Experimental Measurement of Coulomb Energy and Intrinsic Dielectric Polarizability of a Multiply Protonated Peptide Ion Using Electrospray Ionization Fourier-Transform Mass Spectrometry

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Received April 26, 1994[⊗]

Abstract: A method to quantitatively measure the Coulomb energy in gas-phase ions with two or more protons by measuring gas-phase basicity is demonstrated. From these measurements and estimates of distance between charge sites, the intrinsic dielectric polarizability (ϵ_r) of isolated peptide and protein ions can be obtained. This method is demonstrated with the peptide gramicidin S; for the $(M + 2H)^{2+}$ ion, we find the Coulomb energy is >27.9 kcal/mol. The distance between the two charges on this ion (9.5 Å) is determined from the lowest energy configuration obtained by molecular modeling. The proposed gas-phase structure of this ion is consistent with experimentally determined rates of H/D exchange for the singly and doubly protonated molecular ions with D_2O. From the Coulomb energy and distance between charges, an upper limit of $\epsilon_r < 1.2$ is obtained. This is decidedly less than the theoretical value of 2−4 predicted for peptides and proteins.

Introduction

Electrostatic interactions are the predominant long-range force in biomolecules and play an important role in their structure and function. These forces, described by Coulomb's law, are inversely proportional to the dielectric polarizability of the medium between the charges. Thus, an accurate measurement of the intrinsic dielectric polarizability (ϵ_r) of biomolecules is necessary to understand the effects of these forces. The explicit values of ϵ_r for isolated peptide and protein molecules have been hotly debated.[1,2] Experimental values of 2−50 have been reported using a variety of techniques.[1] The larger values are attributed to the effects of water, which has a bulk dielectric constant of 78.38 (298 K).[3] Calculated values of ϵ_r between 2 and 4 have been reported for peptides and proteins in the absence of solvent,[2] although values as high as 10 have been reported for localized portions of a protein.[2e]

Gas-phase multiply protonated ions, such as those produced by electrospray ionization,[4] are ideally suited for studying electrostatic interactions between charge sites in peptides and proteins *in the absence of solvent*. The charge-induced destabilization of multiply protonated ions has been shown to effect both ion reactivity[5−7] and ion stability.[8,9] Fenn and co-workers have proposed a model for the maximum charge on an ion,[10] which is obtained when the Coulomb repulsion by other charges exceeds the binding energy of a charged species. McLuckey, Van Berkel, and Glish[5] have measured proton-transfer rates for different charge states of cytochrome *c* ions with dimethylamine and found these rates to decrease with lower charge state. This is attributed to increasingly basic sites of protonation and decreasing Coulomb repulsion between charges as the charge state of the ion decreases. Smith and co-workers[6a] found that the centroid of the charge distribution of cytochrome *c* shifted from +15 to +10 upon introducing gas-phase H_2O into the electrospray interface. They attribute this to Coulomb destabilization of the ion driving the proton transfer to water to form H_3O^+.

Proton transfer between a multiply protonated ion and neutral molecule has a large reverse activation energy due to contributions of Coulomb, ion−dipole, and ion−induced dipole interactions. Calculations by Bursey and Pederson[11] for the reaction of doubly protonated 1,3-diaminopropane with ammonia and *tert*-butylamine indicate that the ΔH values for bringing two protonated product ions from infinite distance to the proton-transfer interaction distance are 77% and 92% of that of a simple point charge Coulomb model for ammonia (reaction distance 7.70 Å) and *tert*-butylamine (reaction distance 8.80 Å), respec-

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1994.

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tively. Effects of multiple charges on proton transfer for the odd electron ion $C_{60}(XH)_n^{\bullet 2+}$ have been discussed by Petrie *et al.*[12] The gas-phase acidity of $C_{60}H^{\bullet 2+}$ was reported to be 124 ± 8 kcal/mol using a Coulomb potential ($\epsilon_r = 1$) to model the reverse activation energy.[12b] Petrie *et al.* estimated[12a] that the contributions of ion−dipole, ion−induced dipole, and dipole−dipole interactions to the reverse activation energy for this system are less than or equal to 25% of the Coulomb repulsion on the basis of both the physical size of the ion and the calculations of Bursey and Pederson.[11]

In addition to increased rates of proton transfer, multiply protonated ions can be dissociated readily.[8,9] This is due to the high-energy deposition possible in the electrospray interface as well as the Coulomb destabilization. The latter effect has been modeled by Rockwood, Busman, and Smith[9] using linear "charges-on-a-string", in which charges are equidistant and $\epsilon_r = 1$.

Here, we present a method to quantitatively measure the Coulomb energies in multiply protonated ions through measurement of their gas-phase basicities. In combination with estimates of distance between charge sites, ϵ_r for an isolated peptide or protein ion can be determined. These values for the $(M + 2H)^{2+}$ ion of gramicidin S, a cyclic decapeptide, are reported.

Experimental Section

Instrumentation. These experiments were performed using an external ion source electrospray ionization Fourier-transform mass spectrometer operated at a magnetic field strength of 2.74 T. The electrospray interface and ion introduction system were developed in collaboration with Extrel FTMS, Millipore Corp. (Madison, WI). An abbreviated description of this instrument follows; full details will be presented elsewhere.[13] Ions are generated at atmospheric pressure by applying ~2.5 kV to a 31 gauge stainless steel syringe needle through which analyte solution is continuously infused at 2 μL/min using a syringe pump (Harvard Apparatus, Model 2, South Natick, MA). Ions are introduced into the mass spectrometer through an 11 cm stainless steel resistively heated capillary[14] (170 °C) and pass through two differentially skimmed regions, evacuated to 1.4 Torr and 30 mTorr by 17 CFM and 50 CFM mechanical pumps, respectively. Three high-vacuum stages of differential pumping reduce the operating pressure in the final stage, pumped with a 300 L/s diffusion pump (Alcatel Crystal 100, Annecy, France) and 230 L/s Varian Starcell ion pump (Varian Associates, Lexington, MA), to 5×10^{-9} Torr. The first and second high-vacuum regions, pumped by a 1600 L/s diffusion pump (Varian VHS-6) and 850 L/s diffusion pump (Varian VHS-4), operate at a pressure of 1×10^{-5} Torr and an estimated 1×10^{-7} Torr, respectively. Ions are accelerated to 2.2 kV in the second high-vacuum stage using a series of electrostatic lenses to guide the ions past the fringing fields of the magnet. These ions are decelerated in the final high-vacuum stage to ground potential prior to introduction into the cell.

