Spectroscopic characterization of mutations at the Phe⁴¹ position in the distal haem pocket of horseradish peroxidase C: structural and functional consequences

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Three mutants of horseradish peroxidase isoenzyme C (HRPC) have been constructed in which the conserved distal aromatic residue Phe⁴¹ has been substituted by Trp, Val or Ala and the properties of the mutant proteins have been compared with that of the wild-type. The ferric and ferrous states have been studied by resonance Raman, electronic absorption and Fourier-transform infrared spectroscopies, together with their respective fluoride and CO complexes as probes for the integrity of the distal haem-pocket hydrogen-bonding network. The catalytic properties of the mutants, most notably the HRPC-mutant Phe⁴¹ → Trp (F41W) variant, were also affected. Structural modelling suggests that the bulky indole group of the F41W mutant blocks the distal cavity, inhibiting the binding of fluoride and CO to the haem iron, severely impairing the reaction of the enzyme with H₂O₂ to form Compound I. Substitution with the smaller side-chain residues Val or Ala resulted in a 2-fold increase in the affinity of the mutants for the aromatic donor benzhydroxamic acid (BHA) compared with the wild-type, whereas the sterically

hindered F41W mutant was not able to bind BHA at all. All the mutations studied increased the amount of a ferric six-coordinate aquo-high-spin species. On the other hand, the similarity in the Fe–Im stretching frequencies of the mutants and wild-type protein suggests that the distal haem-pocket mutations do not cause any substantive changes on the proximal side of the haem. Spectra of the HRPC mutant Phe⁴¹ → Ala–CO and the HRPC mutant Phe⁴¹ → Val–CO complexes strongly suggested a weakening of the interaction between CO and Arg³⁸ due to a secondary rearrangement of the haem relative to helix B. The effects observed for these HRP mutants were somewhat different from those noted recently for the analogous *Coprinus cinereus* peroxidase (CIP) mutants, particularly the Trp mutant. These differences can be reconciled in part as being due to the smaller size of the distal cavity of HRP compared with that of CIP.

Key words: CO-adducts, distal mutants, haem protein, horseradish peroxidase, resonance Raman spectroscopy.

INTRODUCTION

The superfamily of haem-containing ‘plant’ peroxidases can be divided into three subclasses. Class I includes the prokaryotic intracellular peroxidases, class II, the fungal secretory peroxidases and class III, the classical extracellular plant peroxidases [1]. The three classes are conserved structurally having some nine conserved α -helices. The catalytic His and Arg residues which play a concerted role in H₂O₂ binding to the ferric-resting state and subsequent 2e[−] oxidation of the enzyme to Compound I (reviewed in [2]) are also conserved. Moreover, the so-called ‘closed-haem architecture’ [3,4] of peroxidase is also provided in part by a conserved distal aromatic residue, located next to the catalytic His⁴² in horseradish peroxidase C (HRPC), Figure 1]. In class-I peroxidases this is a Trp residue, e.g. Trp⁵¹ in cytochrome *c* peroxidase (CCP), whereas in class-II and -III peroxidases it is a phenylalanine, namely Phe⁵⁴, in *Coprinus cinereus* peroxidase (CIP) and Phe⁴¹ in [5]. The aromatic ring is orientated almost parallel to the plane of the haem, at a distance of 3.3 Å from the haem iron in HRPC and both the distal Phe⁴¹ and Arg³⁸ are 1–2 Å closer to the haem iron than in other

peroxidases, reflecting the smaller distal cavity of HRPC [6]. In CIP, the conserved Phe residue is located 5.3 Å from the iron atom, above and towards the α -meso haem edge and close to vinyl 2 [7]. Recently, it has been shown [8] that two CIP mutant Phe⁵⁴ → Val (F54V) and Phe⁵⁴ → Gly (F54G) cause an increase in the ν (C=C) vinyl stretching frequencies and have blue-shifted Soret absorption bands. Removal of the bulky aromatic ring allows vinyl 2 to rotate, resulting in decreased conjugation between the C=C vinyl and the porphyrin macrocycle [9].

Mutagenesis of the conserved distal aromatic residue in CCP and CIP has significant effects on the co-ordination and ligand-binding properties of the mutants. In CCP, substitution of Trp⁵¹ with Phe, Met, Thr, Cys, Ala or Gly results in a higher proportion of ferric six-coordinate high spin (6cHS) and an increased tendency to form a low-spin haem [9–14]. This has been explained by an increased affinity of one of the distal water molecules for the haem iron due to the loss of the hydrogen bond between the indole nitrogen and the distal water molecule [9,10,15]. The effect of the hydrogen-bonding interactions between cavity residues and a distal water on the co-ordination state of the CIP F54V, F54G and Phe⁵⁴ → Trp (F54W) mutants has also been studied

Abbreviations used: ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonate); BHA, benzhydroxamic acid; 5c, five coordinate; 6c, six coordinate; CCP, cytochrome *c* peroxidase; CIP, *Coprinus cinereus* peroxidase, expressed in *Aspergillus oryzae* (identical with *Arthromyces ramosus* peroxidase); CT1 band, long-wavelength (> 600 nm) porphyrin-to-metal charge-transfer band; HRPC, horseradish peroxidase isoenzyme C; F41A, HRPC mutant Phe⁴¹ → Ala; F41V, HRPC-mutant Phe⁴¹ → Val; F41W, HRPC-mutant Phe⁴¹ → Trp; F54G, CIP-mutant Phe⁵⁴ → Gly; F54V, CIP-mutant Phe⁵⁴ → Val; F54W, CIP-mutant Phe⁵⁴ → Trp; FTIR, Fourier-transform infrared; Vis, visible; HRP A1, horseradish peroxidase isoenzyme A1; HS, high spin; Mops, 3-(*N*-morpholino)propanesulphonic acid; QS, quantum mechanically mixed spin; RR, resonance Raman.

