Mass Spectrometry of Proteins

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# Multiple Soft Ionization of Gas-Phase Proteins and Swift Backbone Dissociation in Collisions with ≤99 eV Electrons**

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Mass spectrometry (MS) is based on the ionization of sample molecules and subsequent measurement of their mass-to-charge ratio $m/z$.[1] For measurements of molecular mass, it is vital to preserve the intact molecular ion after ionization, that is, to perform "soft ionization".[2] Since the end of the 1980s, large (>10 kDa) biomolecules have been subjected to soft ionization by either electrospray ionization (ESI)[3] or matrix-assisted laser desorption ionization (MALDI),[4] which both add protons to neutral molecules in the positive-ion mode. With the emergence of ESI and MALDI, the challenge has shifted to informative fragmentation of polypeptides.[5] In particular, "native mass spectrometry"[6] produces intact protein–protein and protein–molecule complexes in low protonation states; such complexes fragment poorly and thus yield little structural information. Facile fragmentation of such species requires the rapid introduction of additional charges or radical sites,[7] or preferably both of these types of bond weakening simultaneously.

Before the emergence of soft-ionization techniques, gas-phase cations of biomolecules were predominantly obtained by electron ionization (EI) in which 70 eV electrons remove one valence electron from gas-phase neutral molecules. Whereas small molecules can be ionized by electrons two or three times,[8,9] for molecules larger than 1 kDa, attempts to obtain even singly charged molecular ions failed initially.[10] However, in the 1990s it was shown that protonated biopolymers can increase their charge state by one unit in gas-phase collisions with electrons that have an energy higher than 11 eV.[11] Recently, our research group demonstrated that above 40 eV, double ionization can occur for molecules as large as ubiquitin (8.6 kDa).[12] However, for small proteins, the yield in EI of intact molecular species was relatively small in comparison to the yield of species derived from the dominant EI-induced dissociation. Herein, we report that for larger (>10 kDa) protein ions, multiple ionization can occur without the fragmentation of molecular species. Moreover, protein–molecule complexes, such as holomyoglobin, retain elements of their tertiary structure even after four ionizations, and when backbone fragmentation occurs, polypeptide fragments can preserve weak interactions with the noncovalently bound molecule.

Figure 1 presents the EI mass spectrum of cytochrome *c*, a 12.4 kDa single polypeptide chain with a covalently attached heme group. Upon irradiation with approximately 50 eV electrons, precursor 8+ ions increased their charge state up to 12+. Even after four ionizations, the most abundant ionic species above $m/z$ 700 were the intact molecular ions. In total, the ionized species together with the remaining precursors accounted for 29% of ionic abundance, backbone fragments for 59%, immonium ions (small fragments) for 9%, reduced molecular species for 2.5%, and heme for 0.5% (the corresponding figures for 99 eV irradiation are 37, 43.5, 18, 1, and 0.5%, respectively).

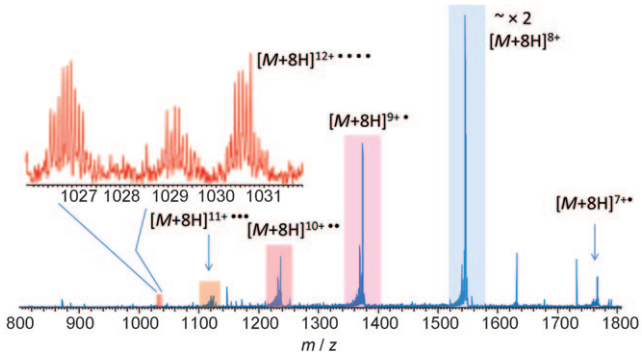


***Figure 1.*** EI mass spectrum (50 eV) of cytochrome *c* 8+ molecular ions obtained by electrospray ionization. Multiple ionization occurs without significant losses from the molecular species.

Holomyoglobin is a 17.6 kDa molecule containing a single polypeptide chain wrapped around a noncovalently bound heme group. As for many weakly bound protein–molecule complexes, the molecular ion of holomyoglobin can only be ESI-produced in a low-charge state from a "native" (ca. pH 7) solution. Collisions with approximately 45 eV electrons ionized some 12+ heme–myoglobin complexes multiple times without dissociation, whereas some other complexes unfolded and released a singly charged heme molecule (Figure 2a). In contrast, slow (ca. 100 ms) heating by 10.6 μm IR photons led only to unfolding without ionization or fragmentation (Figure 2b). Rapid unfolding hampers the determination of the site of heme attachment by conventional tandem mass spectrometry.

