Gas-phase spectroscopy of biomolecular ions: Porphyrins and metalloporphyrins

Steen Brøndsted Nielsen
Department of Physics and Astronomy, Aarhus University, Ny Munkegade 120, DK-8000 Aarhus C, Denmark
sbn@phys.au.dk

Abstract. The electronic structure of a biochromophore (i.e., light absorber) is strongly perturbed by its environment, e.g., water or amino acid residues within protein pockets. To reveal the intrinsic electronic properties, it is therefore necessary to study isolated molecules in vacuo. Many biochromophores are ionic in their natural environment, which renders experiments complicated as it is not possible to produce enough absorbing species for traditional light transmission spectroscopy. In Aarhus we have developed state-of-the-art apparatus to record gas-phase absorption spectra. Some recent results for porphyrin and metalloporphyrin ions are presented, including both electronic ground state ions and electronically excited ions. Fragmentation channels are found from quickly switching the ring voltages after photoexcitation to store particular daughter ions.

1. Introduction
Electronic absorption by biochromophore ions in the gas phase is studied intensively by several research groups to reveal the intrinsic electronic properties of isolated ions. This is useful to understand how the electronic structure is perturbed by a nearby environment such as a solvent or nearby amino acids if the chromophore is buried inside a hydrophobic pocket of a protein. Gas-phase spectra also serve to benchmark theoretical models that predict the energies of electronically excited states.

The conventional way to record absorption spectra of molecules in the gas phase is to measure the light before and after the sample to quantify the loss in intensity. However, in contrast to vaporizable molecules, it is not possible to produce a vast enough number of ions to detect any change in light intensity, and instead photon absorption is identified from ionic fragmentation (action spectroscopy). In our laboratory gas-phase spectroscopy of fragile biomolecular ions is realized from the combination of an electrospray ion source, the electrostatic ion storage ring in Aarhus (ELISA), and pulsed lasers (figure 1) [1, 2]. Ions are photoexcited in the ring, and their lifetimes measured with respect to dissociation. Fits to exponential decays provide numbers that are proportional to the actual number of photoexcited ions at all wavelengths as detailed elsewhere [3].
Figure 1. The electrostatic ion storage ring in Aarhus (ELISA) equipped with an electrospray ion source and lasers. Experiments may involve one or both lasers.

Here we review some results obtained for isolated protoporphyrin IX and heme ions, which were recently published [3-6]. Heme is derived from protoporphyrin IX with two protons replaced by an iron atom in the center (see figure 2) and is found within pockets of heme proteins such as hemoglobin and myoglobin where there is minimal access to water. The iron is coordinated to the four nitrogens of the porphyrin ring. Heme proteins are ubiquitous in nature and are responsible for key biological processes such as dioxygen transport and storage, and sensing of small molecules like CO. They display characteristic UV/vis spectra, and, importantly, the spectral features depend on the iron oxidation state, peripheral substituents, axial ligands, coordination state, spin state, and nearby amino acid residues. Absorption spectroscopy therefore provides detailed information on the microenvironment of the heme. Porphyrins themselves have high triplet quantum yields which make them useful within the field of photodynamic therapy for treatment of cancer.

Figure 2. The structure of the protoporphyrin IX anion and the Fe(III)-heme cations that are subjects for study in this work.

2. Instrumental setup
The electrostatic ion storage ring in Aarhus is shown in figure 1 [1, 2]. It is equipped with an electrospray ion source to produce biomolecular ions in the gas phase. Ions are accumulated in a 22-
pole ion trap and thermally equilibrated by collisions with a helium buffer gas therein. The ions are accelerated as an ion bunch to kinetic energies of 22 keV per charge, and a bending magnet is used to select the appropriate ions according to their mass-to-charge ratio. Following injection into the ring, the ions are stored for tens of milliseconds before being irradiated by a nanosecond light pulse from a tunable EKSPLA laser. This is an Nd:YAG laser where the third harmonic (355 nm) pumps an optical parametric oscillator (OPO). The visible output from this OPO is frequency doubled in a crystal providing UV light. The repetition rate of the experiment is 10 Hz. Lifetimes with respect to dissociation are obtained from measurements of the yield of neutrals hitting the microchannel plate (MCP) detector located at the end of the straight section opposite to the side where photoexcitation is performed, that is, the setup samples delayed dissociation. The pressure in the ring is close to $10^{-10}$ mbar, which sets an upper limit of seconds on the storage time.

In other experiments, photoexcited ions are irradiated with a second laser pulse from another OPO laser in the same region as before. This laser pulse is delayed in time relative to the first one. A dichroic mirror that reflects blue light (pump pulse) and transmits red light (probe pulse) is used to align the beams. If green light is used for the pump pulse, the dichroic mirror is removed and a nearly parallel beam is introduced for the probe beam relative to the pump beam using two different mirrors.

To identify the dissociation channels after photoexcitation, ring voltages are changed at a particular time after photoexcitation to store daughter ions with appropriate kinetic energy. This product ion mass spectrometry is possible as ELISA has been equipped with pulsed power supplies of microsecond response times [7]. After a specific number of revolutions, the product ions are dumped into the MCP detector. The signal from the detector as a function of the scaling of the ring voltages provides the product ion mass spectrum. To increase resolution, a beam scraper can be put in on one side of the ring.