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Figure 1 The distal haem pocket of horseradish peroxidase

(a) Comparison of the wild-type structure (red) (1ATJ [6]) with that predicted for the F41W mutant after conformational refinement by molecular-mechanics calculations (green). (b) The disposition of the F41 side chain in the wild-type enzyme shown as a van der Waals surface. (c) The predicted disposition of the W41 side chain shown as a van der Waals surface to illustrate the blocked distal cavity.

[8]. These mutations resulted in a decrease in the pK_a that controls the alkaline transition between the ferric five-coordinate 5cHS and 6cHS forms. Interestingly, all these mutations resulted in tighter fluoride binding than in wild-type CIP, where this ligand is hydrogen-bonded to the distal Arg and His residues [16]. The increased affinity of the CIP F54W mutant for fluoride was explained by the stabilizing effect resulting from an extra hydrogen-bond interaction between the indole nitrogen and the fluoride anion. Moreover, the increased affinity of the CIP F54V and F54G mutants for fluoride pointed to small structural changes, possibly resulting from a movement of the distal His closer to the haem, thus stabilizing the binding of HF. Secondary structural changes in these mutants, rather than perturbation of the haem-linked hydrogen-bonding network, were deemed responsible for controlling the co-ordination of the distal water to the haem iron [8]. The functional effects of these mutations in CIP have not yet been studied.

In the present work, we report the characterization of the HRPC mutants Phe⁴¹ → Trp (F41W), Phe⁴¹ → Val (F41V) and Phe⁴¹ → Ala (F41A) by resonance Raman (RR), Fourier-transform infrared (FTIR) and electronic absorption spectroscopies. The functional properties of the mutants are also reported. The effects of these mutations on the ferric and ferrous states, fluoride and CO complexes are compared with those reported for the corresponding mutants of the class-II peroxidase, CIP. The aim was to understand better the role of the conserved distal aromatic residue in the context of the smaller distal cavity of HRPC and its effects on enzyme functionality. The influence of this residue position (Phe⁴¹) on ligand binding, substrate binding and fine-tuning of the electronic properties of the haem via its interaction with the vinyl substituents was of particular interest.

MATERIALS AND METHODS

Site-directed mutagenesis and production of recombinant HRPC

A PCR-based method was used for site-directed mutagenesis, which utilized the synthetic HRPC gene [17] exactly as described in [18]. The primer encoding the mutation at position 41 in the HRPC protein sequence was replaced with an identical primer containing the randomized codon NNG/C at the same position. Potentially, all possible amino-acid substitutions were recoverable. Three clones corresponding to F41W, F41A and F41V were chosen for further study and subjected to DNA sequencing by the dideoxy-chain-termination method on both strands of the mutant-bearing DNA insert. Only the expected sequence changes were detected.

Overexpression, isolation of inclusion bodies, refolding and purification of both the wild-type and F41-variant proteins were as previously described [17,18]. Transformed strains were grown in 20 litres of Terrific broth [19] in a Micros 20 fermenter [New Brunswick Scientific (U.K.) Ltd., Hatfield, Herts., U.K.] at a dissolved O_2 tension of 5%. Induction took place after 1–2 h growth (from a 5% inoculum) at a D_{600} of 1.0–2.0 with 0.5 mM isopropyl β -D-thiogalactoside. Harvesting of ≈ 500 g of wet weight *Escherichia coli* took place 4–5 h after induction.

Determination of kinetic and physical parameters

Steady-state turnover with 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) as substrate

Initial rates were measured by following the increase in absorbance at 405 nm resulting from the formation of the ABTS cation radical product with a saturating (1.5 mM) concentration of H_2O_2 and 0.3 mM ABTS in 50 mM phosphate citrate buffer at pH 5.0, as described in [18].

Measurement of the second-order rate constants for Compound I formation

The bimolecular rate constant for Compound I formation (k_1) was determined in an Applied Photo Physics SX 18MV stopped-flow system under pseudo-first-order conditions in 10 mM sodium phosphate buffer, pH 7.0, and 25 °C. Plots of k_{obs} versus H_2O_2 concentration were linear over the range studied (5–20 μ M) and k_1 was obtained from the slope.

Measurement of molar absorption coefficients

Molar absorption coefficients at the Soret maxima were determined in triplicate by the pyridine haemochrome method [20].

Benzhydroxamic acid (BHA)-binding studies

The dissociation constants of the HRPB–BHA complexes were determined exactly as described in [21] by applying a weighted-least-squares error-minimization procedure (Sigma Plot, Jandel Scientific, Madera, CA, U.S.A.) to the equation

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

where the absorbance change at 408 nm resulting from BHA of concentration L binding to a total protein concentration P is determined, but allowing the K_d and maximum absorbance change at saturation (A_{∞}) to vary. All data were corrected for dilution during substrate addition. If P was also allowed to vary (a three-parameter fit) then an independent estimate of the enzyme concentration and hence its molar absorbance coefficient could be made.

