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Absorption by Isolated Ferric Heme Nitrosyl Cations In Vacuo**

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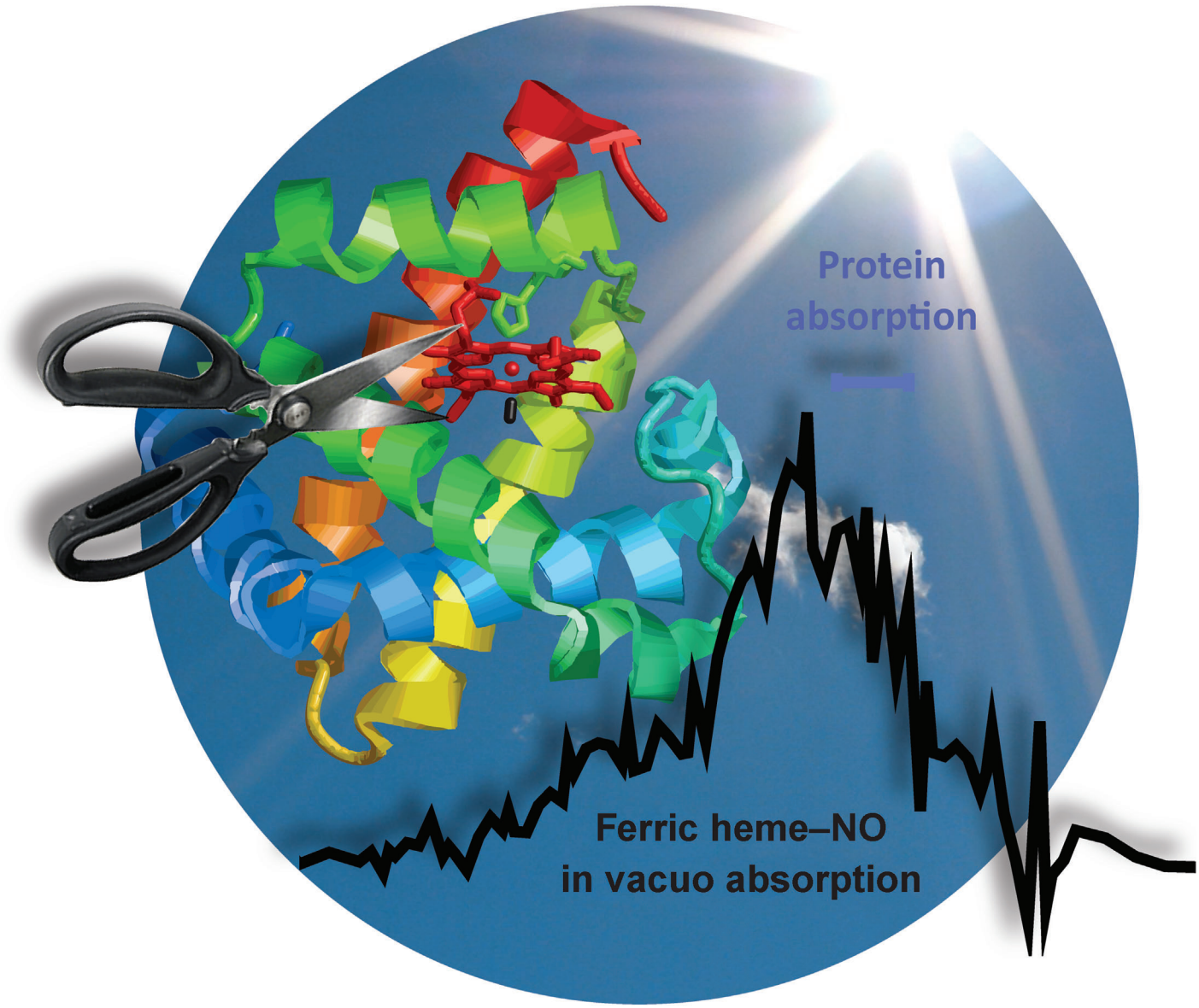

Nitric oxide is a diatomic radical and an important molecule in biology because it binds to the iron centers of certain heme proteins. Indeed, heme nitrosyl proteins play a key role in many physiological functions including blood clotting, blood pressure regulation, immune response, nerve signal transduction, and vasodilation upon the bite of blood-sucking insects.[1–3] The heme prosthetic group is located within a hydrophobic pocket with limited access to water molecules. Still its microenvironment, for example, proximal and distal ligands, differs significantly among the proteins, intimately linking the electronic structure of the heme nitrosyl unit with the biological functions. While the Fe^{II}–NO moiety is bent, Fe^{III}–NO is linear when the proximal ligand is histidine. This linearity is caused by electron transfer from NO to Fe^{III},[4–7] and a more proper description of the unit is Fe^{II}–NO^+. On the other hand, an electron-rich axial ligand such as cysteinate retards electron donation and instead facilitates back-donation from iron to NO to give a slightly bent and tilted coordination geometry.[4,5]

