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Photodissociation of protonated amino acids and peptides in an ion storage ring. Determination of Arrhenius parameters in the high-temperature limit

J. U. Andersen,[a] H. Cederquist,[b] J. S. Forster,[c] B. A. Huber,[d] P. Hvelplund,[a] J. Jensen,[b] B. Liu,[a] B. Manil,[d] L. Maunoury,[d] S. Brøndsted Nielsen,[a] U. V. Pedersen,[a] J. Rangama,[a] H. T. Schmidt,[b] S. Tomita[a] and H. Zettergren[b]

[a] *Institute of Physics and Astronomy, University of Aarhus, DK-8000, Aarhus C, Denmark*
[b] *Physics Department, Stockholm University, SCFAB, S-106 91, Stockholm, Sweden*
[c] *Département de Physique, Université de Montréal, Montréal, Québec H3C 317, Canada*
[d] *Centre Interdisciplinaire de Recherche Ions Lasers, rue Claude Bloch, B.P. 5133, F-14070, Caen Cedex 5, France*

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We have measured the time dependence of the fragmentation of protonated amino acids and peptides upon UV excitation in an electrostatic ion storage ring. After absorption of a 266 nm photon, protonated Trp ($TrpH^+$) has a lifetime of 10–20 μs but also a component with a millisecond lifetime is present. The long lifetime may be due to fluorescence, which leads to ions with lower excitation energy, or to the decay of the $Trp^{+\bullet}$ radical cation formed after prompt hydrogen loss in the laser interaction region. Only one component with a lifetime of about 10 μs was detected for $TyrH^+$. The lifetime of photoexcited $PheH^+$ is even shorter with an upper limit of a few microseconds. For the singly protonated tripeptides ($LysTrpLysH^+$ and $LysTyrLysH^+$), the decay curves are found to consist of a single component that can be reproduced with an assumption of statistical decay after equilibration of the photon energy among all vibrational modes. The rate constant is expressed in the Arrhenius form in terms of the microcanonical temperature, and the decay rate is obtained by integration over the energy distribution, which has a spread corresponding to the canonical energy distribution at room temperature. The resulting deviation from exponential decay makes it possible to determine the decay parameters from a measurement at a single photon wavelength. Activation energies of $E_a = 1.24 \pm 0.07$ and 1.5 ± 0.4 eV were determined for $LysTrpLysH^+$ and $LysTyrLysH^+$, respectively, with pre-exponential factors of $A_d = 10^{11.1\pm0.5}$ and $10^{12.9\pm2.6}$ s^{-1}.

Introduction

Peptides containing the aromatic amino acids tryptophan, phenylalanine, or tyrosine have strong absorption in the UV region, and their photodissociation can be studied with conventional lasers.[1–8] The excitation energy after photon absorption is well defined in the gas phase if the initial energy is known, and this is an advantage compared with methods based on excitation in collisions with other molecules or with a surface, *e.g.* threshold measurements of collision-induced dissociation.[9]

Dissociation can also be induced by heating, in high-temperature mass spectrometry[10] and with the BIRD technique (blackbody infrared radiative dissociation).[11–14] This technique has been used successfully to study dissociation of large biomolecules, including both proteins and oligonucleotides. The decay temperature of the molecules is equal to the temperature of the surrounding walls in the rapid energy exchange (REX) limit, where the rate of absorption and emission of infrared photons is higher than the dissociation rate. It was shown that for ion masses greater than 1.6 kDa, the measured BIRD kinetics will be in the REX limit at temperatures below 520 K.[15] For smaller ions, the REX limit depends on the dissociation parameters.

The BIRD technique and other thermal-dissociation techniques are limited to fairly low temperatures. Typically there are many competing pathways for dissociation, and complex processes with low activation energy and fairly small Arrhenius pre-factor may at high temperatures be replaced by more direct processes with higher activation energy and larger pre-factor. Measurements at the higher temperatures obtained by laser excitation are therefore useful to obtain a more complete picture of the statistical dissociation dynamics. In addition, there may be direct (non-ergodic) fragmentation channels after the electronic excitation.

