Resonance Raman Study of the Active Site of *Coprinus cinereus* Peroxidase†

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ABSTRACT: Resonance Raman (RR) spectra for the resting state ferric and the reduced ferrous forms of recombinant *Coprinus cinereus* peroxidase (CIP), obtained with different excitation wavelengths and in polarized light, are reported. The spectra are compared with those obtained previously for cytochrome *c* peroxidase expressed in *Escherichia coli* [(CCP(MI)] and horseradish peroxidase (HRP-C). Although the enzymic properties of CIP and HRP-C are similar, the RR data show that, in terms of the heme cavity structures, CIP and CCP(MI) are much more closely related to each other than to HRP-C. The ferric state of CIP at neutral pH is characteristic mainly of a five-coordinate high spin heme. However, the lower frequency of the ν_2 mode and a higher frequency of the ν(C=C) vinyl stretching modes for CIP as compared to CCP, indicate a higher degree of vibrational coupling between the two modes in CIP. In addition, CIP is rather unstable under low laser power irradiation as an irreversible transition to a six-coordinate high spin heme followed by a second transition to a six-coordinate low spin heme is observed. This instability of CIP as compared to CCP(MI) is proposed to be a consequence of the presence of a distal Phe54 in CIP rather than the homologous Trp51 in CCP, as Trp51 is hydrogen-bonded to a distal water molecule located above the heme Fe thereby preventing its coordination in CCP. In CIP the Fe^{II}−His RR band has two components with frequencies at 230 and 211 cm^{-1}. The 230-cm^{-1} band is the more intense at neutral pH, whereas at alkaline pH the band at 211 cm^{-1} increases at the expense of the band at 230 cm^{-1}. This shift may reflect structural changes associated with protonation of the proximal His residue. The ν(Fe−Im) stretching mode at 230 cm^{-1} suggests the presence of a hydrogen-bonded imidazole with weaker imidazolate character than in CCP(MI), whereas the 211-cm^{-1} band suggests the presence of a fairly weak hydrogen-bond interaction between the N_δ-H of the proximal His183 and the O atom of the Asp245 side chain. The structural parameters influencing the frequency of the Fe-Im stretching mode are discussed.

Heme-containing peroxidases from bacteria, fungi, and plants are evolutionarily related and can be divided into three structural classes that show less than 20% amino acid sequence identity (Welinder, 1992; Welinder & Gajhede, 1993). Members of the class I peroxidases appear to be of prokaryotic origin and include mitochondrial cytochrome *c* peroxidase (CCP)[1] from yeast (fungus), chloroplast and cytosol ascorbate peroxidases (plant), and gene-duplicated bacterial peroxidases (bacterium). The conversion of toxic hydrogen peroxide into water seems to be the major biological role of this class of peroxidases. Class II peroxidases comprise extracellular fungal peroxidases, such as lignin and manganese peroxidases from white-rot fungi which participate in lignin degradation. The biological role of the inkcap *Coprinus cinereus* peroxidase (CIP), which is 40−45% identical in amino acid sequence to lignin and manganese peroxidases, is unknown, however (Baunsgaard et al., 1993). Class III peroxidases include secretory plant peroxidases such as the classical horseradish peroxidases (HRP-C). Class III peroxidases as well as class I and class II peroxidases can oxidize a broad spectrum of aromatic substrates producing radicals which may initiate polymerization or depolymerization processes and hence give rise to a wide range of biological functions.

In the present study the heme cavity of representatives of class I (CCP), class II (CIP), and class III (HRP-C) peroxidases has been compared by resonance Raman spectroscopy (RR) of ferric and ferrous forms. Although the enzymic properties of CIP and HRP-C are rather similar (Andersen et al., 1991b), the RR data show that the heme environment of CIP is more similar to CCP than to HRP-C.

A second aim of the present study has been to assign the RR bands for CIP and lay the foundations for work in progress on CIP mutants. Fungal CIP has been shown to be identical to the commercial *Coprinus macrorhizus* peroxidase (CMP) and *Arthromyces ramosus* peroxidase (ARP)

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[1] Abbreviations: ARP, *Arthromyces ramosus* peroxidase; CIP, wild-type fungal *Coprinus cinereus* peroxidase and recombinant *Coprinus cinereus* peroxidase expressed in *Aspergillus oryzae*; CCP, yeast cytochrome c peroxidase; CCP(MI), cytochrome c peroxidase expressed in *E. coli* containing Met-Ile at the N-terminus; CCP(MKT), cytochrome c peroxidase expressed in *E. coli* containing Met-Lys-Thr at the N-terminus; Trp51Phe, Trp51 → Phe CCP(MI) mutant; Asp235Asn, Asp235 → Asn CCP(MI) mutant; HRP-C, horseradish peroxidase isoenzyme C; Mb, myoglobin; HEPES, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; 5-c and 6-c, five-coordinate and six-coordinate hemes; HS and LS, high and low spin; NMR, nuclear magnetic resonance; RR, resonance Raman; dp, depolarized; np, nonpolarized; p, polarized; ip, inverse polarized.

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both in its covalent structure and enzymic properties (Kjalke et al., 1992). Recombinant CIP expressed in the filamentous fungus *Aspergillus oryzae* is also identical to fungal CIP in all respects, including sites of glycosylation and N-terminal processing (Baunsgaard et al., 1993; Limongi et al., manuscript submitted). The identity of recombinant CIP and ARP was recently confirmed by X-ray crystallography of CIP (same batch as used for the present study) (Petersen et al., 1994) and ARP (Kunishima et al., 1994). The latter paper, however, suggested (in a footnote) the presence of an additional glycine residue near residue 4 of ARP, which means that ARP residue numbers are one higher than the same CIP residue numbers. To avoid confusion regarding nomenclature, we refer to CIP residues (1−343) throughout this paper, and write "ARP/CIP residue number" when referring to information taken from the crystal structure of ARP.

