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Multiple Electron Ejection from Proteins Resulting from Single Photon Excitation in the Valence Shell

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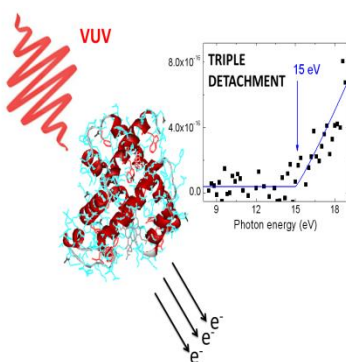
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ABSTRACT. One photon multiple-ionization is a signature of dynamical electron correlations in atoms and small molecules, as observed in the Auger process when Auger electron emission follows core-shell ionization. In such a process, the high energy needed to remove several electrons is due to the strong Coulombic attraction between the last departing electron(s) and the ionic core. Multiply negatively charged molecules offer the possibility to overcome the Coulombic attraction opening the way for multi-electron photodetachment following valence shell excitation. Here, photodetachment studies have been performed on electrosprayed protein polyanions using vacuum ultra-violet synchrotron radiation coupled to a radiofrequency ion trap. Double, triple and quadruple electron emissions from protein poly-anions resulting from single photon excitation in the valence shell were observed with ionization thresholds below 20 eV photon energy. This suggests the existence of large electronic correlations in proteins between weakly-bound electrons standing on distant sites. Besides, the resulting multiradical polyanions appears to be remarkably stable, an important issue in radiobiology.

TOC GRAPHICS



KEYWORDS. PHOTOIONIZATION; MOLECULES; NEGATIVE-IONS; CHARGED IONS; ENERGY-RANGE; PROTEIN POLYANIONS; VACUUM-ULTRAVIOLET; DYNAMICS

Electron correlations play an essential role in a wide range of fundamentally important many-body phenomena in modern physics and chemistry. The advent of short pulses from the vacuum ultraviolet (VUV) up to the extreme ultraviolet (XUV) wavelength range reinforced this area of research with new perspectives for the observation of the correlated electron dynamics in one of its striking evidence: multi-ionization of atoms and molecules.^{1,2} Double ionization of neutral atoms and molecules following either inner-shell excitation with X-ray light or via a direct valence-shell process is now a well-documented mechanism.^{3,4} Mass spectrometry and various electron/electron and ion/ion coincidence techniques, coupled to the Synchrotron Radiation (SR) have been used for the study of inner-shell triple-photoionization on lithium,⁵ rare gas atoms^{4,6} and small molecules.⁷⁻⁹

When the initial targets are negatively charged ions, the ejection of one electron after light excitation is referred to as photodetachment, a process that requires little energy (less than a few eV) due to the weak Coulomb attraction experienced by the outgoing electron. In the past, this process has been widely studied on small atomic and molecular systems in the visible and near UV using lasers. Also, inner-shell double, triple, and quadruple photo-detachment has been recently reported for atomic Ru anion using merged beams techniques at synchrotron radiation facilities.¹⁰

Developments on electrospray ionization sources have made possible the study of photodetachment from biomolecular polyanions (protein, peptides, nucleic acids...) using tunable UV lasers.^{11,12} However, very few studies have been performed using VUV for electron photodetachment from negatively charged ions. These include our recent report on the photodetachment spectroscopy of closed and open shells protein anions in the VUV.¹³ In

particular, we evidenced a double electron detachment (with a threshold at 11.4 eV) in the case of the multiply deprotonated (5- charge state) insulin protein anions.¹⁴

In this work, we aim at exploring for the first time the possibility of valence-shell direct triple and quadruple photodetachment on various protein polyanions, resulting from single photon excitation in the valence shell. To get some insights onto the mechanisms leading to multiple electron emission, we also document threshold values for valence shell double detachment on a large array of proteins of different sizes and holding different charge states.

The experimental setup is based upon a linear ion-trap mass spectrometer¹⁵ coupled to the DESIRS VUV beamline¹⁶ of the SOLEIL synchrotron radiation facility (France). After irradiation of selected $[M-nH]^{n-}$ anions, $[M-nH]^{(n-m)-\bullet}$ ions resulting from the emission of m electrons are detected. As we have already observed in the case of insulin,¹⁴ one-photon multi-ionization process (eq. 1) is in competition with multiphoton processes due to sequential absorption of multiple photons.



