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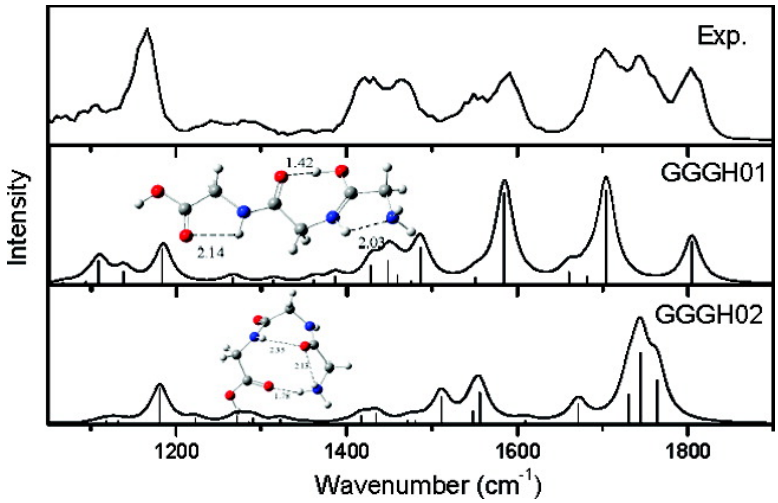

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Infrared Multiple Photon Dissociation Spectroscopy as Structural Confirmation for GlyGlyGlyH$^+$ and AlaAlaAlaH$^+$ in the Gas Phase. Evidence for Amide Oxygen as the Protonation Site

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Proton transfer and protonation are ubiquitous in biological systems and are well-known to be a dominant factor in determining structure, property, and function.[1] For amino acids and small peptides, based on the proton affinities of various possible sites, protonation is generally presumed to occur at the amine nitrogen of the N-terminus or a nitrogen of a basic side chain. Nevertheless, there is some continuing speculation that an amide oxygen may serve as the protonation site as the size of the peptide increases.[2] Direct experimental evidence to support this conjecture is still lacking, however. Thus, it is fundamentally very important to understand the protonation site and the structure of peptides in the gas phase.[3]

Here, the protonation sites and structures of protonated GlyGlyGly and AlaAlaAla have been investigated using infrared multiple photon dissociation (IRMPD) spectroscopy, which is a very powerful technique to elucidate clearly the structures of gas-phase ions in combination with electronic structure calculations.[4] Experiments have been carried out using the Free Electron Laser (FEL) facility, CLIO, in Orsay, France, coupled to an electrospray ionization-ion trap mass spectrometer (Bruker Esquire3000+).[5] The IRMPD spectrum of GlyGlyGlyH$^+$ is shown in Figure 1. The dominant fragment appears at *m*/*z* 115 (i.e., a b2 ion), consistent with previous observations.[6] Both y2 (*m*/*z* 133) and y1 (*m*/*z* 76) ions also appear; however, their intensities are markedly weaker than that of the b2 ion. The formation of a y1 ion is due to the cleavage of the same amide bond as in formation of the b2 ion. The much stronger b2 peak can be attributed to a proton transfer thermal equilibrium. According to the lowest threshold energy of 54.5 kcal mol^{-1} to fragment the parent ion,[7] at least 11 photons of 1745 cm^{-1} corresponding to the carbonyl stretching are required to dissociate the parent ion.

To identify the structure, the calculated spectra of the two most stable isomers are also included. The corresponding structures are shown in Figures 2 and S1, and relative energies are summarized in Table 1. The calculated spectra have been determined at B3LYP/6-311+G(d,p),[8] scaling a factor of 0.995. The calculated frequencies and intensities were convoluted assuming a Lorentzian profile with a 25 cm^{-1} full-width at half-maximum.

