Characterization of the Electronic Properties and Geometric Environment of the Iron Atom in the 'Myoglobin Hydrogen-Peroxide' Complex by Soret-excited Resonance Raman Spectroscopy

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The low temperature (ca. 80 K) Soret-excited resonance Raman spectrum of the 'myoglobin hydrogen-peroxide' complex has been obtained and compared to those of Fe(II) and Fe(III) myoglobin. The RR spectra suggest that the iron atom in the 'myoglobin hydrogen-peroxide' complex is formally in the Fe(IV) state with a t_{2g}^4 low-spin configuration. The iron–pyrrole-nitrogen (Fe–N_p) and iron–imidazole-nitrogen (Fe–N_{im}) bond lengths in the 'myoglobin hydrogen-peroxide' complex have been estimated from the wavenumbers of the iron–pyrrole and iron–imidazole stretching modes around 345 and 375 cm^{-1} using the Fe–N_p and Fe–N_{im} distances in Fe(II) myoglobin available from the X-ray crystallographic data in the literature. The distance from the centre of the porphyrin to the pyrrole nitrogen atoms (Ct–N_p) was estimated from the position of the anomalously polarized band around 1560–1590 cm^{-1} by comparison with data in the literature on model metalloporphyrins. The Fe–N_{im}, Fe–N_p and Ct–N_p distances for the 'myoglobin hydrogen-peroxide' complex were found to be 2.09, 2.08 and 2.01 Å; the distance from the iron atom to the plane of the pyrrole nitrogen atoms was determined by triangulation and found to be $0.53^{+0.14}_{-0.18}$ Å. With laser lilumination of 200 mW power, the low-spin t_{2g}^4 'myoglobin hydrogen-peroxide' complex apparently undergoes a two-electron photoreduction, with a rate constant of ca. 2.5 × 10^{-4} s^{-1} at ca. 80 K, to high spin $t_{2g}^4 e_g^2$ Fe(II) myoglobin, since there is no evidence for Fe(III) myoglobin.

Introduction

It has long been known that the addition of hydrogen peroxide to Fe(III) myoglobin in the pH range 8.0–9.0 results in the formation of a stable 'myoglobin hydrogen-peroxide' complex characterized by an absorption maximum at 422 nm in the Soret region, together with a maximum at 547 nm, a weak band at 580–590 nm and a low shoulder at 510–520 nm in the α, β region [1]. Mössbauer spectroscopy of the 'myoglobin hydrogen-peroxide' complex gave a chemical isomer shift very close to zero and a temperature-independent sharp quadrupole splitting over the 4.2–1.95 K range [2]. The unusually small chemical isomer shift of 0.07 mm s^{-1} [2] indicates that the s-electron character near the iron nucleus of the 'myoglobin hydrogen-peroxide' complex is similar to that of metallic iron and that there is no shielding by the d orbitals. This is consistent with an Fe(IV) state in the 'myoglobin hydrogen-peroxide' complex. The value of the quadrupole splitting of 1.56 mm s^{-1} [2] implies non-symmetric electron density at the iron nucleus which, for a d^4 configuration, is consistent with a low spin (S = 1) state. Very similar results have been obtained for hydrogen peroxide complexes of Japanese-radish peroxidase [2], horse-radish peroxidase B [3], and cytochrome c peroxidase (ES) [3]. A low spin state for the 'myoglobin hydrogen-peroxide' complex is also consistent with the finding that the room temperature magnetic susceptibility is 2.85 B.M. [4], which is very close to the spin-only value for S = 1. No ESR signals [5] have been observed for the 'myoglobin hydrogen-peroxide' complex.

Resonance Raman (RR) spectroscopy provides a particularly powerful tool with which to examine haemoproteins. Metalloporphyrins have two $\pi \rightarrow \pi^*$

transitions [6] in the visible and near ultraviolet regions which are both of E_u symmetry in the D_{4h} point group which applies approximately to metalloporphyrins. Being nearly degenerate, they are subject to strong configuration interaction, giving rise to the intense Soret absorption band in the 400–430 nm region ($\epsilon \sim 10^5 M^{-1} cm^{-1}$), and to the weaker α band and vibronic side band (the β band) in the 500–630 nm region. Soret-excited RR spectra are dominated by A-term scattering whereas the α and β bands give rise to B-term scattering [7]. The Raman bands most resonantly enhanced occur between 1100–1650 cm^{-1} and arise from porphyrin ring modes [8], the wavenumbers of which are perturbed by the oxidation and/or spin state of the iron atom [9].

