Heme Pocket Interactions in Cytochrome *c* Peroxidase Studied by Site-Directed Mutagenesis and Resonance Raman Spectroscopy†

Giulietta Smulevich,*,‡ J. Matthew Mauro,§ Laurence A. Fishel,§ Ann M. English,∥ Joseph Kraut,§ and Thomas G. Spiro*,⊥

Dipartimento di Chimica, Università di Firenze, Via G. Capponi 9, 50121 Firenze, Italy, Department of Chemistry, University of California, San Diego, La Jolla, California 92093, Department of Chemistry, Concordia University, Montreal, Quebec, Canada H3G 1M8, and Department of Chemistry, Princeton University, Princeton, New Jersey 08544

Received January 28, 1988; Revised Manuscript Received April 4, 1988

ABSTRACT: Resonance Raman spectra are reported for Fe^{II} and Fe^{III} forms of cytochrome *c* peroxidase (CCP) mutants prepared by site-directed mutagenesis and cloning in *Escherichia coli*. These include the bacterial "wild type", CCP(MI), and mutations involving groups on the proximal (Asp-235 → Asn, Trp-191 → Phe) and distal (Trp-51 → Phe, Arg-48 → Leu and Lys) side of the heme. These spectra are used to assess the spin and ligation states of the heme, via the porphyrin marker band frequencies, especially ν_3, near 1500 cm^{-1}, and, for the Fe^{II} forms, the status of the Fe–proximal histidine bond via its stretching frequency. The Fe^{II}–His frequency is elevated to ~240 cm^{-1} in CCP(MI) and in all of the distal mutants, due to hydrogen-bonding interactions between the proximal His-175 Nδ and the carboxylate acceptor group on Asp-235. The Fe^{II}–His RR band has two components, at 233 and 246 cm^{-1}, which are suggested to arise from populations having H-bonded and deprotonated imidazole; these can be viewed in terms of a double-well potential involving proton transfer coupled to protein conformation. The populations shift with changing pH, possibly reflecting structure changes associated with protonation of key histidine residues, and are influenced by the Leu-48 and Phe-191 mutations. A low-spin Fe^{II} form is seen at high pH for the Lys-48, Leu-48, Phe-191, and Phe-51 mutants; for the last three species, coordination of the distal His-52 is suggested by a ~200-cm^{-1} RR band assignable to Fe(imidazole)$_2$ stretching. For the proximal Asn-235 mutant, the ~240-cm^{-1} RR band is missing and is replaced by another band at 205 cm^{-1}, attributed to the Fe–His stretch associated with non-H-bonded imidazole. Thus, the replacement of Asp-235 by Asn completely eliminates the H-bond from His-175. The Fe^{III} proteins are low spin at high pH, probably due to hydroxide binding except in the Lys-48 mutant for which coordination of the amine side chain is suggested by the low pH, 4.5, required for formation of appreciable high-spin heme. In the low-pH high-spin forms the hemes are 5- or 6-coordinate depending on access to the distal water molecule. 6-Coordination is favored in the Phe-51 mutant, showing that the Trp-51 H-bond to the distal H_2O inhibits coordination to the Fe, and in the Asn-235 mutant, showing that the His-175–Asp-235 interaction restrains the Fe from entering the heme plane and binding to distal H_2O. The H_2O binding is sufficiently strong in the Asn-235 mutant to produce a high/low-spin mixture. Loss of the Asp-235–Trp-191 interaction in the proximal Phe-191 mutant produces a minority 6-coordinate population, indicating partial freeing of the normal Fe restraint. CCP(MI), like bakers' yeast CCP, develops an appreciable 6-coordinate population at low pH. A protonation-induced conformation change of Arg-48, perhaps to an orientation which stabilizes the bound H_2O via H-bonding, is suggested by the absence of any low-pH 6-coordinate species in the Leu-48 or Lys-48 mutants.

Cytochrome *c* peroxidase (ferrocytochrome *c*:hydrogen-peroxide oxidoreductase, EC 1.11.1.5) is a mitochondrial heme protein of yeast that catalyzes the reduction of hydrogen peroxide by reduced cytochrome *c* (Yonetani, 1976). High-resolution crystal structures of the bakers' yeast protein (Poulos et al., 1980; Finzel et al., 1984) and the fluoride adduct (Edwards et al., 1984) are available. Moreover, there is much information about the enzymatic mechanism from extensive kinetic and spectroscopic studies (Yonetani, 1976). The enzyme has become a paradigm for the interaction between a protein and its prosthetic group in biological O_2 activating systems.

The gene for CCP has been isolated and cloned, and the protein can now be expressed in large quantities in *Escherichia coli* (Fishel et al., 1987). The isoenzyme prepared in this way has two residue changes relative to the bakers' yeast protein, Asp-152 → Gly and Thr-53 → Ile. Both involve replacements at positions distant from the heme group (Poulos et al., 1980; Finzel et al., 1984). The activity of this enzyme, designated CCP(MI), is essentially the same as that of bakers' yeast CCP (Fishel et al., 1987). Mutants of CCP(MI) have been prepared (Fishel et al., 1987) with the aim of elucidating the functional role of residues in or near the heme pocket. In this study we examine resonance Raman (RR) spectra of CCP(MI) and of five mutants obtained by site-specific mutagenesis: Asp-235 → Asn, in which the aspartate carboxylate group which acts as H-bond acceptor (Poulos et al., 1980; Finzel et al., 1984) to the proximal His-175 ligand of the heme Fe is replaced by a carboxamide group; Trp-191 → Phe, in which

† This work was supported by NIH Grant GM 33576 (to T.G.S.), NATO Grant 86/0453 (to G.S., A.M.E., and T.G.S.), NSF Grant DMB-8511656 (to J.K.), and NSRA Postdoctoral Fellowship PHS GM 10292-02 (to J.M.M.).

* Authors to whom correspondence should be addressed.
‡ Università di Firenze.
§ University of California, San Diego.
∥ Concordia University.
⊥ Princeton University.

0006-2960/88/0427-5477$01.50/0

a proximal Trp which is H-bonded to Asp-235 is replaced by the non-H-bonding residue Phe; Trp-51 → Phe, in which a distal tryptophan which is H-bonded to a water molecule sitting above the Fe atom in the ferric structure is again replaced by the non-H-bonding residue Phe; Arg-48 → Leu and Arg-48 → Lys, in which the arginine which can interact with distal ligands (Edwards et al., 1984) is replaced by a shorter nonpolar side chain and by a side chain of about the same length but with an amine instead of a guanidinium group at the end.

Resonance Raman spectroscopy has been extensively applied to heme proteins in general (Spiro, 1985) and peroxidases in particular (Rakhit & Spiro, 1974; Rakhit et al., 1976; Felton et al., 1976; Teraoka & Kitagawa, 1981; Teraoka et al., 1983; Shelnutt et al., 1983; Desbois et al., 1984; Andersson et al., 1985; Kuila et al., 1985; Terner et al., 1985; Sitter et al., 1985a,b, 1986; Hashimoto et al., 1986a,b; Evangelista-Kirkup et al., 1985, 1986; Smulevich et al., 1986a,b). The technique is capable of giving specific structural information with regard to ligation and spin state of the heme. Substantial variations in these respects are found for the mutants in the present study, which provide insight into heme pocket interactions that are important for the enzymatic mechanism. The succeeding paper is concerned with exploring the heme surroundings by use of the RR and IR signatures of heme-bound CO as a probe.

Experimental Procedures

Mutant CCP DNA was prepared by oligonucleotide-directed site-specific mutagenesis in m13mp8, and the proteins were expressed in *E. coli* with the pUC8 expression system we previously described (Fishel et al., 1987). Phage DNA was completely sequenced for each mutant with ^{35}S dideoxy methodology (Biggin et al., 1983) in order to verify that the proper mutation had been obtained and to demonstrate that the remainder of the CCP gene remained unchanged.

