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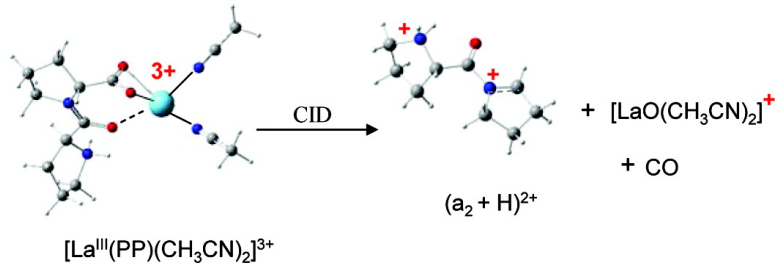

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Abundant Dipositively Charged Protonated a_2 and a_3 Ions from Diproline and Triproline

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Abundant $(a_2 + H)^{2+}$ from diproline and $(a_3 + H)^{2+}$ from triproline were observed via collisionally activated charge disproportionation of $[La(peptide)(CH_3CN)_{1,2}]^{3+}$. These small, dipositive ions with the charges formally located on the peptide backbone are stabilized by charge delocalization onto the pyrrolidine-derived rings, making their formation competitive against other monopositive ions. The $(a_2 + H)^{2+}$ and $(a_3 + H)^{2+}$ ions from diproline and triproline are major products in contrast with those derived from triglycine, whose unprecedented and surprising observations were recently reported (Shi et al. *Angew. Chem., Int. Ed.* 2008, *47*, 8288−8291).

1. Introduction

Collision-induced dissociations (CIDs) of multiply charged peptides have become the cornerstone of gas-phase microsequencing in proteomics.[1] Coulombic explosion plays a major role in CID and results in typically singly charged fragment ions whose mass-to-charge (*m*/*z*) values are employed in peptide and protein identification.[2] A protein, as a macromolecule, can accommodate a large number of protons in the gas phase, albeit this association is often accompanied by concomitant denaturation and swelling of the protein to decrease the charge density.[3]

Oligopeptides, with the exception of those containing basic amino acid residues, are too small to accept more than one proton in the gas phase. Electrospraying a tryptic digest (of a protein) produces abundant doubly protonated peptides terminating in a lysine or arginine residue at the C-terminus. CID of these peptides results in y-, b-, and a-fragment ions as common products.[2a] Formation of doubly charged (protonated) fragment ions is rarely observed and invariably involves longer fragment ions; protonation of smaller fragment ions is hindered by the large Coulombic repulsion encountered by the mobile proton in the vicinity of the incipient fragment ion, thus making formation of small dipositive ions energetically unfavorable. An alternative strategy for introducing multiple charges onto a small peptide is via metal/peptide complexes.[4] Recently, we observed the triply charged complexes $[La^{III}(MetArg)]^{3+}$ and $[La^{III}(MetArg)^2]^{3+}$, with the latter containing an 8-coordinate La^{3+} ion and consequently being very stable toward CID.[5] As an extension of that study, Shi et al.[6] reported that fragmentation of $[La^{III}(GlyGlyGly)(CH_3CN)_2]^{3+}$ resulted in the surprising and unprecedented observation of dipositive protonated a_3 and protonated a_2 ions of triglycine. The $(a_3 + H)^{2+}$ ion was produced via charge disproportionation of the $[La(GlyGlyGly)(CH_3CN)_2]^{3+}$ complex; isolation of $(a_3 + H)^{2+}$ and CID then produced $(a_2 + H)^{2+}$ in low yield. Here, we report the first direct production of a dipositive protonated a_2 ion, that of diproline (ProPro), formed in high abundance in the CID of $[La^{III}(ProPro)(CH_3CN)_2]^{3+}$. The amino acid proline has a modest proton affinity (PA) of 220.0 kcal mol^{-1} [cf. PA(glycine) = 211.9 kcal mol^{-1} and PA(alanine) = 215.5 kcal mol^{-1}];[7] this is likely a result of steric constraints of the ring system, preventing effective delocalization of the charge from the NH_2^+ unit onto the carboxyl group via hydrogen bonding, thereby offsetting the intrinsic basicity of the secondary amine group. In the protonated a_n ions, stabilization by internal hydrogen bonding is less of a factor because of Coulombic repulsion, and the intrinsic basicity of the secondary amine then plays a larger role.

2. Experimental Section

2.1. Experimental Details. Experiments were performed on an MDS SCIEX (Concord, ON) API 3000 prototype triple-quadrupole mass spectrometer. The lanthanum-containing complex ions were generated by electrospraying 1 mM diproline or triproline and 0.1 mM $La(NO_3)_3$ in 50:50 H_2O/CH_3CN at a flow rate of 3 μL/min. Deuterium labeling at the exchangeable hydrogen positions, resulting in d_2-ProPro and d_2-ProProPro, was accomplished by replacing H_2O by D_2O. Diproline and triproline were purchased from Bachem Bio-Sciences (King of Prussia, PA); lanthanum(III) nitrate and solvents were available from Sigma-Aldrich (St. Louis, MO). CID experiments were performed by mass-selecting the precursor ion of interest using the first quadrupole, colliding it with nitrogen in the second quadrupole, and mass-analyzing using the third quadrupole. The nitrogen collision gas was boiled off from liquid nitrogen, which contained water as an unavoidable contaminant.[8]

