Resonance Raman Investigation of Ferric Iron in Horseradish Peroxidase and Its Aromatic Donor Complexes at Room and Low Temperatures†

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ABSTRACT: Resonance Raman (RR) spectra of the acidic form of Fe^{III} horseradish peroxidase (HRP) were obtained at room and low temperatures using B- and Q-band excitation. At 296 K, HRP exhibits two sets of porphyrin skeletal stretching frequencies which are attributed to a thermal mixture of 5- and 6-coordinate high-spin Fe^{III} states. When the temperature is lowered, the observed bands shift to higher frequencies, and these are assigned to intermediate- and low-spin states. Addition of 40% glycerol has no effect on the spectra at 296 K, but at 20 K, all four frequency sets are observed corresponding to the two forms observed at room and low temperature in the absence of glycerol. The 296 K RR spectrum of the HRP–hydroquinone complex is similar to that of free HRP, but conversion to the intermediate- and low-spin states is complete at a higher temperature than in the free enzyme. Addition of benzohydroxamic acid (BHA) to HRP shifts the RR frequencies to those corresponding to a 6-coordinate high-spin species at both room and low temperature. Two ν(C=C) stretching modes are observed for HRP and its donor complexes, indicating that the vinyl groups are inequivalent. On BHA binding, one of the vinyl modes and ν_{37} (E_u) are enhanced, suggesting symmetry lowering of the heme site.

Horseradish peroxidase (HRP)[1] has been the subject of numerous physical studies. Spectroscopic (Tamura, 1971; Schonbaum, 1973; Rakshit & Spiro, 1974; Maltempo et al., 1979; La Mar et al., 1980; Teraoka & Kitagawa, 1981; Kitagawa et al., 1983; Terner & Reed, 1984; Schulz et al., 1984; Evangelista-Kirkup et al., 1985; Palaniappan & Terner, 1989) and magnetic susceptibility (Tamura, 1971; Schonbaum, 1973) studies have been undertaken to probe the status of the heme Fe and the interaction of residues in the heme cavity with the heme. Also, the effect of aromatic donor molecules on the properties of HRP has been investigated by a number of workers (Schonbaum, 1973; Leigh et al., 1975; Schejter et al., 1976; Paul & Ohlsson, 1978; Maltempo et al., 1979; Morishima & Ogawa, 1979; Teraoka & Kitagawa, 1981; Kitagawa et al., 1983; Schulz et al., 1984; Sakurada et al., 1986; Thanabal et al., 1987).

The present RR study of ferric HRP and its donor complexes with hydroquinone (HQ) and benzohydroxamic acid (BHA) was undertaken since the reported Fe^{III} spin and coordination states are not all in agreement. In particular, the amount of intermediate-spin (IS) state reported for ferric HRP at low temperature varies considerably, and Maltempo et al. (1979) ascribed this to differences in isolation and handling procedures. Considering the stability of ferric HRP under a variety of experimental conditions (4–353 K, pH 3–12), the reported sensitivity of the Fe^{III} spin and coordination to handling is rather surprising. In light of this, the present work reports a reinvestigation of the nature of the Fe^{III} atom in HRP under different conditions. HRP from two major commercial suppliers was used with and without further purification, and resonance Raman (RR) was used to probe its heme. The sensitivity of this technique in detailing the coordination of the Fe atom in hemoproteins and model systems has been well documented (Spiro & Li, 1988). Also, as previously demonstrated in studies on cytochrome *c* peroxidase (CCP) (Smulevich et al., 1989), RR is the technique of choice to probe the effect of temperature on the heme since room temperature and low-temperature RR spectra can be readily obtained. Therefore, we examine here the effect of temperature on the RR spectra of HRP at pH 7 and 4.

It has been reported that glycerol alters the Fe^{III} state in CCP (Smulevich et al., 1989). Since many low-temperature spectroscopic studies are carried out in the presence of a high concentration of glycerol, we also present here the effect of this "antifreezing" agent on the RR spectra of HRP at room and low temperature. Finally, the effect of the donors HQ and BHA on the RR spectra was examined. From their EPR studies, Maltempo et al. (1979) reported that at low temperature HQ binding increases the population of the IS state in ferric HRP, whereas in the BHA complex the Fe^{III} atom is high spin (HS). However, from room temperature RR studies, it was concluded that HQ complexation does not affect the spectrum of ferric HRP but BHA binding converts the Fe^{III} atom from 5- to 6-coordinate (6c) within the HS state (Teraoka & Kitagawa, 1981; Kitagawa et al., 1983). Since these RR reports did not examine the spectra of HRP at low temperature, it is not possible to compare the published EPR and RR data.

Experimental Procedures

Materials. HRP was obtained from Sigma (type VI-A, RZ = 3.1, activity = 310 units/mg of solid; type VI, RZ = 3.2, activity = 325 units/mg of solid) and Boehringer Mannheim (grade I, RZ = 3.1, activity = 263 units/mg of solid). The activities are those reported by the suppliers using pyrogallol (Sigma) and guaiacol (Boehringer Mannheim) as donor

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[1] Abbreviations: HRP, ferric horseradish peroxidase; HQ, hydroquinone; BHA, benzohydroxamic acid; HRP-HQ, HQ complex of HRP; HRP-BHA, BHA complex of HRP; RR, resonance Raman; CCP, cytochrome *c* peroxidase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EPR, electron paramagnetic resonance; 5c or 6c, 5- or 6-coordinate; HS, high spin; IS, intermediate spin; LS, low spin.

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substrates. Sigma type VI-A and Boehringer Mannheim HRP were subjected to cation-exchange chromatography which was used by Shannon et al. (1966) to separate HRP isoenzymes. HRP was eluted from a 1.0 × 12 cm CM-Sepharose CL-6B (Pharmacia) column equilibrated with 5 mM sodium acetate, pH 4.4, using an acetate gradient (5–300 mM, pH 4.4) at a flow rate of ~10 mL h^{-1}. The main peak in each case is assumed to be mainly isoenzyme *c* which is the major isoenzyme present in HRP (Shannon et al., 1966). For the RR studies, both purified (main peak) and untreated HRP samples were used. Glycerol (100%, spectroscopic grade), HQ (98% pure), and BHA (99% pure) were obtained from Aldrich, and were used without further purification.

