Fragmentation of peptide negative molecular ions induced by resonance electron capture

Yury V. Vasil'ev,^{1,2,a)} Benjamin J. Figard,³ Jeff Morré,⁴ and Max L. Deinzer^{1,4,a)}

¹Department of Chemistry, Oregon State University, Corvallis, Oregon 97331, USA

²Department of Physics, Bashkir State Agriculture University, Ufa 450001, Russia

³Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, USA

⁴Environmental Health Science Center, Oregon State University, Corvallis, Oregon 97331, USA

(Received 19 May 2009; accepted 2 July 2009; published online 27 July 2009)

A simple robust method to study resonance gas-phase reactions between neutral peptides of low volatility and free electrons has been designed and implemented. Resonance electron capture (REC) experiments were performed by several neutral model peptides and two naturally occurring peptides. The assignment of negative ions (NIs) formed in these gas-phase reactions was based on high mass-resolving power experiments. From these accurate mass measurements, it was concluded that fragment NIs formed by low (1–2 eV) energy REC are of the same types as those observed in electron capture/transfer dissociation, where the positive charge is a factor. The main feature resulting from these REC experiments by peptides is the occurrence of z_n-1 ions, which are invariably of the highest abundances in the negative ion mass spectra of larger peptides. $[M-H]^-$ NIs presumably the carboxylate anion structure dominate the REC spectra of smaller peptides. There was no evidence for the occurrence of the complementary reaction, i.e., the formations of c_n+1 ions. Instead, c_n ions arose without hydrogen/proton transfer *albeit* with lower abundances than that observed for z_n-1 ions. Only the amide forms of small peptides showed more abundant ion peaks for the c_n ions than for the z_n-1 ions. The mechanisms for the N–C $_{\alpha}$ bond cleavage are discussed. © 2009 American Institute of Physics. [DOI: 10.1063/1.3186747]

I. INTRODUCTION

Electron-ion interactions used in mass spectrometric techniques such as electron capture dissociation (ECD),¹ electron transfer dissociation (ETD),² and electron capture induced dissociation (ECID) (Ref. 3) in proteomics are growing in popularity.⁴ The combination of electrospray ionization (ESI) and Fourier transform-ion cyclotron resonance-mass spectrometry coupled with ECD led to a powerful tool for sequencing peptides and proteins and locating weakly bonded post-translational modifications in the biomolecules.⁵ In contrast with vibrational activation methods in tandem mass spectrometry where formation of b or y ions⁶ via cleavage of the peptide bonds is the most effective fragmentation pathway of positive multiply charged peptides, reaction of these species with electrons leads to the generation of mainly c' and z' ions in which the peptide bonds remain intact, while neighboring N–C $_{\alpha}$ bonds break. The mechanism of this complementary decay reaction in charged peptides has been a subject of many experimental and theoretical investigations,⁷ where one of the main challenges was the difficulties associated with determining the internal energy of the breakdown products.

New insight into the mechanism of fragmentation can be gleaned by studying interactions of monoenergetic, low energy electrons with neutral peptides resulting in the formation of peptide negative ions (NIs) via resonance electron

capture (REC). These reactions, important in themselves, are useful for clarifying mechanistic issues of ECD/ETD reactions since, in the absence of protonated sites in neutral gas-phase peptides, they can directly highlight the role of amide OCN π^* molecular orbitals (MOs) in low-energy electron-peptide interactions. Moreover, the energetics of the REC processes can be controlled easily and they are well known for many different classes of compounds. The main impediment for using the method in the case of peptides has been their limited volatility. Indeed, only some of the most volatile amino acids have been studied with free low-energy electrons⁸ and no studies of this kind have been reported for even very small peptides.^{9,10} To be sure recent high-energy collision-induced neutralization/ionization spectra¹¹ indicated ECD-type fragmentation occurred in peptide NIs, but these NI fragments were prepared *a priori* in two steps when the initial collisions between Cs-atom vapor and the positively charged peptide beam resulted in neutralized peptide fragments followed by secondary electron transfer from the Cs atoms to the fragments; obviously, the secondary processes have been facilitated and occurred only because of the high Cs-vapor pressure. This process therefore cannot be considered in the same light as the fragmentations of *intact* NI peptides produced with free electrons.

In order to achieve the objective of free electron interactions with peptides of low-volatility reported here, the principles behind the so-called in-beam technique¹² were employed. This approach, involving direct or desorption chemical and electron ionization (DCI, DEI), was explored prior to the invention and popularization of fast atom bom-

^{a)}Authors to whom correspondence should be addressed. Electronic addresses: y.vasil'ev@orst.edu and max.deinzer@oregonstate.edu.

FIG. 1. Schematic of the ion chamber of the JEOL 600H-MS instrument with the DE wire fitted into the stock direct insertion (DI) probe. F is filament, R is ion repeller, FC is Faraday cup (electron collector), and N and S are the poles of the collimating magnet.

bardment, matrix-assisted laser desorption/ionization, and ESI in the 1980s. Baldwin and McLafferty¹³ first demonstrated that MH^+ ions from peptides with two to six residues could be detected at temperatures 150 °C cooler than conventional heating by using the DCI technique. Two years later, Dell *et al.*¹⁴ employed a similar technique in an electron ionization source (i.e., DEI) for studying the structure of echinomycin. The “in-beam” technique involves simply coating the nonvolatile sample onto the surface, e.g., a wire loop on an extended direct insertion probe and inserting it into the ion source close to the electron beam (DEI) or ion plasma (DCI). Positive ion mass spectra obtained in such a way are in reality a combination of CI and EI events that are often characteristic of protonated MH^+ species.

These early and all but forgotten findings were an inspiration for the design of a direct exposure (DE) probe in the present work to overcome limited sample volatility for studying REC processes of peptides at two fixed energy ranges. Model peptides and two small naturally occurring peptides were examined and the results compared with similar data reported in the literature that were produced by low-energy interactions of positively charged peptides with different carriers of the negative charges, such as free electrons (ECD), atomic species with relatively loosely bound electrons (ECID), or anions (ETD).

