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Time resolved photodissociation of small peptide ions

Combining laser desorption with ion trap/reflectron TOF mass spectromerty

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Abstract. A new instrument has been constructed whose purpose is to study time-resolved photodissociation (TRPD) of small peptide ions. Laser desorption of neutral peptides is combined with laser photoionization in an ion trap followed by thermalization, laser photodissociation and time of flight mass analysis. Ionization and excitation take place through an aromatic chromophore at the C-terminus of the peptide whereas dissociation produces the immonium ion at the N-terminus. The long-range purpose is to uncover the role of IVR in unimolecular fragmentations of peptide radical cations. One avenue is the determination of microcanonical rate constants at a well-defined internal energy E for a series of peptides possessing the same chromophore, undergoing the same fragmentation but having a variable number of degrees of freedom. This paper demonstrates the feasibility of the method and provides the first attempts at rate measurements for the peptide leucyl tyrosine (LeuTyr).

PACS. 82.80.Ms Mass spectrometry (including SIMS, multiphoton ionization and resonance ionization mass spectrometry, MALDI) – 82.39.-k Chemical kinetics in biological systems – 87.15.Mi Spectra, photodissociation, and photoionization; luminescence

1 Introduction

There is increasing interest in recent years in anhydrous protein and peptide ions [1]. The combination of lasers with mass spectrometry has been a particularly powerful tool in analyzing biomolecules. Laser desorption (LD) and ejection of biomolecules from the condensed phase [2] has allowed the transfer of these, otherwise thermally labile species, intact without breakup, into the gas phase. The desorbed species can be ionic or neutral. The strong desorption of neutrals opened up the possibility of post-ionization techniques, particularly multiphoton ionization (MPI) by a second laser – so-called "two-step laser mass spectrometry" [3]. The combination of LD of neutrals, entrainment in a supersonic expansion and laser ionization provides control over the energy deposited in the ion.

The development of mass spectrometry of biomolecules has raised again some old questions. For example, does intramolecular vibrational redistribution (IVR) precede fragmentation in very large proteins, having an extremely large number of degrees of freedom? Does electronic energy relaxation take place or is there site selectivity and charge directed reactivity [4]?

We have taken upon ourselves the study of the extent of statistical *versus* site selective fragmentation of small peptide radical cations. The objective is to find out to what degree does the mode of preparation of the ions and the initial site of excitation affect the type, degree and rate of fragmentation. A major question that has been posed is: does a peptide behave as a collection of amino acids, like beads on a string [4,5], or as a viable super-molecule, namely an ordinary organic molecule albeit with a large number of degrees of freedom. It has recently been suggested theoretically that the charge acts as a scout to find the preferred fragmentation site and IVR does not necessarily precede fragmentation. This contradicts the general wisdom in mass spectral statistical theories such as RRKM/QET and has far reaching implications concerning the fragmentation of large bio-molecules that are now the central topic of analytical research in mass spectrometry. The theoretical effort coincided with some very nice experiments done on small peptides having a chromophore through which laser MPI and photodissociation (PD) proceed [5]. Fragmentation of these peptides has been observed as a direct outcome of the initial ionization and excitation at a specific site, followed by intramolecular charge transfer.

Our plan has been to use controlled laser desorption to evaporate the neutral peptides and to cool them by entraining them in a supersonic expansion. Ionization can

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then follow through the chromophore by laser MPI followed by laser PD. The fragmentations can be studied by time of flight (TOF)/reflectron mass spectrometry. The peptides we have chosen for study were among the ones studied by Weinkauf *et al.* [5], namely $(X)_n{-}Y$, where X = leucine and Y = tyrosine. As a first step we have chosen to study mass spectral fragmentations, and to determine rate-energy ($k(E)$) dependencies.

