Carbamino Group Formation with Peptides and Proteins Studied by Mass Spectrometry

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At high pH and in the presence of dissolved CO_2, the N-terminus and ε-amino groups of amino acids, peptides, and proteins can form carbamino adducts with CO_2, $R\text{-}NH_2 + CO_2 \leftrightarrow R\text{-}NHCOO^- + H^+$. We report the first study of carbamino group formation by electrospray ionization (ESI) mass spectrometry (MS). Angiotensin II, bradykinin, substance P, and insulin have been studied. A careful optimization of the instrumental parameters was necessary to allow the transfer of the fragile adducts into vacuum for mass analysis. Particularly, dissociation of the adducts in the ion sampling process and pH changes in ESI must be minimized. With these precautions, levels of carbamino group formation of angiotensin II and bradykinin determined from mass spectra agree with those expected to be in solution, calculated from literature equilibrium constants. Thus, ESI MS can quantitatively measure ratios of carbamino adduct to total peptide concentration in solution. Values of equilibrium constants for carbamino group formation with substance P ($pK_c = 4.77 \pm 0.18$) and insulin ($pK_c = 4.99 \pm 0.05$) are reported for the first time. (J Am Soc Mass Spectrom 2010, 21, 1500–1505) © 2010 American Society for Mass Spectrometry

At high pH and in the presence of dissolved CO_2, the N-terminus and ε-amino groups of amino acids, peptides, and proteins can form adducts with CO_2, known variously as carbamino groups, carbamino acids, carbamates, carbamides, or carbo amino groups [1]. The reaction is governed by four simultaneous equilibria:

$$R\text{-}NH_3^+ \leftrightarrow R\text{-}NH_2 + H^+$$

$$K_Z = \frac{[R-NH_2][H^+]}{[R-NH_3^+]} \quad (1)$$

$$R\text{-}NH_2 + CO_2 \leftrightarrow R\text{-}NHCOO^- + H^+$$

$$K_C = \frac{[R-NHCOO^-][H^+]}{[R-NH_2][CO_2]} \quad (2)$$

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$$

$$K_1' = \frac{[HCO_3^-][H^+]}{[CO_2]} \quad (3)$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+$$

$$K_3' = \frac{[CO_3^{2-}][H^+]}{[HCO_3^{2-}]} \quad (4)$$

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where K_z is the acid dissociation equilibrium constant for the amino group, K_c is the association equilibrium constant for formation of the carbamino adduct (including the dissociation of the carbamic acid), K_1' is the equilibrium constant for the hydration of CO_2 and its dissociation to bicarbonate, and K_2' is the equilibrium constant for the dissociation of bicarbonate to carbonate [2].

The formation of carbamino groups alters the electrostatic properties of peptides and proteins and can have many different effects. For example, carbamino groups can change peptide conformation [3], influence peptide degradation and receptor binding [2], and increase the neurotoxicity of amino acids [4]. Carbamino groups increase the stability of insulin dimers [5] and are required for metal binding at the active sites of ribulose-1,5-biphosphate carboxylase oxygenase (RUBISCO) [6] and urease [7]. Carbamino group formation at the N-termini changes the oxygen binding properties of hemoglobin [8–12] and allows hemoglobin to transport carbon dioxide [13–15]. Nearly any peptide or protein that does not have the N-terminus blocked is subject to the formation of carbamino groups [2, 16–18].

