Fe⁺ Chemical Ionization of Peptides

J. Paul Speir, Greg S. Gorman, and I. Jonathan Amster

Department of Chemistry, University of Georgia, Athens, Georgia, USA

Laser-desorbed peptide neutral molecules were allowed to react with Fe⁺ in a Fourier transform mass spectrometer, using the technique of laser desorption/chemical ionization. The Fe⁺ ions are formed by laser ablation of a steel target, as well as by dissociative charge-exchange ionization of ferrocene with Ne⁺. Prior to reaction with laser-desorbed peptide molecules, Fe⁺ ions undergo 20–100 thermalizing collisions with xenon to reduce the population of excited-state metal ion species. The Fe⁺ ions that have not experienced thermalizing collisions undergo charge exchange with peptide molecules. Iron ions that undergo thermalizing collisions before they are allowed to react with peptides are found to undergo charge exchange and to form adduct species [M + Fe⁺] and fragment ions that result from the loss of small, stable molecules, such as H₂O, CO, and CO₂, from the metal ion–peptide complex. (*J Am Soc Mass Spectrom* 1993, 4, 106–110)

We have recently demonstrated that laser-desorbed peptide neutral molecules, containing up to 10 amino acids, can undergo chemical ionization in the analyzer region of a Fourier transform mass spectrometer [1]. We are developing this technique, laser desorption/chemical ionization (LD/CI) [2–5], for use in determining the amino acid sequence of peptides by mass spectrometry. The extent of fragmentation and the type of ions that are observed in a mass spectrum can be varied by changing the enthalpy of the reaction used to ionize peptide molecules. The enthalpy of ionization, and thus the appearance of the mass spectrum, can be controlled by the selection of the reagent ion [1]. In principle, any reagent species that can be used in conventional chemical ionization experiments can be used in LD/CI studies. To date, proton transfer [1–5] and charge-exchange reactions [6] have been used to postionize laser-desorbed molecules.

We report the first observation of a gas-phase reaction of a peptide molecule with a transition metal ion. To ionize efficiently the analyte in a LD/CI experiment, a metal ion must undergo a rapid reaction with laser-desorbed neutral molecules that are produced in a fleeting pulse that passes through the reaction region of the Fourier transform mass spectrometer in 1 μ s or less. Prior experiments involving the reaction of metal ions with small molecules reveal a wide range of reactivities. The endothermic reaction of Fe⁺ with CH₄ has been found to be very slow ($k \ll 10^{-12}$ cm³/molecule/s) [7]. More relevant to this study, reactions of small, functionalized molecules with metal ions are often found to occur close to the ion-molecule collision

rate, as has been observed for a variety of metal ions with alkyl chlorides and aliphatic alcohols, with rate constants in the range $0.2\text{--}1 \times 10^{-9}$ cm³/molecule/s [8]. On the basis of the latter observations, it seems reasonable to expect that many metal ions will react rapidly with peptide molecules.

The use of metal ions and metal complex ions for the chemical ionization of organic molecules has been suggested by others [9–13]. Transition metal ions are known to undergo unusual and selective reactions with small organic molecules [14]. Our interest in transition metal ions is for their possible use as reagents for controlling the dissociation of peptide molecules so as to enhance the sequence information that can be obtained by mass spectrometry. The chief aim of this study was to determine the type of reactions that a transition metal ion (Fe⁺) would undergo with peptide molecules. The Fe⁺ ion has been observed to react with carbonyl compounds by cleavage of C–C bonds adjacent to the carbonyl group [15]. Such reactivity appears to be promising for the dissociation of peptide molecules into analytically meaningful fragment ions. For this reason, Fe⁺ was selected first for reactions with peptides. The techniques described here are general and can be used to study the reactions of any metal ion with peptide molecules.

