Haem-linked interactions in horseradish peroxidase revealed by spectroscopic analysis of the Phe-221 → Met mutant

Barry D. HOWES*, Nigel C. VEITCH†, Andrew T. SMITH‡, Christopher G. WHITE‡ and Giulietta SMULEVICH*¹

*Dipartimento di Chimica, Università di Firenze, Via G. Capponi 9, 50121 Firenze, Italy, †Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, U.K., and ‡School of Biological Sciences, University of Sussex, Brighton, BN1 9QG, U.K.

A gene encoding a Phe-221-to-Met substitution in the haem enzyme horseradish peroxidase has been constructed and expressed in *Escherichia coli*. In the wild-type enzyme the side chain of Phe-221 is tightly stacked against the imidazole ring of His-170, which provides the only axial ligand to the haem iron atom. The Phe-221 → Met enzyme is active, and forms characteristic complexes with typical peroxidase ligands (CO, cyanide, fluoride), and with benzhydroxamic acid. Significant differences between the mutant and wild-type enzymes can be detected spectroscopically. These include a change in the Fe(III) resting state of the enzyme to an unusual quantum mechanically mixed-

spin haem species, a marked decrease in the pK_a of the alkaline transition and a reduction in enzyme stability at alkaline pH for both Fe(III) and Fe(II) forms. The perturbation of the haem pocket in the mutant can be attributed to several factors, including the increased steric freedom and solvent accessibility of the His-170 ligand, as indicated by ¹H-NMR data, and the loss of the π - π interaction between His-170 and Phe-221.

Key words: haem enzyme, NMR spectroscopy, resonance Raman spectroscopy, site-directed mutagenesis.

INTRODUCTION

Horseradish peroxidase (HRP) is one of a small number of commercially important haem proteins that continue to be studied in detail. The X-ray crystal structure of the major HRP isoenzyme (HRP C) has been solved [1], and the ability to construct site-directed mutants allows questions about structure and function to be investigated, as well as the optimization of desirable qualities for biotechnological applications [2]. HRP C catalyses the conversion of H₂O₂ into H₂O, a process that requires the concomitant oxidation of reducing substrates such as aromatic phenols or amines [3]. The recombinant enzyme expressed in *Escherichia coli* has a single polypeptide chain of 308 amino acid residues, a haem prosthetic group (iron protoporphyrin IX) and two calcium ions. Disulphide bridges link Cys residues between sequence positions 11–91, 44–49, 97–301 and 177–209 [4]. The eight carbohydrate side chains characteristic of the plant enzyme are absent from this recombinant form. Plant peroxidases have been placed in class III of the plant peroxidase superfamily, which also includes haem peroxidases of bacterial (class I) and fungal (class II) origin [5].

Important features of the haem environment of recombinant wild-type HRP C are illustrated in Figure 1 [1]. The distal residues Arg-38 and His-42 are primary targets for site-directed-mutagenesis studies, due to their role in peroxide catalysis, while Phe-41, Glu-64 and Asn-70 have also been investigated [2,6]. One significant outcome is the discovery of a hydrogen-bonding network connecting the distal and proximal regions of the haem pocket, and the two calcium-binding sites (Figure 1). Comparable studies on proximal site residues of HRP C are limited [2]. Little is known about the role of Phe-221, despite its proximity to His-170, the residue which is co-ordinated directly to the haem iron atom (Figure 1). Phe-221 is conserved in most class-III plant

peroxidases, as well as in lignin and manganese peroxidases (LIP and MnP, respectively; class II) [5,7]. Site-directed-mutagenesis studies of the corresponding residue in MnP (Phe-190) indicate a role in stabilization of the haem environment [8,9]. In structural terms, Phe-221 of HRP C is equivalent to Trp-191 of yeast cytochrome *c* peroxidase (CCP; class I), the site of the protein-based radical in the high-oxidation-state intermediate (compound I) formed by CCP on reaction with H₂O₂ [2]. Replacement of Phe-221 of HRP C with Trp (F221W HRP C) facilitates the generation of a similar protein-based radical site [10].

In the present investigation the role of Phe-221 in HRP C has been examined critically by constructing a new mutant where this residue is replaced by methionine (F221M HRP C), a substitution that occurs naturally in some class-III plant peroxidase isoenzymes from *Arabidopsis thaliana* [11]. The F221M enzyme has been characterized in solution in different oxidation states, at various pH values, in the presence of ligands and as a complex with the aromatic donor molecule, benzhydroxamic acid (BHA). Kinetic and spectroscopic parameters have also been acquired for direct comparison with analogous data for wild-type HRP C and other related plant peroxidases.

MATERIALS AND METHODS

Site-directed mutagenesis and expression of recombinant proteins

A novel PCR-based method utilizing high-fidelity *Pfu* polymerase exactly as described by Doyle et al. [12] was used to construct the F221M HRP C mutant, except that the synthetic HRP C gene cloned in the plasmid pSD18 was used as template [13]. Oligonucleotide primers F221-1 (5'-GACVTGGATCTGCGGACCC-CA-3') and F221-2 (5'-GACTAGTGCAACTGAGGTTG-3'; where mutated codons are shown in bold, and any other base changes introduced to create an additional restriction site are

Abbreviations used: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate); BHA, benzhydroxamic acid; BP1, barley grain peroxidase; CCP, cytochrome *c* peroxidase; HRP A2, horseradish peroxidase isoenzyme A2; HRP C, HRP isoenzyme C; F221M HRP C, Phe-221 → Met mutant of HRP C; F221W HRP C, Phe-221 → Trp mutant of HRP C; LIP, lignin peroxidase; MnP, manganese peroxidase; SBP, soybean peroxidase; HS, high-spin; LS, low-spin; RR, resonance Raman; QS, quantum mechanically mixed-spin; 5-c, five-coordinate; 6-c, six-coordinate; NOESY, nuclear Overhauser enhancement spectroscopy; Im, imidazole ligand.

¹ To whom correspondence should be addressed (e-mail smulev@chim.unifi.it).

Figure 1 Structural features of the haem pocket of HRP C

This diagram was produced from the X-ray crystal structure of the recombinant wild-type enzyme (Brookhaven accession code 1ATJ) [1]. The haem group is shown in red, and is linked to the apoenzyme by a co-ordinate bond from the proximal residue, His-170 (in green). The distal (top left) and proximal (bottom centre) Ca atoms are represented as blue spheres. Note that the side-chain of Phe-221 (shown in blue) is stacked parallel to that of His-170. An extensive hydrogen-bonding network links the proximal and distal regions of the haem pocket and the two Ca atoms (key hydrogen bonds are shown as dotted lines).

underlined) were designed for the point mutation at position 221. 'V' signifies degeneracy to the nucleotides G, C or A, which allows recovery of the Val, Leu and Met codons at position 221, respectively. A restriction site for the enzyme *AclI* was engineered into the sequence so that it spanned the site of ligation and could be used to screen for correctly ligated mutant constructs. A mutational efficiency of 25% was achieved, and the expected sequence changes encoding the F221M mutant were confirmed by automated DNA sequencing (Applied Biosystems). No extraneous mutations were introduced by the PCR procedure. Transformed strains were grown in 16 litres of terrific broth supplemented with 0.2% (w/v) glucose in a LH2000 series fermenter [12]. Induction with 0.5 mM isopropyl β -D-thiogalactoside took place after 1–2 h growth (from a 5% inoculum) at a D_{670} of 1.0–2.0. Harvesting of approx. 300 g (wet weight) of *E. coli* took place 4–5 h after induction. Isolation of inclusion bodies, refolding and purification of both wild-type and F221M HRP C were carried out as described previously [13,14].

Preparation of peroxidase samples

Plant HRP C and HRP isoenzyme A2 (HRP A2) were obtained from Biozyme Laboratories as salt-free lyophilized powders (codes HRP4B and HRP5, respectively). These preparations had RZ (reinheitszahl) or purity index values of 3.1 and 3.8, respectively, and were used without further purification. Soybean

peroxidase (SBP, a gift from Professor Karen Welinder, University of Aalborg, Aalborg, Denmark) was obtained originally from Enzymol International USA (RZ = 1.5) and purified further to give a preparation with an RZ value of 3.1 [15]. This sample was stored in 3 M ammonium sulphate at 4 °C. Purified recombinant enzymes (F221M and wild-type HRP C) were stored at –70 °C as frozen solutions in 10 mM Mops buffer at pH 7.0. Enzyme concentrations were determined spectrophotometrically using molar absorption coefficients of 98, 110 and 107 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ at the Soret maxima for wild-type HRP C, F221M HRP C and plant HRP A2, respectively. Molar absorption coefficients at the Soret maxima of wild-type and F221M HRP C were determined in triplicate using the pyridine haemochrome method.

