Complexes of Silver(I) With Peptides and Proteins as Produced in Electrospray Mass Spectrometry

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Silver(I) forms aqueous phase complexes with both sulfur and nonsulfur containing peptides and proteins. These complexes were introduced into the gas phase via electrospray, and their structures probed by means of tandem mass spectrometry. Experiments with di-, tri-, and oligopeptides show that the abundance of silver(I)-containing ions increases relative to that of proton-containing ions as peptide length increases. This increase is much more dramatic for methionine-containing peptides. Collision-induced dissociation of silver-peptide complexes yields a multitude of product ions that are silver containing. However, even for methionine-containing peptides, very few of these product ions contain the methionine residue. The solution-phase structure and the gas-phase structure of the silver/peptide complex are not identical. The methionine sulfur acts as the silver anchoring point in solution. Desolvation in the gas phase leads to a rearrangement of the silver/peptide complex such that the silver ion becomes chelated to the nitrogen and oxygen atom on the peptide backbone in addition to the methionine sulfur. This rearrangement decreases the importance of the silver/sulfur bond to the extent that it is frequently broken upon collision activation and leads to the formation of silver/peptide product ions that are nonsulfur bearing. (J Am Soc Mass Spectrom 1997, 8, 781–792) Crown copyright 1997

Mass spectrometric investigations of metal-ion binding to peptides and proteins in the gas phase are frequently prompted by the desire to compare the same binding in solution [1–21]. Aside from its sensitivity and selectivity, mass spectrometry, because it measures the properties of an analyte in vacuum, offers a view of the intrinsic metal binding properties of proteins, unencumbered with solvation. A number of groups have devoted much time and effort on complexes of alkali [1–10], alkaline-earth [8, 11–14], and transition metals [15–21]. While most of these investigations centered on cationic metal complexes [1–6, 8, 9, 16–21], some involved anionic complexes as well [7, 10–15]. In earlier studies, fast atom bombardment (FAB) was the sample-introduction technique of choice due to its ease of producing a significant population of metal ions within the source. In later studies, the choice was between FAB and electrospray, the latter of the two, of course, offers the added convenience and advantage of being amenable to aqueous solutions of metal ions and proteins [9, 10, 14, 16–19].

The silver(I) ion is considered a "soft" cation according to Pearson's classification, and as such it prefers binding to soft ligands, ligands that are relatively polarizable [22]. Of all the functional groups of a protein, the softest groups are the sulfur ligand on the side-chain of cysteine and that of methionine [23]. Because the number and location of sulfur-containing residues are often desired information in the determination of unknown peptides, any specific metal binding to these sulfur ligands is potentially analytically useful with the metal ions serving as flags or indicators of the sulfur-containing residues. Furthermore, the fragmentation pathways of metallated peptides are often quite different from those of protonated peptides [2, 5, 6, 24–26], and some studies (e.g., [2]) were carried out partially with the goal of peptide sequencing in mind.

As adduct reagents for proteins, transition-metal ions have received some attention lately because their binding chemistry tends to be different from that of alkali and alkaline metal ions. Tang et al. [24] studied the product ion spectra of a number of proton-, alkali metal-, and silver-containing peptides generated via cesium-ion bombardment on solid peptide samples deposited on silver. They noted marked differences between the spectra of peptides containing different

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cations and suggested that Ag^+ was likely chelated to the N-terminal nitrogen and the carbonyl oxygen of the second residue (this is to be contrasted with Na^+ which was chelated only to carbonyl oxygens). Gross et al. [15, 20, 21] investigated complexes of oligopeptides with Co(II), Mn(II), Ni(II), Fe(II) (sometimes along with alkaline-earth metal ions). Chelation of doubly charged transition metal ions to the N-terminal amino group, deprotonated amide nitrogens, and the C-terminal carboxylate group in formation of anionic complexes was emphasized [15]. Interaction between the transition metal ion and the aromatic ring of a residue was also observed [20]. For cysteine-containing peptides, specific interactions between Fe(II) and the cysteine sulfur have been noted [21]. Loo et al. [16] examined Zn(II) complexes of angiotensin peptides and proposed coordination of Zn(II) to the histidine residue and carbonyl oxygens. Turecek et al. [17–19] reported amino-acid and peptide complexes with copper(II) and diimine ligands. They showed that the amino acids bind Cu(II) via their amino as well as carboxylate terminus.

This article describes efforts to study silver(I) adducts of peptides and proteins. Our interest stemmed from the softness of the cation and its possible selective binding to sulfur in peptides. These complexes were introduced into the gas-phase by means of electrospray. We will show by means of comparisons between the mass spectra of methionine-containing peptides and their nonsulfur containing analogues that methionine-containing peptides exhibit a much higher degree of silver binding than their nonmethionine containing counterparts. The nature of silver binding to peptides will be discussed in light of results of collision-induced dissociation (CID).

Experimental

Experiments were carried out on two triple quadrupole mass spectrometers. The first one was a SCIEX TAGA 6000E equipped with a custom-made electrospray source; the second one was a PE-SCIEX API 300 electrospray mass spectrometer. For the TAGA, the electrospray probe was fabricated from an approximately 3-cm-long, 33-gauge stainless steel tube (Hamilton, ~100 μm i.d.) that had been attached to a length of ~1.59 mm (1/16 in.) o.d. stainless steel tube with epoxy glue. The probe tip was electropolished prior to use. Biasing of the probe tip was achieved via a 50-MΩ current-limiting resistor in series with a high-voltage power supply (Tennelec, Model TC 950) set typically between 2.5 and 3.0 kV. The electrospray current was monitored via a custom-built microammeter that could be floated above ground. The API 300 was equipped with pneumatically assisted electrospray; the spray probe was stainless steel, approximately 27-gauge, with a coaxial tube for air delivery. Samples were infused via a 150-μm-o.d., 75-μm-i.d. fused silica tube inserted within the 27-gauge spray probe and terminated flush with the probe tip. The electrospray probe was biased typically at 5–5.5 kV; 4 kV positive with respect to the interface plate. The optimum probe positions on both instruments were established from time to time, but were typically with the tip about 1–2 cm from the interface plate and with the spray off-axis from the orifice.

Mass spectra were acquired in the positive-ion detection mode under optimal ion transmission conditions with a scan speed of 10–50 ms per m/z unit. Typically, ten scans were summed to obtain a mass spectrum. Low energy CID was carried out in the second quadrupole with argon as the collision gas at a typical thickness [27, 28] of approximately 2.0×10^{14} atoms cm^{-2} for the TAGA, and with nitrogen at a thickness of approximately 20×10^{14} molecules cm^{-2} for the API 300. The collision energy at the laboratory frame of reference (E_{lab}) was typically approximately 40–80 eV.