GGGH01, in which the proton is bound to the amide oxygen at the N-terminus, is found to be the most stable isomer at the B3LYP/6-311+G(d,p) level of theory. Formation of this structure is promoted by the additional strong hydrogen bond to the second amide oxygen with a short contact of only 1.42 Å. The cyclic structure, GGGH02, in which a proton is bound to the N-terminal nitrogen, with formation of two intramolecular hydrogen bonds to the adjacent amide oxygen and the carbonyl oxygen of the C-terminus, is 3.3 kcal mol^{-1} higher in energy than that of GGGH01 at the B3LYP/6-311+G(d,p) level of theory. However, at the MP2-(full)/6-311++G(2d,2p)// B3LYP /6-311+G(d,p) level, it is more stable than GGGH01. With entropy considerations, the free energy

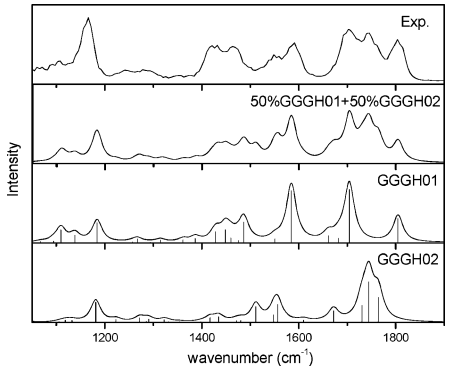

Figure 1. IRMPD spectrum of GlyGlyGlyH$^+$ and calculated spectra of the two most stable isomers (GGGH01, GGGH02) and a simulated mixture.

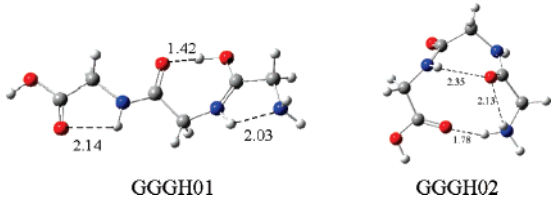

Figure 2. The structures of the two most stable isomers of GlyGlyGlyH$^+$ obtained at the B3LYP/6-311+G(d,p) level of theory.

Table 1. Relative Calculated Enthalpy and Entropy Changes (298 K) for the Different Isomers of GlyGlyGlyH$^+$

	B3LYP/6-311+G(d,p)		MP2(full)/6-311++g(2d,2p)// B3LYP/6-311+G(d,p)	
	$\Delta\Delta H_{298}$ (kcal mol^{-1})	$\Delta\Delta S$ (cal mol^{-1} K^{-1})	$\Delta\Delta H_{298}$[a] (kcal mol^{-1})	$\Delta\Delta G_{298}$[b] (kcal mol^{-1})
GGGH01	0	0	0	0
GGGH02	3.3	−1.9	−1.1	−0.5
GGGH03	3.7	4.7	2.7	1.3
GGGH04	2.9	3.1	3.2	2.3

[a] ZPE and thermal energy correction at 298 K obtained at B3LYP/6-311+G(d,p). [b] ΔH is from the single point energy, and ΔS is from B3LYP/6-311+G(d,p).

of GGGH02 is still 0.5 kcal mol^{-1} lower than that of GGGH01. However, GGGH01 has been reported to be 1.2 kcal mol^{-1} more stable than GGGH02 using B3LYP/6-31++G(d,p).[2b] The computational results are thus ambiguous, and some doubt remains as to which isomer exists and at which site protonation occurs. Another isomer, GGGH03, also involves protonation at the amine terminus, but without hydrogen bond induced cyclization. However, GGGH03 is 2.7 kcal mol^{-1} higher in energy than GGGH01. Proton transfer from the amino group in GGGH03 to the adjacent amide oxygen gives rise to a new isomer, GGGH04, which is 0.5 kcal mol^{-1} less stable than GGGH03.

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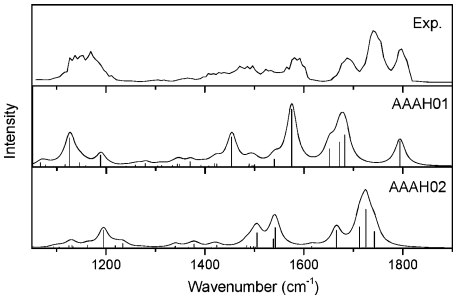

Figure 3. IRMPD spectrum of AlaAlaAlaH$^+$ and calculated spectra of the two most stable isomers (AAAH01 and AAAH02).

