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Ion–ion reactions with fixed-charge modified proteins to produce ions in a single, very high charge state

Brian L. Frey[a], Casey J. Krusemark[b], Aaron R. Ledvina[a], Joshua J. Coon[a], Peter J. Belshaw[a,b], Lloyd M. Smith[a,*]

[a] Department of Chemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, WI 53706, United States
[b] Department of Biochemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, WI 53706, United States

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ABSTRACT

Electrospray ionization (ESI) of denatured proteins produces a mass spectrum with a broad distribution of multiply charged ions. Attaching fixed positive charges, specifically quaternary ammonium groups, to proteins at their carboxylic acid groups generates substantially higher charge states compared to the corresponding unmodified proteins in positive-mode ESI. Ion–ion reactions of these modified proteins with reagent anions leads to charge reduction by proton transfer. These proton transfer reactions cannot remove charge from the quaternary ammonium groups, which do not have a proton to transfer to the anion. Thus, one might expect charge reduction to stop at a single charge state equal to the number of fixed charges on the modified protein. However, ion–ion reactions yield charge states lower than this number of fixed charges due to anion attachment (adduction) to the proteins. Charge reduction via ion–molecule reactions involving gas-phase bases also give adducts on the modified protein ions in low charge states. Such adducts are avoided by keeping the ions in charge states well above the number of fixed charges. In the present work protein ions were selectively "parked" within an ion trap mass spectrometer in a high charge state by mild radiofrequency excitation that dramatically slows their ion–ion reaction rate—a technique termed "ion parking". The combination of ion parking with the fixed-charge modified proteins permits generation of a large population of ions in a single, very high charge state.

1. Introduction

Electrospray ionization (ESI) of proteins creates gas-phase ions in a number of relatively high charge states, and thereby allows for accurate measurements of even high molecular weight proteins with most types of mass spectrometers. Consequently, ESI has greatly enhanced the analysis and characterization of intact proteins by mass spectrometry (MS). Several factors affect the number and distribution of charge states in a protein ESI mass spectrum including the protein's primary structure and molecular conformation, as well as electrospray solvent and instrumental factors [1,2]. All of these factors, however, offer only rather limited control over the charge states obtained by ESI of proteins. Additional control over protein charge states would be advantageous, especially if substantially fewer and substantially higher charge states could be obtained.

Several methods have been developed that alter the charge states of protein ions after electrospray ionization. Many of these techniques aim to concentrate the ions into fewer charge states, both to simplify spectra of protein mixtures and to improve signal-to-noise ratios. Neutral bases, acting in the gas-phase, have been used to abstract protons from protein ions and thereby reduce the charge states [3–6]. Ion–ion reactions in the gas-phase are also used to charge reduce protein ions by removing protons with anions [7,8]. We previously developed a method involving ion–ion reactions at atmospheric pressure for charge reduction electrospray mass spectrometry (CREMS) [9–11]. The method utilizes a corona discharge or a ^{210}Po α-particle source to generate ions for proton transfer reactions near the inlet of a mass spectrometer. CREMS of protein mixtures produces greatly simplified mass spectra by concentrating the ions into a single (1+) or a few low charge states. McLuckey and co-workers have investigated a number of types of ion–ion reactions within a modified ion trap mass spectrometer for charge reduction of proteins, among other purposes [7,8]. In conjunction with their ion–ion reactions, they developed a technique termed "ion-parking," which concentrates ions into a single charge state [12,13]. All of these ion–molecule and ion–ion methods *lower* the charge states of protein ions (with the exception

* Corresponding author. Tel.: +1 608 262 9207; fax: +1 608 265 6780.
E-mail addresses: bfrey@chem.wisc.edu (B.L. Frey), smith@chem.wisc.edu (L.M. Smith).

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of the charge inversion technique mentioned in the next paragraph).

High charge states are often more desirable than low ones for analysis of proteins by mass spectrometry, yet few methods have been developed to increase the charging of proteins. A multi-step charge inversion technique using ion–ion reactions has been shown to increase the charge on peptide ions from singly to doubly charged [14–16]. Additional charging of ions by the production of radical cations has been demonstrated by electron impact or by collisions with molecular oxygen in the gas phase [17,18]. Williams and co-workers have produced some increases in charging, termed "supercharging", over normal ESI conditions by the addition of solvents with high surface tension, such as glycerol and *m*-nitrobenzyl alcohol [2,19]. McLafferty and co-workers demonstrated the benefits of increased charging by employing glycerol addition to generate highly charged ions of a 29 kDa protein. Electron capture dissociation (ECD) of these high charge states provided cleavage of 97% of the interresidue bonds [20].

