Binding mode of benzhydroxamic acid to *Arthromyces ramosus* peroxidase shown by X-ray crystallographic analysis of the complex at 1.6 Å resolution

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Abstract The crystal structure of *Arthromyces ramosus* peroxidase (ARP) in complex with benzhydroxamic acid (BHA) as determined by X-ray analysis at 1.6 Å shows unambiguously how BHA binds to ARP. BHA is located in the distal heme pocket. Its functional groups are held by three hydrogen bonds to $His^{56}N_{\varepsilon}$, $Arg^{52}N_{\varepsilon}$, and $Pro^{154}O$, but are too far away to interact with the heme iron. The aromatic ring of BHA is positioned at the entrance of the channel to the heme pocket, approximately parallel to the heme group. Most water molecules at the active site of the native enzyme are replaced by BHA, leaving a ligand, probably a water molecule, at the sixth position of the heme. Results are compared with spectroscopic data.

Key words: Peroxidase; Heme enzyme; Benzhydroxamic acid; X-ray crystallography; *Arthromyces ramosus*

1. Introduction

The generally accepted, primary function of heme peroxidases, including *Arthromyces ramosus* peroxidase (ARP), is to catalyze the conversion of hydrogen peroxide to water [1]. The reaction, initiated by hydrogen peroxide, provides two oxidizing equivalents to the resting heme group and yields compound I, an oxy-ferryl-[Fe(IV) = O] porphyrin radical cation. Compound I returns to the resting state, via an another intermediate of compound II, completing the catalytic cycle by the use of such reducing agents as halide ions, aromatic donor molecules, and electron transfer proteins, depending on the particular system. The concomitant oxidative reactions which activate halide ions or aromatic molecules are related to such other functions of peroxidases as the syntheses of hormones and lignin [2–4].

To understand the enzyme reaction mechanism, it is necessary to determine the binding sites of the reducing substrates on peroxidases. Recently, using X-ray analysis [5], we showed unequivocally the iodide binding site on ARP, a class II peroxidase of the plant peroxidase superfamily [6]. Sufficient spectroscopic data have been accumulated for the aromatic donor molecules to assume that they form complexes with peroxidases [7–12]. In particular, benzhydroxamic acid (BHA, Fig. 1), a competitive inhibitor for the usual donor molecules to horseradish peroxidase (HRP), has been used in studies of the aromatic donor molecule binding site because it forms a tight complex with HRP [7–13]. The NMR study of HRP by Morishima and Ogawa [8] showed that the unique binding site of the aromatic donors is located near the heme edge. Subsequent NMR studies of HRP, including studies using indole-3-propionic acid [10] and methyl substituted BHAs [11], have provided more detailed information about the binding modes of aromatic compounds [9,12]. The hydrogen bonding of BHA with active site residues and the environment of the heme iron also have been investigated for the recombinant HRP-C and its mutants by resonance Raman spectroscopy [14]. Some of the findings, however, are incompatible. For example, Sakurada et al. reported that the aromatic ring is nearly perpendicular to the heme plane [9], whereas later research suggests that they are roughly parallel to each other. Strangely, few X-ray crystallographic studies have been made on peroxidases that are in a complex with aromatic compounds; only the human myeloperoxidase-salicylhydroxamic acid (SHA) complex has been studied [15].

Here we report the clear determination of the binding mode of BHA to ARP as shown by the crystal structure of the complex. This is the first report of a member of the plant peroxidase superfamily in a complex with an aromatic compound. We also compare our structural findings with the structure of the myeloperoxidase-SHA complex and discuss the spectroscopic data for complexes of HRP with aromatic compounds.

2. Experimental

2.1. Preparation of BHA complex crystals

ARP was purified and crystallized as described elsewhere [16]. The crystals of ARP in a complex with BHA were prepared by soaking native ARP crystals for 19 h in 50 mM ammonium acetate buffer adjusted to pH 5.5 and containing 21 mM BHA and 35% saturated ammonium sulfate. Its crystals belong to space group $P4_22_12$ with $a = b = 74.5$ Å and $c = 118.0$ Å, and it has one ARP molecule in the asymmetric unit.

