Intrinsic Interactions between Alkaline-Earth Metal Ions and Peptides: A Gas-Phase Study

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Received October 23, 1989

Abstract: The gas-phase chemistry of $(M - H + Cat)^+$ complexes formed between alkaline-earth metal ions and simple peptides is reported. The preferred sites of metal ion complexation are determined from collision-induced decompositions of the complexes that occur in the first field-free region of a normal-geometry mass spectrometer. Mechanisms for formation of N-terminal a- and c-sequence ions primarily require the $(M - H + Cat)^+$ complexes to contain the alkaline-earth metal ion coordinated to a deprotonated amide nitrogen. The C-terminal y-sequence ions appear to arise exclusively from $(M - H + Cat)^+$ ions that contain either a deprotonated and cationized C-terminal carboxylate or an amide. The C-terminal z-sequence ions, however, can arise from deprotonation and coordination of the metal ion either to a C-terminal carboxylate or amide or to an intervening deprotonated amide group. Changes in fragmentation patterns with increasing size of alkaline-earth metal ion, with substitution of the C-terminal carboxylate for a C-terminal amide, and with changes in side-chain structure suggest that interactions with the metal ions also may be via more extensive intramolecular complexes that are affected by coordination geometry of the metal ion. The collision-induced decompositions of the peptide–alkaline-earth ion complexes are analytically useful for providing peptide sequence information.

There are many metal ions that serve vital roles in a variety of biomolecular functions. The metal ions include alkali, alkaline-earth, and transition metals. Their function in protein chemistry ranges from stabilizing tertiary structure, to transporting ions through membranes, to activating enzymes.[1] Some interesting features of metal ion interactions with proteins are that some ions, such as Ca^{2+}, may inhibit enzyme activity whereas a closely related metal ion, such as Mg^{2+}, may activate it, and vice versa.[1b,d] Interactions between metal ions and proteins are related to the specificity of metal ion binding sites that are frequently defined by specific conformations of the ligands that maximize bonding to the metal ion. In hydrophobic interiors of large proteins, where aqueous solvation plays either a limited or nonexistent role in metal ion binding, the intrinsic bond strengths of potential binding sites in addition to conformation would define the preferential location of metal ion bonding.[2]

Most research that addresses metal ion interactions with peptides or proteins involves studying the chemistry in either the solution phase or the solid phase. Although some of the aqueous-phase studies of metal ion–peptide interactions give information about some intra- and intermolecular binding sites, the results frequently reflect interactions that involve deprotonated carboxylate (C) terminii.[3] The C terminus of proteins, however, is usually not a preferred binding site for metal ions, and other reactions predominate that involve either amide or side-chain groups.[3a,b,4] X-ray absorption spectroscopy, especially EXAFS, has provided new insight into metal ion binding sites in interiors of proteins.[5] These methods give no information, however, about intrinsic differences in binding sites that ultimately define metal ion–protein interactions.

Intrinsic chemical interactions can be investigated either theoretically by using molecular orbital calculations or experimentally by using gas-phase chemistry in which solvation plays no role. There have been many theoretical investigations, and some gas-phase studies, designed to probe the reasons behind, and the preferred geometries of, cation bonding to amide and carboxylate groups. The intrinsic chemistry of the amide group has been of particular interest because of its duality as a simultaneous Lewis acceptor and Brønsted donor and its importance as the peptide bond. It is this duality that makes the amide moiety well suited for stabilizing protein tertiary structure. The acceptor site of the amide functionality is the basic carbonyl oxygen,[6-9] whereas the donor site is the acidic NH group.[10] Intrinsically and in solution, an amide carbonyl oxygen is more basic than analogous ester or acid carbonyl oxygens.[1b,d,7,8d,11] The amide NH group is in-

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trinsically similar in acidity to a carboxylic acid OH group,[10] but it is significantly less acidic in solution, by ~13 pK_a units.[3a] An amide NH group intrinsically becomes more acidic with proton or metal ion bonding to the carbonyl oxygen,[12] and this is also reflected in solution-phase behavior. That is, in solution, protonating an amide oxygen significantly increases the acidity of the amide NH group by ~8 pK_a units.[3a] Furthermore, in solution, bonding some transition-metal ions to the amide carbonyl oxygen can result in deprotonation of the NH group and exchange of the metal ion for the proton.[3a,4,13]

It would be difficult to perform theoretical calculations to address relative intrinsic strengths of metal ion binding sites in structures as large as peptides. Intrinsic reactivities of different metal ion binding sites can be studied, however, with gas-phase ion chemistry. There are some previous investigations that address intrinsic gas-phase interactions between metal ions and polypeptides. These earlier studies address the gas-phase chemistry of $(M + Cat)^+$ complexes formed between peptides and some of the alkali metal ions,[14] Ag^+,[14d,g] and Cu^+.[14g] Two general interpretations have been proposed to account for fragmentation patterns that arise from collision-induced dissociations of $(M + Cat)^+$ ions of peptides, in which Cat is an alkali metal ion. The earlier interpretation is that alkali metal ions preferentially bond to neutral peptides at sites that have the highest proton affinities so that the alkali ion carries the charge, bonding involves the N-terminal amino group and amide nitrogens (assuming no interaction with side chains), and there is little interaction between the metal ion and the C-terminal carboxylate.[14b-e] A newer interpretation is that the alkali metal ion instead predominantly coordinates to the C-terminal carboxylate group.[14f,g] Here, the $(M + Cat)^+$ ions, which are initially formed from zwitterionic species, contain the metal ion bonded to the deprotonated C-terminus, and the charge in the ions is instead carried by either a protonated amino or another group. It is clear that these two interpretations implicitly require entirely different types of intrinsic gas-phase interactions between the metal ion and the peptide.

There are no data on gas-phase interactions between alkaline-earth metal ions and peptides. There are, however, some theoretical and solution-phase studies of interactions between alkaline-earth metal ions and simple amides. Intrinsically, alkaline-earth metal ions bond to the carbonyl oxygen of simple amides preferentially on an axis along the amide CO bond with calculated bond energies of ≥100 kcal mol^{-1}.[8c,e,9a,b,15] Bonding of alkaline-earth cations increases the double-bond character that is already in the amide OC–N bond and further reduces bond rotation.[9a,b] Solution-phase chemistry generally reflects the intrinsic chemistry.[6b,9a,b]

Interactions between alkaline-earth cations and peptides and proteins, which contain several to many amide groups, a variety of side chains, an N-terminal amine, and a C-terminal carboxylate, are more complex than are interactions with simple amides. In solution, alkaline-earth metal ions usually interact with simple, small peptides by bonding to the anionic carboxylate terminus of peptide zwitterions.[3b-d] In contrast, interactions with proteins generally do not invove bonding to the C-terminal carboxylate but involve specific coordination with deprotonated acidic side chains and amide carbonyl oxygens.[3b] Deprotonation of a peptide amide NH as a result of an amide CO–alkaline-earth metal bond has not been observed in solution.[3a]

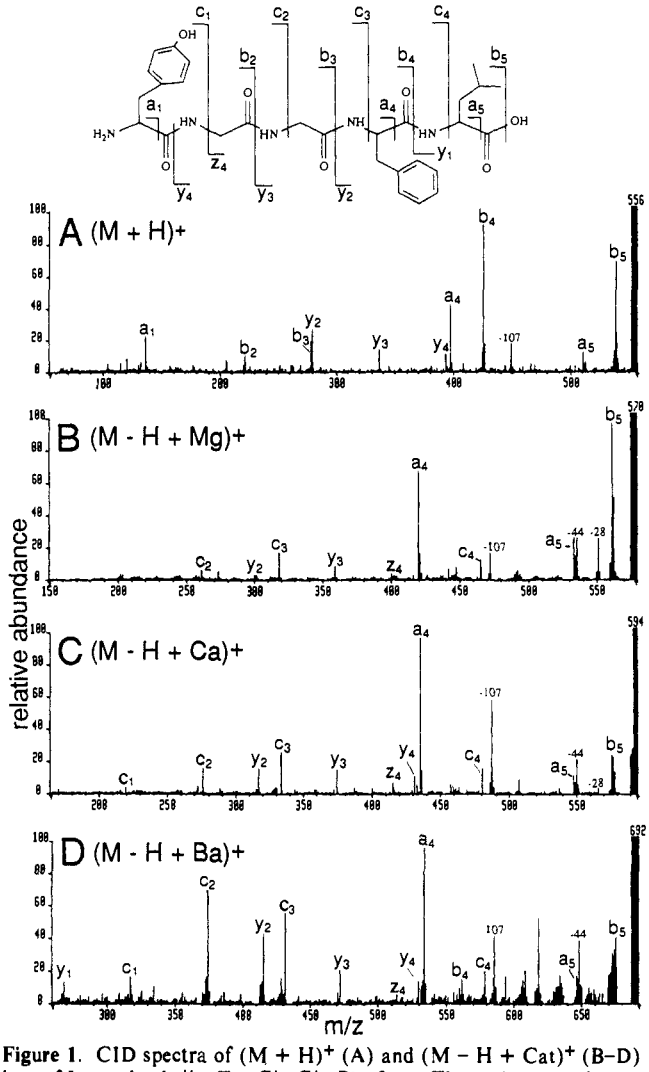

Figure 1. CID spectra of $(M + H)^+$ (A) and $(M - H + Cat)^+$ (B–D) ions of Leu-enkephalin, Tyr-Gly-Gly-Phe-Leu. The a-, b-, c-, and y-sequence ions are described in Table I. The z_4 ion in spectra B–D is a $(z_4 - H + Cat)^{*+}$ ion.

Here we present results of a study of the gas-phase chemistry of $(M - H + Cat)^+$ complexes formed between Mg^{2+}, Ca^{2+}, and Ba^{2+} and simple peptides that do not contain acidic side chains. The $(M - H + Cat)^+$ complexes that contain alkaline-earth metal ions, as opposed to $(M + Cat)^+$ complexes that contain alkali metal ions, inherently require deprotonation of the peptide molecule for formation. This study thus addresses whether the preferential site of deprotonation and cationization is at the C-terminal carboxylate, at amide nitrogens, or at other sites in the peptide molecule. Although intrinsic, gas-phase chemistry of complexes between metal ions and peptides may not completely reflect solution-phase chemistry, gas-phase interactions may mimic hydrophobic metal ion bonding that occurs in interiors of proteins.[2a,c] The gas-phase chemistry thus may delineate the chemistry that controls competitive intramolecular complexation between metal ions and potential metal ion bonding sites and provide evidence regarding metal-ion induced structural conformations.

