Direct Binding of Hydroxylamine to the Heme Iron of Arthromyces ramosus Peroxidase

SUBSTRATE ANALOGUE THAT INHIBITS COMPOUND I FORMATION IN A COMPETITIVE MANNER*

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The interaction of hydroxylamine (HA) with Arthromyces ramosus peroxidase (ARP) was investigated by kinetic, spectroscopic, and x-ray crystallographic techniques. HA inhibited the reaction of native ARP with \( \text{H}_2\text{O}_2 \) in a competitive manner. Electron absorption and resonance Raman spectroscopic studies indicated that pentacoordinate high spin species of native ARP were converted to hexacoordinate low spin species upon the addition of HA, strongly suggesting the occurrence of a direct interaction of HA with ARP heme iron. Kinetic analysis exhibited that the apparent dissociation constant is 6.2 mM at pH 7.0 and that only one HA molecule likely binds to the vicinity of the heme. pH dependence of HA binding suggested that the nitrogen atom of HA could be involved in the interaction with the heme iron. X-ray crystallographic analysis of ARP in complex with HA at 2.0 Å resolution revealed that the electron density ascribed to HA is located in the distal pocket between the heme iron and the distal His\(^{39} \). HA seems to directly interact with the heme iron but is too far away to interact with Arg\(^{22} \). In HA, it is likely that the nitrogen atom is coordinated to the heme iron and that hydroxyl group is hydrogen bonded to the distal His\(^{39} \).

In the past few years, there has been a rapid growth in crystallographic structural information on fungal and plant peroxidases. In addition to cytochrome \( c \) peroxidases, whose structure has been known for over 10 years (1), the structures of lignin peroxidase (LiP)\(^1\) (2, 3), Arthromyces ramosus peroxidase (ARP) (4), manganese peroxidase (5), peanut peroxidase (6), chloroperoxidase (7), barley peroxidase 1 (8), ascocarype peroxidase (9), and horseradish peroxidase (HRP) (10) are now available. These peroxidases exhibit great topological similarity to each other, despite a low level of sequence identity (10). Even though the arrangement of helices among peroxidases resembles each other from fungi to plants, the detailed structures of the active sites as well as the catalytic features of peroxidases differ significantly. The crystal structures of ARP (Coprinus cinereus peroxidase) and LiP are highly similar, exhibiting 1.25 Å root mean square difference in Ca atoms; nevertheless, the substrate specificity and optimal pH are considerably different (3, 4, 11–14). Extensive studies on the structure-function relationship, especially the elucidation of substrate binding sites and detailed binding mechanisms, would be a key to better understand the catalytic mechanism of peroxidases. From the inhibitory damage to the heme of HRP, it has been suggested that the binding sites of the aromatic substrates are located near the heme in the vicinity of the \( \delta \)-meso carbon and 8-methyl group of the heme (15, 16). This region is exposed to solvent in many peroxidases. Very recently, the substrate oxidation sites of LiP were suggested by utilizing a surface plasmon resonance spectroscopy (17) and a site-directed mutagenesis technique (18). However, limited information on the substrate binding mode hampered the explanation of these results.