To react with CO_2, the amine must be in its neutral form (equilibrium 2). Therefore, a relatively high pH, determined by pK_z (equilibrium1), is required. Peptides and proteins have lower values of pK_z (7–8) than amino acids (ca. 9) and thus carbamino groups can be formed at lower pH (7–8). In addition, the N-terminus of a protein generally has a lower pK_z (7–8) than lysine side chains (pK_z = 9–12), and thus more readily forms carbamino adducts near pH = 8.0 The concentration of CO_2 available for the formation of the carbamino adduct depends on the pH and the concentration of bicarbonate (equilibria 3 and 4). Because $K_2' = 1.74 \times$

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10^{-10} ($pK_2' = 9.76$), dissociation of bicarbonate to carbonate is negligible at pH < 8. If the peptide/protein concentration is low compared to the bicarbonate concentration, the concentration of dissolved CO_2 can be estimated from equilibrium 3 ($K_1' = 6.61 \times 10^{-7}$, $pK_1' = 6.18$) [2]. For example, at pH = 7, $[HCO_3^-]/[CO_2] = 6.6$. At higher pH, this ratio increases, and the concentration of CO_2 decreases.

By combining equilibria 1–3, one can relate K_c, K_1', K_z, $[H^+]$, the total amine concentration ($TA = [RNH_2] + [RNH_3^+] + [RNHCO_2^-]$), the total bicarbonate concentration ($TC = [CO_2] + [HCO_3^-] + [RNHCO_2^-]$) and the mole fraction of adduct ($Z = [RNHCO_2^-]/TA$) by [2]

$$K_c = \frac{Z(K_Z K_1' + (K_1' + K_Z)[H^+] + [H^+]^2)}{K_Z(Z^2(TA) - Z(TC + TA) + (TC))} \quad (5)$$

From eq 5, at a given pH, K_c can be calculated from a measured Z value, or a Z value can be calculated from a known K_c value. For example, at pH 8, in 20 mM HCO_3^-, with angiotensin II, ($pK_z = 7.55$ and $pK_c = 4.80$ [2]), Z = 0.26.

Carbamino group formation has been studied with glycine [3] and other amino acids [4], bradykinin [2], and angiotensin [2], substance P [18], insulin [5], myoglobin (Mb) [3, 19, 20], and hemoglobin (Hb) [8–15, 21, 22]. With proteins such as Mb and Hb, side-chain ε-amino groups can also form carbamino acids. These have higher pK_z values (typically 8.7–12) [23] than the N-terminus, but carbamino acid formation with ε-amino groups of Mb [19] and Hb [24] has been seen in NMR studies. Individual residues could not be resolved, but with Hb at pH 8.5, up to 70% of the carbamino group formation was assigned to ε-amino groups [24].

Formation of carbamino groups has been studied mostly by NMR and various titrimetric methods. In this work, we report the first study of the formation of carbamino groups by electrospray ionization (ESI) mass spectrometry (MS). Bradykinin, angiotensin II, substance P, and insulin have been studied. The equilibrium constants for formation of carbamino groups with the N-termini of angiotensin II and bradykinin have been measured by NMR [2] and are shown in Table 1. We find that despite the high bicarbonate concentrations (20–200 mM) necessary for these experiments, it is possible to record useful ESI spectra and measure ratios of carbamino adduct to free peptide. We show that changes in pH in ESI of sprayed solutions must be minimized, and that the carbamino bond is relatively labile, and so care must be taken to avoid dissociation of the CO_2 adducts in the ion sampling region of the mass spectrometer. With these precautions, levels of adducts to angiotensin and bradykinin agree with calculations from the literature values of pK_c and pK_z. Thus, ESI-MS can be used to quantitatively measure levels of carbamino adducts in solution. Values of pK_c for substance P and insulin are determined for the first time.

Table 1. Equilibrium constants for the formation of carbamino groups with angiotensin II and bradykinin (from [2])

	pK_z	pK_c
Angiotensin II	7.55 ± 0.05	4.80 ± 0.10
Bradykinin	7.14 ± 0.05	5.02 ± 0.10

Experimental

Acetic acid, ammonium hydroxide, ammonium acetate, ammonium bicarbonate, angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, M_r = 1046.2 Da), bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, M_r = 1060.2 Da), substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Gly-Leu-Met-NH_2, M_r = 1347.6 Da), and porcine insulin dimer (M_r = 5778.4 Da) were from Sigma-Aldrich, Vancouver, BC, Canada. Nitrogen, UHP-grade, manufacturer's stated purity 99.999%, was from Praxair, Vancouver, BC, Canada.

