Benzohydroxamic Acid−Peroxidase Complexes: Spectroscopic Characterization of a Novel Heme Spin Species

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Abstract: A distinctive characteristic of the class III heme peroxidases of the plant peroxidase superfamily is the presence of a pentacoordinate quantum mechanically mixed-spin heme state resulting from the admixture of $S = {}^5/_2$ and $S = {}^3/_2$ states. This is not observed in class I or II peroxidases and, in fact, is a very rare heme spin state. The corresponding hexacoordinate quantum mechanically mixed-spin state is even more uncommon and has not been observed in heme proteins. The presence of the pentacoordinate form in the class III peroxidases suggested that they could be ideal candidates to display also the six-coordinate quantum mechanically mixed-spin state. With this possibility in mind, the benzohydroxamic acid complexes of the class III peroxidases horseradish isoenzyme C and A2 and soybean peroxidase are studied by electronic absorption, resonance Raman, and EPR spectroscopy at room and low temperatures. The results are compared with those obtained for *Coprinus cinereus* peroxidase which belongs to class II. The binding of benzohydroxamic acid to horseradish peroxidase A2 and soybean peroxidase is found to be 400- and 500- fold weaker, respectively, than that to horseradish peroxidase C. The data show that the binding of benzohydroxamic acid induces the formation of a six-coordinate heme and that the complex is not appreciably altered by lowering the temperature. The *C. cinereus* peroxidase−benzohydroxamic acid complex shows resonance Raman and EPR spectra which are readily identified as being due to six-coordinate high-spin heme, whereas the benzohydroxamic acid complexes with the other peroxidases are characterized by Raman core size marker bands at higher frequencies than expected for six-coordinate high spin. The magnitude of the increase in frequency of the resonance Raman bands follows the order horseradish peroxidase C, soybean peroxidase, to horseradish peroxidase A2, and, concomitantly, the $g_\perp$ EPR value decreases in the same order. It is proposed that these spectroscopic features are characteristic of a six-coordinate form in a quantum mechanically mixed-spin state, with an increasing contribution from an $S = {}^3/_2$ state in the same order as the increasing Raman frequencies.

Introduction

Peroxidases are heme-containing enzymes which catalyze the one-electron oxidation of aromatic substrates by hydrogen peroxide. The binding of a nonphysiological substrate, such as benzohydroxamic acid (BHA), to horseradish peroxidase isoenzyme C (HRPC) has been extensively investigated by various spectroscopic methods[1−11] at both room and low temperatures with the aim of probing the aromatic donor binding site. Recently, the X-ray crystal structure of the complex has been solved.[12] It showed that BHA binds in the distal cavity and revealed a water molecule located at about 2.6 Å from the iron atom. Resonance Raman (RR) spectra on the single crystal and the solution demonstrated that the water molecule was bound to the iron atom, giving rise to a six-coordinate heme.[11] The same conclusion was drawn for the complex between *Arthromyces ramosus* peroxidase (ARP) (a peroxidase essentially identical to *Coprinus cinereus* peroxidase, CIP) and BHA in solution,[11] whose crystal structure revealed the presence of a water molecule at 2.7 Å from the iron atom.[13]

Plant, fungal, and bacterial peroxidases are evolutionarily related and constitute the plant peroxidase superfamily of enzymes, which can be divided into three classes on the basis of their structural divergences.[14] Class I peroxidases are intracellular and are of prokaryotic origin. These include chloroplast and cytosol ascorbate peroxidases and yeast mitochondrial cytochrome *c* peroxidase (CCP). Class II comprises extracellular fungal peroxidases such as CIP and lignin degrading peroxidases. Class III contains a great variety of secretory plant peroxidases, typified by the classical HRPC. Recent studies on

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class III peroxidases have revealed the presence of a hitherto unusual spin state in heme proteins, a pentacoordinate quantum mechanically mixed-spin (5-c QS) species.[15−19] This spin state, which results from a quantum mechanical admixture of intermediate-spin ($S = {}^3/_2$) (IS) and high-spin ($S = {}^5/_2$) (HS) states, has not been noted for class I or class II peroxidases. This interesting observation prompted us to exploit the formation of a hexacoordinate heme in peroxidases upon BHA binding to determine whether this distinctive feature of pentacoordinate class III peroxidases is maintained in the hexacoordinate form, with the generation of a hexacoordinate quantum mechanically mixed-spin (6-c QS) state. The 6-c QS state has never been observed in a heme protein and, to our knowledge, no structure of a 6-c QS complex has been solved. The detection of such a spin state in class III peroxidases, but not in those of class I and II, would offer the opportunity to investigate further the structural and electronic factors which govern the occurrence of the QS state that remain unclear at present.[17]

The application of various complementary spectroscopic techniques is highly desirable if a rigorous investigation is to be obtained. In particular, EPR spectroscopy at low temperature has been crucial for the identification of the quantum mechanically mixed-spin state in heme proteins.[20] On the other hand, it is important to have a direct comparison between EPR and measurements made with other techniques carried out under physiological conditions at room temperature. In this context, optical spectroscopy is a very appropriate tool as one is able to carry out experiments in the entire range from room to liquid helium temperatures. Moreover, an aspect of the spectroscopic study of proteins, which at times has been underestimated, is the potential offered by the use of low temperatures to gain further information. In regard to the present work, advantages of low temperature may be illustrated by the narrowing of the spectral bands and the identification of temperature-dependent conformational changes.[21]

In this work we study the complexes between horseradish peroxidase isoenzyme A2 (HRPA2) and soybean peroxidase (SBP) with BHA at both room and low temperatures by electronic absorption, RR, and EPR spectroscopy. The results are compared with those obtained for the corresponding BHA complexes formed with HRPC and CIP. The peculiar spectroscopic features exhibited by the BHA complexes of class III peroxidases enable us to conclude that they are characteristic of six-coordinate forms in a quantum mechanically mixed-spin state, with an increasing contribution from an $S = {}^3/_2$ state following the order HRPC to SBP to HRPA2.