2.2. Data collection

Diffraction data on the complex crystal were collected at 20°C on a screenless Weissenberg camera for macromolecular crystals and by synchrotron radiation [17] at the BL6A2 of the Photon Factory, the National Laboratory for High Energy Physics. X-rays were focused with a cylindrical-bent asymmetric cut Si(111) monochromator. The collimator had a diameter of 0.1 mm. The radius of the film cassette, which contained a Fuji Imaging Plate (IP) with a detection area of 40 cm × 20 cm, was 286.5 mm. The complex crystal was stable to X-rays, and intensity data were collected for one crystal. Diffraction data recorded on each IP were read out at 100 μm intervals using a Fuji BAS2000 then processed with the program systems DENZO [18] and SCALEPACK [19]. The conditions and results of data collection are shown in Table 1A.

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Abbreviations: ARP, *Arthromyces ramosus* peroxidase; BHA, benzhydroxamic acid; HRP, horseradish peroxidase; SHA, salicylhydroxamic acid; IP, imaging plate

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Table 1
Summary of data collection and crystallographic refinement

(A) Conditions and results of data collection	
Wavelength (Å)	1.00
Oscillation angle/IP (°)	2.5
Exposure time/IP (s)	25
Number of IPs	25
Resolution limit (Å)	1.5
Measured reflections	225 152
Independent reflections	47 193
Completeness (%)[a]	87.6
R_{merge} (%)[b]	4.7
(B) Results of crystallographic refinement	
Resolution range (Å)	7.0–1.6
Number of atoms refined[c]	2 789 (242)
Number of reflections ($F > 2\sigma_F$)	39 924
R-factor (%)	18.9
R-free (%)	22.7
Root-mean-square deviations from ideal values	
Bond distances (Å)	0.018
Angle distances (Å)	0.049
Planar groups (Å)	0.024
Chiral volumes (Å^3)	0.135
Torsion angles of ω (°)	3.2

[a]After scaling and averaging the intensities of the equivalent reflections, those with $F < \sigma_F$ were rejected.
[b]$R_{merge} = \Sigma_i \Sigma_h |I_i(hkl) - <I(hkl)>| / \Sigma_i \Sigma_h <I(hkl)>$
[c]Numerals in parentheses are the number of water molecules.

2.3. Structure determination

The atomic parameters of ARP at pH 7.5, refined at 1.9 Å resolution (PDB code 1ARP) [20], were used to start 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 XPLOR [21]. The $(2F_O - F_C)$ and $(F_O - F_C)$ maps, in which F_C and the phase angles were calculated with the resulting parameters, clearly located the BHA molecule. A BHA model [22] was fitted manually to the maps with TURBO-FRODO [23] and an IRIS 4D/35GT computer graphics system. The occupancy of BHA was assumed to be 1.0.[1] The protein model and locations of water molecules were revised by alternate cycles of XPLOR refinement and inspection of the $(2F_O - F_C)$ and $(F_O - F_C)$ maps. The final model contains 242 water molecules in addition to the protein and BHA. The results of crystallographic refinement are shown in Table 1B. The mean coordinate error was estimated to be about 0.2 Å from a Luzatti plot [25]. The atomic parameters have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY (PDB code 1HSR).

3. Results and discussion

3.1. Binding mode of BHA to ARP

The difference Fourier map shown in Fig. 2 shows the significant electron density ascribable to BHA. The density is clear enough to determine the orientation of BHA unambiguously, and the BHA model is easily fitted to it. The BHA binding mode to ARP subsequently was refined and confirmed with the program XPLOR, in which no restraint was given to the torsion angle of the C(1)–C(7) bond of BHA.

The mode of BHA binding to ARP is shown in Fig. 3. The ARP crystal soaked in BHA solution was nearly isomorphous with the native one. No significant conformational change occurred on BHA binding, except for the three residues surrounding it. The BHA molecule is located in the cavity on the distal side of the heme and is nearly parallel to the heme. All three functional groups of BHA are involved in hydrogen bonds with the amino acid residues of ARP, His^{56}, Arg^{52}, and Pro^{154}. It is noteworthy that on BHA binding the side and main chains of Arg^{52}, His^{56}, and Pro^{154} shifted to form hydrogen bonds with BHA that had more favorable geometries: $His^{56}N_\varepsilon$, $Arg^{52}N_\varepsilon$, and $Pro^{154}O$ moved, respectively, by 0.16, 0.74, and 0.21 Å relative to their positions in the native state. The benzene ring of BHA is situated at the entrance of the heme cavity. When viewed down to the heme plane, the center of BHA is above the methyl group bonded to the 18 position of the heme (Fig. 3).

