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Letter

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Hydration of Guanidinium Ions: An Experimental Search for Like-Charged Ion Pairs

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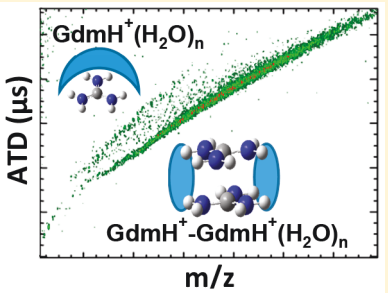
Supporting Information

ABSTRACT: Guanidinium ions ($GdmH^+$) are reported to form stable complexes ($GdmH^+/GdmH^+$) in aqueous solution despite strong repulsive interactions between the like-charged centers. These complexes are thought to play important roles in protein folding, membrane penetration, and formation of protein dimers. Although $GdmH^+$ ions are weakly hydrated, semiempirical calculations provide evidence that these like-charged complexes are stabilized by water molecules, which serve important structural and energetic roles. Specifically, water molecules bridge between the $GdmH^+$ ions of $GdmH^+/GdmH^+$ complexes as well as complexes involving the guanidinium side chains of arginine. Potential biological significances of like-charged complexes have been largely confirmed by *ab initio* molecular dynamics simulations and indirect experimental evidence. We report cryo-ion mobility−mass spectrometry results for the $GdmH^+/GdmH^+$ ion pair confined in a nanodroplet—*the first direct experimental observation of this like-charged complex*. A second like-charged complex, described as a water-mediated complex involving $GdmH^+$ and H_3O^+, was also observed.



Protein dynamics, structure, and function are directly coupled to interactions with the local environment, which include other peptides/proteins, small molecules (osmolytes), ions, and solvent (water). The hydrophobic effect is an important folding effector that has been extensively studied and is reasonably well understood.[1−3] Interactions between hydrophilic groups and water, which include both short- and long-range interactions, strongly influence the structure(s) of both the molecule as well as that of the surrounding water molecules.[4] The influence of hydration is probably strongest for polar amino acid side chains located on the protein surface, but the solvation of polar side chains within the protein core is also an important determinant for protein folding.[3] Electrostatic interactions involving basic and acidic amino acid side chains have been extensively studied, especially those involving the side chain of arginine, i.e., guanidinium ($GdmH^+$), with aspartic and glutamic acid,[5] but interactions of $GdmH^+$ ions with Trp, Arg, and Gln (π-stacking type interactions)[6,7] and with hydrophobic amino acid side chains have also been reported.[8] The classic papers by Scheraga revealed that pairing of positively charged $GdmH^+$ ions on the arginine side chains form stable complexes in aqueous solutions—*seemingly unlikely considering potential effects of strong Coulombic repulsion*.[9,10] Dimers such as ($GdmH^+/GdmH^+$) involving two arginines have also been implicated in many structure/function relationships, including enhancement of passive cell-penetrating actions.[11−13] Scheraga's evidence for this seemingly unlikely like-charge dimer was derived from database searches,[9,14,15] but strong evidence was also recently obtained from X-ray absorption spectroscopy experiments.[16] More recently, higher level quantum mechanical calculations underscore the important role of water in stabilizing these like-charged $GdmH^+/GdmH^+$ dimers,[10,17] and *ab initio* MDS suggest that the like-charge ion pairs are stabilized by amphiphilic behavior and van der Waals interactions. *It is important to note, however, that to our knowledge there has been no reported direct experimental evidence for hydrated like-charged $GdmH^+/GdmH^+$ interactions*.[16−19]

Our current understanding of how water influences structure, function, and dynamics, with few exceptions, is based largely on studies of bulk solvents.[20] In a series of recent studies we have taken advantage of the evaporative cooling, viz., freeze-drying,[21] inherent in electrospray ionization (ESI) and the cryogenic (80 K) operating temperatures of cryogenic ion mobility−mass spectrometry (cryo-IM−MS) for studies of protonated molecules ($[M + xH]^{x+}$) that are hydrated by small numbers (n) of H_2O molecules, i.e., $[M + xH]^{x+}(H_2O)_n$. The cold IM drift tube provides a means to capture ions having limited numbers of H_2O adduction, viz., H_2O molecules that comprise the first few hydration shells that can be interrogated further by IM-MS, i.e., size-to-charge and mass-to-charge. Most relevant to this study are the results on the hydration of the diammonium alkyl cations where the effects of Coulombic repulsion were evidenced by a distinct unfolding transition that occurs over a small range of numbers of hydrating H_2O molecules. For example, the transition from hydration by a single droplet to individual droplets for each ammonium ion of the 1,7-diammonium alkyl ion ($H_3N^+-(CH_2)_n-NH_3^+$; n = 7, 8, and 10) within a single droplet occurred over a range of 16−18 water molecules, whereas the transition occurred at 18−20

