The Distal Cavity Structure of Carbonyl Horseradish Peroxidase As Probed by the Resonance Raman Spectra of His 42 Leu and Arg 38 Leu Mutants†

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Received June 15, 1998; Revised Manuscript Received July 28, 1998

ABSTRACT: CO ligation to horseradish peroxidase C (HRPC) was studied by means of site-directed mutagenesis and resonance Raman spectroscopy. The CO complexes of HRPC His 42 → Leu and Arg 38 → Leu mutants were characterized at pH values ranging from 3.6 to 9.5. The vibrational frequencies of the Fe−C stretching and Fe−C−O bending modes have been identified by isotopic substitution. Both His 42 → Leu and Arg 38 → Leu adducts with CO displayed a single Fe−C stretching band, whereas both recombinant and wild-type HRPC−CO have two bands, corresponding to different conformers. This comparison suggests that CO is H-bonded either to the distal Arg or to the distal His in the two conformers. An acid transition, common to the wild-type protein, was observed for both mutants. This indicates that these distal amino acids do not influence the acid transition. On the contrary, an alkaline transition was only observed for the Arg 38 → Leu mutant, which suggests that distal His is involved in the alkaline transition of HRPC−CO complex. The spectroscopic information is found to be consistent with the X-ray structure of ferric HRPC. A comparison with the CO complexes of cytochrome *c* peroxidase and myoglobin is performed, which displays the functional significance of the structural differences between peroxidase classes I and III and between peroxidases and globins, respectively.

The study of CO ligation to heme proteins is attracting growing interest since CO can act as a sensitive probe of the active site, where the natural substrates, oxygen for oxygen carriers and hydrogen peroxide for peroxidases, bind to iron. CO forms a relatively stable bond with Fe (II) proteins (*1*). This allows for steady-state spectroscopic measurements to be performed. In particular, vibrational spectroscopy can detect the vibrational modes involving the Fe−C and C−O bonds. The C−O stretching mode is more easily detected by infrared (IR) absorption rather than by resonance Raman (RR)[1] spectroscopy, while the Fe−C stretching and Fe−C−O bending modes are practically detectable only by RR spectroscopy (*2*). The main results obtained by vibrational spectroscopy for heme peroxidases can be schematically summarized: (i) the CO complexes of heme peroxidases strongly differ from sterically hindered model CO−porphyrin complexes and display the interactions between CO and adjacent amino acids; (ii) multiple, pH-dependent conformers are present in the CO complexes of horseradish peroxidase (HRP) (*3*−*6*) and of cytochrome *c* peroxidase (CCP) (*7*); and (iii) structural information inferred from RR and IR spectra of the CO complexes is also useful for understanding the molecular basis of the catalytic activity of peroxidases (*8*). The study of CO−peroxidase complexes has benefitted greatly from the availability of site-directed mutants. In fact, comparisons between wild-type and mutated proteins have enabled a clarification of which CO−amino acid interactions are most important. On the other hand, CO complexes give important insights for the structures of the site-mutated variants which are especially important when high-resolution X-ray determinations are not available.

In this study, we have considered two site-mutated derivatives of recombinant HRP isoenzyme C (HRPC*) which differ from the native enzyme in two key distal amino acids, His 42 and Arg 38 (*9*−*13*). Both amino acids are directly involved in the enzymatic activity. Polar interactions and hydrogen-bonding were proposed to occur between these amino acids and peroxide in HRP. We have measured the RR spectra of the CO complexes of His 42 → Leu (H42L) and Arg 38 → Leu (R38L) HRPC* mutants in solution at various pH values ranging from 3.5 to 10, since the structure of HRP and its CO complex is known to be dependent on pH. The results allow us to definitively assign the RR spectra of the HRP−CO complex. The various conformers are correlated with different interactions between the oxygen

† This work was supported by the Italian Consiglio Nazionale delle Ricerche (CNR) and Ministero Università e Ricerca Scientifica e Tecnologica (MURST ex 60% and ex 40%, Cofin. MURST97 CFSIB) (A.F., G.S.), Ministerio de Educacion y Ciencia (Spain) Project ALI96-1111-C04-01 (J.N.R.-L.), the EC HCM program (Contract ERB CHRX-CT 92-0012), and the U.K. Biotechnology and Biological Sciences Research Council (R.N.F.T.).

