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The Use of Chromium(III) to Supercharge Peptides by Protonation at Low Basicity Sites

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Abstract

The addition of chromium(III) nitrate to solutions of peptides with seven or more residues greatly increases the formation of doubly protonated peptides, $[M+2H]^{2+}$, by electrospray ionization. The test compound heptalaanine has only one highly basic site (the N-terminal amino group) and undergoes almost exclusive single protonation using standard solvents. When Cr(III) is added to the solution, abundant $[M+2H]^{2+}$ forms, which involves protonation of the peptide backbone or the C-terminus. Salts of Al(III), Mn(II), Fe(III), Fe(II), Cu(II), Zn (II), Rh(III), La(III), Ce(IV), and Eu(III) were also studied. While several metal ions slightly enhance protonation, Cr(III) has by far the greatest ability to generate $[M+2H]^{2+}$. Cr(III) does not supercharge peptide methyl esters, which suggests that the mechanism involves interaction of Cr(III) with a carboxylic acid group. Other factors may include the high acidity of hexaaquochromium(III) and the resistance of Cr(III) to reduction. Nitrate salts enhance protonation more than chloride salts and a molar ratio of 10:1 Cr(III):peptide produces the most intense $[M+2H]^{2+}$. Cr(III) also supercharges numerous other small peptides, including highly acidic species. For basic peptides, Cr(III) increases the charge state (2+ versus 1+) and causes the number of peptide molecules being protonated to double or triple. Chromium(III) does not supercharge the proteins cytochrome c and myoglobin. The ability of Cr(III) to enhance $[M+2H]^{2+}$ intensity may prove useful in tandem mass spectrometry because of the resulting overall increase in signal-to-noise ratio, the fact that $[M+2H]^{2+}$ generally dissociate more readily than $[M+H]^+$, and the ability to produce $[M+2H]^{2+}$ precursors for electron-based dissociation techniques.

Keywords

Electrospray ionization; supercharging; chromium(III); peptide; charge state

Introduction

The ability to protonate a molecule using electrospray ionization (ESI) [1] or matrix-assisted laser desorption ionization (MALDI) [2] is an important step in the structural analysis of peptides and other biomolecules by mass spectrometry. While singly charged ions are

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Supplemental Material

Additional ESI spectra obtained from solutions of various peptides with Cr(III) are provided, along with ESI spectra for the protein myoglobin mixed with Cr(III) and with the organic supercharging reagent m-NBA.

observed almost exclusively in MALDI experiments on small biomolecules, ESI has the ability to produce multiply charged ions. In the sequencing of peptides and proteins by tandem mass spectrometry (MS/MS) [3–5], multiple charging can have several advantages. These include shifting the m/z of ions to a range of the spectrum where resolution is optimal [6] and increasing the ion intensity for mass spectrometers in which the signal detected is proportional to charge [7,8]. In addition, higher charge state ions from peptides and proteins generally require less energy to initiate dissociation and often provide more sequence-informative fragmentation than lower charge state ions [9–12]

The ability to multiply charge a peptide is particularly important for the MS/MS techniques electron capture dissociation (ECD) [13–15] and electron transfer dissociation (ETD) [16–18]. In ECD and ETD, an electron is transferred to the precursor ion, resulting in a radical species that dissociates to yield structurally-informative product ions. ETD and ECD generally require that the precursor ion be multiply positively charged because addition of an electron to a singly charged ion forms a neutral species whose dissociation products are also neutrals and are not detected by mass spectrometry. An issue that has somewhat limited the utility of ECD and ETD in proteomics research is that acidic or neutral peptides may not doubly (or sometimes even singly) protonate by ESI [19]. However, the analysis of natural and post-translational peptides containing acidic groups is important. For example, acidic peptides are common in biological processes related to neurology [20–22], blood coagulation [23,24], and HIV infection [25]. In addition, acidic peptides have been studied as potential vaccines for malaria [26] and HIV [27,28].

Williams and coworkers [29] have developed a method for increasing the protonation of biomolecules by the addition of “supercharging” reagents to the solution being electrosprayed. The supercharging reagent is usually a small organic molecule and its presence causes the number of protons added to the analyte to increase, leading to more highly charged ions. Compounds found to promote supercharging include *m*-nitrobenzyl alcohol (*m*-NBA) [29–32], glycerol [32], tetramethylene sulfone (sulfolane) [33–35], dimethyl sulfoxide (DMSO) [36], dimethylformamide (DMF) [37], and several benzyl alcohol and nitrobenzene derivatives [34]. These organic reagents enrich in concentration as the more volatile solvent evaporates during ESI. A mechanism proposed by Williams and coworkers for experiments involving denaturing solvents (e.g., methanol) is that the presence of the organic supercharging reagent increases the surface tension of the evaporating droplet, which leads to an increase in protein charging [29,32,36,38]. For non-denaturing solvents (e.g., water) that leave proteins in more folded native-like conformations, the solvent surface tension may be higher than the surface tension resulting from the presence of the organic reagent. In this case, Williams and coworkers proposed that concentration of the organic reagent during droplet evaporation results in chemical or thermal denaturation of the protein [30,35,36,39]. A consequence of a more denatured (unfolded) conformation for a peptide or protein is that a greater number of basic sites are accessible to protons and the charge state distribution produced by ESI increases. Another mechanism, involving interaction of the supercharging reagent with the biomolecule, has been proposed by several groups [33,40,41]. Flick and Williams have also reported that lanthanum(III) chloride can supercharge proteins [41]. In addition, an electrothermal supercharging method has been developed that involves ESI from aqueous ammonium or

sodium salt solutions, with the extent of protonation being dependent on solution conditions and on the ESI capillary temperature and spray potential [42–45]. An important factor is protein unfolding induced by gas bubbles arising from the heated salt solutions [45]. A unique feature of electrothermal supercharging is that the number of protons added to the protein is often greater than the number of highly basic sites; in contrast, organic supercharging reagents normally do not protonate beyond the number of highly basic sites on the protein or peptide.

