Original Article

Formation of Benzyl Carbanion in Collision-Induced Dissociation of Deprotonated Phenylalanine Homologues

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The fragmentation behavior of deprotonated L-phenylalanine (Phe) and its homologues including L-homophenylalanine (HPA) and L-phenylglycine (PG) was investigated using collision-induced dissociation mass spectrometry coupled with a negative ion atmospheric pressure corona discharge ionization (APCDI) technique. The deprotonated molecules $[M-H]^-$ fragmented to lose unique neutral species, *e.g.*, the loss of NH_3 , CO_2 , toluene and iminoglycine for $[Phe-H]^-$; styrene and ethenamine/ CO_2 for $[HPA-H]^-$; and CO_2 for $[PG-H]^-$. All of the fragmentations observed are attributable to the formation of intermediates and/or product ions which include benzyl carbanions having resonance-stabilized structures. The carbanions are formed *via* proton rearrangement through a transition state or *via* a simple dissociation reaction. These results suggest that the principal factor governing the fragmentation behavior of deprotonated Phe homologues is the stability of the intermediate and/or product ion structures.

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INTRODUCTION

Amino acids are the critically important constitutive units of various organic macromolecules such as proteins. One of the most widespread techniques for amino acid analysis is based on chromatographic separation either following or preceding the derivatization of the amino acids with ultraviolet light absorptive or fluorescent functional group detection,^{1,2)} or ion-exchange chromatography.³⁾ Measurements of underivatized amino acids, in contrast, have been performed *via* mass spectrometry (MS) using various ionization techniques including electron ionization,^{4,5)} field desorption,⁶⁾ chemical ionization,⁷⁻¹¹⁾ fast atom bombardment,^{12,13)} electrospray ionization (ESI),¹⁴⁻¹⁸⁾ and atmospheric pressure chemical ionization (APCI).^{19,20)} ESI and APCI, typical atmospheric pressure ionization techniques, have widely contributed to the success of liquid chromatography coupled to MS for the fast analysis of underivatized amino acid.^{14,16,17,19)} Collision-induced dissociation (CID) methods have been utilized in order to better understand the fragmentation behavior of (de)protonated amino acids $[M\pm H]^\pm$.^{12-14,16,17,20)} It has been reported that CID of protonated α -amino acids $[M+H]^+$ can be interpreted simply by the loss of specific neutral species such as NH_3 , H_2O , and CO_2H_2 consisting of CO and H_2O , which depends on the various side chains.¹²⁾ On the other hand, deprotonated amino acids $[M-H]^-$ fragment irregularly to lose H_2 , NH_3 , H_2O , CO_2 ,

and CO_2H_2 , although the aliphatics retain a characteristic preference for CO_2H_2 loss. Thus, these previous reports have noted that α -amino acids other than aliphatics cannot be classified according to specific neutral fragment loss in the CID of $[M-H]^-$.¹²⁾ In order to develop a more efficient MS method useful for amino acid analysis, it is necessary to discover the crucial factors governing the fragmentation behavior of deprotonated amino acids, and especially that of the non-aliphatic ones.

Herein is presented an investigation of the fragmentation behavior of L-phenylalanine (Phe) (an aromatic amino acid) and its homologues, L-homophenylalanine (HPA) and L-phenylglycine (PG), using negative ion atmospheric pressure corona discharge ionization (APCDI) CID-MS. The analytes used have the same functionality involving phenyl, amino and carboxyl groups. The difference between Phe and HPA/PG is simply the possession of one methylene unit more or less. While individual deprotonated molecules $[Phe-H]^-$, $[HPA-H]^-$, and $[PG-H]^-$ dissociate to lose unique neutral species, all of the fragmentations observed can be attributed to the formation of benzyl carbanions as intermediates and/or product ions. The formation of the benzyl carbanion may be a key structural element in the process of fragmenting deprotonated Phe homologues. This report discusses in detail the fragmentation behavior of $[Phe-H]^-$, $[HPA-H]^-$, and $[PG-H]^-$ involving the formation of benzyl carbanions.