Cell growth, addition of bovine hemin (Sigma type I), and purification procedures were identical for CCP(MI) and the five mutants and were conducted as previously described (Fishel et al., 1987). All protein used in the experiments was crystallized 2 times by dialysis of 0.5–1 mM protein solutions dissolved in 30 mM potassium phosphate (pH 6.0) against distilled water at 4 °C. Twice crystallized proteins were judged to be homogeneous by SDS–polyacrylamide gel electrophoresis. Protein was stored at cryogenic temperatures as crystal suspensions in water.

Visible absorption spectra of the ferric proteins were recorded at 23 °C. Buffers used for visible spectra had a constant ionic strength of 0.1 M and had the following compositions: pH 4.5 and 5, sodium acetate; pH 6 and 7, potassium phosphate; pH 8.4, sodium glycylglycine.

Extinction coefficients were determined according to the basic pyridine hemochromogen method of Paul et al. (1953), assuming 1 mol of heme/mol of mutant protein. The following millimolar extinction coefficients were used to determine the concentrations of the proteins (pH 6.0, 0.1 M potassium phosphate): CCP(MI), $\epsilon_{407.3nm}$ = 102; Asn-235 CCP(MI), $\epsilon_{413.2nm}$ = 110; Leu-48 CCP(MI), $\epsilon_{405.3nm}$ = 107; Lys-48 CCP(MI), $\epsilon_{410.9nm}$ = 105; Phe-191 CCP(MI), $\epsilon_{407.9nm}$ = 109; Phe-51 CCP(MI), ϵ_{408nm} = 141.

A more detailed description of the biochemical properties of CCP(MI), the *E. coli* produced wild-type protein used in these studies, and its Phe-51 variant has been previously published (Fishel et al., 1987). A more detailed description of the biochemical properties of the other mutants will be published elsewhere.

For Raman spectroscopy, crystals of the proteins were dissolved in 0.1 M acetate (pH 4.5–6), phosphate (pH 6–7) and Tris-HCl (pH 8–9.3) buffers to give protein concentrations of 0.3–0.5 mM. The Fe^{II} forms were prepared by adding a minimum volume of dithionite solution to the deoxygenated buffered solutions. Since CCP is reported to be unstable above pH 8, the Fe^{II} forms at alkaline pH were prepared by adding degassed buffer and dithionite solutions to the crystals, and the spectra were recorded immediately.

RR spectra were obtained as described in Smulevich et al. (1986a,b) with excitation from the 413.1-nm line (Fe^{III} species) of a Kr^+ laser (Spectra Physics 171) and the 441.6-nm line (Fe^{II} species) of a He/Cd laser (Liconix). The backscattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Spex 1401) equipped with a cooled photomultiplier (RCA) and photon-counting electronics. The spectra were obtained at room temperature.

Results and Discussion

Cytochrome *c* peroxidase expressed in the *E. coli* system we have developed using the CCP gene derived from *Saccharomyces cerevisiae* by Goltz et al. (1982) is referred to as CCP(MI) (Fishel et al., 1987). For expression in the *E. coli* system, the codons specifying the amino acids −2 and −1 of the truncated CCP leader sequence (Kaput et al., 1982) were replaced with codons specifying the amino acids Met-Ile; these two residues are therefore appended onto the N-terminus of the mature CCP protein as isolated from *E. coli*. In addition, CCP(MI) as well as the mutant proteins studied in these experiments has two other primary sequence variations compared with bakers' yeast CCP (Takio et al., 1980; Kaput et al., 1982), Gly-152 instead of Asp and Ile-53 instead of Thr. The combined effects of these two differences in primary sequence, which lie away from the heme pocket in the folded structure, as well as the two amino acids Met-Ile appended onto the N-terminus, had resulted in no significant observed differences in the spectroscopic properties of CCP(MI) compared to the enzyme isolated from bakers' yeast prior to the present work.

The locations of the side chains which are believed to control the heme reactivity in CCP are depicted in the structural diagram of the heme pocket of bakers' yeast CCP (Finzel et al., 1984) shown in Figure 1.

Proximal Linkage in Fe^{II} CCP. In Figures 2–7 we compare RR spectra, obtained with Soret band excitation (Spiro, 1985), for the Fe^{II} forms of the proteins included in this study.

Replacement of Asp-235 with Asn produces a dramatic change in the Fe–His stretching mode of the reduced protein. The broad and complex band at ~240 cm^{-1} in the CCP(MI) RR spectrum (Figure 2) which has been assigned to this mode in the essentially identical spectrum of bakers' yeast CCP (Hashimoto et al., 1986a,b) is replaced by a band of normal width at 205 cm^{-1} in the Asn-235 mutant (Figure 6). This observation strongly supports the view (Stein et al., 1980; Teraoka et al., 1981) that the unusually high frequency of ν_{Fe-His} in Fe^{II} CCP is due to the H-bonding interaction between the proximal His-175 and the carboxylate of Asp-235 (Poulos & Kraut, 1980; Finzel et al., 1984). Model studies of 5-coordinated Fe^{II} porphyrins with the 2-methylimidazole ligand (the 2-methyl group providing steric hindrance to the Fe moving into the porphyrin plane and forming a 6-coordinated complex) have demonstrated the sensitivity of the Fe–imidazole frequency to H-bonding (Stein et al., 1980; Hori & Kitagawa, 1980). The frequency is ~220 cm^{-1} in H_2O but ~205 cm^{-1} in non-H-bonding solvents such as benzene and methylene

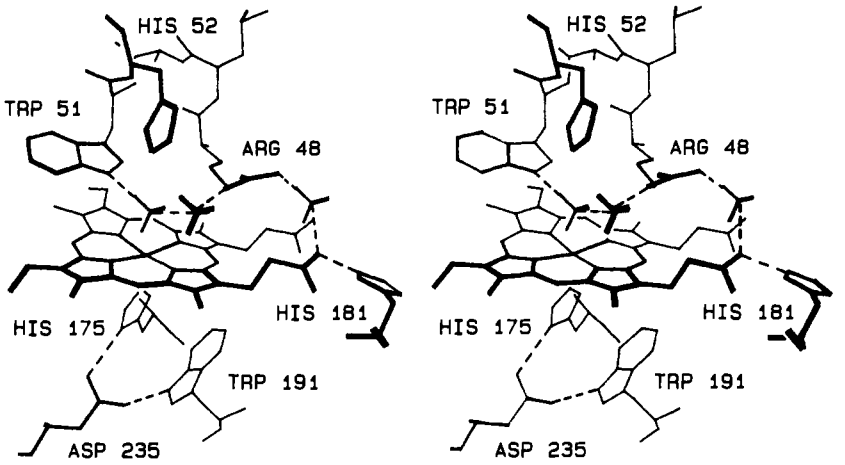

FIGURE 1: Heme crevice of ferric bakers' yeast cytochrome *c* peroxidase from Finzel et al. (1984). Stereoscopic view showing amino acids which are important in the present study. Dotted lines indicate inferred hydrogen bonds, on the basis of distance criteria. Three fixed water molecules are also shown.

