Fifteen Years of Raman Spectroscopy of Engineered Heme Containing Peroxidases: What Have We Learned?

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ABSTRACT

Spectroscopic techniques have been fundamental to the comprehension of peroxidase function under physiological conditions. This Account examines the contribution to our understanding of heme peroxidases provided by electronic and resonance Raman spectroscopies in conjunction with site-directed mutagenesis. The results obtained over 15 years with several heme peroxidases and selected mutants have provided important insights into the influence exerted by the protein in the vicinity of the active site via key amino acids on the functionality and stability of the enzymes. Moreover, resonance Raman spectroscopy has revealed that a common feature of heme peroxidases is the presence of an extensive network of H-bonds coupling the distal and proximal sides, which has a profound influence on the heme ligation, affecting both the fifth and the sixth coordination sites.

Introduction

In 1993, Dr. Michael Smith received the Nobel Prize in Chemistry for "his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies". He developed the technology of site-directed mutagenesis, which allows the DNA sequence of any gene to be altered in a designated manner. Due to Smith's novel findings, new opportunities for studying the structure−function relationships in proteins have been opened.[1−3] Dr. Smith was also involved in the site-directed mutagenesis of yeast cytochrome *c* peroxidase (CCP). In the early 1980s, the isolation[4] and sequence determination[5] of the CCP gene from a standard strain of *Saccaromyces cerevisiae* opened the door to structure−function studies of this protein using mutagenesis[6,7] together with resonance Raman (RR) spectroscopy.[8] Moreover, the availability of the X-ray structure of CCP, the first obtained for any peroxidase,[9] allowed the design of meaningful mutagenesis experiments. Since 1995 several structures of heme peroxidases belonging to the superfamily of plant peroxidases have been solved,[10] and as predicted on the basis of the amino acid alignment,[11] all contain amino acids in the heme cavity that are invariant and considered important for catalytic activity. The role played by these key amino acids was studied by kinetic methods and spectroscopic techniques in a number of peroxidases that were successfully produced by heterologous expression.[12] RR spectroscopy has been widely applied to study heme proteins.[13] The extensive data based on the X-ray structures and RR spectra demonstrate that in the Fe^{3+} resting state of plant peroxidases, the heme iron sixth coordination site is vacant or bound weakly to water, emphasizing that one coordination site must be free to enable peroxides or other ligands to bind and react with the protein.[10] However, comparison between the spectroscopic data of the protein in solution and crystal forms shows that the coordination can be different from that determined by X-ray crystallography.[10,14,15] Since the coordination state of peroxidases can be dramatically affected by the physical and chemical conditions, this finding suggests that a careful comparison between the data obtained on a protein in solution and that as a single crystal is necessary. Therefore, after a crystal structure has been solved, work on structure−function analysis by spectroscopic techniques is not over. Moreover, critical information on protein flexibility or, for example, possible alternative conformations existing in solution is lost if only crystallographic data are considered. The results obtained over the last 15 years from electronic absorption and RR spectra of several heme peroxidases and selected mutants give insight into the influence exerted on protein architecture in the vicinity of the active site by key amino acids providing information both on the functionality of the enzymes and on their stability.

Peroxidases catalyze the two-electron oxidation of a variety of substrates at the expense of H_2O_2 via the formation of oxy-ferryl catalytic species.[16,17] The postulated mechanism of peroxide decomposition relies on the concerted roles played by the conserved distal histidine and arginine, through H-bonds and charge stabilization.

A comprehensive analysis of information presently available from absorption, RR spectra, and X-ray crystallography of wild-type peroxidases and mutants involving residues close to the heme, together with the finding of novel spectroscopic markers, enables some general con-

