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Decharging of Globular Proteins and Protein Complexes in Electrospray

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Abstract: Electrospray ionization mass spectrometry (ESI-MS) is a valuable tool in structural biology for investigating globular proteins and their biomolecular interactions. During the electrospray ionization process, proteins become desolvated and multiply charged, which may influence their structure. Reducing the net charge obtained during the electrospray process may be relevant for studying globular proteins. In this report we demonstrate the effect of a series of inorganic and organic gas-phase bases on the number of charges that proteins and protein complexes attain. Solution additives with very strong gas-phase basicities (GB) were identified among the so-called "proton sponges". The gas-phase proton affinities (PA) of the compounds that were added to the aqueous protein solutions ranged from 700 to 1050 kJ mol^{-1}. Circular dichroism studies showed that in these solutions the proteins retain their globular structures. The size of the proteins investigated ranged from the 14.3 kDa lysozyme up to the 800 kDa tetradecameric chaperone complex GroEL. Decharging of the proteins in the electrospray process by up to 60% could be achieved by adding the most basic compounds rather than the more commonly used ammonium acetate additive. This decharging process probably results from proton competition events between the multiply protonated protein ions and the basic additives just prior to the final desolvation. We hypothesize that such globular protein species, which attain relatively few charges during the ionization event, obtain a gas-phase structure that more closely resembles their solution-phase structure. Thus, these basic additives can be useful in the study of the biologically relevant properties of globular proteins by using mass spectrometry.

Keywords: electrospray ionization · mass spectrometry · noncovalent protein complexes · proteins · proton sponges

Introduction

Mass spectrometry has recently become an important tool in structural biology for the study of globular proteins and protein complexes.[1–3] In particular, electrospray ionization (ESI) has emerged as a powerful technique for generating ions of complex macromolecular species, in which the globular structure is at least partially retained by the ions.[4] By maintaining the proteins in a physiologically relevant buffer amenable to electrospray (e.g., aqueous ammonium acetate) prior to the ionization process, even noncovalent protein assemblies such as protein oligomers,[5–8] ribosomes,[9,10] and small viruses[11,12] can be transferred intact into the gas phase. The retention of globular shape by ions in the gas phase has been demonstrated by ion mobility measurements, in which protein ions with a limited number of charges exhibit collisional cross-sections comparable to those calculated from their crystal structures.[13–17] In addition, gas-phase hydrogen/deuterium exchange experiments of protein ions have revealed that low-charged proteins in particular preferentially retain compact conformations.[17–19] Studies on the gas-phase proton transfer rates of multiply protonated lysozyme ions suggest that the reactivity of the lower charge states corresponds to a gas-phase compact structure resembling the crystal structure.[20]

Because mass spectrometric measurements are carried out on ionized analytes in the gas phase, it is important to bear in mind that during the transfer from the solution to the gas phase the proteins become desolvated and often highly charged. It is expected that the charged residue mechanism (CRM) is dominant in the production of macro-

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ions of proteins and protein complexes. This mechanism was originally proposed by Dole[21] and validated by others.[22,23] On the basis of the CRM model, de la Mora,[22] and Peschke and co-workers[24] argued that the number of charges, Z_R, that globular proteins or protein complexes obtain upon ionization is governed by the Rayleigh limit of the droplet, and can simply be predicted from the radius of the droplet, which is assumed to be the radius of the protein. This is depicted in Equation (1), in which γ is the surface tension of the water droplet, ε_0 is the electrical permittivity of a vacuum, e is the elementary charge, and R is the radius of the droplet.

$$Z_R = (8\pi/e)(\gamma\varepsilon_0 R^3)^{1/2} \quad (1)$$

By regarding protein structures as spherical entities and by assuming that the radius is directly correlated to the molecular weight of a protein, and that the density of a protein is similar to that of water, an even simpler equation can been derived [Eq. (2)], in which M is the mass of the complex in Dalton.

$$Z_R = 0.078\, M^{1/2} \quad (2)$$

Considerable experimental data of the ESI of globular proteins and protein complexes has confirmed that Equation (2) has some validity.[3,22,23] Furthermore, the charge distributions observed in the mass spectra are sometimes used as indicators of protein conformations in solution.[25–29] In such studies it is generally assumed that basic residues, which are internally situated and involved in intramolecular interactions (e.g., self-solvation), are unlikely to become charged during the ionization process. Therefore, (partly) unfolded open structures will attain more charges than compact, folded structures. It has also been argued that these intramolecular interactions are strongly dependent on the number of available basic and acidic amino acids[30] in the protein and that, therefore, the median charge observed is dependent on the difference in the number of basic and acidic amino acids.

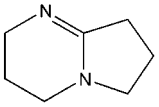
The CRM model also predicts that the charging of globular proteins and protein complexes varies if the buffer salt is changed or if other additives are present in the solution. Indeed, it has been shown that the use of volatile salts of bases, such as ethylammonium acetate and triethylammonium bicarbonate (TEAB), which have higher gas-phase proton affinities (PA), results in the formation of ions with significantly fewer charges than is seen with the more commonly used ammonium acetate (NH_4OAc).[31,32] These observations suggest that extensive proton transfer reactions between the generated protein ions and the buffer base and vice versa may occur in the source region.[32] It has been proposed that these relatively low-charged protein ions are more stable in the gas phase, making such spray solutions more suited for the analysis of macromolecular noncovalent complexes.[31] A systematic study of the effect of buffer cations on the charge states of globular proteins has recently been reported;[32] however, this was limited to small model proteins (ubiquitin, cytochrome C, and lysozyme) and to alkyl ammonium buffer salts, with PA values of up to 982 kJ mol^{-1} in the case of TEAB. A continuous decrease in the charge states of the proteins in the ESI mass spectra was observed as the PA of the salt was increased.

