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Review

Fungal peroxidase: its structure, function, and application [1]

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Abstract

Arthromyces ramosus, a novel hyphomycete, extracellularly produces a single species of a heme-containing peroxidase. The *A. ramosus* peroxidase, ARP, shows a broad specificity for hydrogen donors and high catalytic efficiency as does the well-known peroxidase from horseradish roots (HRP). However, it also exhibits unique catalytic properties. These features permit a wide range of applications for ARP, including high-sensitivity chemiluminescent determination of biological materials, protein cross-linking, and dye-transfer inhibition during laundering. The primary and tertiary structures of ARP are very similar to those of the class (II) lignin and manganese peroxidases of the plant peroxidase superfamily. Mechanistic studies of the ARP-catalyzed reaction revealed that it also proceeds with the classical peroxidase cycle; the native ferric ARP undergoes two-electron oxidation by hydrogen peroxide to yield compound (I), followed by two successive one-electron reductions by the hydrogen donor. X-ray crystallography, site-directed mutagenesis, and spectral analyses of ARP have afforded detailed information on the molecular mechanism of the ARP catalysis, and revealed the roles of active site amino acid residues and dynamic features of coordination as well as spin states of heme iron during catalysis.

Keywords: *Arthromyces ramosus*; *Coprinus cinereus*; Peroxidase; Reaction mechanism; Dye-transfer inhibition

1. Introduction

Peroxidase (donor: H_2O_2 oxidoreductase [EC1.11.1.7]) catalyzes the oxidation of a wide variety of compounds (denoted by AH_2) by hydrogen peroxide:

$$AH_2 + H_2O_2 \rightarrow A + 2H_2O.$$

The enzyme occurs in plants, animals, and microorganisms, and its specificity and biological functions vary with sources of the enzyme. Peroxidase has attracted industrial attention because of its usefulness as a catalyst in clinical examinations and other applications. Perhaps,

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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the best-known peroxidase is that from horseradish root (HRP) which, due to its broad specificity for hydrogen donors and its high catalytic efficiency, has been used widely in spectrophotometric determinations of biological materials.

In 1986, Yamada et al. discovered a novel peroxidase which is produced extracellularly by a novel hyphomycete, *Arthromyces ramosus*, and pointed out its potential usefulness in clinical tests [1]. Later, a similar peroxidase was also found in the culture filtrate of an ink cap basidiomycete, *Coprinus cinereus* [2]. During the past 12 years, unique applications of these peroxidases have been developed. Currently, these fungi are known as new practical sources of peroxidase for industrial purposes, in addition to horseradish roots. Meanwhile, primary and tertiary structures of these fungal peroxidases have also been determined and have provided important information on the specificity and mechanism of these novel peroxidases. In this review, we will describe the current knowledge on the enzymology of the fungal peroxidases with emphasis on their structure/function relationships. We will also mention applications, which have recently been expanded into household uses.

2. The peroxidases of A. ramosus and C. cinereus are essentially identical

It has been shown that the *A. ramosus* peroxidase (ARP) and the *C. cinereus* peroxidase (CiP) are essentially identical to each other. Primary and tertiary structure analyses showed that the amino acid sequences of ARP and CiP are 99% identical [3] and their crystal structures are also essentially identical [4,5]. The following minor structural differences between ARP and CiP have been identified [3]. (i) A tetraglycine segment in the *N*-terminal glycine-rich extension of CiP is replaced by a pentaglycine segment in ARP. (ii) Hence, the polypeptide chain of ARP is longer than that of the CiP by one amino acid residue. (iii) An amino acid residue corresponding to Val99 of CiP is substituted by Ile in ARP as is observed for the minor isozyme of CiP. (iv) They also differ from each other in the degree of *N*-glycosylation [6]. These differences seem to cause no detectable difference in the physicochemical, immunochemical, and catalytic properties [6]. These enzymes have indistinguishable molecular weights and isoelectric points; their molecular activity and optimum pH were the same when examined with different substrates, and their catalytic competence during chemiluminescent reactions is also the same [7].

The taxonomic characteristics of *A. ramosus* and *C. cinereus* are also similar; the only notable difference is that *C. cinereus* has a perfect stage during its life cycle whereas *A. ramosus* does not [3]. [2] These fungi show essentially no genetic polymorphisms with respect to the peroxidase gene, which is in striking contrast with the polymorphisms observed for other plant peroxidases. *A. ramosus* produces a single molecular species of peroxidase; no other isoform has been identified in the culture filtrate [1]. This is further confirmed by Southern blot analysis, which shows that the *A. ramosus* genome encodes only a single copy of the ARP gene [3]. The *C. cinereus* peroxidase gene shows a limited genetic polymorphism encoding one major and one minor isoform of the peroxidase [8]. These observations led to the proposal that *A. ramosus* is an isolate of the imperfect stage of a *Coprinus* and that ARP is one of the possible variants that arose from the polymorphism of the CiP [3,6,8].

Both ARP and CiP have been studied independently by several groups. In order to avoid possible confusion in some discussions, this review mainly deals with the results obtained from ARP studies. We will also mention the

[2] The term 'perfect stage' refers to a stage of sexual reproduction in the fungal life cycle and 'imperfect stage' is that of asexual reproduction. Hyphomycetes, a class of fungi, do not show the perfect stage but reproduce asexually throughout their life cycles.

results obtained from studies of CiP if supplementary information is available from them.

3. Production, purification, and molecular properties

The absence of genetic polymorphism of ARP, along with the fact that ARP is produced extracelluarly by an 8-day aerobic liquid cultivation [1], has been envisioned to be an advantageous feature in the industrial production and application of this peroxidase. In contrast, HRP consists of at least 12 isoforms with different catalytic properties; the composition of these isoforms depends on the conditions of plant cultivation, which extends over a few months, and isolation of a specific isoform requires laborious procedures [9–12].

