Identification of a Critical Phenylalanine Residue in Horseradish Peroxidase, Phe179, by Site-Directed Mutagenesis and ^{1}H-NMR: Implications for Complex Formation with Aromatic Donor Molecules†

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ABSTRACT: The functional and structural significance of Phe179 of horseradish peroxidase isoenzyme C (HRP C) has been investigated by site-directed mutagenesis. This residue is located in a structurally variable insertion between helices F and G, a motif unique to peroxidases of higher plants. Results obtained for three recombinant enzymes, with Phe179 substituted by Ala, His, or Ser, provide the first demonstration of the importance of this side chain for the binding of aromatic donor molecules. Experimental parameters for direct comparison with the wild-type enzyme were obtained by extensive solution state characterization using both optical and ^{1}H-NMR spectroscopy. Significant chemical shift variations for resonances associated with the exposed heme edge, notably heme methyl C18H$_3$ and heme propionate C17^{1}H$_2$, were recorded in NMR spectra of both the resting and cyanide-ligated states of the three Phe179 mutants. Furthermore, comparison of NOE connectivities in NOESY spectra of cyanide-ligated wild-type and mutant enzymes enabled the elusive assignment of the aromatic side chain in close proximity to heme methyl C18H$_3$ to be made to Phe179. Replacement of Phe179 by Ala resulted in an 80-fold decrease in the binding affinity of the cyanide-ligated enzyme for benzhydroxamic acid, with a K_d value similar to that determined for cyanide-ligated HRP A2 (an acidic isoenzyme with valine at position 179). The binding affinity of Phe179→Ser was similarly decreased, while that of Phe179→His was partially restored relative to wild-type HRP C. Cyanide-ligated Phe179→His HRP C exhibited a unique pH-dependent spectral transition associated with a pK_a value of 6.5 ± 0.2, assigned to the His179 side chain. Two closely related enzyme forms exhibiting different affinities for benzhydroxamic acid were observed at neutral pH and above, indicating that the protonation state of His179 gave rise to microheterogeneity in the aromatic donor molecule binding site.

Horseradish peroxidase is a notable example of a heme enzyme which finds wide use in a growing number of industrial, biomedical, and biotechnological processes. Specific applications, which include waste water remediation, biosensor construction, and bioluminescent and immunodiagnostic assays, exploit the ability of the enzyme to oxidise a structurally diverse range of substrates. In this context, fundamental studies with the objective of characterizing interactions between substrates and the enzyme have assumed additional importance. The general reaction scheme of the most abundant and widely used C isoenzyme of horseradish peroxidase (HRP C)[1] has been well described and proceeds through two high oxidation state intermediates, compounds **I** and **II**, following addition of hydrogen peroxide to the resting state of the enzyme. These intermediates are reduced rapidly by substrates in one electron transfer reactions (*1*).

Complexes formed between HRP C and so-called aromatic donor molecules in the absence of hydrogen peroxide have been known for some time (*2*−*4*), with dissociation constants ranging from 0.2 μM for 2-naphthohydroxamic acid (*3*), to mM values for many phenolic compounds (*2*, *4*). The complexes, while intrinsically interesting in themselves, are also useful systems within which our understanding of the substrate interaction site can be developed. In particular, since there is no evidence for substantial conformational change in peroxidase structure on formation of compounds **I** and **II** (*5*, *6*), it is reasonable to assume that complexes formed between phenols or other substrates and the enzyme can be viewed as structurally representative of transient pre-electron transfer complexes involved in the reduction of compounds **I** and **II**. Many complexes are particularly amenable to spectroscopic characterization, with techniques such as resonance Raman and NMR finding wide application (*7*, *8*). The consensus view, which is supported by additional

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[1] Abbreviations: F68A HRP C, the Phe68→Ala mutant of HRP C; F142A HRP C, the Phe142→Ala mutant of HRP C; F143A HRP C, the Phe143→Ala mutant of HRP C; F179A HRP C, the Phe179→Ala mutant of HRP C; F179H HRP C, the Phe179→His mutant of HRP C; F179S HRP C, the Phe179→Ser mutant of HRP C; HRP A2, horseradish peroxidase isoenzyme A2; HRP C, horseradish peroxidase isoenzyme C; PNP, the major cationic isoenzyme of peanut peroxidase; 5-c HS, five-coordinate high-spin; 6-c HS, six-coordinate high-spin.

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results from suicide inactivation experiments (*9*), is that substrates or aromatic donor molecules (the two terms are often used interchangeably) bind close to the "exposed" heme edge, that is to say, in the vicinity of heme methyl $C18H_3$. Benzhydroxamic acid, although not a physiological substrate of HRP C, has been found to be an excellent structural probe in such studies, much as originally envisioned by Schonbaum (*3*), while NMR spectroscopy has been demonstrated to be one of the most versatile solution state techniques for characterizing the complexes formed (*10*−*12*).

The identification of other structural components of the binding site, such as those provided by heme pocket amino acid side chains, has been reviewed recently (*8*). Site-directed mutagenesis studies have also confirmed that distal Arg38 and His42 are important for the binding of benzhydroxamic acid, where they engage in hydrogen bonded interactions with the hydroxamate group (*13, 14*). It is also known that the presence of the side chains of one, or possibly two, Phe residues in the site makes an additional contribution to the overall binding affinity for many molecules (*8*). The most important of these Phe side chains lies within NOE distance of heme methyl $C18H_3$ (*15, 16*). Specific assignment has proved difficult in the absence of three-dimensional crystal structure data for HRP C, although some suggestions have been forthcoming from the results of homology modeling of the structure (*17*). Recently, Schuller et al. published a 2.7 Å resolution structure of the major cationic isoenzyme of peanut peroxidase (PNP), the first complete three-dimensional structure of a heme peroxidase from higher plants (*18*). These authors noted however that the substrate access channel of PNP appeared to lack a clearly distinguished pocket which would constitute a specific binding site for aromatic substrates, in contrast to that established for HRP C. This emphasizes the continuing importance of site-directed mutagenesis studies in HRP C to identify key residues and assess their relative importance for substrate binding, dynamics, and catalysis. Three site-directed mutants, with Phe68, Phe142, and Phe143 each substituted by Ala (F68A, F142A, and F143A HRP C, respectively), have been examined previously (*19*−*21*). While there is some modulation of both binding affinity and the dynamics of the interaction with benzhydroxamic acid for F68A and F142A HRP C, it is clear from the magnitude of the changes that these residues are peripheral to the binding site and exert only an indirect influence (*19, 21*). The behavior of the F143A HRP C mutant was found to be almost identical to that of the wild-type enzyme (*19, 20*).

In the present investigation, a hypothesis proposed from preceeding studies that the identity of the key side chain in contact with the heme is Phe179, has been examined critically (*17, 19*). Three site-directed mutants, with Phe179 substituted by Ala, His, and Ser (F179A, F179H, and F179S HRP C, respectively), have been characterized in solution by optical and NMR spectroscopy in both resting (high-spin) and cyanide-ligated (low-spin) states. Dissociation constants for benzhydroxamic acid complexes with resting and cyanide-ligated enzymes have been determined from titration data obtained using optical and NMR spectroscopy, respectively. The results are compared with those obtained for the wild-type enzyme, related HRP isoenzymes, and selected site-directed mutants.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Mutagenic oligonucleotide primers designated F1 (5′-GTCCATGATGG[A/C/T]GCGA-CACTGG) and F2 (5′-GTCCATGAT[G/C]T[G/C]CCG-GCACTGGTTCTT) encoded several mutants, including F179A and F179S HRP C and F179H HRP C, respectively. Each of these was used in conjunction with an adjacent nonmutagenic primer F3 (5′-CGGTTATACAATTTCAG-CAAC), to amplify the whole expression plasmid including the mutant HRP gene with *Pfu* polymerase (Stratgene Ltd.) by an in-house PCR procedure which will be described in detail elsewhere. The primers were deprotected as previously described (*22*). The DNA sequence of the mutated HRP gene was verified by automated DNA sequencing (Applied Biosystems) and no unexpected sequence changes were detected. Growth and induction of HW1110 (F179A HRP C), HW1110 (F179S HRP C), and HW1110 (F179H HRP C) and refolding and activation were as previously described (*22, 23*) except that the HRP C polypeptide isolated from *Escherichia coli* inclusion bodies was not treated with 30 mM dithiothreitol prior to refolding since it was found to be in the reduced state when expressed (*24*).