Sample preparation

For RR, electronic absorption and FTIR spectroscopies the experimental conditions were as reported in the captions to Figures. The fluoride complexes were obtained in 0.1 M NaF (Merck) and 25 mM sodium citrate, pH 5.0 or, for HRPB F41W, by adding solid NaF to the buffered solution. Although the ferric F41A mutant was found to be less stable eventually losing one of its Ca^{2+} ions (N. Brissett and A. T. Smith, unpublished results), we did not observe a decrease in the free-protein bands when 0.25 mM CaCl_2 was added to prevent loss of Ca^{2+} from the protein. Moreover, upon fluoride binding, the resultant complex was very stable in the laser beam.

The ferrous forms were made by adding 5 μl of fresh sodium dithionite solution (10 g/l) to 35 μl of deoxygenated protein solution. The CO complexes were prepared by flushing the protein solution with ^{12}CO (Riviera, Milan, Italy) and reducing with dithionite as described above or by flushing ferrous protein with ^{13}CO (Fluorochem, Old Glossop, Derbyshire, U.K.). Deuterated CO complexes for FTIR were prepared by dissolving plant HRPB in buffered deuterium oxide ($^2\text{H}_2\text{O}$) (Merck, 99.8%) or redissolving a lyophilized HRPB F41V or F41A sample in $^2\text{H}_2\text{O}$. The pH was adjusted to compensate for the 0.5 unit increase in the pK values in $^2\text{H}_2\text{O}$. The protein concentrations were 15–100 μM for the RR samples, 10–85 μM for the electronic absorption spectra (using a 1 mm path-length cuvette) and 0.1–0.9 mM for the FTIR samples.

Spectroscopy

RR spectra of the ferric- and ferrous-CO forms were obtained by excitation with the 406.7 and 413.1 nm lines of a Kr^+ laser (Innova 90/5; Coherent, Santa Cruz, CA, U.S.A.). The spectra of the ferrous forms were obtained by excitation with the 457.9 nm line of an Ar^+ laser (Innova 90/5; Coherent) or the 441.6 nm Hg/Cd laser (Liconix, Santa Clara, CA, U.S.A.). The back-scattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG 2S), equipped with a cooled photomultiplier (RCA C31034 A) and photon-counting electronics. The RR spectra were calibrated to an accuracy of $\pm 1 \text{ cm}^{-1}$ for intense isolated bands, with indene as standard for the high-frequency region and with both indene and CCl_4 for the low-frequency region. The rotating NMR tube was cooled to approx. 15 °C by a gentle flow of N_2 gas passed through liquid N_2 . Electronic absorption spectra were recorded at 22 °C with a double-beam Cary 5 spectrophotometer, both prior to

and after RR measurements. Infrared spectra were recorded at 22 °C with a Bruker IFS120 FTIR spectrophotometer. The CO complexes were transferred by a gas-tight CO-flushed Hamilton syringe into a vacuum-tight CaF_2 infrared cell of 0.1 mm path length that had also been flushed with CO. The spectra were recorded after evacuation of the sample chamber, together with appropriate reference spectra.

To determine the H/ ^2H isotopic shifts of the $\nu(\text{CO})$ frequencies, the FTIR spectra of the F41V–CO complex were deconvoluted by curve fitting. Best fits were obtained using mixed lineshapes (51% Lorentzian and 49% Gaussian) with a bandwidth of 11.0 cm^{-1} . The RR spectra of the F41V–CO complex were deconvoluted as described in the caption to the Figure.

Molecular modelling of the HRPB-mutant structures

The structures of the F41W, F41V and F41A HRPB mutants were modelled using the crystal structure of the recombinant wild-type HRPB (1ATJ [6]) as a starting point. The appropriate side chain was introduced at position 41 using the ‘edit-protein’ facility embedded with the QUANTA molecular modelling package (Molecular Systems Inc., San Diego, CA, U.S.A.) and the torsion angles giving the lowest relative potential energy selected manually using the ‘bumps’ option. Hydrogen atoms were added using the CHARM/M force field in residue topology file mode and the conformation refined to convergence with 500 cycles of Adopted Basis Newton–Raphson energy minimization on an SGI Onyx 2 computer. This was followed by 10 ps of unrestrained room-temperature molecular dynamics and reminimization as above. The last structure in the trajectory was compared with the wild-type structure subjected to the same procedure.

RESULTS

Comparison of kinetic and physical parameters for the HRPB variants

Table 1 shows that all the mutants at position 41 investigated spectroscopically in this study had lower specific activities with ABTS when compared with the wild-type protein, as shown previously for the F41V mutant [18]. All were nevertheless functional peroxidases. The F41W mutant was notable in that it underwent rapid autoactivation (within 200 s) under standard

Table 1 Comparison of kinetic and physical parameters for Phe⁴¹ mutants

Measurements of the parameters were as described in the Materials and methods section.

Enzyme variant	Specific activity (units/mg)	Compound I formation ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Soret molar absorption coefficient ($\text{mM}^{-1} \cdot \text{cm}^{-1}$)	K_d BHA: enzyme complex (μM)
Recombinant wild-type	860 ± 40	$(1.6 \pm 0.1) \times 10^7$	96 ± 3	2.6 ± 0.2
F41A HRPB	125 ± 10	$(2.2 \pm 0.2) \times 10^7$	207 ± 11	1.3 ± 0.3
F41V HRPB*	197 ± 12	$(2.0 \pm 0.2) \times 10^6$	124 ± 7	1.2 ± 0.1
F41W HRPB	$0^\dagger - 88^\ddagger$	$\approx 1.0^\S$	113 ± 4	> 10000

* Taken from ref. [18].