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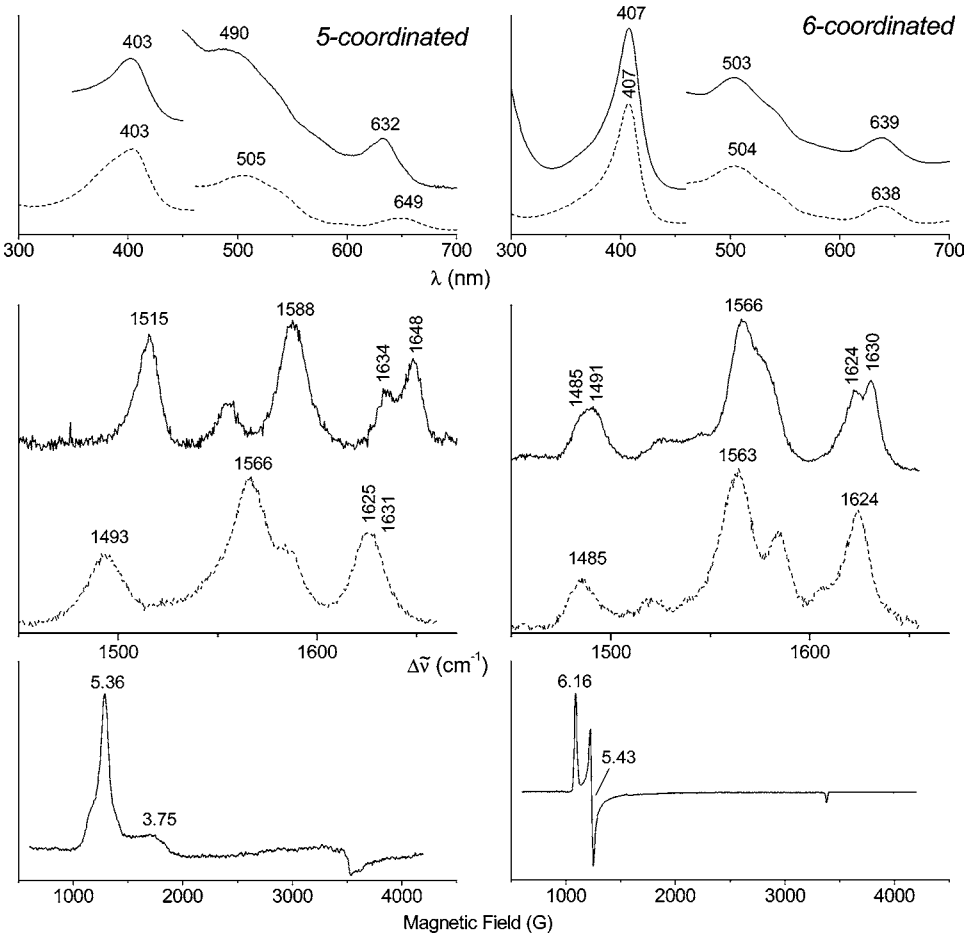


FIGURE 1. From top to bottom, absorption, RR, and EPR spectra at 10 K (—) and room temperature (RT) (— — —): (left) BP1 at 10 K (5cQS) and CIP/ARP at RT (5cHS); (right) HRPC−BHA at 10 K (6cQS) and CIP/ARP−BHA at RT (6cHS) (for abbreviations, see the text).

clusions to be reached concerning peroxidase active sites, which can also be extended to other heme proteins.

Spectroscopic Markers

Sequence alignment has revealed the existence of three classes within the superfamily of plant peroxidases. Class I includes the prokaryotic intracellular peroxidases, class II the fungal peroxidases, and class III the classical secretory plant peroxidases.[11] While the resting state of the proteins belonging either to class I or II is high-spin Fe^{3+}, a distinctive characteristic of the class III peroxidases is a pentacoordinate quantum mechanically mixed-spin (5cQS) state. It is characterized[18,19] (Figure 1, left) by absorption spectra similar to those of a pentacoordinate high-spin (5cHS) heme but with shorter wavelength $\pi \rightarrow \pi^*$ transitions, a band at 630−635 nm due to the porphyrin to iron charge-transfer transition (CT1), a high frequency of the RR core-size marker bands, which cannot be assigned to either 5cHS or hexacoordinate high-spin (6cHS) hemes but may be mistaken as characteristic of low-spin heme, and electron paramagnetic resonance (EPR) spectra with g_{12} values in the range $4 < g_{12} < 6$, typical only of a QS heme.[20] The binding of benzohydroxamic acid (BHA) to class III peroxidases induces the formation of a hexacoordinate quantum mechanically mixed-spin (6cQS) heme[18,19] (Figure 1, right). It is characterized by absorption spectra similar to those of a 6cHS heme but differs from the 5cQS in that the Soret band red-shifts by about 4 nm and sharpens with a ∼35% increase of the extinction coefficient, high frequency of the RR core-size marker bands resembling those of a 5cHS system, and EPR spectra with g_{12} values in the range $4 < g_{12} < 6$.

The QS state reflects a quantum mechanical admixture of intermediate ($S = {}^3/_2$) and high ($S = {}^5/_2$) spin states and is very unusual in biological systems. It has been found to be a common feature of cytochrome *c*′ from various bacterial sources at neutral pH.[21,22] The structural origin and functional significance of the QS states remain elusive. The most widely suggested origins, a weak axial ligand field[23] or a saddle-shaped heme deformation,[24] are not sufficient to cause the QS states.

