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Information for proteomics: ESI-MS titration by sodium ions gives the number of carboxylate groups in peptides

Oleg Timofeev[a,1], Mei M. Zhu[b], Michael L. Gross[b,*]

[a] *Department of Pharmaceutics, College of Pharmacy, Rutgers University, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA*
[b] *Department of Chemistry, Center for Biomedical and Bioorganic Mass Spectrometry, Campus Box 1134, Washington University, One Brookings Drive, St. Louis, MO 63130, USA*

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Abstract

We studied the electrospray ionization (ESI) of various peptides containing amino acids with acidic side chains to test whether the pattern of molecular ion peaks provides information on the number of acidic side chains. When we increased the concentration of sodium salt in the ESI solution containing a peptide, a characteristic pattern arose, and it represents the various sodium salts of carboxylic acid and an additional sodium ion to add charge to the species. Both C-terminal and side chain carboxylic acid groups readily form sodium carboxylates. After reaching full substitution of all acidic hydrogens for sodium ions, further increases in the concentration of sodium cations do not lead to the attachment of additional sodium atoms. This effect allows counting of acid residues in a peptide. This information is easily acquired and may be an appropriate supplement to exact mass measurements and partial sequence data that are currently used in proteomic database searches.

Keywords: Peptides; Sodium ion interactions; Carboxylate groups; ESI-MS; Proteomics

1. Introduction

Objectives for studying alkali metal ion adducts of neutral molecules are to evaluate the mechanism and reaction rates of the association reactions, to understand the site and nature of the binding, and to measure alkali metal ion affinities for a variety of functional groups in inorganic, organic, and biologically important molecules [1–5]. It is possible that the fragmentations of metal ion-bound species are complementary to those of protonated or deprotonated species and provide another source of structural information. For peptides, this information could prove to be a valuable input to database searching in proteomics. Thus, uncovering the kinetics and mechanisms of fragmentation of the complexes is an important motivation for studying metal ion complexes.

The analytical use of metal ions in the gas phase as a structural probe for biologically important molecules has attracted the attention of many laboratories [5–13]. The metal ions and other simple cations represent a broad range of Lewis acids. Their sites of the attachment to a neutral molecule and their ability to react preferentially with various functional groups are varied and interesting. The attachment of a metal ion followed by activation often results in specific fragmentation pathways of the cationized molecules.

The results are important in various fields involving atmospheric chemistry, chemistry of solvents, catalysis, and ion transport through biological membranes. The important, and by now well known, advantage of measurement in the gas phase is that the properties revealed are intrinsic and can be used for modeling of biological and other processes where metal cations play an important role. The two alkali metal ions, Na^+ and K^+, are widely involved in interactions with peptides, proteins, and nucleic acids, and the study of such interactions has a 30-year history [1].

Mass spectrometric determination of the masses and primary structures of peptides is of interest in many areas of biochemistry and biology but particularly in proteomics. Enzyme digestion followed by mass spectral analysis of the resulting mixture of peptides and database searching based on the masses, accurate masses, and/or sequences of the pep-

* Corresponding author. Tel.: +1-314-935-4814; fax: +1-314-935-7485.
E-mail address: mgross@wustl.edu (M.L. Gross).
URL: http://www.chemistry.wustl.edu/~msf.
[1] Present address: Mylan Pharmaceuticals Inc. 3711 Collins-Ferry Road, Morgan town, WV 26505, USA.

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tides is now an accepted procedure for protein identification [14–20]. Even with this extensive information, several alternative proteins are often found in the database search, and that brings uncertainty in protein identification. The number of possible candidates dramatically decreases when additional information on a protein under investigation is available. One example is accurate mass tags [21], and another is the count of basic sites in peptides from the extent of addition of HI to peptide cations in the gas phase [22].

The strategy for determining primary structure of peptides is overwhelmingly one in which $[M + H]^+$ or multiply protonated species are submitted to fragmentation. Other approaches that would give complementary information are charge derivatization, isotopic labeling, and chemical modification of either N- or C-termini or side chains [13,23]. The source of complementary information, however, should be one that is easily obtained. One source is the fragmentation of deprotonated species (e.g., $[M - H]^-$) [24,25]. Another involves introducing adducts of peptides with metal ions, which are often present in the biological sample or can be easily added. The MS/MS of alkali metal adducts is certainly different than that of protonated peptides and may permit stepwise sequencing from the C-terminus of the peptide [26–28].

In this article, we report preliminary results on a simple mass spectrometric approach to form complexes of peptides and alkali metal ions. No MS/MS is required. Rather the number of metal ions that are added to form a series of adducts reveals in a simple way the number of acidic amino acids that are present in the peptide.