† F41W HRPB exhibited no activity in the standard ABTS assay at time zero (the moment the assay was started by the addition of enzyme) but underwent a rapid activation under the assay conditions until after 200 s a maximum specific activity was achieved characteristic of a new activated state.

‡ The autocatalytic activation of this mutant has been described previously [22].

§ A slow unimolecular process independent of H_2O_2 concentration (see text).

Figure 2 Ferric HRPc and the distal F41V, F41A and F41W mutants in 10 mM Mops/NaOH, pH 7.0

(A) Electronic absorption spectra. The region between 460 and 700 nm has been expanded 5-fold. (B) RR spectra. Experimental conditions: 406.7 nm excitation wavelength and 5 cm^{-1} spectral resolution. Wild type, F41V and F41W: 6 s/ 0.5 cm^{-1} collection interval and 30 mW laser power at the sample. F41A: 12 s/ 0.5 cm^{-1} collection interval and 15 mW laser power at the sample.

turnover conditions, as previously reported [22], although it was almost completely inactive before treatment with the peroxide. The reason for this last observation became clear when its reactivity towards H_2O_2 was investigated under stopped-flow conditions. In contrast with the wild-type and other F41 mutants (Table 1), the reaction of F41W with peroxide was very slow ($\approx 1/\text{s}$) and independent of the peroxide concentration (results not shown), suggesting that access of peroxide to the haem iron is initially very slow and is presumably blocked by the bulky Trp side chain (Figures 1a–1c). It also failed to show any evidence of complex formation with the aromatic donor BHA.

The F41A variant exhibited a small but significant increase in its bimolecular reaction rate with H_2O_2 compared with the wild-type protein, despite its 6cHS character (see below). A notable characteristic of the F41A mutation was the extraordinarily high molar-absorption coefficient and sharper Soret peak (consistent with a 6cHS state), which was even higher than that reported for the other 6cHS haem systems, e.g. lignin peroxidase [23]. The molar-absorption coefficient of the F41A mutant was verified by an independent method. The free enzyme concentration was allowed to float as an additional parameter during the fitting of the F41A–BHA titration data (see the Materials and methods section). This gave a calculated value of $216 \pm 25 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the Soret molar-absorption coefficient, in reasonable agreement with the value determined directly by the pyridine haemochrome method ($207 \pm 25 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, see Table 1). The origin of these and other UV–visible (Vis) spectroscopic changes were subsequently investigated by extensive RR and FTIR studies (see below).

Ferric state

Figure 2(A) compares the electronic absorption spectra of wild-type HRPc and its mutants F41W, F41V and F41A at neutral pH. All the mutants have Soret bands that are slightly red-shifted and more intense than the wild-type protein. The molar-absorption coefficient determined for the F41A mutant was 2-fold higher than that of the recombinant wild-type (Table 1). Furthermore, the long-wavelength ($> 600 \text{ nm}$) porphyrin-to-metal charge transfer band (CT1 band) observed at 642 nm in HRPc was blue-shifted by 5, 7 and 12 nm in the F41V, F41A and F41W

mutants, respectively. These changes suggest an increase in the proportion of 6cHS haem at the expense of the mainly 5cHS form characteristic of the wild-type. In fact, the 6cHS species can be distinguished partially from the 5cHS one by an increase in the Soret molar-absorption coefficient of about 40%, a concomitant decrease in intensity of the shoulder at 390 nm and a blue-shift of the CT1 band [24].

Figure 2(B) compares the corresponding RR spectra collected with Soret excitation. As previously reported, HRPc is mainly 5c with a small amount of 6c species [25]. Some of the frequencies of the RR core size-marker bands were found to be higher than those normally attributed to the 5cHS and 6cHS states of model compounds [26]. Recently, a comparison of the NMR hyperfine-shift patterns of HRPc, HRPcA1 (horseradish peroxidase isoenzyme A1) and HRPcA2 (horseradish peroxidase isoenzyme A2) has led to the proposal that the major spin state is in fact a 5c quantum mechanically mixed-spin (5cQS) species [27]. In comparison, the F41V and F41W mutations resulted in the appearance of a new species characterized by RR bands at 1486, 1565 and 1613 cm^{-1} assigned to the ν_3 , ν_2 and ν_{10} modes of a 6cHS species, respectively. This species is formed at the expense of the 5cQS form [25] and its presence is consistent with the UV–Vis spectra. In the ν_2 region of the RR spectra, the F41A mutant also shows a band at about 1571 cm^{-1} , which is assigned to a 5cHS or 6cQS form. In addition, as judged by the presence of a shoulder at 1505 cm^{-1} in the ν_3 band and the slight red-shift of the Soret band in the UV/Vis spectrum, F41W contains a small amount of a low-spin species [25]. Unfortunately, in contrast with the wild-type mutant, the relative intensity and/or frequency of the $\nu(\text{C}=\text{C})$ vinyl stretching bands were apparent for neither the F41A nor for the F41V mutant. This was because both the 6cQS and 5cQS species gave rise to ν_{10} bands which overlap the vinyl modes at 1624 and 1631 cm^{-1} , respectively [28,29]. It is therefore difficult to assign this region unambiguously, even by polarization experiments (results not shown).

Ferrous state

The electronic absorption spectra of the ferrous proteins are typical of 5cHS ferrous haem, with Soret bands at 437, 436 and 435 nm for wild-type, F41V and F41A mutants respectively and bands at 557 and 587 nm for wild-type and both mutants (results not shown). Interestingly, the F41W mutant was only partially reduced, and then very slowly, probably because of the blocked distal pocket.