MATERIALS AND METHODS

Recombinant CIP was obtained by expression in transformed *A. oryzae* (Dalbφge et al., 1992). The peroxidase was precipitated with 2 M ammonium sulfate from filtered fermentation broth, dialyzed, and purified on a column of HiLoad Q-Sepharose (Pharmacia Biotech) eluted with linear gradient of 50−250 mM NaCl in 10 mM bis-Tris buffer, pH 6.0 (Reinheitszahl, RZ, 2.7). Highly purified and homogeneous CIP was obtained by concanavalin A chromatography followed by rechromatography on HiLoad Q-Sepharose eluted with a linear gradient of 75−200 mM NaCl in 10 mM NaAc, pH 4.8. The pH of the pooled peroxidase fractions (RZ, 2.9) was increased to pH 7 with 2 M Tris base prior to storage at 4 °C as an ammonium sulfate precipitate. Fungal CIP (*C. macrorhizus* peroxidase, lot 146015 from Schweizerhall Inc., South Plainfield, NJ) was purified by HiLoad Q-Sepharose chromatography at pH 4.8 as above to an RZ value of 2.8. Horseradish peroxidase isoenzyme C (HRP-C) was purchased from Sigma (type VI, RZ 3.2) and purified as previously described (Smulevich et al., 1991a). Cytochrome *c* peroxidase expressed in *Escherichia coli* [(CCP(MI)] was obtained as described previously (Fishel et al., 1987; Smulevich et al., 1988).

CIP preparations in the form of ammonium sulfate precipitates were centrifuged, and the supernatant was discarded. The precipitate was dissolved in Milli Q water, the pH adjusted to 7 with 2 M Tris, and extensive dialysis against Milli Q water carried out. Before the spectroscopic experiments, the pH was adjusted with 0.1 M phosphate buffer, 0.001 M $CaCl_2$, pH 7, or 0.025 M borate buffer, 0.001M $CaCl_2$, pH 9.7. The ferrous form was prepared by adding a minimum volume of fresh sodium dithionite solution to the deoxygenated buffered solution. Sample concentration were determined spectrophotometrically using an extinction coefficient of 109 cm^{-1} mM^{-1} at 405 nm (Andersen et al., 1991a) and were 0.1−0.4 mM for RR spectra and 10 times more diluted for UV-visible absorption spectra.

Absorption spectra were measured with a Cary 5 spectrophotometer. The RR spectra were obtained with excitation from the 406.7, 413.1, 530.9, and 568.9 nm lines of a Kr^+ laser (Coherent, Innova 90/K) and the 457.9, 476.5, and 514.5 nm lines of an Ar^+ laser (Coherent, Innova 90/5). The backscattered light from a slowly rotating NMR tube 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 RR spectra were calibrated with indene and CCl_4 as standards to an accuracy of ±1 cm^{-1} for intense isolated bands.

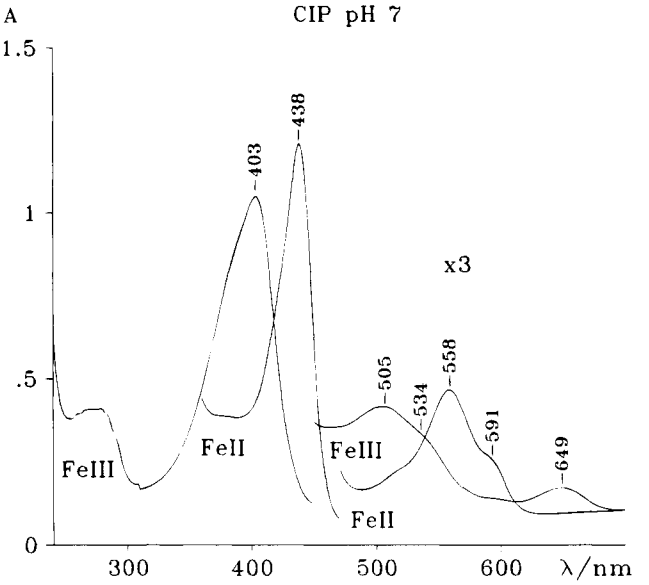

FIGURE 1: Electronic absorption spectra of ferric (a) and ferrous (b) CIP at pH 7.

All the absorption electronic spectra were collected at room temperature. The resonance Raman spectra were collected at about 15 °C. In fact, to minimize the heating effect induced on the protein by the laser beam, the rotating NMR tube was cooled by a gentle flow of N_2 gas passed through liquid N_2.

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 using a rotating NMR tube with 180° back-scattered geometry. The values obtained, 0.73 and 0, compare favorably with the theoretical values of 0.75 and 0, respectively.

RESULTS

Ferric Heme. Figure 1 shows the electronic absorption spectra of the ferric and ferrous forms of recombinant CIP, at pH 7. The enzyme gives an absorption spectrum which is identical to those obtained previously from the original fungal sources (Morita et al., 1988; Andersen et al., 1991a). The RR spectra are also identical for recombinant CIP and purified commercial CMP (data not shown), confirming the high degree of similarity of the two samples as also concluded from NMR studies (Veitch et al., 1994). Furthermore, the RR spectra of CIP (RZ = 2.7) obtained by only one step of anionic chromatography and highly purified CIP (RZ = 2.9) were indistinguishable.

Figure 2 compares the RR spectra in the high frequency region for the ferric forms of horseradish peroxidase isoenzyme C (HRP-C) (a), cytochrome *c* peroxidase expressed in *E. coli* [(CCP(MI)] (b), and CIP (c), obtained with Soret excitation. RR spectroscopic studies have been published previously for CCP(MI) (Smulevich et al., 1988; Smulevich,

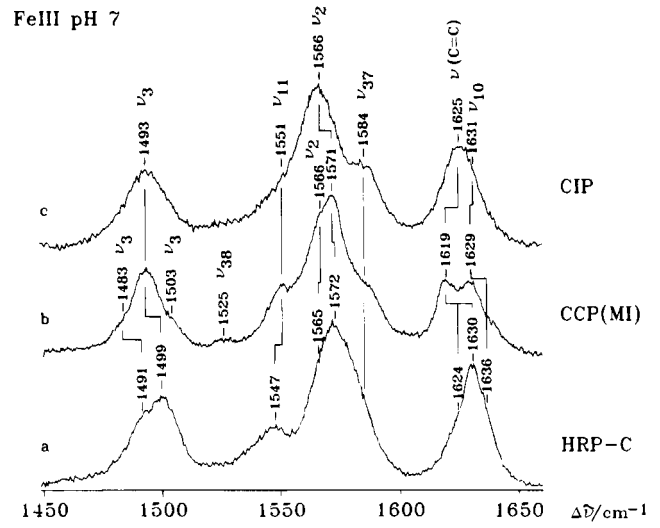

FIGURE 2: RR spectra of ferric HRP-C (a), CCP(MI) (b), and CIP (c) at pH 7. Experimental conditions: (a) 406.7 nm excitation wavelength, 5 cm^{-1} resolution, 6 s/0.5 cm^{-1} collection interval, and 30 mW laser power at the sample; (b) 413.1 nm excitation, 5 cm^{-1} resolution, 5 s/0.5 cm^{-1} collection interval, and 30 mW laser power at the sample; (c) 413.1 nm excitation wavelength, 5 cm^{-1} resolution, 15 s/0.5 cm^{-1} collection interval, and 10 mW laser power at the sample.

