains. For example, carbonyl (CO) and amide (NH) groups along the backbone have lone-pair electrons and thus high gas-phase basicities that stabilize protons,<sup>80</sup> which has the effect of fixing their positions within the gas-phase conformation. Highly charged gas-phase protein structures may be influenced by nearby protons and undergo Coulomb-induced unfolding; however, it is anticipated that protons that are farther than ~10 Å apart are not influenced by one another.<sup>81–84</sup> In general, the maximum charge deposited onto a protein structure in the gas-phase is usually no greater than the total number of basic residues and N-termini in the sequence.<sup>72,80,85</sup> A previous model by Williams and collaborators has been used to elucidate the influence of Coulomb repulsion on the gas-phase basicities of common proteins, showing that the low gas-phase basicities of highly charged proteins causes solvent ions to be protonated instead of basic protein sites.<sup>86</sup> Such Coulomb forces can also be exploited to stabilize local rearrangements of protein solution structures into the gas-phase by use of counterions.<sup>37,87,88</sup>

Upon activation of peptides or proteins by collisions, photons, temperature, etc., protons that were originally sequestered become mobile. A mobile proton is hungry for an electron and thus weakens nearby electron-dense regions associated with covalent bonding interactions, which facilitates dissociation. Wysocki, Gaskell, Harrison, and others describe this as the *mobile proton model* that results in peptide fragments that are valuable for determining oligomer sequences.<sup>89</sup> We note that in order to take advantage of the sensitivity of ESI to monitor protein conformations in solution, careful attention to instrument tuning is necessary. Efforts toward developing instrumental protocols to reduce the amount of ion heating associated with measurements of protein solution conformations have been presented and are ongoing.<sup>90</sup>

## CONCLUSIONS

We have explored the Grotthuss mechanism and its implications on protonation of specific macromolecular structures in solution. This mechanism would predict that small changes in structure are nearly instantaneously accompanied by changes in protonation configuration/state. Because of the differences in time scales of proton migration and protein structural changes, changes in the equilibrium distribution of structures in solution may appear as changes in the ESI charge state distribution. One outcome of these considerations is that ESI may be exquisitely sensitive to protein and peptide conformations in a manner that has not been appreciated previously. As such, theoretical treatments of proteins in solution should consider the rapid changes in protonation states that accompany small changes in structure, an idea that is currently not practical in molecular dynamic simulations.

While these simple considerations of the motions of protons and proteins is useful when analyzing electrospray charge states and conformations, we note that there are several factors that influence the preservation of native-like protein structures into the gas-phase environment that we have not discussed in detail here and are still poorly understood. Instrumental conditions, such as applied electric field, distance from MS inlet, capillary temperature, and numerous other factors influence the desolvation and emergence of protein ions into the gas-phase. Solvent effects, such as using a high-salt buffer or organic solvent, will also influence solution structures and MS charge states.

It is instructive to close this critical insight by considering the influence of the Grotthuss mechanism in understanding the velocities of electrons.<sup>10</sup> As J. J. Thomson was “discovering the electron”, he used an analogy with Grotthuss chains in 1889 to explain the very high velocities of electrons through gases.<sup>91</sup> That is, he considered the existence of a polarized chainlike structure within the gas as a means of conducting electrons. This extension of the ideas of Grotthuss also applies as we consider electronic motion and nuclear motion in small molecules. Born and Oppenheimer realized that nuclear motion in molecules was so much slower than electronic motion that in theory the two are separable. Like protons in proteins, for small molecules we would say that *electrons are fast and smart; nuclei are slow and dumb*. The irony of this is while the Grotthuss mechanism for protons has essentially been manifested in quantum chemical theories for calculating molecular structure (i.e., Born’s and Oppenheimer’s approximation which allows electronic structure to be treated separately in calculations from nuclear motion), the

implications have not yet been realized for theoretical treatment of macromolecules in the solution environment for which it was first proposed.

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### Notes

The authors declare no competing financial interest.

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