In the corresponding low-frequency RR spectra of the wild-type and mutant HRPs, the frequency of the $\nu(\text{Fe}–\text{Im})$ stretching mode (where ‘Im’ is imidazole) originating from the bond between the iron and the $\text{N}\epsilon$ of the proximal ligand His¹⁷⁰ ($243 \pm 1 \text{ cm}^{-1}$), showed no significant change. The high frequency of this band is due to a hydrogen bond between $\text{N}\delta$ of His¹⁷⁰ and Asp²⁴⁷, which gives rise to considerable imidazolate character. The similarity in the $\nu(\text{Fe}–\text{Im})$ frequencies indicate that the distal mutations do not significantly affect the protein structure on the proximal side of the haem, in agreement with the findings for the corresponding mutations in CIP [8] and CCP [9].

Ligand binding

The spectroscopic study of the binding of small exogenous ligands to the haem iron can give useful information on the nature of the hydrogen-bond interaction between distal protein residues (most notably His⁴² and Arg³⁸ in HRPc) and bound ligands. In peroxidases, these distal residues are extremely

Figure 3 Electronic absorption spectra of the CO adduct of ferrous HRPc and the F41V, F41A and F41W mutants

(A) HRPc in 100 mM sodium phosphate, pH 7.0, F41V in 10 mM Mops/NaOH, pH 7.1 and F41A in 10 mM Mops/NaOH and 0.25 mM CaCl₂, pH 6.6. The region between 480 and 620 nm has been expanded 5-fold. (B) F41W in 10 mM Mops/NaOH, pH 7.1. The solid line was recorded immediately after adding CO and the dotted line after 2 h. The maxima in the visible region are found at 540 and 570 nm.

important in stabilizing both bound fluoride and carbon monoxide ligands [16]. Recently, for CIP it was found that the F54W, F54V and F54G mutants had an increased affinity for fluoride compared with the wild type [8]. Moreover, in CCP the X-ray structures of the CO and F complexes show that the distal Trp³¹ is hydrogen bonded to these ligands [30]. For the present HRP mutants, the UV/Vis and RR spectra of the fluoride complexes (results not shown) demonstrate that the wild-type protein and the F41V bind fluoride completely and the F41A mutant almost completely, whereas the fluoride binding of F41W mutant is very poor. The Soret bands of the F41V and F41A fluoride complexes are 1–2 nm blue-shifted respectively relative to the wild type. In this case, the RR spectra of the fluoride complexes allow the vinyl bands to be assigned, because the ν_{10} band (at 1607 cm⁻¹) of the 6cHS–fluoride complex no longer overlaps the vinyl bands as they do for the 5cQS and 6cQS states of the wild-type (see above). These are clearly seen at 1620 and 1631 cm⁻¹ for the wild type, and 1623 and 1628 cm⁻¹ for the F41V and F41A mutants.

The ferrous forms of the wild-type enzyme and the F41V and F41A mutants bound CO instantaneously, giving rise to electronic absorption spectra that were increasingly blue-shifted in the order wild-type > F41V > F41A (Figure 3A). Completely contrary to this, when CO was added to the only partially ferrous F41W, the intensity of the Soret band reached its maximum only after 2 h incubation (Figure 3B, dotted line), indicating that CO bound only very slowly to this mutant.

Figure 4(A) shows the RR spectra of the CO adducts in the Fe–C stretching and Fe–C–O bending frequency regions of HRPc and all three mutants. Figure 4(B) shows the corresponding FTIR spectra in the region of the $\nu(\text{CO})$ stretch. Plant HRPc shows two separate $\nu(\text{Fe–C})$ stretching bands, which have been assigned to two separate CO conformers [31,32]. For the conformer with $\nu(\text{Fe–C})$ at 516 cm⁻¹ and $\nu(\text{CO})$ at 1933 cm⁻¹, CO is hydrogen-bonded to His⁴². The second conformer gives rise to a RR band at 539 cm⁻¹ and a corresponding IR band at 1905 cm⁻¹ and the $\delta(\text{Fe–C–O})$ bending mode is observed at 589 cm⁻¹ [31–33]. These have been assigned to the conformer in which a strong hydrogen bond is made with Arg³⁸ and the Fe–C–O bond is bent

Figure 4 CO adducts of ferrous HRPc and the F41A, F41V and F41W mutants

(A) RR spectra. Experimental conditions: 413.1 nm excitation wavelength and 5 cm⁻¹ spectral resolution. Wild-type HRPc: 100 mM sodium phosphate, pH 7.0, 40 s/0.5 cm⁻¹ collection interval and 5 mW laser power at the sample. F41V and F41W: 10 mM Mops/NaOH, pH 7.1, 10 s/0.5 cm⁻¹ and 5 mW laser power at the sample. F41A: 10 mM Mops/NaOH and 0.25 mM CaCl₂, pH 6.6, 65 s/0.5 cm⁻¹ and 2.5 mW laser power at the sample. (B) FTIR spectra. Experimental conditions: spectral resolution of 0.2 cm⁻¹, Mops/NaOH, pH 7.1 (10 mM for HRPc and F41V, 50 mM for F41A), 1500 scans accumulated for plant HRPc, 600 scans accumulated for F41V and 12500 scans accumulated for F41A.