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EXPERIMENTAL

Analytes

L-Phenylalanine (M_r 165) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). L-Homophenylalanine (M_r 170) and L-phenylglycine (M_r 151) were purchased from Tokyo Chemical Industry (Tokyo, Japan). The deuterium (D) labeled derivative of L-phenylalanine (M_r 167), which has 2 D atoms at the benzylic position, was purchased from Taiyo Nippon Sanso Corp. (Tokyo, Japan). All of the analytes were used without further purification.

APCDI and CID-MS

Discharge experiments using point-to-plane electrodes were performed at atmospheric pressure in unadulterated laboratory air, composed of common air constituents N_2 , O_2 , H_2O , and CO_2 . The laboratory temperature (298 K) and relative humidity (40–70%) were controlled using an air conditioner. The corona needle used as the point electrode was a headless stainless steel pin (Shiga, Tokyo, Japan) with a diameter of 200 μm , 20 mm in length. The needle tip had a radius of curvature of *ca.* 1 μm and the shape of the needle tip surface was adequately approximated by a hyperboloid of revolution. The opposite electrode was the stainless steel orifice plate of a TSQ-7000 triple-quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The discharge gap between the electrodes was adjusted to 3 mm using a manipulator. The DC corona voltage and needle angle with respect to the orifice hole axis used were -1.9 kV and $\pi/2$ rad., respectively. A ceramic micro-heater was placed between the needle and the orifice plate in order to vaporize condensed-phase analytes. Analytes diffused into the discharge area and were negatively ionized. The resulting gas-phase analyte ions were introduced into the orifice hole of the mass spectrometer. The collision gas and energy (lab.) used in the CID experiments were argon at 2.0×10^{-3} Torr and 5–40 eV, respectively.

RESULTS AND DISCUSSION

Collision-induced dissociation of adduct ions $[M+R]^-$

The corona voltage and needle angle used led to a low electric field strength on the needle tip surface ($\approx 10^7$ V m^{-1}) and resulting NO_3^- -free discharge area.²¹⁾ As previously reported,²¹⁾ an NO_3^- -free area can bring about the predominant formation of atmospheric negative ions R^- with relatively high proton affinities (PAs), including O_2^- ($PA(O_2^-) = -\Delta H^\circ(O_2^- + H^+ \rightarrow HO_2) = 1477.0 \pm 2.9$ kJ mol^{-1}),²²⁾ HCO_3^- ($PA(HCO_3^-) < 1551.0 \pm 9.2$ kJ mol^{-1}),²³⁾ and NO_2^- ($PA(NO_2^-) = 1423.4 \pm 9.2$ kJ mol^{-1}).²⁴⁾ Figure 1 shows that those R^- ions ionize analytes *M* such as L-phenylalanine (Phe), L-homophenylalanine (HPA), and L-phenylglycine (PG) to form adducts $[M+R]^-$. The $[M+R]^-$ ions can be produced *via* the following three-body reactions with a third body *P* such as N_2 or O_2 in the discharge area.⁷⁾

It has been reported that CID experiments on the adducts $[M+R]^-$ formed in an NO_3^- -free area make it possible to analyze the fragmentation behavior of deprotonated analytes

$[M-H]^-$.²⁵⁾ That is, the $[M+R]^-$ ions initially dissociate, accompanied by proton abstraction from *M* by R^- due to the higher proton affinity of R^- vs. *M*, resulting in the generation of $[M-H]^-$ (Reaction 2).

The $[M-H]^-$ product ions formed *via* Reaction 2 have the same structure as $[M-H]^-$ ions generated in an APCDI source. When the $[M-H]^-$ product ions have excess energy, further fragmentation of $[M-H]^-$ can occur. The fragmentation behavior of $[M+R]^-$ adducts formed in an NO_3^- -free area has been described in detail elsewhere.^{25,26)}