The determination of the microcanonical rate constant, $k(E)$ *via* time-resolved photodissociation (TRPD) is the subject of this presentation. TRPD is particularly well suited for energy resolved measurements of slow dissociation processes [6–12]. Traditionally TRPD, which has been developed by Dunbar and coworkers, has employed the Fourier transform ion cyclotron resonance (FTICR) technique for ion trapping and mass spectrometric measurements. In TRPD ions are produced by an electron ionization pulse or by a laser MPI pulse. They are allowed to relax to their ground state for a pre-selected time, are then photoexcited by a short laser pulse and the time resolved appearance curve of a mass selected product ion is followed. This can be done for different photon energies provided the ions absorb in the right wavelength region. Microcanonical rate constants are deduced by this method for time ranges that are impossible to achieve by photoelectron photoion coincidence (PEPICO) methods, namely in the millisecond range.

The FTICR is not a necessary prerequisite for TRPD measurements and simpler ion trapping devices may suffice. Neusser and coworkers [13] succeeded in measuring the unimolecular decay of perdeuterated benzene$^{\bullet+}$ with a cylindrical ion trap (CIT), the simplest version of an ion trap. The suitability of our instrumentation – a quadrupole ion trap/reflectron TOF device – for decay time investigations has been demonstrated first through repeating the previously published single-laser one-color photodissociation of perdeuterated benzene [13,14]. This was followed by a two-color two-laser photodissociation experiment on naphthalene that was compared with the previous TRPD results from FTICR experiments [14,15]. It was demonstrated that the results from the quadrupole ion trap are in good agreement with the literature data. Once this was established we have chosen to test the simplest pair of polycyclic aromatic hydrocarbon (PAH) isomers, for which we had previously carried out *ab initio* computations [16] – naphthalene$^{\bullet+}$ and azulene$^{\bullet+}$. Microcanonical $k(E)$ data on this pair of isomers, derived using TRPD on the quadrupole ion trap/reflectron device were deduced and have been published [14]. The present paper deals with our first attempts at measurements of microcanonical rate constants for peptide fragmentations.

2 Experimental

2.1 The instrument

The experimental apparatus is shown schematically in Figure 1. It consists of three vacuum chambers, for laser desorption, for the quadrupole ion trap and for a reflectron time-of-flight (TOF) analyzer, respectively. The latter two parts have been employed by us and have been described in detail in the past [14,17–19]. The reflectron TOF mass spectrometer with a quadrupole ion trap (from R.M. Jordan Company) has been developed originally by Lubman and coworkers [20] to combine the storage capabilities of an ion trap with the speed and resolution of a TOF device. The goal of our research employing such a combination is similar to that of Neusser's, namely the observation of unimolecular decay of energy selected ions on the ms time scale combined with a fast mass detection process [21].

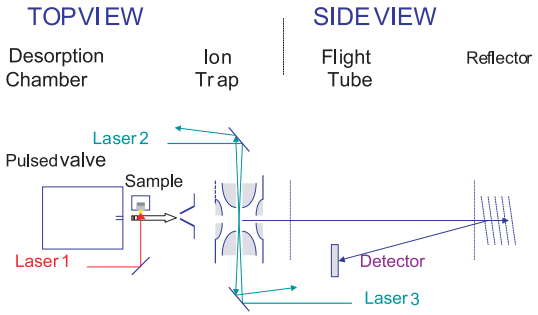


Fig. 1. Quadrupole ion trap/reflectron time-of-flight mass spectrometer. Laser no. 1 causes desorption of the peptide sample, laser no. 2 causes ionization and laser no. 3 causes dissociation.

The first chamber is constructed on axis with the other two. Laser desorption (LD) followed by jet cooling is carried out in this chamber in a similar manner to the one described by de Vries and coworkers [22–24]. LD of peptides is performed by the 1 064 nm fundamental output of a Nd:YAG laser (Minilite from Continuum, 1 mJ/pulse, 10 ns duration) which is focused loosely to a spot of the order of 1.5 mm diameter within 4 mm in front of the nozzle (0.5 mm diameter) of a pulsed supersonic valve (R.M. Jordan Company, 3 bar backing pressure, 4200 Amp operating current). A telescope is used for alignment of the desorption laser spot with respect to the position of the nozzle. A graphite rod is used as a substrate covered evenly with the peptide sample. The sample substrate is moved translationally by a stepping motor to ensure that fresh surface is provided at fixed intervals. The desorbed neutrals are entrained and cooled by collisions with CO_2 or Ar gas through the nozzle. The neutral molecules are introduced into the second chamber downstream, through a 3 mm diameter skimmer.

