Multiple ionization of free ubiquitin molecular ions in extreme ultraviolet free electron laser pulses

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Abstract: The advent of hard X-ray free electron lasers opened up the opportunity of protein structure determination from nanocrystals and potentially even from free single protein molecules, using “diffraction before destruction” approaches. While this allows for investigation of proteins that cannot be grown into large crystals, structural dynamics on the femtosecond time scale present a fundamental limitation. We have investigated the fragmentation of free 10-fold protonated ubiquitin in intense 70 femtosecond pulses of 90 eV photons from the FLASH facility. Mass spectrometric investigation of the fragment cations produced after removal of many electrons, revealed fragmentation predominantly into immonium ions and related ions, with yields increasing linearly with intensity. Ionization clearly triggers a localized molecular response, occurring before excitation energy equilibrates. In line with this interpretation, the effect is almost unaffected by the charge state, as fragmentation of 6-fold de-protonated ubiquitin leads to a very similar fragmentation pattern. Ubiquitin responds to EUV multiphoton ionization, as an ensemble of small peptides.

The investigation of complex biological molecules by means of scattering or absorption of energetic photons from high brilliance light sources is a very powerful approach to address the molecules' chemical and electronic structure and dynamics.

Established techniques such as X-ray photoelectron spectroscopy (XPS) or near edge X-ray absorption fine structure (NEXAFS) spectroscopy involve small absorption cross sections which usually dictate the use of dense condensed-phase targets^{[1][2]}. Soft X-ray spectroscopy has successfully been used for protein identification and mapping^[3,4]. However, in the condensed phase, structural and dynamic molecular properties are often strongly influenced by intermolecular interactions such as ultrafast energy transfer^[5] or the inevitable radiation damage^[6,7]. With the promise held by ultrabright X-ray free electron lasers (FEL), of imaging before destruction, it is crucial to establish how and how fast single gas-phase or nanocrystalline biomolecular systems respond to simultaneous absorption of many energetic photons^[8]. Pioneering experiments proved the feasibility of femtosecond (fs) X-ray FEL diffraction imaging of biomolecular nanocrystals in a liquid jet, indicating that photodestruction proceeds slower than the imaging process^{[9][10]}. It is still under debate, whether this also holds for single free proteins. Experimental studies on the response of single proteins to fs energetic photon pulses are urgently called for.

Mass spectrometric techniques combining electrospray ionization (ESI) and radiofrequency (RF) ion traps have recently been introduced for investigation of photoionization dynamics in gas phase protein ions. First soft X-ray photoionization studies at the C, N and O K-edges were performed on nine-fold protonated cytochrome c (104 amino acid residues)^[11]. Mainly non-dissociative single and double ionization was observed, with spectral features similar to NEXAFS data from solid protein films. Protein backbone scission was very weak. Only CO₂ loss appeared as additional dissociation channel. The predominance of non-dissociative processes is remarkable, because core ionization/excitation leads to substantial local excitation. In contrast, soft X-ray photoionization of the small gas-phase protonated leucine encephalin (5 residues) leads almost exclusively to extensive fragmentation^[12]. Here, small sidechain related fragments dominate the breakup pattern. These findings can be understood in a framework of ionization induced fast dissociation through repulsive states *before* internal vibrational redistribution (IVR) of the excitation energy^[13]. Vacuum ultraviolet (VUV) photoionization studies on a series of synthetic protonated peptides of variable size (2-12 residues) confirmed this scenario^[14]. From these measurements it was determined that ionization along the peptide backbone followed by fast charge migration towards an aromatic sidechain is the main underlying process. VUV photoionization of larger multiply protonated proteins such as cytochrome c, leads almost exclusively to non-dissociative ionization^[15].