Here we explore the possibility of using very strong organic bases to reduce the net charging in the positive electrospray of proteins and protein complexes. Of particular interest are the so-called strained systems, such as the proton sponges,[33] which are known to remain strong bases in the gas phase.[34–37] Their high PA is partly due to intramolecular stabilization between two neighboring proton acceptors. The properties of these organic base molecules in solution have been reviewed.[38] Reducing the net charging may in turn reduce the number of electrostatic repulsions in the protein ions, giving gas-phase structures that resemble more closely those found in the solution phase. To neutralize even the very basic sites in proteins we explored the use of the proton sponges 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and 1,5,7-triazabicyclo[4.4.0]dec-5-ene (HPP); the amino acid arginine; isopropanol; and some basic inorganic salts as additives. Although the proton-sponge-containing solutions were in practice somewhat more troublesome for generating stable electrospray conditions, good mass spectrometric data could be obtained even for very large 800 kDa macromolecular protein complexes. The net charging of the proteins in the electrospray process could be reduced by up to 60% by using these strongly basic additives.

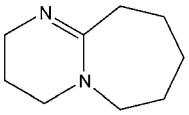
Results

In order to probe the degree of protein protonation during the ESI process, a variety of inorganic and organic compounds with a wide range of gas-phase basicities (GB) were selected as solution additives. Aqueous solutions were prepared from the following compounds, named in increasing order of GB values: pure water in the absence of any additive, isopropanol, ammonium acetate, TEAB, DBN, arginine, DBU, and HPP (Figure 1). The PA and GB values of these compounds are given in Table 1. It has been shown[32] that the effect of an additive is somewhat dependent on its concentration within the range of 0.5 to 10 mM, with a maximum decharging effect at 10 mM and above. Therefore, we decided to prepare all of our electrospray solutions in water with 20 mM additive. The pH was adjusted to 7 with acetic acid.

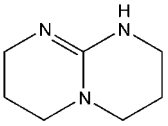
We initiated our ESI experiments with the model protein lysozyme at a fixed concentration of 5 μM. The production of protein ions became more difficult as the GB of the additive, together with the size of the protein, increased. The signals were particularly suppressed in cases with arginine as an additive, due in part to the highly abundant, protonated arginine clusters (discussed below). Figure 2 shows the ESI mass spectra of lysozyme measured in the presence of the



DBN = 1,5-diazabicyclo[4.3.0]non-5-ene, 1006 kJ mol^{-1}



DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, 1015 kJ mol^{-1}



HPP = 1,5,7-triazabicyclo[4.4.0]dec-5-ene, 1022 kJ mol^{-1}

Figure 1. Chemical structures of the additives used in this study. Corresponding gas-phase basicities (GB) are indicated.

Table 1. Gas-phase proton affinities (PA) and gas-phase basicities (GB) of the basic amino acids and additives used. All data is from the NIST database (http://webbook.nist.gov/).

	PA [kJ mol^{-1}]	GB [kJ mol^{-1}]
water	691	660
isopropanol	793	763
ammonia	854	819
triethylamine	982	951
histidine	988	950
lysine	996	951
arginine	1051	1007
DBN	1038	1006
DBU	1048	1015
HPP	1055	1022

different additives. Our data for the uses of pure water, ammonium acetate, and TEAB are in agreement with previously reported data.[32] A clear decrease in the charge states of the protein ions was observed by increasing the GB of the additives used. Lysozyme ions acquired on average ten charges when sprayed from pure water and isopropanol (20 mM) and only three charges when sprayed from the protein sponges DBU (20 mM) or HPP (20 mM). The calculated mean charge states are summarized in Table 2.

We continued our studies with the heme-containing protein myoglobin. The binding of the noncovalent heme group to myoglobin can be used to monitor the conformational state of the protein, as holomyoglobin and apomyoglobin have a molecular mass difference of 616 Da. The ESI mass spectra of myoglobin (5 μM) showed a trend similar to that observed for lysozyme; a clear decrease was observed in the mean charge states of the protein ions as the GB of the additives increased (Figure 3 and Table 2). Whereas myoglobin ions acquired on average 10–11 charges when sprayed from either pure water or isopropanol (20 mM), the number of charges was reduced to only 3–4 when sprayed from solutions containing the proton sponges DBN (20 mM) or DBU (20 mM). For all of the solutions used, only holomyoglobin

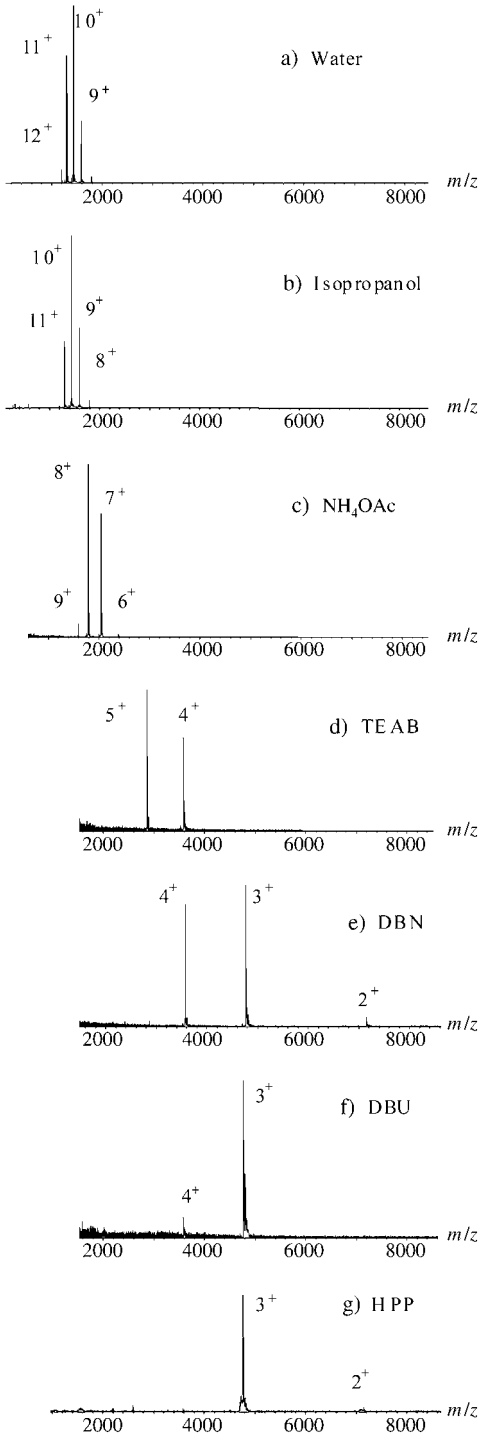


Figure 2. Nanoflow ESI mass spectra obtained from aqueous lysozyme solutions (5 μM) in the presence of different additives (20 mM) at pH 7. The different charge states of the 14.3 kDa protein are indicated.

