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Recombinant horseradish peroxidase isoenzyme C: the effect of distal haem cavity mutations (His42→Leu and Arg38→Leu) on compound I formation and substrate binding

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Abstract Horseradish peroxidase isoenzyme C (HRPC) mutants were constructed in order to understand the role of two key distal haem cavity residues, histidine 42 and arginine 38, in the formation of compound I and in substrate binding. The role of these residues as general acid-base catalysts, originally proposed for cytochrome *c* peroxidase by Poulos and Kraut in 1980 was assessed for HRPC. Replacement of histidine 42 by leucine [(H42L)HRPC*] decreased the apparent bimolecular rate constant for the reaction with hydrogen peroxide by five orders of magnitude ($k_1 = 1.4 \times 10^2$ $M^{-1}s^{-1}$) compared with both native-glycosylated and recombinant forms of HRPC ($k_1 = 1.7 \times 10^7$ $M^{-1}s^{-1}$). The first-order rate constant for the heterolytic cleavage of the oxygen-oxygen bond to form compound I was estimated to be four orders of magnitude slower for this variant. Replacement of arginine 38 by leucine [(R38L)HRPC*] decreased the observed pseudo-first-order rate constant for the reaction with hydrogen peroxide by three orders of magnitude ($k_1 = 1.1 \times 10^4$ $M^{-1}s^{-1}$), while the observed rate constant of oxygen bond scission was decreased sixfold ($k_2 = 142$ s^{-1}). These rate constants are consistent with arginine 38 having two roles in catalysing compound I formation: firstly, promotion of proton transfer to the imidazole group of histidine 42 to facilitate peroxide anion binding to the haem, and secondly, stabilisation of the transition state for the heterolytic cleavage of the oxygen-oxygen bond. These roles for arginine 38 explain, in part, why dioxygen-binding globins, which do not have an arginine in the distal cavity, are poor peroxidases. Binding studies of benzhydroxamic acid to (H42L)HRPC* and (R38L)HRPC* indicate that both histidine 42 and arginine 38 are involved in the modulation of substrate affinity.

Key words Peroxidase · Protein engineering · Distal residues · Compound I formation

Abbreviations *HRP* horseradish peroxidase · *HRPC* horseradish peroxidase isoenzyme C · *HRPC** non-glycosylated recombinant horseradish peroxidase · *(H42L)HRPC** His42→Leu HRPC* · *(R38L)HRPC** Arg38→Leu HRPC* · *(R38 K)HRPC** Arg38→Lys HRPC* · *CcP* yeast cytochrome c peroxidase · *ARP Arthromyces ramosus* peroxidase · *BHA* benzhydroxamic acid · *MOPS* 3-morpholinopropanesulfonic acid

Introduction

Peroxidases are ubiquitous, haem-containing enzymes that are present in plants, in some animal tissues, and in micro-organisms. They carry out a variety of biosynthetic and degradative reactions using peroxides, especially H_2O_2, as a source of two oxidising equivalents. They operate by a "ping-pong" type mechanism in which H_2O_2 initially oxidises the porphyrin ring of the haem to a cation radical and the ferric ion to an oxyferryl species (compound I formation). The porphyrin cation radical of compound I then undergoes a one-electron reduction by substrate to form compound II, which is itself subsequently reduced by substrate at its oxyferryl centre to regenerate the ferric enzyme state [1].

A common feature of peroxidases is the strong conservation of amino acids in the regions of the two histidine residues located on either side of the haem binding pocket. The proximal histidine 170 provides an imidazolate ligand that is covalently bonded to the haem