The water molecules in the active site of ARP were rearranged when BHA bound, four water molecules in the distal heme pocket in the native ARP being replaced by BHA. Consequently, only one significant peak (415) was present on the $(F_O - F_C)$ map on the distal side of the heme,[2] but its position deviates by 0.56 Å from that of the water molecule bound to $His^{56}N_\varepsilon$ in the native state. In the complex 415 forms a hydrogen bond with the hydroxyl group of BHA. Although in the native ARP the water molecule that formed a hydrogen bond with $His^{56}N_\varepsilon$ was too far away to interact with the heme iron [26], in the complex a hydrogen bond between 415 and $His^{56}N_\varepsilon$ is unlikely, 415 being close to the heme iron. It looks as if BHA pushed the distal water molecule above the heme iron and disrupted its hydrogen bond with $His^{56}N_\varepsilon$. It should be noted that none of the BHA atoms are in direct contact with the heme iron.

3.2. Comparison of the complex structures

Davey and Fenna reported on the binding of SHA to human myeloperoxidase [15], a heme peroxidase in which the arrangement of the residues comprising the active site differs markedly from that of the plant peroxidase superfamily. In the myeloperoxidase-SHA complex, the functional groups of SHA are located inside the distal pocket, the aromatic ring being approximately parallel to the heme plane. In this com-

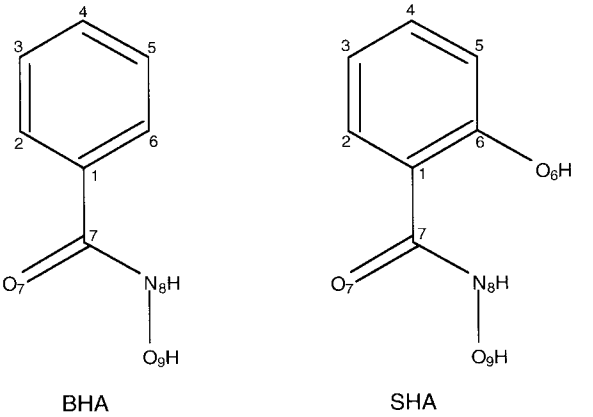

Fig. 1. The BHA and SHA structures with the atom numberings used in the text.

[1] The K_d value for *Coprinus cinereus* peroxidase, a peroxidase nearly identical to ARP, is reported to be 3.7 mM [24]. A similar K_d value was obtained for ARP (T. Hosoya, personal communication). Assuming that K_d=3.7 mM, the ratio of the BHA bound form of ARP is estimated to be about 85%.

[2] The electron density peak at the sixth position of the heme iron is probably that of a water molecule. The possibility, however, cannot be excluded that this peak is due to bound ammonia derived from the ammonium sulfate used as the precipitant because the heme iron of ARP tends to bind to ammonia [26].

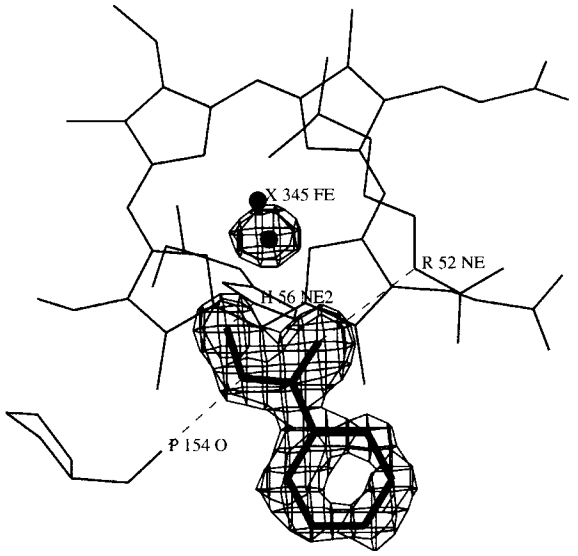

Fig. 2. (F_O-F_C) map for the BHA-bound form of ARP. The F_C and phase angles were calculated from all the atomic parameters except those of BHA and the solvent molecule. The final model is superimposed. The contour level for the electron density is 4.5σ. The peak height of the density at the sixth position of the heme iron is 9.7σ.

plex the hydroxyl group forms a hydrogen bond with the N_ε of the distal histidine, as in the ARP-BHA complex, but SHA extends farther into the active site than does the ARP-BHA complex. In the myeloperoxidase-SHA complex the hydroxyl group is above the heme iron, and there is no water molecule on the distal side of the heme iron.