In the present study we have examined the low temperature (*ca.* 80 K) RR spectrum of the 'myoglobin hydrogen-peroxide' complex excited in the Soret region, and compared this to the RR spectra of Fe(II) and Fe(III) myoglobin in order to obtain a better understanding of the electronic properties and geometric environment of the iron atom in the 'myoglobin hydrogen-peroxide' complex. Our results extend the findings of Harami *et al.* [2] obtained by Mössbauer spectroscopy, and confirm that the iron atom of the 'myoglobin hydrogen-peroxide' complex is formally in the low spin Fe(IV) state. In addition we have calculated the iron–pyrrole-nitrogen (Fe–N_p) and iron–imidazole-nitrogen (Fe–N_{im}) bond lengths, the distances from the centre of the porphyrin ring to the pyrrole-nitrogen atoms (Ct–N_p) and, by triangulation, the displacement of the iron atom from the plane of the pyrrole-nitrogen atoms (Ct–Fe). Furthermore, we demonstrate the two-electron photoreduction of the 'myoglobin hydrogen-peroxide complex' to Fe(II) myoglobin at *ca.* 80 K using high intensity monochromatic light, a step which apparently bypasses Fe(III) myoglobin.

Experimental

Solid sperm-whale myoglobin (Sigma type II) was dissolved in 0.05 *M* tris(hydroxymethyl)aminomethane buffer pH 8 and passed through a G25 Sephadex column to remove traces of fluorescent material. Fe(II) myoglobin was then prepared by the addition of small quantities of sodium dithionite. Fe(III) myoglobin was prepared by the addition of a five-fold molar excess of $K_3Fe(CN)_6$ followed by the removal of excess of $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ by passage through a G25 Sephadex column. The 'myoglobin hydrogen-peroxide' complex was prepared by the addition of a ten-fold molar excess of H_2O_2 to Fe(III) myoglobin. The concentration of myoglobin ranged from 1.0 to 1.5 m*M*. To minimize Rayleigh scattering all solutions were milli-
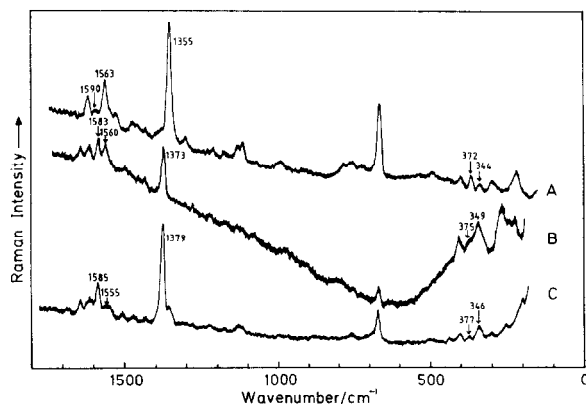

Fig. 1. Soret-excited RR spectra at *ca.* 80 K of Fe(II) myoglobin (A), Fe(III) myoglobin (B) and the 'myoglobin hydrogen-peroxide' complex (C) at low laser power. Conditions of RR spectroscopy: power = 20 mW, time constant = 1 s, scanning rate = 50 cm^{-1} min^{-1}, spectra slit width = 8 cm^{-1}; excitation wavelengths are 436.9 nm (A), 457.9 nm (B) and 422.1 nm (C).

pored (pore size 0.45 μm) prior to recording RR spectra. All samples were checked by optical spectroscopy using a Cary 14 spectrometer and all chemicals were of analytical grade.

RR spectra were recorded on a Spex 1401 spectrometer. Exciting radiation was provided by a Coherent model 12 Ar^+ ion laser and a model 490 tunable dye laser employing the dye stilbene 3. The samples were held between a thin teflon cell and a glass cover slip on a cold finger maintained at *ca.* 80 K by liquid nitrogen. Band wavenumbers were determined by reference to the emission lines of neon. Laser powers quoted are those at the sample (*ca.* 40% of the power at the laser).

Results and Discussion

The Soret-excited RR spectra of Fe(II) myoglobin, Fe(III) myoglobin and the 'myoglobin hydrogen-peroxide' complex are shown in Fig. 1 and the wavenumbers of the bands together with their assignments are given in Table I.