H-Bond Networks

The RR work on CCP mutants[8] provided new insight into heme pocket interactions important for the enzymatic mechanism. The most fascinating result of that work was the striking revelation that both proximal and distal side chains have a profound influence on heme ligation due to an extensive network of H-bonds coupling the distal and proximal sides,[8,25,26] which was later found to be a common feature of heme peroxidases. In particular, in

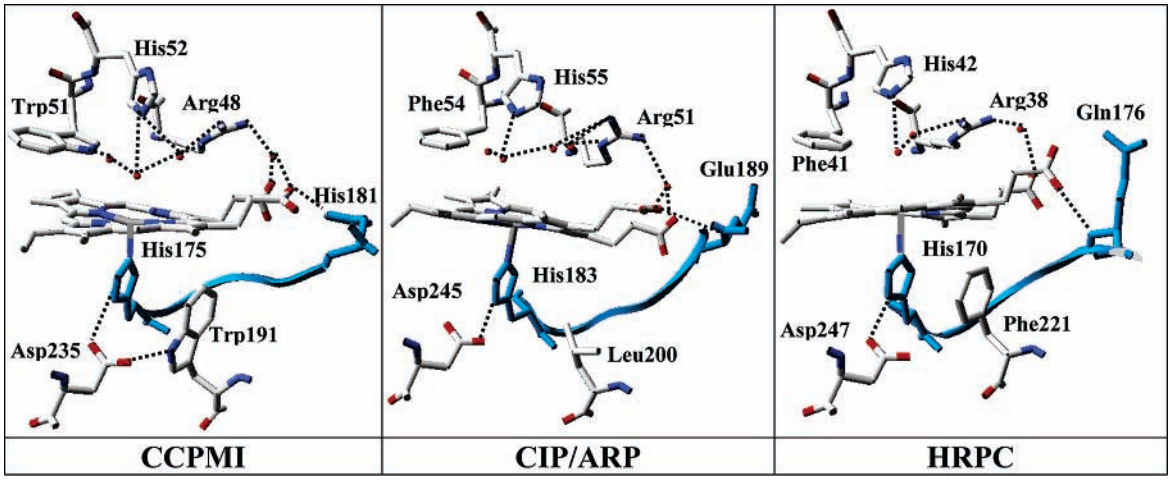


FIGURE 2. Structural diagram of the heme pocket of recombinant CCP (CCPMI) (PDB 1CCP), CIP/ARP (1GZB), and HRPC (1ATJ). Dotted lines indicate H-bonds. Water molecules are also shown. The loop connecting the proximal Fe-ligand with the residue H-bonded to the heme propionyl is shown in blue.

CCP the H-bond chain that preserves protein function and maintains intact the coupling between the proximal and distal residues is constituted by Trp51−distal water molecules−Arg48−heme propionate−His181, connected by a five-residue loop to His175.[8,26,27] On the basis of their X-ray structures, similar H-bond networks were inferred for the other peroxidases (Figure 2).[28]

Below the heme, the conserved H-bond between the N_δ atom of the imidazole ligand and the carboxylate of an aspartic side chain, which acts as H-bond acceptor, imparts an imidazolate character to the histidine ligand (Figure 2). Based on RR studies of model compounds, which have demonstrated the sensitivity of the Fe-imidazole frequency to H-bonding,[29,30] RR and site-directed mutagenesis of CCP clearly indicated that the His175−Asp235 H-bond is a critical interaction, modulating the Fe−His bond strength and restraining the Fe from moving into the heme plane and binding a distal water.[8,25,26] The broad Fe−His stretching (ν_{FeHis}) band[31,32] is composed of two ν_{FeHis} modes, assigned to tautomers of the H-bond between the His175 $N_\delta H$ proton and the Asp235 carboxylate chain (Figure 3).[33] In one tautomer, the proton resides on the imidazole (ν_{FeHis} 233 cm^{-1}), while in the other, the proton is transferred to the carboxylate (ν_{FeHis} 246 cm^{-1}). The influence of the His175−Asp235 H-bond in CCP is dramatically illustrated by the replacement of the Asp235 carboxylate with the asparagine amide. The heme coordination and spin states changed and the RR ν_{Fe-Im} frequency was lowered by about 40 cm^{-1}.

