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Tailored photocleavable peptides: fragmentation and neutralization pathways in high vacuum

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Photocleavable tags (PCTs) have the potential for excellent spatio-temporal control over the release of subunits of complex molecules. Here, we show that electrosprayed oligopeptides, functionalized by a tailored ortho-nitroarylether can undergo site-specific photo-activated cleavage under UV irradiation (266 nm) in high vacuum. The comparison of UV photodissociation (UVPD) and collision-induced dissociation (CID) points to the thermal nature of the cleavage mechanism, a picture corroborated by the temperature dependence of the process. Two competing photodissociation pathways can be identified. In one case a phenolate anion is separated from a neutral zwitterion. In the other case a neutral phenol derivative leaves a negatively charged peptide behind. To understand the factors favoring one channel over the other, we investigate the influence of the peptide length, the nature of the phenolic group and the position of the nitro-group (ortho vs. para). The observed gas phase cleavage of a para-nitro benzylic ether markedly differs from the established behavior in solution.

1 Introduction

The charge state of peptides and proteins affects their chemical and biological behavior through intermolecular electrostatic interactions as well as by modulation of their geometry and folding, electronic and vibrational energy structure, and their electro-optical or collisional properties. Spectroscopic studies of biomolecules in the gas phase are interesting as they specifically allow identifying the role of matrix effects.

The combination of both aspects, i.e. charge control of biomolecules in the gas phase is relevant for molecular trapping, optical and photo-electron spectroscopy, as well as for electron or femto-second x-ray diffraction. Several methods for charge manipulation have been studied in the past, such as atomic collisions, chemical reactions, and low-energy electron attachment.

Laser-induced processes are intriguing since they are compatible with ultra-high vacuum requirements, can achieve high efficiency and combine high spatial resolution with sub-nanosecond timing. UV electron photodetachment (ED) has recently been successfully demonstrated on insulin polyanions, however, in complex molecules it competes with photodissociation (PD).

It has been shown that photocleavage can be optimized using tailored tag molecules that respond to UV light and visible light, also for peptides with ionization energies exceeding the photon energies of table-top lasers. The heterolytic removal of the leaving group (LG) from a singly charged photo-tagged peptide anion is a promising strategy for the controlled generation of neutral zwitterions in the gas phase (Scheme 1) and can be relevant for proteomics.

Our work aims at developing tools that enable the generation of continuous beams of neutral, slow and internally cold peptides and proteins for matter-wave interferometry. Such controlled beams are valuable for fundamental tests of quantum physics, enable new measurements of molecular electronic, optical and magnetic properties, as well as optical and infrared spectroscopy under controlled interaction-free conditions.

Here, we study tailored oligopeptides with a photocleavable tag in an electrospray mass spectrometer, aiming at the controlled
Scheme 1 Photocleavable tags (PCT), reaction scheme, oligopeptides and leaving groups (LG) used in this study. Upon irradiation with 266 nm UV light the functionalized peptides can undergo either heterolytic cleavage or dissociation with simultaneous proton transfer. The functionalized peptides differ in their amino acid sequence Lys-Ala-(Leu-Gly-Ala)ₙ-Leu and in their leaving group (LG) 1a, 1b, 1c, 1d. The index n = 0-3 labels the oligopeptides from a tripeptide 1a to the dodecapeptide 4a.

Fig. 2a and 2b show the UVPD (a) and CID (b) mass spectra of the tripeptide 1a. The fragment at 229 u/e results from heterolytic cleavage of the leaving group a. Both mass spectra show the desired LG-anion a as the only fragment, suggesting that UVPD and CID follow a similar mechanism.

The experiments are performed using a customized ESI-Q-TOF mass spectrometer, as shown in Fig. 1. The electro-sprayed ions are guided into high vacuum by a stack of ring electrodes. They are mass-selected by a quadrupole ion filter, temperature-controlled by the buffer-gas in the first hexapole ion guide (marked in blue in Fig. 1), photo-activated by UV laser light inside the second hexapole ion guide (without buffer gas, marked in red) and detected using a time-of-flight mass spectrometer. A pulse-tube cooler was fitted to the first hexapole, allowing to set a temperature of between T = 60-300 K. Pulsed ultraviolet laser light (λ = 266 nm, 10 ns, < 1 mJ per pulse) was aligned to be collinear and counter-propagating to the ion beam.