Absorption spectroscopy is one of the most utilized methods to analyze heme proteins since spectral features are affected by the oxidation state of the iron center, its axial ligands, its spin state, and the nearby amino acid residues. Extensive studies have been conducted on nitrosylated ferric heme proteins, and we have compiled some of the spectroscopic data in the Q-band region (see Table 1). Here the excitation is a π–π^* transition in the porphyrin macrocycle, and a second band is due to a vibronic transition. Most proteins have either histidine or cysteinate as the proximal heme ligand. For the former, the Q_0 and Q_1 bands are in the ranges 549–574 nm and 518–538 nm while for the latter they are red-shifted to 571–585 nm and 533–549 nm. This difference is linked to the σ-*trans* effect of the negatively charged thiolate.[5,6] Differences within each group are due to the heme microenvironment, particularly at the distal site.

To better understand how the proximal ligand modulates the electron distribution in the porphyrin macrocycle, spectroscopic investigation of the five-coordinate (5c) ferric nitrosyl complex (Figure 1) is needed. This coordination geometry is, however, impossible to realize in solution, and no 5c ferric heme nitrosyl proteins have been found. Hence investigations of such simple but fundamental complexes rely on gas-phase experiments in which the axial ligands can be added in a controlled way, and in which there are no perturbations from environmental factors such as solvent molecules or counterions. This is the topic of the present work.

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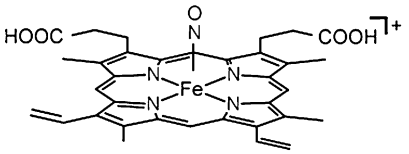

Figure 1. Ferric heme nitrosyl cation.

Absorption spectroscopy of heme in the gas phase has been limited to 4c ferric heme ions and 5c analogues where the fifth ligand is either His or dimethyl sulfoxide.[8–10] The spectrum of intact cytochrome c ions in vacuo was recently reported by Dugourd and co-workers[11] in the Soret band region, and, interestingly, it was found to be similar to that of the native protein in solution. Beautiful work was done by Fornarini and co-workers[12,13] in the characterization of ferric heme nitrosyl ions and other Fe^{III}-NO model complexes based on infrared multiphoton photodissociation. Their work agreed with the formulation of the ground state as Fe^{II}–NO^+ and ruled out the Fe(η^1-ON) linkage isomer. The η^2-NO isomer is more than 1 eV higher in energy than the η^1-NO isomer.[13]

Here we report for the first time on the absorption in the Q-band region by the genuine 5c ferric heme$(NO)^+$ ions, formed in ion–molecule reactions in an octopole. Stabilization of the associative complex is afforded by radiative emission and not ternary collisions.[14] The gas-phase-synthesis approach eliminates the problems of reductive nitrosylation processes in solution phase which lead to reduced iron species.[14–16] Also we succeeded in preparing 6c heme(amino acid)$(NO)^+$ complexes and subjected them to mass spectrometry experiments using a homebuilt sector instrument (Figure 2).

The only photoinduced fragment of heme$(NO)^+$ was heme$^+$ (low photon fluxes). Its formation yield depended linearly on the laser power in agreement with the NO-loss reaction only requiring about 1 eV.[13,17] Importantly, the internal energy of the ions increases by more than 2 eV after photon absorption, most likely driving the dissociation to completion within the flight time to the analyzer (a few μs). NO^+ was not observed in accordance with a change in electronic state after excitation from Fe^{II}–NO^+ to the nearby Fe^{III}–NO(radical) state that is dissociative along the Fe–NO coordinate, which accounts for the weak binding of NO to ferric heme.[6,18]

The action spectrum for NO loss as a function of excitation wavelength is shown in Figure 3 together with that obtained after trapping the complexes in a multipole filled with helium buffer gas for 25 ms. The two spectra are similar, and hence a contribution from long-lived electronically excited ions is irrelevant. A prominent band with its maximum at (561 ± 4) nm is evident and corresponds to the Q_0 band. There also seems to be a shoulder at about 523 nm. For comparison, heme$^+$ has a band with a maximum at 525–530 nm,[9] and NO ligation redshifts the absorption by at least 31 nm. Absorption extends all the way up to 700 nm, the region of ligand-to-metal charge-transfer excitations.

