*Graphical Abstract

UV AND IR SPECTROSCOPY OF COLD PROTONATED LEUCINE ENKEPHALIN

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Abstract:

We have applied ultraviolet and infrared-ultraviolet (IR-UV) double resonance photofragment spectroscopy in a tandem mass spectrometer for the spectroscopic characterization of cryogenically-cooled protonated leucine enkephalin (H^+ -YGGFL), for the purposes of elucidating its three-dimensional structure. The primary UV-induced photofragmentation pathway following excitation of the tyrosine chromophore is loss of the tyrosine side chain (107 Da). IR-enhanced photofragmentation via this channel makes IR-UV depletion spectroscopy difficult, and IR photofragment gain spectroscopy is used instead to record the infrared spectrum in the hydride stretch and amide I/II regions. By comparing the experimental spectrum with the predictions of DFT M05-2X/6-31+G(d) calculations, a single backbone structure was assigned that is similar to, but distinct from, that assigned in the recent work of Polfer et al. [N. C. Polfer et al. *J. Am. Chem. Soc.* 129 (2007) 5887]. Additionally, the assigned structure's theoretical cross-section is comparable to previous ion mobility results. The structure is characterized by a compact hydrogen-bonding architecture in which the peptide backbone self-solvates the N-terminal ammonium group carrying the charge. In addition to H-bonds to the tyrosine π cloud and the second glycine carbonyl oxygen, the ammonium group is involved in a series of cooperatively strengthened H-bonds between the N and C termini, linking the COO-H group to the F-L peptide bond. The resulting structure suggests some relevance to the fragmentation pathways of protonated YGGFL.

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1. Introduction

The measurement of ion mass provides valuable constraints on the elemental composition of a molecule of interest but provides no information on ion structure. A variety of tools have been developed to probe ion structure. Bond connectivity information is usually obtained by inducing fragmentation and determining product ion masses. Fragmentation patterns are the means by which primary structure information is derived from peptide ions, for example, within the context of a tandem mass spectrometry (MS/MS or MSⁿ) experiment^[1,2,3]. Higher order structural information, however, is not directly forthcoming from fragmentation data, although the three-dimensional structure of the ion may well influence the observed fragmentation patterns. Tools for deriving higher order structural information include ion mobility spectrometry (IMS)^[4,5,6,7,8], and hydrogen-deuterium exchange (HDX)^[9,10,11], with the predictions of theoretical calculations providing a primary point of comparison^[12,13,14].

Recently, there has been a growing interest in ion spectroscopy to probe the intramolecular interactions associated with peptide ions. The infrared (IR) spectra of ions can be compared to the spectra predicted by *ab initio* or density functional theory (DFT) calculations to predict the three-dimensional structure(s) of the ions present in the mass spectrometer. Multiple methods have been demonstrated for measuring the IR spectra of gas-phase ions including wavelength dependent IR multi-photon dissociation (IRMPD)^[15,16,17,18,19,20], “messenger spectroscopy” where the ion of interest is “tagged” with messenger atoms/molecules that are lost following IR absorption^[21,22,23,24,25], and infrared-ultraviolet (IR-UV) double resonance techniques^[26,27,28,29,30].

At room temperature, spectral congestion arises from overlapping IR spectra from multiple, energetically-accessible conformations and from a Boltzmann distribution of the initial

vibrational levels of the ion. These factors lead to a degree of spectral complexity that can interfere with direct comparison to theoretical calculations and, thus, complicate unambiguous spectral assignments. In “messenger spectroscopy,” ions are cryogenically cooled to eliminate temperature related spectral complexity and the IR spectrum is measured as the photo-induced loss of the inert messenger “tag.” Furthermore, IR-IR double resonance experiments can be used to carry out conformation-specific analysis^[24].

An alternative approach for elucidating conformationally distinct peptide structures is to use IR-UV double resonance spectroscopy in a cryogenically cooled ion trap. As with the messenger technique, cooling the ions before irradiation collapses the population down to the zero-point vibrational levels of the low energy conformers present in the mass spectrometer, reducing the spectral complexity associated with the number of conformational isomers, hot bands and sequence bands. The highly resolved electronic spectra of cold ions enable, in many cases, conformation-specific vibrational spectra to be taken via double resonance methods and compared with theoretical predictions from quantum mechanical frequency calculations. This approach is analogous to that used for the elucidation of neutral peptide structures and hydrogen bonding motifs^[31,32,33]. Rizzo, Boyarkin, and co-workers have demonstrated the extraordinary potential of IR-UV double resonance methods in their ground-breaking work on peptide ions over the past several years^[26, 27, 28, 29, 30].

