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Spectroscopy of Protonated Peptides Assisted by Infrared Multiple Photon Excitation

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We report here a new technique for spectroscopic studies of protonated, gas-phase biomolecules and demonstrate its utility by measuring highly resolved electronic and infrared spectra of peptides of up to 17 amino acids. After UV excitation of an aromatic chromophore of a protonated peptide, a CO_2 laser further excites the molecules, increasing their vibrational energy and hence their dissociation rate, allowing detection of the UV excitation by monitoring the resulting photofragments. We show that addition of the CO_2 laser excitation increases the fragmentation yield on the time scale of our experiments by as much as 2 orders of magnitude, significantly enhancing the sensitivity of UV photofragment spectroscopy. We also demonstrate that this approach can be applied in an IR-UV double-resonance scheme, allowing measurement of conformer-specific infrared spectra of protonated peptides.

The biological functions of proteins are largely determined by the 3-dimensional structures that they adopt in vivo. Unraveling these structures, and the interactions that determine them, is thus a major focus of biophysical research. Protein structures typically measured in crystals or in vitro may differ substantially from those in biological environments, where interactions with other species influence their conformation. Theory has the potential to calculate peptide structures and reveal the nature of the forces that control them, but calculations need to be verified by experiment. This can be done by predicting structures of small species in an isolated environment, then extending to larger but still isolated peptides and proteins and finally to solvated species including interactions with other molecules. At each step, theoretical approaches should be validated and perhaps adjusted by comparison with experimental data. Infrared (IR) spectroscopy of biomolecules isolated in the gas phase can provide such data in the form of sets of vibrational frequencies. Each set, if measured on the same conformer of a molecule, constitutes a spectroscopic signature that can be used to test and calibrate theory. Thus from an experimental point of view, one would like to measure highly resolved, conformer-specific IR spectra of gas-phase biomolecules over a wide frequency range. Several groups employ a form of IR-UV laser double resonance to measure conformer-selective IR spectra.[1−7] This approach can be combined either with cooling of neutral molecules in supersonic jets[1−6] or protonated species in ion traps to suppress spectral congestion.[8−10] The latter approach, when coupled with electrospray ionization, has proven to be a particularly powerful spectroscopic tool to investigate small peptides.[11−13] One challenge for spectroscopic studies of charged biomolecules in the gas phase is their low concentration, which is limited by space-charge effects. This makes the use of spectroscopic detection techniques such as laser-induced fluorescence or cavity-ringdown difficult if not impossible. In this case, one can use photofragmentation of the excited ions with subsequent collection of charged fragments as a highly sensitive means of spectroscopic detection. For aromatic amino acids[9] and small peptides containing these chromophores,[11−13] UV excitation puts the molecule above the dissociation threshold, where it can fragment either directly from an excited electronic state or, subsequent to internal conversion, from highly vibrationally excited levels of the ground electronic state. For large molecules one expects the latter process to be statistical, in which case the dissociation rate drops steeply with increasing size.[14] For a fixed observation time, which is fundamentally limited by the lifetime of spontaneous IR emission, this will make photofragment spectroscopy of large ions inefficient.

We report here a spectroscopic technique that allows an increase in the photofragmentation yield of large molecules of more than 2 orders of magnitude on the time scale of our experiment, significantly extending the utility of IR/UV photofragment spectroscopy. In this approach, illustrated schematically in Figure 1, protonated species that have been first excited electronically in the UV undergo infrared multiple photon excitation (IRMPE) by a pulsed TEA CO_2 laser. Because statistical dissociation rates increase upon additional vibrational excitation, we expect an increase in fragmentation yield of the excited species during the 10 ms that we allow for dissociation before detecting the fragments. Controlling the CO_2 laser fluence permits selective fragmentation of the UV pre-excited species without noticeable dissociation of electronically unexcited molecules.

Our experimental apparatus has been described in detail elsewhere.[13,15] Briefly, we generate protonated peptides in the gas phase from solution using a nanospray ion source. The ions of interest are selected by a quadrupole mass filter and transferred to a cold ion trap, where they are cooled to ∼10 K in collisions with helium and stored. After a delay of 40−90

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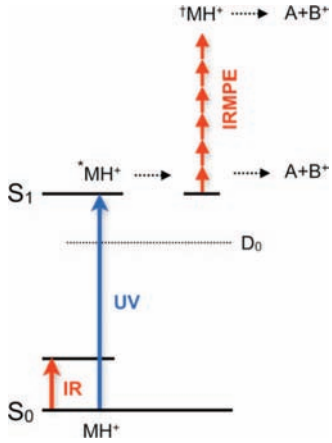

Figure 1. Simplified diagram of excitation in the IR-UV depletion experiment, assisted by IRMPE, as applied to a singly protonated peptide MH^+. The delay between each of the three laser pulses is 200 ns. Only the most energetic cations $^{\dagger}MH^+$ dissociate within the 10 ms delay between UV excitation and fragment detection.