Indeed, for long irradiation times (several hundreds of milliseconds) of the trapped ions, the protein can sequentially eject several electrons by a multi-steps mechanism, in which the product ion from single photodetachment $[M-nH]^{(n-1)-\bullet}$ is stored long enough to reabsorb another photon and then lose an additional electron leading to $[M-nH]^{(n-2)-\bullet\bullet}$, etc... Competition between these two mechanisms is exemplified here for the case of double electron detachment from cytochrome C $[M-11H]^{11-}$ —a protein comprising a sequence of 104 amino acid residues. To disentangle both mechanisms, the ion abundance of the $[M-11H]^{9-\bullet\bullet}$ ion of cytochrome C has been monitored as a function of the irradiation time at 9.5 and 15 eV photon energy as shown in

figure S1 b) and c) in supporting information, respectively. It appears that at 9 eV photon energy (fig. S1b), a nearly zero intensity accounting for the vanishing non-zero background is observed for irradiation time shorter than 100 ms, with an overall pure quadratic dependence with the irradiation duration. This clearly indicates, that observation of double photodetachment at 9 eV comes from a sequential two-photons two-steps mechanism. In contrast, at 15 eV, for low irradiation duration (ie up to ~80 ms), the data are adequately fitted by a linear function. In this range, the one-photon process is dominant. At longer irradiation time, the trend becomes non-linear and the sequential two-photon mechanism is favored. Therefore, in the following, the relative cross sections obtained for protein polyanions have been acquired at 90 ms irradiation time corresponding mainly to the linear regime. Hence, for 90 ms irradiation time, the double photodetachment relative cross section of $[M-IH]^{9-}$ ion of cytochrome c shown in figure S1a) may entirely be ascribed to a valence double photodetachment. A clear onset is observed at 12 eV as derived from a threshold linear Wannier-type fitting.¹⁷ The Wannier fitting is used here only as a convenient mean of extracting the threshold value without physical assumption of the underlying physics.

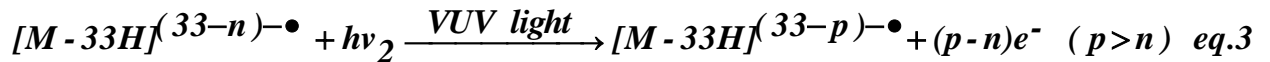
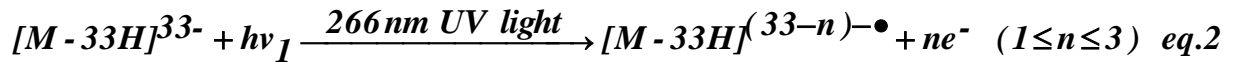
As reported in Table I, thresholds for the double photodetachment are very similar for all protein polyanions. They slightly increase with the size of the protein (from 11.4 eV for insulin to 12.2 eV for larger proteins like myoglobin and BSA). However, the electron density in the studied polyanions is different and decreases when the size of the protein increases (5 charges for 5810 Da for insulin vs 33 charges for 66420 Da for BSA). This slight increase in the thresholds for the double photodetachment might be correlated with the slight decrease of the electron density in the protein polyanion. Interestingly, for a given protein (cytochrome c), the thresholds for the double photodetachment are found to be little dependent on the charge state (they range

from 11.4 eV for 7- to 12 eV for 12-, see Table I). The adiabatic electron affinities (AEA) of the cytochrome c (CytoC) protein have been experimentally determined by Vonderach et al.¹⁸ and are roughly constant from 6- to 10- charge states and then slightly decrease from 10- to 12- charge states. This is due to the fact that the additional charges in protein polyanions can be easily accommodated by a partial unfolding of the CytoC. This partial unfolding prevents a significant increase in the coulomb repulsion as observed with the adiabatic electron affinities and may also explain the almost constant value for double detachment threshold. Partial unfolding already occurs for charge state 12- (see ref.¹⁸) which is an indication that conformation of the protein is a not a key factor for the multiple detachment process.

One objective of this work is to explore the possibility to photodetach more than two electrons with a one-photon VUV valence shell excitation. While cytochrome C only leads to double detachment after VUV photon irradiation in the explored energy range, larger proteins, such as myoglobin and bovine serum albumin (BSA, 66.5 kDa), allow for valence shell triple photodetachment. As an illustration, the single, double and triple photodetachment relative cross sections of myoglobin $[M-9H]^{9-}$ ion (i.e. leading to $[M-9H]^{8-}$, $[M-9H]^{7-}$, $[M-9H]^{6-}$, respectively) in the 8 eV to 19 eV range, are reported in Fig. 1. For the single detachment yield, which threshold is located well below 8 eV, a monotonic increase in detachment yield from 8 to 19 eV, with superimposed structures is visible, as already observed for photodetachment yields of other protein polyanions.¹³ For double and triple photodetachment relative cross sections, sharp thresholds are clearly visible and were determined at 12.2 eV and 15 eV (see Fig. 1b and c). The detachment yields as a function of irradiation time show that the double and triple photodetachment relative cross section (for 90 ms of irradiation time) may entirely be ascribed to a valence-shell double and triple photodetachment (see Fig. S2 in supporting information). In our