Table 1: Mode, Symmetry, and Polarization of the Principal Resonance Raman Bands (cm^{-1}) Observed for Ferric and Ferrous CIP

mode	symmetry	polarization	Fe^{III}	Fe^{II}
ν_{10}	B_{1g}	dp	1631	1604
ν(C=C)		p	1625	1621
ν_{37}	E_u	p	1584	1587
ν_{19}	A_{2g}	ip	1567	1554
ν_2	A_{1g}	p	1566	1559
ν_{11}	B_{1g}	dp	1551	1547
ν_3	A_{1g}	p	1493	1469
δ(=CH_2)		dp	1428	1423
ν_4	A_{1g}	p	1371	1355
$\delta(C_\beta C_a C_b)$		p	405	404
$\delta(C_\beta C_c C_d)$		p	380	374
ν_8	A_{1g}	p	349	347
γ_6	A_{2u}	dp	334	323
ν(Fe−Im) pH 7		p		230
ν(Fe−Im) pH 9.7		p		211

1993) and for HRP-C (Rakshit & Spiro, 1974; Kitagawa et al., 1983; Palaniappan & Terner, 1989; Smulevich et al., 1991a, 1994). Recently, it was found that HRP-C (Smulevich et al., 1994) shows a splitting of the core size marker bands into two components, suggesting the existence of two forms assigned to five- and six-coordinate high spin (5- and 6-c HS). Some RR bands of HRP-C, namely, ν_3 and ν_{10}, appear at considerably higher frequencies (1491 and 1620 cm^{-1} for the 6-c HS and 1499 and 1636 cm^{-1} for the 5-c HS, respectively) [Table 1 of Smulevich et al. (1994)] than those of model HS hemes. This discrepancy was attributed to a different distortion of the heme from planarity in HRP-C, due to external constraints such as steric contacts with the apoprotein. CCP(MI) is mainly five-coordinate high spin heme (5-c HS) as observed by the ν_3 at 1493 cm^{-1}, ν_2 at 1571 cm^{-1}, and ν_{10} at 1629 cm^{-1}. Small amounts of six-coordinate high and low spin (6-c HS, and LS) forms are present at neutral pH, as shown by the presence of ν_3 at 1483 and 1503 cm^{-1}, respectively.

CIP shows RR bands at 1493, 1566, 1584, and 1625 cm^{-1} upon Soret excitation. In order to assign the bands, we

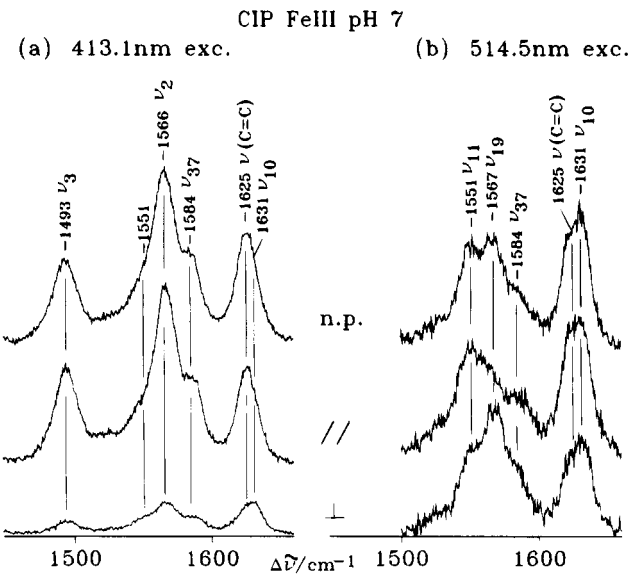

FIGURE 3: RR spectra of ferric CIP at pH 7, taken with 413.1 (a) and 514.5 (b) excitation wavelength. Experimental conditions: 5 cm^{-1} resolution; (a) 10 mW laser power at the sample; 15 s/0.5 cm^{-1} (nonpolarized, np), 12 s/0.5 cm (parallel polarization, ||), and 24 s/cm^{-1} (perpendicular polarization, ⊥) collection interval; (b) 40 mW laser power at the sample; 30 s/0.5 cm^{-1} (np), and 24 s/0.5 cm^{-1} (|| and ⊥) collection interval.

undertook a study using different excitation wavelengths and polarized light as shown in Figure 3. With Soret excitation, polarized (p) bands, due to the totally symmetric porphyrin modes (A_{1g}), and the ν(C=C) vinyl stretching modes are enhanced via the A-term (Franck-Condon mechanism). The depolarized (dp) bands, due to the non-totally symmetric modes (B_{1g}), are also observed in the Soret excitation by enhancement via the Jahn-Teller mechanism. In addition, B_{1g} modes and the inversely polarized (ip) bands (A_{2g}) are selectively enhanced via the B-term (vibronic mixing) in Q band excitation (Choi et al., 1982; Spiro & Li, 1988). Figure 3 shows the RR spectra in polarized light taken with Soret (413.1 nm) and Q band (514.5 nm) excitation. Based on their depolarization ratios, $\rho = I_\perp/I_\parallel$, the bands were assigned as follows: ν_3 at 1493 cm^{-1} (A_{1g}), ν_2 at 1566 cm^{-1} (A_{1g}), ν_{11} at 1551 cm^{-1} (B_{1g}), ν_{37} at 1584 cm^{-1} (E_u), ν(C=C) at 1625 cm^{-1}, and ν_{10} at 1631 cm^{-1} (B_{1g}), respectively. The frequencies of the core size marker bands of CIP are very similar to those observed in CCP(MI) (Figure 2), apart from the ν_2 and the ν(C=C) modes. The ν_2 band appears down-shifted by 5 cm^{-1}, and the ν(C=C) band up-shifted by 6 cm^{-1} as compared to those of CCP(MI). The complete assignment and the symmetry species are reported in Table 1. It is interesting to note that the ν(C=C) stretching modes are also observed in Q band excitation. This is consistent with previous observations for CCP, both in single crystal and solution studies (Smulevich et al., 1990), and in HRP-C studies (Smulevich et al., 1994), despite the fact that vinyl stretching modes are not expected to be enhanced *a priori* with visible excitation.