The methods mentioned above fall primarily into two categories: those that lower the charge state while potentially narrowing the distribution of charge states, and those that raise the charge state of at least some of the ions but do not narrow the distribution. Ideally, protein ions could be generated in a single (or a few) high charge states, as opposed to a few low charge states, for a number of reasons. First, narrowing the distribution by charge reduction to predominantly the 1+ charge state will cause complete charge neutralization of some ions making them undetectable [11,21]. Second, ions in higher charge states tend to acquire fewer adducts; or more accurately, adducts are removed more effectively from higher charge state ions due to their more energetic collisions in the source (nozzle-skimmer) region of the instrument [22]. Third, high charge states correspond to lower m/z values, which are preferable for most mass analyzers and detectors. Some types of instruments have an upper range of 2000 m/z or even lower, some achieve higher resolution at lower m/z (e.g., FT-ICR and orbitraps), and nearly all suffer from poorer detection sensitivity as m/z increases. Finally, more highly charged ions tend to fragment more efficiently. Examples of this trend have been shown for ECD of a peptide [23] and a protein [20]. Electron transfer dissociation (ETD) efficiency improves with fewer residues per charge [24], which for a given peptide or protein is accomplished with higher charge states. Collision induced dissociation (CID) leads to more fragmentation for more highly charged protein ions, and the highest charge states tend to fragment by fewer pathways, which may be beneficial for the first stage of an MS^n top-down protein sequencing strategy [25–27]. For these reasons, creating protein ions in a single, or a few, high charge states is a worthy goal.

We recently reported that proteins with chemically attached fixed charges produce substantially higher charge states than the corresponding unmodified denatured proteins by electrospray ionization [28]. In this paper, we hypothesized that charge reduction of these fixed-charge modified proteins could produce the desired narrow distribution of high charge states. Potentially, proton transfer reactions would remove all labile protons, but not affect the fixed charges, and thereby yield a single relatively high charge state equal to the number of fixed charges. The four proteins used in this study include the reduced and alkylated versions of lysozyme and ribonuclease A (RA-lysozyme and RA-RNase) as well as fixed charge modified versions of them (FC-lysozyme and FC-RNase). The general chemical structures for these proteins and the modification reactions are shown in Fig. 1, and some additional pertinent information is listed in Table 1. These proteins were subjected to ion–ion and ion–molecule charge reduction reactions as well as ion parking to achieve the goal of generating a single high charge state for a protein.

2. Experimental methods

2.1. Materials

The standard proteins bovine ribonuclease A (RNase) and chicken lysozyme were obtained from Sigma (St. Louis, MO). All synthetic reagents were obtained from Aldrich (Milwaukee, WI) unless specified otherwise. The compound (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) was purchased from Applied Biosystems (Foster City, CA). The fixed-charge reagent, (4-aminobutyl)trimethylammonium dichloride, was prepared as described previously [28]. Burdick & Jackson HPLC-grade water, methanol, and acetonitrile were used, and the formic acid was Suprapur® grade from EM Science. The following precursor compounds, used to generate gas-phase reagents, were all obtained from Aldrich: benzoic acid (99.5%), fluoranthene (99%), 1,5,7-triazabicyclo [4.4.0] dec-5-ene (HPP) (98%), and triethylamine (TEA) (99.5%).

2.2. Protein derivatization

Protein modifications were performed similarly to those described previously [28]. Briefly, thiol reduction and alkylation was performed in 60 mM NH_4HCO_3 buffer with 6 M guanidine–HCl and 40 mM DTT at 5 mg/mL protein concentration at 37 °C for 1 h. These reactions were diluted to 2.5 mg/mL with buffer containing 50 mM iodoacetamide and allowed to proceed at room temperature in the dark for 1.5 h. Amine reductive methylation was performed on proteins previously thiol-reduced-and-alkylated. Protein was dissolved in 300 mM triethanolamine buffer at pH 7.5 with 6 M guanidine–HCl to 1.25 mg/mL and then diluted with methanol to 1 mg/mL. Pyridine–BH_3 and formaldehyde were added to final concentrations of 30 mM and 20 mM, respectively. Reactions were sonicated briefly and allowed to react at room temperature for 2 h. Incorporation of fixed charges was performed by acid amidation of proteins that had been previously thiol-reduced-and-alkylated and amine-methylated. Protein was dissolved in wet DMSO (~5% H_2O) to 1.5 mg/mL. Then, 4-(aminobutyl)trimethylammonium chloride hydrochloride and *N*-methylmorpholine were added to yield concentrations of 500 mM and 350 mM, respectively. Along with sonication and gentle heating, water was added in the minimum amount necessary to solubilize the amine salt (~10% additional H_2O). Solid PyAOP was added to the protein solution to a final concentration of 60 mM. Reactions were allowed to proceed at room temperature for 2 h and then an equal volume of H_2O was added. Proteins with multiple modifications were purified and desalted by HPLC between reaction steps to minimize adverse effects from previous reaction components. Proteins were purified by reverse-phase chromatography on a YMC C_{18} ODS-A 100 mm × 10 mm column on a Beckman Coulter System Gold HPLC.