work, the two (three) electron process has a yield of ~50 (~200) times lower than the one observed for single electron ejection (as reported in Fig. 1), as it can be seen in Fig.2a (whose vertical axis is logarithmic not linear). In organic molecules, the ratio of single to double ionization has been reported to be around 2%,¹⁹ which is of the same magnitude as our findings in the case of photodetachment. Similarly, for BSA protein polyanions (33- charge state), thresholds at 12.2 eV and 15 eV were obtained for double and triple photodetachments (see Table I). A valence shell quadruple photodetachment for BSA polyanion (33- charge state) was observed (see Fig. 2) with a threshold estimated at ~18.2 eV.

The crucial issue of sequential absorption of multiple photons versus single photon process was further examined by measuring the photodetachment yields for the photodetachment of $[M-33H]^{(33-n)-\bullet}$ oxidized products of BSA within a complementary two-color scheme experiment combining a UV laser at 266 nm to the VUV light as we already used in Ref.²⁰



The measured photodetachment yield, corresponding to the process described by equation 3 for the photo-generated $[M-33H]^{(33-n)-\bullet}$ radicals ($1 \leq n \leq 3$) (produced via eq. 2) is compared to that of the corresponding closed-shell species $[M-33H]^{33-}$ in figure S3. It shows that oxidized ions have qualitatively very similar absorption yields as their precursor ions. A sequential mechanism due to the absorption of several photons would result in a multiple photodetachment yield equal to the product of the detachment yields recorded for the different steps (Figure 2b) for BSA) The calculated yield corresponding to this sequential absorption of multiple photons is compared in Figure 2 to the experimental yield of the BSA charge state 30^- as produced by a one-VUV

photon triple detachment, from 33^- BSA ions. The clear discrepancy between the two curves confirms, beyond irradiation time dependences, the existence of a multiple detachment due to the absorption of a single VUV photon.

In order to eject n electrons, the photon energy has to be higher than the binding energies of the n electrons of the precursor protein ion. Because the process involves polyanions (for both precursors and products), the deposited energy has also to allow to overcome the Coulombic barrier which results from repulsive interaction between negative charges.²¹ Possibly, the Coulomb barrier for such simultaneous emission of two (or more) electrons may be significantly lower than the barrier for the emission of just one electron, as it was demonstrated for the two-electron emission after photoexcitation of metal-cluster dianions.²² This may explain the relative efficiency of multiple detachment in polyanions and the possibility to disentangle it from multistep mechanisms.

Several processes might account for such a multidetachment after single photon excitation in the valence shell. A knock-out process has been proposed by several authors to account for double photoionization in atoms and rather small molecules.^{19, 23} In this process, an emitted electron transfers part of its kinetic energy to another electron resulting in the emission of an additional electron. It involves electron-electron collisions which cross sections are expected to be low in proteins, owing to the large distances between the negative charges spread over the whole protein skeleton on acidic sites leading to small collision solid angles between the charges. This would lead to a particularly non-efficient process for the triple and quadruple electron losses. As an example, we have calculated the electron-electron distance distribution in the myoglobin protein (9- charge state) assuming a native structure. Both electron interdistance between (i) two negative charges (held by carboxylate groups) and between (ii) a charge on a