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[1] Abbreviations: RR, resonance Raman; IR, infrared; HRPC, horseradish peroxidase isoenzyme C; HRPC*, recombinant horseradish peroxidase isoenzyme C; CCP, cytochrome *c* peroxidase; CCP(MI), recombinant cytochrome *c* peroxidase carrying N-terminal Met and Ile; H42L, His 42 → Leu HRPC* mutant; R38L, Arg 38 → Leu HRPC* mutant; ν_{FeC}, Fe−C stretching vibrational mode; ν_{CO}, C−O stretching vibrational mode; δ_{FeCO}, Fe−C−O bending vibrational mode; H52L, His 52 → Leu CCP(MI) mutant; R48L, Arg 48 → Leu CCP(MI) mutant; H64L, His 64 → Leu myoglobin mutant.

S0006-2960(98)01399-3 CCC: $15.00
Published on Web 09/05/1998

atom of CO and the distal amino acids. The results are compared with the previous data obtained for the CCP−CO complex and discussed in the light of the recently solved X-ray structure of HRPC* (*14*).

MATERIALS AND METHODS

The procedure for the production of HRPC* and its mutants has been described elsewhere (*9, 15*). Protein concentrations, pH values, and buffer types are reported for each individual sample in the figure captions. The ionic strength of all the buffers was adjusted to 0.1 M considering the concentrations and the dissociation constants of the components, while no additional salts were added. The pH was measured directly in the sample after the RR measurement by means of a microelectrode. The CO complexes were prepared by first flushing the ferric protein solution with nitrogen, then flushing with ^{12}CO (Rivoira, Italy) or adding ^{13}CO (FluoroChem, UK) and reducing the protein with 2−10 μL of a fresh sodium dithionite (20 mg/mL) solution.

The RR spectra were obtained by excitation with the 413.1 nm line of a Kr^+ laser (Coherent, Innova 90/K). The backscattered light from a slowly rotating NMR tube was collected and focused into a double monochromator (Jobin-Yvon HG2S 2000), equipped with a cooled photomultiplier (RCA C31034A). The power at the sample was 1.8 mW. We checked by the RR spectra in the ν_4 mode region that the amount of 5-coordinated ferrous species due to laser photolysis was low enough not to appreciably contribute to the RR spectra in the 450−630 cm^{-1} region. The RR spectra were calibrated with the intense low-frequency bands of indene and CCl_4 to an accuracy of 1 cm^{-1} for isolated bands.

RESULTS

Figure 1 shows the low-frequency RR spectra of the CO complexes of HRPC* and its H42L and R38L mutants. The spectrum of HRPC*−CO, measured at pH 6.3, is similar to the spectra of commercial HRPC samples at pH 6.0 (*4*) or pH 7.0 (*5, 6*), with minor differences in the relative band intensities. Four bands are observed at 497, 516, 539, and 590 cm^{-1}. The bands at 516 and 539 cm^{-1} have been shown to shift to lower wavenumbers when natural abundance CO was substituted with either $^{13}C^{16}O$ or $^{12}C^{18}O$ and therefore have been assigned to two ν_{FeC} modes, while the band at 497 cm^{-1} has been assigned to a porphyrin mode since it is not sensitive to the isotopic substitution (*5*). The band at 590 cm^{-1} is a superposition of a porphyrin band and of a band which shifts to lower wavenumbers with $^{13}C^{16}O$ substitution but not with $^{12}C^{18}O$ substitution. The latter band has been therefore assigned to the δ_{FeCO} fundamental mode (*4, 5*). An alternative assignment of the 570−590 cm^{-1} band in the CO complexes of heme proteins to a δ_{FeCO} overtone (*16*) or combination mode (*17*) has been confuted on the basis of the comparison with metal carbonyl complexes (*18*) and FT-IR data on a model compound (*19*). The bands at 497 and 590 cm^{-1} are also observed in the spectra of both mutants, while the two ν_{FeC} bands are replaced by a single intense band at 515 and 525 cm^{-1} for R38L and H42L, respectively. The effect on the mutant complexes of substituting ^{12}CO with ^{13}CO is also shown. The band at 525 cm^{-1} of H42L−CO displays an isotopic shift of 8 cm^{-1}, while the band at 515 cm^{-1} of R38L−CO shifts to 510 cm^{-1}. These bands are therefore assigned to the ν_{FeC} mode. In R38L−CO, there is an additional isotope-sensitive band. The shoulder at approximately 579 cm^{-1}, which overlaps with the 590 cm^{-1} band, shifts to 565 cm^{-1}. The position and the relatively large isotopic shift of this band allow its assignment to the δ_{FeCO} mode. The corresponding band for H42L−CO may be too weak to be detected.