Table 2. Average charge states observed in the ESI spectra of several proteins. For comparison, the charge states predicted from Equation (2) are also quoted.

	MW [kDa]	H_2O 660[a]	Isopropanol 763[a]	NH_3 819[a]	TEAB 951[a]	DBN 1006[a]	DBU 1015[a]	HPP 1022[a]	Predicted from [Eq. (2)]
lysozyme	14.3	10	10	7.8	4.7	3.5	3.1	3	9.3
myoglobin	17.5	10	10.8	8.2	5.7	3.7	3.5	3	10.3
PHBH[b]	90	26.5	21.8	18.9	13.8	9.8	9.4	–	23.1
GltS[c]	166	–	32.5	24.8	17	11	–	–	31.8
GroEL (heptamer)	400	–	–	45	32.5	–	–	–	49.3
GroEL (tetradecamer)	801	–	–	69.5	46.5	–	–	–	69.8

[a] GB [kJ mol^{-1}]. [b] PHBH = *p*-hydroxybenzoate hydroxylase. [c] GltS = glutamate synthase.

ions were observed. The exception was the solution (20 mM) of the most basic proton sponge HPP, in which primarily myoglobin was observed as the triply charged apoprotein. This indicates that the solution with the HPP additive destabilized the globular structure of the protein. A stable spray was difficult to obtain with arginine as a solution additive at concentrations greater than 5 mM. The protein signals seemed to be heavily suppressed by the very intensely protonated arginine clusters. Evaluation of the spectra obtained for myoglobin from neutral solutions containing 1, 2, and 5 mM arginine revealed a continuous charge reduction to an average of four charges at 5 mM arginine, accompanied by the release of heme from myoglobin. At a concentration of 5 mM arginine, the apomyoglobin ion signal was already four times as intense as the holomyoglobin signal. Moreover, we observed that arginine formed intense adducts, not only with itself, but also with the apo and holo form of myoglobin (data not shown). Similar observations were made with lysozyme (see above); therefore, arginine was not used in our further experiments.

To evaluate whether lysozyme and myoglobin remained folded in aqueous solutions that contained any of the additives used, we used circular dichroism in the near-UV wavelength region (260–320 nm) to analyze their tertiary structures. For both lysozyme and myoglobin the circular dichroism spectra were almost independent of the additive in the solution. Only in the case of HPP did both myoglobin and lysozyme exhibit less tertiary structures (Figure 4, only lysozyme data shown). Thus, this data is in agreement with the mass spectra, which suggest that the HPP additive (partially) unfolds myoglobin and lysozyme molecules already in solution. Other additives such as isopropanol, DBN, and DBU did not noticeably influence the tertiary structure of myoglobin and lysozyme.

To probe whether larger protein complexes can be studied by electrospray using the basic additives and proton sponges, and to examine the corresponding decharging effect, we extended the mass range of the proteins studied. We measured the ESI mass spectra of the flavin-containing *p*-hydroxybenzoate hydroxylase (90 kDa), the flavin– and iron–sulfur-containing glutamate synthase (166 kDa), and the chaperone complex GroEL (800 kDa). Size-exclusion chromatography and X-ray data have shown that *p*-hydroxybenzoate hydroxylase is biologically active as a homodimer of approximately 90 kDa.[39] ESI mass spectra of a 1 μM solution of the enzyme sprayed from different additive solutions are shown in Figure 5. *p*-Hydroxybenzoate hydroxylase was exclusively detected as a dimer, which included two flavin adenine mononucleotide cofactors. The protein dimer obtained an average of 27 charges when sprayed from pure water, compared to an average of only ten charges when sprayed from the proton sponges DBN (20 mM) or DBU (20 mM) (Table 2). Thus, by using basic additives we observed around 60% decharging of the *p*-hydroxybenzoate hydroxylase dimeric ions.

Next, we investigated glutamate synthase, a protein that contains a flavin mononucleotide and an iron–sulfur cofactor (total mass 166230 kDa).[40,41] The mass spectrum of glutamate synthase (1 μM) sprayed from ammonium acetate showed that the enzyme is in an equilibrium between the monomeric and dimeric conformations. The mass determined for the monomeric ions, 166200 ± 50 Da, revealed that the cofactors were indeed bound to the protein. When glutamate synthase was sprayed from solutions containing ammonium acetate, TEAB, and DBN, we observed average charge states of the monomeric protein ions of 24, 18, and 11, respectively (Table 2 and Figure 6).

This data is in accordance with the decharging observed for the other proteins investigated. Intriguingly, in the mass spectra relating to the additives TEAB and DBN, we observed ions originating from only monomeric hologlutamate synthase. This may indicate that the additives influence the monomer–dimer equilibrium. Solutions containing the very basic proton sponges were difficult to spray; therefore, this data is not presented.

Finally, we studied the tetradecameric chaperone complex GroEL (800 kDa).[42] In the case of the GroEL complex sprayed from ammonium acetate we obtained spectra very similar to those reported previously,[43] with ions covering about ten different charge states with a mean charge of 67 (Figure 7 and Table 2). Upon spraying the protein complex from TEAB (20 mM), we observed significant decharging to an average charge state of 46. Although 46 charges are regarded as many, this corresponds to just a single charge per 18000 Da, or some 3.5 charges per GroEL monomer. In both ammonium acetate and TEAB we also observed some weaker protein signals of the single heptamer ring of about 400 kDa (Figure 7). The average charge states observed for these protein ions were 44 and 33 in ammonium acetate and TEAB, respectively.