Methods. HRP samples for RR spectroscopy were prepared by dissolving the solids in 0.1 M potassium phosphate buffers (pH 6.0 and 7.0), 0.1 M MOPS buffer (pH 7.0), and 0.1 M citrate (pH 4.0). The samples that were further purified by cation exchange were transferred from acetate buffers to 0.1 M phosphate buffers using ultrafiltration cells with a 10000 molecular weight cutoff filter (Amicon Centricon microconcentrators with YM 10 filter). Samples in 40% glycerol (v/v) were prepared by adding glycerol to the HRP samples in buffer. The donor complexes were formed by adding 25 mM BHA and 100 mM HQ to HRP in 0.1 M phosphate, pH 7. The K_d's for the BHA and HQ complexes are 2.4 μM (Schonbaum, 1973) and 3.2 mM (Paul & Ohlsson, 1978), respectively.

Room temperature RR spectra were obtained for samples in a rotating NMR tube as described previously (Smulevich et al., 1989). The low-temperature RR spectra were obtained by using a closed-cycle He cryotip with automatic temperature control. The samples (10–20 μL) were transferred by syringe into a small groove in the copper cold finger of the cryostat at 270–280 K (or 240 K for the glycerol samples) under N_2 flow. N_2 was passed over the samples until the temperature decreased to 200 K, but the sample chamber was not put under dynamic vacuum above 180 K to prevent sample lyophilization. The temperature was slowly decreased to 80 or 10 K, and RR spectra were obtained at these temperatures. The annealing process was repeated to ensure that the spectra obtained were those of thermally equilibrated HRP.

The back-scattered light from the NMR tube or low-temperature cell was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG-2S) equipped with a cooled photomultiplier (RCA C31034 A) and photon counting electronics. The 406.7- and 530.9-nm lines of a Kr^+ laser (Coherent Radiation Innova 90 K) and 457.9-, 496.5-, 501.7-, and 514.5-nm lines of an Ar^+ laser (Coherent Radiation Innova 90/5) were used as radiation sources. For each spectrum, 2–10 scans were averaged by using a data acquisition system obtained from Jobin-Yvon. The RR spectra were calibrated with indene as a standard (Hendra & Loader, 1968), and the frequencies are accurate to ±1 cm^{-1} for the intense isolated bands.

Polarized spectra were obtained at room and low temperature by inserting a polaroid analyzer between the sample and entrance slit of the spectrometer. To check the reliability of the polarization measurements using a rotating NMR tube with 180° back-scattering geometry, the polarization of the totally symmetric streching mode of CCl_4 at 458 cm^{-1} was measured, and the value obtained, 0.12, compares favorably with the theoretical value of 0.125.

Results

Dependence of RR Spectra on Annealing. The RR spectra of all the HRP samples examined here and those of their donor complexes were found to be sensitive to the rate of cooling. For example, deposition of ~20 μL of HRP on the cold finger at 80 K gave rise to a 10 K RR spectrum which is essentially identical with the 296 K spectrum. Deposition at 180 K yielded a 10 K spectrum which is a mixture of the room temperature and low-temperature spectra. However, deposition at 270 K followed by slow cooling to 10 K allowed the samples to anneal properly, and reproducible spectra were obtained at 10 K and intermediate temperatures following a number of cooling–heating cycles. The samples which contained 40% glycerol were deposited at 240 K because of the lower freezing point of buffer/glycerol mixtures. Again, no changes were observed in the 40% glycerol spectra on repeating the cooling–heating process as long as rapid freezing was avoided.

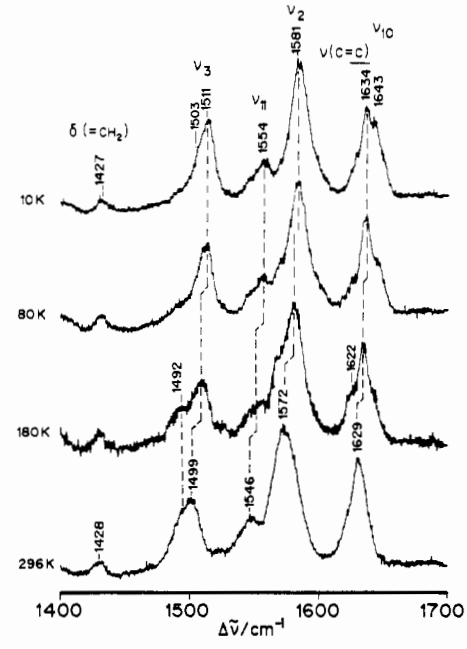

FIGURE 1: Resonance Raman spectra of HRP (1 mM) in 0.1 M phosphate buffer, pH 7, at different temperatures obtained with 406.7-nm excitation. Experimental conditions: 5 cm^{-1} spectral resolution; 15-mW laser power at the sample; 0.5 cm^{-1}/3 s accumulation time.

Identical RR spectra were obtained for all the HRP samples used in this study. No difference was found between the HRP samples from the two suppliers, nor did CM-Sepharose chromatography of the samples change their RR spectra. The observed sensitivity of the RR spectra to the cooling rate, and hence the relative populations of different heme species, may explain the variation in the EPR spectra reported by various workers (Maltempo et al., 1979) as discussed below.

RR Spectra of HRP. The porphyrin skeletal stretching modes, which are observed between 1400 and 1700 cm^{-1} following 406.7-, 501.7-, and 514.5-nm excitation at room and low temperatures, are shown in Figures 1 and 2. The room temperature spectra are similar to those previously reported (Rakshit & Spiro, 1974; Teraoka & Kitagawa, 1981; Kitagawa et al., 1983; Terner & Reed, 1984; Evangelista-Kirkup et al., 1985; Palaniappan & Terner, 1989).