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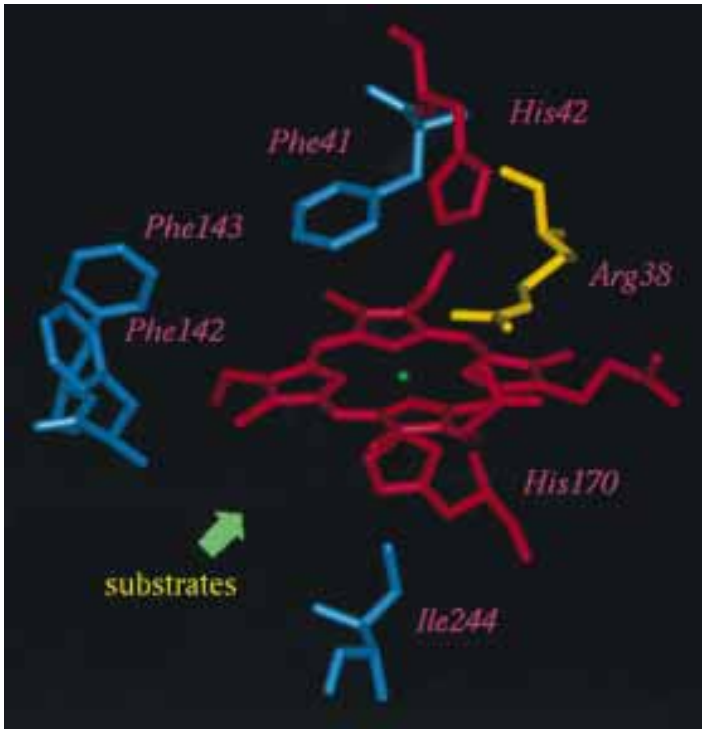

Fig. 1 Energy-optimised homology model of the haem cavity of HRPC as described in [4] and [5]. The two residues mutated to leucine in this study, His42 and Arg38, are shown in the distal haem pocket, where they catalyse compound I formation and modulate the affinity of the enzyme for BHA

iron. The distal histidine 42 (His 52 in C*c*P) is not coordinated to the iron, but is thought to play a major role as a general acid-base catalyst in facilitating peroxide anion binding to the haem and in subsequent heterolytic cleavage of the oxygen-oxygen bond during compound I formation [2]. The conserved arginine 38 (Arg 48 in C*c*P), by providing a positively charged guanidinium group close to the coordinated peroxide anion, is thought to stabilise the developing negative charge on the β-oxygen during heterolytic cleavage of the peroxide to yield compound I [2]. Although a high-resolution crystal structure of HRP is not yet available [3], model-building studies [4, 5], in conjunction with NMR studies [6], show that the general disposition of residues in the haem pocket is similar to that of C*c*P, whose structure is available at atomic resolution [7, 8]. Figure 1 shows the architecture of the haem cavity in HRPC, modelled on that of ARP [4] with the catalytic residues His 42 and Arg 38 shown on the distal side of the haem.

Recent studies with C*c*P variants have been designed to investigate the roles of His 52 and Arg 48 in the partial reactions that comprise the catalytic cycle of C*c*P and to verify the postulates of the Poulos-Kraut mechanism [9–11]. Thus, replacement of His52 by leucine in C*c*P has a pronounced effect on the rate of compound I formation, the apparent bimolecular rate constant being decreased by five orders of magnitude relative to the values for both the native and the recombinant wild-type enzymes. However, the C*c*P variants R48 K and R48L react with H_2O_2 to form compound I only 2- and 200-fold slower, respectively, than does native C*c*P. It was concluded that a basic residue at position 48 is not essential for the formation of a ferryl intermediate and that the distal histidine plays a far more significant role [9].

We repeated this type of experiment with HRPC* variants for several reasons. HRPC* variants have only recently become available, following the development, in these laboratories, of solubilisation and refolding procedures from inclusion bodies isolated from *Escherichia coli* when the synthetic HRPC gene is over-expressed [12]. There are clearly differences in substrate specificity between HRPC and C*c*P and increasing evidence that changes in distal residues affect the kinetics not only of compound I formation but also of substrate binding and oxidation [13, 14]. Recent experiments carried out with (R38 K)HRPC* have shown that the second-order rate constant for compound I formation for this variant is decreased 500-fold and the rate of ABTS oxidation is ca. 10-fold slower [13]. These data suggest that Arg38 could have a more important function in HRPC than in C*c*P. Surprisingly, chemical modification of His 42 in HRPC by diethyl pyrocarbonate suggests that a functional, distal imidazole group is not essential for compound I formation [15].

In this paper, we report a detailed kinetic investigation of compound I formation for (H42L)- and (R38L)HRPC* and the effect of these mutations on BHA binding to the ferric form of the enzyme. The results show not only that His42 and Arg38 are both key residues for the rapid formation of compound I in HRPC but also that they modulate substrate (BHA) binding.

