Aromatic donor molecule binding sites of haem peroxidases

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Introduction
The primary function of haem peroxidases is generally considered to be the catalysis of the conversion of \( \text{H}_2\text{O}_2 \) into \( \text{H}_2\text{O} \). However, these enzymes also have a much wider biological significance. This relates to the concomitant oxidative reactions that occur during catalysis and which involve biomolecules ranging from simple plant hormones to electron transfer proteins, depending on the particular system. In the plant peroxidases, for instance, reducing equivalents are provided by aromatic donor molecules \( \text{AH}_2 \). The well characterized catalytic cycle includes two successive one-electron reduction steps which return the enzyme to the resting state following the formation of the high oxidation state intermediate, Compound I. Overall, the reaction catalysed by plant peroxidases can be written as \( \text{H}_2\text{O}_2 + 2\text{AH}_2 \rightarrow 2^*\text{AH} + 2\text{H}_2\text{O} \) [1]. It is essentially this relationship which links peroxidases with hormone synthesis and degradation, examples being the plant peroxidases and indole acetic acid, prostaglandin endoperoxide synthase and eicosanoids, and thyroid peroxidase and thyroxine [2-4]. In this context, there is considerable interest in understanding the structural features which characterize the interaction of haem peroxidases with other biomolecules.

Abbreviations used: CCP, cytochrome \( c \) peroxidase; HRP, horseradish peroxidase; HRP A2, C and E5, HRP peroxidase isoenzymes A2, C and E5 respectively; CIP, Coprinus cinereus peroxidase; NOE, nuclear Overhauser enhancement.

The purpose of this paper is to summarize progress made from past and continuing studies of the aromatic donor molecule binding sites of haem peroxidases, although the emphasis will be directed towards plant peroxidases. The latter are known to bind a wide spectrum of aromatic donor molecules, many of which form 1:1 complexes with peroxidase in the absence of \( \text{H}_2\text{O}_2 \). These are particularly suitable candidates for investigation by spectroscopic techniques [5-7]. In contrast, it should be noted that other haem peroxidases such as cytochrome \( c \) peroxidase (CCP) interact with a macromolecule, in this case cytochrome \( c \) [8]. Site-directed mutagenesis has been used to modify this enzyme to provide an artificial cavity for small molecule binding [9]. One example is the W191G CCP mutant, which is found to contain a cavity at the Trp-191 site where a variety of imidazole derivatives are able to bind. Of related interest in the area of peroxidase interactions with biomolecules are the lignin peroxidases. These participate in the breakdown of the complex phenylpropanoid polymer, lignin, a process which is considered to involve mediation by veratryl alcohol (3,4-dimethoxybenzyl alcohol) [10]. A putative binding site for this aromatic molecule has already been proposed based on the lignin peroxidase crystal structure [11].

The aromatic donor molecule binding site of horseradish peroxidase

One of the major difficulties in locating the aromatic donor molecule binding site of horseradish peroxi-
The lack of high-resolution crystallographic data. Even today, only a low-resolution 2.8 Å structure of the isoenzyme HRP E5 is available, although an investigation of the structure of recombinant HRP C is in progress [12,13]. This situation has encouraged the full exploitation of other methods, notably proton NMR spectroscopy. The latter technique has been extensively applied to solution studies of haem peroxidases, as highlighted by recent review articles [14,15]. A summary of NMR data recorded for a representative range of complexes of HRP C in resting and cyanide-ligated states is presented in Table 1, together with relevant dissociation constants determined by optical and, in

### Table 1

**Summary of binding and structural data for complexes formed between HRP C and aromatic donor molecules**

The data presented are derived from both optical and proton NMR studies. In each example, information relating to the resting state complex precedes that for the cyanide-ligated state. Dissociation constants were obtained using either optical or NMR methods. Distances reported under 'relaxation data' represent those between the haem iron atom and protons of the aromatic donor molecules, designated 'ArH', unless specifically assigned. Each nuclear Overhauser enhancement (NOE) constraint indicates a maximum interproton distance of 5 Å between the protein amino acid side-chain or haem group substituent (given first) and the aromatic donor molecule proton. The NOE data were obtained from both one-dimensional and two-dimensional (NOESY) experiments. Note that the haem group protons designated C18H3 and C17'4H are equivalent to haem methyl 8-CH3 and haem propionate 7-H, respectively, in the older Fischer notation. A NOE connectivity with 'ArH' indicates that the aromatic donor protons were not specifically assigned in the complex. Phe A and Phe B are the phenylalanine side-chains of two residues participating in the binding site of aromatic donor molecules. Their sequence-specific assignment remains to be confirmed.