II. EXPERIMENTAL METHODS

The DE probe was constructed using filament wire and an electrical pin connector fitted into the stock EI/CI probe for a mass spectrometer, i.e., a JEOL 600H-MS (Fig. 1). Peptide samples in solvent were deposited onto the surface of the wire loop and dried. The DE probe was inserted into the ion chamber of the JEOL 600H-MS and heated by radiation from the hot walls of the ion source that in turn was held

at 200–250 °C. For less volatile compounds, the probe holder was independently heated to increase sample vapor density. The positioning of the DE probe relative to the electron beam was critical in the sense that mass spectra were recorded only when the probe was proximate to and not in the electron beam. When the DE probe was in the electron beam, no ion signal was detected. Except for variations of ion signal intensities, there were no significant differences between mass spectra recorded at distances between the electron beam and the tip of the DE probe of 5, 10, or 15 mm. The fact that the spectra were independent of distance is taken as evidence for the absence of CI processes¹⁵ that might otherwise be likely at small (≤ 5 mm) distances as a result of interactions with high densities of NH_2^- or OH^- produced by REC reactions of peptides. At greater distances, the plasma density of these ions is supposedly too low for the occurrence of CI-type ion formation. To eliminate any chances for the occurrence of chemical ionization, all experiments were carried out with a configuration in which the distance between the DE probe tip and the electron beam was ~ 10 mm. The extended distance ensured that peptide NIs occurred only via REC as proved by the observed energy dependencies of the ion production processes (see Sec. III). Moreover, there were no apparent differences between NI mass spectra of small, volatile peptides produced by either the DE probe or a conventional direct insertion probe. To be sure that NIs were not formed by pyrolysis, the filament was turned off several times during acquisition of the spectra. The ion signals disappeared immediately thus proving that sample heating did not result in conditions producing ions by pyrolytic action. However, partial thermodegradation of the samples forming neutral species followed by electron capture to produce NIs could not be ruled out.

Approximate calibration of the electron energy for these experiments was obtained from the effective yield curves for ions, m/z 35, 70, and 82 from CCl_4 whose yield maxima are ~ 0 and 0.78, 1.23, and 6.1 eV, respectively. The present experiments with peptides were carried out at two fixed electron energies, 1–2 and 5–6 eV. The exact error for the electron energies could not be determined, but at ~ 1 eV it was estimated to be ± 0.5 eV based on the electron energy calibrated with CCl_4 . Mass scale calibration was done using both external and internal procedures based on the NI mass peaks from perfluorokerosene (PFK) for low mass resolution experiments and in the case of high mass resolution experiments, the NI mass peaks from CCl_4 , tribromoethylene, and methyl iodide were used as internal calibrants together with PFK. All compounds were purchased from Sigma/Aldrich (St. Louis, MO) and New England Peptide (Gardner, MA) and used without further purification.

III. RESULTS

A. Dipeptides

Initial experiments were performed on dialanine (Ala_2 , A_2) in the NI mode of the JEOL 600H-mass spectrometer (Fig. 2). At 1–2 eV, peaks of three intense ions were observed with m/z 159, 72, and 87. The mass peak at m/z 159 results from loss of the carboxylic acid hydrogen atom

FIG. 2. REC mass spectra produced from dialanine (A_2) with electron energy of (a) 1–2 and (b) 5–6 eV. The inset in (b) shows results of accurate mass measurements. (c) REC mass spectrum of diglycine recorded with electron energy of 1–2 eV.

$(M-H)^-$ and, as is typically observed in REC-MS of amino acids, it is the most intense peak.^{8,16} At 5–6 eV several additional peaks were present. Happily, the ions produced at these two energies are practically identical to those¹⁷ obtained with the electron monochromator reflectron time-of-flight mass spectrometer (EM-rToF-MS) (Ref. 18), where electron energies can be controlled to within 5–10 meV and NI formation cross sections versus electron energy can be obtained with a small (1–2 meV) energy variation step. This is the most convincing evidence that the method provided sufficient sample vapor just above the probe surface to allow low energy electrons to interact with the molecules and yield REC spectra.

The assignment of peaks at m/z 72 and 87 was initially problematic because there were two possibilities that matched the nominal mass (m/z) of the ions. By using internal standard ions $^{35}\text{Cl}_2^-$, $^{35}\text{Cl}^{37}\text{Cl}^-$, and $^{37}\text{Cl}_2^-$ from CCl_4 , high-resolution mass measurements under REC conditions were made on A_2 . The accurate mass measured for the first ion was 72.0210 Th [Fig. 2(b), inset], which fits the empirical formula $\text{C}_3\text{O}_2\text{H}_4$ with an error of -1.8 ppm relative to the calculated value. This empirical formula leads to the assignment of the NI with m/z 72 as a $z-1$ ion; i.e., C-terminal z type ion, corresponding to the $\text{N}-\text{C}_\alpha$ bond cleavage with loss of one hydrogen atom [Fig. 2(a) inset; the Roepstorff-Fohlman⁶ nomenclature was used throughout as used previously for NIs^{19,20}].

The exact mass of the peak at m/z 87 was determined as 87.0561 Th, using Br^- from tribromoethylene and I^- from methyl iodide as internal calibrants, both of which have effective yield maxima near 0 eV. An empirical formula of $\text{C}_3\text{O}_2\text{H}_7\text{N}_2$ with an error of 3.3 ppm was obtained for this peak, which identifies it as the c ion. The intense ion peak produced from A_2 at m/z 88 with 5–6 eV electrons is the y ion. In the case of low-energy (1–2 eV) experiments, these ions can also be $[M-H]^-$ from mono-Ala that was either

FIG. 3. REC mass spectra of alanine-phenylalanine, AF, (a), diphenylalanine, FF, (b) and dilysine, KK, (c) all recorded with electron energy of 1–2 eV.

initially present as an impurity or more probably formed via thermodegradation of dialanine into Ala monomer during heating.

Other fragmentation channels in the REC mass spectrum of A_2 observed were associated with loss of one or more neutral particles with common masses 17, 19, 45, 46, 61, and 62. Accurate mass measurements for these ions were not performed, however, high resolution experiments for analogous fragmentation channels in the case of larger alanine-based peptides (see below) proved that these were associated with loss of NH_3 , $(\text{H}$ and $\text{H}_2\text{O})$, COOH , HCOOH , $(\text{NH}_3$ and $\text{CO}_2)$, $(\text{NH}_3$ and $\text{CO}_2\text{H})$, respectively.

REC experiments in the case of diglycine (Gly_2, G_2) resulted in similar data [Fig. 2(c)]. Again, accurate mass measurements confirmed efficient formation of $[M-H]^-$ together with $z-1$ and c ions. These experiments also showed that G_2 is thermally more labile than A_2 and, for this reason, experimental conditions for G_2 were much more difficult to maintain. Clean REC mass spectra of G_2 nearly free from mass peaks arising from sample thermodestruction were possible to obtain only at low mass resolving power [Fig. 2(c)]. Unfortunately the same was true of larger glycine-based peptides in comparison to their counterparts in the alanine family.

