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Conformational analysis of cyclo(Phe-Ser) by UV–UV and IR–UV double resonance spectroscopy and *ab initio* calculations

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We present the resonant two-photon ionization (R2PI) spectra as well as the UV–UV and IR–UV double resonance spectra for the cyclic dipeptide Phe-Ser. The R2PI spectrum shows five strong transitions in the region of 37 500–37 900 cm^{-1}. By performing UV–UV double resonance spectroscopy, we distinguished 5 different conformers. For each of these conformers, the origin is the most intense transition. In addition, we performed IR–UV double resonance measurements in the region 3200–3800 cm^{-1} to analyse the NH and OH modes of each conformer. We compared the measured IR spectra to frequencies from *ab initio* calculations to assign each conformational structure. We found two structures in which the hydroxyl group of the serine residue forms a strong hydrogen bond with the carboxyl group of the same residue. One structure shows only a weak hydrogen bond and for the remaining two structures, the hydroxyl group is 'free'.

1. Introduction

Cyclic peptides are a family of molecules that occur both naturally and synthetically. These types of molecules can have important biological functions. For example, they can act as antibiotics, toxins, ion-transport regulators, protein binding inhibitors and enzyme inhibitors [1–4]. As a result, a lot of attention has been paid to designing these molecules with different sizes and different sequences [5]. To understand the specific function of each peptide, it is necessary to determine their structure. By doing so in the gas phase, one can study isolated molecules and determine their intrinsic properties [6, 7]. Furthermore, gas phase measurements provide an optimum test for *ab initio* calculations. By performing resonant two-photon-ionization spectroscopy (R2PI) we can obtain information about the excited state. By applying UV–UV double resonance spectroscopy we can determine the number of conformers and we can look at the low frequency vibrations (torsions) of each conformer. These vibrations provide insight into the flexibility of each conformer. IR–UV double resonance spectroscopy provides information about the ground state of each conformer, and from these data in combination with *ab initio* calculations we can determine the structure of each conformer.

The smallest cyclic peptides that can be built are cyclic dipeptides for which the C terminal is reacted with the N terminal producing an extra peptide bond with loss of a water molecule. The resulting peptide is severely constrained in comparison to the original linear peptide. Recently Weinkauf and co-workers published the R2PI and the UV–UV double resonance spectroscopy of the cyclic Trp-Gly [8]. They found one conformer for this molecule. In addition, they used density functional theory (DFT) calculations with different basis sets in order to find the lowest energy structures. In all the structures they found the cyclic ring folds on top of the indole ring of the tryptophan residue and the structures are stabilized either by C–H$\cdots\pi$ or by NH$\cdots\pi$ interactions.

When employing DFT calculations to determine lowest energy structures it is very desirable to be able to compare the results with experimental variables such as vibrational frequencies. In this paper, we report such comparisons for the cyclic peptide Phe-Ser. We obtained isomer selective vibrational frequencies for 5 low energy conformations by IR–UV double resonance spectroscopy and compare those with DFT calculations to determine conformational structures. We will show that the peptide bond in the cyclic dipeptide is in the *cis* form which is energetically unfavourable in the corresponding linear peptide. This study is part of a larger study focusing on peptides containing phenylalanine or tyrosine to investigate the intermolecular forces (e.g. hydrogen bonding and

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dispersion forces) that lead to a preference for specific conformations.

2. Experimental method

The experimental set-up has been described elsewhere [9]. We obtained Cyclo(Phe-Ser) from Sigma-Aldrich and used it without further purification. In brief, we prepare samples by applying the neat compound to the surface of a graphite substrate. To bring the molecules into the gas phase, we employ laser desorption using a Nd:YAG laser operating at its fundamental wavelength (1064 nm). The laser is attenuated to $1\,mJ\,cm^{-2}$ and focused to a spot approximately 0.5 mm diameter within 2 mm in front of the nozzle. We translate the sample in order to expose fresh sample to successive laser shots. The nozzle consists of a pulsed valve with a nozzle diameter of 1 mm and a backing pressure of about 5 atm of argon drive gas.