Production of ARP has also been achieved by genetic engineering approaches. Cloned ARP cDNA has been expressed heterologously in the yeast, *Saccharomyces cerevisiae*, under the promoter of the glyceraldehyde 3-phosphate dehydrogenase gene, yielding a relatively low level of a secreted active peroxidase [3]. On the other hand, CiP was heterologously expressed in the *Asperguillus oryzae* transformants [13], whose enzyme productivity is currently said to be at industrially viable levels.

The ARP was purified to homogeneity from the culture filtrate of *A. ramosus* by ammonium sulfate fractionation and anion exchange and gel filtration chromatographies, with an activity yield of 57% [1]. Molecular properties of the purified ARP are summarized in Table 1. ARP is a monomeric glycoprotein with a molecular weight of 41,000 and a carbohydrate content of approximately 5%. UV–visible absorption spectra show that ARP contains 1 mol protoheme IX per mole of enzyme [1].

4. Catalytic properties

Table 2 summarizes the substrate specificity of ARP as determined by spectrophotometric and chemiluminescent assays. ARP shows very

Table 1
Molecular properties of ARP

Molecular weight	41,000 (sedimentation equilibrium) [1]
	38,000 (SDS–PAGE) [6]
Number of subunits	1 [1]
Amino acids/subunit	344[a,b] [3,4]
Number of disulfide bonds	4[b] [4]
pI	3.4 [1]–3.5 [6]
Optimum pH	6.0–7.0[c] [1], 5.0–8.0 [6], 8.8–9.0[d] [7,14]
Optimum temperature	40°C[c] [1]
pH stability	pH 5.0–9.0 (at 30°C for 16 h) [1]
Thermal stability	up to 50°C (at pH 7.0 for 30 min) [1]
Cofactor	one protoheme IX/enzyme [1]
Absorption maxima	280, 415, 540, 640 nm (oxidized form) [1]
	280, 438, 557, 585 nm (reduced form) [1]
RZ value[e]	2.7 [1]
Sugar content and glycosylation sites	5% [1], Asn143 and Ser339[b] [4]
Metal ion requirement	two endogenous Ca^{2+} ions[b] [4]
Cellular localization	extracellular [1]

[a] From primary structure analysis.
[b] From X-ray crystallography.
[c] Oxidative coupling of phenols with 4-aminoantipyrine.
[d] Chemiluminescent reaction with luminol.
[e] RZ (Reinheitszahl) value is the absorbance ratio with absorption at λ_{max} of Soret band/absorption at 280 nm.

Table 2
Substrate specificity of ARP

(A) Hydrogen donor	Relative color intensity (%)
Phenol	100
4-Chlorophenol	743
2,4-Dichlorophenol	936
2,4-Dibromophenol	402
2,4,6-Trichlorophenol	624
Pyrocatechol	98
Sodium 4-hydroxybenzoate	167
N,*N*-dimethylaniline	1.4
N,*N*-diethylaniline	7.4

(B) Substrates	ARP		HRP	
	K_m^a	V_{max}	K_m^a	V_{max}
2,4-Dichlorophenol	0.19	1244[b]	0.24	418[b]
ABTS	0.71	2110[b]	0.017	540[b]
Luminol	0.007	810[c]	0.016	1.5[c]

(A) The specificity for phenolic and anilinic hydrogen donors was expressed as a relative color intensity at 500 nm due to their oxidative coupling with 4-aminoantipyrine. The assay was done at pH 7.0 and 37°C for 1 min. For other conditions, see Ref. [1].
(B) Comparison of kinetic constants for hydrogen peroxide as assayed with different hydrogen donors. 2,2′-Azinobis[3-ethyl-2,3-dihydrobenzothiazole-6-sulfonic acid] (ABTS) and luminol are the chromogenic and luminogenic hydrogen donors, respectively. ARP, *A. ramosus* peroxidase. HRP, horseradish peroxidase. For experimental details, see Ref. [7].
[a]Millimolar for H_2O_2.
[b]Units per milligram of protein. One unit of enzyme is defined as the enzyme amount which reduces 1 μmol of H_2O_2 per min at 37°C.
[c]Arbitrary units per microgram of protein.

broad specificity for aromatic hydrogen donors as does HRP, but has higher specific activities than HRP. Luminol also serves as a very good hydrogen donor [7,14]; the V_{max} of the ARP for the chemiluminescent reaction is 500 times greater than that of HRP, although the K_m values of ARP and HRP for luminol are similar [7]. This allows the highly sensitive enzyme assays which detect less than 10 ng ml^{-1} of the peroxidase [7] and also allows highly sensitive determination of hydrogen peroxide (Refs. [7,14], see below). It should be noted that recent NMR analyses of the luminol-bound and -unbound forms of ARP and HRP suggest that luminol is accessible to heme and interacts with it in ARP but not in HRP [15]. This observation may explain why ARP catalyzes the chemiluminescent reaction far more efficiently than HRP. The enzyme showed maximum activity at pH 5.0–8.0, varying with the hydrogen donor used, in the spectrophotometric assay [1,6], and at pH 8.8–9.0 in the chemiluminescent assay [7,14].

Many peroxidases are also known to catalyze the so-called ‘peroxidase–oxidase’ reaction, in which the enzyme uses molecular oxygen instead of hydrogen peroxide as an electron acceptor for the oxidation of a limited number of substrates (AH_2; e.g., NADH and dihydroxyfumaric acid) [16]:

$$2AH_2 + O_2 \rightarrow 2A + 2H_2O.$$

Under certain conditions, this reaction shows several types of non-linear kinetics such as those which are called ‘damped oscillations’ [16], ‘sustained oscillations’ [17], ‘period doubling bifurcations’, ‘quasi-periodicity’, and ‘chaos’. CiP (= ARP) was also shown to exhibit peroxidase–oxidase activity when NADH was used as a substrate [17,18]. However, its oscillatory dynamics were different from those observed with HRP [16] and the other peroxidases [19], suggesting that the mechanism of the peroxidase–oxidase reaction catalyzed by CiP differs from that of the other peroxidases.