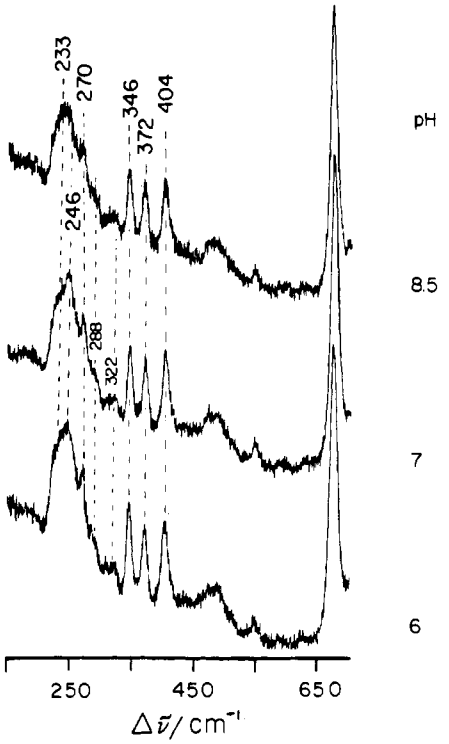

FIGURE 2: Resonance Raman spectra of Fe^{II} CCP(MI) at different pHs obtained with 441.6-nm excitation. Conditions: 20-mW laser power at the source; 5-cm^{-1} spectral slit width; 2 s/0.5 cm^{-1} collection interval.

chloride, while deprotonation at the Nδ position shifts the frequency up to ~240 cm^{-1} (Stein et al., 1980; Teraoka & Kitagawa, 1981). In myoglobin, where the Nδ proton is H-bonded to a neutral backbone carbonyl group, ν_{Fe-His} = 220 cm^{-1} (Kitagawa et al., 1979). The RR spectrum of the Asn-235 mutant provides clear evidence for the disruption of the proximal His-175 H-bond. While some residual H-bonding to the Asn-235 amide group might have been expected, perhaps on the order of the H-bond to the backbone carbonyl in myoglobin, the very low 205-cm^{-1} frequency, unprecedented for a heme protein, implies the absence of any H-bond. We infer that the amide carbonyl group is oriented away from the His-175 ligand in the Asn-235 mutant. The Asp-235 → Asn replacement also produces changes in bands assigned to porphyrin modes; the 345-cm^{-1} band weakens, the 322-cm^{-1} band intensifies, the 270-cm^{-1} band shifts under the 286-cm^{-1} band, and a new band appears at 184 cm^{-1}.

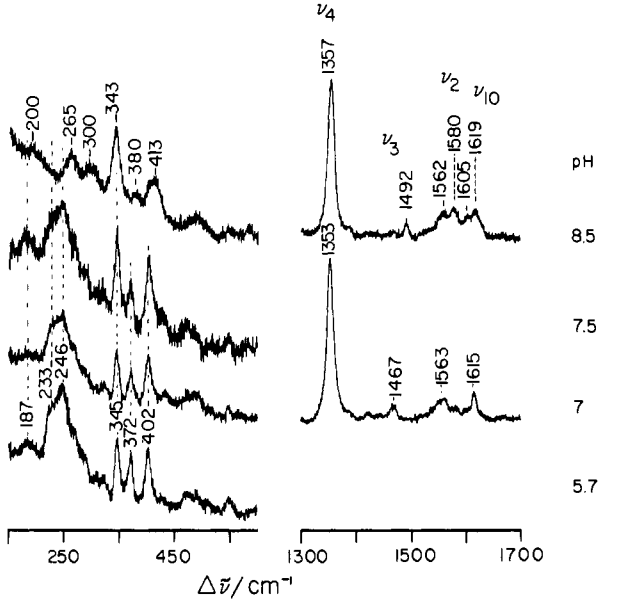

FIGURE 3: RR spectra of Fe^{II} CCP(MI-Phe-51) at different pHs. Same conditions as Figure 1.

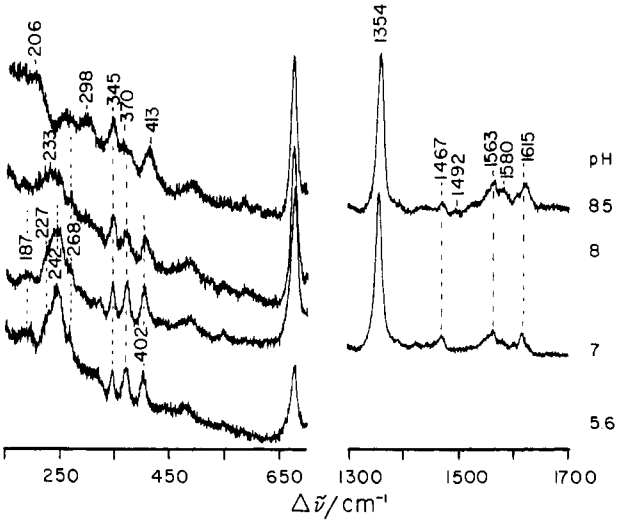

FIGURE 4: RR spectra of Fe^{II} CCP(MI-Leu-48) at different pHs. Same conditions as Figure 1.

It is evident that there are alternative states of the Asp-235–His-175 linkage in CCP(MI) from the fact that the ~240-cm^{-1} Fe–His RR band is a composite of two compo-

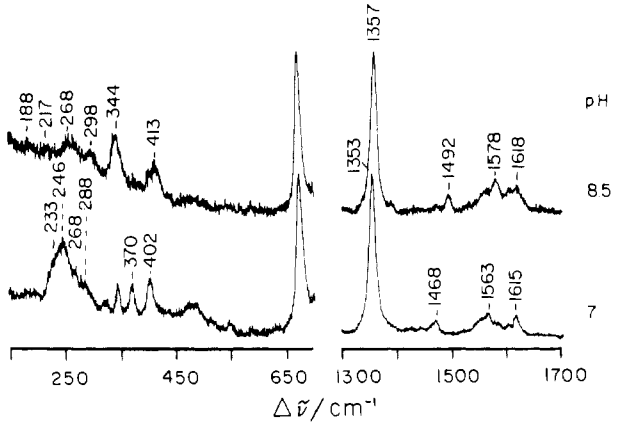

FIGURE 5: RR spectra of Fe^{II} CCP(MI-Lys-48) at different pHs. Same conditions as Figure 1.

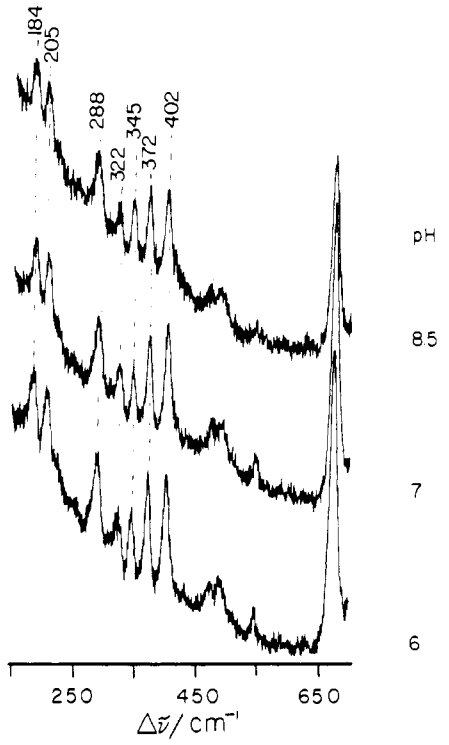

FIGURE 6: RR spectra of Fe^{II} CCP(MI-Asn-235) at different pHs. Same conditions as Figure 1.

nents, at 233 and 246 cm^{-1}. This can be seen from the shape of the band and its variation with pH and among the mutants. From the model compound data, one judges that the 233-cm^{-1} frequency implies a fairly strong H-bond, while the 246-cm^{-1} frequency suggests essentially complete deprotonation of the imidazole ligand. We note that in the analogous enzyme horseradish peroxidase (HRP) the imidazole proton is detectable as a hyperfine-shifted peak in NMR spectra of the reduced form, so that complete deprotonation of the imidazole is ruled out in this case (LaMar & deRopp, 1982) even though the Fe–His RR band is at ~240 cm^{-1} (Teraoka & Kitagawa, 1981) as in CCP. This observation would not, however, be incompatible with a mixture of H-bonded and deprotonated states, especially if they equilibrate rapidly on the NMR time scale. The HRP imidazole ligand has been shown by NMR to be completely deprotonated in the CN^- adduct of Fe^{III} HRP (deRopp et al., 1985).