2.2. Computational Details. Geometry optimizations and harmonic frequency analyses for all minima and transition structures were performed with the Gaussian 03 package[9a] employing Becke's three-parameter B3LYP hybrid functional.[9b,c] For calculations on the La-containing complexes, the 6-31++G(d,p) basis set[9d,e] was used for the main-group elements, and the Stuttgart/Cologne relativistic effective core potential basis set[9f,g] was used for La. For the smaller $(a_2 + H)^{2+}$ and $(a_3 + H)^{2+}$ ions, the triple-ζ 6-311++G(d, p) basis set[9d,e] was employed. All minima and transition structures were verified as such by harmonic frequency analyses. Zero-point vibrational energies were evaluated directly using the normal-mode frequencies without anharmonic scaling. The local minima associated with each transition structure were identified using the intrinsic reaction coordinates method.[9h]

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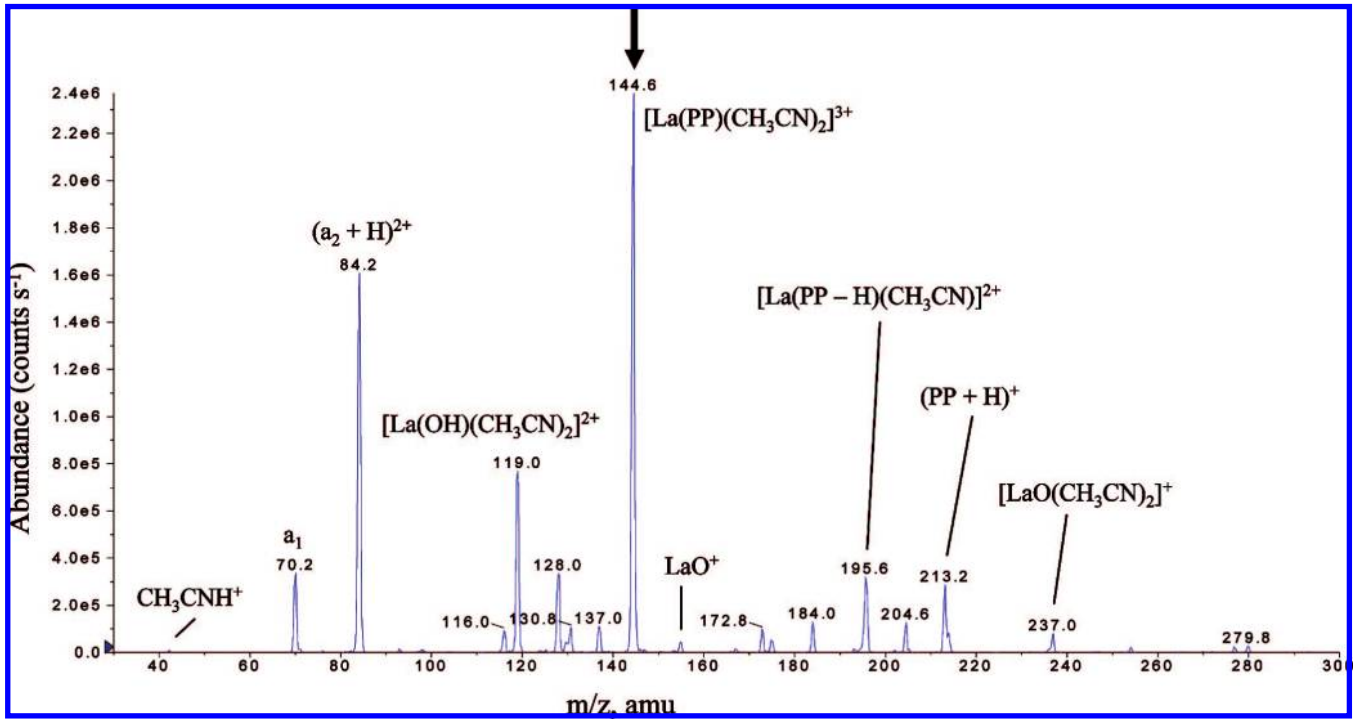


Figure 1. Collision-induced dissociation spectrum of $[La^{III}(ProPro)(CH_3CN)_2]^{3+}$ at a laboratory collision energy of 21 eV. Pro (proline) is abbreviated as P in this and subsequent figures. The vertical arrow indicates the precursor ion. An ion at *m/z* 196, $[LaO(CH_3CN)]^+$, is also present (as indicated by $[LaO(CD_3CN)]^+$ in Figure S1b in the Supporting Information), but for clarity the label is omitted here.