Figure 3 shows the average charge-state increase in electron-ionized cytochrome *c* ions as a function of the irradiation time. There are three distinct regions: 1) a rapid increase in charge state below 3 ms, 2) a constant region

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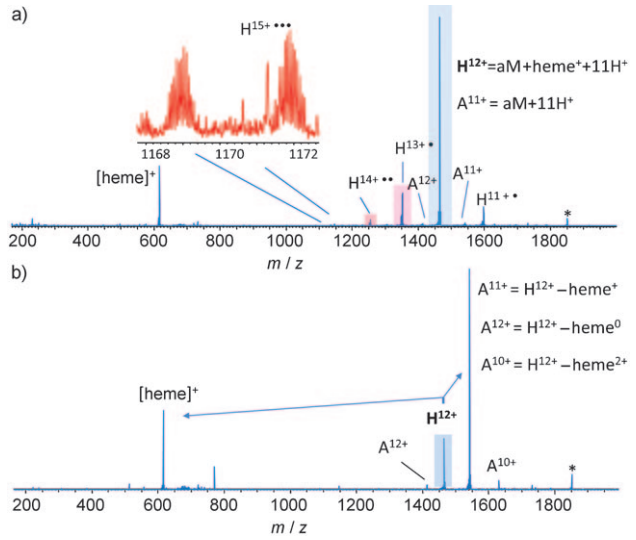


***Figure 2.*** Irradiation of intact holomyoglobin 12+ molecular ions produced by ESI from a "native" (ca. pH 7) solution with a) 45 eV electrons and b) 10.6 μm IR photons. aM = apomyoglobin.

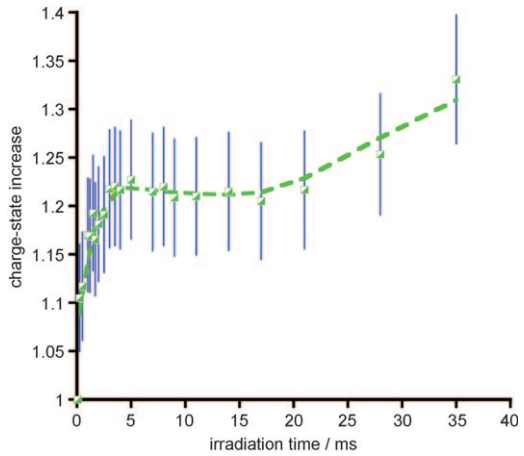


***Figure 3.*** Average charge-state increase of 8+ cytochrome *c* molecular ions upon their reaction with 55 eV electrons as a function of the irradiation time. The value 1.0 corresponds to singly ionized species.

between 3 and 17 ms, and 3) a moderate increase in charge state above about 17 ms. After the stable ion–electron-interaction regime is established (i.e. at interaction times above 3 ms), the average charge state of the ionized molecular species remains constant as long as the dominant event is a single collision with a 55 eV electron. At interaction times above 15–17 ms, the probability for a given molecule to undergo consecutive collisions with two electrons becomes significant, and the average charge state of ionized species increases linearly with irradiation time.

The ionization energies of C, N, O, and S atoms (10.3–14.5 eV) are much smaller than the threshold energies for double and triple ionization (23–35 eV and 35–47 eV, respectively), whereas quadruple-ionization energies (47–78 eV) are more than four times higher than single-ionization thresholds.[13] For small molecules, triple-ionization energies are also high: 54 eV[14] for $CS_2$ and 56 eV[15] for $C_3H_4$; these values are more than three times higher than single-ionization thresholds. However, for large molecules, the thresholds for multiple ionizations are greatly diminished and approach the same low value in the infinite-mass limit, because large species can be ionized at different, spatially distant sites.

As the middle region in Figure 3 shows, the elementary process for the ionization of cytochrome *c* 12+ ions by 55 eV electrons is multiple ionization (the average charge increase is by about 1.2 units), which involves the creation of one or several radical positive charges ("holes"). A subsequent secondary process involves intramolecular charge transfer that separates the charges (holes and ionizing protons) and positions them at the sites with the lowest potential energy.[16] This potential energy includes not only the ionization energies of atoms, but also the repulsive coulombic energy of charge–charge interactions.