Low-energy reactions of a dipeptide with different amino acid residues, Ala-Phe (AF), similar to A_2 and G_2 , showed loss of the hydrogen atom as the most efficient decay channel of molecular NIs [Fig. 3(a)]. The peaks for the $z-1$ and c ions also were rather intense in the low-energy mass spectrum, however, NIs associated with the side chain, $(M-\text{CH}_2\text{Ph})^-$, were observed with higher intensity. The complementary ions, CH_2Ph^- , have been found to be formed at higher energies; this observation is similar to that observed for the REC mass spectrum of Phe.¹⁶ The low-energy peaks associated with y ions again have exactly the same mass as the $[M-H]^-$ NIs from Phe, which were either present in the sample as a byproduct or formed through sample thermodegradation.

Diphenylalanine (Phe_2 , FF) was similar to AF in that it showed less efficient formation of the $z-1$ and c ions in

FIG. 4. REC mass spectra of trialanine, A_3 , (a), glycine-glycine-leucine, GGL, (b) and YFF amide (c) all recorded with electron energy of 1–2 eV. The ion peak in panel (c) marked with a star corresponds to loss of a hydrogen atom and two water molecules; ion peaks with two and three stars are tentatively assigned, respectively, on the basis of losses of two and three side chains, whose formation mechanisms are not yet clear.

comparison to NIs associated with loss of the side chain $[M-CH_2Ph]^-$ [Fig. 3(b)]. The most abundant fragmentation pathways of FF⁻ molecular NIs were, however, loss of a hydrogen atom, $[M-H]^-$, and that associated with the sequential or simultaneous loss of a water molecule, $[M-H-H_2O]^-$. The last reaction was previously observed and discussed for simple amino acids.¹⁶ The low-energy NI mass spectrum of FF indicated an analogous fragmentation channel associated with the involvement of the FF side chain instead of a water molecule, $[M-H-HOCH_2Ph]^-$.

Dilysine, (Lys₂ or KK) was difficult to study due to its high thermal lability. Intense signals of the $[M-H-H_2O]^-$ NIs instead of the expected $[M-H]^-$ NIs in the low electron-energy mass spectrum of the compound [Fig. 3(c)] might actually be due to thermal excitation of the compound. KK showed NIs analogous to those for FF through the involvement of the side chain, $[M-H-HONH_2(CH_2)_4]^-$. The loss of part of the side chain, $[M-NH_2CH_2]^-$, is even more abundant than the loss of the entire side chain, $[M-NH_2(CH_2)_4]^-$. Unfortunately, it was impossible to examine these observations more accurately under the conditions of the present experiment due to the thermal lability of KK. As in the other dipeptides studied, the $z-1$ NIs were observed at low electron-energies [Fig. 3(c)].

B. Tripeptides

The low-energy REC mass spectrum of trialanine, A_3 , shows three pronounced mass peaks for $[M-H]^-$, z_2-1 , and c_2 NIs [Fig. 4(a)]. Intensities of the $[M-H_2]^-$ ions has been found dependent on sample thermal excitation. The N-terminal NIs associated with the N–C_α bond cleavage, c_2 , shows high intensities at both low and high (results not indicated in Fig. 4) energies. Accurate mass measurements were carried out with some ion mass peaks to prove the presence of the following ions: $[M-H]^-$ (nominal mass m/z

230; error 2.8 ppm), $[M-NH_3]^-$ (m/z 214; 2.5 ppm), $[M-NH_3-H_2O]^-$ (m/z 196; 12 ppm), $[M-HCOOH]^-$ (m/z 185; −1.8 ppm), $C_6H_{11}N_2O_3$, i.e., y_2 (m/z 159; 11 ppm), $C_6H_{12}N_3O_2$, i.e., c_2 (m/z 158; 5.4 ppm), $C_6H_9NO_3$, i.e., z_2-1 (m/z 143; −6.7 ppm), C_5H_9NO , i.e., z_2-1-CO_2 (m/z 99; 40 ppm).

Triglycine, G_3 , was not studied in the present experiments, however, its REC spectrum obtained with the EM-rToF-MS (Ref. 17) was found to be practically identical to that of A_3 . A mixed tripeptide with glycine residues in the structure, GGL, showed very abundant $[M-H]^-$ NIs in the low-energy REC spectrum [Fig. 4(b)]. The y_2 and y_1 ions were observed here with slightly higher intensities than the z_2-1 and z_1-1 ions. However we cannot rule out contributions to the signals of these ions from the $[M-H]^-$ ions generated from the dipeptide, GL, and leucine, respectively, possibly formed during sample heating. The c_1 and c_2 ions were also observed, albeit with lower intensities than their counterpart C-terminal ions, z_n-1 .

The YFF amide was studied to determine whether ions could be observed in small peptides without the C-terminal carboxylic acid group. The most intense ion observed was the c_2 ion but the c_1 , z_2-1 , and $(M-H)^-$ ions were also detected at an electron energy of ~1 eV [Fig. 4(c)]. Clearly the absence of the carboxylic acid hydrogen reduces the abundance of $(M-H)^-$ ions derived from YFF amide. The observed $(M-H)^-$ ion is likely due to loss of the phenolic hydrogen from the tyrosine residue.¹⁶ The absence of z_1-1 in the mass spectrum indicates the crucial role of the formation of the carboxylate ion structure in the production of z_1-1 ions.

C. Tetrapeptides

Tetrapeptides are the smallest peptides that can form a 3₁₀-helix structure and, if the mole fraction of the helices in the gas phase is large enough, one might expect some differences in their REC spectra with respect to the smaller peptides. However, a comparison of the low-energy mass spectra of A_3 [Fig. 4(a)] and tetra-alanine, A_4 , [Fig. 5(a)] did not indicate any unusual differences except for the appearance of a new fragmentation channel, z_2-1-18 , in A_4 . Although alanine-based peptides are known to have higher helix-forming tendencies than glycine peptides,²¹ the low-energy REC mass spectra of A_4 [Fig. 5(a)] and tetraglycine, G_4 , [Fig. 5(b)] are found to be similar. Indeed, both spectra are characterized by very intense z_n-1 ions, with z_2-1 ions being more intense than $[M-H]^-$ NIs. Complementary ions with localization of the negative charge on the N-terminus, i.e., c_n ions, were again observed with lower intensities at low electron energies. The z_n-1 ions probably decompose further into NIs associated with the loss of 18 and 44 Da, i.e., water and the CO₂-group, respectively. Accurate mass measurements were not carried out for A_4 but the interpretations are based on analogous NIs observed in A_3 and A_5 (see below). Because of the thermal lability of G_4 , all attempts to gain higher mass resolution for G_4 ions by increasing the temperature for higher sensitivity resulted in sample degradation. The ion assignment for G_4 ions [Fig. 5(b)] was there-

FIG. 5. REC mass spectra of tetra-alanine, A₄, (a), tetraglycine, G₄, (b) and glycine-proline-glycine-glycine peptide, GPGG, (c) all recorded with electron energy of 1–2 eV.