2.3. Mass spectrometry

2.3.1. Ion–ion and ion–molecule reactions at atmospheric pressure

These experiments were conducted with a Mariner orthogonal acceleration time-of-flight mass spectrometer (Applied Biosystems). Samples were electrosprayed from a fused silica capillary (280/100 μm o.d./i.d., Polymicro Technologies, Phoenix, AZ) using a platinum wire at ~2700 V, which was in contact with the sample solution through a MicroTee (Upchurch Scientific, Oak Harbor, WA). The protein samples at a concentration of 10 μM in 1:1 H_2O:MeOH containing 0.2% formic acid were infused with a syringe pump at a flow rate of ~250 nL/min. The nozzle potential was 400 V except where noted otherwise, and the nozzle temperature was 140 °C

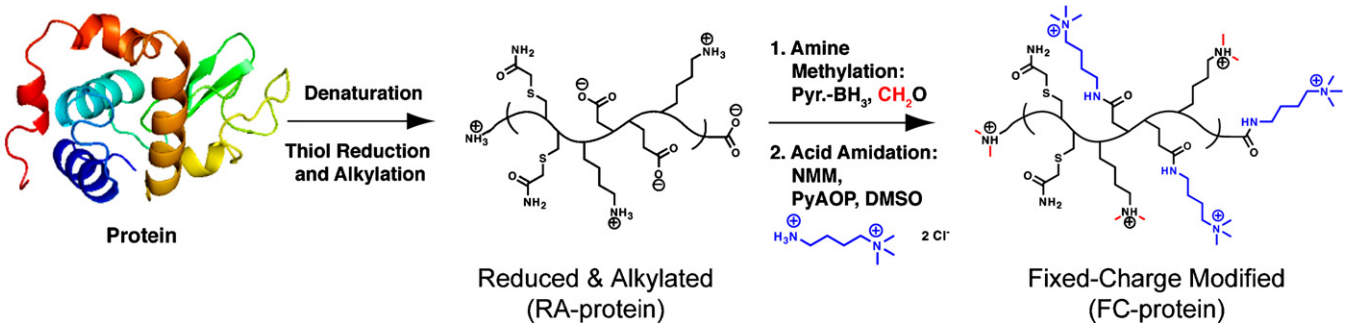

Fig. 1. The chemical modifications are illustrated for the two types of proteins employed: reduced and alkylated (RA) proteins and fixed-charge (FC) modified proteins.

Table 1
The numbers of various functional groups on each of the four proteins used in this study

Group	RA-lysozyme (MW 14,760)	FC-lysozyme (MW 16,088)	RA-RNase (MW 14,137)	FC-RNase (MW 15,690)
Carboxylic acid (D, E, C-term)	10	0	11	0
Fixed charge	0	10	0	11
Guanidinium (Arg)	11	11	4	4
Amine (Lys, N-term)	7	7 (methylated)	11	11 (methylated)
Imidazole (His)	1	1	4	4
Phenol (Tyr)	3	3	6	6

Each fixed-charge modification adds 113 Da, and each dimethylation of an amine adds 28 Da.

except for the TEA ion–molecule experiments, which were conducted at 175 °C. A nozzle extension was mounted to the inlet of the mass spectrometer, as described in detail previously [11], and this allowed introduction of either corona discharge generated anions or TEA vapor for the ion–ion and ion–molecule reactions, respectively. Spectra were obtained as either five or ten co-added scans of 10 s each. Mass spectra were smoothed with Data Explorer software (Applied Biosystems), and an eleven point Gaussian smoothing algorithm was chosen to improve signal-to-noise without artificially broadening the peaks.

2.3.2. Ion–ion reactions within an ion trap, including ion-parking

For these experiments a Thermo Scientific LTQ linear ion trap mass spectrometer was modified to accept a chemical ionization (CI) source on the back of the instrument opposite the nanospray source as previously described [24]. Negative CI of benzoic acid produced benzoate anions (m/z 121). Negative CI of fluoranthene produced radical anions (m/z 202), but also the presence of nitrogen gas led to another anion at m/z 216. While the 202 Da anion is useful for electron transfer, the 216 Da anion engages in proton transfer reactions. These species were introduced through a batch inlet system consisting of a GC oven and heated transfer line (SRI Instruments, Torrance, CA). The ETD-enabled modified LTQ places a mass filter between the CI source and trap so as to only inject the desired anion into the ion trap. The LTQ was further modified to apply a radio frequency trapping voltage to the end lenses of the linear trap. By applying these frequencies in both the radial (applied to the quadrupoles) and axial (applied to the end lenses) directions, ions of opposing charge can be trapped in the same space simultaneously (charge-sign independent trapping). Protein samples (10 μM) were directly infused into the LTQ using PicoTip™ static nanospray tips (New Objective) at a voltage of ~1.2 kV, and the tube lens voltage was 125 V.

The typical scan function in this section of the experiment involved cation injection (~15 ms), anion injection (2–3 ms), mutual ion/ion storage time (100–200 ms) and mass analysis. Ion parking was accomplished by applying an off-resonance excitation voltage (~1.5 V) during the mutual ion/ion storage time. This excitation targeted the 22+ charge state of the FC-RNase (m/z 714.1). The 216 Da anion was used for the ion parking experiments.