Solutions were 20 μM peptide or protein in 20 to 180 mM ammonium acetate or ammonium bicarbonate (water/methanol 90/10 vol/vol). The pH was measured with an Accumet model 15 pH meter (Fisher Scientific, Nepean, ON, Canada) and adjusted to 7.0 or 8.0 with ammonium hydroxide or acetic acid. The concentration of CO_2 in solution was controlled at a given pH by the concentration of bicarbonate (equilibrium 3). Solutions were not degassed. All experiments were done with solutions at room temperature, 22 ± 2 °C. The equilibrium pressure of CO_2 above an aqueous solution of CO_2 is given by $P_{CO2} = K_H x_{CO2}$ where x_{CO2} is the mole fraction of CO_2 in solution, and K_H is Henry's constant. At 22 °C, $K_H = 1.14 \times 10^6$ Torr (or K_H = 26.6 atm M^{-1}) [25]. The concentration of dissolved CO_2 in equilibrium with atmospheric CO_2 ($P_{CO2} = 3.3 \times 10^{-4}$ atm) is calculated to be 1.1×10^{-5} M, considerably less than the concentrations of CO_2 (3.0×10^{-4} to 2.2×10^{-3} M) estimated from equilibrium 3 with bicarbonate 20–180 mM as used here. These calculated concentrations of CO_2 give equilibrium partial pressures of CO_2 of 8.0×10^{-3} to 5.9×10^{-2} atm. Thus CO_2 should come out of solution. Nevertheless, solutions were found to give stable ratios of carbamino intensity to free peptide peak intensity for at least 2 d after preparation, presumably because of the low rate of diffusion of CO_2 out of solution. (The diffusion coefficient of CO_2 in water at 295 K is 1.76×10^{-5} cm^2 s^{-1} [26]; the time for CO_2 to diffuse 1 cm is ca. 8 h).

A home-built ESI triple quadrupole mass spectrometer described previously [27–29] and modified to increase sensitivity was used. Samples are infused into an ESI source with a syringe pump (Harvard Apparatus, Holliston, MA, USA) at 0.5–5 μL/min. Ions formed by pneumatically-assisted electrospray (sprayer voltage = 4500 V) pass through a 2.4 mm diameter aperture in a curtain plate (800 V), a dry nitrogen gas, an ion sampling orifice (0.25 mm diameter) (200–400 V), into a region with a background pressure of 0.6 Torr, through a skimmer (aperture diameter 2.90 mm) (150 V), into a quadrupole ion guide Q0 (9×10^{-3} Torr, N_2) (offset =

120–150 V), through a short radio frequency (rf) quadrupole [30] (offset = 100 V), into a mass analyzing quadrupole Q1 (offset = 117 V). A collision cell with a quadrupole ion guide, Q2 (offset = 108 V), and another quadrupole Q3 (offset = 90 V), follow Q1. Quadrupoles Q2 and Q3 were operated in rf only mode as ion guides. Ion counting was used for detection.

Results and Discussion

Angiotensin II and Bradykinin

Figure 1 shows mass spectra of angiotensin II (Figure 1a) and bradykinin (Figure 1b) in 20 mM ammonium acetate (plain lines) and in 20 mM ammonium bicarbonate (dashed lines), at pH = 8.0. Only doubly charged peptide ions are seen with either salt (ammonium acetate or ammonium bicarbonate). In the presence of 20 mM bicarbonate, adduct peaks higher in *m/z* by 22 Th, corresponding to a mass increase of 44 Da are seen, in addition to the free peptide peaks. The carbamino group is expected to be in the negatively charged carboxyl form at pH 8.0, which would give a mass increase of 43 and a shift to the + 1 charge state, neither of which are seen. Apparently, the negative carboxyl group recombines in ESI. The adduct ion abundance increased with bicarbonate concentration, as expected from equilibrium 2 (see below).