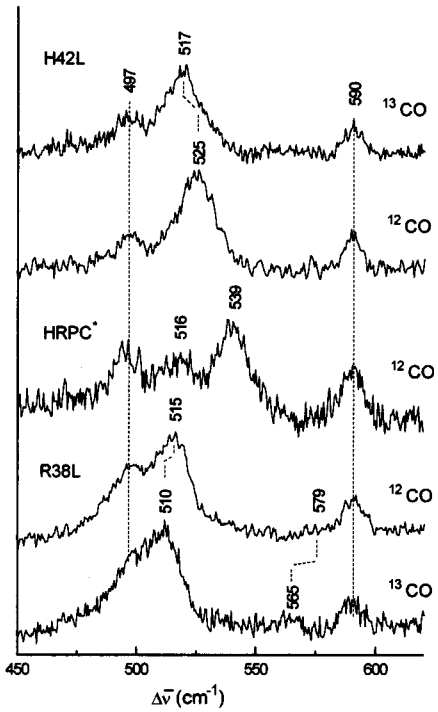

FIGURE 1: (from top to bottom) RR spectra of the complexes of H42L (pH 6.1, 90 μM) with ^{13}CO and ^{12}CO, HRPC* (pH 6.3, 100 μM) with ^{12}CO, and R38L (pH 6.1, 80 μM) with ^{12}CO and ^{13}CO. All samples were in sodium phosphate buffer solution. All spectra were measured with excitation wavelength 413.1 nm and spectral resolution 5 cm^{-1}. The integration times were 40 s/0.5 cm^{-1} (H42L−^{13}CO), 71 s/0.5 cm^{-1} (H42L−^{12}CO), 58 s/0.5 cm^{-1} (HRPC*−^{12}CO), 86 s/0.5 cm^{-1} (R38L−^{12}CO), and 28 s/0.5 cm^{-1} (R38L−^{13}CO).

Figure 2 displays the pH dependence of the CO complexes of HRPC* and both mutants. The HRPC*−CO spectrum at pH 3.6 is identical to the spectrum of the CO complex of commercial HRPC (*6*). The bands at 492 and 524 cm^{-1} have been assigned as the ν_{FeC} of two species which are present at acid pH. The 492 cm^{-1} species is a stable, six-coordinated complex, while the 524 cm^{-1} species is a metastable form where the heme iron is either bound to water or not bound on the proximal side. The spectra of H42L−CO and R38L−CO are essentially unchanged at this pH with respect to the native enzyme, with only slight frequency (490 and 522 cm^{-1}) and intensity differences. At alkaline pH, the CO complexes of HRPC* and the mutants behave very differently. The spectrum of HRPC*−CO is similar to the published RR spectrum of commercial HRPC−CO at alkaline pH (*5*). The two bands at 516 and 539 cm^{-1} at pH 6 merge into a broad band with a maximum at about 530 cm^{-1} at pH 9. The other bands are not changed. R38L−CO samples at alkaline pH display time-dependent RR spectra. In the first scans, the most intense band is broad and centered at about 500 cm^{-1}. This is possibly a superimposition of the bands at 497 and 515 cm^{-1} of the

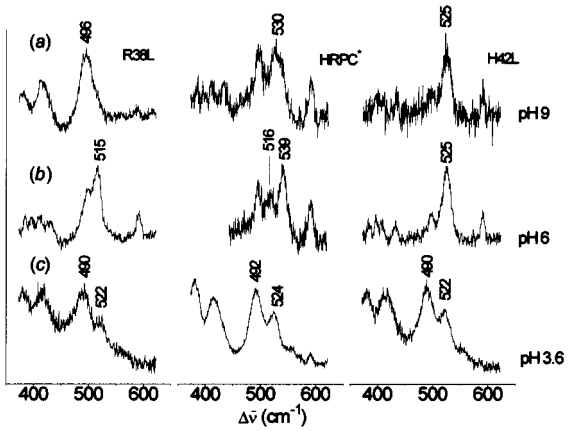

FIGURE 2: (from top to bottom and from left to right) RR spectra of the CO complexes of (*a*) R38L pH 9.0, 80 μM, HRPC* pH 9.2, 200 μM, and H42L pH 9.5, 90 μM, in borate buffer; (*b*) R38L, HRPC*, and H42L, same pH and concentrations as in Figure 1; and (*c*) R38L, 20 μM, HRPC*, 300 μM, and H42L, 20 μM, in formate buffer, pH 3.6. All spectra were measured with excitation wavelength 413.1 nm and spectral resolution 5 cm^{-1}. The integration times were (*a*) 24 s/0.5 cm^{-1} (R38L), 95 s/0.5 cm^{-1} (HRPC*), and 22 s/0.5 cm^{-1} (H42L); (*b*) same as in Figure 1; and (*c*) 26 s/0.5 cm^{-1} (R38L), 18 s/0.5 cm^{-1} (HRPC*), and 4 s/0.5 cm^{-1} (H42L).