fore based on principles similar to those postulated for the ion formation processes in other glycine- and alanine-containing peptides. It is interesting that the relative intensities of the z_n-1-18 and z_n-1-44 NIs are reversed in REC spectra of A₄ and G₄. The origin of the z_3-18 ions in the spectrum of G₄ is not entirely clear but perhaps they are associated with the partial dehydration of the sample during heating followed by REC and fragmentation analogous to what is observed in high-energy collisions of peptide ions with high pressure Cs-vapor.¹¹

The tetrapeptide with one proline residue, GPGG, showed a very intense peak for the y_2 ion in its low electron energy REC spectrum [Fig. 5(c)]. The [M-H]⁻ and ions associated with amide bond cleavages, z_2-1 , z_1-1 , c_3 , and c_2 were of lower intensities than the y_2 ion and the presence of the proline residue precludes the formation of z_3-1 and c_1 ions. There are several unassigned mass peaks in the GPGG mass spectrum whose origin is unknown at this time, but it is possible they are formed via REC by fragments after sample thermodegradation. The intense y_2 ions in the low-energy REC spectrum [Fig. 5(c)] can be explained as arising by this pathway.

D. Pentapeptides

In theory, pentapeptides can adopt both the 3_{10} - and α -helix structures and for this reason it was of interest to compare their low-energy reactions with free electrons with those of shorter peptides. In general, there were no signifi-

FIG. 6. REC mass spectra of penta-alanine, A₅, (a) and pentaglycine, G₅, (b) both recorded with electron energy of 1–2 eV.

cant differences between the low-energy REC spectra of penta-alanine, A₅, [Fig. 6(a)], pentaglycine, G₅ [Fig. 6(b)], and the smaller peptides. As in the case of G₄, it was not possible to conduct accurate mass measurements on G₅. For A₅ high mass resolution was performed on selected NIs that were not present in the mass spectra of smaller peptides. These experiments provided proof for the empirical compositions of the following new type of ions: z_4-1 with an error of 0.1 ppm, c_4 (−7 ppm), z_4-1-H_2O (12 ppm), and a_4-1 (−9 ppm). Moreover, high mass resolving power was applied to z_1-1 (0.4 ppm) and c_1 (8 ppm) NIs to make sure that ions of the same nominal masses had the same elemental composition in all alanine-based peptides. These accurate mass measurements proved again that z_n-1 ions were dominant in the REC spectrum of A₅. Assuming similarities in the ion formation processes in these two pentapeptides, the same conclusions can be drawn for G₅. In comparison to tetrapeptides, formation of z_n-1-18 ions in both pentapeptides was more abundant than z_n-1-44 ions. As in G₄, z_n-18 ($n=3,4$) ions were observed in the mass spectrum of G₅. The z_n-18 ions may arise from either normal REC processes or, as suggested earlier, through the initial loss of water during sample heating followed by electron capture and N–C_α bond cleavage. It should be noted that [M-18]⁻ NIs detected for G₅ are highly temperature dependent. Analogous ion peaks were of much lower intensities in the REC spectra of A₅ and A₆, probably because these compounds are much more thermally stable.

E. Hexapeptides

Of the two hexapeptides studied, only hexa-alanine, A₆, produced a REC spectrum [Fig. 7(a)]. In general, it was similar to that of the smaller peptides. The spectrum of G₆ consisted mostly of unassignable peaks that probably resulted from thermal degradation [Fig. 7(b)]. Two of the most prominent peaks in the spectrum of G₆ were loss of 18 and 36 mass units that were presumably associated with elimination of one and two water molecules, respectively, followed by

FIG. 7. REC mass spectra of hexa-alanine, A_6 , (a) and hexaglycine, G_6 , (b) both recorded with electron energy of 1–2 eV.

electron capture. The $[M-H]^-$ ion was not obvious, but peaks for possible z_n-1 ions were still recognizable [Fig. 7(b)]. Accurate mass measurements for ions of A_6 were carried out for z_5-1 , z_4-1 , and its neighboring peak [Fig. 7(a) inset for z_4-1 and its counterpart]. These measurements confirmed the assignments of the ion peaks as z_n-1 ($n=4,5$) and z_4-2 with an average accuracy within 6 ppm. This means that z_4-2 NIs are associated with the loss of two hydrogen atoms during cleavage of the $N-C_\alpha$ bond. This ion formation process showed moderate temperature dependence and accurate mass measurements [Fig. 7(a) inset] were conducted at a higher temperature of the ion chamber in order to get more abundant z_4-2 NIs.

F. Natural peptides

Two naturally occurring peptides, methionine enkephalin (YGGFM), known for regulating pain and nociception in the body²² and substance P (RPKPQQFFGLM-NH₂; SP-amid) a neuropeptide that acts as a neurotransmitter and a neuromodulator²³ were also studied by REC. Methionine enkephalin [Fig. 8(a)] was measured in the manner described for alanine- and glycine-based peptides and a spectrum was recorded at electron energy of 1–2 eV. All possible z_n-1 ions, i.e., with m/z 132, 279, 336, and 393 were observed as were y ions with m/z 148, 295, 352, and 409. Observation of the entire z_n-1 series with z_2-1 as the main ion peaks in the spectrum is noteworthy as it shows that electrons initiating fragmentation are close in energies for all peptide $C_\alpha-N$ bonds. All these ions are accompanied by a series of ions 18 Da less as observed for the model peptides. Analogous to dilysine [Fig. 3(c)], there were NIs associated with loss of a part of the side chain, $[M-2H_2-SCH_3]^-$. No $[M-H]^-$ NIs were registered under the experimental conditions.

It was expected that SP-amid [MW=1347; Fig. 8(b)] should show similar behavior to YFF amide [Fig. 4(c)] under REC conditions and indeed the z_1-1 ions were not observed in the spectrum. However, in contrast with the YFF amide derivative, no c ions were observed but the extended series of z_n-1 , for $n=2-7$ was present with the z_5-1 ion dominat-

FIG. 8. The REC mass spectra of methionine enkephalin, YGGFM, (a) and Substance P, RPKPQQFFGLM-NH₂, (b) both recorded with electron energy of 1–2 eV.

ing. Proline in the sequence would preclude observation of the z_8-1 and $z_{10}-1$ ions as was observed for GPGG [Fig. 5(c)] but the z_9-1 ion was not present either. The z_7-1 was the highest mass peak observed in the low-energy REC spectrum. No $[M-NH_3]^-$ nor $[M-H]^-$ were detected. The interaction of higher energy electrons (5–6 eV) with SP-amide did not result in formation of any NIs. REC analysis of the SP-free acid produced z_n-1 ions and an abundance of unassignable ion peaks presumably resulting from products of sample thermodegradation; something to be expected because of the high polarity of the acid form. However what is worth noting is that in contrast with the SP-amide, the z_1-1 ion along with other z_n-1 ions was observed.