Ions are trapped in a $5 \times 5 \times 10$ cm^3 rectangular cell using a static trapping potential[15] of 6.0 V. This potential is reduced to 1.0 V during detection. Nitrogen is introduced through a pulsed valve to a pressure of $\sim 2 \times 10^{-6}$ Torr for 5 s during ion accumulation to enhance collisional trapping and thermalization of the ions. All spectra are acquired using an Odyssey Data System (Extrel FTMS, Millipore Corp.). Ions are isolated using SWIFT[16] and are detected using a rf sweep (120 V peak−peak, 1100 Hz/μs, low-to-high frequency). 256K data points are acquired, and 16K data points are tranformed unless otherwise noted. A mechanical beam block is used to prevent ions from passing through the system during ion reaction and detection. Reactant bases (Table 1)[17] (Aldrich Chemical Co., Milwaukee, WI) are degassed using several freeze−pump−thaw cycles and introduced into the mass spectrometer through a sapphire leak valve (Varian Vacuum Products) to a pressure of 8×10^{-8} Torr. Gramicidin S, a cyclic decapeptide (*cyclo*[-Pro-Val-Orn-Leu-D-Phe-]$_2$), is electrosprayed from methanol at a concentration of 1.5×10^{-4} M.

Table 1. Gas-Phase Basicities of Reference Bases

reference base	gas-phase basicity[a] (kcal/mol)
pyrrole	200.3
2-fluoropyridine	202.8
3-fluoropyridine	206.2
isopropylamine	211.0
pyridine	213.1
tert-butylamine	213.0
diethylamine	217.7
di-*n*-propylamine	222.0
triethylamine	224.5
tri-*n*-butylamine	227.0
TMG[b]	234.9
DBN[b]	237.4
DBU[b]	239.7
MTBD[b]	243.3

[a] GB values (ref 17) are reported at 300 K. [b] 1,1,3,3-Tetramethylguanidine (TMG), 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and 1,3,4,6,7,8-hexahydro-1-methyl-2*H*-pyrimido[1,2-*a*]pyrimidine (MTBD).

Gas-Phase Basicity. The apparent gas-phase basicity (GB^{app})[12] is measured using both the bracketing[18] and kinetic[19] methods. In the bracketing method, an ion of unknown basicity is isolated and reacted with neutral bases of known GB. The occurrence of proton transfer, as shown in eq 1, indicates the relative GB^{app} of the two species. The

$$(M + nH)^{n+} + B \rightarrow (B + H)^+ + (M + (n-1)H)^{(n-1)+} \quad (1)$$

apparent basicity of the ion is assigned to one-half the difference between the least basic neutral that undergoes proton transfer and the most basic neutral that does not undergo proton transfer. During the course of the ion accumulation, ions undergo an average of approximately 200 collisions with N_2. Doubling the time in which N_2 is introduced does not affect the ion reactions that we observe. We conclude that ions are sufficiently thermalized so that excess ion kinetic and internal energy does not interfere with these measurements. Molecular ions of gramicidin S are reacted with all reference bases listed in Table 1.

For measurement of GB by the kinetic method, a proton-bound dimer of the ion and base is dissociated; the more basic species in the mixed

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dimer will retain the proton upon dissociation. The GB of the molecule is determined from the relative abundance of the protonated species. In this study, a proton-bound dimer of the singly protonated gramicidin S molecular ion and the base MTBD is formed by trapping ions in the FTMS cell in which the pressure of MTBD is 5×10^{-7} Torr. The $(M + H + MTBD)^+$ ion is isolated and excited to a radius of 0.5 cm (7.0 eV) using SWIFT.[16] A 5 s dissociation delay is used prior to detection.

H/D Exchange. The vacuum chamber of the mass spectrometer is exchanged with D_2O (Aldrich Chemical Co.) at a pressure of 1×10^{-6} Torr for 3 h. Molecular ions of gramicidin S are reacted simultaneously at a D_2O pressure of 7×10^{-7} Torr for variable reaction times. From the resolved isotopes of the molecular ion signal, the contribution of ^{13}C is subtracted to obtain abundances of deuterated species. Rate constants are determined from the best fit of the kinetic data by a program written in Mathematica (Wolfram Research Inc., Champaign, IL) and generously provided by E. Gard and C. Lebrilla (Chemistry Department, UC Davis).[20] From this algorithm, we find that the fractional exchange in the vacuum chamber is 0.8.

Molecular Modeling. All calculations are done with the Insight II molecular modeling package, v.2.3.0 (Biosym Technologies, San Diego, CA), running on an IBM RISC 6000. Structures are minimized in Discover (v.2.9) with the CVFF force field to an rms derivative of less than 0.05 kcal, and dynamics are run at temperatures up to 800 K for 1000 steps (1 ps) between each minimization. Both the $(M + 2H)^{2+}$ and $(M + H)^+$ ions are minimized with an ϵ_r of 1.0 using 10 dynamics-minimization cycles. The distance between the charged ornithine nitrogens is determined from the lowest energy structure. This, combined with the experimentally measured Coulomb energy, is used to obtain a new value of ϵ_r. Structures are minimized with this new value, and the process is repeated iteratively until there is no significant change in structure.

Results and Discussion

Coulomb Energy from GB. For ion–molecule proton transfer reactions, we propose that the "apparent" binding energy of a proton on an ion with n protons (PA_{sp}^{app}), each separated by ≥ 10 Å (vide infra), is quantitatively given by eq 2, in which

$$PA_{sp}^{app} = PA_{intrinsic} - \sum_{ij} \frac{q_i q_j}{4\pi\epsilon_0\epsilon_r r_{ij}} \quad (2)$$

$PA_{intrinsic}$ is the intrinsic proton affinity of the site of protonation, *i.e.*, the PA of the singly charged ion protonated at that site, and the second term is the sum of Coulomb interactions between this charge and all other charges in the ion (q is the charge on an electron, ϵ_0 is the vacuum permittivity, ϵ_r is the dielectric polarizability of the medium between the charges, and r is the distance between the charges). The PA_{sp}^{app} of an ion $(A + nH)^{n+}$ ($n \geq 1$) is greater than its actual PA by the value of the reverse activation energy for removing a proton, *i.e.*, the energy of bringing a proton from infinite distance to the transition state of the $(A + (n-1)H)^{(n-1)+} \cdots H^+$ complex.[12] The PA^{app} of the multiply protonated ion is then that of the site of lowest PA^{app}.