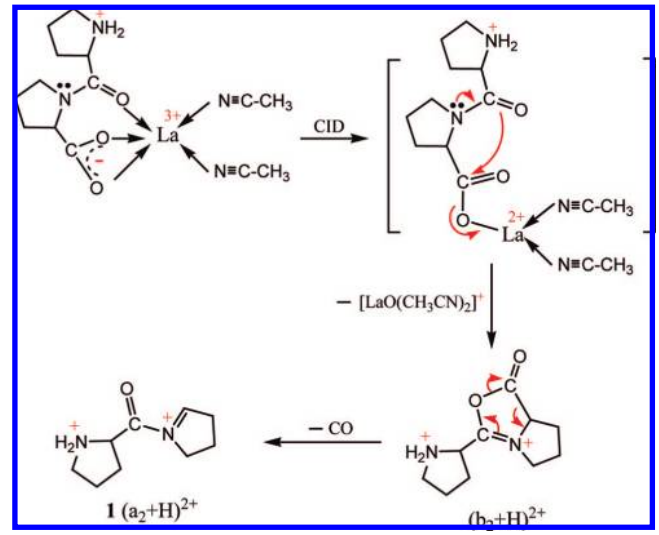
In our preliminary investigations on the structure of the metal complexes, we found that the salt-bridged complex was much lower in energy than the charge-solvated form. In addition, the constraints imposed by the ring structure and the requirement to separate the charges resulted in very few structures at minima for all species: the initial metal complex, $(a_2 + H)^{2+}$, and $(a_3 + H)^{2+}$. Structures for each ion were initially optimized at the B3LYP/6-31G(d)/sdd level, and then the lower-energy structures were further optimized at the B3LYP/6-31++G(d,p)/sdd level of theory. Finally, the lowest-energy structures of $(a_2 + H)^{2+}$ and $(a_3 + H)^{2+}$ were optimized at B3LYP/6-311++G(d,p).

3. Results and Discussion

3.1. $(a_2 + H)^{2+}$ of ProPro. Figure 1 shows the CID spectrum of $[La^{III}(ProPro)(CH_3CN)_2]^{3+}$ (*m/z* 144.6) featuring the $(a_2 + H)^{2+}$ ion of diproline at *m/z* 84 as the most abundant product. The $(a_2 + H)^{2+}$ ion of ProPro has structure **1** (shown in Scheme 1), where the two charges are on separate pyrrolidine-derived rings bridged by a carbonyl group. Identification of the $(a_2 + H)^{2+}$ ion was supported by the use of d_2-ProPro, deuterated at the amino and carboxyl positions, and formed by hydrogen/deuterium exchange in D_2O/CH_3CN as the solvent; this peptide yielded d_2-$(a_2 + D)^{2+}$ at *m/z* 85 (with both deuteriums on the amino nitrogen). As expected, the CID of $[La^{III}(ProPro)(CD_3CN)]^{3+}$ (*m/z* 146.4) resulted in d_0-$(a_2 + H)^{2+}$ at *m/z* 84 (see Figure S1 in the Supporting Information for details). Formation of $(a_2 + H)^{2+}$ from $[La^{III}(ProPro)(CH_3CN)_2]^{3+}$ involves LaO^+, CO, and two CH_3CN molecules as byproducts; these are observed in Figure 1 (where Pro is abbreviated as P in the figure) in various degrees of association, e.g., $[LaO(CH_3CN)_2]^+$ at *m/z* 237 and $[LaO(CH_3CN)]^+$ at *m/z* 196. Replacing ProPro by ProAla (PA) gave the same fragmentation results: $(a_2 + H)^{2+}$ (*m/z* 71) was formed (see Figure S2 in the Supporting Information).

Other fragmentation channels of $[La^{III}(ProPro)(CH_3CN)_2]^{3+}$ are also evident in Figure 1. First, a dissociative proton-transfer

SCHEME 1



reaction from diproline to acetonitrile gives $[La^{III}(ProPro - H)(CH_3CN)]^{2+}$ at *m/z* 195.6 and $[CH_3CN + H]^+$ at *m/z* 42. The abundance of the latter is quite low, owing to mass discrimination of low-*m/z* ions within the quadrupole filter. Second, hydration of $[La^{III}(ProPro)(CH_3CN)_2]^{3+}$ followed by dissociative proton transfer results in $[La^{III}(OH)(CH_3CN)]^{2+}$ at *m/z* 119 and protonated diproline $[ProPro + H]^+$ at *m/z* 213. Hydration of $[Pb^{II}(CH_3CN)]^{2+}$ has been shown to yield vibronically excited $[Pb^{II}(H_2O)(CH_3CN)]^{2+}$ that dissociates spontaneously, giving $PbOH^+$ as a major product.[8] A similar scenario is postulated here. Hydration of $[La^{III}(OH)(CH_3CN)]^{2+}$ is also evident as $[La^{III}(OH)(CH_3CN)(H_2O)]^{2+}$ at *m/z* 128 and $[La^{III}(OH)(CH_3CN)(H_2O)_2]^{2+}$ at *m/z* 137.

Formation of $(a_2 + H)^{2+}$ from $[La^{III}(ProPro)(CH_3CN)_2]^{3+}$ requires first collisionally activated charge disproportionation, giving $(b_2 + H)^{2+}$ and $[LaO(CH_3CN)_2]^+$ (Figure 2). Density