Samples of ferrous enzymes for electronic absorption and resonance Raman (RR) spectroscopy were prepared by addition of 2 μl of dithionite (20 $\text{mg}\cdot\text{ml}^{-1}$) to 50 μl of deoxygenated peroxidase solution. The fluoride complex was prepared by addition of 1 M sodium fluoride (Merck) to the enzyme samples in 25 mM citrate, pH 4.9, to give a final concentration of 0.1 M fluoride. BHA complexes were prepared by adding aliquots of 0.2 M BHA (Sigma) in 10 mM Mops, pH 7.0, to the enzyme samples, to a final (saturating) concentration of 5 mM.

Enzyme samples intended for NMR experiments were thawed if required and subjected to several cycles of solvent exchange and concentration at 4 °C using Amicon Centricon 10 micro-concentrators. The solution conditions employed were either 20 mM $\text{KH}_2\text{PO}_4/{}^2\text{H}_2\text{O}$, pH* 7.0, or 20 mM $\text{KH}_2\text{PO}_4/15$ mM $\text{KCN}/{}^2\text{H}_2\text{O}$, pH* 7.6, for preparation of resting and cyanide-ligated states of the enzymes, respectively. A similar procedure was applied in the case of plant HRP A2, except that the enzyme was dissolved directly in the solution required prior to solvent exchange and concentration. A Radiometer pH26 meter was used for pH measurements, which were uncorrected for the small deuterium isotope effect. Specific readings in ${}^2\text{H}_2\text{O}$ solutions are denoted pH* within the text.

Kinetic measurements

Peroxidase activity was measured in 20 mM phosphate/citrate buffer at pH 5.0 using the substrate 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) [13]. Initial rates were measured by following the increase in absorbance at 405 nm resulting from the formation of the ABTS cation radical product. A saturating concentration of 1 mM H_2O_2 was used for the assays, which were initiated by addition of enzyme to a concentration of 0.125 nM. The second-order rate constant for compound-I formation (k_1) was determined under pseudo-first-order conditions in 10 mM sodium phosphate buffer at pH 7.0 and 25 °C (Applied Photophysics SX18MV stopped flow system). Plots of k_{obs} against H_2O_2 concentration were linear over the range studied (5–20 μM), and k_1 was obtained from the gradient.

Electronic absorption and RR spectroscopy

Electronic absorption spectra were measured with a Cary 5 spectrophotometer using a cuvette of 1 mm path length. RR spectra were obtained at room temperature with excitation from the 406.7 and 568.2 nm lines of a Kr^+ laser (Coherent, Innova 90/K), and from the 441.6 nm line of a HeCd laser (Liconix). The back-scattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG2S) equipped with a cooled photomultiplier (RCA C31034A) and photon-counting electronics. To minimize local heating of the protein by the laser beam, the

sample was cooled by a gentle flow of N₂ gas passed through liquid N₂. RR spectra were calibrated to an accuracy of 1 cm⁻¹ for intense isolated bands, with indene as the standard for the high-frequency region and with indene and CCl₄ for the low-frequency region. Polarized spectra were obtained by inserting a polaroid analyser between the sample and the entrance slit of the monochromator. The depolarization ratios of the bands at 314 and 460 cm⁻¹ were measured to check the reliability of the polarization measurements. The values obtained, 0.73 and 0.00, compared well with the theoretical values of 0.75 and 0.00, respectively. Peak intensities were determined using a curve-fitting program to simulate experimental spectra with Lorentzian line shapes.

NMR spectroscopy

¹H-NMR data were acquired using a Varian 500 MHz instrument. One-dimensional spectra of resting-state enzymes were obtained with a spectral width of 100 kHz, 8–16 K transients and a recycle time of 5 s⁻¹. For cyanide-ligated enzymes, a spectral width of 40 kHz, 2 K transients and a recycle time of 2 s⁻¹ were used. The 90° pulse width was typically 5.2–6.5 μs and a 45° flip angle was used in the acquisition of spectra of resting-state enzymes. Line-broadening factors of 30 and 10 Hz were introduced on processing one-dimensional spectra of resting and cyanide-ligated states of the enzymes, respectively. Baseline correction was carried out for all spectra. Two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) data were obtained as described previously [16]. All NMR experiments were carried out at 30 °C and referenced to 1,4-dioxan as an internal standard with a resonance at 3.74 p.p.m. relative to 2,2-dimethyl-2-silapentane-5-sulphonate.

RESULTS

Kinetic and physical parameters of ferric enzymes

The catalytic properties of F221M HRP C compare favourably with those of wild-type HRP C, to the extent that enzyme activity is not greatly compromised by the substitution (Table 1). The second-order rate constant for the reaction of resting-state enzyme with H₂O₂ to give the high-oxidation-state intermediate, compound I, was also close to that obtained for wild-type enzyme, as indicated in Table 1. Compound I of F221M HRP C did not decay spontaneously to compound II any faster than compound I of the wild-type enzyme. Steady-state kinetic parameters obtained using ABTS as substrate revealed only minor changes, the most significant of which was a decrease in $V_{\max}/[E_0]$ from 1070 ± 20 s⁻¹ to 730 ± 20 s⁻¹. The compensatory decrease in the apparent K_m for ABTS gave a value for the effectiveness ratio ($k_{\text{cat}}/2K_m$) that was almost unchanged by the Phe → Met substitution. The redox potential for the Fe(III)/Fe(II) couple at neutral pH was almost unaltered between wild-type HRP C and the F221M mutant (Table 1).

Figure 2 Electronic absorption and RR spectra of ferric peroxidase enzymes

(A) Electronic absorption spectra of 100 μM wild-type (WT) HRP C at pH 7.0 in 10 mM Mops, 80 μM HRP A2 at pH 7.3 in 0.1 M sodium phosphate, 140 μM SBP at pH 7.0 in 0.1 M sodium phosphate, and 70 μM F221M HRP C at pH 7.0 in 10 mM Mops. The visible region is expanded 6-fold. (B) RR spectra of 30 μM WT HRP C at pH 7.0 in 10 mM Mops, 32 μM HRP A2 at pH 7.3 in 0.1 M sodium phosphate, 33 μM SBP at pH 6.9 in 0.1 M sodium phosphate, and 35 μM F221M HRP C at pH 7.0 in 10 mM Mops, recorded with 406.7 nm excitation. Experimental conditions: 5 cm⁻¹ resolution; 6 s/0.5 cm⁻¹ collection interval, 30 mW laser power at the sample (HRP C); 9 s/0.5 cm⁻¹ collection interval, 10 mW laser power (HRP A2); 20 s/0.5 cm⁻¹ collection interval, 15 mW laser power (SBP); 14 s/0.5 cm⁻¹ collection interval, 20 mW laser power (F221M HRP C).

Electronic absorption spectroscopy of ferric enzymes

The electronic absorption spectrum of resting-state F221M HRP C at pH 7.0 is shown in Figure 2(A), together with the cor-

Table 1 Comparison between kinetic and physical parameters determined for wild-type and F221M HRP C

E_0 is the reduction potential for the Fe(II)/Fe(III) couple at pH 7.6. K_d (BHA) is the apparent dissociation constant for BHA in 20 mM sodium phosphate, pH 7.0 and 25 °C.

Enzyme	ϵ (mM ⁻¹ ·cm ⁻¹)	k_1 (M ⁻¹ ·s ⁻¹)*	Apparent K_m (μM)†	$V_{\max}/[E_0]$ (s ⁻¹)	$k_{\text{cat}}/2K_m$ (μM ⁻¹ ·s ⁻¹)	E_0 (mV)	K_d (BHA) (μM)
Wild type	98 ± 3	(1.7 ± 0.1) × 10 ⁷	140 ± 10	1070 ± 20	3.8	-240 ± 10	2.0 ± 0.1
F221M	110 ± 3	(1.5 ± 0.1) × 10 ⁷	110 ± 10	730 ± 20	3.3	-260 ± 20	4.3 ± 0.1

* In 10 mM sodium phosphate, pH 7.0 and 25 °C.