2. Experimental

2.1. Materials

The peptides were prepared by solid-phase synthesis by using fluorenylmethoxycarbonyl chemistry on a Synergy 432A peptide synthesizer (Applied Biosystems, Foster City, CA, USA) or Symphony/Multiplex peptide synthesizer (Protein Technologies, Tucson, AZ, USA). The synthesized peptides were purified by C18 reverse-phase HPLC, and the purity of each peptide was verified by MALDI mass spectrometry on a Voyager RP-DE MALDI-TOF (PerSeptive Biosystems, Framingham, MA, USA). The various salts and solvents were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Protocol

The sodium ion adducts were formed in a series of experiments in which the concentration of a sodium salt was increased in mixtures containing the various peptides. The ratio of the peptide concentration to that of the alkali metal salt was varied from 1:1 to 1:20.

For Na titrations conducted at Rutgers University, the intention was to spray the peptides from ammonium formate solutions at various pH values. Stock solutions of the peptides in water (~1 mM) were diluted in 1:1 water:methanol solution containing 0.1% formic acid. The pH of the solution was adjusted by adding sufficient ammonium hydroxide to give pH values of 6.2, 7.0, and 8.0. The final concentration of the peptide was of 50 μM. The pH was adjusted by adding sufficient ammonium hydroxide to give a pH in the range of 6.2–8.0. Titration by NaCl was carried out by adding 10 mM aqueous NaCl in 2-μl increments to the peptide solutions. After mixing for 5 m, the resulting solution was infused into the electrospray ionization (ESI) ion source of a PE Sciex API 365 mass spectrometer (Applied Biosystems) in the Q1 scan mode via a syringe pump with a flow rate of 3 μl/min.

For the first-round experiments at Washington University (called "LCQ-A"), the titrations were carried out as described above except sodium nitrate was used instead of sodium chloride and a LCQ Classic ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) was used. For the second round of experiments at Washington University (called "LCQ-B"), a ratio of 1:20 for peptide to NaOAc was used. 5 μl of 1 mM peptide aqueous stock solution was mixed with 5 μl of 20 mM NaOAc aqueous stock solution. The resulting solution was diluted 50 times with H_2O to prepare 500 μl of a final solution containing 20 μM peptide and 400 μM NaOAc (pH ~ 8). For analysis, 5 μl was used in each loop injection, and this plug of peptide solution was carried into the ESI source of the LCQ with 50:50:1 CH_3CN:H_2O:CH_3COOH at a flow rate of 5 μl/min. The positive-ion, full-scan mode was used. The spray voltage was 5.0 kV, and the capillary temperature was 200 °C. Data, which were taken while sodium acetate solution was sprayed, were processed using Xcalibur 1.3; 35 scans were summed and smoothed (7-point, boxcar smoothing) for each acquisition.

3. Results and discussion

The hypothesis tested in this work is that the number of sodium ion adducts that can be detected by ESI mass spectrometry is related to the number of acidic functions in a peptide. To test the hypothesis, we synthesized a set of eight pentapeptides of the sequences $D_nV_{(5-n)}$ ($n = 0$–4) and two other pentapeptides. We then obtained the ESI mass spectra on two different mass spectrometers (see Table 1). The information base for testing the hypothesis and for utilizing this idea is simply the appearance of a cluster of various ions, $[M + H]^+$, $[M + Na]^+$, $[M - H + 2Na]^+$, etc. No more than the normal mass spectrum would be required, allowing one to conduct a relatively simple experiment to obtain data that would be complementary to that obtained when principally protonated species are followed.

The basis for the hypothesis is that at a sufficient concentration of Na^+, all carboxyl groups will be converted to sodium salts. Thus, a peptide with a single carboxyl group at the C-terminus (RCOOH) will be introduced into the gas

Table 1
Molecular ion patterns resulting from ESI-MS of various pentapeptides in the presence of sodium salts

Number of acidic	Peptide	Instrument[a]	Relative abundance (%)						
			0 Na[b]	1 Na[b]	2 Na[b]	3 Na[b]	4 Na[b]	5 Na[b]	6 Na[b]
0	STWYV	LCQ-B	100	27	27				
	VVVVV	LCQ-B	100	19	29	8			
1	ARNDC	LCQ-B	100	22	14	12			
	DVVVV	API 365	5	64	32	100			
		LCQ-B	100	70	27	48	5		
2	DDVVV	LCQ-A	26	100	31	26	58		
		LCQ-B	100	55	20	19	19	3	
	DVDVV	API 365	18	100	29	25	27		
		LCQ-B	100	46	19	14	14	1	
	DVVDV	LCQ-A	39	100	39	28	32		
		LCQ-B	100	37	16	15	16	4	
	DVVVD	LCQ-B	99	100	36	24	27	5	
3	DDDVV	API 365	9	100	48	42	34	46	5
		LCQ-B	100	25	12	12	9	9	
4	DDDDV	LCQ-B	100	34	13	12	11	9	7