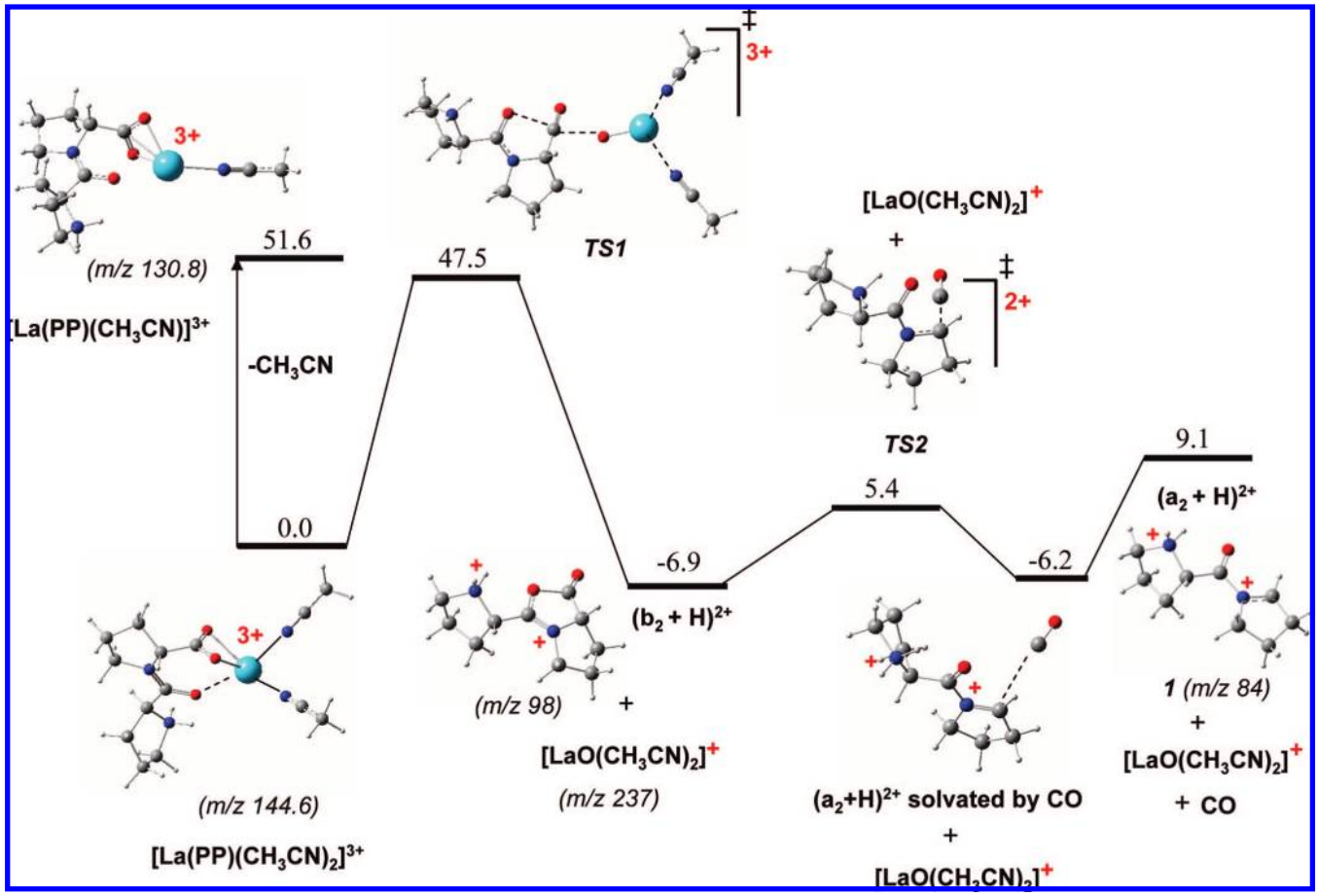


Figure 2. Potential energy surface for the dissociation of $[La^{III}(ProPro)(CH_3CN)_2]^{3+}$. Energies are $\Delta H°_0$ values in kcal mol^{-1}; TS = transition structure.

functional theory (DFT) with the hybrid functional B3LYP and the 6-31++G(d,p) basis set shows this reaction to have a relatively high barrier ($\Delta H°_0{}^{‡}$) of 47.5 kcal mol^{-1}; the products are vibronically excited and, as all subsequent steps have lower barriers, could further dissociate to give $(a_2 + H)^{2+}$. The lowest-energy structure of $[La^{III}(ProPro)(CH_3CN)_2]^{3+}$ is a salt-bridged complex, which is lower in enthalpy than the charge-solvated form by 13.3 kcal mol^{-1} (see Figure S3 in the Supporting Information). Charge disproportionation and formation of the incipient $(b_2 + H)^{2+}$ ion is facilitated by the high affinity of La^{3+} for O^-, stabilized by the solvating acetonitrile.[6] The vibronically excited $(b_2 + H)^{2+}$ can easily lose CO to give $(a_2 + H)^{2+}$; this step is endothermic by only 16.0 kcal mol^{-1}, and the product is 38.4 kcal mol^{-1} lower in energy than the rate-determining transition state, **TS1**. Compared with the $(a_2 + H)^{2+}$ ion of triglycine, the $(a_2 + H)^{2+}$ ion of diproline is more stable due to charge delocalization afforded by the proline rings. Both N atoms of dication **1** are more heavily substituted than those of the $(a_2 + H)^{2+}$ ion obtained from GlyGlyGly, thereby allowing for more charge delocalization by means of the inductive effect as well as hyperconjugation.[10] It should be emphasized here that the $(a_2 + H)^{2+}$ ions of diproline and triglycine carry their charges on the amino-acid backbone, which are subject to larger Coulombic repulsion than any analogous dipositive a_n ions comprising basic residues (arginines, lysines, and histidines) that can locate the charges on their expansive side chains.

