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Increasing Charge While Preserving Noncovalent Protein Complexes for ESI-MS

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Abstract

Increased multiple charging of native proteins and noncovalent protein complexes is observed in electrospray ionization (ESI) mass spectra obtained from nondenaturing protein solutions containing up to 1% (v/v) *m*-nitrobenzyl alcohol (*m*-NBA). The increases in charge ranged from 8% for the 690 kDa $\alpha_7\beta_7\beta_7\alpha_7$ 20S proteasome complex to 48% additional charge for the zinc-bound 29 kDa carbonic anhydrase-II protein. No dissociation of the noncovalently bound ligands/subunits was observed upon the addition of *m*-NBA. It is not clear if the enhanced charging is related to altered surface tension as proposed in the “supercharging” model of Iavarone and Williams (Iavarone, A. T.; Williams, E. R. *J. Am. Chem. Soc.* **2003**, 125, 2319–2327). However, more highly charged noncovalent protein complexes have utility in relaxing slightly the mass-to-charge (*m/z*) requirements of the mass spectrometer for detection and will be effective for enhancing the efficiency for tandem mass spectrometry studies of protein complexes.

Keywords

electrospray ionization; noncovalent complexes; proteins; supercharging

Introduction

A hallmark of electrospray ionization (ESI) for the mass spectrometric analysis of large biomolecules is the multiple charging phenomenon described by the early work of John Fenn and others [1]. This feature is most easily accessed by proteins that have been structurally denatured in ESI-friendly solvents, such as acidified water augmented with acetonitrile or methanol. Extensive multiple charging is observed also for native proteins and noncovalent protein complexes, but the multiple charging is reduced and the charge distribution is shifted to increasing *m/z*. The near-physiological solution pH needed to retain the fidelity of the structure of the native protein complex does not typically promote as extensive multiple charging as the acidic regime.

Experimentally, there are many other ways to shift the ESI charge state distribution (CSD) to lower charge. Increasing the energy of gas-phase collisions, e.g., in the atmosphere-vacuum interface of the ESI mass spectrometer or other regions downstream from the ESI source, will shift the CSD to higher *m/z* because the higher charged molecules are more susceptible to

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collisional dissociation than the lower charged molecules [2,3] and/or the more energetic collisions result in endothermic proton transfer to the solvent molecules [4]. Rather than the ammonium acetate or ammonium bicarbonate solution buffers commonly employed to maintain neutral pH, buffers of stronger gas phase basicity such as methylammonium acetate [5] or triethylammonium acetate/bicarbonate [6,7] have been reported to reduce charging for native proteins. Ion-molecule reactions with proton scavengers of high gas phase basicity, such as diethylamine, reduce protein charging effectively [8–10]. Very effective neutralization of the multiply charged ESI ions can be achieved with a bipolar neutralizer composed of a ^{210}Po α -particle source that ionizes air to produce a high concentration of bipolar (positively and negatively charged) ions or with a corona discharge source [11,12]. Work from McLuckey's lab demonstrated the capabilities of ion-ion reactions to effectively reduce protein charge as a means to reduce the spectral complexity of ESI mass spectra [13].

However, there have been few studies reporting effective means to increase charging beyond that measured under normal ESI experimental conditions. Increased charging would further reduce the m/z requirements of the analyzer, and higher charged molecules are more effectively dissociated in MS/MS-type experiments [3]. Li and Cole demonstrated increased charging for polypeptides with decreasing ESI tip orifice diameter [14]. A series of papers by Iavarone and Williams reported that applying high surface tension, low relative volatility solvents could enhance ESI charging, or "supercharge" [4,15–18]. For 12 kDa cytochrome *c* under denaturing solvent conditions (3% acetic acid and organic solvents), maximum charge increased from 20–21+ to 24+ with the incorporation of *m*-nitrobenzyl alcohol (*m*-NBA) [18]. Because electron capture cross sections increase quadratically with charge [19], addition of one more charge can dramatically enhance the efficiency of electron capture dissociation (ECD) and electron transfer dissociation (ETD), especially for small peptides. This strategy was exploited by Jensen and coworkers, who reported that adding *m*-NBA to the solvent of an LC-ETD-MS/MS platform increased the abundance of the 3+-charged tryptic peptides, improving ETD performance for peptide sequencing and identification [20]. Recently, we reported that *m*-NBA increased charging of small proteins in the ambient desorption/ionization method, electrospray-assisted laser desorption/ionization (ELDI) [21].