Experimental Section

Sample Preparation. HRPA2 and HRPC were purchased from Biozyme, UK, batch 898 DX (Reinheitszahl, RZ = 3.75) and batch S35A (RZ = 3.1), respectively, in freeze-dried form. The proteins were used without further purification and were dissolved in the appropriate buffer before use. SBP from soybean seed coat was purchased from Enzymol International (Ohio, USA) (RZ = 1.45) and purified as previously described (RZ = 3.4).[16] Recombinant CIP was obtained by expression in transformed *Aspergillus oryzae*[22] and purified as previously reported.[23]

The buffers were either 0.1 M bicine at pH 7.5 or 0.1 M phosphate at pH 7.0. Only bicine buffer was used for the solutions of the free protein at low temperature. The concentrations of the proteins in the resting state were calculated from the absorbance using the following extinction coefficients: SBP, 90 mM^{-1} cm^{-1} at 403 nm;[16] CIP, 109 mM^{-1} cm^{-1} at 405 nm;[24] HRPC, 102 mM^{-1} cm^{-1} at 403 nm;[25] HRPA2, 107 mM^{-1} cm^{-1} at 404 nm.[26] Protein concentrations for the electronic absorption and RR spectra at low temperature were in the range 14−150 μM and 180−200 μM, respectively. The concentration range was 4−150 μM for both the electronic absorption and RR room temperature measurements. The concentration of all the samples for the EPR measurements was 100 μM. The complexes with benzohydroxamic acid (BHA, Sigma) were made by adding BHA to give a final concentration corresponding to ca. 90% protein saturation. The dissociation constants, K_d, were measured following the procedure of Smith and co-workers.[27,28] The UV−vis spectra were recorded using a quartz cuvette (1 cm optical path). Titrations were carried out at 23° C by making successive additions of a BHA stock solution to a solution of the enzyme (4−7 μM in 10 mM MOPS at pH 7.0, 2.5 mL) to a final BHA concentration of 8−13 mM for HRPA2, CIP, and SBP, and 45 μM for HRPC. The dissociation constants of the BHA complexes were determined by fitting the data using a weighted least-squares error minimization procedure.[27,28] All data were corrected for the dilution upon substrate addition.

Spectroscopy. The absorption spectra were recorded with a Cary 5 spectrophotometer. The RR spectra in solution were obtained with excitation from the 406.7 nm line of a Kr^+ laser (Coherent) and the 496.5 nm line of an Ar^+ laser (Coherent, Innova/5). The backscattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG2S) equipped with a cooled photomultiplier (RCA C31034 A) and photon-counting electronics. Polarized spectra were obtained by inserting a polaroid analyzer between the sample and the entrance slit of the spectrometer. The depolarization ratios of the bands at 314 and 460 cm^{-1} of CCl_4 were measured to check the reliability of the polarization measurements. The values obtained, 0.73 and 0.0, compare favorably with the theoretical values, 0.75 and 0.0, respectively. To minimize the heating effects induced on the protein by the laser beam, the room temperature RR spectra were collected using a rotating NMR tube cooled by a gentle flow of N_2 gas passed through liquid N_2.

The low temperature experiments were carried out using a closed-cycle cryostat with automatic temperature control. The absorption and RR spectra were obtained using a quartz microcuvette (100 μL, 0.3 cm optical path). The cuvette was mounted on the cold finger of the cryostat and the protein solution carefully added at 90 K under nitrogen. Identical RR spectra were obtained when droplets, deposited on the cold finger at 90 K, were used instead of the microcuvette. The temperature was then lowered to 12 K under vacuum.

The RR spectra were calibrated with indene as standard for the high-frequency region, and with indene and CCl_4 for the low-frequency region. The frequencies were accurate to ± 1 cm^{-1} for the intense isolated bands, and to about ± 2 cm^{-1} for overlapped bands or shoulders.

EPR spectra were recorded on a Bruker ER 200D-SRC instrument (John Innes Centre, Norwich, UK), equipped with an NMR gaussmeter

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Table 1. Binding of Benzohydroxamic Acid to Resting State Peroxidases

peroxidase	benzohydroxamic acid apparent dissociation constant (K_d) (μM) previous work	present work
HRPC	2.1 ± 0.1[28]	2.5 ± 0.3
HRPA2	2480[31]	930 ± 156
SBP		1343 ± 170
ARP/CIP	3717 ± 213[28, 13]	3535 ± 35

and a microwave frequency counter. An Oxford Instruments ESR 900 cryostat was used to obtain low temperatures. The spectra were recorded under nonsaturating conditions at 5 K, 9.35 GHz microwave frequency, 1 mW microwave power, and 1 mT modulation amplitude. All samples were frozen rapidly by immersion of the EPR tube into isopentane cooled in liquid nitrogen. The EPR simulation program used to determine the g values has been reported previously,[29] and is appropriate for effective $S = {}^1/_2$ systems without g-strain. While simulation programs accounting for g-strain have been applied to spectra of $S_{eff} = {}^1/_2$ states in hemoproteins,[30] to our knowledge one which can provide a complete description of a QS system with g-strain has not been written yet.

Results

Benzohydroxamic Acid Binding. Table 1 reports the benzohydroxamic acid dissociation constants (K_d) for the peroxidases under investigation. BHA binds to HRPA2, SBP, and ARP/CIP approximately 400-, 500-, and 1500-fold more weakly than to HRPC. It should be noted that a previous measurement[31] of the K_d for HRPA2 gave a value about 2.5 times larger than that found from the present study.

Spectroscopic Studies. Figure 1 shows the electronic absorption spectra of CIP, HRPC, SBP, and HRPA2, complexed with BHA at pH 7.0. Prior to complexation all the proteins are mainly five-coordinate, with the Soret maximum at about 402−403 nm, a shoulder at about 385 nm, and the CT1 bands at 635 (SBP),[16] 639 (HRPA2),[15] 643 (HRPC),[11] and 649 nm (CIP).[23] Upon addition of BHA the electronic absorption spectra show marked changes. The Soret band red-shifts and sharpens with an increase of the extinction coefficient by about 35%, the shoulder at lower wavelength disappears, and the CT1 band is in the range between 636 (SBP) and 639 nm (HRPC). All these changes are indicative of the formation of a six-coordinate species.[32]

Figure 2 compares the corresponding RR spectra of the BHA complexes taken with Soret excitation. In agreement with the apparent dissociation constants (Table 1), only HRPC binds the substrate completely at relatively low BHA concentration. The bands due to the unbound protein are indicated with an asterisk.