2.2 Photoionization and photodissociation

Photoionization and photodissociation take place in the second chamber where a quadrupole ion trap (a "Paul-trap" [25]) is used as the ion source. The quadrupole ion trap consists of two endcap electrodes and a ring electrode situated between them. The inside surfaces of the trap are hyperbolic. There are six 3.5 mm diameter apertures. Two apertures are in the two endcaps, one of which is used as the inlet of the laser desorbed neutral sample and the other for ion ejection. Four are in the ring electrode with two at the top and bottom (vertical direction), and the other

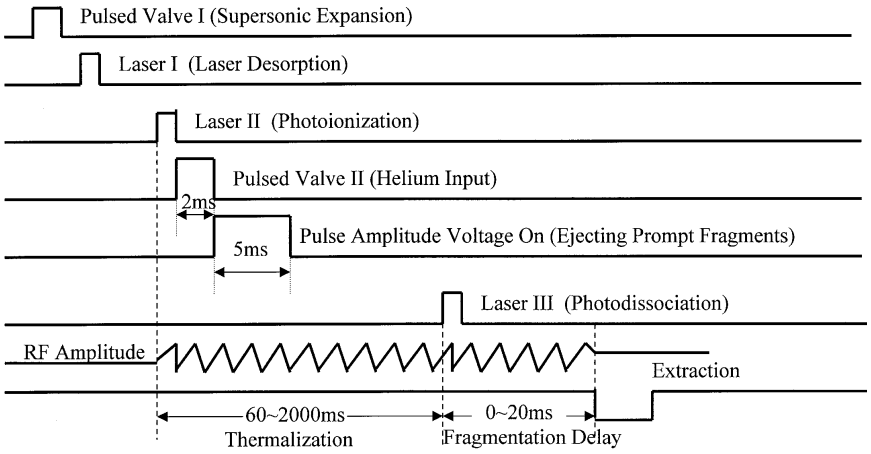


Fig. 2. Pulse sequence for the LD/TRPD experiment.

two in the horizontal direction for the laser beams. About 1 cm above the top aperture is the nozzle of a General Valve pulsed valve that can provide pulsed helium buffer gas. During operation, the RF voltage is applied to the ring electrode while both endcaps are held at 0 V. The RF field serves to trap ions present within the volume of the trap until they are ejected by application of a DC extraction pulse to the exit endcap. During extraction, the RF voltage is turned off.

Cold intact neutral molecules undergo two-photon ionization by either one of the following methods: the frequency-doubled output of a dye laser (ND6000, 275–290 nm, 0.3–0.8 mJ/pulse) pumped by a Nd:YAG laser (Surelite from Continuum), or alternatively the 266 nm fourth harmonic of a Nd–YAG laser (Minilite from NewWave, 1.3 mJ/pulse). In one-color experiments, the photon employed for photodissociation has the same wavelength as is used for the two-photon ionization. Photodissociation is achieved in two-color experiments by absorption in the visible wavelength region (530–580 nm, 2–2.4 mJ/pulse). Both the photoionizing- as well as the photodissociating-laser are aligned to pass through the center of the ion trap, but from opposite directions (see Fig. 1). Both laser beams are focused. Setting the lenses at well-defined positions out of focus changes the laser spots to increase or decrease the laser intensities.

2.3 Time-resolved photodissociation (TRPD)

Two digital delay/pulse generators control all events. Figure 2 shows the timing sequence employed in the measurements. Laser desorption takes place after the opening of the nozzle of the Jordan pulsed valve. The desorbed neutrals are transported into the ion trap where they are photoionized. The pulse to the ionization laser is synchronized with the RF pulse. The storage time of the ions is controlled by another output of the pulse generator by triggering the extraction pulser that serves the dual purposes of ejecting the ions and providing the start-time reference of the TOF mass analysis. The data are collected by a LeCroy Waverunner LT262 digital oscilloscope.