<table>
<thead>
<tr>
<th>Aromatic donor molecule</th>
<th>Binding data</th>
<th>Relaxation data</th>
<th>NOE data</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. para-Cresol (4-methylphenol)</td>
<td>( K_{a,RS} = 2.8 ) mM</td>
<td>ArH: 8.5 – 11.0 Å</td>
<td>C18H3 ↔ C2H, 6H, Phe A ↔ C3H, 5H</td>
<td>5, 17</td>
</tr>
<tr>
<td></td>
<td>( K_{a,RS} = 3.5 \pm 0.4 ) mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( K_{a,CN} = 5.7 \pm 0.9 ) mM</td>
<td>C2H: 9.1 Å</td>
<td>C18H3 ↔ ArH</td>
<td>5, 17, 20</td>
</tr>
<tr>
<td></td>
<td>( K_{a,CN} = 7.1 \pm 1.2 ) mM</td>
<td>C3H: 8.4 Å</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( K_{c,RS} = 4.0 \pm 0.5 ) mM</td>
<td>C3H: 9.2 Å</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( K_{c,CN} = 7.8 \pm 0.5 ) mM</td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>2. Resorcinol (3-hydroxyphenol)</td>
<td>( K_{a,RS} = 4.4 ) mM</td>
<td>C3H: 11.0 Å</td>
<td>C18H3 ↔ ArH</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CSH: 9.0 Å</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C6H: 10.0 Å</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>OCH3: 10.5 Å</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH3: 8.8 Å</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( K_{c,CN} = 3.8 ) mM</td>
<td></td>
<td>Phe A ↔ C1'H, 2H, 6H</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C18H3 ↔ C2H</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phe A ↔ C1'H, 2H, 5H, 6H</td>
<td>This work</td>
</tr>
<tr>
<td>3. 2-Methoxy-4-methylphenol</td>
<td>( K_{a,RS} = 2.4 ) μM</td>
<td>C2H: 9.7 Å</td>
<td>C18H3 ↔ ArH</td>
<td>6, 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C3H: 10.9 Å</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C4H: 12.0 Å</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( K_{a,CN} = 0.15 ) mM</td>
<td></td>
<td>C18H3, C17'4H, His-42CqH ↔ ArH</td>
<td>6, 22, 23</td>
</tr>
<tr>
<td>4. Ferulic acid (4-hydroxy-3-methoxy-cinnamic acid)</td>
<td>( K_{a,RS} = 1.7 ) mM</td>
<td>ArH: 9.3 – 11.2 Å</td>
<td>Phe A ↔ C2H, 3'H2</td>
<td>18, 19, 21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phe B ↔ C2H, 3'H2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( K_{a,RS} = 6.2 \pm 2.9 ) mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( K_{a,CN} = 2.0 ) mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Benzhydroxamic acid</td>
<td>( K_{a,RS} = 2.8 ) mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Indole-3-propionic acid</td>
<td>( K_{a,RS} = 3.5 \pm 0.4 ) mM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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some cases, NMR methods [5-7,16-18]. In the majority of examples selected, both relaxation [16,19,20] and nuclear Overhauser enhancement (NOE) data [16,20-23] are available. Analysis of Table 1 allows the following general conclusions to be stated. (1) The dissociation constants for the donor molecules are in the millimolar range, with the notable exception of benzhydroxamic acid. The donor molecules bind more weakly to the six-coordinate low-spin cyanide-ligated state of peroxidase. (2) Haem iron to aromatic donor molecule proton distances fall in the range 8.4-12.0 Å. (3) The NOE data indicate that donor molecules bind relatively close to haem methyl C18H3 in agreement with enzyme inactivation studies [22]. The side-chains of His-42 and two Phe residues, Phe A and Phe B, are also implicated in the binding site, as has been previously discussed [21-23,25].