For the simplest case, consider a diprotonated peptide in which the protons reside on identical sites, *i.e.*, a molecule with a plane of symmetry between the charge sites. The GB and PA of the neutral molecule, A, are defined[21] in eq 3. Similarly, the GB and the PA of the singly charged ion are given in eq 4.

$$A + H^+ = AH^+, \quad -\Delta G_1 = GB(A) = RT \ln \frac{[AH^+]}{[A][H^+]}, \quad -\Delta H_1 = PA(A) \quad (3)$$

$$AH^+ + H^+ = AH_2^{2+}, \quad -\Delta G_2 = GB(AH^+) = RT \ln \frac{[AH_2^{2+}]}{[AH^+][H^+]}, \quad -\Delta H_2 = PA(AH^+) \quad (4)$$

If the sites of protonation are identical, or more specifically have identical intrinsic PA ($-\Delta H_1 = PA_{intrinsic}$), and the structure of the singly charged ion is independent of the method of formation, *i.e.*, the structure of AH^+ is the same if formed by removing a proton from AH_2^{2+} or by adding a proton to A, then $-\Delta G_3$ for reaction 5 is equal to the difference in GB

$$\begin{aligned} AH_2^{2+} + A = 2AH^+, -\Delta G_3 &= RT \ln \frac{[AH^+]^2}{[AH_2^{2+}][A]} \\ &= -\Delta G_1 - (-\Delta G_2) \\ &= GB(A) - GB(AH^+) \\ &= -\Delta H_1 + \Delta H_2 - T(\Delta S_2 - \Delta S_1) \end{aligned} \quad (5)$$

between A and AH^+. For reaction 5 to be observed in our experiment, the activation barrier must be small. This occurs when the basicity of A is greater than or equal to $GB(AH^+)$ plus the reverse activation energy ($GB^{app}(AH^+)$). From eq 2, the Coulomb energy can be expressed as the difference in the apparent binding energies of the proton in the monoprotonated and diprotonated ions (eq 6). For systems in which entropic

$$\begin{aligned} \text{Coulomb energy} &= \Delta H_2^{app} - \Delta H_1 \\ &= GB(A) - GB^{app}(AH^+) + T(\Delta S_2^{app} - \Delta S_1) \end{aligned} \quad (6)$$

effects are negligible, or for which $\Delta S_1 = \Delta S_2$, then $\Delta G_3 = \Delta H_3 = 0$ for reaction 5 in the limit of infinite separation of charge in the AH_2^{2+} ion. At finite charge separation, the difference $\Delta H_2^{app} - \Delta H_1 = PA(A) - PA^{app}(AH^+) = GB(A) - GB^{app}(AH^+)$ is a quantitative measure of the Coulomb energy in the AH_2^{2+} ion.

The use of structurally different amines as reference bases in this study has been shown to introduce negligible error in measurements of PA.[19b] However, entropic effects can be substantial with small molecules in which intramolecular cyclization occurs.[22] This is due primarily to symmetry changes and loss of internal rotations upon protonation. For example, protonation of 1,5-diaminopentane results in cyclization of the ion with a corresponding entropy of cyclization of −20 cal/(mol·K) ($T\Delta S_{cyc} = -7.0$ kcal/mol at 300 K).[22a] For large molecules such as peptides and proteins with many polarizable groups, cyclization or solvation of the charge sites is expected to occur. If the charge sites are independently solvated through localized intramolecular solvation, then the ΔS_1 of cyclization should be approximately equal to that for ΔS_2. Thus, entropic effects of cyclization cancel for reaction 5. The contribution to $T\Delta S$ from symmetry change for reaction 5 for large peptides and proteins should be small compared to the error in our experimentally measured value of GB.[23] The assumption that ΔS for reaction 5 is negligible could be tested by measuring

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(23) We estimate that this value is less than 1.2 kcal/mol for gramicidin S.

the temperature dependence (van't Hoff plots) of the difference in ΔG for the proton-transfer reactions of the AH^+ and AH_2^{2+} ions.

We present results for gramicidin S for which these conditions appear to be satisfied. For molecules in which charge sites interact to solvate the proton in the AH^+ ion, ΔS_1 will not equal ΔS_2, and these values as well as enthalpy of cyclization must be determined in order to find the Coulomb repulsion. Results for such a system will be presented elsewhere.

Effects of Multiple Charges on Ion–Molecule Reactions. In the bracketing method for determining GB, proton transfer between an ion of unknown GB and a neutral of known GB (or vice versa) indicates the relative GB of these species. For multiply protonated ions, equating the reverse activation energy to Coulomb energy requires that effects of multiple charge on the well depth of the ion–molecule reaction be negligible. For these near-thermal reactions, the interaction potential ($V(r)$) between an ion and a neutral molecule with a permanent dipole moment for an impact parameter of zero is given by eq 7,[24] in

$$\overline{V(r,\cos\theta)} = Ae^{-cr} - \frac{\alpha q^2}{2r^4} - \frac{q\mu_D}{r^2}\overline{\cos\theta(r)} \qquad (7)$$

which r is the separation distance, A and c are constants, α is the polarizability of the neutral molecule, μ_D is the dipole moment of the molecule, and θ is the angle the dipole makes with the line of centers of the collision. Ae^{-cr} represents short-range repulsion due to electron cloud interpenetration, $-\alpha q^2/2r^4$ represents the point-charge induced dipole attractive potential, and $-(q_D/r^2)\cos\theta(r)$ represents the ion–permanent dipole potential. Thus, this interaction potential depends on the ion charge and the polarizability and dipole moment of the neutral molecule. Because of the $1/r^4$ and $1/r^2$ dependencies, this attractive potential is a relatively short-range interaction. The equilibrium hydrogen bond length between the ion and neutral molecule is ~1.4 Å for two species of comparable GB.[25] For a diprotonated ion, if the charges are separated by 10 Å, the effects of the second charge on the depth of the potential well between the first charge site and the neutral molecule must be *less* than 2.7%.[26] This difference in well depth is small compared to the ±2 kcal/mol error in our GB measurement. Thus, the reactivity of diprotonated ions in which the charges are separated by ≥10 Å will be determined by both the intrinsic reactivity and Coulomb repulsion at each charge site. For ions with identical charge sites, the net collisional cross section should be <2 times that of a singly protonated ion.[27]

Figure 1 illustrates a qualitative interaction potential between an AH_2^{2+} ion and a neutral base, B. If the GB of B is lower than the GB^{app} of AH^+, proton transfer from the AH_2^{2+} ion to neutral B will not occur, due to the high activation barrier. As the GB of B approaches the GB^{app} of AH^+, curve crossing to the highly exothermic reaction channel for the formation of AH^+ and BH^+ becomes possible. The potential for separation of AH^+ and BH^+ is given by Coulomb's law (Figure 1, short-dashed line, at a charge separation distance of >11.4 Å). The gas-phase basicity of A can be measured by observing proton transfer from the AH^+ ion to a more basic neutral, B′. If B and B′ have comparable values of α and μ_D, the well depths for these ion–molecule reactions will be nearly the same. Thus, the difference between the GB of A and the GB^{app} of AH^+ is a direct measurement of the Coulomb potential at a distance r between the two charges on the AH_2^{2+} ion and separation of these charges at infinite distance.[28] This value corresponds to the Coulomb energy between the two charges on the AH_2^{2+} ion.