Preparation of Peroxidase Samples for NMR Spectroscopy. Wild-type plant HRP C was obtained from Biozyme Laboratories Ltd as a salt-free lyophilized powder (Biozyme HRP-4B) with an RZ (Reinheitzahl), or purity index, value of 3.2. Purified recombinant enzymes F179A, F179H, F179S, and wild-type HRP C were stored at −70 °C as frozen solutions in 10 mM MOPS buffer, pH 7.0. After thawing, these samples were subjected to several cycles of concentration and solvent exchange, carried out at 4 °C in Amicon Centricon 10 microconcentrators. The solvent systems used were 20 mM KH_2PO_4, D_2O, pH 7.0, and 20 mM KH_2PO_4, 15 mM KCN, D_2O, pH 7.6, for preparation of resting and cyanide-ligated states of the enzymes, respectively. A similar procedure was applied in the case of wild-type plant HRP C, except that the enzyme was dissolved directly in the solvent of interest. The solvent system used for the pH titration of cyanide-ligated F179H HRP C was 20 mM KH_2PO_4, 15 mM KCN, D_2O, pH 5.5. pH adjustments were made by addition of 20-fold diluted NaOD. A Radiometer pH26 meter was used for pH measurements which are uncorrected for the small deuterium isotope effect. Specific readings in D_2O solutions are denoted as pH* in the text. Enzyme concentrations were determined spectrophotometrically using extinction coefficients of 98, 102, 102, 101, and 115 cm^{-1} mM^{-1} at the Soret maxima for wild-type recombinant, wild-type plant (*25*), F179A, F179H, and F179S HRP C respectively. Extinction coefficients at the Soret maxima of wild-type recombinant HRP C and F179 HRP C mutants were determined by the pyridine hemochrome method (*26*). The concentration of recombinant enzyme samples was typically 0.30−0.50 mM.

NMR Spectroscopy. All ^{1}H-NMR experiments were recorded using either Varian or Bruker 500 MHz instruments. One-dimensional spectra of resting state enzymes were obtained with a spectral width of 100 kHz, 8−16K transients, and a recycle time of 5 s^{-1}. In the case of cyanide-ligated enzymes, a spectral width of 40 kHz, 2K transients and a recycle time of 2 s^{-1} were standard. The 90° pulse width was typically 6.2−6.6 μs, with a 45° flip angle used for acquisition of spectra of the resting state enzymes. Spectral processing of one-dimensional spectra introduced line-

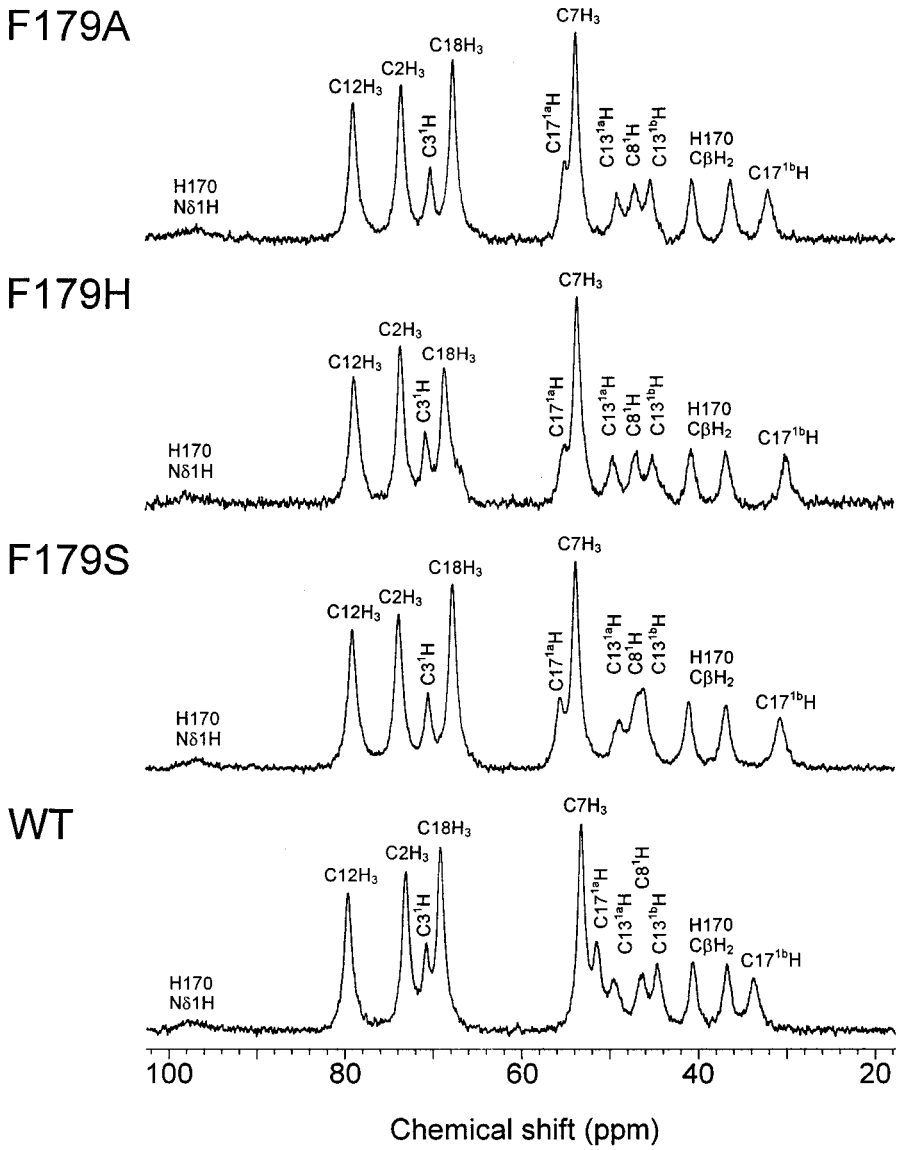

FIGURE 1: Hyperfine-shifted regions from the ^{1}H-NMR spectra of resting state wild-type recombinant HRP C and F179 HRP C mutants. A number of heme and proximal His170 proton resonance assignments are given for reference. Spectra were recorded at 500 MHz with solution conditions of 20 mM potassium phosphate, D_2O, at pH 7.0 and 30 °C.

broadening factors of 30 and 10 Hz for resting state and cyanide-ligated enzyme samples, respectively. Baseline correction was carried out for all spectra. Two-dimensional spectra were obtained using the general procedures described previously (*27*). All experiments were carried out at 30 °C and referenced to 1,4-dioxan as an internal standard with a resonance at 3.74 ppm relative to 2,2-dimethyl-2-silapentane-5-sulfonate.

Determination of Dissociation Constants. The dissociation constants (K_d) of complexes formed between resting state enzymes and benzhydroxamic acid were determined by titration of the Soret region of the visible spectrum as described previously (*22*). The solution conditions used were 10 mM sodium phosphate at pH 7.0 and 25 °C. K_d values were calculated by fitting the data to eq 1 using a weighted least-squares error minimization procedure.

$$A = 2A_\infty L/\{(L + K_d + P) + [(L + K_d + P)^2 - 4PL]^{1/2}\} \quad (1)$$

The absorbance change at 408 nm resulting from benzhydroxamic acid of concentration L binding to a total protein concentration P was determined, while allowing the remaining K_d and maximum absorbance change at saturation (A_∞) to float. Dissociation constants for complexes formed between cyanide-ligated enzymes and benzhydroxamic acid were determined using chemical shift data obtained from the titration profiles of selected resonances in ^{1}H-NMR spectra as described previously (*19*). Experiments were carried out by direct addition of small aliquots (typically 2−20 μL) of titrant to a known concentration and volume of enzyme contained within an NMR tube. The stock titrant solution was 50.0 mM benzhydroxamic acid (obtained from Sigma as a crystalline substance) in 20 mM KH_2PO_4, 15 mM KCN, D_2O, pH 7.6. All data were corrected for dilution during substrate addition. A modified strategy was employed for the determination of K_d values in the case of the complex formed between cyanide-ligated F179H HRP C and benzhydroxamic acid, due to its heterogeneity. The relative concentrations of the two species present were obtained by integration of the corresponding $C18H_3$ resonances in the ^{1}H-NMR spectrum. The chemical shift profile of the $C18H_3$ resonance representing the major species was fitted as described above to obtain an initial estimate of K_d. This value was then used to calculate the concentration of free

benzhydroxamic acid experienced by the minor species and subsequently estimate its K_d. Values for the concentrations of free benzhydroxamic acid were then recalculated, taking into account the effect of the minor species. These were used to revise the initial estimate of K_d for the major species. This iterative procedure was continued until successive calculations showed no further significant change in free benzhydroxamic acid concentration or the K_d values of both species.