Materials and methods

Reagents

HRP (EC 1.11.1.7) isoenzyme C (Type VI) was purchased from Sigma (Poole, UK) and used without further purification. HRPC* was purified from a strain of *E. coli* over-expressing a synthetic HRPC gene as described by Smith et al [12]. The concentrations of HRPC* and HRPC were determined spectrophotometrically using $_{403nm}$ = 92 $mM^{-1}cm^{-1}$ and $_{403nm}$ = 102 $mM^{-1}cm^{-1}$, respectively [12, 16]. Reagent grade H_2O_2 (30% v/v) was obtained from BDH/Merck (Poole, UK) and its concentration was determined by iodide titration with HRPC [17]. BHA was purchased from Sigma. All other chemicals were of analytical grade and supplied by BDH/Merck.

Genetic construction of HRPC* variants

Site-directed mutagenesis of the distal histidine 42 and arginine 38 of recombinant HRPC* was carried out using the polymerase chain reaction (PCR) and a synthetic HRPC gene previously over-expressed in *E. coli* [13]. The construction of the mutant plasmids used in this study has been described elsewhere [18].

The DNA sequence of the mutant HRPC* genes were confirmed by the dideoxy-chain-termination method.

Preparation of recombinant peroxidases

Growth and induction of *E. coli* strains over-producing recombinant HRPC* variants were as previously described [14]. Folding and activation of HRPC* variants recovered from *E. coli* inclusion bodies were achieved essentially by the method of Smith et al [12] with the modifications subsequently described [14, 18]. The enzymes were purified by FPLC using cation-exchange chromatography on a Mono-S HR 5/5 column (Pharmacia) equilibrated with 20 mM acetate buffer (pH 4.0) and eluted with sodium chloride (0–0.3 M gradient). Purified enzyme was desalted using Sephadex G-25 (Pharmacia) into 10 mM sodium MOPS buffer (pH 7.0) and stored in liquid nitrogen. The Soret extinction coefficients of the enzyme variants were determined after haem content determination by the pyridine haemachrome method [19]. These were $_{403nm} = 96.5\ mM^{-1}cm^{-1}$ for (H42L)HRPC* (Rz = 3.4) and $_{398nm} = 86\ mM^{-1}cm^{-1}$ for (R38L)HRPC* (Rz = 3.2). All experiments gave reproducible data when performed with enzyme from at least two seperate preparations.

Spectrophotometry

Single-wavelength transient kinetics were monitored using a stopped-flow spectrophotometer (model SF-51, Hi-Tech Scientific, Salisbury, UK) in 10 mM sodium phosphate buffer (pH 7.0). Data were recorded through an RS232 interface on a micro-computer and analysed by fitting absorbance-time curves to exponential functions using a least-squares minimisation programme supplied by HI-Tech Scientific. Stopped-flow rapid-scan spectrophotometry was carried out with the same apparatus modified with a diode-array detection system (MG-6000, Hi-Tech Scientific). Ultraviolet/visible absorption spectra were recorded in quartz cuvettes (1 cm) on a Shimadzu UV-2101PC spectrophotometer with a spectral band width of 1 nm and a scan speed of 120 nm/min. Steady-state assays of peroxidase activity were carried out with the same instrument by measuring the appearance of products. Tetraguaiacol formation was followed at 470 nm ($_{470nm} = 26600\ M^{-1}cm^{-1}$) and dopaminechrome at 480 nm ($_{480nm} = 3300\ M^{-1}cm^{-1}$). The assay medium was the same as that used for pre-steady-state kinetics. The values of k_{cat} were obtained varying the concentration of reducing substrate at constant and saturating hydrogen peroxide (1 mM). Kinetic data on rate of product formation versus reducing substrate concentration were analysed using a non-linear regression programme. All transient and steady-state kinetic studies were at 25 °C with the spectrophotometers thermostatted using a Techne C-400 circulating water bath with an integral heater-cooler system.