Results

Gas-phase $(M - H + Cat)^+$ complexes between Mg^{2+}, Ca^{2+}, and Ba^{2+} and 36 hexa-, penta-, tetra-, tri-, and dipeptides were studied with fast atom bombardment (FAB) and collisional activation mass spectrometry. Some of the peptides contain either a N-terminal benzoyl or a C-terminal amide, alcohol, or ester

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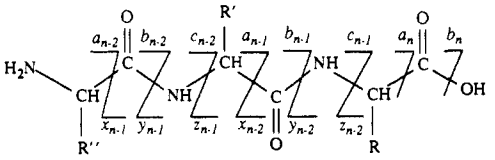
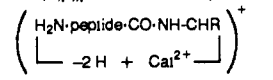
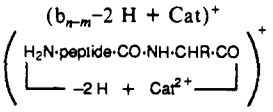
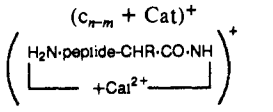
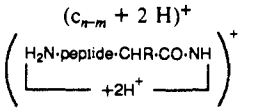
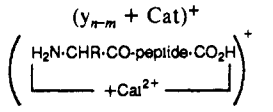
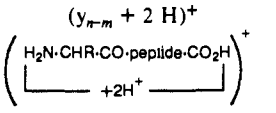
Table I. Sequence Ions Formed from Collision-Induced Decompositions of $(M - H + Cat)^+$ and $(M + H)^+$ Ions of Peptides[a]



fragment ions from $(M - H + Cat)^+$[b]	analogous fragment ions from $(M + H)^+$[b]
$(a_{n-m} - 2\,H + Cat)^+$ 	$(a_{n-m})^+$ $(H_2N\text{-peptide-CO-NH-CHR})^+$
$(b_{n-m} - 2\,H + Cat)^+$ 	$(b_{n-m})^+$ $(H_2N\text{-peptide-CO-NH-CHR-CO})^+$
$(c_{n-m} + Cat)^+$ 	$(c_{n-m} + 2\,H)^+$ 
$(y_{n-m} + Cat)^+$ 	$(y_{n-m} + 2\,H)^+$ 

[a] Other fragment ions also appear in spectra of $(M - H + Cat)^+$ ions that do not have direct analogies to fragment ions formed from $(M + H)^+$ ions. [b] Here, n refers to the total number of amino acids in the peptide and m is from 0 to $n-1$.

group. One pentapeptide contains a methylated amide nitrogen. The formation of peptide–metal ion complexes, which were prepared in situ on the FAB probe tip, was maximized with one of several FAB matrices that was saturated with an alkaline-earth metal hydroxide. The gas-phase complexes were accelerated from the ion source and fragmented by 8-keV collisions with helium in the first field-free region of a normal-geometry mass spectrometer. Fragment ions from collision-induced decompositions (CIDs) were observed with B/E-linked scans that provided mass resolution of approximately 1500 and better than unit mass measurement.

CID spectra of the pentapeptide Leu-enkephalin complexed with H^+, Mg^{2+}, Ca^{2+}, and Ba^{2+} show typical types of fragment ions that can arise from $(M + H)^+$ ions and from $(M - H + Cat)^+$ ions of some polypeptides (Figure 1). Gas-phase fragmentations of $(M + H)^+$ ions have been used extensively for sequencing polypeptides,[16,17] and mechanisms for the fragmentations have been proposed.[17] Relationships between some fragment ions that arise from $(M - H + Cat)^+$ and from $(M + H)^+$ ions of peptides are shown in Table I. The N-terminal sequence ions contain the amino terminus and are labeled from a_{n-m} to c_{n-m}, whereas C-terminal sequence ions contain the carboxylate terminus and are labeled from x_{n-m} to z_{n-m}. The numerical subscript refers to the number of amino acid side chains that are contained in the fragment ion, in which n is total number of amino acids in the peptide chain and m is from 0 to $n - 1$.

Fragment ions that contain alkaline-earth metal ions that are shown in Table I are isoelectronic with analogous fragment ions that arise from protonated peptides. For instance, c ions formed from $(M - H + Cat)^+$ precursors contain a covalent bond, and also perhaps a coordinate covalent bond, to a doubly charged alkaline-earth cation. Analogous c ions from $(M + H)^+$ ions contain a covalent bond to one proton and a coordinate covalent bond to the other proton. The $(M - H + Cat)^+$ ions of some peptides fragment to give $(z_{n-m} - 2\,H + Cat)^+$ ions, and they are analogous to $(z_{n-m})^+$ ions formed from $(M + H)^+$ ions. Not all fragment ions that arise from $(M - H + Cat)^+$ ions of peptides, however, have direct analogies to fragment ions formed from $(M + H)^+$ ions. Some peptide $(M - H + Cat)^+$ ions fragment to produce a few $(b_{n-m} + Cat)^+$, $(z_{n-m} - H + Cat)^{\cdot+}$, $(z_{n-m} + Cat)^+$, and $(x_{n-m} - H + Cat)^{\cdot+}$ ions.

Interactions between peptides and protons (Figure 1A) can trigger similar types of fragmentations as interactions between peptides and alkaline-earth metal ions (Figure 1B–D). The fragment ion abundances, however, may be very different. Protonated peptides, such as Leu-enkephalin in Figure 1A, generally preferentially fragment to give $(b_{n-m})^+$ N-terminal sequence ions and $(y_{n-m} + 2\,H)^+$ C-terminal sequence ions,[16,17] although there also are three $(a_{n-m})^+$ ions in the spectrum in Figure 1A. The alkaline-earth metal ions (Figure 1B–D) primarily induce cleavages to give N-terminal $(a_{n-m} - 2\,H + Cat)^+$ and $(c_{n-m} + Cat)^+$ ions and an abundant $(b_n - 2\,H + Cat)^+$ ion. The most prevalent C-terminal ions are $(y_{n-m} + Cat)^+$ ions. In general, neither protonated peptides[16,17] nor most larger peptides complexed with alkaline-earth metal ions fragment to give abundant C-terminal x or z ions, although there is a weakly abundant $(z_4 - H + Cat)^{\cdot+}$ fragment ion in spectra of Leu-enkephalin complexed with alkaline-earth metal ions (Figure 1B–D). For Leu-enkephalin in Figure 1, all CID spectra show a radical-cation fragment ion that results from loss of the tyrosine side chain (−107 u). Complexes between peptides and alkaline-earth metal ions also generally fragment to give an ion that results from loss of 44 u, which is primarily loss of CO_2, and an ion that results from loss of 28 u (CO). (These elemental compositions were deduced by comparing fragmentations of all the peptides studied.)

Peptides that contain a C-terminal amide instead of a C-terminal carboxylate fragment in noticeably different ways (Figure 2). The $(M + H)^+$ ions of Leu-enkephalin amide (Figure 2A) still fragment to give $(a_{n-m})^+$ and $(b_{n-m})^+$ N-terminal ions, but now C-terminal $(z_4)^+$ and $(z_3)^+$ fragment ions replace the $(y_4 + 2\,H)^+$ and $(y_3 + 2\,H)^+$ fragment ions, respectively. The $(M - H + Cat)^+$ ions of Leu-enkephalin amide (Figure 2B–D) still fragment to give N-terminal $(a_{n-m} - 2\,H + Cat)^+$, $(c_{n-m} + Cat)^+$, and $(b_n - 2\,H + Cat)^+$ ions. A significant change, however, is that C-terminal amides fragment to give more highly abundant C-terminal $(y_{n-m} + Cat)^+$ ions. For Leu-enkephalin, a new C-terminal $(x_{n-m} + Cat)^+$ series of ions and a $(z_3 + Cat)^+$ ion now also arise. Loss of the tyrosine side chain (107 u) is still abundant, although loss of 44 u (C_3H_8) now arises from cleavage of the side chain of leucine.

Relative abundances of fragment ions change with changing size of alkaline-earth cation, as shown in Figures 1 and 2. For both C-terminal carboxylates and amides, absolute relative abundances of lower mass C-terminal y ions and lower mass N-terminal c ions increase with increasing size of alkaline-earth metal ion. Abundances of C-terminal z ions, however, are not significantly affected. There are other changes with changing size of alkaline-earth cation that are a result of side-chain structure. Abundances of lower mass a ions sometimes increase with increasing size of alkaline-earth cation, but this effect is influenced by the structure of the N-terminal amino acid. Some new C-terminal y ions may appear with increasing size of alkaline-earth cation, but this is affected by the structure of the side chain of the C-terminal amino acid. Increases in abundances of a and x ions that arise via cleavages of bonds in the central amino acids

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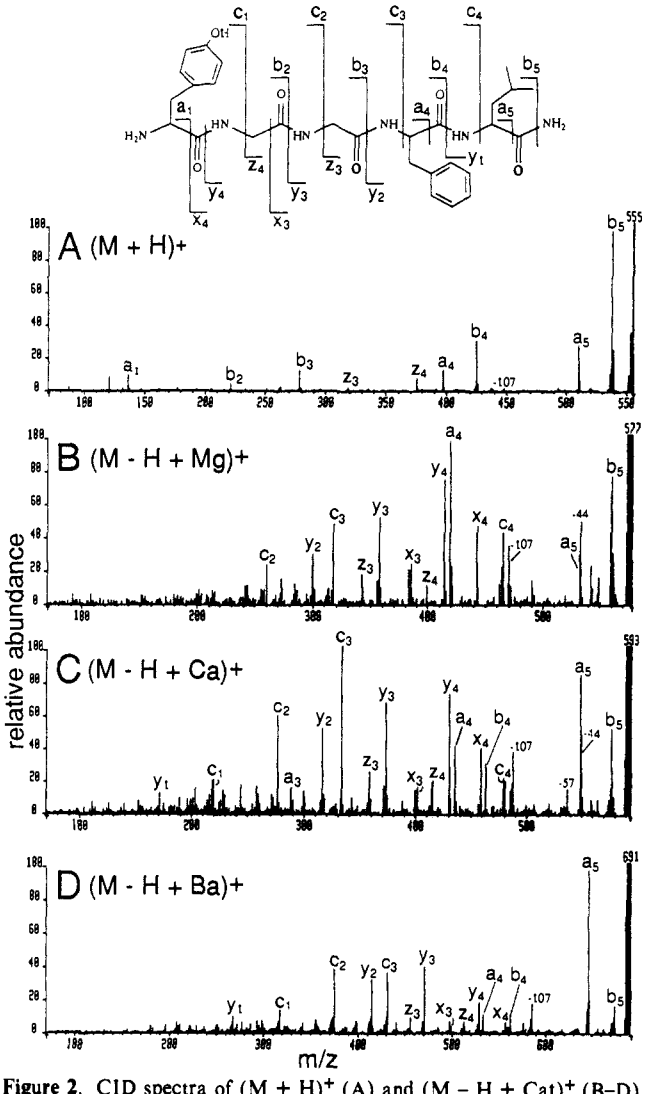


Figure 2. CID spectra of $(M + H)^+$ (A) and $(M - H + Cat)^+$ (B–D) ions of Leu-enkephalin amide, Tyr-Gly-Gly-Phe-Leu-NH_2. The a-, b-, c-, and y-sequence ions are described in Table I. The spectrum in A also shows two $(z_{n-m})^+$ ions. Spectra in B–D also show some $(x_{n-m} + Cat)^+$ ions, a $(z_4 - H + Cat)^{\bullet+}$ ion, and a $(z_3 + Cat)^+$ ion.

of the peptide chain are dependent upon structures of the side chains that are adjacent to the site of cleavage. Increased abundances of the a_5 ion with increasing size of the alkaline-earth metal ion in spectra of Leu-enkephalin amide (Figure 2) are not a trend seen in spectra of all peptide amides.

