Introduction

Haem-containing peroxidases from plants, fungi and bacteria are evolutionarily related [1]. They are built from two structural domains enveloping ferric protoporphyrin IX and contain 11 conserved helices. The two domains, it appears, originated from an early gene duplication event [2]. The general picture of these peroxidases has emerged largely from the crystal structure of mitochondrial yeast cytochrome c peroxidase (CCP) [3] and the alignment of a large number of amino acid sequences. The latter also showed that three distantly related classes or evolutionary branches of peroxidases exist: intracellular peroxidases of prokaryotic origin (class I), fungal (class II) and plant (class III) peroxidases targeted for the secretory pathway via the endoplasmic reticulum [1]. Figure 1 shows examples of two distinct peroxidases from each class. Each pair shows very different reactivity, despite the fact that they are 40-45% identical in amino acid sequence. In the present paper we discuss the structural basis for these distinct differences. We show that the electrostatic potential imposed by the apo-protein on the haem active site can be very different for such a pair of similar peroxidases.

Methods

Atomic co-ordinates for CCP (2CYP) [3], Phanerochaete chrysosporium peroxidase (PCL) (1LGA) [4] and Coprinus cinereus peroxidase (CIP) (1ARP) [5,6] were obtained from the Brookhaven Protein Database. CIP and Arthromyces ramosus peroxidase (ARP) were identical [5-7], despite the fact that ARP residue numbers [5] are listed as one higher than for CIP [6]. ARP was claimed to contain an additional Gly near the N-terminus [5]. However, ARP bought from Sigma shows the same N-terminal amino acid sequence as CIP obtained from various sources (Figure 1) (K. G. Welinder, unpublished work), in accord with previous results [8]. Incomplete side chains of charged residues were restored in a minimum energy conformation. The electrostatic potential calculations were based on the Poisson–Boltzmann equation and performed using the DelPhi program (version 2.5; Biosym Technologies Inc., San Diego, CA, U.S.A.) [9-11]. In this model the protein interior was treated as a continuum and assigned a dielectric constant of 2. The surrounding water was assigned a dielectric constant of 80, and an ionic strength of 0.145 M. A full charge approximation was applied by setting His to +1, Lys, Arg and the N-terminus (of PCL) to +1, Asp, Glu and the C-terminus to -1, and the two calcium ions of PCL and CIP to +2. As the purpose of this study was to compare the influence of the different apo-proteins on the haem active site, haem and water molecules of the distal cavity were uncharged and treated as part of the protein continuum. The marked polarity of α-helices was assumed to contribute equally to the potentials of the three peroxidases, as the helices are conserved. The electrostatic potentials at the sites of distal Arg-48 or proximal Asp-235 were calculated in the same way, except that the site of analysis was uncharged. CCP residue numbers are used throughout unless otherwise noted.

Results and discussion

Conserved residues

In Figure 1 invariant and conserved residues are indicated that are common to all known peroxidases. Helices B and F sandwich the haem prosthetic group and are particularly conserved, for structural and for functional reasons. A number of glycine and proline residues are conserved in support of proper backbone bending. Cystine bridges are absent in class I peroxidases and differently located in class II and III peroxidases. In contrast, a salt bridge and H-bonding network provided by Ser-103, Asp-106, Gly-129 and Arg-130 [12] are invariant, and fix and direct the long loop connecting the two structural domains of peroxidases. The conserved phenylalanine/tyrosine posi-
Amino acid sequence alignment of selected peroxidases

Class I peroxidases CCP (cytochrome c peroxidase) and PEA (pea ascorbate peroxidase), fungal class II peroxidases PCL (Phanerochaete chrysosporium lignin peroxidase) and CIP (Coprinus cinereus peroxidase), and plant class III peroxidases HRP C (horseradish peroxidase) and BP I (barley peroxidase) are aligned [2]. The first line shows the α-helices observed in CCP [3], and conserved in CIP [5,6] and PCL [4]. The Ca²⁺ ligands are observed in CIP and PCL and predicted in the plant peroxidases [2]. The second line shows invariant residues in capital letters, and highly conserved residues in lower-case letters. A gap is shown by a dash, and every tenth residue is underlined. The fungal peroxidases extend into a small C-terminal domain, the sequence of which is not shown.