Although these data highlight some properties of the binding site, many questions remain unanswered. In the first instance it is necessary to obtain the specific assignment of amino acid side-chains participating in the site, a problem which is being tackled by the study of both related plant peroxidases and site-directed mutants of HRP C [26,27]. There is also interest in the dynamic properties of the interaction between peroxidase and aromatic donor molecules. These and other points are illustrated with reference to the specific examples discussed in detail below.

**NMR studies of the complex formed between ferulic acid and cyanide-ligated HRP C**

The use of the cyanide-ligated form of HRP C for NMR studies of peroxidase complexes offers the advantage that the effects of donor molecule binding on haem and haem-linked resonances can be assessed using available proton assignments [28,29]. A titration of cyanide-ligated HRP C with ferulic acid results in perturbations to a number of proton resonances as shown in Figure 1. Haem methyl C18H3, Arg-38 CβH, Phe A and Phe B (not shown) resonances are all affected, with a smaller perturbation noted to Arg-38 CβH. A dissociation constant of 3.8 mM was calculated for the interaction based on the chemical shift titration for the C18H3 resonance, which exhibits a maximum chemical shift change of ~0.36 p.p.m. at saturation. Two-dimensional NOESY experiments were recorded with a complex of the composition ferulic acid/cyanide-ligated HRP C of 3:1 and the connectivities listed in the legend to Figure 1 assigned.

Intra-complex NOE connectivities are essentially conserved between resting and cyanide-ligated states, although in the latter a number of additional but weak connectivities were resolved [22]. Only the C2H proton of ferulic acid gave a detectable NOE connectivity to the haem methyl C18H3. It is also noteworthy that the NOE connectivities normally observed between haem methyl C18H3 and Phe A aromatic protons were absent in the complex [21].

**NMR studies of the complexes formed between benzhydroxamic acid and cyanide-ligated isoenzymes of HRP**

Of all the donor molecule complexes of HRP C studied to date, that formed with benzhydroxamic acid is perhaps the most intriguing. Not only does this molecule bind more strongly to the resting and cyanide-ligated states of the enzyme than other donors (see Table 1), but it also alters the haem group from a five-coordinate high-spin to a six-coordinate high-spin state, most probably with a water molecule acting as the sixth ligand at the unoccupied distal site [30]. When a titration of the cyanide-ligated enzyme with benzhydroxamic acid is followed using proton NMR (Figure 2) a number of key resonances are perturbed, notably those of haem methyl C18H3, haem propionate C17νH, Arg-38, His-42 and an isoleucine methyl group (probably lle-244) [22,23]. Analysis of the lineshape and chemical shift changes for the haem methyl C18H3 group also supports the assertion that there are two possible binding modes for benzhydroxamic acid [23]. It is of interest to ascertain whether these observations are unique to isoenzyme C of HRP or, on the contrary, common to plant peroxidases in general. This can be assessed by performing similar experiments with a second plant peroxidase such as one of the acidic isoenzymes from horseradish, HRP A2.

Comparison of the amino acid sequences of isoenzymes A2 and C indicates that they show only 54% identity [31]. In fact, HRP A2 is closer to other acidic peroxidases such as tobacco peroxidase A than to HRP C. Reimann and Schönbaum reported that the binding of benzhydroxamic acid to the resting state of the acidic HRP isoenzyme, HRP A2, was a factor of 104 weaker than for resting state HRP C [32]. The interaction between benzhydroxamic acid and cyanide-ligated HRP A2 is also different from that with cyanide-ligated HRP C, as analysis of the data in Figure 2 shows. One striking feature is the contrast between the haem C18H3 resonances in the spectra of the bound states of the
**Figure 1**

