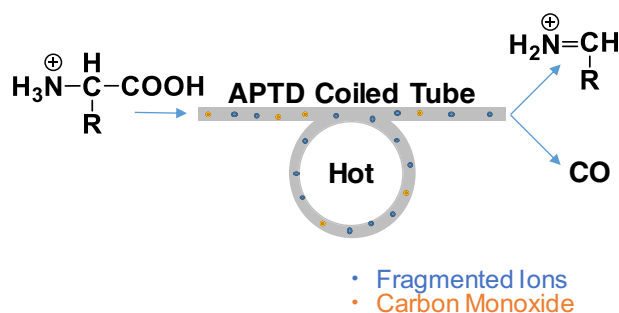


RESEARCH ARTICLE

Detection of Neutral CO Lost During Ionic Dissociation Using Atmospheric Pressure Thermal Dissociation Mass Spectrometry (APTD-MS)

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Abstract. Elucidation of ion dissociation patterns is particularly important to structural analysis by mass spectrometry (MS). However, typically, only the charged fragments from an ion dissociation event are detected in tandem MS experiments; neutrals are not identified. In recent years, we have developed an atmospheric pressure thermal dissociation (APTD) technique that can be applied to dissociate ions at atmosphere pressure and thus provide one way to characterize neutral

fragments. In this paper, we focus on the detection of neutral CO resulting from amino acid and peptide ion dissociation. In the first set of experiments, several protonated amino acids (e.g., +1 ion of phenylalanine) were found to undergo loss of a neutral (s) of total mass 46 Da, a process leading to iminium ion formation. We successfully detected the neutral species CO by using a CO sensor, UV-Vis and MS analysis following selective CO trapping with a rhodium complex. The capture of CO from dissociation of protonated amino acids supports the assignment of the loss of 46 Da to neutral losses of CO and H₂O, rather than loss of formaldehyde or dihydroxycarbene, other possible fragmentation pathways that have been subject of debate for a long time. In a second experiment, we used the APTD method in combination with the CO detection technique, to demonstrate the formation of CO in the conversion of *b* ions to *a* ions during peptide ion dissociations. These results showed the potential of APTD in the elucidation of ion dissociation mechanisms, using simple home-built apparatus.

Keywords: Neutral fragment detection, Carbon monoxide, peptide and amino acid, Mass spectrometry

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Introduction

Ionic dissociation is the usual basis for structural analysis by MS [1–8]. However, a significant limitation is that only charged fragments from ion dissociation events are detected while neutral fragments are simply lost. Researchers began to

pay attention to the “lost” neutral fragments several decades ago. Analysis of neutral species by mass spectrometric techniques became possible when neutralization-reionization mass spectrometry (NRMS) [9–11] and neutral fragment reionization (N_rR) [12] were introduced, based on reionization of the resulting neutrals by collision with another target (e.g., O₂) via electron transfer or charge stripping. However, although this type of technique is very powerful, it requires specialized instrumentation and it is implemented in vacuum, which limits its utility. Recently, we reported our efforts to solve this problem by developing atmospheric pressure neutral reionization mass spectrometry (APNR) [13–17]. In APNR, analyte ions are thermally dissociated using atmospheric

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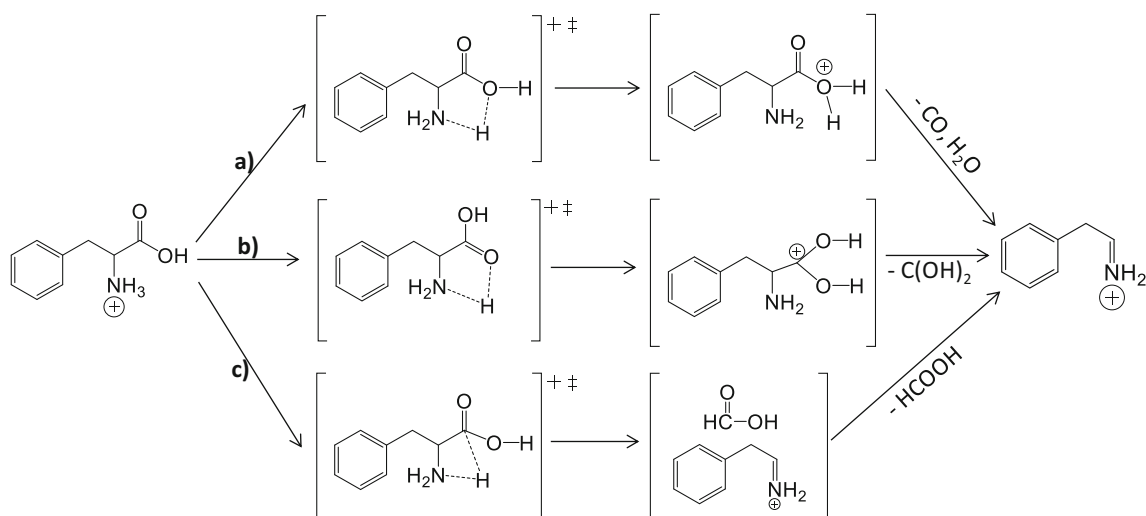
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pressure thermal dissociation (APTD) followed by online ambient reionization using electrosonic spray ionization (ESSI) or corona discharge. In this study, we report that neutral CO species lost during fragmentation can be characterized by several techniques including online CO sensor monitoring, and offline UV-Vis or MS detection following complexation with a binuclear rhodium complex. Detection of CO is significant as CO was proposed to be produced from two important ion dissociation pathways: the dissociation of protonated amino acids to form iminium ions (loss of 46 Da) and the conversion from peptide *b* ions to *a* ions (loss of 28 Da).