The band of heme$(NO)^+$ at 561 nm is redshifted compared to that of model complexes in aqueous solution (see

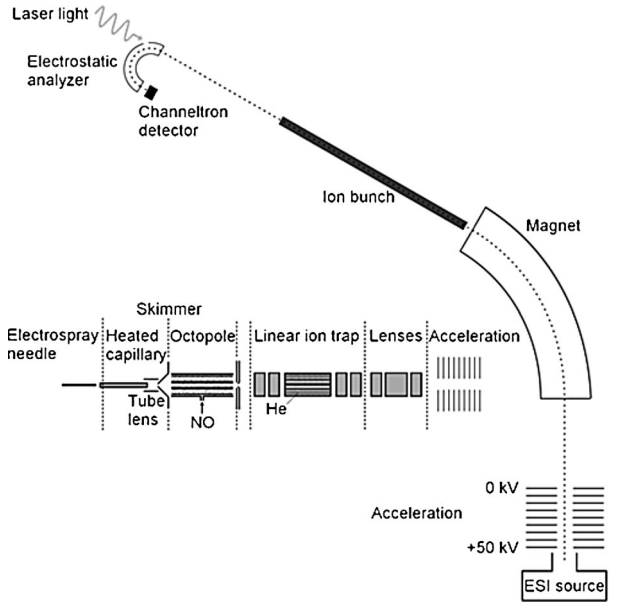

Figure 2. Schematic representation of the experimental apparatus. See the text and the Experimental Section for details.

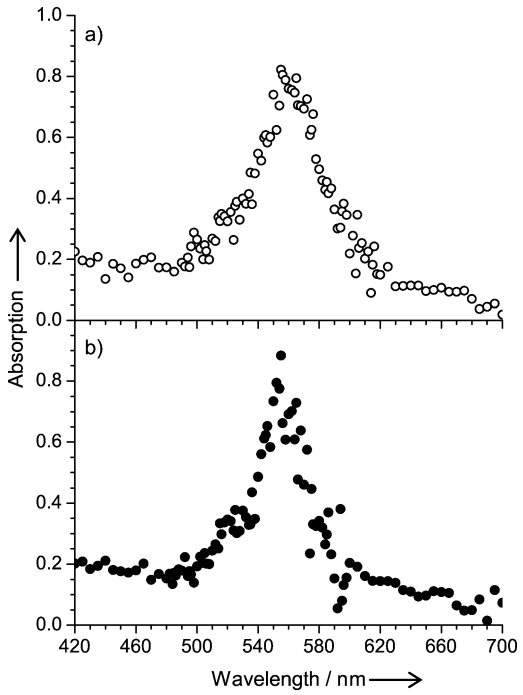

Figure 3. Action spectra of heme(NO)$^+$ sampling heme$^+$ fragments. a) Ions were trapped in an octopole filled with NO gas. b) Ions were trapped in the octopole as in (a) and the ion bunch was subsequently trapped in a 14-pole trap filled with helium.

Table 1: Band maxima in the Q-band region for ferric heme nitrosyl complexes. Mb = myoglobin, Hb = hemoglobin, Cyt = cytochrome, HRP = horse radish peroxidase, PGHS = prostaglandin H synthase, IDO = indoleamine 2,3-dioxygenase, NOS = nitric oxide synthase, His = histidine, Cys = cysteine, Tyr = tyrosine, Met = methionine. All values are in nm.

	Proximal ligand	Q_1 band	Q_0 band	Ref.
Model complexes				
$[Fe^{III}(TMPS)]$[a]	H_2O		541	[24]
$[Fe^{III}(TPPS)]$[b]	H_2O		533	[25]
Proteins				
Mb[c]	His	536	570	[25]
Mb[d]	His	536	574	[26]
Mb	His	534/532	565/574	[27, 28]
Hb[e]	His	532/533	563/566	[25, 28]
Hb_α[e,f]	His	532	562	[28]
Hb_β[e,f]	His	538	568	[28]
Hb_m[g,h]	His	524	572	[28]
Hb_h[g,i]	His	524	566	[28]
Hb[j]	His	534	565	[27]
Cyt c′[k]	His	528.5	562	[29]
Cyt c′[l]	His	528.8	562.4	[29]
Cyt c′[m]	His	528.4	562	[29]
Cyt c′[n]	His	526.8	560	[29]
Cyt c′[o]	His	530.4	564.4	[29]
Cyt c peroxidase	His	537	571	[30]
Cyt c[p]	His	518/529	549/563	[25, 29]
PGHS	His	537	570	[31]
HRP	His	533	568	[30, 32]
MQH_2:NO oxidoreductase	His	536	562	[33]
IDO	His	534	568	[29]
Catalase[q]	Tyr	537	574	[25]
Cyt $P450_{cam}$[r]	Cys	540	571	[34]
Cyt $P450_{LM}$[s]	Cys	543	574	[34]
Cyt $P450_{LM2}$[t,u]	Cys	543	575	[34]
Cyt $P450_{LM4}$[t,v]	Cys	542	571	[34]
mNOS	Cys	549	585	[34]
$iNOS_{ox}$	Cys	547/549	585/580	[35, 36]
inducible NOS[w]	Cys	549	585	[34]
NOS[x]	Cys	545	576	[37]
NOS[y]	Cys	549	580	[38]
P450nor	Cys	539	572	[39]
heme in vacuo	none	523	561	this work
	Met	524	563	–