Dissociation of $(a_2 + H)^{2+}$ gives only one major product, protonated 1-pyrroline, the a_1 ion of proline at *m/z* 70 (see Figure S4a in the Supporting Information). This pathway is unusual in that it involves transferring a hydrogen from the protonated pyrrolidine ring of structure **1** to the nitrogen that formally carries a positive charge in the pyrroline ring. Two pathways were considered: migration of a hydrogen from the NH_2^+ and migration of a hydrogen from the α-carbon (Figure 3). DFT calculations showed that transfer of the N—H hydrogen is energetically more favorable ($\Delta H°_0{}^{‡} = 37.5$ and $\Delta G°_{298}{}^{‡} = 36.5$ kcal mol^{-1}) than that of the $C_α$—H hydrogen ($\Delta H°_0{}^{‡} = 58.5$ and $\Delta G°_{298}{}^{‡} = 56.0$ kcal mol^{-1}). This prediction is supported by CID of d_2-$(a_2 + D)^{2+}$, giving exclusively d_1-iminium ion at *m/z* 71 (see Figure S4b in the Supporting Information). In the transition state for the transfer of the hydrogen from NH_2^+, the C—N bond is broken, the C—CO bond remains intact, and the pyrroline ring has migrated to the NH_2^+ and begun to form a N—H bond. Dissociation of this complex leads to two protonated 1-pyrroline ions and CO.

3.2. $(a_3 + H)^{2+}$ of ProProPro. The CID spectrum of $[La^{III}(ProProPro)(CH_3CN)]^{3+}$ (*m/z* 163) is shown in Figure 4. A major fragmentation channel is again charge disproportionation to give $(a_3 + H)^{2+}$ at *m/z* 132.6 and $[LaO(CH_3CN)]^+$ at *m/z* 196 and CO.[6] LaO^+ is also present at *m/z* 155. A second major charge disproportionation reaction is the one that gives a_1 at *m/z* 70, $[La^{III}(ProProPro - a_1)]^{2+}$ at *m/z* 189, and CH_3CN. The CID of $(a_3 + H)^{2+}$ shows product ions $(a_2 + H)^{2+}$ and a_1 (vide infra). Compared to the results of Shi et al.,[6] Figures 1 and 4 are exceptional in that the $(a_2 + H)^{2+}$ ion of ProPro is observable at all under the current experimental conditions, and in such high abundance.

Figure 5a shows the CID spectrum of $(a_3 + H)^{2+}$, giving protonated 1-pyrroline (a_1 and its isobaric internal iminium ion)

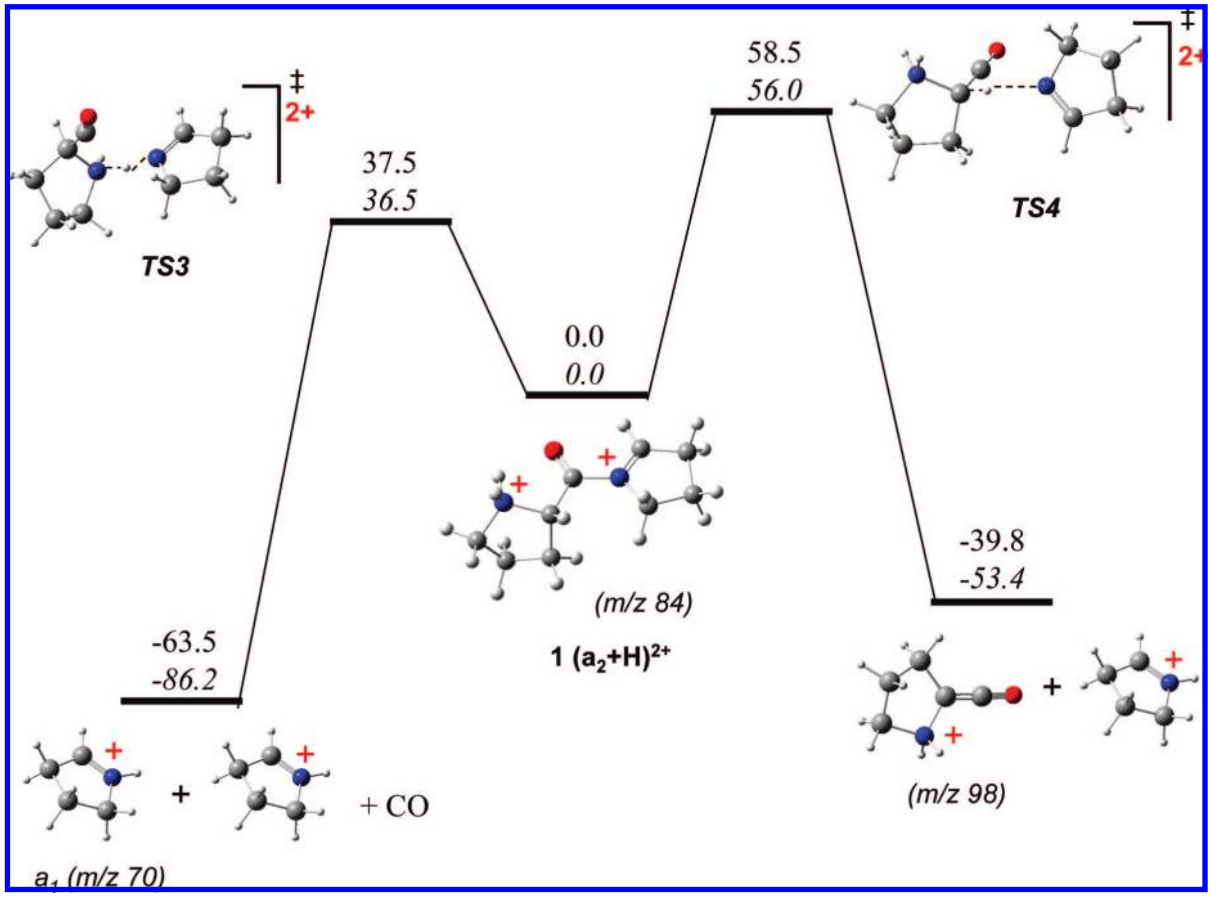


Figure 3. Energy profile for fragmentation of $(a_2 + H)^{2+}$ at the B3LYP/6-311++G(d,p) level of theory ($\Delta H°_0$, normal font; $\Delta G°_{298}$, italicized font).