An examination of the experimental IRMPD spectrum leads to the conclusion that the bands may be best assigned based on a combination of the calculated spectra of GGGH01 and GGGH02, as shown in Figure 1. The bands at 1108 and 1166 cm^{-1} are best assigned as the bending vibration of the hydrogen-bonded OH of the amide group and the free OH of the carboxyl group. The broad band from 1410 to 1480 cm^{-1} is related to the CH_2 and NH_2 rocking and scissors modes. Each CH_2 group has a somewhat different environment, resulting in the broad band. The amide II mode appears at 1589 cm^{-1}, which matches very well with the calculated NH bending and CN stretching vibration of 1585 cm^{-1} for GGGH01. The band at 1550 cm^{-1} cannot be assigned to GGGH01; however, it corresponds very well with the amide II mode of GGGH02. Compared to this band in GGGH02, the frequency for that in GGGH01 is blue shifted about 40 cm^{-1}. This is also consistent with the difference of the calculated values due to the different protonation sites and structures. The amide II mode of GGGH02 is similar to that in protonated GlyGly (∼1540 cm^{-1}), in which the protonation site is at the amine nitrogen.[9]

The bands between 1650 and 1850 cm^{-1} are usually due to the carbonyl stretching vibrations, called the amide I modes, which are the most important modes to identify the conformation of peptides. In the experimental spectrum, the bands at 1703 and 1803 cm^{-1} are due to the two carbonyl stretching vibrations in the amide and carboxyl groups of GGGH01, respectively. The band at 1745 cm^{-1} cannot be assigned to any band of GGGH01; however, it matches very well with the amide I mode of GGGH02. The three corresponding calculated modes closely overlap around 1745 cm^{-1}. According to the calculated structure, the three C═O bond lengths are very similar at 1.23 Å for the amide carbonyl in the N-terminus (1730 cm^{-1}), 1.22 Å for the carboxyl group (1744 cm^{-1}), and 1.21 Å for the internal amide carbonyl (1764 cm^{-1}). However, for GGGH01, the two carbonyl groups are markedly different due to the proton being bound to one of the amide oxygens and the formation of a linear structure. The corresponding C═O bond lengths are 1.20 and 1.25 Å in the carboxyl and amide groups (1805 and 1704 cm^{-1}), respectively. These two bands of GGGH01 fit very well with the corresponding bands in the experimental IRMPD spectrum.

Both GGGH03 and GGGH04 have higher energies than those of GGGH01 and GGGH02 using the two different calculation methods. In addition, it is clear that the experimental spectrum is different from their calculated spectra. According to the experimental and computational results, both GGGH01 and GGGH02 thus exist as major species under the experimental conditions. However, minor amounts of other isomers cannot be completely excluded. Therefore, the present work constitutes the first experimental confirmation of the existence of a protonated peptide in which the proton is bound to the amide oxygen.

The IRMPD spectrum of AlaAlaAlaH$^+$ is shown in Figure 3, together with the calculated spectra. The structures of the different isomers are shown in Figure S2, and the relative energies are summarized in Table S1. Only isomers of L-trialanine have been considered here. The energy order for the different isomers of protonated trialanine is similar to that for protonated triglycine.

As found for GGGH$^+$, the experimental IRMPD spectrum of AAAH$^+$ may be assigned to a mixture of AAAH01 and AAAH02. Compared with the corresponding bands of GGGH01, those in AAAH01 are red shifted by 10−20 cm^{-1}, which is consistent with the structural variations expected for the substitution of hydrogens by methyl groups. The strong bands at 1687 and 1796 cm^{-1} fit very well with the simulated bands of the amide I modes of AAAH01 at 1683 and 1794 cm^{-1}, with the exception that the intensity order is reversed. The strongest band at 1741 cm^{-1} is in very good agreement with the overlap of the three carbonyl stretching vibrations of AAAH02. According to the experimental intensities, it appears that the population of AAAH02 is somewhat greater than that of AAAH01. Although direct deduction of the relative amounts of species from the experimental intensities is problematic,[5a,10] from the calculated energies, presuming a Boltzmann distribution of possible isomers, the amounts of AAAH01, AAAH02, AAAH03, and AAAH04 are 37, 61, ∼1, and ∼1%, respectively, as shown in Table S1.

In conclusion, the experimental IRMPD spectra of GGGH$^+$ and AAAH$^+$ indicate clearly that two main isomers coexist under the experimental conditions. The first is a linear structure, and the other is cyclic due to hydrogen bonding. For the first time, IR spectra provide direct evidence that an amide oxygen may serve as the protonation site for peptides in the gas phase.

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Supporting Information Available: Table S1, Figure S1 and S2, and the complete refs 3−5 and 8. This material is available free of charge via the Internet at http://pubs.acs.org.

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