The majority of supercharging studies have involved proteins. Only four studies [11,12,46,47] have included smaller peptides. Madsen and Brodbelt [12] used m-NBA to increase the charge on ions produced for a peptide with twelve residues; however, the number of protons added did not exceed the number of basic residues. Jensen and coworkers [11] incorporated 0.1% m-NBA into the solvent system during a liquid chromatography (LC) analysis of 33 peptides from a tryptic digest. They found that the average charge state for the peptide ions increased by approximately +0.25. Consequently, many peptides could be dissociated from the 3+ charge state rather than 2+, which caused an increase in ETD fragmentation efficiency and a better success rate in identifying the peptide sequences. (With the exception of a 16-residue peptide, these peptides did not protonate in excess of the number of highly basic sites in their sequences.) Tysbin and coworkers [46] demonstrated a dual-sprayer process for supercharging using several large proteins and also substance P, an 11-residue peptide that has three highly basic sites. The intensity of the 3+ charge state for substance P was tripled, but additional protonation along the backbone to form more highly charged ions did not occur. Meyer and Komives [47] performed LC-MS/MS experiments on proteolytic peptides eluted from mobile phases containing m-NBA or DMSO. They found that m-NBA increased the peptide ion charge states by approximately +1, while DMSO decreased peptide charging and resulted in charge state coalescence.

During a recent study of the collision-induced dissociation (CID) of peptides cationized by the addition of metal salts to the electrosprayed solution, we noted that chromium(III) nitrate, $\text{Cr}(\text{NO}_3)_3$, caused the neutral peptide heptaalanine (A7) to doubly protonate [48]. In contrast, when Cr(III) was not present in the solution, A7 produced exclusively singly charge peptide, $[\text{M}+\text{H}]^+$. This formation of $[\text{M}+2\text{H}]^{2+}$ was unexpected because A7 has only one highly basic site, the N-terminal amino group. In general, ESI protonates peptides at only the most basic sites, which are the N-terminal amino group and the side chains of arginine, lysine, and histidine residues [49]. (Although this generalization does not always hold true, the exceptions are usually proteins and not small peptides [50,51].) Because the side chain of alanine is a methyl group that will not protonate, the second proton must be located at an amide group of the peptide backbone or at the C-terminus.

In the present study, the use of Cr(III) as a supercharging reagent for small peptides was explored. Several other metal ions were also evaluated as supercharging reagents, although none proved to be as effective as Cr(III).

Experimental

Peptides and Reagents

The peptides A7, AAAAAGA, and EEEEGDD were purchased from Biomatik (Cambridge, Ontario, CA). Cytochrome c from bovine heart (12.3 kDa) and myoglobin from equine heart (17.0 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other peptides were synthesized in our laboratory with an Advanced ChemTech (Louisville, KY, USA) Model 90 automated peptide synthesizer using standard Fmoc procedures [52]. The peptides were used as synthesized, without further purification (which accounts for impurity peaks in the mass spectra shown). All peptide synthesis reagents were purchased from Advanced ChemTech or VWR (Radnor, PA, USA). Peptide methyl esters were produced by acid-catalyzed esterification with methanol, as discussed previously [53].

All metal salts were purchased from VWR or Thermo Fisher Scientific (Waltham, MA, USA). The organic supercharging reagents, DMSO and m-NBA, and the ESI solvents, HPLC grade acetonitrile and methanol, were also purchased from VWR. Deionized and distilled water was produced with a Barnstead E-pure system (Dubuque, IA, USA).

Peptides were dissolved to 1 mg/ml in a solvent of methanol:water at a 50:50 volume ratio. From these stock solutions, the peptide solutions studied by ESI were generated at a final peptide concentration of 10 μ M in acetonitrile:water at a volume ratio of 50:50. The proteins cytochrome c and myoglobin were studied at a concentration of 5 μ M in both acetonitrile:water and methanol:water solutions, each a 50:50 volume ratio. Prior to ESI, metal salts or acids were added to these peptide or protein solutions at the concentrations discussed below. The pH of the solutions being electrosprayed was measured with a Thermo Scientific Orion pH meter.

Mass Spectrometry

All studies were performed using a Bruker (Billerica, MA, USA) HCTultra PTM Discovery System high capacity quadrupole ion trap mass spectrometer. The ESI source has a grounded needle. A high voltage of -3.5 kV was placed on the platinum coating at the entrance to a glass capillary and also on the stainless steel endplate and capillary entrance cap. The capillary exit voltage was 103 V. The drying gas was nitrogen with a temperature of 300°C and a flow rate of 5–10 L/min. The nebulizer gas was also nitrogen and the pressure was optimized between 5–10 psi to obtain the best ESI signal. Samples were infused at a flow rate of 2.5 μ L/min with a KD Scientific (Holliston, MA, USA) syringe pump. Single stage mass spectra were acquired in the positive ion mode. All spectra shown here were obtained from signal averaging of 600 scans. The ETD experiment for EEEEGDD was also performed on this instrument using fluoranthene as the reagent anion and a cation/anion reaction time of 200 ms.

For nanospray (nanoESI) experiments, sample was introduced using a syringe pump connected to fused silica transfer tubing (360 μ m x 50 μ m) and emitters (360 μ m x 75 μ m x 15 μ m). NanoESI emitters were purchased from New Objective (Woburn, MA, USA). A sample flow rate of 5–20 μ L/h was employed. There was no nebulizer gas. Nitrogen was the drying gas, used at a temperature of 120–150°C and a flow rate of 3–10 L/min.

Results and Discussion

Effects of Selected Metal Ions on Supercharging of A7

Heptaalanine, A7, was employed as the test peptide to study the ability of various metal ions to promote supercharging. Trivalent salts of aluminum (Al), chromium (Cr), iron (Fe), rhodium (Rh), lanthanum (La), and europium (Eu) were studied, as well as a tetravalent salt of cerium (Ce) and divalent salts of manganese (Mn), iron (Fe), copper (Cu), and zinc (Zn). Table 1 lists the metal salts studied and provides absolute signal intensity of $[M+2H]^{2+}$ for several replicate trials using solutions with a peptide concentration of 10 μ M and a metal ion concentration of 100 μ M (metal:peptide molar ratio of 10:1). Table 1 also includes the measured pH for all solutions, information on the intensities of metal adduct ions found in the mass spectra, and reference values for pertinent physical properties of the metal ions.