IV. DISCUSSION

One of the most characteristic fragmentation channels of low-energy gas-phase electron-peptide reactions is the one that leads to the loss of a hydrogen atom, i.e., $[M-H]^-$. The process should not be confused with deprotonation of peptides when NIs are formed by a different mechanism⁹ even if the NIs have the same final structure. Another typical ion forming pathway is the one associated with the $N-C_\alpha$ bond cleavages accompanied by hydrogen atom loss that eventually leads to the production of the z_n-1 ions. Relative ion yields within these two channels behave in opposite directions with size of the peptides. The $[M-H]^-$ ion abundances decrease and the z_n-1 ions increase with increasing peptide size. The absence of the z_1-1 ion peaks in the mass spectra of peptides with the amide group at the C-terminus indicates that the formation of peptide carboxylate anions constitutes the z_1-1 ions. Other mechanisms are probably involved in the formation of z_n-1 ions ($n>1$), however, the carboxylate structure should play a significant stabilizing role for the smallest dimers and trimers where formation of helical peptides is not possible. Evidence in support of this mechanism is the relative height of the z_2-1 ion peak, which is dramatically decreased in comparison to the c_2 ions in the REC spectrum of YFF-amide. Since this was not the case for the SP-amide, it is concluded that for larger peptides there is

FIG. 9. Total energy difference in atomic units (a.u.) of Gly (top panel) and Ala (bottom panel) conformers. Energies of all conformers were normalized with respect to the energies of β -strand C5 conformers (black triangles). Relative energies of statistical coils are depicted as black squares, 3_{10} -helices as red squares, α -helices as blue circles, and PPII helix of Ala₂ as green circle.

another mechanism for producing z_n-1 ions. It is not entirely clear from the present experiments whether z_n-1 or $[M-18]^-$ and $[M-44]^-$ are precursors for the weak z_n-1-18 and z_n-1-44 NIs. However, relative abundances of the z_n-1-18 ions grow faster than that of z_n-1-44 ions with increasing peptide sizes and this fact favors z_n-1 ions as precursors. Indeed, loss of the CO₂ group from the z_n-1 NIs can hardly occur in the absence of carboxylate ions. It is interesting to note that high¹⁵ and low-energy²⁰ CID spectra of singly and multiply¹⁹ deprotonated peptides also show abundant yields of NIs associated with loss of CO₂ groups where deprotonation of carboxylic acid group was possible. Zubarev and co-workers²⁴ observed this process from singly charged NIs formed from dianions after collisions with high energy electrons; the process was named electron detachment dissociation. The same group²⁵ reported on the UV laser-induced loss of CO₂ from singly protonated peptides in zwitterionic states, $[(+)\dots(-)\dots(+)]$, with a negatively charged carboxylate group.

$[M-H]^-$ ions produced from amino acids by capture of electrons of ~ 1 eV are associated with carboxylate anion structures through involvement of the so-called dipole-bound states.⁸ If the same mechanism works for peptides, it is no wonder that the abundance of the $[M-H]^-$ ions decreases with increasing peptide size. Clearly, the carboxylate ion plays a diminishing role in larger peptides when compared to those with C-terminal amide groups. To rationalize the fragmentation mechanisms of free peptide NIs produced with low energy electrons, *ab initio* UHF and RHF 6-31G* calculations were performed on Gly- and Ala-based oligomers up to octamers with extended (β -strand, C5), random or statistical coil (bent structures), 3_{10} -(C10), and α helical (C13) conformations (Fig. 9). PPII helices were also considered for Ala-based peptides, however, a stable structure was predicted only for Ala₂, whereas other Ala-oligomers starting with the initial PPII geometry inevitably turned to β -strand conform-

ers after geometry optimization. For some Gly- and Ala-based oligomers, γ -turn (C7) conformers were also calculated. By analogy to that previously reported,²⁶ optimized α -helical conformers for peptides smaller than Gly₈ and Ala₈ were predicted not to exist. Oligomers with $n=5, 6$, and 7 shown as α -helices (Fig. 9) are actually hybrid $\alpha/3_{10}$ -helical structures. The same problems associated with small α -helices have been discussed earlier.²⁷ Some conformers of triglycine were also examined using density functional theory (DFT) at the UB3LYP/6-311+G** level.

The RHF 6-31G* level of theory together with the scaling procedures^{28,29} was applied here mainly for studying electronic structures of the peptides, particularly for predictions of π^* shape resonance energies on the basis of Koopmans' theorem. It is important to note that the reliability of this theoretical approach has been proved in the case of simple amino acids,^{8,16,28} where excellent matches of theoretically predicted and experimentally determined energies of π^* shape resonances have been found.

Before discussing the results of the present calculations in relation to experimental data, one should recall that Sobczyk *et al.*³⁰ reported higher level UHF calculations with aug-cc-pVDZ basis sets applied to dialanine. The so-called stabilization method for probing metastable NIs to predict resonance energies for vertical electron capture was applied. It was shown that direct attachment of an electron to a peptide σ^* orbital localized mainly in the amide bond should occur at ~ 6 eV; electron attachment to the π^* orbital localized on the same group was predicted to occur at 2.5–2.8 eV,³⁰ which is, however, higher than the experimental value (2.05 eV) for formamide.³¹ Furthermore, the calculations³⁰ predicted that electron capture into the OCN π^* orbital should result in a significantly lower energy barrier for cleavage of the C $_{\alpha}$ -N bond and formation of more energetically favorable products than the cleavage of other bonds. Based on these results,³⁰ a predissociative or indirect dissociative electron attachment mechanism was proposed for ECD when the activation barrier was determined by crossing of π^* and σ^* states. The inclusion of electron correlation at the MP2 level resulted in a slight lowering of the resonance energy and activation barrier. However, according to calculations,³⁰ an electron energy of ~ 2.5 eV is still generally required to break the C $_{\alpha}$ -N bond via electron capture by neutral peptides.

The RHF 6-31G* calculations in the present report predict that π^* shape resonance energies for Gly- and Ala-type peptides, when based on Koopmans' theorem and a scaling procedure,^{28,29} should be different depending on peptide size or conformation (Fig. 10). Indeed, the difference in the nature of the lowest unoccupied MO (LUMO) becomes especially apparent with increasing peptide size. LUMOs of β -strands are mainly localized at the carboxylic acid group, whereas helical conformers have a larger wave function amplitude at the N-terminus. LUMO energies were found to decrease with increasing peptide size for all conformers (Fig. 10). In the case of di-Ala and di-, tri- and tetra-Gly, C7 conformers were examined at the RHF 6-31G* level and the calculations predicted that LUMO energies for some of these conformers are rather low (ca. 1.2–1.3 eV).