We have recently combined an electrospray source with a 22-pole ion trap, in which the ions are bunched and reach thermal equilibrium with a trapping gas before injection into an ion storage ring. In this paper we show that lifetime measurements in a storage ring may provide new information regarding the dissociation parameters. The lifetimes after conversion of electronic energy to vibrational energy give Arrhenius parameters for statistical dissociation, which can be compared with results from BIRD measurements. A branching between decays with different lifetimes can be observed but the detection does not distinguish between different decay products. It is therefore important to correlate with experiments which determine the decay products. The importance of long-lived electronic states and fluorescence is discussed.

Experimental

The experimental setup is described in detail in ref. 16, except for the 22-pole ion trap[17,18] which recently has replaced a

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cylindrical ion trap. In brief, a water/methanol (1:1) solution of the amino acid or peptide is electrosprayed with a home-made ion source (Fig. 1) that is held at a voltage of 22 kV. The source is equipped with a 22-pole ion trap, which accumulates ions for 100 ms. During this time the ions are equilibrated with a He buffer gas at the temperature of the ion trap. The 22-pole ion trap follows the design of Gerlich.[17,18] It has a length of about 4 cm and the 22 rods with 1 mm radius form a cylinder with a radius of 5 mm. There are electrodes at each end to stop and extract ions. The five thin ring electrodes located around the 22 electrodes produce a weak electrostatic field in the axial direction for manipulation of the ions inside the trap. The 22 electrodes with alternating potential produce an effective potential with a wide flat region in the middle of the trap. The ions trapped in this region have very small micro-motion, and therefore heating due to this motion is much reduced compared to a simple cylindrical trap. The RF voltage applied to the multipole electrode has an amplitude of 100 V and a frequency of 3 MHz. To avoid heating due to collisions after extraction from the trap, the ions pass through one more differential pumping stage before the 22 kV acceleration.

After mass analysis with a magnet, the extracted ions are injected into an electrostatic storage ring[19,20] with a kinetic energy of 22 keV (Fig. 2). The ring consists of two straight sections about 3 m long, and the ions are deflected at the ends by two 10° deflectors and a 160° cylindrical deflector. Typically, a bunch of ions is injected every 100 ms, and neutrals produced by unimolecular decay or by collisions with the residual gas are detected by a micro-channel-plate (MCP) in the pulse-counting mode. The pressure in the ring is a few times 10^{-11} mbar and collisions with the residual gas limit the storage lifetime to about 10 s. A few tens of milliseconds after injection of an ion bunch, the ions are irradiated with a pulse of UV photons. The neutral fragments from dissociation after photon absorption are detected by the micro-channel plate. Since it is mounted at the side opposite to the laser, this detector only records fragmentation processes delayed by more than about 1/4 of the revolution time after photon absorption. The signals are recorded by a multi-channel scaler, triggered at the time of injection of an ion bunch. To monitor fast decay processes, a channeltron detector is installed just after the 10° deflector at the same side of the ring as the laser.

Photo-excitation was performed at 243 nm, 260 nm, and 266 nm. The source of 243 nm and 260 nm photons was a third-harmonic, pulsed alexandrite laser (PAL101 from LightAge, Somerset, New Jersey), operating at 10 Hz. It was tuned to generate either 729 nm or 780 nm light, which was frequency doubled to 365 nm and 390 nm, respectively. This light was mixed with the fundamental to generate the third-harmonic output. A prism was used to select the light of the proper wavelength. The source of 266 nm photons was the fourth harmonic of a Nd:YAG laser (Quanta-Ray, Spectra-Physics), also operating at 10 Hz; the fundamental of 1064 nm was frequency doubled to 532 nm and again doubled to 266 nm. The UV light

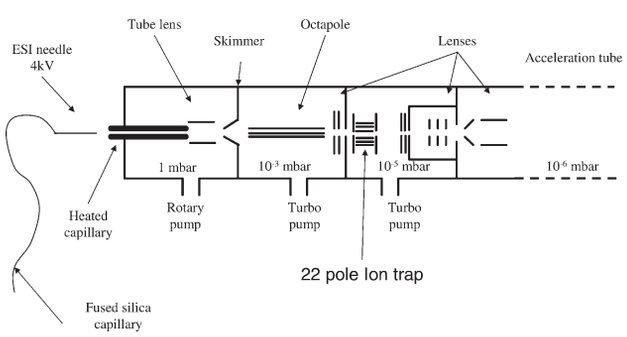

Fig. 1 Schematic drawing of the electrospray source equipped with a 22-pole ion trap.