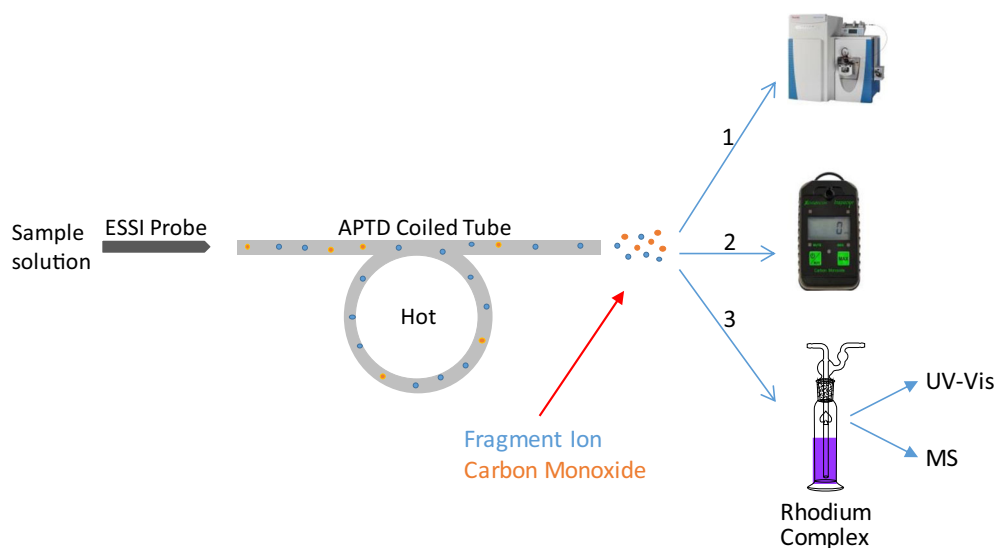
Although gas-phase ion dissociation of protonated amino acids has been extensively studied under a variety of ionization conditions and methods, [18–27] the mechanism for the formation of iminium ion during amino acid or peptide ion dissociation [28–30] has been the subject of debate. Three main mechanisms have been previously proposed in different studies (see Scheme 1 (a) losses of H₂O and CO; (b) loss of dihydroxycarbene, C(OH)₂, and (c) loss of formic acid, HCOOH [31]. However, none of these studies have given unambiguous experimental evidence regarding the structure of the lost neutrals. In the report by Harrison et al., which proposed pathway **a** (Scheme 1), the authors demonstrated that the reaction was analogous to the retro-Koch reaction and required a translocation of the proton from nitrogen to the OH group with a subsequent loss of CO and H₂O [27]. Pathway **b** (Scheme 1), proposed to produce C(OH)₂, was supported by Kulik and coworkers via metastable ion and collisional activation fast atom bombardment (FAB) technique [32]. Unfortunately, the result showed no evidence for an intact C(OH)₂ neutral product. Pathway **c** (Scheme 1) was proposed by Meot-Ner and Field, and formic acid was suggested to be produced. However, this mechanism requiring a four-centered transition state was expected to be thermodynamically unfavorable [33]. Later, O'Hair and coworkers explored this ion dissociation mech-

anism through a computational study. Based on ab initio and density functional theory calculations, the losses of CO and H₂O neutrals were shown to be both thermodynamically and kinetically favored over the alternative HCOOH or C(OH)₂ loss process [31]. Furthermore, the unimolecular chemistry of protonated amino acids was also explored in detail by Wesdemiotis et al [1]. However, direct characterization of CO neutral resulting from amino acid ion dissociation has not previously been achieved and that is the focus of the current study.

In this paper, we applied the APTD technique to dissociate protonated amino acids such as +1 ion of phenylalanine, generated by electrosonic spray ionization (ESSI, a soft ionization method similar to electrospray ionization ESI [34]), to generate both ionic and neutral fragments at atmospheric pressure. As APTD operates outside the mass spectrometer rather than in vacuum, it should be possible to access the neutrals and characterize them. As shown in Scheme 2, an amino acid (e.g., phenylalanine) was sprayed by ESSI into a coiled and heated tube outside the mass spectrometer where it underwent thermal dissociation to produce fragment ions and neutrals. The coiled shape increases gas turbulence, facilitating ion dissociation. Our results showed that neither formic acid (HCOOH) nor dihydroxycarbene C(OH)₂ was detected during APTD of phenylalanine. On the other hand, CO was successfully detected in the process. This study provides direct evidence to support the production of carbon monoxide in the process for the formation of iminium ion from amino acid ion dissociation. In addition, upon ion activation such as collision-induced dissociation (CID), *b* ions are often produced as a result of peptide amide backbone cleavage. It is also well accepted that *a* ions are generated from *b* ions via further dissociation by loss of CO (Scheme 3) [35–38]. However, CO was not characterized previously in this ion dissociation process. This experiment also was



Scheme 1. Three proposed amino acid fragmentation pathways toward the formation of iminium ion (illustrated using phenylalanine as an example): (a) losses of CO and H₂O molecules, (b) loss of dihydroxycarbene C(OH)₂, and (c) loss of formic acid HCOOH



Scheme 2. Schematic drawing of the APTD apparatus and processes: (1) direct detection of ionic fragments from the APTD coiled tube by MS, (2) use of CO sensor to directly detect carbon monoxide emerging from the APTD coiled tube, and (3) use a binuclear rhodium complex solution to trap CO molecules followed with offline characterization by MS and/or UV/Vis

attempted to provide evidence for this widely accepted assumption.

Experimental

General Materials

Phenylalanine, leucine, histidine, tryptophan, ^{13}C -labeled phenylalanine, phenethylamine, and rhodium acetate dimer were purchased from Sigma-Aldrich (St. Louis, MO). Gly-His-Gly and Gly-Trp-Gly peptides were bought from MP Biomedicals, LLC (Solon, OH) and Chem-Impex Int'l Inc., (Wood Dale, IL), respectively. HPLC-grade acetonitrile was purchased from Fisher Scientific (Hampton, NH). Acetic anhydride was purchased from Spectrum Chemical (New Brunswick, NJ). Ammonium hydroxide was purchased from GFS Chemicals (Powell, OH). Deionized water for sample preparation was obtained from a Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA).