Experimental

The experiments were performed in a Fourier transform mass spectrometer that was designed and constructed in this laboratory specifically for LD/CI studies. The magnetic field used in these experiments as 1 T, produced by an electromagnet (Walker Scientific, Worcester, MA) with a 24-cm pole cap diameter. The analyzer cell of the mass spectrometer (4.4-cm

Address reprint requests to I. Jonathan Amster, Department of Chemistry, University of Georgia, Athens, GA 30602.

cube) was housed in an ultra-high-vacuum chamber pumped to a base pressure of 2×10^{-10} torr by a turbomolecular pump. The transmitter and receiver plates of the analyzer cell have 1.3-cm-diameter holes drilled through their centers. These openings are aligned with magnesium fluoride windows on the vacuum chamber so that the UV output of an excimer laser (Questek, Model 2110, Billerica, MA) can be directed through the cell, along the two principal axes that are perpendicular to the magnetic field, at targets for peptide desorption and metal ion formation. In-volatile peptide samples are applied to a 1-cm-diameter stub, as described below, and introduced into an antechamber that is evacuated with a cryopump before being transferred to the main vacuum chamber. A homebuilt manipulator delivers the peptide-coated stub to an externally controlled jaw mounted on the analyzer cell that supports the sample 5 mm outside an opening in an analyzer cell plate. Volatile samples can be admitted into the vacuum chamber through a sapphire-sealed leak valve (Varian Associates, Lexington, MA) or through one of two pulsed valves (General Valve, Series 9, Fairfield, NJ). The timing of valve, laser, and ion excitation pulses and the detection and processing of the transient signal are handled by a commercial data system (IonSpec, Irvine, CA).

The peptide samples used in this study were obtained commercially (Sigma Chemical Co., St. Louis, MO) and used without further purification. Peptide samples were dissolved in methanol and applied as thin films (100 ng/mm²) to the substrate by the electrospray technique [16]. The substrate was a Macor stub sputter coated with 75 nm of gold/palladium. Desorption of peptide neutral molecules was accomplished by focusing a fraction (100 μ J) of the 193-nm output of the excimer laser (10-ns pulse) to a spot size of approximately 0.3 mm $\times$ 0.8 mm. The laser irradiance (5×10^6 W/cm²) was controlled by using neutral density filters and by adjusting the focus of the laser beam to change the spot size. The irradiance of the laser was tuned to cause peptide neutral molecules to desorb without forming ions directly with the laser, for example, by multiphoton ionization. The conditions used for laser desorption caused the exclusive formation of intact peptide neutral molecules. Prior work has established that no neutral fragments are formed with the desorption conditions used in this study [4]. This was confirmed by examining the LD/CI mass spectrum produced with NH₄⁺ as a reagent ion in which only the protonated molecule of the peptide was observed. In addition, exothermic protonation of the peptides by a series of increasingly acidic reagent ions produces fragmentation patterns that are consistent with protonation of a molecular species [1, 4, 5].

Iron ions were formed by two methods. The first was laser ablation of a steel target with the same excimer laser that was used for peptide desorption. A diagram of the instrument arrangement used for this experiment is shown in Figure 1. The output of the

Figure 1. Diagram of the optical arrangement used for metal ion LD/CI experiments. The output of the excimer laser is mixed with light from an He-Ne laser (not shown) at the first beam splitter. The second beam splitter passes a majority of the energy of the laser to a metal target for generating metal ions. A fraction of the laser beam is reflected by the second beam splitter and is used for laser desorption of peptide neutral molecules. Electronically controlled, solenoid-driven shutters are used to pass or block the laser beams, so that only one beam reaches its respective target per laser shot.

excimer laser was split with a UV-grade, fused-silica plate. The transmitted light (92% of the incident energy) was tightly focused ($> 10^8$ W/cm²) onto the metal target to form Fe⁺ ions that were trapped in the analyzer cell. The reflected beam (8% of the incident energy) was used for peptide desorption. Solenoid-driven electronic shutters that have rectangular apertures that match the dimensions of the excimer beam block or pass the two beams, so that successive laser shots can be directed alternately at the metal target and at the peptide-coated sample stub. This arrangement allowed a single excimer laser to be used both for metal ion formation and for peptide desorption. Metal ions formed by laser ablation were allowed to undergo 20–100 collisions with Xe admitted through a pulsed valve in an effort to reduce the presence of excited-state species.