Relative abundances and types of fragment ions also change with increasing size of the peptide. An example of this phenomenon is shown in Figure 3 for $(M - H + Ca)^+$ ions of di- through pentaphenylalanine. CID spectra of dipeptides are generally quite varied and depend upon side-chain structures (Figure 3A). Some dipeptide $(M - H + Cat)^+$ ions fragment to give very abundant C-terminal $(z_1 - H + Cat)^{\bullet+}$ ions, others fragment to give abundant internal fragment ions that arise from concomitant losses of both N- and C-terminal groups, and others fragment to give weakly abundant ions that are members of the more typical a, b, c, and y series. Loss of H_2O to produce the $(b_2 - 2\,H + Cat)^+$ ion is frequently the most facile fragmentation of $(M - H + Cat)^+$ ions of dipeptides. The spectrum in Figure 3A shows that the most abundant fragment ion produced from CIDs of $(M - H + Ca)^+$ ions of diphenylalanine is an internal fragment I that arises by loss of NH_2 and CO_2H (61 u).

Tripeptide $(M - H + Cat)^+$ ions fragment to give a greater number of sequence ions although their exact composition is strongly dependent upon side-chain structure (Figure 3B). An abundant z_1 ion is generally not seen in spectra of tripeptides or larger, and N-terminal a, b, and c ions become of greater importance. Although there are no abundant C-terminal $(y_{n-m} + Ca)^+$ ions formed from $(M - H + Ca)^+$ ions of triphenylalanine (Figure 3B), other tripeptides do fragment to give sequence-specific y ions. Tripeptides that contain glycine as the N-terminal amino acid also generally fragment to give a facile loss of 29 u (presumably $HN{=}CH_2$) to form C-terminal $(x_2 + Cat)^+$ ions. As shown in Figure 3B, the most abundant N-terminal sequence ions, which in this case are a_2 and b_2 ions, arise from cleavages more distant from the amino terminus.

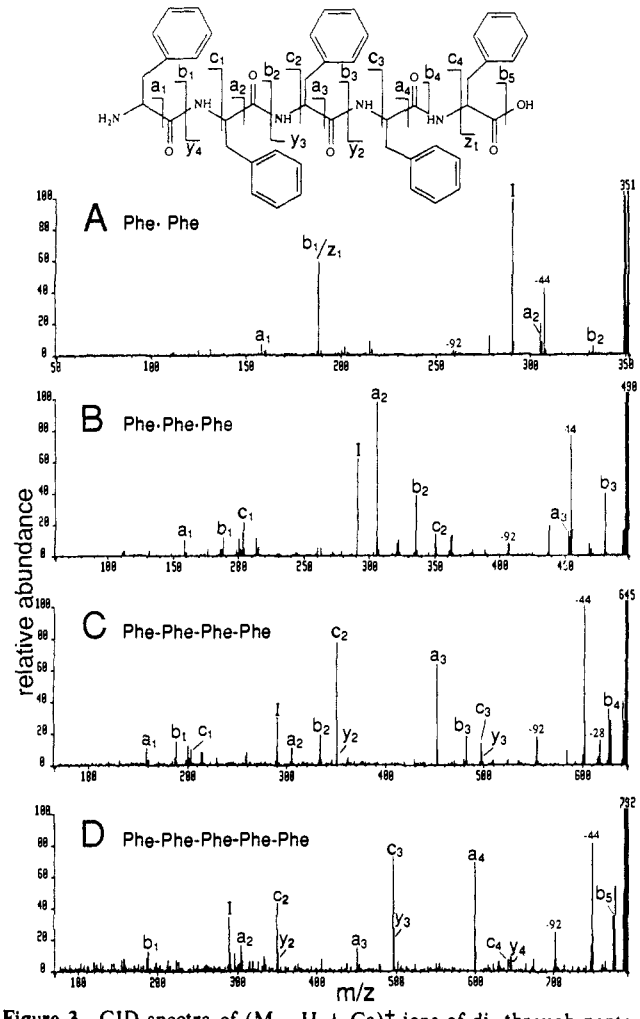


Figure 3. CID spectra of $(M - H + Ca)^+$ ions of di- through pentaphenylalanine. The a-, c-, and y-sequence ions are described in Table I. The b_n ion is a $(b_n - 2\,H + Cat)^+$ ion, whereas the other b ions are $(b_{n-m} + Cat)^+$ ions. The z_1 ion in A is a $(z_1 - H + Cat)^{\bullet+}$ ion. The ion labeled I is an internal fragment ion that is of m/z 290.

There is a transition on going from tri- to tetra-, penta-, and hexapeptides. One feature of this transition is that for the tetrapeptides and larger, the $(a_{n-1} - 2\,H + Cat)^+$ ion and the $(c_{n-2} + Cat)^+$ ions generally become that most abundant N-terminal sequence-specific fragment ions in their respective series (Figure 3C,D). Another general feature is that C-terminal $(y_{n-2} + Cat)^+$ ions generally become the most abundant fragment ions in the y series. Furthermore, the most abundant c and y ions generally are ones that arise from cleavages through bonds in the middle of the peptide chain. As discussed above, however, relative abundances of the members of the y series are significantly affected by the size of the alkaline-earth metal ion. The lowest mass members of each series of sequence ions generally become less abundant with increasing size of the peptide.

As mentioned above, some trends in relative ion abundances are subject to influences from side-chain structure. Peptides that we have studied here do not include ones that have acidic side chains. Some of the peptides, however, do contain basic side chains such as histidine, arginine, and lysine. One example of fragmentations of a peptide that contains basic side chains is shown

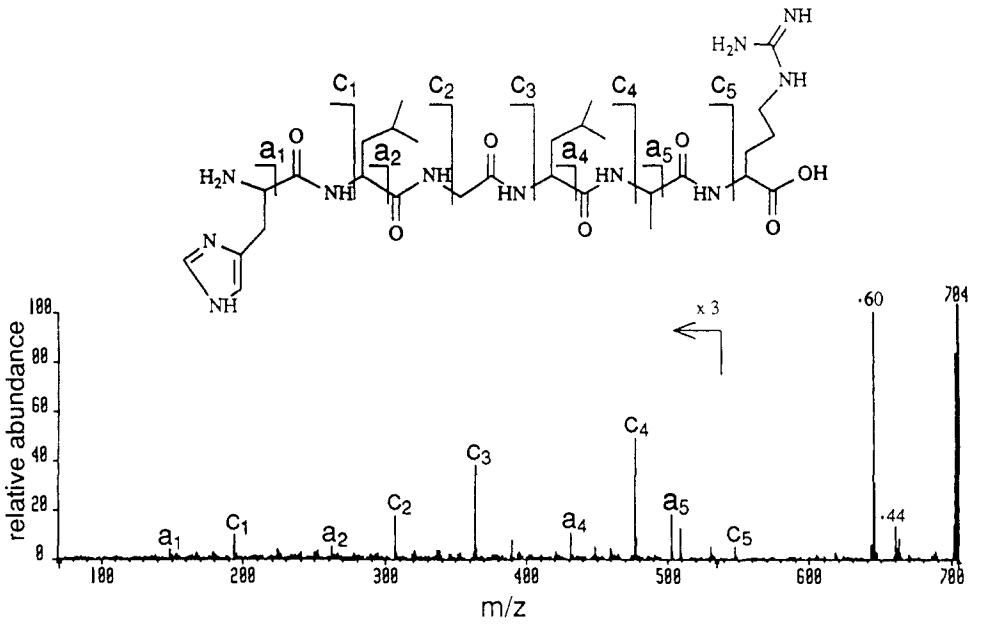


Figure 4. CID spectra of $(M - H + Ca)^+$ ions of the hexapeptide His-Leu-Gly-Leu-Ala-Arg. The a- and c-sequence ions are described in Table I.

in Figure 4 for $(M - H + Cat)^+$ ions of His-Leu-Gly-Leu-Ala-Arg. The presence of a basic amino acid can serve to direct the fragmentation so that cleavages in immediate proximity to the basic amino acid become more abundant. In Figure 4, the histidine residue on the amino terminus results in increased abundances of $(c_1 + Cat)^+$ and $(a_1 - 2\,H + Cat)^+$ fragment ions that typically would be difficult to detect from cleavages of a hexapeptide. As shown in Figure 4, however, the arginine residue on the carboxylate terminus does not direct fragmentation to give C-terminal sequence ions. Fragmentations of other peptides that contain two basic amino side chains indicate that histidine also directs fragmentation preferentially to lysine, and arginine directs fragmentation preferentially to lysine.