carboxylate group and electron from aromatic rings. Data are reported in Fig. S4 in supporting information. Electron interdistances from two negative charges range from 10 Å to 45 Å, with an average value around 30 Å. Interestingly, the interdistance between a charge on a carboxylate group and electron from an aromatic ring is shorter with an average value around 24 Å. This may favor electronic couplings between aromatic amino-acid group and carboxylate groups to the detriment of a particule-particule collision scheme such as a “knock-out process”. We may then postulate that multiple photodetachment arises from a one-photon excitation of several electrons. This multi-electron excitation process would either result in the concerted emission of several electrons due to direct excitations into the continuum (figure 3a) or, following the previously-described resonant mechanism for single photodetachment,¹² to auto-ionization after excitation of resonant bound states (figure 3b). Another possibility would be a one-photon excitation of a single electron resulting in a highly excited state that would then decay via multiple autoionization into the multiple photodetachment continuum (figure 3c). An electron can be ejected following VUV single photon absorption. Following this primary event, the ejection of two other electrons may occur through an Auger-like process (figure 3d). Interestingly, Zubarev and coworkers²⁴ have evidenced that electron detachment from multiply charged anions could be obtained by interactions with fast electrons (>10 eV). The onset of the polypeptide chain ionization leads to creation of a positive radical charge (hole). This hole is mobile, with the driving forces for its transfer being the Coulombic attraction to the negative charges as well as the difference in local ionization energies of amino acid residues. Mutual neutralization of the hole and an electron results in electronic excitation that causes backbone bond cleavage. One may speculate that mutual neutralization of the hole and an electron might induce ejection of low-binding energy electrons as suggested in mechanisms c) and d).

Assuming that 15 eV in a protein like myoglobin (possessing 1569 atoms) is redistributed in all the vibrational modes would lead to an increase in temperature of 0.12 K. This increase of temperature is of course not sufficient to induce thermoionic processes. However, it has been shown in clusters (in particular with the pioneering works of E. Campbell on fullerenes)²⁵ that the combination of a high density of vibrational states together with strong rovibronic couplings and a low electron binding energy is particularly favorable for the development of delayed electron emission. Matheis et al.¹¹ have shown that delayed emission is a competitive process to direct detachment in polypeptides. However, they reported that in the far-UV (213 nm), a dense manifold of excited states of the monoanion becomes accessible (corresponding to remaining π - π^* excitations), increasing the direct detachment cross section. We may expect that the direct detachment cross section continues to increase in the VUV range. These considerations permit to rule out the thermionic emission.

Beyond the above mechanistic discussion, another approach for the understanding of the striking results we observed consists in considering the energetic of the multi-detachment process. Indeed, phenomenologically, for the n th photodetachment process in protein polyanions the array of results that we gathered and which are summarized in Table 1 suggest that an empirical threshold (PD n) law could be written as :

$$PD^n \cong 8.5 + (n - 1) \times 3.2 \text{ eV} \quad (2 \leq n \leq 4) \quad \text{eq.4}$$

These PD n threshold value appears to roughly match the sum of the electron affinity of the carboxylate ($\sim 3.2\text{eV}$)²⁶ and the ionization energy of aromatic amino-acids ($\sim 8 \text{ eV}$)^{27, 28}. Thus, it may be suggested that multi-electron emission by photodetachment requires first the energy for

ionization of one electron localized onto a peptide bond or a neutral amino acid and then an additional energy for the detachment of extra electrons from carboxylate groups. Eq. 4 shows that the detachment mechanism (concerted multi-electron process or high energy excitation of a single electron coupled to multi-electron emission, see Figure 3) is only efficient when it involves excitation of an electron from peptide bonds or amino-acids (other than low binding electrons on carboxylates). Eq. 4 appears to be quite universal, valid for small (insulin) or very large (BSA) proteins, and for a huge range of charge state (here from 5^- to 33^-). The multi-photodetachment is a clear signature of strong electronic correlations occurring within the protein. Eq. 4 suggests a correlation between the carboxylate electrons and the electrons on other chromophores, which are distributed on distant sites, as exemplified for 9- charge state of myoglobin protein (see fig. 4). The evidence for excitations delocalized on the whole protein and its influence on charge and information flows, have still to be assessed.

Besides, a striking observation on the recorded mass spectra is the stability of the oxidized protein (exhibiting very little fragmentation) over the time scale of the experiment (up to 10 s), even in the case of multiple photodetachment. Protein oxidized anions are long-lived species, a feature of great importance in biology, such as in aging. Moreover, multiple-photodetachment could be a major process by which VUV irradiation of proteins may lead to the emission of numerous low energy electrons possibly leading to specific radiation damages.²⁹

Our experimental findings open the way for electron correlation studies in molecules in an unprecedented mass and size domain, i.e. on full proteins, with the emission of three and four electrons localized on distant sites, well beyond the atomic or molecular (up to tri-atomics) cases previously studied. They open a new window for extremely rich basic research, with exploration of quantum effects in bioorganic matter. In particular, the energy correlation between the n

electrons emitted and the exact electronic structure of the ion produced should be studied in detail in the future, ideally by electron spectroscopy, in order to understand both the dynamics of the process and its implications in terms of structure and stability. This means understanding how the energy deposited into the system is distributed among the different electronic and nuclear degrees of freedom, which may or may not lead to the fragmentation of the molecule.