The core size marker band frequencies of the CIP−BHA complex have been previously shown to be 1485 (ν_3), 1542 (ν_{11}), 1559 (ν_{19}), 1563 (ν_2), and 1615 cm^{-1} (ν_{10}),[11] which are typical of 6-c HS hemes. The other three peroxidases show the presence of two species: one characterized by a weak ν_3 at 1485 cm^{-1} (the other core size marker bands of this species cannot be clearly assigned due to overlap with other bands), as seen for CIP, and the other species characterized by a ν_3 which appears at a progressively higher frequency in the order HRPC (1491 cm^{-1}), SBP (1493 cm^{-1}), HRPA2 (1495 cm^{-1}). The ν_2 band follows the same trend, with a frequency of 1566 cm^{-1} in HRPC and 1568 cm^{-1} in HRPA2. In SBP this band is very broad and centered at 1571 cm^{-1} due to overlap with the ν_2 of the unbound protein at 1575 cm^{-1} (see Figure 2). After subtracting this contribution the band appeared at about 1569 cm^{-1} (data not shown). In the region between 1600 and 1650 cm^{-1} it can be seen that the two ν(C=C) stretching modes overlap at 1624 cm^{-1} in CIP, but give rise to two bands in the other three peroxidases. On the basis of the spectra taken in polarized light (data not shown) they are assigned at 1624 and

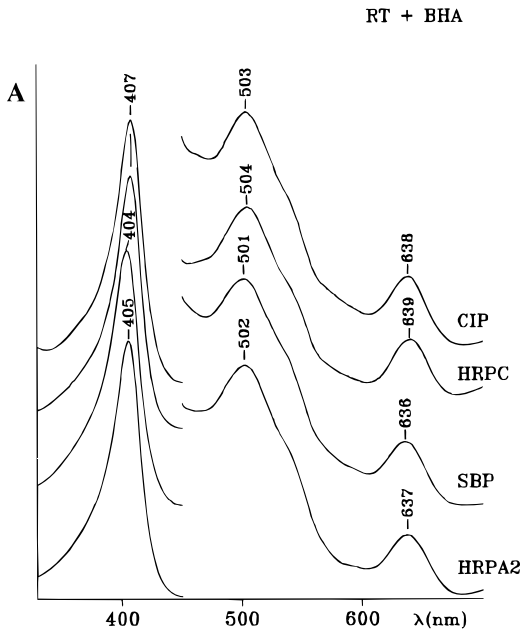

Figure 1. Electronic absorption spectra of CIP−BHA (7.1 μM CIP), HRPC−BHA (4.7 μM HRPC), SBP−BHA (12 μM SBP), and HRPA2 (7.5 μM HRPA2) at room temperature. The spectra have been scaled arbitrarily and the region between 450 and 700 nm has been expanded 8-fold with respect to the Soret band.

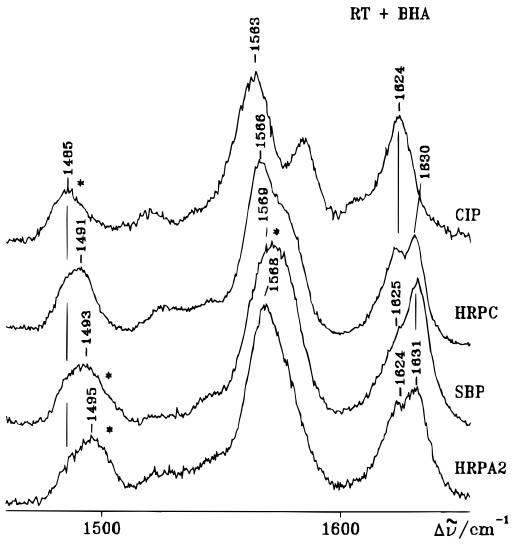

Figure 2. Resonance Raman spectra of CIP−BHA, HRPC−BHA, SBP−BHA, and HRPA2-BHA at room temperature. Experimental conditions: 5 cm^{-1} spectral resolution; 406.7 nm excitation. CIP: 10 mW laser power at the sample, 6 s/0.5 cm^{-1} accumulation time. HRPC: 30 mW laser power at the sample, 4 s/0.5 cm^{-1} accumulation time. SBP: 15 mW laser power at the sample, 10 s/0.5 cm^{-1} accumulation time. HRPA2: 10 mW laser power at the sample, 8 s/0.5 cm^{-1} accumulation time. The asterisk indicates the noncomplexed protein.

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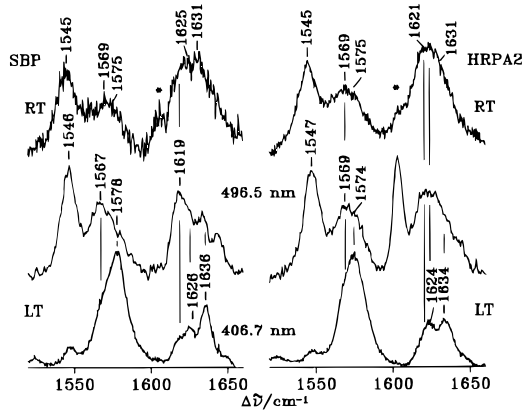

Figure 3. Resonance Raman spectra of SBP−BHA (left) and HRPA2−BHA (right) complexes at room temperature (RT) and 12 K (LT). Experimental conditions: 5 cm^{-1} spectral resolution. SBP: RT, 496.5 nm excitation, 50 mW laser power at the sample, 12 s/0.5 cm^{-1} accumulation time; LT, 496.5 nm excitation, 90 mW laser power at the sample, 30 s/0.5 cm^{-1} accumulation time; LT, 406.7 nm excitation, 20 mW laser power at the sample, 3 s/0.5 cm^{-1} accumulation time. HRPA2: RT, 496.5 nm excitation, 70 mW laser power at the sample, 25 s/0.5 cm^{-1} accumulation time; LT, 496.5 nm excitation, 100 mW laser power at the sample, 20 s/0.5 cm^{-1} accumulation time; LT, 406.7 nm excitation, 20 mW laser power at the sample, 4 s/0.5 cm^{-1} accumulation time. The asterisk indicates the band due to BHA.