Discussion

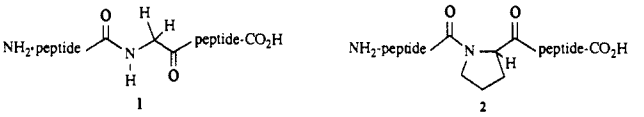
CID spectra in Figures 1–4 show that fragmentation patterns of $(M - H + Cat)^+$ ions change with increasing size of alkaline-earth metal ion, with converting the C-terminal carboxylate into an amide, and with altering the structures of the side chains. This can be the result of two distinct phenomena. One is that the $(M - H + Cat)^+$ precursor ions may be a mixture of isomeric species that contain the alkaline-earth metal ion complexed to different sites in the peptide molecule. Thus, if a mechanism of fragmentation required an alkaline-earth cation to be in a specific location, relative abundances of fragment ions that would arise via the mechanism would be proportional to the number of precursors that contain the metal ion in the specific location. The other phenomenon that would affect relative ion abundances would be changes in relative rates of different fragmentation reactions as a result of the presence of a particular alkaline-earth cation and as a result of the presence of a particular substituent.

To address relationships between changes in fragmentation patterns with changes in metal ion or with changes in substituent, the mechanisms of reaction first must be understood. Here, fragmentations of many structurally diverse peptides are evaluated, and evidence for mechanisms that account for production of the most abundant sequence ions, the $(a_{n-m} - 2H + Cat)^+$, $(c_{n-m} + Cat)^+$, and $(y_n + Cat)^+$ fragment ions, is described. There also is sufficient evidence to support a mechanism for production of $(z_n - H + Cat)^{\cdot+}$ ions even though the z ions usually are in low relative abundances.

Reaction mechanisms discussed below provide information about the metal ion binding site in the peptide. Furthermore, the mechanisms provide information that can be used to predict fragmentations and, to a limited extent, relative ion abundances. Some mechanisms are consistent in that they require the metal ion to be bonded at a specific location in the peptide chain. Other mechanisms only require the metal ion to be located toward either the N or C terminus. The mechanisms of fragmentation are supported by shifts in mass of fragment ions that are a result of various structural changes in the peptides. In addition, by changing the alkaline-earth metal ion, the mechanisms also are supported by the presence of analogous fragment ions that are shifted in mass as a result of the change in mass of the alkaline-earth metal ion.

Formation of $(a_{n-m} - 2\,H + Cat)^+$ Sequence Ions. The N-terminal $(a_{n-m} - 2\,H + Cat)^+$ sequence ions arise from cleavages through HRC–CO bonds (Table I), and the mechanism of fragmentation must involve transfer of hydrogen to a departing neutral fragment. The most abundant a_{n-m} sequence ions containing the N terminus are generally the $(a_n - 2\,H + Cat)^+$ and $(a_{n-1} - 2\,H + Cat)^+$ fragment ions unless either glycine or proline are the $n - m$ amino acids. The $(a_n - 2\,H + Cat)^+$ fragment ion arises by loss of 46 u from peptides that contain C-terminal carboxylates, by loss of 45 u from C-terminal amides, and by loss of 60 u from C-terminal methyl esters. Loss of 46 u from C-terminal carboxylates does not always compete favorably with loss of 44 u (CO_2) (Figures 1, 3, and 4). Loss of 45 amu from C-terminal amides, however, can produce one of the more abundant ions in the spectra (Figure 2B–D).

CID spectra of peptide–alkaline-earth metal ion complexes that contain either glycine (**1**), alanine, proline (**2**), or a methylated amide nitrogen give important clues about the mechanism for formation of $(a_{n-m} - 2\,H + Cat)^+$ ions. The effect of glycine,

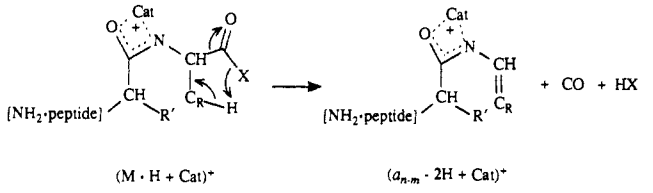


1 2

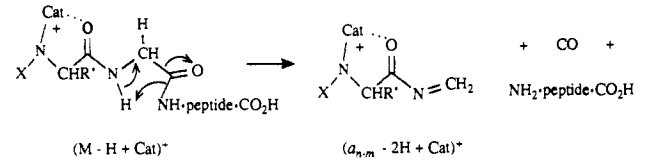
in which R = H (**1**), can be seen in spectra of $(M - H + Cat)^+$ ions shown in Figures 1, 2, and 4. No a ions that would arise via cleavages through glycine H_2C–CO bonds, which would be a_3 and a_2 ions in Figures 1 and 2 and a_3 ions in Figure 4, are detected. In contrast to glycine, cleavage of an alanine $H(CH_3)C$–CO bond is facile, as shown by the presence of the $(a_5 - 2\,H + Cat)^+$ ion in Figure 4. The presence of glycine as the $n - m$ amino acid, however, does not inhibit formation of either higher or lower mass a ions. These data indicate that an α-hydrogen on the side chain of the $n - m$ amino acid is required in the reaction mechanism, but hydrogen transfer from an R group on an adjacent amino acid is not involved.

Neither the amide N of proline (**2**) nor an amide N of an amino acid that has been methylated can be a source of hydrogen for transfer in the mechanism. Furthermore, the lack of an amide hydrogen in these species means that deprotonation and cation-

Scheme I



Scheme II



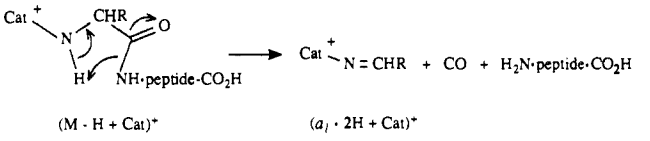
ization cannot occur at their respective amide N atoms. Proline does have, however, an α-hydrogen on its side chain that could be involved in the mechanism. CID spectra of peptides that contain proline nonetheless reveal that proline as the $n - m$ amino acid completely stops formation of $(a_{n-m} - 2\ H + Cat)^+$ ions. Formation of $(a_{n-m} - 2\ H + Cat)^+$ ions also is stopped by replacing the amide NH in the $n - m$ amino acid with an amide NCH_3. Neither proline as the $n - m$ amino acid nor an $n - m$ amino acid that contains a methylated amide group, however, inhibits formation or either higher or lower mass a ions. These results indicate that the amide hydrogen of the $n - m$ amino acid is required for formation of $(a_{n-m} - 2\ H + Cat)^+$ ions, but amide hydrogens in adjacent amino acids are not involved in the reaction mechanism.

The experimental observations thus are reconciled by a mechanism that requires the $n - m$ amino acid both to contain an amide NH group that can be deprotonated and cationized and to contain an α-hydrogen on the side chain that can be transferred to a neutral leaving group (Scheme I). Here, X can be OH, NH_2, OCH_3, or NH-peptide-CO_2H. The mechanism requires the amide NH that is deprotonated and cationized to be in immediate proximity to the site of reaction. If this were not the case, then a_{n-m} ions would still be observed in CID spectra of peptides that contain proline as the $n - m$ amino acid.

The mechanism shown in Scheme I requires breaking an OC–X bond that has partial double-bond character.[3a,9c] Acyl chlorides, however, are believed to undergo thermolytic fragmentation via an analogous transition state to give CO, an alkene, and HCl.[18] Thermal decompositions of acyl chlorides are characterized by first-order rate constants and occur as concerted reactions in which activation energies range from 50 to 55 kcal mol^{-1} and Arrhenius A factors[19] are $\sim 10^{14}$ s^{-1}.[18] Chloride in acyl chlorides is a much better leaving group than OH, NH_2, or OCH_3. Thus, the activation energy for the reaction in Scheme I may be greater than the activation energy for thermal fragmentations of acyl chlorides. There are extra driving forces for the reaction shown in Scheme I, however, that are a result of the reduced charge density on the carbon that is α to the amide nitrogen as a result of metal ion complexation[12] and of the stability of the resonance-stabilized product ion. The bridged O–Cat–N bond structure depicted in Scheme I would be more stable than a nonbridged N–Cat bond by $\sim$50 kcal mol^{-1}, in analogy to bonding between the formate anion and Ca^{2+}.[20a] Furthermore, the bridged O–Ca–O bonds in the formate anion have a calculated bond strength of $\sim$290 kcal mol^{-1}.[20a,b]

The reaction shown in Scheme I explains the formation of the most abundant a ions. There are some cases, however, in which

Scheme III



weakly abundant $(a_{n-m} - 2\ H + Cat)^+$ fragment ions can be observed even though glycine is the $n - m$ amino acid. For instance, weakly abundant $(a_2 - 2\ H + Cat)^+$ ions arise from cleavage of one glycine H_2C–CO bond in Gly-Gly-Leu. Furthermore, weakly abundant a_3 and a_2 ions arise from cleavages of two glycine H_2C–CO bonds in *N*-benzoyl-Gly-Gly-Gly.

The mechanism shown in Scheme II, in which X = H_2N-peptide-CO, benzoyl, or H, explains the experimental results. For X = H_2N-peptide-CO or benzoyl (C_6H_5CO), the mechanism involves an alkaline-earth cation still bonded to a deprotonated amide nitrogen as originally depicted in Scheme I. An additional interaction, however, between the alkaline-earth cation and the carbonyl oxygen on the C-terminal side of the deprotonated and cationized amide N is involved. The CO–Cat bond strength in the five-membered chelate shown in Scheme II would be considerably less than the bond strength in the bridged O–Cat–N structure originally depicted in Scheme I.[20,21] Nevertheless, the charge density of the amide N that is involved in the transition state in Scheme II would be more polarized as a result of the adjacent CO–Cat interaction so that there would be increased double-bond character in the amide OC–NH bond and the amide H would be more acidic.[3a,9a,b,12] This resulting increased acidity of the amide NH thus would be an inducement for the reaction shown in Scheme II.

The a ions that are described as arising via Scheme II are significantly reduced in abundance in comparison to a ions that are described by Scheme I. The Arrhenius A factor for the reaction in Scheme II, however, should be similar to the A factor for the reaction in Scheme I because the five-membered transition state depicted in Scheme II is directly analogous to the one in Scheme I except for the source of the hydrogen that is transferred to the departing neutral. Furthermore, the product ions from the reaction in Scheme II also are resonance stabilized as are product ions from the reaction in Scheme I. Therefore, reduced abundances of a ions formed via Scheme II may be partially related to a higher activation energy for the reaction in Scheme II as compared to the activation energy for the reaction in Scheme I. The preferential fragmentation of the $(M - H + Cat)^+$ ions to form a_{n-m} ions via Scheme I would thus result in a competitive shift[22] that would reduce detectability of fragment ions formed via Scheme II.