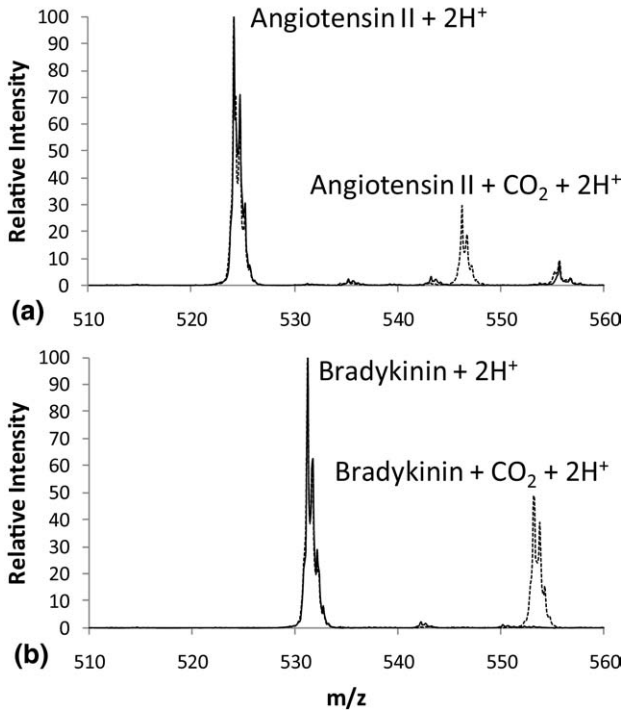

Figure 1. Mass spectra of 20 μM (**a**) angiotensin II and (**b**) bradykinin in 20 mM ammonium acetate (plain lines) and 20 mM ammonium bicarbonate (dashed lines) (water/methanol 90/10 vol/vol, pH = 8.0).

Effects of Operating Conditions

The observed carbamino ion abundance, i.e., the ratio of carbamino peak intensity to total peptide peak intensity, $(RNHCOOH + 2H^+)/((RNHCOOH + 2H^+) + (RNH_2 + 2H^+))$, depends on the operating conditions. The effects of sprayer flow rate (Figure 2a), orifice-skimmer voltage difference (Figure 2b) and skimmer-Q0 offset voltage difference (Figure 2c) on the carbamino ion abundance of bradykinin were investigated. Similar effects were seen with angiotensin II, substance P, and insulin.

Figure 2a shows that the observed level of adduct remains constant for solution flow rates above about 3 μL/min. At lower flow rates the observed level of adduct decreases with decreasing flow rate. This may be due to a decrease of the pH of the sprayed solution. When positive ions are formed in ESI, excess protons are forced into the sprayed solution or formed by electrolytic production, lowering the pH [31]. This change in pH increases as the flow rate decreases. A change of pH from 8.0 to 7.0 can cause the calculated ratio in solution $[RNHCOO^-]/([RNHCOO^-] + [RNH_2] + [RNH_3^+])$ to decrease by ×2 because of a greater degree of protonation of the N-terminus (a decrease of pH by 1 causes $[H^+]$ to increase by ×10, but also $[CO_2]$ to increase ×10 (equilibrium 3). These effects are offsetting). This change is well within the range calculated to occur in ESI [31].

Figure 2b shows the effect of the orifice-skimmer voltage difference on the abundance of the bradykinin carbamino adduct ion. Voltage differences above about 150 V cause the abundance of the adduct ion to decrease. This decrease is attributed to dissociation of the carbamino bond by ion activation in the orifice-skimmer region of the mass spectrometer. Under the conditions where the carbamino bond is dissociated, no additional dissociation of the peptide is seen, indicating that the carbamino bond is weaker than a peptide bond.

Figure 2c shows that as the voltage difference between the skimmer and the offset of Q0 increases, the abundance of the adduct ion decreases. This decrease is also attributed to dissociation of the carbamino bond through ion activation by collisions in Q0, analogous to the injection of ions into the collision cell of a triple quadrupole system.