Figures 2–4 show the CID spectra of the adduct ions $[M+R]^-$ (R^- : O_2^- , HCO_3^- , and NO_2^-) for Phe, HPA, and PG, respectively, obtained at a collision energy of 25 eV. The CID of $[Phe+R]^-$, $[HPA+R]^-$, and $[PG+R]^-$ resulted in the formation of individual deprotonated analytes $[M-H]^-$ as well as the product ions at m/z 147, 103, 91, and 72 (∇ in Figs. 2(a)–(c)), 91 and 74 (∇ in Figs. 3(a)–(c)), and 106 (∇ in Figs. 4(a)–(c)), respectively. According to a previous study,²⁵⁾ the product ions marked as ∇ in Figs. 2–4 most likely originate from the deprotonated analytes $[M-H]^-$. Other studies using ESI tandem MS and fast atom bombardment collisional activation MS have also reported the generation of product ions at m/z 147, 103, 91, and 72 in the CID of $[Phe-H]^-$,^{12,14)} which is consistent with the present results (Fig. 2). On the basis of the CID spectra of the adduct ions $[M+R]^-$ for Phe, HPA, and PG obtained here, the fragmentation behavior of the deprotonated analytes $[Phe-H]^-$, $[HPA-H]^-$, and $[PG-H]^-$ are discussed below.

Fragmentation characteristics of $[M-H]^-$

The $[Phe-H]^-$ at m/z 164 fragments to form the product ions at m/z 147, 103, 91, and 72, as shown in Fig. 2. The abundances of those product ions varies with varying collision energy. Figure 5(a) shows the relative intensities of $[Phe-H]^-$ and the resulting four product ions as a function of collision energy, obtained from the CID spectra of the O_2^- adducts $[Phe+O_2]^-$. The relative intensities (*RI*) of individual ions at a given collision energy were calculated from the measured absolute peak intensities $AI(Y^-)$ (Y^- : $[Phe+O_2]^-$, $[Phe-H]^-$, and product ions at m/z 147, 103, 91, and 72) in the CID spectra using the following equation:

$$RI(Y^-) = (AI(Y^-)) / \sum AI(Y^-) \times 100 \quad (3)$$

The intensity patterns of individual ions with changing collision energy are independent of the atmospheric ion species R^- of the precursor ions $[Phe+R]^-$. At low collision energies (10–20 eV), the product ion at m/z 147 is dominant, likely formed *via* the loss of NH_3 (17 Da) from $[Phe-H]^-$. With increasing collision energy, the relative intensity of the product ion at m/z 103 increases while the ion at m/z 147 decreases. This result suggests that the product ion at m/z 147 having excess internal energy can be further fragmented *via* the loss of CO_2 (44 Da) to form the product ion at m/z 103. In order to understand the detailed fragmentation behavior of $[Phe-H]^-$ to form the product ions at m/z 147 and 103, CID experiments using an isotope-labeled analyte, Phe- d_2 , having two deuterium (D) atoms at the benzyl position (see Fig. 1(b)), were performed. The CID spectrum of $[Phe-d_2+O_2]^-$ at m/z 199 obtained at a collision energy of 25 eV is shown in Fig. 2(d). The product ions at m/z 148 and 104 are shifted by

Fig. 1. Negative ion mass spectra of (a) Phe, (b) Phe- d_2 , (c) HPA, and (d) PG. A_{BP} represents the absolute abundance (arbitrary units) of the base peak in each mass spectrum.

Fig. 2. APCDI-CID spectra of the precursor ions (a) $[Phe + O_2]^-$, (b) $[Phe + HCO_3]^-$, (c) $[Phe + NO_2]^-$, and (d) $[Phe-d_2 + O_2]^-$ obtained at a collision energy of 25 eV.

Fig. 3. APCDI-CID spectra of the precursor ions (a) $[\text{HPA}+\text{O}_2]^-$, (b) $[\text{HPA}+\text{HCO}_3]^-$, and (c) $[\text{HPA}+\text{NO}_2]^-$ obtained at a collision energy of 25 eV.

Fig. 4. APCDI-CID spectra of the precursor ions (a) $[\text{PG}+\text{O}_2]^-$, (b) $[\text{PG}+\text{HCO}_3]^-$, and (c) $[\text{PG}+\text{NO}_2]^-$ obtained at a collision energy of 25 eV. The ion peaks having an asterisk (*) represent the atmospheric ions as background.