Titration of cyanide-ligated HRP C with ferulic acid monitored by proton NMR

One-dimensional $^1$H NMR spectra were recorded at 600 MHz and 30°C in 20 mM potassium phosphate, 15 mM KCN, $^2$H$_2$O solution at pH 7.6. The initial enzyme concentration was 2.2mM. In a NOESY experiment carried out with a 3:1 complex of ferulic acid/cyanide-ligated HRP C, the following chemical shift perturbations and NOE connectivities were noted (s and w refer to strong and weak respectively):

<table>
<thead>
<tr>
<th>Proton</th>
<th>Free</th>
<th>Bound</th>
<th>$\Delta\delta$</th>
<th>NOE connectivities</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$^{11}$H</td>
<td>6.36</td>
<td>6.46</td>
<td>+0.10</td>
<td>Phe A ArH (s)</td>
</tr>
<tr>
<td>C$^{5}$H</td>
<td>6.90</td>
<td>7.05</td>
<td>+0.15</td>
<td>Phe A ArH (w)</td>
</tr>
<tr>
<td>C$^{6}$H</td>
<td>7.09</td>
<td>7.19</td>
<td>+0.10</td>
<td>Phe A ArH (s)</td>
</tr>
<tr>
<td>C$^{2}$H</td>
<td>7.21</td>
<td>7.34</td>
<td>+0.13</td>
<td>Phe A ArH, haem methyl C$^{18}$H (w)</td>
</tr>
<tr>
<td>C$^{18}$H</td>
<td>7.30</td>
<td>7.40</td>
<td>+0.10</td>
<td>Phe A ArH (w)</td>
</tr>
<tr>
<td>OCH$_{3}$</td>
<td>3.88</td>
<td>4.03</td>
<td>+0.15</td>
<td>None detected</td>
</tr>
<tr>
<td>Phe A ArH</td>
<td>7.78</td>
<td>7.62</td>
<td>-0.15</td>
<td>C$^{1}$ and C$^{6}$H (s), C$^{5}$H, C$^{2}$H and C$^{12}$H (w)</td>
</tr>
<tr>
<td>Phe A ArH</td>
<td>7.84</td>
<td>7.71</td>
<td>-0.13</td>
<td>C$^{1}$H (w)</td>
</tr>
</tbody>
</table>

It should be noted that the saturation point is reached at a significantly lower donor-to-enzyme ratio with HRP C than with HRP A2, although the overall chemical shift changes for C$^{18}$H$_{3}$ are comparable between HRP A2 ($-2.95$ p.p.m. at a benzhydroxamic acid to enzyme ratio of 150 to 1) and HRP C ($-2.37$ p.p.m. (major form) and $-2.79$ p.p.m. (minor form)). The pattern of chemical shift changes for other haem-linked resonances on benzhydroxamic acid binding shows some differences between the two isoenzymes. This is particularly the case for the haem propionate C$^{17}$H resonance, which is shifted by $-0.75$ p.p.m. and $+2.29$ p.p.m. for HRP A2 and HRP C respectively.
Comparison between the binding of benzhydroxamic acid to cyanide-ligated HRP A2 (a) and C (b) isoenzymes

NMR spectra were recorded at 500 MHz and 30°C in 20 mM potassium phosphate, 15 mM KCN, H2O solutions at pH 7.6. Peroxidase samples were obtained from Biozyme Laboratories Ltd. (HRP 4B and 5), and initial enzyme concentrations of 2.5 mM (HRP C) and 2.1 mM (HRP A2) were used. The upper trace of each pair shows the appearance of the 1H NMR spectrum of the cyanide-ligated form towards saturation point. New 1H resonance assignments for cyanide-ligated HRP A2 were obtained by standard two-dimensional NMR techniques as previously described [14,27]. These are given in p.p.m. as follows, with the overall change in chemical shift for the benzhydroxamic acid-bound form in parentheses: C18H, 29.47 (-2.95); C7H, 27.08 (+0.06); C17' H, 22.88 (-0.75); His-I70 Cβ,H, 21.00 (-0.25); C8'H, 18.99 (+0.62); His-I70 Cβ,H, 16.53 (-0.08); His-42 Cr,H, 12.60 (-0.22) (NB this resonance overlaps with His-I70 NH in the unbound form); Arg-38 Cβ,H, -4.40 (+0.73) and Arg-38 Cδ,H, -7.23 (+1.21).