The imidazolate character of the proximal heme ligand has been proposed to stabilize the ferryl intermediate due to its electron-donating properties. However, while the Asp235 cannot be replaced without impairing reactivity,[34] replacing the histidine ligand with glutamine or glutamate has little effect on the rate of compound I formation.[35] Moreover, peroxide activity of His175Gly is restored under conditions where imidazole is bound to the iron, forming a weaker hydrogen bond to Asp235 than that observed in the wild-type protein.[36,37] Therefore, the studies of the

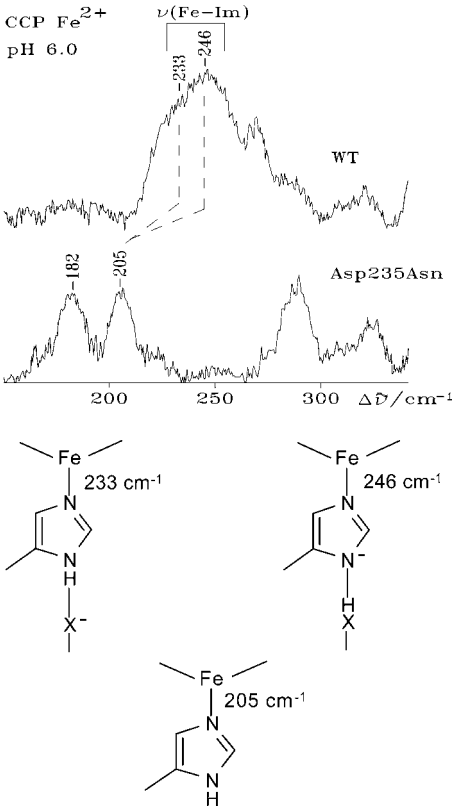


FIGURE 3. Low-frequency RR spectra (top) of ferrous CCP(MKT) and the Asp235Asn mutant and H-bond tautomers (bottom) formed with the proximal ligand.

proximal variants of peroxidases clearly indicate that the "electron push" effect may be not so important for the activity since the strength of the proximal histidine−aspartate hydrogen bond can be modulated without serious effects on the peroxide cleavage step.

Above the heme, the conserved positively charged guanidinium of an arginine residue and an H-bond from the distal histidine N_δ atom to a nearby asparagine depresses the histidine pK_a constraining N_ϵ to act as a H-bond acceptor during the catalytic cycle. In the resting state, the heme sixth coordination site is vacant or bound

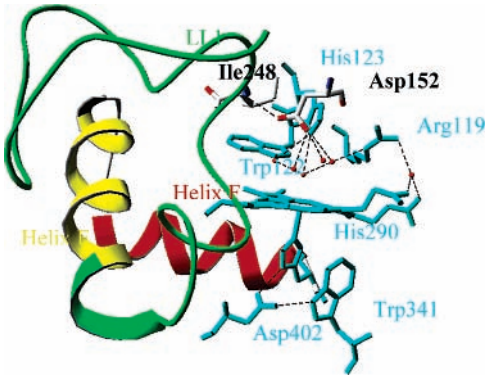


FIGURE 4. Structural diagram of the heme pocket of KatG from *Synechocystis* according to the crystal structure of KatG from *Haloarcula marismortui* (1ITK). Dotted lines indicate H-bonds. Water molecules are also shown.

weakly to water (Figure 2). However, the coordination state of peroxidases can be dramatically affected by the physical and chemical conditions suggesting that a careful comparison between the data obtained on a protein in solution and that as a single crystal is necessary. This remarkable sensitivity is a consequence of the high polarity of the distal cavity and the extended H-bond network involving water molecules and conserved distal and proximal amino acids. Moreover, the H-bonded distal water network is preserved by the distal histidine and arginine. Mutation of these residues modifies the heme coordination and perturbs the proximal Fe−Im bond strength, as a consequence of pH-dependent changes, charge distribution, size, and H-bonding acceptor/donor properties of the substituting amino acid.[8,27,38−41]

