New Reagents for Enhanced Liquid Chromatographic Separation and Charging of Intact Protein Ions for Electrospray Ionization Mass Spectrometry

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Abstract

Electrospray ionization produces multiply charged ions, thereby lowering the mass-to-charge ratio for peptides and small proteins to a range readily accessed by quadrupole ion trap, orbitrap, and ion cyclotron resonance (ICR) mass analyzers (m/z = 400–2,000). For Fourier transform mass analyzers (orbitrap and ICR), higher charge also improves signal-to-noise ratio, mass resolution, and mass accuracy. Addition of *m*-nitrobenzyl alcohol (*m*-NBA) or sulfolane has previously been shown to increase the charge states of proteins. Moreover, polar aprotic dimethylformamide (DMF) improves chromatographic separation of proteolytic peptides for mass analysis of solution-phase protein hydrogen/deuterium exchange for improved (78–96%) sequence coverage. Here, we show that addition of each of various modifiers (DMF, thiodiglycol, dimethylacetamide, dimethylsulfoxide, and N-methylpyrrolidone) can significantly increase the charge states of proteins up to 78 kDa. Moreover, incorporation of the same modifiers into reversed-phase liquid chromatography solvents improves sensitivity, charging, and chromatographic resolution for intact proteins.

Keywords

Charge state; Fourier transform mass spectrometry; ion cyclotron resonance; FTMS; FT-ICR; quadrupole ion trap; orbitrap

INTRODUCTION

Electrospray ionization (ESI)[1] coupled with mass spectrometry (MS) is extensively used for analysis of large biopolymers[2] and protein complexes.[3] The principal advantage of ESI is to form and detect multiply charged analyte molecules. Low flow ESI such as microelectrospray[4] and nanoelectrospray[5] further enhances ESI through improved ionization efficiency and sensitivity. ESI facilitates the detection of large biomolecules with quadrupole and trapped-ion mass analyzers by lowering the mass-to-charge ratio. Moreover, the charge state distribution is affected by analyte gas-phase basicity,[6–8] solution pH,[9] solvent composition,[6] droplet size,[10] instrument parameters,[11] and protein conformation.[12–17]

Buffers with strong gas-phase basicity such as triethyl ammonium acetate/bicarbonate[18] or methyl ammonium acetate[19] have been reported to *decrease* charging of proteins. High gas-

phase basicity additives, such as diethylamine and other bases can significantly reduce protein charging by removing protons.[20, 21] Ion/molecule[22] and ion/ion reactions[23] with neutral bases or anions can reduce protein charge: an advantage for low-resolution mass analyzers, by reducing the number of charge states and thus simplifying the mass spectrum. Higher skimmer voltage or capillary temperature[11] can further promote charge reduction due to collisions between analyte ions and neutrals or an electrode.

Higher charge state is desirable for peptides and proteins because: (a) Ion signal-to-noise ratio, mass resolving power, and mass accuracy are proportional to charge state for the highest resolution mass analyzers for which detection is based on induced-charge (orbitrap[24] and ion cyclotron resonance[25, 26]) and (b) For both electron capture dissociation (ECD)[27] and electron transfer dissociation (ETD),[28] MS/MS efficiency increases as the square of ion charge.[29] Attempts to increase peptide/protein charge state have included variation in solvent[30] and acid concentration,[12] as well as disulfide bond reduction by dithiothreitol (DTT).[31] The charge state distribution for test proteins shifts to lower m/z with decreased tip orifice diameter.[32] Addition of m-nitrobenzyl alcohol (m-NBA) to create charge enhancement, or "super-charging" has been systematically studied by Williams et al. under denaturing conditions.[33–36] Loo et al. have characterized enhanced charging of proteins and protein complexes by m-NBA, analogous molecules, and sulfolane in non-denaturing solvent with ammonium acetate buffer.[37, 38] For example, sulfolane produces a higher average charge state (61%) than m-NBA (21%) for myoglobin, under non-denaturing conditions.[38] Jensen and coworkers reported improved ETD MS/MS efficiency for peptide sequencing and identification by addition of m-NBA to the mobile phase for on-line LC ETD MS/MS of tryptic peptides: to our knowledge, the first such use of a "supercharging" modifier. However, in that report, signal-to-noise ratio decreased due to greater chromatographic peak width and consequently lower peak height.[39]