The CIP enzyme was found to be very unstable in the laser beam. Figure 4 shows four consecutive RR spectra of ferric CIP collected at room temperature and neutral pH. Each spectrum required 15 min for collection. It can be seen that the protein undergoes slow, irreversible changes in spin and coordination states upon irradiation. Core size marker bands indicative of 6-c HS heme (ν_3 at 1485 cm^{-1}, ν_2 at 1562

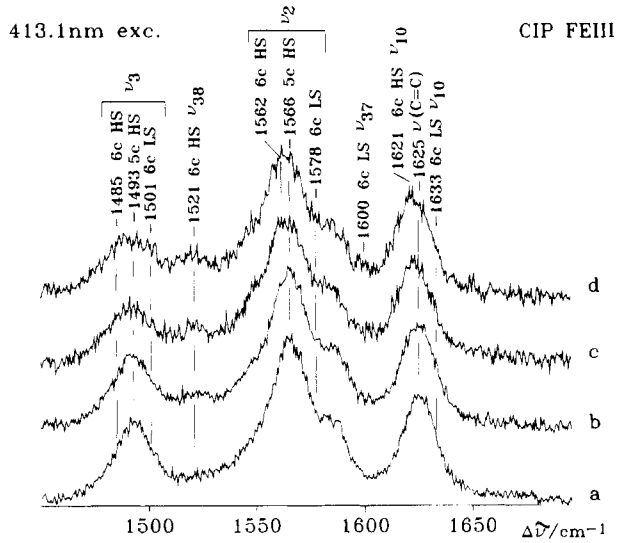

FIGURE 4: Decay of ferric CIP excited at 413.1 nm at pH 7. Four consecutive RR spectra of (a–d), each scan requiring about 15 min. Experimental conditions: 5 cm^{-1} resolution, 1 s/0.5 cm^{-1} collection interval, and 20 mW laser power at the sample. The spectra were obtained without a flow of nitrogen gas.

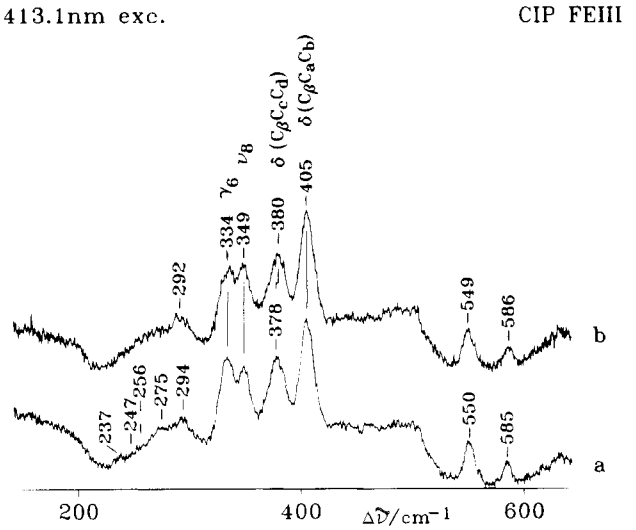

FIGURE 5: RR spectra of ferric CIP at pH 7, taken with 413.1 nm excitation wavelength. Experimental conditons: 5 cm^{-1} resolution, 5 s/0.5 cm^{-1} collection interval. Spectrum a: 10 mW laser power on a fresh sample. Spectrum b: 30 mW laser power at the sample, 1 h of exposure.

cm^{-1}, and ν_{10} at 1621 cm^{-1}) and of 6-c LS heme (ν_3 at 1501 cm^{-1}, ν_2 at 1578 cm^{-1}, and ν_{10} at 1633 cm^{-1}) appear. The low frequency region changes accordingly, as shown in Figure 5. The spectrum of the fresh solution (Figure 5a) shows strong bands at 334, 349, 378, and 405 cm^{-1} assigned to γ_6, ν_8, the $\delta(C_\beta C_c C_d)$ bending mode of the propionyl groups, and the $\delta(C_\beta C_a C_b)$ bending mode of the vinyl groups, respectively. The spectrum taken after 1 h of exposure to the laser beam (Figure 5b) shows a decrease in intensity of the band at 334 cm^{-1} and a small upshift of the band due to the propionyl groups.

Ferrous Heme. The electronic absorption spectrum of the reduced protein at neutral pH is shown in Figure 1. The overall spectrum appears very similar to those observed for CCP (Conroy et al., 1978) and HRP-C (Keilin & Hartree, 1951), with the Soret band at 438 nm and the Q bands at 559 and 590 (shoulder) nm. The spectrum of the reduced

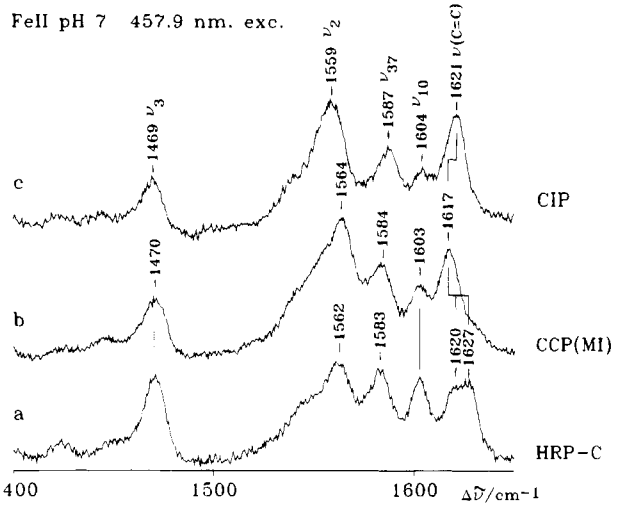

FIGURE 6: RR spectra in the high frequency region of ferrous HRP-C (a), CCP(MI) (b), and CIP (c) at pH 7. Experimental conditions: 457.9 nm excitation wavelength, 5 cm^{-1} resolution, (a) 9 s/0.5 cm^{-1} collection interval and 30 mW laser power at the sample; (b) 5 s/0.5 cm^{-1} collection interval and 30 mW laser power at the sample; (c) 8 s/0.5 cm^{-1} collection interval and 15 mW laser power at the sample.