2 Results and discussion

Fig. 2c and 2d trace the UV photodepletion efficiency for the tripeptide 1a as a function of the laser fluence and for two different molecular temperatures. The molecules interact with a buffer gas at 300 K (c) or 60 K (d) prior to the PD experiments. We define the UVPD efficiency as $1 - S/S_0$. It measures the reduction of the parent ion signal in the presence ($S$) or absence ($S_0$) of the UV light. Its dependence on the laser fluence $F$ is derived from kinetic rate equations:

$$1 - S/S_0 = 1 - \alpha + \alpha(1 + \gamma \sigma F)e^{-\sigma F}$$

with $\alpha$ as the spatio-temporal overlap between the UV laser beam and the ion beam, $\gamma$ the fraction of two-photon processes and $\sigma$
the PD cross section as a lower bound to the absolute absorption cross section (see Fig. S1, ESI†). The temperature of the buffer gas determines whether one ($\gamma = 0$) or at least two photons ($\gamma = 1$) are needed to deplete the parent ion signal. From Fig. 2a, we extract $\alpha = 0.4 \pm 0.1$ and $\sigma = 0.4 \pm 0.1 \text{Å}^{-2}$. While single-photon cleavage prevails at 300 K, the data are best fitted by a 70% probability for a two-photon process when the molecules are 60 K cold.

This suggests that the cleavage process depends on the molecular heat capacity, which increases with peptide length (Fig. 3b). More photons are then required for heterolytic cleavage to occur on the experimental time scale (Fig. S3, ESI†). This hypothesis is corroborated by the observation that the character of the dissociation changes with peptide length. Fig. 3a shows that for the tripeptide 1a heterolytic cleavage of the LG-anion a at 229 u/e is the only observed dissociation channel. However, for all longer oligopeptides we find the additional channel involving the transfer of a proton, which results in the separation of a neutral leaving group LG-H from a negatively charged peptide (Fig. 3b and Scheme 1). While the hexapeptide 2a still shows partial heterolysis, the longer peptides 3a and 4a dissociate exclusively under proton transfer, with the fragment at $m/z = (M - 230)$ u/e. The proton transfer reaction is always accompanied by the formation of a second fragment at $m/z = (M - 246)$ u/e.

In contrast to that we have never observed proton transfer in our collision induced dissociation experiments (Fig. S5, ESI†). Instead, the CID spectrum of the hexa- and nonapeptide 2a and 3a yield about 5% of heterolytic cleavage at 300 K, and the nonapeptide spectrum shows the appearance of some backbone fragments.

A systematic variation of the leaving group a, b, c, d at the tripeptide 1, confirmed our design hypothesis that the electron withdrawing fluorine substituents stabilize the negative charge on the LG phenolates and enable heterolytic cleavage. We correlate the heterolytic cleavage efficiencies with density functional theory (DFT) calculations (ESI†) to shed light on our experimental findings. Initial conformations used in DFT calculations are modeled in terms of chemical constitution and further locally relaxed using manually created conformations. Short ab initio molecular dynamics (AIMD) simulations at 300 K further helped us to explore the potential energy surface (PES) for candidates while the electronic potential is provided by DFT at the PBE0/3-21G level of theory. Several conformational candidates are found locally optimized at 0 K at the PBE0/Def2TZVP level and lowest energy conformations are used in the following calculations. The energetics of the photocleavage process is addressed by relating heterolytic bond dissociation energies (BDE) vertical electron detachment energies (VDE) fragment yields and $\Delta H_{\text{e}}$ values. Additionally, mean thermal energies are estimated from calculated vibrational spectra in the harmonic approximation (Table S1, ESI†).