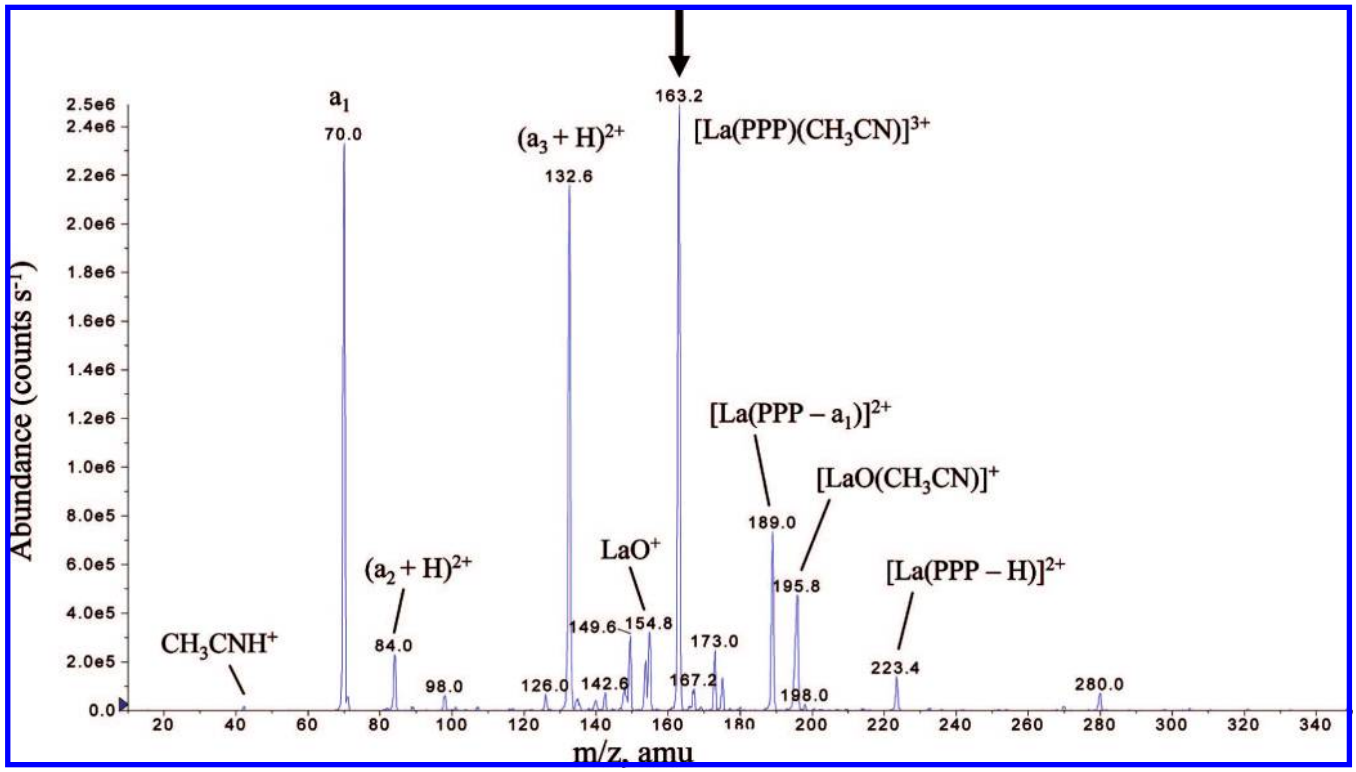


Figure 4. Collision-induced dissociation spectrum of $[La^{III}(ProProPro)(CH_3CN)]^{3+}$ at a laboratory collision energy of 30 eV. The vertical arrow indicates the precursor ion.

and $(a_2 + H)^{2+}$ as abundant fragment ions. This contrasts with the CID spectrum of the triglycyl $(a_3 + H)^{2+}$ ion, where the singly charged a_2 ion is the most prominent product.[6] Both b_2 and a_2 ions are observed as minor products, along with ions at

