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Soret Band of the Gas-Phase Ferri-Cytochrome *c*

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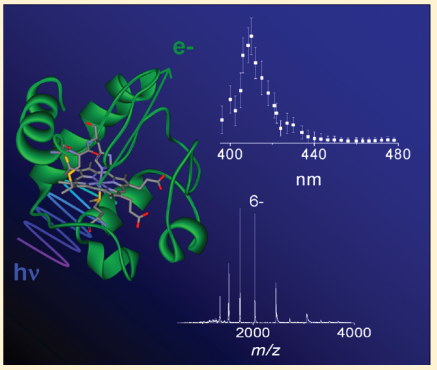
Supporting Information

ABSTRACT: We report the first visible spectrum of a heme-protein in the gas phase. The aim of this work was to provide a reference for the optical absorption of an isolated heme-protein to better understand the influence of protein conformation and fluctuation and of solvent on its optical properties. After laser irradiation of gas-phase cytochrome *c* (cyt *c*), electron emission is observed. Electron photodetachment yield of cyt *c* 6- was recorded in the region of the Soret band of the porphyrin group, showing a maximum at 410 nm. Our results are compared with optical spectra of gas-phase heme and of cyt *c* in solution. We discuss the influence of the polypeptide chain and of the solvent on both the position and the broadening of the Soret band. Action spectrum of gas-phase cyt *c* is close to the absorption of native cyt *c* in solution, suggesting an efficient protection of the heme group from solvent accessibility by the polypeptide chain and similar interactions between the two moieties in solution and the gas phase.



SECTION: Kinetics, Spectroscopy

Cytochrome *c* (cyt *c*) has long served as a model protein, both in solution[1−6] and in vacuo,[7−16] for developing new concepts and approaches in proteins folding. The presence of a heme group, covalently linked to the polypeptide, gives the protein its reddish-brown color and provides a spectroscopic probe.[17−19] The heme group is a porphyrin bearing an iron atom bound to the four ring nitrogens and displays characteristic absorption bands (δ, Soret, Q0 and Q1 bands).[20] The absorbance of cyt *c* is highly sensitive to the heme environment; it depends on the oxidation and ligation state of the iron as well as on the conformation of the protein.[3,19,21−23] Absorption spectroscopy is then essential to understand the conformational changes and the associated dynamics in heme proteins. In native cyt *c*, the heme is located in a hydrophobic protein pocket with minimum access to solvent. Gas-phase experiments are therefore useful to provide benchmark spectra of unsolvated hemes to validate or reject the validity of calculations and to elucidate the influence of the protein environment on its optical properties with regards to its intrinsic properties. Whereas optical properties of cyt *c* and of its heme in solution are well-documented, previous spectroscopic works in vacuo have focused on the isolated heme[24−26] as well as on other prosthetic groups.[27−30] Midinfrared action spectra for different charge states of cyt *c* have also been reported.[31] The general picture that arises from the pioneer work of Nielsen and coworkers is that the absorption of four-coordinate [Fe(III)-heme]$^+$ shows a blue shift of 30 nm as compared with the one recorded for cyt *c* in non-denaturating solution (for both the Soret and Q-band regions).[24,25] To understand the possible role of small molecules entering the heme pocket and influence of ligation to proximal amino acids on the heme group structure, spectroscopic experiments on heme groups complexed with different molecules including amino acids were conducted.[24,25,32,33] We recently reported action spectra of entire proteins in the gas phase.[34−36] In line with the strategy of expanding the environment around the heme group to understand shifts in the absorption profiles due to changes in hosting environments, we document for the first time a spectrum of the Soret band in gas-phase holo-cyt *c*. To the best of our knowledge, this is the first example of a visible spectrum in the gas phase of a prosthetic group attached to its protein.

cyt *c* contains 104 amino acids of which 13 bear acidic groups that can be easily deprotonated in the gas phase. (It also contains 2 arginine and 18 lysine basic amino acids.) The mass spectrum obtained by spraying in the negative mode a native solution of cyt *c* is displayed in an inset in Figure 1. It shows a series of peaks due to the holo-protein with a charge state distribution centered around 6-. This charge distribution centered on low charge states is expected for folded cyt *c*,[11,37] whereas charge-state distribution ranges from 7- to 15- in denaturing buffer (data not shown).[37] We used high-resolution mass spectrometry to confirm the oxidation state of iron. The high-resolution mass spectrum distribution (see inset in Figure 1) shows that the oxidized [Fe(III)] state is observed.[38,39] The linear ion trap was used to isolate gaseous cyt *c* 6- [M-6H]$^{6-}$ species that was subsequently irradiated with the laser. Figure 1