As the Soret transition is polarized in the plane of the porphyrin, only in-plane Raman bands are enhanced on resonance therewith [10]. If the peripheral substituents are ignored, the porphyrin macrocycle has 81 normal modes of vibration of which 55 are in-plane and classify as

$$\Gamma_{in-plane} = 7a_{1g} + 6a_{2g} + 7b_{1g} + 7b_{2g} + 14e_u$$

under the D_{4h} point group. The e_u vibrations, however, are inactive in Raman scattering although they might contribute to the observed RR spectra if substi-

TABLE I. Wavenumbers (cm^{-1}) of Observed Raman Bands for Fe(II) Myoglobin, Fe(III) Myoglobin and the 'Myoglobin Hydrogen-Peroxide' Complex Excited in the Soret Region[a] at *ca.* 80 K.

Fe(II) Myoglobin	Fe(III) Myoglobin	'Myoglobin hydrogen-peroxide' Complex	Assignment [ref.] and Relative Contributions of Specified Modes
1690[b](w)		1684(w)	
	1639(w)		
1616(m)	1613(w)	1613(w)	b_{1g} $C_aC_m > C_bC_b > C_aC_b$ [8]
1590(w)[c]	1583(m)[c]	1585(s)[c] ⎫	a_{2g} $C_aC_m > \delta CH > C_aC_b$ [8]
1563(s)[c]	1560(m)[c]	1555(w)[c] ⎭	
1527(w)	1495(w)	1506(w)	
1472(w)		1471(w)	
1429(w)	1431(w)	1432(w)	
1390(w, sh)			
1355(vs)[d]	1373(s)[d]	1379(vs)[d]	a_{1g} $C_aC_b > C_aC_m > C_bC_b$ [8]
1301(w)		1301(w)	
1213(w)	1224(w)	1227(w)	b_{1g} $C_aC_b > \delta CH$ [8]
1175(w)	1175(w)	1180(w)	
1133(m)	1130(w)	1131(w)	
1116(m)			
1072(w)	1082(w)		
993(w)	978(w)		
786(w)	798(w)		
755(w)	755(w)	756(w)	
726(w)			
671(s)	671(w)	674(m)	$C_aC_b > \delta C_bC_aN$ [22]
500(w)		504(w)	
444(w)	444(w, sh)	443(w)	
404(w)	410(w)	409(w)	
372(w)	375(w, sh)	377(w)	Fe–imidazole stretch [16]
344(w)[e]	349(m)[e]	346(w)[e]	Fe–pyrrole stretch [16]
303(w)		305(w)	
	273(m)	257(w)	
225(m)	236(w)	206(w)	

[a]Excited at 436.9, 457.9 and 422.1 nm for Fe(II) myoglobin, Fe(III) myoglobin and the 'myoglobin hydrogen-peroxide' complex respectively. [b]Observed using 422.1 nm excitation. [c]C(ap) band which is a spin-state marker independent of oxidation state [9, 10]. [d]A(p) band which is an oxidation-state marker independent of spin state [9, 10]; C_aN stretching may also be involved. [e]Although this mode will be coupled to other modes of the Fe porphyrin system, the bond length changes (Table II) are not very sensitive to the extent of this coupling.

All bands wavenumbers are accurate to 1–2 cm^{-1}, with the exception of the strong bands which are accurate to within 1 cm^{-1}. vs = very strong, s = strong, m = medium, w = weak, sh = shoulder.

tuents destroy the effective symmetry centre of the porphyrin macrocycle. The a_{1g} modes are polarized (p), the b_{1g} and b_{2g} modes depolarized (dp) and the a_{2g} modes anomalously polarized (ap).

The available evidence on a series of Fe(II) and Fe(III) haemoproteins and model haem compounds indicates that the polarized band in the region 1355–1382 cm^{-1}, known as the A(p) band, is an oxidation-state marker independent of spin state [9, 10]. As the A(p) band is totally symmetric, it is Soret enhanced and indeed it is the most prominent feature in the high frequency region of the Soret-excited RR spectra (Fig. 1). Normal coordinate analysis [8] has shown that the A(p) band mainly involves breathing of the outer porphyrin ring and is composed of a mixture of C_a–C_b, C_a–C_m and C_b–C_b stretches (where C_m is the methine carbon atom, and C_a and C_b are the α- and β-carbon atoms of pyrrole, respectively, (Fig. 2)). Increasing the charge on the central metal atom results in contraction of its d orbitals with a consequent reduction in their overlap with the porphyrin π* orbitals, and an increase in σ-donation from the porphinato-pyrrole-nitrogen atoms. The depopulation of the porphyrin π* orbitals strengthens the symmetric outer porphyrin ring modes and increases their force constants and vibrational frequencies. Thus, the wavenumber of the A(p) band may be viewed as an indicator of π electron density

TABLE II. Electronic Properties and Geometrical Environment of the Iron Atom in Fe(II) Myoglobin, Fe(III) Myoglobin and the 'Myoglobin Hydrogen-Peroxide' Complex.