5. Reaction mechanism and its kinetic and thermodynamic aspects

Scheme 1 shows a generally-accepted mechanism of the peroxidase-catalyzed reaction in which each of compounds (I) and (II) undergoes a one-electron reduction by hydrogen donor, AH_2 [20].

Hydrogen peroxide removes two electrons from peroxidase, yielding a highly oxidized state of peroxidase, the so-called 'compound (I)', which is green in color [Scheme 1, step (1)]. Spectral characteristics suggest that compound (I) contains an oxygenated Fe(IV)- (i.e., oxyferryl-) based porphyrin radical cation. Compound (I) then undergoes two successive one-electron reductions by AH_2 as follows: an AH_2 molecule adds one electron to compound (I), converting it to compound (II), which is red in color [step (2)]. Compound (II) is also suggested to contain an oxyferryl state of the heme iron but lacks the porphyrin radical cation; compounds (I) and (II) differ by only one electron on the porphyrin ring. Another AH_2 molecule subsequently reacts with compound (II), adding a second electron to it and yielding the native peroxidase [step (3)]. Disproportionation between two AH · molecules gives rise to AH_2 and an oxidation product A, or, in some cases, dimerization of two AH · molecules occurs to yield A_2H_2 [step (4)].

Several lines of evidence indicate that ARP catalysis also proceeds via the one-electron transfer mechanism described above, as does HRP [21,22]. However, the redox properties of compounds (I) and (II) of ARP are quite different from those of HRP [21]. The stabilities of compounds (I) and (II) of ARP depend on pH and buffer concentration. At neutral pHs, the reaction of ferric ARP with H_2O_2 gives rise to a stable compound (I) [step (1)]. The titration of compound (I) with ascorbate or hydroquinone (used as AH_2) produces the ferric ARP without the apparent formation of compound (II), in contrast with HRP catalysis in which compound (II) formation can be seen under the same conditions [steps (2) and (3)]. These observations are due to the fact that the ARP compound (II) is too unstable to be detected and is rapidly reduced to the ferric ARP. The instability of compound (II) is due to its unusually high reduction potential (E_0'). The E_0' values of compounds (I) and (II) of ARP were estimated from redox equilibria coupled with an $IrCl_6^{2-}/IrCl_6^{3-}$ system. The E_0' values are dependent on pH and buffer concentration, as expected, and are 0.915 V for compound (I) and 0.982 V for compound (II) at pH 7. For comparison, the E_0' values for HRP are 0.880 V and 0.900 V, respectively. The E_0' value of the ARP compound (II) is the highest one-electron reduction potential ever reported for biological oxidants [21]. The observed dependence of E_0' values on pH and buffer concentration may be related to the protonation states of compounds (I) and (II) of the peroxidase.

The rate constants, k_1, k_2, and k_3 (see Scheme 1), and their pH dependences were also

$$\text{ARP [Fe(III)]} + H_2O_2 \xrightarrow{k_1} \text{compound I [Fe(IV)·]} + H_2O \quad (1)$$

$$\text{compound I [Fe(IV)·]} + AH_2 \xrightarrow{k_2} \text{compound II [Fe(IV)]} + AH\cdot \quad (2)$$

$$\text{compound II [Fe(IV)]} + AH_2 \xrightarrow{k_3} \text{ARP [Fe(III)]} + AH\cdot + H_2O \quad (3)$$

$$2AH\cdot \xrightarrow{k_4} A + AH_2 \text{ (or } A_2H_2) \quad (4)$$

Scheme 1.

determined by pre-steady and steady state methods [22]. The k_1 value, the rate of compound (I) formation, is constant at neutral and alkaline pHs and decreases at acidic pHs, being governed by one ionization with a pK_a value of 5.0. The estimated k_1 values at neutral and alkaline pHs are $6–10 \times 10^6$ M^{-1} s^{-1} by both methods, and are very large, similar to those of the other peroxidases. The values of k_2 and k_3, the rates of reduction of compounds (I) and (II) by hydrogen donor, are also obtained from pre-steady state analysis. The k_2 value shows a bell-shaped pH dependence with a maximum ($> 120 \times 10^6$ M^{-1} s^{-1}) at pH $\sim$ 8.0 with pK_a values of $\sim$ 7 and $\sim$ 9. The k_3 value also shows a bell-shaped pH dependence with a maximum (48×10^6 M^{-1} s^{-1}) at pH 8.0 and pK_a values which are similar to those of the k_2 values. Although the decreases in the k_2 and k_3 values at alkaline pHs may be in part related to the lower reduction potentials of compounds (I) and (II) [21], it would also be possible that the observed pH dependences are due to catalytically important ionizations (see Section 8). The k_2 and k_3 values were similar to each other at pH 5–6. This observation was unusual for a peroxidase because k_2 is normally at least 10 times larger than k_3 for peroxidases, and the observed large k_3 likely arises from the unusually high reduction potential of compound (II) as reported by Farhangrazi et al. [21].

6. Cloning, primary structure, and sequence similarity

A 500-bp internal stretch of the ARP cDNA was amplified by PCR (polymerase chain reaction) with *A. ramosus* cDNAs as a template, using primers which were designed on the basis of the determined amino acid sequence. Using the 500-bp cDNA fragment as a probe, cDNA and genomic DNA libraries were screened for the ARP gene by plaque hybridization. Several positive clones were obtained, and clones containing a full-length ARP gene were sequenced [3].