Amino Acid Sequence Nomenclature in Plant Peroxidases. It should be noted that the sequence of PNP has a single site deletion corresponding to Arg159 of HRP C (*28*). Consequently, the residues from Thr159 to Asn188 in PNP are shifted in sequence number by −1 in some publications relative to the corresponding residues in HRP C (*18*). Amino acid sequence numbering used in the present study follows that of HRP C for ease of reference (*29*).

RESULTS

Resting State Enzymes. The hyperfine-shifted resonances from ^{1}H-NMR spectra of resting state wild-type and mutant enzymes are compared in Figure 1. These resonances, which appear typically between 20 and 100 ppm for high-spin heme peroxidases, are highly sensitive to changes in the immediate heme environment of the enzymes. An extensive series of studies involving reconstitution with deuterated hemes, one-dimensional NOE experiments, and analysis of two-dimensional NOESY spectra has allowed specific assignments to be made for these resonances in plant HRP C (*30*−*32*), the only high-spin peroxidase for which they are currently available. The spectra of wild-type plant and recombinant HRP C have been shown to be identical (*33*), although a distinct improvement in both linewidth and resolution of the hyperfine-shifted resonances is afforded by use of the latter enzyme due to a 20% reduction in molecular mass (recombinant HRP C produced in *E. coli* is nonglycosylated). Each of the spectra presented in Figure 1 comprises one very broad resonance between 95.0 and 100.0 ppm, four heme methyl resonances, and eight heme resonances each with the intensity of a single proton. This set of resonances is most clearly resolved in the spectra of F179A and F179H HRP C. While the spectra of the F179 mutants resemble the spectrum of the wild-type overall, there are a number of significant chemical shift differences evident for certain resonances, as summarized in Table 1. The greatest variation in chemical shift is shown by the heme propionate C17^{1}H$_2$ protons of the mutants, with +3.4 to +4.1 ppm and −1.6 to −3.9 ppm for C17^{1a}H and C17^{1b}H, respectively, compared to wild-type. The heme methyl C18H$_3$ resonances of the mutants also exhibit significant shifts of between −0.7 and −1.5 ppm compared to wild-type. In addition, the C18H$_3$ resonance of F179H HRP C is of reduced intensity relative to the corresponding resonances of F179A, F179S, and wild-type HRP C, with a shoulder visible to slightly higher field at 66.8 ppm. Relatively small shifts of less than ±1.0 ppm are found for the remaining protons of the heme group and His170, with the exception of one of +1.4 ppm for C13^{1b}H of F179S HRP C, compared to the wild-type value.

Cyanide-Ligated Enzymes. All three F179 mutant enzymes readily formed six-coordinate low-spin cyanide-ligated complexes in the presence of cyanide at pH 7.6. The hyperfine-shifted resonances from the ^{1}H-NMR spectra of the cyanide-ligated enzymes are compared with those of the wild-type enzyme in Figure 2, and the corresponding assignments summarized in Table 2. This set also includes a number of resonances assigned to amino acid residue side chains in the heme pocket such as Arg38, His42, and Ile244, in contrast to the spectrum of the resting state enzyme where only His170 is represented. The assignments were obtained by identifying established patterns of connectivities in NOESY spectra (*16*, *33*). It is evident from Figure 2 that while each spectrum comprises the same set of resonances, there are many significant chemical shift changes as a consequence of the substitutions at position 179. The most strongly perturbed heme resonances are once again those of heme methyl C18H$_3$ and heme propionate C17^{1a}H, with maximum shift changes of −1.12 (C18H$_3$) and +1.95 (C17^{1a}H) ppm recorded in the spectra of cyanide-ligated F179A and F179H HRP C, respectively, compared with cyanide-ligated wild-type enzyme. Heme pocket amino acid residue protons are also affected, notably Arg38 Cβ1H and Cδ1H, which show considerably greater chemical shift variation than His42 Cϵ1H and Ile244 CδH$_3$. The most upfield shifted resonance, that of the proximal ligand ring proton His170 Cϵ1H, exhibits the greatest overall chemical shift perturbations, namely, −1.9, −2.0, and −3.3 ppm, for F179H, F179S, and F179A HRP C, respectively. One general trend which can be observed from the data in Table 2 is that where significant chemical shift perturbations of ±0.40 ppm or more are observed, their magnitude varies according to the order F179A > F179S > F179H, with the exception of the heme propionate proton C17^{1a}H, for which this order is reversed.

pH Dependence of Cyanide-Ligated F179H HRP C. The ^{1}H-NMR spectrum of cyanide-ligated F179H HRP C recorded at pH 7.6 and shown in the spectral comparison of Figure 2 is anomalous due to the appearance of an additional broad resonance at 29.07 ppm located between the resonances of C18H$_3$ and C7H$_3$ and to the apparent decrease in intensity and increase in linewidth of the C18H$_3$ resonance itself. These features are absent from the corresponding spectra of cyanide-ligated F179A, F179S, and wild-type HRP C. Their origin was investigated by titration of the cyanide-ligated enzyme, the results of which are illustrated in Figure

Table 1: Comparison between ^{1}H-NMR Assignments for Hyperfine-Shifted Resonances of Resting States of F179 Mutants of Horseradish Peroxidase and the Wild-Type Enzyme[a]

proton[b]		chemical shift data in ppm			
		WT	F179A	F179H	F179S
His170 Nδ1H	—	97.5	−0.7	−0.3	−0.6
C12H$_3$	5-CH$_3$	79.7	−0.7	−0.9	−0.6
C2H$_3$	1-CH$_3$	73.1	+0.5	+0.4	+0.7
C3^{1}H	2-H$_\alpha$	70.8	−0.6	−0.1	−0.3
C18H$_3$	8-CH$_3$	69.2	−1.5	−0.7	−1.5
C7H$_3$	3-CH$_3$	53.3	+0.5	+0.2	+0.5
C17^{1a}H	7-H$_\alpha$	51.5	+3.6	+3.4	+4.1
C13^{1a}H	6-H$_\alpha$	49.5	−0.3	0.0	−0.6
C8^{1}H	4-H$_\alpha$	46.5	+0.7	+0.4	+0.4
C13^{1b}H	6-H$_{\alpha'}$	44.7	+0.7	+0.3	+1.4
His170 Cβ1H	—	40.7	0.0	−0.1	+0.3
His170 Cβ2H	—	36.8	−0.5	−0.1	0.0
C17^{1b}H	7-H$_{\alpha'}$	33.8	−1.6	−3.9	−3.1

[a] Chemical shift data obtained at 30 °C with solution conditions of 20 mM potassium phosphate, D_2O at pH 7.0. The chemical shift values for ^{1}H resonances of the F179 mutants are expressed as the difference in ppm from the wild-type value. [b] The older Fischer nomenclature for heme protons is also given for reference.