Property	Fe(II) Myoglobin	Fe(III) Myoglobin	'Myoglobin Hydrogen-Peroxide' Complex
A(p) band/cm^{-1}	1355[a]	1373[a]	1379
Formal charge on the iron atom	+2	+3	+4
C(ap) band[b]/cm^{-1}	1563	1560	1585
Ratio of band intensities ca. 1585/ca. 1560 cm^{-1}	0.2	1.1	2.1
Spin state	high spin	high/low spin[c]	low spin
Electronic configuration	$t_{2g}^4 e^2$	$t_{2g}^3 e_g^2 / t_{2g}^5$ [c]	t_{2g}^4
Ct–N$_p$ distance[d] (R)/A	2.04$_7$	2.05$_2$[e]	2.00$_7$
Fe–imidazole stretch/cm^{-1}	372[f]	375	377
Fe–N$_{im}$ stretching force constant/mdyn A^{-1} [g]	0.913	0.928	0.938
Fe–N$_{im}$ distance (r)/A	2.10$_0$[h]	2.09$_2$	2.08$_6$
Fe–pyrrole stretch/cm^{-1}	344[f]	349	346
Fe–N$_p$ stretching force constant/mdyn A^{-1} [g]	0.964	1.004	0.921
Fe–N$_p$ distance (r')/A	2.060[i]	2.039	2.083
Ct–Fe distance[j] (d)/A	$0.23^{+0.24}_{-0.00}$ [k]	$0.00^{+0.35}_{-0.00}$ [k]	$0.56^{+0.14}_{-0.18}$

[a]The wavenumbers of the A(p) band for frozen Fe(II) and Fe(III) myoglobin are the same as those found by Yamomoto *et al.* for solutions of Fe(II) and Fe(III) myoglobin at room temperature [14]. [b]Only the wavenumber of the C(ap) band of the majority species is given. [c]The low spin component of Fe(III) myoglobin at pH 8.0 arises mainly from the presence of 10% low spin Fe(III) myoglobin hydroxide [15]. [d]The Ct–Fe distances are calculated on the basis of their very strong correlation with the wavenumber of the C(ap) band by comparison with the data for model metalloporphyrin compounds [12]. [e]This Ct–Fe distance refers to the majority species, high spin aquo Fe(III) myoglobin, and not low spin Fe(III) myoglobin hydroxide. [f]The wavenumbers for the Fe–imidazole and Fe–pyrrole stretches for Fe(II) myoglobin given here are identical to those found by Kincaid *et al.* [16] using solutions of Fe(II) myoglobin at room temperature. [g]1 mdyn A^{-1} = 10^2 N m^{-1}. The force constants are calculated on the basis of a ligand mass of 14. [h]Fe–N$_{im}$ bond length in Fe(II) myoglobin, as determined by X-ray crystallography [19]. [i]Fe–N$_p$ distance for Fe(II) myoglobin, as determined by X-ray crystallography [19]. [j]Errors are calculated assuming errors of ±0.02 A for the Fe–N$_p$ and Ct–N$_p$ distances, and ±2 cm^{-1} for the Fe–pyrrole stretch. [k]The Ct–Fe distances in Fe(II) and Fe(III) myoglobin, determined by X-ray crystallography, are 0.42 ± 0.1 and 0.27 ± 0.1 A respectively [18, 19].
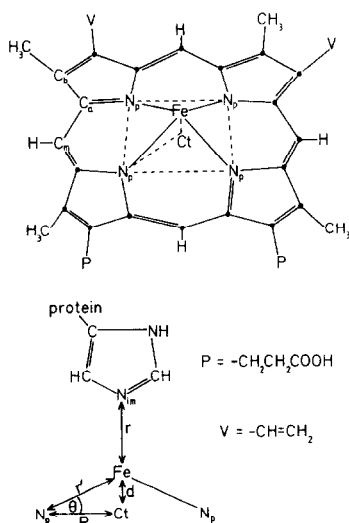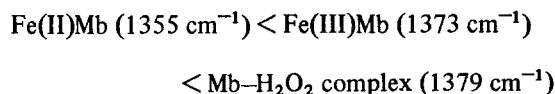

