Resonance Raman spectroscopy shows different temperature-dependent coordination equilibria for native horseradish and cytochrome *c* peroxidase

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Resonance Raman spectra are reported for native horseradish peroxidase (HRP) and cytochrome *c* peroxidase (CCP) at 290, 77 and 9 K, using 406.7 nm excitation, in resonance with the Soret electronic transition. The spectra reveal temperature-dependent equilibria involving changes in coordination or spin state. At 290 K and pH 6.5, CCP contains a mixture of 5- and 6-coordinate high-spin Fe^{III} heme while at 9 K the equilibrium is shifted entirely to the 6-coordinate species. The spectra indicate weak binding of H_2O to the heme Fe, consistent with the long distance, 2.4 Å, seen in the crystal structure. At 290 K HRP also contains a mixture of high-spin Fe^{III} hemes with the 5-coordinate form predominant. At low temperature, a small 6-coordinate high-spin component remains but the 5-coordinate high-spin spectrum is replaced by another which is characteristic either of 6-coordinate low-spin or 5-coordinate intermediate spin heme. The latter species is definitely indicated by previous EPR studies at low temperature. This behavior implies that, in contrast to CCP, the distal coordination site of HRP is only partially occupied by H_2O at any temperature and that lowering the temperature significantly weakens the Fe-proximal imidazole bond. Consistent with this inference, the 77 K spectrum of reduced HRP shows an appreciable fraction of molecules having an Fe-imidazole stretching frequency of 222 cm^{-1}, a value indicating weakened H-bonding of the proximal imidazole.

Resonance Raman spectroscopy *Horseradish peroxidase* *Cytochrome c peroxidase* *Coordination equilibrium*

1. INTRODUCTION

Resonance Raman (RR) spectroscopy has proved to be a useful probe of the heme ligation and spin state in heme proteins [1]. An early puzzle uncovered by the technique concerned the clear spectral difference between methemoglobin (metHb) and native horseradish peroxidase (HRP) [2], both of which are high-spin Fe^{III}-containing heme proteins with proximal histidine ligands. It was subsequently recognized [3] that the metHb spectrum was assignable to a 6-coordinate high-spin heme while that of HRP corresponded to a 5-coordinate high-spin heme. Other spectroscopic characteristics [4,5] confirm the absence of a water molecule firmly bound to the heme of HRP, in contrast to metHb. On the other hand, cytochrome *c* peroxidase (CCP) has also been suggested to contain 5-coordinate heme on the basis of its RR spectrum [6] though the X-ray crystal structure does show a water molecule on the distal side of the heme group [7]. The H_2O-Fe distance, 2.4 Å [8], is, however, appreciably longer than in metHb (2.0 Å) [9]. The energetics of H_2O coordination to the Fe is relevant to the peroxidase mechanism. Fe^{III}-bound H_2O is the final product of substrate reduction of the high-valent peroxidase intermediates and must be displaced by peroxide in the catalytic cycle.

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To examine the nature of the distal water interaction, we have recorded RR spectra for both HRP and CCP at ambient and low temperatures. Consistent with weak binding of H_2O, CCP shows a mixture of 5- and 6-coordinate high-spin hemes at 290 K, with the 6-coordinate form becoming dominant at low temperature. HRP also shows a small fraction of 6-coordinate high-spin heme at 290 K, but this fraction does not increase at low temperature. The dominant 5-coordinate high-spin spectrum is replaced, at low temperature, by one characteristic of a 5-coordinate intermediate-spin heme, consistent with previously reported EPR data [10]. Since the intermediate spin state becomes stable when the axial ligand field is weak, this transition implies a weakening of the Fe-proximal histidine bond at low temperature in HRP. This inference has been confirmed for reduced HRP which shows an increasing population of heme with a lowered frequency for the Fe-imidazole stretching mode.

2. EXPERIMENTAL

HRP (Sigma, type VI) was used without further purification. CCP was purified and crystallized from pressed baker's yeast as described [11]. The proteins were dissolved in 0.1 M sodium phosphate buffer of the desired pH. Fe^{II} protein was prepared by adding a minimal amount of a sodium dithionite solution to a deoxygenated sample. Low-temperature samples were obtained as frozen solution in phosphate buffer (Fe^{III}-CCP and Fe^{III}-HRP) or in glycerol/buffer (75%/25%) glasses (Fe^{II}-HRP).

