THE OPTICAL PROPERTIES OF HEME *a*: RESONANCE RAMAN SCATTERING WITH VISIBLE EXCITATION

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1. Introduction

Heme *a*, the iron-containing prosthetic group of the respiratory protein, cytochrome oxidase, has unusual substituents at two pyrrole carbons: a formyl at position 8 and a hydroxyfarnesylethyl at position 2 [1]. As a consequence, the optical properties of heme *a* are significantly different from those of protoheme [2].

In vivo, two hemes *a* occur per protein molecule. One is associated with cytochrome *a* and is low-spin, while the second is identified with cytochrome a_3 and is high-spin [3]. The protein optical properties are thus a convolution of those of one low-spin and one high-spin heme *a*. The possible modulation of these basic features by protein-mediated interaction effects has rendered the absorption spectrum of cytochrome oxidase controversial [4,5] and several suggestions, ranging from an independent chromophore model [4,6] to an $a{:}a_3$ exciton interaction model [7], have been made. The possibility that the longest wavelength visible transition in low-spin heme *a* compounds has porphyrin π to formyl carbonyl π^* charge transfer character has also been raised [8]. The latter hypothesis is of particular interest in light of current models for the role of the formyl group in enzyme function [9].

Resonance Raman spectroscopy is able to provide insight into the electronic properties of heme *a* in solution and in situ [10–12]. In the experiments reported here we have investigated the Raman spectra of low-spin heme *a* complexes obtained with laser excitation in resonance with the 590 nm optical band. Our data demonstrate that this band corresponds to the heme *a* Q_{00} transition; no evidence of carbonyl involvement was found. The visible spectrum of reduced cytochrome oxidase is discussed with reference to the unusually high α band oscillator strength of low-spin heme *a* complexes. A comparison of the low-spin model compound Raman data we have obtained with analogous data reported for the protein [13] shows a close correspondence between the two. We also note that the depolarization ratios for heme *a* vibrations enhanced by α band excitation are much lower than observed for protoheme compounds.

2. Materials and methods

Heme *a* was isolated from beef heart cytochrome oxidase, its concentration determined and low-spin model compounds prepared in an aqueous buffer system (0.1 M sodium phosphate, 0.001 M sodium EDTA, 0.07 M SDS and 0.6 M *N*-methyl imidazole) as in [12]. Reduction was achieved by adding aqueous dithionite to the degassed solution. Oxidized samples of heme *a* in aprotic organic solvents were converted to the low-spin state by adjusting *N*-methyl imidazole to 0.6 M. An aprotic reducing system consisting of the 18–crown–6 complex of sodium dithionite in the appropriate solvent [14] was used to reduce heme *a* to its ferrous state. In the organic solvent system we were able to obtain samples of heme *a* in which hydrogen bonding to the formyl carbonyl is minimized [12]. Aggregation effects occur for aqueous low-spin heme *a* in the absence of SDS; addition of SDS eliminates this phenomenon and the low-spin heme *a* complexes in both the aqueous buffer system

above and in DMSO follow Beer's law to a concentration of at least 510 μM.

Raman spectra were recorded by using a Spex 1401 double monochromator and the associated Ramalog electronics. Excitation in the 600 nm region was obtained from a dye laser with Rhodamine 6G dye pumped by a Spectra Physics Model 164 argon ion laser as in [15]. Reported frequencies are accurate to ± 2 cm^{-1}; other instrument conditions are reported in the figure legend. Depolarization ratios were measured by using a polaroid analyzer followed by a polarization scrambler to record the spectrum for light scattered parallel or perpendicular to the incident light polarization. As a check on this procedure, depolarization ratios were recorded for cytochrome c^{2+}(λ_{ex} = 514.5 nm) and DMSO (λ_{ex} = 591.5 nm) and found to agree with literature values. Samples (250–500 μM in heme *a*) were prepared in 5 mm pathlength cuvettes; optical spectra were recorded with a Cary 17D spectrophotometer before and after each Raman experiment to monitor sample integrity.