1 Da relative to the ions produced *via* the CID of $[\text{Phe}+\text{R}]^-$ (m/z 147 and 103 in Figs. 2(a)–(c)). This fact indicates that both of the product ions at m/z 148 and 104 contain one D atom, *i.e.*, $[\text{Phe}-\text{H}]^-$ initially loses the amino group and one H atom from the benzylic position to form the product ion at m/z 147. This phenomenon can occur due to the rearrangement of a proton from the benzylic position to a carboxyl group *via* a five-membered ring transition state, resulting in the formation of an intermediate which possesses a carbanion at the benzyl position. Thus, the fragmentation pathway of $[\text{Phe}-\text{H}]^-$ to form the product ions at m/z 147 and 103 can be presumed to be as shown in Scheme 1(a).

The $[\text{Phe}-\text{H}]^-$ ion activated at higher collision energy led to the formation of product ions at m/z 91 and 72 (Fig. 5(a)). It should be noted that CID of $[\text{Phe}-d_2-\text{H}]^-$ resulted in product ions at m/z 93 and 72, suggesting that they contain two and no D atoms, respectively. It is likely therefore that the product ion at m/z 91 corresponds to a benzyl anion (BA) originating from the side-chain of $[\text{Phe}-\text{H}]^-$, while the ion at m/z 72 is attributable to the backbone moiety. In order to explain the formation of the product ions at m/z 91 and 72, it is reasonable to propose that $[\text{Phe}-\text{H}]^-$ can be converted into an ion-neutral complex consisting of BA and iminoglycine (IG, 73 Da) *via* rearrangement of a proton from the amino group to the carboxyl group through a five-

membered ring transition state, as shown in Scheme 1(b)-i. When the ion-neutral complex simply dissociates into BA and IG, the BA can be produced as the product ion at m/z 91 (Scheme 1(b)-ii). In contrast, it could be expected that the ion-neutral complex dissociates accompanied by proton abstraction from IG by BA, because of the high proton affinity of BA ($1587 \pm 8.8 \text{ kJ mol}^{-1}$).²⁷ This process leads to the formation of deprotonated IG, $[\text{IG}-\text{H}]^-$, at m/z 72 as an alternate product ion (Scheme 1(b)-iii). The formation of $[\text{IG}-\text{H}]^-$ (m/z 72) dominates as compared to that of BA (m/z 91) with increasing collision energy, as shown in Fig. 5. This result may indicate that the activation energy for the dissociation reaction accompanied by proton transfer from IG to BA (Scheme 1(b)-iii) is higher than that for the simple dissociation into BA and IG (Scheme 1(b)-ii). Figure 5(a) also shows that the reactions presented in Scheme 1(a) can begin to occur at lower collision energy than those in Scheme 1(b), while both Schemes 1(a) and (b) originate from proton rearrangement *via* transition states and intermediates including carbanions at the benzyl position. This can be interpreted by energy values related to proton release from the benzyl position and the amino group in $[\text{Phe}-\text{H}]^-$, estimated approximately using the gas-phase acidities (GAs) of toluene ($\text{C}_6\text{H}_5\text{CH}_3$) and ethylamine ($\text{C}_2\text{H}_5\text{NH}_2$). The GA of toluene (ΔH° ($\text{C}_6\text{H}_5\text{CH}_3 \rightarrow \text{C}_6\text{H}_5\text{CH}_2^-$ (BA) + H^+), 1587 ± 8.8 ,²⁷) is lower

Fig. 5. Relative intensities of (a) [Phe-H]⁻ and the product ions at *m/z* 147, 103, 91, and 72, (b) [HPA-H]⁻ and the product ions at *m/z* 91 and 74, and (c) [PG-H]⁻ and the product ion at *m/z* 106 as a function of collision energy, obtained using the CID spectra of [M+O₂]⁻ for individual analytes M.

than that of ethylamine (ΔH° (C₂H₅NH₂→C₂H₅NH+H⁺), 1671±4.6 kJ mol⁻¹.²⁸) It is therefore suggested that a proton at the benzyl position can be released under less energetic conditions as compared to one at the amino group in [Phe-H]⁻, resulting in the occurrence of reactions originating from proton rearrangement from the benzyl position (Scheme 1(a)) at lower collision energy.