The Fe⁺ ions could also be formed by dissociative charge exchange of ferrocene. For this process, neon was admitted through a pulsed valve and ionized by 70-eV electron bombardment. Ferrocene was introduced into the system through a second pulsed valve. The charge-exchange reaction between Ne⁺ and ferrocene is highly exothermic ($\Delta H = -14.6$ eV) and leads to the exclusive formation of Fe⁺. Because the ferrocene is admitted in a short-duration burst, the reaction of Fe⁺ with ferrocene to form the ferrocenium ion does not occur to an appreciable extent [17].

Results and Discussion

Initial attempts to allow Fe⁺ to react with laser-desorbed peptides utilized metal ions that were formed by laser ablation of a steel target and were not colli-

sionally thermalized. The reaction of the dipeptide Val-Ala with a metal ion prepared in this fashion did not lead to the formation of any metal-containing product ions, as shown in the mass spectrum of Figure 2, which is typical of the reactions of nonthermalized metal ions with laser-desorbed peptide molecules. The base peak of the mass spectrum corresponds to the protonated molecule of the peptide. Unreacted Fe^+ also appears in the mass spectrum. Some of the mass spectra obtained with these experimental conditions also exhibited a peak at m/z 72, corresponding to the immonium ion of valine. The formation of a protonated ion can be postulated to occur by a mechanism in which the metal ion undergoes charge exchange with the peptide (reaction 1), followed by hydrogen transfer between the odd-electron peptide molecular ion and a second peptide molecule (reaction 2):

The observation of the protonated ion rather than an odd-electron ion suggests that several collisions occur between a trapped ion and the desorbed peptide neutral molecule. A simple order-of-magnitude analysis of the desorption process suggests that an ion undergoes 1-1000 collisions with laser-desorbed peptide neutral molecules. The laser desorbs 1000 monolayers of peptide per laser shot. For a laser spot size of 1 mm^2 , 10^{15} molecules are desorbed per laser shot. The desorbed neutral molecules travel 2 cm before encountering the ions in the analyzer cell. The density of neutral molecules in the vicinity of the ions depends on their angular spread during desorption. Consider the limiting cases of 0° (forward directed) and 45° solid angle. If the neutral molecules leave the surface with a solid angle of 45° , after traveling 2 cm, they will be spread out over an area of approximately 1 cm^2 and produce a flux of 10^{15} molecules/ cm^2 . Assuming an ion-molecule reaction cross section of 10^{-14} cm^2 ($100 \text{ \AA}^2$), ions will undergo 10 collisions (flux $\times$ cross section). If the desorbed neutral molecules do not spread out before reacting with the ions, they will pass through a

1-mm^2 area and have a flux of 10^{17} molecules/ cm^2 . This will result in 1000 collisions of an ion with the neutral molecules. If the ion-molecule reaction cross section is a factor of 10 smaller, the number of collisions will be between 1 and 100. Considering the various uncertainties, 1-1000 collisions seems to be a reasonable approximation and is consistent with the experimental results. In further support of the proposed sequence of reactions, we find that charge-exchange ionization of peptides by rare gas ions often leads to the formation of a protonated molecule [18] and that an odd-electron molecular ion is observed if the flux of laser-desorbed neutral molecules is reduced by using substrate-assisted laser desorption [1]. We have not been able to apply the latter desorption method to Fe^+ chemical ionization studies, as discussed later.