Table 2 Vibrational frequencies of ferrous–CO complexes (see also Figure 5)

	ν_{CO} (cm ⁻¹) H ₂ O (pH 7.1)	$\Delta^2\text{H}_2\text{O}$ (pH 7.6)	$\nu_{\text{Fe–C}}$ (cm ⁻¹) ¹² C	$\Delta^{13}\text{C}$	$\delta_{\text{Fe–C–O}}$ (cm ⁻¹) ¹² C	$\Delta^{13}\text{C}$
HRPc	1905	–2	539	–4*†	589	–16*†‡§
	1933	–1	516	–5*†		
HRPc F41A	1926		525	–5	579	–16
	1934		516	–5		
HRPc F41V	1912	–1	526	–5	579	–16
	1919	–1	519	–5		
HRPc F41V, alkaline form	1937	–1	511	–5		
HRPc, alkaline§	1934		530			
HRPc, pH 3¶	1967		492	–5	–	
HRPc H42L††	1924‡		525§	–7§		
CCP, pH 7**	1922		530	585		
CCP, alkaline**	1948		503	575		

* ref. [31].

† ref. [33].

‡ ref. [44].

§ ref. [32].

|| broad band.

¶ ref. [37].

** ref. [45].

†† HRPc mutant Phe⁴² → Leu.

[32]. The dominant band in the RR spectrum of the F41A–CO complex at neutral pH (Figure 4A) was at 516 cm⁻¹ with a shoulder at 525 cm⁻¹. On the basis of the isotopic shift observed with ¹³C (Table 2), these were assigned to two $\nu(\text{Fe–C})$ modes.

The RR spectrum of the F41V–CO complex was more complicated. Deconvolution of the broad feature around 490–550 cm⁻¹ revealed the presence of four bands with peaks at 501,

Figure 5 Spectra of CO complexes of ferrous HRPc F41V

(A) RR spectra. Experimental conditions: 413.1 nm excitation wavelength, 5 mW laser power at the sample and 5 cm^{-1} spectral resolution. pH 8.2 [50 mM *N,N*-bis-(2-hydroxyethyl) glycine/NaOH]: 15 s/ 0.5 cm^{-1} collection interval. pH 7.1 (10 mM Mops/NaOH): 10 s/ 0.5 cm^{-1} collection interval. pH 5.9 [50 mM 2-(*N*-morpholino)ethanesulphonic acid/NaOH]: 20 s/ 0.5 cm^{-1} collection interval. The 470–550 cm^{-1} regions of the spectra were deconvoluted by curve-fitting, using Lorentzian lineshapes and band widths of 15.5 cm^{-1} . (B) FTIR spectra. Experimental conditions: 0.2 cm^{-1} spectral resolution, 6100 scans accumulated at pH 8.2, 600 scans accumulated at pH 7.1 and 7200 scans accumulated at pH 5.9.

511, 519 and 526 cm^{-1} . The bands at 511, 519 and 526 cm^{-1} , which display isotopic shifts of 5 cm^{-1} with ^{13}CO (Table 2), were assigned to the $\nu(\text{Fe}-\text{C})$ modes. A strong feature seen at 495 cm^{-1} in wild-type HRPc was shifted to 497 cm^{-1} for F41A and F41W and 501 cm^{-1} for F41V. These did not show any isotopic shifts and were therefore assigned to a porphyrin mode. A pH titration of the F41V–CO RR spectrum (Figure 5) showed that the bands at 519 and 526 cm^{-1} strengthened at pH 5.9, whereas the band at 511 cm^{-1} which overlaps the porphyrin mode at 501 cm^{-1} was still the dominant $\nu(\text{Fe}-\text{C})$ band at pH 8.2. The band at 579 cm^{-1} in the spectra of both F41A and F41V showed an isotopic downshift of 16 cm^{-1} (Table 2) and has been assigned to the $\delta(\text{Fe}-\text{C}-\text{O})$ bending mode.

The FTIR spectrum of F41V at neutral pH showed three $\nu(\text{CO})$ bands, at 1937, 1919 and 1912 cm^{-1} . All gave small but significant isotopic downshifts in $^2\text{H}_2\text{O}$ (Table 2) and are comparable with those observed for plant HRPc (see Table 2). At pH 5.9, two bands at 1919 and 1912 cm^{-1} dominate the spectrum, whereas at pH 8.2 these bands are almost completely absent (Figure 4B). Therefore we assign the $\nu(\text{CO})$ band at 1933 cm^{-1} and the $\nu(\text{Fe}-\text{C})$ at 507 cm^{-1} to an alkaline form, and the bands at 519 and 526 cm^{-1} in the RR spectra at acidic pH to two $\nu(\text{CO})$ conformers at 1919 and 1912 cm^{-1} , respectively. At pH 8.2, the relative intensities of the $\nu(\text{Fe}-\text{C})$ band at 489 cm^{-1} (RR) and the $\nu(\text{CO})$ band at 1965 cm^{-1} (FTIR) increased during data accumulation, indicating that the F41V mutant was slowly denaturing at alkaline pH.

By using the vibrational signatures of the CO adducts we were able to probe the haem environment in further detail. For a large class of CO–porphyrin adducts, including haem proteins and model compounds, a linear correlation between the frequencies of the $\nu(\text{Fe}-\text{C})$ and the $\nu(\text{CO})$ stretching modes has been established [31–39] and is explained in terms of π back-bonding of the Fe–CO unit. Back-donation from the Fe $d(\pi)$ to the CO π^* orbitals increases the Fe–C bond order and decreases the C–O bond order, thereby increasing the $\nu(\text{Fe}-\text{C})$ stretching frequency and decreasing the $\nu(\text{CO})$ stretching frequency. The back-bonding is enhanced by many factors, such as steric hindrance of the bound CO, increased polarity of the environment (positive

Figure 6 Plot of the $\nu(\text{Fe}-\text{C})$ versus $\nu(\text{CO})$ frequencies in the CO complexes.