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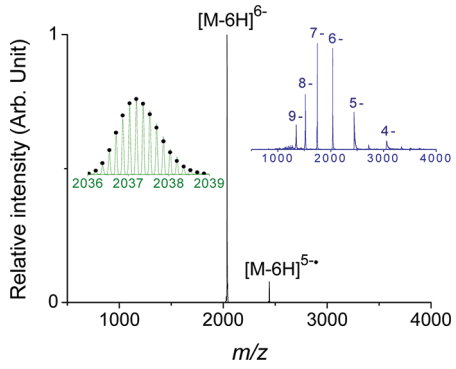

Figure 1. Photodetachment spectrum of isolated $[M-6H]^{6-}$ after 200 ms of irradiation at 415 nm (~200 μJ/pulse). The inserts show the full mass spectrum obtained after electrospraying a native solution of cyt *c* (blue) and the isotopic distribution of the 6- charge state precursor ion (green). Circles show the theoretical isotopic distribution for $[Fe(III)cyt\ c\text{-}6H]^{6-}$, which establishes Fe(III) as the correct oxidation state.

shows the mass spectrum obtained after irradiation at 415 nm during 200 ms (two laser shots). Only the $[M-6H]^{5-\bullet}$ oxidized product ion is observed with a yield of 7% and obtained by one electron loss from the parent ion. No other fragment ions resulting from the fragmentation of the $[M-6H]^{6-}$ precursor ion are detected. Electron photodetachment is the only event observed after irradiation.

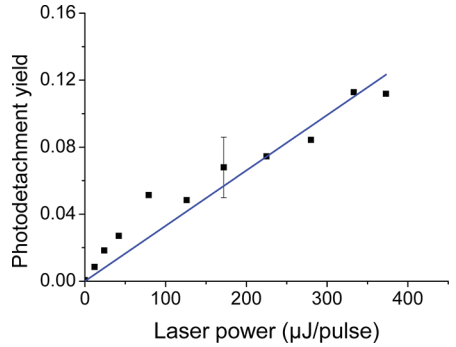

Figure 2. Electron photodetachment yield ($[M-6H]^{5-\bullet}/([M-6H]^{5-\bullet} + [M-6H]^{6-})$) of $[M-6H]^{6-}$ ion plotted as a function of the laser power (irradiation time 200 ms, laser wavelength 415 nm). The laser power was controlled using a half-wave plate and a polarizer. The blue line is a linear fit of the data.

Figure 2 shows the evolution of the electron detachment yield (i.e., $[M-6H]^{5-\bullet}/([M-6H]^{5-\bullet} + [M-6H]^{6-})$) measured as a function of the laser power for an irradiation time of 200 ms at 415 nm. A linear increase in the photodetachment yield is observed at low fluence. It indicates that the electron photodetachment is a monophotonic process. A single visible photon absorption is sufficient to oxidize the Fe(III)-cyt *c* protein in the gas phase. The heme group is responsible for absorption bands at 410 nm (B or Soret Band) due to a $\pi-\pi^*$ porphyrin transition. In solution, an ultra-fast electronic relaxation occurs through a charge transfer from the porphyrin to a d orbital of the metal (ligand metal charge transfer, LMCT), followed by intrametal relaxation and a slower electron back transfer and eventually bond cleavage.[26,40,41] Here, electronic excitation results in an electron detachment, from either the metallo-porphyrin or the polypeptide chain. Let us consider now the two hypotheses. In the ferric form first, the relaxation pathway to the partially occupied metal d orbital is very favorable[40] and is probably faster than coupling of the S2 state of porphyrin to an autoionizing state that would lead to electron detachment directly from the porphyrin before LMCT. Ionization potentials of different free and liganded iron ferrous porphyrins have been measured and are roughly equal to 6 ± 0.5 eV depending on the ligands.[42,43] This is much higher than the excitation energy. However, the repulsive Coulombic interaction of the d electrons with the six excess electrons of the protein decreases electron binding energies in gas-phase 6- cyt *c* as compared with isolated porphyrins, and electron emission may occur by tunneling through the repulsive coulomb barrier.[44−47] The second hypothesis is a transfer of excitation from the porphyrin to the polypeptide chain, leading to the detachment of one of the excess electrons whose binding energy is much lower. Indeed, whereas the electron affinity of COO group in a neutral molecule is close to 3.25 eV,[48] binding energies are much lower in highly charge proteins, which was recorded at 0.9 eV in the case of folded cyt *c* 6-.[14] This value is lower than the photon energy at 415 nm. Electron detachment from large negatively charged ions following visible excitation and probably excitation transfer between different moieties of the system was already observed, for example, in the case of ligand attached to DNA strands and of metal clusters protected by ligands.[49,50] In cyt *c*, energy transfer between tryptophan and the heme group is very efficient,[40,51,52] and a reverse transfer from heme group to autoionizing state leading to electron loss from a carboxylate group may be observed here. Note that in the case of excitation at 4.66 eV modelization of experimental results by Vonderach et al.[14] with a simple electrostatic model are in favor of detachment from a carboxylate group. (Detachment from the heme group would lead to higher binding energies.) However, relaxation after excitation of the Soret band may follow different pathways, and it is not possible to conclude definitively here. Note that the electron-loss mechanism observed here is, a priori, different from electron transfer from cyt to cyt oxidase in the respiratory chain, which involves coupled electron−proton transfer reactions.[53]