The substrates for most plant and fungal peroxidases are small molecules such as aromatics, organic acids, halides, and metal complexes. Elucidation of the binding modes of such small molecules to peroxidases, except for a few examples, has not been successful because of their weak binding. Benzydrolamie acid (BHA) is known to form kinetically tight complexes with most peroxidases, in particular with HRP isozyme C (19). The crystallographic analyses of ARP-BHA complex (20) and HRP isozyme C-BHA complex (21) showed unequivocally how BHA binds to the distal pocket in both enzymes. The hydrophilic portion of BHA forms hydrogen bonds to the distal catalytic His and Arg and to backbone oxygen of Pro in ARP. The hydrogen bonds to these residues were also observed in HRP isozyme C-BHA complex, although the side chain atom of Arg involved in the hydrogen bond differs from that in ARP. A large reorientation of Phe\(^{39} \) near the access channel upon BHA binding was exhibited in HRP isozyme C (21), which may account for extraordinary high affinity of BHA to the enzyme. Recently, the binding mode of salicylhydroxamic acid to ARP was reported to be very similar to that of BHA to ARP (22). The crystal structure of ARP-iodide complex prepared by the soaking method showed that a single iodide ion is located at the entrance of the access channel to the distal pocket and lies 12.8 Å apart from the heme iron (23). Interestingly, the binding site of iodide is different from that of BHA. For HRP, the binding mode of the naturally occurring substrate, ferulic acid, to the
enzyme-CN complex was recently reported, indicating the involvement of the distal Arg in the stabilization of substrate binding (24). On the other hand, the binding mode of the oxidizing substrate to peroxidases is still unclear.

In the present work, we have found that hydroxylamine (HA) specifically binds to ARP during the course of surveying other organic substrate analogues bound to ARP. HA is an interesting probe to characterize the substrate binding site, because its structure resembles the hydrophilic moiety of BHA (Scheme 1). In addition, because the molecular size and shape of HA somewhat resembles H₂O₂, the binding mode of this small compound to ARP may suggest the binding mode of H₂O₂ to peroxidases. Therefore, we investigated in more detail the inhibitory effect and binding mode of HA using kinetic, spectroscopic, and x-ray crystallographic techniques. Here, we report that HA is located between the distal His and heme iron and that it directly binds to the heme iron, causing a competitive inhibition against the oxidizing substrate.

MATERIALS AND METHODS

Enzymes—ARP was isolated from the extracellular culture medium of A. ramosus as described previously (25). All the purified proteins exhibited a single peak on Mono Q column (Amersham Pharmacia Biotech) chromatography. The sample had an RZ value (A₄₀₀nm/A₂₈₀nm) of 2.5. The concentration of ARP was determined using ε₄₇₀ of 109 mM⁻¹ cm⁻¹ (26).

The enzyme was crystallized as described previously (25). The crystals of ARP in complex with HA were prepared by soaking the native ARP crystals. HA Binding—UV-visible absorption spectra were recorded using Perkin-Elmer Lambda 19 spectrophotometer at room temperature. To measure difference spectra, both the reference and sample cuvettes were obtained on a SpectraPro-300i (Acton Research Co.) spectrophotometer at room temperature. To measure difference spectra, both the reference and sample cuvettes were obtained on a SpectraPro-300i (Acton Research Co.) spectrophotometer. The (2ₐ) and ABTS oxidation was determined at 469 nm using 8-quino- linol (Wako Pure Chemicals) (27, 28). 8-Quinolinol was recrystallized from ethanol/hexane before use.

Steady State Kinetics—The initial rate of 2,6-dimethoxyphenol (DMP) and ABTS oxidation was determined at 469 nm using ε of 49.6 mM⁻¹ cm⁻¹ (29) and 36.8 mM⁻¹ cm⁻¹ at 414 nm (30), respectively. Reaction mixtures contained ARP (3 μM), HA (50–800 μM), DMP (0–20 mM), or ABTS (50–300 μM) in 50 mM phosphate, pH 7.0. The reaction was initiated by the addition of H₂O₂. Initial rate versus 1/[H₂O₂], 1/[DMP], or 1/[ABTS] was determined at various fixed concentrations of HA (0–400 μM).