RR spectra were obtained with the 406.7 nm line of a Kr^+ laser (Spectra Physics 171) via backscattering from a slowly rotating NMR tube at room temperature (290 K) or off the surface of a frozen protein solution held in vacuo on a liquid N_2-cooled cold finger (77 K) [12]. The 9 K spectra were obtained using an Air Products model 202 liquid helium cryogenic system. The spectrometer consisted of a Spex 1401 double monochromator fitted with a cooled RCA 31034 photomultiplier. Spectra were collected using photon counting electronics and an MINC (DEC) computer. Band positions were calibrated using the 1000 cm^{-1} band of toluene (high frequency), or the 459 cm^{-1} band of CCl_4 (low frequency), and are accurate to $\pm$ 1 cm.

3. RESULTS AND DISCUSSION

Figs 1 and 2 show RR spectra of aqueous samples of native CCP and HRP at 290, 77 and 9 K. (The temperatures are nominal; local heating by the laser beam no doubt increases the temperature of the frozen samples appreciably.) The samples were prepared at pH 6.0 (HRP) and 6.5 (CCP) in 0.1 M phosphate buffer, but similar spectra were obtained over the range pH 3.0–7.0 for HRP and 6.0–7.0 for CCP. The spectral region above 1450 cm^{-1} contains porphyrin skeletal modes whose frequencies are sensitive to the porphyrin core size [3,13] which is influenced by the oxidation, ligation and spin states. Particularly useful as a diagnostic marker is the ν_3 band at 1470–1515 cm^{-1}, which falls in a clear region of the spectrum.

The 290 K CCP spectrum (fig.1) shows 2 distant ν_3 bands at 1477 and 1493 cm^{-1} that are characteristic of 6- and 5-coordinate high-spin Fe^{III} hemes, respectively [3,13]. As the temperature is lowered, the 1493 cm^{-1} band diminishes while the 1477 cm^{-1} band increases. At 9 K, the

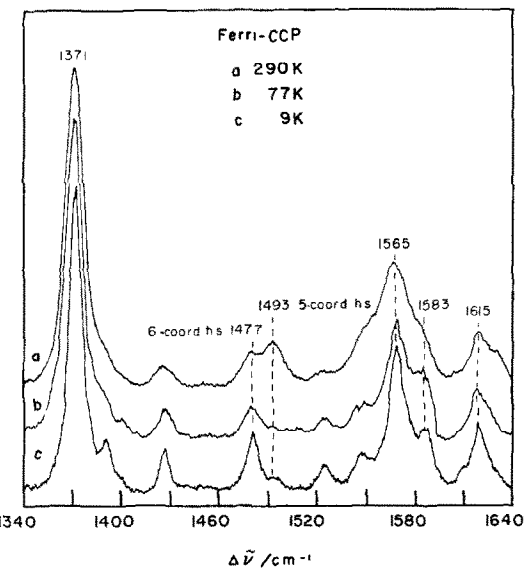

Fig.1. 406.7 nm-excited Raman spectra of CCP (1 mM) in 0.1 M phosphate buffer, pH 6, in liquid (a) 290 K and frozen solution; (b) 77 K; (c) 9 K nominal temperatures of the probe. Conditions: 50 mW laser power at the sample, 5 cm^{-1} spectral slitwidth, 3 s/0.5 cm^{-1} accumulation intervals.

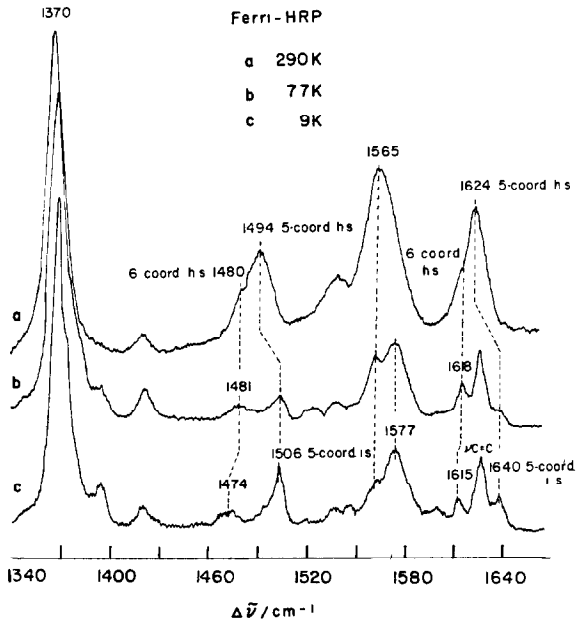

Fig.2. As fig.1, but for HRP.