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and 21−24 water molecules for 1,8- and 1,10-diammonium alkyl ions, respectively.[22]

Such studies provide new avenues for addressing an important fundamental question: do soluble, polar molecules alter the surrounding water structure or does the presence of water alter the structure of the polar molecule? In the case of the latter, water has been found to play an important role in maintaining the structure of $[M + 3H]^{3+}$ ions of substance P (SP), an important tachykinin neuropeptide (RPKPQQFFGLM). For example, the ions formed by slow dehydration have a more compact conformation whereas the most stable gas-phase conformer has an extended conformation.[23,24] Similarly, water-mediated formation of a noncovalent dimer of the small protein ubiquitin was attributed to formation of water bridges between the polar side chains that surround the I44 hydrophobic patch.[25,26] Alternatively, stable "magic number" water clusters have been reported for ornithine- and lysine-containing peptides, which are indicative of water-structuring around the peptide, whereas no evidence for magic numbers are found for arginine-containing peptides.[21,27−29] Collectively, the results for small molecules, peptides and small proteins underscore the importance of solute−solvent interactions on conformational preferences of solute as well as the structure of the surrounding water network.[30−33]

Here, cryogenic ion mobility−mass spectrometry (cryo-IM−MS) is used to investigate hydrated guanidinium ($GdmH^+$) and arginine ($ArgH^+$) ions, specifically how the size/shape of hydrated cluster ions change as a function of the number (n) of water molecules, $(GdmH^+)(H_2O)_n$ and $(ArgH^+)(H_2O)_n$. The cryo-IM-MS instrument has been described previously,[27,34] and it is important to note that cryogenic cooling of the drift tube is essential for retaining the weakly bound water molecules. Briefly, ions are generated ("freeze-dried")[21] by static ESI emitter tips (∼3−5 μm outer diameter) and guided into a cold (∼80 K) ion mobility drift tube that is housed within a liquid nitrogen dewar. The hydrated cluster ions are first separated on the basis of size-to-charge using IM followed by mass-to-charge (m/z) analysis by time-of-flight (ToF) MS.

Figure 1 contains plots of the IM arrival-time distribution (ATD) vs m/z of the hydrated $GdmH^+(H_2O)_n$ and $ArgH^+(H_2O)_n$ ions. Note that abundant $GdmH^+(H_2O)_n$ cluster ions range from $n \sim 1$−30, whereas $ArgH^+(H_2O)_n$ cluster ions for $n > 50$ are observed. Beauchamp et al. showed evidence that the hydrated ions are formed by stepwise elimination of single H_2O molecules from larger hydrated ions,[27,35] and this appears to also be the case for $GdmH^+(H_2O)_n$ and $ArgH^+(H_2O)_n$ ions. In a recent study, Kim et al. reported MDS results that suggest that some water loss occurs by ejection of small neutral clusters from the nanodroplet.[36]

For both $GdmH^+(H_2O)_n$ and $ArgH^+(H_2O)_n$ ions the ATD decreases as the number of water molecules decreases, forming smaller droplets with no change in the existing ion or droplet structure. This is the expected behavior for hydrated ions where loss of H_2O is purely an evaporative process and is most apparent for both $GdmH^+(H_2O)_n$ and $ArgH^+(H_2O)_n$ where n = 1−3. Regions of discontinuity, as observed for n = 5−10, are indicative of either changes in size of the hydrated ions owing to a change in the structure of the ion or orientation of the hydrating H_2O molecules. Because the structure of the $GdmH^+$ ion is rather rigid, we attribute this to changes in the orientation of hydrating water molecules. The water network