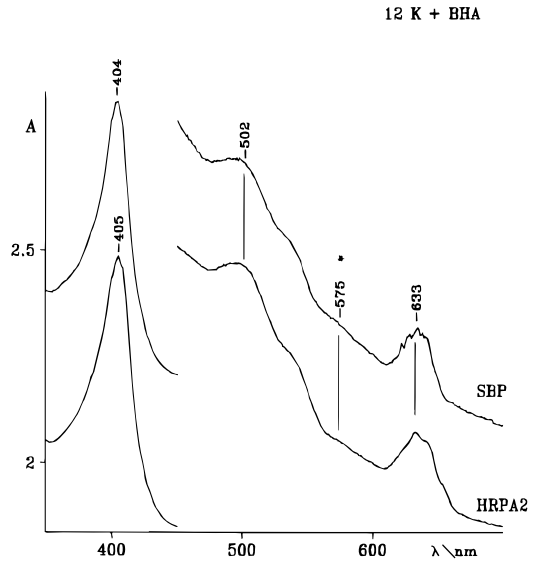

Figure 4. Electronic absorption spectra of SBP−BHA (7.1 μM SBP) and HRPA2−BHA (7.1 μM HRPA2) at 12 K. An offset has been subtracted from the spectra and the region between 450 and 700 nm has been expanded 8-fold with respect to the Soret. The asterisk indicates a 6-c LS heme resulting from the unbound protein.[18,19]

1630 cm^{-1} in HRPC,[7] at 1625 and 1631 cm^{-1} in SBP, and at 1624 and 1631 in HRPA2.

Figure 3 (top) shows the spectra of SBP−BHA (left) and HRPA2-BHA (right) obtained with visible excitation at room temperature. The spectra in polarized light (data not shown) indicate that the band at 1569 cm^{-1} is inversely polarized and, therefore, is assigned to the ν_{19} mode. Moreover, in the 1600−1650 cm^{-1} region a depolarized band at 1619 (SBP) and 1621 cm^{-1} (HRPA2) is observed. This band is assigned to the ν_{10} mode. A polarized band is also observed at 1631 cm^{-1} in both SBP and HRPA2, due to the ν(C═C) stretching mode of the vinyl groups which are still Raman active with this excitation, as previously observed for other peroxidases.[7,15,16,23,33,34]

It has been previously shown[7] that upon lowering the temperature of the HRPC−BHA complex, the shoulder at 1485 cm^{-1} disappeared, giving rise to a RR spectrum characterized by a sharp ν_3 at 1491 cm^{-1}, but no other major changes were detected in the other core size marker bands. Thus, one may infer that the spin and coordination states of the Fe in the HRPC−BHA complex are temperature independent,[7] in agreement also with the recent finding that the electronic absorption spectrum of the HRPC−BHA complex is almost identical at both 296 and 80 K.[11] Therefore, we undertook a detailed analysis of HRPA2 and SBP complexed with BHA at low temperature to take advantage of the consequent sharpening of the bands, which facilitates the assignment of the core size marker bands.

Figure 4 shows the electronic absorption spectra of the SBP− and HRPA2−BHA complexes obtained at 12 K. It can be seen that whereas the $\pi\rightarrow\pi^*$ transitions are not affected by the lower temperature, giving rise to Soret and Q-bands very similar to those obtained at room temperature (Figure 1), the CT1 band is markedly affected. In both complexes it blue-shifts by 3−4 nm. Moreover, in the HRPA2−BHA complex the band shows a fine structure.

Figure 5 shows the high-frequency region RR spectra of the complexed peroxidases at room and low temperatures obtained with Soret excitation. The spectra of the BHA complexes are compared with those of the free proteins, which enables an easier assignment of the bands due to the noncomplexed heme. It can be seen that at low temperature the spectrum of the unbound protein should not interfere with the bands due to the complex, as judged from the relative intensity of the weak ν_3 at 1511 (HRPA2) and 1513 cm^{-1} (SBP) which in the free protein is very intense.[18,19] For both proteins, the decrease in temperature causes the disappearance of the shoulder at 1485 cm^{-1}, as previously observed for HRPC.[7] Concomitantly, the band due to the ν_2 mode sharpens and upshifts to 1578 (SBP−BHA) and 1574 cm^{-1} (HRPA2-BHA). The ν_{37} mode, expected in this region, is not clearly evident. This band is probably overlapped with the ν_2 mode. In fact, in the HRPC−BHA complex, lowering the temperature to 12 K causes this band to upshift from 1574 and 1579 cm^{-1} accompanied by a marked increase in intensity.[7] In the region of the vinyl stretching modes major changes are observed. In particular, whereas the vinyl mode at lower frequency does not change appreciably its frequency, the other vinyl stretching mode, observed at 1631 cm^{-1} at room temperature in both the complexed proteins, upshifts by 5 and 3 cm^{-1} at low temperature for the SBP and HRPA2 complexes, respectively. Figure 3 compares the RR spectra taken with visible excitation (496.5 nm) at room and low temperatures of SBP−BHA (left) and HRPA2-BHA (right) complexes, with the corresponding spectra taken with Soret excitation (406.7 nm). It can be seen that for the ν_{11} (1545−1547 cm^{-1}), ν_{19} (1567−1569 cm^{-1}), and ν_{10} (1619−1621 cm^{-1}) modes the decrease in temperature does not appreciably affect the frequency whereas, as has been noted previously,[18,19] the higher frequency vinyl upshifts at 12 K. The assignment of the core size marker bands, obtained with the help of spectra recorded in polarized light at room temperature (data not shown), is reported in Table 2.

The following conclusions can be drawn from an analysis of the data: (i) The RR bands of the BHA−peroxidase complexes do not change their frequency upon lowering the temperature, indicating that there is no change of the core size. (ii) The only exception appears to be represented by the ν_2 mode which upshifts by about 9 and 6 cm^{-1} (neglecting any contribution due to the ν_{37} mode in determining the frequency of the composite band) when the temperature is reduced from 296 to 12 K in SBP−BHA and HRPA2−BHA, respectively (Figure 5