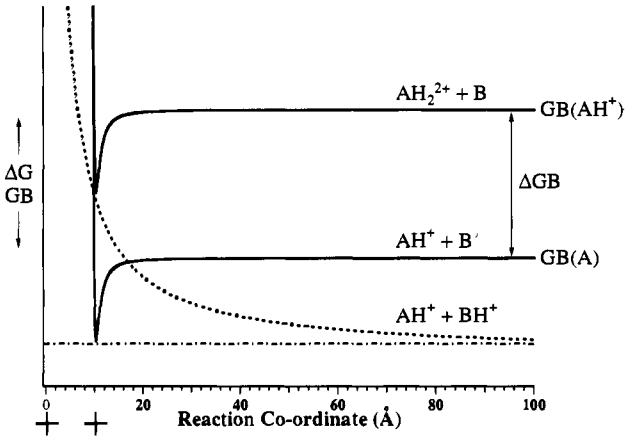

Figure 1. Qualitative interaction potential for the reaction of doubly and singly protonated ions with neutral bases, B and **B′**, for an ion in which the charge sites are separated by 10 Å. The solid curves are ion–permanent dipole potentials and the short-dashed curve is a Coulomb potential between the charge sites.

Measured GB of Gramicidin S. Gramicidin S is a symmetric, cyclic decapeptide with two basic ornithine residues. The two identical side-chain nitrogens are the most basic sites in this molecule and thus the most favorable sites of protonation. Both singly and doubly protonated ions are readily produced by electrospray ionization.

Ion abundances for the reactions of $(M + 2H)^{2+}$ ions with the bases *tert*-butylamine (TBA, GB = 213.0 kcal/mol) and diethylamine (DEA, GB = 217.7 kcal/mol) for reaction times up to 60 s are shown in Figure 2. With TBA, <3% proton transfer is observed for reaction times up to 60 s (Figure 2, top). With DEA, proton transfer to form $(M + H)^+$ and $(DEA + H)^+$ ions is 81% complete after 60 s (Figure 2, bottom).[29] Thus, the GB^{app} of the $(M + H)^+$ ion is between those of TBA and DEA, or 215.4 ± 2 kcal/mol. Note that proton transfer from the $(M + 2H)^{2+}$ ion indicates the GB^{app} of the $(M + H)^+$ ion as defined in eq 4. Reaction rates for proton transfer from $(M + 2H)^{2+}$ ions to the bases listed in Table 1 correlate directly to GB^{app}. This indicates that differences in α and μ_D for the bases have a negligible effect on reactivity and thus do not significantly effect the well depths of these ion–molecule reactions.

In the reaction of $(M + 2H)^{2+}$ with neutral DEA, $(DEA + H)^+$ and $(M + H)^+$ are formed in exactly equal abundance. Thus,

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(26) Assuming that the molecule is directly between the charges, the effects of a second charge on the attractive ion–induced dipole well depth will be $\leq[(1.4)/(10 - 1.4)]^2 = 2.7\%$. For a sterically constrained system, such as a peptide, the effects of a second charge on this well depth will be less than $(1.4/10)^2 = 2.0\%$. For reactions involving molecules without a permanent dipole, the effects of a second charge on this well depth will be less than $(1.4/10)^4 = 0.04\%$.

(27) The actual cross section is expected to be less than twice that for the singly protonated ion since one degree of freedom, corresponding to the distance between the charges in the doubly charged ion, is frozen. In addition, the physical (hard-sphere) cross section which is relatively independent of charge becomes increasingly significant for larger ions.

(28) This assumes that $\Delta S_1 = \Delta S_2$ and that the differences in ΔS for the formations of $(B + H)^+$ and $(B' + H)^+$ are negligible.

(29) These data fit a single exponential curve as expected for pseudo-first-order kinetics, with a rate constant of $k = 1.4 \times 10^{-13}$ cm^3 molecule^{-1} s^{-1}.

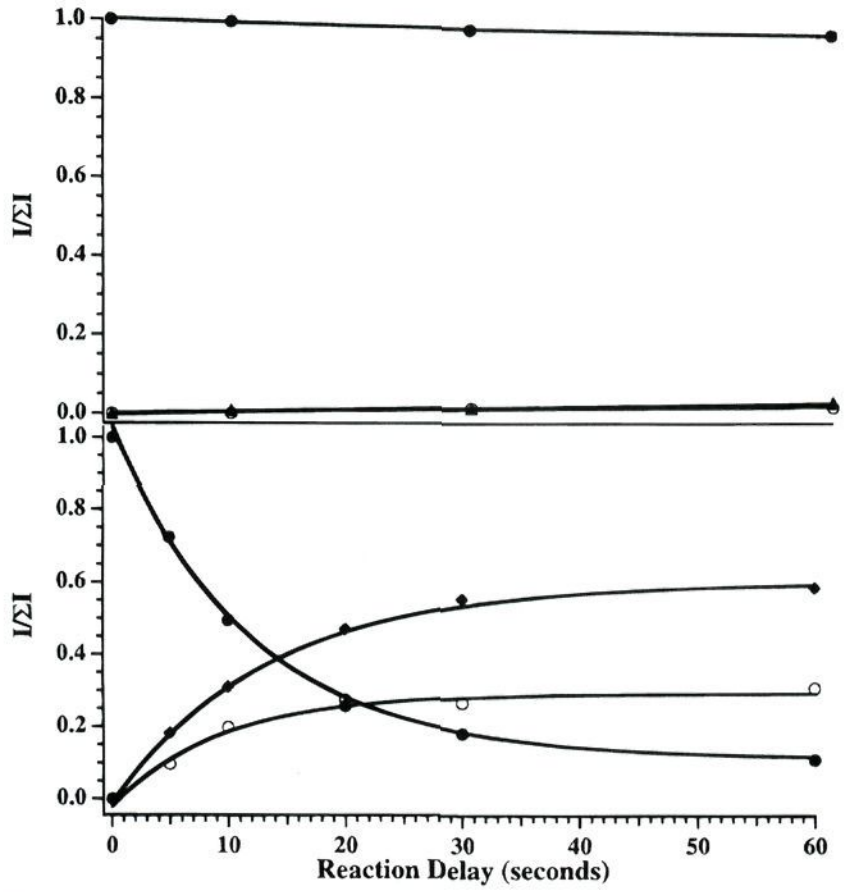

Figure 2. Ion abundances versus time for the reactions of $(M + 2H)^{2+}$ with *tert*-butylamine (TBA) (top) and diethylamine (DEA) (bottom): ○, $(M + H)^+$; ●, $(M + 2H)^{2+}$; ▲, $(TBA + H)^+$; ◆, $(DEA + H)^+$.