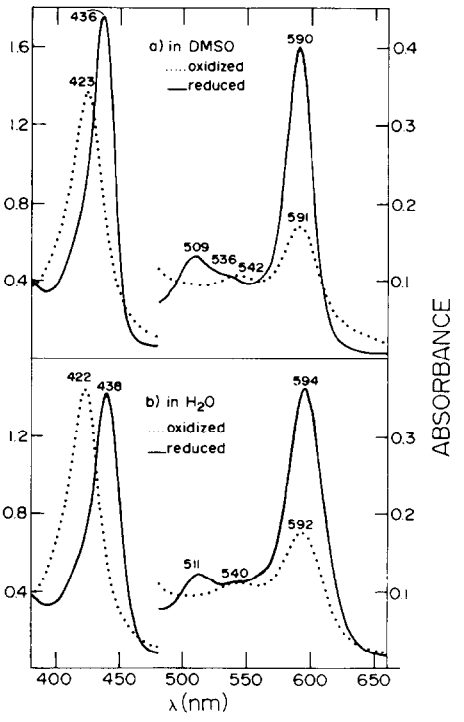

Fig.1. Optical spectra of oxidized and reduced heme *a* $(NMeIm)_2$ in (a) DMSO and (b) H_2O. 17 μM heme *a* was used in both samples.

3. Results

The optical spectra recorded for oxidized and reduced low-spin heme *a* complexes in water and in the aprotic solvent, DMSO, are shown in fig.1. In the oxidized species the longest wavelength visible transition occurs near 590 nm, with a weaker transition ~1500 cm^{-1} to higher energy, at 540 nm. Upon reduction the band at 590 nm doubles in intensity, a new band appears at ~510 nm and the Soret shifts from 425 to 435 nm. That three transitions occur in the visible region for reduced, low-spin heme *a* is unusual; typically low-spin hemes and heme proteins show only the Q_{00} (α) and Q_{01} (β) transitions. Moreover, the energy splitting between the two longest wavelength bands in heme a^{2+} is ~1600 cm^{-1}, significantly greater than the 1000–1200 cm^{-1} α–β band splitting found in other heme proteins [16].

Raman spectra in the 1000–1700 cm^{-1} region, obtained for the samples of fig.1 with excitation in resonance with the 590 nm optical band, are shown in fig.2. Depolarization ratios for the more prominent vibrations are listed in table 1. Several bands were observed in the low frequency region although the resonance enhancement was weaker, by a factor of ~3, for the strongest of these vibrations compared to the higher frequency bands.

In contrast to Raman spectra of heme *a* obtained with Soret excitation [12], the carbonyl stretching vibration in the 1670–1680 cm^{-1} region is not observed upon visible excitation even in the aprotic DMSO solvent system. We have also failed to observe the carbonyl band for heme *a* complexes dissolved in CH_2Cl_2 or CH_3CN when λ_{ex} = 590 nm. The vibrational bands observed are, for the most part, typical of those seen with other hemes and heme proteins under α band excitation [16]. Very striking differences are observed, however, when the depolarization ratios for heme *a* complexes are compared to those of heme *b* or *c* compounds. For the latter, only depolarized (dp) and anomalously polarized (ap) (ρ = 3/4 and $\rho > 3/4$, respectively, in D_{4h} symmetry) modes are strongly enhanced when λ_{ex} is in resonance with the α band [16]. Moreover, the ρ values for ap modes are typically $\gg 1$. For heme *a*, we observe no depolarization