Fig. 2. Labelling scheme for the porphyrin skeleton of proto-porphyrin IX, the porphyrin of myoglobin.

and aromaticity of the porphyrin. Looking at Table II we find that the wavenumber of the A(p) band increases in the order:

$$Fe(II)Mb\ (1355\ cm^{-1}) < Fe(III)Mb\ (1373\ cm^{-1})$$

$$< Mb-H_2O_2\ complex\ (1379\ cm^{-1})$$

which is suggestive of a formal Fe(IV) state in the 'myoglobin hydrogen-peroxide' complex, characterized by increased σ donation from the porphinato-pyrrole-nitrogen atoms and decreased π electron density and aromaticity of the porphyrin. We also note that the A(p) band of compound II of horse radish peroxidase and compound ES (the enzyme substrate complex) of cytochrome c peroxidase, both of which are thought to contain Fe(IV) on the basis of Möss-bauer spectroscopy [2, 3] and magnetic susceptibility measurements [4], both lie at 1382 cm^{-1} [11].

The wavenumber of the anomalously polarized band in the region 1555–1590 cm^{-1}, known as the

C(ap) band, has been shown to be correlated both with spin state [9, 10] and the Ct–N_p distance [12] for a wide range of metalloporphyrins. The C(ap) band is composed of a mixture of C_a–C_m and C_a–C_b stretches [8]. Increasing the Ct–N_p distance results in expansion of the inner 16-membered porphyrin ring; if the geometry of the pyrrole is retained, then there will be an increase in the C_a–C_m bond length and C_a–C_m–C_a angle. Thus increasing the Ct–N_p distance decreases the force constant of the C_a–C_m bond resulting in a decrease in the wavenumber of the C(ap) band. It is generally found that the Ct–N_p distance increases on going from low to high spin iron so that for high spin haemoproteins the C(ap) band lies around 1555–1575 cm^{-1} and for low spin haemoproteins between 1580–1590 cm^{-1} [9–13]. If two spin states are present in equilibrium, then two C(ap) bands are seen, one for each species [13, 14], and the ratio of the intensity of the C(ap) band at *ca.* 1585 cm^{-1} to that at *ca.* 1565 cm^{-1} provides a good measure of spin state with ratios $\lesssim 0.6$ indicative of high spin and ratios $\gtrsim 2.0$ of low spin [14]. From Fig. 1, we find the ratios to be *ca.* 0.2, 1.1 and 2.1 for Fe(II) myoglobin, Fe(III) myoglobin and the 'myoglobin hydrogen-peroxide' complex respectively. Thus Fe(II) myoglobin and the 'myoglobin hydrogen-peroxide' complex classify as essentially pure high and low spin respectively, in agreement with the magnetic susceptibility data [4]. Fe(III) myoglobin, on the other hand, consists of high and low spin species, the latter mainly arising from the presence of ~10% low spin Fe(III) myoglobin hydroxide at pH 8 (calculated using a value of 8.95 for the pK of the acid–alkaline transition for Fe(III) myoglobin [15]).

Thus, the iron atom in the 'myoglobin hydrogen-peroxide' complex may be viewed as a d^4 system in the t_{2g}^4 configuration. The ground state for such axially compressed t_{2g}^4 systems is a $^3A_2$ state. The absence of ESR signals for the 'myoglobin hydrogen-peroxide' complex [5] indicates that spin degeneracy is removed by spin-orbit coupling and that only the lowest state, characterized by a spin singlet $S_Z = 0$, is significantly occupied at low temperatures.

The C(ap) band of high spin Fe(II) and Fe(III) myoglobin and of the low spin 'myoglobin hydrogen-peroxide' complex lies at 1563, 1560 and 1585cm^{-1} respectively. From the results of Huong [12], the wavenumbers of these modes can be used to calculate the Ct–N_p distances, R, using the empirical relationship:

$$R = 4.860 - 0.0018\,\tilde{\nu} \qquad (1)$$

This gives rise to Ct–N_p distances of 2.047, 2.052 and 2.007 Å for high-spin Fe(II) myoglobin, high-spin Fe(III) myoglobin, and the low spin 'myoglobin hydrogen-peroxide' complex, respectively.