Why is fast sidechain loss suppressed in larger systems, even though it is thought to occur before IVR happens? What is the role of the photoinduced charge(s)? To answer these questions, we systematically investigated multiple ionization of tenfold protonated ubiquitin [ubi+10H]¹⁰⁺ (m=8565 u, 76 amino acid residues) in extreme ultraviolet (EUV) fs pulses from the FEL FLASH^[16]. Ubiquitin has no disulfide bridges and its tertiary structure thus is not very rigid. Tenfold protonation is known to induce substantial structural relaxation driven by Coulomb repulsion of the protonation sites^[15]. The gas-phase conformation of [ubi+10H]¹⁰⁺ can be classified as elongated (with maximum elongation reached for 13-fold protonation)^[17]. Charge or energy transport upon photoabsorption in this system therefore most likely proceeds solely along the protein backbone.

The experiments were performed by interfacing a home-built tandem mass spectrometer with beamlines at the FEL FLASH (DESY, Hamburg, Germany) and the synchrotron facilities BESSY II (HZB, Berlin, Germany) and MAXII (Maxlab, Lund, Sweden) (see supporting information for details).

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A typical mass spectrum of $[\text{ubi}+10\text{H}]^{10+}$ products after interaction with 70 fs FEL pulses of 90 eV photons is shown in Figure 1. The pulse energies were attenuated to 0.1 μJ for single photon absorption conditions. As seen before for soft X-ray^[11] and VUV single photon absorption^[15] of gas phase protonated proteins, non-dissociative single ionization into $[\text{ubi}+10\text{H}]^{11+}$ (labeled: 11+) is the dominant process, in part accompanied by CO_2 loss ($m=44$, labelled -44). Other large fragment channels are weak. Non-dissociative double ionization (labeled: 12+) and associated CO_2 loss are significantly weaker. In the mass over charge range between the trap cut-off ($m/z=75$) and $m/z=150$ a group of peaks shows up with intensities exceeding those of $[\text{ubi}+10\text{H}]^{12+}$. Almost all of them can be assigned to immonium (related) ions (internal fragments containing a single sidechain formed by two adjacent backbone cleavages). This is in line with our previous VUV photoionization studies on much smaller singly protonated peptides^[13, 14]. Ubiquitin primary and secondary structure are shown in the circle around the spectrum. The one-letter-codes are also used to label the immonium peaks. Amino acids observed in the spectrum are labeled in color and unobserved ones are shown in black. Amino acids outside the mass range of the system are shown in grey.

Figure 1 One-letter-code sequence of ubiquitin (colored letters) and secondary structure (β -sheets and α -helices). Bottom spectrum: mass spectrum for $[\text{ubi}+10\text{H}]^{10+}$ photoionization by 0.1 μJ 70 fs EUV pulses at 90 eV. Inset: Zoom into the region of immonium-ion related fragments.

Most amino acids contribute to the immonium peaks but their relative intensities do not reflect the amino acid molar ratio of ubiquitin: For instance, the Methionine peak ($m=104$, 1 residue, Clearly, almost all amino acids contribute to the immonium peaks but their relative intensities do not reflect the amino acid molar ratio of ubiquitin: For instance, the Leucine/Isoleucine peak ($m=86$, 16 out of 76 residues) is only 4 times stronger than the Methionine peak ($m=104$, 1 residue), since just a single backbone scission is required for immonium formation from the NH_2 terminal M.

In Figure 2 the same mass spectrum (Figure 2a) is compared to 5 photoionization mass spectra obtained under other conditions. All spectra have been normalized to the total photoabsorption cross section at the respective photon energy to suppress cross sectional effects. An EUV single-photon absorption reference spectrum obtained with synchrotron radiation of 90 eV (Figure 2d) shows features qualitatively and quantitatively similar to the 0.1 μJ FEL spectrum at $h\nu=90$ eV (Figure 2a), albeit on a 30% lower relative intensity scale. At lower photon energy (45 eV, Figure 2c), single photon conditions induce slightly less double ionization and fragmentation. At 45 eV, only about 25% of the

photoabsorption involves strongly bound atomic 2s electrons. At 90 eV this fraction increases to 45%^[18]. As a consequence, excitation energies are on average markedly higher.