Effect of Bicarbonate Concentration

Based on the results of Figure 2, the solution flow rate was set to 5 μL/min, the orifice-skimmer voltage difference was set to 75 V, and the skimmer-Q0 voltage difference was set to 16 V. These conditions gave reasonable sensitivity and, most importantly, preserved the CO_2 adducts. The intensities of the adduct ion peaks and the free peptide ion peaks were measured at different bicarbonate concentrations. Figure 3 shows the ratio of carbamino peak intensity to the total intensity of the peptide peaks for angiotensin II (Figure 3a) and

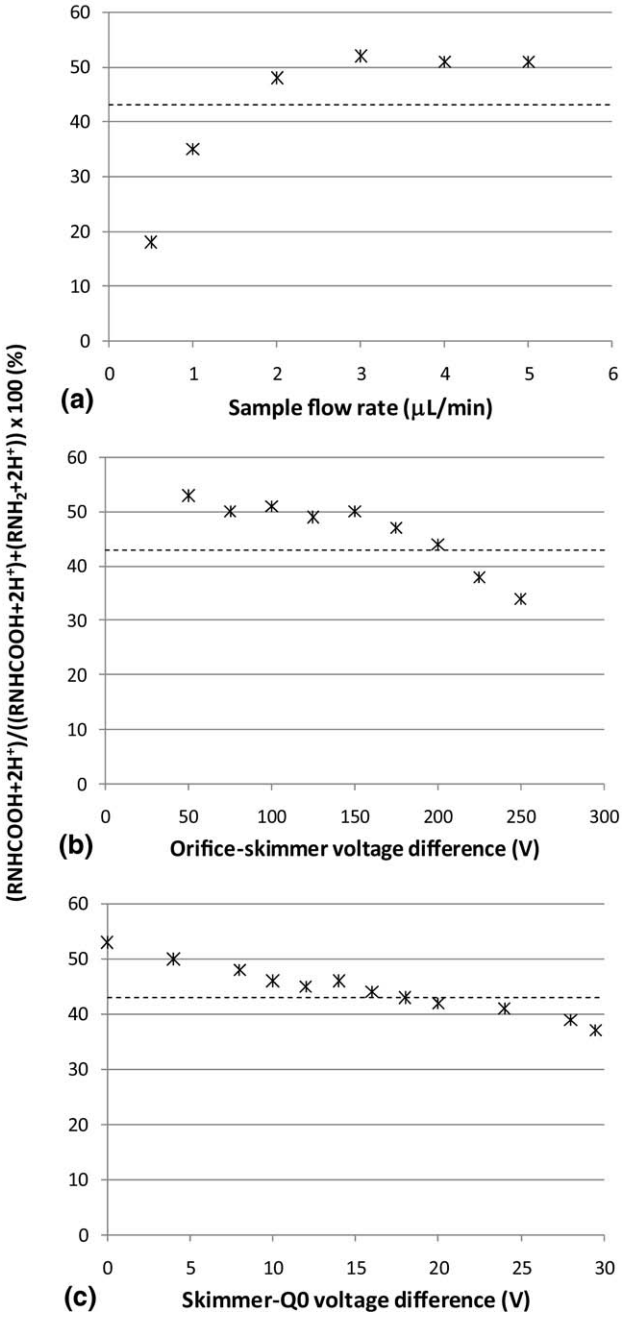

Figure 2. Ratios of intensities (RNHCOOH + $2H^+$)/((RNHCOOH + $2H^+$) + (RNH_2 + $2H^+$)) versus (**a**) sample flow rate, (**b**) voltage difference between the orifice and skimmer, and (**c**) voltage difference between the skimmer and Q0 for 20 μM bradykinin in 60 mM ammonium bicarbonate (water/methanol 90/10 vol/vol, pH 8.0). Dashed lines: concentration ratio in solution, Z, calculated from eq 5 using the equilibrium constants of Table 1.