Dynamic aspects of aromatic donor molecule binding to HRP

One difficulty inherent in discussing the data in Table 1 is the tendency to assume a static view of aromatic donor molecule binding. This is far from reality, however, as the donor molecules are in a state of dynamic exchange between the binding site and the external surroundings of the enzyme. In the case of the complex between ferulic acid and cyanide-ligated HRP C, the resonances of binding site groups such as C18H3 remain sharp during the titration and as single components, indicating a fast exchange regime (i.e. > 10^1 s^-1 [33]). A characteristic feature of the binding of benzhydroxamic acid to cyanide-ligated HRP C is the broadening of binding site proton resonances and the existence of two components of the C18H3 resonance in the
bound state. Analysis of linewidth and chemical shift changes indicates that the major and minor components are in states of intermediate and slow exchange respectively [33]. This is in agreement with the finding of La Mar that the two ternary complexes (i.e. of benzhydroxamic acid, cyanide and peroxidase) exhibit on and off rates differing by a factor of approximately 10 [23]. The broadening of the same C18H3 resonance in cyanide-ligated HRP A2 is less severe and the linewidth passes through a maximum (results not shown), indicating that the so-called 'moderately fast' exchange regime is operative [33]. These observations highlight the complexity of binding site interactions which evidently differ both between peroxidase isoenzymes and with donor molecules. This does not necessarily indicate that the essential features of the binding site alter greatly within the plant peroxidases. In fact the general pattern of haem-linked resonances in the NMR spectra, and their perturbation on benzhydroxamic acid binding, are partially conserved between cyanide-ligated HRP C and A2, as inspection of Figure 2 shows. The weaker perturbation of the same resonances in cyanide-ligated HRP C by ferulic acid may indicate that, in this case, interactions with binding site groups are primarily hydrophobic. It is likely that benzhydroxamic acid additionally engages in hydrogen-bonded interactions, and there is growing evidence that binding causes disruption or changes to the hydrogen bonding network linking the proximal and distal regions around the haem group [27,34].

One additional factor which must directly influence the nature of donor binding is the participation of amino acid side-chains such as those of two Phe residues and one Ile residue already alluded to in the context of HRP C. As the same 'core' groups such as haem methyl C18H3, haem propionate C17\textsuperscript{4}H, and distal His-42 are always available to assist donor binding in peroxidases, it seems likely that benzhydroxamic acid additionally engages in hydrogen-bonded interactions, and there is growing evidence that binding causes disruption or changes to the hydrogen bonding network linking the proximal and distal regions around the haem group [27,34].

A small number of HRP C mutants have been characterized by proton NMR and limited studies made of their interaction with benzhydroxamic acid [27]. Two of these, R38K and F41V HRP C, comprise substitutions to highly conserved residues in the immediate vicinity of the haem group and on the distal side of the haem plane. The resting state of R38K HRP C peroxidase does not bind benzhydroxamic acid while, in contrast, resting-state F41V HRP C binds it twice as strongly as the plant enzyme. A third mutant, F41W HRP C, also shows no affinity for benzhydroxamic acid [26,36]. NMR studies show, as expected, that neither Phe A nor Phe B is Phe-41. However, titration of cyanide-ligated F41V HRP C with benzhydroxamic acid highlights differences in both the perturbation of haem-linked resonances and dynamic properties of the interaction, as compared with cyanide-ligated plant HRP C [27]. It is of interest that an amino acid substitution made to a residue situated in the haem binding region which does not directly participate in the donor molecule binding site nevertheless has a measurable effect on donor binding.