form at pH 9.7 is identical to that at neutral pH (data not shown). Figure 6 compares the RR spectra in the high frequency region of reduced HRP-C (a), CCP(MI) (b), and CIP (c), all obtained at neutral pH. CIP gives rise to a spectrum characterized by core size marker bands very similar to those observed for the other two enzymes (ν_3 at 1469 cm^{-1}, ν_2 at 1559 cm^{-1}, ν_{37} at 1587 cm^{-1}, ν_{10} at 1604 cm^{-1}), characteristic of a 5-c HS heme (Choi et al., 1982). The complete assignment, also based on the RR spectra in polarized light taken with 530.9 and 568.2 nm excitation (data not shown), is reported in Table 1. From Figure 6 it appears that the three spectra differ essentially in the frequency of the ν_2 and the ν(C=C) modes. As observed in the ferric form, for ferrous CIP the ν_2 mode is down-shifted by 5 and 3 cm^{-1} with respect to CCP(MI) and HRP-C. On the other hand, the ν(C=C) stretch up-shifts by 4 cm^{-1} with respect to CCP(MI), and by 1 cm^{-1} with respect to the vinyl mode at 1620 cm^{-1} of HRP-C. This latter protein shows a second vinyl mode at 1627 cm^{-1}.

The corresponding spectra in the region 150–360 cm^{-1} are shown in Figure 7. The low frequency region RR spectra of five-coordinate ferrous hemoproteins are characterized by the presence of a strong band due to the ν(Fe-Im) stretching mode in the region 200–250 cm^{-1}. A common characteristic of peroxidases is the polar hydrogen-bond between the N_δ atom of the fifth ligand and an anionic proximal side chain which gives the proximal ligand imidazolate character and results in both a strengthening of the Fe–ligand bond and a higher frequency of the associated vibrational mode, as compared to the other hemeproteins.

The ν(Fe–Im) stretching mode has been detected at 244 cm^{-1} in HRP-C (Teraoka & Kitagawa, 1981) and at 247 cm^{-1} in CCP (Hashimoto et al., 1986) based on the ^{54}Fe isotopic shift. Recently, a second ν(Fe–Im) stretching mode has been detected in CCP(MKT) at 233 cm^{-1} at neutral pH (G. Smulevich, S. Hu, K. R. Rodgers, D. B. Goodin, K. Smith, and T. G. Spiro, unpublished results). Figure 7 shows that the bands observed in CCP(MI) are also present in CIP. CIP, in addition, shows a new band at about 211 cm^{-1}.

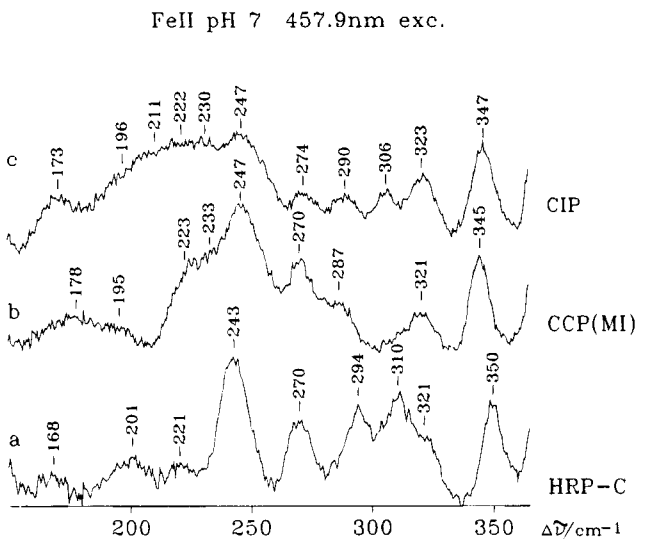

FIGURE 7: RR spectra in the low frequency region of ferrous HRP-C (a), CCP(MI) (b), and CIP (c) at pH 7. Experimental conditions: 457.9 nm excitation wavelength, 5 cm^{-1} resolution, (a) 5 s/0.5 cm^{-1} collection interval and 40 mW laser power at the sample; (b) 6 s/0.5 cm^{-1} collection interval and 30 mW laser power at the sample; (c) 30 s/0.5 cm^{-1} collection interval and 15 mW laser power at the sample.

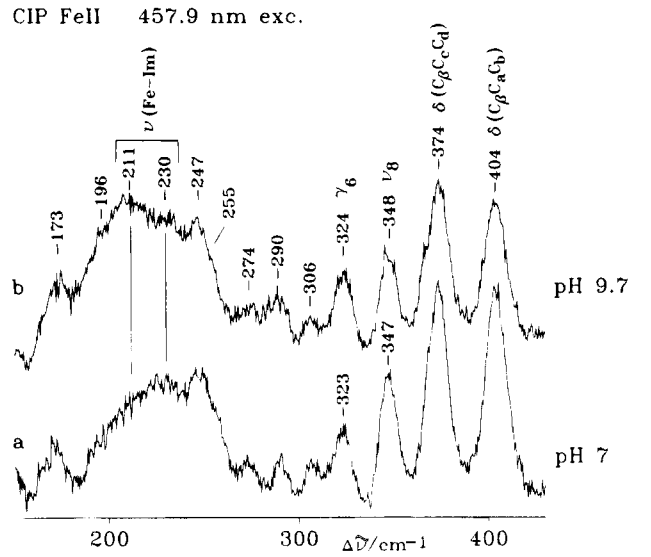

FIGURE 8: RR spectra in the low frequency region of ferrous CIP at pH 7 (a) and 9.7 (b) taken with 457.9 nm excitation wavelength. Experimental conditions: 5 cm^{-1} resolution, 15 mW laser power at the sample; (a) 18 s/0.5 cm^{-1} and (b) 36 s/0.5 cm^{-1} collection interval.

Peroxidases show a pH-dependent frequency shift of the ν(Fe−Im) stretching mode. The band down-shifts to 241 cm^{-1} in HRP-C at pH 8 (Teraoka & Kitagawa, 1981) and to 233 cm^{-1} in CCP (Hashimoto et al., 1986; Dasgupta et al., 1989) and CCP(MI) at pH 8.5 (Smulevich et al., 1988). In order to assign the ν(Fe−Im) stretching mode in CIP, we undertook a study of the ferrous form at different pH values. Between pH 6 and 10 the electronic absorption spectra do not change indicating that the coordination and spin state of the heme are not affected by the pH. Figure 8 compares the RR spectra in the low frequency region of CIP at pH 7 and pH 9.7 and shows a change in the relative intensity of the bands. In particular at alkaline pH, the bands at 347, 374, and 404 cm^{-1} assigned to ν_8, the $\delta(C_\beta C_c C_d)$ bending mode of the propionyl groups, and the $\delta(C_\beta C_a C_b)$ bending mode of the vinyl groups, respectively, appear to be much weaker than the broad band centered at 211 cm^{-1}. In addition, intensity changes are also observed in the region 200−250 cm^{-1}. At alkaline pH the band at 211 cm^{-1} increases in intensity with a concomitant weakening in intensity of the band at 230 cm^{-1}.