Fifty nine oligopeptides, having two to eleven residues, plus a few proteins were examined for complexing with silver(I) although only a few selected examples will be detailed in this paper. Approximately 50% of the oligopeptides contained one or several methionine residues; 30% contained only residues having neutral side-chain groups. All peptides and proteins were commercially available from Sigma Chemical Co. and were used as received. Samples for analysis on the TAGA were typically 10 μM–2 mM in peptide and 1–10 mM in silver(I) nitrate in a solution of 50 mM acetic acid in 50/50 water/methanol. A fraction of the samples was also analyzed without acetic acid addition. Samples for the API 300 were typically tenfold more dilute in peptide and silver(I) nitrate concentration. The solution to be analyzed was continuously infused into the electrospray probe by means of a syringe pump (Harvard Apparatus, Model 22) at a typical flow rate of 1 μL min^{-1}. Electrical isolation of the pump from the spray probe was achieved with poly(tetrafluoroethylene) (Teflon®) or PEEK tubing.

Molecular modeling was performed by means of ZINDO, a semiempirical quantum mechanical calculation-based program available in Hyperchem (Hypercube, Inc., Guelph, Ontario). The structures were found by means of an iterative process where the geometry of the complex under consideration was optimized via a minimization of the complex's total energy.

Results and Discussion

Figure 1 shows an electrospray mass spectrum of a solution of bovine insulin and silver(I). Four clusters of ions, ranging from 5+ to 8+, are readily discernible. Within each cluster, a number of peaks of identical charge but different combinations of H and Ag may be observed. For instance, in the 6+ cluster, there are six peaks: $[M + 6H]^{6+}$, $[M + 5H + Ag]^{6+}$, $[M + 4H + 2Ag]^{6+}$, $[M + 3H + 3Ag]^{6+}$, $[M + 2H + 4Ag]^{6+}$, and $[M + H + 5Ag]^{6+}$. In the 7+ and 8+ cluster, the first ions in the series (lowest m/z values) are $[M + 6H + Ag]^{7+}$ and

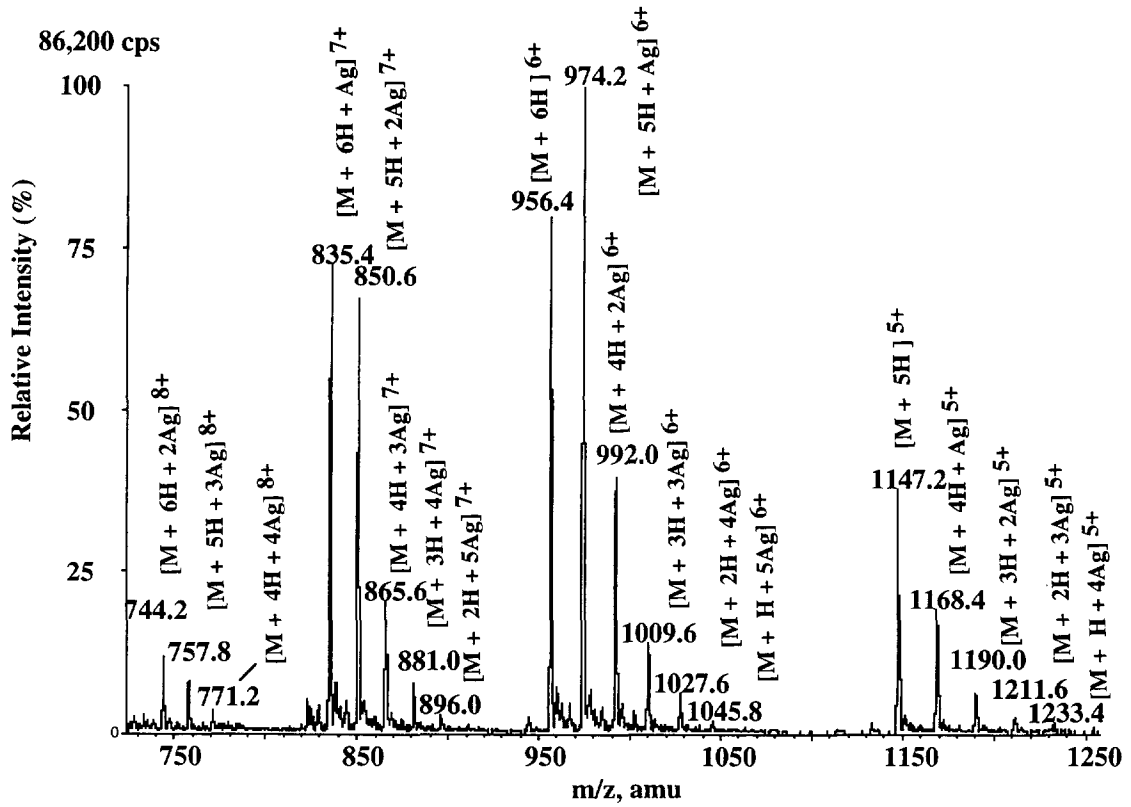

Figure 1. Electrospray mass spectrum of insulin + Ag(I): 50 μM insulin and 1 mM silver(I) nitrate in 50 mM acetic acid, 50/50 water/methanol.

$[M + 6H + 2Ag]^{8+}$, respectively. Bovine insulin has two N-terminal amino groups and four basic residues, the typical maximum charge state that we observe in the absence of silver(I) is +6H. In the presence of silver(I), hypercharging—attaining a charge state that is higher than that achievable with protons alone—is apparent. This observation suggests that some sites of Ag^+ attachment are likely different from those of H^+ attachment. Insulin has six cysteine residues engaged in three internal disulfide linkages; the sulfur group in these residues is a potential candidate for silver(I) attachment given the preference of this ion for soft ligands [23].

This speculation appeared confirmed in a subsequent experiment in which results of silver(I) attachment to methionine enkephalin (YGGFM) and leucine enkephalin (YGGFL) were compared (Figure 2). These two pentapeptides differ only in the C-terminal residue—methionine for the former and leucine for the latter—and their electrospray spectra are quite different. There are several points of note: First, the most prominent ion for methionine enkephalin electrosprayed from a solution of pH 3 was the hypercharged $[M + H + Ag]^{2+}$, whereas that for leucine enkephalin was $[M + H]^+$. Second, singly charged $[M + Ag]^+$ and $[M–H + 2Ag]^+$ ions were present in both spectra although they were less abundant relative to $[M + H + Ag]^{2+}$ for methionine enkephalin and to $[M + H]^+$ for leucine enkephalin. Third, significant silver(I) attachment was possible even for a nonsulfur-containing peptide. Fourth, the spectra from pH 7 solutions (insets) were very similar to those from pH 3 solutions. The effect of increasing the solution pH from 3 to 7 was a small increase of the ratio of the abundance of silver-containing to that of the nonsilver containing (i.e., purely protonated) peptide ions (from 12.1 to 14.6 for methionine enkephalin, and 0.33 to 0.49 for leucine enkephalin).