For resonant C 1s- π^* excitation at $h\nu=288.5$ eV (Figure 2e) subsequent Auger electron emission is the main ionization mechanism. Again, the mass spectrum resembles the low intensity FEL spectrum (Figure 2a), albeit with lower overall intensities. Single-photon C core ionization at 310 eV (Figure 2f) which is followed by Auger ionization leads to two-electron removal. From Figure 2f it is evident that the $[\text{ubi}+10\text{H}]^{12+}$ peak

Figure 2. Photoionization mass spectra of $[\text{ubi}+10\text{H}]^{10+}$ in 70 fs EUV pulses at 90 eV at 0.1 μJ a) and at 2.3 μJ b) pulse energy. For comparison, data for single photon absorption (obtained at the BESSY II synchrotron) at 45 eV (c), 90 eV (d), 288.5 eV (e) and 310 eV (f) are shown. The intensities are normalized to the respective total photoabsorption cross section

is stronger than $[\text{ubi}+10\text{H}]^{11+}$ one, which is strongly suppressed.

For massive multiphoton absorption in 2.3 μJ , 70 fs FEL pulses (Figure 2b) immonium related fragment ions with small m/z dominate the spectrum. Their distribution is very similar to the one at 0.1 μJ (Figure 2a). The $[\text{ubi}+10\text{H}]^{11+}$ and $[\text{ubi}+10\text{H}]^{12+}$ peaks remain visible. These ions cannot be formed in the focus of the beam, where single and double photoabsorption conditions are ruled out, considering the orders of magnitude of photon fluence and photoabsorption cross section. Accordingly, $[\text{ubi}+10\text{H}]^{11+}$ and $[\text{ubi}+10\text{H}]^{12+}$ are most likely formed in the low-intensity halo surrounding the high-intensity focus of the beam.

It is instructive to quantitatively analyse partial ion yields as a function of FEL pulse intensity. The spatial beam profile perpendicular to the beam axis is approximately Gaussian, and

the (time averaged) intensity profile around the focus can thus be described as^[19]:

$$I(r, z) = \frac{4 \ln 2}{\pi \Delta^2(z)} \exp\left(-\frac{4 \ln 2}{\Delta^2(z)} r^2\right) I_0 \text{ with } \Delta(z) = \Delta_0 \sqrt{1 + \left(\frac{z}{z_0}\right)^2} \quad (1)$$

with $\Delta(z)$ the beam width at a distance z from the focus and r the radial distance from the beam axis. The Gaussian width of the beam in the focus (beam waist) is $\Delta_0 = 25 \mu\text{m}$ and the Rayleigh length (representing beam divergence) is $z_0 = 0.84 \text{ mm}$. Details are given in the supporting information.

The estimation of photoabsorption yields using this equation requires the ubiquitin photoabsorption cross section at 90 eV. This photon energy well exceeds the binding energy of the deepest O 2s valence orbitals (34.3 eV in glycyl-glycine^[20]). The $[\text{ubi}+10\text{H}]^{10+}$ ionization energy can be extrapolated from the value for $[\text{ubi}+9\text{H}]^{9+}$ ($\sim 13.9 \text{ eV}^{[15]}$) to be 14.2 eV. Well above the ionization threshold, photoionization cross sections are usually reproduced within a few percent by summation of the atomic cross sections^[21]. For ubiquitin (378 C, 105 N, 118 O, 1 S, 629 H atoms), a total cross section of $\sigma_{\text{total}}(90 \text{ eV}) \sim 6.7 \times 10^{-18} \text{ cm}^2$ is obtained when using tabulated atomic data^[18].

Figure 4 . a) total ion photoionization yield as a function of FEL pulse intensity and b) non-dissociative single ionization ($[\text{ubi}+10\text{H}]^{11+}$ and $[\text{ubi}+10\text{H}-\text{CO}_2]^{11+}$) (blue) and non-dissociative double ionization ($[\text{ubi}+10\text{H}]^{12+}$ and $[\text{ubi}+10\text{H}-\text{CO}_2]^{12+}$) (red). The symbols represent experimental data and the lines have been calculated using equation (1) for a focus fwhm of $25 \mu\text{m}$ (see text).