Here we present the application of IR-UV double resonance spectroscopy in a cryogenically-cooled 22-pole ion trap for the structural investigation of protonated leucine enkephalin (YGGFL, $m/z = 556$). Leucine enkephalin is a biologically active endogenous opioid peptide that has been extensively studied as a model peptide in mass spectrometry^[34]. The mechanisms and energetics of its gas-phase fragmentation pathways have been experimentally

determined using a wide variety of instrumental setups and a wide variety of ion activation methods. From this wealth of experimental data, it has been well-established that the lowest energy fragmentation process is cleavage of the F-L amide bond, resulting in formation of the b_4 ion ($m/z = 425$)^[8, 35, 36]. From the b_4 ion, a reaction cascade can occur that leads to various secondary fragments in the mass spectra, such as the facile loss of CO to form the a_4 ion ($m/z = 397$)^[36, 37]. As such, the intensity ratio of a_4 and b_4 ions has been used as a measure of the internal energy content in the ion^[38, 39]. The structure of protonated YGGFL and its low energy fragments have been studied with a variety of methods, including IMS^[8] and spectroscopy^[15, 30]. Although room temperature IRMPD data^[15] (1000-1800 cm^{-1}) have been reported for protonated leucine enkephalin and its fragments and cold IR-UV double resonance data^[30] have been reported for its b- and a-type fragments, this work presents the first cold UV photofragment and IR-UV double resonance spectra of the intact parent peptide ion. Interrogation of the ion at cryogenic temperatures as opposed to room temperature leads to a revised structural assignment for this prototypical protonated peptide ion.

2. Experimental Details

The experimental details are briefly described here. Aspects of the apparatus and control hardware/software have been described previously^[40]. Here we emphasize certain experimental details unique to the present study.

2.1 UV and IR Spectroscopy

A schematic diagram of the Instrument for Cold Ion Spectroscopy (ICIS) at Purdue is shown in Figure 1.

Figure 1. Schematic diagram of the instrument (not to scale). The quadrupoles designated q1, q2 and q3 make up the triple quad axis of the instrument, and the transport quadrupoles labeled q_{trans}1 and q_{trans}2 along with the 22-pole cold trap make up the spectroscopy axis (nESI – nano-electrospray ionization). Adapted from reference [40].

Briefly, the instrument consists of two orthogonal axes, a mass spectrometric axis and a spectroscopic axis. The mass spectrometric axis consists of three quadrupoles. The second and third quadrupoles (q2 and q3) have been modified to be operated as linear ion traps (LITs). After ions are generated via nano-electrospray ionization (nESI) and transported through an ion funnel, they are turned by the first DC quadrupole ion deflector, transported through q1 and trapped in q2. While in q2, the parent ion of interest is isolated via a notched chirp broadband waveform, which is sourced from the instrument's Griffin Analytical Technologies Argos control module (FLIR, West Lafayette, IN). The isolated parent ion is then turned by the second ion deflector between q2 and q3 down the spectroscopic axis, which contains a 22-pole ion trap typically held

at 5K by a closed cycle helium cryostat (Sumitomo Heavy Industries, Tokyo, Japan). A 50 W cartridge heater coupled with a temperature controller allows for precision temperature control of the 22-pole trap from 4 K to 120 K (Lakeshore, Westerville, OH). To trap and cool the ions, a pulse of ultra-pure He (99.9999%) is introduced directly to the trap via a pulsed valve (Parker series 9 general valve) 1–3 ms before the ions are introduced. The He atoms are cooled to near the trap temperature via collisions with the copper trap enclosure. Collisions between the parent ions and the cold He buffer gas result in efficient cooling of translational and internal degrees of freedom. We estimate the internal vibrational temperatures of the photofragment ions to be in the range of 10-16 K, from the ratio of cold to hot band intensities in the UV spectrum of the b_4 ion of YGGFL in our apparatus. After cooling, the parent ions are interrogated via UV and IR lasers in the 22-pole ion trap. Resonant absorption of a UV photon promotes the ion from its ground electronic state to an excited electronic state. From the excited electronic state, the ion can fragment via a curve crossing to a dissociative electronic state (electronic predissociation) or via internal conversion to excited vibrational levels of the ground electronic state followed by unimolecular dissociation (vibrational predissociation). In IR-UV experiments, resonant absorption of an IR photon can increase or decrease the number of UV-generated photofragments, depending on experimental conditions, as will be described in greater detail shortly.

The parent ion and any resulting photofragments are transported back through the second ion deflector to q3. In q3 a waveform generator (33220A, Agilent Technologies, Santa Clara, CA) applies a broadband waveform calculated by SxWave software, which has been described previously^[41]. This waveform serves to eject any parent ions remaining after irradiation, leaving only photofragments in q3. At this point, the photofragments can be mass analyzed via mass

selective axial ejection (MSAE) or can be dumped as an ion packet into the channeltron detector by applying an attractive potential on the q3 exit lens. The latter method results in much smaller ion losses during detection and also allows for the collection of a photofragmentation spectrum that is the sum of the ion signal from all of the individual photofragment mass channels. All UV and IR spectra shown here were acquired in this total photofragment mode. As discussed previously^[40], an overlapped scan function was developed that involves the simultaneous and independent trapping of ions in q2 and the cold trap/q3. This allows for an effective filling time for q2 of 60 ms, a q3 analysis ramp of 60 ms and a cooling time of 25 ms in the 22-pole cold trap, all within a 100 ms timeframe, which accommodates coupling with the 10 Hz repetition rate of the lasers.