Even though the energy of a single 266 nm photon (4.7 eV) is smaller than the BDE of 1a (6.9 eV), it adds to a mean thermal energy of 1.4 eV at 300 K and thus to a total internal energy of 6.1 eV, which is close enough to the BDE for fragmentation to occur after some intra-molecular reorganization. At lower temperature, here at 60 K, the total internal energy of 4.8 eV is far below the BDE value. This is consistent with the observation in Fig. 2b that at 60 K two or more photons are required in most
be realized with electrons and atoms of the photolinker, that is without the involvement of solvent molecules, site-specific dissociation should also be possible in the gas phase, as seen in our experiments. To decipher the role of the nitro group, isomers of the tripeptide 1a and the nonapeptide 3a, (p-1a, p-3a) were synthesized with the nitro-group in the para-position of the benzylic ether function (Fig. 5). We find that p-1a and 1a cleave with a comparable heterolytic efficiency, corroborating the thermal nature of the process. This is markedly different from the behavior in solution (DMSO-d₆) where irradiation of p-1a at 254 nm does not yield any cleavage, while it does for 1a (Fig. S8, ESI†). However, the modified nonapeptide p-3a does not cleave under conditions where 3a dissociates. This indicates that the proton transfer pathway resembles the accepted solution phase mechanism and can be suppressed by repositioning the nitro group. The heterolytic channel, on the other hand, is too slow for the para-functionalized nonapeptides. For the short peptides 1a-1b, the sum of photon (4.7 eV) and thermal energies (≈ 1.3 eV) is sufficient to release a negatively charged LG, and the heterolytic mechanism is observed. For larger peptides, however, the heterolytic dissociation cannot be entirely excluded, given the computed VDE values of 4.6 eV, especially since the experimental fragment collection efficiency is not exactly known.

Apparently, for some LGs heterolytic cleavage becomes less probable than a dissociation involving proton transfer (1c). Heterolytic cleavage must leave a zwitterionic peptide behind which might be favored by the formation of a tropylium cation. Preliminary DFT calculations (ESI†) indicate that this structure is of comparable stability to the corresponding benzyl cation. It remains, however, an open task to model detailed reaction pathways and to evaluate the barriers for the intermediates. We also find that the trend in fragmentation yields for compounds 1a-1d (Fig. 4b) correlate with the pKₐ values of the protonated leaving groups LG-H (Table S1, ESI†), even though the latter also include ion solvation energies.

To compare the optical response of the tripeptides 1 with different LGs, time-dependent density functional theory (TDDFT) calculations were performed at the same level of theory as before, involving 100 excited states. Gaussian convolutions to the calculated line spectra show strong absorption around 250 nm for 1a-1d (Fig. 4b). Electronic excitation analysis based on natural transition orbitals (NTO) confirms that the UV light excites the PCT rather than the peptide. We also find that the absorption spectra do not significantly change upon exchange of the LG.

The photocleavage of o-nitrobenzylethers and related nitroaroyls in solution is well documented in the literature. Since cleavage of the 2-phenoxymethyl-nitrobenzene can already

![Fig. 3](image_url)

Fig. 3 (a) UVPD mass spectra for peptides 1a-4a for maximum laser fluence of 3.3 J cm⁻². The fragment at 229 u/e results from heterolytic cleavage of the LG. Red arrows indicate the fragments formed due to proton transfer dissociation (M-LG-H and M-LG-H-16). (b) Fragment yield for LG a (dark circles) and for fragments due to proton transfer dissociation (red squares) as a function of the peptide length. Points and error bars represent experimental values, full lines display curve fitting using the exponential depletion function.

![Fig. 4](image_url)

Fig. 4 a) LG fragment yield of the functionalized tripeptide 1 as a function of the laser fluence. The four difference curves correspond to the same PCT core with four different leaving groups (a, b, c and d). b) The oscillator strength of the tripeptides 1a - 1d is obtained by TDDFT. For simplicity we show a Gaussian convolutions to the line spectrum only for peptide 1a. The arrow points to the NTOs with the largest eigenvalue for the transition close to 266 nm. For the particular LG a the calculations find efficient charge transfer from the absorbing PCT towards the LG in the transition from the ground state |g⟩ to the excited state |e⟩.

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Both the neutral and the charged dissociation pathways are interesting and useful for gaining optical control over the motional states of molecules. Photo-cleavage can be realized with high spatial control and very precise timing. This technique may be used for post-neutralizing singly charged anions beams, which have been previously guided and cooled in a buffer gas environment. The optically induced gas phase photo-depletion of the parent peak is also promising for realizing coherent beam splitters based on photo-depletion of a molecular beam with nanometer resolution. This will become important for quantum optics and metrology experiments with complex neutral biomolecules.

Conflicts of interest

There are no conflicts to declare.

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Notes and references