On B-band excitation (406.7 nm), the selectively enhanced peaks observed in Figure 1 at 1499, 1572, and 1629 cm^{-1} in the 296 K spectrum have been assigned to ν_3, ν_2, and the vinyl C=C stretching modes, respectively (Rakshit & Spiro, 1974). These peaks clearly shift to higher frequencies as the temperature is lowered, and ν_3, ν_2, and ν(C=C) appear at 1511, 1581, and 1634 cm^{-1}, respectively in the 10 K spectrum. Also apparent in the 180 K spectrum (and in the 296 K spectrum

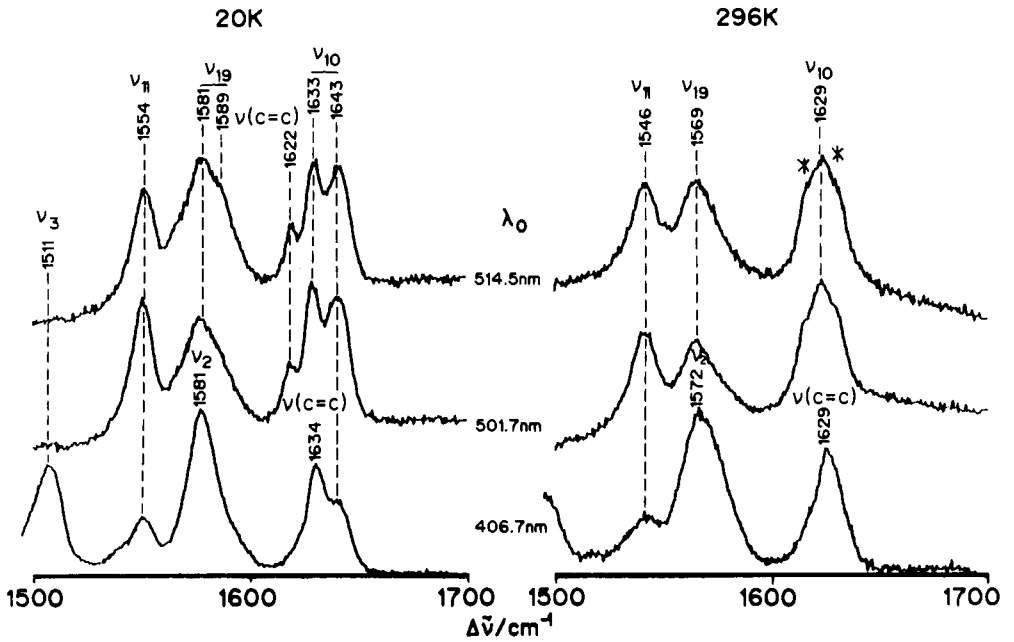

FIGURE 2: Resonance Raman spectra of HRP in 0.1 M phosphate buffer, pH 7, at 20 K ([HRP] = 1 mM) and 296 K ([HRP] = 0.07 mM) obtained with different excitation frequencies. Experimental conditions: 5 cm^{-1} spectral resolution; 406.7-nm excitation; 15-mW laser power at the sample; 501.7- and 514.5-nm excitation; 200- and 100-mW laser power at the sample at room and low temperature, respectively; 0.5 cm^{-1}/10 s accumulation time. Asterisks indicate water bands.

as poorly resolved shoulders) are a second ν_3 band at 1492 cm^{-1} and a shoulder at 1622 cm^{-1}. On further cooling, both these bands diminish in intensity, and at 10 K, a new shoulder on the ν(C=C) peak is visible at ~1643 cm^{-1}.

These results agree in part with those obtained at pH 6 by Evangelista-Kirkup et al. (1985) allowing for the fact that *all* their reported frequencies are ~5 cm^{-1} lower than those reported here. However, on cooling to 77 and 9 K, they observed bands corresponding to a 6-coordinate high-spin (6cHS) heme species that is not observed here. Also, they do not report a 5 cm^{-1} shift to higher frequency of ν(C=C) at low temperature. These differences cannot be ascribed to either a pH or a buffer ion effect. Spectra obtained between pH 7 (phosphate or MOPS) and pH 4 are identical with those shown in Figure 1, with the exception that at pH 4 the 1492 cm^{-1} shoulder is still visible on ν_3 at 10 K. Since the low-temperature spectra are highly sensitive to the cooling rate, a more likely explanation for the observed differences at low temperature is the method of sample cooling.

The nontotally symmetric modes are resonance-enhanced on Q-band excitation between 500 and 600 nm (Spiro & Li, 1988). Figure 2 compares the RR spectra obtained on 406.7-, 501.7-, and 514.5-nm excitation at 296 and 20 K in the region between 1500 and 1700 cm^{-1}. The intense modes expected in this region are ν_{11} (B_{1g}), ν_{19} (A_{2g}), and ν_{10} (B_{1g}) in order of increasing frequency. Hence, the bands observed at 1546, 1569, and 1629 cm^{-1} on 514.5-nm excitation at 296 K are assigned to ν_{11}, ν_{19}, and ν_{10}, respectively. The two shoulders on the mode at 1629 cm^{-1} are due to a broad water band. At 20 K, ν_{11} appears at 1554 cm^{-1}, and bands are now visible at 1581, 1589 (sh), 1622, 1633, and 1643 cm^{-1}.

The spectra of HRP in the presence of 40% glycerol at 296 K are shown in Figures 3 and 4. A comparison with the corresponding 296 K spectra in Figures 1 and 2 reveals that 40% glycerol has no detectable effect on the Fe coordination in HRP at room temperature. Spectra taken in 66% glycerol are also identical with those in Figures 3 and 4, except that a fluorescent impurity in the glycerol reduces the quality of the spectra. When the temperature is lowered, however, the

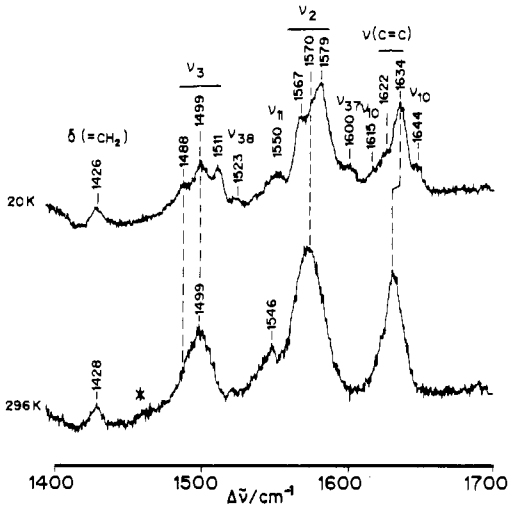

FIGURE 3: Resonance Raman spectra of HRP (1 mM) in 40% glycerol/0.1 M phosphate buffer, pH 7, at 296 and 20 K obtained with 406.7-nm excitation. Experimental conditions as in Figure 1. The asterisk indicates a glycerol band.