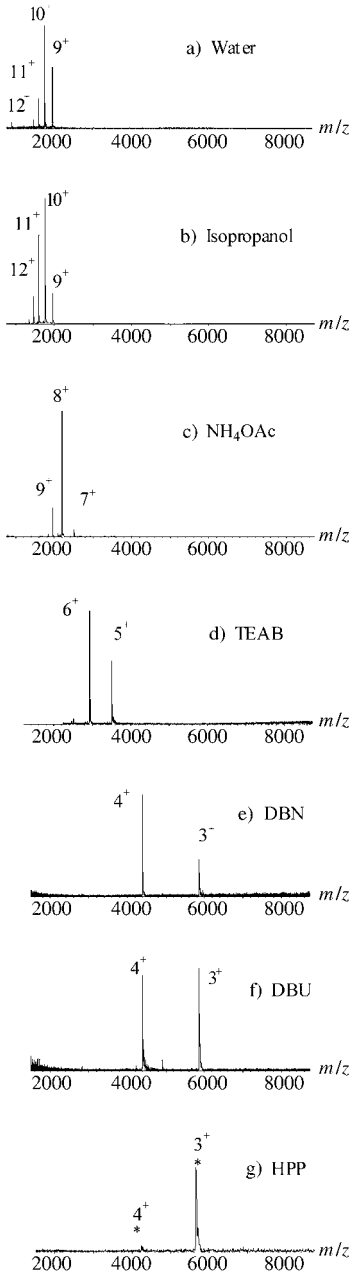


Figure 3. Nanoflow ESI mass spectra obtained from aqueous myoglobin solutions (5 μM) in the presence of different additives (20 mM) at pH 7. The different charge states are indicated. In spectra (a) to (f) the ions originate from holomyoglobin (17.5 kDa). The asterisks in spectrum (g) indicate that the ions originate from apomyoglobin (16.9 kDa).

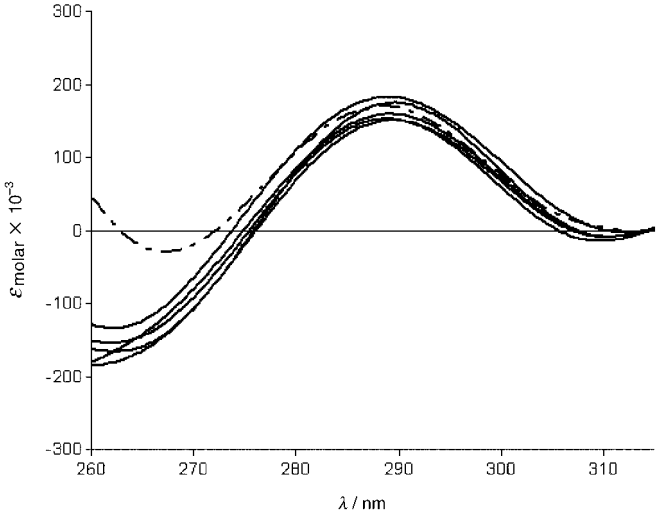


Figure 4. Near-UV circular dichroism spectrum of lysozyme (1 mg mL^{-1}) in different additive solutions (20 mM) at pH 7. The five similar continuous lines are the circular dichroism spectra recorded in water, isopropanol, TEAB, DBN, and DBU. The deviating dashed line corresponds to the spectrum of lysozyme in HPP.

Discussion

ESI mass spectrometry in the presence of aqueous volatile buffer solutions avoids the unfolding and/or denaturing of proteins and is, therefore, an ideal method for the mass spectrometric study of folded proteins and protein complexes.[2,3] Protein ions observed during ESI are often multiply charged and these multiple charges, together with the complete desolvation of the protein, are expected to influence the quaternary and tertiary structure of the protein. Consequently, it may be difficult to relate mass spectrometric data, such as that used to measure the activation energies for collision-induced dissociation or thermal decomposition in the gas phase, to the free energy of dissociation in solution (for a discussion, see references [44,45]). Some of the structural changes occurring in the gas phase may be related to Coulombic repulsions between neighboring charges in the protein ions. It is, therefore, desirable to limit the number of charges in the positive ion mode, which can be achieved by using the method described in this report.

The origin of the charges on the proteins can be understood by considering the mechanism of electrospray. The CRM model is expected to be dominant in the production of protein ions.[21–23] This model predicts that firstly, droplets formed in the electrospray process will be charged at the surface mostly by ammonium ions (or other ions obtained from the added buffer salt), and secondly, that the charging of globular proteins and protein complexes is affected by buffer salts other than ammonium acetate. Our observations are in good agreement with those of previous reports[32] and indicate that extensive proton competition reactions between the generated protein ions and the buffer base and vice versa may occur at the end of the desolvation process. To confirm this hypothesis, we plotted the average charge that the protein displays in the ESI mass spectra of the proteins lysozyme, myoglobin, *p*-hydroxybenzoate hydroxylase, and glutamate synthase as a function of the GB of the additive (Figure 8). This figure clearly shows that there is an almost linear relationship ($R^2 > 0.98$ for all linear fits) between the observed charge and the GB (or PA) of the additive. This validates our hypothesis that the protein decharging observed originates from proton transfer reactions between the protonated proteins and the additive molecules, probably during the last desolvation step. We speculate that