<table>
<thead>
<tr>
<th>Class</th>
<th>Enzyme</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CCP</td>
<td>TTPFMNHIEQST QYIVVYYLLEL RGMREM DFGT QRTS</td>
</tr>
<tr>
<td>I</td>
<td>PEA</td>
<td>TTPFMNHIEQST QYIVVYYLLEL RGMREM DFGT QRTS</td>
</tr>
<tr>
<td>II</td>
<td>PCL</td>
<td>TTPFMNHIEQST QYIVVYYLLEL RGMREM DFGT QRTS</td>
</tr>
<tr>
<td>II</td>
<td>CIP</td>
<td>TTPFMNHIEQST QYIVVYYLLEL RGMREM DFGT QRTS</td>
</tr>
<tr>
<td>III</td>
<td>HRP C</td>
<td>TTPFMNHIEQST QYIVVYYLLEL RGMREM DFGT QRTS</td>
</tr>
<tr>
<td>III</td>
<td>BP I</td>
<td>TTPFMNHIEQST QYIVVYYLLEL RGMREM DFGT QRTS</td>
</tr>
</tbody>
</table>

The first line shows the α-helices observed in CCP [3], and conserved in CIP [5,6] and PCL [4]. The Ca²⁺ ligands are observed in CIP and PCL and predicted in the plant peroxidases [2]. The second line shows invariant residues in capital letters, and highly conserved residues in lower-case letters. A gap is shown by a dash, and every tenth residue is underlined. The fungal peroxidases extend into a small C-terminal domain, the sequence of which is not shown.

Proposed role of the conserved distal and proximal His–Asx H-bonding networks

Crystallographic structure determination of PCL [4] and CIP [5,6] confirmed the presence of a conserved distal His-52–Asn-82 H-bond, and of a conserved proximal His-175–Asp-235 H-bond, observed first in CCP [3]. At both of these sites the H-bonded networks are in fact conserved in more extended forms. At the distal side, two side chains and one carbonyl backbone are conserved: ND₁(His-52)-OD₁(Asn-82)ND₂-O(Glu-76). Conserved Glu-76 might be important to the electrostatic field applied to the distal H-bonding network, assisting in pulling the proton away from distal His as needed during the various reactions at the active site. At the proximal side, two invariant side chains, a buried water and a hydroxy side chain are conserved in all: ND₁(His-175)-OD₁(Asp-235)-H₂O₅₃⁵-O(Thr-234). CCP is unique in that OD₂(Asp-235) is H-bonded to Trp-191. In CCP Trp-191 carries a cation radical in Compound I. In classes I and III the cation radical is located on the porphyrin ring, and a more extensive H-bonded proximal network seems to exist as observed in the crystal structures of CIP and PCL: ND₁(His-176)-OD₁(Asp-238)-H₂O₅₃⁵-O(Thr-237) and OD₁(Asp-238)-H₂O₅₃⁵-O(Thr-237) (PCL residue numbers). We suggest that these conserved active-site H-bonded networks function to stabilize the different charges at the redox centre that develop during peroxidase reactions. Charges are delocalized very efficiently through H-bonds and minimize
the free energy of the system. The delocalization is extremely fast as compared with rates that involve diffusion and formation or cleavage of chemical bonds. A H-bonding network is also likely to give rise to abnormal pK<sub>A</sub> values and difficulties in assignment of formal pK<sub>A</sub> values to specific groups such as distal His, Arg and water, two haem propionates, and proximal His and Asp. Furthermore, pK<sub>A</sub> values will change with the redox state, as is well known for peroxidases [13–15].

Electrostatic potentials, pK<sub>A</sub> values and redox potentials

The electrostatic potentials of the two fungal peroxidases, PCL and CIP, and of mitochondrial CCP in solution at neutral pH, are shown in Figure 2. The electrostatic potentials at selected sites imposed by the apo-proteins, treating haem as an uncharged protein continuum, are compared in Table 1, which also lists pl and net charges of the peroxidases at neutral pH. Although PCL and CIP have similar isoelectric points and are 45% identical in their amino acid sequences (Figure 1), the electrostatic potentials at their haem active sites are strikingly different. CIP has a positive, PCL a negative and CCP an electrostatic potential intermediate between the two. Comparing these potentials allows us to draw qualitative conclusions as to the influence of the apo-protein on active-site pK<sub>A</sub> values and redox potentials.