All of the previous supercharging reports of peptides and proteins used denaturing solvent systems. We show that low concentrations of *m*-NBA can also enhance ESI multiple charging of proteins and noncovalent protein complexes in nondenaturing solvent systems. Increased charge ranged from +8% to a high of +48% for the 6 proteins measured.

Experimental

Positive ion electrospray ionization mass spectra were acquired with two different systems: a hybrid quadrupole time-of-flight (QTOF) mass spectrometer with a Triwave (three traveling wave-enabled stacked ring ion guides) ion mobility (IM) separator (QTOF/IM; Waters Synapt HDMS, Manchester, UK), and an LTQ-FT Ultra mass spectrometer (Thermo Fischer Scientific) [22]. The nanoESI source using borosilicate glass capillaries with Au/Pd coatings (Proxeon Biosystems, Odense, Denmark) was operated at low analyte flow conditions (50 nL/min). Increased vacuum backing pressure in the atmosphere/vacuum region of up to 5 mbar was applied on the QTOF instrument for detection of the large proteins and complexes, and 2 mbar for smaller proteins.

Enolase (yeast), carbonic anhydrase II (bovine), phosphorylase *b* (glycogen phosphorylase, rabbit muscle), myoglobin (horse), alcohol dehydrogenase (yeast), and *m*-NBA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The *Methanosarcina thermophila* 20S proteasome sample was obtained from Calbiochem (San Diego, CA) [23]. All protein samples were desalted with 20 mM ammonium acetate prior to analysis using centrifugal filter devices

(10,000 or 30,000 molecular weight cutoff, Microcon and Amicon Ultra, Millipore Corporation, Billerica, MA, USA). The final protein concentration for ESI-MS measurements was approximately 5 μ M in 20 mM ammonium acetate, pH 6.8 unless noted otherwise. The addition of *m*-NBA to 1% did not alter the pH of the solutions significantly (less than 0.2 pH units).

Results and Discussion

The addition of 0.1–1.0% (v/v) *m*-NBA to the 6 noncovalent protein complexes tested increased modestly the average and maximum charge states measured. At the highest *m*-NBA concentration tested for each protein complex, typically 0.5%, the increase in multiple charging ranged from 8% for the 690 kDa $\alpha_7\beta_7\beta_7\alpha_7$ 20S proteasome complex (Supplemental Figure 1) to a 48% increase in charging for zinc-bound 29 kDa carbonic anhydrase (Table 1). Charge state increases with increasing *m*-NBA concentration up to approximately 0.5% v/v. For example, the average charge of the 93 kDa enolase dimer complex increases from +18.5 in 0% *m*-NBA to +22.4 (0.1% *m*-NBA) to +24.0 (0.5% *m*-NBA); increasing the concentration of *m*-NBA to 0.7% did not further increase the average charge state. Concentrations above 1.0% were not tested, as *m*-NBA has limited solubility in near-neutral pH aqueous ammonium acetate.

For the noncovalent protein complexes tested, adding *m*-NBA did not directly induce dissociation of bound ligands or protein subunits. For example, increasing *m*-NBA to 1.0% increased average charging from +10.0 to +14.8 for zinc-metalloenzyme carbonic anhydrase II (Figures 1a and 1b). Zinc-bound $[M + Zn + (n-2)H]^{n+}$ molecules obtained were retained even with *m*-NBA. In contrast, ESI-MS of carbonic anhydrase in denaturing solutions (e.g., 50% acetonitrile with 3–5% acetic acid or 0.1–1% formic acid) yields ions for the apo-protein exclusively (data not shown).

Figures 1c and 1d show ESI mass spectra of the 195 kDa phosphorylase *b* dimer. A 12.5% increase in average charging to +32.3 is observed from the addition of 0.1% *m*-NBA, without disruption of the dimer complex. Although the number of charges on proteins in near-neutral pH, buffered aqueous solutions is relatively low compared to protein mass spectra acquired from denaturing, acidic solutions, multiply charged molecules remain susceptible to collisional dissociation [2]. Care must be taken to minimize the collision energies endured by the higher charged proteins in the various regions of the instrument, for example, in the atmosphere/vacuum interface [3] or lower pressure regions downstream from the source. This can be accomplished by reducing the cone voltage in the ESI interface or the collision energy in the trap-cell of the Triwave ion mobility separator of the QTOF/IM instrument (Waters Synapt HDMS) or the capillary lens voltage of the ion trap-Fourier transform ion cyclotron resonance system (Thermo LTQ-FT Ultra).