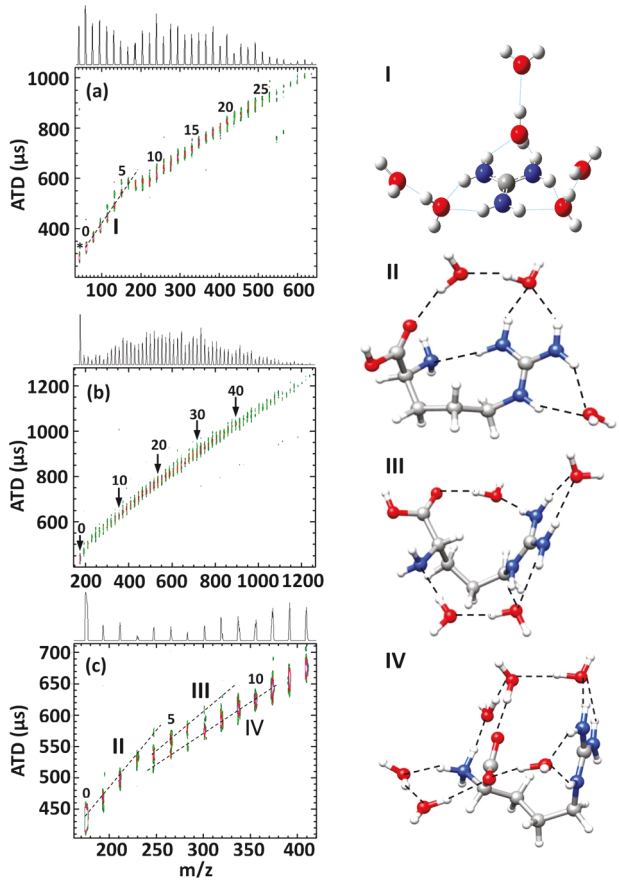

Figure 1. Two-dimensional contour plots of ATD vs m/z plots of (a) $GdmH^+(H_2O)_n$, (b) $ArgH^+(H_2O)_n$, and (c) $ArgH^+(H_2O)_n$ ions for n = 1−13 were obtained from solutions of 200 μM GdmHCl or 300 μM arginine solutions in 18.2 MΩ H_2O. Structure **I** shows a proposed structure populated by $GdmH^+(H_2O)_n$ ions where n = 6. (adapted from ref 39). Structures **II**, **III**, and **IV** are proposed structures for the ions that fall on the respective ATD trendlines in (c) (adapted from ref 37). The peak labeled with an asterisk denotes a fragment carbocation.

transitions from a dome-like structure to a planar-like structure (structure **I**, Figure 1) for n = 6−9, as there is insufficient H-bonding to maintain the hemispherical network above the carbon atom. Instead of an abrupt transition from interstitial NH_2 bonding to single H-bonding with NH_2 at n = 9, we find it more likely that the first hydration shell rearrangement occurs over the same dome-like to planar-like H-bond network transition. It appears H-bonding with the interstitial sites only becomes energetically favorable upon sufficient dehydration.

It is interesting to compare the ATD vs m/z plots of $GdmH^+(H_2O)_n$ to that for $ArgH^+(H_2O)_n$ (Figure 1b). The ATD vs m/z in the regions $n > 10$ follow a single trendline, but the ATD for n = 4 is shifted downward relative to that for n = 5. Although the signals are relatively weak, it appears that multiple signals are detected for n = 4, 5, and 6; these shifts are highlighted by the dashed lines in Figure 1c. These differences are interpreted in terms of greater conformational diversity for the $ArgH^+(H_2O)_n$ ions, as previously suggested by Bowers.[37] (see structures **II**−**IV**, Figure 1). The salt bridging (SB) structure (**IV**) is more compact and the charge solvated (CS) structure (**III**) is more extended. In addition, a third conformer family (**II**) is observed at n = 4 where limited hydration causes intramolecular charge solvation to dominate, and structure **III**