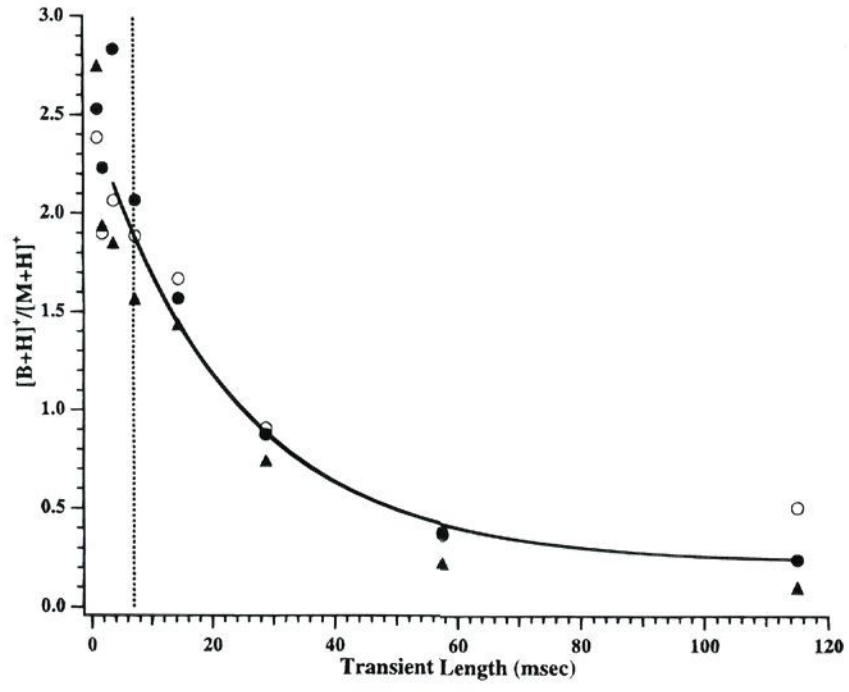

Figure 3. Ratio of signal intensity of $[DEA + H]^+/[M + H]^+$ as a function of transient length for the reaction of gramicidin S $(M + 2H)^{2+}$ with DEA. The vertical dashed line at 7 ms corresponds to the average time between collisions: ○, 60 s reaction; ●, 30 s reaction; ▲, 10 s reaction.

the observed intensities of these ions are a direct measurement of their relative detection efficiencies. Under these experimental conditions, the measured abundances of these ions change as a function of transient detection length (Figure 3). With 16K data points (7 ms), the average signal for $(DEA + H)^+$ (*m/z* 74) at three reaction times is 1.8 times that for $(M + H)^+$ (*m/z* 1142). With 128K data points (58 ms), this ratio is reduced to 0.3. Loss of $(DEA + H)^+$ ion signal over the course of our detection transient is likely due to higher collisional energy loss and scattering for this lighter ion, as well as resonant proton transfer to neutral DEA present in the system. By extrapolating to transient times shorter than 7 ms, the average time between collisions, effects of ion signal loss are minimized. For these short transients, we find the relative detection efficiency of *m/z* 74 ions to be 2.3 times greater than for *m/z* 1142 ions.

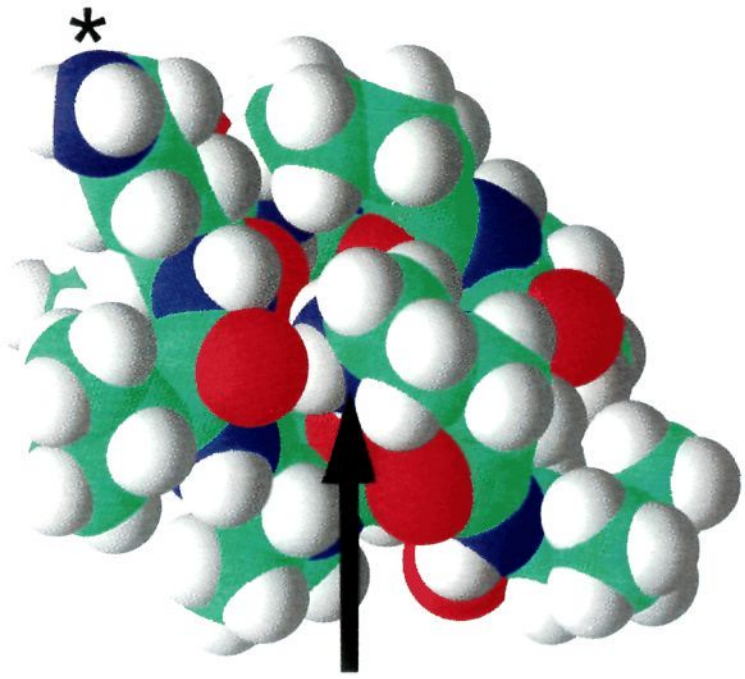

Figure 4. Lowest energy structure of gramicidin S $(M + H)^+$ obtained by molecular modeling, showing five backbone carbonyl oxygens solvating the charged ornithine side-chain nitrogen: green, carbon; white, hydrogen; red, oxygen; blue, nitrogen. The protonated ornithine side chain ($-CH_2CH_2CH_2NH_3^+$) is indicated with an arrow. The uncharged ornithine side chain is indicated with a star.

For the $(M + H)^+$ ion, we observe no significant proton transfer with any of the bases in Table 1, indicating that M is more basic than MTBD (GB = 243.3 kcal/mol),[17] the most basic molecule for which a GB has been reported. With MTBD, the abundance of the $(MTBD + H)^+$ ion is 2.5% that of the $(M + H)^+$ after 60 s reaction. To determine if steric effects (vide infra) prevent the interaction of this base with the charged ornithine side-chain nitrogen on the molecular ion of gramicidin S, the proton-bound dimer ion $(M + H + MTBD)^+$ was formed. Collisional dissociation of this complex results in the exclusive formation of $(M + H)^+$; no $(MTBD + H)^+$ is observed above the background (<4%). From the bracketing and kinetic measurements, we conclude that gramicidin S is more basic than MTBD, *i.e* has GB > 243.3 kcal/mol. This enables us to place a lower limit on the Coulomb energy of >27.9 kcal/mol.