Here, we report the screening of dimethylformamide (DMF), dimethylacetamide (DMAA), N-methylpyrrolidone (NMP), dimethylsulfoxide (DMSO), and thiodiglycol (TDG) "super-charging" reagents. Compared with conventional solvents (water/acetonitrile/formic acid), some of the modifiers improved the signal-to-noise ratio (S/N) by enhanced charging of intact proteins. Furthermore, DMF and DMSO are LC-compatible and improve chromatographic resolution for intact proteins as well. Finally, we discuss physico-chemical properties that may contribute to charge enhancement.

EXPERIMENTAL

Materials

Dimethylformamide, dimethylacetamide, N-methylpyrrolidone, dimethylsulfoxide, thiodiglycol, bovine ubiquitin (8.5 kDa), equine cytochrome c (12.8 kDa), chicken lysozyme (14.3 kDa), equine myoglobin (16.8 kDa), bovine carbonic anhydrase (29 kDa), and transferrin (78 kDa) were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water and acetonitrile were purchased from J. T. Baker (Philipsburg, NJ).

Sample Preparation

Protein samples (10 μM) were first prepared in conventional ESI solution: H$_2$O/acetonitrile/formic acid, 50/50/0.5 (v/v/v). All protein samples were then diluted to 1 μM in conventional ESI solution with varying concentrations of DMF, DMAA, DMSO, TDG, NMP, and m-NBA to yield the same final protein concentration for ESI FT-ICR MS.

Direct Infusion, Liquid Chromatography, and Mass Spectrometry

Direct infusion was performed with an Advion Biosystems TriVersa Nanomate (Ithaca, NY).[40] Liquid chromatography was performed with a Jasco HPLC/SFC instrument (Jasco, Easton,

MD). Sample injection (10 μL of 500 fmol/μL protein mixture) was performed with a LEAP robot (HTS PAL, Leap Technologies, Carrboro, NC). Reversed-phase liquid chromatography (RPLC) was performed with conventional (A = H_2O/acetonitrile/formic acid, 95/5/0.5 v/v; B = acetonitrile/H_2O/formic acid, 95/5/0.5, v/v) and modified (A = H_2O/acetonitrile/DMSO/ formic acid, 90/5/5/0.5 v/v/v; B = acetonitrile/H_2O/DMSO/formic acid, 90/5/5/0.5 v/v). Separation was conducted with either a Jupiter C_{18} or C_5 column (Jupiter™, Phenomenex, Torrance, CA, USA; 1×50 mm, 5 μm particle size, 300 A pore size) at 50 μL/min. Column effluent was split 1/1000 for efficient microelectrospray ionization.[4]

Mass spectrometry was carried out with a hybrid 14.5 T LTQ FT-ICR mass spectrometer (LTQ-FT, Thermo Fisher Corp., Bremen, Germany).[41] External ion accumulation[42] was performed in the front end linear ion trap with a target ion population of one million charges for each FT-ICR measurement. Ions were then transferred (~1 ms transfer period) through three octopole ion guides (2.2 MHz, 250 V_{p-p}) to a capacitively coupled[43] closed cylindrical ICR cell (55 mm i.d.) for analysis. Data was collected with Xcalibur 2.0 software (Thermo Fisher). FT-ICR mass spectra were acquired from m/z 390–2000 at high mass resolving power (m/$\Delta m_{50\%}$ = 200,000 at m/z 400, in which $\Delta m_{50\%}$ denotes mass spectral peak full width at half-maximum peak height). The total data acquisition period for each sample was 3 min for direct infusion and 80 min for LC/MS.