neutral form with a new band at 496 cm^{-1}. After about 1 h, the band at 496 cm^{-1} reaches its maximum intensity and the spectrum does not change further. The sample eventually aggregates and precipitates. The absorption spectra of R38L also show a time-dependent behavior (data not shown). The maximum of the Soret band shifts from 422 to 421 nm within 2 h after the complex formation, while the β band shifts from 541 to 539 nm. The changes observed in the RR spectra are possibly related to the absorption changes. Additional effects could be induced by the laser irradiation during the RR spectra acquisition. The behavior of the H42L−CO complex at alkaline pH markedly differs from that of HRPC*−CO and R38L−CO since its RR spectrum is identical to the spectrum at pH 6.

Figure 3 shows pH-dependent measurements which demonstrate the reversibility of the changes induced by acid pH in HRPC*−CO and by alkaline pH in R38L−CO. In (a), HRPC*−CO was prepared at acid pH and its RR spectrum was measured. Then, the sample was flushed with nitrogen gas for 2 h in order to remove CO. After flushing, the pH was changed to 6.7 with a concentrated buffer solution. The sample was again saturated with CO, and more dithionite was added. The spectrum of the resulting complex is comparable to the HRPC*−CO spectra at acid to neutral pH. It is noteworthy that if CO is not removed before changing the solution pH to 6.7, the neutral form is not obtained. The only change is a shift of the 492 cm^{-1} band to 496 cm^{-1} and an intensity decrease of the 524 cm^{-1} band. In (b), a similar protocol was applied to R38L−CO. The sample was prepared at pH 10, and the RR spectrum was measured after 1 h in order to obtain the stable form shown in Figure 2. Then, the sample was flushed with nitrogen, its pH was changed to 6, and CO and dithionite were added. Even if the spectrum of R38L−CO at acid pH is not fully recovered, the band at 515 cm^{-1} is clearly detectable. It was not possible to extend the study, for example, with a longer nitrogen flushing time, since the sample tends to precipitate some time after the preparation. Removing CO

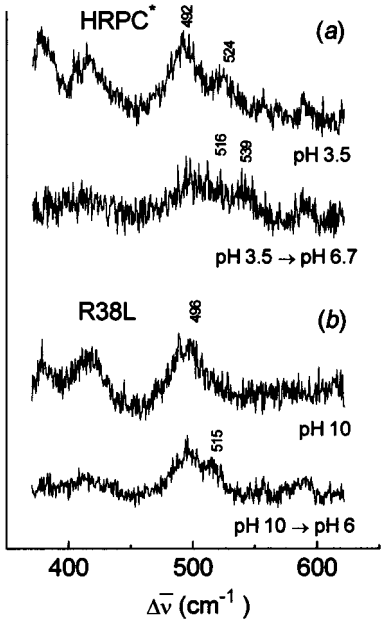

FIGURE 3: (*a*) RR spectra of a HRPC*−CO sample at pH 3.5 and of the same sample after flushing it with nitrogen, changing the pH to 6.7, and allowing CO to bind again; (*b*) RR spectra of an R38L−CO sample at pH 10 and of the same sample after flushing it with nitrogen, changing the pH to 6.3, and allowing CO to bind again.

was an essential step as for HRPC*−CO at acid pH. If R38L−CO formed at pH 10 was not flushed with nitrogen gas, its RR spectrum did not change with pH down to pH 4, where some amount of the acid form appears.

DISCUSSION

(a) Structural Features of the Two HRPC−CO Conformers. The presence of two conformers of the HRPC−CO complex at neutral pH was evident both in the IR (*20*) and in the RR spectra (*5*). The RR ν_{FeC} bands at 519 and 541 cm^{-1} of the two conformers have been shown to correspond to the IR ν_{CO} bands at 1932 and 1906 cm^{-1}, respectively. Our results give a clear indication that the existence of these two conformers derives from different CO−protein interactions, which also influence the vibrational frequencies. It has been shown (*18*, *21*) that a negative correlation exists between ν_{FeC} and ν_{CO} frequencies for a large class of CO−porphyrin adducts (including both model porphyrins and heme proteins), depending on the extent of π back-bonding, that is, electron back-donation from the Fe d_π to the CO π^* orbitals. Back-bonding increases the Fe−C bond order and decreases the CO bond order, thereby increasing ν_{FeC} vibrational frequencies and decreasing ν_{CO} frequencies. Figure 5 shows a plot of ν_{FeC} versus ν_{CO} frequencies for HRPC*−CO, R38L−CO, H42L−CO, and other CO−protein complexes selected for comparison. Within this frame we can propose a structural model for the two HRPC−CO conformers.