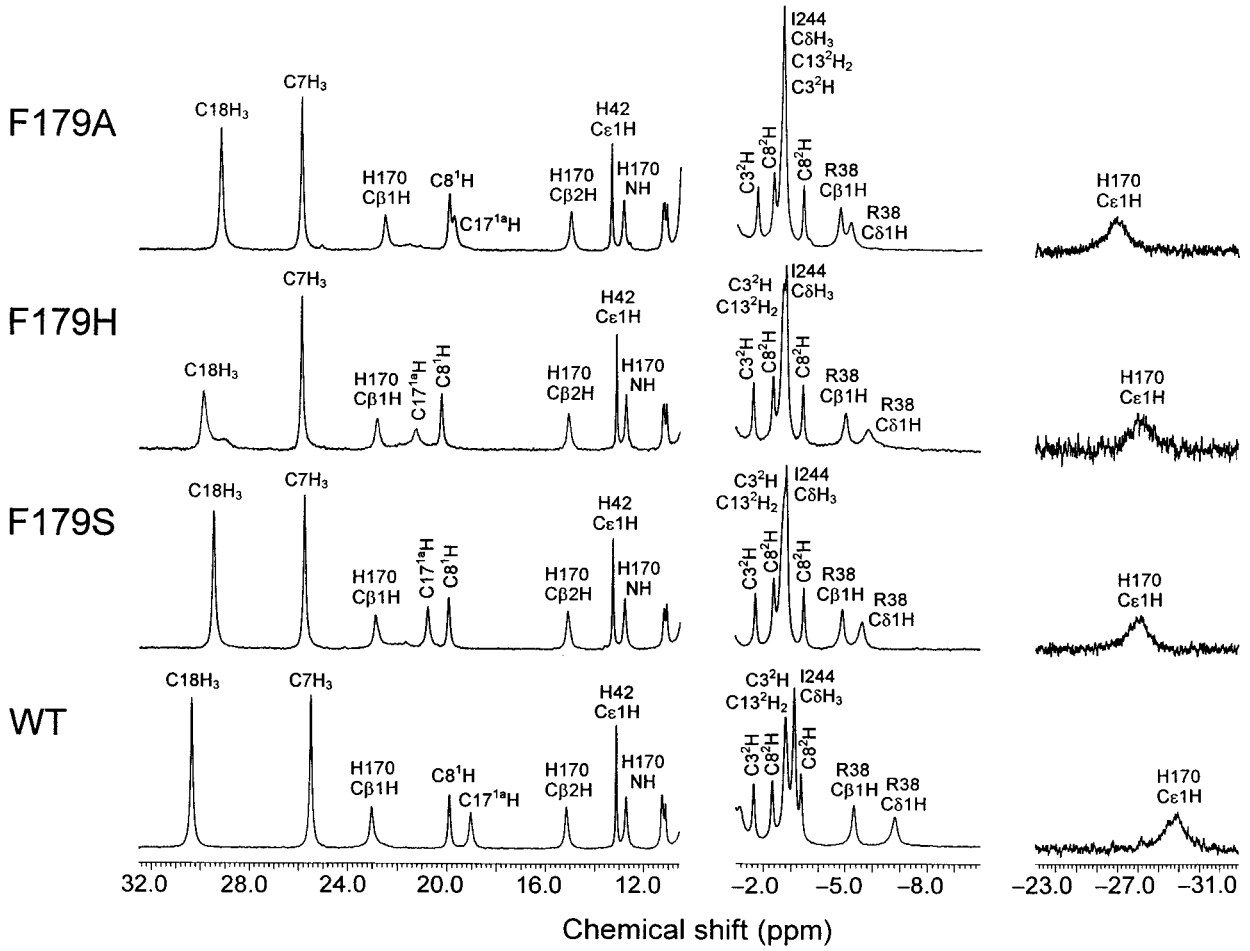

FIGURE 2: Hyperfine-shifted regions from the ^{1}H-NMR spectra of cyanide-ligated wild-type recombinant HRP C and F179 HRP C mutants. A number of heme and heme-linked proton resonance assignments are given for reference. The spectral region containing the broad, upfield-shifted resonance of His170 Cϵ1H is plotted at $\times$8 intensity relative to the two other regions. Spectra were recorded at 500 MHz with solution conditions of 20 mM potassium phosphate, 15 mM potassium cyanide, D_2O, at pH 7.6 and 30 °C.

Table 2: Comparison between ^{1}H-NMR Assignments for Hyperfine-Shifted Resonances of Cyanide-Ligated States of F179 Mutants of Horseradish Peroxidase and the Wild-Type Enzyme[a]

proton[b]		chemical shift in ppm			
		WT	F179A	F179H	F179S
$C3^2H$	2-H$_\beta$	−1.61	−1.76	−1.63	−1.73
$C3^2H$	2-H$_{\beta'}$	−2.77	−2.72	−2.72	−2.80
$C7H_3$	3-CH_3	25.39	25.89	25.90	25.80
$C8^1H$	4-H$_\alpha$	19.87	19.94	20.27	19.98
$C8^2H$	4-H$_\beta$	−2.26	−2.35	−2.34	−2.39
$C8^2H$	4-H$_{\beta'}$	−3.29	−3.43	−3.43	−3.48
$C13^{2a}H$	6-H$_\beta$	−2.77	−2.72	−2.72	−2.77
$C13^{2b}H$	6-H$_{\beta'}$	−2.77	−2.72	−2.72	−2.77
$C17^{1a}H$	7-H$_\alpha$	19.34	19.76	21.29	20.82
$C18H_3$	8-CH_3	30.31	29.19	29.90	29.50
Arg38 Cβ1H		−5.31	−4.79	−5.01	−4.93
Arg38 Cδ1H		−6.87	−5.16	−5.83	−5.65
His42 Cϵ1H		13.17	13.36	13.16	13.31
His170 NH		12.74	12.87	12.78	12.82
His170 Cβ1H		23.18	22.54	22.84	22.91
His170 Cβ2H		15.23	15.01	15.11	15.14
His170 Cϵ1H		−29.2	−25.9	−27.3	−27.2
Ile244 CδH_3		−3.14	−2.72	−2.81	−2.85

[a] Chemical shift data obtained at 30 °C with solution conditions of 20 mM potassium phosphate, 15 mM potassium cyanide, D_2O at pH 7.6. [b] The older Fischer nomenclature for heme protons is also given for reference.

3. Analysis of an additional ^{1}H-NMR spectrum recorded at pH 9.7* indicated the onset of more profound changes to the integrity of the enzyme, and higher pH measurements were not pursued. Chemical shift values of key resonances differed by no more than the experimental margin of error, ± 0.01 ppm, between pH* 8.6 and 9.7.

At pH* 5.5, the heme methyl $C18H_3$ resonance at 29.71 ppm has a linewidth significantly greater than that of the neighboring $C7H_3$ resonance. As the pH is increased, the $C18H_3$ resonance becomes downfield-shifted, reaching a chemical shift value of 29.93 ppm at pH* 8.6, and its linewidth increases, passing through a maximum close to pH* 6.4. In addition, a second component, appearing slightly upfield of this resonance, can be detected in the spectra above pH* 6.4. Its linewidth decreases as the pH is raised above this value, and a chemical shift value for the resonance of 29.03 ppm is attained at pH* 8.6. The heme propionate $C17^{1a}H$ resonance also shows similar titration behavior, with a chemical shift of 20.64 ppm at pH* 5.5 and an overall perturbation of +0.70 ppm. The chemical shift data for the $C18H_3$ resonance were used to derive a pK_a value of 6.5 ± 0.2 for this transition, as illustrated in Figure 4. There were no comparable pH-dependent chemical shift changes noted to other resonances appearing in the hyperfine-shifted region of the spectrum.

The two resonances appearing at 29.93 and 29.03 ppm in the spectrum at pH* 8.6 have an integrated intensity which is equal to that of the heme methyl $C7H_3$ resonance, and a relative intensity ratio of 4:1 respectively. The premise that both resonances might be derived from heme methyl $C18H_3$ was verified by analysis of a NOESY spectrum of cyanide-

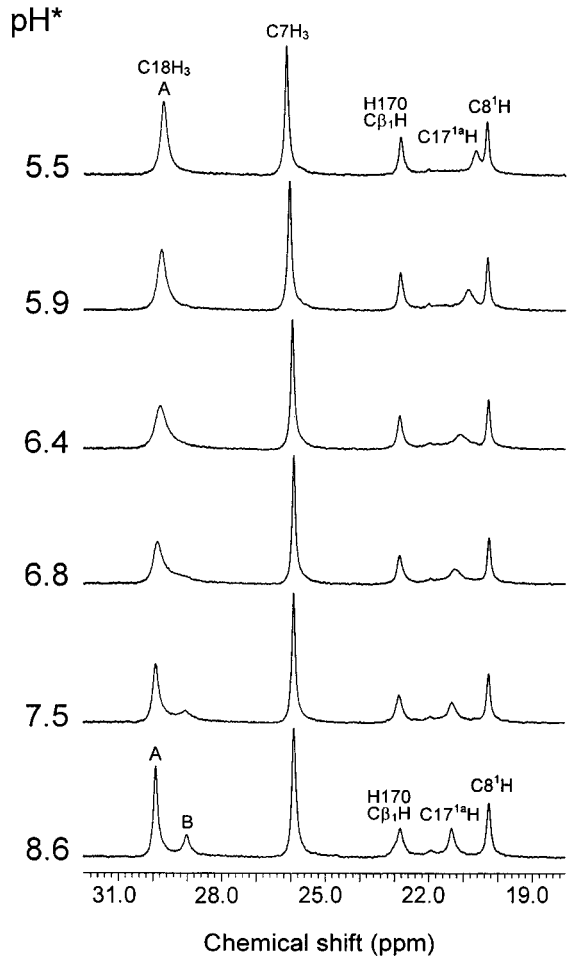

FIGURE 3: pH dependence of a selected subset of hyperfine-shifted resonances in the ^{1}H-NMR spectrum of cyanide-ligated F179H HRP C. Resonance assignments are indicated, including those of heme methyl C18H$_3$ and heme propionate C17^{1a}H at the exposed heme edge. The major and minor components of the C18H$_3$ resonance visible above pH* 6.4 are denoted A and B, respectively. Spectra were recorded at 500 MHz with solution conditions of 20 mM potassium phosphate, 15 mM potassium cyanide, D_2O, at 30 °C. Note! *Measurements were not made below pH* 5.5 due to safety considerations.*