The cloned cDNA for ARP contained an open reading frame of 1092 bp that encodes a protein consisting of 364 amino acid residues (Fig. 1). Sequence analysis of the ARP genomic DNA revealed that the structural gene for ARP contains 14 introns. This was one of the highest number of introns found in the genes coding for proteins that are similar in size to ARP [3].

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-20  Met Lys Leu Ser Leu Phe Ser Thr Phe Ala Ala Val Ile Ile Gly Ala Leu Ala Leu Pro
  1  Gln Gly Pro Gly Gly Gly Gly Gly Ser Val Thr Cys Pro Gly Gly Gln Ser Thr Ser Asn
 21  Ser Gln Cys Cys Val Trp Phe Asp Val Leu Asp Asp Leu Gln Thr Asn Phe Tyr Gln Gly
 41  Ser Lys Cys Glu Ser Pro Val Arg Lys Ile Leu Arg Ile Val Phe His Asp Ala Ile Gly
 61  Phe Ser Pro Ala Leu Thr Ala Ala Gly Gln Phe Gly Gly Gly Gly Ala Asp Gly Ser Ile
 81  Ile Ala His Ser Asn Ile Glu Leu Ala Phe Pro Ala Asn Gly Gly Leu Thr Asp Thr Ile
101  Glu Ala Leu Arg Ala Val Gly Ile Asn His Gly Val Ser Phe Gly Asp Leu Ile Gln Phe
121  Ala Thr Ala Val Gly Met Ser Asn Cys Pro Gly Ser Pro Arg Leu Glu Phe Leu Thr Gly
141  Arg Ser Asn Ser Ser Gln Pro Ser Pro Pro Ser Leu Ile Pro Gly Pro Gly Asn Thr Val
161  Thr Ala Ile Leu Asp Arg Met Gly Asp Ala Gly Phe Ser Pro Asp Glu Val Val Asp Leu
181  Leu Ala Ala His Ser Leu Ala Ser Gln Glu Gly Leu Asn Ser Ala Ile Phe Arg Ser Pro
201  Leu Asp Ser Thr Pro Gln Val Phe Asp Thr Gln Phe Tyr Ile Glu Thr Leu Leu Lys Gly
221  Thr Thr Gln Pro Gly Pro Ser Leu Gly Phe Ala Glu Glu Leu Ser Pro Phe Pro Gly Glu
241  Phe Arg Met Arg Ser Asp Ala Leu Leu Ala Arg Asp Ser Arg Thr Ala Cys Arg Trp Gln
261  Ser Met Thr Ser Ser Asn Glu Val Met Gly Gln Arg Tyr Arg Ala Ala Met Ala Lys Met
281  Ser Val Leu Gly Phe Asp Arg Asn Ala Leu Thr Asp Cys Ser Asp Val Ile Pro Ser Ala
301  Val Ser Asn Asn Ala Ala Pro Val Ile Pro Gly Gly Leu Thr Val Asp Asp Ile Glu Val
321  Ser Cys Pro Ser Glu Pro Phe Pro Glu Ile Ala Thr Ala Ser Gly Pro Leu Pro Ser Leu
341  Ala Pro Ala Pro
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Fig. 1. Amino acid sequence of ARP deduced from nucleotide sequence of ARP cDNA. The underlined amino acid sequence is the suggested signal sequence for secretion, and the *N*-terminus of the mature form of the peroxidase is pyroglutamic acid, shown in *boldface*. The glycosylation sites are double-underlined.

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                          □       ○                                   ▽
ARP      47 V R K I L R I V F H D A I 59------175 D E V V D L L A A H S L A 187
BAP      39 A H Q A I R L T F H D A V 51------168 L E T V W G L I A H T V G 180
PCP      38 A H E S I R L V F H D S I 50------167 L E L V W M L S A H S V A 179
MnP      37 A H E V I R L T F H D A I 49------164 F E V V S L L A S H T V A 176
Turnip   33 G A S I L R L F F H D C F 45------159 R D M V A L S G A H T I G 171
CCP      43 G P V L V R L A W H T S G 55------165 R E V V A L M G A H A L G 177
HRP      33 A A S I I R L H F H D C F 45------161 S D L V A L S G G H T F G 173
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Fig. 2. Conserved amino acid residues. The amino acid sequences near the invariant His and Arg residues of several peroxidases are aligned with each other. The proximal and distal His and essential Arg residues of ARP are indicated by (▽), (○), and (□), respectively, above the sequences. The enzymes are as follows: ARP, *A. ramosus* peroxidase [3]; BAP, lignin peroxidase of *Bjekandera adusta* [48]; PCP, lignin peroxidase of *P. chrysosporium* [49]; MnP, manganese peroxidase of *P. chrysosporium* [49]; Turnip, turnip peroxidase [50]; CCP, *S. cerevisiae* cytochrome *c* peroxidase [51]; and HRP, horseradish peroxidase [52].

The deduced amino acid sequence for residues −20 to −1, which is rich in hydrophobic amino acids and contains a basic amino acid residue (Lys) at position −19, is a signal sequence for secretion. The mature form of the enzyme consists of 344 amino acid residues and its amino terminus is pyroglutamic acid. The amino acid sequence of ARP contains one potential glycosylation site (Asn143). The sugar chain analysis and X-ray crystallography of ARP revealed that a sugar chain, $Man_7GlcNAc_2$, is indeed attached to this site [4,6]. The crystallographic studies also revealed the presence of an *O*-glycosyl moiety linked to Ser339 [4].