The H-bonded and deprotonated states can be viewed as representing two minima in a double-well potential for the imidazole proton (Joesten & Schaad, 1974). If, as is usually

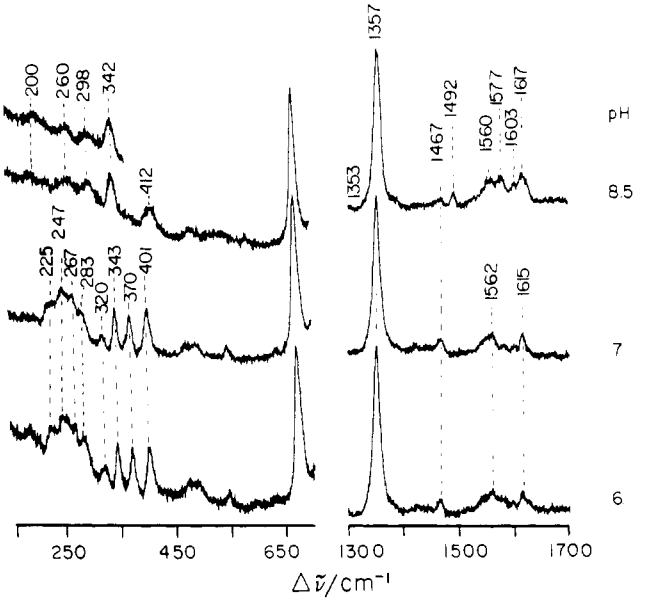

FIGURE 7: RR spectra of Fe^{II} CCP(MI-Phe-191) at different pHs. Same conditions as Figure 1, except 1 s/0.5 cm^{-1} collection interval at pH 8.5. The inset is a second pH 8.5 scan taken 30 min later, showing the development of the 200-cm^{-1} band.

the case, there is a substantial potential barrier for proton transfer (Schroeder & Lippincott, 1957; Smulevich et al., 1987; Smulevich & Foggi, 1987), then a double-well potential is expected, and the residence time at either of the minima would be long on the vibrational time scale (~10^{-13} s). The two wells correspond to the proton residing on the imidazole N atom (233-cm^{-1} component—the high frequency, relative to the 205-cm^{-1} Asn-235 value, reflecting the strength of the H-bond) or the carboxylate O atom (246-cm^{-1} component). Since the two RR components are of comparable intensity, the two minima cannot be very different in energy.

This inference is at first sight very surprising because of the pK_a disparity between imidazole and carboxylic acids. Protonation of carboxylates occurs at pH ~4; imidazolium ion deprotonates at pH ~7, but the pK_a for neutral imidazole is >14. In Fe^{II} CCP the His-175 imidazole is bound to Fe^{II}, which is expected to lower the pK_a but not by as much as a proton does in imidazolium ion. Fe^{II} heme model complexes with 2-methylimidazole ligand can be deprotonated in nonaqueous solvent but remain protonated in alkaline aqueous solution (Stein et al., 1980; Teraoka & Kitagawa, 1981). The actual pK_a is impossible to measure because of decomposition reactions at sufficiently high pH. It is clear, in any event, that many pH units separate the pK_a's of a carboxylic acid and of Fe^{II}-bound imidazole. Solvation by water is a critical determinant of these pK_a's, however, and the proximal His-175–Asp-235 linkage in CCP is well isolated from the solvent. The Asp carboxylate group represents a buried negative charge. This charge would be neutralized by proton transfer from His-175, and the resulting imidazolate anion would be neutralized by the bound Fe^{2+}.

An instructive parallel is offered by the celebrated charge relay system of serine proteases, which have an Asp carboxylate interacting with an active site His imidazole. In this case the proton is on the imidazole, not the carboxylate (Robillard & Shulman, 1974a,b; Bachovin & Roberts, 1978), but the reason seems to be that the carboxylate group is stabilized by three additional H-bonds from neighboring residues. Were it not for these interactions, the proton would transfer to the carboxylate group in order to reduce charge separation, according to ab initio calculations (Allen, 1981). In the present

case the Asp-235 carboxylate has one additional H-bond, from the indole NH group of Trp-191 (Finzel et al., 1984). This interaction seems not to be of critical importance to the status of the His-175–Asp-235 H-bond, however, since the Phe-191 mutant still shows a two-component Fe–His RR band (Figure 7), although the lower frequency component is shifted somewhat, from 233 to 225 cm^{-1}. Thus, the His-175–Asp-235 interaction is perturbed by the replacement of the indole side chain with a smaller and non-H-bonding phenyl ring, but alternative deprotonated and H-bonded states are still seen. A similar perturbation is seen for the Leu-48 mutant (Figure 4); in this case both components shift down slightly, to 242 and 227 cm^{-1}. Since this mutation is on the distal side of the heme, it is clear that the His-175–Asp-235 interaction is sensitive to long-range as well as short-range effects. The other distal mutants Phe-51 (Figure 3) and Lys-48 (Figure 5) show essentially the same band profile as does CCP(MI).

In summary, the Asp-235 carboxylate group seems to be well enough isolated from stabilizing H-bond interactions to compete on a nearly equal basis with the His-175 imidazole for the proton shared between them. The alternative states, with the proton on the carboxylate or on the imidazole, can be described by a double-well potential. The position of the proton may well be coupled to protein motions, via the electrostatic potential, and distinct protein conformations for the two states are quite likely. In this view, the double-well potential would represent a multidimensional coordinate of the protein as well as of the proton. Alterations of these states via the Phe-191 or Leu-48 substitutions can be seen as perturbing the protein part of the double-well potential.

The H-bond potential is also affected by the solution pH. A downshift in the average frequency of the Fe^{II}–His RR band with increasing pH has been reported for HRP (Teraoka & Kitagawa, 1981) as well as CCP (Hashimoto et al., 1986a,b), and the pK_a, near 7.5, has in each case been attributed to the titration of a distal histidine residue. It is clear from the spectra in Figure 2, however, that the effect of increasing the pH is not a frequency shift but an intensity loss of the 246-cm^{-1} component, i.e., the component associated with complete transfer of the proton from His-175 to Asp-235. This transfer is evidently inhibited by the pH ~7.5 deprotonation step. While this pK_a may be associated with the distal His-52, another candidate, His-181, may have a more direct effect on the proximal linkage. His-181 is only six residues away from the proximal His-175 and is H-bonded to a heme propionate group in the bakers' yeast Fe^{III} CCP structure (Figure 1). This anchoring H-bond would be lost upon His-181 deprotonation, and the resulting chain flexibility in the proximal region might account for diminished proton transfer from His-175 to Asp-235. A key role is also implied for the distal Arg-48 residue by the evidence that not only proton transfer but the His-175–Asp-235 H-bond itself are lost at pH 8.5 in the Leu-48 mutant (Figure 4), both components of the ~240-cm^{-1} band being replaced by a band at ~206 cm^{-1}, the same frequency as observed for the Asn-235 mutant (whose spectrum is independent of pH). Arg-48 holds a water molecule which is H-bonded to the same heme propionate group to which His-181 is H-bonded (Figure 1).

Low-Spin Fe^{II} Species. The theme of protein flexibility is elaborated in the Lys-48, Phe-51, and Phe-191 mutants, all of which are converted to low-spin Fe^{II} species at pH 8.5, as seen in the high-frequency region of the RR spectra, which contain porphyrin marker bands whose frequencies are characteristic of the coordination and spin states (Spiro, 1985). The well-isolated ν_3 band, at 1470 cm^{-1} for high-spin Fe^{II} and 1492 cm^{-1} for low-spin Fe^{II}, is particularly diagnostic. The other spin-marker bands are overlapped with one another and are harder to resolve, but in no case is the band pattern inconsistent with the ν_3 position. The high-spin to low-spin conversion is reversible; in all cases the pH 7 spectrum can be regenerated by lowering the pH. Consequently, the conversion is inferred to be associated with ligation changes in the heme pocket. In the case of Phe-191, development of the low-spin species at pH 8.5 takes ~0.5 h (see Fig. 7, upper left side) but remains reversible.