Recently, catalase−peroxidases[41] (KatGs), have raised considerable interest, since, despite their striking sequence homologies to CCP, they are the only members of the plant peroxidase superfamily exhibiting peroxidase and substantial catalase activities. Extensive RR studies have been performed on these novel proteins to understand their dual enzymatic activity.[41−45] Marked differences in the structural role of the conserved amino acids and H-bond networks in KatG with respect to the other plant peroxidases were found. The proximal amino acids play a major role in the stability of the protein architecture, since disruption of the proximal tryptophan−aspartate H-bond by mutation weakens the heme binding to the protein. Furthermore, mutagenesis of both proximal and distal side residues of KatG affects selectively the function inhibiting mainly the catalase but not the peroxidase activity.[46,47] Interesting results were obtained upon mutation of the distal aspartate residue, conserved in all KatGs analyzed so far. In *Synechocystis* KatG, this residue is the distal Asp152. Its carboxyl oxygen points toward the heme pocket and is H-bonded to two distal water molecules.[48] Moreover, Asp152 is H-bonded to the nitrogen atom of Ile248, which is part of the KatG-specific insertion LL1 (Figure 4, green) connecting the distal side with the proximal E and F helices (Figure 4, yellow and red, respectively), the latter carrying the proximal histidine ligand. This distal H-bond represents a novel link between the proximal and distal sides of the heme cavity and is important for the stability of the heme pocket and for maintaining the proximal H-bond strength. Exchange of Asp152 with serine, present in CCP, drastically alters the proximal histidine−aspartate H-bond interaction making KatG structurally similar to CCP, increasing the peroxidase activity with a concomitant dramatic reduction of the overall catalase activity.[42,49]

The present results suggest that small structural perturbations and disruption of the H-bond networks have only a small impact for compound I formation, which is common to both catalatic and peroxidatic activity, but a defined extended H-bond network is crucial for selectively guiding and binding the (second) peroxide molecule necessary for the catalatic activity.[42,49]

Vinyl−Protein Interaction

Despite the largely invariant coordination and spin state of the plant peroxidases and the close similarity of their RR spectra, the Soret band wavelength varies from 398 nm in barley peroxidase (BP1)[18] to 408 nm in CCP[50] as a consequence of the degree of conjugation between the porphyrin macrocycle and the vinyl substituents.[51] The conjugation can induce an upshift of the UV−vis maxima of up to 10 nm.[52] To rationalize this effect, we analyzed the absorption and RR spectra of selected mutants and fluoride complexes of various peroxidases. The vinyl stretching frequencies of the proteins markedly differ. Their X-ray structures revealed that vinyl orientation differs mainly at position 2 as a consequence of different steric hindrances imposed by the protein matrix. As the constraint on the 2-vinyl diminishes, the conjugation between the vinyl and the porphyrin decreases, the 2-vinyl $\nu_{C=C}$ stretching frequency increases, and a large frequency separation is observed between the two $\nu_{C=C}$ stretching modes in the RR spectra. When the protein matrix exerts weak or no constraints on the 2-vinyl group, two distinct $\nu_{C=C}$ stretching modes are found in the RR spectra. The $\nu_{C=C}$ vinyl stretching frequency, therefore, is a sensitive probe of the degree of electronic conjugation between vinyl and porphyrin. We established a relationship between the $\nu_{C=C}$ stretching frequency and the torsion angle, τ, of the 2-vinyl group, involving the relative orientations of vinyl (C_b-C_a) and pyrrole ($C_\beta-C_\alpha$) C=C double bond π-planes. The vinyl and pyrrole double bond π-planes parallel ($\tau = 140°$) and perpendicular ($\tau = 50°$) give rise to maximum and minimum conjugation between the vinyl group and the porphyrin macrocycle (Table 1). Three limiting vinyl conformations (trans, twist, and cis) are sketched in Figure 5.[28] The amino acid that interacts with vinyl 2 is possibly that at position 172 (CCP numbering), which is methionine in CCP, leucine in class II peroxidases, and serine in ascorbate peroxidase (APX) and class III peroxidases (Figure 5). The repulsive interaction between the vinyl group and the amino acid is strongest in CCP (class I) and weakens on passing from class II to class III peroxidases and APX (class I).

On the basis of the spectroscopic data, amino acid sequence alignment, and the distances between the vinyl