Figure 1 shows typical mass spectra obtained for several mixtures of A7 with metal salts. For comparison, Figure 1(a) provides the ESI mass spectrum of a 10 μ M solution of A7 in a solvent system of 50:50 (v:v) acetonitrile:water with no added metal ion. In the absence of metal ion, $[M+2H]^{2+}$ is not produced. As seen in Figure 1(b), the presence of Cr(III) causes $[M+2H]^{2+}$ to form in an intensity roughly equal to that of $[M+H]^+$. Addition of the other metal ions generally produces $[M+2H]^{2+}$, but in an intensity too low to be analytically useful; see, for example, the spectra of Figure 1(c)–(e). Mn(II), Zn(II), and Rh(III) are the only metal ions investigated that do not generate $[M+2H]^{2+}$. Although not included in the current study, Co(II), Ni(II), and Cu(I) did not significantly supercharge A7 in our previous CID study of metal-A7 adducts [48].

Optimal Conditions for Supercharging A7 Using Cr(III)

Several molar ratios of Cr(III) to A7 were tested to determine the best conditions for supercharging. With a metal:peptide molar ratio of 5:1 or less, a slight increase in $[M+H]^+$ intensity was observed, although very little formation of $[M+2H]^{2+}$ occurred. The maximum intensity of $[M+2H]^{2+}$ was found at a 10:1 metal:peptide ratio. Greater concentrations of Cr(III) generally did not increase the $[M+2H]^{2+}$ intensity further but instead lead to a gradual decline in intensities for both 2+ and 1+ species. Also, addition of Cr(III) to the solution caused peptide adduct ions containing sodium or potassium ions to greatly decrease in intensity, although Cr(III) adduct ions formed instead. The most commonly observed Cr(III) adduct ion is $[M+Cr-H]^{2+}$.

Using Cr(III) and A7 in a 10:1 molar ratio, two organic solvent systems were tested, 50:50 (v:v) acetonitrile:water and 50:50 (v:v) methanol:water. The relative intensities of doubly and singly protonated A7 ions were similar with both solvents. However, with acetonitrile:water the absolute intensity for both ions was approximately double that found for methanol:water. This is probably because, even without addition of Cr(III), the intensity for singly protonated A7 ions was greater in acetonitrile:water. (For small peptides, the ESI source used here generally yields a more intense signal with the acetonitrile:water solvent.) These experiments suggest that either solvent system is suitable for supercharging. Because the overall ion intensity was greatest with acetonitrile:water, this solvent was used for all other experiments involving peptides.

The identity of the negative counter ion was considered because anions affect protonated ion generation by ESI in ways that relate to their structure and their acid/base properties [54–56]. As the intensity data in Table 1 show, the nitrate salts of chromium(III) and iron(III) produced an $[M+2H]^{2+}$ signal for A7 that was almost twice as intense as the signal produced with the corresponding chloride salts. Chloride anions are more basic than nitrate anions [56], which suggests that chloride anions may be less effective in enhancing peptide protonation. Acetate buffers are often employed in LC-MS experiments due to the volatility of the resulting neutrals and their ability to minimize salt-induced signal suppression in ESI [57,58]. An attempt was made to study the acetate salt for Cr(III), $Cr(OAc)_3 \cdot H_2O$. However, the solubility of $Cr(OAc)_3 \cdot H_2O$ in aqueous solutions is low [59] and we were unable to dissolve this salt to an adequate extent for the present study. Also, a low solubility salt is not desirable as an ESI solution additive because solid can clog the needle.

Ion production by nanoESI [60] was studied because this technique has a lower sample flow rate into the source and a greater tolerance for salt impurities than ESI. Low flow rates have been reported to increase ion intensity [61]. The flow rate in our ESI experiments was $\sim 2.5 \mu L/min$, while the nanoESI flow rate was $\sim 0.2 \mu L/min$. Without Cr(III), almost no $[M+2H]^{2+}$ was produced using either nanoESI or ESI. Addition of Cr(III) to the A7 solution increased the $[M+2H]^{2+}$ intensity to the same extent for both nanoESI and ESI. No advantage was seen in using nanoESI for supercharging A7. Therefore, all other experiments in this study employed ESI.

Supercharging Other Peptides with Cr(III)

In addition to A7, the ability of Cr(III) to supercharge peptides was studied using a variety of neutral, basic, and acidic peptides. These experiments employed the nitrate salt, $Cr(NO_3)_3 \cdot 9H_2O$, with a 10:1 molar ratio of Cr(III) to peptide. In all cases for peptides of seven residues or more, the addition of Cr(III) greatly enhanced the production of $[M+2H]^{2+}$.

Peptides with five residues or less were not observed to form $[M+2H]^{2+}$. The small peptides studied were the tripeptides GAA and SSS and the pentapeptides GGGGG (G5) and AAAAA (A5). The short chain lengths of these peptides may inhibit their ability to accept two protons due to Coulomb repulsion of neighboring protons. Douglass and Venter have shown empirically that production of a protein or peptide charge state in the highest intensity requires that there be at least three uncharged residues between the charge sites [62].

The ability of hexapeptides to undergo enhanced protonation depends on the sequence. The peptides GGGGGG (G6), AAAAAA (A6), and ASSAAA did not supercharge. Several other hexapeptides (as discussed below) produced $[M+2H]^{2+}$, although usually in lower intensities than were seen for similar hepta- and larger peptides.

