Communication

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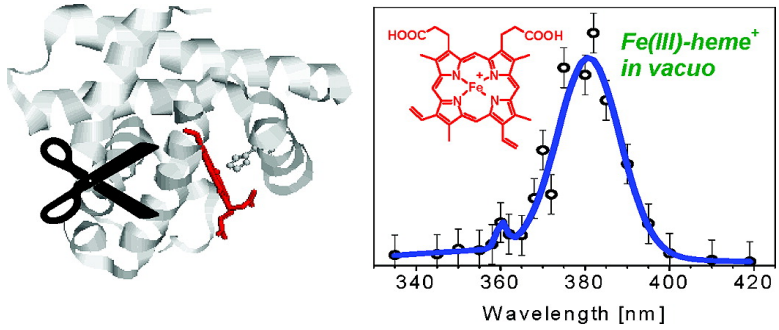


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# A Soret Marker Band for Four-Coordinate Ferric Heme Proteins from Absorption Spectra of Isolated Fe(III)-Heme$^+$ and Fe(III)-Heme$^+$(His) Ions in Vacuo

Morten Køcks Lykkegaard, Anneli Ehlerding, Preben Hvelplund, Umesh Kadhane, Maj-Britt Suhr Kirketerp, Steen Brøndsted Nielsen,* Subhasis Panja, Jean Ann Wyer, and Henning Zettergren

*Department of Physics and Astronomy, University of Aarhus, Ny Munkegade, DK-8000 Aarhus C, Denmark*

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Heme proteins are readily identifiable from their characteristic UV/vis spectra ($\delta$, Soret, $Q_0$, and $Q_1$ bands).[1−6] Spectral features depend on the iron oxidation state, peripheral substituents, axial ligands, coordination state, spin state, and nearby amino acid residues. Absorption spectroscopy is essential for comprehending the conformational changes that occur upon ligation or reduction/oxidation of the heme iron, and transient absorption is used extensively to monitor the dynamics of geminate recombination or larger protein conformational changes after, for example, photolysis of an iron-ligand bond.[6,7] Often heme or other biochromophores are located in hydrophobic protein pockets or crevices with minimum access to water. These pockets are in certain cases well simulated by a vacuum.[8] It requires gas-phase experiments to remove the influence of a nearby environment, determined by the protein folding state, from the inherent properties of the heme.

Here we report the absorption spectra in the Soret band region of isolated four-coordinate (4c) Fe(III)-heme$^+$ and five-coordinate (5c) Fe(III)-heme$^+$(His) ions in vacuo from action spectroscopy. Fe(III)-heme$^+$ refers to iron(III) coordinated by the dianion of protoporphyrin IX. We find that the absorption of the 5c complex is similar to that of 5c metmyoglobin variants with hydrophobic binding pockets except for an overall blueshift of about 16 nm. In the case of 4c iron(III), the Soret band is similar to that of 5c iron(III) but much narrower. To our knowledge this is the first unequivocal spectroscopic characterization of 4c ferric heme.

Spectroscopy studies on gaseous heme$^+$ ions are sparse and include the absorption spectrum in the Q-band region of Fe(III)-heme$^+$(DMSO)[9] and a recent vibrational characterization of Fe(III)-heme$^+$(NO) and Fe(III)-heme$^+$(imidazole).[10]