To confirm our observations, another pair of oligopeptides was examined. Substance P (RPKPQQFFGLM–NH_2) and [Nle^{11}]–substance P (RPKPQQFFGL–Nle–NH_2) are a pair of undecapeptides that differ once again only in their C-terminal residues, methionine for the former and norleucine for the latter; both C-terminal functional groups are amide instead of the more common carboxylate. Figure 3 shows electrospray mass spectra of substance P in the absence (upper panel) and in the presence of Ag^+ (lower panel) whereas Figure 4 shows the same for [Nle^{11}]–substance P; the solutions' pH was 3 for both peptides. The difference between the two sets of spectra is evident and striking. In the presence of silver(I), the most prominent ion for substance P was $[M + 2H + Ag]^{3+}$, while that for [Nle^{11}]–substance P was $[M + 2H]^{2+}$. For solutions of neutral pH, the most prominent ion remained $[M + 2H + Ag]^{3+}$ for substance P and $[M + 2H]^{2+}$ for [Nle^{11}]–substance P (spectra not shown).

This difference between methionine and nonmethionine containing peptides was also apparent for shorter peptides, such as di- and tripeptides, although the mag-

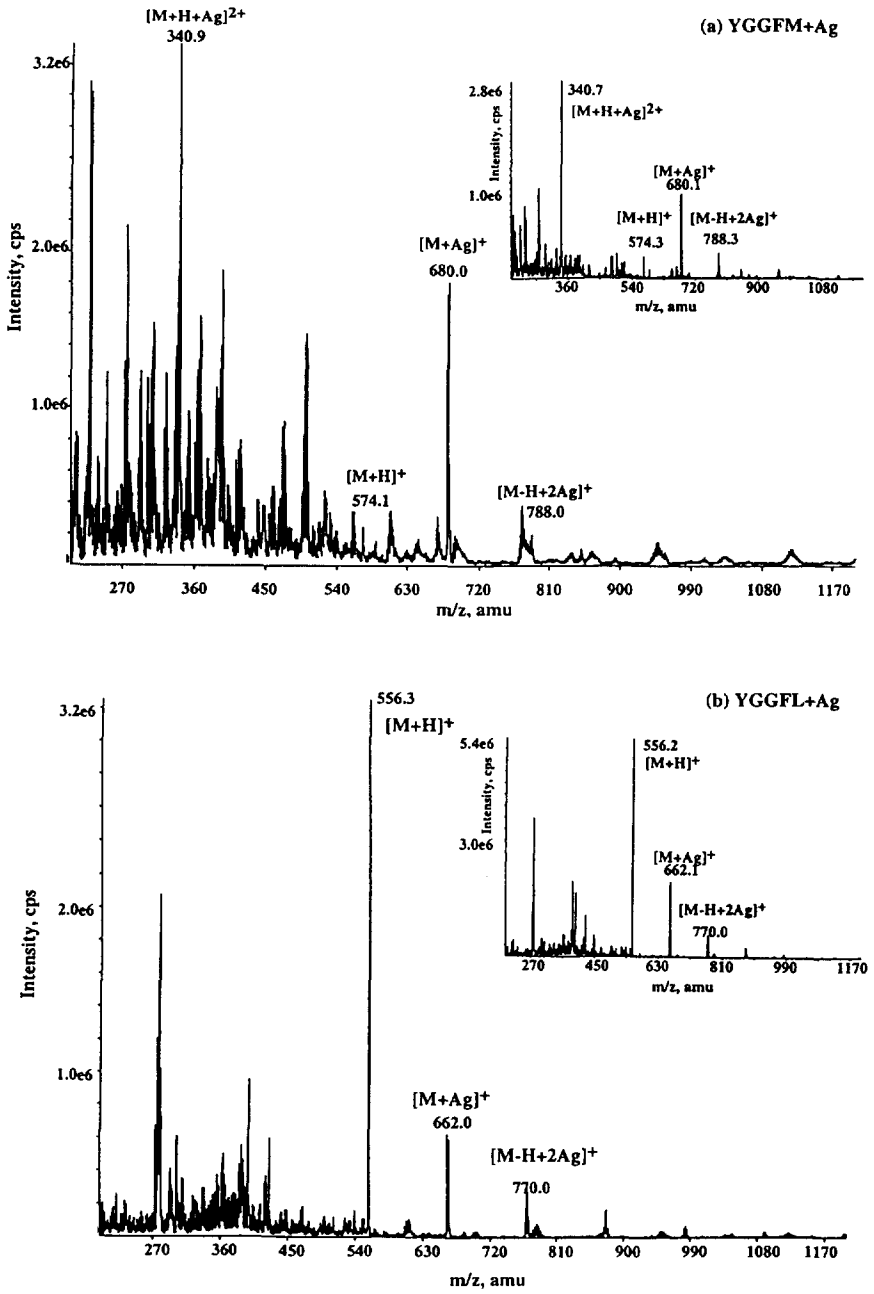

Figure 2. Electrospray mass spectra of (**a**) methionine enkephalin + Ag(I) and (**b**) leucine enkephalin + Ag(I): 10 μM peptide and 1 mM silver(I) nitrate in 50/50 water/methanol; main spectra, solutions acidified with 50 mM acetic acid; insets, neutral solutions.

nitude was smaller. For a set of 24 di- and tripeptides that was examined in solutions of pH 3, the average response ratios between the $[M + Ag]^+$ ion and the $[M + H]^+$ ion was 0.39 for peptides that contained one methionine residue, 0.05 for peptides that were nonsulfur containing, and 5.9 for methionylmethionine, which contained two sulfur groups. For comparison, the equivalent response ratio between silver-containing and nonsilver-containing peptide ions for methionine enkephalin was 12.1; leucine enkephalin, 0.33; substance P, 13.4; and $[Nle^{11}]$–substance P, 0.12. What is evident from these results is that although the presence of methionine enhances silver binding, binding still occurs in the absence of sulfur.

The silver–peptide complex is apparently formed only in solutions where silver(I) and the peptide can react. In an experiment similar in design to that of Ogorzalek-Loo and Smith [29, 30], we electrosprayed a silver(I) nitrate solution and a methionine enkephalin solution separately into two chambers, the exits of which terminated in a common transfer tube positioned directly in front of the orifice of the mass spectrometer (Figure 5). In this experiment, no silver complex of