To obtain a resonant two-photon ionization (R2PI) spectrum, we use a frequency doubled dye laser and detect the photo-ions in a time-of-flight mass spectrometer. By monitoring specific mass peaks while varying the two-photon ionization wavelength, we obtain mass selected excitation spectra. We perform double resonance spectroscopy by applying two successive laser pulses separated by a delay of about 200 ns [10, 11]. As a result of this delay we obtain two peaks in the time-of-flight spectrum that can be monitored individually. The first laser pulse serves as an intense 'burn' laser and is scanned over the desired wavelength region, while the delayed laser is used as the 'probe' laser and is fixed on one resonance. The burn laser depletes the ground state and when both lasers are tuned to a resonance of the same conformer, this causes a decrease in the signal of the probe laser. To obtain IR spectra for each conformer, we use IR–UV double resonance spectroscopy by employing an IR laser as the burn laser. For this purpose we use an OPO system (LaserVision) pumped by a Nd:YAG laser. The output of the OPO system is $8\,mJ\,pulse^{-1}$ and the bandwidth is $3\,cm^{-1}$.

3. Theoretical method

We performed calculations on the neutral form of the Cyclo(Phe-Ser) using a two step approach, calculating candidate structures using a simple molecular mechanics force field followed by geometry optimization using density functional theory. We used the Amber force field as implemented in the Amber 7 program suite to perform simulated annealing. Low energy candidate structures from Amber were then used as starting structures for subsequent optimization with the B3LYP hybrid density functional using the program Gaussian03. We built the preliminary structure using the GaussView 2.1 program. We then minimized this structure in Gaussian03 using the B3LYP functional and the 6-31G* basis set in order to generate the charges needed in Amber. We used the Amber 7 program *antechamber* to generate charges for Amber from the Gaussian03 output. While the charges were only calculated for a single conformation, they were sufficiently accurate for the purpose of calculating candidate structures with simulated annealing. We performed simulated annealing using a simple protocol of running at high temperature (800 K) for 30 ps followed by 10 ps of stepwise cooling, and a final energy minimization. This cycle was repeated 500 times, and results were then sorted by energy and classified according to structure. We obtained about 100 different structures based on the sorted energies. Those structures were subsequently minimized using the B3LYP functional and the 6-31G* basis set. Next we chose the lowest 30 structures to minimize using the B3LYP density functional and the 6-31G** basis set. Finally we calculated the frequencies of all the optimized structures and compared those with the experimental data from IR–UV double resonance spectroscopy.

4. Results and discussion

Figure 1 shows the structure of Cyclo(Phe-Ser). The dashed line in the middle shows the two parts (phenylalanine and serine) from which this molecule is built. NH(S) and C=O(S) belong to the serine, and NH(P) and C=O(P) belong to the phenylalanine, and we will use these notations in our analysis of the structures below.

4.1. *R2PI and UV–UV double resonance spectra*

Figure 2 (*a*) shows the R2PI spectrum, recorded using an attenuated laser beam to ensure that we are not saturating any transitions. The R2PI spectrum shows 5 main transitions in which 3 peaks are very close to each other in the region of $37\,600\,cm^{-1}$, and the other 2 transitions are higher in energy by about $150\,cm^{-1}$. The inset shows a portion of the R2PI spectrum recorded with a higher laser flux, which reveals additional transitions to the blue. We assign these transitions as torsional vibrations of the flexible side chain. We also recorded the R2PI spectrum of the linear dipeptide Phe-Ser, which is shown in figure 2 (*b*) for comparison.

To find the number of conformations and the origins of their respective spectra, we applied UV–UV double resonance spectroscopy, with the results displayed as the top traces in figure 3. Each of the 5 traces represents

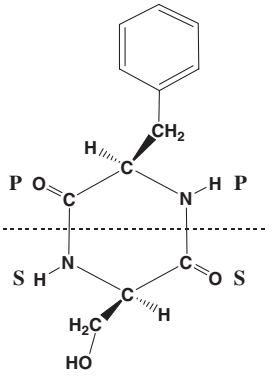

Figure 1. Scheme showing the structure of the Cyclo(Phe-Ser). The notation (P) represents the phenylalanine residue and (S) represents the serine residue.