bradykinin (Figure 3b) versus bicarbonate concentration. The solid lines show the concentration ratios in solution, Z, calculated from eq 5 using the equilibrium constants (pK_z and pK_c) of Table 1. The dashed lines show the highest and lowest ratios calculated from the combined uncertainties of the values of pK_c and pK_z (Table 1). For ammonium bicarbonate concentrations higher than 20 mM, the observed ratios agree within 20% with ratios calculated from the literature pK_c values. Conversely, the K_c values of angiotensin and bradykinin determined from these ratios, [$(1.10 \pm 0.10) \times 10^{-5}$ and $(1.15 \pm 0.22) \times 10^{-5}$ respectively], agree with the literature K_c values [$(1.56 \pm 0.3) \times 10^{-5}$ and $(9.55 \pm 0.2) \times 10^{-6}$ respectively], within about 25%. The corresponding average pK_c value for each peptide calculated from ratios of carbamino peak to total peptide peaks for each bicarbonate concentration agree within 3% with the literature values. Thus, ESI-MS can quantitatively measure the ratio of carbamino adduct concentration to total peptide concentration in solution, provided pH changes in ESI and dissociation of the adducts in the ion sampling process are minimized.

Substance P and Insulin

We have also observed carbamino group formation with substance P and insulin. Carbamino group formation on the α-amino groups (N-termini) of substance P [18] and insulin [5] has been observed previously by NMR, but values of pK_c have not been reported.

Figure 4 shows the mass spectra of 20 μM substance P in 20 mM ammonium acetate (plain line) and in 20 mM

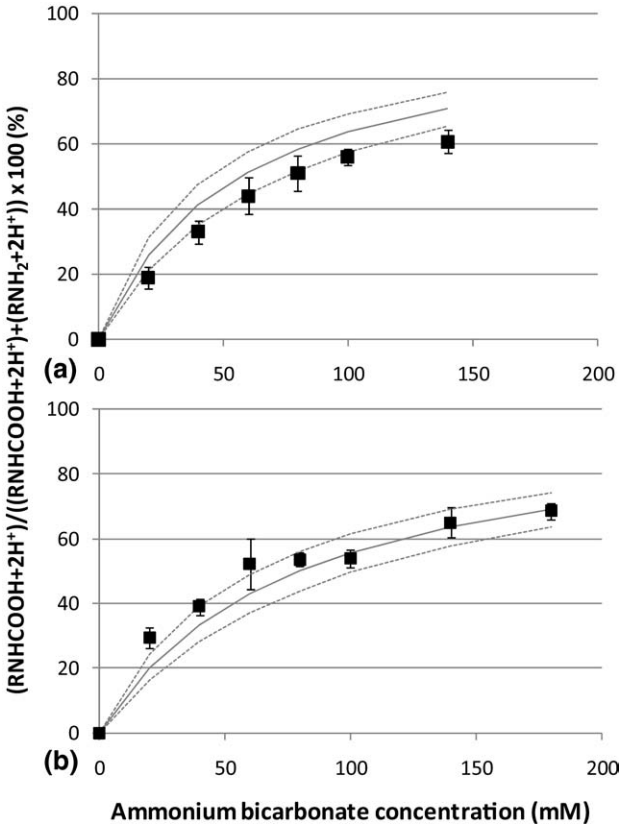

Figure 3. Ratios of intensities (RNHCOOH + $2H^+$)/((RNHCOOH + $2H^+$)+(RNH_2 + $2H^+$)) for 20 μM (**a**) angiotensin II and (**b**) bradykinin in ammonium bicarbonate (water/methanol 90/10 vol/vol, pH = 8.0) versus ammonium bicarbonate concentration. Filled squares: average values and standard deviations of three measurements. Plain grey lines: values calculated from literature K_z and K_c; dashed lines: values calculated from the uncertainties of the literature values.