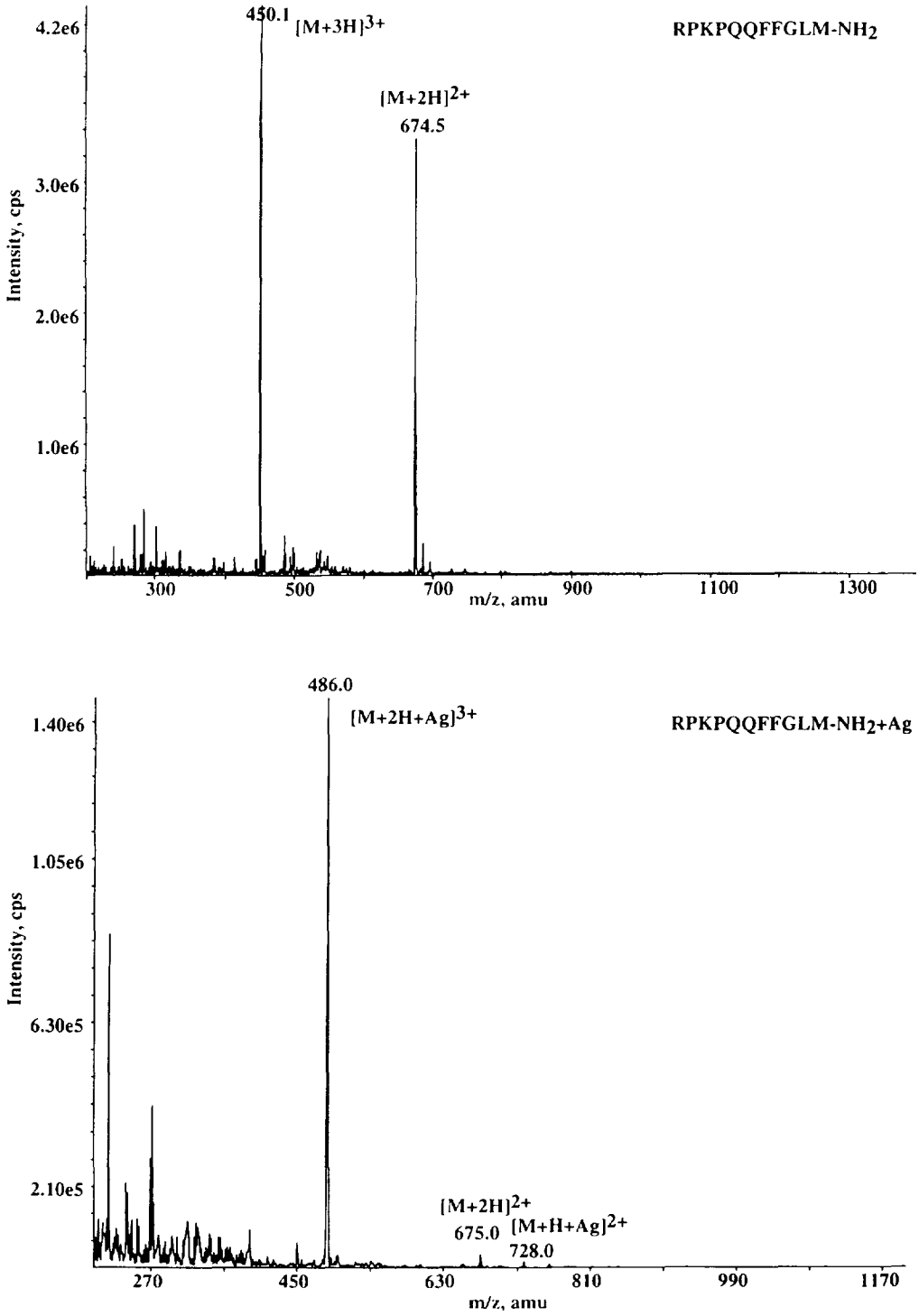

Figure 3. Electrospray mass spectra of substance P and substance P + Ag(I): Upper panel, 10 μM substance P in 50 mM acetic acid, 50/50 water/methanol; lower panel, otherwise identical substance P solution plus 1 mM silver(I) nitrate.

methionine enkephalin was observed. Evidently, adducts do not form in the 1-atm or the "lens" region of the mass spectrometer and can only form in solution. This is, perhaps, not surprising in view of the high Coulombic energy barrier for cation/cation reaction in the gas phase.

Collision-Induced Dissociation

To gain some knowledge on the structure of the silver–peptide complexes, we performed collision-induced dissociation of them. We began with the simpler di- and tripeptides. To designate fragment ions, conventional Ro-

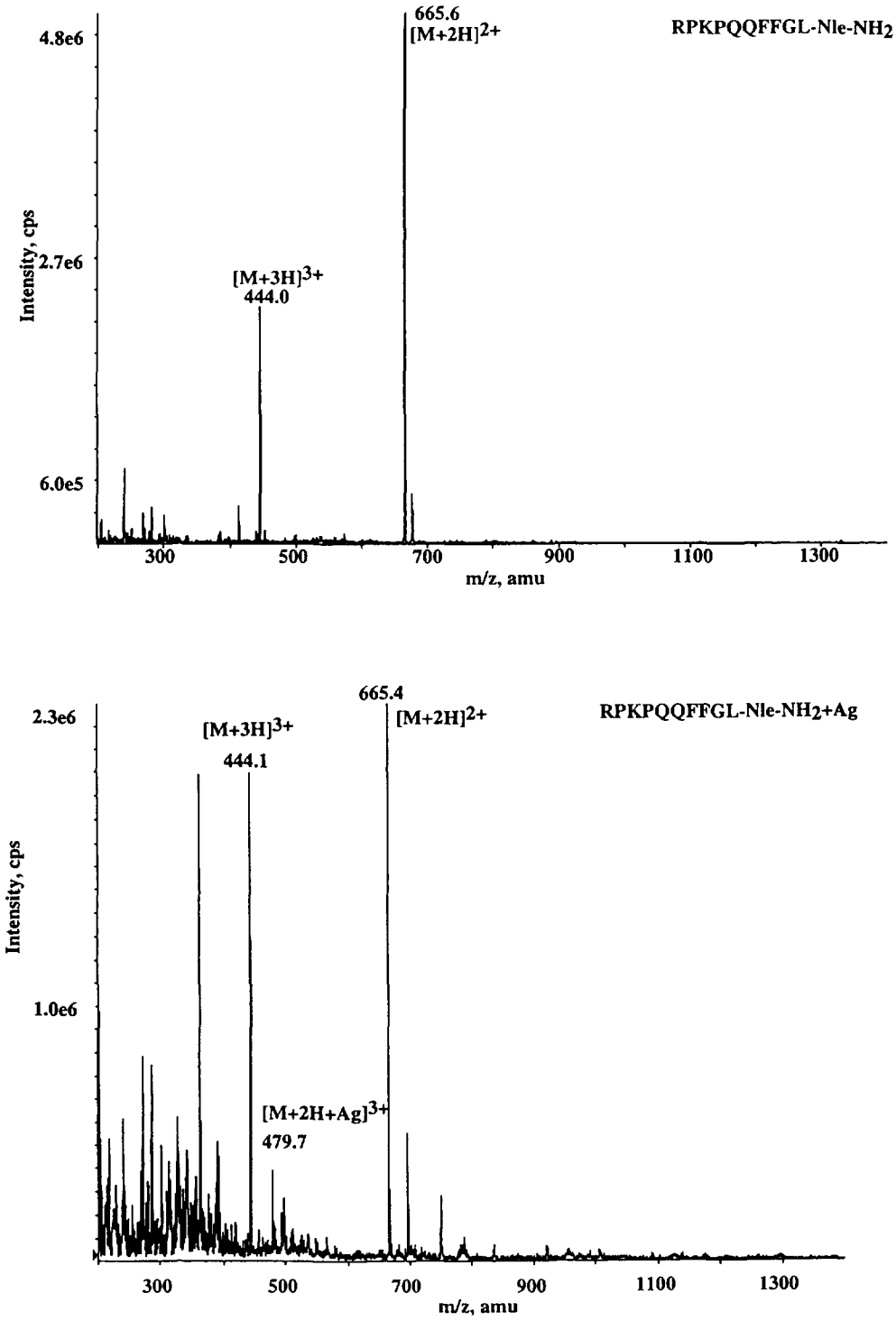

Figure 4. Electrospray mass spectra of [Nle[11]]–substance P and [Nle[11]]–substance P + Ag(I): upper panel, 10 μM [Nle[11]]–substance P in 50 mM acetic acid, 50/50 water/methanol; lower panel, otherwise identical [Nle[11]]–substance P solution plus 1 mM silver(I) nitrate.

epstorf/Biemann notation was used [31, 32] with explicit hydrogen and silver. For example, a_2 of glycylalanylalanine (GAA) is $NH_2CH(CH_3)CONHCH(CH_3)$ and, therefore, $[a_2–H + Ag]^+$ is $[NH_2CH(CH_3)CONHCH(CH_3)–H + Ag]^+$.