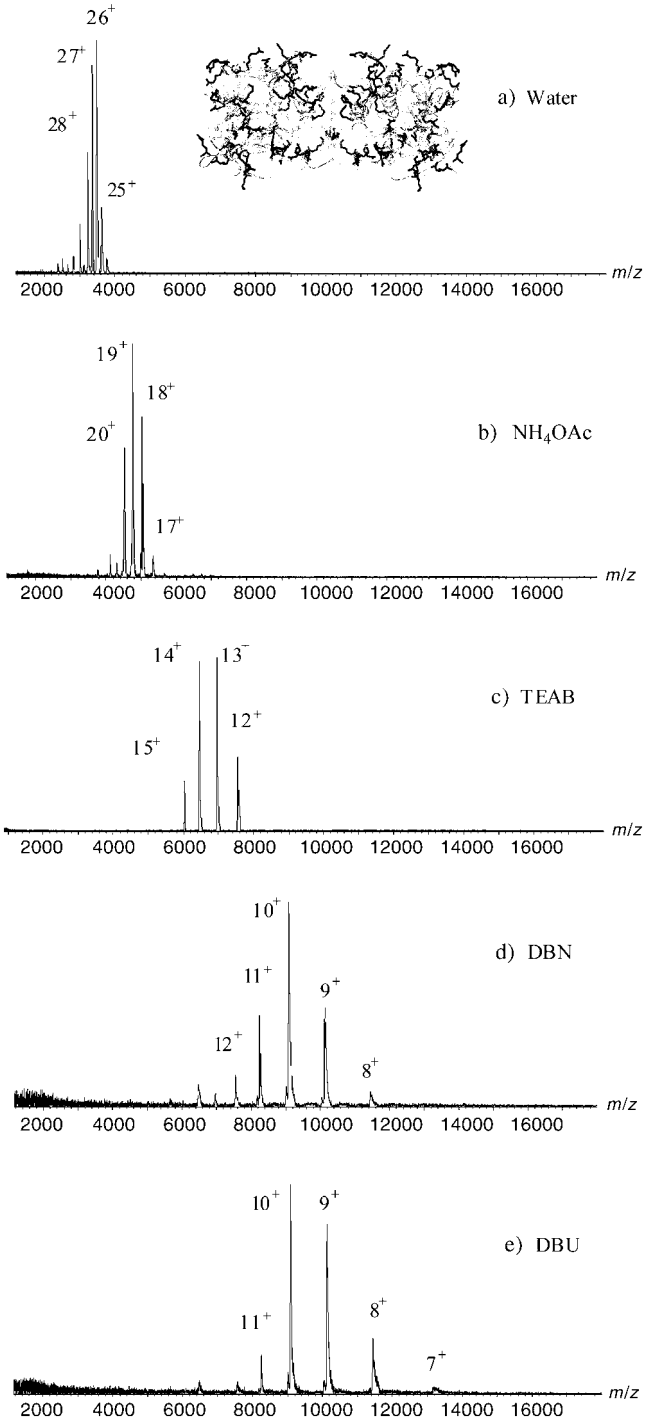


Figure 5. Nanoflow ESI mass spectra obtained from aqueous *p*-hydroxybenzoate hydroxylase solutions (1 μM) in the presence of different additives (20 mM) at pH 7. The different charge states of the dimer (90 kDa), which includes two cofactors, are indicated. The crystal structure of the *p*-hydroxybenzoate hydroxylase dimer is also shown.

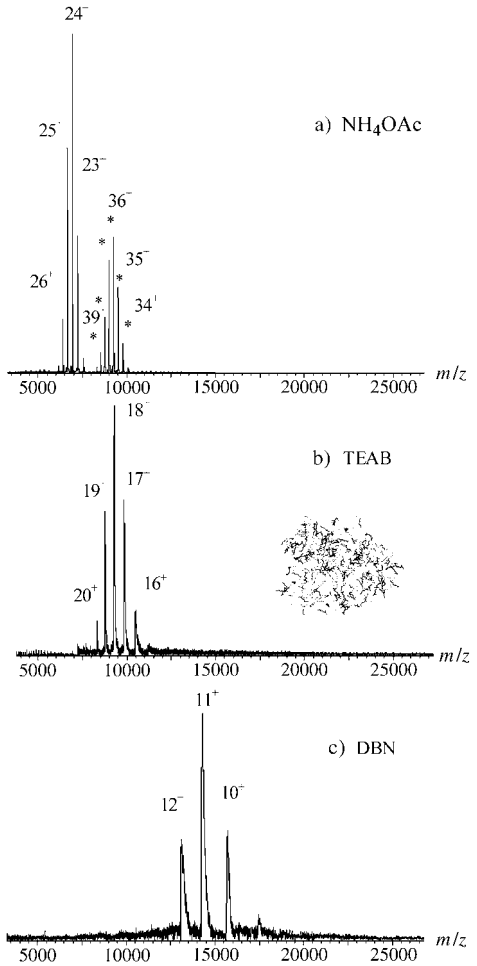


Figure 6. Nanoflow ESI mass spectra obtained from aqueous glutamate synthase solutions (1 μM) in the presence of different additives (20 mM) at pH 7. The different charge states of the monomer (166 kDa) are indicated. The asterisks indicate that the ions originate from the glutamate synthase dimer (332 kDa). The crystal structure of the glutamate synthase monomer is also shown.

in some of the final steps in the desolvation process the protein is still "solvated" by several molecules of the additive, which bind to the protein in a proton-bound manner, as shown schematically in Figure 9. In the final desolvation step, the additive ligand dissociates from the protein and either takes the proton with it or leaves it at the protein, depending on the difference in the proton affinities of the additive and the specific site on the protein. The only discrepancy is the behavior observed in pure water for the proteins lysozyme and myoglobin, which obtain a significantly lower number of mean charges than predicted from the linear plots in Figure 8 (see Table 2). This deviation can be explained: In these cases, charging is restricted only by the Rayleigh limit [Eq. (1)], which predicts for lysozyme and myoglobin a maximum of 13 charges in pure water. Therefore, the potential average charge would be reduced to 10–11, as observed in our experiments.

Although this explains the observed behavior reasonably well, other parameters were also considered. In electrospray ionization the surface tension of the solution may be an important parameter in the charging process, as indicated by Equation (1) and suggested by others.[46,47] The surface tension is evidently related to the constituents of the spraying solution. In general, droplets with a higher surface tension