spectrum shows skeletal mode frequencies which are all characteristic of 6-coordinate high-spin Fe^{III} heme [12], as seen in the comparison shown in table 1 with the frequencies observed for $Fe^{III}PP(DMSO)_2^+$ (PP, protoporphyrin IX; DMSO, dimethyl sulfoxide). The 290 K spectrum shows considerable broadening in the region above 1520 cm^{-1}, due to overlapping contributions from 5- and 6-coordinate hemes; the expected frequencies for the former are shown in table 1 for $Fe^{III}PP(Cl)$. In view of this clear evidence for a mixture of 5- and 6-coordinate species, we take the 2.4 Å of the H_2O–Fe distance in the CCP crystal structure [8] as representing an average over bound and unbound water molecules, with expected distances to Fe of ~2.0 and ~3.0 Å. At low temperature, the bound water is stabilized and the 6-coordinate heme is the dominant species. The distal water may be weakly H-bonded to the distal histidine [8] but the geometry is evidently not optimal for simultaneous coordination to the Fe atom. When fluoride binds, the heme is 6-coordinate at room temperature [2,3]; a distal arginine residue moves into position to H-bond to the bound ligand [14]. Other ligands, including peroxide, may likewise induce motion of the arginine side chain, which is suggested to participate in the heterolysis of the peroxide O–O bond [14].

The situation for HRP is different. Its 290 K spectrum (fig.2) is characteristic of mainly 5-coordinate high-spin heme, as reported in [3]. There is, however, a detectable 6-coordinate high-spin component giving rise to low-frequency shoulders on the ν_4 and ν_{10} bands (1480 and 1618 cm^{-1}). At low temperature, the 6-coordinate bands persist and appear to shift slightly to lower frequencies, but the 6-coordinate population does not increase, as it does for CCP. However, the 5-coordinate high-spin spectrum is replaced by another spectrum with distinctly higher frequencies for ν_3 (1506 cm^{-1}), ν_2 (1577 cm^{-1}) and ν_{10} (1640 cm^{-1}). (Note that a band remains at 1624 cm^{-1} due to the protoheme vinyl C=C stretch, which is accidentally coincident with ν_{10} in the 5-coordinate high-spin spectrum [13].) These frequencies are characteristic either of 6-coordinate low-spin or 5-coordinate intermediate-spin hemes, as shown by the comparisons in table 1. The 1375 cm^{-1} shoulder on the ν_4 band is best interpreted as an intermediate spin contribution, in view of the distinctly elevated ν_4 frequency shown by such species (table 1 and [15]). EPR spectra of frozen HRP solutions definitely show a mixture of high- and intermediate-spin hemes [10], with negligible low-spin species, provided the solution pH is below the alkaline transition. Consequently, assignment of the high-frequency RR bands to a low-spin heme is precluded. For CCP, however, the low-temperature EPR spectra are purely high-spin in character [16,17] (at solution pH values below the alkaline transition [18]), consistent with the present RR data.

One possible explanation for these differences between CCP and HRP is that CCP has a tryptophan adjacent to the iron-linked water molecule while HRP has a phenylalanine [7]. The indole ring nitrogen of the tryptophan in CCP denotes a hydrogen bond to its axial water ligand in CCP [8] which could further stabilize the 6-coordinate form of CCP at low temperature relative to HRP.

Structural and magnetic studies of iron porphyrins have shown that the intermediate spin state is attained when the axial ligands are absent (e.g. $Fe^{II}TPP$ [19]) or exert a weakly ligand field (e.g. $Fe^{III}OEP(ClO_4)$ [20], $Fe^{III}PPP(ClO_4)$ [21]). In this situation the axial Fe orbital, d_{z^2}, is stabilized suf-

Table 1

Porphyrin skeletal mode frequencies (cm^{-1}) for peroxidase and model Fe^{III} hemes in different coordination and spin states

Mode[a]	ν_4	ν_3	ν_{11}	ν_2	ν_{37}	ν_{10}
6-coordinate high-spin						
$Fe^{III}PP(DMSO)_2^{+}$[b]	1370	1480	1545	1560	1580	1610
CCP (9 K)	1371	1477	1545	1565	1583	1615
HRP (9 K)	1372	1474	–	1565	–	1615
5-coordinate high-spin						
$Fe^{III}PP(Cl)$[b]	1373	1491	1553	1570	1591	1626
CCP (290 K)	1371	1493	–	–	–	1625
HRP (290 K)	1370	1494	1555	–	–	1624
5-coordinate intermediate spin						
$Fe^{III}OEP(SbF_6)$[c]	1377	1513	1558	1581	–	1646
HRP (9 K)	1375	1506	–	1577	–	1640
6-coordinate low-spin						
$Fe^{III}PP(ImH)_2^{+}$[b]	1373	1502	1562	1579	1602	1640