The low-spin state at pH 8.5 implies coordination by a distal ligand with a strong ligand field. For the Lys-48 mutant the deprotonated distal Lys side chain is the likely sixth ligand. For Phe-51 and Phe-191, however, the only sixth ligand candidates would appear to be the distal His-52 residue or a water molecule or hydroxide ion. Coordination of His-52 is suggested by the appearance of a band at 200 cm^{-1} (Figures 3 and 7) which could be assigned to Fe(imidazole)$_2$ stretching (Mitchell et al., 1987); the resulting bis-imidazole complex would be low spin. The crystal structure of bakers' yeast CCP (Figure 1) shows His-52 to be well out of reach of the Fe atom (Finzel et al., 1984), however, and its coordination would require a substantial structural reorganization.

Water or hydroxide are not generally strong field ligands. A bis-hydroxide Fe^{II} heme complex can be prepared and is known to be high spin (Parthasarathi et al., 1987). Coordination of water or hydroxide to Fe^{II} heme with an imidazole fifth ligand has not been modeled, however. The local dielectric constant in the CCP heme pocket might be low enough to induce hydroxide binding to Fe^{II} with sufficient coulomb energy to induce a low-spin configuration. Since low-spin complex formation is not seen for CCP(MI) or Leu-48, the forces favoring a low- vs a high-spin structure at pH 8.5 are evidently closely balanced. The Phe-51 mutation would lower the local dielectric constant in the distal region while the Phe-191 mutation, by releasing the Asp-235–Trp-191 H-bond interaction, would allow easier movement of the Fe atom into the heme plane, a requirement for 6-coordinate low-spin complex formation. It is puzzling, however, that the Asn-235 mutation, which allows still easier Fe atom access to a sixth ligand (see next section), does *not* support low-spin Fe^{II} complex formation at pH 8.5.

Fe^{III} Proteins: Control of Water or Hydroxide Binding. Figures 8–13 show RR spectra with Soret band excitation for the Fe^{III} form of the protein. In this form proximal ligation cannot be monitored directly since the Fe–His stretching mode is insufficiently enhanced to be detected in the RR spectra (Spiro, 1985). The high-frequency region of the spectra, however, shows a marked diversity of behavior reflecting the influence of the residue substitutions on the coordination state of the Fe^{III} ion.

At high pH all the Fe^{III} proteins are converted to low-spin forms, as judged by the high frequency of the ν_3 band, ~1505 cm^{-1}, as well as by the other marker bands. For the Lys-48 mutant (Figure 10) this conversion occurs at an unusually low pH, ~5, and is attributable to distal ligation by the Lys amine side chain, its pK_a lowered substantially by coordination to Fe^{III}. For the other proteins, candidate distal ligands include the His-52 imidazole or hydroxide ion. Low-spin Fe^{III} heme formation by hydroxide coordination is precedented in hydroxymethemoglobin (Iizuki & Kotani, 1969) and seems likely for the CCP variants examined here.

At low pH the proteins are mainly high spin, but there are wide differences in the proportions of 5-coordinate (5-c) and 6-coordinate (6-c) species. The band patterns differ appre-

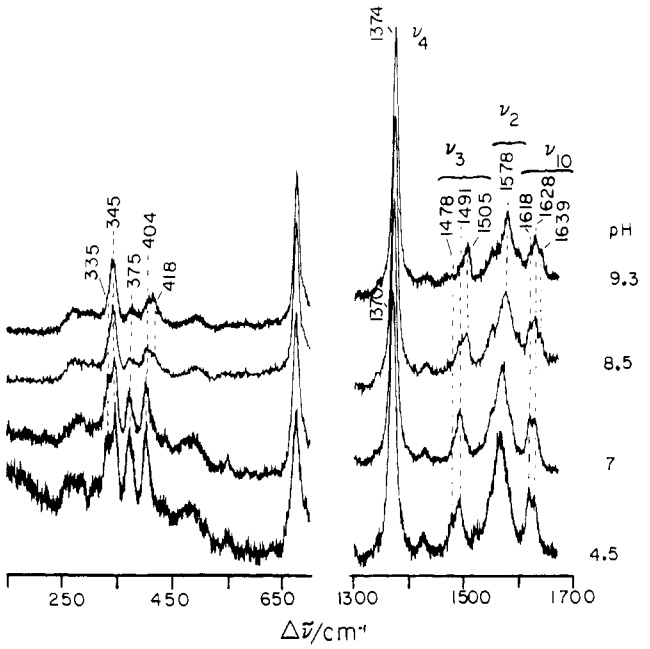

FIGURE 8: RR spectra of Fe^{III} CCP(MI) at different pHs obtained with 413.1-nm excitation. Conditions: 50-mW laser power at the source; 5-cm^{-1} spectral slit width; 1 s/0.5 cm^{-1} collection interval.

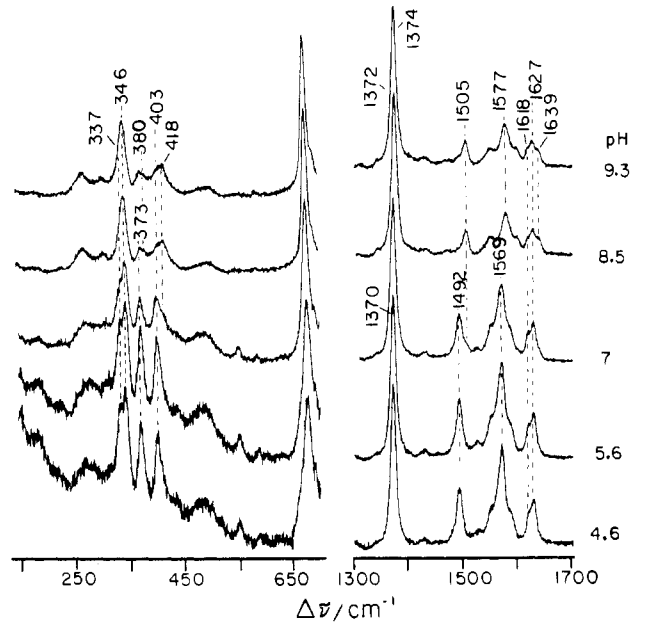

FIGURE 9: RR spectra of Fe^{III} CCP(MI-Leu-48) at different pHs. Same conditions as Figure 7.

ciably between these two coordination states because of the core expansion associated with binding a sixth ligand to high-spin Fe^{III} (Spiro, 1985). The characteristic ν_3 frequency is 1491 cm^{-1} for 5-c but 1478 cm^{-1} for 6-c complexes. We infer that the fraction of 6-c species depends on the tendency to bind a water molecule; there are no plausible distal side chains for a low-field sixth ligand at low pH. In the crystal structure (Figure 1) of the Fe^{III} form of bakers' yeast CCP, a water molecule is located above the Fe atom but at a distance, ~2.4 Å (Finzel et al., 1984), too long for bonding. This water molecule is H-bonded to the Trp-51 indole side chain. It is therefore very interesting that the Phe-51 mutant (Figure 11) shows predominantly 6-coordination, while CCP(MI) (Figure 8) and bakers' yeast CCP (Hashimoto et al., 1986a,b; Smulevich et al., 1986a,b) are mainly 5-c, at least at pH 7.