A variety of neutral peptides with exclusively alkyl side chains were studied. These included the 7-residue peptides AGAAAAA, AAAAAGA, AGGAAAA, AAIAAAA, AALAAAA, AAVAAAA, and GGAVAAA; the 8-residue peptide AGGAAAAA; the 9-residue peptide AGGAAAAAA; the 13-residue peptide A13; and the 14-residue peptide A14. All neutral

peptides produced only $[M+H]^+$ in the absence of Cr(III), and abundant $[M+2H]^{2+}$ when Cr(III) was added to the solution. (As an example, the spectrum for AGGAAAA can be found in Supplemental Figure 1.) The 6-residue peptide AVGIGA also generated $[M+2H]^{2+}$ in the presence of Cr(III), but in low abundance. While addition of Cr(III) to the heptapeptides generally produced $[M+2H]^{2+}$ in an abundance nearly equal to that of $[M+H]^+$, for the hexapeptide AVGIGA the intensity of the 2+ ion was only about 10% of the intensity of the 1+ ion upon Cr(III) addition. In addition, two hexapeptides with neutral side chains containing heteroatoms, TAAAAA and AAAAAN, undergo a limited amount of supercharging.

The analysis of peptides containing basic residues can also benefit from the addition of Cr(III). Figure 2 shows mass spectra for the heptapeptide AHAAAAA, where H is the basic histidine residue. As seen in Figure 2(a), a low intensity of $[M+2H]^{2+}$ formed even without Cr(III), which is consistent with the fact that the sequence includes highly basic sites at the N-terminal amino group (residue 1) and at the side chain of histidine (residue 2). However, the absolute intensity of $[M+2H]^{2+}$ increased from 0.2×10^6 in the absence of Cr(III) to 5.8×10^6 in the presence of Cr(III). This is a nearly 30-fold increase in $[M+2H]^{2+}$ intensity due to the use of Cr(III) as a supercharging reagent. A similar affect was observed for the heptapeptide, GVAKAAAA, which has a basic lysine residue (K). For both peptides, not only did Cr(III) shift the predominant charge produced from 1+ to 2+, but the number of peptide molecules being protonated doubled or tripled. This is illustrated in Figure 2, where the combined 1+ and 2+ absolute intensity in the absence of Cr(III) is 2.4×10^6 , but is 6.6×10^6 when Cr(III) is added. For neutral and acidic peptides, Cr(III) shifts the charge state (causing 2+ to form in place of 1+), but a dramatic increase in the overall intensity of protonated ions does not always occur.

Peptides containing acidic residues can be difficult to analyze by MS/MS in the positive ion mode because they may form protonated ions in low abundance. However, our studies show that acidic peptides can be readily supercharged in the presence of Cr(III). Again, these peptides produce little or no $[M+2H]^{2+}$ in the absence of Cr(III), but form $[M+2H]^{2+}$ upon addition of Cr(III). Our studies included 7-residue peptides of the types XAAAAAA, AAAXAAA, and AAAAAAX, where X = aspartic acid (D) and glutamic acid (E). (As an example, Supplemental Figure 1 includes the ESI mass spectrum for AAEEAAA with addition of Cr(III).) In addition, with Cr(III) the following 6-residue peptides supercharged: DAAAAD, DAAADA, EAAAEA, EAAAAE, and AAEEEA. For the hexapeptides, glutamic acid residues facilitated more supercharging than aspartic acid residues. In general, with glutamic acid the intensity of $[M+2H]^{2+}$ is 70–100 % of the intensity of $[M+H]^+$, while this value is only 10–30 % for aspartic acid. The side chain of glutamic acid has one more methylene group than the side chain of aspartic acid. The longer glutamic acid side chain may better coordinate to Cr(III) and facilitate supercharging.

Several highly acidic peptides were found to produce sufficient $[M+2H]^{2+}$ for further study by MS/MS techniques upon addition of Cr(III). These peptides include heptaaspartic acid (D7), heptaglutamic acid (E7), and the biological peptide hirudin(54–65), which is involved in blood clotting and has the sequence GDFEEIPEEYLQ. In addition, the highly acidic peptide EEEEGDD readily supercharged, as is shown in Figure 3. The intensity of $[M$

+2H]²⁺ increased by about 25-fold when Cr(III) was added to the solution and the number of peptide molecules being protonated doubled [Figure 3(a) versus Figure 3(b)]. With added Cr(III), [M+2H]²⁺ was sufficiently intense to be studied by MS/MS techniques and, as an example, the ETD spectrum of this ion is shown in Figure 3(c). The signal-to-noise ratio (S/N) is excellent and cleavage occurs at every residue, allowing the peptide to be readily sequenced from this spectrum. EEEEGDD is a fragment of low-molecular weight chromium-binding substance (LMWCr, also known as chromodulin), a biological peptide found in humans and animals that is involved in metabolism [63–65] and may have utility in diabetes treatment [66]. We have recently sequenced a biological form of this peptide, pEEEGDD [67]. This was a challenge because the high acidity of the peptide resulted in only deprotonation by ESI and MALDI. The negative ion mode CID and post-source decay (PSD) spectra contain an inconsistent mixture of backbone cleavage ions that often include intense water loss. We interpreted these spectra and sequenced the peptide conclusively only after synthesizing two candidate peptides and comparing their negative ion MS/MS spectra to those of the biological peptide. The project would have been much simpler if Cr(III) had been used to protonate the peptide, followed by either CID or ETD in the positive ion mode.

Comparison of Cr(III) to Organic Supercharging Reagents

The ability of Cr(III) to supercharge cytochrome c (bovine heart) and myoglobin (equine heart) was explored. Cytochrome c is a 102-residue protein with 24 highly basic residues (including the N-terminus), while myoglobin has 122-residues with 31 highly basic residues (including the N-terminus). These proteins were selected for study because they have been the subject of several reports using organic supercharging reagents [29,31,33,37,38,46]. Addition of Cr(III) had no effect on the protonation of either cytochrome c or myoglobin; the spectra did not show significant addition of even one extra proton in comparison to the spectra of solutions without added Cr(III). Spectra involving myoglobin are provided in Supplemental Figure 2. These experiments were performed in two solvent systems, 50:50 (v:v) methanol:water and 50:50 (v:v) acetonitrile:water. In both solvents, the protein concentration was 5 μ M and Cr(III) to protein molar ratios of 10:1, 20:1, and 30:1 were employed.