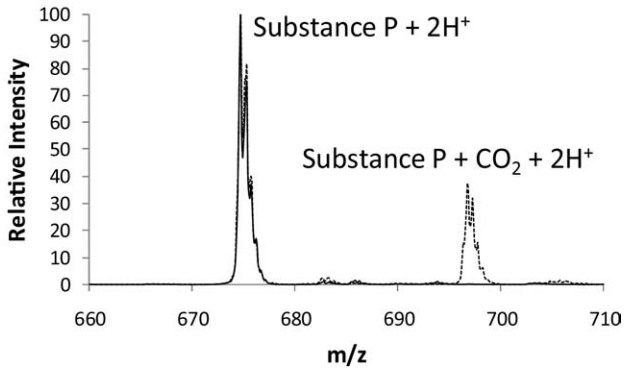

Figure 4. Mass spectra of 20 μM substance P in 20 mM ammonium acetate (plain lines) and 20 mM ammonium bicarbonate (dashed lines) (water/methanol 90/10 vol/vol, pH = 7.0).

ammonium bicarbonate (dashed line), at pH = 7.0. Doubly charged ions dominate the spectra and the adduct corresponding to a mass increase of the peptide ion of 44 Da is seen, as with bradykinin and angiotensin II.

The mass spectra of 20 μM insulin in 23 mM ammonium acetate (plain lines) and in 23 mM ammonium bicarbonate (dashed lines), at pH = 8.0 are shown in Figure 5a. Charge states +3 and +4 dominate the spectrum. Figure 5b shows a blow-up of the region near the +4 peak in the mass spectrum. In ammonium acetate, a peak corresponding to a mass increase of 17 Da (attributed to an NH_4^+ adduct) can be observed, in addition to the free protein peak. This adduct is also seen with the +3 peak, in a similar proportion. Other minor peaks (<10%), presumably corresponding to various salt adducts, are detected. They are neglected in this study. In ammonium bicarbonate, an adduct corresponding to a mass increase of 44 is seen for the free insulin peak and for the NH_4^+ adducts. Only one CO_2 adduct is seen. Three residues in insulin can form carbamino adducts near pH 7.0: Phe B1, pK_z = 7.2 [32]; Gly A1, pK_z = 7.9 [32]; Lys B29, pK_z 7.8 [32]. Because Phe B1 has the lowest pK_z, it may be responsible for most of the carbamino formation at pH = 7.0, but we cannot rule out formation of adducts to Gly A1 or Lys B29.

Figure 6 shows the relative abundance of carbamino ions versus bicarbonate concentration for substance P (Figure 6a) and insulin (Figure 6b). For insulin, the