Binding studies

Difference spectra of the Soret region (350–450 nm) for the HRPC* variants with various concentrations of BHA were recorded in 1-cm quartz micro-cuvettes (400 µl) in 10 mM MOPS (pH 7.0). Titrations were carried out at room temperature by making successive additions (4 µl) of BHA (50 mM) to enzyme solutions (400 µl, 5–10 µM) to a final BHA concentration of 6–25 mM. The dissociation constant (K_d) of the HRPC-BHA complex was determined as described previously [14].

Results and discussion

Reaction between (H42L)HRPC* and hydrogen peroxide

Peroxidase compound I can be distinguished from the resting state of the enzyme by its decreased absorbance in the Soret region. A rapid-scan stopped-flow time course for the reaction of (H42L)HRPC* with 5 mM H_2O_2 is shown in Fig. 2. Compound I formation for this variant is much slower than for HRPC*, the reaction being complete after 25 s. The time course of the absorbance change at 395 nm (isobestic point for compound I and compound II) was biphasic (Fig. 3). The data were fitted to a two-exponential function, $A(t) = A_A\exp(-k_At) + A_B\exp(-k_Bt)$. Under pseudo-first-order conditions with H_2O_2 in large excess, k_A increased linearly with increasing hydrogen peroxide concentration (0.3–5 mM; Fig. 4A). The second expon-

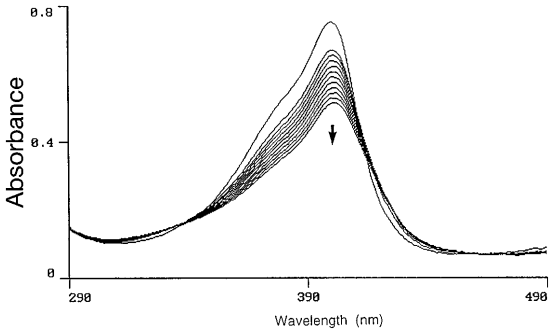

Fig. 2 Rapid-scan stopped-flow time course of the reaction of (H42L)HRPC* (8.0 µM) with H_2O_2 (5 mM) in 10 mM phosphate buffer, pH 7.0. The first scan was taken 20 ms after the flow stopped, and the subsequent scans were at 2.2-s intervals. The *arrow* shows the direction of absorbance change with time

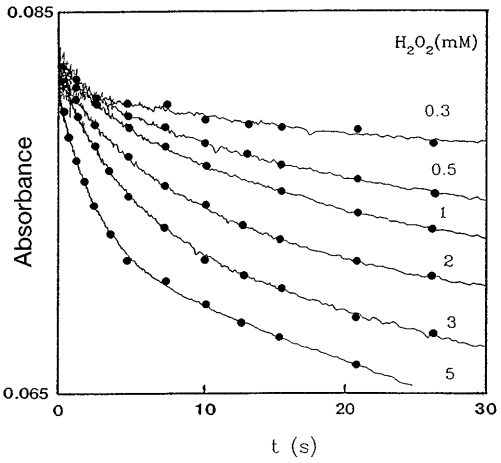

Fig. 3 Stopped-flow time courses at 395 nm for the reaction of (H42L)HRPC* (1.0 µM) with H_2O_2 in 10 mM phosphate buffer pH 7.0. The *filled circles* are simulated data points using the rate constant values from Table 1 and Eq. 1

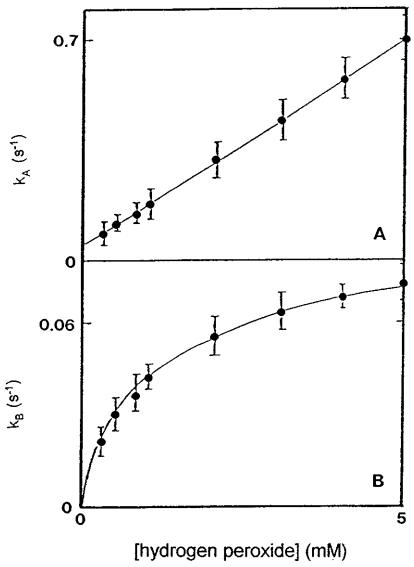

Fig. 4 Dependence of apparent first-order rate constant k_A (**A**) and k_B (**B**) for (H42L)HRPC* compound I formation as a function of $[H_2O_2]$ at pH 7.0