FIG. 10. Experimental [formamide (FA, magenta strip), Gly and Ala taken, respectively, from Refs. 31 and 28] and calculated π^* resonance energies for FA (magenta strip) and Gly- (red strips) and Ala- (blue strips) based peptides with β -strand (a) and 3_{10} -helical (b) conformations. In the case of dimers and trimers, statistical coil conformations optimized from initial geometry parameters typical for helical conformers (not shown) and some bent conformers (not shown) have been found to have lower π^* resonance energies than these conformers (see text).

According to UHF 6-31G* calculations, β -strands are the most stable conformers up to pentamers both for Gly- and Ala-oligomers (Fig. 9) and that they should possess the smallest dipole moment among those conformers studied particularly in comparison to helices. These results are in line with those in other reports.^{27,32} Further examination of the nature of peptide dipoles has shown that both the magnitude and directions of the dipole moments are different depending on the conformer. Indeed, calculations predict that β -strands have positive polarity near the carboxyl group, whereas dipoles of helices are opposite in direction, i.e., with the positive end oriented toward the N-termini. The dipoles of γ -turns or statistical coils with bent structures orient themselves orthogonally to the peptide backbone and are near the middle of the sequence. This theoretical finding correlates well with the behavior observed for the abundance of $[M-H]^-$ NIs that appear in REC spectra of peptides during the initial scans and then disappear with time and are not present in the final scans; a phenomenon that contrasts with the behavior of the z_n-1 ions whose intensities remain constant throughout the experiment. The first scans are recorded when the temperature of the DE probe is low, a condition favoring the survival of relatively rigid structures like β -strands or helices in the gas phase. By the time the last scans are recorded, the temperature of the DE probe has

become much hotter, which causes melting of the β -strands and helices into expanding globules, as proved by the Jarrold group³³ in the case of protonated peptides. This observation was much more apparent in some REC studies carried out with our EM-rToF-MS equipped with a modified DE probe when the DE probe temperature was varied over much wider temperature ranges and at different rates.¹⁷ Thus, as a result of the temperature dependent disappearance of the β -strands in the gas phase, the apparent intensity of the $[M-H]^-$ decreases since only β -strands have positive dipoles near the carboxylic acid group. These observations also help support the formation of a dipole-bound anion state mechanism postulated earlier for amino acids.⁸ The tendency for the relative intensities of the $[M-H]^-$ ions to decrease in later scans, i.e., with time, and as the peptide sizes increase may in fact be caused by two factors: (1) melting of the β -strands into expanding globules with increasing temperature (large peptides require higher temperatures for evaporation) and (2) decreased mole fraction of β -strands in the gas phase on going to larger oligomers as predicted by calculations (Fig. 9).

The calculations based on the DFT UB3LYP/6-311+G** level of theory did not change the order of the most stable conformers for either dialanines or diglycines. However, in contrast with UHF 6-31G*, the DFT UB3LYP/6-311+G** calculations predict that C7 conformer of Gly₃ have comparable or even slightly (ca. 2–3 kcal/mol) lower total energy than that of the β -strand; nevertheless, the free energies of C5 and C7 conformers are predicted to be almost isoenergetic at room temperature. Notably, similar DFT calculations with four different density functionals applied to dipeptide analogs of Gly and Ala also showed³⁴ lower energies for the C7 conformers with respect to the C5 counterparts; one should note, however, that due to an acetyl group at the N-terminus and an acetamide group at the C-terminus, these dipeptide analogs should be compared with common tripeptides rather than with dipeptides. The C7 conformer with internal hydrogen bonds could be an ideal precursor for the formation of z_n-1 ions with or without carboxylate anion structure (Fig. 11). A similar mechanism without involvement of the carboxylate anion can be visualized for helical conformers. In both these scenarios, proton transfer takes place to neutralize the negative charge after electron capture at a generic amide group [Figs. 11(a) and 11(b)]. Significantly, this type of proton transfer can be accompanied by simultaneous hydrogen atom transfer from a different place on the peptide backbone of larger helices. Such H-transfer would most likely occur if a hydrogen atom that can form a hydrogen bond at some place removed from the cleavage sites is closer to a charged fragment than it is to its neutral counterpart during this fragmentation process (Fig. 12). This type of decay of peptide's resonances is probably decisive in forming z_4-2 ions in Ala₆. In the case of electron capture into a virtual MO localized mainly at the carboxyl group, fragmentation with formation of the z_1-1 NIs involves a hydrogen atom (Fig. 13) rather than a proton (Fig. 11) transfer and this fact differentiates the z_1-1 ions from other z_n-1 ($n > 1$) ions.

Many of the peptides studied showed $[M-17]^-$ and $[M-35]^-$ NI peaks in their REC spectra. Accurate mass mea-

FIG. 11. Idealized mechanisms for the N- C_α bond cleavages caused by low-energy electron capture into amid OCN π^* MO resulting in formation of z_n-1 ions with (a) and without (b) carboxylate anion structure and c_k ion (c).

measurements proved that the first ions are associated with loss of the amino group and the second with additional consecutive or simultaneous loss of water. Elimination of the amino group formally occurs via cleavage of the C_α -N bond and hydrogen atom transfer and the corresponding ions therefore can be considered as pseudo- z_n-1 NIs. This type of mechanism is perhaps the basis for the rather high abundance of these ions in REC spectra. Fragmentation mechanisms resulting in the formation of $[M-NH_3]^+$ NIs may be similar to those producing z_1-1 ions, however, initial electron capturing mechanisms are presumably different in both these cases. Only accurate determinations of their cross sections over the entire resonance energy range¹⁷ can help answer this question.

Formation of c_n ions through electron capture into π^* orbitals localized on either the amide or carboxyl group does

not involve hydrogen/proton transfer as they are either radical- or charge-site driven reactions (Figs. 11 and 13). Although z_n-1 and c_n NIs have been considered complementary species, in reality only c_n+1 NIs should be considered as complementary to z_n-1 NIs. The present experiments did not produce any evidence for formation of these odd-electron c_n+1 NIs. Calculations of different structures of c_1+1 and c_2+1 species in glycine-based peptides using UHF 6-31G* level of theory predicted negative electron affinities, EA, for them. Even if a higher level of theory was to predict that their EAs are positive, it is difficult to imagine that their magnitude would be large enough to compete kinetically with electron autodetachment. These conditions may be responsible for the c_n+1 species not being observed from Ala- and Gly-based peptides.

As for larger Gly- and Ala-based peptides whose

FIG. 12. Idealized mechanisms for the N- C_α bond cleavages in Ala₆ caused by low-energy electron capture into amid OCN π^* MO followed by proton transfer without and with hydrogen movement (hydrogen atom is highlighted in red) that resulted in formation of z_4-1 and z_4-2 ions, respectively.