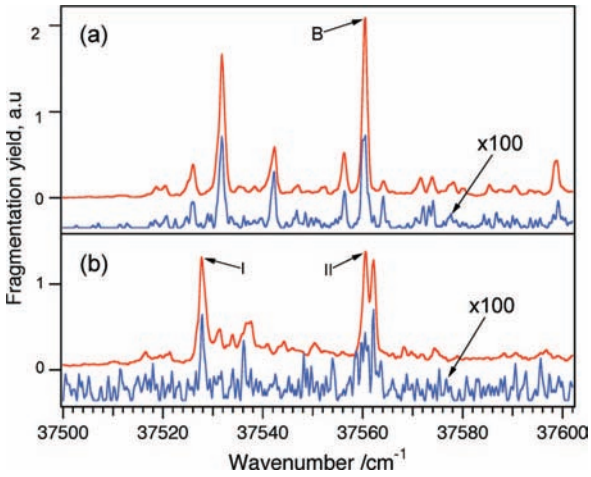

Figure 2. Electronic spectra of (a) Ac-Phe-$(Ala)_5$-Lys-H^+ and (b) Ac-Phe-$(Ala)_7$-Lys-H^+-$(Pro)_2$-$(Ala)_5$-Lys-H^+ obtained by UV fragmentation (blue lines) and by UV excitation assisted by IRMPE (red lines). The monitored fragments with mass-to-charge ratio of (a) 600 and (b) 710 correspond to the loss of phenylalanine side chain. The CO_2 laser is tuned to the 9P(16) line (1050.1 cm^{-1}).

ms, during which time the helium is pumped out, the peptides are excited successively with a UV and a CO_2 laser pulse. The latter consists of an intense 150 ns leading peak followed by a 2 μs long tail. We allow 10 ms for the laser-activated ions to dissociate before ejecting the contents of the trap and detecting a fragment of particular *m/z* using a quadrupole mass spectrometer. An excitation spectrum is generated by recording the fragment signal as a function of the frequency of the UV laser. We obtain a vibrational spectrum by monitoring the depletion of the UV-induced fragment signal as a function of the frequency of an IR laser that we fire 200 ns before the UV, keeping the frequency of the latter tuned to a transition in the electronic spectrum.

Figure 2 compares UV photofragment spectra of two protonated peptides, $AcFA_5K\cdot H^+$ and $AcFA_7K\cdot H^+P_2A_5K\cdot H^+$, obtained with and without CO_2 laser assistance. Both peptides contain a phenylalanine chromophore that absorbs in the near-UV spectral region. The spectra are recorded by detecting protonated fragments that correspond to the loss of the phenylalanine side chain. At the CO_2 laser fluence used to obtain these spectra (3−4 J/cm^2) we observe none of these fragments when the UV laser is blocked. The fragmentation yield changes

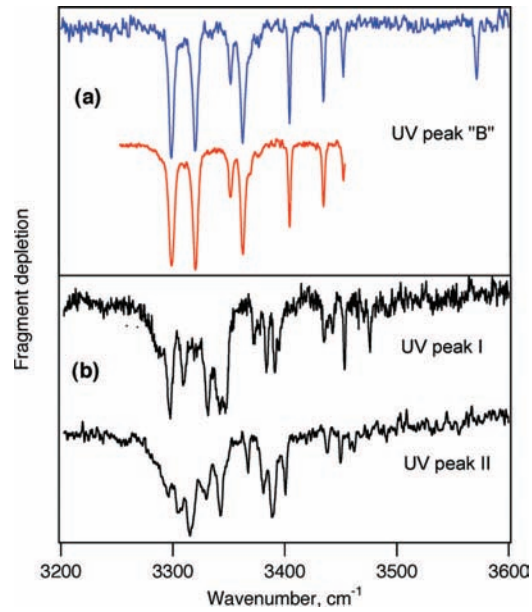

Figure 3. IR-UV depletion spectra of (a) Ac-Phe-$(Ala)_5$-Lys-H^+ and (b) Ac-Phe-$(Ala)_7$-Lys-H^+-$(Pro)_2$-$(Ala)_5$-Lys-H^+, obtained by fixing UV laser wavenumber at (a) peak "B" and (b) peaks I and II of Figure 2. The maximum level of the depletion is (a) 91% and (b) 62%. All spectra, except the upper trace of panel a, have been obtained with the assistance of IRMPE.