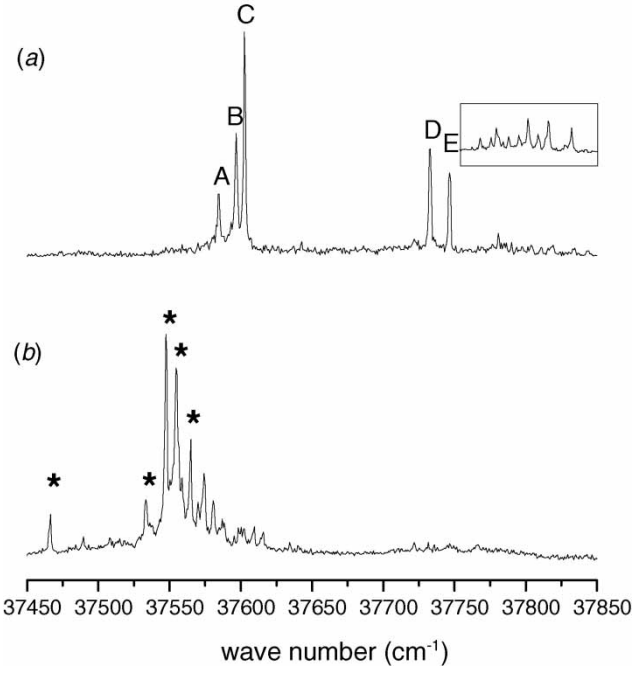

Figure 2. R2PI spectra of (*a*) cyclic Phe-Ser and (*b*) linear Phe-Ser. The inset shows part of the R2PI at higher UV laser flux.

a UV–UV spectrum recorded with the probe laser tuned to each one of the five main transitions in the R2PI spectrum. This molecule exhibits very low intensity vibrations compared to those of linear peptides. We also observed five different conformers for the linear Phe-Ser, the origins of which are indicated by asterisks in figure 2 (*b*). The R2PI spectrum of the linear Phe-Ser is richer in low frequency vibrations than that of the cyclic one. This can be explained by the increased rigidity of the cyclic structure as compared to the non-cyclic one. The blue-shift of conformers D and E of about 150 cm^{-1}

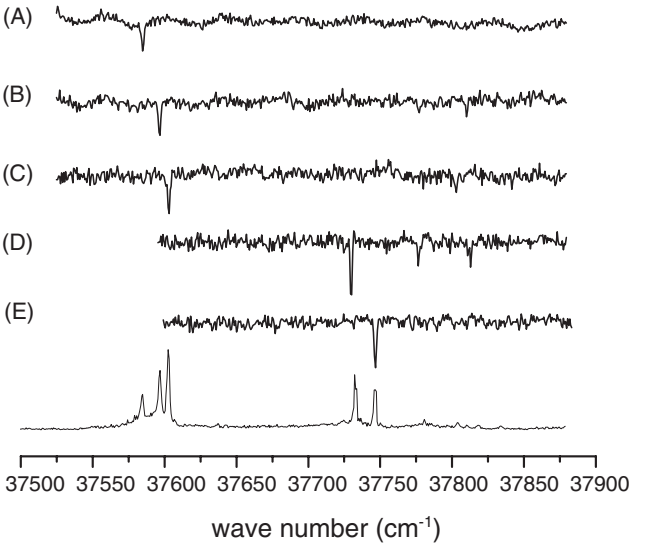

Figure 3. UV–UV double resonance spectroscopy of Cyclo(Phe-Ser). Traces A–E represent the spectra of the five different conformers.