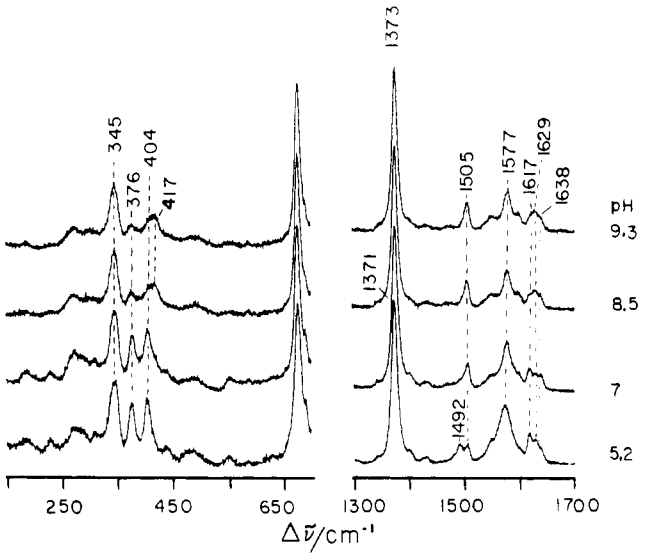

FIGURE 10: RR spectra of Fe^{III} CCP(MI-Lys-48) at different pHs. Same conditions as Figure 7.

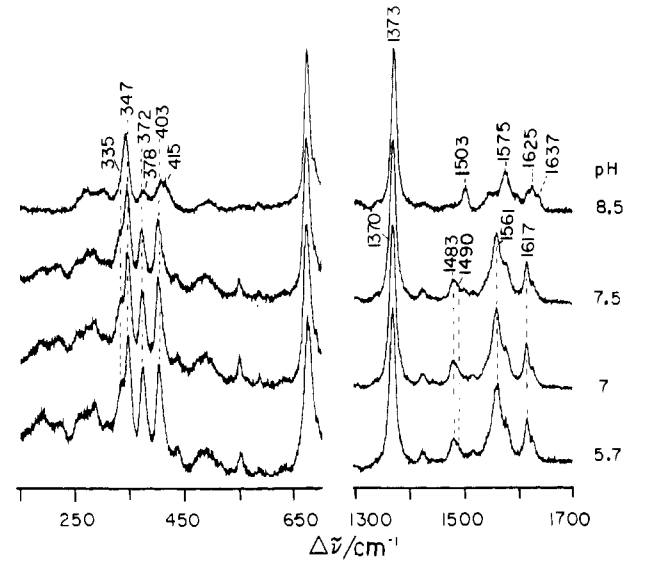

FIGURE 11: RR spectra of Fe^{III} CCP(MI-Phe-51) at different pHs. Same conditions as Figure 7.

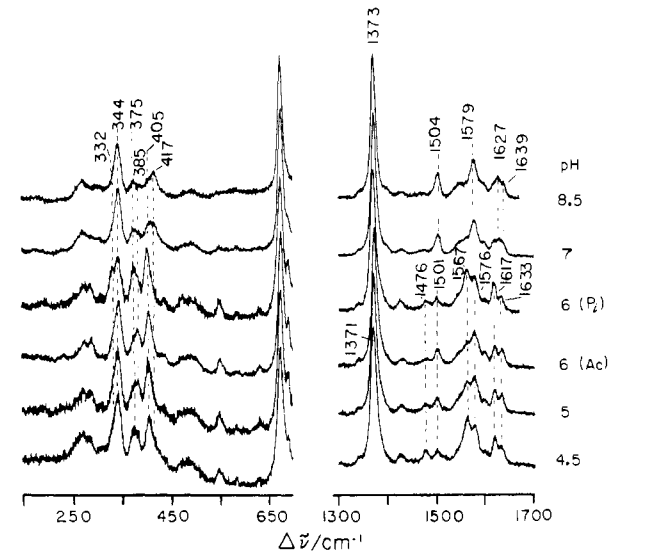

FIGURE 12: RR spectra of Fe^{III} CCP(MI-Asn-235) at different pHs. At pH 6 a buffer effect is seen in the spectral intensity differences for phosphate (P_i) vs acetate (Ac). Same conditions as Figure 7.

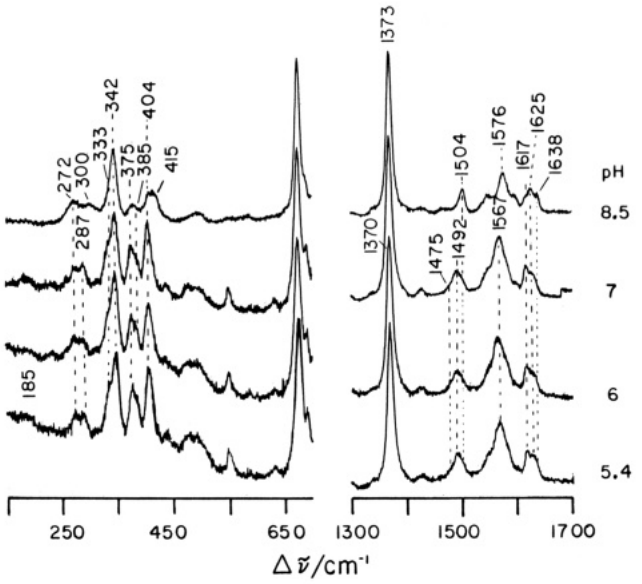

FIGURE 13: RR spectra of Fe^{III} CCP(MI-Phe-191) at different pHs. Same conditions as Figure 7.

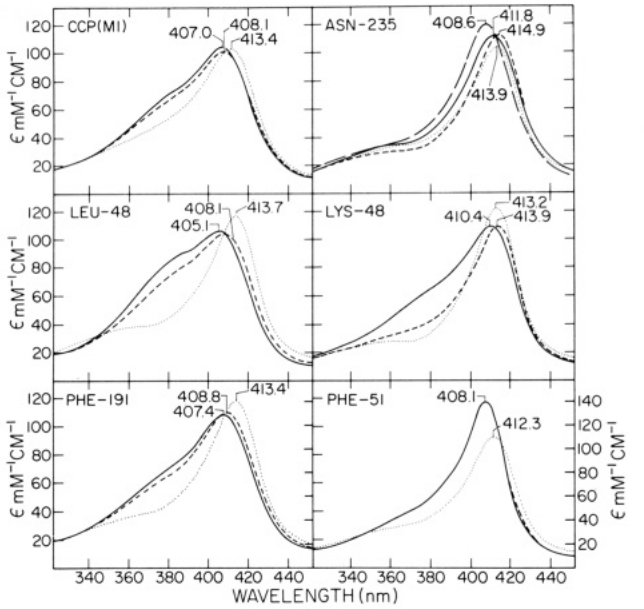

FIGURE 14: Absorption spectra of ferric CCP(MI) and mutants in the Soret region: (−−) pH 4.5; (—) pH 5; (---) pH 7; (···) pH 8.4.

Loss of the Trp-51 H-bond evidently allows the distal water molecule to bind to the heme Fe, although a minority 5-c population can still be seen in the Phe-51 spectra.

The proximal Asn-235 mutant, on the other hand, shows exclusive 6-coordination, and in this case there is a substantial low-spin population, even at pH 4.5 (Figure 12). However, the low-spin ν_3 frequency is distinctly lower at pH values less than 6 than at pH values above 7, 1501 vs 1504 cm^{-1}. We interpret these spectra as reflecting the presence of a low-spin hydroxide complex above pH 7 but a mixed-spin aquo complex below pH 7. Spin mixtures are well established for aquomethemoglobin and myoglobin and indeed provided an early example of the ability of RR spectroscopy to distinguish spin mixtures from intermediate spin states (Strekas & Spiro, 1974). The change from a mainly 5-c heme in CCP(MI) to a mixed-spin aquo complex in the Asn-235 mutant must be due to the loss of the His-175–Asp-235 H-bond, documented in the dramatic change of the Fe–His RR band in the Fe^{II} form as discussed above. Breaking this interaction allows the Fe atom to approach the heme plane more readily, and the result is strong binding of the distal water molecule. A buffer anion effect is also noticeable in the spectra taken at pH 6; the intensity ratio of the low- to the high-spin ν_3 bands is significantly greater when the buffer is acetate than when it is phosphate. Selective binding of acetate to the distal residue, or to the heme Fe itself, is suggested. Anion effects on the 5-c vs 6-c high-spin populations in CCP were noted by Hashimoto et al. (1986a,b).