Lignin peroxidase PCL at all sites is more negative than CCP and much more negative than CIP (Table 1). This means that the acid forms of Arg, His, Asp and haem propionates are more favoured in PCL than in CCP and CIP, or that the pK<sub>A</sub> values of the active-site groups show the relationship PCL &gt; CCP &gt; CIP, except for distal His where PCL = CCP &gt; CIP. The electrostatics calculations also predict that proximal His of PCL has less imidazolate character than that in CCP and CIP, which are similar at this site. The absolute changes in electrostatic potentials and pK<sub>A</sub> values cannot be calculated for different peroxidases, as they will depend on (minor) structural differences. However, they can be calculated by comparing a wild-type enzyme and its mutants [11].

Different electrostatic potentials imposed on the redox centre will also affect the redox potentials of the couples ferric/ferrous (3/2), ferryl (cpdII)/ferric (4/3) and porphyrin π-cation-ferryl (cpdI)/ferryl (cpdII) (5/4). The more negative potential of PCL, than of CCP and CIP at the iron and the haem (Table 1) will stabilize a positive iron, and hence increase the ability of PCL to abstract an electron from a substrate relative to CCP and CIP, meaning that <i>E</i><sub>0</sub> is predicted to be more positive (CIP &lt; CCP &lt; PCL). The known <i>E</i><sub>0</sub> values [13,15,16] seem to support this prediction. The <i>E</i><sub>0</sub> of the redox couples are linked with active-site pK<sub>A</sub> values in such a way that removing a proton (increase in pH) will ease also removing an electron (<i>E</i><sub>0</sub> decreased) from the redox centre [13–15], for thermodynamic reasons.

Different reactivities of pairs of similar peroxidases

Haem-type and active-site residues are invariant in the plant peroxidase superfamily, as illustrated by CCP, PEA, CIP, PCL, HRP C and BP 1 in Figure 1, and cannot account for the different reactivities of these peroxidases with peroxides and haem ligands. The class I peroxidases CCP and PEA are highly specific in their ways of eliminating hydrogen peroxide from their respective intracellular compartments, as indicated in their names. Both may abstract electrons from cytochrome c or ascorbate via Trp-191, and have nearby substrate binding sites at the most accessible haem edge [17].

The class II peroxidases lignin-degrading PCL, and CIP which is rather similar to HRP C in its reactions, differ clearly at their substrate binding sites. PCL has a narrow and CIP a wide channel to the haem distal cavity [5,6]. However, the most striking differences between the two are (i) their pH ranges, as PCL reacts with peroxide over a wide range but only near pH 3 with reducing substrates, whereas CIP reacts with peroxide and reducing substrates pH 5–11, and (ii) PCL can oxidize veraaryl alcohol and has higher oxidation potential than CIP [15,16]. The higher redox potentials of PCL compounds are supported by the marked negative electrostatic potential exerted by apo-PCL at the redox centre (Table 1). The difference in pH-dependence is most likely the result of structural differences near the haem, although we predict that all PCL active-site pK<sub>A</sub> values are higher than for CIP. PCL has three additional pH-sensitive groups: Asp-183 interacting with propionate A, and an ion pair His-82–Glu-146 fencing the haem access channel [4]. In CIP the Soret absorption maximum wavelength changes from 405 nm at pH 6.0 to 393 nm at pH 3.6 and is accompanied by loss of activity [18]. The structural change around pH 5 could be a combination of the rather open haem cavity in CIP and the positive electrostatic potential that we see even at pH 7.
Figure 2

Stereoscopic view of three peroxidases and their calculated electrostatic potentials

The Cα tracing of the structures is seen perpendicular to the haem edge along the channel leading to the distal cavity. Active-site residues are emphasized in 'ball and stick' style. Positive electrostatic potential surfaces are dotted at \( \pm 2.5 \text{ kJ/mol} \). (a) CCP, (b) CIP, and (c) PCL. The negative electrostatic potentials (not shown) are dominating and cover the rest of the molecules. The calculated potentials at selected sites are shown in Table 1.
Plant Peroxidases, Structure and Molecular Biology

Table I

Net charge, isoelectric point, redox potential and calculated electrostatic potentials at selected sites of peroxidases

Redox potentials at pH 7 are from [16]. Electrostatic potentials at pH 7 are calculated as described in Methods and averaged over the non-hydrogen atoms of guanido (4 atoms), imidazole (5 atoms), carboxylate (3 atoms) and haem (43 atoms).