DISCUSSION

The RR spectra of both the ferric and ferrous states of recombinant and fungal CIP are identical (data not shown), indicating that their heme pockets are the same as was also concluded by a previous study based on the NMR data (Veitch et al., 1994). The ferric state of CIP is characteristic of a five-coordinate high spin heme. However, CIP is rather unstable under low laser power irradiation. Local heating induced by the laser beam (Figure 4) causes an irreversible transition to a 6-c HS heme and then a 6-c LS heme. This peculiar instability has not been observed for CCP, CCP-(MI), or HRP-C.

The heme pocket structures of CCP (Poulos et al., 1980; Finzel et al., 1984) and CCP(MI) (Wang et al., 1990) are very similar and show a nonbound distal water molecule at 2.4 Å (CCP) and 2.7 Å [CCP(MI)] above the heme iron, respectively. The heme pocket structures of CIP at 2.6-Å resolution (Petersen et al., 1994) and of ARP at 1.9-Å resolution (Kunishima et al., 1994) are also very similar and with a presumably nonbound water 2.96 Å (in ARP) above the heme iron in agreement with the 5-c high spin heme found in the present study. The RR spectra of CIP were repeated under the same conditions that were used for the crystal growth of CIP, i.e., in 18% (w/v) poly(ethylene glycol) with average molecular weight 6000, 0.35 M $MgCl_2$, buffered at pH 7.0 with 0.1 M HEPES, or ARP, i.e., in a high concentration of ammonium sulfate. The RR spectra are still characteristic of 5-c HS heme (data not shown), indicating that the different procedures used to obtain the crystals do not affect the coordination of the ARP/CIP heme.

Comparison of the heme cavities of CCP and ARP/CIP reveals that they are very similar. The key catalytic residues in CIP (His55, Arg51, Asn92, His183, and Asp245) and in CCP (His52, Arg48, Asn82, His175, and Asp235) are conserved within this superfamily of peroxidases (Welinder, 1992), whereas CIP contains a distal phenylalanine (Phe54) at the position of the Trp51 of CCP. The distal water molecule 415 sitting above the Fe atom of ARP/CIP is hydrogen-bonded to another water molecule 374 which, in turn, is hydrogen-bonded to Arg51. Arg51 is also hydrogen-bonded to water molecule 394 which is further hydrogen-bonded to the propionyl groups. This hydrogen-bond network is also present in CCP and differs only by the presence of an extra hydrogen-bond between the distal water molecule 595 and the Trp51. Loss of this hydrogen-bond in the Trp51Phe mutant of CCP(MI) increases the ability of the distal water to bind to heme iron giving rise to 6-c HS heme in solution at pH 7 (Smulevich et al., 1988; Smulevich, 1993). Therefore, the absence of this anchor in ARP/CIP might explain the appearance of a 6-c heme in CIP upon laser exposure. In HRP-C Phe41 is homologous to ARP/CIP Phe54, and wild-type HRP-C is in fact a mixture of 5- and 6-c HS hemes at neutral pH and room temperature (Smulevich et al., 1994).

When the high frequency region RR spectra of HRP-C, CCP(MI), and CIP are compared (Figure 2), it appears that

the frequencies of the core size marker bands of CIP are similar to those of CCP(MI). The only differences are the lower frequency of the ν_2 mode and the higher frequency of the ν(C=C) modes in CIP (at 1566 and 1625 cm^{-1}, respectively) with respect to CCP(MI) (at 1571 and 1619 cm^{-1}, respectively). Although two bands are expected for the vinyl stretching modes, as observed in HRP-C (at 1624 and 1630 cm^{-1}), only one band was discernible in CCP due to accidental frequency degeneracy as a consequence of equivalent orientation of the two vinyl groups (G. Smulevich, S. Hu, K. R. Rodgers, D. B. Goodin, K. Smith, and T. G. Spiro, unpublished results). A similar situation appears to occur in CIP, and the polarized band observed at 1625 cm^{-1} is therefore assigned to two vinyl stretches. The same behavior is observed in the ferrous forms of CCP(MI) and CIP, as only one band is observed in CCP(MI) (at 1617 cm^{-1}) and in CIP (at 1621 cm^{-1}) whereas, in HRP-C, two ν(C=C) vinyl stretching modes are observed (at 1620 and 1627 cm^{-1}). The ν_2 mode of CIP is at lower frequency than in the other two enzymes. The ν_2 mode is coupled to the vinyl ν(C=C) mode in protohemes, and saturation of the vinyl groups raises the ν_2 frequency by about 10 cm^{-1} (Choi et al., 1982). Therefore, the lower frequency of the ν_2 mode and the higher frequency of the ν(C=C) vinyl stretches in CIP as compared to CCP indicate a higher degree of vibrational coupling between the two modes in CIP. The corresponding vinyl bending modes $\delta(C_\beta C_a C_b)$ of CIP and CCP(MI), which cannot be vibrationally coupled with the ν_2 mode, are also observed at about the same frequencies for both the ferric and ferrous hemes.

The other strong CIP bands observed in the low frequency region at frequencies very similar to those observed for the other peroxidases and heme proteins are assigned to the bending mode $\delta(C_\beta C_c C_d)$ of the propionyl group (at 380 cm^{-1} for ferric and 374 cm^{-1} for ferrous), and ν_8 (at 349 cm^{-1} for ferric and 348 cm^{-1} for ferrous). It is interesting to note the appearance of a strong band at 334 cm^{-1} in the ferric spectrum. This band, which decreases upon conversion of the heme from 5-c HS to 6-c HS (Figure 5) and disappears in the 6-c LS heme (data not shown), is assigned to the γ_6 out-of-plane mode, in agreement with the band observed in CCP(MKT) at 335 cm^{-1}. The intensity of this band in CCP-(MKT), assigned to the out-of-plane mode γ_6 from its large *meso-d*$_4$ shift, appeared particularly sensitive to the ligation state of the heme (G. Smulevich, S. Hu, K. R. Rodgers, D. B. Goodin, K. Smith, and T. G. Spiro, unpublished results). Out-of-plane modes are not expected to be enhanced in the RR spectra of heme proteins. Their appearance is related to the distortion of the porphyrin skeleton, as shown by the study of the ruffled tetragonal crystal form of nickel octaethylporphyrin, which exhibits numerous out-of-plane modes (Li et al., 1989).