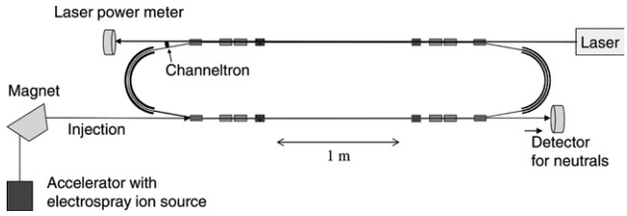

Fig. 2 Illustration of the electrostatic storage ring (ELISA).

was selectively transferred to the apparatus by use of five mirrors that reflect only UV photons.

Results and discussion

Protonated amino acids: $TrpH^+$, $TyrH^+$ and $PheH^+$

We have measured the time dependence of dissociation in the storage ring after photo-excitation at 266 nm (4.66 eV) for three protonated amino acids. The results for $TrpH^+$ and $TyrH^+$ are shown in Fig. 3. The signal before time zero, defined as the time for firing the laser, is due to neutral particles from dissociation induced by collisions with the residual gas. Since the lifetime of the stored beam is several seconds, this yield is constant on the time scale of the measurements. After the laser pulse, there is for both ions a very fast component disappearing after a couple of revolutions for $TrpH^+$ (revolution time 58 μs) and after one revolution for $TyrH^+$ (revolution time 54 μs). For $TrpH^+$ there is an additional slowly decaying component which is discussed below. The power dependence indicated that both components are due to one-photon absorption.

The very different ratios of the signal from metastable excited ions to the background of collision induced dissociation can be explained by differences both in the absorption cross section[21] and in the lifetime after photon absorption. In solution at pH 7 the extinction coefficient is about a factor of five larger for Trp than for Tyr. The cross section is even smaller for phenylalanine (about a factor of 25 lower than

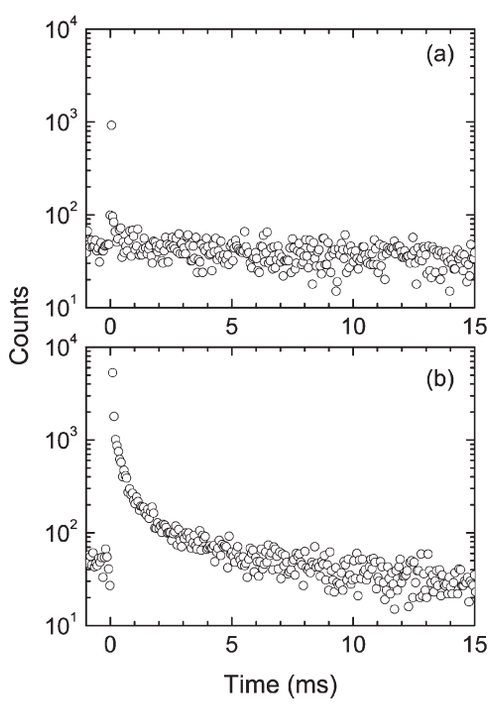

Fig. 3 Time dependence of photofragmentation of $TyrH^+$ (a) and $TrpH^+$ (b) with 266 nm photons.

for tryptophan) and we did not observe any signal at all in the MCP detector from laser excitation of $PheH^+$. However, there is photon absorption in the molecules since a large fragment signal was observed in the channeltron detector just after the laser pulse (see Fig. 2). This signal had disappeared already after one revolution in the ring, so only an upper limit of a few microseconds can be set on the lifetime for the dissociation. For $TrpH^+$ the lifetime can be estimated from the ratio of the counts in the first and second revolutions to be of the order of 10–20 μs. For $TyrH^+$ only one point is seen in the time spectrum and the lifetime is less than for $TrpH^+$; a rough estimate is about 5 μs.