3. Results and discussion

3.1. Ion–ion reactions of modified proteins

We applied the method of atmospheric pressure charge reduction electrospray mass spectrometry (AP-CREMS) to fixed-charge modified proteins in order to investigate the effect on the charge state distribution. We hypothesized that proton transfer reactions in the gas phase could remove the labile protons, but not affect the fixed charges, and thereby yield simplified spectra of a single, or a few, relatively high charge states. Fig. 2a and b show the normal ESI mass spectra for the protein lysozyme in its reduced and alkylated (RA) form as well as its fixed-charge (FC) modified form, respectively. The results for AP-CREMS of FC-lysozyme are shown in Fig. 2c, and substantial charge reduction to lower charge states (higher m/z peaks) is observed. Unfortunately the charge state distribution does not become significantly narrower with charge reduction (12+ to 24+ becomes 5+ to 15+). Similar results were obtained (but not shown) for the fixed-charge modified version of another protein, FC-RNase.

An intriguing result occurs for charge reduction of these fixed charge modified proteins; specifically, the spectra show sizable peaks for several charge states lower than the number of fixed charges. For example, the CREMS spectrum in Fig. 2c shows charge states down to 5+ even though the FC-lysozyme protein contains 10 fixed charges. The 10 quaternary ammonium groups on this protein cannot be affected by the proton transfer reactions occurring during CREMS, and so the only explanation for the charge states below 10+ is the presence of negative charges along with the 10 positive charges. Carboxylate groups would be the most likely negatively charged functional group on the protein itself; but recall that this protein was amidated at all of its carboxylic acid groups, thereby eliminating the possibility of their deprotonation. Rather than deprotonation of a group on the protein itself, another possibility is ion attachment (adduction) of a negatively charged species to the protein ion. It is evident from the multiple peaks within

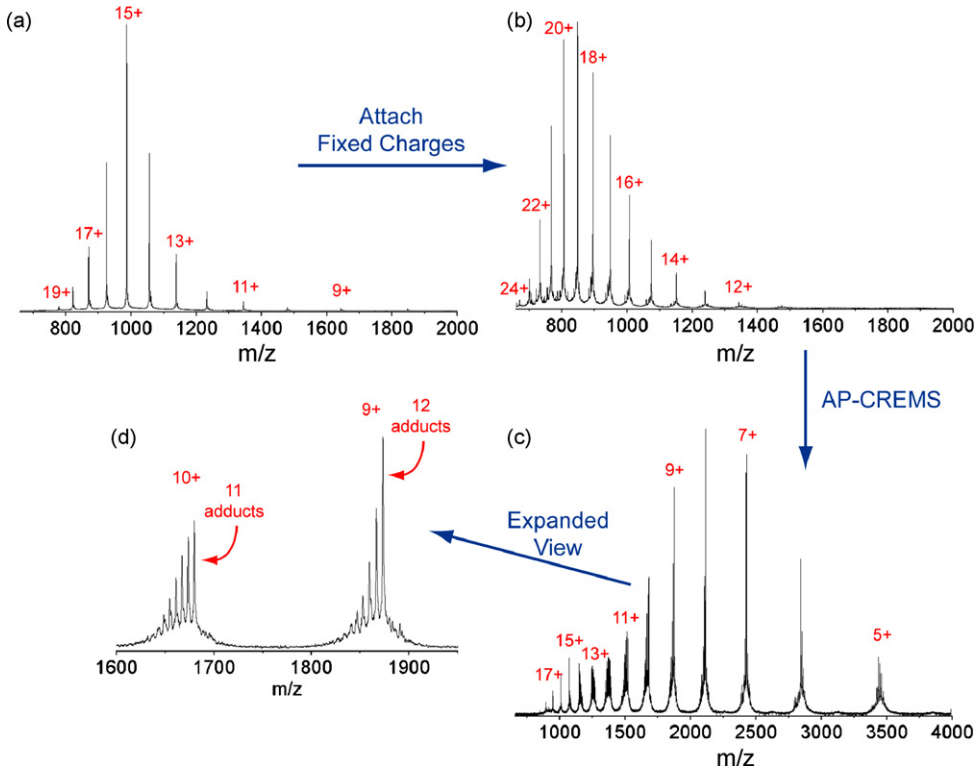

Fig. 2. Mass spectra are shown for (a) RA-lysozyme, (b) FC-lysozyme, and (c and d) charge reduction of FC-lysozyme. The charge state distribution is shifted to substantially higher charge states by modifying the protein to contain fixed-charges. Charge reduction by the ion–ion reactions of AP-CREMS leads to lower charge states, including some less than the number of fixed charges (10). The expanded view clearly shows several adduct peaks for each charge state.

each charge state envelope that significant adduction is occurring (Fig. 2d). The 63 Da mass difference between adjacent adduct peaks indicates that nitrate anions are forming a complex, or adduct, with the protein cations. We previously observed nitrate adduction during CREMS, and we demonstrated that the nitrate forms from N_2 and O_2 in the corona discharge [11]. Avoiding nitrate adduction in the past was accomplished by using methanol vapor in N_2 rather than air as the gas flowing through the corona discharge. In the present study with fixed-charge modified proteins, however, prevention of adducts proved to be more difficult. Nitrate adducts still were observed while using methanol vapor in N_2, presumably from imperfect purging of O_2 from the system. Rather than expending additional effort to prevent nitrate formation during atmospheric pressure CREMS, we chose to perform the charge reduction within an ion trap, which provides more control over anion selection. Benzoate anions were reacted with ions of the FC-lysozyme protein in the ion trap mass spectrometer, and the resulting spectrum is shown in Fig. 3a. Numerous benzoate adduct peaks were observed for these charge-reduced ions, despite past observations that benzoate anions act as proton transfer reagents [29].