Two modes of low wavenumber are also of particular interest. By the use of model compounds and isotopic labelling experiments, Kincaid *et al.* [16] have assigned the polarised bands near 375 and 345 cm^{-1} as Fe–imidazole and Fe–pyrrole stretches respectively. In the case of the former mode, an approximate value for the Fe–N_{im} stretching force constant (k) is related to the wavenumber, $\tilde{\nu}$, by the expression:

$$k = \left(\frac{\tilde{\nu}}{1303}\right)^2 \bigg/ \left(\frac{1}{m_{Fe}} + \frac{1}{m_N}\right) \qquad (2)$$

where m_{Fe} and m_N are the atomic masses of the iron and nitrogen atoms respectively. Use of Badger's modified rule [17] then allows the determination of the change (Δr) in the Fe–N_{im} distance (r), resulting from a change in the force constant from k_1 to k_2 given by:

$$\Delta r = r_1 - r_2 = (k_1^{-1/3} - k_2^{-1/3})(a_{ij} - d_{ij}) \qquad (3)$$

where a_{ij} is the 'standard' bond length and d_{ij} is the distance of closest approach (for a first row atom, N, bonded to a third row atom, Fe, $d_{ij} = 0.85$ Å and $a_{ij} = 2.35$ Å). It should be noted that, although Badger's rule does not yield accurate bond lengths, the errors involved cancel in estimating small changes in distances from wavenumber shifts so that eqn. (3) yields reasonably accurate values for Δr [16, 17]. If the Fe–N_{im} bond length in one of the species is known from X-ray crystallography then the Fe–N_{im} bond length in the other species may be estimated. In the case of the Fe–pyrrole stretch a similar procedure will yield values for the Fe–N_p bond lengths (r'). From the values of R and r', the displacement (d) of the iron atom from the plane of the pyrrole nitrogen atoms (*i.e.* the distance Ct–Fe) may be calculated by triangulation:

$$d = \sqrt{r'^2 - R^2} \qquad (4)$$

The situation is complicated, however, by the fact that the reduced mass is a function of d, since the more out of plane the iron atom is, the more it moves in the Fe–N_p normal coordinate. Hence, using Wilson's method for calculating G matrix elements, the Fe–N_p stretch force constant (k') is given by:

$$k' = \left(\frac{\tilde{\nu}}{1303}\right)^2 \bigg/ \left(\frac{4\sin^2\theta}{m_{Fe}} + \frac{1}{m_N}\right) \qquad (5)$$

where θ is the angle Ct–N_p–Fe. Using a similar expression for $\Delta r'$ as in eqn. (3) and substituting k_1 and k_2 by the expression for k' in eqn. (5), and d/r' for $\sin\theta$, gives

$$r'_1 = r'_2 + \left[\left(\frac{1303}{\tilde{\nu}_1}\right)^{2/3}\left(\frac{(d_1/r'_1)^2 + 1}{14}\right)^{1/3} - \right.$$

$$\left. \left(\frac{1303}{\tilde{\nu}_2}\right)^{2/3}\left(\frac{(d_2/r'_2)^2 + 1}{14}\right)^{1/3}\right](a_{ij} - d_{ij})$$

(6)

Thus, if r'_2 is known, d_2 can be calculated from eqn. (4) and the only unknowns in eqn. (6) are r'_1 and d_1. r'_1 can be calculated assuming $d_1 = 0$ and a better value for d_1 can then be estimated from eqn. (4). From these values of r'_1 and d_1 a new value of r'_1 can be calculated and the calculation repeated by iteration until convergent values of r'_1 and d_1 are obtained.

The results of these calculations are summarised in Table II. The Fe–imidazole stretch lies at 372, 375, and 377 cm^{-1} for Fe(II) myoglobin, Fe(III) myoglobin, and the 'myoglobin hydrogen peroxide' complex. From these values the Fe–N_{im} bond length is 0.00_8 and 0.01_4 Å shorter in Fe(III) myoglobin (2.09_2 Å) and in the 'myoglobin hydrogen-peroxide' complex (2.08_6 Å) respectively, than in Fe(II) myoglobin (2.10_0 Å). The difference in Fe–N_{im} bond lengths between Fe(II) and Fe(III) myoglobin is in agreement with X-ray crystallographic data [18, 19] where no difference in bond length could be detected within the accuracy (± 0.05 Å) of the estimation. If we assume that the force constant for the Fe–N_{im} bond is proportional to the bond energy (which is borne out empirically where data for comparable bonds are available [20]) and use the binding enthalpy (40.2 kJ mol^{-1}) [16] of 1-methylimidazole to Co(II) protoporphyrin IX as a reasonable estimate for the Fe–imidazole bond energy in myoglobin (given that Fe and Co are neighbours and the globin cavity is hydrophobic), then the force constant changes of 0.015 mdyn Å^{-1} and 0.010 mdyn Å^{-1} on going from Fe(II) to Fe(III) myoglobin and from Fe(III) myoglobin to the 'myoglobin hydrogen-peroxide' complex, rexpectively, translate into bond energy changes of 0.6 and 0.4 kJ mol^{-1} respectively.