† In phosphate/citrate buffer, pH 5.0 and 25 °C.

Table 2 RR frequencies (cm⁻¹) for haem spin states of F221M HRP C

A minor 6-c HS species is also present, for which only the ν_3 mode at 1485 cm⁻¹ can be detected.

Mode	6-c HS [#]	5-c HS [#]	QS
ν_3	1492	1499	1505
ν_2	1566	≈ 1572	1575
ν_{11}^*	1548	1548	1548
ν_{37}^*	1582	1582	1582
$\nu(\text{C}=\text{C})$	1623	1623	1623
$\nu(\text{C}=\text{C})$	1631	1631	1631
ν_{10}	1623	—	1637†

* Only the mean frequency can be determined, due to overlap of the modes from the three spin states.

† This mode may overlap with that of the 5-c HS[#] state [18].

responding spectra of the wild-type enzyme and two other plant peroxidases, HRP A2 and SBP, obtained under the same conditions. The absorption maxima for F221M HRP C are blue-shifted with respect to those of the wild-type, and the molar absorption coefficient is increased (Table 1). In principle, this can arise either as a consequence of the degree of vinyl group conjugation with the porphyrin macrocycle, or from the presence of a quantum mechanically mixed-spin (QS) state of haem iron [17]. The absorption spectra of HRP A2 and SBP are also blue-shifted compared with wild-type HRP C, and it is notable that the absorption maxima of SBP are similar to those of F221M HRP C. There is no evidence from the spectra in Figure 2(A) to support the presence of any accompanying low-spin (LS) haem species in these enzymes at neutral pH.

RR spectroscopy of ferric enzymes

A comparison between the high-frequency RR spectra (obtained with Soret excitation at 406.7 nm) of the resting states of four peroxidase enzymes is presented in Figure 2(B). The RR spectra of wild-type HRP C, HRP A2 and SBP have been analysed in detail previously [15,18,19]. In the case of wild-type HRP C, the RR spectrum indicates that a mixture of five-coordinate high-spin (5-c HS[#]) and six-coordinate HS (6-c HS[#]) haem species is present. The frequencies of the core-size marker bands of these species are anomalous, as they are higher than those normally observed for 5-c and 6-c HS haems; this distinction is emphasized by the [#] notation [18]. The most striking features of the RR spectrum of F221M HRP C (compared with the spectrum of the corresponding wild-type enzyme) are that the core-size marker bands are shifted to higher frequencies, and a new band appears at 1637 cm⁻¹. The broad ν_3 band has two components at 1492 and 1505 cm⁻¹, and a third, less prominent component at 1485 cm⁻¹, whereas the ν_2 region is characterized by a broad peak at 1575 cm⁻¹ composed of three overlapping bands at 1566, 1575 and 1582 cm⁻¹ (curve-fitting analysis not shown). Three distinct bands to higher frequency are also observed at 1623, 1631 and 1637 cm⁻¹. The detailed assignment of these RR bands is given in Table 2, based principally on core-size marker-band frequencies for both model haem compounds and wild-type HRP C [18], HRP A2 [19], SBP [15] and barley grain peroxidase (BP1) [20]. Note that a haem species characterized by high RR band frequencies has also been found as a component of resting state HRP A2, SBP and BP1, and identified as a QS state. On this basis, the bands at 1566 and 1575 cm⁻¹ can be assigned to the ν_2 modes of 6-c HS[#] and QS haem species, respectively, and the band at 1582 cm⁻¹ to the ν_{37} mode.

RR spectra recorded in polarized light (results not shown) reveal that while the bands at 1631 and 1637 cm⁻¹ are polarized and depolarized, respectively, the band at 1623 cm⁻¹ has an intermediate polarization (0.4). This suggests that there are two overlapping bands at this frequency, one polarized and one depolarized. The bands at 1631 and 1637 cm⁻¹ can therefore be assigned to a vinyl stretching mode and a ν_{10} mode, respectively, whereas the band at 1623 cm⁻¹ can be considered to derive from a superposition of a second vinyl (polarized) and a ν_{10} (depolarized) mode. The band at 1548 cm⁻¹ is depolarized, and can be assigned to a ν_{11} mode.

The ν_3 component at 1492 cm⁻¹ has two possible origins, either a 6-c HS[#] haem, as in wild-type HRP C, or a 5-c HS haem with normal RR band frequencies. The anomalous polarization of the band at 1623 cm⁻¹, suggesting a ν_{10} mode at this frequency, and the presence of a ν_2 band at 1566 cm⁻¹ favour the first of these two alternatives. The ν_3 band at 1505 cm⁻¹, together with the ν_2 at 1575 cm⁻¹ and the ν_{10} at 1637 cm⁻¹ are consistent with the presence of a QS haem species. In addition, the unexpectedly low frequency of the ν_{11} mode at 1548 cm⁻¹ (only the mean frequency of this band can be quoted, due to overlap of the ν_{11} bands from the haem species present), compared with the high frequencies observed for the other modes, is a further demonstration that the QS haem species is the predominant component of the resting state of the F221M mutant. In fact, for all the peroxidases in which the QS state has been observed the RR frequencies of the QS modes are high, with the exception of the ν_{11} mode [15,19–21]. It is interesting to note that the low-frequency RR spectrum of the mutant, obtained with excitation at 406.7 nm, is effectively unchanged with respect to that of wild-type HRP C (results not shown). This spectral region is characterized by out-of-plane haem modes and the bending modes of the haem vinyl and propionate groups [17], indicating that no appreciable changes in haem deformation result from the substitution of Phe-221 by Met.

RR spectra recorded with excitation at 568.2 nm (results not shown) uncover a small increase in frequency for the ν_{11} (1551 cm⁻¹) and ν_{10} (1640 cm⁻¹) modes, observed at 1548 and 1637 cm⁻¹, respectively, with Soret excitation. The ν_{19} band is centred at 1580 cm⁻¹, and the vinyl bands are visible at 1621 and 1631 cm⁻¹, as observed previously for other haem proteins on excitation in the visible region [17]. The excitation wavelength of 568.2 nm is in resonance with the α band of a 6-c LS haem species; thus the frequency shifts observed may result from enhancement of a LS species with band frequencies that are slightly higher than those of the QS species. This LS species is present in F221M HRP C in such small amounts that it cannot be detected in either the electronic absorption spectrum or in RR spectra obtained at other excitation wavelengths.

¹H-NMR spectroscopy of ferric F221M HRP C

The hyperfine-shifted resonances from the ¹H-NMR spectra of ferric (resting-state) wild-type HRP C, plant HRP A2 and F221M HRP C are compared in Figure 3. The chemical-shift values of these resonances are very sensitive to any changes which affect the environment of the haem group. The spectral region displayed includes the resonances of the four haem methyl groups, which can be distinguished easily from the remaining single-proton intensity resonances. The latter correspond to protons of the vinyl and propionate substituents, as well as to proximal His C β H₂ [22]. An additional, very broad resonance appears in the spectra of wild-type HRP C and plant HRP A2 in the region between 95.0 and 100.0 p.p.m. This resonance has been assigned to proximal His-170 N δ 1H, and is of interest

Figure 3 Hyperfine-shifted regions from the ^1H -NMR spectra of wild-type HRP C, plant HRP A2 and F221M HRP C

Spectra were obtained for ferric (resting) states of the enzymes at 500 MHz in 20 mM $\text{KH}_2\text{PO}_4/\text{H}_2\text{O}$ at $\text{pH}^* 7.0$ and 30°C . Known proton resonance assignments for the haem group and proximal His residue are given for reference [16,22].

Table 3 Haem methyl ^1H -NMR assignments and mean chemical-shift values recorded for the resting states of selected plant peroxidases

Chemical-shift data were obtained at 30°C with solution conditions of 20 mM $\text{KH}_2\text{PO}_4/\text{H}_2\text{O}$ at $\text{pH}^* 7.0$; F41V [24], F68A (N. C. Veitch, unpublished work), F179 mutants [16], SBP (N. C. Veitch and K. G. Welinder, unpublished work).