Resonance Raman Spectroscopic Analysis—Resonance Raman (RR) spectra were obtained on a SpectraPro-300i (Acton Research Co.) spectrograph (operating at 3600-groove grating) using a Spectra-Physics Beamlock 2060 Kr ion laser (413.1 nm), a Kaiser Optical holographic spectrophotograph (operating at 3600-groove grating) using a Spectra-Physics Beamlock 2060 Kr ion laser (413.1 nm), a Kaiser Optical holographic spectrophotograph and a Princeton Instruments (LN-1100PB) liquid N₂-cooled CCD detector. Incident laser power at the sample was 15 mW. Spectra were collected in a 90° scattering geometry from solution samples contained in glass spinning cell (2-cm diameter, 1500 rpm) tubes for 5 min at room temperature. Spectral resolution was set to 3 cm⁻¹. Peak frequencies were calibrated relative to indene and accurate to ± 0.1 cm⁻¹. During Raman experiments, UV-visible spectra were simultaneously collected on Photal MCD-2000 diode array spectrometer with Photal MC-2530 as a light source. Data were calibrated and analyzed with GRAMS/386 spectroscopic software (Galactic Industries Corp.). The concentration of ARP for RR studies was 100 μM in 100 mM phosphate buffer, pH 7.0, in the presence or absence of 100 μM HA.

Diffraction Data Collection—Diffraction data on the complex crystal were collected at room temperature on a R-AXIS IV imaging plate area detector. CuKα radiation from a Rigaku rotating anode generator was monochromatized with nickel-filter and focused with double-bent mirrors. The diffraction data recorded in each imaging plate were read out at 100-mm intervals and then processed using PROCES (31). Intensities of the partial reflections recorded on two adjacent imaging plates were combined to obtain the integrated intensities. The conditions and results of data collection are shown in Table I.

Structure Determination—The atomic parameters of ARP at pH 4.5 refined at 1.8 Å resolution (32) were used for the structural refinement, several water molecules near the heme being excluded. The parameters were refined using the observed diffraction data of the complex by the program XPAR (33). The locations of water molecules were revised by alternate cycles of XPAR refinement and inspection of the (2Fₒ - Fᵣ) and (Fᵣ - Fₛ) maps with TURBO-FRODO (34) and Indigo2 workstation. The (2Fₒ - Fᵣ) and (Fᵣ - Fₛ) maps, in which Fᵣ and the phase angles were calculated with the resulting parameters, clearly showed electron density ascribable to the HA molecule. A HA model was fitted manually to the maps. The final model contains 211 water molecules in addition to the protein and HA. The results of crystallographic refinement are shown in Table I. There is no unusual conformation as indicated by the Ramachandran plot. The atomic parameters have been deposited in the Protein Data Bank (code 1CSI).

RESULTS AND DISCUSSION

Interaction of Hydroxylamine to ARP—Upon the addition of the excess HA to native ARP, the Soret peak was shifted from 403 to 414.5 nm with a change in the visible region (500 to 540 and 570 nm), suggesting the occurrence of a change in heme environment (Fig. 1). The Soret peak of HA complex was not shifted in the pH range of 5–8 and was stable for several hours
competitive inhibition, from which $Ki$ that HA is a true competitive inhibitor for H$_2$O$_2$, because inhibitions of the secondary plot, slope versus $A_D$ were calculated from the following equation, $KD(app)$ was calculated from Equation 1.

\[ \Delta A = \Delta A_a - \Delta A_i = h \cdot \log [S] + \log K_{D(app)} \] (Eq. 2)