The Fe–pyrrole stretching frequencies for Fe(III) myoglobin and the 'myoglobin hydrogen-peroxide' complex are 349 cm^{-1} and 346 cm^{-1} respectively. From these values, the Fe–N_p bond lengths in Fe(III) myoglobin (2.03_9 Å) and in the 'myoglobin hydrogen-peroxide' complex (2.08_3 Å) are 0.02_1 Å shorter and 0.02_3 Å longer, respectively, than in Fe(II) myoglobin (2.06_0 Å). We note that the Fe–N_p bond length in Fe(III) myoglobin determined by X-ray crystallography, is 2.04 ± 0.005 Å [18] so that agreement is close.

The displacement of the iron atom from the plane of the pyrrole nitrogen atoms (d) was calculated to be $0.23^{+0.24}_{-0.00}$, $0.00^{+0.35}_{-0.00}$ and $0.56^{+0.14}_{-0.18}$ Å for Fe(II) myoglobin, Fe(III) myoglobin and the 'myoglobin

hydrogen-peroxide' complex respectively. As the Ct–N_p and Fe–N_p distances are each much larger than the Ct–Fe distance, errors in the first two distances will induce relatively large errors in the Ct–Fe distance, which is accordingly not particularly accurate. The value for Fe(II) myoglobin and Fe(III) myoglobin are, however, in reasonable agreement with the values obtained by X-ray crystallography *viz.* 0.42 ± 0.1 and 0.27 ± 0.1 Å [18, 19] respectively.

It is of particular interest that the iron atom in the 'myoglobin hydrogen-peroxide' complex lies significantly out of the plane of the pyrrole-nitrogen atoms, despite the fact that the ionic radius of Fe(IV) is significantly smaller than that of Fe(III) or Fe(II). We suggest the following explanation: EXAFS studies on Fe(II) haemoglobin and the so-called 'picket fence porphyrins' [21] together with high quality *ab initio* calculations including electron correlation (many body) effects [23] have shown that the dominant factor determining the position of the iron atom with respect to the plane of the pyrrole-nitrogen atoms lies in the balance of the repulsive non-bonded interactions between the bond pairs of the proximal imidazole and the nitrogen orbitals of the porphyrin, and the five attractive Fe–N interactions. The former will be very sensitive to ring size, the smaller the ring the larger the effect. The radius of the porphyrin hole, $d(Ct–N_p)$, in the 'myoglobin hydrogen-peroxide' complex is significantly smaller than that of either high spin Fe(II) or Fe(III) myoglobin by 0.04 to 0.05 Å resulting in a significant increase in the non-bonded interactions (see Table II). No significant difference, however, can be detected in the Fe–N_p bond lengths of high spin Fe(II) and Fe(III) myoglobin and the low spin 'myoglobin hydrogen-peroxide' complex. As a result, the iron atom of the 'myoglobin hydrogen-peroxide' complex moves out of the plane of the pyrrole-nitrogen atoms until the repulsive non-bonded interactions are balanced by the five attractive Fe–N interactions.

The ligand in the sixth coordination position of the iron atom in the 'myoglobin hydrogen-peroxide' complex is not known. A likely possibility is OOH$^-$, which should have its associated Fe–O vibrations around 420–580 cm^{-1}, *cf.* for MbOH, νFeO occurs at 490 cm^{-1} [24]. However, we failed to detect any vibrations in the low wavenumber RR spectrum of the 'myoglobin hydrogen-peroxide' complex which were not present in either the Fe(II) or Fe(III) myoglobin RR spectra. This may not be surprising as an Fe–OOH vibration, on account of the relatively high degree of ionic character to the Fe–O bond, would be expected to give rise to only a weak Raman band.