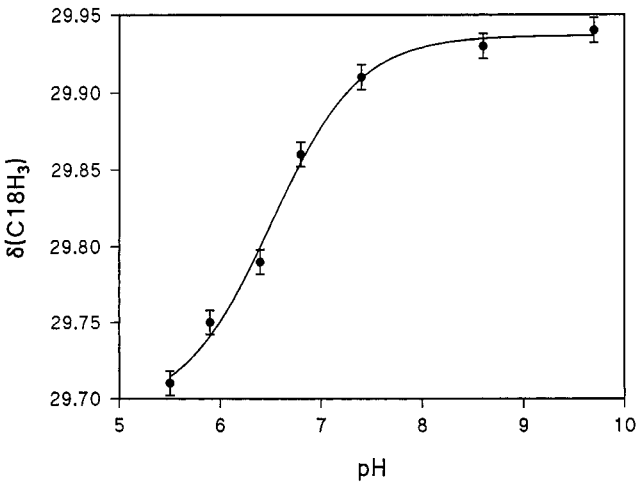

FIGURE 4: pH dependence of the chemical shift of the major component of heme methyl C18H$_3$ in the ^{1}H-NMR spectrum of cyanide-ligated F179H HRP C. The continuous line of best-fit represents a single-site protonation step associated with a pK_a value of 6.5 ± 0.2. Solution conditions correspond directly to those described in conjunction with the experimental data in Figure 3.

ligated F179H HRP C recorded at pH* 8.6, one region of which is shown in Figure 5. Two exchange-coupled cross-

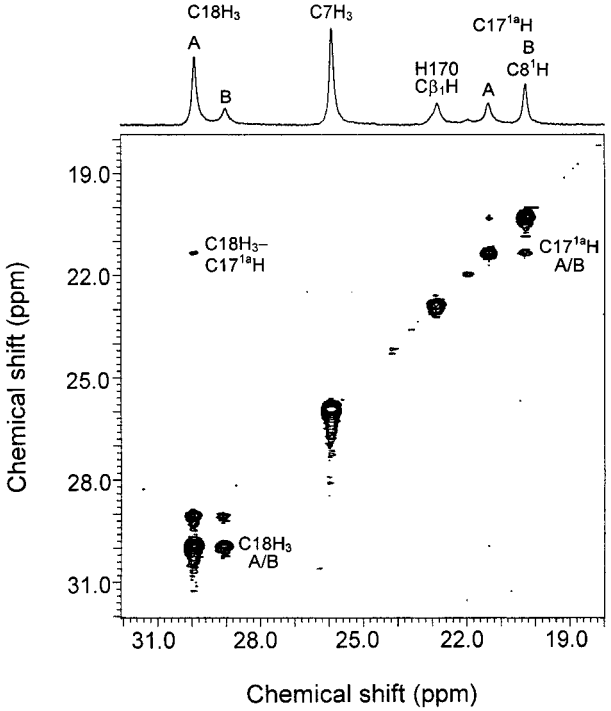

FIGURE 5: Downfield-shifted region from the two-dimensional NOESY spectrum of cyanide-ligated F179H HRP C at pH* 8.6. Exchange and NOE cross-peaks are labeled below and above the diagonal, respectively. The corresponding one-dimensional ^{1}H-NMR spectrum is also displayed, together with relevant resonance assignments. The major and minor components of the C18H$_3$ and C17^{1a}H resonances are denoted A and B, respectively. Spectra were recorded at 500 MHz with solution conditions of 20 mM potassium phosphate, 15 mM potassium cyanide, D_2O, at 30 °C. The mixing time employed in the NOESY experiment was 30 ms.

peaks are found in addition to the NOE connectivities expected between the hyperfine-shifted resonances. The first, between the resonances at 29.93 and 29.03 ppm, indicates that both represent heme methyl C18H$_3$. Confirmation of the assignment of this and other resonances follows from their characteristic NOE connectivities, one of which is indicated in Figure 5. A second exchange-coupled cross-peak links the C17^{1a}H resonance at 21.33 ppm with one at 20.27 ppm. The existence of this second component of the C17^{1a}H resonance could not have been anticipated from the one-dimensional spectral profiles in Figure 3, as it is coincident with the heme vinyl C8^{1}H resonance.

A ^{1}H-NMR analysis of the pH dependence of cyanide-ligated wild-type plant enzyme has been reported previously (*34*). Spectral changes were found to be minimal between pH 5.5 and 9.0 and were in contrast to those reported here for cyanide-ligated F179H HRP C. The linewidth and intensity of the C18H$_3$ resonance remain essentially unaltered, and only a single component is observed throughout this pH range. Although the resonances of both C18H$_3$ and C17^{1a}H exhibit small upfield and downfield chemical shift changes, respectively, with increasing pH, there is no evidence to support any spectral transition between pH 5.0 and 9.0. The spectra of cyanide-ligated wild-type plant and recombinant HRP C are known to be essentially identical at pH* 7.6 (*33*). A spectrum of cyanide-ligated recombinant HRP C obtained at pH* 5.5 for direct comparison with the data presented in Figure 3 confirmed the observations of the earlier study on wild-type plant enzyme.

Heme Pocket Solution Structure. Two-dimensional NOESY spectra were acquired for samples of both cyanide-ligated

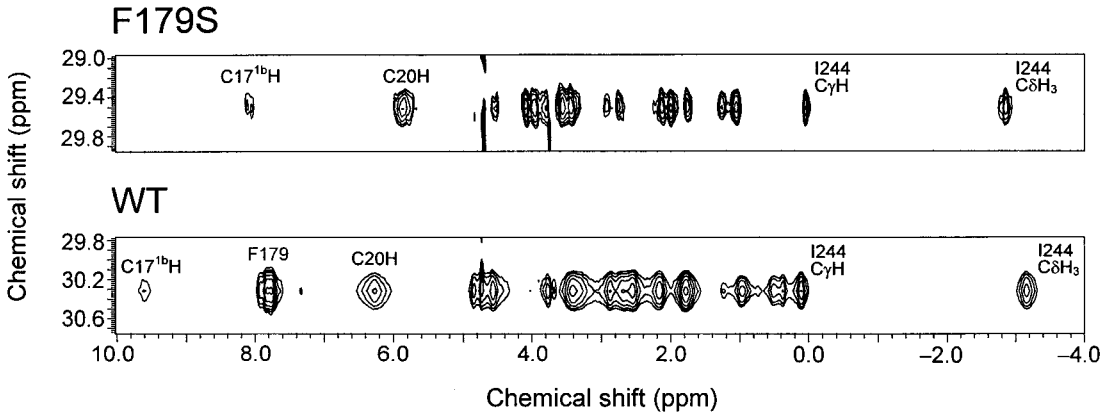

FIGURE 6: NOE connectivities associated with heme methyl C18H_3, as extracted from the two-dimensional NOESY spectra of cyanide-ligated F179S and wild-type plant HRP C. Assignments of key cross-peaks are given for reference. Spectra were acquired at 500 MHz under identical solution conditions of 20 mM potassium phosphate, 15 mM potassium cyanide, D_2O, pH 7.6 and 30 °C. Mixing times of 30 ms were used in both experiments.

F179S and wild-type plant HRP C to determine the effect of mutating this residue on the solution structure of the heme pocket. Comparative analysis of these spectra indicated that in general, the pattern of NOE connectivities observed in cyanide-ligated wild-type plant HRP C was retained in the mutant (*16*). This was the case when both intraheme, and heme to heme pocket residue side chain connectivities were considered. One outstanding difference evident from the spectra is illustrated by the data presented in Figure 6. Each spectral region summarizes NOE connectivities associated with the heme methyl resonance, C18H_3. In the case of cyanide-ligated wild-type plant HRP C, these connectivities have been documented and assigned previously on the basis of the results of both one- and two-dimensional NOE experiments (*16*, *33*, *35*). The main feature of interest is the strong NOE to aromatic ring protons appearing as a cross-peak at 7.78 ppm. Two additional NOE cross-peaks appear in the region between 5.00 and 10.00 ppm, assigned to the heme meso proton C20H (6.22 ppm) and heme propionate proton C17^{1b}H (9.59 ppm). It is immediately apparent that the same region in the spectrum of cyanide-ligated F179S HRP C contains only two NOE cross-peaks, which can be assigned with confidence to C20H (5.84 ppm) and C17^{1b}H (8.05 ppm). There are no NOE connectivities to any aromatic protons from C18H_3 in cyanide-ligated F179S HRP C. One point of possible contention, namely, that the resonance at 8.05 ppm might in fact represent an aromatic residue, can be dismissed by the additional evidence presented in Figure 7. This figure shows traces taken from the NOESY data set at the resonance positions of C18H_3 and C17^{1a}H in cyanide-ligated F179S HRP C and provides a clear demonstration of the assignment of the C17^{1b}H proton.