weakly with the frequency of our line-tunable CO_2 laser and exhibits a maximum around the 9P(16) line (1050.1 cm^{-1}) for both peptides. The two spectra of the smaller, singly charged peptide, $AcFA_5K\cdot H^+$, shown in Figure 2a, are essentially identical in all main features, demonstrating that IRMPE is insensitive to the details of the UV excitation and thus does not distort the spectrum. The striking feature of these results is the increase of the dissociation yield by more than 2 orders of magnitude induced by the addition of the CO_2 laser. This improvement allows UV photofragment spectroscopy of the larger, doubly charged peptide, $AcFA_7K\cdot H^+P_2A_5K\cdot H^+$ (Figure 2b), which contains 220 atoms. We also obtained significant enhancements in the yield of the side-chain loss channel for protonated peptides that contain tyrosine and tryptophan ($AcYA_5K\cdot H^+$, $AcYA_4FK\cdot H^+$, $AcWA_5K\cdot H^+$, $AcYA_7K\cdot H^+P_2A_5K\cdot H^+$), verifying that this effect is not specific to phenylalanine. The recorded UV spectra have high signal-to-noise ratio and are thus suitable for conformer-selective, IR-depletion spectroscopy.

Figure 3a compares the IR spectra of the smaller peptide, $AcFA_5K\cdot H^+$, measured with and without CO_2-laser assistance. The UV laser is tuned to the peak labeled "B" in the spectrum of Figure 2a, which we previously assigned as belonging to a conformer "B" of this peptide,[12] and thus the IR spectra of Figure 3a reflects the absorption spectrum of only this conformer. The two spectra are essentially identical, with no distortion induced by CO_2-laser-assisted dissociation. Figure 3b shows two IR depletion spectra of $AcFA_7K\cdot H^+P_2A_5K\cdot H^+$ obtained with CO_2-laser assistance and by fixing UV laser frequency on peaks I and II in the electronic spectrum in Figure 2b. It is important to note that no depletion was detected without CO_2-laser assistance, due to the poor signal-to-noise ratio of the UV-only fragmentation signal. The two IR spectra are clearly different, indicating that they arise from two different conformers (or perhaps two distinct groups of conformers if there are overlapping transitions in the UV spectrum). We thus demonstrate that IRMPE subsequent to UV pre-excitation extends

conformer-specific IR-UV depletion spectroscopy to cold, protonated peptides of up to 17 amino acids. These spectra present a clear challenge to theory, which needs to demonstrate the ability to calculate structures of such large species with accuracy sufficient to reproduce the measured vibrational frequencies in order to establish credibility.

The detection technique presented here is similar to what we have previously called Infra Red Laser Assisted Photofragmentation Spectroscopy (IRLAPS), which we employed for detecting weak overtone transitions in small, neutral polyatomics,[16,17] and which Lee and co-workers applied to protonated water clusters.[18] There are some qualitative differences in applying this general scheme to such large systems, however. In smaller molecules, the ability to discriminate against dissociation of ground-state molecules with the CO_2 laser was based primarily on two factors: the anharmonic red-shift in the CO_2-laser absorption frequency and lower dissociation threshold fluence for pre-excited molecules.[19] For the larger systems explored here, the enormous density of vibrational states will lead to a low average occupation number of most vibrational modes, even at the energy of UV excitation. In this case one expects only a small anharmonic shift of the resonant frequency pumped by the CO_2 laser and insignificant statistical broadening in the IR absorption spectra,[20,21] in contrast to the case in small molecules.[22] This limits our ability to discriminate against CO_2 laser dissociation of ground-state molecules, although it is likely to lower the fluence required to dissociate pre-excited molecules. Moreover, the IR spectra of peptides should exhibit several IR active vibrations around 10 μm that may overlap, and this will increase the cross section for IRMPE. This is consistent with the relatively low threshold CO_2 laser fluence (3−4 J/cm^2) required for fragmentation of the UV-pre-excited peptides on the 10 ms time scale of our experiment.

There are two observations, however, that suggest that the dissociation of the peptides investigated here may not be statistical as we initially assumed. First, the fragmentation channel that is always greatly enhanced by IRMPE of UV pre-excited molecules is the loss of the neutral, aromatic side chain via cleavage of the $C_\alpha-C_\beta$ bond, which is not the weakest in these molecules. The latter is evident from the fact that IRMPE of our cold peptides with only the CO_2 laser yields several *b*- and *x*-type fragments, but no fragments that correspond to the loss of an aromatic side chain. Second, this fragmentation occurs promptly after CO_2 laser excitation, since the dissociation yield does not drop when we shorten the time allowed for dissociation from 10 ms down to ∼0.2 ms—the minimum delay possible in our experiment. These two observations lead us to suggest that the fragmentation induced by IRMPE of the UV-pre-excited protonated peptides occurs not on the electronic ground surface, but rather on an electronically excited-state surface or in a promptly formed intermediate species. Recent, detailed studies of the UV photodissociation mechanism of gas-phase aromatic amino acids suggest the involvement of an electron transfer process followed by H-atom detachment or formation of a biradical with subsequent $C_\alpha-C_\beta$ bond cleavage.[23,24] Whether a similar mechanism governs the fragmentation dynamics in the larger peptides investigated here is currently a subject of detailed study in our laboratory.

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