The ARP shows the highest sequence similarities to the lignin and manganese peroxidases of *Phanerochaete chrysosporium* (43 and 41% identity, respectively) and lignin peroxidase of *Phlebia radiata* (41%). A lower sequence identity (25%) was found with isozyme C of HRP [3]. Welinder [23] classified the plant peroxidase superfamily, consisting of the distantly related heme-containing peroxidases of plant, fungal, and bacterial origins, into three categories on the basis of their amino acid sequences: class (I), yeast and prokaryotic peroxidases including cytochrome *c* peroxidase; class (II), extracellular fungal lignin and manganese peroxidases; and class (III), plant peroxidases typified by HRP. The amino acid sequence of ARP clearly indicates that the ARP is a class (II) enzyme; however, ARP shows no detectable lignin and manganese peroxidase activities [3].

Heme-containing peroxidases have been shown to contain two His residues and one Arg residue which are invariant among the peroxidases [23]. It has been shown in many peroxidases that one of these His residues is the proximal ligand to the heme iron, and the other His as well as the Arg residues lie distal to the heme and play important roles during catalysis. In ARP, His184 and His56 correspond to the proximal and distal His, respectively, and Arg52 to a distal Arg (Fig. 2). This identification was confirmed by crystallographic studies [4] (Fig. 4).

7. Stereostructure

The stereostructure of ARP has been determined by the multiple isomorphous replacement method at a 1.9 Å resolution [4]. Fig. 3 shows the overall stereostructure of ARP drawn with emphasis on the helical segments and domain structure. ARP is an α-protein consisting of 10 major and two short helices and a few short β-strands. All eight cysteine residues in ARP form disulfide bonds. The heme group occupies a crevice between two large domains. These spatial arrangements of secondary and domain structures are very similar to those of the other class (II) peroxidases [24,25], being consistent with their high sequence similarities. Crystallographic studies also revealed that ARP contains two Ca^{2+} sites (not shown in Fig. 3; for details,

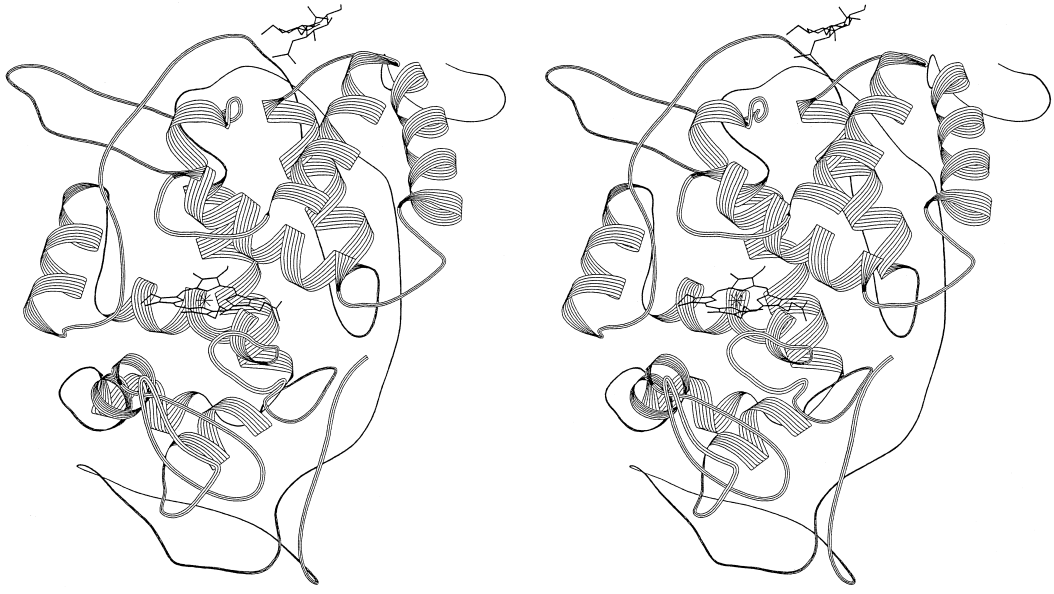

Fig. 3. Overall stereostructure of ARP. Cited from Ref. [4] with permission.

see Ref. [4]). The endogenous Ca^{2+} ion is also found in HRP, in which Ca^{2+} is suggested to play a role in maintaining the protein structure in the heme environments and spin state of the heme iron in favor of catalytic activity [26]. This may also be the case for ARP.

The heme group is bound to the protein through noncovalent interactions [4]. The propionate groups of protoporphyrin IX are bound to the protein backbone through hydrogen bonds, some of which are mediated by a water molecule. A N^{ε} atom of the proximal His184 coordinates as the fifth ligand to a heme iron. Hydrophobic interactions are also predicted to stabilize the heme–protein interaction. Fig. 4 shows a view of the heme environment of ARP [4]. Along with the distal His56 and proximal His184 residues, some other amino acid residues which are conserved among peroxidases are present in close proximity to the heme. Among these, Asn93 and Arg52 lie distal to heme and Asp246 lies proximal to the heme. The roles of these amino acid residues in the catalytic functioning of the peroxidase are discussed along with the reaction mechanism in Section 8.

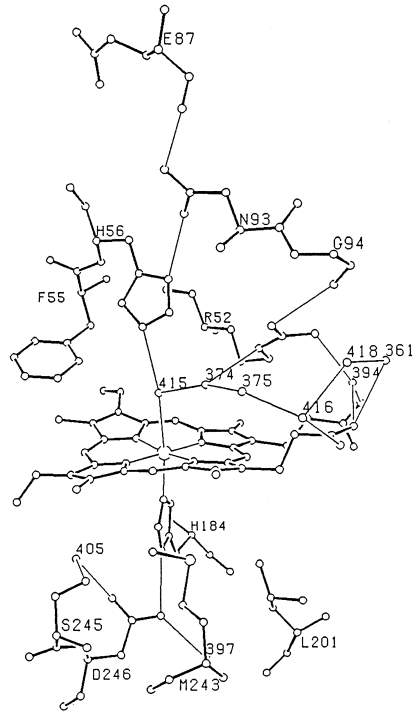

Fig. 4. Heme environment of ARP [4]. Recent analysis revealed that the electron density (shown with '415') observed at the sixth coordination site of heme at pH 7.5 was an ammonia molecule which arose from the precipitant used for crystallization. The ARP heme is in a pentacoordinated state under the physiological conditions [27].