Ionization can produce the parent ions in a variety of excited states. It can also lead to prompt fragmentations. In two-color TRPD, the ionization and excitation steps are separated in time so that the parent ions can undergo radiative and collisional thermalization to lose their excess internal energy acquired in the ionization step. In some of the experiments we are applying two additional pulses between the ionization and excitation laser pulses. Pulsed helium buffer gas is provided for the dual purpose of bringing the ions into the center of the trap and for enhancing collisional thermalization. This event is triggered by the second digital delay/pulse generator that is triggered by the Q-switch output of the ionization laser, which makes sure the nozzle opens after the ionization event [26]. The bulk of the helium gas has to be pumped out by the time the excitation laser leading to photodissociation is turned on to ensure that the photoexcited ions undergo no further relaxing collisions. A second pulse serves to eject any prompt fragments that are formed. This is achieved by temporarily increasing the amplitude of the RF voltage that is applied to the ring electrode [27].

Following thermalization the photoexcitation laser is fired to excite the thermalized ions to well defined internal energies. This is followed by measurements of the fragment ion buildup as a function of the trapping delay time leading to the TRPD curve. Each time point along the TRPD curve is the result of 500 laser shots. Time dependent daughter ion intensities are normalized *via* the corresponding parent ion intensities, taking the parent intensity at the shortest trapping time as the reference point, in order to take into account fluctuations in light intensity for different laser shots.

The dissociation kinetics of the photoexcited peptide ions can be deduced by fitting the measured buildup curve of their fragment ions.

Dipeptides and tripeptides were purchased from Sigma (Israel) and were used without further purification.

3 Results and discussion

3.1 Mass spectra

The mass spectra of a series of peptides were determined and compared with previous data by Weinkauf *et al.* [5]. This established the ability to combine laser desorption

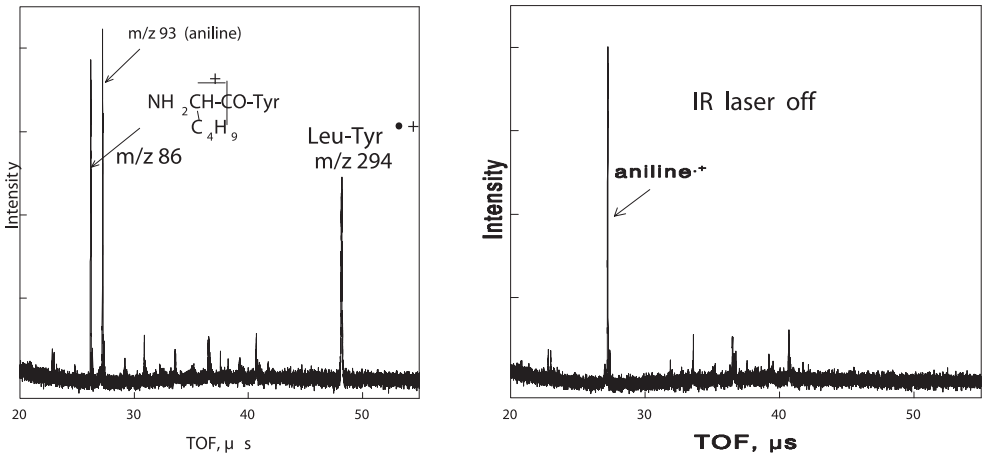
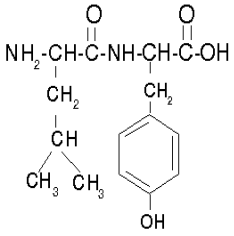


Fig. 3. Resonant MPI fragment mass spectra of the dipeptide LeuTyr; one-color experiment at 281 nm. The right-hand panel is with the desorption laser off whereas the left-hand panel is with the desorption laser (1 064 nm) on. The carrier gas is spiked with aniline.