FIG. 13. Idealized mechanisms for the N- C_{α} bond cleavages caused by low-energy electron capture into carboxylic $\pi^*(\text{COO})$ MO resulting in formation of z_1-1 ions (a) and c_n ions (b).

$[\text{M}-\text{H}]^-$ ion abundances decrease with peptide size, SP, and methionine enkephalin did not show peaks for these ions in the low-energy electron range. As noted for model peptides, the absence of $[\text{M}-\text{H}]^-$ ions can be attributed to the low mole fraction of gas phase β -strands. Secondary structures of SP in solution are highly dependent on the medium³⁵ and SP probably displays an extended chain structure only in water, whereas in other solvents it adopts a helical structure in the midregion section. Reliable data on gas-phase structures of neutral SP are not available, but the present data certainly do not favor an extended conformer for SP in the gas phase and probably not for methionine enkephalin either. Indeed, quantum chemical calculations on enkephalins³⁶ predicted the lowest energy for bent structures.

V. CONCLUSIONS

The main aim of the present work was to adopt a simple robust method to study resonance gas-phase reactions between neutral peptides and free electrons. Answers to questions concerning electron interaction with neutral peptides is of fundamental importance in its own right, but in addition studies in this area will provide useful information on the mechanism of electron capture by peptides into amide MO as it relates to the ECD mechanism. The present results proved that peptide backbone cleavages occur under REC conditions and that energies of electrons in the range of 1–2 eV are sufficient to form c and $z-1$ fragment ions from neutral peptides. The results from these studies indicate that a relatively simple probe can be adapted to vaporize peptides into the gas phase and make it possible to explore their interactions with low-energy electrons. Such a probe implemented on the EM-rToF-MS instrument that delivers energy resolved electrons of better than 10 meV should provide a wealth of additional information¹⁷ and thereby help to elucidate more precisely the mechanisms of electron-peptide interactions. From the present accurate mass measurement studies, it is concluded that fragment ions formed by low energy REC are of the same types as those observed in ECD/ETD experiments

where the positive charge is a factor. The main difference in the present experiments is the occurrence of z_n-1 ions that are more efficiently formed than z_n ions. No evidence for the occurrence of the complementary reaction of c_n+1 ion formation was found. Instead, c_n ions appear to arise without hydrogen/proton transfer *albeit* of lower abundances than that observed for z_n-1 ions. Only amide forms of small peptides showed more abundant ion peaks for the c_n ions than for the z_n-1 ions. A different mechanism for N- C_{α} bond cleavage was evident for larger amide-containing peptides, however, z_n-1 ions still predominated. The fragmentation pathways from gas-phase REC for two naturally occurring peptides, methionine enkephalin, and substance P, were similar to those observed for the model peptides.

During preparation of this manuscript, a paper³⁷ appeared on the REC of small peptides containing cysteine residues. $[\text{M}-\text{H}]^-$ NIs and c -ions were observed even with electrons of energies less than 1 eV but there was no mention of $z-1$ NIs. The absence of the $z-1$ ions in these experiments³⁷ is in apparent contrast with the present data and needs clarification. The reason may have something to do with the different methods of introducing peptides into the ion chamber, as unlike the desorption approach used here, polydisperse aerosols were used for creating molecular peptide beams.³⁷ The aerosol method presumably generates much cooler molecules prior to electron attachment and their internal energies are therefore too low to overcome a potential barrier required for proton transfer to produce the $z-1$ NIs. The difference in the results from the two experimental approaches is noteworthy and certainly calls for further studies. To this end Mihalca *et al.*³⁸ reported a significant difference between the ECD of multiprotonated peptides recorded at 86 K and that at room temperature and Tsybin *et al.*³⁹ found decreased intensity of N-terminal c -ions and increased intensities of C-terminal z -ions in ECD experiments of protonated peptides when internal energies were increased by infrared irradiation.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Environmental Health Sciences (NIEHS) Grant No. RO1 ES 009536 and an award from the Keck Foundation. This publication was made possible in part by Grant No. P30 ES00210 from the NIEHS and by the CRDF Grant No. RUC1-2908-UF-07. The authors wish to acknowledge the Mass Spectrometry Facility of the Environmental Health Sciences Center at Oregon State University. The authors also wish to thank Dr. Valery Voinov for many insightful suggestions.

¹R. A. Zubarev, N. L. Kelleher, and F. W. McLafferty, *J. Am. Chem. Soc.* **120**, 3265 (1998).

²J. E. P. Syka, J. J. Coon, M. J. Schroeder, J. Shabanowitz, and D. F. Hunt, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 9528 (2004); J. J. Coon, J. E. P. Syka, J. C. Schwartz, J. Shabanowitz, and D. F. Hunt, *Int. J. Mass Spectrom.* **236**, 33 (2004); S. J. Pitteri, P. A. Chrisman, J. M. Hogan, and S. A. McLuckey, *Anal. Chem.* **77**, 1831 (2005).

³P. Hvelplund, B. Liu, S. B. Niesen, and S. Tomita, *Int. J. Mass Spectrom.* **225**, 83 (2003).

⁴R. A. Zubarev, *Mass Spectrom. Rev.* **22**, 57 (2003).