8. Heme iron states and roles of amino acid residues during catalysis

The observed mechanistic and structural conservations between ARP and the plant peroxidase superfamily enzymes allow us to discuss the roles of the active site amino acids during ARP catalysis on the basis of the crystal structures of ARP and its complexes, along with the known catalytic mechanism of peroxidases belonging to the same superfamily (Fig. 5).

8.1. Coordination and spin states of resting form of ARP

Spectral and X-ray crystallographic analyses showed that, at physiological pHs (i.e., pH 6–7), the resting form of ARP contains a pentacoordinated high-spin ferric state of the heme iron; whereas, at alkaline pHs, it also contains a low-spin hexacoordinated state [27,28]. The proximal His184 serves as the fifth ligand to the heme iron and is suggested to play a role to stabilize the higher oxidation states of the iron necessary for the catalytic cycle [29]. The N^{δ} atom of the proximal His is hydrogen bonded with Asp246 which is well conserved among peroxidases and has been suggested to be functionally important in cytochrome *c* peroxidase [30]. Studies using a site-directed CiP mutant with Asn that substitutes for this Asp showed that this amino acid substitution significantly weakens the ligation of the proximal His to the heme iron and affects the heme iron's coordination and spin states as well as its pH dependences, suggesting that this conserved Asp is

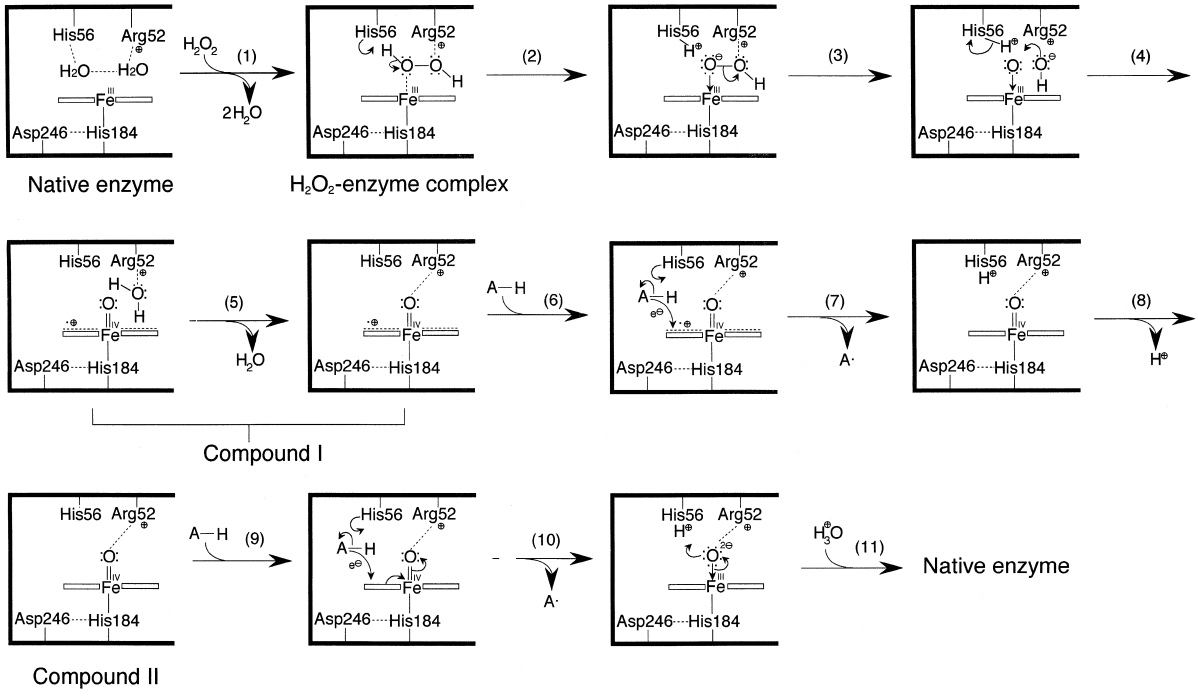

Fig. 5. Proposed mechanism of ARP catalysis as depicted by Fukuyama et al. [33] [for compound (I) formation, steps (1)–(4)] and Abelskov et al. [22] [for reductions of compounds (I) and (II), steps (6)–(11)] with modifications. To tie both proposed mechanisms, step (5) has been introduced in this scheme. The bold line represents the ARP protein and the horizontal open rectangles indicate the heme plane. The broken lines indicate weak interactions including hydrogen bonding. Spectral studies of peroxidases show that compound (I) contains an oxygenated Fe(IV) with the cation radical (shown by $\cdot\oplus$) delocalized on the protoporphyrin ring, as shown by the dotted line above the heme plane. It should be noted that the distal His is an alternative candidate as a hydrogen bond partner with the ferryl oxygen in compound (II), as suggested from HRP studies [21,53–55].

important in maintaining the heme iron atom in the pentacoordinated high-spin state with its sixth coordination site being vacant [28,31].