Electronic spectra of all conformational isomers present in the 22-pole cold trap are acquired by measuring the total photofragment signal as a function of UV wavelength. Two approaches are available to acquire infrared spectra. In one, the UV laser wavelength is fixed and an IR pulse is spatially overlapped with and counter-propagating the UV laser, entering the 22-pole trap 100 ns before the UV laser pulse. In a method analogous to that used for neutral species, resonant ion dip infrared spectroscopy (RIDIRS), the UV laser is fixed on one of the observed transitions in the electronic spectrum of the parent ion, while the IR frequency is tuned through the spectral region of interest.^[42,43] By vibrationally exciting the parent ions before they are electronically excited, the ground state is depopulated, causing a decrease in UV photofragmentation efficiency. Only IR transitions arising from conformers sharing the probed UV transition will give rise to depletions in the photofragment signal, resulting in a conformation specific IR spectrum (see Figure 2b). However, in addition to the depopulation of the ground state, absorption of a resonant IR photon can lead to intramolecular vibrational energy

redistribution (IVR). This redistribution of energy among accessible vibrational levels results in statistical inhomogeneous broadening of the UV spectrum to the red of the electronic origin. This phenomenon can be exploited in an alternate approach for acquiring IR spectra, referred to as IR fragment ion gain spectroscopy, where the UV laser is fixed at a wavelength to the red of the lowest energy electronic origin in the UV spectrum. Vibrational excitation acts to induce UV absorption at this wavelength, resulting in a gain in UV photofragmentation signal (Figure 2c). This method is not conformation specific because the UV laser is fixed to the red of any conformation specific transitions, which results in an IR spectrum of all conformers present. However, because it is a measurement of a signal above an essentially zero background it will typically have a higher signal-to-noise ratio than the IR dip method, which is a measurement of depletion of the parent signal. As such, IR gain is appropriate as an initial survey method, and in cases where only one conformer is present, it can also serve as the final, conformation-specific spectrum. The IR gain method is analogous to the infrared ion-gain spectroscopy (IRIGS) used in neutral species^[44,45] and was recently demonstrated for ions by Nagarnova et al.^[46]. Both methods are summarized schematically in Figure 2.

Figure 2. Schematic diagram for the IR-UV double resonance techniques discussed here (a) with example spectra for the IR depletion experiment (b) and the IR gain experiment (c).

In this work, all reported IR spectra were taken with the IR gain method. In the depletion measurements (Supplemental Material) the size of the observed depletion was small compared to previous studies. Indeed, as we shall see, IR-UV holeburning scans, in which the IR wavelength is fixed while the UV photofragment laser is tuned, indicate that the UV photofragmentation signal of protonated YGGFL is enhanced significantly by IR excitation. These spectra demonstrate that the processes responsible for IR depletion and IR gain are occurring in competition with one another at the wavelength used for IR-UV depletion spectroscopy. We note in passing that the degree of IR-induced UV spectral broadening, and thus the propensity for observing IR-induced depletion, appears to be highly ion dependent. In cases such as this, where

the UV spectrum broadens in such a way that the IR-induced gain makes it difficult to observe the IR-induced depletion of the ground state when on resonance, the signal-to-noise ratio of IR depletion suffers. For [YGGFL+H]⁺, IR gain measurements are reported because they were much higher in signal-to-noise ratio and because the IR spectrum indicated the presence of only one conformer/conformational family.

2.2 Materials

Leucine enkephalin was purchased from Sigma–Aldrich (St. Louis, MO). Acetic acid and methanol were purchased from Mallinckrodt (Phillipsburg, NJ). A YGGFL stock solution of 2.0 mg/mL was prepared in a 50:50:1 methanol:water:acetic acid solution. The stock solution was diluted approximately four-fold to produce the nESI working solution. Pulled borosilicate glass capillaries (Sutter Instruments, Novato, CA) were filled with 10 μ L of the working solution for nESI.

2.3 Laser Systems

The UV spectroscopy presented in this paper utilizes the frequency doubled output of a pulsed dye laser (ScanMatePro, Lambda Physik) pumped by the second harmonic of a Q-switched Nd:YAG laser (Brilliant B, Quantel). The UV power is limited to ~ 1.5 mJ/pulse with a beam diameter of approximately 3 mm, which is larger than the ion cloud. These power densities provide adequate ion photofragment signal without overly saturating the UV spectrum. The IR spectroscopy reported here employs the tunable output of a seeded Nd:YAG pumped optical parametric oscillator/optical parametric amplifier (OPO/OPA, LaserVision). IR gain spectra were recorded in the hydride stretch ($2800\text{--}3700\text{ cm}^{-1}$), amide I ($1600\text{--}1800\text{ cm}^{-1}$) and amide II ($1400\text{--}1600\text{ cm}^{-1}$) regions. For generation of IR light in the amide I/II regions, an

AgGaSe₂ crystal was fixed down-field of the OPO output. The laser wavelength scans for both lasers and the data acquisition are controlled via LabVIEW (LabView 8.2, National Instruments). Each population of ions in the 22-pole trap is subjected to either a single UV laser shot or one IR laser shot and one UV laser shot.