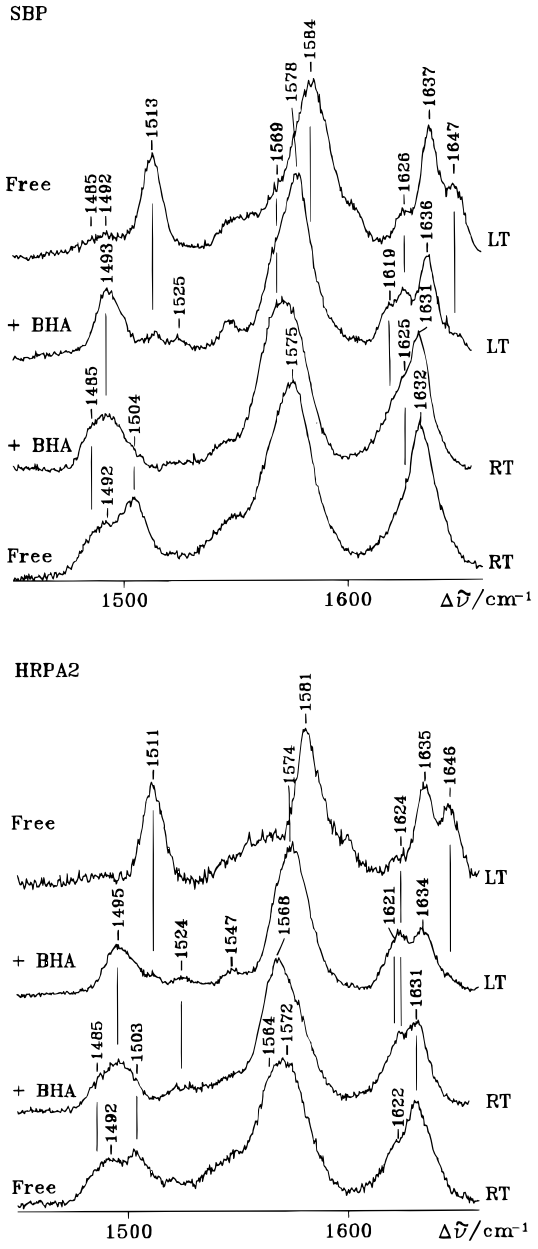

Figure 5. Resonance Raman spectra of SBP and the SBP−BHA complex at room temperature (RT) and 12 K (LT) (top) and of HRPA2 and the HRPA2−BHA complex at room temperature (RT) and 12 K (LT) (bottom). Experimental conditions: 5 cm^{-1} spectral resolution and 406.7 nm excitation. SBP: RT, 15 mW laser power at the sample, 20 s/0.5 cm^{-1} accumulation time. SBP−BHA: RT, 15 mW laser power at the sample, 10 s/0.5 cm^{-1} accumulation time. SBP: LT, 30 mW laser power at the sample, 3 s/0.5 cm^{-1} accumulation time. SBP−BHA: LT, 20 mW laser power at the sample, 3 s/0.5 cm^{-1} accumulation time. HRPA2 and HRPA2−BHA: RT, 10 mW laser power at the sample, 8 s/0.5 cm^{-1} accumulation time. HRPA2: LT, 15 mW laser power at the sample, 10 s/0.5 cm^{-1} accumulation time. HRPA2−BHA: LT, 20 mW laser power at the sample, 4 s/0.5 cm^{-1} accumulation time.

and Table 2). These data can be explained if one recalls that this mode is coupled to the vinyl ν(C=C) mode in protohemes, as shown by the fact that saturation of the vinyl groups increases the ν_2 frequencies by about 10 cm^{-1}.[35] Since at least one vinyl stretch mode upshifts at low temperature a concomitant upshift of the ν_2 frequencies is expected. These effects have also been recently observed in selected distal mutants of CIP when the distal Phe54 was replaced by the small Gly residue.[36] However, no blue-shift of the Soret band is observed upon lowering the temperature to 12 K in the HRPA2- and SBP−BHA complexes, which is expected when the conjugation between the double bonds of the vinyl and the macrocycle decreases.[32,37] This might be an effect due to the presence of unbound proteins, as judged by the shoulder at 575 nm observed in Figure 4 (see Experimental Section). In fact, at room temperature the free protein displays a Soret maximum at 402 (SBP) and 403 nm (HRPA2), whereas at low temperature a temperature-induced transition to a low-spin (LS) state drives the Soret maximum to about 410 nm.[18,19] In accord with this hypothesis, it is noted that if the amount of unbound protein is increased by slightly decreasing the BHA concentration, the Soret band undergoes a slight red-shift (data not shown). (iii) Despite the similarity of the electronic absorption spectra, all characteristic of 6-c HS heme, only CIP−BHA shows the core size marker band frequencies typical of this coordination and spin state; all the other complexes show marker band frequencies which are all upshifted with respect to CIP−BHA, and move to higher frequency in the order HRPC < SBP < HRPA2.

Figure 6 shows the EPR spectra of the BHA complexes of the four peroxidases and their g values, determined by simulation of the spectra (Table 2). It is immediately apparent that, with the exception of CIP−BHA, the g values indicate that the electronic ground states of the BHA complexes are not typical of HS states. This is particularly evident if one evaluates g_{12} {where $g_{12} = (g_1 + g_2)/2$}. It can be seen that g_{12} (Table 2) decreases from metmyoglobin (MetMb) and CIP−BHA, which are 6-c HS hemes, to HRPA2, in the same order as the observed frequency increase of the core size marker bands: MetMb = CIP > HRPC > SBP > HRPA2. One should note that the line shape of the SBP−BHA and HRPA2−BHA spectra is not simulated as well as that of the other complexes, particularly for the g_2 feature. The large line width (~5 mT) associated with these two complexes has been noted previously for QS systems.[17,19,38,39] EPR line widths result from homogeneous broadening due to electronic relaxation and from inhomogeneous broadening due to g-strain or unresolved hyperfine structure.[40,41] In the present case, g-strain is likely the origin of the line broadening. The asymmetric line shape evident in the spectra is also a typical feature of g-strained systems.[40,42] The simulated spectra, shown under the corresponding experimental spectra in Figure 6, were computed using a program appropriate for effective $S = {}^1/_2$ systems without g-strain; therefore, the discrepancy between the experimental and simulated spectra of systems which depart significantly from these criteria (i.e., SBP−BHA and HRPA2−BHA) is not unexpected. Hence, the g values presented in Table 2 for SBP−BHA and HRPA2−BHA should be regarded only as approximate values. However, the g_{12} values of the three class III peroxidases are sufficiently

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Table 2. EPR g Values and Frequencies of the Most Intense RR Bands in the High-Frequency Region of Various BHA Peroxidase Complexes Obtained at Low Temperature, Compared with the Values Obtained for MetMb

	EPR				resonance Raman (cm^{-1})					
complex	g values			mean g_{12}[a]	ν_3	ν_{11}	ν_{19}	ν_{10}	ν_2	ν(C=C)
MetMb				5.92[73]	1483[74]	1544[74]	1562[75]	1611[75]	1558[74]	1621[74]
CIP−BHA[11]	6.04	5.74	2.00	5.89	1485	1542	1559	1615	1563	1624
HRPC−BHA	6.16	5.43	1.99	5.80	1491[7]	1547	1567	1616	1569	1622, 1634
SBP−BHA	6.01	5.43	1.99	5.72	1493	1546	1567	1619	1578[b]	1626, 1636
HRPA2−BHA	5.93	5.36	1.99	5.65	1495	1547	1569	1621	1574[b]	1624, 1634

[a] $g_{12} = (g_1 + g_2)/2$. [b] Overlapped with the ν_{37} mode.