The assignment of the resonance at 7.78 ppm to the aromatic ring protons of Phe179 using NOE data prompted a closer examination of the corresponding one-dimensional NMR spectrum of cyanide-ligated wild-type HRP C. The region between 5.00 and 8.50 ppm is characterized by a number of relatively narrow linewidth resonances superimposed on a broad envelope of proton intensity. Two resonances of the former category at 7.78 and 7.84 ppm have been proposed previously to correspond to the Phe side chain in close contact to heme methyl C18H_3 (referred to as either Phe A or Phe W) (*15*, *16*), now shown to be Phe179. The spectrum of cyanide-ligated F179S HRP C contains three such resonances at 7.66, 7.71, and 7.77 ppm. Furthermore, comparison of the same spectral region in resting state F179S

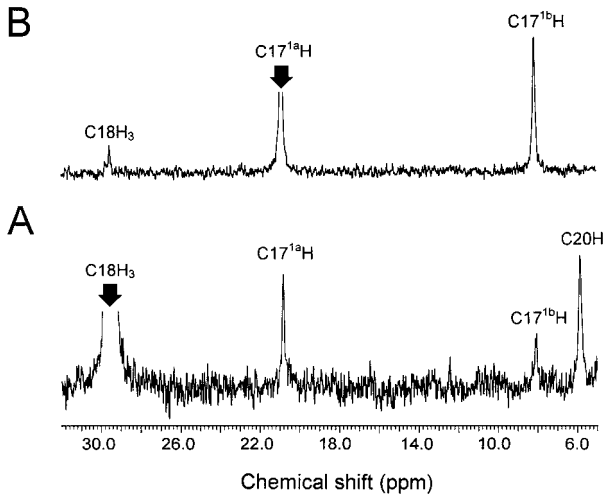

FIGURE 7: Traces extracted from the two-dimensional NOESY spectrum of cyanide-ligated F179S HRP C confirming the assignment of heme propionate C17^{1b}H by comparison of NOE connectivities associated with (A) heme methyl C18H_3 and (B) heme propionate C17^{1a}H. Spectra were acquired at 500 MHz with solution conditions of 20 mM potassium phosphate, 15 mM potassium cyanide, D_2O, pH 7.6 and 30 °C. A mixing time of 30 ms was used.

and wild-type HRP C confirms that the resonances assigned to Phe A are present in both cases (data not shown). This indicates that the resonance assigned to Phe179 at 7.78 ppm (Phe W) is coincident with that belonging to an additional Phe side chain (Phe A). The close proximity of Phe179 to the heme group, and thereby the paramagnetic heme iron atom, leads to the expectation that any proton resonances associated with it will suffer considerable broadening, and are unlikely to be well-resolved in the ^{1}H-NMR spectrum (*36*). A similar situation has been reported previously for the side chain proton resonances of Phe41, which were found to be subject to very strong paramagnetic relaxation and could not be visualised in normal one-dimensional spectra (*16*). This residue is known to be located in close proximity to heme methyl C7H_3 (*18*).

Complex Formation between Benzhydroxamic Acid and Resting State F179 Mutants. The interaction between benzhydroxamic acid and the resting states of the enzymes was evaluated using optical spectroscopy. Each of the F179 mutants exhibited the characteristic spectral transition from a five-coordinate to six-coordinate high-spin state on titration with benzhydroxamic acid (*37*, *38*). In this respect their

Table 3: Summary of Dissociation Constants Determined for Complexes between Benzhydroxamic Acid and Horseradish Peroxidase[a]

peroxidase	apparent dissociation constant K_d expressed in μM: resting state	cyanide-ligated state
F68A HRP C[b]	11.4 ± 0.3	310 ± 20
F142A HRP C[c]	8.7 ± 0.2	216 ± 12
F179A HRP C	72.4 ± 2.5	7500 ± 200
F179H HRP C (major)	13.9 ± 0.4	1860 ± 130
F179H HRP C (minor)		1200 ± 80
F179S HRP C	42.5 ± 1.1	5100 ± 100
wild-type HRP C[d]	2.1 ± 0.2	95 ± 5
wild-type HRP A2[e]	2480	9600 ± 250

[a] Data were obtained with solution conditions of either 10 mM sodium phosphate, pH 7.0 and 25 °C, or 20 mM potassium phosphate, 15 mM potassium cyanide, D_2O, pH 7.6 and 30 °C for resting and cyanide-ligated states of the F179 HRP C mutants, respectively. [b] Taken from (*21*). [c] Taken from (*19*). [d] Taken from (*3, 12*). [e] Taken from (*8, 41*).

behavior was identical to that of the wild-type enzyme (*3*). The apparent dissociation constants obtained from the titration data for the three mutants are given in Table 3. Values of 13.9 ± 0.43, 42.5 ± 1.08, and 72.4 ± 2.45 mM represent a weakening of binding affinity of ×7, ×20, and ×35 for F179H, F179S, and F179A HRP C, respectively, compared to wild-type enzyme.

Complex Formation between Benzhydroxamic Acid and Cyanide-Ligated F179 Mutants. Cyanide-ligated enzyme samples were titrated to saturation point with benzhydroxamic acid, and complex formation was monitored by recording successive ^{1}H-NMR spectra. Those recorded before the addition of benzhydroxamic acid and subsequently at the end of the titration are compared in Figure 8 for two of the three F179 mutants and for wild-type plant HRP C.

The pattern of chemical shift perturbations experienced by the hyperfine-shifted resonances shown in Figure 8 is similar, both for the mutants and for wild-type HRP C. However, in the case of selected resonances, notably heme methyl $C18H_3$, there is considerable variation in the magnitude of chemical shift change incurred on benzhydroxamic acid binding to the enzymes. Apparent dissociation constants were calculated from the titration profiles of this resonance, as illustrated by the data in Figure 9 obtained with cyanide-ligated F179H HRP C, and are listed in Table 3. It is evident from this data that the parameters associated with the formation of benzhydroxamic acid complexes are greatly altered by substitution of Phe179. As a point of comparison, results obtained previously using identical techniques for the mutants F68A and F142A HRP C are included in Table 3.

The two cyanide-ligated mutant enzymes, F179A and F179S HRP C, show almost identical behavior with respect to benzhydroxamic acid complex formation. This is reflected by similar K_d values of 7.5 ± 0.2 and 5.1 ± 0.1 mM respectively, which represent approximately 80- and 55-fold weaker binding compared to cyanide-ligated wild-type enzyme. The K_d value for cyanide-ligated F179A HRP C approaches that determined for the cyanide-ligated state of the acidic horseradish peroxidase isoenzyme, HRP A2, reported to be 9.6 ± 0.25 mM (*8*). Cyanide-ligated F179H HRP C represents a special case, as both the major and minor components of the heme methyl $C18H_3$ resonance titrate with benzhydroxamic acid. Their respective K_d values of 1.9 ± 0.1 and 1.2 ± 0.1 mM represent approximately 20- and 13-fold weaker binding, respectively, compared to cyanide-ligated wild-type enzyme.