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>Net charge</th>
<th>pi</th>
<th>$E_0$ (mV) (3/2)</th>
<th>Distal</th>
<th>Proximal</th>
<th>Carboxylate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Arg</td>
<td>His</td>
<td>His</td>
</tr>
<tr>
<td>CCP</td>
<td>-13</td>
<td>5.0</td>
<td>-194</td>
<td>-42</td>
<td>+21</td>
<td>-121</td>
</tr>
<tr>
<td>CIP/ARP</td>
<td>-12</td>
<td>3.5</td>
<td>?</td>
<td>+25</td>
<td>+50</td>
<td>-117</td>
</tr>
<tr>
<td>PCL</td>
<td>-19</td>
<td>3.5</td>
<td>-137</td>
<td>-80</td>
<td>+13</td>
<td>-151</td>
</tr>
</tbody>
</table>

The class III peroxidases HRP C and BP 1 have very different pH optima and peroxide reactivities [19,20], despite the fact that they carry similar net charges at neutral pH (+3 and +4 respectively). In contrast to other known plant peroxidases, BP 1 only reacts with peroxide below pH 5 and shows increasing rates as the pH decreases, until acid protein denaturation takes over [19]. Moreover, the rate constant for the BP 1 reaction with peroxide increases 10-40-fold in the presence of phenolic substrates [20]. Since the crystal structures are unknown for plant peroxidases, the contributions to these distinct differences from structure and from electrostatic potentials cannot be evaluated at the present.

Conclusions

We suggest (i) that the conserved His-Asx H-bonding networks at the distal and proximal sides of haem in peroxidases serve to delocalize charges during peroxidase reactions, thereby reducing the free energy or stabilizing both resting state and intermediates, and (ii) that essential properties of haem peroxidases such as redox potentials, pH optima and stability are strongly influenced also by medium- and long-range electrostatic forces and can be modulated in a predictable way by mutation of charged residues.

We thank H. Lundbeck A/S for providing computer facilities, the Carlsberg Foundation (92-0309/20), the Danish Research Academy (8940173), the Danish Technology Research Council (1989-133/1-890009) and the EU Human Capital and Mobility Programme (ERBCHRX-CT92-0012) for support of the reported research, and Dr. G. Smulevich, University of Florence, for stimulating discussions.


1995
Radical cation cofactors in lignin peroxidase catalysis

P. J. Harvey* and L. P. Candea†

*University of Greenwich, Wellington Street, London SE18 6PF, and †CRC Gray Laboratory, P.O. Box 100, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, U.K.

Introduction

It is becoming increasingly apparent that catalysis by the extracellular fungal peroxidase, lignin peroxidase (LiP), involved in the oxidative degradation of polymeric lignin, can be significantly enhanced by the addition of the fungal secondary metabolite veratryl alcohol (VA; 3,4-dimethoxybenzyl alcohol). LiP is a haem-containing glycoprotein, structurally similar to horseradish peroxidase (HRP) (one iron protoporphyrin IX in the high-spin pentacoordinate ferric state with histidine co-ordinated as the fifth ligand [1,2]; similar sequence of amino acids at the active site [3,4]) and with a similar overall three-dimensional structure to cytochrome c peroxidase [5]. It reacts with H₂O₂ similarly, to form Compound I, an oxy-ferryl high-spin complex with porphyrin radical cations (VA⁺). However, a satisfactory rationale which is able to explain all the observed data has not yet been achieved due to uncertainty over the mechanism by which VA is oxidized by LiP, the properties of VA⁺, and whether VA⁺ are indeed products of the LiP-catalysed oxidation of VA.

The simplest explanation for reaction enhancement of LiP catalysis by VA derives from the realization that radical cations can act as charge transfer agents [24] (eqn. 1) and that the immediate products of the LiP-catalysed oxidation of VA are radical cations (VA⁺).

\[ VA^{++} + S \rightleftharpoons VA + S^{++} \]  

\[ (1) \]

However, a satisfactory rationale which is able to explain all the observed data has not yet been achieved due to uncertainty over the mechanism by which VA is oxidized by LiP, the properties of VA⁺, and whether VA⁺ are indeed products of the LiP-catalysed oxidation of VA.

LiP catalytic mechanism with VA as reductant

The first step in the oxidation of VA catalysed by LiP involves oxidation of the native enzyme with H₂O₂, for which the second-order rate constant \[ k = (4.2-6.5) \times 10^{-5} \text{ M}^{-1} \text{s}^{-1} \] has been determined [25-27]. This step is controlled by an acidic

---

Abbreviations used: LiP, lignin peroxidase; VA, veratryl alcohol; VA⁺, VA radical cations; HRP, horseradish peroxidase; 4-MA, 4-methoxymandelic acid.

Received 9 November 1994