Photoreduction of the 'Myoglobin Hydrogen-Peroxide' Complex by High Intensity Light

Figure 3 shows the effect on the RR spectrum of the 'myoglobin hydrogen-peroxide' complex of 200
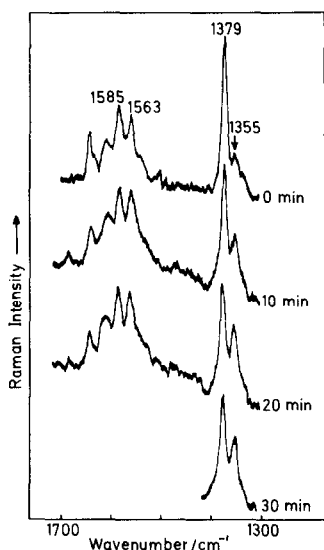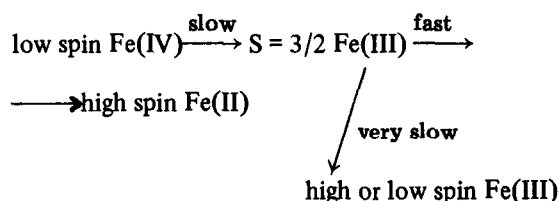
Fig. 3. Soret-excited RR spectra at *ca.* 80 K showing the photo-reduction of the 'myoglobin-hydrogen peroxide' complex to Fe(II) myoglobin at high laser power. Scanning proceeds from right to left and the three spectra were recorded in succession. Note the strong intensity of the C(ap) band at 1563 cm^{-1} characteristic of high spin Fe(II) myoglobin in the first scan, approximately 6 min after illumination. Conditions of RR spectroscopy: power = 200 mW, time constant = 1 s, scanning rate = 50 cm^{-1} min^{-1}, spectral slit width = 8 cm^{-1}, excitation wavelength = 457.9 nm.

mW irradiation at 457.9 nm. It is clearly seen that the intensity of the A(p) band at 1379 cm^{-1}, characteristic of the 'myoglobin hydrogen-peroxide' complex decreases, whereas the intensity of the A(p) band at 1355 cm^{-1}, characteristic of Fe(II) myoglobin, increases. At the same time there is an increase in the C(ap) band at 1563 cm^{-1} characteristic of high spin Fe(II) myoglobin. No evidence for the formation of Fe(III) myoglobin (A(p) band at 1373 cm^{-1}, C(ap) band at 1560 cm^{-1}) could be found. We interpret this as being indicative of conversion of the low-spin 'myoglobin hydrogen-peroxide' complex to high-spin Fe(II) myoglobin. At *ca.* 80 K this process, as monitored by the decrease in intensity of the 1379 cm^{-1} or the increase in intensity of the 1355 cm^{-1} band, proceeds with a rate constant of ~2.5 × 10^{-4} s^{-1} at a laser power of 200 mW. At laser powers below 20 mW photoreduction is minimal. On the basis of our data, we cannot determine whether photoreduction proceeds by a simultaneous two-electron reduction or by two sequential single-electron transfer steps. In the latter case, the first electron-transfer step must be rate limiting as we can find no direct evidence for Fe(III) myoglobin.

A possible explanation for the above findings may be as follows. The configuration of the t_{2g} sub-shell remains unchanged in low spin Fe(IV) (t_{2g}^4), high spin Fe(II) ($t_{2g}^4 e_g^2$) and intermediate (S = 3/2) spin Fe(III) ($t_{2g}^4 e_g^1$). On the other hand, the configuration of the t_{2g} subshell in either high ($t_{2g}^3 e_g^2$) — or low (t_{2g}^5) — spin Fe(III) is different from that in low spin Fe(IV). Therefore, if two sequential electron-transfer steps are involved, the intermediate species is likely to be intermediate (S = 3/2) spin Fe(III) which is unstable and would therefore be rapidly reduced to high spin Fe(II). This can be represented by the following scheme:

$$\text{low spin Fe(IV)} \xrightarrow{\text{slow}} S = 3/2 \text{ Fe(III)} \xrightarrow{\text{fast}}$$

$$\longrightarrow \text{high spin Fe(II)}$$

$$\text{very slow} \searrow$$

$$\text{high or low spin Fe(III)}$$

The mechanism of photoreduction of the 'myoglobin hydrogen-peroxide' complex is not known but it may involve electron abstraction either from the porphyrin or the axial ligands. Of the two possibilities, we favour the latter, since electron abstraction from the porphyrin would be expected to result in changes in the wavenumbers of the porphyrins ring modes; this however, is not observed.

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