In the present work, Fe(III)-heme$^+$ ions were formed by electrospray of hemin chloride in methanol and $CH_2Cl_2$ (1:1), and to produce the histidine-coordinated complex, histidine was added to the solution. Ions were thermalized in a 22-pole ion trap with helium buffer gas at room temperature, accelerated as an ion bunch to 22-keV kinetic energies, mass-to-charge ($m/z$) selected by a magnet, and injected into the electrostatic ion storage ring in Aarhus, ELISA (Figure 1).[8,11] In one experiment, the magnet was set to allow only heme$^+$ ions of $m/z$ 616 to enter the ring and in a second experiment, only heme$^+$(His) ions of $m/z$ 771. This mass selection assured that we had the appropriate ions in the ring. After storage in the ring for 35 ms, the ions were irradiated with light from a pulsed tunable EKSPLA laser at one side of the ring. The width of the laser pulse was about 3.2 ns, and wavelengths were scanned from 330 to 419 nm. Absorption of light leads to vibrationally hot ions, and in separate experiments it was observed that these ions decay primarily by loss of $CH_2COOH$ from heme$^+$ and by loss of His from heme$^+$(His).[12] For absorption spectroscopy, neutrals formed in the side opposite to the photoexcitation region were measured in a microchannel plate detector. Delayed dissociation occurred on a microsecond to millisecond time scale (Figure 2), as has been discussed earlier in the case of protoporhyrin ions.[13] A signal proportional to the absorption cross section was obtained as the yield of neutrals divided by the number of photons in the pulse and normalized to the neutrals yield from residual gas collisions (action spectroscopy). The spectra were the same whether only the first laser peak or the first 10 (see Figure 2) were used, which indicates that the change of dissociation lifetimes with excitation wavelength can be neglected.

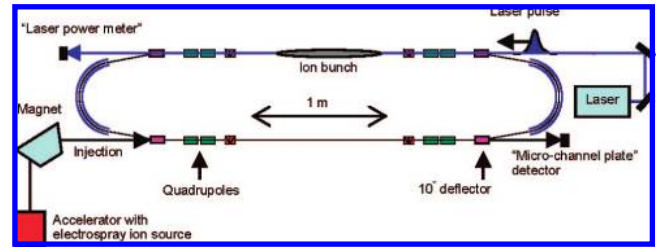


***Figure 1.*** The electrostatic ion storage ring in Aarhus, ELISA, in combination with a laser system. The photofragment yield was obtained as a function of wavelength from the signal of neutrals hitting the detector.

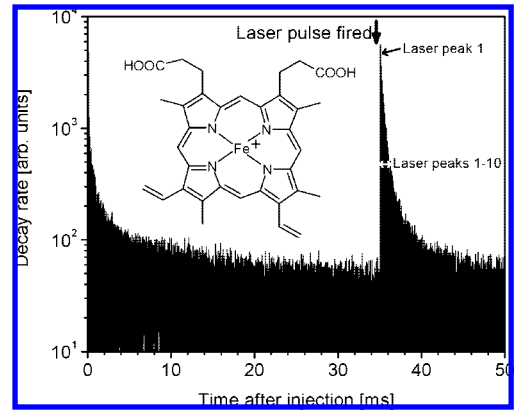


***Figure 2.*** Decay spectrum of Fe(III)-heme$^+$. The high number of neutrals detected immediately after injection is due to metastable ions that have been excited during extraction from the ion trap. After a few milliseconds the signal is dominated by collisional decay in the ring. The pressure was of the order of $10^{-11}$ mbar. After 35 ms the ions were photoexcited with 390-nm light, which resulted in a large number of neutrals from delayed dissociation. The revolution time of the ions in the ring was 100 $\mu$s. The photo yield was obtained from summation over the first 10 laser peaks, indicated by the white double-headed arrow.

In Figure 3 we present the absorption spectra in the Soret band region of Fe(III)-heme$^+$ and Fe(III)-heme$^+$(His). The isolated five-coordinate complex displays a broad absorption band with a maximum at 379 nm, and a pronounced shoulder on the high energy side, which


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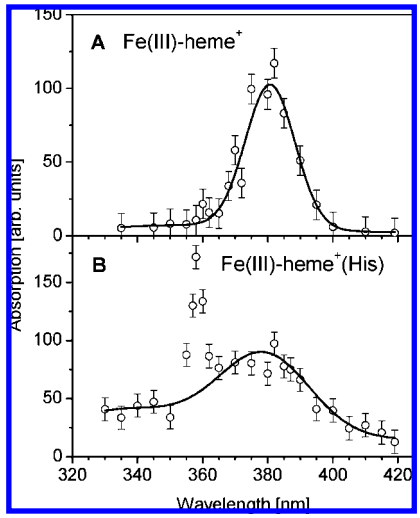