Scheme 1. The dipeptide LeuTyr.

with laser ionization in our system. Figure 3 shows the mass spectra for the dipeptide leucyl tyrosine, LeuTyr (Scheme 1) with the ionizing laser in the UV on (single color experiment at 281 nm). The right-hand panel is with the desorption laser off whereas the left-hand panel is with the desorption laser (1 064 nm) on. Without desorption what is seen is a peak due to aniline with which the argon carrier gas is spiked. With the desorption laser on there are two major peaks − the parent ion at mass to charge ratio m/z 294 and the immonium ion [28] at m/z 86. This demonstrates as has been known [5] that although ionization is at the aromatic chromophore of the C-terminus there is rapid charge transfer and dissociation occurs at the N-terminus. Similar spectra were observed for the higher peptides, $(X)_n{-}Y$, where X = leucine and Y = tyrosine and $n > 1$, namely a TOF spectrum made up of the parent ion at the appropriate m/z ratio and an immonium ion at m/z 86. As expected, lowering the power of the ionizing UV laser reduces the immonium ion intensity.

We tested next the pulsing scheme shown in Figure 2. The mass spectra of LeuTyr were measured under different pulsing conditions as shown in Figure 4. Each of these spectra were measured with the supersonic expansion (pulsed valve I) on, the desorption laser pulse on, the UV photoionization laser on and the pulsed helium valve on. The top panel shows a spectrum obtained as before in a single color experiment. The photodissociation laser (III) is turned off and prompt fragments formed by the ionizing laser are not ejected. A relatively high fragmentation yield due to a high ionizing laser power is observed. In the middle panel the amplitude of the RF voltage that is applied to the ring electrode was temporarily increased. This led to ejection of the unwanted fragments at m/z 86. In the bottom panel, the RF pulse that leads to ejection of prompt fragments was left on but the photodissociation laser pulse III was turned on either in the UV (at 266 nm, Fig. 4a) or in the visible (at 532 nm, Fig. 4b). This led to the reappearance of immonium fragment ions at m/z 86 due to photodissociation of thermalized parent ions. Similar results were obtained (Fig. 5) for the tripeptide LeuLeuTyr. The appearance of the immonium fragment upon photodissociation in the visible is in seeming contradiction with previous data. No two-photon absorption of visible light was observed in the early experiment [5a] as judged by the absence of the immonium fragment ion from the spectrum. This was interpreted as indicating charge migration after first photon absorption eliminating the positive charge at the aromatic chromophore. The positive charge at the aromatic chromophore is a prerequisite for absorption of light in the range of wavelengths of 520–560 nm. The major difference between the present experiment and the previous one lies in the time scales. Whereas the spectra presented in Figures 4 and 5 were taken after a fragmentation delay time of 10 ms, those measured by Weinkauf *et al.* [5a] are for a time scale of several microseconds. Our interpretation is that the different behavior is because we are able to measure the slow dissociation rate that is the result of single photon dissociation whereas experiments on the microsecond timescale are not able to observe such slow dissociations. We have verified the notion of dissociation following single photon absorption in the visible by observing for the photodissociation step first order laser power dependencies of the daughter ion signals. Nonetheless, it is quite obvious from Figures 4a and 4b that there is considerably more fragmentation in the UV than in the visible as expected from the results of the Weinkauf group [5a].