APTD-MS Setup

The APTD device was home-made, as a one-loop stainless steel tube (3.2 mm o.d., 1.6 mm i.d., 20 cm length, and 2.5 cm loop diameter) wrapped by heating tape [17]. Either a LCQ DECA XP MAX (Thermo Finnigan, San Jose, CA) or a Thermo Fisher Orbitrap QE Plus mass spectrometer (San Jose, CA) was used to analyze the APTD-produced ions. A 90-V voltage was applied to the heating tape and the

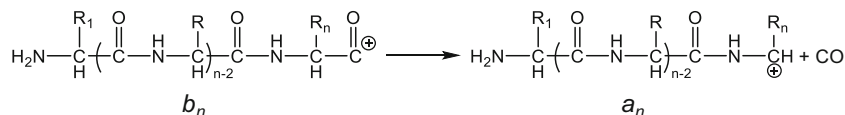
stainless steel tube was heated to 300 °C. The temperature of the APTD tube was measured using an infrared thermometer (Tenma, Taiwan). The sample solution was sprayed from an ESSI source operated at the flow rate of 10 $\mu\text{L}/\text{min}$ with the assistance of +5 kV voltage and 170 psi nebulizing N_2 gas.

Synthesis of Binuclear Rhodium Complex *Cis*-[$\text{Rh}_2(\text{C}_6\text{H}_4\text{PPh}_2)_2(\text{O}_2\text{CCH}_3)_2](\text{HOAc})_2$

The synthesis followed a reported procedure [43]. A 100-mg quantity of rhodium acetate dimer $\text{Rh}_2(\text{O}_2\text{CCH}_3)_4$ and 150 mg of triphenylphosphine were added into 10 mL of acetic acid in a flask under a nitrogen gas atmosphere. The mixture solution was refluxed for 2 h during which the blue coloration was replaced by red-brown, and then a dark purple color. The solution was concentrated to half of its initial volume and the purple solid deposit was filtered off and washed by ACN. The ^1H -NMR for this compound was δ 1.17 (s, $[\text{O}_2\text{CCH}_3]$, 6H), 2.15 (s, CH_3COOH , 6H), 6.4–7.7 (m, $[(\text{C}_6\text{H}_5)_2\text{P}(\text{C}_6\text{H}_4)]$, 28H) (Signal of the carboxylic acid proton of CH_3COOH was not observed due to proximity to Rh).

MS Analysis of Carbon Monoxide Trapped by Binuclear Rhodium Complex Solution

A 25-mM phenylalanine in ACN/ H_2O /HOAc solution was sprayed by an ESSI source and dissociated in the APTD tube, and the species flowing out of the tube was then directed into a



Scheme 3. Scheme showing dissociation from peptide (b) ion to a ion by the loss of neutral CO. Note that a and b ions are drawn in the way shown in their original definitions; their true structures may well be cyclic [39–42]

trap containing 5 mL of 3 mM dinuclear rhodium complex solution in chloroform for 3 min. The amino acid sample injection flow rate was 10 $\mu\text{L}/\text{min}$ and the nebulizing gas pressure used was 100 psi. After trapping, the solution was diluted by ACN with 0.1% HOAc (1:1 dilution), and then subject to ESI-MS analysis. The ESI-MS spectra were collected by using a Thermo Fisher Orbitrap QE Plus mass spectrometer (San Jose, CA) in the positive ion mode.

UV-Vis Analysis of Carbon Monoxide Trapped by Binuclear Rhodium Complex Solution

The dinuclear rhodium complex solution (3 mL, 1 mM) was used to capture the carbon monoxide from the phenylalanine APTD dissociation. The trapping period was 3 min for each sample. The amino acid sample injection flow rate for APTD was 10 $\mu\text{L}/\text{min}$ and the nebulizing gas pressure used was 100 psi. The UV spectra were collected by using an Agilent 8453 UV-visible spectrophotometer (Santa Clara, CA).

Results and Discussion

APTD of Phenylalanine

The dissociation of the amino acid phenylalanine (Phe) by APTD was first investigated at several different temperatures (Fig. 1). At relatively low temperature (45 $^{\circ}\text{C}$, APTD coiled tube temperature), the $[\text{Phe} + \text{H}]^+$ peak (m/z 166) was dominant, which meant that most of $[\text{Phe} + \text{H}]^+$ had not dissociated

at this temperature. Only a small peak at m/z 120 was seen, corresponding to the iminium ion $[\text{Phe} + \text{H} - \text{CO}_2\text{H}_2]^+$ from dissociation of $[\text{Phe} + \text{H}]^+$ via loss of a neutral (s) with chemical formula CO_2H_2 (total mass 46 Da). When the temperature was increased, the intensity of $[\text{Phe} + \text{H} - \text{CO}_2\text{H}_2]^+$ peak (m/z 120) increased as well. At 300 $^{\circ}\text{C}$, the $[\text{Phe} + \text{H} - \text{CO}_2\text{H}_2]^+$ peak (m/z 120) was abundant and the $[\text{Phe} + \text{H}]^+$ peak (m/z 166) had disappeared from the spectrum. As expected, when phenylalanine was replaced by ^{13}C -labeled phenylalanine sample (the carboxylic carbon is labeled with ^{13}C), a major fragment ion $[\text{Phe} + \text{H} - \text{CO}_2\text{H}_2]^+$ (m/z 120) was also observed, resulting from dissociation of $[\text{Phe} + \text{H}]^+$ via loss of a neutral (s) with chemical formula $^{13}\text{CO}_2\text{H}_2$ (total mass 47 Da, Fig. 1S, Supporting Information). Indeed, in this ion dissociation experiment, although the fragment ion of m/z 120 was observed, the corresponding neutrals were not detected, and they could be either $\text{CO} + \text{H}_2\text{O}$, HCOOH or dihydroxycarbene $\text{C}(\text{OH})_2$.