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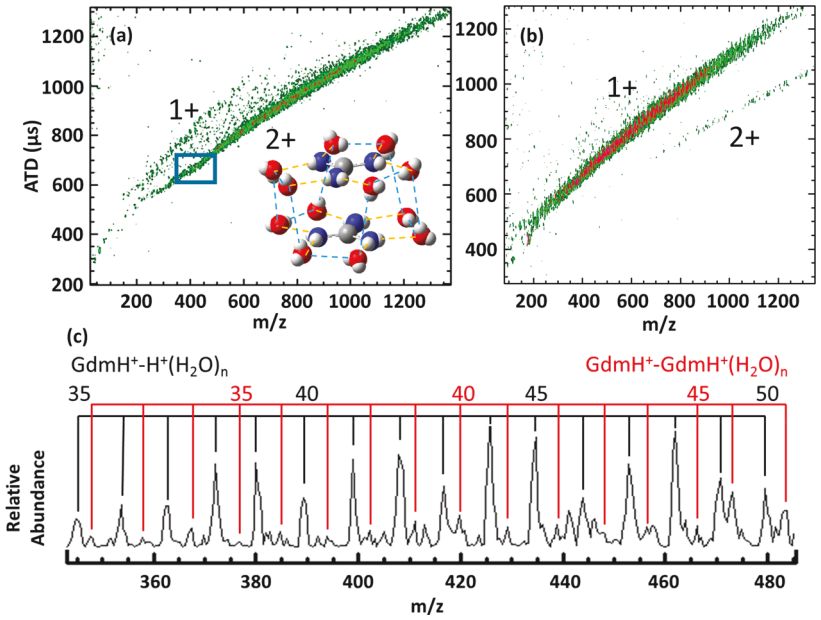

Figure 2. Two-dimensional contour plots of ATD vs m/z of (a) $GdmH^+(H_2O)_n$ and (b) $ArgH^+(H_2O)_n$ ions obtained from solutions of 400 μM GdmHCl in 0.1% formic acid or 300 μM arginine in 18.2 MΩ H_2O. A proposed structure of $GdmH^+{-}GdmH^+(H_2O)_{12}$ like-charged complex is shown in the inset; N−H---O and water−water hydrogen bonds are shown with orange and blue dashed lines, respectively. This structure is similar to that reported by Vazdar et al.[17] (c) Mass spectrum extracted from the region between m/z 340 and 485 showing hydrated like-charged ion pairs ($GdmH^+{-}GdmH^+(H_2O)_n$, red) and ($GdmH^+{-}H^+(H_2O)_n$, black).

transitions to structure **II**. We note that Gao et al. comment that the SB and CS states become nearly isoenergetic at n = 7.[37] Our data suggest the CS structure becomes dominant at n = 4, the CS and SB forms are nearly equal in abundance at n = 5−6, and the SB structure becomes dominant for clusters with 7 or more water molecules. To test the hypothesis that a proton is transferred from the N- to C-terminus, the pathway by which **IV** transitions to **III**, we examined the arginine methyl ester arginine ($MeRH^+(H_2O)_n$) (Figure S1). The ATD vs m/z plot for $MeArgH^+(H_2O)_n$ closely resembles that for $GdmH^+(H_2O)_n$.

Previous studies have described $GdmH^+$ ions as being weakly hydrated owing to low charge density; i.e., the positive charge is delocalized over each of the $-NH_2$ groups. Sharp et al. described $GdmH^+$ as having amphiphilic character where the partially positively charged hydrophilic $-NH_2$ groups bind water strongly and the central carbon atom possesses hydrophobic character (structure **I**, Figure 1).[38] Heiles et al. argued that the strongest hydration occurs at small water cluster sizes, such as when $GdmH^+$ might be approaching or already in close contact with another molecule. They suggest the formation of the second hydration shell and a rearrangement of the first shell upon addition of a fourth and ninth water molecule, respectively.[39] Understanding these strongly hydrated structures then assumes new levels of importance in describing the binding interactions of $GdmH^+(H_2O)_n$; that is, the solvation of the $GdmH^+$ ion can be altered by interactions from other groups such as in ion pair formation. Structure **II** shows how the $GdmH^+$ ion can be intramolecularly solvated while structure **IV** shows how the ion forms a solvent bridge with the C-terminus. The methyl ester arginine inhibits the latter interaction and does not form the more compact ion conformation in structure **IV**.