ν_3 region in Figure 3 contains three distinct peaks at 1488, 1499, and 1511 cm^{-1} corresponding to at least three different heme species. Likewise, extra bands appear in the ν_2 and the 1600–1650 cm^{-1} regions of the 20 K spectrum. Furthermore, on Q-band excitation, the 20 K spectra (Figure 4) exhibit congested ν_{10} and ν_{19} regions.

RR Spectra of HRP–BHA. Figures 5 and 6 show the RR spectra for the BHA complex of HRP. The spectrum shown in Figure 5 at 296 K is similar to those reported previously (Teraoka & Kitagawa, 1981; Kitagawa et al., 1983). The ν_3 maximum shifts to 1491 cm^{-1} on binding BHA and does not change in frequency at low temperature, indicating that the spin and coordination of the Fe in HRP–BHA are temperature-independent. Two bands are evident in the vinyl region of the 296 K spectrum. At 80 K and below, the 1630 cm^{-1} band shifts to 1634 cm^{-1}, giving rise to a clearly resolved doublet of equal intensity, and a shoulder appears at 1618 cm^{-1}.

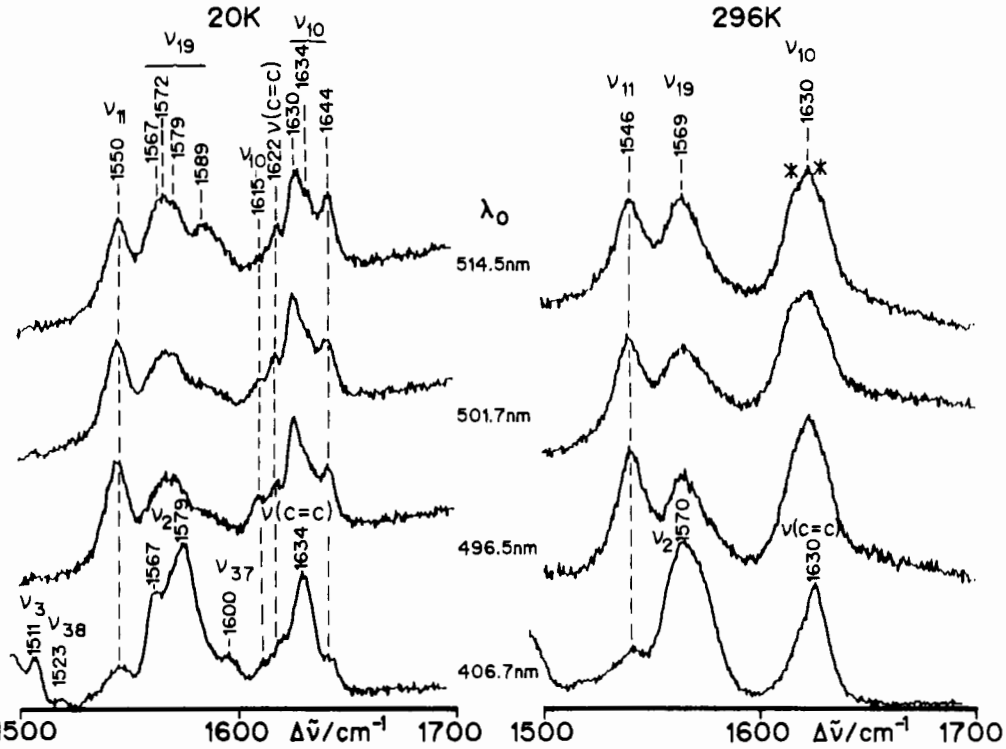

FIGURE 4: Resonance Raman spectra of HRP in 40% glycerol/0.1 M phosphate buffer, pH 7, at 20 and 296 K obtained with different excitation frequencies. Experimental conditions as in Figure 2: 496.5-nm excitation; 200- and 100-mW laser power at the sample at room and low temperature, respectively. Asterisks indicate water bands.

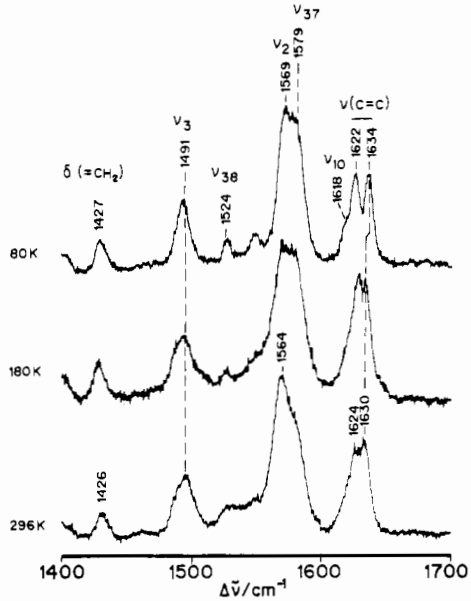

FIGURE 5: Resonance Raman spectra of HRP (1 mM) + BHA (25 mM) in 0.1 M phosphate buffer, pH 7, at different temperatures obtained with 406.7-nm excitation. Experimental conditions as in Figure 1.

Polarization measurements (not shown) reveal that the bands at 1622 and 1634 cm^{-1} are polarized but the shoulder is depolarized. The relative intensities of the peaks on Q-band excitation (Figure 6) confirm the assignment of the 1618 cm^{-1} shoulder (1616 cm^{-1} at 20 K) to a nontotally symmetric mode, and the doublet to symmetric modes.

RR Spectra of HRP–HQ. The B-band RR spectra of HRP–HQ are shown in Figure 7 as a function of temperature. The spectrum at 10 K is identical with that at 80 K so it is not included in Figure 7. A comparison of Figures 1 and 7 reveals that the spectra of HRP in the presence and absence of HQ are similar at room temperature as reported previously by Kitagawa et al. (1983). The 20 K RR spectra of HRP–HQ on 406.7- and 514.5-nm excitation are compared in Figure 8. These spectra are similar to those obtained in the absence of HQ, but the intensity ratio of the modes at 1633 and 1641 cm^{-1} is different from that observed for the corresponding modes in Figure 2, and ν_{19} does not show the intense shoulder at 1589 cm^{-1} observed for free HRP.