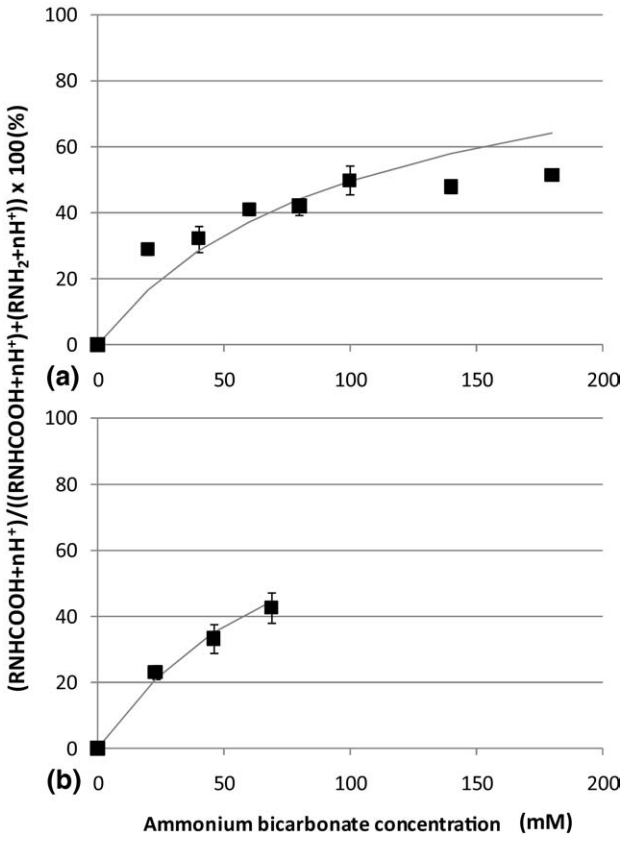

Figure 6. Ratios of intensities (RNHCOOH + nH^+)/((RNHCOOH + nH^+)+(RNH_2+nH^+) for 20 μM (**a**) substance P and (**b**) insulin in ammonium bicarbonate (water/methanol 90/10 vol/vol), (**a**) pH = 7.0 and (**b**) pH = 8.0, as a function of ammonium bicarbonate concentration. (**a**) n = 2, (**b**) n = 3 and 4. Black squares: average values and standard deviations of three measurements. Grey lines: calculated ratios for (**a**) pK_c = 4.77, pK_z = 7.10 and (**b**) pK_c = 4.99, pK_z = 7.5.

intensity ratio (r) is measured on background subtracted spectra as follows:

$$r = \frac{\sum_{n=3}^{4}((RNHCOOH + nH^+) + (RNHCOOH + NH_3 + nH^+))}{\sum_{n=3}^{4}((RNHCOOH + nH^+) + (RNHCOOH + NH_3 + nH^+)) + \sum_{n=3}^{4}((RNH_2 + nH^+) + (RNH_2 + NH_3 + nH^+))}$$

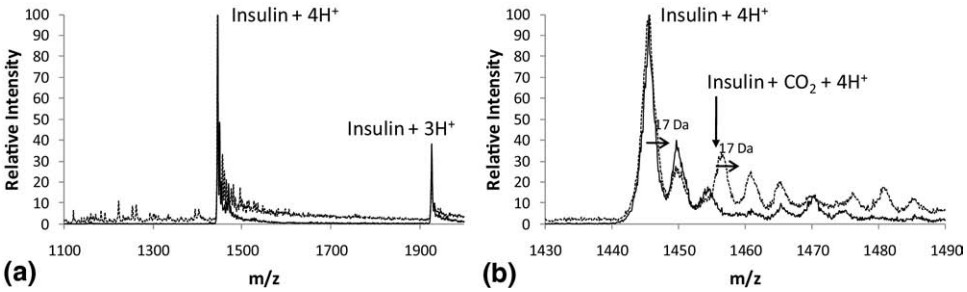

Figure 5. Mass spectra of 20 μM insulin in 23 mM ammonium acetate (plain lines) and 23 mM ammonium bicarbonate (dashed lines) (water/methanol 90/10 vol/vol, pH = 8.0). (**a**) Full mass range and (**b**) +4 charge state region.

With insulin the highest carbonate concentration was limited to 69 mM. At higher concentrations, the ion intensities were too low to allow useful measurements. From the observed levels of carbamino adducts and the known pK_z values of substance P (7.10 [33]) and porcine insulin (7.5, average value between the pK_z of the two insulin chains: 7.2 and 7.9 [32]) a value of pK_c can be calculated for each data point. Averaging these values, we determine pK_c of substance P is 4.77 ± 0.18 and of insulin 4.99 ± 0.05. The solid lines in Figure 6 show the ratios calculated from these pK_c values.

This work shows that ESI-MS offers a new method for the quantitative study of carbamino group formation in peptides and small proteins. In comparison to the NMR and titrimetric methods used previously, smaller amounts of material are required and the measurements can be done more rapidly. Attempts were made to observe carbamino group formation with larger proteins, especially myoglobin. Unfortunately, with the gentle ion sampling conditions needed for this work, the formation of other adducts (Na^+, NH_4^+, bicarbonate) hinders the detection of CO_2 adducts. Higher mass resolution, such as provided by ion cyclotron resonance or other mass spectrometers may be required in this case. In the future, it may be possible to study formation of carbamino groups and the binding of metal ions to carbamino groups [34] with larger proteins such as hemoglobin.

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