8.2. Mechanism of compound (I) formation

The distal side of the heme provides the catalytic site for peroxidase, and the conserved His and Arg residues therein have been shown to play very important roles during the catalysis of peroxidases [32]. Fig. 5 [steps (1)–(4)] outlines the mechanism of compound (I) formation proposed by Fukuyama et al. based on the results from their crystallographic studies of the ARP · I_3^- complex in which I_3^- mimics $^-$OOH [33]. Hydrogen peroxide enters into the distal cavity, breaking hydrogen bonds to displace two water molecules. During the binding of hydrogen peroxide to the distal pocket, the imidazole ring of the distal His56 rotates so that its N^{ε} atom moves toward the iron atom [33]. The distal His56 then serves as a general acid–base catalyst to translocate a proton from one oxygen atom to the other of the hydrogen peroxide to assist the coordination of $^-$OOH to heme iron and heterolytic cleavage of its O–O linkage, thus yielding compound (I). In the case of cytochrome *c* peroxidase, the distal Arg residue is suggested to move upon the binding of hydrogen peroxide and neutralize the negative charge of the bound $^-$OOH ion [34]. X-ray crystallographic studies suggested that this role of the distal Arg52 is also possible in ARP catalysis, although the movement of Arg52 during substrate binding is restricted by multiple hydrogen bonds [33]. The importance of this distal Arg in the anion binding has also been demonstrated from mutagenesis studies of peroxidases including CiP [32]; the stabilization of the anion-bound complex by interaction of the distal Arg's positively charged guanidium group with the bound anion is estimated to be 4 kcal mol^{-1}. The distal Asn93 in ARP corresponds to the conserved Asn residue that has been suggested for HRP to assist in O–O bond scission and also to play a role in the one-electron reduction of compound (I) by a hydrogen donor (see below) [35].

8.3. One-electron reductions of compounds (I) and (II)

In ARP, compound (I) undergoes two successive one-electron reductions by the hydrogen donor to yield the native peroxidase. It is assumed that several structural features of compounds (I) and (II) of cytochrome *c* peroxidase and HRP are also conserved in ARP [22]; the reduction of compounds (I) and (II) by a hydrogen donor probably occurs at the heme edge [36], and the ferryl oxygen is suggested to be hydrogen-bonded with the distal Arg throughout these processes [37–39]. Abelskov et al. analyzed the pH dependence of the reduction rates of CiP compounds (I) and (II) and attempted to identify the ionizable groups responsible for each catalytic process [22]. They found that the reduction rates of both compounds (I) and (II) are governed by two ionizations with pK_as of ~ 7 and ~ 9 (see Section 5 for details). They assigned these ionizations to the distal His and phenolic hydroxyl, respectively. On the basis of these observations, along with the possible structural conservations of compounds (I) and (II) among the heme peroxidases, they proposed reduction mechanisms for compounds (I) and (II) as shown in Fig. 5 [steps (6)–(11)] [22]. Distal His acts as a general acid–base catalyst during the two successive one-electron reduction processes; it abstracts a proton from hydrogen donor, denoted here by AH, followed by a concurrent one-electron transfer from AH to the porphyrin π-electron system and the release of A · [22]. This role of the distal His may be assisted by the conserved hydrogen-bonding network which is mediated by Asn93. The proton acquired from the first substrate is released to the solvent water, and the proton from the second substrate is available for the oxygen ligand of the ferryl iron, which is reduced to a ferric ion by accepting one electron from the second substrate. Finally, the second A · is re-

leased to the solvent and the ARP structure returns to the resting form.

The crystal structure of ARP in the complex with benzhydroxamic acid, which is an analog of aromatic hydrogen donor, has recently been elucidated [40], and afforded important information for insights into the interaction of the aromatic hydrogen donor with functional groups at the active site. The bound benzhydroxamic acid is positioned in the distal pocket, near the heme edge, with its hydroxamate function being hydrogen-bonded with His56N$^{\varepsilon}$, Arg52N$^{\varepsilon}$, and Pro154O. The aromatic ring of the bound benzhydroxamic acid is at the entrance of the channel to the heme pocket and in an approximately parallel orientation to the heme group, and is too far away from the heme iron to interact.

9. Applications

9.1. General considerations

Peroxidase has been used as an enzyme label for immunoconjugates and nucleic acid probes in enzyme immunoassay and non-radioisotopic hybridization techniques, respectively [41]. Although HRP had long been used for these purposes, ARP can also be used because it exhibits higher specific activities than does HRP while maintaining a broad substrate specificity for hydrogen donors as does HRP.

Other important applications of peroxidase include its use as a coupled enzyme in clinical examinations; peroxidase has been used in conjunction with oxidase-catalyzed reactions for the specific and sensitive determination of a variety of oxidase substrates of medical interest [42]. Oxidation of the substrate by a specific oxidase yields a stoichiometric amount of hydrogen peroxide. This then serves as a substrate for the peroxidase-catalyzed oxidation of a chromogenic hydrogen donor, the formation of which can be followed spectrophotometrically. Blood sugar (glucose) and serum cholesterol, for example, have been determined with glucose oxidase and cholesterol oxidase, respectively, according to this principle. ARP and HRP are equally efficient catalysts in these systems with the appropriate choice of a hydrogen donor. The oxidase/peroxidase system can also be assayed electrochemically. This is exemplified by the amperometric determination of D-amino acids using biosensors based on a carbon paste electrode chemically co-immobilized with ARP and D-amino acid oxidase [43]. The D-amino acid biosensor works successfully within the optimal potential ranges for amperometric sensors. A linear response range was obtained between 0.1 and 1.4 mM for D-phenylalanine and between 5 and 1000 μM for hydrogen peroxide at an applied potential of −50 mV.

HRP has been shown to be capable of removing toxic aromatic compounds in wastewater depending on the nature of the compounds. The *C. macrorhizus* peroxidase (= CiP and ARP) has been used in a batch reactor for this purpose [44]. The use of the fungal peroxidases would be of more practical advantage because their production on an industrial scale is more feasible.

Besides these general applications, the use of ARP allows the following unique applications, which are difficult to achieve with HRP.