Peroxidase	Chemical shift data (p.p.m.)					Mean
	C12H ₃	C2H ₃	C18H ₃	C7H ₃	Total	
HRP C F41V	83.5	74.0	70.5	54.1	282.1	70.5
HRP C F68A	79.7	73.3	69.3	53.3	275.6	68.9
HRP C wild type	79.6	73.1	69.2	53.2	275.1	68.8
HRP C F179S	79.1	73.8	67.7	53.8	274.4	68.6
HRP C F179H	78.8	73.5	68.5	53.5	274.3	68.6
HRP C F179A	79.0	73.6	67.7	53.8	274.1	68.5
Plant SBP	76.5	68.5	65.7	54.7	265.4	66.4
Plant HRP A2	78.1	68.6	64.6	52.6	263.9	66.0
HRP C F221M	76.0	69.1	66.7	54.7	262.2	65.6

because it is an exchangeable proton which is still present in $^2\text{H}_2\text{O}$ solutions of these enzymes [22,23]. It is noteworthy that this resonance is absent from the spectrum of F221M HRP C acquired under identical solution conditions. The pattern of the remaining hyperfine-shifted resonances shows some variation as

judged by the spectra illustrated in Figure 3, but it is the changes to the chemical-shift values of the haem methyl resonances that are of greater interest. These are summarized in Table 3, from which a systematic variation in the arithmetic mean of the haem methyl chemical shifts can be discerned. Additional ^1H -NMR data for the resting states of SBP and site-directed mutants of HRP C at Phe-41 (a residue that contributes to the closed-haem architecture of the enzyme), and Phe-68 and Phe-179 (residues that contribute to the aromatic donor-molecule binding site), are also included in this analysis [16,24]. Previous work has shown that the resting state of F41V HRP C is predominantly 6-c HS [14], whereas the resting states of F68A HRP C and the F179 mutants are 5-c HS [16]. Replacement of either Phe-68 or Phe-179 has a negligible effect on the mean value of the haem methyl chemical shift, and the identity of the residue introduced appears to be unimportant in this respect. The most significant feature of these data therefore is the decrease in the value of this parameter in the order F41V HRP C > HRP C > SBP > HRP A2 > F221M HRP C.

Electronic absorption and RR spectroscopy of F221M HRP C at alkaline pH

The electronic absorption spectra of resting-state F221M HRP C at pH 10.1 and the wild-type enzyme at pH 10.0 and 12 are presented in Figure 4. The different behaviour of the mutant is

Figure 4 Electronic absorption spectra of ferric F221M and wild-type HRP C at alkaline pH

Spectra were recorded with 70 μ M F221M HRP C at pH 10.1 in 0.1 M glycine, 70 μ M HRP C at pH 12.0 in 0.01 M NaOH, and 70 μ M HRP C at pH 10.0 in 0.1 M glycine.

demonstrated clearly by comparison of the spectra of the enzymes at pH 10, which indicate that F221M HRP C and wild-type HRP C are essentially LS and HS, respectively. These data show that the pK_a for the alkaline transition in F221M HRP C is lowered by approx. 2 pH units from the value determined for HRP C of close to 11.0 [25]. The RR spectra of the mutant (both high- and low-frequency regions) obtained at pH 10.1 were also found to be very similar to those of the wild-type enzyme recorded at pH 12 (results not shown) [26]. The Fe-OH stretching frequency at 503 cm^{-1} in the RR spectrum of the mutant is unchanged with respect to wild-type enzyme, although there is a small decrease of 2 cm^{-1} in the frequency of the propionyl bending mode. It was not possible to obtain either electronic absorption or RR spectra recording the complete alkaline transition of F221M HRP C due to the instability of the enzyme at higher pH values, particularly in the laser beam. The weak CT1 band at 639 nm and the blue-shift and broadening of the Soret band in the electronic absorption spectrum of the mutant at pH 10.1 may indicate that the alkaline transition is not complete at this value. However it is more likely that these effects are due to the presence of free haem, evidence for which can be found in the high-frequency region of the RR spectrum at the same pH value.

Electronic absorption and RR spectroscopy of ferrous enzymes

The electronic absorption and RR spectra of the reduced states of the enzymes are shown in Figures 5(A) and 5(B), respectively. At pH 5.8, the electronic absorption spectrum of Fe(II) F221M HRP C indicates that some LS haem may be present, due to the blue-shift and broadening of the Soret band compared with the spectrum of wild-type enzyme (which corresponds to that of a 5-c HS haem species). As the pH is raised, the presence of a LS component becomes clear from the increasing blue-shift of the Soret band and the appearance of bands at 540 and 558 nm (note that the latter overlaps with, and therefore intensifies, the band due to the HS haem component). However, the high-frequency RR spectra of Fe(II) F221M HRP C at pH values up to 9.5 are very similar to those of the wild-type enzyme, and there are no clear indications of any bands characteristic for a LS haem species (results not shown). The latter is not unexpected, since the excitation wavelength of 441.6 nm is in resonance with the HS species. Wild-type HRP C remains HS at alkaline pH values [27].

Figure 5 Electronic absorption and RR spectra of ferrous forms of F221M and wild-type HRP C

(A) Electronic absorption spectra of 70 μ M ferrous HRP C (WT) at pH 7.0 in 0.1 mM Mops, 70 μ M ferrous F221M HRP C at pH 5.8 in 0.1 M citrate and at pH 9.6 in 0.1 M glycine, and the ferrous-CO complex of F221M HRP C (40 μ M) at pH 6.5 in 10 mM Mops. (B) Low-frequency RR spectra of 100 μ M ferrous HRP C (WT) at pH 7.0 in 0.1 M Mops, and 35 μ M ferrous F221M HRP C at pH 5.8 in 0.1 M citrate and pH 9.5 in 0.1 M glycine, recorded with 446.1 nm excitation. Experimental conditions: 5 cm^{-1} resolution; 10 s/0.5 cm^{-1} collection interval (HRP C); 5 s/0.5 cm^{-1} collection interval (F221M HRP C at pH 5.8); and 15 s/0.5 cm^{-1} collection interval (F221M HRP C at pH 9.5). In each case, 20 mW laser power was used at the sample.

The intense band observed at 243 cm^{-1} in the low-frequency RR spectrum of Fe(II) HRP C has been assigned previously to a stretching mode between the haem iron atom and the imidazole ligand (Im) of the proximal histidine residue, His-170 [28]. Reference to Figure 5(B) indicates that there is a significant increase in $\nu(\text{Fe-Im})$ between wild-type Fe(II) HRP C (the value at pH 5.8 has been measured as 244 cm^{-1}) [27], and the mutant (248 cm^{-1} at pH 5.8). The bands at 312, 348, 372 and 406 cm^{-1} (which are unchanged in frequency) can be assigned to γ_6 , ν_8 and the bending modes of the propionyl and vinyl groups, respectively. The frequency of $\nu(\text{Fe-Im})$ in the mutant is pH-dependent (Figure 5B), in common with the wild-type Fe(II) enzyme. This pH sensitivity is typical for the Fe-Im stretching mode of peroxidases belonging to the plant peroxidase superfamily, and is due to the strong hydrogen bond between the

Figure 6 Hyperfine-shifted regions from the ^1H -NMR spectra of cyanide-ligated wild-type (WT) and F221M HRP C

Spectra were acquired at 500 MHz in 20 mM KH_2PO_4 /15 mM $\text{KCN}/^2\text{H}_2\text{O}$ at pH* 7.6 and 30 °C. Haem and haem-linked proton resonance assignments are indicated. The upfield spectral region containing the broad resonance of His-170 Cε1H is plotted at $\times 8$ relative intensity.

proximal His and Asp residues (see Figure 1) [17,28]. The band in the RR spectrum of the mutant at 268 cm^{-1} is also sensitive to pH, with a decrease in frequency of 5 cm^{-1} observed between pH 5.8 and 9.5. This band can therefore be assigned to an internal mode of the imidazole ligand which is enhanced in the RR spectrum by vibrational coupling with the Fe-Im mode. The frequencies of the remaining bands in the RR spectrum appear to be unaffected by pH changes over this range.

