The functional role of the key residues in the active site of peroxidases
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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 hydrogen-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$^{II}$ 5-co-ordinate high-spin haem the $\nu$(Fe-Im) 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

Abbreviations used: CCP, cytochrome c peroxidase; CIP, Coprinus cinereus peroxidase; HRP, horseradish peroxidase; ARP, Arthromyces ramosus peroxidase; RR spectroscopy, resonance Raman spectroscopy; 5-c HS, 5-co-ordinate high spin (etc.); I.S, low spin; CCP(MI), CCP expressed in Escherichia coli.
Figure I
Haem crevices of (a) CCP [3] and (b) ARP [6] adapted to CIP
Dotted lines indicate the inferred hydrogen bonds.

proteins compared with the enzyme isolated from baker's yeast [11-14]. However, the co-ordination and spin states are markedly affected by the mutation of the side chains of both the proximal and the distal sides of the haem. On the distal side, the absence of the anchoring H-bond between the N atom of the indole ring of the Trp-51 side chain and the distal water molecule renders the protein extremely sensitive to minor changes in the distal cavity. In fact, whereas in phosphate buffer at pH 7 the W51F mutant is mainly characteristic of 6-coordinate high spin (6-c HS) haem [14] (implying that the water molecule is bound to the Fe atom), the same protein in phosphate buffer in the presence of 30% 2-methyl-2,4-pentanediol (necessary to induce crystallization) or as a single crystal is predominantly 5-c HS [15,16]. Other mutations on the distal side, such as the replacement of His-52 or Arg-48 with Leu, do not affect the co-ordination of the iron, but change the capability of the protein to bind external ligands. For example, the R48L mutant does not bind F⁻ (G. Smulevich, unpublished work).

On the proximal side, CCP, besides the H-bond between the imidazole side chain of the fifth ligand and the aspartate carboxylate group of Asp-235, which is present in all peroxidases, shows an additional H-bond between the Asp-235 residue and the indole group of Trp-191. Disruption of one of these two H-bonds gives the Fe atom some flexibility, as shown by the RR studies on Asn-235 [14,15], Phe-191, Tyr-191, Glu-191 and Gln-191 variants (G. Smulevich, unpublished work). At neutral pH these mutants show the presence of 6-c HS and LS (low-spin) haems, indicating that these two H-bonds in the native protein restrain the Fe atom from moving into the haem plane and binding the distal water molecule.

HRP C
Although the crystal structure of HRP C has not yet been resolved, the comparison of its sequence with that of CCP shows that HRP C differs from CCP by the absence in the cavity of the two Trp side chains which are replaced by Phe (Phe-41 and -221, according to HRP sequence numbers) (Welinder et al., Fig. 1, this issue, p. 258).

The RR spectra of recombinant HRP C, in both solution [17] and single crystal form (G. Smulevich, unpublished work), are identical to those of the plant HRP C. The spectra demonstrate that the protein exists in two forms, assigned to 5-c and 6-c HS with a different degree of distortion of the haem with respect to other peroxidases or haemoproteins studied so far. In fact, some Raman bands appear at higher frequency than those normally observed for the corresponding HS mode. This discrepancy is partially relieved upon mutation of the distal side chains of Phe-141 or Arg-38, suggesting that the distortion of the haem results from external forces such as steric contacts with the protein [17].
Distal mutations cause changes in the coordination and spin states of the haem. The F41W and F41V variants are characterized by an increase in the amount of 6-c HS haem at the expense of the 5-c HS, and the R38K mutant by an increase of both 6-c HS and LS. Mutations on the distal side affect also the ability of the protein to bind substrates such as benzohydroxamic acid. In particular, F41W and R38K lose the ability to bind the donor, whereas the F41V mutant has a 2-fold higher affinity. These results suggest that the aromatic binding site is found towards the distal rather than the proximal side of the haem [17]. In addition, R38K loses the capability to bind anionic ligands such as F- (Sanders, S. A., Burke, J. F., Thorneley, R. N. F., Bray, R. C. and Smith, A. T., unpublished work).

**CIP**

Fungal CIP is identical to the recombinant protein expressed in *Aspergillus oryzae* [19-21], which is in turn almost identical to ARP, differing from CIP only by the presence of an additional glycine residue near residue 4 [6]. The structures of both ARP [6] and recombinant CIP [7] have been solved with 1.9 and 2.6 Å resolution respectively. The structure of ARP adapted to that of CIP (i.e. using CIP residue numbers) is shown in Figure 1.

The RR spectra of CIP in phosphate buffer at pH 7 or in the media used for crystallization are characteristic of a 5-c HS haem, indicating that the procedure used to obtain the crystal does not affect the co-ordination of the haem iron and that the distal water molecule W415 is not ligated to the iron atom. Moreover, the spectra are very similar to those obtained for CCP, but very different from those of HRP C [21]. However, contrary to the other proteins, CIP is extremely unstable in the laser beam, giving rise to 6-c HS and LS haems upon low-power irradiation.