Discussion

Correlations of observed frequencies between 1450 and 1700 cm^{-1} with structure have shown that all porphyrin skeletal frequencies above 1450 cm^{-1} exhibit a linear dependence on porphyrin core size (Parthasarathi et al., 1987). In Table I, the frequencies reported for 5cHS, 6cHS, 6cLS, 5cIS, and 6cIS model hemes are presented. On the basis of these data, we assign the frequencies observed for HRP under different experimental conditions to different spin and coordination states of Fe^{III}. A detailed rationale for these assignments will now be given.

HRP. From its room temperature RR spectra, HRP has been assigned a 5cHS heme (Rakshit & Spiro, 1974; Teraoka & Kitagawa, 1981; Kitagawa et al., 1983; Terner & Reed, 1984; Palaniappan & Terner, 1989). In addition, a small amount of 6cHS heme has been reported based on the low-frequency shoulder on ν_3 (Evangelista-Kirkup et al., 1985). The 296 K spectra reported here are consistent with this assignment since a shoulder is observed at 1492 cm^{-1} (Figure 1). However, on Q-band excitation (Figure 2), only one ν_{10} peak is observed at 1629 cm^{-1} which is broadened by the contribution of the bending mode of H_2O. Since the ν_{10} modes for 5cHS and 6cHS species are expected at ~1626 and 1610 cm^{-1}, respectively (Table I), the latter could be masked by the water band. The presence of some 6cHS heme may also be inferred from the low frequencies observed here for the ν_{11} and ν_{19} modes which are ~5 cm^{-1} lower than those reported by

Table I: Predicted Skeletal and Vinyl Stretching Mode Frequencies (cm^{-1}) for Model Hemes and Observed Frequencies for Different HRP States

	ν_3, A_{1g}	ν_{11}, B_{1g}	ν_2, A_{1g}	ν_{19}, A_{2g}	ν_{10}, B_{1g}	ν(C=C)
$(DMSO)_2PPFe^{III}$, 6cHS[a]	1480	1545	1559	1560	1610	1621
$(Cl)PPFe^{III}$, 5cHS[a]	1491	1553	1570	1571	1626	1626
$(ImH)_2PPFe^{III}$, 6cLS[a]	1502	1562	1579	1586	1640	1620
$(OEP)Fe^{III}(py)ClO_4$, 6cIS[b]	1506	1567	~1585	~1584	1637	
$(OEP)Fe^{III}ClO_4$, 5cIS[b]	1513	1560	1581	1588	1645	
HRP, 6cHS[c]	1491	1544	1564	1564	1618	1624, 1630
HRP, 5cHS[d]	1499	1546	1572	1569	1629	1629
HRP, 6cLS[e]	~1503		1581	1589	1643	
HRP, IS[e]	1511	1554	1581	1581	1633	1622, 1634

[a] Data from Choi et al. (1982). [b] Data from Figure 8 of Teraoka and Kitagawa (1980). [c] HRP-BHA at 296 K. [d] HRP at 296 K. [e] HRP and HRP-HQ at 20 K.

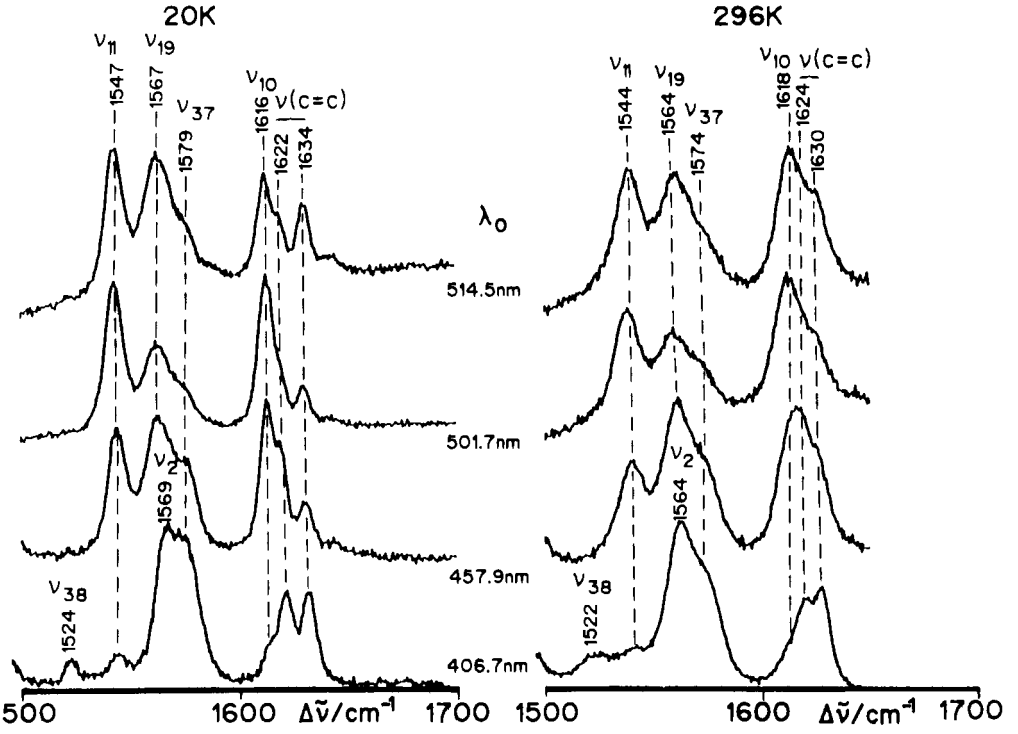

FIGURE 6: Resonance Raman spectra of HRP (1 mM) + BHA (25 mM) in 0.1 M phosphate buffer, pH 7, at 20 and 296 K obtained with different excitation frequencies. Experimental conditions as in Figure 2.