The low frequency region RR spectra of 5-c HS ferrous heme is characterized by a strong band due to the stretching ν(Fe−Im) of the bond between the iron atom and the proximal imidazole side chain (His183 in CIP). The RR spectra of all peroxidases studied so far show the presence of this mode at a relatively higher frequency than for other heme proteins and model compounds (Kimura et al., 1981; Teraoka & Kitagawa, 1981; Kitagawa et al., 1983; Desbois et al., 1984; Kuila et al., 1985; Hashimoto et al., 1986; Manthey et al., 1986; Smulevich et al., 1988, 1991b; Dasgupta et al., 1989; Smulevich, 1993). This behavior has been interpreted as reflecting the imidazolate character of the proximal axial ligand, induced by a polar N$^-$···HO hydrogen bond between the axial His ligand and a buried Asp side chain. In fact, the presence of this polar hydrogen bond is a peculiarity of all the peroxidases studied so far, and it has been suggested to have an important role in stabilizing the high oxidation state intermediates of the heme iron (Finzel et al., 1984). The X-ray structure of ARP/CIP shows that the distance between His183 and Asp245 is about 2.9 Å, very close to that observed in CCP. Therefore, a polar hydrogen bond between the fifth ligand and the carboxylate group of Asp245 should occur also for ARP/CIP.

By comparing the 200−250 cm^{-1} region of the RR spectra of CCP(MI) and CIP at pH 7, we can distinguish in the latter a very broad band resulting from the overlap of several bands. In particular the presence of an extra band at around 210 cm^{-1} is observed as compared to the spectrum of CCP(MI). As a consequence of the polar hydrogen bond, the frequency ν(Fe−Im) stretching mode of peroxidases down-shifts at alkaline pH, due to a weakening of the hydrogen bond. This effect was observed in HRP-C (Teraoka & Kitagawa, 1981), CCP (Hashimoto et al., 1986), and CCP(MI) (Smulevich et al., 1988). Moreover, in the latter case two bands at 247 and 233 cm^{-1} were observed at acidic pH, and only the 233 cm^{-1} at alkaline pH. A similar behavior is exhibited by CIP. At alkaline pH the band at 211 cm^{-1} increases in intensity at the expense of the band at 230 cm^{-1}. Therefore, we assign these two bands to two ν(Fe−Im) stretching modes. A down-shift of this mode at alkaline pH with respect to pH 7 is also confirmed by its relative intensity increase with respect to the bands occurring between 340 and 410 cm^{-1}. In particular the ν_8 at 348 cm^{-1} at alkaline pH upshifts by 1 cm^{-1} and weakens in intensity. The same behavior has been previously obtained in the Asp235Asn mutant of CCP(MI) (Smulevich et al., 1988). In this case the replacement of the carboxylate group by a carboxamide group caused the shift of the Fe−His stretching frequency to 205 cm^{-1}. Therefore, the ν_8 is coupled with the ν(Fe−Im) stretching mode as is also demonstrated by its isotopic shift upon $^{54}Fe/^{56}Fe$ substitution in the RR spectra of CCP(MKT) (G. Smulevich, S. Hu, K. R. Rodgers, D. B. Goodin, K. Smith, and T. G. Spiro, unpublished results).

More uncertain is the nature of the band at 247 cm^{-1} in ferrous CIP. This band could be due to another ν(Fe−Im) stretching mode, as in CCP (Hashimoto et al., 1986) and CCP(MI) (Smulevich et al., 1988). Even if this hypothesis cannot be completely ruled out, this band could be assigned also to a heme mode which is enhanced by the coupling with the near Fe−Im stretching mode at 230 cm^{-1}. A similar effect has been previously observed for ferrous Mb, with a ν(Fe−Im) stretching mode at 221 cm^{-1} and a relatively strong band at 240 cm^{-1} with Soret excitation (Bangcharoen-paurpong et al., 1984). A weak band at 247 cm^{-1} is present in the ferric CIP form. In this case the band is certainly due to a porphyrin mode.

The presence of a ν(Fe−Im) stretching mode of CIP at 230 cm^{-1} suggests that the imidazolate character of the proximal ligand is weaker than in CCP (at 247 cm^{-1}) and HRP-C (at 243 cm^{-1}). The band at 211 cm^{-1} at alkaline pH corresponds to a ν(Fe−Im) stretching mode with a fairly weak hydrogen bond between the N_δ−H group and the oxygen atom of Asp245.

NMR studies on the cyanide complexes of CIP (Lukat et al., 1989; Veitch et al., 1994) revealed that the proximal histidyl C_ϵ1H signal has a smaller upfield shift (−22.2 ppm at 25 °C) than for HRP-C [−29.9 ppm at 25 °C (La Mar et al., 1982)], whereas the result is similar to the one reported for CCP [−20.6 ppm at 28 °C, (Banci et al., 1991); −22.0 ppm at 22 °C (Satterlee & Erman, 1991)]. A comparison between NMR and RR data is complicated by the fact that the former method utilized the cyanide adducts of the ferrous form of the proteins (which are 6-c LS), whereas the RR spectroscopy provides information directly of the ferrous form (which are 5-c HS). RR spectroscopy provides a direct measure of the Fe−Im bond strength via the ν(Fe−Im) stretching frequency, whereas proton NMR probes the imidazole nonexchangeable C_ϵ1H proton whose hyperfine shift correlates with the state of the protonation of the axial imidazole (La Mar et al., 1982; Banci et al., 1993, and references therein). Studies on ferrous model porphyrins containing imidazole derivatives as fifth ligands show that the RR frequency of the ν(Fe−Im) stretching mode is sensitive to the bond strength which is influenced by the status of the proton on the bound imidazole (Stein et al., 1980; Hori & Kitagawa, 1980). The lowest frequency (196 cm^{-1}) has been observed for a Fe^{II}-protoporphyrin IX containing a His derivative as fifth ligand and should correspond to an imidazole ring bound to the heme iron with no hydrogen bond interaction (Othman et al., 1993). The hydrogen bond involving the N_δH proton is expected to increase the electronegativity of the imidazole group, the ligand field strength, and therefore the frequency of the ν(Fe−Im) stretching mode. Accordingly, the highest frequency of this mode was observed at 258 cm^{-1} for the mono (His^-) complex to Fe^{II}-protoporphyrin IX (Desbois & Lutz, 1985).