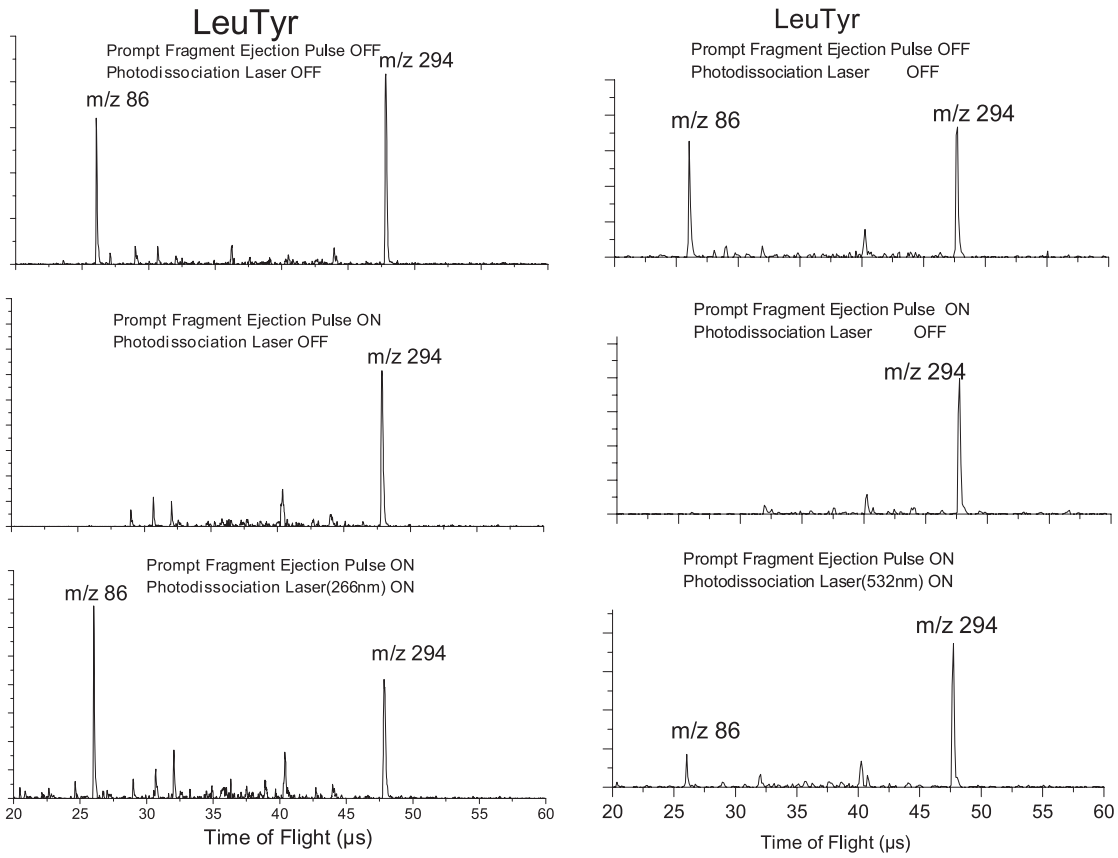


Fig. 4. Laser desorption/laser ionization/laser photodissociation mass spectra of LeuTyr. (a) Resonant MPI at 281.77 nm and photodissociation at 266 nm; (b) resonant MPI at 281.77 nm and photodissociation at 532 nm; see text.

3.2 One-color photoionization and time-resolved photodissociation (TRPD)

The one color photoionization and time-resolved photodissociation spectrum for LeuTyr obtained at 266 nm is presented in Figure 6. The experimental results can be fitted with an exponential rise of the normalized yield of the immonium fragment ion with a rate constant $k = (1.3 \pm 0.3) \times 10^3$ s^{-1}. There is a large non-zero intercept of the fragment ion yield at zero trapping time. This is ascribed to a higher-order, faster multiphoton process than the one depicted by the time dependence on the millisecond time scale. Resonant two-photon ionization at 266 nm forms the $C_6D_6^{\bullet+}$ in perdeuterated benzene with no or very little excess internal energy and the third photon then leads to excitation to a well-defined internal energy [13,14]. This is not the case for LeuTyr. The ionization energy of the tyrosine chromophore is 8.0 eV [5a]. Two 266 nm photons, each contributing 4.66 eV, leave the parent LeuTyr$^{\bullet+}$ ion with an excess energy of up to ∼1.32 eV. The maximum excess internal energy is below the estimated dissociation threshold of 1.5 eV [5a]. However the ions possess thermal energy as well. Absorption of a third photon leads to an internal energy in the excited chromophore in the range of 4.66 eV to 5.98 eV. The rather low dissociation rate constant observed $k = (1.3 \pm 0.3) \times 10^3$ s^{-1} is quite surprising for such a high internal energy. At the excess energy corresponding to a one-photon excitation, fragmentation on a nanosecond time scale is reasonable [5a]. Furthermore, it is not a truly microcanonical rate constant because the internal energy is not well-defined. It is plausible that some, or all of the intercept at zero trapping time is due to a three photon process and that the time-dependence observed in the millisecond range is due to the least internally excited ions in the distribution. These are the ions formed by two-photon ionization and excitation just slightly above the dissociation threshold. A close interaction of dissociation and charge transfer has been demonstrated and the N-terminal fragmentation channel that we observe is open only if charge is transferred [5a]. If our interpretation of the data presented in Figure 6 is correct, it would mean that charge transfer from the tyrosine chromophore to the N-terminus occurs already at the very low internal energy of 1.3–1.5 eV, even lower than the previously assumed lower limit of 2.0–2.4 eV [5a]. Furthermore, there must be some ionization events in which the electron does not carry off all the excess energy as kinetic energy. If in all of the ionization processes most of the excess energy were transferred to the leaving electron [5a]