[a] TMPS = tetra-*meso*-(sulfonatomesityl)porphinate. [b] TPPS = *meso*-tetrakis(*p*-sulfonatophenyl)porphyrin). [c] Whale skeletal muscle. [d] Equine heart. [e] Human. [f] Subunit. [g] *Glycera dibranchiata*. [h] Monomers. [i] Oligomers. [j] Opossum, no distal His. [k] *Rhodobacter capsulatus* B100. [l] *Rb. capsulatus* ATCC 11166. [m] *Rhodopseudomonas palustris* ATCC 17001. [n] *Rhodospirillum rubrum* ATCC 11170. [o] *Chromatium vinosum* ATCC 17899. [p] Horse heart. [q] Distal site of the heme is free. [r] *Pseudomonas putida*. [s] Rat liver microsomes. [t] Rabbit liver microsomes. [u] Phenobarbital-induced P450. [v] 5,6-Benzoflavone-induced P450. [w] Murine macrophages. [x] Rat brain. [y] Neuronal.

Table 1) but is very close to that observed for proteins with a proximal His residue as most of the spectral data in Table 1 range between 560 and 572 nm. Hence the effect of His is limited, and small differences are more likely due to variations at the distal site. This conclusion is supported by the results on 6c complexes (vide infra). That His coordination plays a minor role in perturbing the electronic structure also follows from previous measurements on heme$^+$ and heme(His)$^+$ ions which showed almost identical absorption in the Q-band region.[9]

Ferric heme nitrosyl proteins with histidine proximal ligands have a Soret band with maxima at 415–422 nm while for the isolated ions it is below 420 nm. We only see the tail of this band which typically has an oscillator strength an order of

magnitude larger than that of the Q bands. For comparison, the Soret bands of isolated heme$^+$ and heme$^+$(His) ions are blueshifted by 16 nm or so compared to the Soret bands of the proteins.

Next we consider the results for heme(Met)(NO)$^+$. Photoinduced loss of Met alone did not occur to any significant degree while NO loss did. The NO-loss spectrum displays two transitions, one at (563 ± 5) nm (Q_0) and the other at (524 ± 5) nm (Q_1) (Figure 4a). Hence axial coordination of Met does not perturb the lowest-energy transition much but it does enhance coupling to vibrational modes. Indeed, two bands of similar magnitude are normally seen for ferric heme nitrosyl proteins (6c heme). The Q_1 band for most proteins with proximal His is in the range 524–538 nm which is again redshifted relative to the analogous band of the gaseous ions.

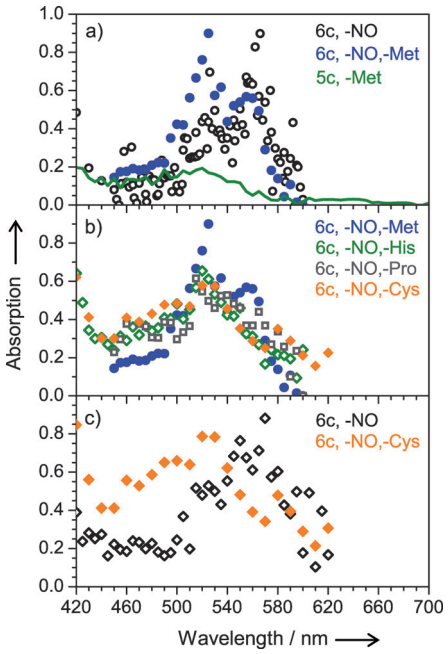

Figure 4. a) Action spectra of heme(Met)(NO)$^+$ sampling either heme(Met)$^+$ or heme$^+$ fragments. The spectrum of heme(Met)$^+$, sampling heme$^+$, is shown for comparison as the dark green curve. b) Action spectra of heme(amino acid)(NO)$^+$, sampling heme$^+$. Amino acid = His, Pro, Met, or Cys. c) Action spectra of heme(Cys)(NO)$^+$ sampling either heme(Cys)$^+$ or heme$^+$.