Materials and methods

The experimental setup is based upon a linear ion-trap mass spectrometer (LTQ XL, Thermo Electron, San Jose, CA, USA) coupled to the DESIRS undulator-based beamline¹⁶ of the SOLEIL synchrotron radiation facility (France) to perform action spectroscopy in the VUV range, and is described in details in refs.^{15, 20, 30, 31}. Briefly, multiply deprotonated insulin, cytochrome C from equine heart, apo-myoglobin and bovine serum albumin (BSA) proteins (Sigma Aldrich) were generated from a 50/50 water/acetonitrile (v/v) solution at a concentration of $\sim 50 \mu\text{M}$ and directly electrosprayed at a flow rate of $5 \mu\text{L}/\text{min}$ for subsequent analysis in a negative-ion mode. For photo-induced electron detachment experiments, the precursor parent ions were selected, and irradiated by the SR for 90 ms, by monochromatized VUV photon with typical flux in the 10^{12} - 10^{13} ph/sec range in a 5-20 meV bandwidth. The high harmonics of the undulator which could be transmitted by the grating's diffraction high order were cut-off by a Ar-filled gas filter³² between 8 and 16 eV. After irradiation of selected anions, the mass spectrum is recorded, leading to the observation of charge reduced ions (see Eq. 1). The yield of the ion product is monitored as a function of $h\nu$. Repeating this procedure over the photon energy range leads to a VUV action spectra which are normalized to the incoming photon flux as measured by a Si

photodiode (IRD, AXUV 100). The two-color-scheme (See also Ref. ²⁰) on oxidized product was achieved by irradiation of the stored ion with the 4th harmonics of a Nd :YAG laser (266 nm), co-propagating with the SR owing to the insertion of a drilled mirror, prior to the photodetachment with the SR.

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Supporting Information. Partial relative cross section in the 8-15.2 eV for 90 ms irradiation duration for double photodetachment of cytochrome c [M-11H]¹¹⁻. Ion abundances for myoglobin for the double and triple photodetachment product ion at 9.5 eV and 18.8 eV recorded as function of the irradiation time. Yields of photodetachment as a function of the SR energy for BSA as a function of the oxidation state. Calculated electron-electron distance distribution in the myoglobin protein (9- charge state). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Protein Name	Molecular weight (Da)	Charge State	Adiabatic electron affinities (eV) (from ref. ¹⁸)	Threshold (eV)		
				Double	Triple	Quadruple
Insulin	5 808	5-		11.4±0.1 (from ref. ¹⁴)		
Cytochrome C	12 384	7-	0.90 ± 0.2	11.4±0.25		
		8-	0.95 ± 0.2	11.4±0.25		
		9-	1.0 ± 0.2	11.5±0.1		
		10-	0.95 ± 0.2	11.6±0.25		
		11-	0.5 ± 0.2	12.0±0.1		
		12-	0.15 ± 0.2	12.0±0.25		
Myoglobin	16 951	9-		12.2±0.25	15±0.25	
BSA	66 430	33-		12.2±0.3	14.8±0.3	18.2±0.3

TABLE I: Molecular weight and charge state of proteins studied in this work. Adiabatic electron energies and thresholds for double, triple and quadruple photodetachment are given.