Figure 2 contains ATD vs m/z plots for 2^+ ions formed from solutions of (a) GdmHCl and (b) arginine. Because the abundances of doubly charged ions were low using small ESI emitter tip sizes (~3−5 μm outer diameter), these data were obtained using larger ESI emitter tip sizes (~15 μm outer diameter) and slightly higher drift tube pressures. These conditions favor formation of larger droplets, which increases the abundances of doubly charged ions as well as the like-charged ion pairs. The doubly charged ion region of Figure 2a contains both hydrated $GdmH^+{-}GdmH^+(H_2O)_n$ and $GdmH^+{-}H^+(H_2O)_n$. The individual ion signals in the mass spectrum (Figure 2c) are sufficiently resolved to allow for assignment of the hydrated 2^+ ions, ranging from approximately 15 to over 130 (165−1200 m/z) water molecules. The signal for 2^+ ion clusters for both $GdmH^+{-}GdmH^+(H_2O)_n$ and $GdmH^+{-}H^+(H_2O)_n$ begins to decrease in abundance n = ~55 and have completely dissociated to 1^+ clusters by n = 15−20. While peaks with m/z corresponding to $ArgH^+{-}ArgH^+(H_2O)_n$ and $ArgH^+{-}H^+(H_2O)_n$ complexes were observed, the 2^+ ion abundances in the spectrum for the arginine solution are very weak and we have not otherwise made attempts to assign the m/z values. Kubíčková et al. showed that the stabilizing interactions of $GdmH^+(H_2O)_n$ that favor formation of $GdmH^+{-}GdmH^+$ complexes are not detected for other positively charged ions.[40] It should be noted that we do not observe any doubly charged ions in ESI mass spectra of solutions of ammonia, specifically $(NH_4^+)_2(H_2O)_n$ clusters (see Figure S2). Thus, our data provide additional evidence that the like-charge guanidinium ion pairs are not artifactual, and the ion pairing behavior observed is specific for guanidinium ions.

The dehydrated (i.e., n = 0) $GdmH^+{-}GdmH^+(H_2O)_n$ like-charged ion pair is not observed in Figure 2a; however, low abundance and reproducible signals for the like-charged ion pair are observed for $n \geq 15$. This observation is consistent with results reported by Vazdar et al.[17] They reported that formation of parallel stacked $GdmH^+{-}GdmH^+$ like-charged ion pairs requires at least 12 water molecules, and that the stability of the complex increases as the number of water

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molecules increases.[17] The surrounding water molecules provide essential enthalpic benefits through an extended H-bonding network, which affords charge solvating interactions that overcome entropic costs and reduce Coulombic repulsion. In addition, chloride anions located on the periphery of the like-charged ion pair have been predicted to stabilize the complex,[41] but chloride adduct ions are not observed experimentally. To test whether Cl^- ions are involved in formation of the ion pair and being lost as HCl,[42] we investigated the hydration of guanidinium acetate and observed similar like-charged ion pairs starting at $n \geq 11$. Vazdar et al.[17] also predicted a T-shape complex as a local minimum, and while the 2^+ water clusters display considerable heterogeneity in their mobility, we are unable to confidently assign a T-shape or parallel stacked ion pair. However, given that 6−7 water molecules per $GdmH^+$ hydrates in a nearly planar fashion (structure **I**, Figure 1a),[43] we propose that the $GdmH^+$−$GdmH^+(H_2O)_n$ like-charged ion pair exists in a stacked, parallel structure similar to that reported by Vazdar et al. (Figure 2a inset).[17] Regardless, typical studies on $GdmH^+$−$GdmH^+$ have suggested the complex is stable due to favorable intermolecular interactions between the $GdmH^+$ ions acting as a counterforce to Coulombic repulsion, but it is apparent that solvent bridging water must be accounted for in the stabilization of the ion pair.

A similar 2^+ trendline was also observed for arginine solution (Figure 2b), but the relative abundances of the ions are very low and the peak resolution is not sufficient for high confidence assignments. These differences probably arise owing to alternative mechanisms for hydrating the $ArgH^+$ ions. Specifically, as the numbers of hydrating water molecules increase, i.e., for $n > 6$, the C-terminus of the molecule can take on a negative charge by forming an ion pair, COO^-−$(H^+(H_2O)_n)$, or through formation of a salt bridge with the N-terminus. In either case these competing mechanisms of hydration might disrupt the arrangements of water molecules that favor formation of the hydration network necessary to bridge two nearby $GdmH^+$ moieties. These interactions may explain why $ArgH^+$−$ArgH^+(H_2O)_n$ complexes are only observed at larger water cluster sizes ($n > \sim 25$).