9.2. Chemiluminescence analysis

Generally, luminescent analysis is 10^2–10^3 times more sensitive than the equivalent spectrophotometric method and shows wider calibration ranges, as exemplified by the luciferase-based bioluminescent determination of ATP. Thus, high-sensitivity determination of the oxidase substrate should, in theory, be possible with the peroxidase-based chemiluminescent system where a luminogenic hydrogen donor is used instead of a chromogenic one. However, in actual practice, this has been achieved using HRP with only limited success, mainly because of the low quantum yields of the HRP-catalyzed chemiluminescence reactions. This problem has been circumvented in part by introducing a

chemiluminescence enhancer, which is a co-substrate, into the HRP-based chemiluminescent system. Discovery of the high catalytic efficiency of ARP in the chemiluminescence reactions in the absence of the enhancer (see Section 4; Table 2) was the breakthrough that overcame these difficulties with HRP, and allowed the composition of the reaction mixture to be simplified. ARP was used in chemiluminescent assays for glucose and cholesterol (Fig. 6), which were 28 times and 134 times more sensitive, respectively, than the corresponding assays involving HRP [7]. ARP was also used for chemiluminescent enzyme immunoassays, in which ARP was shown to have a long-lived, high signal intensity with a high signal-to-noise ratio which permitted a high detection sensitivity [14].

9.3. Protein cross-linking

An ARP-catalyzed efficient protein cross-linking technique has been developed using bovine brain calmodulin as a model substrate [45]. Protein cross-linking is mediated by intermolecular dityrosine formation, and ARP was the only peroxidase with such catalytic competence. This can be considered as 'zero-length cross-linking', that is, the length of cross-linker is essentially zero. Oxidation by H_2O_2 of calmodulin with ARP under optimum conditions yielded a mixture of a variety of soluble dimers through the hexamers of calmodulin with a substantial degree of biological activity. This technique would be potentially useful in the food sciences because the cross-linking of food proteins has been shown to improve the physicochemical and functional properties of food materials [46].

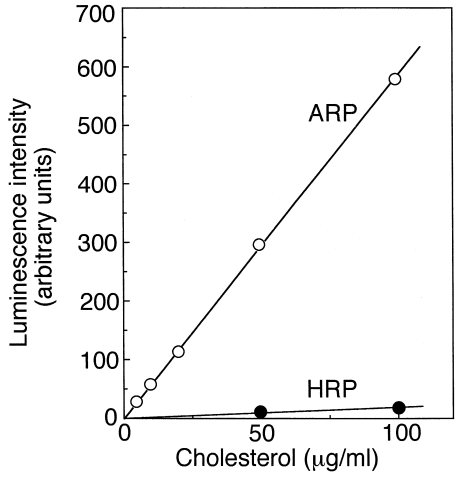

Fig. 6. Chemiluminescent determination of cholesterol with cholesterol oxidase/peroxidase systems. For experimental details, see Ref. [7].

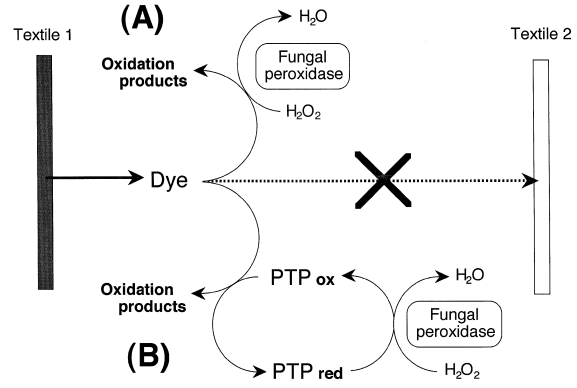

Fig. 7. Mechanism of enzymatic dye-transfer inhibition system. (A) Mechanism without phenothiazine-10-propionic acid (PTP). (B) PTP-mediated mechanism.

9.4. Enzymatic dye-transfer inhibition technology

A peroxidase-based system for the inhibition of dye transfer during washing with laundry detergents has recently been developed [47]. CiP (or ARP) has been selected as the best catalyst for this system on the basis of its excellent catalytic properties, stability, and commercially viable productivity.

Fig. 7 shows the reaction mechanism of the dye-transfer inhibition system. Dyes released from textiles during washing are oxidized by

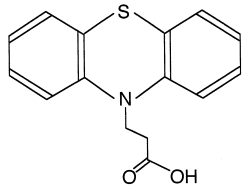

Fig. 8. Structure of phenothiazine-10-propionic acid.

hydrogen peroxide with the aid of CiP and subsequently decolorized [Fig. 7, mechanism (A)]. Although a great many colored substances are resistant to oxidation by this mechanism (A), introducing another substrate for CiP, called the 'mediator', to the system has been found to enhance greatly the oxidation reaction. This is probably due to the mechanism in which CiP oxidizes the mediator with hydrogen peroxide and the resultant activated oxidized form of the mediator efficiently oxidizes colored substances [mechanism (B)]. Phenothiazine-10-propionic acid (Fig. 8), for example, has been shown to be an excellent mediator, which shows very good performance in the dye-transfer inhibition system and is toxicologically and ecologically safe at the levels present. The overall effect of dye transfer inhibition, in reality, should arise from both mechanisms (A) and (B). It should be noted that the activity of the peroxidase system towards dyes on the fabrics is negligible.

Until now, a nonenzymatic method has been employed to prevent dye transfer during washing. This nonenzymatic method uses synthetic polymers, such as poly(1-vinyl-2-pyrrolidone), which are thought to complex the dye molecules in solution, thereby reducing the affinity of dyes for the textile fibers. A comparison of the enzymatic and nonenzymatic dye-transfer inhibition systems showed that the enzymatic method is, in many cases, more effective than the nonenzymatic method [47].

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