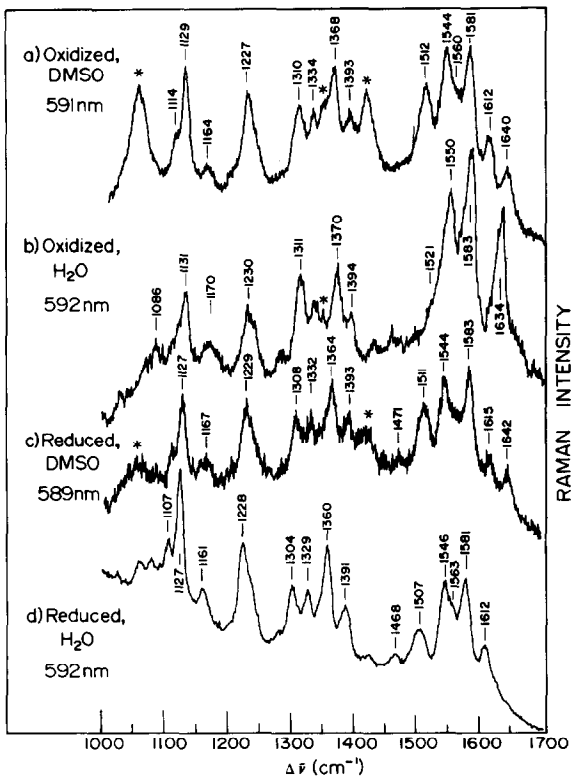

Fig.2. Resonance Raman spectra of oxidized and reduced heme *a* $(NMeIM)_2$ in DMSO (a,c) and in H_2O (b,d). A 1 s time constant and 50 cm^{-1}/min scan rate were used in (a) and (c); in (b) and (d) these parameters were 2.5 s and 20 cm^{-1}/min. Slitwidths were used which provided 6 cm^{-1} resolution; the laser power incident on the samples was 150 mW. The exciting wavelength is given for each spectrum. Solvent or ligand vibrational modes are marked by an asterisk.

ratios > 1 and only two modes, 1580 cm^{-1} and 1310 cm^{-1}, have ρ values indicative of anomalous polarization; the remaining bands are polarized under D_{4h} symmetry.

For the reduced heme *a* complexes, the nature of the solvent has little effect on the Raman bands observed: only the 1640 cm^{-1} band, already weak in DMSO, is absent in water. For oxidized heme *a* a significant influence of the solvent is apparent. The 1512 cm^{-1} vibration is absent for aqueous heme a^{3+}, while the 1634 cm^{-1} vibration is strongly enhanced. In DMSO the intensity of bands in the 1000–1400 cm^{-1} region are roughly the same as those around 1550 cm^{-1}, whereas in water the lower frequency vibrations are less strongly enhanced. Reduction of heme a^{3+} in water results in a marked change in the Raman spectrum; on the other hand, for the DMSO samples perturbations to the Raman spectrum by the reduction process are minimal. The redox process is

Table 1
Low-spin heme *a* depolarization ratios

Heme a^{3+} $(NMeIm)_2$ in DMSO		Heme a^{3+} $(NMeIm)_2$ in H_2O		Heme a^{2+} $(NMeIm)_2$ in H_2O	
Band (cm^{-1})	ρ	Band (cm^{-1})	ρ	Band (cm^{-1})	ρ
1640	.45	1634	.37		
1612	.38			1612	.58
1581	.86	1583	.81	1581	.92
1544	.50	1550	.48	1546	.53
1512	.33			1507	.28
		1394	.44	1391	.43
1368	.43	1370	.44	1360	.42
1310	.70	1311	.83	1307	.95
1227	.39	1230	.52	1228	.33
1129	.54	1131	.60	1127	.37

Values of $\rho = I_\perp/I_{//}$ for the major bands of the species in fig.2. The depolarization ratios for reduced heme *a* in DMSO were similar to the values recorded for the water sample; only the latter are reproduced here

reflected in changes in the oxidation marker band region (1360–1370 cm^{-1}) in both systems, although the band shifts (4–10 cm^{-1}) are much smaller than those observed upon Soret excitation [11]. This behavior probably indicates the presence of a second, redox-insensitive, band in this region [16].