An analysis of the thermodynamics of charge transfer between metal ions and peptides suggests that excited-state species are involved. The enthalpy of reaction 1 is equal to the ionization energy of the peptide minus the recombination energy of the metal ion. The recombination energy of Fe^+ in its ground state (^6D , $4s3d^6$) is 7.8 eV [19]. The ionization energy of peptides can be estimated to be close to that of dimethyl formamide (9.1 eV) or *N*-methylacetamide (8.9 eV) [20]. Reaction 1 is therefore endothermic for ground-state metal ions and ground-state peptide molecules. The observation of charge exchange between Fe^+ and peptide molecules requires that the metal ion or the peptide, or both, are in an excited state, because endothermic ion-molecule reactions should occur too slowly to form an appreciable abundance of a product ion. It is not unreasonable to expect that high irradiance 193-nm laser ablation of the steel target should cause the formation of highly electronically excited Fe^+ . There are several excited states of Fe^+ that are expected to be long-lived and that occur up to 2 eV above the ground state of the ion (^4P is 1.8 eV above the ground state; ^4D , 1 eV; ^4F , 0.3 eV) [21]. Additionally, the conditions used for laser desorption of peptide neutral molecules in this experiment (193-nm laser, thin peptide film on a thin metal film substrate) have been shown to cause the formation of gas-phase neutral molecules with 1 eV of internal energy [1], which would also cause the charge-exchange reaction with Fe^+ to be exothermic. It is likely that a combination of these two factors lead to the predominance of charge-exchange ionization for the reaction of Fe^+ with peptide molecules, as in Figure 2.

Substantially different results were obtained when the Fe^+ ions were allowed to undergo thermalizing collisions prior to their reaction with laser-desorbed peptides. Figure 3 shows the mass spectrum of the products of the reaction of Val-Pro-Leu with Fe^+ , which has first undergone collisions with xenon. Ions containing iron are clearly evident in the mass spectrum. The adduct species $[\text{M} + \text{Fe}]^+$ and fragments formed by

Figure 2. Mass spectrum of the products of the reaction of Val-Ala with Fe^+ formed by laser ablation and without collisional thermalization of the metal ion.

Figure 3. Mass spectrum of the products of the reaction of Val-Pro-Leu with Fe⁺ formed by laser ablation and with thermalization of the metal ion with xenon collisions. The designations a_n, b_n, c_n, and y_n follow the Roepstorff convention.

loss of small molecules from the iron adduct, [M + Fe - H₂O]⁺, [M + Fe - CO]⁺, and [M + Fe - CO₂]⁺, are the prominent high-mass ions. A peak 16 mu above [M + Fe]⁺ may possibly be [M + Fe + O]⁺, resulting from reaction of the metal-peptide adduct with additional peptide neutral molecules; [M + Fe + X]⁺ species, where X = CO or CO₂, have been observed in the Fe⁺ LD/CI mass spectra of this and other peptides. The intensities of the latter ions are irreproducible and are often negligible, as in the mass spectra reported here. The fragment ions [b₂ + Fe]⁺, [y₂ + Fe]⁺, [c₂ + Fe]⁺, and [y₂ - CO₂H + Fe]⁺ are also present in the mass spectrum. The metal-containing fragment ions result from cleavage of an amide bond (b₂ and y₂) and a bond adjacent to an amide bond (c₂). In addition, ions that contain no metal are also found in the mass spectrum, including the molecular ion, fragments resulting from the loss of water, CO, and CO₂, as well as the ions a₂, b₂, c₂, y₂, and a₁ (where a_n, b_n, c_n, x_n, and y_n are Roepstorff designations [22]). The ions that contain no metal apparently result from a charge-exchange reaction of Fe⁺ with the peptide molecule and in fact are found in the LD/CI mass spectrum of this peptide, resulting from charge-exchange ionization with rare gas ions [18].

Figure 4 shows the mass spectrum of the products of the reaction of the dipeptide Val-Ala with Fe⁺ formed by dissociative charge-exchange ionization of ferrocene. The metal-containing ions include [M +

Figure 4. Mass spectrum of the products of the reaction of Val-Ala with Fe⁺ formed by dissociative charge-exchange ionization of ferrocene by neon ions.