Detection of Carbon Monoxide by Sensor

Loss of a neutral fragment (s) with mass 46 Da from $[\text{Phe} + \text{H}]^+$ (m/z 166) yielded the fragment ion $[\text{Phe} + \text{H} - \text{CO}_2\text{H}_2]^+$ (m/z 120) upon APTD of phenylalanine at 300 $^{\circ}\text{C}$. O'Hair et al. [31] proposed that protonated aliphatic amino acids could lose neutral H_2O and CO (the combined mass is 46 Da) upon fragmentation in the gas phase, based on computation. Previously established methods for carbon monoxide detection include gas chromatography [44], carboxy-myoglobin (Mb-CO) assay [45, 46], electrochemical assays [47, 48], colorimetric

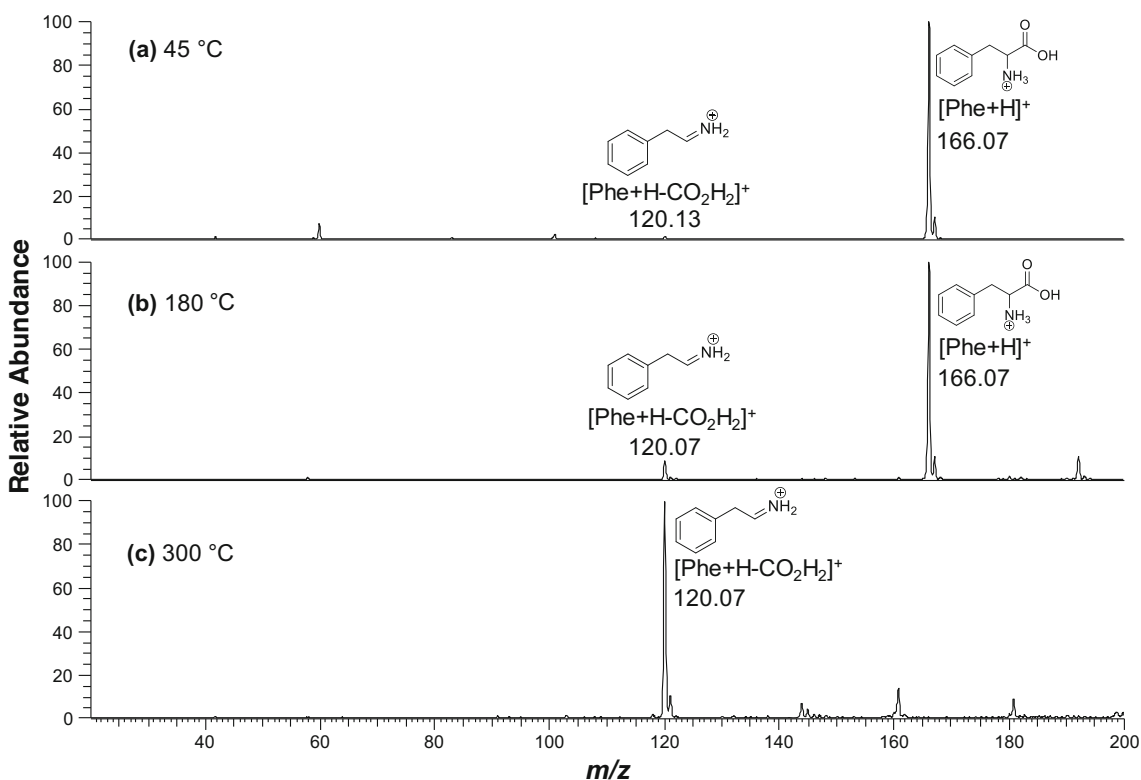


Figure 1. APTD-MS spectra for phenylalanine at (a) 45 $^{\circ}\text{C}$, (b) 180 $^{\circ}\text{C}$, and (c) 300 $^{\circ}\text{C}$. 0.25 mM phenylalanine solution in ACN/ H_2O /HOAc (50:50:0.1 by volume) was sprayed by ESSI (spray voltage: +5 kV) into a hot coiled tube at a flow rate of 10 $\mu\text{L}/\text{min}$

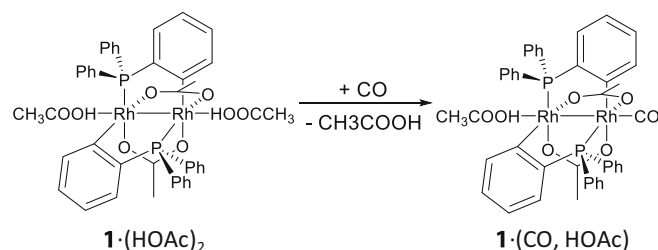
CO sensing [49, 50], and laser infrared absorption methods [51]. In this study, a Sensorcon carbon monoxide detector, a household product for monitoring the presence of CO due to safety concerns, was used to detect CO generated in the amino acid APTD process. The detection mechanism of the CO sensor is that CO is oxidized on a platinum working electrode. The current generated from the electrochemical reaction gives a measure of the concentration of CO in the atmosphere [52–54]. In this experiment, the CO detector was held about 1 cm from the outlet of APTD tube. All of the blank solvents and samples were measured in triplicates (Fig. 2). While CO was not detected by the sensor from APTD of the blank solvent (Fig. 2(1)), phenylalanine sample did show a positive reading from CO detector (15 ppm on average, Fig. 2(3)). In addition, phenethylamine, structurally similar to phenylalanine except for the absence of a carboxylic acid group, was also sprayed by ESSI and underwent dissociation in APTD tube. As expected, no CO was detected by the sensor reading (Fig. 2(2)). As phenethylamine and phenylalanine only differ in a terminal carboxylic group, the CO sensor detection result strongly suggested that CO was produced from the loss of the carboxylic group of phenylalanine.