The electron photodetachment yield of cyt *c* was recorded as a function of a laser wavelength from 390 to 480 nm. The resulting action spectrum is shown in Figure 3. It shows a main

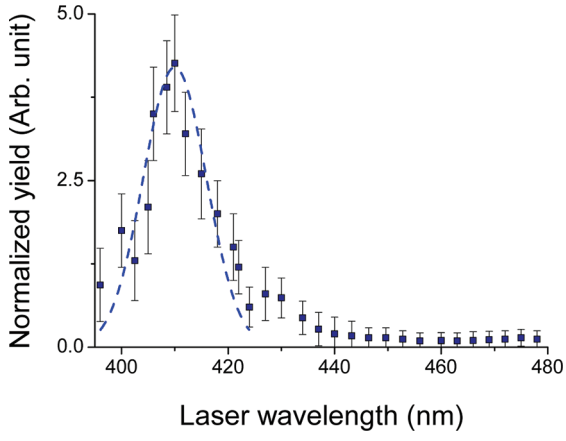

Figure 3. Laser-normalized electron photodetachment yield (see eq 1) measured as a function of the laser wavelength for $[M-6H]^{6-}$ cyt *c*. The laser energy curve measured as a function of the wavelength is displayed in the Supporting Information.

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band centered around 410 nm with a full width at half-maximum of ∼15 nm and a shoulder at 428 nm. Absorption of heme protein in the visible part of the spectrum is due to excitation of the porphyrin and, in the case of the Soret band observed here, to the $\pi-\pi^*$ S2 ← S0 transition.[20] Optical spectrum of gas-phase cyt *c* can then be compared with spectra recorded in solution for native and unfolded cyt *c* as well as with gas-phase heme spectrum. The Soret band for gas-phase ferric four-coordinate $[Fe(III)\text{-}Heme]^+$ was observed at 380 nm.[24] As seen in Figure 3, the bonding of the heme to the protein induces a shift of 30 nm, although interaction with one histidine residue broadens the absorption spectrum but did not result in any significant shift.[24] Building up the whole protein pocket around the heme group and not only one amino acid axial ligand appears to be important to restore optical properties of the heme-protein. The protein pocket has an influence on the coordination of iron, exact geometry of the heme, and also the charge landscape around the optical active group. Maxima for the Soret Band are observed at 409 and 399 nm for native and unfolded cyt *c* in solution at pH ∼5, respectively.[3] At this pH value, the protein is positively charged in solution (isoelectric point: ∼10.5), but the charges are screened by the solvent and counterions. The maximum of the band for the native structure in solution is very close to the value reported here for the gas-phase protein. Considering the significant hypochromism of unfolded protein in solution and the features of gas-phase heme spectrum together suggests: (i) that the protein binding motifs around the heme group are very similar between the solution and the gas phase and that the conformation and the six-coordinated motif of heme are preserved and (ii) that the polypeptide chain protects the chromophore from solvent molecules that would alter its spectrum in solution as compared with our results. Whereas the position of the band is not affected by the solvent-free condition of the gas phase, our spectrum is sharper than the ones recorded in solution.[3,18,19] The broadening of optical spectra in solution as compared with gas phase is usual for small molecules but is rather surprising for such a large molecule with a chromophore located in a hydrophobic pocket. Broadening of the Soret band is due to anharmonic couplings with vibrational modes and conformational heterogeneity that results in distortions of the heme and a dynamically changing local electric field around the heme.[54] Even small changes in positions of the atoms of the prosthethic group or of the atoms near the active group induced by thermal activity can have a large influence on absorption properties.[55] The difference between gas phase (where the effective ion temperature is close to room temperature[56]) and solution measurements (performed at room temperature) outlines the enduring question of the relationship of protein internal motion to the solvent fluctuation and viscosity. In solution, protein fluctuations can be separated into two types: a contribution from solvent-coupled motions and a contribution from motions uncoupled from solvent motions. Our results suggest that both effects contribute to broadening in solution with anharmonic motions of the solvent efficiently transmitted to the motion of the metal-porphyrin system through nonbonded interactions.[57] Electronic perturbations, in particular, changes in partial charges due to a change in the dielectric environment or in the total charge borne by the protein, may also contribute to changes in conformation and electronic transitions. Finally, the shoulder observed at 427 nm may result from the coexistence of several noninterconverting conformations in gas-phase cyt *c* 6-. Three isomers are resolved for this charge state in ion mobility experiments.[7,8,14] Changes in protein conformation may induce different porphyrin geometric and electronic structures of the iron-heme complex,[58] which would result in different optical spectra resolved here due to the small broadening of the bands. Gas-phase experiments provide new data to go further in the understanding of the interactions between the prosthetic heme groups and the protein matrix and its influence on the modulation of the chromophore, and we hope that this will favor the development of simulations.