These results suggest that fixed-charge modified proteins have a high propensity for adduction with the anions employed during charge reduction ion–ion reactions. This result can be understood by considering the possible outcomes from this gas-phase reaction between oppositely charged species: (i) the anion may abstract a proton from the cation and leave as a neutral (proton transfer), (ii) the anion may transfer an electron to the cation (electron transfer), or (iii) a complex can be formed between the ions (adduction). Each of these three reaction types has been observed with multiply charged protein cations, and the extent of each mechanism is affected most by the nature of the anion [8,30,31]. In the present case, however, the complex formation is mostly a result of the nature of the modified protein cation, specifically its fixed charges.

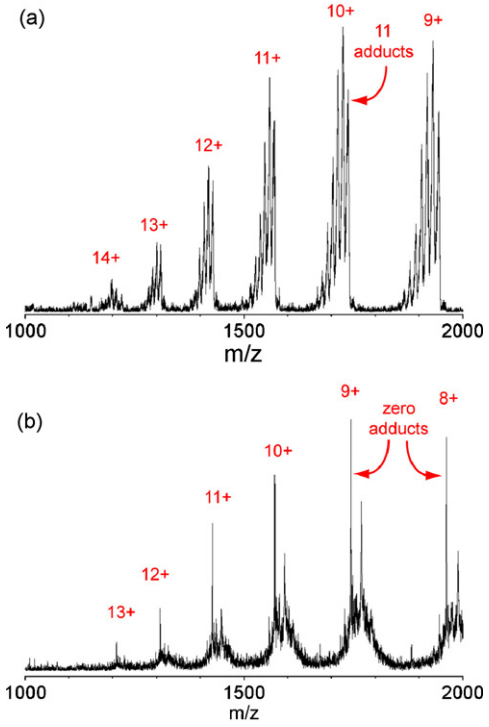

Fig. 3. Charge reduction by ion–ion reactions in an ion trap mass spectrometer. (a) Benzoate anions were employed for charge reduction of the 28+ charge state of FC-lysozyme. Numerous adduct peaks are observed. (b) The 216 Da anions were employed for charge reduction of FC-RNase. Proton transfer reactions, rather than adduction, dominate in this case, as evidenced by the tallest peaks having zero adducts.

Table 2
Mass spectral results are given from three different experimental conditions for charge reduction of FC-lysozyme

Experiment:	1		2		3	
	AP-CREMS (nitrate adducts, 300 V)		Ion Trap (benzoate adducts)		AP-CREMS (nitrate adducts, 400 V)	
Charge state	# adducts	S_{az}	# adducts	S_{az}	# adducts	S_{az}
17+	0	17			0	17
16+	0	16			0	16
15+	0	15			0	15
14+	0	14	7	21	0	14
13+	1	14	8	21	0	13
12+	5	17	9	21	0	12
11+	10	21	10	21	0	11
10+	11	21	11	21	0	10
9+	12	21	12	21	1	10
8+	13	21			2	10
7+	14	21			3	10
6+	15	21			7	13
5+	17	22			16	21

Experiments 1 and 2 correspond to data from Fig. 2c and 3a, respectively. (The spectrum corresponding to Experiment 3 is not shown.) The number of adducts observed in the tallest peak for each charge state are listed along with the S_{az} value, which is the sum of adducts plus charges. There is a propensity for S_{az} values to equal the number of fixed charges plus arginines (21), or the number of fixed charges (10), as indicated by the shaded areas.

These proteins run out of labile protons while still remaining in a relatively high charge state, which provides a strong Coulombic attraction for additional anions. These "extra" anions form a complex with the protein because (i) proton transfer is no longer an option, (ii) electron transfer is highly improbable for these types of anions, and (iii) breaking apart the anion–cation complex in the gas phase is not energetically favorable.

A particular pattern is observed for the number of adducts occurring on fixed-charge modified protein ions in various charge states. As shown in Experiment 1 of Table 2, the sum of the charge state and the number of adducts giving rise to the tallest peak for that charge state repeatedly yields the same value across much of the charge state distribution. This sum of adducts plus charges (S_{az}) is 21 in the case of fixed-charge modified lysozyme, which corresponds to the total number of fixed charges (10) plus arginine residues (11). Charge reduction of FC-lysozyme with benzoate anions in the ion trap also produced an S_{az} of 21, as seen in Experiment 2 of Table 2. Furthermore, an S_{az} value of 15 is obtained for another protein, FC-RNase, which has 11 fixed charges and 4 arginine residues (data not shown). Since the S_{az} value reproducibly corresponds to the total of fixed charges plus arginines for these charge reduction experiments, it must be thermodynamically favorable for the nitrate and benzoate anions to form complexes with the quaternary ammonium and guanidinium groups. Therefore, the S_{az} value could be used to determine the numbers of certain residues (e.g., Arg) for unknown denatured proteins. This type of characterization has been demonstrated in the literature with other adduction phenomena [32,33].