UV-Vis Analysis of Carbon Monoxide after Binding with a Binuclear Rhodium Complex

In order to more thoroughly examine our hypothesis, a binuclear rhodium complex *cis*-[Rh₂(C₆H₄PPh₂)₂(O₂CCH₃)₂](HOAc)₂, **1** (HOAc)₂, was chosen to trap the APTD-generated carbon monoxide gas, and the resulting complex **1** (CO, HOAc) was then analyzed by UV-Vis spectroscopy. This binuclear rhodium complex was first synthesized by Cotton et al. [43], and further developed into a colorimetric sensing method by Esteban et al [49, 50]. The molecule contains two cyclometalated phosphine ligands and two acetic acid molecules attached to the two rhodium atoms. When this binuclear rhodium complex is exposed to carbon monoxide gas, one acetic acid molecule can be replaced by a CO molecule (Scheme 4). Upon CO binding, a characteristic

#	Structure	Triplicated Trials	Avg. Readings (ppm)
1	blank solvent		0
2			0
3			15

Figure 2. Measurements of carbon monoxide produced from APTD of (1) ACN/H₂O/HOAc (50:50:0.1) blank solvent, (2) 25 mM phenethylamine in ACN/H₂O/HOAc (50:50:0.1), (3) 25 mM phenylalanine in ACN/H₂O/HOAc (50:50:0.1). All of measurements were made in triplicate as shown



Scheme 4. Selective binding of CO by the binuclear rhodium complex **1**·(HOAc)₂. The complex product **1**·(CO, HOAc) forms after CO binding

UV-Vis absorption peak at 480 nm can be seen. This complex is highly selective to CO, and does not bind other gases like CO₂, N₂, O₂, or Ar [49].

In this experiment, the UV-Vis spectrum of the pure dinuclear rhodium complex **1**·(HOAc)₂ in chloroform (1 mM) was measured first and the absorption at 480 nm was low (line a, Fig. 3). When 25 mM phenylalanine in ACN/H₂O/HOAc (50:50:0.1) was sprayed into the coiled tube at 300 °C for APTD and the gaseous species flowing out of the APTD tube was bubbled through the trapping solution of dinuclear rhodium complex **1**·(HOAc)₂ in chloroform (1 mM), the UV-Vis spectrum of the resulting solution showed a pronounced increase in absorption at 480 nm (line d, Fig. 3). The duration for the CO collection was 3 min. The result indicates the formation of CO during APTD of phenylalanine. Several control experiments were also conducted. When no phenylalanine was in the

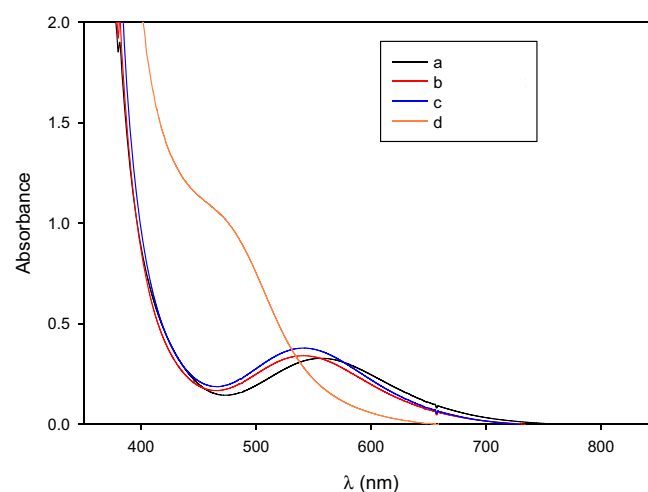


Figure 3. UV-vis spectra of (a) 1 mM dinuclear rhodium complex **1**·(HOAc)₂ in chloroform (black line a), (b) 1 mM dinuclear rhodium complex **1**·(HOAc)₂ in chloroform after trapping gaseous species flowing out of the APTD when a blank solvent ACN/H₂O/HOAc (50:50:0.1) was sprayed into the APTD tube at 300 °C (red line b), (c) 1 mM dinuclear rhodium complex **1**·(HOAc)₂ in chloroform after trapping species flowing out of the APTD when 25 mM phenethylamine in ACN/H₂O/HOAc (50:50:0.1) was sprayed into the APTD tube at 300 °C (blue line c), and (d) 1 mM dinuclear rhodium complex **1**·(HOAc)₂ in chloroform after trapping species flowing out of the APTD when 25 mM phenylalanine in ACN/H₂O/HOAc (50:50:0.1) was sprayed into the APTD tube at 300 °C (orange line d)

spray solvent for APTD, no increase in 480 nm absorption was noted (line b, Fig. 3). Furthermore, when phenethylamine solution was sprayed for APTD, no change in 480 nm absorption was detected (line c, Fig. 3). Similar to the CO sensor measurement result mentioned above, these UV-Vis data showed that CO is formed from APTD of phenylalanine and originates from the carboxylic acid group of the amino acid.

MS Analysis of Carbon Monoxide after Binding with Binuclear Rhodium Complex

MS should be an accurate, sensitive, and reliable tool to detect small molecules like carbon monoxide. However, the mass of CO is smaller than the cutoff mass of our mass spectrometers. Therefore, direct online analysis of CO from APTD was not an option in this case. We reasoned that CO could be detectable after selective trapping with the binuclear rhodium complex. Figure 4a displays the ESI-MS spectrum of 3 mM dinuclear rhodium complex **1**·(HOAc)₂ in chloroform. The peak of m/z

786.99 corresponding to the binuclear rhodium complex cation $[1\text{-OAc}]^+$ (theoretical mass 786.9909, observed mass 786.9882, mass error 3.43 ppm), was detected. Notably, when the gaseous species flowing out of the heated tube after APTD of phenylalanine was trapped by the dinuclear rhodium complex **1**·(HOAc)₂, analysis of the rhodium complex revealed a new peak at m/z 814.99 (Fig. 4d), corresponding to $[1\text{-OAc} + \text{CO}]^+$ (theoretical mass 814.9859, observed mass 814.9852, mass error 0.86 ppm). CID of m/z 814.99 gave rise to fragment ions at m/z 786.99 and 726.97 (Fig. 2S-a, Supporting Information), by consecutive losses of CO and HOAc, consistent with its structural assignment. Furthermore, when standard carbon monoxide gas was bubbled into the dinuclear rhodium complex **1**·(HOAc)₂, the adduct ion $[1\text{-OAc} + \text{CO}]^+$ (m/z 814.99) was also observed (Figure 4e). These results show that APTD of phenylalanine does produce CO. Meanwhile, when only solvent or the structurally similar compound phenethylamine was sprayed for APTD and subsequent trapping by the rhodium complex, no peak at m/z 814.99 was detected (Fig. 4b, c).