In one conformer, the oxygen atom of bound CO mainly interacts with Arg 38 via hydrogen bond (Figure 4*a*). In fact, its ν_{FeC} band at 539 cm^{-1} disappears when Arg 38 is substituted with leucine (Figure 1). A direct evidence for H-bonding is the H/D shift (ca. 2 cm^{-1}) of the IR ν_{CO} band at 1906 cm^{-1} (*5*, *10*, *20*, *22*). H-bonding from Arg 38 has been proposed also for the complexes of HRPC with OH$^-$

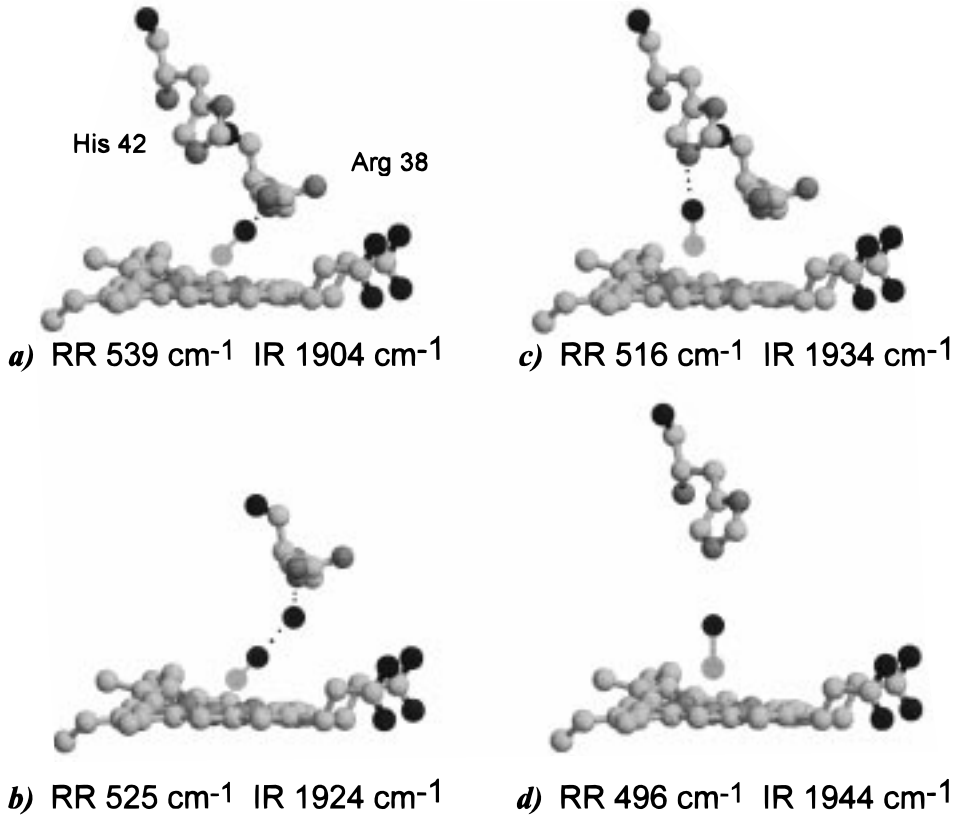

FIGURE 4: Proposed structures for the different conformers in HRPC*−CO (*a* and *c*) and H42L−CO (*b*) at neutral pH, and in R38L−CO at alkaline pH (*d*). The vibrational frequencies are from Table 1. Proposed hydrogen bonds are represented by dots. The Fe−C bond is not drawn for graphical reasons. In *a* and *c*, the heme and amino acid disposition for HRPC* are taken from the ferric protein structure deposited at the Brookhaven Protein Data Bank, PDB code 1ATJ (ref *14*).

(*23*), CN^- (*24*), and F^- (*25*). Moreover, positive interactions with CO have been observed to be most effective in increasing π back-bonding (*18*). In this context, it is reasonable to conclude that CO is directly H-bonded to the positively charged guanidinium group of Arg 38 in the 539 cm^{-1} conformer, as previously proposed (*10*).

The ν_{FeC} shift from 539 to 525 cm^{-1} (the latter corresponding to $\nu_{CO} = 1924$ cm^{-1} (*10*)) observed in the H42L mutant indicates that the substitution of His42 with leucine also influences the Arg 38−CO interaction. Both the ν_{FeC} and the ν_{CO} frequencies of H42L−CO are close to the acid form of the CO complex of baker's yeast CCP (*7*). Considering that the distal Arg is extremely flexible in peroxidases (*26*), a possible explanation is that the His 42 → Leu mutation induces a rearrangement of the distal pocket so that CO is H-bonded with a water molecule, which is in turn H-bonded with Arg 38 (Figure 4*b*), as in CCP−CO (see below).