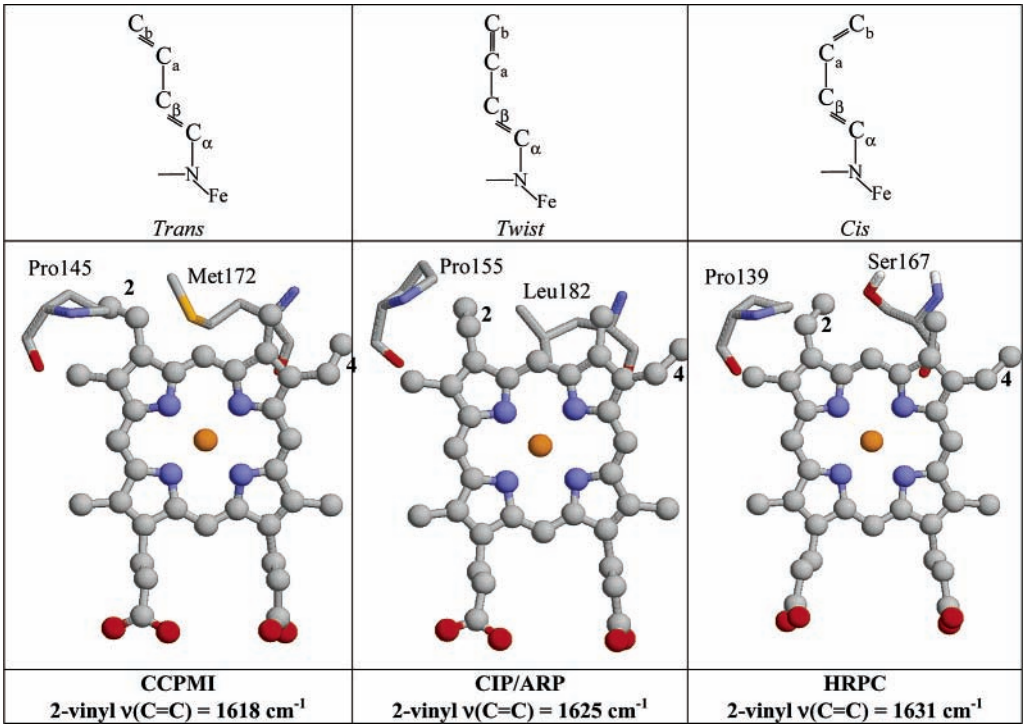


FIGURE 5. Schematic representations (top) of the different 2-vinyl conformations and structures (bottom) viewed along the *z*-axis, perpendicular to the heme plane, of CCPMI, CIP/ARP, and HRPC.

Table 1. Spin and Coordination States, 2-Vinyl Stretch Frequencies, Torsion Angles, τ, and Soret Band Maxima of Peroxidases[28]

	coordination spin states	2-vinyl[a] $\nu_{C=C}$ (cm^{-1})	τ (deg)	Soret λ_{max} (nm)
CCP	5cHS	1618	152.1	408
APX	5cHS	1629	28.8	403
CIP/ARP	5cHS	1625 s	88.3	403
Phe54Gly CIP/ARP	6cHS	1635 w		402
HRPC	6cQS/5cQS	1631 s	36.9	403
A. thaliana A2	5cQS/5cHS/6cHS	1631	43.4	403
soybean peroxidase	5cQS/5cHS/6cHS	1632	42.3	402
BP1	5cQS	1631 s	−64.3	398

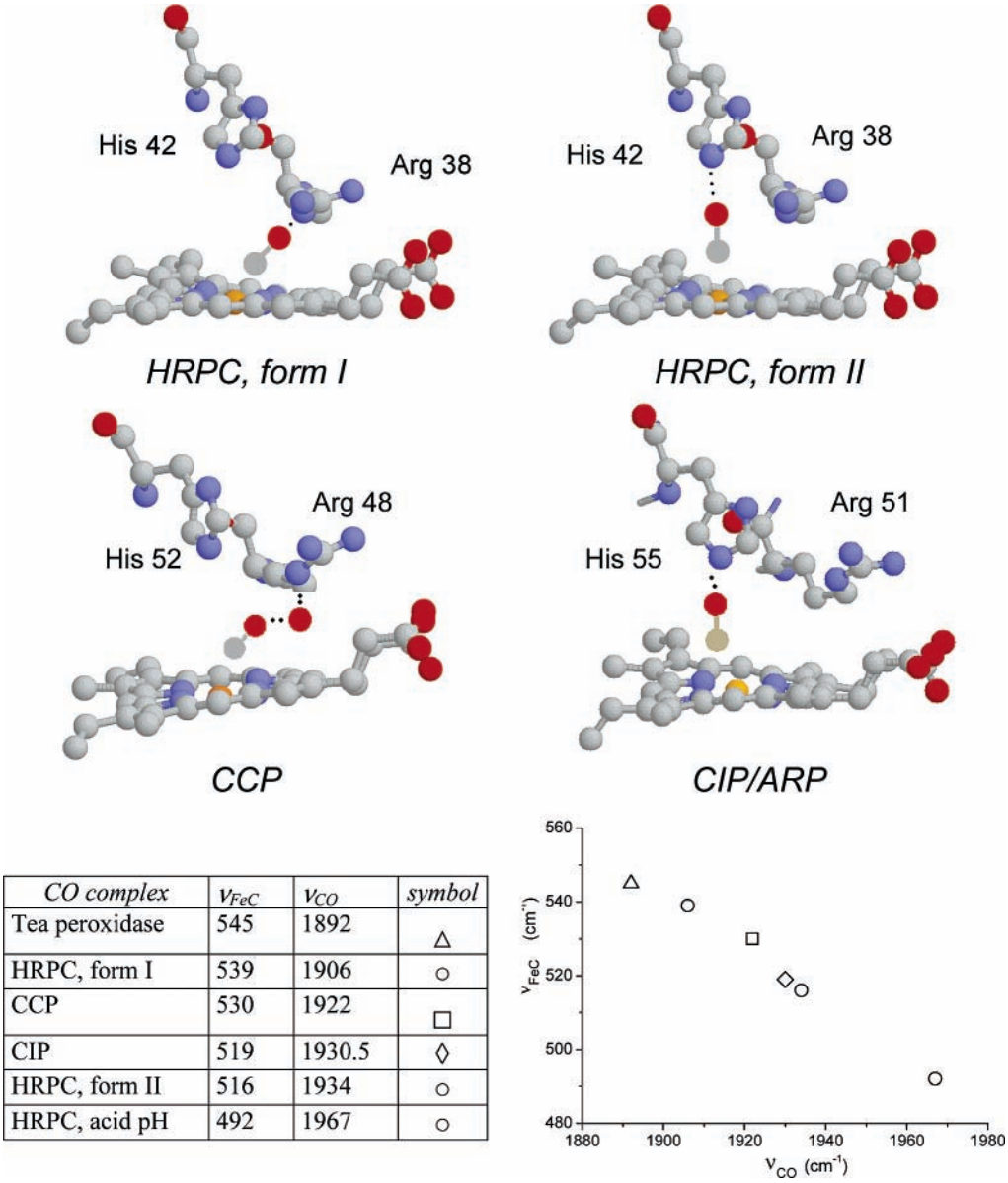
[a] s and w indicate a strong and weak RR band, respectively.