□, HRPc; ○, HRPc mutants; △, CCP. The frequencies are listed in Table 2.

charges) around the CO, and hydrogen-bonding between the CO oxygen atom and a distal residue. Moreover, the strength of the *trans* ligand has been found to affect the $\nu(\text{Fe}-\text{C})$ frequency. A weak or absent *trans* ligand increases the frequency due to a strengthening of the Fe–C σ bond, whereas a strong *trans* ligand has the opposite effect [33,36].

Figure 6 shows a plot of $\nu(\text{Fe}-\text{C})$ versus $\nu(\text{CO})$ frequencies for the CO adducts of CCP, HRPc and HRPc mutants. The point on the upper left-hand side of the plot is the HRPc–CO conformer in which CO is strongly hydrogen-bonded to Arg³⁸ [32]. At pH 3, the HRPc–CO adduct is found at the other end of the plot. This complex is characterized by a linear, non-hydrogen-bonded Fe–CO unit [37]. The HRPc–CO conformer, present at neutral pH, in which the oxygen of the bound CO interacts mainly with the distal His⁴², is intermediate between these two extremes. The frequencies observed for the acid form of HRPc are typical of a linear CO adduct with little or no π back-bonding. This is consistent with no, or only very slight, interaction with the surrounding distal pocket. Similar frequencies are also observed for the myoglobin (Mb) mutant His(E7)Gly and the acid form of Mb (A_0 state) [40–42], for which the crystal structure shows that the distal pocket has an open conformation in which the His residue swings out of the pocket upon protonation [43]. The F41W variant bound CO very slowly. The complex was characterized by a broad low-frequency porphyrin band at 418 cm^{-1} , a $\nu(\text{Fe}-\text{C})$ band at 489 cm^{-1} , and no band from the $\delta(\text{Fe}-\text{C}-\text{O})$ bending mode. This ‘fingerprint’ is very similar to the spectrum of HRPc–CO at pH 3 [37]. This suggests that the extreme steric hindrance generated by the bulky Trp residue prevents immediate binding and that the subsequent slow binding of CO is dependent on a slow conformational adjustment which opens the cavity.

The F41A and F41V substitutions also markedly affect CO binding. The conformers found for these mutants have vibrational signatures that differ from those obtained for the wild-type protein. In particular, the F41A mutant gave rise to two CO conformers, of which one was very similar to the wild-type conformer in which the CO is hydrogen-bonded to His⁴². The other has frequencies that are similar to those measured for the HRPc mutant His⁴² → Leu [32]. The present study suggests that the F41A mutation induces a rearrangement of the distal pocket in such a way that the CO cannot interact directly with the distal Arg, but instead the hydrogen bonds via an intervening

water molecule. Such an arrangement has been observed in the crystal structure of the CCP–CO complex [30]. For the F41V mutant, three CO bound forms were identified. However, pH titration showed that the conformer with $\nu(\text{Fe–C})$ at 511 cm^{-1} and $\nu(\text{CO})$ at 1937 cm^{-1} exists mainly at alkaline pH. The acid to alkaline transition has a $\text{pK}_a \leq 7$, compared with 8.5 for the CO complex of wild-type HRPC [31]. The alkaline form of F41V–CO is quite different from that of the corresponding species observed for wild-type HRPC. This is characterized by $\nu(\text{Fe–C})$ at 530 cm^{-1} and $\nu(\text{CO})$ at 1934 cm^{-1} [32,44]. The relative position of the alkaline form of the F41V–CO complex in Figure 6 compared with that of the alkaline CCP–CO complex [45] is indicative of a strong polar interaction between the bound CO and the surrounding peptide. Both the F41V–CO forms present at acid pH appear below the correlation line in Figure 6 and to the left of the position of the F41A–CO complex. This indicates that the Fe–C bond of the F41V–CO adduct has been weakened slightly. This could be ascribed to a *trans*-axial effect probably as a direct consequence of a slightly stronger Fe–Im bond in the F41V–CO complex compared with that of the F41A–CO complex.

DISCUSSION

Structural and catalytic consequences of the Phe⁴¹ mutation

As previously found for CCP and CIP, mutation of the HRPC distal residue at position 41 results in the formation of an aquo-6cHS haem. Moreover, the electronic absorption spectra of the ferric and ferrous forms, as well as those of the fluoride and CO adducts, all show an increasing blue-shift in the order Phe > Val > Ala, suggesting a slight reorientation of at least one haem-vinyl substituent. In the analogous CIP mutants a blue-shift in the Soret band was found with a concomitant increase in the RR stretching frequency for one of the vinyls and an upshift of the ν_2 mode [9]. In the present case, however, the vinyl stretching and ν_2 frequencies of the HRPC mutants do not shift to the same extent found in CIP. This is not surprising considering that Phe⁵⁴ in CIP is much closer to vinyl 2 ($4.1\text{ \AA}$) [7] than in HRPC Phe⁴¹ ($4.9\text{ \AA}$) [6]. Therefore for CIP the aromatic side chain locks vinyl 2 in position and replacement of the Phe⁵⁴ allows its reorientation [9].