In the second HRPC−CO conformer, the oxygen of the bound CO molecule mainly interacts with His 42 (Figure 4*c*). Its ν_{FeC} frequency (516 cm^{-1}) is unaffected by the Arg 38 → Leu mutation (Figure 1). The question arises whether the interaction with His 42 is only of a polar nature or if it also involves H-bonding. An H/D isotopic shift (ca. 1 cm^{-1}) has been reported for the IR ν_{CO} band of this conformer at 1934 cm^{-1} (*5*, *10*, *20*, *22*). H-bonding from His 42 to CO implies that $N_{\epsilon 2}$ is protonated at pH 6−7, where the 516 cm^{-1} conformer is present. On the other hand, $N_{\delta 1}$ of His 42 at neutral pH is H-bonded to the $O_{\delta 1}$ of Asn 70 in HRPC (*14*) as well as in all the other peroxidases so far studied via X-ray diffraction (*27*). Mutagenesis of Asn 70 has highlighted the role of Asn 70 in maintaining the basicity of distal His (*28*, *29*). If the $N_{\delta 1}$−H bond is conserved in the CO complex, then His 42 must have a pK_a > 7 to be a H donor to CO. There is evidence both from the literature and from our results that the pK_a of His 42 in HRP−CO is in fact > 7. This issue is discussed in section (b). Moreover, H-bonding from His 42 to CO is geometrically favorable, as can be judged from the X-ray structure of ferric HRP (*14*). Since His 42 $N_{\epsilon 2}$ is 6.0 Å above the Fe atom in a nearly axial position, CO can fit in betweeen the central Fe and His 42 and build a linear H bond with $N_{\epsilon 2}$. Therefore, we suggest that CO is H-bonded to His 42 in the second conformer.

(b) pH Dependence of the Interactions between CO and the Distal Amino Acids. It is evident from the spectra shown in Figure 2 that the mutations of the distal amino acids Arg 38 and His 42 do not have effects on the structure of the CO complexes at acid pH. In fact, the RR spectra at pH 3.5 of the native enzyme and both mutants are very similar. Previous work (*6*) has shown that an acid transition takes place in HRPC−CO. The very low frequency of the ν_{FeC} (492 cm^{-1}), together with a high frequency of the ν_{CO} (1967 cm^{-1}), points to very weak π back-bonding (Figure 5), consistent with a slight or even absent interaction with the surrounding amino acids. This result clearly contradicts the proposal (*22*) that His 42 is involved in the acid transition of HRPC−CO with a pK_a of about 4 as in the ferric enzyme. On the other hand, His 42 deprotonation can be correlated instead with the *alkaline* transition of HRPC−CO with pK_a

= 8.3 (*5*). This interpretation is further supported by the RR (Figure 2) and IR spectra (*10*) at alkaline pH, showing that H42L−CO lacks an alkaline transition. Therefore, we exclude a role for the distal histidine in the acid transition, and favor instead the previous proposal (*30*) that the origin of the acid transition is the protonation of a heme propionate.

There is a discrepancy between the RR results at acid pH and the corresponding IR results (*10*). In fact, the IR spectra show differences among the mutated and the wild-type enzyme at acid pH, but there are no changes between pH 6 and 3.3 for R38L−CO and H42L−CO. This divergence in the results must be ascribed to the different way of preparing the CO complexes. In fact, it has been shown that the final six-coordinated form of HRPC−CO at acid pH is dependent on the procedure followed. If the CO adduct was formed at pH 7 and then the pH was changed to a value higher than 3.1, the transition from the neutral to the acid form was incomplete or did not take place (*6*, *30*). The final six-coordinated acid form was observed only when CO was directly added to a reduced protein at acid pH (*6*). The samples for RR spectra were reduced and bound with CO already at acid pH; therefore the protein had the necessary time to reach the acid stable conformation. On the other hand, the CO complexes for IR were prepared at neutral pH, and the pH was subsequently changed with a concentrated buffer. It is thus conceivable that the proteins were still in their neutral forms within the measurement time necessary for the IR spectra (few minutes). This explains why the IR spectra of neither R38L−CO nor H42L−CO display any change between pH 3.3 and 6.0. Moreover, IR measurements of H42L−CO and R38L−CO at longer times (Rodriguez-Lopez, J. N., George, S. J., and Thorneley, R. N., unpublished experiments) show that a band at about 1965 cm^{-1} grows after 40 min. This band has been shown (*6*) to correspond to the 492 cm^{-1} RR band in HRPC−CO, and it can be safely related to the band at 490 cm^{-1} in the mutant complexes. We also observed that the acid transition occurs faster in the RR experiments than in the IR experiments, possibly because of the laser irradiation. In fact, R38L−CO and H42L−CO samples which were prepared the same way as that of the IR measurements displayed the features of the neutral species only in the first 10 min (data not shown).