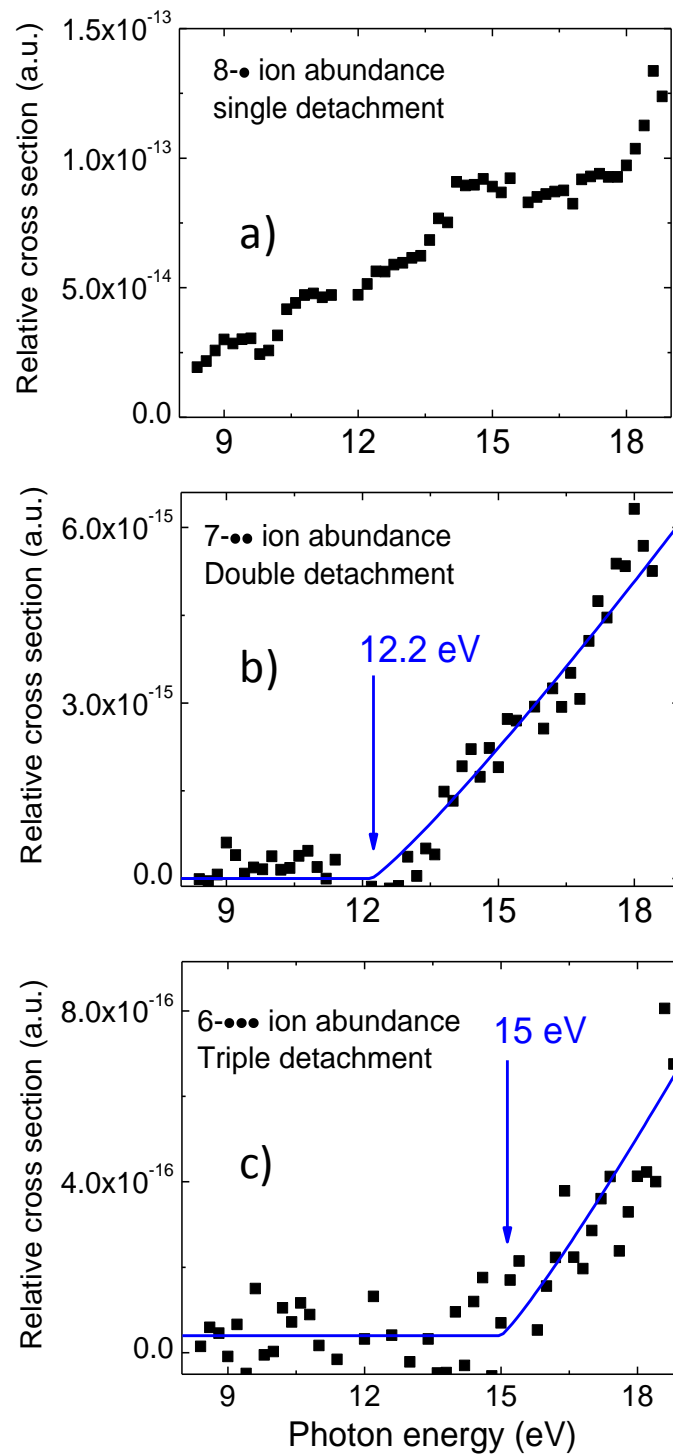


Figure 1 : relative cross section for 90 ms irradiation duration for a) single, b) double and c) triple photodetachment for the 9- charge state of myoglobin protein anions. The solid line is a Wannier-type linear fit to the data.