Unlike the other mutants studied here, the F41W HRPC mutant was unable to react rapidly with H_2O_2 . The k_{obs} for formation of Compound I was independent of the concentration of H_2O_2 between 2 and $20\text{ }\mu\text{M}$. Reaction with peroxide was probably limited by a slow protein conformational change of $\approx 1/\text{s}$, the limiting value for its reaction with peroxide. Under steady-state conditions, the F41W mutant activated autocatalytically. This process was dependent on prior exposure to H_2O_2 and presumably resulted in some kind of oxidative destruction of the Trp side chain. A blocked distal pocket with the F41W mutant is consistent with its failure to bind the aromatic donor BHA. In contrast, the F41V and F41A mutants had twice the affinity for BHA compared with the wild type (Table 1). The crystal structure of the HRPC–BHA complex shows that the distance between Phe⁴¹ C α 2 and BHA O6 is $3.10\text{ \AA}$ [46]. Therefore a smaller residue at position 41 may allow the BHA molecule to penetrate the distal cavity more deeply resulting in more favourable hydrogen-bonding interactions with Arg³⁸ and His⁴² and/or more favourable hydrophobic interaction with Phe¹⁷⁹ [47].

To gain more insight into the likely structural perturbations induced by the F41W mutation, in particular the extent to which it might block the distal haem cavity, we modelled the position of the new side chain using the $2.15\text{ \AA}$ X-ray structure of the wild type [6]. Figure 1 compares the predicted structure of the

mutant with that of the wild type after conformational refinement using molecular mechanics calculations. The Trp residue is very restricted and is predicted to lie in a plane almost parallel to the haem. A comparison of the van der Waals surfaces of the wild-type Phe residue at position 41 with that of Trp in the mutant, clearly shows how the larger size of the Trp residue would block the distal cavity. This in turn would impair substrate access. The HRPC distal haem pocket is inherently smaller than that of CCP. Therefore to accommodate the substitution a substantial upward movement of the distal His away from the haem iron and an accompanying small downward movement of the haem group and proximal His is predicted. Modelling of the F41A and F41V structures did not predict the same sort of substantial conformational rearrangements described above for the F41W mutant and no attempt was made to predict these changes in any detail.

Fluoride and CO as structural probes for hydrogen-bonding interactions

In peroxidases, the distal Arg and His residues are directly involved in the stabilization of the ligand by hydrogen-bond interactions. In particular, for the fluoride complexes, the catalytic Arg is the main factor controlling ligand binding due to the strong hydrogen bond between the positively charged guanidinium group and the anion. The distal His also contributes to the stability of the fluoride complex, probably by accepting a proton from HF and by providing additional hydrogen bonding via an intervening water molecule to the bound anion [16]. In the class-I peroxidases, the distal Trp (Trp⁵¹ in CCP) is also hydrogen-bonded to the anion [16,24]. This latter interaction was shown to be particularly important for CIP. Wild-type CIP shows a low affinity for the fluoride ligand, probably due to a relatively weak stabilizing interaction from the distal Arg [8,16]. The F54W CIP mutation increased the stability of the fluoride complex by approx. 4500-fold and caused a red-shift of the CT1 band maximum, in a manner very similar to that seen in the CCP–fluoride complex. This indicates that the higher affinity for fluoride was due to the extra hydrogen bond provided by the indole side chain [8,16].

Wild-type HRPC already has a higher affinity for fluoride compared with CIP, due to a strong hydrogen bond with the much closer Arg³⁸. The replacement of the distal Phe⁴¹ by Val or Ala does not affect the binding of fluoride. On the other hand, unlike CIP F54W, HRPC F41W binds fluoride weakly, despite the additional hydrogen bond that the indole group could in principle provide. Therefore in conjunction with the modelling studies, we conclude that the indole side chain renders the small distal cavity of HRPC inaccessible to the fluoride ligand. Accordingly, the extreme steric hindrance generated by the bulky Trp residue prevents immediate binding of CO and blocks Compound I formation with H_2O_2 . Both CO binding and reaction with peroxide must involve a slow and significant rearrangement of the distal pocket.

Unlike fluoride binding, replacement of Phe⁴¹ by Ala or Val appears to weaken the interaction between bound CO and Arg³⁸ without markedly affecting its interaction with His⁴². We propose this to be a direct consequence of the co-localization of the Phe⁴¹, His⁴² and Arg³⁸ residues in helix B and the constrained orientation of Phe⁴¹ (parallel to and almost in van der Waals contact with the haem). This presumably places the haem-binding pocket of wild-type HRPC under some degree of conformational strain. We speculate that the removal of the bulky residue at position 41 allows a relaxation of this strain, which in turn causes an adjustment of the haem relative to helix B, resulting in an increase in the distance between the bound CO and Arg³⁸.

The present results have shown that the size of the residue at position 41 plays an important role in maintaining the haem iron co-ordination state and its reactivity. The HRP F41W mutant is very unusual in its behaviour compared with the analogous substitution in CIP. The spectroscopic modelling and kinetic studies suggest that this is due to the extreme steric hindrance caused by the bulky Trp side chain in the context of the smaller distal haem cavity of HRP. Whether a transient Trp radical may exist in this mutant after exposure to H_2O_2 remains to be established. However, it is interesting to note that bacterial catalase-peroxidases also contain a Trp residue at this position and that this Trp has been shown to be essential for Compound I reduction by H_2O_2 [48].

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