Fe]⁺, [M + Fe + CO]⁺, [M + Fe - CO]⁺, and [M + Fe - CO₂]⁺. The dominant peak in the mass spectrum corresponds to [M + H]⁺, an ion that does not contain iron, presumably formed by the charge-exchange mechanism described previously. Another product that does not contain iron is the immonium ion of valine (a₁), which has been observed in LD/CI mass spectra of this compound by using either proton-transfer or charge-exchange reagent ions. The low-abundance m/z 186 ion is most likely ferrocenium, resulting from charge-exchange ionization of ferrocene by the iron cation.

We have not been able to obtain mass spectra in which metal ion attachment occurs without also observing the competing process of charge exchange ionization, even with our best efforts to reduce the population of excited state Fe⁺ and peptide molecules. The Fe⁺ have been allowed to undergo thermalizing collisions with molecular gases such as methane prior to the reaction with laser-desorbed peptide neutral molecules and found to yield similar spectra to those reported here. We have tried to reduce the internal energy of the laser-desorbed peptides in an effort to reduce charge-exchange ionization of the peptides by the metal ions by using the recently reported method of substrate-assisted laser desorption of neutral peptides [1]. This technique has been shown to form gas-phase neutral molecules of Val-Pro-Leu that contain 1 eV less energy than by the desorption method used in the experiments reported here. Using substrate-assisted laser desorption, neither cationization nor charge exchange was observed for peptides or amino acids with Fe⁺ chemical ionization. One interpretation of these data is that the reaction of thermalized Fe⁺ with thermalized peptides, either to form an adduct or to undergo charge exchange, may be too slow to be observed by the LD/CI technique. This interpretation seems to run counter to the many observations of rapid reactions between metal ions and small, functionalized organic molecules [8-13]. Clearly, the dependence of the reaction rate on the internal energy of the reactants needs further study. For the conditions reported here, the rate of these reactions appears to be sufficiently rapid only when the peptide is vibrationally or electronically excited by a minimum of 1 eV. On the other hand, when a large amount of excess internal energy is present in both the peptide and the metal ion, charge exchange is exothermic and rapid compared with metal attachment (as shown in Figure 1) LD/CI of Val-Ala with Fe⁺ formed by laser ablation, with no thermalizing collisions of the metal ion.

Although iron-cationized species are less abundant than the ions that do not contain iron in the mass spectra reported here, the relative ratio of the two types of ions (metal containing versus metal free) is variable. In some mass spectra that were recorded for this study, [M + Fe]⁺ gave rise to the base peak of the LD/CI mass spectrum, whereas in other mass spectra of the same compounds, charge-exchange products

were dominant. The variation in the type of products that are observed results from changes in the relative rate constants for cationization versus charge exchange. Such results highlight the difficulty in reproducibly desorbing peptide neutral molecules with a fixed amount of internal energy with the experimental conditions used here (193-nm light; no organic substrate).

Conclusions

The transition metal ion Fe^+ is found to react with laser-desorbed peptide molecules through two main channels—metal-ion attachment and charge exchange—to yield both metal-containing and metal-free peptide ions. The relative yield of the two types of reactions depends on the internal energy of both the metal cation and the desorbed peptide neutral molecule. Metal-containing fragment ions are formed by the loss of small molecules (H_2O , CO , and CO_2) from the metal adduct molecular species. In addition, the reaction of Fe^+ with Val-Pro-Leu yields metal-containing peptide fragment ions formed by cleavage of amide bonds and the bonds adjacent to the amide bonds. These results demonstrate that laser-desorbed peptide molecules do react with bare metal ions. Future studies will investigate the reactions of other first-row transition metal ions with peptide molecules.