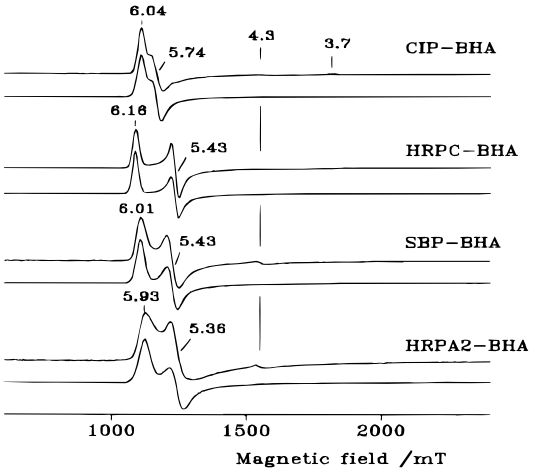

Figure 6. Low-field EPR spectra of CIP−BHA, HRPC−BHA, SBP−BHA, and HRPA2−BHA. All samples were 100 μM in 0.1 M bicine at pH 7.7. The spectra were recorded at 5 K, 1 mW microwave power, 9.35 GHz microwave frequency, and 1 mT modulation amplitude. Simulated spectra, obtained using a simulation program appropriate for $S_{eff} = {}^1/_2$ systems without g-strain, are shown under each experimental spectrum. The feature at $g = 4.3$ results from a non heme iron impurity.

different that their order is not expected to change even if the line shape could be satisfactorily simulated. Attempts to simulate the SBP−BHA and HRPA2−BHA spectra with more than one species failed, consistent with the evidence from the RR data for only one QS species at low temperature. Furthermore, the use of very large line widths to obviate the lack of a suitable simulation program, as previously reported for some QS systems,[39] proved inappropriate in this case.

The weak feature present in all the spectra at $g = 4.3$ corresponds to a non heme iron impurity often seen in protein samples.[43] The weak feature at $g = 3.7$ (g_2 and g_3 are not observed) in the CIP−BHA spectrum is assigned to a LS heme due to the uncomplexed protein. Such large g_{max} LS EPR signals have been reported to be associated with bis-imidazole iron coordination in both model complexes and heme proteins in which the imidazole ligand planes approach perpendicular orientation.[44−47] A LS species in the CIP−BHA complex was also seen at low temperature by electronic absorption and RR studies.[11] In the present case, such a complex would presumably result from a temperature-induced movement of the distal His toward the heme iron, enabling it to bind. An alternative explanation in which the features at $g = 4.3$ and 3.7 are considered to result from the ground state of an $S = {}^3/_2$ impurity is excluded as CIP in the resting state has features at 4.3 and 3.41, not at 3.7.[19] The LS signal observed at 77 K in the case of the BHA and salicylhydroxamic acid (SHA) myeloperoxidase complexes ($g = 2.66, 2.22, 1.81$),[48] in which the hydroxamic acid was proposed to be the sixth ligand generating the LS state, is clearly very different from that reported here for the CIP−BHA complex. In the former, however, the crystal structure of the SHA complex[49] showed that the SHA molecule extends much further into the active site than is the case for the HRPC− and ARP−BHA complexes[12,13] and that there is no distal water molecule present, facilitating binding at low temperature. The absence of LS signals in the other BHA complexes is likely a consequence of the significantly lower affinity of CIP for BHA compared to the other proteins as discussed above. Therefore, there is a higher proportion of uncomplexed heme present in CIP which is able to form LS heme states at low temperature as previously reported.[11,19] The higher g value of the LS species observed in the CIP−BHA complex (3.70) compared to the free protein (3.41) possibly reflects a perturbation of the sixth ligand orientation in the presence of BHA leading to a more closely perpendicular disposition of the imidazole planes.

Discussion

BHA Binding. The X-ray structures, solved for the HRPC−BHA[12] and ARP−BHA[13] complexes, are very similar. Both structures show that BHA is located in the distal heme pocket, with the aromatic ring nearly parallel to the heme. The functional groups are held by hydrogen bonds to the N_ϵ of the distal Arg, the N_ϵ of the distal His, the O of a Pro residue, and a water molecule. This latter, which is located at about 2.6−2.7 Å from the heme iron, is in turn hydrogen-bonded to the distal His and Arg. In the HRPC−BHA complex, hydrophobic contacts between BHA and various side chains were found. In particular, the closest amino acid residue to BHA is Phe179.[50] Its substitution by site-directed mutagenesis significantly increases the K_d of the HRPC−BHA complex.[51] Since this residue is substituted by an aliphatic side chain in HRPA2[14] and, moreover, the structural motif that contributes Phe179 to the binding site of HRPC is lacking in ARP/CIP, it is suggested that this residue plays an important role and can partly account for the decreased affinity for BHA.[52] The amino acid sequence of SBP is not yet available, but these data suggest that Phe 179 is missing also in this protein.