The charge-state increase has a beneficial effect for subsequent swift dissociation, for example, by electron capture dissociation (ECD), which produces backbone N–$C^{\alpha}$ cleavages.[17] The primary bond cleavage in ECD is swift and possibly nonergodic, as evidenced by the losses observed in Figure 2 of small, covalently attached groups from the ionized species without weak-structure unfolding and heme loss. ECD can preserve noncovalent interactions,[18] but conventional ECD of holomyoglobin 8+ ions produced no backbone fragmentation because of the low charge state. However, EI-accompanying[10,11] electron capture by the ionized holomyoglobin complex gave several backbone fragments with masses that indicated that the heme group was still attached (Figure 4). Thus, multiple electron ionization is likely to find its place in the arsenal of tandem MS techniques as a step in the structural characterization of large protein–molecule complexes in their native (low) protonation state.

In summary, we have demonstrated the multiple ionization of intact protonated protein–molecule complexes by less than 100 eV electrons. Depending upon the exact scenario of the ion–electron collision, the complexes can be ionized multiple times without unfolding, can lose a small group, or can undergo backbone fragmentation with or without the loss of the noncovalently attached molecule. It is not clear what determines the exact scenario; the different outcomes are possibly due to the presence in holomyoglobin solutions of two populations, which differ in the orientation of the heme group by rotation by 180° and thus have different gas-phase stabilities.[19]

The most intriguing outcomes are the possibilities of soft ionization and "soft fragmentation", which may indicate the direction of further development for native mass spectrometry of protein complexes. Another possibility may be to combine the traditional soft-ionization techniques MALDI and ESI with EI for the analysis of smaller molecules.[20]

## Experimental Section

The proteins were dissolved in water with 1% methanol to a concentration of $10^{-4}$ M and electrosprayed from a metal-coated pulled-glass capillary (Proxeon). The MS experiments were performed on an LTQ FT mass spectrometer (Thermo Fisher) equipped

  

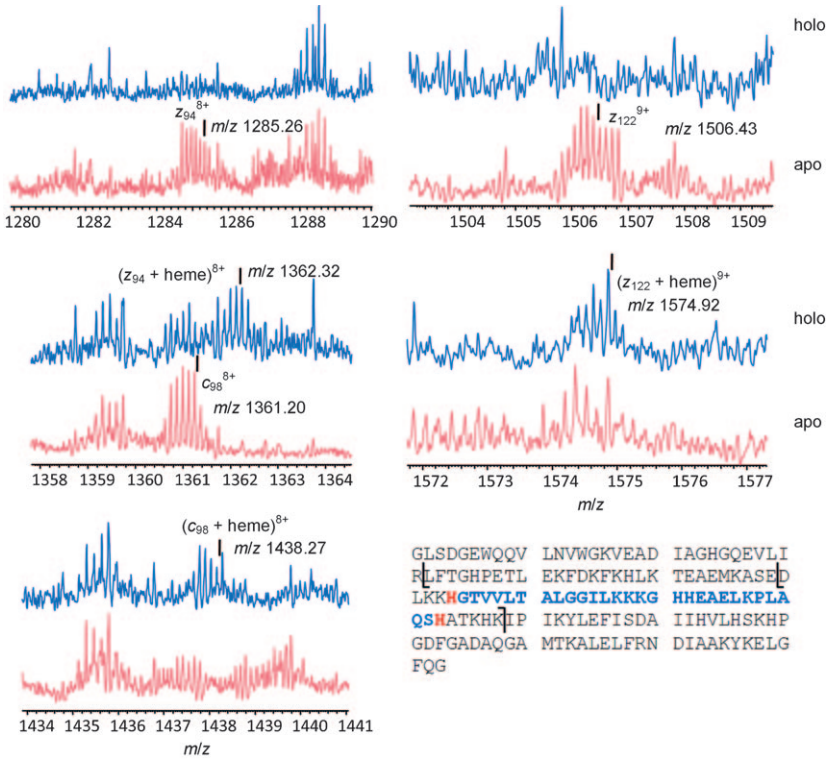


***Figure 4.*** Backbone fragments derived from the irradiation with 50 eV electrons of 12+ molecular ions of apomyoglobin (no heme) and the holomyoglobin complex with the heme group. The masses of the fragment ions indicate that the heme group is still attached following backbone fragmentation of the holomyoglobin complex.

with an indirectly heated dispenser cathode (HeatWave) as a source of low-energy electrons. The electron current was 30 μA.

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