The effect of the Phe-191 mutation is in the same direction but is more attenuated (Figure 13). A single broad ν_3 band is seen, centered at the 5-c frequency, but with appreciable intensity at the 6-c high- and low-spin positions. Thus, loss of the Trp-191–Asp-235 interaction allows the Fe atom some flexibility but not nearly as much as loss of the His-175–Asp-235 interaction.

The low-pH form of the Leu-48 mutant shows exclusive 5-coordination (Figure 9). A strong sharp ν_3 band is seen at 1492 cm^{-1} between pH 7 and pH 4.5. In contrast, CCP(MI), which is mainly 5-c at pH 7, shows a substantial 6-c ν_3 component at pH 4.5 (Figure 8). An even greater conversion to 6-coordination at low pH has been reported by Hashimoto et al. (1986a,b) for bakers' yeast CCP, and a pK_a of 5.5 was determined for the process. They discussed the possibility that this pK_a is associated with protonation of His-52, or else Asp-235. The absence of 6-coordination in the Leu-48 mutant also points to an important role for Arg-48, although not, of course, in the protonation per se. The Arg-48 side chain can occupy alternative orientations in the protein. In the bakers' yeast CCP–fluoride adduct, it swings down from the position shown in Figure 1 and forms a H-bond directly to the F^- ligand (Edwards et al., 1984). In this adduct the distal His-52 is probably protonated since anion binding to Fe^{III} peroxidases is accompanied by uptake of a proton. We propose that His-52 protonation in the absence of bound anion induces a similar conformation change, allowing Arg-48 to stabilize a bound water molecule. The elimination of 6-coordination in Leu-48 would then have a natural explanation since the stabilizing Arg-48 interaction would be absent.

Yonetani and co-workers (Yonetani & Anni, 1987) have recently presented evidence that the amount of low-pH-inducible 6-c heme bakers' yeast CCP correlates with the age of the protein and suggested that freshly prepared protein contains no 6-c high-spin heme. If this is the case, then the proposed Arg-48 stabilization of the bound water may depend on some irreversible modification of the protein.

We note that there are changes in the low-frequency region of the RR spectra which correlate with the coordination and spin state changes. Thus the relative intensity of the 335-cm^{-1} band is lower for 6-c than for 5-c high-spin heme, as also noted by Hashimoto et al. (1986a,b). For low-spin heme this band disappears, while the 375-cm^{-1} band intensity diminishes and the 404-cm^{-1} band broadens and shifts to 418 cm^{-1}.

Figure 14 shows absorption spectra in the Soret band region for CCP(MI) and the five mutants, across the pH range 5–8.4. These spectra are readily interpretable on the basis of the RR results as arising from variable contributions from 5-c and 6-c high- and low-spin species. The pH-dependent Soret maximum is ~412 nm for 6-c low-spin heme (e.g., Lys-48) and ~408 nm for 6-c high-spin heme (e.g., Phe-51 at pH 6). Conversion of high-spin heme from 6-c to 5-c is accompanied by a weakening of the Soret peak absorptivity (ϵ_{408} = 141 mM^{-1} cm^{-1} for Phe-51 vs ϵ_{405} = 107 mM^{-1} cm^{-1} for Leu-48) and development of a strong shoulder at ~370 nm.

Table I summarizes the spin and coordination states found for the various mutants in both Fe^{II} and Fe^{III} forms.

Table I: Summary of Heme Coordination and Spin States[a] in CCP Mutants for Fe^{III} and Fe^{II} Forms at Various pH Values

		pH				
		~5 (acetate)	6 (phosphate)	7 (phosphate)	8.5 (Tris-HCl)	9.3 (Tris-HCl)
CCP (bakers' yeast)[b]	Fe^{III}	6c hs	6c hs			
		5c hs (pH 4.3)	5c hs	5c hs	5c hs	5c hs
					6c ls	6c ls
	Fe^{II}		5c hs	5c hs	5c hs	5c hs
CCP(MI)	Fe^{III}	6c hs				
		5c hs		5c hs	5c hs	5c hs
					6c ls	6c ls
	Fe^{II}		5c hs	5c hs	5c hs	
Asn-235	Fe^{III}	6c hs	6c hs	6c hs		
		5c hs	5c hs			
		6c ls (pH 4.5)	6c ls	6c ls	6c ls	
	Fe^{II}		5c hs	5c hs	5c hs	
Lys-48	Fe^{III}	5c hs		5c hs		
		6c ls (pH 5.2)		6c ls	6c ls	6c ls
	Fe^{II}			5c hs		
					6c ls	6c ls
Leu-48	Fe^{III}	5c hs (pH 4.6)	5c hs	5c hs		
				6c ls	6c ls	6c ls
	Fe^{II}		5c hs	5c hs	5c hs	
					6c ls	6c ls
Phe-51	Fe^{III}	6c hs	6c hs	6c hs		
		5c hs (pH 4–5.7)	5c hs	5c hs		
					6c ls	
	Fe^{II}		5c hs	5c hs		
					6c ls	
Phe-191	Fe^{III}	6c hs	6c hs	6c hs		
		5c hs	5c hs	5c hs		
		6c ls	6c ls	6c ls	6c ls	
	Fe^{II}		5c hs	5c hs	5c hs	
					6c ls	

[a] 6c and 5c, 6- and 5-coordinate; hs and ls, high spin and low spin. The dominant form is underlined. [b] From Hashimoto et al. (1986).

Conclusions

The present results illustrate the power of site-directed mutagenesis combined with structural probes to illuminate the molecular interactions at enzyme active sites. The RR spectrum of the CCP heme group proves to be markedly sensitive to alterations in the surrounding side chains which interact with the heme ligands. Identifying the target side chains from the protein crystal structure is, of course, a central step in the strategy.

Both proximal and distal side chains have profound influences on the heme ligation. On the proximal side, the His-175–Asp-235 H-bond is a critical interaction, modulating the Fe–His bond strength and restraining the Fe atom from moving into the heme plane and binding a distal water molecule. Both effects are dramatically demonstrated by the Asn-235 mutant, in which His-175 H-bonding is apparently absent. The Fe–His stretching frequency is found at a low, non-H-bonded value, and in the Fe^{III} form a mixed-spin aquo complex is found. The His-175–Asp-235 interaction in the parent enzyme allows partial transfer of the proton to the Asp carboxylate group. The Fe–His RR band has two components, with frequencies corresponding to H-bonded and deprotonated imidazole, judging from model studies. The interaction is suggested to be described by a double-well potential with nearly isoenergetic minima, the coordinate involving proton transfer being coupled to the protein conformation. This proximal interaction is altered by raising the pH to 8.5, especially in the Leu-48 mutant, suggesting a role for His-181 in anchoring the proximal chain via interactions with a heme propionate group and with Arg-48.