Acknowledgments

Financial support by the National Science Foundation (grant no. CHE-9024922) is gratefully acknowledged. Acknowledgment is made to the donors of the Petroleum Research Fund for partial support of this work. Financial support was also provided by a research award from the American Society for Mass Spectrometry, sponsored by Finnigan MAT.

References

1. Speir, J. P.; Amster, I. J. *Anal. Chem.* **1992**, *64*, 1041–1045.
2. Cotter, R. J. *Anal. Chem.* **1980**, *52*, 1767–1770.
3. Amster, I. J.; Land, D. P.; Hemminger, J. C.; McIver, R. T. Jr. *Anal. Chem.* **1989**, *61*, 184–186.
4. Speir, J. P.; Gorman, G. S.; Cornett, D. S.; Amster, I. J. *Anal. Chem.* **1991**, *63*, 65–69.
5. Speir, J. P.; Gorman, G. S.; Amster, I. J. In *Mass Spectrometry in the Biological Sciences: A Tutorial*; Gross, M. L., Ed.; Kluwer: Dordrecht, The Netherlands, 1991; pp. 199–212.
6. Speir, J. P.; Gorman, G. S.; Amster, I. J. Presented at the 11th International Mass Spectrometry Conference, Amsterdam, August 25–29, 1991; Poster WeA-A28.
7. Schultz, R. H.; Elkind, J. L.; Armentrout, P. B. *J. Am. Chem. Soc.* **1988**, *110*, 411–423.
8. Allison, J.; Ridge, D. P. *J. Am. Chem. Soc.* **1979**, *101*, 4998–5008.
9. Hodges, R. V.; Beauchamp, J. L. *Anal. Chem.* **1976**, *48*, 825–829.
10. Burnier, R. C.; Byrd, G. D.; Freiser, B. S. *Anal. Chem.* **1980**, *52*, 1641–1650.
11. Peake, D. A.; Gross, M. L. *Anal. Chem.* **1985**, *57*, 115–120.
12. Chowdhury, A. K.; Cooper, J. R.; Wilkins, C. L. *Anal. Chem.* **1989**, *61*, 86–88.
13. Nuwaysir, L. M.; Wilkins, C. L. *Anal. Chem.* **1989**, *61*, 689–694.
14. (a) Freiser, B. S. *Talanta* **1985**, *32*, 697–708; (b) Ridge, D. P. In *Structure/Reactivity and Thermochemistry of Ions*; Ausloos, P.; Lias, S. G., Eds.; Reidel: Dordrecht, The Netherlands, 1987; pp. 165–175; (c) Armentrout, P. B. In *Gas Phase Inorganic Chemistry*; Russell, D. H., Ed.; Plenum: New York, 1989; pp. 1–42; (d) Schwarz, H. *Acc. Chem. Res.* **1989**, *22*, 282–287; (e) Eller, K.; Schwarz, H. *Chem. Rev.* **1991**, *91*, 1121–1177.
15. Burnier, R. C.; Byrd, G. D.; Freiser, B. S. *J. Am. Chem. Soc.* **1981**, *103*, 4360–4367.
16. McNeal, C. J.; Macfarlane, R. D.; Thurston, E. L. *Anal. Chem.* **1979**, *51*, 2036–2039.
17. Foster, M. S.; Beauchamp, J. L. *J. Am. Chem. Soc.* **1975**, *97*, 4814–4817.
18. Speir, J. P.; Amster, I. J., unpublished results.
19. Moore, C. E. Atomic Energy Levels; NSRDS–NBS 35; U.S. Department of Commerce; Washington, D.C., 1971; pp. 55–58.
20. Rosenstock, H. M.; Draxl, K.; Steiner, B. W.; Herron, J. T. *J. Chem. Phys. Ref. Data* **1977**, *6*, 1–328.
21. Lang, K. R. *Astrophysical Formulae*; Springer-Verlag: Berlin, 1980; p. 142.
22. Roepstorff, P.; Fohlman, J. *Biomed. Environ. Mass Spectrom.* **1984**, *11*, 601.