Collision-induced dissociation of [HPA-H]⁻ ions (*m/z* 178) led to the formation of product ions at *m/z* 74 and 91 (Fig. 3), which most likely correspond to the glycine enolate and benzyl anions originating from the backbone and side-chains of [HPA-H]⁻, respectively. Taking into account the fragmentation behavior of [Phe-H]⁻ shown in Scheme 1(a), it seems likely that the glycine enolate anion (*m/z* 74) occurs *via* initial proton rearrangement from the benzyl position

to the carboxyl group through a six-membered ring transition state, followed by subsequent loss of styrene (104 Da) from [HPA-H]⁻, as shown in Scheme 2(a). The benzyl anion at *m/z* 91 is likely formed *via* simple dissociation reactions including the successive loss of CO₂ (44 Da) and ethenamine (43 Da) (Scheme 2(b)). The dominant formation of the glycine enolate anion at *m/z* 74, independent of precursor ion species and collision energy (Figs. 3 and 5(b)), indicates that preferential proton rearrangement *via* the six-membered ring transition state (Scheme 2(a)) takes place as compared to the simple dissociation reaction (Scheme 2(b)). In contrast, the [PG-H]⁻ ion at *m/z* 150 can only be fragmented to form the product ion at *m/z* 106 *via* the loss of CO₂, regardless of collision energy (Fig. 5(c)). A proposed fragmentation mechanism for [PG-H]⁻ is shown in Scheme 3.

It should be noted here that all of the fragmentations observed in the CID of [Phe-H]⁻, [HPA-H]⁻, and [PG-H]⁻ involve intermediates and/or product ions which include carbanions at the benzyl position (Schemes 1–3). The carbanions are most likely stabilized by resonance.⁶⁾ It is therefore concluded that the principal factor governing the fragmentation characteristics of deprotonated Phe homologues is the stability of the structure of intermediates and/or product ions. That is, fragmentations proceed to form benzyl carbanions having resonance-stabilized structures *via* proton rearrangement through transition states and/or simple dissociation reactions.

CONCLUSION

The fragmentation behavior of deprotonated L-phenylalanine (Phe) and its homologues L-homophenylalanine (HPA) and L-phenylglycine (PG) was investigated using CID-MS combined with NO₃⁻-free APCDI. Analytes M in the NO₃⁻-free discharge area were ionized by atmospheric negative ions R⁻ having high proton affinities, *e.g.*, O₂⁻, HCO₃⁻, and NO₂⁻, to form [M+R]⁻ adducts. The CID of individual adducts [M+R]⁻ led to the formation of product ions originating from deprotonated analytes [M-H]⁻. The observed fragmentation behavior of [Phe-H]⁻, [HPA-H]⁻, and [PG-H]⁻ lead to the following conclusions:

1. The principal factor governing the fragmentation behavior of the deprotonated Phe homologues [M-H]⁻ is the stability of the structure of intermediates and/or product ions. That is, all of the precursor ions [M-H]⁻ dissociate to form a carbanion at the benzyl position, having a resonance-stabilized structure, *via* proton rearrangement through a transition state and/or *via* simple dissociation reactions.
2. If a given precursor ion [M-H]⁻ is capable of generating a benzyl carbanion *via* both proton rearrangement and a simple dissociation reaction, the proton rearrangement proceeds preferentially as compared to the simple dissociation reactions.
3. If there are some different proton rearrangement pathways generating benzyl carbanions in the CID of a certain [M-H]⁻ ion, the pathway originating from the position at which a proton can be released less energetically proceeds under lower collision energy conditions.

The present study contributes to an understanding of the fragmentation behavior of deprotonated aromatic amino

Scheme 1. Proposed fragmentation behavior of deprotonated (D labeled) L-phenylalanine $[\text{Phe (Phe-}d_2\text{)}-\text{H}]^-$ to form (a) the product ions at m/z 147 (148) and 103 (104) and (b) m/z 91 (93) and 72.

Scheme 2. Proposed fragmentation behavior of deprotonated L-homophenylalanine $[\text{HPA}-\text{H}]^-$ to form (a) the product ions at m/z 74 and (b) m/z 91.

Scheme 3. Proposed fragmentation behavior of deprotonated L-phenylglycine $[\text{PG}-\text{H}]^-$ to form a benzyl carbanion at m/z 106.

acids.

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