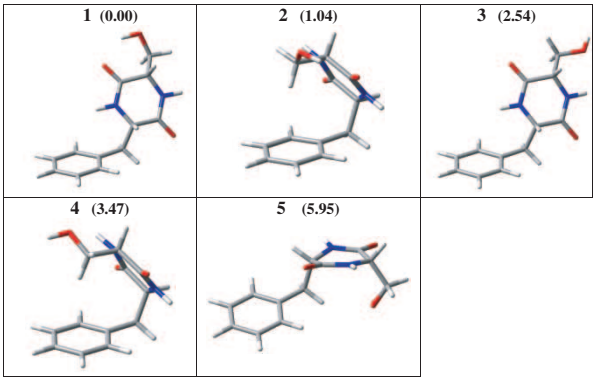

Figure 4. Lowest conformations as calculated by DFT/6-31G**. The relative energy in kcal mol^{-1} of each structure is listed above each structure.

relative to conformers A–C is an indication of a changed environment of the chromophore that we are probing (the benzene ring) as will be discussed below.

4.2. *DFT calculations and the IR–UV double resonance spectra*

Figure 4 shows the calculated 5 minimum energy structures with the relative energy listed above each structure in kcal mol^{-1}. We observe three different types of structures. In the first, the hydroxyl in the R group of the serine residue is hydrogen bonded to the carboxyl (O–H···O=C) of the same residue. Structures 1 and 2 belong to this type. In the second type, seen in structures 3 and 4, the hydroxyl group is 'free'. In the third type,

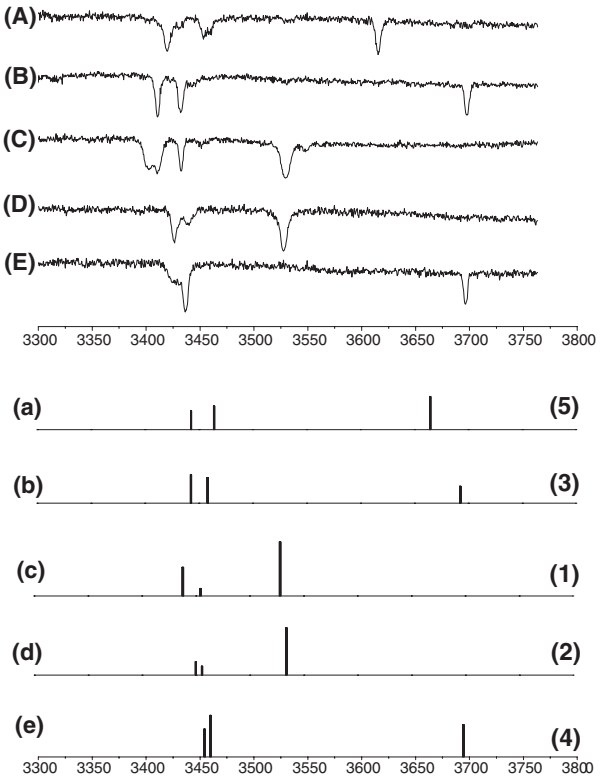

Figure 5. Top: experimental IR–UV double resonance spectra of the five different conformers of Cyclo(Phe-Ser). Bottom: calculated frequencies for the five structures from figure 4.

the hydroxyl group is slightly interacting with the carboxyl group and this is the case for structure 5. From our theoretical calculations, we measured the distance between the carboxyl and the hydroxyl groups in the serine residue and we found that in structures 1 and 2 the distance is about 1.95 Å which is typical for strong hydrogen bonding. In structure 5 we found the distance to be 2.76 Å consistent with weak hydrogen bonding as is also shown in the experimental IR data below.

As shown in figure 4, structures 1 and 3 are very similar: the only difference is a rotation of the R group of the serine residue that leads to hydrogen bonding between the hydroxyl and C=O in structure 1, while in structure 3 this rotation leaves the hydroxyl free of hydrogen bonding. The same phenomenon can be seen in the comparison between structures 2 and 4.

Figure 5 shows the IR–UV double resonance spectra as the top traces and the calculated frequencies of the assigned structures as stick spectra below. In the region we scanned, we expect to observe three modes, the hydroxyl in the R group of the serine residue, and two modes for two NH groups. In the experimental data we observe the free hydroxyl stretch at 3696 cm^{-1}. In conformer A the hydroxyl frequency is red-shifted to 3615 cm^{-1}. This shift indicates a weak hydrogen bond and corresponds to the calculated frequency of structure 5. The other two bands in conformer A appear at 3453 and 3419 cm^{-1}. This is a typical frequency for the NH band, and based on the calculation we assign the band at 3453 cm^{-1} to be the NH(S) and 3419 cm^{-1} to be NH(P).