Molecular Modeling. The three-dimensional structure of gramicidin S, investigated by crystallography,[30] ESR,[31] and solution-phase NMR,[32] indicates that this peptide forms a β-pleated sheet with a turn at both proline residues and four intramolecular hydrogen bonds across the backbone ring. In solution, the charged ornithine side chains are above the ring, approximately 8−10 Å apart,[31] where they are solvated by water. In the absence of water, this structure is expected to be highly energetically unfavorable due to Coulomb repulsion between charges.

To determine the structure of this peptide in the gas phase, we use molecular modeling to obtain the minimum-energy conformation. The lowest energy structure for the singly protonated gramicidin S ion shows that the protonated ornithine side-chain nitrogen is highly solvated by five carbonyl oxygens of the backbone (Figure 4). Thus, in the absence of solvent, it is highly energetically favorable for the molecule to "self-

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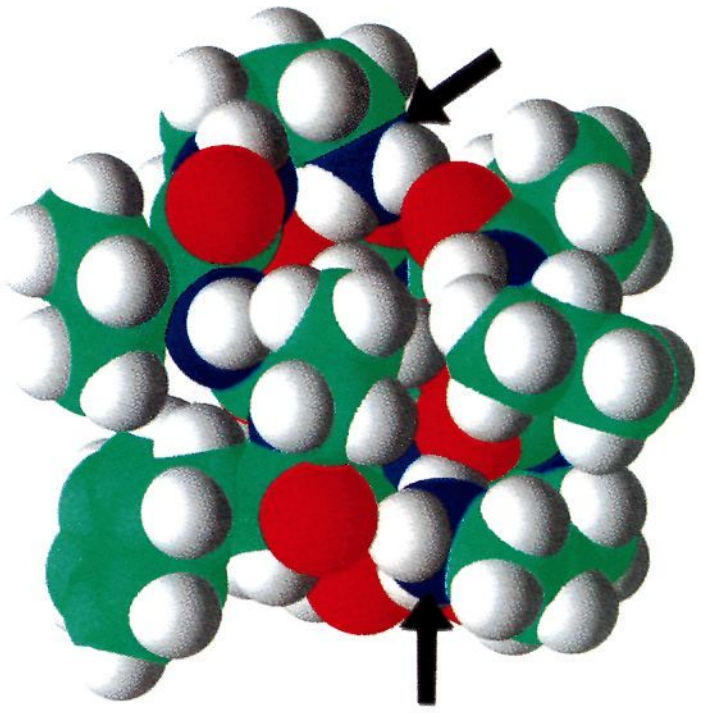

Figure 5. Lowest energy structure of gramicidin S $(M + 2H)^{2+}$ obtained by molecular modeling, showing the hydrogens on the ornithine side-chain nitrogens each hydrogen-bonded to a backbone carbonyl oxygen: green, carbon; white, hydrogen; red, oxygen; blue, nitrogen. The protonated ornithine side chains ($-CH_2CH_2CH_2NH_3^+$) is indicated with an arrows.

solvate" the charge. This accounts for the significantly greater basicity of the $(M + H)^+$ of gramicidin S (>243.3 kcal/mol) as compared to that of an isolated ornithine residue (222.5 kcal/mol).[33] This difference, >20.8 kcal/mol, represents the stabilization free energy due to these additional intramolecular interactions.

For the doubly protonated ion, the protonated ornithine side-chain nitrogens are independently solvated, with each of the hydrogens hydrogen-bonded to a backbone carbonyl oxygen (Figure 5). The hydrogen-bond distance for five of these hydrogens range from 1.8−2.0 Å. One hydrogen is significantly less solvated, and has a hydrogen-bond distance of 2.3 Å. The charged nitrogens on the ornithine side chains are separated by 9.5 Å.

H/D Exchange. Simultaneous reaction of both the singly and doubly protonated molecular ions of gramicidin S with D_2O introduced through a pulsed valve at 2.2×10^{-6} Torr results in a maximum exchange of five and six hydrogens for deuterium for these ions, respectively, after 60 s (Figure 6). This is consistent with exchange occurring at the ornithine side-chain nitrogens, the expected sites of highest hydrogen lability. In a separate experiment, D_2O was introduced though a leak valve at a constant pressure of 7×10^{-7} Torr and allowed to react with the molecular ions for various reaction times up to 600 s (Figure 7). Relative rate constants, extracted from these measurements and normalized to both the slowest exchange rate and the site multiplicity, are given in Table 2.

For the $(M + H)^+$ ion, all five hydrogens exchange at comparable rates. Thus, hydrogen exchange takes place at approximately the same rate on both the charged and the uncharged ornithine nitrogens. Interaction between the $(M + H)^+$ ion and D_2O is expected to occur primarily at the charge site. These similar rates of exchange indicate that interaction and scrambling of hydrogens between these two side chains occurs. This is consistent with the pseudo-first-order deuterium exchange rates observed by McLafferty and co-workers[34] for larger multiply protonated proteins.

For the $(M + 2H)^{2+}$ ion, the exchange rates are ~3−4 times those for the $(M + H)^+$ ion after taking into account the factor

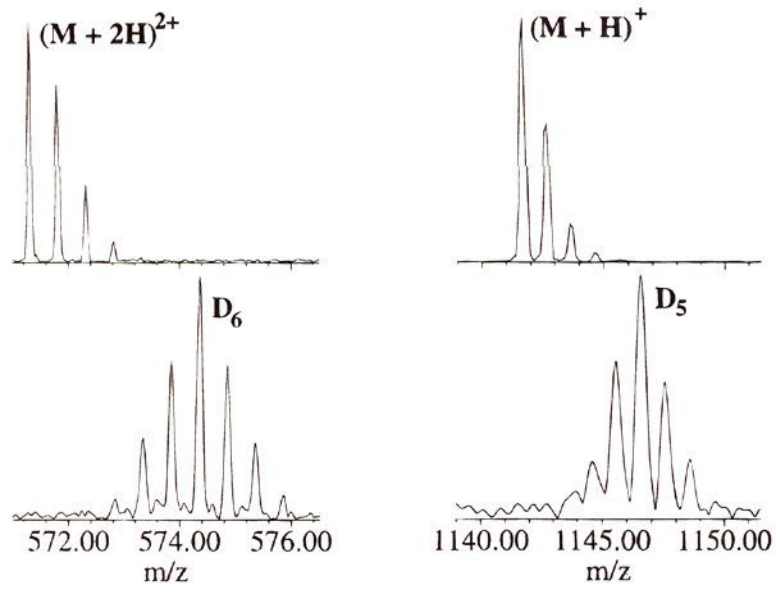

Figure 6. ESI-FTMS spectra of the singly (right) and doubly (left) protonated molecular ions of gramicidin S with no reaction (top) and 60 s reaction (bottom) with D_2O introduced through a pulsed valve at a pressure of 2.2×10^{-6} Torr. Expansions of molecular ion regions are shown.