As mentioned above, sometimes very weakly abundant $(a_2 - 2\ H + Cat)^+$ fragment ions are formed via the reaction shown in Scheme II in which X = H. Here, the precursor ions would contain a deprotonated N-terminal amine instead of a deprotonated amide. Evidence for a deprotonated N-terminal amine is also provided from fragmentations that produce weakly abundant $(a_1 - 2\ H + Cat)^+$ ions. The $(a_1 - 2\ H + Cat)^+$ ions can be detected from CIDs of some di- and tripeptides and from CIDs of a few larger peptides such as those shown in Figures 3C and 4. It should be noted that abundances of $(a_1 - 2\ H + Cat)^+$ ions in Figure 3 and 4 may be influenced by the structure of the side chain in the N-terminal amino acid, as discussed above.

The $(a_1 - 2\ H + Cat)^+$ ions, $CHRNCat^+$, can be described as arising via a five-membered transition state (Scheme III) that is analogous to the reactions shown in Schemes I and II. This mechanism explains why tripeptides that contain glycine as the N-terminal amino acid can fragment to give weakly abundant $(a_1 - 2\ H + Cat)^+$ ions whereas *N*-benzoyl-Gly-Gly-Gly does not. That is, deprotonation and cationization of the N-terminal amide

(18) (a) Lennon, B. S.; Stimson, V. R. *J. Am. Chem. Soc.* **1969**, *91*, 7562–7564. (b) Lennon, B. S.; Stimson, V. R. *Aust. J. Chem.* **1970**, *23*, 525–531.

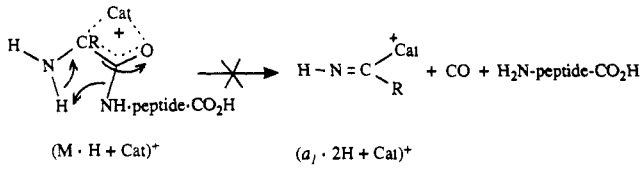
(19) Robinson, P. J.; Holbrook, K. A. *Unimolecular Reactions*; Wiley-Interscience: London, 1972; p 236.

(20) (a) Gottschalk, K. E.; Hiskey, R. G.; Pedersen; Koehler, K. A. *THEOCHEM* **1982**, *87*, 155–159. (b) Gresh, N.; Pullman, A.; Claverie, P. *Int. J. Quantum Chem.* **1985**, *28*, 757–771. (c) Berthod, H.; Pullman, A. *J. Comput. Chem.* **1981**, *2*, 87–95.

(21) (a) Gottschalk, K. E.; Hiskey, R. G.; Pedersen, L. G.; Koehler, K. A. *THEOCHEM* **1981**, *76*, 197–201. (b) Gottschalk, K. E.; Hiskey, R. G.; Pedersen, L. G.; Koehler, K. A. *THEOCHEM* **1981**, *85*, 337–342.

(22) Lifshitz, C. *Mass Spectrom. Rev.* **1982**, *1*, 309–348.

Scheme IV



nitrogen in the *N*-benzoyl derivative would leave no hydrogen available on the N-terminal nitrogen to be transferred to a departing neutral as shown in Scheme III.

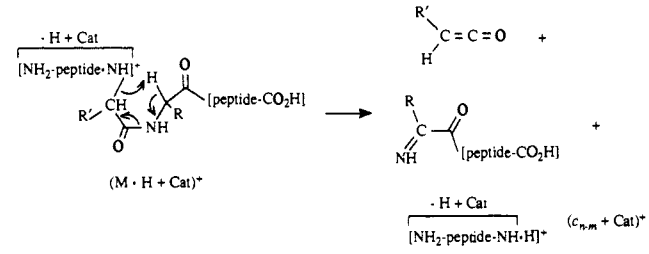
It is unlikely that the $(a_1 - 2\ H + Cat)^+$ ions arise from precursor $(M - H + Cat)^+$ ions that contain a deprotonated and cationized N-terminal α-carbon even though gas-phase deprotonation of the α-carbon of $H\text{–}CH_2CON(CH_3)_2$ is favored over deprotonation of NH_3 by ~30 kcal mol^{-1}.[10b,d] Assuming that the relative bond strengths between an alkaline-earth cation and C of $-CR_3$ vs N of $-NR_2$ are comparable to the relative bond strengths to Li^+,[8e,9a,b] deprotonation and cationization can be approximated to be favored at the amine N by at most ~62 kcal mol^{-1} or disfavored by at most ~8 kcal mol^{-1}.[23] Even if some precursor $(M - H + Cat)^+$ ions contained a deprotonated α-carbon, the alkaline-earth cation most likely would be bonded in a bridged structure so that the C–CO bond had partial double-bond character, as depicted in Scheme IV. Breaking the partial C–CO double bond as required to form the $(a_1 - 2\ H + Cat)^+$ fragment ions, as implied in Scheme IV, would be excessively energy intensive. Thus, even if some alkaline-earth metal ions were bonded to a deprotonated α-carbon, it is unlikely that $(a_1 - 2\ H + Cat)^+$ fragment ions would be observed to arise from such species.

Initial formation of $(M - H + Cat)^+$ precursor ions that contain a deprotonated N-terminal amino group bonded to an alkaline-earth cation can be approximated to be less favored by ~42 kcal mol^{-1} than bonding an alkaline-earth cation to a deprotonated amide.[25] This would reduce the number of isomeric $(M - H + Cat)^+$ ions that would contain a deprotonated and cationized N-terminal amino group, which would account for low abundances of a_1 and a_2 ions in spectra of tri- and dipeptides and for their frequent absence in spectra of tetra- and larger peptides. Another factor that can account for low abundances of a_1 ions is that sometimes there is a competitive shift[22] that is a result of preferential formation of c ions. This is discussed further below in relation to formation of $(c_{n-m} + Cat)^+$ ions.

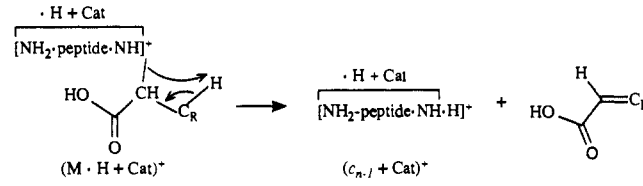
Mechanisms of formation of all $(a_{n-m} - 2\ H + Cat)^+$ fragment ions discussed above require the alkaline-earth metal ion to be bonded in close proximity to the site of reaction. The proximity of the alkaline-earth cation serves to polarize bonds and thus lower the transition-state energies for the reactions. This feature, however, is not the case for other types of fragment ions that arise from $(M - H + Cat)^+$ ions, to be discussed below.

Formation of $(c_{n-m} + Cat)^+$ Sequence Ions. The N-terminal $(c_{n-m} + Cat)^+$ sequence ions arise from cleavages through OCN(H)–CHR bonds (Table I), and the mechanism for their formation must require transfer of hydrogen from a neutral leaving group to the ionic fragment. These sequence ions, which contain the amino terminus, are quite useful analytically. As shown in Figures 1–4, the $(c_{n-m} + Cat)^+$ fragment ions are the most complete series of N-terminal sequence ions that are detected. Spectra of larger peptides frequently do not show the lowest mass $(c_1 + Cat)^+$ ion unless the N-terminal amino acid has a basic side chain. In general, the highest mass $(c_{n-1} + Cat)^+$ ion also is in lesser abundance than other $(c_{n-m} + Cat)^+$ ions.

Scheme V



Scheme VI



Fragmentations of structurally diverse peptides provide evidence for two mechanisms that give rise to $(c_{n-m} + Cat)^+$ ions. Glycine (**1**) does not inhibit formation of c-type ions except that no $(c_{n-1} + Cat)^+$ ion is formed if glycine is the C-terminal amino acid. Alanine, in which R = CH_3, however, never inhibits formation of c ions. These results indicate that as long as $m > 1$, an R group with an α-hydrogen is not needed in the mechanism for formation of $(c_{n-m} + Cat)^+$ ions. An R group with an α-hydrogen is needed, however, to form $(c_{n-1} + Cat)^+$ ions that arise from cleavage of the OCN(H)–CHR bond in the C-terminal amino acid. *N*-Benzoyl derivatives fragment to give an abundant low-mass c-type ion, $C_6H_5CONH\text{–}Cat^+$, that would arise from $(M - H + Cat)^+$ precursor ions that contain a deprotonated N-terminal amide. This suggests that the hydrogen that is transferred to the fragment ion from the departing neutral is transferred to the amide N atom of the cleaved OCN(H)–CHR bond. Furthermore, peptides that contain a methylated amide group in the $n - m + 1$ amino acid still fragment to give $(c_{n-m} + Cat)^+$ ions and higher and lower mass members of the c series of ions. Thus, hydrogen tht is transferred in the mechanism is not arising from an amide NH, and the amide N that is involved in the cleavage reaction is not needed as the site of deprotonation and cationization. No $(c_{n-m} + Cat)^+$ ions arise in cases in which proline is the $n - m + 1$ amino acid because cleavage through the CON–C bond of proline (**2**) would not cleave the peptide chain.

All these results are reconciled by two mechanisms, both of which require $(M - H + Cat)^+$ precursor ions to have a deprotonated amide nitrogen that does not have to be in immediate proximity to the site of reaction. The mechanism shown in Scheme V explains formation of $(c_{n-m} + Cat)^+$ fragment ions in which $m > 1$. This mechanism involves a six-membered transition state and is similar to the mechanism proposed for pyrolytic decompositions of alkyl chloroformates to give CO_2, an alkene, and HCl.[26] Activation energies for thermal decompositions of alkyl chloroformates range from 25 to 38 kcal mol^{-1}, and Arrhenius *A* factors are from 10^8 to 10^9 s^{-1}.[26] The activation energy for the reaction in Scheme V should be greater than the activation energies for cleavage of alkyl chloroformates because in Scheme V an

(23) The $H\text{–}CH_2CON(CH_3)_2$, $H\text{–}CH_3$, and $H\text{–}NH_2$ heterolytic bond dissociation energies are 373.5, 416.6, and 403.6 kcal mol^{-1}, respectively.[10b,d] The $Li\text{–}NH_2$ and the $Li\text{–}CH_3$ bond dissociation energies are calculated to be 62 and 45 kcal mol^{-1}, respectively.[24] The bond dissociation energy of $Li\text{–}CH_2CON(CH_3)_2$ should be significantly less than that of $Li\text{–}CH_3$ in analogy to $H\text{–}CH_2CON(CH_3)_2$ and $H\text{–}CH_3$. The ratio of the $H\text{–}CH_2CON(CH_3)_2$ bond strength to the $H\text{–}CH_3$ bond strength can be used in conjunction with the $Li\text{–}CH_3$ bond strength to obtain a maximum approximated bond strength of 40 kcal mol^{-1} for the $Li\text{–}CH_2CON(CH_3)_2$ bond.