2.4 Computational Methods

A conformational search was performed with the MACROMODEL suite of programs to generate a set of starting structures^[47]. The Amber* force field was used to find stable minima within a 50 kJ/mol window of the global minimum^[48]. The 50 lowest energy structures were then optimized using density functional theory (DFT). After geometry optimization, harmonic frequency calculations were performed in the Gaussian09 program^[49]. To best account for any dispersive interactions, Truhlar's hybrid density functional M05-2X was used with the 6-31+G(d) basis set^[50]. On the basis of accepted values^[51,52], hydride stretch, amide I, and amide II vibrational modes are scaled by 0.937, 0.955, and 0.945 respectively, to account for anharmonicity in the calculated harmonic vibrational modes. These scaling factors bring the calculated fundamentals into best agreement with those observed in the experimental vibrational spectrum. As a means of comparing our results with ion mobility studies, the DFT optimized geometries and atomic charges of the optimized structures were used to calculate theoretical collision cross-sections via the trajectory method in the MOBCAL program developed by the Jarrold group^[53].

3. Results and Discussion

In this section we present the UV photofragment spectrum and IR gain spectra for cold, protonated leucine enkephalin. An overview of the UV spectrum is given first, followed by a

discussion of the IR spectra and their comparison to theoretical predictions. We then examine the assigned structure of protonated leucine enkephalin, including a comparison to the structure assigned from room-temperature IRMPD data as well as to ion mobility data. This is followed by an analysis of its photofragmentation mass spectra and a discussion of the possible implications of the assigned structure on the observed fragmentation pathways.

3.1 UV Spectroscopy

YGGFL has two possible chromophores available for UV excitation, the aromatic rings on the tyrosine (Y) and phenylalanine (F) side chains. The UV spectrum taken in the region of the Y chromophore for protonated YGGFL is shown in Figure 3. The electronic band origin appears at $35,684\text{ cm}^{-1}$, which represents a blue shift compared to those of bare protonated tyrosine ($35,081\text{ cm}^{-1}$, $35,111\text{ cm}^{-1}$, $35,186\text{ cm}^{-1}$, and $35,235\text{ cm}^{-1}$)^[26,40]. This blue shift is in agreement with previous UV spectroscopic data on the CID fragments of YGGFL, which showed an increasing blue shift relative to bare protonated tyrosine with increasing fragment ion size^[30].

Figure 3. UV photofragment spectrum of $[\text{YGGFL+H}]^+$ in the region near the S_0 - S_1 origin.

The UV spectrum has strong transitions present in the low energy portion of the spectrum and very dense vibronic structure that extends to higher wavenumbers. The three sharp transitions between 35,830 and 35,860 cm^{-1} yield peak widths of FWHM of 3 cm^{-1} , indicating low rotational temperatures of 10 K or below. The electronic band origin at 35,684 cm^{-1} has a FWHM of 10 cm^{-1} and shows splitting when the spectrum is taken at higher resolution (Figure S1), indicating overlapping transitions. This observation suggests the presence of more than one electronic origin due to conformers with similar environments about the tyrosine UV chromophore. However, as we shall see, the infrared gain spectrum can be accounted for via a single peptide backbone conformation. This suggests that the low-energy conformers contributing to the unresolved transitions near 35680 cm^{-1} have similar local environments for the tyrosine side chain, and likely also share a similar peptide backbone structure. To the red of the assigned electronic origin, a small background UV-induced photofragmentation signal decays to zero over a range of $\sim 450 \text{ cm}^{-1}$, indicating the possible presence of a structure that modulates the tyrosine interaction more directly. Much of the observed low-frequency activity in the UV spectrum is attributable to Franck-Condon activity involving low-frequency vibrations involving motion of the peptide backbone against the tyrosine ring. In many of the low energy conformations, the lowest frequency modes have frequencies near 10 cm^{-1} , comparable to the observed spacing in the spectrum of Figure 3. The rapid decay of intensity in the first set of transitions indicates weak Franck-Condon progressions, and as such a relatively small geometry change between ground and excited states along these large-amplitude vibrational coordinates.

3.2 IR Spectroscopy

Figure 4. IR ion-gain spectra of $[\text{YGGFL}+\text{H}]^+$ in the amide I/II regions (a), in the hydride stretch region (b) and the two assigned low energy structures (c). A table of the assigned vibrations is shown in the Supplemental Material (Table S1).

The IR gain spectra obtained in the amide I/II and hydride stretch regions for protonated YGGFL are shown in Figures 4a and 4b, respectively.