Figure 6 shows the product ion spectrum of the $[M + {}^{107}Ag]^+$ ion of GAA. The majority of product ions, especially those that have relatively high m/z values, contain silver. Silver has two natural isotopes (106.9 and 108.9) of almost equal abundance; a comparison of the product ion spectra of $[M + {}^{107}Ag]^+$ and $[M + {}^{109}Ag]^+$ quickly reveals the product ions that contain silver. In

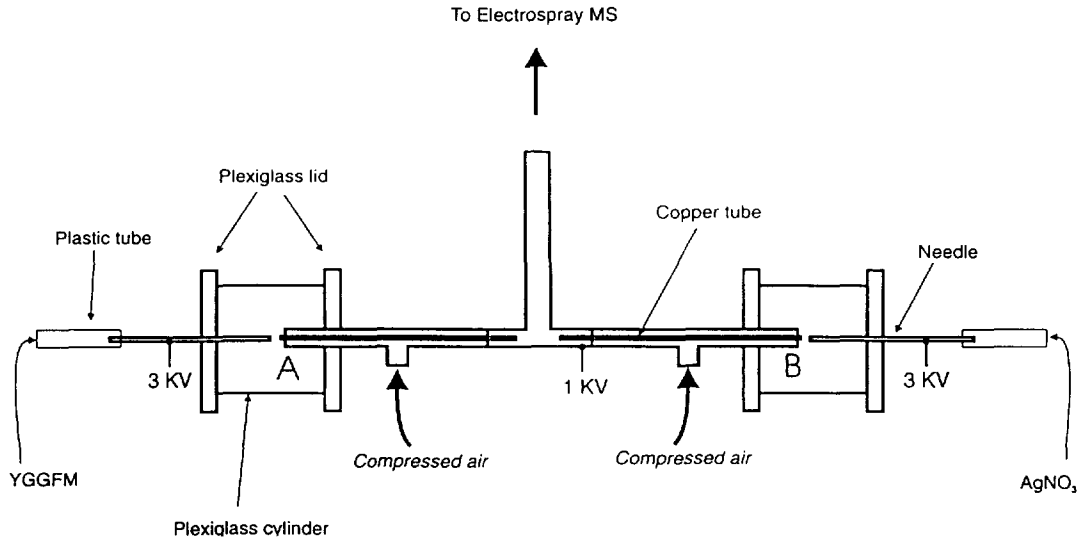

Figure 5. Dual chamber electrospray apparatus.

this report, all product ion spectra shown are those from ^{107}Ag-containing adducts, and the isotope will not be specified hereafter. In general, the following series of products ions are observed: $[a_n–H + Ag]^+$, $[b_n–H + Ag]^+$, $[y_n + H + Ag]^+$, and $[b_n + OH + Ag]^+$. With the exception of the $[b_n + OH + Ag]^+$ ions, all product ions are commonly observed in low-energy fragmentation of singly protonated peptide ions with Ag^+ substituting for H^+ in the ionic product. The equivalent of $[b_n + OH + Ag]^+$ ions are common in the fragmentation of alkali metal-containing peptides [2, 5, 6, 24–26]; $[b_n + OH + Ag]^+$ ions were identified in the pioneering work of Tang et al. [24].

Figure 7 shows the product-ion spectrum of the $[M + Ag]^+$ ion of glycylmethionine. If the Ag^+ in the precursor ion were attached only to the methionine sulfur, then one would expect that any N-terminal fragment ion shorter than and including b_1, which cleaves at the amide bond, be nonsulfur containing. The assignment for the product ion at m/z 182 is $[b_1 + OH + Ag]^+$; the precursor ion fragmenting to this product ion must have its silver ion bound to functional groups on the

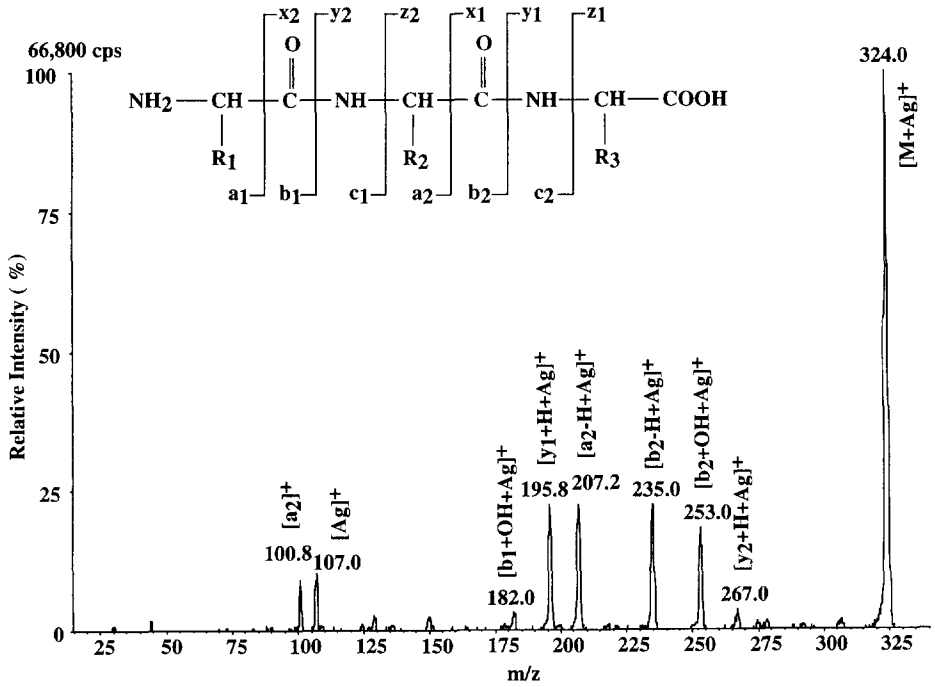

Figure 6. Product-ion spectrum of $[M + Ag]^+$ ion of GAA, E_{lab} = 40 eV.