For comparison, we separately added two organic supercharging reagents, m-NBA [29–32] and DMSO [36], to cytochrome and myoglobin solutions. Extensive supercharging consistent with literature reports [29,31,33,37,38,46] was observed using both the methanol:water and the acetonitrile:water solvents. Following the procedure of Tsybin and coworkers [46], the solutions also contained 0.1 % formic acid. The concentration (by volume) of supercharging reagents was 0.5 % for m-NBA and 1–5 % for DMSO.

Attempts were also made to supercharge cytochrome c and myoglobin by adding m-NBA and Cr(III) to the same solution. Both solvents systems were tested, the m-NBA concentration was 0.5 %, and the Cr(III):protein molar ratio was 10:1. The addition of Cr(III) to the solutions had no effect on supercharging. The ESI spectra for the protein solutions with supercharging from added m-NBA were virtually identical both with and without Cr(III).

In the converse experiment, the organic supercharging reagents m-NBA (at 0.5 %) and DMSO (at 1 %) were added to solutions of our test peptide A7. Again, experiments were conducted with both solvent systems. The result was an extremely minor formation of $[M+2H]^{2+}$, which was not of sufficient intensity to be useful in MS or MS/MS experiments. This greatly contrasts the ability of Cr(III) to produce $[M+2H]^{2+}$ in high abundance for A7.

These results suggest that organic supercharging reagents (e.g., m-NBA and DMSO) work best for protonation of larger molecules such as proteins, while Cr(III) excels at adding protons to smaller molecules such as peptides. Thus, organic supercharging agents and Cr(III) probably affect protonation by different mechanisms.

Factors that May Contribute to the Ability of Cr(III) to Supercharge

The metal ions studied were selected to test the effects of specific properties on supercharging ability. These properties are discussed below, with pertinent reference and experimental values given in Table 1.

One factor to consider in $[M+2H]^{2+}$ formation is the acidity of the solution. In aqueous solutions, metal ions promote acidity by undergoing hydrolysis:

Cr(III) forms the hexaaquachromium(III) ion, $[Cr(H_2O)_6]^{3+}$, which has an hydrolysis dissociation constant (pK_1) in the range of 3.8–4.2 [68,69]; this is the acid dissociation constant (pK_a) of the aquo-metal complex. This low pK_1 value is consistent with our observation that the addition of Cr(III) to an A7 solution lowers the pH to 5.3 ± 0.3 ; in contrast, the pH of the A7 solution without metal ion is 7.8 ± 0.3 . As the values in Table 1 indicate, aquo-metal complexes of divalent metal ions have higher pK_1 values than complexes of trivalent metal ions. Thus, divalent metal ions produce less acidic solutions than trivalent metal ions, which is consistent with our pH measurements. The divalent metal ions that were studied provide only a weak to non-existent enhancement of $[M+2H]^{2+}$ intensity for A7, which may indicate that these ions do not provide an adequately acidic environment for supercharging. However, acidity is not the only factor affecting protonation. The most acidic metal ion in our study is Ce(IV), which has a pK_1 of -1.1 for its aquo-complex [68] and a solution pH of 3.6 ± 0.2 when mixed with A7; however, Ce(IV) produced only a low intensity of $[M+2H]^{2+}$ for A7.

Acidifying the peptide solution in the absence of a metal ion also does not doubly protonate A7. Experiments were performed in which the A7 solution was acidified with 0.5–2.0 volume % of acetic acid, trifluoroacetic acid, hydrochloric acid, nitric acid, and an acidic buffer. A pH of 5.0 in the absence of the metal ions does not cause A7 to supercharge. Even when the pH was lowered to a highly acidic value of pH 2.0 using nitric acid, only a very low abundance of $[M+2H]^{2+}$ forms. Also, addition of acetic acid to the solution containing A7 and Cr(III) does not result in a greater increase in protonation relative to the use of Cr(III) without added acid.

A noteworthy feature is that Cr(III) does not readily reduce. The standard reduction potential, E^0 , for Cr(III) reduction to Cr(II) is -0.407 V [70]. In contrast, Fe(III) much more readily reduces to Fe(II), with E^0 of $+0.771$ V [70]. As the data in Table 1 indicate, Fe(III) was found to doubly charge A7 in an intensity roughly one-third that of Cr(III). Of the metal ions studied, Fe(III) is second only to Cr(III) in the ability to supercharge A7. However, we did not find Fe(III) salts to hold promise as supercharging reagents because the $[M+2H]^{2+}$ intensity that they generated was unstable. There was a pronounced ion intensity fluctuation and the $[M+2H]^{2+}$ signal sometimes went away entirely. This is illustrated by the mean $\pm$ standard deviation ion intensity values of Table 1, which are 3.7 ± 2.2 ($\times 10^5$) for iron(III) chloride and 5.1 ± 4.5 ($\times 10^5$) for iron(III) nitrate. With this same ESI source, we have also observed unstable Fe(III)-peptide ion signals during studies of metallated peptides [71].

Electrochemical processes are known to occur during ESI [72–74] and it is possible that reduction of the metal ion is causing instability of the $[M+2H]^{2+}$ signal generated using Fe(III). In the positive ion mode for the ESI source used here, the sample solution sprays out of a grounded needle into an electric field resulting from a high negative voltage applied at the end plate and capillary entrance region. Using this general ESI source design, Hoppilliard and coworkers [75] observed reduction of Cu(II) to Cu(I) (E^0 of $+0.158$ [70]) in metal-peptide complexes. These researchers found that the electron capture was dependent on the ESI source voltages and current, and noted that reducing conditions could be obtained easily and inadvertently. In fact, Hoppilliard and coworkers stated that the ideal system for studying this effect is Fe(III) reducing to Fe(II), which occurs more readily than reduction of Cu(II) to Cu(I). With regard to our present work, Ce(IV) very readily reduces to Ce(III) with an E^0 of $+1.72$ [70], which may limit the ability of Ce(IV) to supercharge peptides. Like Cr(III), the trivalent ions of Al, La, Eu, and Rh do not readily reduce. However, unlike Cr(III) and Fe(III), they do not promote supercharging for A7. Our conclusion is that the ability of a metal ion to resist reduction does not cause a metal ion to be a supercharging agent, but may allow the metal to remain in a form that is optimal for supercharging.