Further mutants, F142A and F143A HRP C, have also been constructed on the reasoned premise that Phe-142 and Phe-143 could be candidates for Phe A and Phe B. Two interesting facts arise from the preliminary NMR studies of these mutants (N. C. Veitch, R. J. P. Williams, N. M. Bone, J. F. Burke and A. T. Smith, unpublished work). Comparison with the NMR spectrum of plant HRP C indicates that Phe A and Phe B cannot be assigned to Phe-143, and furthermore that Phe A cannot be assigned to Phe-142. However, the proton resonances of Phe A undergo both chemical shift and linewidth changes, as noted from the NMR spectrum of resting-state F142A HRP C. This substitution also affects the ability of the enzyme to bind benzhydroxamic acid, with a 4-fold decrease in affinity with respect to the plant and F143A HRP C enzymes. As with F41V HRP C, this phenylalanine substitution clearly affects aromatic donor molecule binding, although Phe-142 is not directly implicated in the binding site from NOE experiments. Further studies of other phenylalanine residue mutants at positions 68, 179, 187 and 221 are in progress to provide definitive identification of Phe A and Phe B.

**Site-directed mutants of *Coprinus cinereus* peroxidase**

*Coprinus cinereus* peroxidase (CIP) is a member of the class of fungal peroxidases and is also allied to the classical plant peroxidases within the plant peroxidase superfamily [38]. Site-directed mutants of this peroxidase have been examined by proton

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**NMR studies of HRP C mutants**

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NMR in order to provide further insights into the nature of aromatic donor molecule binding [39]. In the first instance, substitutions have been made to residues located in three short peptide segments believed to control access of substrates to the haem site, a premise in agreement with analysis of the recent crystal structures solved for CIP [40,41]. The three mutants G156F, N157F and G156F:N157F CIP, have Phe residues replacing Gly-156 and Asn-157. These residues are aligned with Phe-142 and Phe-143 of HRP C respectively, and additionally, Gly-156 is aligned with Phe-148 of lignin peroxidase, a residue in the predicted veratryl alcohol binding site [11]. Comparison of NMR and reactivity data for the CIP mutants indicates once again that these residue positions only exert an indirect influence on substrate preference and binding [39,42].

**Concluding remarks**

A comprehensive description of the aromatic donor molecule binding site of plant peroxidases is a realistic goal through the use of a combination of NMR and other techniques to characterize wild-type and mutant enzymes. The analysis is complicated by the need to take into account the dynamic properties of peroxidase complexes, both from the point of view of exchange processes and the inherent mobility of binding site groups themselves. Hydrogen-bonded interactions are also of importance with certain classes of donor molecules such as the benzhydroxamic acids. This is highlighted by the inability of the R38K HRP C mutant to bind benzhydroxamic acid.

The binding site interactions between peroxidase and donor molecules exemplified by HRP C can be usefully divided into three categories or levels of significance. At the 'core' of the binding site is the so-called 'exposed' haem edge, including haem methyl C18H1, as well as the conserved residues Arg-38 and His-42. These groups are likely to play a major role in aromatic donor binding throughout all the plant peroxidases, although their degree of importance will depend to a certain extent on the specific chemical characteristics of the aromatic donor molecule. A second level of interaction includes amino acid side-chains such as those of Phe A and Phe B and the tentatively assigned Ile-244. These residues may be located in or adjacent to the three peptide segments proposed to be situated towards the entrance of the binding site and to control substrate access to the haem. The final level includes residues which are not directly implicated in aromatic donor molecule binding. These can be defined as a category on the basis of a lack of NOE connectivities between amino acid residue side-chains and donor molecules. Nevertheless, substitutions made to these residues result in changes to the affinity of binding of aromatic donor molecules and also perturb the chemical shift or linewidth of resonances of clearly identified binding site groups.

In the case of HRP C this category includes the side-chains of Phe-41 and Phe-142. How these effects are transmitted to the immediate region of the binding site is an important question in itself. Local conformational change induced by the substitution of one amino acid side-chain for another may be responsible, although this presupposes a degree of flexibility or mobility in the binding site as a whole. It may well be that a binding site with these characteristics is required to accommodate a wide variety of aromatic donor molecules, including those which are relatively bulky (such as the naphthohydroxamic acids [6]), as well as those which can adopt two modes of binding. Differences in aromatic donor binding between plant peroxidases no doubt reflect changes in the binding site at the second and third levels. This is likely to be the explanation for the differences observed in the binding of benzhydroxamic acid by the two HRP isoenzymes, HRP A2 and HRP C.