Loss of both Met and NO was observed at high laser power after the stepwise absorption of two photons (not coherent). This is reasonable as it requires about 1.2 eV to lose His from heme(His)$^+$,[19] and the total cost of losing both NO and the amino acid is likely about 2 eV. Minor fragmentation would occur within the limited time window for sampling fragmentation after one-photon absorption. The action spectrum has a band maximum at about 559 nm (Q_0) (Figure 4a). The spectrum is skewed towards the blue with Q_1 now being more intense. However, if NO loss happens before the second photon is absorbed, that is, within a few nanoseconds, the action spectrum represents the absorption cross section of heme(Met)(NO)$^+$ multiplied by that of heme(Met)$^+$. For comparison, we have included the spectrum of heme(Met)$^+$ (Figure 4a), which shows that as expected this 5c complex absorbs at shorter wavelengths than heme(NO)$^+$, and that most fragmentation occurs close to the Q_1 band of heme(Met)(NO)$^+$.

Figure 4b shows the spectra for heme(His)(NO)$^+$ and heme(Pro)(NO)$^+$ obtained by monitoring heme$^+$. It is evident that these ions absorb in the same region as heme(Met)(NO)$^+$. However, exact band maxima are uncertain since the signal for NO loss alone was too small to be recorded.

The spectrum for NO loss from heme(Cys)(NO)$^+$ is similar to that of heme(Met)(NO)$^+$ (Figure 4c) but somewhat broadened at longer wavelengths. If Cys binds as a thiolate to the iron and the amino group is protonated to account for the overall +1 charge, a spectral redshift is expected based on the protein data. Hence the ion population may contain two dominant isomers, one of which adopts a binding mode similar to that in heme(Met)(NO)$^+$ while the other has an iron thiolate ammonium ion structure. Sampling the loss of both ligands provides a spectrum that is similar to those for the other amino acids but slightly broader (Figure 4b), but again we caution that we are measuring a combined action spectrum of heme(Cys)(NO)$^+$ and heme(Cys)$^+$ formed after instant NO loss.

In conclusion, our work has shed light on the inherent electronic properties of ferric heme nitrosyl complexes. The absorption spectra of six-coordinate complexes with NO and Met or Cys are similar to that of the 5c heme$^+$(NO) ion but with a more pronounced Q_1 band. Since the absorption spectra of ferric heme nitrosyl proteins with histidine as proximal ligands are similar to those of the gaseous complexes or slightly redshifted, the protein microenvironment does not perturb the lowest-energy transition of the porphyrin macrocycle much. In addition to being of importance in protein biospectroscopy, these data are useful to benchmark quantum chemical calculations of ferric heme nitrosyl complexes and guide new developments in this field. Such calculations are nontrivial and challenging even for ground-state electronic structures[20–23] and need to be tested against experimental data such as those presented here where environmental effects are disentangled from inherent properties.

Experimental Section

All compounds were purchased from Sigma–Aldrich. Hemin was diluted in CH_3OH/CH_2Cl_2 (1:1), and amino acids were added to form complexes. Ions were produced by electrospray ionization and passed through a heated capillary and tube-lens/skimmer region into an octopole that is operated as a trap by proper pulsing of the lens directly following it. The pressure in the octopole was 0.01–0.1 mbar. There, ions were allowed to react with NO for 25 ms. In one experiment, heme$^+$(NO) ions extracted from the octopole were trapped again for 25 ms in a 14-pole (multipole) trap filled with He at room temperature. Ions were accelerated to a kinetic energy of 50 keV, and those of interest were selected by an electromagnet and subsequently irradiated with light (420–700 nm) from an EKSPLA laser system. This is an Nd:YAG laser that pumps an optical parametric oscillator with a repetition rate of 20 Hz. Ion bunches were about 10 μs wide and laser pulses less than 10 ns. An electrostatic analyzer was scanned to identify the fragment ions; these were

counted by a channeltron detector. Since the ion source was running at 40 Hz, only every second ion bunch was irradiated to obtain the laser-induced signal. The formation of fragments with the laser off was due to either dissociation of metastable ions or to collisions with residual gas in the beam line. In cases with high count rates this yield was used to correct for small fluctuations in the intensity of the parent ions. The photo-induced yield of a fragment ion was measured as a function of wavelength. Yields were corrected for photon fluxes to obtain action spectra. As fragmentation is a result of absorption, these spectra are taken to represent absorption by the ions.

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