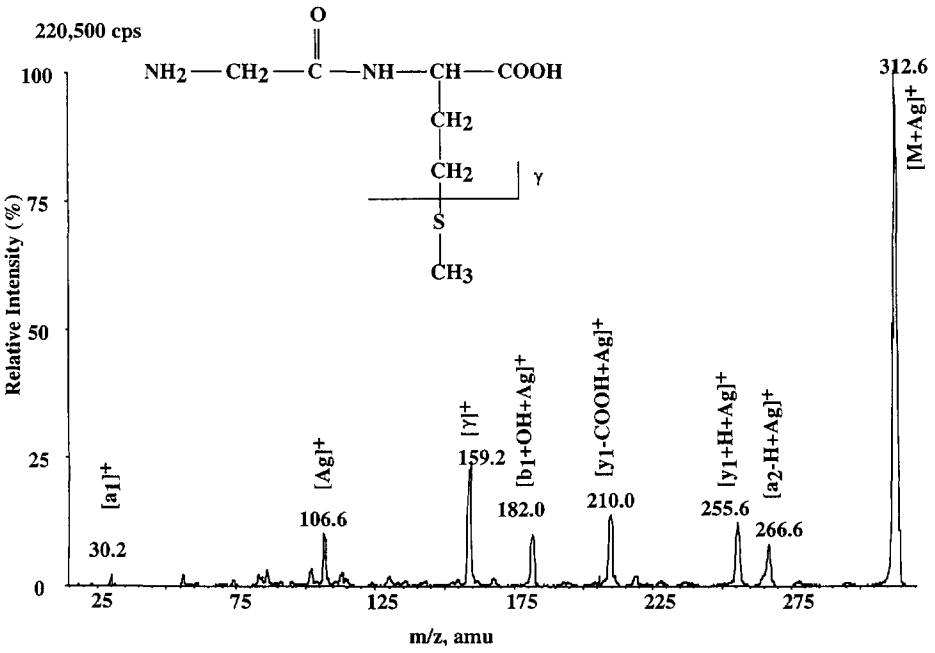

Figure 7. Product-ion spectrum of $[M + Ag]^+$ ion of glycylmethionine, E_{lab} = 40 eV.

N-terminal side of the point of cleavage (transfer of the silver ion from the neutral to the ionic product during fragmentation is ignored here). The existence of gas-phase precursor ions where Ag^+ is bound to functional groups other than the methionine sulfur in glycylmethionine is supported by results of molecular modeling. The three lowest energy structures determined using ZINDO were shown in Figure 8. Whether the three structures shown are indeed the three lowest energy structures of the $[M + Ag]^+$ ion of glycylmethionine is not central to the argument at this point. What is central, however, is that the silver ion in all three structures appears to bind to the methionine sulfur and to a number of other functional groups, including the carbonyl oxygen, the amide nitrogen and the N-terminal nitrogen. In other words, the silver ion is chelated to a number of functional groups including the methionine sulfur. This is in accord with observation of silver complexes of nonsulfur containing peptides. Geometry optimizations of the $[M + Ag]^+$ ions of tri- and longer peptides resulted in structures containing identical types of bonding.

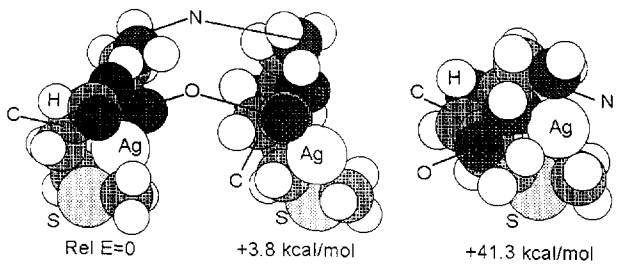

Figure 8. Structures of $[M + Ag]^+$ ion of glycylmethionine calculated by means of ZINDO; the numbers show the binding energies of the structures in excess of the lowest energy structure on the left.

Chelation of Ag^+ to multiple sites was confirmed on longer peptides. Figure 9a shows the product-ion spectrum of the $[M + Ag]^+$ ion of methionine enkephalin. It is evident that most product ions are N-terminal fragment ions (e.g., $[a_4–H + Ag]^+$, $[b_3–H + Ag]^+$ and $[b_3 + OH + Ag]^+$) that do not contain the methionyl side-chain but do contain the silver ion. The y ions, $[y_3 + H + Ag]^+$ and $[y_4 + H + Ag]^+$, contain sulfur, but their abundances are relatively low. When the C-terminal methionine group was replaced with leucine, the y ions were completely absent. Figure 9b shows the production spectrum of leucine enkephalin; evidently only N-terminal products ions are present. The product ions that are reported here as well as the presence of the y ions for methionine enkephalin and the absence of them for leucine enkephalin are identical to those reported by Tang et al. [24] for the metastable-ion decay of the $[M + Ag]^+$ ion of these two peptides when desorbed by means of Cs^+ bombardment.

The product-ion spectrum of the $[M + H + Ag]^{2+}$ ion of methionine enkephalin (Figure 10) is significantly different from that of the $[M + Ag]^+$ ion (Figure 9a) and has never been reported. For the doubly charged ion, one may expect that, to minimize coulombic repulsion, a short peptide that is devoid of basic residues (such as methionine enkephalin) would have its proton residing at the N-terminal nitrogen and the silver ion near the C terminus. A comparison of Figures 9a and 10 supports this hypothesis; relatively more abundant silver-containing y ions and less abundant b ions are apparent from the fragmentation of $[M + H + Ag]^{2+}$.