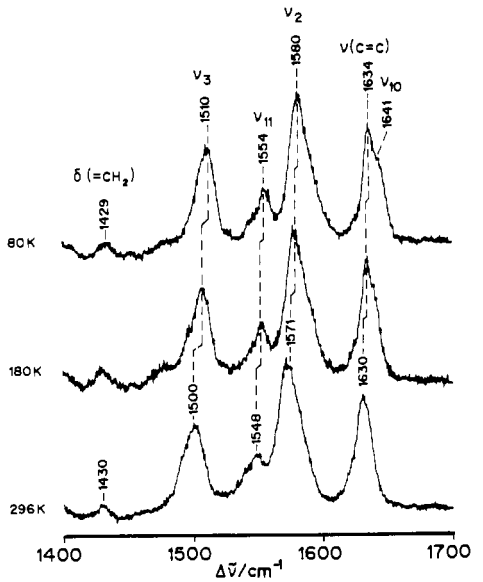

FIGURE 7: Resonance Raman spectra of HRP (1 mM) + HQ (100 mM) in 0.1 M phosphate buffer, pH 7, at different temperatures obtained with 406.7-nm excitation. Experimental conditions as in Figure 1.

Rakshit and Spiro (1974) and Terner and Reed (1984). The width of the ν_{19} band is also consistent with a mixture of two HS forms, but since the ν_3, ν_2, and ν_{10} modes of the 5cHS species dominate the spectrum in Figure 1, this is assumed to be the major species in free HRP at 296 K.

The 10 K spectrum in Figure 1 shows bands at higher frequencies relative to the 296 K spectrum, indicating a transition to either LS or IS heme (Table I). A similar shift to high frequency is observed for the nontotally symmetric modes on Q-band excitation at 20 K (Figure 2). A surprising feature of these Q-band spectra is the high relative intensity of the peaks at 1622 and 1633 cm^{-1} which are assigned to ν(C=C) modes in the B-band spectra (Figure 1). Vinyl ν(C=C) modes are not expected in the Q-band spectra, and Terner and Reed (1984) did not assign may in their room temperature Q-band spectra of HRP isoenzymes A-1, B, or C. Polarization measurements (not shown) obtained with 514.5-nm excitation at 20 K indicate that the 1554, 1634, and 1643 cm^{-1} bands are depolarized, whereas the band at 1581 cm^{-1} is inverse-polarized, and the band at 1622 cm^{-1} has a polarization ratio of ~0.4. On this basis, we assign the intense bands at 1633 and 1643 cm^{-1} to ν_{10} modes, and the 1622 cm^{-1} band to a vinyl mode that gains intensity from the 1633 cm^{-1}

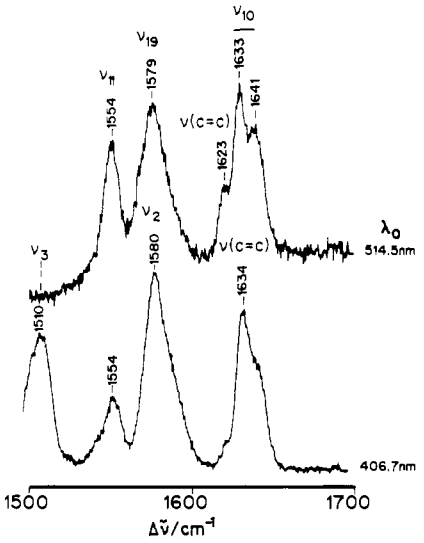

FIGURE 8: Resonance Raman spectra of HRP (1 mM) + HQ (100 mM) in 0.1 M phosphate buffer, pH 7, at 20 K obtained with 406.7- and 514.5-nm excitation. Experimental conditions as in Figure 2 except for 50-mW laser power at the sample with 514.5-nm excitation.

ν_{10} mode, as was observed for CCP (Smulevich et al., 1990).

The presence of two ν_{10} modes is consistent with the observation of two ν_3 modes at 1511 and 1503 (sh) cm^{-1}, and two ν_{19} modes at 1581 and 1589 (sh) cm^{-1}. This latter shoulder could also arise from ν_{37} (E_u), but E_u modes are generally observed as weak features only with B-band excitation (Choi et al., 1982; Parthasarathi et al., 1987). Therefore, on the basis of the frequencies observed for model complexes, we attribute the 1511, 1581, and 1633 cm^{-1} bands to IS heme and the 1503 (sh), 1589 (sh), and 1643 cm^{-1} bands to 6cLS heme (Table I). The assignment of these latter bands to 6cLS heme agrees with the low-temperature optical (Tamura, 1971) and EPR (Schonbaum, 1973) spectra of free HRP which also show signals due to LS heme. However, the presence of two IS species at low temperature cannot be ruled out on the basis of the RR frequencies (Table I).

Wang and Van Wart (1989) examined the temperature dependence of the RR spectra of microperoxidase. Their findings are similar to those obtained here, since at room and low temperature, both peroxidases are largely HS and IS, respectively.

HRP + 40% Glycerol. Addition of 40% glycerol to solutions of HRP has no effect on the 296 K spectra. This is in marked contrast to the effects of glycerol addition on the room temperature RR spectra of CCP mutants (Smulevich et al., 1989). However, glycerol suppresses in part the transition to the low-temperature species as was also observed for CCP. The presence of 6cHS and 5cHS heme species, as well as IS heme, is clearly evident from the ν_3 (1488, 1499, 1511 cm^{-1}) and ν_2 (1567, 1570, 1579 cm^{-1}) regions in Figure 3. The ν_{10} region of the Q-band spectra in Figure 4 shows extra peaks at 1615 cm^{-1} (6cHS) and 1630 cm^{-1} (5cHS) in addition to those assigned to IS (1634 cm^{-1}) and LS (1644 cm^{-1}) at 20 K in buffer. Accordingly, the ν_{19} region shows bands at 1589 cm^{-1} (6cLS) and 1572 cm^{-1} (5cHS) with two shoulders at 1567 cm^{-1} (6cHS) and 1579 cm^{-1} (IS). Thus, in 40% glycerol at 20 K, all *four* species observed for free HRP between 296 and 10 K coexist.