Ligand binding to F221M HRP C

A useful approach for probing differences between the distal haem cavity of wild-type HRP C and a specified mutant is to study ligand binding at the sixth co-ordination site of haem iron. Ligands such as fluoride and cyanide bind to the resting-state enzyme in their protonated forms, whereas CO binds only to the reduced Fe(II) state [3]. The electronic absorption spectrum of fluoride-ligated F221M HRP C (results not shown) is similar to that of wild-type enzyme [29,30], although the absorption maxima of the Soret (403 nm) and CT1 (610 nm) bands are blue-shifted by 1 nm. The RR spectra are also very similar to the wild-type (results not shown) [30]. Likewise the electronic absorption (Figure 5A) and RR spectra of the Fe(II)-CO complex of F221M HRP C show close similarity to the corresponding spectra of the analogous complex with the wild-type enzyme (results not shown). These results demonstrate that the reduced form of the mutant binds CO fully. The bands observed in the low-frequency RR spectrum of the Fe(II)-CO complex of wild-type HRP C at 497, 516, 539 and 590 cm^{-1} [31,32], assigned previously to a porphyrin mode, two ν_{FeC} modes and a bending δ_{FeCO} mode overlapped with a porphyrin mode, respectively, are present at the same frequencies in Fe(II)-CO F221M HRP C.

F221M HRP C readily forms a 6-c LS cyanide-ligated complex. This state of the enzyme is more amenable to analysis by ^1H -NMR than by RR spectroscopy. The two sets of hyperfine-

Table 4 Comparison between ^1H -NMR assignments for hyperfine-shifted resonances of cyanide-ligated states of F221M HRP C and the wild-type enzyme

Chemical-shift data were obtained at 30 °C with solution conditions of 20 mM KH_2PO_4 /15 mM $\text{KCN}/^2\text{H}_2\text{O}$ at pH* 7.6. Haem proton nomenclature is given according to both IUB/IUPAC and Fischer (in parentheses) conventions.

Proton	Chemical shift (p.p.m.)		
	Wild type	F221M	$\Delta\delta$
C3 ² H (2-H _β)	-1.61	-1.07	+0.54
C3 ² H (2-H _β)	-2.77	-2.32	+0.45
C7H ₃ (3-CH ₃)	25.39	25.07	-0.32
C8 ¹ H (4-H _α)	19.87	21.11	+1.24
C8 ² H (4-H _β)	-2.26	-2.11	+0.15
C8 ² H (4-H _β)	-3.29	-2.95	+0.34
C13 ^{2a} H (6-H _β)	-2.77	-2.95	-0.18
C13 ^{2b} H (6-H _β)	-2.77	-3.07	-0.30
C17 ^{1a} H (7-H _α)	19.34	19.22	-0.12
C18H ₃ (8-CH ₃)	30.31	33.23	+2.92
Arg-38 Cβ1H	-5.31	-6.05	-0.74
Arg-38 Cδ1H	-6.87	-9.92	-3.05
His-42 Cε1H	13.17	12.62	-0.55
His-170 NH	12.74	12.62	-0.10
His-170 Cβ1H	23.18	22.44	-0.74
His-170 Cβ2H	15.23	15.18	-0.05
His-170 Cε1H	-29.2	-31.5	-2.3
Ile-244 CδH ₃	-3.14	-3.64	-0.50

shifted ^1H -NMR resonances compared in Figure 6 were obtained from samples of cyanide-ligated F221M and wild-type HRP C studied under identical solution conditions. Proton resonance assignments for cyanide-ligated F221M HRP C were obtained using standard methods [33], and are summarized in

Figure 7 Electronic absorption and RR spectra of BHA complexes of wild-type and F221M HRP C

(A) Electronic absorption spectra of BHA complexes of 40 μ M HRP C (WT) and F221M HRP C at pH 7.0 in 10 mM Mops. (B) RR spectra of the BHA complexes of 40 μ M HRP C (WT) and 40 μ M F221M HRP C at pH 7.0 in 10 mM Mops with 406.7 nm excitation. Experimental conditions: 5 cm^{-1} resolution; 4 s/0.5 cm^{-1} collection interval, 30 mW (HRP C) and 20 mW (F221M HRP C) laser power at the sample.

Table 4 together with data for the wild-type enzyme [16]. On careful examination of the spectra of mutant and wild-type enzyme it can be seen that each contains the same set of resonances, but with significant differences in chemical-shift values. What is curious about the spectrum of cyanide-ligated F221M HRP C is that both the sign and the magnitude of several important resonances are perturbed in a manner that is unique to this mutant. In particular, the resonances of both haem methyl C18H₃ (+2.92 p.p.m.) and His-170 C ϵ 1H (−2.3 p.p.m.) are shifted significantly in a direction (downfield and upfield, respectively) opposite to that recorded for other mutants of HRP C studied previously as their cyanide-ligated complexes. These include substitutions made at haem-pocket residues Ser-35 and Gln-176 [34], Arg-38 and Phe-41 [24], Glu-64 [6], Asn-70 [35] and Phe-179 [16].

Nuclear Overhauser enhancement connectivities between protons represented by the resonances assigned in the spectra of Figure 6 were identified from NOESY spectra of cyanide-ligated F221M HRP C and compared with those recorded previously for cyanide-ligated wild-type HRP C [16]. No appreciable differences were detected between the two sets of spectra.

BHA binding to F221M HRP C

The aromatic donor molecule BHA is a useful probe of the structural integrity of the distal haem pocket of HRP C due to the hydrogen-bonded interactions made by the hydroxamic acid side chain with Arg-38, His-42, Pro-139 and a distal water molecule co-ordinated to the haem iron atom (the complex is principally 6-c HS) [36,37]. The electronic absorption and RR spectra of resting-state F221M HRP C in the presence of saturating amounts of BHA are shown in Figure 7, together with the corresponding spectra of the wild-type enzyme. Although the electronic absorption spectra are similar overall, that of the BHA complex of F221M HRP C is slightly blue-shifted compared with the wild type, notably with respect to the CT1 band, for which a difference of 3 nm was recorded. The high-frequency RR spectra of the BHA complexes of F221M and wild-type HRP C are identical (Figure 7B), with the exception of two bands at 1499 and 1572 cm^{-1} , which are only present in the former, and whose presence was confirmed by curve-fitting of the spectra. These bands are assigned to the ν_3 and ν_2 modes of a minor 5-c HS⁺ haem species characterized by anomalous core-size marker-band frequencies, which is also observed in the RR spectrum of wild-type HRP C [18]. This species is almost certainly present in the absence of BHA, but one of the characteristic bands in the high-frequency RR spectrum is obscured by the dominant QS haem species with a ν_3 mode at 1505 cm^{-1} . Note that the frequencies of the vinyl stretching modes at 1623 and 1631 cm^{-1} are not altered by the substitution of Phe-221 by Met, indicating that the orientation of the vinyl groups is unchanged. In addition, the low-frequency RR spectra of the BHA complexes of F221M and wild-type HRP C were also found to be identical (results not shown). The fact that only small differences can be detected between the binding of BHA to F221M and wild-type HRP C is also reflected by the K_d values for the complexes reported in Table 1. These show that the Phe-221 $\rightarrow$ Met substitution gives rise to only a 2-fold decrease in binding affinity for this aromatic donor molecule.

DISCUSSION

F221M HRP C is a fully functional peroxidase with a reactivity profile towards H₂O₂ and reducing substrates (such as ABTS) almost identical with that of the wild-type enzyme. The stability of compound I is unaffected by the substitution, and no protein-based radical is formed. This contrasts with the Trp-221 radical detected as a second intermediate during the reaction of F221W HRP C with H₂O₂ [10]. The ability of the F221M HRP C mutant to form complexes with either typical peroxidase ligands or BHA is unimpaired. Nevertheless, a number of significant differences from the wild-type enzyme have been detected by using a combination of electronic absorption, RR and ¹H-NMR spectroscopies. These provide evidence for the existence of some global haem-linked changes in the enzyme, which are discussed in detail below.