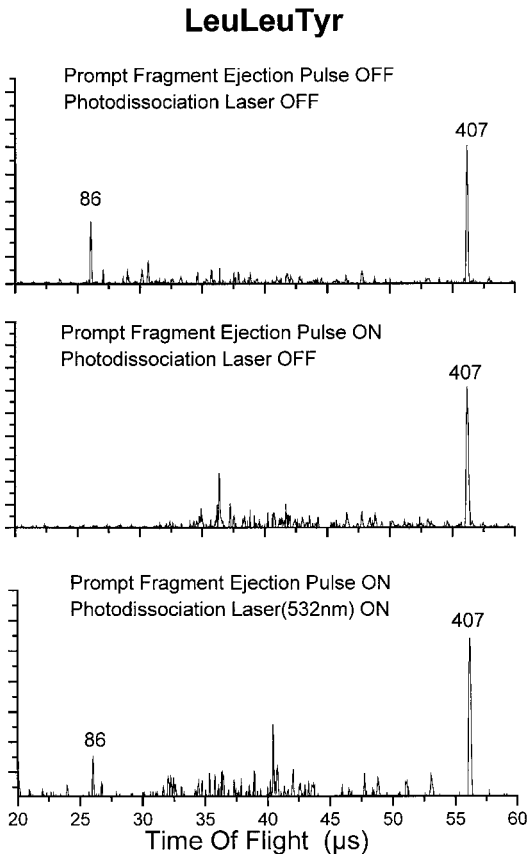


Fig. 5. Laser desorption/laser ionization/laser photodissociation mass spectra of LeuLeuTyr. Resonant MPI at 281.77 nm and photodissociation at 532 nm; see text.

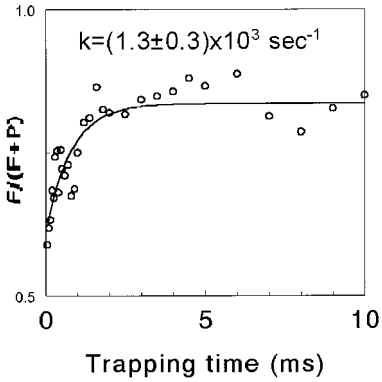


Fig. 6. Normalized signal of the immonium ion from the reaction LeuTyr$^{\bullet+}$ $(P, m/z\ 294) \rightarrow$ immonium^{+} $(F, m/z\ 86)$ in the dipeptide leucyl tyrosine with increasing trapping time after one-color (266 nm) two-photon ionization and excitation described in the text. The experimental points are fitted with a single-exponential curve (solid line) yielding an inverse time constant (*i.e.* rate constant) $(1.3 \pm 0.3) \times 10^3\ \mathrm{s}^{-1}$.