While the RR spectra at acid pH are not affected by mutation of Arg 38 and His 42, the spectra of the CO complexes of HRPC*, R38L, and H42L at alkaline pH show differing behavior. H42L−CO is not subject to changes in the pH range 6.0−9.5. This is consistent with CO being H-bonded (through water) to Arg 38 in this mutant, since Arg 38 has a higher pK_a than 9.5. On the other hand, R38L−CO is less stable at alkaline pH than H42L and HRPC* and its RR spectra point to a definite change in geometry of the bound CO with respect to pH 6. The ν_{FeC} frequency (496 cm^{-1}) is close to the value of the acid forms of HRPC−CO (*6*) and myoglobin−CO (*31*), for which an open structure has been proposed. This suggests that alkaline R38L−CO is also an open form, with no interaction with His 42 (Figure 4*d*). An open geometry is also consistent with the recombination kinetics of R38L−CO at pH 8.5 after laser photolysis (*9*). The alkaline transition for this mutant, which was also observed in the IR spectra (*10*), can be ascribed to the deprotonation of His 42.

Table 1: Vibrational Frequencies (cm^{-1}) of the ν_{FeC} and ν_{CO} Modes of the CO Complexes of Various Heme Proteins[a]

	ν_{FeC}	ν_{CO}
HRPC*, pH 6	539[b]	1906[c]
CCP, pH 7 (form II)	530[d]	1922[d]
HRPC*, alkaline pH	530[b]	1934[c]
HRPC* (H42L), pH 6−9.5	525[b]	1924[c]
CCP (H52L), pH 9	522[e]	1928[e]
HRPC*, pH 6	516[b]	1934[c]
HRPC* (R38L), pH 6	515[b]	1941.5[c]
CCP (H52L), pH 6	508[e]	1944[e]
CCP (form I), alkaline pH	503[d]	1948[d]
CCP (R48L), acid pH	500[f]	1941[f]
CCP (R48L), alkaline pH	500[f]	1951[f]
HRPC* (R38L), alkaline pH	496[b]	1944[c]
HRPC, acid pH	492[g]	1967[g]
Myoglobin (H64L)	489[h]	1966[i]

[a] The ν_{FeC} frequencies are plotted versus ν_{CO} frequencies in Figure 5. [b] This work. [c] Ref 10. [d] Ref 7. [e] Ref 34. [f] Ref 33. [g] Ref 6. [h] Ref 38. [i] Ref 37.

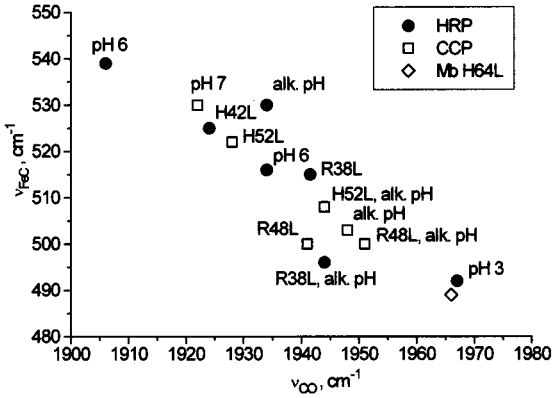

FIGURE 5: Plot of observed ν_{FeC} frequencies versus ν_{CO} frequencies in the CO complexes of HRPC* and its mutants (●), of CCP and its mutants (□), and of the His 64 → Leu mutant of myoglobin (◇). The frequencies are from Table 1.

It is not easy to reconcile the results for the mutants at alkaline pH with the RR spectrum of HRPC*−CO at pH 9 (Figure 2*a*). This spectrum is characterized by a very broad ν_{FeC} band centered at about 530 cm^{-1} which corresponds to a broad IR ν_{CO} at 1933 cm^{-1} (*3*, *22*). We tentatively assign the RR band to a superposition of a conformer which is similar to H42L−CO, that is, H-bonded to Arg 38 through water (Figure 4*b*), and of a conformer which still interacts with His 42 but is not H-bonded. On the basis of the above considerations concerning the interaction between His 42 and CO, His 42 deprotonation seems to be the trigger for this alkaline transition.