Some peak broadening due to clustering of *m*-NBA molecules onto the protein molecules is observed. Distinct adducts due to *m*-NBA clustering is evident for smaller proteins, such as myoglobin (Supplemental Figure 2). With 0.5% *m*-NBA, the average charge for myoglobin is approximately +10, but significant adduct formation with *m*-NBA (separated by 153 Da, the molecular weight of *m*-NBA) is observed especially for the higher charged molecules. Increasing the energy in the trap-cell of the Triwave from +15V to +40V (Supplemental Figure 2) dissociates neutral *m*-NBA from the protein and enhances the signal for the higher charged molecules. However, higher collision energies can also dissociate the weakly-bound heme cofactor molecule from the holo-protein to yield a small amount of the apo-protein form.

The mechanism of the increased charging observed for native protein complexes in the presence of *m*-NBA is unclear. The supercharging mechanism postulated by Iavarone and

Williams dictates that adding low volatility, high surface tension solvents such as *m*-NBA (50 mN/m) to denaturing solvent systems of high volatility (e.g., water/methanol/acetic acid) increases the surface tension, and therefore the charge density on the droplets, and thus making more charges available to the desorbed analyte [18]. However, in solvent systems composed largely of water (72 mN/m), adding *m*-NBA lowers the surface tension overall; because water has a higher relative vapor pressure, charging is not expected to increase. Previous ESI-MS studies added *m*-NBA to denaturing solutions containing methanol and acetic acid for protein analytes, although aqueous solutions were employed with polyethylene glycol, dendrimers, and diaminoalkanes [16,18]. Increased charging was not observed from *m*-NBA-containing aqueous solutions, except with a larger 7.2 kDa dendrimer, where it was attributed to the presence of multiple structural conformers [18]. Moreover, work by Šamalíková and Grandori appears to question the precise role of surface tension on ESI protein charging, as they found that the CSDs of proteins do not respond to changes in surface tension [24,25].

Protein conformation may play some role in the increased charging. Alcohols and other organic solvents can affect the structure and function of proteins, and their effect on ESI charge states has been noted previously [26]. Whether the addition of *m*-NBA to aqueous solutions can affect the conformation of the proteins studied is unclear currently. Preliminary experiments using circular dichroism and hydrogen/deuterium exchange MS techniques showed no measurable effect on the solution structure of aqueous myoglobin with 0.5% *m*-NBA (data not shown).

It is clear, however, that the addition of *m*-NBA modestly enhances ESI multiple charging of native protein complexes, and that the noncovalent complexes survive intact for their measurement by ESI-MS. This feature can be exploited potentially by ECD and ETD to identify the sites of noncovalent ligand binding [22] and to probe the structures of gas phase protein complexes [11,27].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. ESI-MS (LTQ-FT) of carbonic anhydrase II (a) and with 1.0% *m*-NBA (b). The insets show expanded views of select charge states and demonstrate capabilities to resolve apo-from holo-forms. (The arrow depicts where a peak for the apo-protein would be found in the spectrum if it were present.) ESI-MS (QTOF/IM) of phosphorylase *b* (c) and with 0.1% *m*-NBA (d).

Table 1Increase in charging observed in ESI-MS of noncovalent protein complexes containing *m*-nitrobenzyl alcohol

Protein	MW ($\times 10^3$)	Charge state ^I	Charge state with <i>m</i> -NBA ^I
Myoglobin	17.6 (+ heme)	8.2 (9)	10.3 (12)
Carbonic anhydrase II	29.1 (+ Zn)	10.0 (11)	14.8 (19)
Enolase	93.3 (dimer)	18.5 (20)	24.0 (27)
Alcohol dehydrogenase	147.2 (tetramer)	23.6 (25)	28.0 (29)
Phosphorylase <i>b</i>	194.9 (dimer)	28.7 (33)	32.3 (35)
20S Proteasome	689.3 ($\alpha_7\beta_7\beta_7\alpha_7$)	53.7 (56)	58.2 (60)

^I Average charge state given by $(\sum q_i I_i) / (\sum I_i)$ from *i* to *N*, where q_i is the charge of the *i*-th charge state, I_i is the intensity of the *i*-th charge state, and *N* is the number of measured charge states in a mass spectrum [15]. The value in parenthesis is the maximum charge state observed in the mass spectrum.