Distal ligand–protein interactions are also clearly revealed by the mutants. Loss of the Trp-51 H-bond to the distal H_2O increases the latter's ability to bind to the heme Fe^{III}. Lowering the pH of CCP(MI) likewise increases distal H_2O binding, and the loss of this effect in Leu-48 suggests a coupling to an Arg-48 orientation favoring H_2O binding in the low-pH form, perhaps via H-bonding to the bound H_2O. When Arg-48 is replaced by Lys, however, the amine side chain binds directly to the Fe, as evidenced by the observation of the lowest pK_a for the transition to the low-spin form. The other proteins are converted to low-spin forms at higher pH, probably via hydroxide binding. Low-spin Fe^{II} hemes are also observed at pH 8.5 for the Phe-191, Phe-51, and Leu-48, as well as the Lys-48, mutants. For the former three (in Lys-48 the amine side chain again provides a plausible ligand) hydroxide binding is a possible explanation for a low-spin Fe^{II} state. Alternatively, His-52 binding is suggested by a ~200-cm^{-1} RR band assignable to Fe(imidazole)$_2$ stretching, but a substantial conformation change would then be required for the His-52 side chain to reach the Fe.

Registry No. CCP, 9029-53-2; Asp, 56-84-8; Asn, 70-47-3; Trp, 73-22-3; Phe, 63-91-2; Arg, 74-79-3; Leu, 61-90-5; Lys, 56-87-1; Fe, 7439-89-6; heme, 14875-96-8.

References

Allen, L. C. (1981) *Ann. N.Y. Acad. Sci. 367*, 383.

Andersson, L. A., Renganathan, V., Chiu, A. A., Loehr, T. M., & Gold, M. H. (1985) *J. Biol. Chem. 260*, 6080.

Andersson, L. A., Renganathan, V., Loehr, T. M., & Gold, M. H. (1987) *Biochemistry 26*, 2258.

Bachovin, W. W., & Roberts, J. D. (1978) *J. Am. Chem. Soc. 100*, 8041.

Bangcharoenpaurpong, O., Schomacher, K. T., & Champion, P. M. (1984) *J. Am. Chem. Soc. 106*, 5688.

Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963.

Choi, S., & Spiro, T. G. (1983) *J. Am. Chem. Soc. 105*, 3683.

deRopp, J. S., Thanabal, V., & La Mar, G. N. (1985) *J. Am. Chem. Soc. 107*, 8268.
Desbois, A., & Lutz, M. (1981) *Biochim. Biophys. Acta 671*, 168.
Desbois, A., Mazza, G., Stretkowski, F., & Lutz, M. (1984) *Biochim. Biophys. Acta 785*, 161
Edwards, S. L., Poulos, T. L., & Kraut, J. (1984) *J. Biol. Chem. 259*, 12984.
Evangelista-Kirkup, R., Poulos, T. L., & Spiro, T. G. (1985) *FEBS Lett. 192*, 2976.
Evangelista-Kirkup, R., Smulevich, G., & Spiro, T. G. (1986) *Biochemistry 25*, 4420.
Felton, R. H., Romans, A. Y., Yu, N.-T., & Schonbaum, G. R. (1976) *Biochim. Biophys. Acta 434*, 82.
Finzel, B. C., Poulos, T. L., & Kraut, J. (1984) *J. Biol. Chem. 259*, 13027.
Fishel, L. A., Villafranca, J. E., Mauro, J. M., & Kraut, J. (1987) *Biochemistry 26*, 351.
Goltz, S., Kaput, J., & Blobel, G. (1982) *J. Biol. Chem. 257*, 11186.
Hashimoto, S., Tatsuno, Y., & Kitagawa, T. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2417.
Hashimoto, S., Teraoka, J., Inubushi, T., Yonetani, T., & Kitagawa, T. (1986b) *J. Biol. Chem. 261*, 11110.
Hori, H., & Kitagawa, T. (1980) *J. Am. Chem. Soc. 102*, 3608.
Iizuki, T., & Kotani, M. (1969) *Biochim. Biophys. Acta 194*, 351.
Joesten, M. D., & Schaad, J. L. (1974) *Hydrogen Bonding*, Marcel Dekker, New York.
Kaput, J., Goltz, S., & Blobel, G. (1982) *J. Biol. Chem. 257*, 15054.
Kitagawa, T., Abe, M., & Ogoshi, H. (1978) *J. Chem. Phys. 69*, 4516.
Kitagawa, T., Nagai, K., & Tsubaki, M. (1979) *FEBS Lett. 104*, 376.
Kuila, D., Tien, M., Fee, J. A., & Ondrias, M. R. (1985) *Biochemistry 24*, 3394.
La Mar, G. N., & deRopp, J. S. (1982) *J. Am. Chem. Soc. 104*, 5203.
Mitchell, M. L., Li, X.-Y., Kincaid, J. R., & Spiro, T. G. (1987) *J. Phys. Chem. 91*, 4690.
Parthasarathi, N., Hansen, C., Yamaguchi, S., & Spiro, T. G. (1987) *J. Am. Chem. Soc. 109*, 3865.
Paul, K. G., Theorell, H., & Akeson, A. (1953) *Acta Chem. Scand. 7*, 1284.
Poulos, T. L., Freer, S. T., Alden, R. A., Edwards, S. L., Skogland, U., Takio, K., Eriksson, B., Xuong, N., Yonetani, T., & Kraut, J. (1980) *J. Biol. Chem. 255*, 575.
Rakhit, G., & Spiro, T. G. (1974) *Biochemistry 13*, 5317.
Rakhit, G., Spiro, T. G., & Uyeda, M. (1976) *Biochem. Biophys. Res. Commun. 71*, 803.
Robillard, G., & Shulman, R. G. (1974a) *J. Mol. Biol. 86*, 519.
Robillard, G., & Schulman, R. G. (1974b) *J. Mol. Biol. 86*, 541.
Saitoh, T., Mori, K., & Itoh, R. (1981) *Chem. Phys. 60*, 161.
Schroeder, R., & Lippincott, E. R. (1957) *J. Phys. Chem. 61*, 921.
Shelnutt, J. A., Satterlee, J. D., & Erman, J. E. (1983) *J. Biol. Chem. 258*, 2168.
Sitter, A. J., Reczak, C. M., & Terner, J. (1985a) *Biochim. Biophys. Acta 828*, 229.
Sitter, A. J., Reczak, C. M., & Terner, J. (1985b) *J. Biol. Chem. 260*, 7515.
Sitter, A. J., Reczak, C. M., & Terner, J. (1986) *J. Biol. Chem. 261*, 8638.
Smulevich, G., & Foggi, P. (1987) *J. Chem. Phys. 87*, 5657.
Smulevich, G., Evangelista-Kirkup, R., English, A., & Spiro, T. G. (1986a) *Biochemistry 25*, 4426.
Smulevich, G., Dasgupta, S., English, A., & Spiro, T. G. (1986b) *Biochim. Biophys. Acta 873*, 88.
Smulevich, G., Foggi, P., Feis, A., & Marzocchi, M. P. (1987) *J. Chem. Phys. 87*, 5664.
Spiro, T. G. (1985) *Adv. Protein Chem. 37*, 110.
Stein, P., Mitchell, M., & Spiro, T. G. (1980) *J. Am. Chem. Soc. 102*, 7795.
Strekas, T. C., & Spiro, T. G. (1974) *Biochim. Biophys. Acta 351*, 237.
Takio, K., Titani, K., Ericsson, L. H., & Yonetani, T. (1980) *Arch. Biochem. Biophys. 203*, 615.
Teraoka, J., & Kitagawa, T. (1981) *J. Biol. Chem. 256*, 3969.
Teraoka, J., Job, D., Morita, Y., & Kitagawa, T. (1983) *Biochim. Biophys. Acta 747*, 10.
Terner, J., Sitter, A. J., & Reczak, C. M. (1985) *Biochim. Biophys. Acta 828*, 73.
Yonetani, T. (1976) *Enzymes (3rd Ed.) 13*, 345–361.
Yonetani, T., & Anni, H. (1987) *J. Biol. Chem. 262*, 9547.