(c) Comparison with the CO Complexes of CCP. In contrast to HRPC, baker's yeast CCP−CO in solution at pH 6.0 shows only one CO conformer whose IR and RR signatures have been assigned to a form with CO H-bonded with a distal residue (*7*). Actually the X-ray structure of CCP−CO (*26*) showed that CO is 12° tilted and H-bonded with a water molecule, which in turn is H-bonded with the distal arginine (Arg 48). Moreover, upon complexation with CO, the Arg 48 terminal nitrogen atoms move away from the ligand by about 0.6 Å with respect to ferric CCP. By comparing the structures of ferric CCP (*32*) and HRPC* (*14*), we noticed that the terminal nitrogen atoms of the distal Arg are located at a shorter distance (about 1 Å) from the heme iron in HRPC* than in CCP. As a consequence of this

structural difference, it can be hypothesized that CO is tilted and directly H-bonded with Arg 38 in HRPC*−CO, as represented in Figure 4*a*. The 9 cm^{-1} upshift of the ν_{FeC} band and the 18 cm^{-1} downshift of the ν_{CO} band of the first conformer of HRPC*−CO as compared to CCP−CO (Table 1 and Figure 5) confirm that the interaction with CO is much stronger in HRPC* than in CCP in solution as well as in crystals. This result agrees with the conclusions obtained in the study of compound I formation for the distal Arg and His mutants of both CCP and HRPC*. In fact, it has been shown that the removal of the positively charged guanidinium group of Arg from the active site is more critical in HRPC* than in CCP (*11*). Therefore, the distal Arg must interact with the incoming ligand much more strongly in HRPC* than in CCP.

The finding that the conserved distal His is not capable of interacting with the incoming CO in CCP is intriguing (*26*). A comparison of the structures of the ferric proteins indicates that not only the distal Arg but also the distal His is more favorably positioned in HRPC* to directly interact with the incoming CO than in CCP (*26*). The conclusion that the distal Arg in CCP is the only residue stabilizing the incoming CO is also evident from the RR data for the Arg 48 → Leu CCP(MI) mutant which show a CO adduct at neutral pH with no H-bond interactions (*33*). Moreover, the results obtained on the His 52 → Leu CCP (MI) mutant (H52L) clearly show that the His 52 residue does not titrate in the acid−alkaline transition (*34*). As a consequence, two different CO forms have been found at acid and alkaline pH for the CO complex of H52L, both closely resembling the corresponding forms of the native enzyme (Table 1, Figure 5).

(d) Comparison with the CO Complexes of Myoglobin Mutants. CO adducts with myoglobins have been more intensely investigated than CO adducts with peroxidases (*1*), particularly with the aim of clarifying the O_2−CO discrimination mechanism of globins. An outstanding feature of CO−myoglobin adducts is the presence of multiple conformational substates, observed in the IR absorption spectra (*35*), despite the fact that only distal histidine can interact with bound CO. A simple molecular model has been proposed for the conformational substates of CO−myoglobin on the basis of distal His tautomerism, which has also been tentatively applied to HRPC (*36*). Our results show that the origin of the band multiplicity in the RR and IR spectra of HRPC−CO is different. The comparison of H42L−CO with the CO complex of the His 64 → Leu myoglobin mutant is particularly dramatic. A single ν_{CO} band at 1966 cm^{-1} (*37*) and a single ν_{FeC} at about 490 cm^{-1} (*38, 39*) are observed. A reduced degree of π back-bonding is evident from these frequencies, indicating little interaction with the distal cavity amino acids. In contrast, H42L−CO exhibits pronounced frequency shifts due to the effect of the distal arginine on π back-bonding (Table 1, Figure 5). The presence of an arginine residue which is able to H-bond the substrate is a key difference between peroxidases and globins, and the many aspects of its functional significance have been discussed in detail (*27*).

CONCLUSIONS

Despite the fact that two distal key residues, His and Arg, are conserved in both CCP and HRPC, as well as in all other heme peroxidases studied so far (*40*), the present results show that they play a different role in stabilizing the exogenous CO ligand. Both residues in HRPC are involved in H-bond interactions with the coordinated CO, whereas for CCP only the distal Arg appears to be involved. The different geometric dispositions and pK_a's of the distal residues in the two proteins are considered to be responsible for the different stabilization mechanisms. In particular, the shorter distance between the distal Arg and the heme iron in HRPC relative to CCP accounts for the stronger H-bond interaction with the bound CO. Accordingly, the distal Arg appears to play a more important role in the binding of hydrogen peroxide in HRPC than in CCP, as shown in earlier kinetic studies on compound I formation (*11*).

ACKNOWLEDGMENT

J.N.R.-L. and R.N.F.T. are grateful to Dr. S. J. George for helpful discussions.

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BI981399V