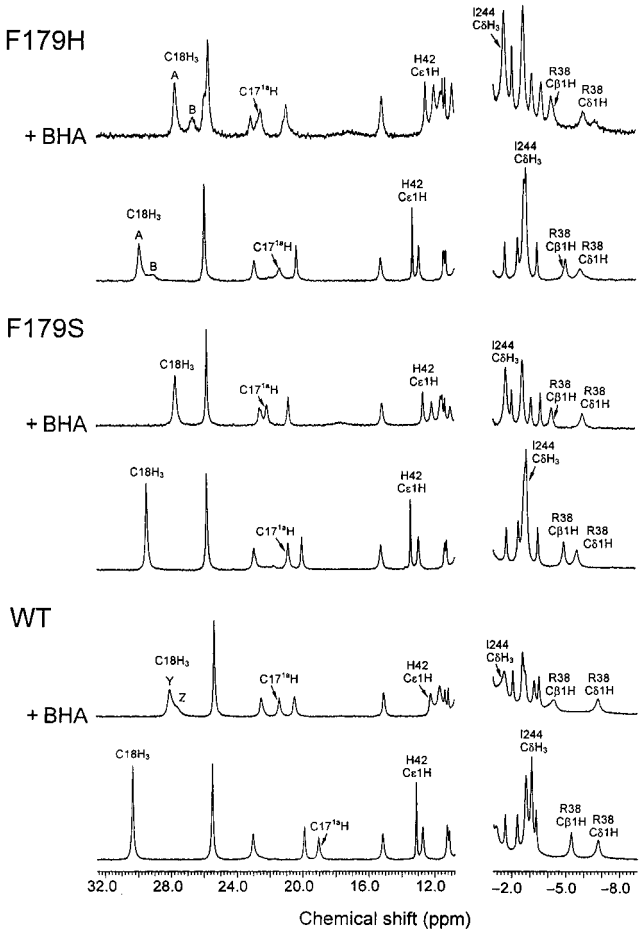

FIGURE 8: Comparison between hyperfine shifted resonances from ^{1}H-NMR spectra of cyanide-ligated F179H, F179S, and wild-type HRP C in the presence and absence of benzhydroxamic acid. The upper trace of each pair shows the appearance of the spectrum close to saturation point with benzhydroxamic acid. Resonance assignments for groups participating in the aromatic donor molecule binding site are given to indicate the magnitude of chemical shift changes occurring on benzhydroxamic acid complex formation. The major and minor components of the $C18H_3$ resonance of cyanide-ligated F179H HRP C are denoted A and B, respectively. The unusual profile of the $C18H_3$ resonance of the complex formed with cyanide-ligated wild-type HRP C is a composite of two components, denoted Y and Z, which are representative of two binding modes for benzhydroxamic acid (*11, 12*). All spectra were acquired at 500 MHz with solution conditions of 20 mM potassium phosphate, 15 mM potassium cyanide, D_2O, pH 7.6 and 30 °C.

The dynamic aspects of the interaction of the enzymes with benzhydroxamic acid also call for comment. In each of the three mutants, the heme methyl $C18H_3$ resonance initially broadens on addition of benzhydroxamic acid, reaches a maximum linewidth, and then narrows again as saturation is reached, an example of the so-called "moderately fast" exchange regime (*39*). The spectra in Figure 8 also show that the $C18H_3$ resonance appears as a single component in the fully-bound states of the mutant enzymes. This is in marked contrast to wild-type HRP C, where there are two components (*11, 12*), but similar to wild-type HRP A2 where again, only one component has been found (*8*).

Spatial Relationship between the Heme Group and Benzhydroxamic Acid in Enzyme Complexes. The location of benzhydroxamic acid with respect to the heme group in complexes formed with cyanide-ligated F179H and F179S HRP C mutants was examined in greater detail using NOESY

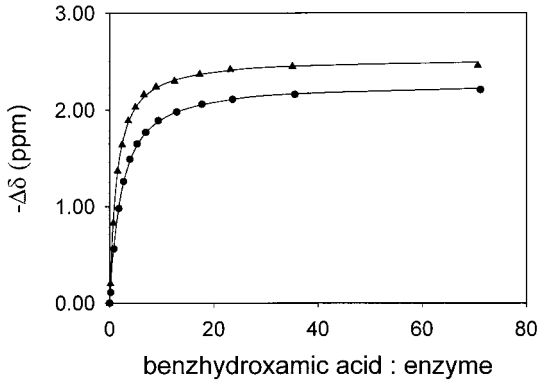

FIGURE 9: Binding curves representing the interaction between benzhydroxamic acid and cyanide-ligated F179H HRP C. Changes in chemical shift for the major and minor components of the heme methyl resonance, $C18H_3$, are plotted as a function of the ratio of benzhydroxamic acid to cyanide-ligated enzyme, and denoted by ● and ▲, respectively. At saturation, the maximal chemical shift changes for these components were −2.21 and −2.46 ppm, respectively. The continuous lines of best-fit represent theoretical dissociation curves corresponding to K_d values of 1.9 ± 0.1 and 1.2 ± 0.1 mM for the major and minor species respectively. Titration data were obtained with solution conditions of 20 mM potassium phosphate, 15 mM potassium cyanide, D_2O, pH 7.6 at 30 °C. The initial enzyme concentration was 0.35 mM.

experiments. An example of the data obtained for cyanide-ligated F179S HRP C is shown in Figure 10, where NOE connectivities from heme methyl $C18H_3$ are given for a benzhydroxamic acid bound state of the enzyme. Heme and heme-linked resonance assignments for $C17^{1b}H$ (8.78 ppm), C20H (5.91 ppm), and Ile244 CδH_3 (−2.00 ppm) in the complex were verified by observation of standard NOE connectivities. The resonances of both $C17^{1b}H$ and Ile244 CδH_3 are shifted downfield by +0.73 and +0.85 ppm, respectively, from their values in the unbound enzyme. Two new cross-peaks appear at 7.57 and 7.76 ppm, of which the latter is the most intense. These arise from transferred NOEs to the CϵH_2 and CδH_2 protons of free benzhydroxamic acid. A similar result was obtained from analysis of the NOESY spectrum of the complex formed between benzhydroxamic acid and cyanide-ligated F179H HRP C. Transferred NOEs from $C18H_3$ to the CϵH_2 and CδH_2 protons of free benzhydroxamic acid were observed as cross-peaks at 7.56 and 7.80 ppm, respectively, with that to 7.80 ppm again the most intense (data not shown). Other NOE connectivities from $C18H_3$ to heme and heme-linked protons were as expected.

DISCUSSION

Spatial Relationship between Phe179 and the Heme Group. When one-dimensional ^{1}H-NMR spectra of both resting and cyanide-ligated states of the Phe179 mutants are compared with corresponding data for wild-type HRP C, the resonances of the substituents of pyrrole IV, heme propionate $C17^1H_2$, and heme methyl $C18H_3$ are found to be significantly perturbed. This localized region of the heme group, which is coincident with the exposed heme edge, is clearly sensitive to the substitution of Phe179 by other amino acid side chains. It is only information from NOESY spectra, however, that provides direct structural evidence for the location of the Phe179 side chain. The abolition of the strong NOE between the protons of heme methyl $C18H_3$ and those of an unidentified aromatic side chain in the NOESY spectrum of cyanide-ligated F179S HRP C is compelling evidence for the assignment of this heme contact to Phe179. The side chain of Phe179 must therefore be in close proximity to heme methyl $C18H_3$. It is not possible to state a quantitative interproton distance for this constraint, as the intensity of the NOE is also influenced in this case by the proximity to the paramagnetic center. However, as this NOE has been confirmed to be primary in nature (*35*), an estimate of the interproton distance as 2.5−3.0 Å can be proposed with some confidence. A previous study, in which the ^{1}H-NMR spectra of cyanide-ligated states of acidic HRP isoenzymes A1 and A2 were compared with that of cyanide-ligated HRP C, concluded that Phe221 was the most likely candidate for the aromatic residue in heme contact with $C18H_3$, although Phe179 was considered as an alternative choice (*40*). A modeling study based on recent structural alignment procedures suggested that two Phe residues, Phe68 and Phe179, were within 5 Å of a proposed benzhydroxamic acid binding site, although the position of Phe179 was noted to be very uncertain due to its location in the untemplated F−G insertion (*17*). The differing conclusions of these investigations emphasize the value of ^{1}H-NMR analysis of site-directed mutants as a powerful method for the unequivocal identification of functionally significant residues such as Phe179.

Evidence for Microheterogeneity in F179H HRP C. Substitution of Phe179 by a His residue confers distinctive properties on the enzyme which are most clearly manifested in the pH-dependence study of the cyanide-ligated state. The presence of a titrating group with a pK_a of 6.5 ± 0.2 in the vicinity of heme pyrrole IV is indicated by the titration behavior of heme methyl $C18H_3$ and $C17^{1a}H$. There is no evidence for a comparable transition in cyanide-ligated wild-type HRP C, which therefore implicates the His179 side chain as the likely source of the titrating group in this mutant. At pH values above the pK_a, two components of both the $C18H_3$ and $C17^{1a}H$ resonances are observed. These also appear as exchange-coupled cross-peaks in NOESY spectra, although the pattern of NOE connectivities from the major component of each of these resonances is unchanged. This indicates that the heme substituents on pyrrole IV effectively experience a locally microheterogenous environment. In addition, the distinctive two-component profile of the $C18H_3$ resonance in resting state F179H HRP C provides further evidence of this phenomenon. The His179 side chain, the most obvious candidate for the source of the heterogeneity (seen only in this mutant), appears to be able to adopt two conformations, the relative populations of which are strongly pH-dependent and may reflect changes in hydrogen-bonded interactions.