4. Discussion

The similarity between the heme *a* Raman frequencies and those of heme *b* or *c* compounds under α band excitation, together with the lack of resonance enhancement of the carbonyl stretching vibration, clearly indicate that the 590 nm optical band corresponds to the heme *a* Q_{00} transition. This conclusion is in agreement with recent LCAO MO SCF calculations which place the formyl carbonyl π^* state at an energy well into the ultraviolet [17]. With this assignment, we can compare the optical properties of heme *a* with heme *c* and protoheme containing complexes. Following [18], we calculated oscillator strengths for α and Soret bands of several porphyrin complexes and list the ratio (*r*) of these in table 2. An increase in *r* indicates a stronger electronic transition dipole for the Q_{00} state which, in the 4 orbital model, results from a greater electronic interaction between the Q and B states [18]. For heme a^{2+}, table 2 shows that the α band transition is more strongly allowed than in other, non-formyl containing porphyrin compounds. Moreover, formylation at a second ring position causes a further increase in *r*. The *r* values for the heme *a* compounds, when juxtaposed with that of reduced cytochrome oxidase, serve to rationalize the optical properties of the protein.

Because the Q band transition for high-spin reduced heme *a* is weak [19], the decreased *r* value for the oxidase is consistent with the presence of one low-spin and one high-spin heme *a* per protein molecule.

A comparison of the α band-excited resonance Raman spectra of low-spin heme *a* with the corresponding spectra obtained [13] for the oxidase shows good agreement between the two. For the in vitro chromophore several of the bands shift to lower frequencies, up to 10 cm^{-1} in the case of the 1520 cm^{-1} vibration, but they can nevertheless be correlated because of similarities in depolarization ratio. (For example, the depolarization ratio of the 1521 cm^{-1} band in vivo is .35 when λ_{ex} = 602 nm [21].) Heme *a* Raman spectra are sensitive to solvent as the data of fig.2 indicate, which may account for much of the frequency shift. The correspondence observed between the low-spin models and the protein Raman data is the expected result if the principal absorber in the visible region is low-spin cytochrome *a*. This interpretation is also consistent with the transition dipole moment strength arguments above.

The primary mechanism for resonance enhancement of porphyrin vibrations in heme proteins under α or β band excitation involves vibronic coupling between the Q and B states via Herzberg-Teller active modes. In D_{4h} symmetry these are A_{2g}, B_{1g} and B_{2g} and consequently only anomalously polarized or depolarized modes are observed in the Raman spectrum [16]. For heme *a* compounds, the Herzberg-Teller mechanism also appears to be active as evidenced by anomalously polarized modes at 1581 and 1310 cm^{-1}. However, the depolarization ratios for the majority of the bands we observe are $< 3/4$ and

Table 2
Metalloporphyrin α band/Soret band oscillator strength ratios

Compound	$r=f_\alpha/f_\gamma$	Ref.
Low-spin heme a^{2+} in H_2O	0.18	This work
Low-spin heme a^{2+} in DMSO	0.17	This work
Reduced cytochrome oxidase	0.10	[4]
Reduced cytochrome *c*	0.06	[16]
Oxyhemoglobin	0.05	[20]
Low-spin, reduced 2,6-diformyl heme	0.25	[17]

Oscillator strengths for α (f_α) and Soret (f_γ) band transitions were calculated as in [18] by using the band halfwidth at half-maximum intensity as a measure of $\Delta\nu$

these modes are classified as polarized under D_{4h} symmetry. There are a number of mechanisms by which this could occur including the following: Franck-Condon overlaps induced by the strong heme a α band transition dipole moment, a perturbation to the basic D_{4h} symmetry such that the contribution of the isotropic tensor invariant to the total polarizability tensor is enhanced for depolarized or anomalously polarized modes, or a more drastic symmetry reduction to, for example, C_{4h} or C_{2v} symmetry [16]. Excitation profiles for vibrational mode enhancements and depolarization ratios are required to elucidate the relative importance of these possibilities and are in progress.

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