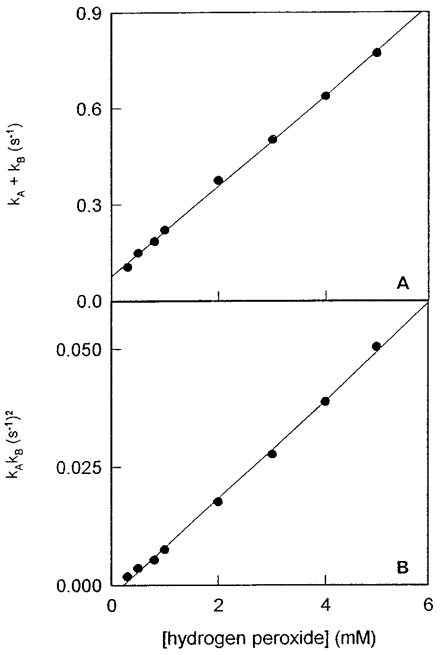

Fig. 5 Plot of the sum (**A**) and the product (**B**) of the apparent first-order rate constants k_A and k_B obtained from Fig. 4, as a function of $[H_2O_2]$

ential, k_B, exhibited a hyperbolic dependence on the concentration of H_2O_2 (0.3–5.0 mM; Fig. 4B). The bi-phasic kinetics observed for (H42L)HRPC* contrasts with the previously reported monophasic behaviour of both wild-type and recombinant HRPC [14].

We considered two mechanisms that are consistent with this bi-phasic kinetic behaviour. The simplest mechanism requires compound I formation to occur *via* two sequential reactions, a pre-equilibrium formation of a precursor complex [(H42L)HRPC*/H_2O_2] followed by a rate-limiting redox step involving heterolytic cleavage of the haem-bound peroxide anion.

$$E + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}H_2O_2 \xrightarrow{k_2} \text{compound I} + H_2O \quad (1)$$

Since the plot of k_B versus $[H_2O_2]$ passes through the origin, the second step must be essentially irreversible, as indicated by the omission of k_{-2} from Eq. 1. Initial estimates of the elementary rate constants for the partial reactions shown in Eq. 1 showed that both steps occur at comparable rates, and therefore, no simplifying assumptions can be made to the rate expression in order to obtain the values of the individual rate constants. A mathematical solution has been given by Bernasconi [20] and the elementary rate constants were obtained from plots of the sum or the product of the first-order rate constants for the two phases versus $[H_2O_2]$ (Fig. 5). The values of these elementary rate constants are given in Table 1. Simulations of the absorbance versus time curves using these values were made using a computer program K-SIM (supplied by Dr. N. Millar). These are shown overlaid on the stopped-flow data in Fig. 3.

Table 1 Kinetic constants for the reaction between HRPC species and hydrogen peroxide at pH 7.0 and 25 °C. The rate constants are defined in Eq. 1; their values were independent of enzyme concentration over the range 0.5–2 μM with hydrogen peroxide present in large excess

Species	k_1 ($M^{-1}\,s^{-1}$)	k_{-1} (s^{-1})	k_2 (s^{-1})	$K_m^{H_2O_2}$ (mM)[a]
HRPC	$(1.7 \pm 0.1) \times 10^7$		780[b]	46×10^{-3}
HRPC*	$(1.6 \pm 0.1) \times 10^7$			
(H42L)HRPC*	$(1.4 \pm 0.1) \times 10^2$	(0.08 ± 0.01)	(0.07 ± 0.01)	2.0 (1.5)
(R38L)HRPC*	$(1.1 \pm 0.1) \times 10^4$		(142 ± 10)	12 (12.9)

[a] Experimental values of $K_m^{H_2O_2}$ obtained from the plot of k_{obs} versus $[H_2O_2]$. The value of $K_m^{H_2O_2}$ for HRPC was taken from Beck and Van Wart [26], who obtained it by extrapolation from the values of $K_m^{H_2O_2}$ obtained at low temperatures. In parentheses the calculated value from the expression $K_m^{H_2O_2} = (k_{-1} + k_2)/k_1$, assuming that $k_{-1} \ll k_2$ in the (R38L)HRPC*