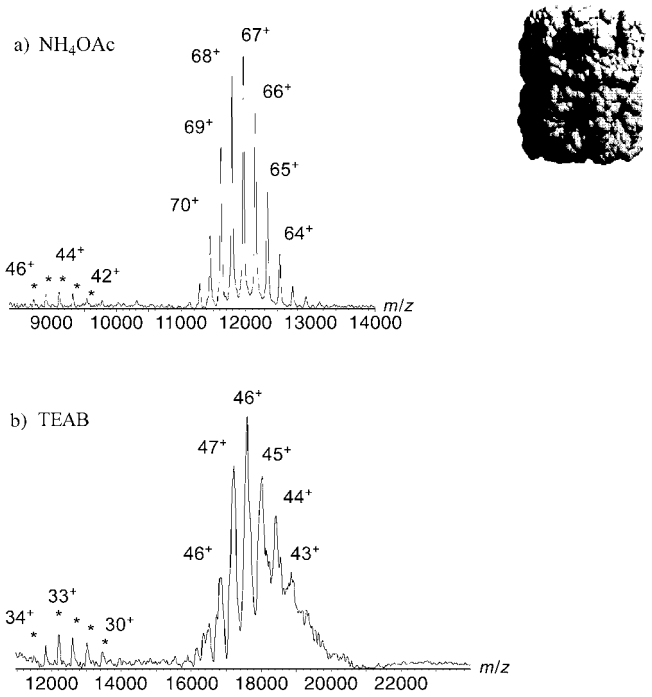


Figure 7. Nanoflow ESI mass spectra obtained from aqueous GroEL solutions (1 μM) in the presence of a) ammonium acetate and b) TEAB at pH 7. The different charge states of the GroEL tetradecamer (800 kDa) are indicated. The asterisks indicate that the ions originate from the single heptameric ring of GroEL (400 kDa). The crystal structure of GroEL is shown.

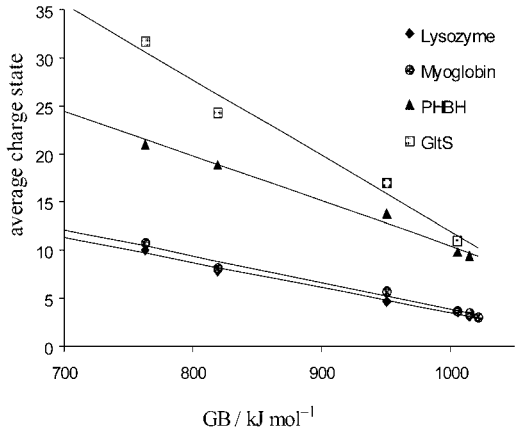


Figure 8. Experimentally determined average charge states of the proteins and protein complexes versus the gas-phase basicities (GB) of the additives used. PHBH = *p*-hydroxybenzoate hydroxylase, GltS = glutamate synthase.

should attain more charges before reaching the Rayleigh limit, resulting in a higher charging of the proteins. It is difficult to estimate the surface tension of the solutions used in the present study. We used aqueous solutions containing up to 0.3% by weight basic additives and up to 0.1% by weight acetic acid (the latter to achieve pH 7). The surface tension γ of pure water is 0.072 N m^{-1}, and those of isopropanol,

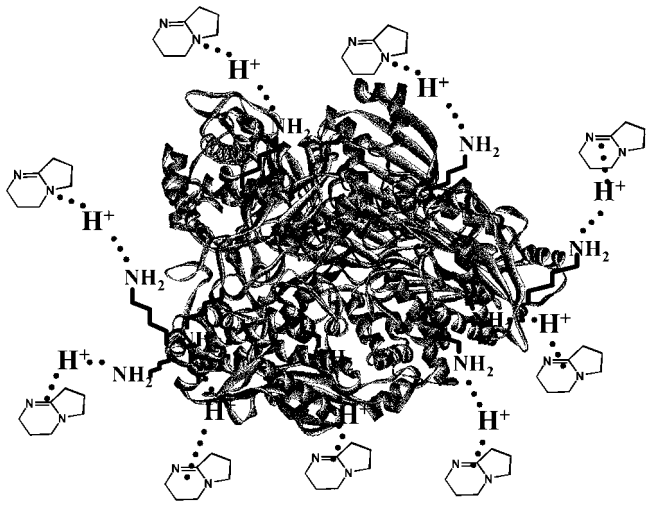


Figure 9. Schematic representation of the proposed final stage of the electrospray desolvation process. Prior to the final steps of desolvation, the protein remains "solvated" by several molecules of the additive that bind in a proton-bound manner. In the final desolvation, the additive ligand dissociates from the protein and either takes the proton with it, or leaves the proton at the protein, depending on the difference in proton affinities of the additive and the specific site on the protein. In this figure glutamate synthase is the protein and proton-bound DBN is the additive.

TEAB, DBN, DBU, and HPP are 0.023, 0.023, 0.046, 0.042, and 0.051 N m^{-1}, respectively.[48] If surface tension was a major factor, we would expect the charging behaviors with the additives isopropanol and TEAB to be similar, as they have identical surface tensions. In addition, 0.1% acetic acid in pure water reduces γ only marginally to 0.0715 N m^{-1},[48] and 0.12% isopropanol reduces γ only slightly further to approximately 0.067 N m^{-1}.[48] When we consider Equation (1), these changes have no significant effect on the charging. Thus, we believe the surface tension of the solution is not a major factor affecting the differences in protein charging in our data.

The present method is not unique for the decharging of proteins in the electrospray process. Smith and co-workers[49,50] developed a special radioactive ^{210}Po source that induces charge reduction during the ESI-MS of proteins. The ions produced were reacted in an aerosol with the particles produced from this source. The multiply charged protein ions thereby react with the bath gas, which contains positively and negatively charged ions. Effective charge reduction using this system was achieved for several medium-sized proteins. The advantage of the method described in this paper is that it can be used without modification on any existing ESI-MS system.

If the charge observed on the protein ions is assumed to be a result of proton competition events between the protein ions and additives, our results may provide an alternative method to evaluate the GB/PA of proteins, and possibly even the GB/PA of specific sites in gaseous protein ions. In fact, the data presented here may be considered to be a sort of titration experiment, somewhat similar to the proton

transfer reactivity measurements of protein ions, usually performed in a Fourier transform mass spectrometer.[20]

Finally, the decreased charging of proteins would minimize the risk of eventual Coulombic repulsions on the multiply charged protein ions; these repulsions would otherwise affect the tertiary and/or quaternary structure. The method presented here could, therefore, be the method of choice to "gently" decharge proteins in so-called "soft-landing" protein arrays, in which an array is made by landing protein ions, prepared by ESI, on a solid surface.[51] Proteins may retain (or regain) their biological activity following a soft landing, even when they have traveled as gaseous ions through the mass spectrometer. The method presented here enables proteins to transfer from solution into the gas phase, during which proteins and/or very large protein complexes attain relatively few charges upon ionization. This results in a gas-phase structure that more closely resembles the solution-phase structure. This method may be useful in the study of the biologically relevant properties of globular proteins by mass spectrometry.