(24) Würthwein, E.-U.; Sen, K. D.; Pople, J. A.; von Ragué Schleyer, P. *Inorg. Chem.* **1983**, *22*, 496–503.

(25) There are no data that describe the ΔH_{rxn} for exchanging a proton for an alkaline-earth metal ion in either amines or amides. The reaction can be approximated, however, by knowing the strengths of the $H\text{–}NH_2$ bond (403.6 kcal mol^{-1}), the H–NRCO bond (355.4 kcal mol^{-1}), and the $Li\text{–}NH_2$ bond (62 kcal mol^{-1}).[10b,d,24] The Li–NRCO bond strength is expected to be approximately [62 × 355.4/403.6], or 55 kcal mol^{-1}.[11c] The bonds between the alkaline-earth metal ion Be^{2+} and the nitrogen ligands should be approximately 100 kcal mol^{-1} greater than the Li^+ bond strengths,[8e,9b] or 162 kcal mol^{-1} for $Be^+\text{–}NH_2$ and 155 kcal mol^{-1} for Be^+–NRCO.

(26) (a) Choppin, A. R.; Compere, E. L. *J. Am. Chem. Soc.* **1948**, *70*, 3797–3801. (b) Lewis, E. S.; Herndon, W. C. *J. Am. Chem. Soc.* **1961**, *83*, 1955–1958.

Table II. Absolute Relative Abundances of $(a_1 - 2\ H + Cat)^+$ and $(c_2 + Cat)^+$ Ions Formed from Collision-Induced Dissociations of $(M - H + Cat)^+$ Ions of Three Proline-Containing Peptides[a]

	$(M - H + Ca)^+$		$(M - H + Ba)^+$	
peptide[b]	a_1 (%)	c_2 (%)	a_1 (%)	c_2 (%)
YPFPG	ND[c]	3	ND	5
GPRP	ND	3	1	8
GPGG	ND	8	ND	16

[a] Absolute relative abundances were calculated by dividing the ion current that is carried by the fragment ion by the total ion current that is carried by all fragment ions and multiplying by 100. [b] The single letter codes for the amino acids are as follows: Y = Tyr, P = Pro, F = Phe, G = Gly, and R = Arg. [c] None detected.

amide partial double bond must be broken. The neutral product that contains the carboxylate terminus would be stabilized by resonance, however, and this might serve to lower the transition-state energy.

The highest mass $(c_{n-1} + Cat)^+$ fragment ions, which are formed by cleavage between the C-terminal β-carbon and the amide nitrogen of the C-terminal amino acid, cannot arise via the six-membered transition state that is depicted in Scheme V. In addition, as discussed above, glycine as the C-terminal amino acid inhibits formation of $(c_{n-1} + Cat)^+$ ions although the fragmentation occurs for alanine as the C-terminal amino acid. These results are explained by the mechanism in Scheme VI in which the α-hydrogen of the R group of the C-terminal amino acid is transferred to the departing fragment ion. This mechanism involves a four-membered transition state that is analogous to the thermolytic 1,2-elimination of NH_3 and isobutene from *tert*-butylamine, which has an activation energy of 67 kcal mol^{-1} and an Arrhenius *A* factor of $\sim 10^{14}\ s^{-1}$.[19] 1,2-Eliminations of HX occur with lower activation energies and lower *A* factors than would be required for free-radical mechanisms, and many of them are no longer considered to be symmetry-forbidden[27a] reactions. Concerted, although not always synchronous, 1,2-eliminations that involve atoms that have nonbonded electron pairs, such as the N atom in Scheme VI, are not truly symmetry-forbidden because the transition state is six-electron instead of four-electron: The nonbonded electron pair takes part in the reaction mechanism.[27b-d]

As mentioned above, $(c_{n-1} + Cat)^+$ ions are always in low relative abundance as compared to other members of the c-ion series even though the *A* factor for the reaction in Scheme VI would be greater than the *A* factor for the reaction in Scheme V. This suggests that the low relative abundances of the $(c_{n-1} + Cat)^+$ ions may be a result of a higher activation energy for the 1,2-elimination reaction than for the reaction in Scheme V.

The $(M - H + Cat)^+$ ions of some peptides fragment to give $(c_{n-m} + Cat)^+$ ions that can only arise from precursors that contain the alkaline-earth cation complexed to a deprotonated N-terminal amino nitrogen. Examples of three such peptides are Tyr-Pro-Phe-Pro-Gly, Gly-Pro-Arg-Pro, and Gly-Pro-Gly-Gly, and each contains proline as the second amino acid from the N-terminal end of the peptide chain. The $(M - H + Cat)^+$ ions of these peptides fragment to give an abundant $(c_2 + Cat)^+$ ion that arises from cleavage of the OCN(H)–CHR bond of the amino acid on the C-terminal side of proline. The mechanism for formation of c_2 ions would be via the six-membered transition state described in Scheme V.

As discussed above in relation to formation of a_1 ions, deprotonation of the N-terminal amino nitrogen would be energetically unfavorable. Nevertheless, $(M - H + Cat)^+$ ions of the three amino acids named above fragment to give $(c_2 + Cat)^+$ ions. In Table II is a list of absolute relative abundances of $(a_1 - 2\ H + Cat)^+$ and $(c_2 + Cat)^+$ ions that are formed from $(M - H + Cat)^+$ ions of the three proline-containing peptides. Included are data for fragmentation of precursor ions that contain either Ca^{2+} or Ba^{2+}. The data in Table II show that even though abundant $(c_2 + Cat)^+$ ions can be detected, the a_1 ions either are more weakly abundant or are not detected.

Because the a_1 and c_2 ions would arise from the same $(M - H + Cat)^+$ precursor ions, the low abundances of the a_1 ions thus are partially a result of a competitive shift.[22] The five-membered transition state that is associated with formation of a_1 ions (Scheme III) would have a higher activation energy than the six-membered transition state that is associated with formation of c_2 ions. This implies that it is the relative activation energies of the two competing reactions, and not the *A* factors, that most strongly influence the relative abundances of the a_1 and c_2 fragment ions.

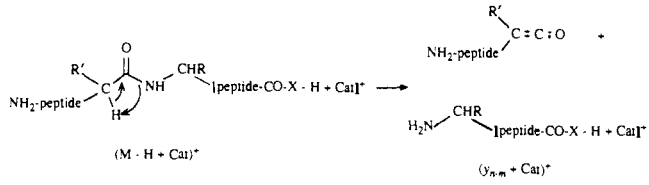
In contrast to mechanisms for formation of a ions, mechanisms that explain formation of $(c_{n-m} + Cat)^+$ fragment ions do not involve deprotonation and cationization of the amide NH that is in immediate proximity to the site of reaction. If a precursor ion structure such as that shown in Scheme I were involved, there would be less of a driving force for the two reactions shown in Schemes V and VI. That is, if the N atom of the OCN(H)–CHR bond were deprotonated and cationized, cation-induced polarization would reduce the charge density of the N atom and it would become a less nucleophilic site for accepting a transferred proton.

Formation of $(y_{n-m} + Cat)^+$ Sequence Ions. The C-terminal $(y_{n-m} + Cat)^+$ sequence ions arise from cleavages through the amide OC–$NHCR_2$ bond (Table I), and the mechanism for their formation must require transfer of hydrogen from a neutral leaving group to the fragment ion. These fragment ions, which contain the carboxylate terminus, are quite analytically useful. They are present in many CID spectra of all but dipeptides, although there are exceptions as shown in Figures 3B and 4. The smallest mass member of the series, $(y_1 + Cat)^+$, is rarely observed in tetrapeptides and larger, although sometimes it is important in spectra of tripeptides. Relative abundances of y_{n-m}-ions increase with increasing size of alkaline-earth cation (Figure 1). Furthermore, the y_{n-m} ions become highly abundant in spectra of C-terminal amides (Figure 2).

Fragmentations of a variety of peptides that contain either a C-terminal carboxylate, amide, ester, or alcohol provide evidence for a mechanism that explains formation of the $(y_{n-m} + Cat)^+$ sequence ions. Neither glycine nor proline as the $n - m$ amino acid inhibits formation of either $(y_{n-m} + Cat)^+$ or higher or lower mass y ions. Thus, the mechanism requires neither transfer of an α-hydrogen from a side chain nor transfer of a hydrogen from an NH group. Furthermore, the amide NH of the $n - m$ amino acid must not be required as the site of deprotonation and cationization. *N*-Benzoyl derivatives of peptides, C_6H_5-CO-NH-peptide-CO_2H, however, do not fragment to give $(y_n + Cat)^+$ ions. This provides evidence that the mechanism involves transfer of hydrogen from the CHR group of the $n - m + 1$ amino acid. In addition, C-terminal amides fragment to produce y ions, although C-terminal esters and an alcohol that we have studied do not fragment to give any y ions.

These results support a mechanism in which the precursor ions have a deprotonated and cationized C-terminal carboxylate or amide (Scheme VII) so that X = OH or NH_2. The 1,2-elimination reaction in Scheme VII shows hydrogen being transferred from the CHR group of the $n - m + 1$ amino acid to the departing fragment ion and the alkaline-earth cation being located distant from the site of reaction (a "charge-remote" fragmentation[28]). The cleavage reaction that gives rise to $(y_{n-m} + Cat)^+$ ions is a

Scheme VII



(27) (a) Woodward, R. B.; Hoffmann, R. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 781–932. (b) Goddard, W. A., III. *J. Am. Chem. Soc.* **1972**, *94*, 793–807. (c) Tvaroška, I.; Klimo, V.; Valko, L. *Tetrahedron* **1974**, *30*, 3275–3280. (d) Hiberty, P. C. *J. Am. Chem. Soc.* **1975**, *97*, 5975–5978.

(28) Adams, J. *Mass Spectrom. Rev.* **1990**, *9*, 141–186 and references therein.

less facile process because it involves breaking the amide bond that intrinsically has partial double-bond character.[3a,9c] It can be seen that if the alkaline-earth metal ion in the $(M - H + Cat)^+$ precursor ions were bonded to the $n - m$ deprotonated amide, as required for a_{n-m}-ions in Scheme I, the double-bond character would be increased even more [8e,9a,b] and would make this an even more energetically unfavorable reaction.