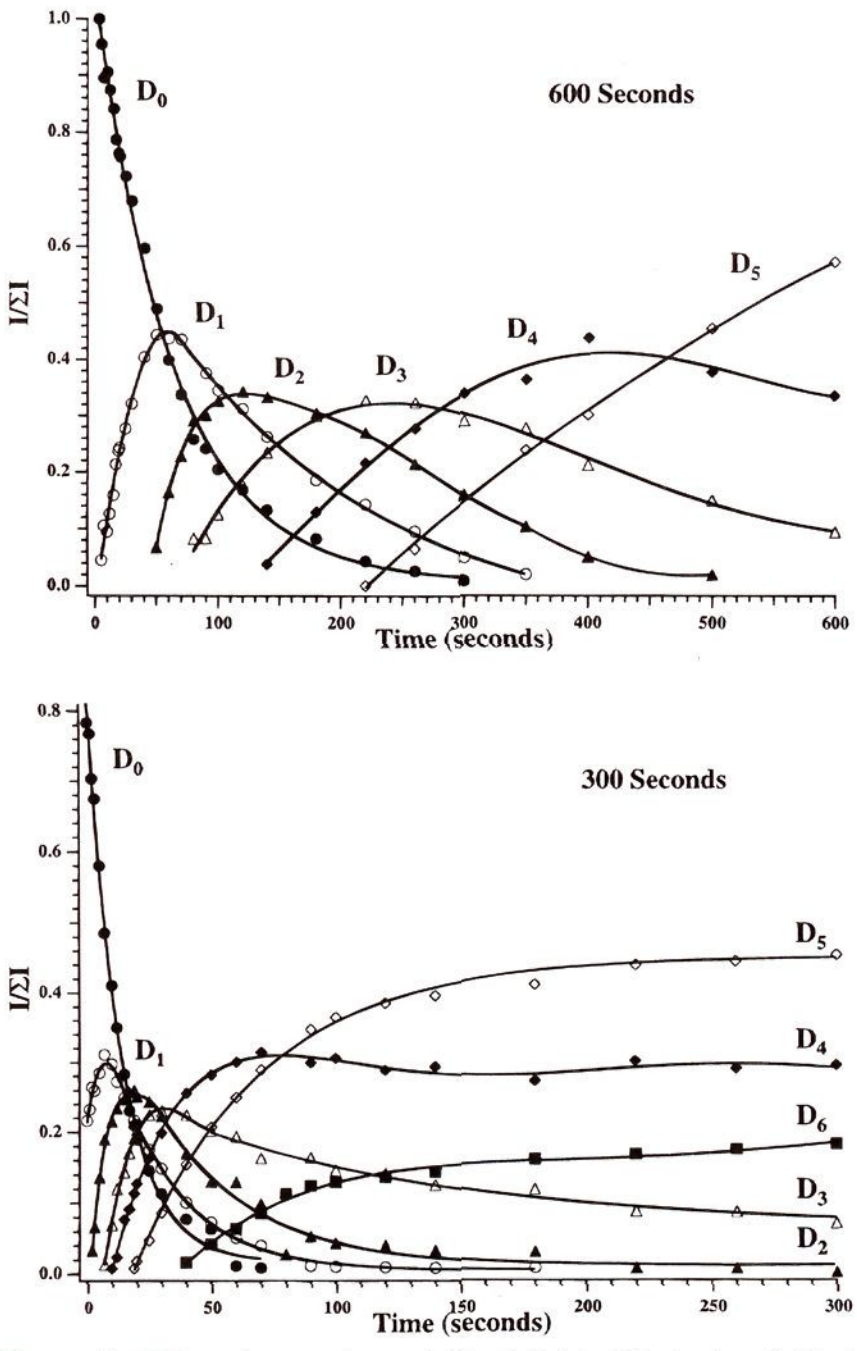

Figure 7. H/D exchange of gramicidin S $(M + H)^+$ (top) and $(M + 2H)^{2+}$ (bottom) ions with D_2O: ●, ○, ▲, △, ◆, ◇, and ■ refer to 0−6 hydrogens exchanged for deuterium, respectively.

(33) The basicity of ornithine is estimated to be approximately the same as that of lysine, reported by Lias and co-workers (ref 7). We are unable to measure this directly; we form only proton-bound dimers, trimers, etc., with electrospray ionization.

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Table 2. Relative Rate Constants for H/D Exchange of Gramicidin S $(M + H)^+$ and $(M + 2H)^{2+}$

relative rate constants[a]	$(M + H)^+$	$(M + 2H)^{2+}$ [b]
k_1	1.0	2.9
k_2	1.2	4.0
k_3	1.4	4.2
k_4	1.5	2.7
k_5	1.2	2.7
k_6		6.3

[a] Relative rate constants are normalized to $k_1(M + H)^+ = 2.69 \times 10^{-12}$ cm^3 molecule^{-1} s^{-1}. Values are corrected for site multiplicity, assuming equivalent hydrogens. [b] Rate constants reported for the $(M + 2H)^{2+}$ ion are divided by 2 in order to account for their increased collisional cross sections.

of <2 increase in the collisional cross section. The higher value for k_6 is likely due to error in fitting our kinetic data at longer reaction times. Lebrilla and co-workers[20] have measured exchange rates of the protonated amino acid lysine with CH_3-OD and found these rates are 1 order of magnitude higher than those we observed for reaction of $(M + 2H)^{2+}$ with D_2O. The proton affinities of methanol (PA = 181.9 kcal/mol[17]) and water (PA = 166.5 kcal/mol[17]) are much closer to each other than to lysine (PA = 230.3 kcal/mol[17]). This indicates that the different exchange rates for the $(M + 2H)^{2+}$ ion of gramicidin S and lysine are due to steric effects. Thus, our measured rates of exchange for the $(M + 2H)^{2+}$ ion suggest a structure in which both charge sites are solvated by the backbone carbonyl oxygens, but less so than for the singly charged ion. These results are consistent with the structures obtained by molecular modeling and indicate that the distance between charge sites must be approximately that of the diameter of the backbone ring (9–10 Å).