Figure 11 shows the product-ion spectra of the [M +

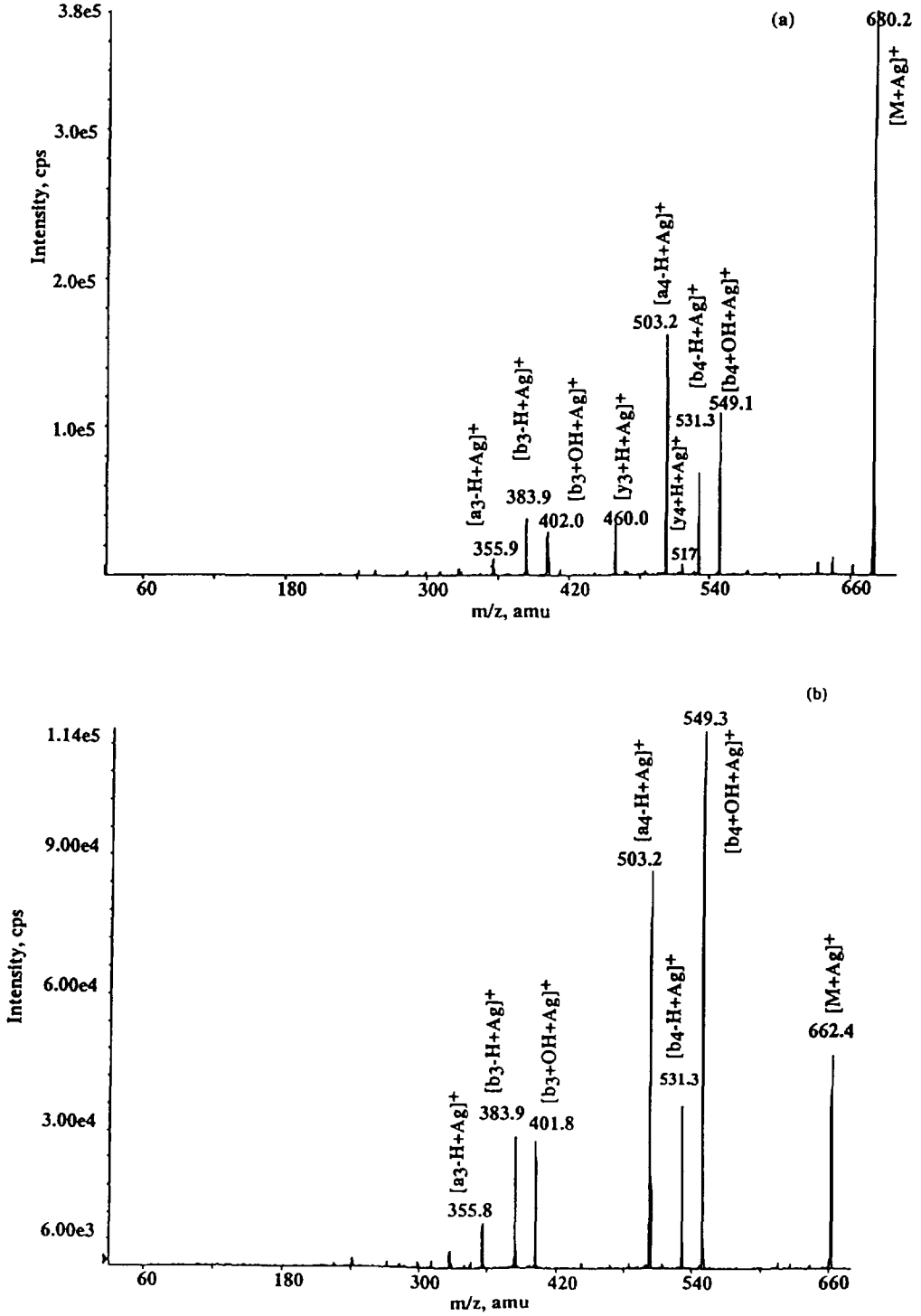

Figure 9. Product-ion spectra of the $[M + Ag]^+$ ion of (**a**) methionine enkephalin and (**b**) leucine enkephalin; E_{lab} = 38 eV for both experiments.

$2H + Ag]^{3+}$ ions of substance P and [Nle^{11}]-substance P. Both spectra are dominated by b ions, such as $[b_{10} + Ag]^{2+}$, $[b_9 + Ag]^{2+}$, etc., that do not contain the C-terminal residue (methionine and norleucine, respectively). The only apparent difference between the two spectra is that the fragment ions produced from substance P include two relatively weak y ions ($[y_6 + H + Ag]^+$ and $[y_5 + H + Ag]^+$), while those from [Nle^{11}]-substance P do not. This observation is in accord with a similar observation on the methionine/leucine enkephalin pair noted above.

During the course of this study, product-ion spectra of the same precursor ions were typically recorded several times; these were found to be reproducible as

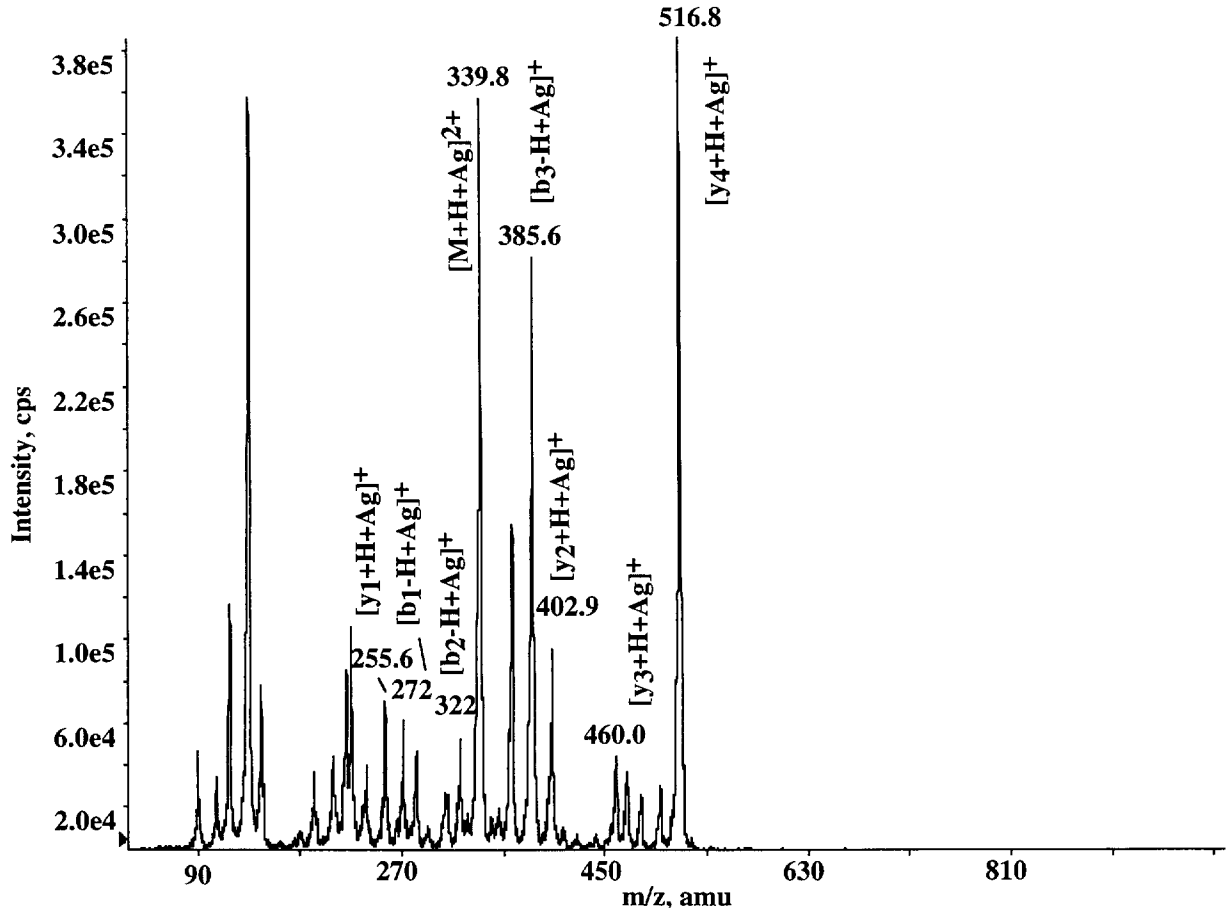

Figure 10. Product-ion spectrum of the $[M + H + Ag]^{2+}$ ion of methionine enkephalin; E_{lab} = 48 eV.

long as the mass spectrometric conditions were identical irrespective of the pH (3 or 7) from which the precursor ions were electrosprayed.