A Critical Role for Phe179 in Benzhydroxamic Acid Binding. Complex formation between HRP C and benzhydroxamic acid is profoundly affected by the substitutions made at Phe179. In each case, binding of the aromatic donor molecule is weakened significantly, although the extent to which this occurs is modulated by the nature of the side chain at position 179 as well as by the coordination and spin state of heme iron. The F179A HRP C mutant displays the lowest affinity for benzhydroxamic acid followed successively by the F179S and F179H HRP C mutants. This general trend is maintained in both resting and cyanide-ligated states of the enzymes. However, the sensitivity of complex formation to the substitutions at Phe179 differs within each of these two series. The cyanide-ligated mutants bind benzhydroxamic acid 2−3 times more weakly, relative to cyanide-ligated

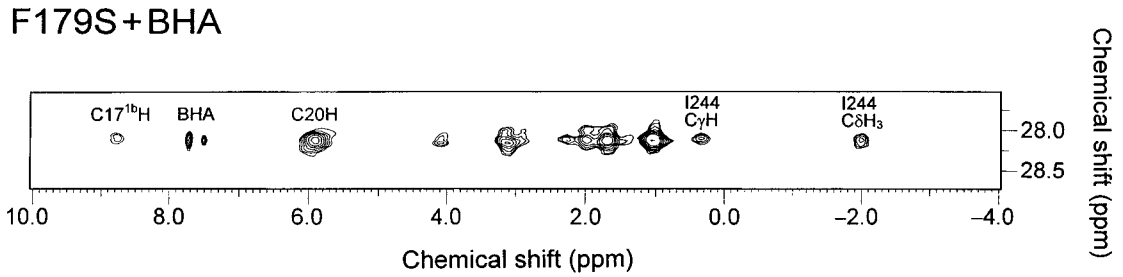

FIGURE 10: NOE connectivities associated with heme methyl C18H_3, as extracted from the two-dimensional NOESY spectrum of a 1:14 complex formed between cyanide-ligated F179S HRP C and benzhydroxamic acid (BHA), respectively. Assignments of key cross-peaks are given for reference. The spectrum was acquired at 500 MHz with solution conditions of 20 mM potassium phosphate, 15 mM potassium cyanide, D_2O, pH 7.6 and 30 °C. A mixing time of 30 ms was used.

wild-type enzyme, than their resting state counterparts. These observations confirm that while the Phe179 side chain is a structural component of the substrate binding site in both states of the enzyme, its influence is greater in the ternary complexes. The relatively minor contribution of other Phe residues, such as Phe68 and Phe142, to the binding site is apparent from consideration of Table 3. Comparison between the K_d values derived for the F68A and F142A HRP C mutants and those derived for the F179A and F179S HRP C mutants provides a clear demonstration of the relative importance of these residues. It is also of interest that although the binding affinity for benzhydroxamic acid is always weaker for cyanide-ligated rather than resting state enzymes, it is most strongly weakened in the case of the F179 HRP C mutants. Conversely it should be noted that even when Phe179 is mutated, benzhydroxamic acid still binds to the cyanide-ligated enzymes in the vicinity of heme methyl C18H_3, according to data derived from NOESY spectra of the complexes. The location of benzhydroxamic acid with respect to the heme group is thus not significantly altered between cyanide-ligated wild-type HRP C and the Phe179 mutants. This is also indicated by the similar patterns of chemical shift perturbations to heme and heme-linked resonances recorded for these enzymes during titration with benzhydroxamic acid.

A further feature of interest in the assessment of the role of Phe179 in benzhydroxamic acid binding concerns the effect of its substitution on dynamic aspects of the interaction. This is highlighted to some extent by the spectra in Figure 8 and, in particular, by the profiles of the heme methyl C18H_3 resonances in the bound states of the cyanide-ligated enzymes. In the cyanide-ligated wild-type enzyme, heterogenous complex fomation is believed to be a consequence of alternative orientations of selected heme pocket side chains (*11*). It has been shown previously that substitution of either Phe68 or Phe142 influences the partitioning of benzhydroxamic acid between the two possible complexes (termed Y and Z) (*19, 21*). When Phe179 is substituted, only the Y complex is formed, and in each case, exchange is characterized by the moderately fast regime. In cyanide-ligated wild-type HRP C, the Y and Z complexes exhibit intermediate and slow exchange, respectively (*11, 12*).

Aromatic Donor Molecule Binding Sites in Horseradish and Other Plant Peroxidases. The present study provides the first direct experimental evidence for the participation of Phe179 in the aromatic donor molecule binding site of HRP C. At the same time, the data obtained for the F179A HRP C mutant offer new insights into the interaction between benzhydroxamic acid and HRP A2. This acidic isoenzyme of HRP shows only 54% amino acid sequence identity with the more abundant C isoenzyme (*29*), although comparative NMR studies indicate that the structures of their heme-linked regions are similar in solution (*8, 40*). It is curious, therefore, that these isoenzymes exhibit such a wide discrepancy in their affinity for benzhydroxamic acid, which is 1200- and 100-fold weaker for resting and cyanide-ligated states of HRP A2 compared to HRP C, respectively (Table 3; *8, 41*). The difference in the affinity for the cyanide-ligated isoenzymes can be ascribed to the presence of Phe179 in HRP C. This residue is replaced by Val in HRP A2 (*29*). It is significant in this respect that the K_d value for benzhydroxamic acid binding to cyanide-ligated F179A HRP C is close to that of cyanide-ligated HRP A2. Although there is no crystal structure data available for HRP A2, it has been demonstrated from NMR studies of the cyanide-ligated enzyme that an aliphatic side chain is in fact within NOE distance of heme methyl C18H_3 (*40*) and thus replaces the Phe residue side chain now shown to be Phe179 in HRP C. This aliphatic residue in HRP A2 can now be assigned with confidence to Val179. The absence of a Phe residue at position 179 in resting state HRP A2 can only partly account for its decreased affinity for benzhydroxamic acid. In resting state HRP C, the driving force for binding derives from a combination of hydrophobic interactions and polyfunctional hydrogen bonding (*3*). It is anticipated that the network of hydrogen-bonded interactions must therefore differ between the resting states of these two enzymes.

Analysis of amino acid sequence alignments for the plant peroxidases (*29*), a group of enzymes more accurately described as class III of the plant peroxidase superfamily (*42*), shows that there is considerable variability at position 179. It is possible to have considerable confidence in the alignment of this sequence region as it follows an invariant Cys residue at position 177 which participates in a conserved disulfide bridge (*42*). Sequence alignments indicate that a Phe residue is more often observed at position 180, as for example in the major cationic isoenzyme of PNP, which has the partial sequence −CTA**F**− between residues 177 and 180 (*28*). What is fascinating in this example is that according to the crystal structure of PNP, the side chain of Phe180 points downward, well away from the exposed heme edge, whereas that of Ala179 points directly toward the heme methyl group C18H_3 (*18*). This is in excellent agreement with the NMR results reported in the present study, which show that HRP C, with the corresponding partial sequence −CR**F**I−, has the side chain of Phe179 situated close to the exposed heme edge at C18H_3. Once again, in HRP A2, which has the corresponding partial sequence, −CGV**F**−, Val179 has been shown to be adjacent to the exposed heme edge, and it is likely that Phe180 will be similarly located

to its counterpart in cationic PNP. On the strength of these observations it may be concluded that the presence of a Phe residue at position 179 contributes significantly to the binding affinity of class III plant peroxidases for benzhydroxamic acid. It should be possible to substantiate this proposal as further data on interactions of plant peroxidases with aromatic donor molecules become available. An interesting corollary is the weak affinity of the class II fungal peroxidase from *Coprinus cinereus* for benzhydroxamic acid, with a K_d of only 3.7 mM (*20*). Fungal peroxidases lack the structural motif which contributes Phe179 to the binding site of HRP C (*42*), a distinguishing feature between class II and III peroxidases, the functional implications of which are under investigation.

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