Section 3.2.1 IR Gain vs. Dip Behavior for Protonated Leucine Enkephalin

It was observed that IR-UV double resonance depletion signal for YGGFL, measured as a fractional depletion, was lower than expected, compared with the depletion of the b_4 fragment ion of YGGFL, which has been previously studied by Rizzo et al.^[30], and which we had reproduced as a preliminary to the present work. Depletion spectra for YGGFL and its b_4

fragment ion in the N-H stretch region are summarized in Figures S2 and S3 in the Supplementary Material. The differences in IR depletion between the two species is probed by resonant “IR-heated” UV spectra for both in Figures S4 and S5. Upon absorption of a resonant IR photon, the discrete quantum of energy imparted by the photon is redistributed among accessible vibrational levels via intramolecular vibrational energy redistribution (IVR). The resulting broadening of the UV absorption profile is an example of statistical inhomogeneous broadening, which is fundamentally different from thermal congestion which arises from a Boltzmann distribution over a range of vibrational energies^[46]. This difference is illustrated in Figure 5, which compares the IR-heated UV spectrum (Figure 5d) with UV photofragment spectra recorded with the 22-pole cold trap held at 25, 50, and 110 K (Figures 5a-c).

Figure 5. The UV spectrum of protonated YGGFL with the 22-pole cold trap held at 5 K is shown in red. Shown in black is the UV spectrum with the 22-pole cold trap heated to 25 K (a), 50 K (b, signal x5), 110 K (c, signal x5) and with the 22-pole cold trap held at 5K with resonant IR excitation (3394 cm^{-1}) prior to UV excitation (d).

The broadening shown in (d) is quite distinct from that due to thermal broadening shown in (a-c), involving a shift in the UV absorption profile to the red in addition to signal broadening. Note also that the IR-induced gain signal is enhanced in integrated intensity relative to that in the absence of IR excitation, a point to which we shall return in the discussion section. The enhanced and red-shifted UV photofragment signal upon IR excitation results in IR gain measurements being significantly higher in S/N ratio than IR depletion measurements. For this reason, the IR spectroscopy presented here was taken via non-conformation specific IR gain measurements as opposed to conformation specific IR depletion. The presence of four amide N-H stretches in the hydride stretch region ($2800\text{-}3700\text{ cm}^{-1}$) and of five carbonyl stretches in the amide I region ($1600\text{-}1800\text{ cm}^{-1}$) indicates a single backbone conformation for the pentapeptide, further validating the use of IR gain measurements.

Section 3.2.2 Conformational Assignment

In Figure 4, the experimental IR gain spectra are compared with the predicted IR transitions of the two lowest energy calculated structures. The two structures (0.00 kJ/mol and 0.33 kJ/mol) are essentially identical in the calculated spectra and thus indistinguishable given the resolution of the instrument. Previous work with the tyrosine chromophore has shown that due to the two possible orientations of the phenolic OH group, the number of low-energy conformers can be doubled, as seen here^[26]. The two structures have the same backbone conformation and differ only in the rotation of the tyrosine side chain, which does not affect the backbone structure or H-bonding. For these reasons, it is likely that both are present in the 22-pole cold trap, and likely contribute to the 10 cm^{-1} breadth near the electronic origin. However, because the backbone conformations are identical, the two structures can be considered members of the same conformational family and treated as equivalent for the sake of this discussion. The

presence of only one conformational family is in agreement with previous IMS-MS data on room-temperature protonated YGGFL ions^[54].

Section 3.2.3 Analysis of Observed IR Transitions

The wavenumber positions and intensity patterns of the calculated and experimental transitions agree quite well after applying standard scaling factors, particularly in the amide NH stretch and amide I regions. Two of the NH stretch fundamentals involving the ammonium group and the carboxylic acid OH, are shifted all the way down to the 2900-3100 cm^{-1} region, and show extraordinary broadening. The calculations reproduce the correct frequency and intensity patterns for these transitions, but underestimate their frequency shift. This is consistent with previous reports on other peptide systems, where the π -bound and C=O bound NH stretch fundamentals of the NH_3^+ group are broadened and the magnitude of their shift to lower frequency is underestimated by DFT.^[30] Despite this, the excellent over-all agreement between experiment and calculation enable a structural assignment to be made. In the IR spectrum, all of the amide N-H stretches are shifted significantly to lower frequency than the “free” position (3450-3500 cm^{-1}), indicating a strongly reinforced hydrogen bonding architecture. In what follows, we use a Cn nomenclature common in the literature^[32] that characterizes a H-bond by the number of atoms ‘n’ in the H-bonded ring that connects the hydrogen of the XH donor and the acceptor atom. Using this nomenclature, the peptide backbone is designated from N-terminal to C-terminal [$\text{NH}_3^+(\text{a})_-(\text{b})_-(\text{c})/ \text{NH}(1)/\text{NH}(2)/\text{NH}(3)/\text{NH}(4)/\text{COOH}$] as $\pi_C17_C11/C14/C7/F/C10/C7(\text{OH})$, as shown in Figure 6. The transitions involving the C7, C10, and C14 hydrogen bonded N-H stretches do not involve the ammonium group directly and occur in the expected ranges previously observed for neutrals^[31,32,33]. The transition involving the $\pi\text{-NH}_3^+$ H-bond is $\sim 50\text{-}75 \text{ cm}^{-1}$ lower in frequency than that observed for a free NH stretch of