where $\Delta A_a$ and $K_{D(app)}$ were calculated from Equation 1. A plot of $\log [\Delta A/(\Delta A_a - \Delta A_i)]$ against log [S] exhibited a straight line with a slope ($h$, Hill coefficient) of 0.99, suggesting the binding of single HA to the native enzyme near the heme. However, a possible binding of other HA molecule(s), which is too far from the heme to affect the Soret absorption, could not be omitted. Inhibitory Effect of Hydroxylamine on ARP Reactions—If HA acts as a substrate analogue, it should inhibit the normal catalytic reaction of ARP. Therefore, the inhibitory effect of HA against either oxidizing substrate or reducing substrates was studied using steady state kinetics. To avoid the kinetic complication, the concentration of one substrate was fixed in excess (at least twice as much as $K_m$). The family of plots, $1/v$ versus $1/[S]$, $1/[DMP]$, or $1/[ABTS]$ at various fixed concentrations of HA intercepted on the ordinate. Furthermore, a linear relationship of the secondary plot, slope versus [HA] demonstrated competitive inhibition, from which $K_D$ was calculated (Table II). Comparing these data with the $K_D(app)$ value, it is concluded that HA is a true competitive inhibitor for H$_2$O$_2$, because $K_i$ and $K_D(app)$ values sit in the same range. HA also acts as an inhibitor for the oxidation of DMP and ABTS, but the inhibition against those reducing substrates occurred with very small $K_i$ values (Table II). HA may act as an apparent competitive inhibitor against DMP and ABTS, probably because HA binding might influence the electron transfer reaction for the oxidation of those reducing substrates. The type of inhibition was somehow different for DMP and ABTS, which might suggest that the binding sites for these two substrates are different. DMP is a phenolic compound so that it is neutral at neutral pH. On the other hand, because of the sulfonate group, ABTS shows a strong anionic character at neutral pH. Very recently, it has been reported that ABTS and other anionic substrates have different oxidation sites on the LiP protein from its preferable substrate oxidation site such as veratryl alcohol (18). Importantly, for ARP, HA acts as a competitive inhibitor against H$_2$O$_2$, which strongly supports the possibility that HA may directly interact with the heme iron.

Resonance Raman Spectroscopy—Because a RR spectral method is very sensitive to the change in spin and coordination states of heme iron, an RR spectrum of ARP-HA was compared with that of native ARP. Fig. 2 shows the RR spectra in the high frequency region for the native ferric ARP and ARP-HA complex with Soret excitation. The RR bands of the native ARP at 1371, 1494, 1567, and 1625 cm$^{-1}$ are identical to previously reported RR bands for C. cinereus peroxidase (36). In that study, it was also mentioned that C. cinereus peroxidase was found to be unstable in the laser beam. However, in the present study, ARP was stable to exhibit the above mentioned native ferric marker bands for several hours even at room temperature and using a higher laser power of 15 mW. We believe that it might be caused by the difference in the efficiency of rotary cells for the measurement but not by the difference in the enzyme samples.

Upon the addition of HA, the marker bands indicative of hexacoordinate low spin heme appeared ($v_2$ at 1504 cm$^{-1}$, $v_2$ at 1575 cm$^{-1}$, and $v_10$ at 1636 cm$^{-1}$) that were almost identical to the hexacoordinate low spin marker bands previously reported (36). On the other hand, the ferric marker band at 1371 cm$^{-1}$ and $v(C=C)$ at 1625 cm$^{-1}$ were not shifted. We reported that the binding of ammonia to the heme iron of ARP (32), and the direct interaction of HA is not surprising.

pH Dependence of HA Binding to ARP—$K_D(app)$ for HA binding to ARP was plotted over the pH range of 5–8 as described above. Plots of $K_D(app)$ against pH are shown in Fig. 3. Above pH 5, $K_D(app)$ decreased with increasing pH, indicating pH-dependent binding of HA to ARP and better binding at higher pH. Possible ionizable group(s) controlling the pH dependence observed in the pH range for this experiment could be the amino group of HA and/or the amino acid residue(s) in heme pocket of ARP. In the distal pocket, there are two ionizable amino acid residues, His$^{56}$ (distal His) and Arg$^{52}$. The $pK_a$ of the distal His in C. cinereus peroxidase has been reported to be lower than 1 (37). The $pK_a$ of Arg seemed to be out of the range utilized here. To simplify the equation for pH dependence of HA binding to the heme of ARP, the ionization of amino acid residues in the distal pocket were omitted, and the binding form of HA was assumed to be NH$_2$OH because of the better binding at higher pH (Fig. 3). Then the binding equation could be described using Equations 3 and 4.