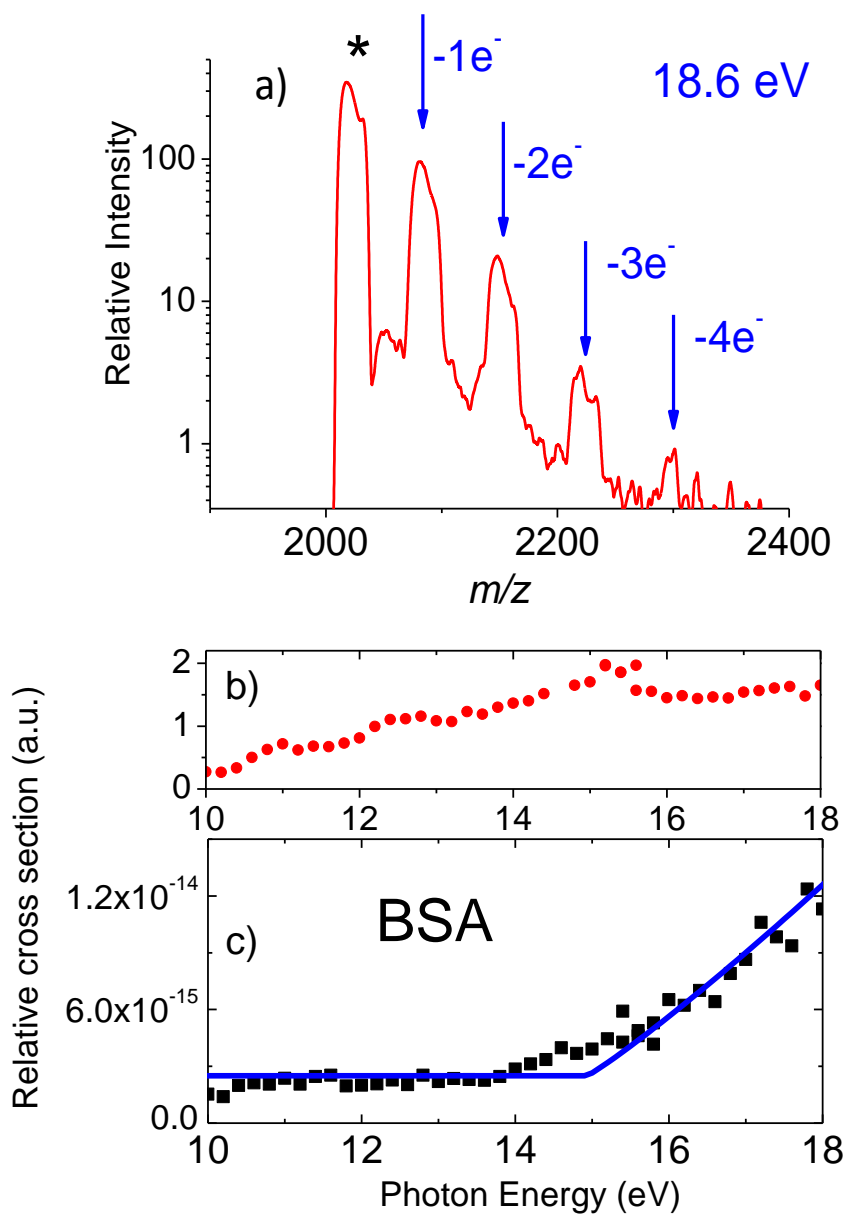
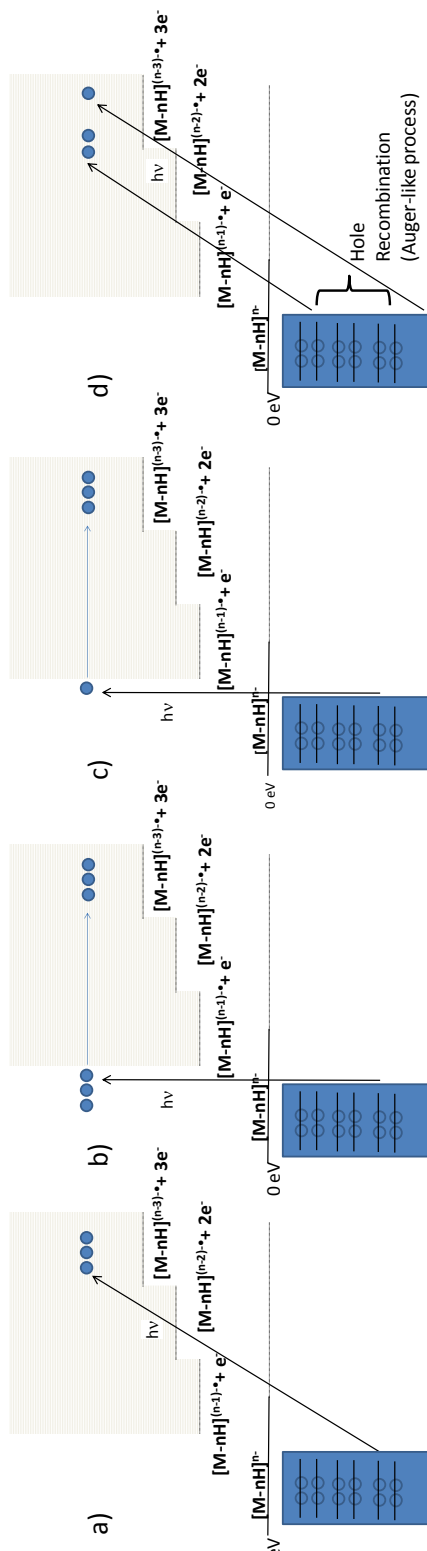


Figure 2 : a) Mass spectrum after irradiation with 18.6eV photons and 90 ms irradiation duration of BSA $[M-33H]^{33-}$ anion. The asterisk corresponds to the precursor ion ($[M-33H]^{33-}$, m/z 2017). b) (red filled circles) Calculated multiple photodetachment yield which would correspond to ionization by sequential absorption of multiple photons. It is equal to the product of the detachment yields recorded for the different steps ($33- \rightarrow 32-\bullet \rightarrow 31-\bullet\bullet \rightarrow 30-\bullet\bullet\bullet$). The curves used to produce this lastest curve are shown in Fig. S3. c) (black filled squares) relative cross section for 90 ms irradiation duration for triple photodetachment for the $33-$ charge state of BSA protein anions. The solid line is a Wannier-type linear fit to the data.