[a] See [13]

[b] PP, protoporphyrin IX; DMSO, dimethyl sulfoxide; ImH, imidazole; data from [13]

[c] OEP, octaethyl porphyrin; data from [14]

ficiently that the Fe valence electrons distribute themselves among the three d_π and the d_{z2} orbitals, leaving only the d_{x2-y2} orbital empty. The emptying of this in-plane orbital contracts the Fe–N(pyrrole) bonds and shifts the porphyrin skeletal frequencies to the neighborhood of those shown by low-spin hemes [3,15]. The high-frequency skeletal modes of the 9 K HRP spectrum likewise imply short Fe–N(pyrrole) bonds for the dominant heme species and the intermediate-spin state established by EPR [10] requires that the axial field be weak. This is a surprising conclusion inasmuch as the axial bond to the proximal imidazole is expected to be strong, especially as the proximal imidazole is partially imidazolate in character due to strong H-bonding, seen for CCP in the crystal structure [8], and detected for HRP via NMR [22] and RR [23–25] spectroscopy in the reduced state.

We find, however, that the Fe^{II} HRP RR spectrum at low temperature gives evidence for an increasing fraction of heme with a weakened Fe–imidazole bond as shown in fig.3. At 77 K, there is a prominent band at 222 cm^{-1}, along with the 243 cm^{-1} band which has been assigned to Fe-imidazole stretching for strongly H-bonded imidazole [23–25]. The 222 cm^{-1} band is not the sharp 229 cm^{-1} ice band seen in dilute frozen solution [12]. The ice band can be suppressed by raising the concentration of the (light-absorbing) protein or by adding glycerol to form a glass; either method produced the spectrum shown in fig.3. The 222 cm^{-1} band frequency is that seen for the Fe-imidazole mode of myoglobin [26] in which the proximal imidazole is only weakly H-bonded to a backbone carbony [9]. At 290 K, the RR spectrum shows a small 222 cm^{-1} feature; its growth in the 77 K spectrum parallels the increasing fraction of intermediate spin heme in the Fe^{III} protein. We infer that the lowering of the temperature induces a weakening of the Fe–proximal imidazole bond for an increasing fraction of the HRP molecules. The likeliest mechanism is loss of the strong H-bonding, perhaps due to a displacement away from the heme of the H-bond acceptor residue. Whether weakly H-bonded imidazole exerts a sufficiently small intrinsic axial field to produce an

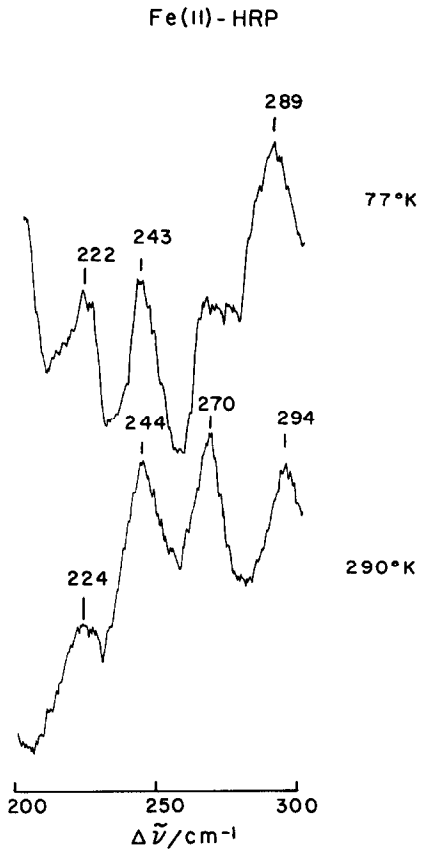

Fig.3. 413.1 nm-excited low-frequency Raman spectra for Fe^{II}HRP (1.5 mM) in 0.1 M phosphate buffer, pH 7, at 290 K (lower spectrum), and 77 K taken in a 4:1 mixture of glycerol and buffer (upper spectrum). Conditions as in fig.1, except 15 s/0.5 cm^{-1} accumulation intervals.

intermediate-spin state in a 5-coordinate Fe^{III} heme is uncertain, no model compound having been tested in this regard, as far as we are aware. It is relevant, however, that cytochrome *c'*, a 5-coordinate heme protein with its proximal imidazole exposed to solvent water [27], gives an intermediate-spin Fe^{III} state at low pH [28–30]. Aside from imidazole H-bonding, it is possible that protein steric forces weaken the axial bond in both cytochrome *c'* and HRP. A weak axial field is not operative in CCP, however, indicating a distinct difference in the heme-linked conformations available to the 2 peroxidases.

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