The proximal haem region of F221M HRP C

The side chain of Phe-221 is stacked approximately parallel to the proximal histidine residue, His-170, as illustrated in Figure 1 [1]. There are no hydrogen-bonding interactions between Phe-221 and other neighbouring groups. The ¹H-NMR data for resting-state F221M HRP C acquired in ²H₂O solution indicate that the exchangeable proton His-170 N δ 1H must be accessible to solvent, as no resonance is observed in the spectral region

between 95.0 and 100.0 p.p.m., in contrast to wild-type HRP C (Figure 3). A broad single-proton intensity resonance is observed in this region in the corresponding spectrum of resting-state F221W HRP C [10]. This indicates that solvent access to His-170 is sterically controlled, and that the bulky side chains of Phe-221 and Trp-221 hinder the exchange of the His-170 Nδ1H proton in $^2\text{H}_2\text{O}$ solutions. The RR spectra of Fe(II) F221M HRP C obtained at pH 5.8 indicate a significant increase in the stretching frequency of the iron-imidazole bond, compared with the wild-type enzyme. This reflects a decrease in the iron-imidazole bond length, and consequently the iron-imidazole bond strength is increased. In structural terms this can be attributed to the reduction in the steric constraints imposed on the proximal histidine residue, and the loss of the π - π interaction between the aromatic rings of His-170 and Phe-221.

The distal haem region of F221M HRP C

F221M HRP C exhibits many properties common to the distal haem region of the wild-type enzyme, including the formation of ligand-bound complexes with CO, cyanide, fluoride and hydroxide, and an aromatic donor molecule complex with BHA. There appear to be no significant changes to the relative locations of distal haem-pocket residues in these ligand-bound complexes. This is supported by the fact that the pattern and intensity of nuclear Overhauser enhancement connectivities between haem and distal-residue protons are unchanged between mutant and wild-type enzymes. Characteristic distal-residue interactions with ligands are also maintained. In the absence of ligands, Fe(II) F221M HRP C exists as a mixture of 5-c HS and 6-c LS species at neutral pH, whereas wild-type Fe(II) HRP C is solely 5-c HS [27]. The 6-c LS component of the mutant becomes more dominant at higher pH values, in contrast with wild-type Fe(II) HRP C, which is 5-c HS at alkaline pH values. The origin of this sixth ligand in Fe(II) F221M HRP C has not been determined definitively, but the side chain of the distal residue His-42 is a likely candidate.

The unique distribution of the hyperfine-shifted resonances in the ^1H -NMR spectrum of cyanide-ligated F221M HRP C (Figure 6) is a notable feature of this enzyme. The pattern of these resonances depends on the orientation of the magnetic axes of the haem unit, a parameter related to the tilt of the Fe-CN fragment [38]. A change to the magnetic axes induced by substitution of a residue in the haem-linked region (or by binding of an aromatic donor molecule) may result in chemical-shift perturbations to resonances not just of neighbouring groups, but also of those outside the immediate substitution site. For example, the altered distribution of hyperfine-shifted resonances observed for the BHA complex of cyanide-ligated HRP C can be correlated with a specific decrease in the tilt of the Fe-CN fragment [38,39]. The physical origins of this decrease lie in the hydrogen-bonding interactions made by the hydroxamic acid side chain with the distal residues Arg-38, His-42 and the N atom of the ligand, which perturb the hydrogen-bonding network in the distal haem region [40]. The Phe-221 → Met substitution in HRP C leads to a comparable change in the magnetic axes of the cyanide-ligated enzyme, but the physical origin is more difficult to define, as Phe-221 does not engage in any hydrogen-bonding interactions with neighbouring residues (Figure 1). In this case it is likely that the proximal histidine residue relays the perturbation to the distal haem pocket, as a small change to either the orientation or position of His-170 (an essential link in the hydrogen-bonding network) would be sufficient to alter the magnetic axes [38]. The loss of the π - π interaction between His-170 and Phe-221 may also be important in this respect.

Presence of a QS haem species in ferric F221M HRP C

Substitution of Phe-221 by Met in HRP C leads to a profound change in the electronic structure of haem iron. Analysis of the data obtained here using spectroscopic techniques indicates that, as with the wild-type enzyme, several haem species constitute the ferric (resting) state of F221M HRP C at room temperature and neutral pH. The major component is the QS haem species, but a 6-c HS^{*} haem species and two minor components (6-c HS and 5-c HS^{*}) are also present. The appearance of a QS haem species is an interesting result. This state derives from the quantum-mechanical mixing of high- and intermediate-spin states ($S = 5/2$ and $S = 3/2$, respectively), and is relatively uncommon in biological systems. Known examples include the neutral-pH form of cytochrome *c*' [41,42] and class-III plant peroxidases such as HRP A2, SBP and BP1 [15,19,20].

The ^1H -NMR data summarized in Figure 3 and Table 3 show that the mean hyperfine shift of the haem methyl resonances decreases in the order F41V HRP C > HRP C > SBP > HRP A2 > F221M HRP C. This agrees with and extends the information provided in an earlier study in which systematic variation in the mean haem methyl chemical shift of the resting states of a series of HRP isoenzymes was considered to be a reflection of the degree of quantum-mechanical mixing of a $S < 5/2$ spin state with the HS ($S = 5/2$) state [22]. A decrease in this parameter was correlated with an increase in the contribution made by the $S < 5/2$ spin state component (probably $S = 3/2$) to the ground state. The ^1H -NMR results obtained here complement the evidence from electronic absorption and RR spectroscopy for an increased proportion of the QS haem species in the F221M HRP C mutant compared with HRP A2, SBP and HRP C. The F41V mutant of HRP C contains no QS haem component, but rather an increased proportion of a 6-c HS species, compared with the wild-type enzyme [18]. The ^1H -NMR spectra of resting-state F221W HRP C acquired under similar solution conditions have been published recently [10]. In this mutant, the mean hyperfine shift of the haem methyl resonances was reduced by 2 p.p.m. compared with wild-type enzyme. This is consistent with the observation that substitution of Phe-221 in HRP C leads to a significant increase in the proportion of the QS haem component in the resting state of the enzyme. The reasons for this increase are not clear at present. It has been suggested previously that a weak ligand to haem iron is responsible for the appearance of a QS haem species [43,44]. However, the present results contradict this view, as the iron-imidazole bond in F221M HRP C is strengthened relative to the wild-type enzyme, and the QS haem species is the main component of the resting state. The presence of a QS haem species does not compromise either peroxidase reactivity with H_2O_2 (Table 1) or the ability of the enzyme to bind ligands or aromatic donor molecules such as BHA. Although many site-directed mutants of HRP C have been examined in detail by spectroscopic techniques, including those involving the distal residues Arg-38, Phe-41, His-42 and Asn-70 [2], F221M is the first example in which the proportion of the QS haem species is increased in the resting state of the enzyme.

Global haem-site interactions in HRP

Substitution of Phe-221 of HRP C by Met influences the distal region of the haem pocket as well as the local proximal region. The corresponding residue in cytochrome *c* peroxidase (Trp-191) is hydrogen-bonded to the proximal Asp residue (equivalent to Asp-247 in Figure 1) and thus linked directly to the hydrogen-bonding network connecting the proximal and distal regions of the haem pocket [45]. In contrast, Phe-221 of HRP C is not

linked directly or indirectly to any other residue within the haem pocket.

There is no evidence from the present study to suggest that the Phe-221 → Met substitution affects either the catalytic or structural roles of the distal residues Arg-38 and His-42. However, the present data show that this substitution leads to some global structural changes in the haem pocket, particularly at alkaline pH. The acid-alkaline transition of the ferric form of the mutant has a pK_a value approx. 2 pH units lower than wild-type enzyme ($pK_a = 10.9$) [25], although in both cases the same Fe-OH haem species is formed (Figure 4). In addition, the onset of denaturation occurs at pH values that are significantly lower than those for wild-type enzyme. In the ferrous form at alkaline pH a LS species appears that is not observed in the wild-type. This probably arises from co-ordination of distal His-42 rather than hydroxide, since it appears that hydroxide is unable to form a LS ferrous complex [46]. Although a number of factors may contribute to alkaline denaturation of proteins, a common origin for the changes observed in F221M HRP C at alkaline pH is likely. This can be described in terms of an overall weakening of the hydrogen-bonding network connecting the proximal and distal haem regions of the enzyme (Figure 1). Site-directed mutagenesis studies in CCP lend support to this proposal, as substitution of residues that participate in the hydrogen-bonding network of this enzyme also results in a decrease of the pK_a of the alkaline transition [47]. Although Phe-221 of HRP C is not hydrogen-bonded to any other residue, the consequences of its substitution (the increased solvent accessibility and steric freedom of His-170, and the loss of the π - π interaction) are sufficient to disrupt the hydrogen-bonding network in the haem pocket.