The signal from neutrals measured on the microsecond timescale is from the decay of vibrationally excited ions in the electronic ground state S_0. This decay may occur after a direct electronic transition to S_0 from the excited singlet state but another route is *via* intersystem crossing to a triplet state, *e.g.*, T_1. This increases the decay time because a further spin-forbidden intersystem crossing from T_1 to S_0 is necessary to obtain sufficient vibrational excitation for statistical dissociation on the microsecond time scale.

Trapping in a triplet state is of importance for all three aromatic amino acids under study. First consider phenylalanine in which the side chain is toluene. The triplet quantum yield is 0.25 for benzene, 0.53 for toluene, and 0.65 for 1,4-dimethylbenzene.[22,23] Substituents on the benzene ring therefore increase the triplet quantum yield considerably. In aqueous solution the triplet-state lifetime of Phe or GlyPheGly is a few microseconds.[24] In the case of tyrosine a hydrogen on the benzene ring is replaced by OH. The triplet-state lifetime of phenol in water is 3.3 μs, it is 5.6 μs for Tyr, and between 3 and 4 μs for TyrGly, TyrGlyGly, and GlyTyrGly peptides.[24] The side chain of Trp is indole. There is disagreement on the triplet-state lifetime of indole in aqueous solution, *e.g.* 12 μs,[24] 40 μs,[25] and 1.2 ms.[26] The 1.2 ms lifetime may, however, be due to photoionization and a slow geminate recombination between an indole radical cation and a solvated electron, generating indole in the triplet state. The probability for $S_1 \rightarrow T_1$ intersystem crossing of indole is approximately 0.28.[27] In the case of Trp, two transient absorption bands after optical excitation at 265 nm have been measured in the time ranges 2–20 μs and 20–45 ns. The first and perhaps also the second band correspond to population of a triplet-state.[24] When Trp is the residue in a tripeptide, GlyTrpGly, the triplet-state lifetime is 12 μs.[24]

It thus appears that the triplet-state lifetimes of amino acids in solution are similar to the lifetimes measured for the photoexcited protonated amino acids *in vacuo*. It is therefore possible that the statistical decay of vibrationally excited ions in the electronic ground state is much faster (submicroseconds). If so, the time window of our instrument does not allow us to identify the fraction of ions that decay directly after internal conversion from S_1 to S_0. We have previously reported such branching ratios in the case of nucleotide[28] and protoporphyrin ions.[29] On the other hand, the measured decay time of the protonated amino acids could be the lifetime for statistical dissociation and the triplet lifetimes could be longer *in vacuo* than in solution.

Interestingly, the long lifetime component of $TrpH^+$ exhibits a multi-component decay curve. In fact, as shown in Fig. 4, the decay curve is well reproduced by the $1/t$ law which applies for an internal energy distribution that is broad enough to give a large spread in lifetimes.[30,31] Photon absorption results in a well-defined excitation energy ($h\nu$ plus the initial internal energy). In the present experiments, $TrpH^+$ was thermally equilibrated at room temperature and the average internal energy E_0 is 0.3 eV. The canonical internal energy distribution has a full width of 0.2 eV which is quite small compared to the photon energy. Therefore there must be an additional process involved. In the following we consider the possible importance

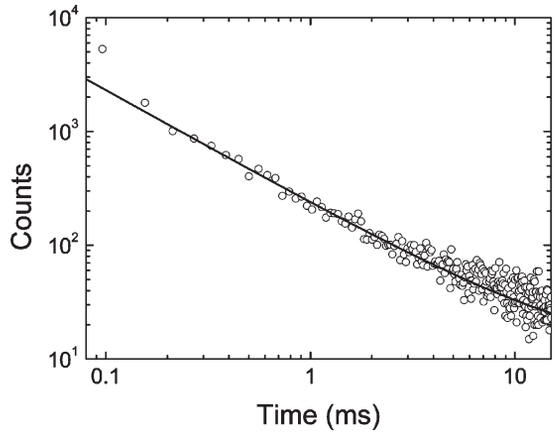

Fig. 4 Decay curve of $TrpH^+$ after 266 nm photon absorption. The solid line is a fit to a $1/t$ decay curve including the backgrounds due to CID.