Experimental Section

Proteins: The *gls*F gene from *Synechocystis* sp. PCC 6803 encoding glutamate synthase was overexpressed in the glutamate auxotrophic *Escherichia coli* strain CLR207 *Rec* in Luria–Bertani medium containing ampicillin (150 $\mu g\,mL^{-1}$) at 37 °C under vigorous aeration.[52] The enzyme was purified according to an established procedure.[41] The gene encoding the Cys116Ser mutant of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* was overexpressed in transformed *E. coli* TG2 in Luria–Bertani medium containing ampicillin (100 $\mu g\,mL^{-1}$) and isopropyl-β-D-1-thiogalactopyranoside (IPTG, 20 $\mu g\,mL^{-1}$) at 37 °C under vigorous aeration. The protein was purified according to the procedure described previously.[53] The gene encoding GroEL was overexpressed in the *E. coli* strain MC1009 in Luria–Bertani medium containing ampicillin (100 $\mu g\,mL^{-1}$) and arabinose (0.0005 % w/v) at 37 °C under vigorous aeration. GroEL was purified according to a previously described protocol,[54] which was slightly modified by the introduction of an acetone precipitation step. Both chicken egg white lysozyme (purity = 95 %) and horse heart myoglobin (purity = 95 %) were purchased from Sigma–Aldrich.

Chemicals: Isopropanol, ammonium acetate (NH_4OAc), triethylammonium bicarbonate (TEAB), arginine, 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and 1,5,7-triazabicyclo[4.4.0]dec-5-ene (HPP) were from Sigma–Aldrich. MilliQ water was used.

Nanoflow electrospray ionization mass spectrometry: Mass spectrometric measurements were performed on an ESI-ToF (LCT) mass spectrometer (Micromass). The instrument was equipped with a Z-nano-electrospray source (Micromass) operating in the positive ionization mode. Nanoflow electrospray needles were made from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments) on a P-97 puller (Sutter instruments, USA). Needles were coated with a thin layer of gold (~500 Å) by using a Scancoat Six sputter coater (Edwards High Vacuum International). All spectra were recorded from aqueous solutions (20 mM) of the different additives, in which acetic acid was used to adjust the pH to 7. Protein concentrations varying from 1 to 5 μM were used for the mass spectrometry measurements. The potential difference between the nanoflow spray needle and the orifice of the mass spectrometer was set to 1.2–1.5 kV and the cone voltage was set to the minimum possible value to detect signals of the protein ions. The cone voltage varied from 30–50 V for the less basic additives and used up to 150 V for the most basic additives, depending on protein size. Source pressure conditions in the mass spectrometer were adjusted for optimal ion transmission. To optimize collision-damping conditions, the pressure in the interface region was adjusted by reducing the pumping capacity of the rotary pump by closing the speed valve, as described previously.[55]

Circular dichroism spectroscopy: A dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems) was used for circular dichroism measurements. The subtractive double-grating monochromator was equipped with a fixed disk, holographic gratings (2400 lines mm^{-1}, blaze wavelength 230 nm), and 1.24 mm slits. Samples were measured in a cell of path-length 1 mm. The protein concentration was 1 $mg\,mL^{-1}$ in aqueous solutions (20 mM) of the different additives, in which acetic acid was used to adjust the pH to 7. Each measurement was the average of five repeated scans (step resolution 1 nm), from which the corresponding background spectrum was subtracted. The measured signals were converted to molar ellipticity (ε_{molar}) on the basis of their molecular masses, concentration, and light path-length.

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[1] J. A. Loo, *Mass Spectrom. Rev.* **1997**, *16*, 1–23.
[2] H. Hernandez, C. V. Robinson, *J. Biol. Chem.* **2001**, *276*, 46685–46688.
[3] A. J. R. Heck, R. H. van den Heuvel, *Mass Spectrom. Rev.* **2004**, *23*, 368–389.
[4] J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, C. M. Whitehouse, *Science* **1989**, *246*, 64–71.
[5] Z. Zhang, A. Krutchinsky, S. Endicott, C. Realini, M. Rechsteiner, K. G. Standing, *Biochemistry* **1999**, *38*, 5651–5658.
[6] W. J. van Berkel, R. H. van den Heuvel, C. Versluis, A. J. Heck, *Protein Sci.* **2000**, *9*, 435–439.
[7] C. V. Robinson, *Nat. Struct. Biol.* **2002**, *9*, 505–506.
[8] M. W. Pinkse, C. S. Maier, J. I. Kim, B. H. Oh, A. J. Heck, *J. Mass Spectrom.* **2003**, *38*, 315–320.
[9] D. R. Benjamin, C. V. Robinson, J. P. Hendrick, F. U. Hartl, C. M. Dobson, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7391–7395.
[10] C. L. Hanson, P. Fucini, L. L. Ilag, K. H. Nierhaus, C. V. Robinson, *J. Biol. Chem.* **2003**, *278*, 1259–1267.
[11] G. Bacher, W. W. Szymanski, S. L. Kaufman, P. Zollner, D. Blaas, G. Allmaier, *J. Mass Spectrom.* **2001**, *36*, 1038–1052.
[12] S. D. Fuerstenau, W. H. Benner, J. J. Thomas, C. Brugidou, B. Bothner, G. Siuzdak, *Angew. Chem.* **2001**, *113*, 559–562; *Angew. Chem. Int. Ed.* **2001**, *40*, 541–544; corrigendum: *Angew. Chem.* **2001**, *113*, 1011; *Angew. Chem. Int. Ed.* **2001**, *40*, 982.
[13] M. F. Jarrold, *Annu. Rev. Phys. Chem.* **2000**, *51*, 179–207.
[14] S. J. Valentine, J. G. Anderson, A. D. Ellington, D. E. Clemmer, *J. Phys. Chem. B* **1997**, *101*, 3891–3900.
[15] R. W. Purves, D. A. Barnett, B. Ells, R. Guevremont, *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 894–901.
[16] R. W. Purves, D. A. Barnett, B. Ells, R. Guevremont, *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 738–745.
[17] S. J. Valentine, D. E. Clemmer, *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 506–517.
[18] D. Suckau, Y. Shi, S. C. Beu, M. W. Senko, J. P. Quinn, F. M. Wampler III, F. W. McLafferty, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 790–793.
[19] T. D. Wood, R. A. Chorush, F. M. Wampler III, D. P. Little, P. B. O'Connor, F. W. McLafferty, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 2451–2454.
[20] D. S. Gross, P. D. Schnier, S. E. Rodriguez-Cruz, C. K. Fagerquist, E. R. Williams, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 3143–3148.