We attempted to remove adducts from protein ions by increasing the internal energy of the ion–ion complexes. The nozzle-skimmer potential was increased from 300 V to its maximum 400 V for the AP-CREMS experiments performed in front of the mass spectrometer inlet. This additional in-source energy led to removal of many adducts. The resulting S_{az} value decreased from 21 to 10 for the FC-lysozyme (Experiment 3 of Table 2), which implies that nitrate anions acquired labile protons from the arginine residues in order to leave as neutral nitric acid molecules. The higher nozzle-skimmer potential produces better spectra (fewer and more intense peaks), but still leaves some adducts on ions in charge states less than the number of fixed charges (i.e., <10+), as expected from the inability of fixed charges to transfer a proton to the anion. An alternative strategy for adding energy to the ion–ion complexes also was performed, namely collisional activation within the ion trap mass spectrometer for the charge reduction experiment involving benzoate anions. Specifically, the 10+ ion having 8 adducts (m/z 1702.9) was collisionally activated in order to remove benzoate adducts (as neutral benzoic acid molecules). The resulting spectrum (not shown) contains predominantly the peak corresponding to the desired 10+ ion without adducts (m/z 1609.7), although a few smaller adduct peaks remained. In both the AP-CREMS and the ion–ion reactions in the ion trap, additional energy does remove some adducts and make the charge reduced spectra cleaner, but it still does not achieve the desired result—a single, relatively high charge state peak for a protein.

We also obtained data for another proton transfer reagent, which apparently is much less prone to ion attachment than nitrate and benzoate ions. This anion reagent has an m/z of 216, and it is generated in a chemical ionization source from fluoranthene in a carrier gas of N_2. The charge reduction spectrum for FC-RNase using this anion is shown in Fig. 3b, and only a small amount of adduction is observed, as evidenced by the tallest peaks from each charge state being those with zero adducts. Note that a small amount of adduction occurs (1 or 2 adducts of 217 Da), but it is much less than that found for nitrate or benzoate anions. Surprisingly, ions without adducts are observed even for charge states (8+ to 10+), which are lower than the number of fixed charges (11) for this FC-RNase protein. We attribute this result to deprotonation of functional groups on the protein to create negative charges along with the 11 fixed positive charges. Since the carboxylic acids were all capped by attaching fixed charges at those sites (Fig. 1), the next most likely sites for deprotonation are the phenol groups (tyrosine) or the amide groups (asparagine, glutamine, or protein backbone) [34,35]. Thus, it appears that charge reducing fixed-charge proteins with ion–ion reactions does not stop at the number of fixed charges, but rather produces a distribution of lower charge states by either anion attachment or deprotonation of even very weakly acidic functional groups.

3.2. Ion–molecule reactions of modified proteins

Ion–molecule reactions were investigated for charge reduction of the fixed-charge modified proteins in order to possibly avoid the adduction issue encountered with ion–ion reactions. Ion–molecule reactions are generally less effective than ion–ion reactions for pro-

ton transfer in the gas phase [36], but significant charge reduction has been shown nonetheless. Recent work by Catalina et al. has demonstrated efficient charge reduction of proteins through the addition of solution additives with high gas-phase basicities [6]. They found the extent of charge reduction correlates to the gas-phase basicity of the additive. We explored charge reduction with these solution additives, including the most highly basic one, 1,5,7-triazabicyclo [4.4.0] dec-5-ene (HPP). Electrospraying unmodified lysozyme from 5 mM HPP at pH 7 produced ions in only the 2+ and 3+ charge states, consistent with Catalina's work. Charge reduction was less significant, however, for the fixed-charge modified lysozyme, which yielded 10+ to 19+ ions. It is promising that no adducts were observed and that these ion–molecule proton transfer reactions appeared to stop at the 10+ charge state (i.e., the number of fixed charges). Nonetheless, a narrow charge state distribution still was not achieved, and there was a dramatic loss in sensitivity combined with significant electrospray stability problems.