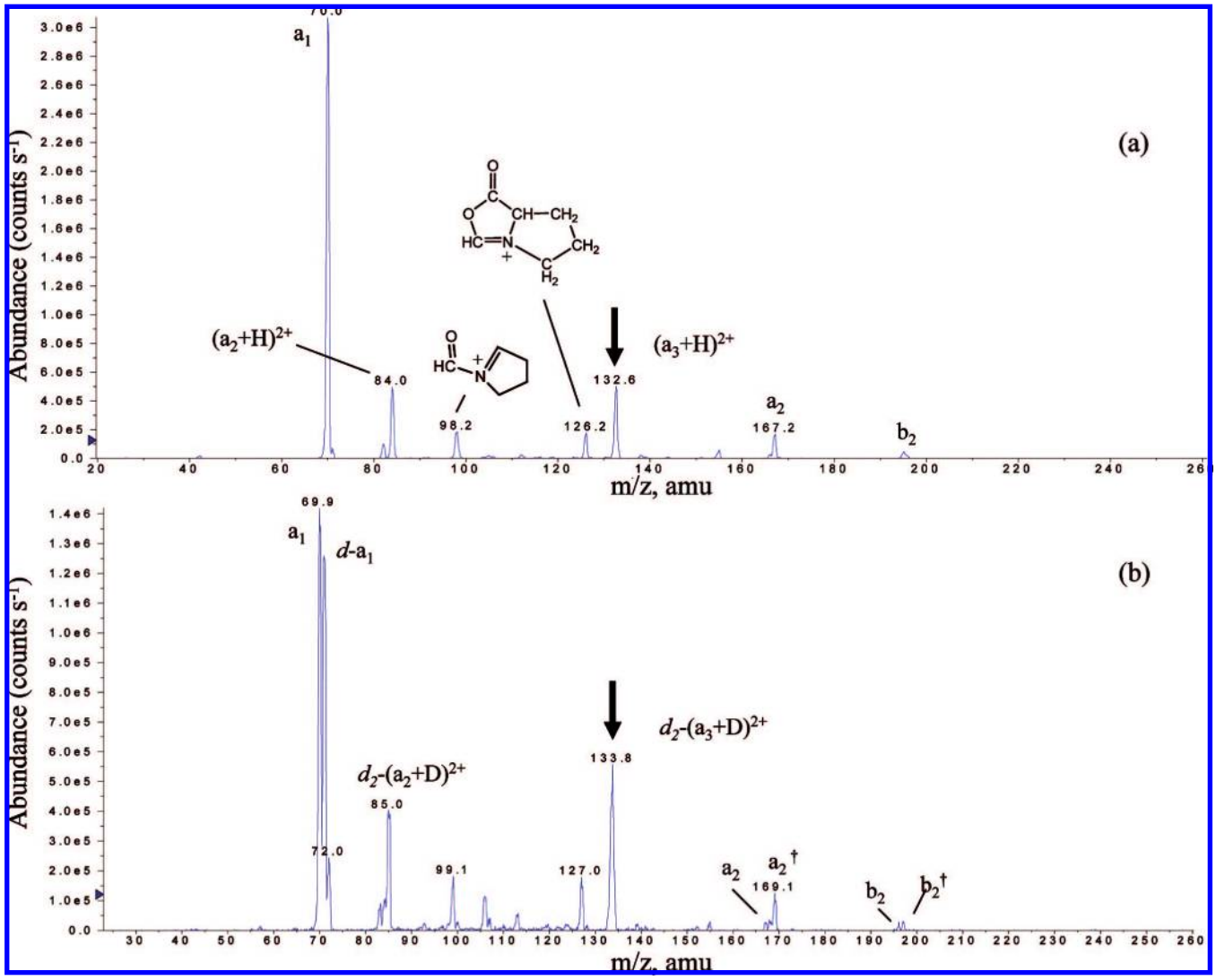


Figure 5. (a) Collision-induced dissociation spectrum of $(a_3 + H)^{2+}$ at a laboratory collision energy of 25 eV. (b) Collision-induced dissociation spectrum of d_2-$(a_3 + D)^{2+}$ at a laboratory collision energy of 25 eV. The vertical arrows indicate the precursor ions.

m/z 126 and 98 (vide infra). The spectrum of d_2-$(a_3 + H)^{2+}$ in Figure 5b shows *two* b_2 ions (denoted $b_2^{\dagger}$ and b_2), one ($b_2^{\dagger}$) containing two deuterium atoms and the other (b_2) containing only one. Both of these ions lose CO to give $a_2^{\dagger}$ and a_2 ions. The ions at *m/z* 126 and 98 in Figure 5a are shifted to 127 and 99 in the d_2-$(a_3 + D)^{2+}$ CID spectrum of Figure 5b, indicating that both product ions contain only one deuterium atom and are probably formed from the b_2 ion by the loss of 1-pyrroline and (1-pyrroline plus CO), respectively.

The energetics of the competing dissociation reactions for triprolyl $(a_3 + H)^{2+}$ are summarized in Figure 6. The initial steps are fragmentations to give $(a_2 + H)^{2+}$ ($\Delta H°_0 = 48.9$ and $\Delta G°_{298} = 25.7$ kcal mol^{-1}), b_2 plus protonated 1-pyrroline ($\Delta H°_0{}^{\ddagger} = 41.9$ and $\Delta G°_{298}{}^{\ddagger} = 39.9$ kcal mol^{-1}), and $b_2^{\dagger}$ plus protonated 1-pyrroline ($\Delta H°_0{}^{\ddagger} = 40.3$ and $\Delta G°_{298}{}^{\ddagger} = 37.6$ kcal mol^{-1}). These energies are much more similar among themselves than those of the analogous reactions for the $(a_3 + H)^{2+}$ ion of triglycine (73.0 and 52.0, 33.0 and 32.2, and 34.6 and 33.1 kcal mol^{-1}, respectively).[6] Consequently, in the dissociation of the triprolyl $(a_3 + H)^{2+}$ ion, formation of $(a_2 + H)^{2+}$ is competitive, in contrast to the case for the analogous triglycyl ions.[6] In addition, the barrier against formation of the $b_2^{\dagger}$ isomer is slightly lower than that against formation of the b_2 ion, the reverse of the situation for the $(a_3 + H)^{2+}$ ion of triglycine.