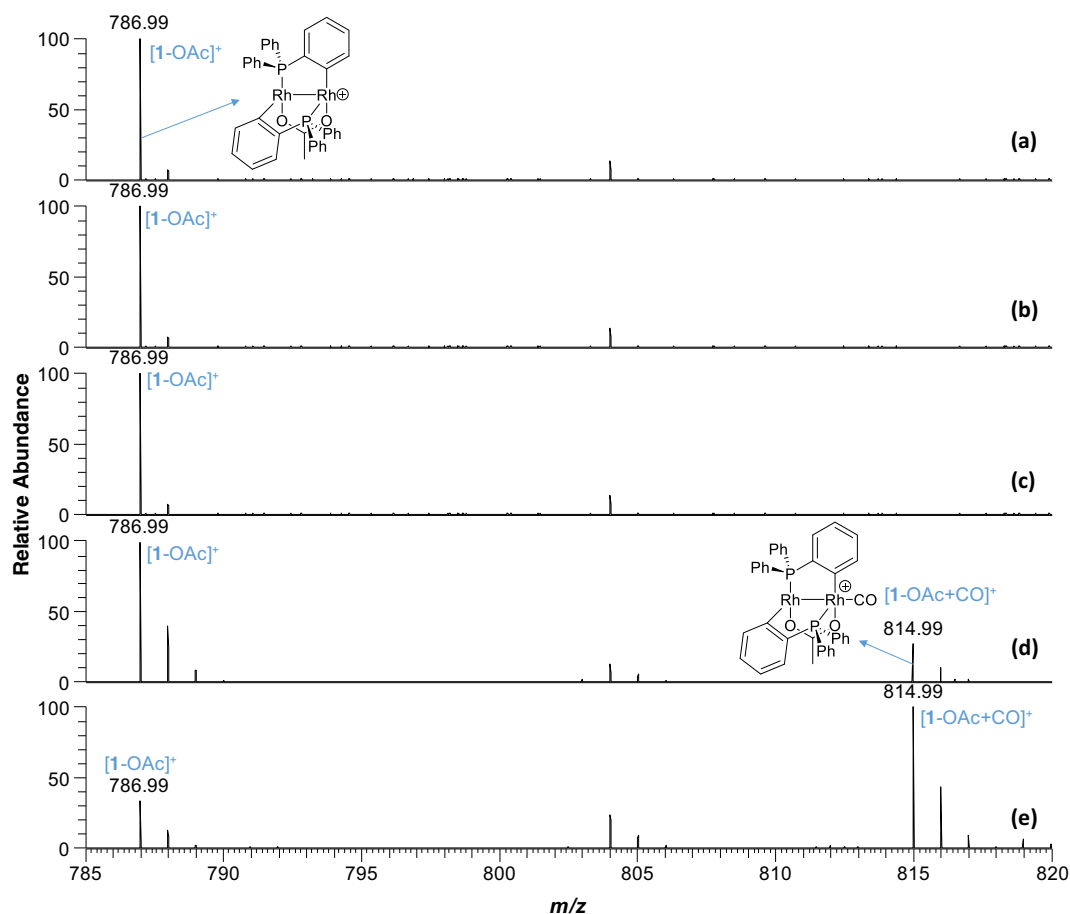


Figure 4. ESI-MS spectra of (a) 3 mM dinuclear rhodium complex **1**·(HOAc)₂ in chloroform, (b) 3 mM dinuclear rhodium complex **1**·(HOAc)₂ in chloroform after trapping gaseous species flowing out of the APTD tube when a blank solvent of ACN/H₂O/HOAc (50:50:0.1) was sprayed into the APTD tube at 300 °C, (c) 3 mM dinuclear rhodium complex **1**·(HOAc)₂ in chloroform after trapping gaseous species flowing out of the APTD tube when 25 mM phenethylamine in ACN/H₂O/HOAc (50:50:0.1) was sprayed into the APTD tube at 300 °C, (d) 3 mM dinuclear rhodium complex **1**·(HOAc)₂ in chloroform after trapping gaseous species flowing out of the APTD tube when 25 mM phenylalanine in ACN/H₂O/HOAc (50:50:0.1) was sprayed into the APTD tube at 300 °C, (e) 3 mM dinuclear rhodium complex **1**·(HOAc)₂ in chloroform after trapping standard carbon monoxide gas (purity 99.99%) for 3 min

In addition, a ^{13}C -labeled phenylalanine sample (the carboxylic carbon is labeled with ^{13}C) was subjected to APTD, and the rhodium complex trapping, a peak of m/z 815.99, corresponding to $[\text{1-OAc} + ^{13}\text{CO}]^+$ (theoretical mass 815.9879, observed mass 815.9883, mass error 0.49 ppm) was detected, as expected (Fig. 3S, Supporting Information). CID of m/z 815.99 produced fragment ions at m/z 786.99 and 726.97 (Fig. 2S-b, Supporting Information), by consecutive losses of ^{13}CO and HOAc, consistent with its structural assignment. This result supported the hypothesis that APTD of phenylalanine produces CO and the CO stems from the carboxylic group of the amino acid.