NH_3^+ [30]. Additionally, the C11 and C17 NH_3^+ NH stretches have large shifts to lower frequency and are broadened from the expected locations of amide $\text{NH}\cdots\text{O}=\text{C}$ H-bonds in neutral systems, further illustrating the effects of the charge site on strengthening the overall hydrogen bonded network of the molecule [31,32,33]. At 3012 cm^{-1} the COO-H stretch is shifted down by almost 600 cm^{-1} from the “free” position of $\sim 3585\text{ cm}^{-1}$ [31], an amount far in excess of a typical H-bond involving an amide group. Indeed, such an extreme shift to lower frequency, when paired with the large breadth and integrated intensity of the band, are reminiscent of the OH stretch infrared transitions of COOH groups when they engage in cooperatively-strengthened H-bonds like those formed in carboxylic acid dimers [55]. This suggests that the C-terminus of YGGFL is involved in an extremely strong hydrogen bond. The assigned backbone structure and the overall hydrogen bonding architecture responsible for these characteristic infrared signatures are discussed in more detail below.

3.3 Structural Analysis

Figure 6. Labeled hydrogen bonding architecture of $[\text{YGGFL}+\text{H}]^+$. The red and blue dotted lines in the chemical structure on the right link the groups connected by H-bonds, with donor-acceptor directions from N-terminal to C-terminal in red and from C to N-terminal in blue.

The optimized geometry for the assigned lowest energy structure of protonated YGGFL is shown in Figure 6 with the hydrogen bonding architecture labeled.

Section 3.3.1 Comparison to the IRMPD Structural Assignment

The assigned structure is different from the one previously assigned to protonated YGGFL by Polfer et al.^[15]. However, that assignment was based upon room temperature IRMPD data, as opposed to the data on cryo-cooled ions presented here. Although the structures are similar in terms of the ammonium hydrogen bonding character, they differ in the orientation and hydrogen bonding arrangement near the C-terminus and the leucine side chain. A comparison of the structures is shown in the Supplemental Material (Figure S6). Geometry optimization and harmonic frequency calculations were performed on the structure assigned by Polfer at the M05-2X/6-31+G(d) level of theory used in this work, resulting in a zero-point corrected relative energy 11.36 kJ/mol above the global minimum, with the higher energy structure providing a much poorer match to the cold experimental data in the hydride stretch region as compared to the global minimum structure assigned here (Figure S7). The spectroscopic differences between the two structures are far less obvious in the amide I/II region, which was the only region investigated in the previous study. These results suggest some caution in assigning structures based on room-temperature IRMPD data in the amide I/II regions alone.

Section 3.3.2 Comparison to IMS Data

The theoretical cross-section of the assigned lowest energy structure under cryo-cooled conditions was calculated to be $166 \pm 9 \text{ \AA}^2$, which is consistent with previous room-temperature IMS-MS measurements (162 and 160 $\text{\AA}^2$)^[8,54]. However, a variety of the higher energy structures also have calculated cross-sections that are consistent with the experimental IMS data,

despite having remarkably different predicted spectroscopic signatures and peptide backbone structures (Supplemental Materials Table S2). This highlights the discriminatory value of the spectroscopic approach relative to, for example, the measurement of a rotationally averaged cross-section alone.

Section 3.3.3 Hydrogen Bonding Arrangements of the Assigned Structure

The structure that is assigned on the basis of cold, IR-UV double resonance data contains a single backbone conformation held together by seven H-bonds involving as donors all three NH_3^+ NH groups, the carboxylic acid OH, and three of the four amide NH groups. The C7 ring involves a hydrogen bond between the amide NH on the second glycine residue (G2) and the carbonyl oxygen on the tyrosine (Y) residue. The amide NH on the first glycine residue (G1) forms a C14 H-bonded ring to the carbonyl oxygen of the C-terminus. The C10 H-bonded ring connects the amide NH on the leucine (L) residue to the carbonyl oxygen on G1. All three hydrogens in the ammonium group are involved in hydrogen bonds, forming C11 and C17 ring with the G2 and C-terminus C=O groups, respectively, and a π hydrogen bond with the tyrosine π cloud. Finally, the *trans* carboxylic acid OH group enables a strong hydrogen bond with the carbonyl oxygen on the phenylalanine residue (F).

Section 3.3.4 Effects of the Charge Site on the Structure

It is clear from this structure that the peptide backbone is wrapping itself around the NH_3^+ site, self-solvating the charge with two of its C=O groups and the tyrosine ring. At the same time, the structure facilitates the formation of a network of other hydrogen bonds, some of which are cooperatively strengthening one another. We have already noted that the carboxylic acid OH group is engaged in a very strong H-bond, as is evident now from the calculated structure, in

which the OH group is flipped from its more typical *cis* position to the *trans* orientation to form an unusually short H-bond (1.72 Å) that is cooperatively strengthened by the NH_3^+ H-bond to its C=O group. Indeed, the $\text{NH}_3^+ \rightarrow \text{O}=\text{C}-\text{OH} \rightarrow \text{O}=\text{C}(\text{F})$ H-bonds are a defining feature of the resulting structure by which the protonated amine site at the N-terminus and the F-L amide groups are linked to one another via strong directional H-bonds.