3. Photodissociation of Fe(III)-heme cations and protoporphyrin IX anions

3.1. Absorption spectra of four-coordinate Fe(III)-heme$^+$ ions

The action spectra of Fe(III)-heme$^+$ (i.e., ferric heme) are shown in figure 3. These represent the gas-phase absorption spectra under the assumption that the quantum yield for light emission after excitation is independent on excitation energy and that any prompt dissociation processes are insignificant. The band with maximum at about 381 nm is denoted the Soret band while the lower-energy bands in the region from 450 nm to 600 nm are the Q bands. There is more than one Q band due to strong coupling with vibrational modes.

![Figure 3. Action spectra of Fe(III)-heme cations. The spectrum is split in two wavelength regions since the exit windows of the laser are different which results in different overlaps between the ion and laser beams. Light below 420 nm is produced by frequency doubling of the output from the OPO.](image-url)
These spectra serve as reference spectra to tell whether four-coordinate Fe(III)-heme is formed within say myoglobin under certain conditions, e.g., after long photoirradiation time or low pH. They could not have been obtained by simply dissolving Fe(III)-heme$^+$ in a solution since the central Fe$^{3+}$ has a strong affinity for anions and water, increasing the coordination number from four to either five or six. A previous attempt to spectroscopically characterize ferric heme involved the synthesis of a cage around the heme to avoid anions or solvent molecules to bind to the iron [8]. In that work the Soret absorption band was found to have its maximum at 400 nm, a redshift of 19 nm compared to our measurement. Our data for the purely isolated heme ion therefore seems to indicate that the bulky environment around the heme is not completely innocent but does cause a redshift in the $\pi\pi^*$ transition energy.

Photodissociation mass spectra of Fe(III)-heme$^+$ cations reveal that the dominant dissociation path is $\beta$-cleavage resulting in loss of CH$_2$COOH (figure 4) in accordance with work by others [9-12]. This reaction has been calculated to require about 2 eV [5], which is less than the photon energies used. There is a peak that corresponds to the fragment formed after loss of both CH$_2$COOH groups; this process requires two photons.

![Figure 4. Photodissociation mass spectrum of Fe(III)-heme cations (532 nm).](image)

3.2. Spectroscopic characterization of photoexcited protoporphyrin IX anions
The time spectrum obtained after photoexcitation of protoporphyrin IX anions with 430-nm light is shown in figure 5a. Two exponential functions are required to account for the decay, and a power dependence study shows that the total decay is due to one-photon absorption. One possible explanation for two time constants is that after photoexcitation, there are two competing pathways [13]: In one, internal conversion to the electronic ground state occurs and vibrationally excited ions are formed that quickly dissociate. In the other, intersystem crossing to a lower-lying triplet state occurs, and the bottleneck for dissociation is now the intersystem crossing from this state to the electronic ground state which requires a spin flip. The probabilities of the two pathways are 40 % and 60 %. This picture is in accordance with the fact that porphyrins have high triplet quantum yields, which is exploited in photodynamic therapy. Triplet state porphyrins can transfer their excitation energy to molecular oxygen, which results in highly reactive singlet oxygen that attacks nearby tissue.

The absorption by molecules in triplet states is redshifted compared to that by ground-state molecules, and we therefore decided to carry out a pump-probe experiment to spectroscopically characterize the long-lived protoporphyrin IX anions. The wavelength of light for the first laser pulse was set to 430 nm while 684-nm light was used for the second. It is evident from figure 5b that the photoexcited ions absorb the 684-nm photons while no absorption occurs if the ions are not initially photoexcited (figure 5c). In another experiment the pump wavelength was set to 535 nm, and the probe wavelength was scanned from 650 nm to 950 nm. The yield of fragments due to the probe laser was recorded to produce the action spectrum (figure 6). This spectrum reveals an absorption band with
maximum at or below 650 nm similar to what is observed for triplet-state excited porphyrins in solution phase [14, 15]. It is difficult to scan lower in wavelength since we then move into the absorption band of ions in their ground state.

**Figure 5.** A. Time spectrum of protoporphyrin IX anions that were photoexcited after 35.19 ms by 430-nm light (pump). B. Same as A but a probe laser pulse (684 nm) was fired 0.67 ms after the pump laser pulse. C. Time spectrum of protoporphyrin IX anions that were photoexcited by the probe laser (684 nm) at 35.86 ms. No prior pump pulse was used, and consequently no absorption was seen after firing the probe laser.

**Figure 6.** Action spectrum obtained as the yield of photoneutrals due to the probe laser pulse as a function of probe wavelength. The pump laser was set to produce light at 535 nm. Time delay between the pump and probe was 0.57 ms.
4. Summary
We have demonstrated that experiments on biochromophore ions at the electrostatic ion storage ring provide detailed information on lifetimes with respect to dissociation, the dissociation channels, and the absorption by both ground-state and electronically excited ions.

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