**TABLE II**

| Inhibitor | Substrate | Type of inhibition | $K_i$ |
|-----------|-----------|--------------------|-------|
| HA        | H$_2$O$_2$| Competitive        | 2.46 mM |
| DMP       | Competitive| 184 μM |
| ABTS      | Mixed     | 215 μM |
| BHA       | H$_2$O$_2$| Noncompetitive    | 171 μM |
| DMP       | Noncompetitive| 44.4 μM |

2 T. Johjima, H. Wariishi, and H. Tanaka, submitted for publication.
The ARP crystal soaked in HA solution was isomorphous with the native crystal. No significant change in the conformation of the main chain and side chains of ARP was observed upon the binding of HA, unlike in HRP, where a large reorientation of Phe$_{68}$ occurred upon the addition of BHA (21). The difference Fourier map (Fig. 4) exhibits the significant electron density at the distal heme pocket between heme iron and the distal His. Since the experimental conditions taken in the present crystallographic study (pH 5.6), ammonia derived from ammonium sulfate does not bind to ARP (32), the electron density observed in ARP-HA complex (Fig. 4) is most likely caused by exogenously added HA. Furthermore, all the data obtained from spectral and kinetic experiments indicated the occurrence of the direct interaction of HA with the heme iron. We assumed that the nitrogen atom of HA binds to the heme iron. Although the current resolution of the x-ray analysis is marginal to define the orientation of HA, HA was nearly parallel to the heme plane. The electron density corresponding to water molecule was not seen near the distal His of ARP-HA complex; although it was clearly seen in the native ARP (32). The distal water molecule may be replaced by HA. As the deviation from the spherical shape of the density was insignificant at the current resolution of 2 Å, the HA model fitted to the density is tentative in its orientation. The current binding mode of HA to ARP is shown in Fig. 5. The nitrogen atom is coordinated to heme iron (2.5 Å), and the hydroxyl group is involved in a hydrogen bond with the distal His$_{56}$ (2.8 Å). The distance between HA and Arg$_{52}$ is too far (>4 Å) to form a hydrogen bond.

**Substrate Binding Site of ARP**—The crystal structure of ARP-BHA complex reported by Itakura et al. (20) suggests that one of the major factors controlling the binding mode of BHA with ARP is the hydrogen bonding network caused by the side chain functional groups and distal amino acid residues. Although HA possesses similar functional groups to those of BHA, it binds to ARP in a totally different way (Fig. 5). The difference between HA and BHA is whether they contain aromatic moiety or not (Scheme 1). Therefore, the comparison of the binding modes of HA and BHA to ARP would provide an important information on the binding site for aromatic substrates. Presumably, the aromatic moiety of BHA plays a more important role in its binding to ARP. The role of aromatic moiety of BHA seems to be the hydrophobic interaction with hydrophobic amino acid residues of ARP. The aromatic ring of BHA is surrounded by Pro$_{31}$, Gly$_{84}$, Ile$_{153}$, Gly$_{155}$, Pro$_{156}$, Gly$_{191}$, Leu$_{192}$, and Phe$_{230}$ (20). These amino acid residues form a hydrophobic circle, likely providing hydrophobic interaction with other hydrophobic compounds like aromatics. Therefore, BHA was fixed near the entrance of the distal heme pocket, whereas HA was not trapped by these hydrophobic residues and directly binds to the heme iron. The involvement of those hydrophobic amino acid residues was recently suggested from the molecular dynamic calculation for the binding of a series of aromatic substrates to ARP (22). The direct binding of HA to the heme iron resulted in the competitive inhibition against the
reaction of H$_2$O$_2$ with native ARP. On the other hand, BHA caused noncompetitive inhibition against the reaction of H$_2$O$_2$ with native ARP (Table II), probably because the distal entrance for H$_2$O$_2$ was concealed. Furthermore, the size of the distal entrance of ARP is the largest among fungal peroxidases whose crystal structures have been clarified (3–5). The broad substrate specificity of ARP would be explained by a loose aromatic binding site.