RESULTS AND DISCUSSION

Direct Infusion

The addition of a relatively low proportion of an appropriate "modifier" reagent to the conventional electrospray denaturing solvent mixture shifts both the average and the maximum charge state of an intact protein to lower *m/z*, as seen in Figure 1 for cytochrome *c*. The most abundant charge state shifts from 15+ with conventional electrospray solvent mixture to 17+, 20+, 20+ and 18+ on addition of DMF, DMSO, TDG or *m*-NBA. Note that DMSO achieved 3 times higher S/N, partly by confining the charge state distribution to a narrower *m/z* range. In contrast, *m*-NBA reduced the S/N by 20%. The *average* charge state shifts from 13.6 (for conventional ESI solvent mixture) to 16, 19.5, 18.6 and 19.1 on addition of DMF, DMSO, TDG or *m*-NBA. Thus, DMSO improves S/N and increases the average and most abundant charge states to yield lower *m/z*. The same DMSO effects are observed for myoglobin (Figure 2) and carbonic anhydrase (Supporting Information Figure S-1).

Liquid Chromatography

For high-throughput top down proteomics it is imperative to test the chromatographic separation performance for various modifiers with a Jupiter C_{18} column for conventional and modified solvents (e.g., 5% DMSO or 0.5% *m*-NBA or 10% DMF added to both mobile phases). (A stepwise gradient (Supporting Information, Figure S-2) resulted in a total run time of 100 min.) For all four solvent systems, proteins eluted in the order of their molecular weight; but with longer shorter retention time and improved separation with DMSO. Addition of *m*-NBA, as reported for *tryptic* peptides,[39] shortened the retention times for *all proteins*, as did DMSO (Supporting Information, Figure S-3). DMF, as we previously reported,[44] increased the protein retention time relative to conventional mobile phase solvents.

We further optimized chromatographic separation by use of a Jupiter C_5 chromatographic column. Figure 3 presents a total ion chromatogram for protein standards separated by conventional and 5% DMSO-modified solvents. Proteins elute in the same order with 5% DMSO, but with improved chromatographic separation and higher peak capacity relative to conventional solvents. As for direct infusion data, enhanced charging and better sensitivity were observed with DMSO.

Figure 4 shows the charge state distributions of transferrin as it elutes from the Jupiter C_5 column with conventional and DMSO-modified solvents. The base peak shifts dramatically from 53+ to 71+ with DMSO. The highest charge state of transferrin shifts from 67+ to 77+ by DMSO incorporation into the mobile phase. Retention time for transferrin increases from 35 min to 37 min with DMSO, as we also found for other protein standards (Figure 3).

Physico-Chemical Properties

The total number of charges, Z, on a spherical droplet of radius, R, at the point of Rayleigh instability is predicted to be:

$$Ze = 8\pi \left(\varepsilon_0 \gamma R^3\right)^{1/2} \tag{1}$$

in which e is the elementary charge, ε_0 is permittivity of a vacuum and γ is surface tension.[45] Hence, the charge state distribution should shift to lower m/z (higher charge) as the solvent surface tension increases. Relative to acetonitrile (27.6 mN/m) all of the presently tested solvents have higher surface tension than conventional ESI solvents and exhibit enhanced charging, consistent with the Rayleigh equation. The modifiers could also affect the ESI droplet size and thus the desolvation process. Similarly, DMAA (32.4 mN/m) yielded lower positive charge states than DMF (35.2 mN/m), DMSO (42.9 mN/m), TDG (54.0 mN/m) and m-NBA (50±5 mN/m). Moreover, the somewhat higher gas-phase basicity for DMAA (877.0 KJ/mol) and NMP (891.6KJ/mol)[46] relative to DMF (856.6 KJ/mol) which may contribute to their lower charging due to proton abstraction.