A distinguishing property of Cr(III) is the kinetic inertness of its complexes in aqueous solution [76–78]. The residence time of a water ligand in the first hydration shell around Cr(III) is 2.0×10^{12} μ s (23 days) [69]. In comparison, the residence time of a water ligand around Fe(III) is only 316 μ s [69]. Residence time for water ligands might correlate to the interactions of the metal ions with oxygens at the peptide backbone. To test this hypothesis, our study also included Rh(III), which like Cr(III), is known for its kinetic inertness to water exchange [79]. The residence time of a water ligand at Rh(III) is 3.2×10^{13} μ s [69]. Al(III), which has a residence time of 6.3×10^6 μ s [69], was also investigated. However, neither Rh(III) nor Al(III) were found to be good supercharging reagents. Thus, the residence time of water and other oxygen-containing functional groups around the metal ion does not significantly affect supercharging.

Metal ion size is another consideration in characterizing supercharging ability. There might be an optimal metal ion size to facilitate interactions of the ion with the peptide backbone and promote protonation. In support of this theory, the two trivalent metal ions that cause the greatest supercharging, Cr(III) and Fe(III), have similar sizes. The ionic radius in solid crystals of Cr(III) is 75.5 pm, while the ionic radius of Fe(III) is 78.5 pm [69]. At 67.5 pm,

the ionic radius of Al(III) is only slightly smaller than that of Cr(III) and Fe(III). All three metal ions also have the same estimated hydrated ionic radius in water, 450 pm [80]. In addition, at 80.5 pm, the ionic radius of Rh(III) is similar to that of Cr(III). However, Rh(III) and Al(III) are poor supercharging reagents. Therefore, while metal ion size may influence supercharging, it is not the major factor that causes Cr(III) and Fe(III) to supercharge.

In comparing the physical properties listed in Table 1 for the metal ions studied, the species most similar to Cr(III) is Rh(III). Yet, Cr(III) induces abundant supercharging, while Rh(III) induces none. This suggests that the primary reason that Cr(III) causes supercharging is not related to a physical property, but a chemical property. Chromium is a first row transition metal, while rhodium is a second row transition metal. Although their physical properties may be similar, the first and second transition series often have very different chemical properties. In general, first row metals are more reactive and form a greater number and diversity of metal-ligand complexes than second row metals [81]. Another factor supporting the premise that the ability of Cr(III) to supercharge relates to a chemical property is the fact Fe(III) also induces facile supercharging (although in an erratic manner). Like chromium, iron is a first row transition metal and their trivalent ions frequently have similar chemical properties; for example, both Cr(III) and Fe(III) are members of the “iron group” of classical qualitative inorganic analysis [82] and both are transported in the human body by the glycoprotein transferrin [83].

Our experiments suggest that Cr(III) and organic supercharging agents enhance protonation by different mechanisms. One supercharging mechanism proposed by Williams and coworkers is that organic supercharging agents cause a protein to undergo conformational changes during droplet evaporation by ESI, which makes highly basic sites more accessible [30,35,36,39,84,85]. This is probably not the mechanism with Cr(III) because the peptides studied here are sufficiently small that backbone folding is unlikely to make basic sites entirely inaccessible. More important, these neutral and acidic peptides do not have a second highly basic site to protonate, but instead are undergoing protonation of a backbone amide group or the C-terminus to form $[M+2H]^{2+}$. Changes in surface tension of the ESI droplets due to the addition of the organic reagents has also been proposed to play a role in protein supercharging [29,32,36,38], although other studies suggest that surface tension may be of limited importance [86,87]. Surface tension is unlikely to be a factor in Cr(III) supercharging because the ability of a metal ion to affect aqueous surface tension primarily relates to ionic charge and not identity (e.g., Ca^{2+} and Mg^{2+} salts have almost the same effect on surface tension) [88].

Supercharging with Cr(III) resembles electrothermal supercharging [42–45] in that both methods can add a greater number of protons than there are highly basic sites on the biomolecule. Electrothermal supercharging is believed to result from conformational changes and denaturation when a biomolecule is exposed to factors that include temperature, voltage, ionic strength, and the development of gas bubbles in solution. However, the “over-protonating” of proteins by electrothermal supercharging probably does not involve the peptide backbone or C-terminus but instead results from protonation of the side chains on less basic residues (i.e., proline, tryptophan, and glutamine) [51]. The alkyl side chain peptides studied here, including A7, have no residues with side chains that will protonate.

The Role of Cr(III)-Peptide Interactions in Supercharging

A proposed supercharging mechanism that is likely to be a factor for Cr(III) is interaction of the supercharging reagent with the biomolecule. Douglass and Venter suggested that the formation of adducts of cytochrome c and sulfolane are responsible for supercharging [33]. Based on data from thermal studies, Chingin et al. proposed that the supercharging mechanism involves direct interaction [40]. Flick and Williams suggested that La(III) adduction to the protein led to increased Coulomb repulsion and caused protein unfolding [41]. For supercharging with Cr(III), metal ion-peptide interaction goes along with the premise that a chemical factor is at work rather than a physical property.

Chromium(III) coordinates in solution to ligands with oxygen [78,89–93] or nitrogen [94–98], although this is not unique among transition metal ions. Chromium(III) is a hard Lewis acid that prefers to bind to oxygen rather than nitrogen [99]. (The other hard metal ions in this study are Fe(III) and Al(III).) The binding of Cr(III) to oxygen ligands is usually strong and often involves coordinate covalent bonds [81]. Little is known of the ability of Cr(III) to bind to peptides, with the exception of work on the chromium-binding peptide found in animals, LMWCr. LMWCr is a small highly acidic peptide of 10–12 residues that tightly binds four Cr(III) [100–102]; in fact, the EEEEGDD fragment of LMWCr (whose mass spectra are shown in Figure 3) binds four Cr(III) in solution [66]. In solution, carboxylic acid groups on the side chains of the acidic residues and at the C-terminus are involved in Cr(III) binding. Also, both Cr(III) and Fe(III) are transported in animals by transferrin, and the two metal ions have very similar binding affinities to this glycoprotein [83].