HRP–HQ. Binding of HQ to the heme cavity of HRP causes changes in the absorption spectrum (Paul & Ohlsson, 1978). However, the RR spectrum of HRP–HQ appears almost identical with that of free HRP at room temperature, except that the ν_3 band at 1500 cm^{-1} is narrower and more intense. Unlike glycerol, HQ induces the transition from HS to IS at higher temperature (compare Figures 1 and 7), such that the RR spectrum does not change between 80 and 20 K. Also, the population of the IS species has grown at the expense of the 6cLS species at 20 K, since the intensity of the ν_{10} IS band (1633 cm^{-1}) relative to the ν_{10} 6cLS band (1641 cm^{-1}) is greater in the HRP–HQ spectra (Figure 8) than in free HRP (Figure 2), and the intense shoulder at 1589 cm^{-1} (ν_{19}, 6cLS) is obvious only in free HRP. These results are in agreement with those of Maltempo et al. (1979), who reported that binding of HQ to HRP increased the amount of IS signal observed in the EPR spectra at 6 K.

HRP–BHA. BHA binds tightly to HRP ($K_d \sim 2.5\ \mu M$) and changes a number of physical properties of the enzyme. The magnetic moment shifts from 5.23 to 5.97 μ_B at room temperature, and the reported EPR spectrum shows mainly HS signals (Schonbaum, 1973). However, while there seems to be little doubt that the Fe^{III} atom is HS in HRP–BHA, the coordination number of the Fe is still a matter of debate. Binding of BHA causes a dramatic change in the absorption spectrum of HRP; the Soret absorption band narrows, and its intensity maximum shifts from 403 nm (ϵ = 102.2 mM^{-1} cm^{-1}) in the native enzyme to 408 nm (ϵ = 154 mM^{-1} cm^{-1}) in the BHA complex (Schonbaum, 1973). The shape and intensity of the Soret band in HRP–BHA are similar to those observed for 6cHS heme in the Phe-51 mutant of CCP (Smulevich et al., 1988) whereas the 5cHS heme of native CCP exhibits a Soret band of lower absorptivity with a low-wavelength shoulder like native HRP. Hence, changes in the Soret band of HRP on binding BHA are consistent with a shift from largely 5cHS to 6cHS heme.

The 296 K RR spectrum shown in Figure 5 is essentially identical with that obtained with 441.6-nm excitation by Kitagawa et al. (1983), who assigned a 6cHS heme to HRP–BHA with ν_3 and ν_{10} at 1491 and 1623 cm^{-1}, respectively. However, polarization measurements at 20 K (not shown) indicate that the two bands at 1622 and 1634 cm^{-1} are polarized and the shoulder at 1618 cm^{-1} is depolarized. Therefore, we assign the two polarized bands to the vinyl ν(C=C) modes and the shoulder to ν_{10} of 6cHS heme. This assignment is further strengthened by the Q-band spectra in Figure 6, since the ν_{10} peak (1616 cm^{-1}) is enhanced, and the 1622 and 1634 cm^{-1} bands, although still observed, are considerably less intense than the ν_{10} mode as expected (Spiro & Li, 1988).

On the basis of NMR studies (Sakurada et al., 1986; Thanabal et al., 1987), a substrate binding site near methyl-8 of the heme has been proposed for HRP. The RR data indicate that occupation of this site by BHA gives rise to a 6cHS heme at room and low temperatures. In contrast, when HQ binds, the Fe^{III} atom remains 5cHS at room temperature, but the 5cHS → IS transition occurs at higher temperatures than in free HRP.

Vinyl Modes. In protoheme complexes and many heme proteins, the two vinyl groups give rise to a single ν(C=C) band at $\sim$1622 cm^{-1} in their RR spectra (Choi et al., 1982). Splitting of the vinyl modes due to interaction with the protein has been reported previously for CTT hemoglobins (Gersonde et al., 1989) and in liver microsomal cytochrome P-450 (Hildebrandt et al., 1989). Furthermore, selective deuteration of the vinyls in CTT hemoglobin shows that the highly localized vinyl ν(C=C) modes at 1624 and 1630 cm^{-1} are uncoupled and inequivalent (Gersonde et al., 1989). Thus, the observation of two vinyl modes at 1622 and 1630 cm^{-1} (296 K) for all the species examined here indicates that the 2- and

4-vinyls are also inequivalent in HRP. Cooling increases the separation between the vinyl peaks since the high-frequency mode shifts to 1634 cm^{-1} at 20 K, indicating that the conformation of only one vinyl group changes at low temperature.

The identical frequencies of the vinyl bands observed for HRP and its donor complexes imply that occupation of the donor binding site does not affect the conformation of the vinyl groups. This is consistent with the proposed substrate binding site being near methyl-8 of the heme (Sakurada et al., 1986) so that direct contact between the substrate and the vinyls is highly unlikely. However, the change in the relative intensities of the vinyl doublet in HRP–BHA (Figure 5) compared to free HRP (Figure 1) or HRP–HQ (Figure 7) is notable. In addition, the E_u mode at 1579 cm^{-1} (ν_{37}, Figure 5) is strongly enhanced in the spectrum of HRP–BHA. E_u modes are Raman inactive under D_{4h} symmetry but become weakly activated on B-band excitation in protohemes due to the vinyl substituents (Choi et al., 1982) which lower the symmetry of the heme. Therefore, BHA binding to HRP must distort the active site such that RR activity of the E_u mode is further enhanced. Normal mode calculations on nickel protoporphyrin show that the ν_{37} and vinyl modes are coupled (K. Kushmeider and T. G. Spiro, personal communication), so it is not surprising that *both* the vinyl mode at 1622 cm^{-1} and the ν_{37} mode at 1579 cm^{-1} are strongly enhanced in HRP–BHA.

ν_3 Frequencies. It should be noted that some of the frequencies for the model HS hemes in Table I differ considerably from those assigned to the corresponding heme species in HRP. This is particularly the case for the HS ν_3 modes which are $\sim$10 cm^{-1} higher for the 6cHS and 5cHS states of HRP compared to the models. However, the assignment of *pure* HS states to HRP does not agree with its reported magnetic moment of 5.23 μ_B (Tamura, 1971; Schonbaum, 1973). Maltempo et al. (1979) have interpreted the deviation of the magnetic moment from the spin-only value of 5.9 μ_B in terms of a quantum mechanical admixture of Fe^{III} IS and HS states, with the latter state predominant, since the magnetic moment is closer to that for HS than IS (3.9 μ_B). Although admixing may shift some of the RR core size marker bands from the frequencies expected for *pure* HS states, it should be pointed out that HRP–BHA, which has a magnetic moment of 5.97 μ_B (Schonbaum, 1973), also exhibits an anomalously high ν_3. Recently, Czernuszewicz et al. (1989) reported that flattening of the porphyrin ring in nickel octaethylporphyrin causes significant increases in some, but not all, of the bands between 1350 and 1650 cm^{-1}. This is consistent with the proposal of Rakshit and Spiro (1974) that the HS heme in HRP may be more planar than in other heme proteins and that this may account for the elevated ν_3 frequencies.