Extensive NMR studies have been done to determine the binding mode of aromatic compounds including BHA to HRP [7–12]. Most support that BHA binds to the distal side of the heme extending to the heme iron and is located near the heme edge (pyrrole D) based on observations that BHA interacts strongly with the heme methyl $C18H_3$ as well as some protons of the distal histidine and arginine residues [11,12]. In addition, involvement of the distal histidine and arginine residues in BHA binding has been shown by resonance Raman spectroscopy [14]. These spectroscopic results are basically consistent with the results obtained in our X-ray analysis. The binding modes of BHA to HRP and ARP, however, are not necessarily the same because the structures of these peroxidases in the heme cavity and entrance to the channel differ markedly [20]. Not only does the orientation of the imidazole ring of the distal histidine of ARP differ from the orientations of other peroxidases with known crystal structures [27–30], but Pro^{154} of ARP, the hydrogen partner of BHA, may not be conserved in other peroxidases, including HRP. Because the crystal structure of peanut peroxidase, a class III peroxidase of the plant peroxidase superfamily, is known [30], clarification of the BHA binding mode of this class is awaited with much interest.

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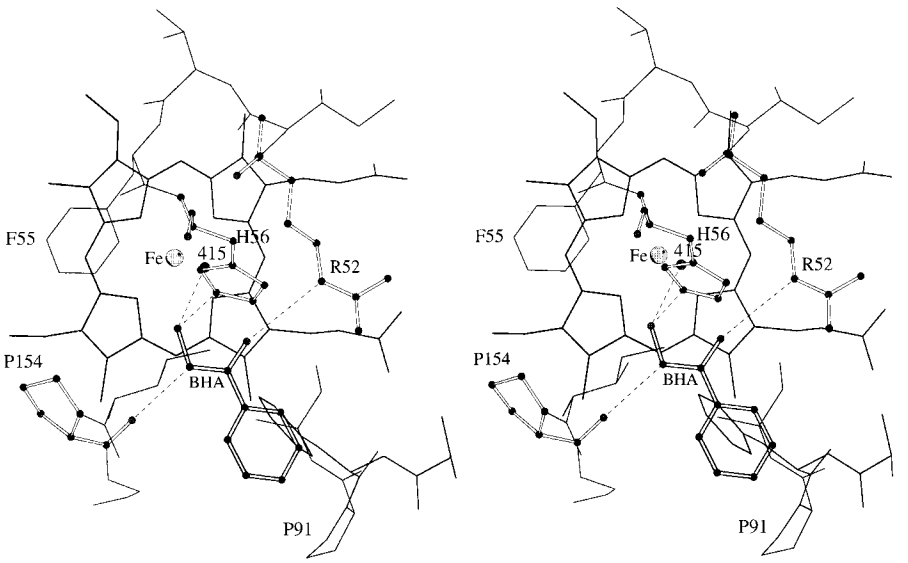

Fig. 3. Environment of the BHA molecule shown by a stereo-pair. Possible hydrogen bonds involving BHA are shown by broken lines. The lower side of the figure is the surface of the ARP molecule. The hydrogen bond distances for BHA are O(7)...$Arg^{52}N_\varepsilon$, 3.3 Å; N(7)...$Pro^{154}O$, 2.8 Å; O(8)...$His^{56}N_\varepsilon$, 2.6 Å; and O(8)...415, 2.7 Å. The respective distances of Fe...415 and Fe...O(8) are 2.7 Å and 4.4 Å. The distance between C(1) in BHA and the methyl carbon bonded to C(18) in the heme is 3.9 Å. The torsion angle about C(1)–C(7) bond of BHA is 14°. The dihedral angle between the benzene ring of BHA and the pyrrole D ring of the heme is 9°.

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