[b] The value of k_2 for the glycosylated enzyme cannot be determined experimentally. The given value is calculated assuming $k_2 = k_1 K_m^{H_2O_2}$

We also considered a second, more complex mechanism that assumes two forms of the enzyme that would have to interconvert slowly (or not at all) compared to the rate of reaction with H_2O_2 (i.e. $k < 0.01\ s^{-1}$). This could arise from the presence of two or more ligation states of the haem in the ferric state (e.g. five- and six-coordinate forms). A much less likely explanation, because of the constraint of a slow rate of interconversion, is heterogeneity in the protein sample consequent on the refolding process. This mechanism is shown in Eq. 2 where E and E' represent the two forms of (H42L)HRPC*.

$$\begin{array}{l} E + H_2O_2 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E - H_2O_2 \quad \searrow\ k_2 \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \text{compound I} + H_2O \\ E' + H_2O_2 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E' - H_2O_2 \quad \nearrow\ k_2 \end{array} \qquad (2)$$

The amplitudes of the two phases require that the more reactive state (E) is present as *ca.* 80% of the total enzyme. Appropriate values for the elementary rate constants in Eq. 2 can simulate the observed linear and hyperbolic dependences of the observed pseudo-first-order rate constants k_A and k_B for each phase on H_2O_2 concentration. We interpreted the data in terms of the simpler and kinetically competent mechanism represented by Eq. 1.

Reaction between (H42L)HRPC* and reducing substrates

The reduction of compound II to native enzyme is usually the rate-limiting reaction in the peroxidase cycle. Glycosylated HRPC has a k_{cat} of 400 and 494 s^{-1} for guaiacol and dopamine, respectively. The activity of the (H42L)HRPC variant is greatly decreased, with k_{cat} values of 0.07 and 0.09 s^{-1} for guaiacol and dopamine, respectively. Thus, k_{cat} is essentially independent of the nature of the reducing substrate. The closeness of these values of k_{cat} to that of $k_2 = 0.07 \pm 0.01\ s^{-1}$ (calculated assuming mechanism 1) strongly suggests that mechanism 1 is operating and that the redox step in compound I formation is rate-limiting for (H42L)HRPC*. Mechanism 2 predicts higher values of k_{cat} if the formation of compound I is rate limiting. If substrate reduction is rate-limiting for mechanism 2, it is difficult to understand why the values of k_{cat} are essentially identical for guaiacol and dopamine.

We conclude that the distal histidine, His42, does play a critical role in the rapid formation of compound I in HRPC, as previously shown for C*c*P following the original proposal of Poulos and Kraut [8, 9]. Our results indicate that histidine 42 has two important functions in compound I formation: (1) Acceptance of a proton from the incoming hydrogen peroxide to facilitate the binding of the peroxide anion to the Fe(III) haem and (2) facilitation of the heterolytic cleavage of the oxygen-oxygen bond in the redox step that generates the ferryl haem. This presumably involves proton donation to the β-oxygen of the bound peroxide anion thereby promoting the elimination of H_2O. These results do not exclude the possibility that His42 is also involved in other steps of the peroxidase cycle; we have observed that compound II of (H42L)HRPC* is more reactive with excess peroxide than is compound II of HRPC* (see also the effect on BHA binding below). Since for (H42L)HRPC* compound I formation appears to be rate limiting, steady state experiments with guaiacol and dopamine tell us little about the proposed role of His42 in regulating electron transfer or hydrogen atom abstraction from the bound donor molecule to the haem in compounds I and II [15]. Experiments to confirm this proposed function of His42 must start with either compound I or compound II under single-turnover conditions. Such experiments were beyond the scope of the present investigation because of the lack of availability of (H42L)HRPC*.

The apparent bimolecular rate constant for the reaction of (H42L)HRPC* with hydrogen peroxide is five orders of magnitude slower than that for wild-type HRPC* and is similar to that for the reaction between met-myoglobin or met-haemoglobin with hydrogen peroxide [21, 22]. However, since both globins and HRPC possess a histidine residue near the peroxide binding site of the haem, it cannot be concluded that a suitably positioned distal histidine alone is sufficient to promote rapid heterolytic cleavage of peroxides to yield compound I like species. It is highly likely that other distal residues, such as arginine 38 are important in compound I formation. We therefore prepared (R38L)HRPC* and investigated the kinetics of its reaction with H_2O_2.