The interaction of HA with the heme iron of LiP and manganese peroxidase occurred, whereas no spectral shift was observed upon the addition of HA to HRP (data not shown). Because the distal entrance of HRP is definitely larger than LiP and manganese peroxidase (3, 5, 10), the binding features of HA to the heme iron of peroxidases cannot be explained by the size of the distal entrance. So far, the direct interaction of HA to the heme iron was observed with fungal peroxidases. Although further studies would be required, HA may be an interesting probe to analyze the distal environment of peroxidases.

**Hydroxylamine as a H$_2$O$_2$ Model**—The molecular size and shape of HA resembles H$_2$O$_2$ (Scheme 1). It has been very difficult to characterize the complex between native peroxidase and H$_2$O$_2$, which was retarded by a fast reaction of the heterolytic cleavage of peroxide by peroxidases to form compound I species. The binding mode of this small compound to ARP may suggest the binding mode of H$_2$O$_2$ to peroxidases.

Because HA acts as a competitive inhibitor against H$_2$O$_2$ and directly binds to heme iron in the distal pocket, ARP-HA complex may serve as a compound 0 model. Compound 0 was proposed to be a hyperporphyrin formed by deprotonation of an H$_2$O$_2$-peroxidase (ferric heme iron) complex (39–41). The cryoenzymological technique was utilized, showing that this intermediate exhibits Soret bands at 360 and 410 nm and a weak band in the visible near 570 nm (40). In the present study, HA-ARP complex exhibits absorption bands at 360, 414.5, 540, and 570 nm. The hydrogen bond between the hydroxyl group of HA and the distal His$^{56}$ may facilitate the deprotonation of HA. Further spectroscopic and kinetic studies as well as high resolution x-ray analysis to clarify the role of HA in the catalytic action are now under way.

A number of works have been directed toward elucidating the substrate binding sites of many peroxidases (15–18, 20, 21, 23, 24, 35, 42–44). In the present study, we found a novel substrate analogue, HA, which forms a stable complex with ARP. Because HA is such a small compound, x-ray was insufficient to identify the electron density observed upon the addition of HA to ARP. We combined kinetic, spectroscopic, and x-ray crystallographic techniques to probe the heme environment and the binding mode of HA to ARP. Comparison of the binding modes of HA and BHA to ARP suggested the binding site for aromatic substrates. In addition, from the similarity of molecular size and shape of HA with those of H$_2$O$_2$ and the binding mode of HA to ARP, we proposed HA-ARP complex as the structural model for peroxidase compound 0.

**REFERENCES**

1. Finzel, B. C., Poulos, T. L., and Kraut, J. (1984) *J. Biol. Chem.* **259**, 13027–13036
2. Piontek, K., Glumoff, T., and Winterhalter, K. (1993) *FEBS Lett.* **315**, 119–124
3. Poulos, T. L., Edwards, S. L., Wariishi, H., and Gold, M. H. (1993) *J. Biol. Chem.* **268**, 4429–4440
4. Kunishima, N., Fukuyama, K., Matsubara, H., Hatanaka, H., Shibano, Y., and Amachi, T. (1994) *J. Mol. Biol.* **235**, 331–344
5. Sundaramoorthy, M., Kishi, K., Gold, H. M., and Poulos, T. L. (1994) *J. Biol. Chem.* **269**, 32759–32767
6. Schaller, D. J., Ban, N., van Huystee, R. B., McPherson, A., and Poulos, T. L. (1996) *Structure* **4**, 311–321
7. Sundaramoorthy, M., Terner, J., and Poulos, T. L. (1995) *Structure* **3**, 1567–1577
8. Henrikson, A., Welinder, K. G., and Gajhede, M. (1998) *J. Biol. Chem.* **273**,