The dependence for formation of the like-charged ion pair on a solvent bridge is supported by recent results invoking solvent bridges in the solvation of both charge groups in alkyl diammonium cations and the noncovalent ubiquitin dimer.[22,44] Additionally, the $GdmH^+$ solvent bridging provides insight into how two arginine residues come together to form solvated ion pairs.[9,10] The observation of a solvent bridge that forms around the hydrophobic region of $GdmH^+$ is similar to results reported by Servage et al.[25] for the hydrated, noncovalently bound ubiquitin dimer. They showed that the dimer does not dissociate to form monomer ions until late in the desolvation process. The formation of the noncovalent ubiquitin dimer was attributed to interactions involving the I44 hydrophobic patch and to solvent bridging involving the positively charged arginine side chain located near the I44 hydrophobic patch.[25] The similarities between ubiquitin and guanidinium ion pairs underscore the potential importance of such binding "hot spots" and provide a simple model system to gain additional insight into these effects on the peptide/protein scale. While it is well-known that hydrophobic patches provide the thermodynamic impetus for dimerization, these data support substantial binding forces resulting from solvent bridges which must be disrupted before dissociation.

We have demonstrated that the H-bonding network from $(GdmH^+(H_2O)_n)$ undergoes a structural shift from $n = 6-9$, in agreement with the transition from a dome-shaped cluster to a more planar cluster. Conversely, the structure of $ArgH^+(H_2O)_n$ behaves quite differently; at large values of n, these ions have a more solution-like structure that is best described as a zwitterionic salt-bridging structure. The charge sites on the zwitterionic termini of the arginine residue remain stable until only 5−6 water molecules remain, and the ion transitions to a gas-phase charge-solvating structure. A second transition was observed, which we attribute to a transition toward intramolecular solvation of the $GdmH^+$ moiety by the N-terminus. This intramolecularly solvated ion conformation becomes favored when there is very little hydration, i.e., $n < 5$.

We report the first direct experimental evidence for solvated like-charged guanidinium complexes ($GdmH^+$−$GdmH^+(H_2O)_n$), that clearly show that a minimum of ∼15 water molecules are required to stabilize this like-charge ion pair. Since the hydrated guanidinium ion is nearly planar for $n = 6-7$ and the charge is delocalized across each $-NH_2$ group, we suggest that the two $GdmH^+$ ions are stacked parallel to one another. Each of the guanidinium ions is solvated by 6 water molecules, and the remaining ∼3 water molecules serve to bridge the $-NH_2$ groups. However, as the number of water molecules increases, the hydrated like-charged complex takes on a number of alternative structures. The enthalpic benefit provided by an H-bonding network bridging the two $GdmH^+$ ions rationalizes how like-charged complexes have been observed between arginine residues and in theoretical studies of guanidinium ion pairs.

We also report an unexpected observation of a heteroion pair involving hydrated H_3O^+ and $GdmH^+$. The like-charged $GdmH^+$−$GdmH^+$ ion pair is stabilized by the network of bridging water molecules (H-bonding), quadrupole−quadrupole, hydrophobic, and van der Waals interactions,[16,18] whereas the stabilizing interactions for the like-charged $GdmH^+$−$H^+(H_2O)_n$ complex are limited to H-bonding, thus the relatively high abundance of the latter is an unexpected result. This suggests that the heteroion pair must receive similar enthalpic benefits from structuring the hydration network, and that this is an important stabilizing interaction. Furthermore, the effects of Coulombic instabilities of the $GdmH^+$−$H^+(H_2O)_n$ complex may be minimized by rapid shuttling of the proton via the Grotthuss mechanism, effectively dispersing the charge density. We argue that the diffuse charge distribution and unique water-structuring capability of the $GdmH^+$ ion play key roles in formation of both the $GdmH^+$−$GdmH^+(H_2O)_n$ and $GdmH^+$−$H^+(H_2O)_n$ complexes. These requirements rationalize the absence of like-charge complexes involving point charge species, specifically NH_4^+ and H^+ ions.[40] Lastly, it is important to recognize that the nanodroplet environment is not an accurate model for bulk water; thus the guanidinium ion pairs may be unique to confinement effects of the nanodroplet. The diffuse charge distribution and unique water-structuring capabilities of the $GdmH^+$ ions may be relevant to its actions as a protein denaturant. The structuring observed here in the confined nanodroplet may have parallels to confinement near the protein surface.[45]

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.9b00268.

Two-dimensional contour plots of ATD vs m/z of hydrated methyl ester arginine and hydrated ammonium ions (PDF)

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Notes

The authors declare no competing financial interest.

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