To avoid the electrospray problems, further ion–molecule reactions were performed by adding a base, triethylamine (TEA), in the vapor phase rather than in the sample solution. Others have performed ion–molecule proton transfer reactions by addition of gas-phase reagents [3–5]. In the present experiments, vapor-phase TEA in a carrier gas of nitrogen was introduced into a nozzle extension mounted to the inlet of the TOF mass spectrometer. The TEA reacts with the electrosprayed protein ions as they travel through this nozzle extension into the mass spectrometer. Charge reduction by vapor-phase TEA does give good signal intensity and a stable electrospray; the spectra are shown in Fig. 4 for both RA-lysozyme and FC-lysozyme. The reduced and alkylated protein was charge reduced to predominantly the 4+ charge state, and so that accomplished the goal of simplifying the electrospray spectrum of a protein. The disadvantage to this method is that fairly low charge state (high m/z) ions are produced, which are often not as desirable as higher charge states because of decreased detection sensitivity, among other issues mentioned above. The fixed-charge modified protein yielded higher charge states (Fig. 4b), however a significant amount of adduction occurred, which once again produces ions in charge states less than the number of fixed charges and distributes those ions among several adduct peaks. The attached anions for these ion–molecule reactions come from the protein sample solution itself; initially it was trifluoroacetate, but then even after buffer exchange to formate, some formate and even TEA adducts were observed. While somewhat unexpected, this result of ion attachment occurring even for ion–molecule reactions has a straightforward explanation (presented here even though direct experimental evidence was not obtained). During electrospray, some anions from solution are associated with the protein, and typically these anions (e.g., formate) acquire protons and then the neutral volatile acid (e.g., formic acid) dissociates from the protein during the gas-phase collisions in the nozzle-skimmer region [33]. Charge reduction by TEA of a fixed-charge modified protein changes this situation. The TEA molecules collide with these protein ions and scavenge labile protons becoming positively charged in the process, which repels them away from the still positively charged protein. Consequently, some anions originally associated with the protein do not find a labile proton and so they remain forever bound to the protein as a complex, which results in adduct peaks.

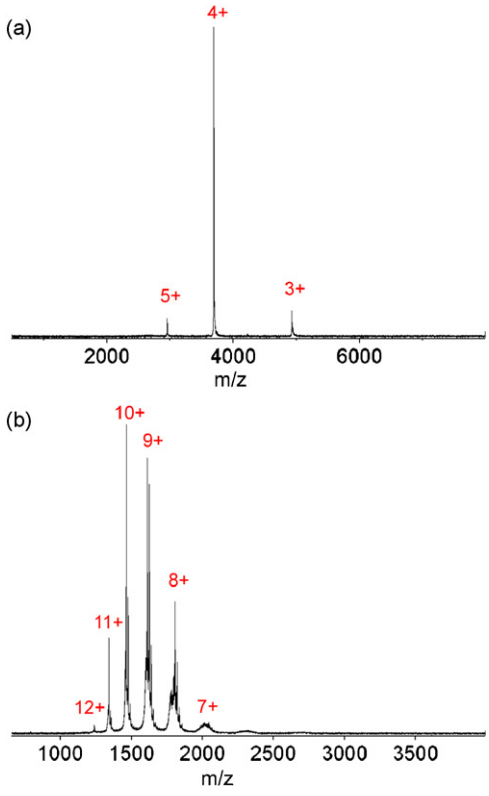

Fig. 4. Charge reduction by ion–molecule reactions with triethylamine in the gas-phase for (a) RA-lysozyme, and (b) FC-lysozyme. Note that the spectra for these proteins *before* the ion–molecule reactions are shown in Fig. 2a and b, respectively.

3.3. Ion parking of fixed-charge modified proteins

The ion–ion and ion–molecule reactions discussed above lead to problems with adduction and do not produce the desired single, high charge state; therefore ion parking experiments were applied to the fixed-charge modified proteins. Fig. 5 shows three ESI-MS spectra: (a) RA-RNase, (b) FC-RNase, and (c) FC-RNase with ion parking occurring during charge reduction. The range of charge states observed for the reduced and alkylated protein is 8+ to 21+ (Fig. 5a), and it is substantially higher for the fixed-charge modified protein (16+ to 28+, Fig. 5b). The ion parking experiment produces nearly a single, high charge state peak (22+, Fig. 5c).

The ion parking experiment involves ion–ion charge reduction reactions in the ion trap combined with application of a low-amplitude waveform of a certain frequency [12,13]. The frequency is chosen to correspond to the m/z value of the desired charge state (714 Da and 22+, in the example shown in Fig. 5). This mild excitation of the 22+ ions allows them to remain in the trap while substantially reducing their rate of reaction with the anions, due to their increased relative velocity and/or reduced physical overlap with the anion cloud [13]. Ions in charge states higher than 22+ react normally with the anions and undergo charge reduction until they become 22+ ions, at which point they essentially stop reacting and accumulate in that charge state. Any ions initially in charge states lower than 22+ react with anions leading to even lower charge state ions, often outside the m/z range of the spectrometer. Small peaks corresponding to lower charge states (8+ to 21+) are observed in the ion-parking spectrum, Fig. 5c. These ions are either: (i) ions "leaking" out of the parked 22+ charge state due to a small but finite reaction rate with reagent anions, or (ii) ions initially in charge states <22+ that did not become charge reduced enough ($\leq$7+) to disappear from the spectrum. Note that the ion–ion reaction rate is proportional to the square of the protein charge, and therefore the reactions become slower as the charge is reduced [7].