[a] API 365: data taken with this mass spectrometer at Rutgers University. LCQ-A: first-round data taken with an ion-trap (LCQ) at Washington University; LCQ-B: second-round data taken with the LCQ at Washington University 2 years later (see Section 2 for details).
[b] 0 Na refers to $[M+H]^+$, 1 Na to $[M+Na]^+$, 2 Na to $[M-H+2Na]^+$, etc.

phase as three ions: $[M+H]^+$, $[M+Na]^+$, $[M-H+2Na]^+$. A different peptide with one acidic side chain and a free carboxyl group at the C-terminus will enter the gas phase as four ions: $[M+H]^+$, $[M+Na]^+$, $[M-H+2Na]^+$, and $[M-2H+3Na]^+$. The peaks are readily identified because they belong to a series whose members are separated by 22 units. The interpretation of the data is also straightforward: the number of free carboxylic acids in an unknown peptide is one less than the maximum number of sodium ions seen in a cluster.

The peptides contained adventitious sodium salts, and their mass spectra expectedly showed peaks corresponding to $[M+Na]^+$ ions. If no measures were taken post synthesis to remove traces of Na^+, the abundance of these ions was typically 10% of the abundance of the base peak, which corresponds to the $[M+H]^+$ ion. Gradual increase of concentration of the sodium salt, such that the concentration of the sodium salt became 10–20 times that of the peptide, decreases the abundance of the $[M+H]^+$ ions and increases the intensity of peaks corresponding to the peptide–sodium adducts. These adduct ions have the general formula: $[M+n\mathrm{Na}-(n-1)\mathrm{H}]^+$. When members of the series contain more than one sodium ion, it is reasonable to propose that the ions are mainly the products of the reaction between carboxyl groups of a C-terminus and those of side chains with the sodium salt. The acidic hydrogen atoms of carboxyl groups are easily exchangeable for Na.

The peptides DDVVV, DVDVV, DVVDV, and DVVVD contain two aspartic acid (D) residues and a total of three carboxylic groups (including that at the C-terminus). We expected a maximum number of readily adducted Na atoms to be four to give an $[M+4Na-3H]^+$ ion. This is achieved as seen in typical spectra (Fig. 1A and B) of two isomeric peptides, each of which can readily take up three Na^+ to give sodium carboxylates, and a fourth Na^+ to serve as the charge carrier. Those spectra were taken with an ion-trap mass spectrometer using NaOAc as the sodium ion source and a spray

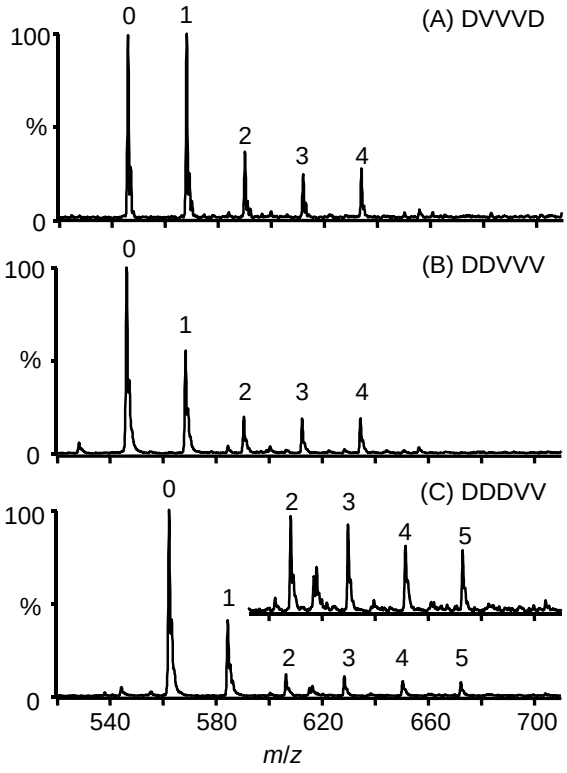

Fig. 1. ESI-MS spectra of three pentapeptides: (A) DVVVD; (B) DDVVV; and (C) DDDVV. 0 refers to $[M+H]^+$, 1 to $[M+Na]^+$, 2 to $[M-H+2Na]^+$, etc.

solvent of acetonitrile and water with a trace of acetic acid. Other examples of this phenomenon are seen in Table 1; for these peptides, either an ion-trap (see solvent conditions above) or a triple quadrupole instrument or both were used. The spray solvent was either acidic acetonitrile/water or acidic methanol/water (see Section 2.2). Both experiments give comparable results, demonstrating that, although the relative abundances of the various $[M + nNa - (n - 1)H]^+$ change, the overall pattern of peaks and the interpretation is consistent from one instrument to another and from one spray solvent to another.