C_a and C_b atoms and the surrounding amino acids as determined by the X-ray structures, the role of a variety of interactions, including van der Waals contacts, $\pi-\pi$ electron interactions, and C−H···O hydrogen bonds, was inferred. The vinyl group orientations appear to depend on the concerted orientations of the distal helix B and proximal helix F axes, controlled by the H-bonds between the distal arginine, a heme propionyl group, and an amino acid on the extended strand adjacent to proximal helix F.

Ligand Binding

Carbon Monoxide. H-bonding and, more generally, polar interactions between the bound CO and the distal amino acids can be related to important structural features of the heme pocket. The strength of these interactions can be inferred from the Fe−C and CO stretching frequencies, which are linked by an inverse correlation.[53] Polar interactions increase the back-donation from the Fe d_π to the CO π^* orbitals, thereby strengthening the Fe−C bond while weakening the CO bond and increasing the FeC stretching frequency while decreasing the CO stretching frequency (table and plot in Figure 6).[54,55] The comparison of the CO complexes of horseradish peroxidase (HRPC), CCP, and *Coprinus cinereus* peroxidase (CIP/ARP, since the covalent structure and enzymatic properties of the peroxidases from *Coprinus cinereus*, CIP, and *Arthromyces ramosus*, ARP, are identical[56]) highlights the considerable sensitivity of CO in probing the heme environment. We found that in these peroxidases the oxygen atom of the bound CO experienced H-bond interactions with the distal amino acids. HRPC displays two conformers, which have been identified on the basis of the RR and IR spectra of distal mutants,[57] one being H-bonded with the distal arginine and the other with the distal histidine. At pH 6, CCP[58] is characterized by only one form in which, according to the X-ray structure, the CO is H-bonded to the distal arginine via a water molecule[59] (Figure 6). In CIP/ARP, the bound CO has a H-bond interaction with the distal histidine, very similar to form II in HRPC, and the data indicate a distal side that is more open or flexible than in other plant peroxidases.[60]

Anionic Ligands: Fluoride and Hydroxide. In general, heme proteins bind fluoride at acid pH and hydroxide at alkaline pH. CCP represents an interesting exception since the protein undergoes an acid−alkaline transition involving a substantial protein conformational change, as revealed by NMR[61] and by perturbations of RR bands associated with the heme vinyl substituents. This change permits the binding of the distal His52 or, as in the case of the Leu52 mutant, the hydroxide to the Fe atom.[27,33] Since the His52 N_ϵ imidazole is ca. 5 Å away from the Fe atom in the crystal structure of the acid form, the change