Reaction between (R38L)HRPC* and hydrogen peroxide

The reaction between (R38L)HRPC* and hydrogen peroxide to form compound I gave monophasic, exponential absorbance versus time curves (Fig. 6). Under pseudo-first-order conditions, with hydrogen peroxide in large excess, the observed first-order rate constant exhibited a linear dependence on hydrogen peroxide concentration (0–0.5 mM) whereas at higher hydrogen peroxide concentrations (up to 50 mM) hyperbolic behaviour was observed with a limiting value of $142 \pm 10\ s^{-1}$ (Fig. 7). These data are also consistent with mechanism 1. Although biphasic kinetics might also have been expected for this mutant with $k_A = k_1[H_2O_2] + k_{-1}$ and $k_B = k_2[H_2O_2]/(K_m + [H_2O_2])$ where $K_m = (k_{-1} + k_2)/k_1$, the values of the elementary rate constants for this mutant only allow the observation of the second exponential, k_B [20]. Although we have observed monophasic kinetics for (R38L)HRPC*,

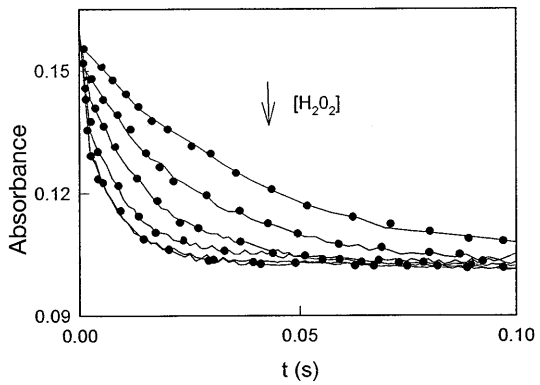

Fig. 6 Stopped-flow time courses at 401 nm for the reaction of (R38L)HRPC* (2 μM) with H_2O_2 in 10 mM phosphate pH 7.0. The concentrations of H_2O_2 used were 2, 4, 10, 20, 40 and 50 mM. The *filled circles* are simulated data points using the rate constants of Table 1

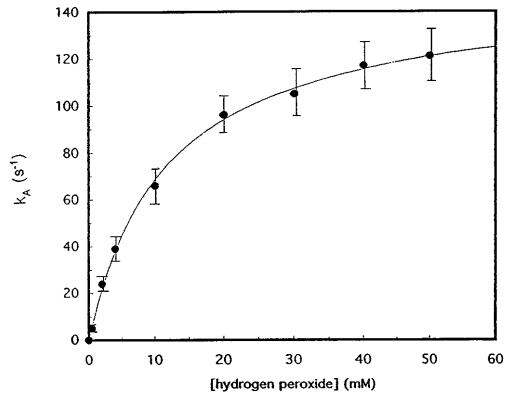

Fig. 7 Dependence of k_A, the pseudo-first-order constant for (R38L)HRPC* compound I formation, on $[H_2O_2]$ at pH 7.0 and 25 °C

the hyperbolic dependence of k_B on peroxide concentration is still consistent with a two-step mechanism. The values of the elementary rate constants assuming the mechanisms shown in Eq. 1, are given in Table 1. A comparison of these rate constants with those of native recombinant HRPC* shows that replacing arginine 38 by leucine significantly decreases both the rates of binding of the hydrogen peroxide to the ferric haem and the subsequent heterolytic cleavage of the oxygen-oxygen bond that yields compound I. As has been proposed previously for C*c*P, the positively charged guanidinium group of Arg38 in HRPC appears to have a role both in the orientation of the hydrogen peroxide at the active centre [23] and in promoting the heterolytic cleavage of peroxide [8]. We therefore conclude that both distal residues, His42 and Arg38, are required in HRP for efficient formation of compound I.