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The fairly long distance (2.6−2.7 Å) between the Fe atom and the distal water molecule noted above for the two BHA complexes might suggest that the latter is not directly bound to the metal.[52] However, the RR spectra obtained for HRPC−BHA in solution and single-crystal forms were found to be identical, indicating that they are consistent with the presence of a six-coordinate species. Similarly, the RR spectra of the CIP−BHA complex in solution[11] and as a single crystal (G. Smulevich, unpublished results) indicate the presence of a 6-c HS species. The RR solution spectra reported here show that at room temperature two six-coordinate species are formed for the peroxidases of class III complexed with BHA, whereas CIP gives rise to a single 6-c HS species. La Mar and co-workers[8] found the ternary HRPC−BHA−CN complex to be heterogeneous, with two forms in slow exchange, attributed to an alternate conformation of a distal residue. Interestingly, substitution of either Phe68 or Phe142 influences the partitioning of benzohydroxamic acid between the two possible complexes.[53,54] Moreover, when Phe179 is substituted only one type of complex is formed.[51] It is not clear if the two forms observed by RR at room temperature in the binary HRPC−, HRPA2−, and SBP−BHA complexes correspond to the two forms observed by NMR in the ternary HRPC−BHA−CN complex. We observe two species which are both six-coordinate; therefore, the difference between the two forms appears to be due to a residue in the heme cavity, as previously suggested for the ternary complex by NMR. Moreover, the invariance of the RR core size frequencies between room and low temperatures indicates that the heme size is not affected by the temperature. In the electronic absorption spectra the temperature-induced blue-shift of the CT1 might indicate a perturbation of the hydrogen-bond network connecting the distal ligand (water molecule) with BHA and the distal His and Arg. In fact, the CT1 wavelength is extremely sensitive to the hydrogen bond status of the sixth ligand.[32] In particular, decreasing hydrogen bond donation toward an anionic sixth ligand determines a blue-shift of the CT1 maximum, as observed in both the fluoride and hydroxyl complexes of peroxidases.[37,55] In the present case, the HRPC−BHA room temperature X-ray structure shows the distal water molecule is at 3.06 Å from the N_ϵ of the guanidinium group of the distal Arg and, thus, hydrogen bonded. This hydrogen bond, therefore, explains the red-shifted maximum of the CT1 band in all the peroxidase−BHA complexes, which is about 5−6 nm higher than that of MetMb. Moreover, the blue-shift of the CT1 upon lowering the temperature might suggest that the distal Arg has a different geometrical disposition with respect to the distal water molecule at room temperature.

Heme State. The present data show that the six-coordinate form present at low temperature in the complexes between class III peroxidases and BHA is characterized by an electronic absorption spectrum which is similar to those typical of a 6-c HS form (such as the CIP−BHA complex or MetMb), but with RR core size marker bands at higher frequencies than expected. The upshift of the RR frequencies follows the order HRPC to SBP to HRPA2 and, concomitantly, the g_{12} EPR value {where $g_{12} = (g_1 + g_2)/2$} decreases in the same order to values progressively less than 6. These observations assume a particular significance when viewed in the light of the spectroscopic characteristics of 5-c QS heme states. Such species are defined by an overall blue-shift of the electronic absorption spectrum compared to typical HS hemes, high RR frequencies of the core size marker bands, similar to those of the 6-c LS hemes, EPR g_{12} values in the range $4 < g_{12} < 6$, and a decrease of the mean hyperfine shifts for both the heme methyls and axial His H_β compared to HS hemes.[2,10,17,32,56] The striking similarities between the RR and EPR properties of the 5-c QS form and those of the hexacoordinate state observed in the BHA complexes of the class III peroxidases immediately prompt the proposal that their spectroscopic features are characteristic of a six-coordinate form in a QS state, with an increasing contribution from an $S = {}^3/_2$ state in the same order as the increasing RR frequencies. This species is also the major form at room temperature. Not many RR data are available on the 6-c QS state. However, one RR study of iron-porphyrin derivatives in the IS state has been reported by Teraoka and Kitagawa,[4] which supports the assignment of the novel species observed in the peroxidase BHA complexes. Although the g_{12} values of MetMb and CIP−BHA differ marginally from 6, which could indicate the admixture of some IS state with the HS state, it is at such a low level (<5%) that their electronic ground states can be well described by a HS electronic configuration.

The observation of such a heme spin state in these proteins is not completely unexpected, since the three proteins in the resting state, as well as barley peroxidase (BP 1), another protein belonging to class III of the peroxidase superfamily, show a 5-c QS at room temperature in equilibrium with HS species. The proportion of the $S = {}^3/_2$ state increases in the order HRPC < HRPA2 < SBP < BP1.[10,15−17] Moreover, the QS species is still present at low temperature.[7,17−19] It is noteworthy that CIP, in the resting state at room temperature, differs from the other peroxidases cited as it exhibits a 5-c HS heme.[23] The presence of this unusual 6-c QS form might explain why the HRPC−BHA complex was assigned by NMR to a five-coordinate high-spin heme on the basis of comparison of the hyperfine shift observed for MetMb.[10] However, a detailed analysis of the NMR spectra of these complexes is necessary before any final conclusion can be drawn concerning the sensitivity with which NMR can detect a 6-c QS species.

This is the first observation of a hexacoordinate QS state in a heme protein and, as has been noted previously,[17] the pentacoordinate QS state is also rare in heme proteins. The origin of the QS state in heme proteins remains elusive. The most widely suggested cause has been a weak axial ligand field;[57] however, a saddle-shaped deformation of the heme has also been proposed to be a prime factor governing the occurrence of the QS state in heme proteins.[58] The weakness of the Fe ligand(s) does not appear to be a completely adequate explanation of the presence of the QS state in heme proteins. All peroxidases belonging to the superfamily of plant peroxidases are distinguished by a proximal His of marked imidazolate character which results in a fairly strong bond between the His N_ϵ atom and the Fe atom. In the present case, the comparison between the structures of the HRPC−BHA[12] and ARP−BHA[13] complexes does not yield further insight into possible causes of the QS state, as the distance between the ligands and the Fe atom are comparable. However, a recent analysis, based on the complete body of structural data available for peroxidases and

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a deconvolution procedure for various heme distortions, found that moderate saddling alone is probably not sufficient to cause the QS state, although some saddling may be necessary for observation of the QS species.[17]

EPR Rhombicity. It is intriguing that observation of rhombicity in ferric hexacoordinate HS heme EPR spectra is also unusual. A noteworthy example of such a case though is that of the mammalian peroxidases. In fact, rhombic ferric HS EPR spectra have been observed for the hexacoordinate mammalian peroxidases lactoperoxidase,[48,59] intestinal peroxidase,[60,61] eosinophil peroxidase,[62,63] and prostaglandin H synthase,[64,65] which have a water molecule as the sixth ligand (as in the present case). Therefore, it is interesting to pose the question of a correlation between the two unusual observations of rhombic EPR spectra and the occurrence of the 6-c QS state in type III peroxidases. In fact, a potential point in common between EPR rhombicity and the QS state can be found in heme deformation. The possible importance of a saddling distortion in generating the QS state has already been noted above. The factors which may contribute to the splitting or broadening of the EPR feature in the region of $g = 6$, which reflects a departure from tetragonal to rhombic heme symmetry, are disruption of the porphyrin structure involving displacements of the heme nitrogen atoms or π electron binding to the iron at the fifth or sixth coordination positions.[66] Indeed, it has been remarked upon previously[67] that ferric HS heme proteins with anionic axial ligands, such as catalase, cytochrome P450, and peroxidases, usually display quite large rhombicity in their EPR spectra.[66,68]