CO complex	ν_{FeC}	ν_{CO}	*symbol*
Tea peroxidase	545	1892	△
HRPC, form I	539	1906	○
CCP	530	1922	□
CIP	519	1930.5	◊
HRPC, form II	516	1934	○
HRPC, acid pH	492	1967	○

FIGURE 6. H-bonding interactions of bound CO in HRPC, CCP, and CIP/ARP. The structure of CO−CCP is adapted from ref. 59. The other structures are pictorial representations based on the RR results. The tilting and bending angles of bound CO are exaggerated for clarity. The X-ray structure of CO−CCP displays a 12° tilt, and that of CO−HRPC displays a bending angle of 171°.[71] The table and plot show the inverse linear ν_{FeC}/ν_{CO} frequency correlation for the above proteins and for the extreme back-bonding cases of CO−HRPC at acid pH[70] and the CO complex of tea ascorbate peroxidase.[72]

generates tension in the protein, as revealed by the unusual photolability of the bound His52 ligand in the protein at alkaline pH.[62]

The comparison of the UV−vis and RR spectra of the fluoride and hydroxide complexes of various peroxidases and selected mutants has highlighted that the complex mechanism of stabilization of anionic ligands exerted by the distal amino acids resembles that of compound I formation during peroxidase catalysis, where ligand stabilization by the distal arginine is coupled to protonation of the distal histidine.[63] Replacing arginine with leucine inhibits fluoride and hydroxyl binding. As previously shown by the X-ray structure,[64] this amino acid is determinant in controlling the anion binding via a strong H-bond between the positively charged guanidinium group and the anion. Concomitantly, the distal histidine accepts a proton and H-bonds probably through a water molecule with the anion.[18,39,51,65−68] A sensitive indicator of axial ligand interactions with the distal amino acids is the wavelength of the charge-transfer transition (CT1). It blue-shifts when the p and/or π donor capability of the axial ligands increases[39,66,69,70] or when the ligand acts as a H-bond donor. The stronger the H-bond and charge donation to the iron atom, the lower is the CT1 wavelength. The opposite effect is observed when the ligand acts as an H-bond acceptor. This trend is illustrated by the effect of distal mutation on the CT1 band of CCP−F[51] and CIP−F (Table 2).[67] The CT1 wavelength of the CCP−F complex, where the bound F^- receives hydrogen bonds from Arg48, Trp51, and a water molecule,[59] is greater than

Table 2. Soret and CT1 Bands (nm) of Peroxidase−Fluoride Complexes[39,51,65,67]

proteins	Soret	CT1	H-bonds with fluoride		
CCP	406	617	H_2O	Trp51	Arg48
His52Leu CCP	406	617	?	Trp51	Arg48
Trp51Phe CCP	406	613	H_2O		Arg48
CIP/ARP	406	615	H_2O		Arg51
Phe54Trp CIP/ARP	407	618	H_2O	Trp54	Arg51
Arg51Gln CIP/ARP	405	613	H_2O		Gln51
HRPC	404	611	H_2O		Arg38
His42Leu HRPC	403	610	?		Arg38
myoglobin	406	610	H_2O	His64	

that observed for CIP−F. In the CCP Trp51Phe mutant, the number of H-bonds is reduced from three to two. The loss of the H-bond donor Trp51 blue-shifts the CT1 band to a position similar to that of the CIP−F complex, which also has only two H-bond donors. Conversely, replacement of Phe54 with tryptophan in CIP causes a red-shift of the CT1 band by 3 nm with respect to the parent enzyme giving rise to an absorption spectrum very similar to that obtained for CCP.

Conclusions

The considerable insight into biological processes that may be gained by the powerful combination of spectroscopic techniques and mutagenesis is amply demonstrated by the case of the peroxidases. The application of spectroscopic methods to peroxidase mutants has been a driving force in unraveling protein function details. Functional studies of distal variants confirmed that the distal histidine and arginine are mainly responsible for the characteristic peroxidase activity, playing a concerted role in the catalytic cycle. However, protein function is affected to a remarkably variable extent by mutation of the key residues. This variability is not due to the different effects of mutation on the heme coordination and spin states since, for example, although replacement of arginine by leucine preserves the 5-coordination state in the mutated peroxidases their catalytic activity varies to a great extent.[8,27,38,39,56] Therefore, it depends on the role played in the catalytic cycle by the key residues and the fact that they are involved in H-bonded networks essential for protein stability and communication between the two sides of the heme pocket.

We gratefully acknowledge the contributions of our collaborators whose names are listed in appropriate references.

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