Substitution of the distal arginine by leucine has a greater effect in HRPC than in C*c*P. The C*c*P variant (R48L)C*c*P reacted with hydrogen peroxide to form compound I only 200 times slower than did native C*c*P [9]. The corresponding substitution in HRPC causes the second-order rate constant for compound I formation to decrease by a factor of 1200. This indicates that the detailed architecture of the distal pockets of HRPC and C*c*P are different. A similar conclusion was drawn when arginine 38 was changed to lysine [13] and in a recent resonance Raman study [27]. In this case, the positively charged side chain of the lysine substituted quite well for the corresponding arginine 48 in C*c*P [9] but not in HRPC [13].

The oxygen-binding globins have very low peroxidase activities. They do not have an arginine corresponding to Arg38 in HRPC (Arg48 in C*c*P), and this is most likely a factor in determining their low peroxidase activity. The greater decreases in peroxidase activity observed when the distal arginine is replaced by leucine or lysine in HRPC, relative to those induced by analogous mutations in C*c*P, may indicate that the distal cavity of HRPC resembles that of the globins to a greater extent than does that of C*c*P. In support of this hypothesis, recent laser flash photolysis studies have shown that the bimolecular rate constant for CO binding to (R38L)HRPC* ($k = 5\times10^6\,M^{-1}s^{-1}$) is three orders of magnitude higher than that with native HRPC* ($k = 4.4\times10^3\,M^{-1}s^{-1}$) and is similar to that of human haemoglobin [18].

Binding of benzhydroxamic acid to mutant enzymes

Aromatic hydrogen atom donor substrates (e.g. guaiacol, *p*–cresol) are thought to bind to a peripheral site(s) on HRPC close to the δ-meso and C18H3 haem edge, about 8–11 Å from the iron (indicated by the arrow in Fig. 1) [24, 25]. Other substrates, such as BHA, are proposed to bind to a distal haem site [5, 13, 25]. However, the precise location of the substrate binding site(s) of HRPC is still not certain (see reference 25 for a short review of NMR data). In order to explore the role of distal residues in substrate binding, we measured the effect of distal mutations on the affinity of HRPC* for BHA (Table 2). (H42L)HRPC* bound BHA 10^3 times less tightly than either the glycosylated or the recombinant form of HRPC. However, an even greater increase was observed in the dissociation constant, K_d, for the binding of BHA to (R38L)HRPC* (ca. 5×10^3 weaker).

Table 2 Dissociation constants for BHA binding to HRPC* and two distal cavity variants, (H42L)HRPC* and (R38L)HRPC*

Enzyme	K_d (mM)[a]
HRPC	$(2.6\pm0.1)\times10^{-3}$
HRPC*	$(2.5\pm0.1)\times10^{-3}$
(H42L)HRPC*	(2.9 ± 0.5)
(R38L)HRPC*	(12.1 ± 0.7)

[a] Dissociation constants for the binding of BHA to HRP mutants determined spectrophotometrically from Soret region difference spectra at 25 °C, pH 7.0, 10 mM MOPS

We have previously interpreted changes in the resonance Raman [27] and absorption spectra of (R38 K)HRPC* induced by BHA (up to 0.5 mM) in terms of hydrogen bonding of the hydroxamic acid moiety of BHA to the distal arginine (Arg38). This interaction was more likely, on the basis of modelling studies, than hydrogen bonding to His42 [13]. However, Table 2 clearly shows that mutating either histidine 42 or arginine 38 greatly weakens BHA binding. These data are more consistent with a model in which the positive charge of arginine 38 interacts electrostatically with the partial negative charge developed on the oxygen of the side chain of BHA and a hydrogen bond is formed with the imidazole group of histidine 42, i.e., both residues act concertedly in a manner not dissimilar to that which is thought to occur during compound I formation.

Conclusions

In this paper we have reported the results of a partial characterisation of two distal HRPC mutants. Clearly, arginine 38 and histidine 42 are involved in modulating not only peroxide binding to the ferric state of HRPC* and the subsequent heterolytic cleavage to form compound I, but also the affinity of the enzyme for BHA. Comparison of these data with those previously reported for C*c*P shows that these mutations induce differential effects for these two highly homologous enzymes that are best explained by differences in the distal cavity architectures. It is proposed that the distal cavity of HRPC has greater similarity to a globin-type haem cavity than to that of C*c*P.

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