of a triplet state and of fluorescence. Also the formation of the tryptophan radical cation after prompt loss of hydrogen from $TrpH^+$ is discussed since its decay spectrum will be superimposed on that of $TrpH^+$.

If the ms lifetime component of the $TrpH^+$ decay is due to a triplet state, there must be solvent quenching in water solution where the triplet-state lifetime is of the order of microseconds. The triplet energy of indole in low-temperature glasses is 2.86 eV.[32] Hence, $TrpH^+$ in the triplet state has an internal energy of $E_0 + 4.66\ \text{eV} - 2.86\ \text{eV} = 2.1\ \text{eV}$. This is still large compared to the initial energy spread of the molecules and therefore the $1/t$ decay function is not explained.

Another possible process after photon absorption is fluorescence from the initially excited Trp. In aqueous solution, the fluorescence quantum yield of Trp is larger than 0.1 and highly dependent on the pH of the solution (low when $pH < 4$).[21] Since tryptophan has a higher fluorescence quantum yield than the other amino acids, its fluorescence is often used to study the structure and dynamics of proteins in solution.[33] The maximum light emission is around 350 nm and the internal energy of $TrpH^+$ is about 1.4 eV after fluorescence. The width of the internal energy distribution includes the spread in the fluorescence energy, which is quite large at room temperature. For example, fluorescence spectra of Trp in electrospray show a full width of 0.6 eV.[34] Combined with the width of the initial thermal energy distribution this gives a width of order 0.8 eV, which is more than sufficient to explain the multi-component ($1/t$) decay function. For example, with an activation energy of 1.2 eV and a pre-exponential factor of $5 \times 10^{13}\ \text{s}^{-1}$ in the Arrhenius expression, the lifetime after fluorescence covers the range from 100 μs to 0.1 s. The dominant statistical decay process could be loss of ammonia which is observed in collisions at a few eV in the centre-of-mass frame.[35] The activation energy for NH_3 loss has been calculated to be 1.0 eV at the B3LYP/6-31G* level of theory.[36]

This explanation is consistent with the fact that no long-lifetime component was observed for $TyrH^+$. The fluorescence quantum yield of Tyr is lower than that of Trp and in addition maximum light emission is at 300 nm, a much shorter wavelength than that of Trp.[21] Therefore $TyrH^+$ would have an internal energy of only 0.5 eV after fluorescence and no decay would be observed.

However, very recent evidence by Kang *et al.*[37] suggests another interpretation. The main dissociation channel of $TrpH^+$ after 266 nm photon absorption appears to be loss of a hydrogen to give the $Trp^{+\bullet}$ radical cation. The mechanism for this is believed to be a prompt direct process that involves an ion where the proton is located on the indole ring and not

on the amino nitrogen. When the indole ring is protonated, fluorescence is strongly quenched. We have calculated the energy required for loss of a hydrogen from protonated 3-methylindole at the MP2/6-311++G(2d,p)//B3LYP/6-31+G(d) level to be 3.6 eV (corrected for zero-point kinetic energies).[38] The remaining average energy in the $Trp^{+\bullet}$ cations is then about 1 eV, and with an additional broadening of the distribution associated with the hydrogen loss we may be in the regime of $1/t$ decay. The radical cation is still stored in the ring as its mass is only one less than that of $TrpH^+$, and the subsequent dissociation of the $Trp^{+\bullet}$ could account for the measured slow decay. The major dissociation channel of $Trp^{+\bullet}$ is a C_α–C_β bond cleavage.[6,37,39]