In ESI, solvents with higher vapor pressure evaporate first. All of the tested solvents have significantly higher boiling point and lower vapor pressure than water (100 °C) thereby increasing the lifetime of the ESI droplets and causing droplet heating, leading to changes in protein conformation as reported by Williams and coworkers.[47] Also, the altered distribution of analyte within the droplet[48] could cause enhanced charging with these polar modifiers (e.g., due to higher surface concentration). Further work is in progress to better understand charge enhancement, improved LC separation, and applicability to higher molecular weight proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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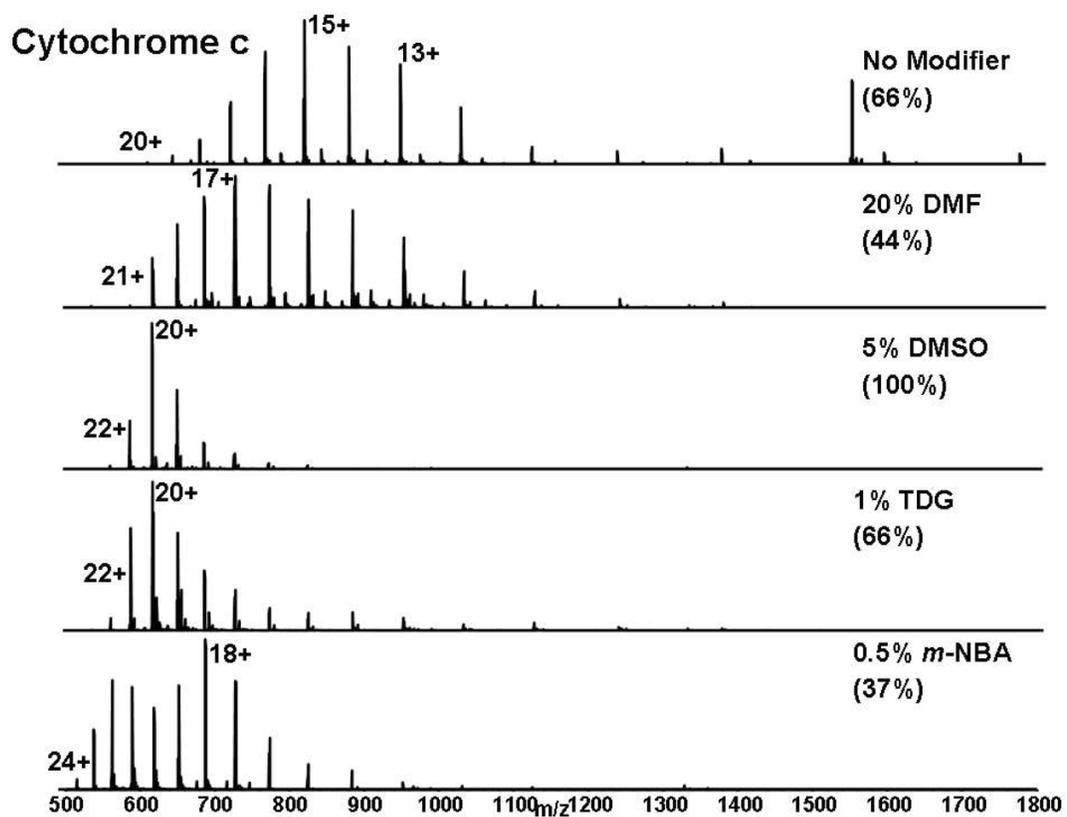48. Cech NB, Enke CG. Anal Chem 2000;72:2717–2723. [PubMed: 10905298]