In conformer B, the hydroxyl mode, measured at 3696 cm^{-1}, fits the frequency for the free hydroxyl. NH(S) is red-shifted to 3432 cm^{-1} and NH(P) is red-shifted to 3410 cm^{-1} compared to the corresponding modes in conformer A. The frequencies calculated for structure 3 represent the best fit to this structure. We observe the NH(S) and NH(P) vibrations in conformer C at the same frequencies as in conformer B, while the hydroxyl frequency in this conformer is red-shifted to 3529 cm^{-1}, indicative of strong hydrogen bonding. An extra peak is overlapped with the frequency of NH(P) with a maximum at 3402 cm^{-1}. This peak could be an overtone of the C=O(S) stretch vibration that is blue-shifted by hydrogen bonding with the hydroxyl group. As a result, the overtone of this frequency would happen to be similar to the frequency of NH(P), resulting in a Fermi resonance. The structure of this conformer is very similar to the structure of conformer B, but in structure B there is no hydrogen bonding and so a shift in the C=O(S) frequency is absent and there should be no Fermi resonance. A direct measurement of the fundamental C=O(S) frequency at about 1700 cm^{-1} could in principle test this model. Based on the current data the frequency of structure 1 exhibits the best fit to conformer C.

The IR–UV double resonance spectrum of conformer D shows a red-shift of the hydroxyl to 3529 cm^{-1}, associated with strong hydrogen bonding as in conformer C. NH(S) and NH(P) in this conformer are blue-shifted to 3439 and 3426 cm^{-1} respectively compared to conformer C. We assign structure 2 to conformer D. The difference between this structure and structure C is that in this conformer, the peptide ring is folded and the serine residue is positioned above the benzene ring of the phenylalanine residue, and one of the hydrogens of the R group of the serine is slightly interacting with the π system of the ring. The NH(S) and NH(P) frequencies in conformer E are at the same frequencies as those in conformer D; the difference here is that NH(S) mode is more intense than NH(P) as opposed to the intensity distribution in conformer D. The other difference is that the hydroxyl in conformer E is free. We assign conformer E to structure 4. We note that the two NH vibrations are strongly coupled in structures 2 and 4 while in the other structures they are not.

The analysis of the IR–UV double resonance spectra shows that structures 2 and 4 correspond to conformers D and E, which are the two conformers that in the R2PI spectrum are blue-shifted by $150\,cm^{-1}$ compared to the other conformers. This suggests that the CH$\cdots\pi$ interaction in those two conformers causes the shift in the electronic spectrum, although a C=O$\cdots\pi$ interaction could also cause such a shift. It is also possible that the bent geometry plays a role by causing a large geometry difference between the excited state and the ground state.

5. Summary

The cyclization of the Phe-Ser, does not change the number of low energy conformers that are frozen in the beam compared to the linear form of this peptide. For both molecules, the cyclic and the linear Phe-Ser, we observed five different conformations. This phenomenon occurs despite the fact that cyclization in general adds rigidity to the molecule, from which one might expect a reduction in the number of conformations. However, the rigidity of the cyclic peptide is evident in the R2PI spectra. The cyclic peptide has very low intensity torsional vibrations as opposed to what is usually observed in flexible molecules. In addition, we were able to characterize the structure of each conformer by using IR–UV double resonance spectroscopy combined with *ab initio* computations. We found that the R group of the serine residue is playing an important role in shaping the structure of each conformer by intramolecular hydrogen bonding involving its hydroxyl group. Furthermore, small interactions of one of the hydrogens of this group with the benzene ring of the phenylalanine can lead to a distinguishable shift of the UV spectrum as observed in conformers D and E. In all cyclic structures the peptide bond is in the *cis* form, which is energetically unfavourable in the corresponding linear peptide. The results show that calculations at the level employed here suffice for describing a relatively rigid molecule with the size of a dipeptide.

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