The aromatic donor molecule binding sites of fungal peroxidases such as lignin and *Coprinus* peroxidase are less well understood, despite the advantage of high-resolution crystal structures. It is still uncertain whether veratryl alcohol forms a true complex with lignin peroxidase or acts only as a small molecule mediator for the oxidation of lignin. Of related interest is the observation made from a comparison of the two crystal structures of these fungal peroxidases that the haem access channel in CIP is much larger than that for lignin peroxidase [41]. This indicates that the CIP aromatic donor molecule binding site may have more features in common with those of the plant peroxidases. Further complementary investigations of CIP and HRP C mutants and of their interaction with a range of aromatic donor molecules should enable this question to be resolved in the near future.

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37 Reference deleted
Peroxidases are haem-containing enzymes which catalyse the reduction of hydrogen peroxide by different organic substrates. Plant, fungal and bacterial peroxidases are evolutionarily related and constitute the plant peroxidase superfamily, which can be divided into three structural classes. Class I, of prokaryotic origin, includes cytochrome c peroxidase (CCP); class II contains secretory fungal peroxidases such as *Coprinus cinereus* peroxidase (CIP); and class III comprises secretory plant peroxidases such as horseradish peroxidase (HRP). Although the three classes show only approx. 20% sequence identity, they have conserved residues in the haem cavity, namely the distal Arg and His, and the proximal His H-bonded with a buried Asp side chain, which are functionally and structurally important [1,2]. In addition, the crystal structures of CCP [3], lignin peroxidase [4,5], *Arthromyces ramosus* peroxidase (ARP) [6] and CIP [7] show that an extended H-bond network connects the proximal to the distal side of the haem cavity through the distal water molecules, the distal Arg, the propionate groups of the haem, and the proximal His H-bonded to the oxygen of an aspartic residue.

Resonance Raman (RR) spectroscopy combined with site-directed mutagenesis is a powerful technique with which to elucidate the molecular interactions at the enzyme active site of haemoproteins. In fact, its selectivity and sensitivity allow one to obtain information on the haem prosthetic group which is the active site of the enzymes. The extended aromatic system of the porphyrin ring gives rise to two $\pi \rightarrow \pi^*$ transitions at about 400 nm (Soret band) and 500–600 nm (Q band). Configuration interactions and vibronic mixing between these transitions produce different resonance effects in the RR spectra which can be selectively studied by exciting the samples with different excitation wavelengths. In particular, the 1300–1700 cm$^{-1}$ region of the RR spectra is characterized by strong bands, called core size marker bands, whose frequencies are markedly affected by the co-ordination, spin and oxidation state of the central Fe atom. In addition, the vibrational modes of the vinyl substituents, which are conjugated to the porphyrin $\pi$ system, are observed [8]. In the Fe$^{III}$ 5-co-ordinate high-spin haems the $\nu$(Fe-IIm) stretching mode gives rise to a fairly strong band. Its frequency provides information on the status of the bond to the proximal imidazole ligand. Therefore the frequencies of the RR active modes give important information on the porphyrin nuclear geometry and electronic structure.

The data obtained for the representatives of the three classes, CCP, CIP and HRP, together with several key single-site mutants in the haem cavity, will be analysed, and their results compared with the known X-ray structures.

**Iron (III) proteins**

**CCP**

The haem cavity structure of CCP is shown in Figure 1 [3]. The distal water molecule, W595, H-bonded to the Trp-51 indole side chain and to the water molecule in position 648 is located 2.4 Å above the Fe atom. The RR spectra of CCP obtained in solution at neutral pH [9], and on the single crystal [10], are characteristic of 5-co-ordinate high spin (5-c HS) haem, clearly indicating that the distal water molecule is not co-ordinated to the haem iron.

Expression of CCP in *Escherichia coli* [CCP(MI)] resulted in no significant differences in the spectroscopic and functional properties of the