The ν_3 frequency assigned to the IS state of HRP at low temperature is between those reported for 5cIS and 6cIS model compounds. Due to the paucity of RR data on IS heme systems, designation of a coordination number to the IS state of HRP based on its RR spectra would be tentative at the present time.

Finally, we would like to consider the most plausible implications of the observed sensitivity of the Fe^{III} spin and coordination to solvent, temperature, and substrate. Glycerol, which stabilizes the tertiary structure of proteins, was found to stabilize the 5cHS form of CCP and its mutants at both room and low temperature (Smulevich et al., 1989). Since the same is observed here for HRP, freezing must induce a change in axial ligation of HRP, giving rise to the HS $\rightarrow$ IS transition at low temperature. Binding of HQ promotes this axial ligation change, whereas BHA binding changes the axial ligation at room temperature, and inhibits temperature-induced conformations in HRP that could lead to further axial ligation changes.

Conclusions

(1) At 296 K in the presence and absence of 40% glycerol, the heme in free HRP is predominantly 5cHS with a small amount of 6cHS species also present.

(2) At 20 K, free HRP is predominantly IS with some 6cLS species. Bands due to HS heme species were observed in the low-temperature spectra in the absence of glycerol only if the samples were not properly annealed.

(3) Glycerol partially suppresses the temperature-induced heme transitions from HS to IS and LS, since HS species are still observed at 20 K in the presence of 40% glycerol. This is consistent with previous results on CCP and its mutants, where the amount of HS heme also increases on addition of glycerol.

(4) Binding of HQ to HRP has the opposite effect to glycerol since it promotes the temperature-induced transitions from HS to IS and LS at higher temperatures than in free HRP. Also, HRP–HQ has a higher population of IS species at 20 K compared to free HRP.

(5) Binding of BHA to HRP gives rise to a 6cHS heme at both room and low temperatures. The presence of an intense E_u mode at 1579 cm^{-1} in HRP–BHA, together with the enhancement of the vinyl ν(C=C) mode at 1622 cm^{-1}, suggests that BHA binding lowers the symmetry of the heme site.

(6) The presence of two vinyl ν(C=C) modes in HRP and its donor complexes indicates that the 2- and 4-vinyls are inequivalent. Also, cooling affects the high-frequency vinyl mode.

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Registry No. HQ, 123-31-9; BHA, 495-18-1; peroxidase, 9003-99-0; heme, 14875-96-8; glycerol, 56-81-5; iron, 7439-89-6.

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Hairpin Formation in the Self-Complementary Dodecamer d-GGTACGCGTACC and Derivatives Containing GA and IA Mispairs

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ABSTRACT: The dodecamer d-GGTACGCGTACC and four derivatives with GA and IA mispairs in the 6,7 and 5,8 positions have been examined in dilute solution and 0.01–0.1 M sodium chloride. Concentration dependence of T_m, gel electrophoresis, and equilibrium centrifugation indicate that these self-complementary oligomers can form hairpins under the present conditions. Thermal transitions measured in the ultraviolet primarily represent melting of hairpin to coil [cf. Scheffler et al. (1968, 1970)]. The T_m values show little or no depression for 6,7 substitution but rather large depression for 5,8 replacement. We interpret the results to indicate that the 6,7 sequences have two-base loops and five base pair stems and that the 5,8 sequences have four-base loops and four base pair stems. A concurrent theoretical modeling study [Raghunathan et al. (1991) *Biochemistry* (following paper in this issue)] provides support for this interpretation.

Study of self-complementary DNA segments of defined sequence provides valuable information on the structure and energetics of these molecules and on the effect of composition and sequence on conformation [see, for example, Aboul-ela et al. (1985), Hilbers et al. (1985), Breslauer et al. (1986), and Orbons et al. (1986)]. The biological relevance of palindromic sequences is clear from their occurrence at functional and at recognition sites in DNA. Controlled perturbations, such as the introduction of nonstandard oppositions at specific points in the chain, provide insights into the chemistry of mispairing and are relevant to the occurrence or correction of biological mutations and to possible effects of mispairing on hairpin–duplex equilibria. We present here a report of the basic sequence d-GGTACGCGTACC, which contains recognition and cleavage sites for the restriction endonucleases *Rsa*I and *Fnu*DII. The effect of four perturbations of this basic sequence containing AG and AI[1] mispairs at the 6,7 and 5,8 positions is examined. In dilute solution and low to moderate concentrations of sodium chloride, we find that hairpin structures play a major role in these molecules. Many sequences used in other model hairpin studies have employed runs of A, C, or T to force hairpin formation. In contrast, members of the present series are not prevented by limitations on pairing specificity from forming paired duplexes. Lower temperatures and higher concentrations of oligomer and salt favor the formation of duplex helices. The complex equilibria governing duplex–hairpin interconversion will be described in a separate report.

Materials and Methods

Each of the five oligomers was prepared by manual synthesis on a 25–30-μmol scale by using cyanoethyl phosphoramidite chemistry (McBride & Caruthers, 1983; Sinha et al., 1984; Atkinson & Smith, 1984). They were purified by DEAE-cellulose chromatography with a 0–0.8 M gradient of ammonium bicarbonate in 7 M urea. Purity of central fractions of main peaks was determined by 5′ ^{32}P labeling followed by electrophoresis on a 20% polyacrylamide gel in 0.1 M Tris–borate buffer, pH 8.3, containing 8 M urea. For electrophoresis under nondenaturing conditions, 8 M urea was omitted.

UV spectra were measured with a Cary Model 118 spectrophotometer interfaced to an LDACS computer system

[1] Abbreviation: I, inosine.