An hypothesis that carboxylic acid groups are involved in the Cr(III) supercharging mechanism is supported by the solution-phase data on Cr(III) binding to LMWCr and by our experimental observations that hexa- and heptapeptides with acidic residues supercharge with the identity of the acidic residue (glutamic acid versus aspartic acid) affecting protonation. To test this hypothesis, we removed the C-terminal carboxylic acid groups of A7, AAVAAAA, and AAIAAAA by converting these peptides to methyl esters. ESI on these peptide methyl esters showed no increased $[M+2H]^{2+}$ signal when Cr(III) was added. In contrast, the acid forms of all three peptides supercharge in abundance upon addition of Cr(III) to produce $[M+2H]^{2+}$ at near equal intensity to $[M+H]^+$.

Although no protonation enhancement occurs upon addition of Cr(III), even without Cr(III) the three methyl esters form $[M+2H]^{2+}$ at ~30% the intensity of $[M+H]^+$. This is interesting because the acid forms of these peptides produce almost no $[M+2H]^{2+}$ without Cr(III). This may be a conformational effect, with the methyl esters having more open structures due to less participation of the C-terminus in hydrogen bonding [53]. Another possibility is that, for peptide acids, zwitterions of the type $[M+2H-H]^+$ are present; that is, some peptide molecules may be doubly protonating by ESI even without addition of Cr(III) but are not observed as 2+ ions due to C-terminal deprotonation. (Several studies have indicated that gas-phase peptide zwitterions involving protonation/deprotonation exist [103–107].) If this is the case, peptide methyl esters might form $[M+2H]^{2+}$ without the addition of Cr(III) because their C-terminus cannot be deprotonated to produce a zwitterion. Also, this leads to the speculation that Cr(III) may be generating $[M+2H]^{2+}$ for peptides by interacting with the

C-terminal carboxylic acid group and hindering its ability to deprotonate and form a $[M + 2H-H]^+$ zwitterion.

Interaction of Cr(III) with peptide C-terminal or side chain carboxylic acid groups appears to be an important step in the supercharging mechanism. Chromium(III) forms almost exclusively hexacoordinate complexes of octahedral geometry [81]. Therefore, it is possible that several carbonyl oxygens along the peptide backbone coordinate at multiple sites of the Cr(III) octahedron in solution or during drying in ESI as coordinating water is stripped from the droplet. Using scale models, we found that at least four carbonyl oxygens on the A7 backbone can coordinate at octahedral sites of Cr(III) without severe distortion of the backbone. Such interaction might assist in transfer of a proton from aquo-Cr(III) complexes to the peptide backbone or the C-terminus.

The mechanism for supercharging may involve a water-solvated complex of chromium(III) and the peptide; for example, $[M+Cr-H+n(H_2O)]^{2+}$. Desolvating collisions in the ESI source could lead to dissociation of the complex to form $[M+Cr-H]^{2+}$ (which is observed in abundance in many of the spectra, see Figures 1(b) and 3(b)) or to form $[M+2H]^{2+}$ through elimination of $Cr(OH)_3$ or $Cr(OH)_3(H_2O)_{n-3}$. Evidence for an intermediate involving water is provided by the presence of $[M+Cr-H+H_2O]^{2+}$ in many of the mass spectra (especially for neutral and acidic peptides). The intensity of this ion relative to $[M+2H]^{2+}$ is variable; in spectra of A7 with Cr(III) obtained over a period of a year, the relative intensity of $[M+Cr-H+H_2O]^{2+}$ ranges from 3–30 % of the intensity of $[M+2H]^{2+}$. In addition, the spectra for several peptides contain a low abundance of $[M+Cr-H+2(H_2O)]^{2+}$.

The Location of the Second Proton in Doubly Protonated Peptides

The proton location in $[M+2H]^{2+}$ for peptides lacking residues with basic side chains is an interesting facet of this work. For such peptides, the most basic site is the N-terminal amino group, which is the presumed location of the proton in $[M+H]^+$. For protonated pentaglycine (GGGGG), Wu and McMahon [108] showed with infrared multiphoton dissociation (IRMPD) spectroscopy that the proton resides at the N-terminal amino group with strong hydrogen bonding to the C-terminal carbonyl carbon of the carboxylic acid group; the result is a macrocyclic structure. This alludes to an importance of the C-terminus in protonation.

Because $[M+2H]^{2+}$ was not observed for peptides with less than six residues, the location of the second proton is probably at the sixth residue (or higher), which places the proton at either the C-terminal residue or the penultimate residue for A7. The second proton might reside on the second most basic site, which high level molecular orbital calculations [108–110] indicate is a carbonyl oxygen of an amide group. For hexaglycine (G6), the carbonyl oxygen of the N-terminal residue is only 8.7 kcal/mol less basic than the N-terminal amino group [109]. In triglycine, the largest peptide for which gas-phase basicity calculations involving all heteroatoms have been performed, protonation at the internal carbonyl oxygen requires 7 kcal/mol more energy than protonation at the N-terminal carbonyl oxygen [110]. For triglycine (GGG), the C-terminal carboxylate carbonyl oxygen is of comparable basicity to the amide nitrogens [110], which are widely considered to be gas-phase protonation sites in the mobile proton model of peptide dissociation [9,111]. The fact that converting the C-terminus to a methyl ester prevents supercharging may mean that the second proton resides

at the C-terminus. While this is not as energetically favorable as backbone protonation at an amide oxygen, the driving factor in supercharging may be coordination of Cr(III) to the acidic C-terminus, with proximity to the coordination site determining the protonation site. Thus, at present there is no conclusive evidence regarding whether the second proton in supercharging of A7 and similar peptides is occurring at the peptide backbone or the C-terminus.