Figure 1.
Representative mass spectra of cytochrome c from solutions containing the listed modifiers, each at concentration optimal for "supercharging". The highest peak in each spectrum is reported as a percentage of the highest peak in the DMSO spectrum.
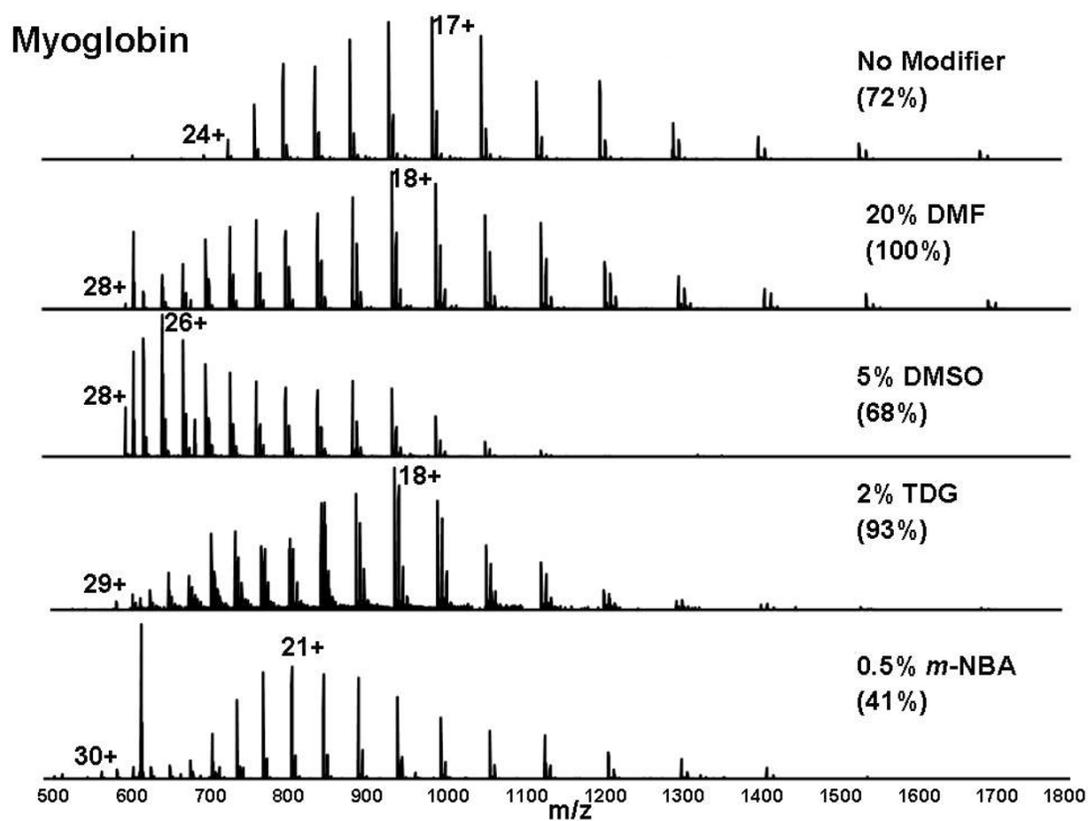
Figure 2.
Representative positive-ion electrospray FT-ICR mass spectra of myoglobin (1 μM), displayed as in Figure 1. The base peak shifts from 17+ with conventional solvents to 26+ with DMSO and 21+ with *m*-NBA. The highest peak in each spectrum is reported as a percentage of the highest peak in the DMF spectrum.