**Iron (II) proteins**

In many haemoproteins the haem iron atom is co-ordinated to the N° imidazole nitrogen of the proximal histidine residue. The v(Fe-Im) stretching mode is RR active only in the Fe° haems and gives rise to a strong band in the 200-250 cm⁻¹ region. The frequency of the v(Fe-Im) stretching mode is a sensitive probe of the bound histidine, providing structural information on the haem moiety. In particular, studies on ferrous model porphyrins, containing imidazole derivatives as the fifth ligand, show that the RR frequency, indicative of the bond strength, is influenced by the status of the proton of the N° of the bound imidazole. The frequency upshifts when the N°-proton is involved in H-bonding and the degree of the shift depends on the H-bond strength.

The RR spectra of all peroxidases studied so far show the presence of the v(Fe-Im) stretching mode at a relatively higher frequency than the other haem proteins and model compounds (Table 1). This behaviour has been interpreted as reflecting the imidazolate character of the proximal ligand, induced by the polar N°-HO hydrogen bond between the N° of the axial His ligand and a buried Asp side chain. However, when comparing the RR frequencies of the v(Fe-Im) stretching mode observed in CCP(MI) [14], HRP C [17,22] and CIP [21] (Table 1) at both acidic and alkaline pHs, some differences are observed. In particular, in CIP the v(Fe-Im) stretching mode is about 15 cm⁻¹ down-shifted with respect to CCP, implying a weaker H-bond between the N° and the oxygen atom of Asp-245. This hypothesis is not confirmed by the structural data of the two proteins, which exhibit comparable distances ( ~2.9 Å) between the two atoms involved in the proximal H-bond. Even if the different imidazolate character could derive from a different geometry of the N°-H-O H-bond, other parameters influencing the frequency of the Fe-Im stretching mode have to be taken into account [21]. Such parameters are the tilt angle θ between the

**Table 1**

<table>
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<th>Alkaline (pH)</th>
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haem normal and the Fe-Im vector, and the azimuthal angle $\phi$, which denotes the rotation of the projection of the His plane on the porphyrin plane with respect to the N(1)-Fe-N(3) axis [23]. These parameters, resulting from the repulsive interaction between the $\pi$ electrons of the porphyrin and the $\sigma^*$ antibonding electron of the Fe-Im bond, are interrelated in that for a given $\theta$, an increase in $\phi$ leads to a decreased $\pi-\sigma^*$ interaction and therefore to a decrease in the Fe-Im stretch frequency. On comparing the structures of CCP and CIP it appears that, whereas the tilt angle is similar, the His plane is approximately co-linear with the N(1)-N(3) axis ($\phi = 7^\circ$) in CCP and rotated by about 40$^\circ$ in CIP (Figure 2). Therefore the observed difference of the frequency of the $\nu$(Fe-Im) stretching modes in the two proteins may depend on the $\phi$ angle.

Proximal and distal mutations affect the frequency of the $\nu$(Fe-Im) stretching mode. The most severe change has been observed for the proximal mutation Asp-235 $\rightarrow$ Asn in CCP(M1), in which the aspartate carboxylate group, which acts as an H-bond acceptor to the proximal His-175, is replaced by a carboxamide group. The stretch frequency shifts down to 205 cm$^{-1}$ confirming the important role played by the His-175-Asp-235 H-bond [14]. On the other hand, the disruption of the H-bond between the Asp-235 and Trp-191 induces a strengthening of the His-175-Asp-235 H-bond, as indicated by the 3 cm$^{-1}$ upshift of the $\nu$(Fe-Im) stretching mode in the RR spectra of the F191, Y191, G191 and Q191 Fe$^{III}$ mutants (G. Smulevich, unpublished work). Moreover, a weakening of the proximal His-Asp interaction is observed upon mutation of the distal residues in both CCP [14] and HRP C [17].

**Conclusions**

The following conclusions can be reached from a comparative analysis of the RR spectra of CCP, HRP C, CIP and their variants.

1. The ferric forms of the three enzymes under investigation are mainly 5-c HS. The H-bond between the indole ring of the Trp-51 residue and the distal water molecule, present in CCP, helps in maintaining the sixth position of the iron in an empty state. In fact, the replacement of the indole ring of the distal Trp with a non-H-bonding phenyl group of a Phe residue drives CCP to a 6-c HS state. Therefore the presence of a 6-c HS haem in HRP C, as well as the peculiar instability of CIP upon laser irradiation, which drives the protein towards a 6-c state, can be ascribed to the lack of this H-bond, the distal Trp of CCP being replaced by a Phe residue in both proteins. The conserved polar H-bond between the proximal His and the buried Asp side chain is also important in restraining the movement of the iron atom towards the haem plane to bind the distal water molecule, imparting an anionic character to the fifth ligand, and strengthening the bond with the iron atom. The extra H-bond between the proximal Asp and the Trp residue, a peculiarity only of CCP, helps in maintaining the iron atom out of the haem plane towards the proximal side of the cavity.

2. The H-bond network connecting the proximal and the distal sides of the haem appears to be conserved in both CCP and HRP C, even if HRP C differs from CCP in having an altered structure of the distal haem cavity.

3. The replacement of the distal Arg affects the ability of the proteins to bind anionic ligands, such as $F^-$, with the ligand stabilization role played by the positively charged guanidinium group being lost. This result appears to confirm the hypothesis that the conserved distal Arg stabilizes the ferryl
centre of compound I during the catalytic cycle [24].

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