Investigation of Other Possible Neutral Species from APTD

At the same time, the possibility of producing dihydroxycarbene $\text{C}(\text{OH})_2$ (pathway b, Scheme 1) was also investigated. Due to the high reactivity of carbene, the dihydroxycarbene would react with water from the spray solvent via insertion into the O-H bond of H_2O , [55] and a product peak at m/z 65, corresponding to $[\text{HC}(\text{OH})_3 + \text{H}]^+$, should be detected by the APTD-MS in the positive ion mode. However, there was no such peak in the acquired MS spectrum, which suggested that dihydroxycarbene was not formed from APTD of phenylalanine (Fig. 4S, Supporting Information).

Furthermore, the possibility of producing formic acid (HCOOH) following APTD of the amino acid (pathway c, Scheme 1) was also examined. To detect neutral species HCOOH , we reasoned that HCOOH could be re-ionized into formate ion HCOO^- (m/z 45) in the negative ion mode, by using the atmospheric pressure neutral re-ionization (APNR) technique. [17] Thus, in our APTD experiment of phenylalanine, a second ESSi sprayer was added downstream so as to spray solvent ACN/ H_2O /ammonia (50:50:0.1). The generated microdroplet could capture neutral species flowing out of the APTD tube and get them ionized. The re-ionized neutrals could be detected by the nearby mass spectrometer (Scheme 1S, Supporting Information). When only solvent ACN/ H_2O /HOAc (50:50:0.1) was sprayed into the APTD tube at 300 °C, APNR-MS spectrum (Fig. 5S-a) shows a background peak at m/z 45 with intensity of 5.09×10^3 (manufacturer's arbitrary unit of counts). When phenylalanine was added into the solvent for APTD, the peak of m/z 45 did not show a significant increase (peak intensity 5.29×10^3 , Fig. 5S-b). This result suggests that no HCOOH was formed from APTD of phenylalanine.

As formic acid HCOOH could decompose into CO and H_2O [56] at elevated temperature, we also performed an experiment to exclude the possibility that the detected CO from APTD of phenylalanine was a HCOOH decomposition product. In a separate experiment, HCOOH was directly sprayed into APTD tube at 300 °C and the gaseous species coming out of the tube was trapped by the dinuclear rhodium complex

solution. After trapping and subsequent ESI-MS analysis, no peak was detected at m/z 814.99 (Fig. 6S, Supporting Information).

APTD of Other Amino Acids

Other amino acids (leucine, histidine, and tryptophan) were also tested for APTD in our study. The amino acids were dissociated first by APTD and the loss of 46 Da from the protonated amino acid was seen in all cases (Fig. S7, Supporting Information). Then the released neutral CO molecules were trapped by rhodium complex solution and successfully detected by MS (Fig. S8, Supporting Information). This information further revealed that CO released from the dissociation of protonated amino acid into iminium ion is a quite general pathway.

APTD of Peptides

The APTD method can also be used to dissociate peptides. Two peptides, Gly-His-Gly (GHG), and Gly-Trp-Gly (GWG), were chosen as test samples in this experiment. Compared to APTD dissociation of amino acids, loss of 46 Da from the two peptide ions was not observed (Fig. 5). However, both *a* and *b* ions are seen in the two cases. It has been long regarded that *a* ions originate from *b* ion, by facile loss of CO. However, no direct experimental evidence is reported in the literature, to our knowledge. Thus, by simply carrying out trapping of gaseous species from APTD of these two peptides using the dinuclear rhodium complex, we were able to detect the characteristic adduct ion $[\text{1-OAc} + \text{CO}]^+$ (m/z 814.99, Fig. 6). This data provides evidence to corroborate the formation of CO during the dissociation of these peptide ions, which is most likely ascribed to the conversion of *b* into *a* ions. Therefore, *a* ions should arise from further dissociation of *b* ions, rather than from direct dissociation of precursor peptide ions, as no CO would be formed in the latter case. (Note that under some circumstances, direct formation of CO during *a* ion and *y* ion formation is possible [57, 58]; however, this appears not to be the case for the peptides that were examined in this study, as neither $[\text{peptide-CO}]^+$ or *y*₁ ions was detected during the APTD process (Fig. 5)).

Furthermore, the approach of CO neutral characterization of this study can be used in a more quantitative manner. A quantitative comparison experiment was run to compare the amount of CO produced from APTD of peptides GHG and GWG. As measured by the CO sensor, the averaged values from APTD of GHG and GWG were 7 ppm and 8.3 ppm, respectively (Table 1S, Supporting Information). The result showed that the dissociation of GWG generated more CO than GHG under the same experimental conditions. Likewise, as shown in Fig. 6b, c, the relative intensity of the adduct ion $[\text{1-OAc} + \text{CO}]^+$ at m/z 814.99 (relative to the binuclear rhodium complex cation $[\text{1-OAc}]^+$ at m/z 786.99) was higher in the case of GWG than that for GHG, which is in line with the CO sensor measurement result. As