For the peptides VVVVV, DVVVV, DDVVV, DVDVV, DVVDV, DVVVD, DDDVV, DDDDV, containing from 0 to 4 aspartic acid residues, the $[M + nNa - (n - 1)H]^+$ ions emerge from the electrospray, where $n = 2$, 3, 4, 5, and 6 for peptides contain 0, 1, 2, 3, and 4 aspartic acid residues, respectively (see Fig. 1C for one example). One may choose sodium acetate, sodium chloride, or sodium nitrate as the source of the sodium ions. Furthermore, the results were not qualitatively sensitive to the variation of the sample pH, which was adjusted over the range of 6.2–8.0, as described in Section 2.2. It is important, however, that the relative concentration of the sodium ion be at approximately 10 times or more that of the peptide. Further increases of concentration of the sodium salt relative to that of the peptide lead to a decrease of the abundance of all the metal–peptide adduct ions. The outcome of these experiments shows that a titration of a peptide solution with sodium salt allows determination of the maximum number of readily exchangeable hydrogen atoms in a peptide. This number can be used to count the number of easily exchangeable hydrogen atoms (acidic hydrogen atoms of the carboxylic groups).

For 6 of the 15 experiments recorded in Table 1, a sodium adduct containing one sodium ion greater than expected was seen at low abundance. The maximum relative abundance was 8% for VVVVV. The additional sodium adduction should not cause a problem in interpretation for two reasons. There is always a precipitous drop in the sodium adduction once the carboxylic acids have been exchanged as can be seen from two points of view. The adduct that reveals the number of acidic side chains is (1) always greater in abundance than that from additional adduction, and (2) of comparable or greater abundance than the adduct with one less sodium (Table 1). Attachment of the additional sodium may occur because the amide linkage itself is acidic and capable of exchanging a proton for a sodium ion. The reduced acidity of an amide with respect to a carboxylic acid, however, usually means that the uptake of sodium ions in excess of the number of carboxyl groups plus one is minimal. We suggest that any automated interpretation could take into account those minor amounts of excessive exchange of H for Na.

We also investigated two additional pentapeptides, STWYV and ARNDC, one of which has no amino acids with acidic side chains and the other that has one amino acid with an acidic side chain (Table 1). Two and three sodium adducts were observed for these two peptides, respectively. These results also suggest that the slightly acidic side chains of S, T, Y, and C do not exchange with Na^+ as effectively as do the carboxylic acid groups of D (and E).

4. Conclusion

Although these results come from a feasibility study, the results do suggest that we can formulate a rule for predicting sodium adduct formation: the total number of carboxylic acid groups (including that at the C-terminus) is equal to the total number of readily adducted sodium ions minus 1. For some peptides, the number of adducted sodium ions is greater than the rule would allow. For example, in the spectrum of peptide DVVVD (Fig. 1A), which contains two aspartic acid residues, there is also a peak corresponding to $[M+5Na-4H]^+$. The ion is of low abundance (~5%), however, and as discussed above, may be due to exchange at an amide group. For these cases, the rule may be reformulated to focus on the relative abundance of the sodium adducts; the first significant drop in the abundance of adducts gives the number for counting acidic residues.

The results suggest that there are a limited number of sodium atoms that can be readily attached to a peptide or exchanged for a hydrogen ion in a peptide molecule. This number depends principally on the number of acidic hydrogen atoms. In fact, the formation of $[M + Na]^+$ ions can also be considered as an exchange of H^+ by Na^+. This charge-carrying hydrogen atom is the most easily exchanged because it is the most acidic H^+ in the ion. The rapid growth of abundance of $[M + Na]^+$ ions together with rapid decrease of abundance of $[M + H]^+$ ions after the initial addition of the sodium salt to the spray solution confirms this suggestion. The preferred sites of the charge-carrying Na^+ are probably delocalized in a manner that is similar to the "mobile proton" in the $[M + H]^+$ ions [29].

Future studies should be aimed at systematic titrations of a large sample of peptides of varying sizes and hydrophilicities under conditions that mimic those used in proteomics to test the generality of the results reported here.

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