Figure 3: Proposed photodetachment, detachment: a) concerted b) multi-electron resonant emission of three electrons; leading to emission of single electron ionization leading Auger-like process, hole ejection of additional



mechanisms for multi-exemplified in the case of triple direct three electron emission; excitation followed by c) single electron excitation three electrons. d) single to formation of a hole. Via an recombination leads to the electrons.

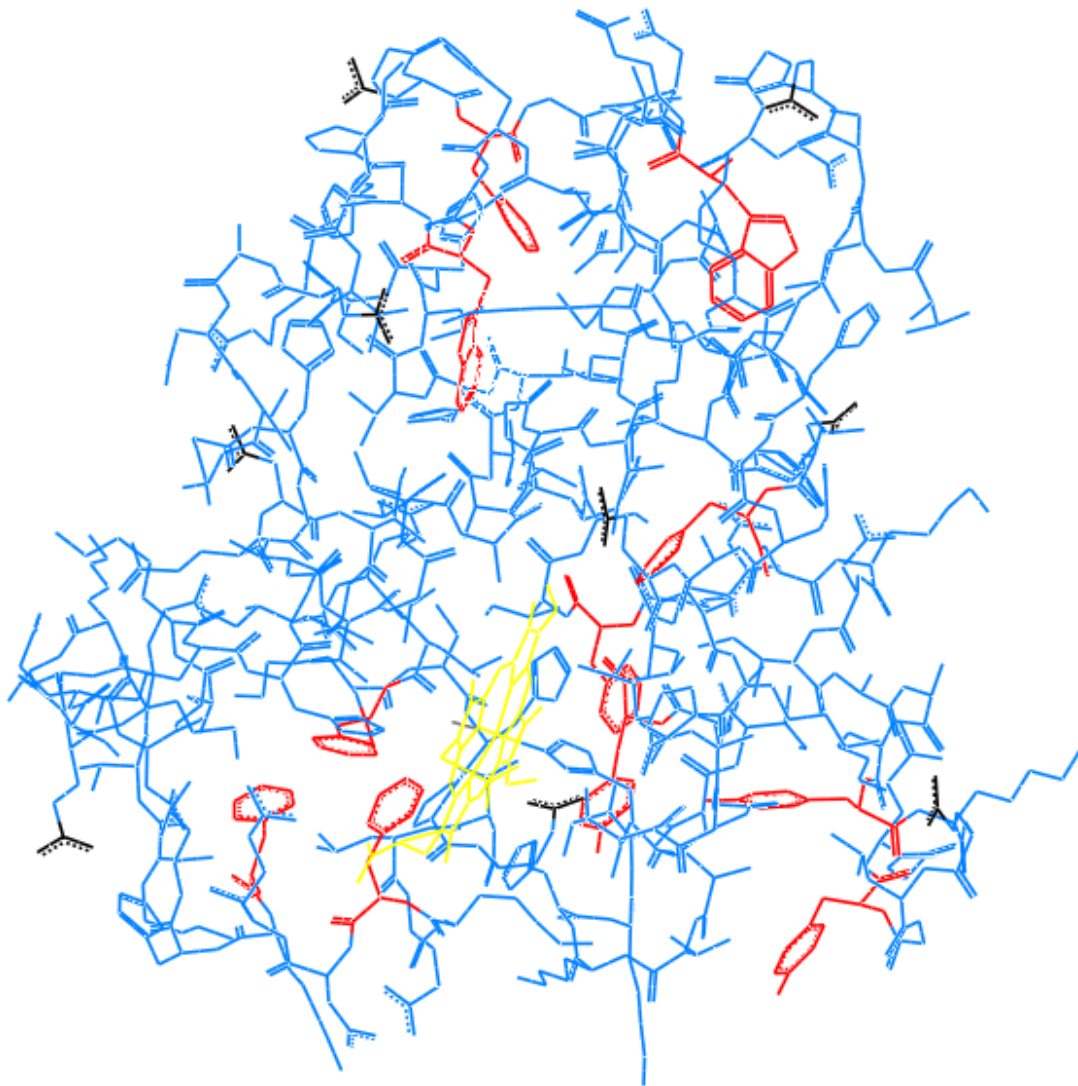


Figure 4: Native structure of myoglobin as obtained from X-ray diffraction, taken from the protein data base pdb (1MBN). The location of the 9 negative charges (determined through minimization of the Coulomb energy) on the oxygen atoms of carboxylic groups of acidic residues are highlighted in black, as well as aromatic groups of aromatic amino-acid highlighted in red. The prosthetic heme group is also shown in yellow. This figure shows that the weakest bond electrons, which are on carboxylate groups are localized on distant sites on the protein.