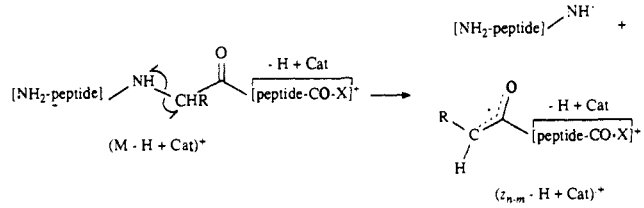
Formation of $(y_{n-m} + Cat)^+$ ions by cleavage of an amide partial double bond via a four-membered transition state would be characterized by a higher activation energy and a higher Arrhenius A factor than mechanisms shown in Schemes I and V for formation of the most abundant N-terminal a- and c-fragment ions, respectively. Experimentally it is observed that C-terminal y ions are generally in lower relative abundances than most N-terminal a- and c-fragment ions for peptides that contain a C-terminal carboxylate (see Figure 1). These data might suggest that the relative activation energies for the different reactions are the most important factors that influence the detectability of the various ions in the CID spectra. This view is too simplistic, however, because it does not take into account the significantly higher relative abundances of y ions that arise from CIDs of C-terminal amides (see Figure 2).

The remarkable increase in abundances of y ions in CID spectra of C-terminal amides vs C-terminal carboxylates may be the result of preferential bonding of the alkaline-earth cation to a C-terminal amide vs a C-terminal carboxylate. An amide carbonyl oxygen has a significantly higher metal ion affinity than either an acid or an ester carbonyl oxygen.[8d,e,11c,e,29] Thus, a peptide C-terminal carboxylate would not be a favored site of cation bonding, and isomeric $(M - H + Cat)^+$ ions would be comprised predominantly of species that contain the alkaline-earth cation bonded to deprotonated peptide amide groups. As a result, there would be fewer precursor ions that would undergo fragmentations that give rise to $(y_{n-m} + Cat)^+$ ions. In contrast, a C-terminal amide would compete more favorably for the cation against other peptide amide groups so that more $(M - H + Cat)^+$ ions would be formed that have a deprotonated and cationized N-terminal amide. In this latter case, abundances of $(y_{n-m} + Cat)^+$ ions vs abundances of a and c ions would be determined both by the number of precursor ions that preferentially have a deprotonated and cationized C-terminal amide and by the relative rates of the different competing fragmentations.

Formation of $(z_{n-m} - H + Cat)^{\bullet+}$ Sequence Ions. The C-terminal $(z_{n-m} - H + Cat)^{\bullet+}$ fragment ions are counterparts to N-terminal $(c_{n-m} + Cat)^+$ ions only in that they are formed by cleavages through OCN(H)–CHR bonds (see Table I). The mechanisms of formation of the z_{n-m} and c_{n-m} series of ions, however, are significantly different because the $(z_{n-m} - H + Cat)^{\bullet+}$ ions are open-shell (radical) ions and do not arise via a hydrogen rearrangement. The $(z_{n-m} - H + Cat)^{\bullet+}$ fragment ions are not as analytically important as the C-terminal $(y_{n-m} + Cat)^+$ ions because they are formed in less abundance and usually are observed as an incomplete series of sequence ions. Fundamentally they are of interest, however, because they are open-shell ions that do not have analogies to fragment ions formed by CIDs of $(M + H)^+$ ions.[16,17]

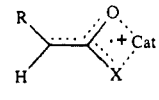
Fragmentations of peptides that contain either a C-terminal ester or alcohol, or a proline as the C-terminal amino acid, give the greatest information about the mechanism of formation of $(z_{n-m} - H + Cat)^{\bullet+}$ fragment ions. Furthermore, fragmentations of these peptides provide evidence regarding the location of the alkaline-earth cation in relation to the cleavage reaction. The $(M - H + Cat)^+$ ions of peptides that contain either a C-terminal ester or an alcohol do not fragment to give a $(z_1 - H + Cat)^{\bullet+}$ ion because formation of z_1 ions requires either a C-terminal carboxylate or an amide that can be deprotonated and cationized. The esters and alcohol, however, can fragment to give higher mass members of the z-ion series as long as there is an amide group that can be deprotonated and cationized that is between the C-terminal ester or alcohol and the site of reaction. Proline as the $n - m$ amino acid inhibits formation of $(z_{n-m} - H + Cat)^{\bullet+}$ ions because cleavage through the OCN–C bond of proline would not cleave the peptide chain (see **2**). Thus, proline as the C-terminal amino acid also prevents formation of $(z_1 - H + Cat)^{\bullet+}$ ions but does not inhibit formation of $(z_2 - H + Cat)^{\bullet+}$ ions even though proline does not contain an amide NH that can be deprotonated and cationized.

Scheme VIII



These results indicate that C-terminal $(z_{n-m} - H + Cat)^{\bullet+}$ ions, in contrast to $(y_{n-m} + Cat)^+$ ions, can be formed from decompositions of $(M - H + Cat)^+$ precursor ions that contain the alkaline-earth metal ion complexed to any site that can be deprotonated and cationized. Furthermore, the site of metal ion complexation does not have to be in immediate proximity to the site of reaction. These observations, and the open-shell nature of the fragment ions, can be explained by a fragmentation mechanism that involves a homolytic cleavage to give rise to distonic radical cation fragment ions (Scheme VIII). Here, X = OH, NH_2, OCH_3, or an alcoholic H.

Distonic radical ions are ones in which the radical and charge sites are located either on different atoms or at least in different molecular orbitals.[30] Distonic radical ions are frequently more stable than their counterparts that contain both radical and charge sites in the same molecular orbital. The α-carboxy radical site in $(z_{n-m} - H + Cat)^{\bullet+}$ fragment ions (Scheme VIII) would be particularly stable because of resonance delocalization. The z ions pictured in Scheme VIII contain the radical and charge sites separated by several atoms. The $(z_1 - H + Cat)^{\bullet+}$ fragment ions, however, would contain both the radical and charge sites delocalized over several molecular orbitals (**3**). Here, X = O or NH. The $(z_1 - H + Cat)^{\bullet+}$ radical cations are analogous to $CH_2CO_2Li_2^{\bullet+}$ radical cations that are formed from CIDs of $(M - H + 2\ Li)^+$ ions of fatty acids.[31]



$(z_1 \cdot H + Cat)^{\bullet+}$

3

Relative abundances of $(z_{n-m} - H + Cat)^{\bullet+}$ fragment ions do not change significantly if a C-terminal carboxylate is exchanged for a C-terminal amide. This is not surprising because the z ions, as opposed to y ions, do not exclusively arise from a deprotonated and cationized C-terminal carboxylate or amide. Relative abundances of different z ions that arise from either the same or isomeric $(M - H + Cat)^+$ precursor ions thus would be determined by the rates of the homolytic fragmentation reactions relative to the rates of competing rearrangement reactions that would give rise to a, c, and y ions. Homolytic cleavages of C–C bonds have activation energies of ~70–90 kcal mol^{-1} and Arrhenius A factors of $\sim 10^{16}$–10^{17} s^{-1}.[32] Both the activation energies and the A factors for the homolytic cleavages would be greater than activation energies and A factors for the rearrangement reactions. Exper-

(29) Kollman, P.; Rothenberg, S. *J. Am. Chem. Soc.* **1977**, *99*, 1333–1342.

(30) Hammerum, S. *Mass Spectrom. Rev.* **1988**, *7*, 123–202, and references therein.

(31) (a) Adams, J.; Gross, M. L. *Anal. Chem.* **1987**, *59*, 1576–1582. (b) Adams, J.; Gross, M. L. *Org. Mass Spectrom.* **1988**, *23*, 307–316. (c) Adams, J.; Gross, M. L. *J. Am. Chem. Soc.* **1989**, *111*, 435–440.

(32) Benson, S. W.; O'Neal, H. E. *Kinetic Data on Gas Phase Unimolecular Reactions*; NSRDS-NBS 21; U.S. Government Printing Office: Washington, DC, 1970.

imentally, the z series of ions is of low abundance relative to the a, c, and y series of ions in CID spectra of most $(M - H + Cat)^+$ ions of peptides. This suggests that it is the higher activation energy for the homolytic cleavage reactions, and thus a competitive shift,[22] that at least partially causes the z ions to be detected in lower relative abundances.

Other Fragment Ions. Other fragment ions besides those that have been discussed in detail above can be seen in CID spectra of some $(M - H + Cat)^+$ ions of peptides. Our current data do not provide enough evidence to propose mechanisms for production of either x- or b-sequence ions. The x ions, which may be either $(x_{n-m} + Cat)^+$ or $(x_{n-m} - H + Cat)^{\bullet+}$ ions, occur sporadically. The $(b_n - 2\ H + Cat)^+$ ions that arise either by loss of H_2O from C-terminal carboxylates or by loss of NH_3 from C-terminal amides are sometimes quite abundant (see Figures 1–3). Other members of this series, however, are usually weakly abundant or not detected at all. Occasionally, $(b_{n-m} + Cat)^+$ and $(z_{n-m} + Cat)^+$ ions are observed. Losses of 44 u as CO_2 and 28 u as CO also occur for most peptides that contain a C-terminal carboxylate (see Figures 1, 3, and 4).

Effects of Alkaline-Earth Metal Ion and Substituents on Fragment Ion Abundances. Specific fragmentations of different numbers of isomeric $(M - H + Cat)^+$ ions determine the relative abundances of different fragment ions in the CID spectra. Preferential formation of one $(M - H + Cat)^+$ precursor ion over another could be determined by differences in the intrinsic metal ion affinities of the different amide oxygens that are competing for the metal ion, if the system were at equilibrium. The intrinsic metal ion affinities of the different amide oxygens would then be controlled by the structures of the side chains on both the C- and N-terminal sides of the amide groups. For instance, increasing alkyl substitution on both the CHR carbon atom on the N-terminal side of the amide oxygen and on the OCNHR nitrogen atom on the C-terminal side of the amide oxygen would increase the intrinsic metal ion affinity.[8] Structures of the side chains also could favor formation of a specific $(M - H + Cat)^+$ ion by providing additional intramolecular interactions with the metal ion that would further stabilize the binding site. Other intramolecular interactions between a bound metal ion and other peptide amide groups also could serve to further stabilize a particular binding site and thus increase formation of a particular $(M - H + Cat)^+$ ion.