Protonated tripeptides: LysTrpLysH$^+$ and LysTyrLysH$^+$

The statistical lifetimes of the three protonated amino acids are clearly too short to be measured accurately in the ring. If the photon energy is distributed over all degrees of freedom before dissociation we can increase the lifetime by increasing the size of the molecule, and small peptides should therefore be easier to study. Moreover, the fluorescence from tryptophan is strongly quenched by side chains of amino acids in a peptide, *e.g.*, protonated amino groups of lysine residues or α-amino groups. Finally, the population of triplet states will have less importance for the time spectrum as the triplet–singlet decay will not be rate-limiting for the dissociation. Results for the tripeptides LysTrpLysH$^+$ and LysTyrLysH$^+$ are presented in the following. The additional proton is expected to be located on the lysine residues because of their high proton affinity and not on the indole ring of the tryptophan residue.

The decay of LysTrpLysH$^+$ ions after absorption of a 266 nm photon is illustrated in Fig. 5. The decay curve is reproduced reasonably well by a single exponential with a lifetime of 1.06 ms plus a constant background due to collisions with the residual gas (dotted line). The deviation from this curve can be explained as a consequence of the finite width of the internal energy distribution, as we shall discuss in the following.

The rate constant for statistical decay of molecules with excitation energy E can be written in the form,[40,41]

$$k(T) = A_\mathrm{d} \exp\left(-\frac{E_\mathrm{a}}{k_\mathrm{B} T_\mathrm{eff}}\right) \qquad (1)$$

where T_eff is an effective decay temperature equal to the microcanonical temperature T corresponding to E, with a finite-heat-bath correction,

$$T_\mathrm{eff} = T - \frac{E_\mathrm{a}}{2C}. \qquad (2)$$

Here C is the microcanonical heat capacity at the temperature T. The canonical caloric curve can easily be evaluated with vibrational frequencies calculated at the semiempirical PM3 level from the Gaussian98 program package,[38]

$$E = \sum \hbar\omega_i \left[\exp\left(\frac{\hbar\omega_i}{k_\mathrm{B} T}\right) - 1\right]^{-1}, \qquad (3)$$

and to a good approximation the microcanonical caloric curve is obtained by a scaling of the energy with a factor $(N-1)/N$, where N is the number of one-dimensional oscillators.[41] The average internal energy of the tripeptide ion is $E_0 = 0.87$ eV at room temperature. If all the photon energy is converted into vibrational excitation, the average energy becomes $E_\mathrm{ex} = E_0 + h\nu$ after absorption of one photon. The average microcanonical temperature of the system after photon absorption, T_ex, may then be determined from the microcanonical caloric curve.

The distribution in internal energy of the ions has a width corresponding to that of the canonical ensemble at the temperature T_0 of the ion trap (room temperature). The distribution is approximated well by a Gaussian and after photoabsorption can be expressed as

$$g(T) \propto \exp\left(-\frac{(T - T_\mathrm{ex})^2}{2\sigma^2}\right)$$
$$\sigma = \frac{\sqrt{C_0 k_\mathrm{B}}}{C} T_0 \qquad (4)$$

where C_0 is the heat capacity at the temperature T_0. The rate of decay, $I(t)$, at time t after photon absorption can be written as

$$I(t) = \int k(T) g(T) \exp(-k(T)t) \mathrm{d}T. \qquad (5)$$

There are two unknown parameters, the pre-exponential factor A_d and the dissociation energy E_a in eqn. (1), and for exponential decay in thermal equilibrium, measurements at several temperatures are required for determination of these parameters. However, here the spread in lifetime associated with the energy spread gives additional information: For combinations of activation energy and pre-factor leading to the same average lifetime, the spread in lifetime will be larger for high activation energy. Thus it is possible to determine both parameters from a single decay curve.