The b_2 ion loses CO to give the a_2 ion, which then dissociates to the a_1 ion. This is the common fragmentation pathway observed in the mass spectra of protonated peptides.[11,12] An additional ion at *m/z* 126 has previously been reported in the CID spectrum of $(ProProPro + H)^+$ and is formed from the b_2 ion.[13] Several possible structures for this ion have been examined; the lowest-energy structure located was a bicyclic ion formed with a barrier slightly higher than that for the initial formation of the b_2 ion ($\Delta H°_0{}^{\ddagger} = 47.4$ and $\Delta G°_{298}{}^{\ddagger} = 35.9$ kcal mol^{-1}) (see Figure 6). A subsequent loss of CO with a relatively low barrier gives the ion at *m/z* 98, and a further loss of CO gives the ubiquitous protonated 1-pyrroline.

The $b_2^{\dagger}$ ion, a ketene, also loses CO to give the $a_2^{\dagger}$ ion, a carbene. An intramolecular proton transfer from NH_2^+ of the $a_2^{\dagger}$ ion and concomitant cleavages of the C—N and C—C bonds yield protonated 1-pyrroline, CO, and neutral pyrroline, products identical to those from dissociation of the a_2 ion. The barrier against dissociation of $a_2^{\dagger}$ is higher than that against dissociation of a_2, consistent with the higher observed abundance of $a_2^{\dagger}$ (Figure 5b).

In conclusion, abundant dipositively charged protonated a_2 and a_3 ions from diproline and triproline can be formed by CID of La-containing complexes. These ions are stable species in the gas phase; DFT calculations support the observations by

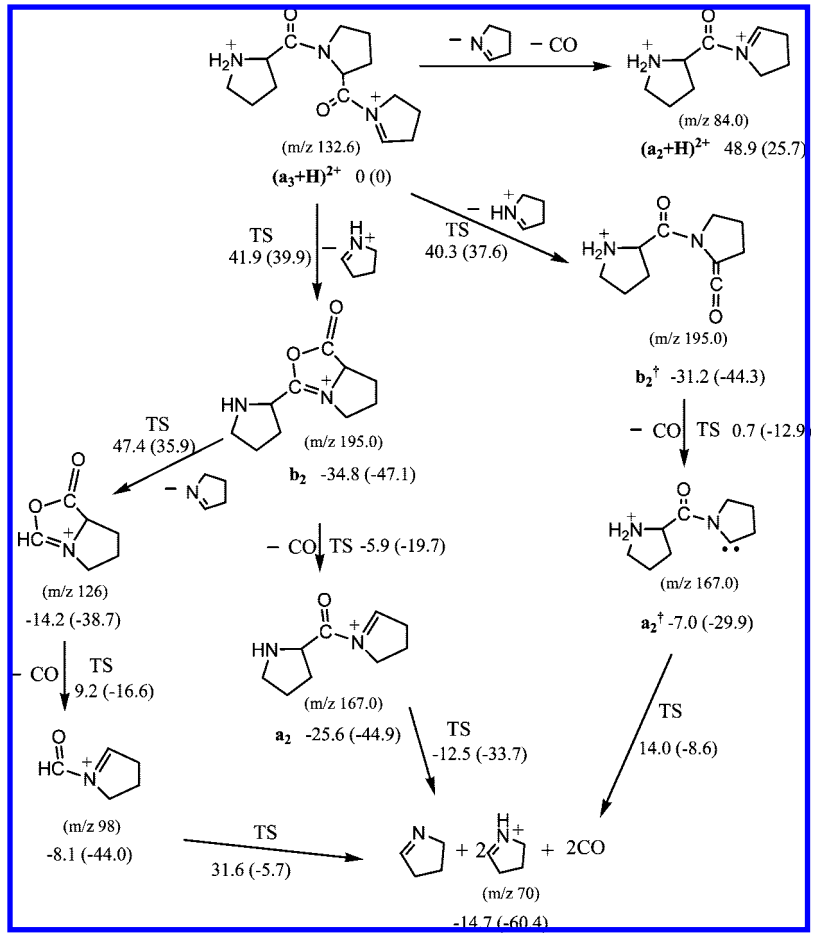


Figure 6. Fragmentation pathways for $(a_3 + H)^{2+}$ at the B3LYP/6-311++G(d,p) level of theory. $\Delta H°_0$ ($\Delta G°_{298}$) values are in kcal mol^{-1}; TS = transition structure.

showing that the barriers against their dissociations are >37 kcal mol^{-1}. The $(a_2 + H)^{2+}$ ion can also be formed in relatively high abundance by CID of $(a_3 + H)^{2+}$. These properties are in sharp contrast to those of the analogous dipositive triglycyl ions. We envisage that the superior stability of these ions is due to the abilities of the secondary amino groups in the prolines to assist in charge delocalization.

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Supporting Information Available: CID spectra of $[La^{III}(d_2\text{-ProPro})(CH_3CN)_2]^{3+}$, $[La^{III}(ProPro)(CD_3CN)_2]^{3+}$, and $[La^{III}(Pro\text{-}Ala)(CH_3CN)_2]^{3+}$; optimized structures of $[La^{III}(Pro\text{-}Pro)(CH_3CN)_2]^{3+}$; CID spectra of $(a_2 + H)^{2+}$, d_2-$(a_2 + D)^{2+}$; and tables of total energies and Cartesian coordinates of the optimized structures and transition states shown in Figures 2, 3, and 6. These materials are available free of charge via the Internet at http://pubs.acs.org.

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