***Figure 3.*** Absorption spectra of four-coordinate Fe(III)-heme$^+$ (A) and five-coordinate Fe(III)-heme$^+$(His) (B) in vacuo.

is likely due to ligand-to-metal charge transfer (LMCT) transitions. The sharp peak at ca. 358 nm is either due to resonant excitation of the $\nu_4$ porphyrin breathing mode[2] or to a different laser beam profile at this wavelength, which has a better overlap with the ion beam. Ignoring this band, the spectrum is similar in shape to that of 5c high-spin Fe(III)-heme in metmyoglobin variants in which the distal histidine is replaced by a nonpolar residue thereby preventing water taking up the available sixth coordinate site.[14] Such protein variants have maximum absorption at about 395 nm, a redshift of 16 nm compared to the isolated complex. According to protein X-ray crystallography, the iron is displaced out of the plane of the porphyrin macrocycle by 0.28 Å to maximize chemical bonding with the histidine.[14a] It should be noted that the propionic acid side chains are ionized in the protein in contrast to the gas-phase species. The spectrum is also similar to that of the adduct between 1,2-dimethyl-imidazole and ferric heme embedded in SDS (sodium dodecyl sulfate) micelles where the maximum is at 400 nm.[15] The environment within the micelle mimics the hydrophobic cavity of heme proteins.

The bare Fe(III)-heme$^+$ absorbs maximally at about 381 nm (Figure 3) like Fe(III)-heme$^+$(His). However, the shoulder toward low wavelengths is absent, and the spectrum appears to be narrower with a full width at half-maximum of 20 nm. This overall change in band shape is linked to the new electronic structure of the heme when the iron occupies the central hole of the porphyrin ring (planar heme). Density functional theory calculations have revealed that the ground-state is a quartet state.[16] For comparison, six-coordinate Fe(III)-heme is either low-spin or high-spin, depending on the axial ligands.[17] Again an indication of a sharp peak at 360 nm is observed. In recent work, Fang et al.[18] synthesized a highly sterically hindered bis-pocket siloxyl Fe(III) porphyrin that is four-coordinate with maximum absorption at 400 nm, red-shifted by 20 nm relative to the gas-phase heme species.

Previous spectroscopic characterization of 4c ferric heme by conventional methods was hampered by the strong affinity of Fe(III) for water and anions. Four-coordinate Fe(III)-heme in Aplysia myoglobin at low pH (<5) was inferred by Giacometti et al.[19] but later abandoned based on resonance Raman experiments.[20] A change in absorption at low pH may be ascribed to both a change in the heme coordination state and its environment due to conformational changes and denaturation. Such problems in the interpretation of absorption spectra are not encountered in gas-phase experiments where ions of interest are selected according to their mass-to-charge ratio by mass spectrometry, and the environment around the heme can be gradually built up in a controlled manner.

Spectroscopic studies on methemalbumin by Kamal and Behere[21] suggest that the heme in this case is devoid of a well-defined heme cavity and that it does not bind to histidine but to a surface residue. The Soret maximum is at 404 nm, and the band is associated with a shoulder to lower wavelengths. A comparison with our absorption spectra indeed supports the assignment of 5c Fe(III)-heme.

In conclusion, our work provides a prediction of the change in absorption of a 5c ferric heme protein upon histidine deligation, namely a sharpening of the band but with a minimal change in the wavelength of maximum absorption. Acid-induced rupture of the iron−histidine bond due to histidine protonation[22] or local heating after multiple photon absorption are possible paths for lowering the coordination state. The spectra also serve as a benchmark for quantum chemistry calculations of excited states.

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