The RR frequency observed in ferrous CIP at 230 cm^{-1} indicates a longer Fe−Im bond than in the other peroxidases. These results agree with the X-ray structure of ferric CCP (Finzel et al., 1984) and ARP (Kunishima et al., 1994), although one must consider that they fall within the limit of experimental error, and also that we are comparing the X-ray data of the ferric species with the RR data obtained for the reduced form of the proteins [the ν(Fe−Im) stretching mode is not active in the RR spectrum of the oxidized form]. In CCP the Fe−N is 2.0 Å, whereas in ARP it appears 0.1 Å longer. As a consequence, in CCP the Fe atom deviates from the heme plane comprised of four pyrrole N atoms, by 0.2 Å, whereas in ARP by only 0.07 Å.

In myoglobin (Mb), in which the N_δ proton of the fifth ligand is hydrogen bonded with a neutral peptide carbonyl group, the ν(Fe−Im) stretch occurs at 220 cm^{-1} (Kitagawa et al., 1979; Argade et al., 1984). The distance obtained from the X-ray structure of deoxy-Mb (i.e., ferrous) (Takano, 1977b) between the N_δH and the O of the carbonyl group of Ser7F is 3.3 Å. Thus, not only is the hydrogen-bonding interaction stronger in CCP and in all peroxidases so far studied by X-ray crystallography, but the electrostatic environment of the proximal His is considerably more negative than in the globins, whose hydrogen bond acceptor is a peptide carbonyl oxygen atom. On the other hand, both CCP and ARP/CIP exhibit comparable distances (2.9 Å) between the N_δ atom of the proximal ligand and the O atom of the carboxylate group of Asp. The different imidazolate character of CCP and CIP could derive from a different geometry, such as linear or bent, of the O···H···N_δ bond which gives rise to a different strength of the hydrogen bond interaction.

Other parameters, deriving from the interaction between the π orbitals of the porphyrin and the σ^* orbitals of the Fe−Im, which influence the frequency of the Fe−Im stretching mode, must also be considered: (1) Protein-induced proximal strain defined by the tilt angle θ between the heme normal and the Fe−Im vector and the displacement of the iron from the heme plane (Bangcharoenpaurpong et al., 1984; Friedman et al., 1990); (2) the azimuthal angle ϕ, which denotes the rotation of the projection of the His plane on the porphyrin plane with respect to the N(1)−Fe−N(3) axis (Bangcharoenpaurpong et al., 1984). These parameters are interrelated in that, for a given θ, a more out-of-plane iron is associated with a smaller ϕ. Comparison of CCP and CIP reveals that the two proteins have a similar tilt angle θ, about 7° for CCP and 3° for ARP/CIP, whereas the iron displacement seems slightly bigger for CCP (0.2 Å) than for ARP/CIP (0.07 Å). The azimuthal angles are quite different, however. In CCP the His plane is approximately colinear with the N(1)−Fe−N(3) heme axis (ϕ about 7°), in ARP/CIP the His plane is rotated about 40°. Therefore, according to the model of Bangcharoenpaurpong et al. (1984), in which a decrease in ϕ leads to an increase in the Fe−Im stretch frequency, the observed differences in Fe−Im stretching frequencies of the two proteins cannot depend on the θ angle but possibly on the ϕ angle. In accord with this view, the frequency of the ν(Fe−Im) stretching mode of *Scapharca inaequivalvis* hemoglobin is about 15 cm^{-1} lower (203 cm^{-1}) than those observed for sperm whale myoglobin (Mb) and human hemoglobin (Song et al., 1993). On the basis of NMR (La Mar et al., 1983, 1985) and X-ray (Takano, 1977a,b; Fermi, 1975) structural data, it appears that the ϕ angle is very small for Mb and human hemoglobin, whereas it is about 40° for *S. inaequivalvis* hemoglobin (McGourty et al., 1989).

CONCLUSIONS

Recombinant CIP gives rise to RR spectra mainly characteristic of a five-coordinate high spin heme. The spectra of both the ferric and the ferrous forms are quite similar to those obtained previously for CCP, except for the frequencies of the ν_2 and ν(C=C) modes which are indicative of a higher degree of vibrational coupling between the two modes in CIP. Comparison of the heme cavity structures of CCP and ARP/CIP reveals that they are very similar. The distal catalytic Arg and His residues are conserved in the two proteins, whereas CIP contains a distal Phe54 at the position of Trp51 in CCP. Absence of a distal Trp hydrogen bond to the water molecule above the heme Fe atom might increase the ability of this water molecule to bind to the heme iron as observed during prolonged laser exposure.

The 200−250 cm^{-1} region of the RR spectra of Fe^{II} CIP is characterized by a very broad profile resulting from the overlap of several bands. On the basis of the relative intensity changes observed in the low frequency spectral region at neutral and alkaline pHs, it has been concluded that the Fe−Im RR band of ferrous CIP has two components at 230 and 211 cm^{-1}. In fact the populations of the two forms change with pH, as the 230-cm^{-1} band is more intense at neutral pH, whereas the 211-cm^{-1} band increases in

intensity at the expense of the band at 230 cm^{-1} at alkaline pH. The fairly strong band at 247 cm^{-1} which seems independent of the pH is assigned to a porphyrin mode which is coupled with the the ν(Fe−Im) stretching mode. However, it cannot be excluded that this band is due to another ν(Fe−Im) stretching mode whose frequency corresponds well to those observed in CCP and HRP.

At neutral pH the RR frequencies indicate that the Fe−Im bond is stronger than at alkaline pH, due to a stronger hydrogen bond between the N_δ−H group of the fifth ligand and the O atom of Asp245. The RR frequency of the ν-(Fe−Im) stretching mode suggests a weaker imidazolate character of the proximal ligand in CIP as compared to CCP and HRP-C. However, additional electronic interactions depending on structural parameters, such as the tilt and azimuthal angles, might shift the RR frequency.

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