Stabilization of the binding site in a particular $(M - H + Cat)^+$ by intramolecular complexation could either increase or decrease relative abundances of fragment ions that arise via fragmentation of the particular $(M - H + Cat)^+$ ion. To detect the product ion of a specific fragmentation, the specific transition state must be accessible and the relative rate of formation of the product ion must compete favorably against rates of formation of other product ions in the time window of the experiment. If intramolecular complexation between an alkaline-earth cation and the peptide molecule were such that a specific transition-state conformation were enhanced, then the rate constant for the corresponding reaction would increase. The opposite effect would occur if complexation were to freeze the molecule into an unfavorable structure that either prevented or reduced attainment of transition-state conformation.

We have insufficient evidence at present to address thoroughly effects on relative ion abundances that may be caused by intramolecular complexation. There are some experimental observations, however, that suggest such interactions may be quite important. These observations include changes in fragmentation patterns with changing size of the alkaline-earth cation, with changing from a C-terminal carboxylate to a C-terminal amide, and with changing side-chain structure.

CID spectra of $(M - H + Cat)^+$ ions of Leu-enkephalin and Leu-enkephalin amide (Figures 1 and 2, respectively) show that changes in alkaline-earth cation result in significant changes in the CID spectra. One change is that, for both amide and acid, $(M - H + Cat)^+$ ions that contain the larger alkaline-earth cations fragment to produce more abundant low-mass ions that arise by cleavage reactions that are in closer proximity to both the N and C termini. This suggests that the larger alkaline-earth metal ions do not bond preferentially either to the C or to the N terminus, but interact with both termini to a greater extent. Consequently, there is a greater probability that the larger alkaline-earth metal ions sample a greater portion of the molecule prior to deprotonation. Metal ion induced conformations have been used to explain why some enzymes preferentially bind, and are activated by, Ca^{2+} but not Mg^{2+}.[3c,33] The ability of the larger alkaline-earth cations to bind with a greater portion of the peptide chain and to interact intramolecularly with a greater number of ligands that have less sterically restricted orientations is a result of their larger coordination numbers and ionic radii.[1a,3b,c,33,34]

Another noticeable change in CID spectra in Figures 1 and 2 is that, for the C-terminal amide, the a_n ion becomes significantly more abundant with increasing size of alkaline-earth metal ion, whereas the a_{n-1} ion becomes less abundant. This trend is not reflected, however, in spectra of $(M - H + Cat)^+$ ions of the C-terminal acid in which absolute abundances of the a_n and a_{n-1} ions change little with size of alkaline-earth metal ion. These data suggest that the amide group on the C terminus may be specifically interacting with the bonded alkaline-earth metal ion to stabilize the bonding site that gives rise to the a_n ions either so that more structurally specific $(M - H + Cat)^+$ ions are formed or so that the transition-state energy is lowered and the rate of fragmentation is increased. Furthermore, because the effect of this interaction changes with size of the alkaline-earth metal ion, the extent of the intramolecular complexation may be controlled by the preferred coordination geometry of the alkaline-earth cation.[1a,3b,34]

The trends in abundances of a_n and a_{n-1} ions in Figures 1 and 2 are completely different, however, if the C-terminal amino acid leucine is exchanged for methionine in which $R = (CH_2)_2SCH_3$. In this case, for the C-terminal acid, the a_{n-1} ion becomes significantly more abundant with increasing size of alkaline-earth metal ion, whereas the a_n ion becomes less abundant. This trend is not reflected, however, in spectra of $(M - H + Cat)^+$ ions of the C-terminal amide in which the absolute abundances of the a_{n-1} and a_n ions change little with size of alkaline-earth metal ion. These data suggest that the C-terminal methionine side chain may be interacting with the bonded alkaline-earth cation in a way that is inhibiting formation of a_n ions so that more a_{n-1} ions are being detected instead. Because abundances of a_n ions are decreasing with size of alkaline-earth cation, the extent of this complexation also may be controlled by the coordination geometry of the metal ions. Interaction of the methionine side chain with the bonded alkaline-earth cation may be more pronounced in the C-terminal acid than in the C-terminal amide because the side chain would compete against the acid group for intramolecular metal ion complexation more effectively than it would compete against the more basic amide group.

Conclusions

This first investigation addresses mechanisms that give rise to the most abundant and analytically useful sequence ions in CID spectra of $(M - H + Cat)^+$ ions of complexes between peptides and alkaline-earth cations. Our evidence is derived from correlating structural changes with changes in fragmentations, as opposed to using isotopic labeling or MS–MS–MS experiments. The mechanisms provide information regarding the location of the alkaline-earth metal ion in relation to the sites of fragmentation. Interactions between alkaline-earth metal ions and tetrapeptides and larger, which do not contain acidic side chains, involve preferential formation of $(M - H + Cat)^+$ ions in which the alkaline-earth cation is bonded to a deprotonated amide group. Initial anchoring of the alkaline-earth metal ion to an amide carbonyl oxygen would trigger deprotonation of the amide nitrogen

(33) (a) Hay, R. W. *Bio-Inorganic Chemistry*; Ellis Horwood: Chichester, 1984; pp 70–79. (b) Deerfield, D. W.; Olson, D. L.; Berkositz, P.; Byrd, P. A.; Kochler, K. A.; Pederson, L. G.; Hiskey, R. G. *J. Biol. Chem.* **1987**, *262*, 4017–4023.

(34) Martin, R. B. In *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker: New York, 1984; Vol. 17, pp 1–49.

to result in a direct bond between the amide nitrogen and the metal ion, as occurs in solution as a result of a bond to a transition-metal ion.[3a,4,13]

Cationization and deprotonation of a peptide amide group produces precursor $(M - H + Cat)^+$ ions that give rise to the most abundant N-terminal a and c ions and C-terminal z ions. This chemistry also is responsible for formation of highly abundant y ions that are formed from peptides that contain C-terminal amides. Formation of a ions requires the alkaline-earth metal ion to be in immediate proximity to the site of reaction. Reactions that give rise to the other sequence ions, however, do not have this requirement. More experimentation with peptides that contain either sequential prolines or sequential amino acids that contain methylated amides will provide evidence regarding the exact location of the alkaline-earth cation in relation to the site of fragmentation for the c and z ions.

The overall fragmentation of $(M - H + Cat)^+$ ions of simple peptides thus reflect intrinsic gas-phase peptide–metal ion bonding as opposed to solution-phase bonding. In analogy to intrinsic proton and lithium ion affinities,[7-11] gas-phase cationization followed by deprotonation should favor amide groups over C-terminal acid and N-terminal amino groups. This trend, as reflected by relative ion abundances, is observed experimentally. In contrast, if the bonding was occurring in solution, the peptides would be present as zwitterions and the predominant $(M - H + Cat)^+$ species would contain the alkaline-earth metal ion complexed to the C-terminal carboxylate. In our gas-phase experiments, some precursor $(M - H + Cat)^+$ ions contain the alkaline-earth metal ion complexed to a deprotonated C-terminal carboxylate, but fragmentations of these species to give y ions do not overwhelm other fragmentations that are observed in the CID spectra. Instead, we observe C-terminal y ions to be generally of lower abundances than N-terminal sequence ions unless the C-terminal carboxylate is changed into a C-terminal amide.

Other evidence is needed to address the role of intramolecular complexation on preferential formation of particular $(M - H + Cat)^+$ ions and on relative fragment ion abundances. Initial results presented here suggest that metal ion specific and structure specific intramolecular complexation may be responsible for changes in fragmentation patterns with increasing size of alkaline-earth cation, with changing a C-terminal carboxylate for an amide, and with changing side-chain structure. Further research is being conducted to address these phenomena because the gas-phase chemistry should reflect intrinsic interactions that occur in hydrophobic interiors of proteins.

Experimental Section

Peptides were obtained either from Sigma or from the Emory University Microchemistry Center. The peptides included HLGLAR; RYLPT, VHLTP, YPFPG, YGGFM, FFFFF, *t*-Boc-YAG[N-Me]FG-ol, YGGFL and YGGFL-NH_2; ALAL, FFFF, AAAA, GPRP, GPGG, VAAF, AGFL, GGFL, AGFM, GGFM, and GGFM-NH_2; GGV, FFF, IPI, GGL, GGI, *N*-benzoyl-GGG, *N*-benzoyl-GHL, *p*-OH-*N*-benzoyl-GHL, AAA-OCH_3; LL, LG, AL, LA, FF, LL-OCH_3, and GL-NH_2. Alkaline-earth metal hydroxides and matrices used for fast atom bombardment, which were 3-nitrobenzyl alcohol, 5:1 dithiothreitol/dithioerythreitol, and 2:1 thioglycerol/glycerol, were from Aldrich.

Samples were prepared for fast atom bombardment (FAB) by mixing small amounts (μg) of the peptides with one of the FAB matrices on a stainless steel FAB probe tip. The $(M + H)^+$ ions were desorptively ionized from one of the matrices used by itself; the $(M - H + Cat)^+$ ions were prepared from matrices that had been saturated with an alkaline-earth metal hydroxide.

Mass spectrometric experiments were performed with a VG 70-S normal-geometry (EB configuration, where E is an electrostatic analyzer or ESA and B is a magnetic sector) mass spectrometer, in which the first field-free region is between the ion source and the ESA. The mass spectrometer is equipped with an Ion Tech saddle-field fast atom bombardment gun and a commercial FAB ion source. FAB-desorbed ions were produced by bombarding the sample with 7-keV Ar atoms at an atom gun current of 2 mA. Ions produced were accelerated to 8-keV translational energy.

Fragment ions that were formed by collision-induced decompositions (CIDs) that occur in the first field-free region were observed with B/E-linked scans. Helium was used as collision gas at a pressure of 1×10^{-6} Torr, as measured by the ion gauge in the first-field-free region. Experiments were performed at a resolution of approximately 1500 (10% valley), and magnet calibration was performed with a mixture of LiI, NaI, RbI, and CsI in H_2O. All spectra were acquired with VG software, and CID spectra are the result of averaging 10–30 scans. Background spectra were acquired for all experiments in order to eliminate artifact fragment ions that might arise from chemical noise.

Acknowledgment. Support for this research was provided in part by the Emory University Research Fund and the Petroleum Research Fund administered by the American Chemical Society. The NIH (Grant S10-RR-02478) is also acknowledged for the use of the VG 70-S as a shared instrument. This paper originally was presented in part at the 37th ASMS Conference on Mass Spectroscopy and Allied Topics, May 1989.