Is There a Common Origin for the QS State and the EPR Rhombicity? A determination of the percentage rhombicity (defined as $(\Delta g/16) \times 100$, where Δg is the difference in the g values of the two components near $g = 6$ [66]) of the HRPC−, SBP−, and HRPA2−BHA complexes reveals that they all have a rhombicity of about 4%, which is very similar to that observed for the uncomplexed proteins.[19,43,69] It is interesting that the EPR spectrum of the CIP−BHA complex contrasts with those of the other BHA complexes, displaying an almost axial EPR signal (1% rhombicity). The uncomplexed CIP protein exhibits two HS signals, one axial and one of 9% rhombicity.[19] If one makes the reasonable assumption that the axial and rhombic forms correspond to the 6-c HS and 5-c HS hemes, respectively, seen in the RR spectra,[19] it is evident that hexacoordination effectively eliminates the rhombicity in CIP. Thus, the binding of a water molecule at the sixth coordination site of the heme iron apparently perturbs the electron distribution at the heme group, possibly leading to reduced anionic character of the proximal His and, consequently, reduced rhombicity. It has not been possible though to draw any firm conclusions from a comparison of the crystal structures of ARP[13,70] and HRPC,[12,50] for either the proteins alone or complexed with BHA, or from the RR data of CIP, CCP, HRPC,[71] and SBP,[16] concerning the importance of axial ligand anionicity and the presence of rhombicity in the HRPC−, SBP−, and HRPA2−BHA EPR spectra. In fact, the distal Fe−H_2O distances in the BHA complexes are 2.6 Å in HRPC and 2.7 Å in ARP while, on the proximal side, the Fe−$N_{(His)}$ distance is 2.2 Å in HRPC and 2.14 in ARP.

The disappearance of EPR rhombicity on binding of a water molecule in CIP−BHA is, in fact, the opposite effect to that observed for some Mb mutants in which loss of the iron bound distal water molecule, present in wild-type MetMb, resulted in rhombic EPR spectra rather than the characteristic axial MetMb EPR spectrum.[67] This effect is apparently absent, or the additional mechanism of heme distortion is active in the three class III peroxidase complexes producing the observed rhombicity. Thus it cannot be excluded that heme distortion gives rise to both the QS state and the rhombicity of the EPR spectra. To our knowledge, EPR studies of 6-c QS heme model compounds are extremely limited. A study of the N-terminal acetylated hemoctapeptide from cytochrome *c*, acetylmicroperoxidase 8 (AcMP8), at 77 K and various pH values has shown that it can be described as a mixture of LS and 6-c QS states in thermal equilibrium.[72] The 6-c QS species was found to exhibit an axial EPR signal and, consequently, suggests that heme distortion is not of prime importance in generating the QS state of the peroxidase BHA complexes reported here. It should be borne in mind, however, that while this may be the case for model compounds, in the highly constrained heme environment within the protein matrix heme distortion may assume greater importance.

It is worth mentioning that all of the heme proteins which display a 5- or 6-c QS state and have been studied by EPR spectroscopy exhibit rhombic EPR signals. Furthermore, they have broad EPR line widths, likely the result of *g*-strain.[17,19,38,39] In other words, QS systems appear to be characterized by heterogeneity of the heme center. If one recalls that the strains are not unidirectional, then coupling of the strain distribution to the axial zero-field splitting tensor **D** will give rise to a lowering of the axial symmetry at the iron site.[40] Therefore, while treating this empirical observation with caution, it seems possible that rhombic EPR spectra may be a characteristic of QS states in heme proteins.

Influence of the Ruffling Distortion. It should be noted that the CIP−BHA complex, which has a nearly axial EPR signal, has a greater degree of ruffling, saddling, and doming heme distortions than HRPC−BHA (Shelnutt, personal communication). At this point it must be borne in mind that both saddling and doming or both ruffling and doming heme distortions must be simultaneously present to lower the heme symmetry to rhombic (C_{2v}). The activation of any one of these distortions alone does not lower the heme symmetry from tetragonal. Thus, one might expect that the CIP−BHA EPR spectrum should be

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even more rhombic than that of HRPC−BHA, if heme distortion is of importance. However, it is noted that ruffling is present in an approximately equal degree to saddling in CIP−BHA and essentially absent in HRPC−BHA (Shelnutt, personal communication). Thus, while moderate saddling is probably necessary, but not a sufficient condition to trigger the formation of the QS state,[17] ruffling can be excluded from having any significant influence in generating the QS state. In fact, the CIP−BHA data suggest that it may even annul the potential influence of the saddling distortion in creating the QS state.

Conclusions

The combined analysis of the spectra of HRPC, HRPA2, and SBP at different temperatures with and without BHA allows us to infer the characteristics of both the 5-c QS [15−17] and 6-c QS states.

A 5-c QS state is characterized by (i) electronic absorption spectra similar to those of a 5-c HS heme but with shorter wavelength $\pi \rightarrow \pi^*$ transitions, which rule out the presence of a LS heme; (ii) a CT1 charge-transfer band at 630−635 nm, which rules out the presence of either 5-c HS or 6-c LS hemes (but possibly might correspond to a 6-c HS heme); (iii) high frequency of the RR core size marker bands, which cannot be assigned to either 5-c or 6-c HS hemes (but possibly might correspond to LS heme); and (iv) EPR spectra with g_{12} values in the range $4 < g_{12} < 6$, typical only of a QS heme.[38]

A 6-c QS state is characterized by the following: (i) An electronic absorption spectrum similar to that of a 6-c HS heme. It differs from the 5-c QS in that the Soret band red-shifts by about 4 nm and sharpens with a ∼35% increase of the extinction coefficient. (ii) High frequency of the RR core size marker bands, which cannot be assigned to either 5-c or 6-c HS hemes, but are much closer to those of a 5-c HS model compound than a 6-c HS heme. (iii) EPR spectra with g_{12} values in the range $4 < g_{12} < 6$.

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