The dominating effect of the protonated amine site is also evident in other ways. For instance, the phenylalanine residue NH is not involved in a H-bond, yet its frequency is at 3380 cm^{-1} , almost 100 cm^{-1} below the typical frequency of a free amide NH group^[31]. This is another consequence of the protonated amine, which is H-bonded to this same amide group's C=O moiety via a C11 H-bond (Figure 6). The fact that the calculations pick up on this shift to lower frequency for the F NH group means that a significant electronic distortion of the amide group is produced by binding of its C=O group to NH_3^+ .

Finally, the large blue shift of the electronic origin and the dense low-frequency vibronic structure in the UV photofragment spectrum result from the NH_3^+ binding directly to the tyrosine ring. It is known from previous work on the UV spectroscopy of phenyl derivatives that π H-bonds produce a shift to the blue in the $\text{S}_0\text{-S}_1$ transition of the phenyl ring. Here we see that the π H-bond involving the NH_3^+ site is an extreme example of this effect, producing a +600 cm^{-1} shift in the UV spectrum, some 5-10 times that of typical protonated solvents such as H_2O or HCl ^[56,57]. Furthermore, since the NH_3^+ group is so strongly solvated by even remote portions of the rest of the peptide backbone, $\pi\text{-}\pi^*$ excitation will lead to Franck-Condon activity in some of the lowest-frequency vibrations in the ion, producing the observed congestion in the UV photofragment spectrum.

3.4 Photofragmentation Mass Spectra

Figure 7. UVPD mass spectra of $[\text{YGGFL}+\text{H}]^+$ with resonant UV excitation at $35,684\text{ cm}^{-1}$ (a) and with non-resonant UV excitation at $35,657\text{ cm}^{-1}$ preceded by resonant IR excitation at 3394 cm^{-1} (b). Identities of the major fragment peaks are indicated. Each spectrum is the average of 150 scans.

The UV spectrum shown in Figure 3 is a measure of the total photofragment ion signal versus UV laser wavelength. The photofragment mass spectrum at the electronic band origin ($35,684\text{ cm}^{-1}$) is shown in Figure 7(a). The UV photodissociation (UVPD) mass spectrum primarily consists of the known low energy CID-type fragment (b_4 , a_4 , y_3 , y_2/b_3) ions for protonated YGGFL, while the lower abundance unlabeled peaks are consistent with the

successive fragmentation of sequence ions and the internal fragments observed for higher energy CID^[34]. The a_4/b_4 intensity ratio is similar to those observed under high energy CID conditions, and is consistent with the large internal energy imparted by absorption of a single UV photon at this wavelength (427 kJ/mol), with b_4 ions undergoing secondary fragmentation via loss of CO to form a_4 ions. Note that the internal energy distribution of these UV-excited cryo-cooled ions is essentially a delta function at the UV wavelength ($E_{\text{int}} = 427$ kJ/mol), since prior to UV excitation the ions are in the ground state zero-point level by virtue of the cooling afforded by the 5 K temperature of the 22-pole trap. This is in stark contrast to a thermalized ion whose internal energy distribution is governed by a Boltzmann distribution.

Electronic excitation also leads to cleavage of the tyrosine C_α - C_β bond (i.e. the loss of a *p*-hydroxy benzyl radical ($m/z = [M+H-107]^+$). This 107 Da loss has been shown to be a common photofragmentation pathway for tyrosine containing peptides subjected to UVPD^[26,27,40]. This fragmentation pathway is thought to originate through an electronic predissociation pathway where an excited ion undergoes a curve crossing onto a dissociative electronic potential energy surface. The common CID-type fragments are thought to originate from a competing pathway where internal conversion of the excited ions from S_1 to a continuum of vibrational levels in the highly excited ground electronic state is followed by unimolecular dissociation. These competing pathways are indicated in Figure 2a.

Figure 7(b) shows the UVPD spectrum generated under the IR gain experiment conditions, where the parent ion first absorbs a resonant IR photon (3394 cm^{-1}) prior to non-resonant UV excitation at a wavelength just red of the S_0 - S_1 origin ($35,657\text{ cm}^{-1}$). The identities of the peaks present in the mass spectra shown in Figure 7 are identical under both resonant UV (a) and resonant IR/non-resonant UV (b) conditions. However, the relative peak intensities are

different. The parent signal, UV laser powers and detection conditions were identical for both experiments, ensuring that the vertical axis accurately portrays peak intensity information. In the two UVPD spectra, the relative intensity ratio for the -107 Da loss peak and the a_4 fragment peak are inverted, with the tyrosine side chain cleavage becoming more prominent in the case of IR and UV irradiation. Note that the integrated intensities of the -107, a_4 , and b_4 photofragment ion peaks is almost the same with or without the IR excitation step, indicating that increased fragmentation via loss of the 107 Da fragment occurs in competition with internal conversion to form the a_4/b_4 products.