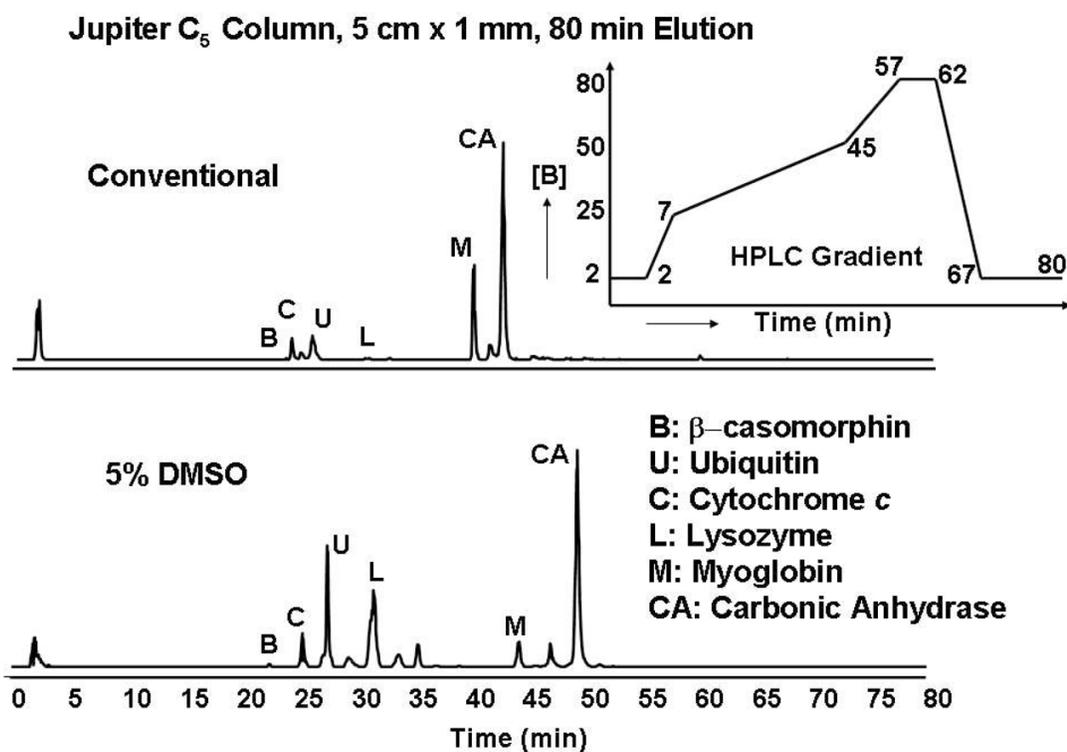
Figure 3.
Elution profiles for 6 standards for a Jupiter C$_5$ column with conventional (top) and 5% DMSO-modified (bottom) electrospray solvents. The gradient elution profile is shown in the inset.
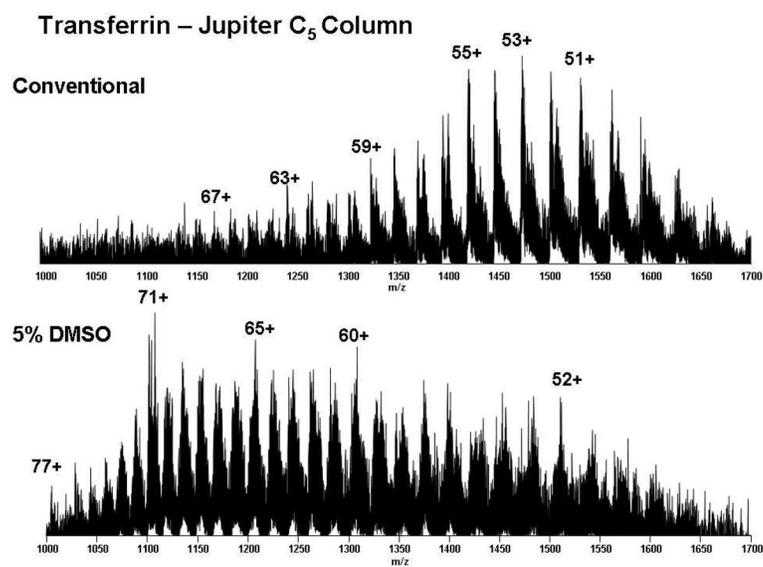
Figure 4.
Charge state distribution for transferrin (78 kDa) eluted from a Jupiter C_5 column with conventional and 5% DMSO-modified solvents. The gradient profile is as in Figure 3.