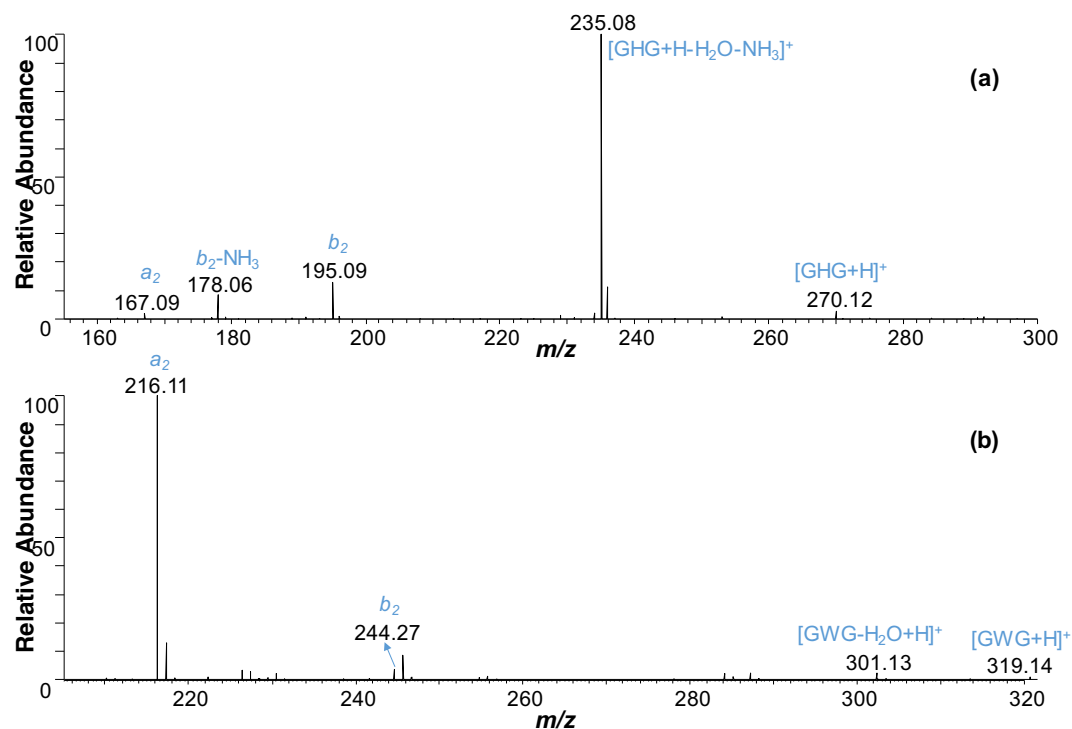


Figure 5. APTD-MS spectra of (a) 5 mM GHG in ACN/H₂O/HOAc (50:50:0.1) and (b) 5 mM GWG in ACN/H₂O/HOAc (50:50:0.1). The temperature of the APTD tube was 300 °C

shown in Fig. 5, the *a* ion appears to be dominant in the case of GWG upon APTD; therefore, this result also

supports the assumption that CO was produced along with generation of the *a* ion for the peptides studied.

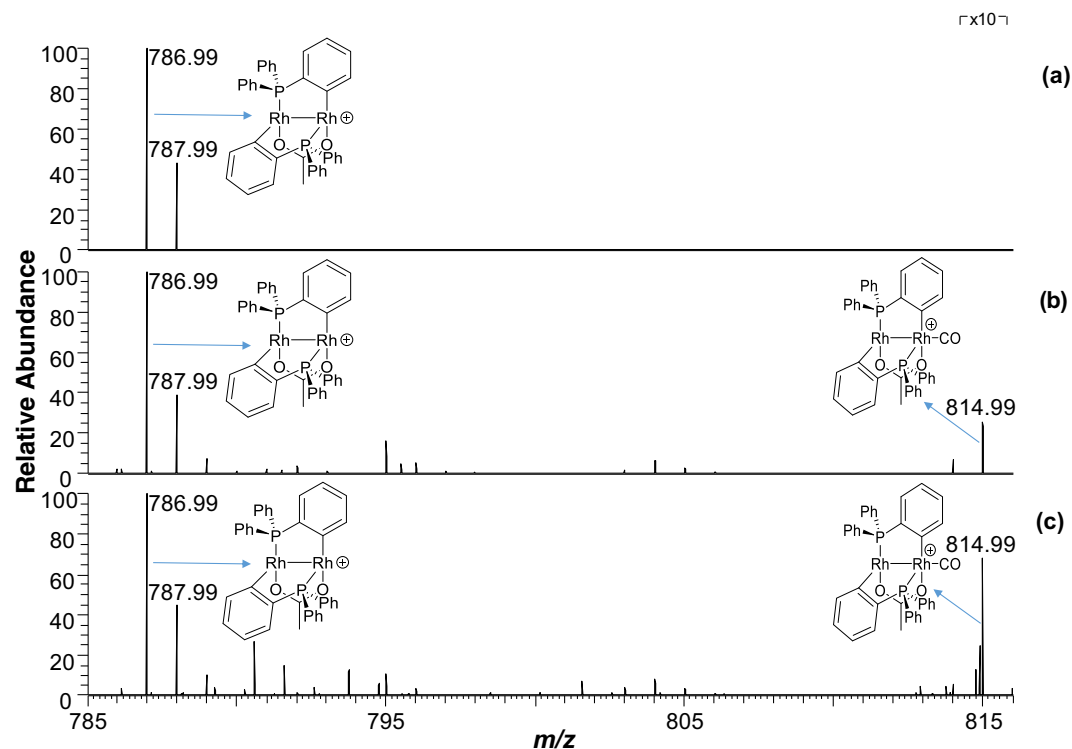


Figure 6. ESI-MS spectra of 3 mM dinuclear rhodium complex **1**·(HOAc)₂ solution after trapping neutral species flowing out of the APTD tube when (a) ACN/H₂O/HOAc (50:50:0.1), (b) 5 mM GHG in ACN/H₂O/HOAc (50:50:0.1), and (c) 5 mM GWG in ACN/H₂O/HOAc (50:50:0.1) was sprayed into the tube for APTD at 300 °C

Conclusions

This study demonstrates a new application of APTD in investigating the amino acid and peptide ion dissociation mechanisms, specifically identification of the neutral loss species CO involved in dissociation of protonated amino acid into iminium ion and peptide ion $b \rightarrow a$ conversion. Due to the fact that ion dissociation in APTD occurs at atmospheric pressure, characterization of neutral species becomes possible, as demonstrated using either online CO sensor or offline spectroscopic/mass spectrometric tools. Our results demonstrate that the fragmentation of protonated amino acids into iminium ions involves losses of CO and H₂O rather than loss of HCOOH or C(OH)₂. Detection of CO from the tested peptide ions demonstrates that a ion is most likely to come from b ion dissociation by loss of CO. Our study once again suggests that APTD has utility for structure analysis and elucidation of ion dissociation mechanism. Due to the neutral fragment characterization could provide increased structural information, this method could be used to elucidate complicated ion dissociation mechanisms, such as the investigation of cyclic peptide or glycan ion dissociation behaviors, which could be helpful to cyclic peptide sequencing and glycan structural analysis.

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