Silver Binding to Peptides

As discussed earlier, an apparent trend in this study is that the relative response between silver-containing ions and protonated ions of the same peptide increases with increasing peptide length, and this increase is much more dramatic for methionine-containing peptides than for nonmethionine-containing ones. Our interpretation of this observation is that the silver ion is bound in solution to a number of functional groups among several amino acid residues, but with the methionine sulfur atom being the anchoring point for silver attachment. A metal ion that binds sulfur in preference to oxygen forms not only strong σ bonds with the readily polarizable sulfur ligand, but also π bonds by back donation of electrons from metal $d\pi$ to ligand $p\pi$ orbitals; silver(I) is such an ion. The electronegativity of sulfur is low, its polarizability is high, and it becomes highly polarized in the field of a small ion, such as a metal ion. The fact that Ag(I), Au(I), and Hg(II) bind readily to sulfur groups is used in the preparation of heavy-atom derivatives in protein crystallography [23]. Our silver-binding experiments were carried out in acidic as well as neutral conditions. Under acidic conditions, the C-terminal carboxylate group is protonated in solution; this significantly decreases the metal-binding capacity of this group [23, 33]. The drastic difference between the mass spectra of silver-containing solutions of substance P and [Nle^{11}]-substance P, two peptides whose C termini contain the amide rather than the carboxylate functionality, provides further evidence that the carboxylate ion plays a minor role in silver binding. In neutral and acidic solution, silver(I) ions would be competing with protons for binding to the N-terminal amino group. Thus, there is little doubt that, in solution, silver binds preferably to the methionine sulfur.

Because the product-ion spectra of silver adducts of methionine-containing peptides and their nonmethionine-containing analogues are very similar (i.e., most fragment ions containing silver do not contain the methionine residue), it was concluded that the gas-phase structures of silver adducts of methionine and nonmethionine-containing peptides are very similar. What this means is that the solution-phase and the gas-phase structure of the silver-peptide complex are not identical, and that the solution-phase structure

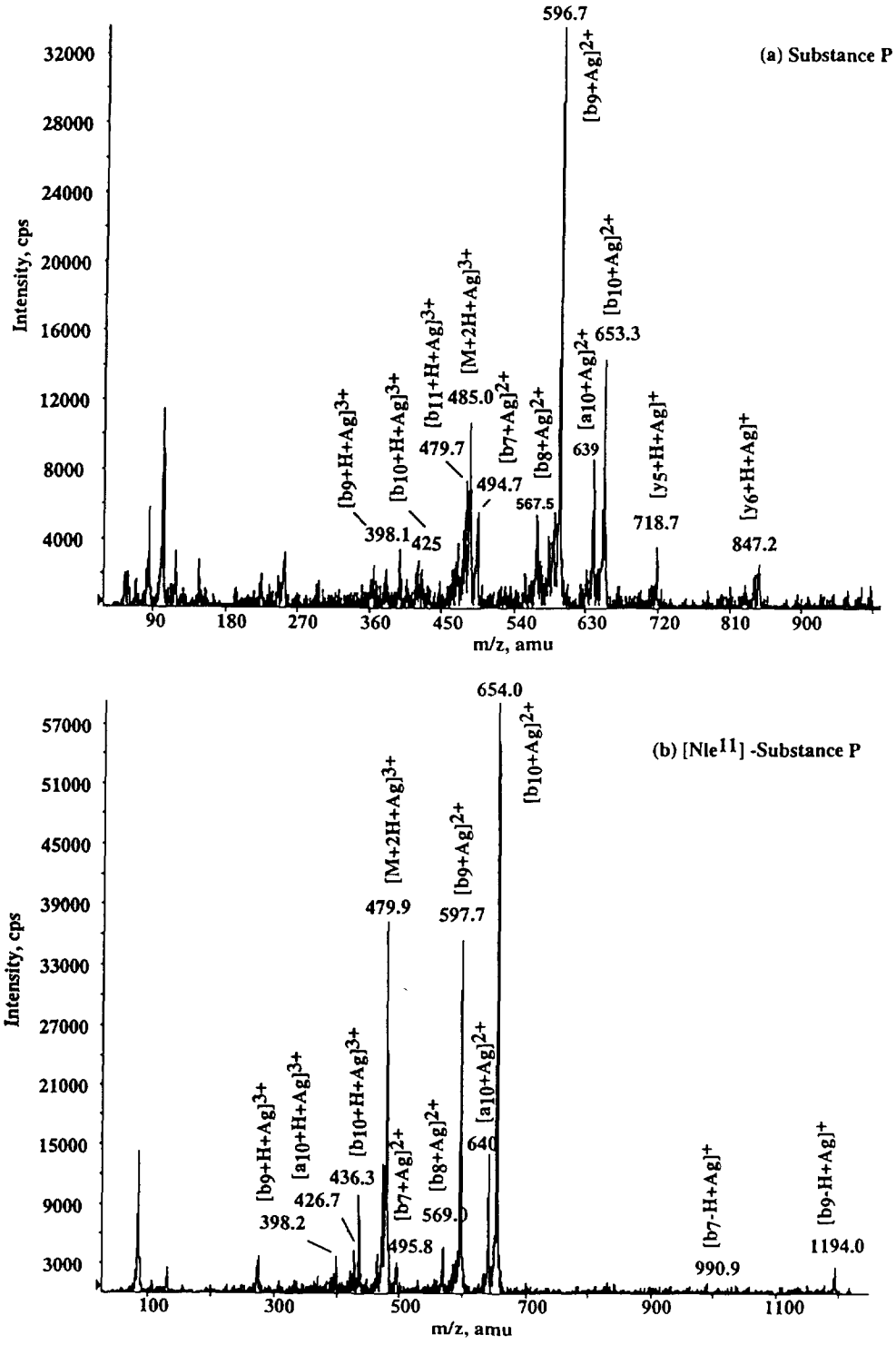

Figure 11. Product-ion spectra of the $[M + 2H + Ag]^{3+}$ ion of (**a**) substance P, E_{lab} = 78 eV and (**b**) [Nle^{11}]-substance P, E_{lab} = 63 eV.

rearranges to the gas-phase structure within the time-frame of a few milliseconds that is available in the electrospray experiment. In this model, the sulfur atom in a methionine-containing peptide serves as the silver ion anchoring point in solution, accounting for the higher silver-peptide complex abundance observed for methionine-containing peptides. However, rearrangement in the gas phase decreases the importance of the silver/sulfur bond to the extent that most product ions do not contain the methionine group.

There is some similarity between this model and the model proposed by Williams et al. [34, 35] for proto-

nated peptides and proteins; protonated sites of peptides in the gas phase are not necessarily identical to those in solution (because of electrostatic effects). For protonated peptides, the driving force for rearrangement is the increase in coulombic repulsion as a consequence of desolvation in the gas phase; for silver-containing peptides, the driving force is unknown. However, one may speculate that water is an effective competitor with the peptide backbone for solvation of silver in solution and this increases the anchoring effect of the methionine sulfur while, in the gas phase after the removal of water, the only effective "solvent" is the peptide backbone. As a result, the peptide wraps around the silver ion and chelates it by using its nitrogen and oxygen atoms on the peptide backbone. This chelation decreases the importance of (and probably weakens [36]) the silver/sulfur bond to the extent that it becomes the preferred cleavage site upon collision activation and consequently leads to silver-peptide product ions that are nonsulfur bearing.

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