Ion parking into such a high charge state eliminates the adduction problems described earlier in this paper, as evidenced by the lack of adduct peaks on the parked 22+ peak of FC-RNase (Fig. 5c). This result may be partially due to the use of the 216 Da anion,

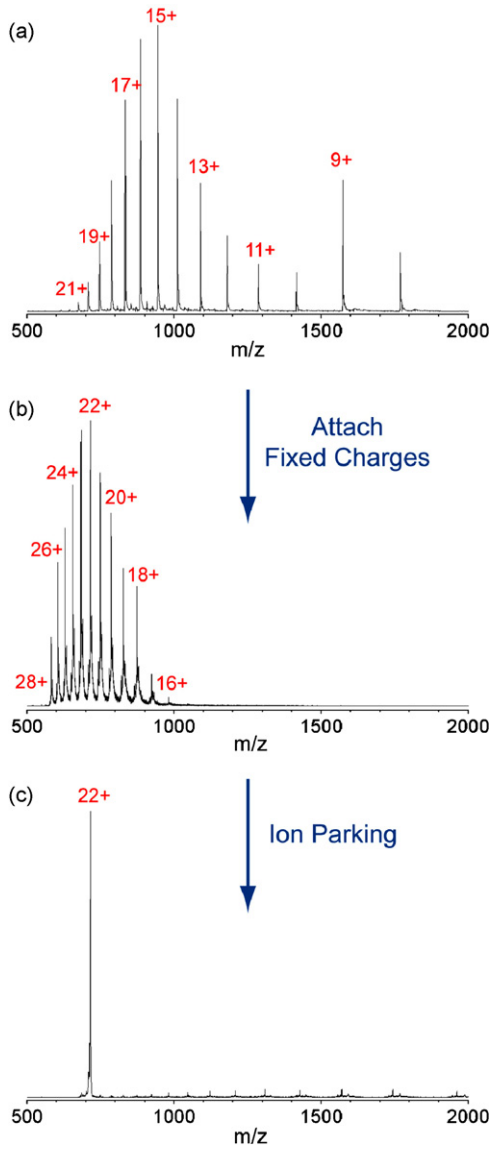

Fig. 5. The mass spectra are shown for (a) RA-RNase, (b) FC-RNase, and (c) FC-RNase with ion parking. Attaching fixed charges leads to significantly higher charge states. Ion parking allows many ions to congregate in a single charge state. The combination of modified proteins with fixed charges and ion parking produces an abundance of ions in a charge state even higher than the highest one present in the ESI spectrum of the original reduced and alkylated protein.

which proved to be less prone to forming adducts, but also it can be attributed to keeping the protein in high charge states. For example, this protein has 11 fixed charges, and so the high charge state ions (>22+) undergoing charge reduction to 22+ still have more than 11 labile protons that can be abstracted by reagent anions. Even the ion attachment reported in the above sections of this paper did not occur for charge state peaks above about 15+ (see Fig. 2c and Table 2).

The utility of performing ion parking on fixed-charge modified proteins is that it leads to production of a large population of ions in a single, very high charge state. We chose to demonstrate this concept by parking the ions in the 22+ charge state, which is not even present for electrospray ionization of the RNase protein without fixed charges (Fig. 5a). The population of ions in the 22+ charge state is increased substantially (3.8-fold) by ion parking, even considering that the 22+ charge state had the most ions prior to parking (Fig. 5b). Since the ion parking experiment moves ions from higher charge states into a lower chosen charge state, the only ions that could be parked at 22+ are the ones initially in 22+ to 28+ of Fig. 5b. Sixty-one percent of these initial ions were parked in the 22+ charge state (during a 100 ms ion-parking experiment), as calculated by the ratio of the parked 22+ peak area to the total of the original 22+ to 28+ peak areas. One could choose to park ions into a charge state near the low end of the distribution (17+ for instance). In that case, nearly the entire original population of ions could be parked into a single charge state, except for ions lost due to inefficiencies.

The abundance of ions generated in an individual high charge state by ion parking of fixed-charge modified proteins can advance several areas of protein mass spectrometry. For instance, whole protein characterization may be improved for mass spectrometers with relatively low m/z cutoffs such as commercial ion traps, or those with higher resolution at smaller m/z values such as FT-ICR instruments and orbitraps. These ions in very high charge states also can aid in the exploration of fragmentation mechanisms. Finally, this method of creating a large population of ions in a single, high charge state may enhance top-down proteomics, especially when employing fragmentation techniques that tend to favor higher charge states such as ETD and ECD.

4. Conclusions

Modifying proteins with fixed charges at their carboxylic acid functional groups leads to substantially higher charge states for these proteins during positive-mode electrospray ionization mass spectrometry. Charge reduction of these proteins by ion–ion or ion–molecule reactions can yield protein ions in charge states below the number of fixed charges. This result is due to either deprotonation of very weakly acidic sites on the protein or anion attachment to the protein. The fixed-charge modified proteins show a strong propensity for anion attachment, which is due to the inability of the fixed-charge groups to transfer a proton to the anion. Applying the technique of ion parking allows the proteins to remain in a charge state well above the number of fixed charges where no adduction is observed. Ion parking of a fixed-charge modified protein can produce a substantial number of ions in a single, very high charge state—a charge state above even the highest one observed for the same protein without the fixed charges.

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