Dielectric Polarizability. The distance between charges is estimated to be 9.5 Å, the length between the two charged ornithine side-chain nitrogens found by molecular modeling. Charge delocalization is expected to be small compared to this distance for multiply protonated systems. This is less likely to be true in odd electron systems.[12b] From this value and the lower limit of Coulomb energy determined from the GB measurements, the upper limit of the intrinsic dielectric polarizability of gas-phase molecular ions of gramicidin S can be determined from eq 2 and is found to be <1.2. This value is significantly below the range of 2–4 predicted by theory for peptides and proteins.[2] This may be due in part to the more constrained structure of this cyclic molecule over that of a linear one. In addition, the intramolecular solvation observed for this ion is expected to limit its possible orientations and hence reduce its polarizability. The value of ϵ_r depends on molecular orientation in an applied electric field. Our measured ϵ_r is for two positive charges located at the ornithine side-chain nitrogens. This is expected to be the most relevant orientation since charges are localized at these sites in both solution and the gas phase.

It should be noted that the magnitude of the value we report depends on the accuracy of the GB values reported in the literature for the reference bases. The GB scale is a topic of some controversy. Using GB values for *tert*-butylamine (GB = 216.7 kcal/mol) and diethylamine (221.4 kcal/mol) reported by Meot-Ner and Sieck[35] normalized to 300 K and to the GB of ammonia reported by Lias *et al.*,[17] values of Coulomb repulsion and ϵ_r of >24.2 kcal/mol and <1.4, respectively, are obtained.

Finally, our values also depend on the assumption that the sites of protonation are independently solvated in both the singly and doubly protonated ions. The lowest energy structures obtained by molecular modeling indicate that this is true. However, our deuterium exchange experiments show that scrambling of the hydrogens between the two nitrogens of the ornithine side chains in the $(M + H)^+$ ion takes place. This could occur through a bridged structure with water. However, if the lowest energy structure for this ion is one in which both ornithine side-chain nitrogens interact to stabilize the charge, contrary to the structure obtained by molecular modeling, then both enthalpic and entropic corrections are required. We consider here briefly the magnitude of this correction.

In order to obtain an estimate of the change in ΔG for reaction 3 for a "bridged" structure in which both side chains stabilize the charge versus that for an independently solvated charged side-chain structure, we compare the enthalpy and entropy of cyclization for the protonation of two molecules that are crudely structurally comparable to the two ornithine side chains in gramicidin S, $H_2N-(CH_2)_6-NH_2$ ($\Delta H_{cyc} = -18.8$ kcal/mol, $T\Delta S_{cyc} = -7.0$ kcal/mol)[22a] for the bridged structure and $H_2N-(CH_2)_3-NH_2$ ($\Delta H_{cyc} = -15.5$ kcal/mol, $T\Delta S_{cyc} = -4.1$ kcal/mol)[22a] for the independently solvated side chain. A bridged structure will be approximately $\Delta(\Delta G) = -18.8 + 15.5 + 7.0 - 4.1 = -0.4$ kcal/mol more basic. If this structure is formed, the Coulomb energy must be corrected to account for this increased basicity, resulting in a value for Coulomb energy of >27.9 − 0.4, or >27.5 kcal/mol. This correction has a negligible effect on ϵ_r ($\epsilon_r < 1.2$). Therefore, our conclusion that ϵ_r is substantially below that predicted by theory is not significantly affected by the structure of the $(M + H)^+$ ion. We are investigating other small peptides in which this type of bridging interaction is not physically possible.

Conclusions

The Coulomb energy in multiply protonated ions, such as those formed by electrospray ionization, can be obtained from the differences in apparent gas-phase basicities of individual charge states. If charges are localized and separated by a distance significantly greater than the interaction distance of an ion–neutral complex, the depth of the interaction potential between a multiply charged ion and a neutral molecule is essentially the same as that for a singly charged species. Thus, conventional methods for determining GB, such as the bracketing and kinetic methods, measure the combined effects of intrinsic basicity and Coulomb energy at the site of protonation. For a molecule with identical charge sites, the difference in apparent PA between the neutral molecule and the protonated ion is a direct measurement of the Coulomb energy in the doubly protonated ion. In systems in which no intramolecular interactions at the charge site occur, or for which the charge sites are independently solvated, the difference in PA^{app} will be approximately equal to the difference in GB^{app}. For the doubly protonated gramicidin S molecular ion, a small cyclic decapeptide with identical charge sites, we find the Coulomb energy is >27.9 kcal/mol. The singly protonated molecular ion is more basic than any molecule in the literature for which a GB has been reported. We attribute this to the extensive intramolecular solvation that stabilizes the charge in this ion. We are investigating the use of larger peptides and proteins as "superbases" which may be useful for extending the GB scale.

The intrinsic dielectric polarizabilities of gas-phase ions can be obtained from the Coulomb energy and estimated distance between charges. For gramicidin S, the distance between charged nitrogens that we obtain from molecular modeling is 9.5 Å. This corresponds to an intrinsic dielectric polarizability of <1.2. To obtain a more accurate value for small peptides,

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we are pursuing these measurements on peptides for which the $(M + H)^+$ ion has measurable GB. Accurate values of dielectric polarizability are of importance for calculations in which Coulomb potential plays a role, such as molecular modeling,[36] intramolecular electron-transfer kinetics,[37] *e.g.* photosynthesis, and dissociation of multiply protonated ions by tandem MS,[8,9] *e.g* for protein[38] and DNA[39] sequencing. We are extending these measurements to larger, multiply protonated protein ions in which the intrinsic basicities of the charge sites differ.

Acknowledgment. The authors are grateful to J. L. Beauchamp, S. Bock, M. T. Bowers, E. Gard, P. R. Kemper, C. B. Lebrilla, P. B. O'Connor, R. A. Mathies, T. O. Robinson, R. J. Saykally, P. D. Schnier, and B. E. Winger for helpful discussions and/or experimental assistance, to the W. R. Grace, Co. for fellowship support (for D.S.G.), and to the Arnold and Mabel Beckman Foundation (M1652), the National Science Foundation (CHE-9258178), the Analytical Chemists of Pittsburgh (M1601), the Exxon Foundation (13605), and Extrel FTMS, Waters Millipore Corp., for generous financial support. We also acknowledge the National Institutes of Health (S10 RR05651-01) for partial support of our computer graphics facility.

JA941269J

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