An example is the analysis of the decay curve for photoexcited LysTrpLysH$^+$ molecules in Fig. 5. The average microcanonical temperature is 818 K after absorption of a 266 nm photon. From a fit to the measurements by a curve calculated from Eqns. (1)–(5) the parameters $A_\mathrm{d} = 10^{11.1 \pm 0.5}$ s^{-1} and $E_\mathrm{a} = 1.24 \pm 0.07$ eV were determined. The dashed curves were obtained with slightly different values of E_a and A_d but with the lifetime fixed at its optimum value, 1.06 ms. The curves illustrate that although there is strong correlation between the parameters, the tail is quite sensitive to the combination of parameters and they can be determined fairly accurately. To check the validity of this analysis, the experiment was repeated with two other wavelengths, 243 nm and 260 nm. The microcanonical temperatures are 855 K and 827 K for 243 nm and 260 nm, respectively. In Fig. 6 the experimental decay curves are shown to be reproduced quite well at both wavelengths.

A similar experiment has been carried out with another protonated tripeptide, LysTyrLysH$^+$. In Fig. 7, the decay curve after 266 nm photon absorption is shown together with a calculated curve. The average temperature of the ions is 839 K

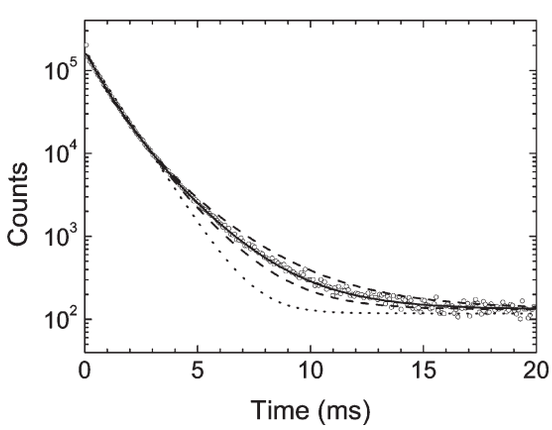

Fig. 5 Decay curve of LysTrpLysH$^+$ after irradiation by 266 nm photons. The solid line is from calculation with $E_\mathrm{a} = 1.24$ eV and $\log(A_\mathrm{d}) = 11.1$. The dashed lines are results of calculations with $E_\mathrm{a} = 1.0$ and 1.5 eV keeping the same lifetime of 1.06 ms. The dotted curve is a fit with a single exponential.

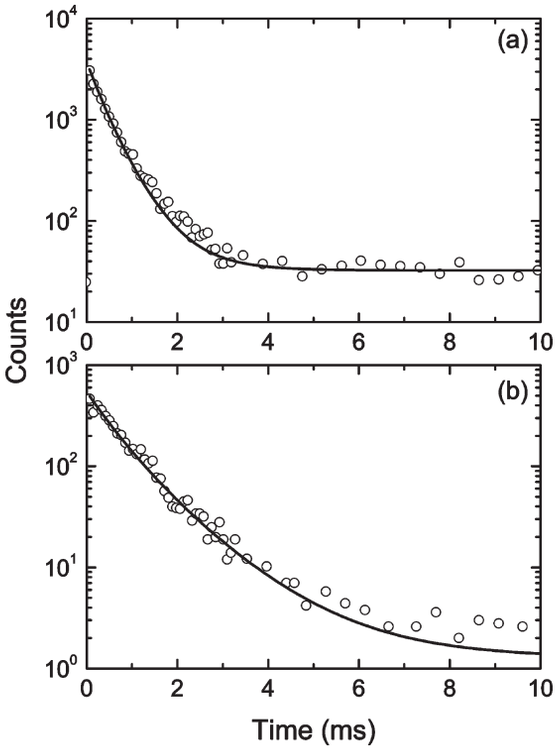

Fig. 6 Decay curves of $LysTrpLysH^+$ after absorption of a photon with wavelength of 243 nm (a) or 260 nm (b). The points after 3 ms are averaged over five points. Solid lines are results of calculations based on eqns. (1)–(4) with dissociation parameters $E_a = 1.24$ eV and $\log(A_d) = 11.1$.

after photon absorption. The Arrhenius parameters obtained from the analysis are $A_d = 10^{12.9\pm2.6}$ s^{-1} and $E_a = 1.50 \pm 0.37$ eV. The optimized lifetime after absorption of 266 nm photon is 0.86 ms. The uncertainties are quite large, and this illustrates that this method for determination of Arrhenius parameters requires high-quality data with good statistics.