- ⁵N. L. Kelleher, R. A. Zubarev, K. Bush, B. Furie, B. C. Furie, F. W. McLafferty, and C. T. Walsh, *Anal. Chem.* **71**, 4250 (1999).
- ⁶See the nomenclature for peptide ions in P. Roepstorff and J. Fohlman, *Biomed. Mass Spectrom.* **11**, 601 (1984).
- ⁷M. Sobczyk, D. Neff, and J. Simons, *Int. J. Mass Spectrom.* **269**, 149 (2008); E. A. Syrtstad and F. Tureček, *J. Am. Soc. Mass Spectrom.* **16**, 208 (2005); N. Leymarie, C. E. Costello, and P. B. O'Connor, *J. Am. Chem. Soc.* **125**, 8949 (2003).
- ⁸Y. V. Vasil'ev, B. J. Figard, D. F. Barofsky, and M. L. Deinzer, *Int. J. Mass Spectrom.* **268**, 106 (2007) (see also references therein.).
- ⁹These processes should not be confused with the well studied and classified fragmentation processes of deprotonated peptide routinely generated by different "soft" mass spectrometric techniques in combination with collision activation; see, for instance, J. H. Bowie, C. S. Brinkworth, and S. Dua, *Mass Spectrom. Rev.* **21**, 87 (2002); D. Bilusich and J. H. Bowie, *ibid.* **28**, 20 (2009).
- ¹⁰Preliminary studies on the subject from our group have been reported recently. Y. V. Vasil'ev, B. Figard, and M. L. Deinzer, in *Encyclopedia of Mass Spectrometry: Molecular Ionization*, edited by M. L. Gross and R. M. Caprioli (Elsevier, Oxford, 2007), Vol. 6, pp. 295–305; Y. V. Vasil'ev, V. G. Voinov, D. F. Barofsky, and M. L. Deinzer, *Resonance Electron Capture by Small Peptides*, Proceedings of the 56th ASMS Conference on Mass Spectrometry and Allied Topics, Denver, CO, 2008 (unpublished).
- ¹¹P. Hvelplund, B. Liu, S. B. Nielsen, S. Panja, J.-C. Pouilly, and K. Støckel, *Int. J. Mass Spectrom.* **263**, 66 (2007).
- ¹²See reviews on the subject. G. D. Daves, Jr., *Acc. Chem. Res.* **12**, 359 (1979); R. J. Cotter, *Anal. Chem.* **52**, 1589 (1980); A. McCormick, "Development and Trends in Mass Spectrometry," in *Mass Spectrometry*, Vol. 6, pp. 153–172 (Royal Society of Chemistry, London, 1981), edited by R. A. W. Johnstone.
- ¹³M. A. Baldwin and F. W. McLafferty, *Org. Mass Spectrom.* **7**, 1353 (1973).
- ¹⁴A. Dell, D. H. Williams, H. R. Morris, G. A. Smith, J. Feeney, and G. C. K. Roberts, *J. Am. Chem. Soc.* **97**, 2497 (1975).
- ¹⁵A. L. C. Smit and F. H. Field, *J. Am. Chem. Soc.* **99**, 6471 (1977); M. Eckersley, J. H. Bowie, and R. N. Hayes, *Int. J. Mass Spectrom. Ion Process.* **93**, 199 (1989); R. J. Waugh and J. H. Bowie, *Rapid Commun. Mass Spectrom.* **8**, 169 (1994); A. M. Bradford, R. J. Waugh, and J. H. Bowie, *ibid.* **9**, 677 (1995).
- ¹⁶Y. V. Vasil'ev, B. J. Figard, V. G. Voinov, D. F. Barofsky, and M. L. Deinzer, *J. Am. Chem. Soc.* **128**, 5506 (2006).
- ¹⁷Y. V. Vasil'ev, D. F. Barofsky, and M. L. Deinzer (unpublished).
- ¹⁸V. G. Voinov, Y. V. Vasil'ev, J. Morré, D. F. Barofsky, M. L. Deinzer, M. Gonin, T. F. Egan, and K. Führer, *Anal. Chem.* **75**, 3001 (2003); V. G. Voinov, Y. V. Vasil'ev, H. Ji, B. J. Figard, J. Morré, T. F. Egan, D. F. Barofsky, and M. L. Deinzer, *ibid.* **76**, 2951 (2004).
- ¹⁹N. P. Ewing and C. J. Cassidy, *J. Am. Soc. Mass Spectrom.* **12**, 105 (2001).
- ²⁰A. G. Harrison and A. B. Young, *Int. J. Mass Spectrom.* **255–256**, 111 (2006); *J. Am. Soc. Mass Spectrom.* **15**, 446 (2004).
- ²¹D. Whitford, *Proteins: Structure and Function* (Wiley, Chichester, 2005).
- ²²T. Yoshimasa, K. Nakao, H. Ohtsuki, S. Li, and H. Imura, *J. Clin. Invest.* **69**, 643 (1982); *Opioid Peptides: Molecular Pharmacology, Biosynthesis, and Analysis*, edited by R. S. Rapaka and R. L. Hawks (National Institute on Drug Abuse Office of Science, Rockville, MD, 1986).
- ²³T. Hökfelt, B. Pernow, and J. Wahren, *J. Intern. Med.* **249**, 27 (2001); C. L. DeVane, *Pharmacotherapy* **21**, 1061 (2001).
- ²⁴B. A. Budnik, K. F. Haselmann, and R. A. Zubarev, *Chem. Phys. Lett.* **342**, 299 (2001).
- ²⁵F. Kjeldsen, O. A. Silivra, and R. A. Zubarev, *Chem.-Eur. J.* **12**, 7920 (2006).
- ²⁶R. Wieczorek and J. J. Dannenberg, *J. Am. Chem. Soc.* **126**, 12278 (2004).
- ²⁷R. Wieczorek and J. J. Dannenberg, *J. Am. Chem. Soc.* **126**, 14198 (2004).
- ²⁸K. Afilaatoni, B. Hitt, G. A. Gallup, and P. D. Burrow, *J. Chem. Phys.* **115**, 6489 (2001).
- ²⁹K. Afilaatoni, G. A. Gallup, and P. D. Burrow, *J. Phys. Chem. A* **104**, 7359 (2000).
- ³⁰M. Sobczyk, I. Anusiewicz, J. Berdys-Kochanska, A. Sawicka, P. Skurski, and J. Simons, *J. Phys. Chem. A* **109**, 250 (2005).
- ³¹M. Seydou, A. Modelli, B. Lucas, K. Konate, C. Desfrançois, and J. P. Schermann, *Eur. Phys. J. D* **35**, 199 (2005).
- ³²M. F. Jarrold, *Phys. Chem. Chem. Phys.* **9**, 1659 (2007).
- ³³M. Kohtani, T. C. Jones, J. E. Schneider, and M. F. Jarrold, *J. Am. Chem. Soc.* **126**, 7420 (2004); M. Kohtani, T. C. Jones, R. Sudha, and M. F. Jarrold, *ibid.* **128**, 7193 (2006).
- ³⁴R. Improta, V. Barone, K. N. Kudin, and G. E. Scuseria, *J. Chem. Phys.* **114**, 2541 (2001); *J. Am. Chem. Soc.* **123**, 3311 (2001).
- ³⁵P. Datar, S. Srivastava, E. Coutinho, and G. Govil, *Curr. Top. Med. Chem.* **4**, 75 (2004).
- ³⁶F. Eisenmenger and U. H. E. Hansmann, *J. Phys. Chem. B* **101**, 3304 (1997); T. M. Watson and J. D. Hirst, *Phys. Chem. Chem. Phys.* **6**, 2580 (2004).
- ³⁷S. Geddes, J. Zaharidis, J. Eisenhauer, and G. A. Petrucci, *Int. J. Mass Spectrom.* **282**, 13 (2009).
- ³⁸R. Mihalca, A. J. Kleinijenhuis, L. A. McDonnell, A. J. R. Heck, and R. M. A. Heeren, *J. Am. Soc. Mass Spectrom.* **15**, 1869 (2004).
- ³⁹Y. O. Tsybin, H. He, M. R. Emmett, C. L. Hendrickson, and A. G. Marshall, *Anal. Chem.* **79**, 7596 (2007).