Conclusion

The addition of chromium(III) nitrate to solutions being electrosprayed is shown to dramatically increase $[M+2H]^{2+}$ intensity for neutral and acidic peptides that normally produce only $[M+H]^+$. In addition, for slightly basic peptides that produce $[M+2H]^{2+}$ in low abundance, addition of Cr(III) both shifts the charge state distribution to predominantly 2+ and causes the number of ions being protonated to double or triple. This greatly enhanced production of $[M+2H]^{2+}$ has several advantages for peptide sequencing by MS/MS. First, production of abundant $[M+2H]^{2+}$ allows ECD and ETD experiments to be performed on peptides that normally could not be studied because of the need for these electron-base techniques to have multiply charged precursor cations. Second, for CID, which is the most common dissociation technique in peptide sequencing, the amount of structurally-informative fragmentation is generally greater for $[M+2H]^{2+}$ than for $[M+H]^+$. This is why biological peptide sequencing by CID in proteomics research usually involves 2+ precursor ions [112,113]. Finally, the large increase in signal intensity for $[M+2H]^{2+}$ precursor ion results in a corresponding increase in S/N of the MS/MS spectra. A limiting factor in the ability of bioinformatics techniques to correctly identify peptides is mass spectra with low S/N [114,115]. This makes selecting peaks from noise difficult and results in false positive identifications for peptides. The ability of Cr(III) to increase both charge and ion intensity in the ESI mass spectra could prove to be very useful in peptide analysis and sequencing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

ESI mass spectra of solutions containing a 10:1 molar ratio of metal:A7 for: (a) no metal, (b) Cr(III), (c) Zn(II), (d) La(III), and (e) Eu(III). For Figure 1(c), the low intensity peak below the arrow labelled “No $[M+2H]^{2+}$ ” is not the ion of interest, but a singly charged impurity.

Figure 2. ESI mass spectra of AHAAAAA with (a) no Cr(III) and (b) Cr(III) at a 10:1 molar ratio of Cr:AHAAAAA.

Figure 3.

ESI mass spectrum of EEEEGDD with (a) no Cr(III), (b) Cr(III) at a 10:1 molar ratio of Cr:EEEGDD, and (c) ETD on $[M+2H]^{2+}$ produced by addition of Cr(III).

Table 1

Absolute signal intensity for $[M+2H]^{2+}$ from heptalaanine (A7), pH of the solutions, and properties of the metal ions.

Metal Salt	Atomic Number of Metal	Ionic Charge	Coordination Number of Metal ^a	Metal Adducts in ESI MS Spectra ^b	$[M+2H]^{2+}$ Absolute Intensity ($\times 10^5$), $x \pm s$	pH of Solution, $x \pm s$	pK ₁ ^c	E ⁰ , V ^d	Residence Time, μs^e	Ionic Radius, pm ^f	Hydrated Ionic Radius, pm ^g
Al(NO ₃) ₃ •9H ₂ O	13	3+	4,5,6	n	1.5±1.4	4.3±0.1	4.97	– ^h	6.3 x 10 ⁶	67.5	450
CrCl ₃ •6H ₂ O	24	3+	6	m	7.9±0.6	5.3±0.3	4.0	–0.407	2.0 x 10 ¹²	75.5	450
Cr(NO ₃) ₃ •9H ₂ O	24	3+	6	m	17.3±3.9	5.2±0.1	4.0	–0.407	2.0 x 10 ¹²	75.5	450
MnCl ₂ •4H ₂ O	25	2+	4,5,6	m	0	7.1±0.1	10.59	–	0.0316	97	300
FeCl ₃ •6H ₂ O	26	3+	4,5,6,8	n	3.7±2.2	3.7±0.6	3.05	+0.771	316	78.5	450
Fe(NO ₃) ₃ •9H ₂ O	26	3+	4,5,6,8	n	5.1±4.5	3.5±0.2	3.05	+0.771	316	78.5	450
FeSO ₄ •7H ₂ O	26	2+	4,6,8	m	2.0±0.4	5.6±0.2	9.5	–	0.32	75	300
CuCl ₂	29	2+	4,5,6	m	1.5±1.1	5.6±0.2	8.0	+0.153	5.0 x 10 ⁴	87	300
Zn(NO ₃) ₂ •6H ₂ O	30	2+	4,5,6,8	w	0	6.6±0.1	8.96	–	0.032	88	300
RhCl ₃	45	3+	6	n	0	6.8±0.3	3.4	–	3.2 x 10 ¹³	80.5	450
La(NO ₃) ₃ •6H ₂ O	57	3+	6,7,8,9,10,12	s	1.8±1.4	5.9±0.6	8.5	–	0.050	117.2	450
(NH ₄) ₂ Ce(NO ₃) ₆	58	4+	6,8,10,12	w	1.4±1.1	3.6±0.2	–1.1	+1.72	–	101	550
Eu(NO ₃) ₃ •6H ₂ O	63	3+	6,7,8,9	w	1.6±1.4	5.9±0.4	7.8	–0.36	–	108.7	450

^aCoordination numbers are from reference [116].

^bThe metal adduct formed with Cr(III) and La(III) is $[M+Met-H]^{2+}$. The metal adduct with Mn(II), Fe(II), Cu(II) and Zn(II) is $[M+Met]^{2+}$. The metal adducts with Eu(III) are $[M+Met]^{3+}$ and $[M+Met-H]^{2+}$. Adduct ion intensity is compared to the intensity of $[M+H]^+$ with n = no adduct formed, w = weak intensity at <30%, m = medium intensity at 30–65%, and s = strong intensity at >65%.

^cpK₁ values for dissociation of the first proton from the aquo-metal complex are at 298.15K and from references [68,69].

^dStandard reduction potentials, E⁰, for the removal of one electron at 298.15K and 1atm are from reference [70].

^eResident times for water exchange from the aquo-metal complex at 25 °C are from reference [69].

^fIonic radii of the metals in a crystalline solid are from reference [117]. The metal ions where low and high spin forms are known, the radii listed is for the spin that is most common for the aquo-complex.

^gEstimated hydrated ion radii are from reference [80].

^h– indicates that the value is not available in the literature, usually because the value cannot be measured