Substitution of Phe-190 of MnP (equivalent to Phe-221 of HRP-C) by either Ile or Ala also lowers the pK_a of the alkaline transition and has a marked destabilizing effect on the enzyme [8]. The transition is characterized by the formation of a 6-c LS bis-histidinyl complex [48]. This requires displacement of helix B to bring distal His-46 within co-ordination range of the haem iron atom. Some authors have argued that mutation of Phe-190 of MnP also results in the loss of the distal calcium ion [9]. There is no evidence in the present study to indicate that the distal calcium ion is lost from F221M HRP C. However, the calcium ions of class-II fungal peroxidases such as LIP and MnP are more labile than those of class-III plant peroxidases, as demonstrated by the fact that the reversible alkaline inactivation of LIP involves the release of both the distal and proximal calcium ions, and bis-histidinyl co-ordination of the haem [49]. We propose that the presence of the proximal Phe in class-II and -III peroxidases is critical for the overall stabilization of the haem architecture under stressed conditions such as those experienced at high pH. Substitution of this residue in both MnP (class II) and HRP C (class III) results in a decrease of the pK_a of the alkaline transition. In MnP this transition is characterized by formation of a bis-histidinyl haem (with the loss of at least the distal calcium ion), whereas in HRP C a hydroxyl-bound LS haem is formed and there is no loss of calcium. The difference between the lability of the distal calcium in class-II and -III peroxidases is due mainly to the disulphide bridge between Cys-44 and Cys-49, which is unique to the latter [5]. This restricts the flexibility of distal helix B and limits movement of His-42 in the distal haem cavity of HRP C.

We thank the MRC Biomedical NMR Centre (National Institute for Medical Research, Mill Hill, London, U.K.) for access to NMR facilities, Dr Brigitte Meunier and Professor Peter Rich (University College London) for measurement of reduction potentials, and Professor Mario Marzocchi (University of Florence, Florence, Italy) for constructive comments. This work was supported by the EU Biotechnology Programme, 'Towards Designer Peroxidases' BIO4-CT97-2031 (to G. S., N. C. V.

and A. T. S.), Italian CNR and MURST Cofin MURST97-CFSIB (to G. S.) and the Biotechnology and Biological Sciences Research Council (BBSRC; Project Grant 85/B11934, to A. T. S.). C. G. W. thanks the BBSRC for a studentship.

REFERENCES

- Gajhede, M., Schuller, D. J., Henriksen, A., Smith, A. T. and Poulos, T. L. (1997) Crystal structure of horseradish peroxidase C at 2.15 Å resolution. *Nat. Struct. Biol.* **4**, 1032–1038.
- Smith, A. T. and Veitch, N. C. (1998) Substrate binding and catalysis in heme peroxidases. *Curr. Opin. Chem. Biol.* **2**, 269–278.
- Dunford, H. B. (1991) Horseradish peroxidase: structure and kinetic properties. In *Peroxidases in Chemistry and Biology*, vol. II (Everse, J., Everse, K. E. and Grisham, M. B., eds), pp. 1–24. CRC Press, Boca Raton.
- Welinder, K. G. (1979) Amino acid sequence studies of horseradish peroxidase. *Eur. J. Biochem.* **96**, 483–502.
- Welinder, K. G. (1992) Superfamily of plant, fungal and bacterial peroxidases. *Curr. Opin. Struct. Biol.* **2**, 388–393.
- Tanaka, M., Ishimori, K. and Morishima, I. (1998) Roles of the highly conserved Glu residue in the heme distal site of peroxidases. *Biochemistry* **37**, 2629–2638.
- Welinder, K. G. (1992) Plant peroxidases: structure-function relationships. In *Plant Peroxidases 1980–1990: Progress and Prospects in Biochemistry and Physiology* (Penel, C., Gaspar, T. and Greppin, H., eds), pp. 1–24. University of Geneva, Geneva.
- Kishi, K., Hildebrand, D. P., Kusters-van Someren, M., Gettemy, J., Mauk, A. G. and Gold, M. H. (1997) Site-directed mutations at phenylalanine-190 of manganese peroxidase: effects on stability, function, and coordination. *Biochemistry* **36**, 4268–4277.
- Banci, L., Bertini, I., Capannoli, C., Del Conte, R. and Tien, M. (1999) Spectroscopic characterization of active mutants of manganese peroxidase: mutations on the proximal side affect calcium binding of the distal side. *Biochemistry* **38**, 9617–9625.
- Morimoto, A., Tanaka, M., Takahashi, S., Ishimori, K., Hori, H. and Morishima, I. (1998) Detection of a tryptophan radical as an intermediate species in the reaction of horseradish peroxidase mutant (Phe-221 → Trp) and hydrogen peroxide. *J. Biol. Chem.* **273**, 14753–14760.
- Simon, P., Capelli, N., Flach, J., Overny, S., Tognoli, M., Penel, C. and Greppin, H. (1996) The peroxidase gene family of *Arabidopsis thaliana*. In *Plant Peroxidases: Biochemistry and Physiology* (Obinger, C., Burner, U., Ebermann, R., Penel, C. and Greppin, H., eds), pp. 179–183. University of Geneva, Geneva.
- Doyle, W. A., Blodig, W., Veitch, N. C., Piontek, K. and Smith, A. T. (1998) Two substrate interaction sites in lignin peroxidase revealed by site-directed mutagenesis. *Biochemistry* **37**, 15097–15105.
- Smith, A. T., Santama, N., Dacey, S., Edwards, M., Bray, R. C., Thorneley, R. N. F. and Burke, J. F. (1990) Expression of a synthetic gene for horseradish peroxidase in *Escherichia coli* and folding and activation of the recombinant enzyme with Ca^{2+} and heme. *J. Biol. Chem.* **265**, 13335–13343.
- Smith, A. T., Sanders, S. A., Thorneley, R. N. F., Bray, R. C. and Burke, R. C. (1992) Characterisation of a haem active-site mutant of horseradish peroxidase, Phe41 → Val, with altered reactivity towards hydrogen peroxide and reducing substrates. *Eur. J. Biochem.* **207**, 507–519.
- Nissim, M., Feis, A. and Smulevich, G. (1998) Characterization of soybean seed coat peroxidase: resonance Raman evidence for a structure-based classification of plant peroxidases. *Biospectroscopy* **4**, 355–364.
- Veitch, N. C., Gao, Y., Smith, A. T. and White, C. G. (1997) Identification of a critical phenylalanine residue in horseradish peroxidase, Phe179, by site-directed mutagenesis and 1H -NMR: implications for complex formation with aromatic donor molecules. *Biochemistry* **36**, 14751–14761.
- Smulevich, G. (1998) Understanding heme cavity structure of peroxidases: comparison of electronic absorption and resonance Raman spectra with crystallographic results. *Biospectroscopy* **4**, S3–S17.
- Smulevich, G., Paoli, M., Burke, J. F., Sanders, S. A., Thorneley, R. N. F. and Smith, A. T. (1994) Characterization of recombinant horseradish peroxidase C and three site-directed mutants, F41V, F41W and R38K, by resonance Raman spectroscopy. *Biochemistry* **33**, 7398–7407.
- Feis, A., Howes, B. D., Indiani, C. and Smulevich, G. (1998) Resonance Raman and electronic absorption spectra of horseradish peroxidase isozyme A2: evidence for a quantum-mixed spin species. *J. Raman Spectrosc.* **29**, 933–938.
- Howes, B. D., Schiadt, C. B., Welinder, K. G., Marzocchi, M. P., Ma, J.-G., Zhang, J., Shelnutt, J. A. and Smulevich, G. (1999) The quantum mixed-spin heme state of barley peroxidase: a paradigm for class III peroxidases. *Biophys. J.* **77**, 478–492.
- Smulevich, G., English, A., Mantini, A. R. and Marzocchi, M. P. (1991) Resonance Raman investigation of ferric iron in horseradish peroxidase and its aromatic donor complexes at room and low temperatures. *Biochemistry* **30**, 772–779.
- De Ropp, J. S., Mandal, P., Brauer, S. L. and La Mar, G. N. (1997) Solution NMR study of the electronic and molecular structure of the heme cavity in high-spin, resting state horseradish peroxidase. *J. Am. Chem. Soc.* **119**, 4732–4739.