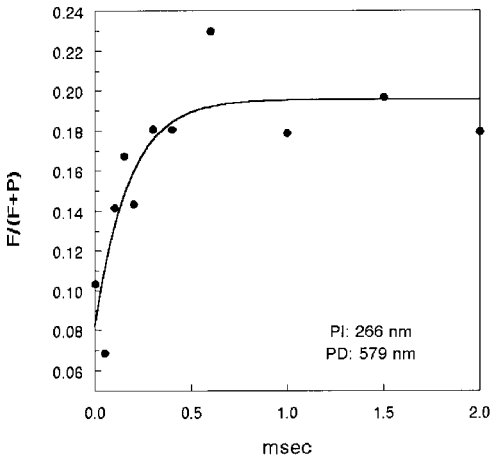


Fig. 7. TRPD points for dissociation of LeuTyr$^{\bullet+}$ by photons at 579 nm (2 mJ). Photoionization at 266 nm (0.4 mJ) is followed by collisional cooling with a helium pulse and by a pulse to the ring electrode that temporarily increases the amplitude of the RF voltage to eject fragment ions formed in the ionization pulse. A total thermalization period of 1 980 ms is applied before the photodissociation pulse is turned on. The solid curve is a single-exponential fit, yielding an inverse time constant $(5.7 \pm 2.6) \times 10^3\ \mathrm{s}^{-1}$.

no dissociation would be observed for the two photon process. Dissociation is observed at the very low energy levels studied, contrary to previous experience [5b], because of the very long time scales employed in our experiment.

3.3 Two-color photoionization and time-resolved photodissociation

In two-color TRPD experiments two-photon ionization in the UV is followed by one-photon excitation in the UV or in the visible. The reactions in which photoionization at 266 nm were followed by a thermalization period and excitation was at 280–289 nm gave dissociations that were instantaneous on the time scale of our instrument. This would mean that the rate constant is $k(4.3\ \mathrm{eV}) \gtrsim 10^5\ \mathrm{s}^{-1}$ in agreement with previous assumptions [5]. We have spiked the helium with aniline and found out that when the helium has not been fully pumped out before the photodissociation pulse has been turned on the rate constants drop considerably due to multiple deactivating collisions of the helium with the peptide ion.

The TRPD spectrum obtained for ionization at 266 nm followed by dissociation at 579 nm is presented in Figure 7. The experiments were performed using a helium pulse following laser photoionization and an RF pulse to eject the prompt fragments formed by the UV ionizing pulse. The intercept at zero trapping time was very low. Two objectives have clearly been achieved: thermalization of the parent ions prior to photodissociation and ejection of the prompt fragments formed by the ionization pulse. We have used a very long thermalization period of 1 980 ms in these experiments and the helium was fully pumped out before the photodissociation pulse was turned on. The internal energy transferred by two 579 nm photons is 4.28 eV. The rate constant $k = 5\,700 \pm 2\,600\ \mathrm{s}^{-1}$ is very low for this energy. However as noted earlier, the second photon in the visible cannot be absorbed if the charge has been transferred away from the aromatic chromophore [5a]. We have

indeed found the photodissociation yield to scale with the first power of the laser intensity. This would mean that the rate constant observed experimentally is for an internal energy of 2.14 eV, $k(2.14\ \text{eV}) = 5\,700 \pm 2\,600\ \text{s}^{-1}$.

4 Conclusions

Classically, mass spectrometry has dealt with cation radicals produced by electron ionization or photoionization of organic molecules. Mass spectra of these radical cations were successfully treated by statistical theories. A very large body of work has been accumulated recently on protonated peptides, cleavages of which were observed to be "charge directed", and a "mobile proton model" has been developed as a framework for understanding these peptide dissociations [29]. Protonated peptides are outside our immediate range of interests. This holds also for electron capture dissociation of multiply charged protein cations, a process that is suggested to be "nonergodic" [30], *i.e.* non-statistical.

We have focused attention in our present study on photodissociation of laser-ionized peptides, that is to say we are in the process of comparing experiment and theory for peptide cation radicals. The measured rate constants we have so far are quite low, demonstrating the occurrence of IVR prior to dissociation. Clearly more work is required in order to establish the internal energy dependence of the rate constants. The present experiments serve mainly to demonstrate the feasibility of the method. In the long run a major aim would be to determine the microcanonical rate constants for the series of peptides, $(X)_n{-}Y$, where X = leucine and Y = tyrosine, at the same internal energy but for increasing n, *i.e.* for an increasing number of degrees of freedom. Once this is achieved one might think of developing new techniques to study ergodicity also for protonated peptides and multiply charged proteins.

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