[21] M. Dole, L. L. Mack, R. L. Hines, R. C. Mobley, L. D. Ferguson, M. B. Alice, *J. Chem. Phys.* **1968**, *49*, 2240–2249.
[22] J. de la Mora, *Anal. Chim. Acta* **2000**, *406*, 93–104.
[23] N. Felitsyn, M. Peschke, P. Kebarle, *Int. J. Mass Spectrom.* **2002**, *219*, 39–62.
[24] M. Peschke, A. Blades, P. Kebarle, *J. Am. Chem. Soc.* **2002**, *124*, 11519–11530.
[25] V. Katta, B. T. Chait, *J. Am. Chem. Soc.* **1991**, *113*, 8534–8535.
[26] A. Dobo, I. A. Kaltashov, *Anal. Chem.* **2001**, *73*, 4763–4773.
[27] E. T. van den Bremer, W. Jiskoot, R. James, G. R. Moore, C. Kleanthous, A. J. Heck, C. S. Maier, *Protein Sci.* **2002**, *11*, 1738–1752.
[28] I. A. Kaltashov, S. J. Eyles, *Mass Spectrom. Rev.* **2002**, *21*, 37–71.
[29] R. Grandori, I. Matecko, N. Muller, *J. Mass Spectrom.* **2002**, *37*, 191–196.
[30] R. Grandori, *J. Mass Spectrom.* **2003**, *38*, 11–15.
[31] D. Lemaire, G. Marie, L. Serani, O. Laprevote, *Anal. Chem.* **2001**, *73*, 1699–1706.
[32] U. H. Verkerk, M. Peschke, P. Kebarle, *J. Mass Spectrom.* **2003**, *38*, 618–631.
[33] S. T. Howard, *J. Am. Chem. Soc.* **2000**, *122*, 8238–8244.
[34] E. D. Raczynska, P. C. Maria, J. F. Gal, M. Decouzon, *J. Phys. Org. Chem.* **1994**, *7*, 725–733.
[35] M. Decouzon, J. F. Gal, P. C. Maria, E. D. Raczynska, *Rapid Commun. Mass Spectrom.* **1993**, *7*, 599–602.
[36] E. D. Raczynska, M. Decouzon, J. F. Gal, P. C. Maria, R. W. Taft, F. Anvia, *J. Org. Chem.* **2000**, *65*, 4635–4640.
[37] J. F. Gal, P. C. Maria, E. D. Raczynska, *J. Mass Spectrom.* **2001**, *36*, 699–716.
[38] H. A. Staab, T. Saupe, *Angew. Chem.* **1988**, *100*, 895–909; *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 865–879.
[39] B. Entsch, W. J. van Berkel, *FASEB J.* **1995**, *9*, 476–483.
[40] M. A. Vanoni, B. Curti, *Cell. Mol. Life Sci.* **1999**, *55*, 617–638.
[41] R. H. van den Heuvel, D. I. Svergun, M. V. Petoukhov, A. Coda, B. Curti, S. Ravasio, M. A. Vanoni, A. Mattevi, *J. Mol. Biol.* **2003**, *330*, 113–128.
[42] P. B. Sigler, Z. Xu, H. S. Rye, S. G. Burston, W. A. Fenton, A. L. Horwich, *Annu. Rev. Biochem.* **1998**, *67*, 581–608.
[43] A. A. Rostom, C. V. Robinson, *J. Am. Chem. Soc.* **1999**, *121*, 4718–4719.
[44] A. van der Kerk-van Hoof, A. J. R. Heck, *J. Mass Spectrom.* **1999**, *34*, 813–819.
[45] M. I. Catalina, N. J. de Mol, M. J. E. Fischer, A. J. R. Heck, *Phys. Chem. Chem. Phys.* **2004**, *6*, 2572–2579.
[46] A. T. Iavarone, E. R. Williams, *J. Am. Chem. Soc.* **2003**, *125*, 2319–2327.
[47] M. Samalikova, R. Grandori, *J. Am. Chem. Soc.* **2003**, *125*, 13352–13353.
[48] *Handbook of Chemistry and Physics*, 51st ed., CRC, Ohio, **1971**.
[49] M. Scalf, M. S. Westphall, J. Krause, S. L. Kaufman, L. M. Smith, *Science* **1999**, *283*, 194–197.
[50] M. Scalf, M. S. Westphall, L. M. Smith, *Anal. Chem.* **2000**, *72*, 52–60.
[51] Z. Ouyang, Z. Takats, T. A. Blake, B. Gologan, A. J. Guymon, J. M. Wiseman, J. C. Oliver, V. J. Davisson, R. G. Cooks, *Science* **2003**, *301*, 1351–1354.
[52] F. Navarro, E. Martin-Figueroa, P. Candau, F. J. Florencio, *Arch. Biochem. Biophys.* **2000**, *379*, 267–276.
[53] W. van Berkel, A. Westphal, K. Eschrich, M. Eppink, A. de Kok, *Eur. J. Biochem.* **1992**, *210*, 411–419.
[54] E. Quaite-Randall, A. Joachimiak, *Methods Mol. Biol.* **2000**, *140*, 29–39.
[55] N. Tahallah, M. Pinkse, C. S. Maier, A. J. Heck, *Rapid Commun. Mass Spectrom.* **2001**, *15*, 596–601.

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