These results suggest two possible reasons for the enhanced site specific cleavage of the tyrosine side chain following UV excitation of the IR-excited ions. In the first case, the change in total energy absorbed by the molecule may result in a higher probability of curve-crossing to the dissociative electronic state that leads to loss of the -107 Da fragment, at the expense of internal conversion to the ground state. In the second case, absorption of an IR photon 100 ns prior to UV irradiation may allow for isomerization to different energetically low-lying conformations prior to photofragmentation. One or more of these newly populated conformations may have a strong absorption to the red of the electronic origin for the original conformer, which is observed as the measured IR gain cross-section. Previous work on the tyrosine chromophore has shown that different conformations of the same molecule can have different primary photofragmentation channels^[26,27,40]. The absorption of the IR photon places an additional 40 kJ/mol in the ion, an amount sufficient to access some 27 calculated conformations from the assigned global minimum structure. Depending on the heights of the barriers separating these minima, isomerization could lead to the observed enhancement of -107 Da loss channel. We hope that

future UVPD spectra on other analytes may contribute further insights into these excited state dynamics.

3.5 Structural Effects on Fragmentation

With a firm structural assignment for cold protonated YGGFL, it is worth considering how this structure might influence the observed strong preference for peptide backbone fragmentation at the b_4/a_4 site. We noted already that, aside from the 107 Da loss channel, the fragment peaks formed by UVPD appear to be similar to those formed in a high collision energy CID experiment, consistent with these fragment ions being produced following internal conversion onto the ground state surface. Interestingly, the assigned structure for protonated YGGFL (Figure 6) links the NH_3^+ site directly to the C-terminus C=O group and the G2 carbonyl group, suggesting that the F carbonyl group relevant to the b_4 ion formation pathway is not protonated by the ammonium charge site directly but by a proton hopping from the adjacent G2 carbonyl oxygen and/or the adjacent C-terminus. Protonation of the G2 oxygen and subsequent hopping to the F-L amide bond oxygen to initiate cleavage follows the traditional model outlined by the mobile proton theory^[58]. While protonation at the carbonyl oxygen of the C-terminus could also follow this model, an alternative proton transfer mechanism is presented by the cooperatively strengthened $\text{NH}_3^+ \rightarrow \text{O}=\text{C}-\text{OH} \rightarrow \text{O}=\text{C}(\text{F})$ hydrogen-bonded network. This network ties together the compact structure for protonated YGGFL and could also serve as a kind of conduit for proton transfer that could drive fragmentation preferentially towards the b_4 site. In this scheme, the mobile proton leading to backbone amide group protonation is not necessarily the charge carrying proton.

However, what is not clear, based on the present data, is whether proton transfer occurs on a timescale faster than conformational isomerization of the energized ion, or whether isomerization precedes proton transfer. If isomerization is fast compared to proton transfer, the compact ion structure will unfold upon activation to a new conformational distribution that may bear little resemblance to the original structure. Alternatively, if proton transfer is faster than isomerization, the starting structure will be directly relevant to the observed fragmentation pattern, and could direct proton transfer down specific pathways. Data of the type provided by this work has the potential to help sort out the circumstances in which the ion finds itself in one limit or the other.

4. Conclusions

The preferred conformation of cold, gas-phase protonated leucine enkephalin was investigated in this work via UV and IR spectroscopy. A comparison of the IR spectra measured in the hydride stretch, amide I and amide II regions to vibrational frequencies calculated via density functional theory indicates the presence of a single backbone conformation. This is a revised structural assignment from that previously assigned based on room-temperature IRMPD data^[15]. The assigned structure is held together with a total of seven H-bonds, three of which self-solvate the NH_3^+ site. Two of the strongest hydrogen bonds form a kind of bridge linking the protonated N-terminus, the C-terminus COOH group, and the phenylalanine C=O group ($\text{NH}_3^+ \rightarrow \text{O}=\text{C}-\text{OH} \rightarrow \text{O}=\text{C}$). Cleavage of the F-L peptide bond results in formation of the b_4 ion, which is a prevalent, low-energy fragmentation pathway for $[\text{YGGFL}+\text{H}]^+$. The ability to unambiguously assign this structure is a result of the cryogenically-cooled IR-UV double resonance spectroscopy used in this work, as opposed to other methods of structural determination. During UVPD through the $\text{S}_0\text{-S}_1$ absorption due to the tyrosine moiety, excited

state processes lead to cleavage of the tyrosine side chain at the C_{α} - C_{β} bond (loss of 107 Da), which occurs in competition with formation of the b_4/a_4 ions that likely are formed following internal conversion to form hot ground state ions. The relationship between ion structure and fragmentation pathway is one that can be uniquely explored using the spectroscopic tools afforded by experiments of this type.

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