With $I(r, z)$ and σ_{total} the mean ionization state of ubiquitin cations in the focus of an FEL pulse can be determined for every space coordinate, assuming independent photoabsorption events with identical cross section. This assumption is based on i) the moderate FEL intensity, that leads to only relatively few ionization events per ubiquitin cation and ii) the small increase in protein ionization potential with charge state, that typically amounts to less than 1 eV per charge state, even when structural relaxation is hampered^[15]. Assuming Poisson distributed ionization states, the distribution of the degrees of photoionization in the total focal volume, is obtained by mere integration.

Figure 3a displays the experimental total photoionization yield, ($[\text{ubi}+10\text{H}]^{10+}$ loss from the trap), as a function of FEL pulse intensity in arbitrary units. The calculated total yield was scaled to fit the experimental data. Figure 3b shows partial ion yields for non-dissociative single and double ionization, and for the associated CO_2 loss channels. For comparison, the calculated yields for one and two photon absorption, respectively are given as solid lines (data scaled with the same factor as for the total yield and with a branching ratio obtained by scaling). For single

ionization, the branching ratios of 25% for the non-dissociative channel ($[\text{ubi}+10\text{H}]^{11+}$) and 19% for additional CO_2 loss ($[\text{ubi}+10\text{H}-\text{CO}_2]^{11+}$) are found. The remaining 56% is likely to give rise to unresolved fragmentation channels in the extended pedestal of the single ionization peaks (see also Figure 1 (inset)). For double ionization, the branching ratios of 12% ($[\text{ubi}+10\text{H}]^{12+}$) and 19% ($[\text{ubi}+10\text{H}-\text{CO}_2]^{12+}$) are substantially lower, because the increased excitation energy accompanying the ionization process induces stronger fragmentation. Non-dissociative triple ionization is not observed. Note, that we have only considered independent single photoionization processes. It is known, that for $h\nu=90 \text{ eV}$, double photoionization contributes significantly (e.g. about 20% for benzene^[22]), partly due to direct simultaneous emission of two electrons and partly due to sequential processes via excited singly charged intermediates.

The increase of single and double ionization yields with pulse intensity is due to the increase of the ionization volume. However, our main interest is multiple ionization in very strong XUV fields. The calculations (see supporting material) show that the distribution of ionization states reached in the beam focus shifts dramatically with beam intensity. At $0.1 \mu\text{J}$ pulse energy, about 97% of the ion signal is due to single ionization, while at $2.3 \mu\text{J}$, much higher initial charge states are formed, for instance 18-fold ionization is predicted to contribute $\sim 1\%$. The bimodal nature of the spectrum in Figure 2b ($2.3 \mu\text{J}$) resembles the superposition of two distinct photoionization regimes identified in gas phase synchrotron experiments the occurrence of: i) non-dissociative single and double ionization^[11] for protonated large proteins ii) fragmentation, with immonium ions dominating^[12, 13], for smaller protonated peptides. The small m/z part of the spectrum (immonium ions) shows up at high intensities indicating that it is related to multiple-photon absorption processes in the high intensity part of the focus.

Figure 3 Photoionization mass spectra of $[\text{ubi}-6\text{H}]^{6-}$ under single 90 eV photon conditions (a, obtained at the BESSY II synchrotron) and for 90 fs EUV pulses at 90 eV at $8.5 \mu\text{J}$ (b). The intensities are normalized to the respective total photoabsorption cross section. In both cases, no small negative fragment ions are observed. The low mass spectrum in (b) (colored in blue) represents positive fragment ions formed from the negative precursor $[\text{ubi}-6\text{H}]^{6-}$.