In summary, following several reports on isolated prosthetic groups, we finally succeeded to report the first visible spectrum of a prosthetic group embedded in a gas-phase protein. Results show that electronic excitation at 415 nm leads to an electron detachment. Action spectra show that the optical properties of gas phase cyt *c* (charge state 6-) are close to the one recorded for native form of cyt *c* in solution but significantly shifted as compared with the gaseous ferric $[Fe(III)\text{-}Heme]^+$. It suggests that the protein environments in gas phase and in solution of native cyt *c* are very similar and that they protect the heme group from the solvent. Influence of charge state on structural and electronic properties of gas-phase protein is an important issue that requires coupling of spectroscopy with ion mobility.

■ EXPERIMENTAL METHODS

The experimental setup consists of a mass spectrometer coupled to a UV−vis optical parametric oscillator (OPO) laser.[59,60] The mass spectrometer is an LTQ quadrupole linear ion trap (Thermo Fisher Scientific, San Jose, CA). A quartz window was fitted on the rear of the LTQ chamber to allow the introduction of the laser beam. The laser is a nanosecond tunable OPO laser (Panther, Continuum) pumped by a Nd:YAG laser. The repetition rate of the laser was 10 Hz. The laser beam passes through two diaphragms (1 mm diameter), lenses and a mechanical shutter electronically synchronized with the mass spectrometer, after which it is injected on the axis of the linear trap. The laser power was controlled using a half-wave plate and a polarizer and was monitored with a power meter located just before the injection in the ion trap on a laser reflection on the chamber quartz window. The mechanical shutter is used to synchronize the laser irradiation with the trapping of the ions. To perform laser irradiation for a given number of laser pulses, we add in the ion trap radio frequency (RF) sequence an MS^n step with an activation amplitude of 0%, during which the shutter located on the laser beam is opened. Mass spectra are recorded after laser irradiation as a function of the laser wavelength. At each laser wavelength, a laser-normalized yield of electron photodetachment is deduced from the mass spectrum through

$$\sigma = \ln((parent + daughter)/parent)/\phi \quad (1)$$

where ϕ is the laser fluence, *parent* is the intensity of the precursor ion, and *daughter* represents the intensity of the product ion peak. Optical action spectra are obtained by plotting the normalized yield of electron photodetachment as a function of the laser wavelength. High-resolution mass spectra were recorded with a Q Exactive (Thermo Fisher Scientific, San Jose, CA).

Cyt *c* from bovine heart was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Cyt *c* was dissolved in 90/10 water/methanol (v/v) at a concentration of 10 μM and directly electrosprayed at a flow rate of 5 μL/min for subsequent analysis in a negative-ion mode. Complementary mass spectra

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experiments have been realized under denaturing conditions using cyt *c* 10 μM diluted in 80/20 methanol/water.

■ ASSOCIATED CONTENT

Supporting Information

Laser energy curve as a function of the laser wavelength. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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