The fragmentation channels are not known with certainty at such high temperature of the ions. For comparison, Arrhenius parameters in the low-temperature region have been reported for several peptides. The activation energies for the formation of b and y ions (peptide bond cleavage) from di-, tri- and tetra-alanine are 2.11 eV, 1.46 eV, and 1.20 eV,[42] similar to the values we found for the two tripeptides. Williams and coworkers have studied the nonapeptide bradykinin in great detail with the BIRD technique.[12,43] In this case the activation

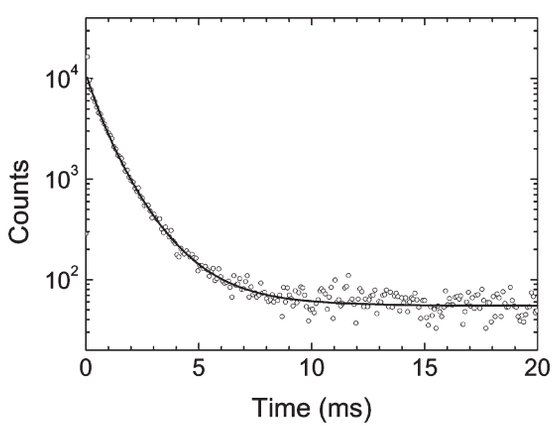

Fig. 7 Decay curve of $LysTyrLysH^+$ after 266 nm photon absorption. The solid line is from a calculation with $E_a = 1.50$ eV and $\log(A_d) = 12.9$.

energies were 1.3 eV and 0.8 eV for the singly and doubly charged ion, respectively, and the pre-exponential factors were found to be $10^{12.59}$ and $10^{6.94}$ s^{-1}. These parameters are for processes that occur in the temperature region between 150 °C and 190 °C. The major dissociation channel of the singly charged bradykinin is loss of ammonia whereas the major dissociation channel of the doubly charged ion is formation of the complementary b_2/y_7 pair.

At higher temperatures, y and b ions are also formed from the singly charged bradykinin ions. A folded ion conformation, in which the two arginine residues interact closely through a salt-bridge structure, has been argued to be important for loss of NH_3. A high temperature can lead to an unfolding of the peptide and switch off this channel in favour of peptide bond breakage. Thus a different trade off between energy and entropy is not the only reason for changes in fragmentation at high temperatures. The pre-exponential factors obtained for the peptides in our experiments are larger than for processes that give b and y ions in bradykinin, and this seems to indicate more direct peptide-bond cleavage (no hydrogen atom movement involved) or loss of a side chain.

Conclusions

In this paper, we have presented the time spectra for dissociation of protonated Trp, Tyr and Phe and protonated LysTrpLys and LysTyrLys tripeptides after photon absorption in an electrostatic storage ring. For the tripeptides, the spectra show a single-component, nearly exponential decay, indicating that the photon energy is converted rapidly to vibrational excitation. From comparison with a statistical description of the decay we have determined the dissociation parameters. The temperature of the photoexcited tripeptides is about 800 K, which is much higher than the temperatures normally used with the BIRD technique. The measurements therefore provide supplementary information on the dependence of the dissociation dynamics on temperature.

For the protonated amino acids, the temperature is so high after photon absorption that the decay is too fast to be studied in detail with the storage ring. For tyrosine and phenylalanine there is only a very fast decay with lifetimes of about 10 μs and a few microseconds, respectively. For tryptophan the fast decay is slightly slower and there is another component in the spectrum, which can be fitted well by a power-law. The power-law decay curve is typical for statistical decay of molecules with a broad internal energy distribution. Further experiments are required to establish the decay mechanisms but we believe that the slow component may be associated either with strong fluorescence from gas phase protonated tryptophan or with decay of $Trp^{+\bullet}$ radical cations that are formed after prompt photolysis of indole N–H.

Acknowledgements

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