Before investigating this phenomenon in more detail, it is important to demonstrate that it is not limited to a small range of protein charge states. To this end, photoabsorption in a multiply de-protonated system $[\text{ubi}-6\text{H}]^{6-}$ was studied. The obtained mass spectra are displayed in Figure 4. Absorption of a single 90 eV photon leads to non-dissociative detachment of one or more electrons, i.e. formation of $[\text{ubi}-6\text{H}]^{5-}$, $[\text{ubi}-6\text{H}]^{4-}$ and $[\text{ubi}-6\text{H}]^{3-}$ (see Figure 4a, right panel). The peaks are broadened, due to loss of small neutral groups, as also observed for $[\text{ubi}+10\text{H}]^{10+}$. The high m/z of $[\text{ubi}-6\text{H}]^{2-}$ (not shown here) prevents unambiguous identification because of low detection efficiency and strong broadening of the peak. No negative small fragment ions are observed (see Figure 4a, left panel).

In analogy to the case of $[\text{ubi}+10\text{H}]^{10+}$, where non-dissociative ionization into $[\text{ubi}+10\text{H}]^{11+/12+}$ was still observed for 70fs and 2.3 μJ pulses (see Figure 2b), also for $[\text{ubi}-6\text{H}]^6$ in slightly more intense FEL pulses (90fs and 8.5 μJ), electron detachment into $[\text{ubi}-6\text{H}]^{5-/4-/3-}$ remains an important channel (see Figure 4b, right panel). As for single photon absorption, small negative fragments are unobserved (data not shown, here). However, Figure 4b (left panel) displays the positive ion mass spectrum for the lower mass range. The mass spectrum is qualitatively and quantitatively very similar to the one obtained from $[\text{ubi}+10\text{H}]^{10+}$ (Figure 2b), with immonium ions as dominating features. Sequence ions, solely due to backbone scission are almost completely absent. If addition of 16 negative charges a single ubiquitin molecular ion does not have a substantial influence on ubiquitin photofragmentation in intense EUV pulses, fragmentation due to Coulomb repulsion can be ruled out.

Figure 5 Partial ion yields as a function of photons per micropulse for 4 of the strongest immonium (and related) ions. The scaling is identical to Figure 4. The other immonium ions follow a linear dependence, as well.

Representative for the low mass part of the spectrum, the yields for 4 immonium (or related) ions as a function of FEL pulse intensity are displayed in Figure 5 and a clear linear dependence is observed (as for the other immonium ions). This is clearly different from the total ion yield (Figure 3a) and in particular from the partial yields for non-dissociative single and double ionization, which saturate at moderate photon fluxes (Figure 3b). The bimodal nature of the mass spectrum (high m/z peaks, mainly due to non-dissociative photoionization processes and low m/z peaks, mainly due to immonium ions) persists when pulse intensity increases. Relatively more immonium ions are formed and relatively less non-dissociative processes occur, but there is no transition region, in which intermediate size fragments due to backbone scission of the protein (sequence ions) prevail. Both findings suggest, that fs EUV multiphoton ionization cannot be viewed as a process in which the number of increased photons translates into a gradual increase of protein total internal energy and charge, ultimately emerging in the fragmentation pattern. The correct image seems to be one, in which the protein undergoes *independent* photoionization processes. Each local photoionization process then induces fast local fragmentation processes in which immonium ions are produced. These processes occur before IVR can set in. The protein thus responds to EUV photoionization, as an ensemble of small, few amino-acid peptides.

We have shown that fs absorption of 10 or more EUV photons by a small protein induces fragmentation into small fragments, independent on the initial charge state of the protein. The molecular dynamics are dominated by fast local structural responses. In the future, these processes will be studied in more

detail by pump-probe type experiments and for the even higher intensities and photon energies, employed in hard X-ray diffraction imaging.

Acknowledgements

The FEL experiments were carried out at the FLASH facility at DESY, a member of the Helmholtz Association (HGF). We would like to thank Stefan Düsterer for assistance in using beamline B3. We also thank the Helmholtz Zentrum Berlin for allocation of synchrotron beamtime and acknowledge funding from the EC's Seventh Framework Programme (FP7/2007-2013, grant nr.312284). GR is supported by the Netherlands Organization for Scientific Research (NWO, Rubicon 68-50-1410)

Keywords: mass spectrometry • photoionization • proteins • electron transport • polycations

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