- 23 La Mar, G. N., De Ropp, J. S., Smith, K. M. and Langry, K. C. (1980) Proton nuclear magnetic resonance study of the electronic and molecular structure of the heme crevice in horseradish peroxidase. *J. Biol. Chem.* **255**, 6646–6652
- 24 Veitch, N. C., Williams, R. J. P., Bray, R. C., Burke, J. F., Sanders, S. A., Thorneley, R. N. F. and Smith, A. T. (1992) Structural studies by proton-NMR spectroscopy of plant horseradish peroxidase C, the wild-type recombinant protein from *Escherichia coli* and two protein variants, Phe41 → Val and Arg38 → Lys. *Eur. J. Biochem.* **207**, 521–531
- 25 Sitter, A. J., Shiflett, J. R. and Terner, J. (1988) Resonance Raman spectroscopic evidence for heme iron-hydroxide ligation in peroxidase alkaline forms. *J. Biol. Chem.* **263**, 13032–13038
- 26 Feis, A., Marzocchi, M. P., Paoli, M. and Smulevich, G. (1994) Spin state and axial ligand bonding in the hydroxide complexes of metmyoglobin, methemoglobin, and horseradish peroxidase at room and low temperatures. *Biochemistry* **33**, 4577–4583
- 27 Teraoka, J. and Kitagawa, T. (1981) Structural implication of the heme-linked ionization of horseradish peroxidase probed by the Fe-histidine stretching Raman line. *J. Biol. Chem.* **256**, 3969–3977
- 28 Kitagawa, T. (1988) The heme protein structure and the iron histidine stretching mode. In *Biological Applications of Raman Spectroscopy*, vol. III (Spiro, T. G., ed.), pp. 97–131, J. Wiley and Sons, New York
- 29 Keilin, D. and Hartree, E. F. (1951) Purification of horseradish peroxidase and comparison of its properties with those of catalase and methemoglobin. *Biochem. J.* **49**, 88–104
- 30 Neri, F., Kok, D., Miller, M. A. and Smulevich, G. (1997) Fluoride binding in hemoproteins: the importance of the distal cavity structure. *Biochemistry* **36**, 8947–8953
- 31 Evangelista-Kirkup, R., Smulevich, G. and Spiro, T. G. (1986) Alternative carbon monoxide binding modes for horseradish peroxidase studied by resonance Raman spectroscopy. *Biochemistry* **25**, 4420–4425
- 32 Uno, T., Nishimura, Y., Tsuboi, M., Makino, R., Iizuka, T. and Ishimura, Y. (1987) Two types of conformers with distinct Fe-C-O configuration in the ferrous CO complex of horseradish peroxidase. Resonance Raman and infrared spectroscopic studies with native and deuteroheme-substituted enzymes. *J. Biol. Chem.* **262**, 4549–4556
- 33 Chen, Z., De Ropp, J. S., Hernández, G. and La Mar, G. N. (1994) 2D NMR approaches to characterizing the molecular structure and dynamic stability of the active site for cyanide-inhibited horseradish peroxidase. *J. Am. Chem. Soc.* **116**, 8772–8783
- 34 Tanaka, M., Ishimori, K. and Morishima, I. (1999) Luminol activity of horseradish peroxidase mutants mimicking a proposed binding site for luminol in *Arthromyces ramosus* peroxidase. *Biochemistry* **38**, 10463–10473
- 35 Tanaka, M., Nagano, S., Ishimori, K. and Morishima, I. (1997) Hydrogen bond network in the distal site of peroxidases: spectroscopic properties of Asn70 → Asp horseradish peroxidase mutant. *Biochemistry* **36**, 9791–9798
- 36 Schonbaum, G. R. (1973) New complexes of peroxidases with hydroxamic acids, hydrazides, and amides. *J. Biol. Chem.* **248**, 502–511
- 37 Smulevich, G., Feis, A., Indiani, C., Becucci, M. and Marzocchi, M. P. (1999) Peroxidase-benzhydroxamic acid complexes: spectroscopic evidence that a Fe-H₂O distance of 2.6 Å can correspond to hexa-coordinate high-spin heme. *J. Biol. Inorg. Chem.* **4**, 39–47
- 38 La Mar, G. N., Chen, Z., Vyas, K. and McPherson, A. D. (1995) An interpretive basis of the hyperfine shifts in cyanide-inhibited horseradish peroxidase based on the magnetic axes and ligand tilt. Influence of substrate binding and extensions to other peroxidases. *J. Am. Chem. Soc.* **117**, 411–419
- 39 Veitch, N. C. and Williams, R. J. P. (1995) The use of methyl-substituted benzhydroxamic acids as structural probes of peroxidase substrate binding. *Eur. J. Biochem.* **229**, 629–640
- 40 Chang, Y.-T., Veitch, N. C. and Loew, G. H. (1998) A theoretical study of benzhydroxamic acid binding modes in horseradish peroxidase. *J. Am. Chem. Soc.* **120**, 5168–5178
- 41 Fujii, S., Yoshimura, T., Kamada, H., Yamaguchi, K., Suzuki, S., Shidara, S. and Takakuwa, S. (1995) Electron paramagnetic resonance studies of ferric cytochrome *c'* from photosynthetic bacteria. *Biochim. Biophys. Acta* **1251**, 161–169
- 42 Othman, S., Richard, P., Vermiglio, A. and Desbois, A. (1996) Evidence for a proximal histidine interaction in the structure of cytochromes *c'* in solution: a resonance Raman study. *Biochemistry* **35**, 9224–9234
- 43 Scheidt, W. R. and Reed, C. A. (1981) Spin-state/stereochemical relationships in iron porphyrins: implications for the hemoproteins. *Chem. Rev.* **81**, 543–555
- 44 Munro, O. Q., de Wet, M., Pollak, H., van Wyk, J. and Helder, M. (1998) Haempeptide models for haemoproteins. Part 3. N-acetylmicroperoxidase-8: EPR, Mössbauer and magnetic susceptibility studies on an iron(III) porphyrin in thermal equilibrium between $S = 3/2$, $5/2$ and $S = 1/2$ states. *J. Chem. Soc. Faraday Trans.* **94**, 1743–1752
- 45 Finzel, B. C., Poulos, T. L. and Kraut, J. (1984) Crystal structure of yeast cytochrome *c* peroxidase refined at 1.7-Å resolution. *J. Biol. Chem.* **259**, 13027–13036
- 46 Parthasarathi, N., Hansen, S., Yamaguchi, S. and Spiro, T. G. (1987) Metalloporphyrin core size resonance Raman marker bands revisited: implications for the interpretation of hemoglobin photoproduct Raman frequencies. *J. Am. Chem. Soc.* **109**, 3865–3871
- 47 Smulevich, G., Miller, M. A., Kraut, J. and Spiro, T. G. (1991) Conformational change and histidine control of heme chemistry in cytochrome *c* peroxidase: resonance Raman evidence from Leu-52 and Gly-181 mutants of cytochrome *c* peroxidase. *Biochemistry* **30**, 9546–9558
- 48 Youngs, H. L., Moënn-Loccoz, P., Loehr, T. M. and Gold, M. H. (2000) Formation of a bis(histidyl) heme iron complex in manganese peroxidase at high pH and restoration of the native enzyme structure by calcium. *Biochemistry* **39**, 9994–10000
- 49 George, S. J., Kvaratskhelia, M., Dilworth, M. J. and Thorneley, R. N. F. (1999) Reversible alkaline inactivation of lignin peroxidase involves the release of both the distal and proximal site calcium ions and bishistidine co-ordination of the haem. *Biochem. J.* **344**, 237–244

Received 24 March 2000/26 September 2000; accepted 30 October 2000