Pentacoordination of the heme iron of *Arthromyces ramosus* peroxidase shown by a 1.8 Å resolution crystallographic study at pH 4.5

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Abstract In the presence of ammonium sulfate the absorption spectra of a peroxidase from the fungus *Arthromyces ramosus* (ARP) showed that the low-spin component increased as the pH increased from 6.0 to 9.0, whereas in its absence ARP remained in the high-spin state in the pH range investigated. The crystal structure of ARP at pH 4.5 in the presence of ammonium sulfate at 1.8 Å resolution showed that the electron density at the 6th position of the heme iron seen at pH 7.5 had disappeared and that the iron atom deviated markedly from the heme plane. These observations strongly suggest that under physiological conditions the heme of ARP is in the pentacoordinated high-spin state and that at a high pH the heme iron is able to bind ammonia, forming the low-spin state. The location of the water molecule at the distal side of the heme in peroxidases is also discussed.

Key words: Peroxidase; Heme enzyme; Coordination of heme; X-ray crystallography; *Athromyces ramosus*

1. Introduction

The fungus *Arthromyces ramosus* secretes a large amount of peroxidase [1], a heme-containing enzyme that catalyzes the oxidation of a variety of substrates by utilizing hydrogen peroxide. *Arthromyces ramosus* peroxidase (ARP) gives a several hundred fold higher chemiluminescence in the oxidation of luminol than does horseradish peroxidase (HRP), therefore ARP has been suggested as useful for very sensitive assays of biological substances [2,3]. Sequence studies of the complementary and genomic clones encoding ARP [4] show that it is a class II peroxidase as are ligin peroxidase (LiP) and manganese peroxidase (MnP) from the fungus *Phanerochaete chrysosporium* [5]. ARP has a single polypeptide protein of 344 residues, one iron protoporphyrin IX as a prosthetic group, two calcium ions, and carbohydrates attached to Asn-143 and Ser-339. ARP and the closely related *Coprinus cinereus* and *Coprinus macrorhizus* peroxidases were characterized by Kjalke et al. [6]. Crystal structures of the native ARP, its cyanide- and triiodide-bound forms, and *C. cinereus* peroxidase have been obtained at high resolution [7–9].

Spectroscopic studies suggest that under physiological conditions yeast cytochrome *c* peroxidase (CcP) and LiP in the resting state are predominantly in the pentacoordinate high-spin state but may convert to the high-spin hexacoordinate or to the low-spin state when the temperature or pH is lowered [10–13]. Crystallographic analyses of CcP, LiP, MnP, and ascorbate peroxidase have shown that the water molecule is located at the sixth coordination position of each heme [14–17]. In the case of ARP, structural analysis of crystals grown at pH 7.5 with ammonium sulfate as the precipitant showed significant electron density at the sixth coordination position of the heme [7]. Moreover, the color of the ARP crystals showed marked pH dependence, being reddish-brown at a high pH and brown at a low one. This indicates a coordination change of the ligand to the heme iron with pH change. The ligand of the ARP heme can not be assumed to be a water molecule because the interaction between water and the heme iron is known to be weak. To clarify the nature of the heme iron of ARP, we determined the crystal structure of ARP at pH 4.5 using diffraction data to 1.8 Å resolution and examined the absorption spectra of ARP solutions. We report clear evidence that under physiological conditions the heme iron of ARP is pentacoordinated and in the high-spin state. On the basis of a structural comparison of peroxidases, we suggest that in peroxidases the water molecule is bound to the imidazole N_ε atom of the distal histidine rather than to the heme iron.

2. Experimental

2.1. Absorption spectra of ARP

Absorption spectra of ARP solutions in the presence of 1.4 M ammonium sulfate, the same concentration as in the crystallization, and in its absence were measured at three pH values (6.0, 7.5, and 9.0). Spectra were recorded with a SHIMADZU UV-3101PC spectrophotometer. The buffers were 50 mM sodium succinate for pH 6.0, 50 mM Tris HCl for pH 7.5, and 50 mM sodium borate for pH 9.0. The spectra are compared in Fig. 1.

2.2. Preparation of the low-pH form of ARP crystals

ARP was purified and crystallized as described elsewhere [18]. The crystals belong to space group $P4_22_12$ with $a = b = 74.57$ Å and $c = 117.47$ Å with one ARP molecule in the asymmetric unit. The low-pH form of the ARP crystal was prepared by soaking parent ARP crystals for 2 h in 50 mM sodium acetate buffer adjusted to pH 4.5 containing 35% saturated ammonium sulfate. Conversion of the form of the crystal could be monitored by the change in color from reddish-brown to brown and appeared to be completed within 1 h.

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Abbreviations: ARP, *Arthromyces ramosus* peroxidase; HRP, horseradish peroxidase; LiP, lignin peroxidase; CcP, cytochrome *c* peroxidase; MnP, manganese peroxidase; IP, imaging plate.

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2.3. Data collection

Diffraction data of the low pH form were collected to 1.8 Å resolution at 20°C on a screenless Weissenberg camera for macromolecular crystals and using synchrotron radiation [19] 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 wavelength was 1.00 Å, and 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 crystal was oscillated around the a+b axis. The oscillation angle and coupling constant for each IP respectively were 4.5 to 5.2° and 1.5 to 1.8°/mm, in order to minimize the overlapping of diffraction spots. The overlap angle between adjacent IPs was 0.2°. The low pH form 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 BA100 then processed with the program system WEIS [20] and PROTEIN [21]. Partial reflections were discarded. Data collection results are shown in Table 1A.

2.4. Structure determination

The atomic parameters of ARP at pH 7.5 refined at 1.9 Å resolution (PDB code 1ARP) were used to start the structural refinement, several water molecules near the heme being excluded. The model was refined by simulated annealing using the program XPLOR [22]. The protein model and locations of the water molecules were revised using $2F_o-F_c$ and F_o-F_c maps with FRODO [23,24] and an IRIS 4D/35GT computer graphics system. The final model contains 285 water molecules in addition to the protein. The final crystallographic R-factor was 18.8% for 27,884 reflections with $F > 2\sigma_F$ in the 7.0–1.8 Å resolution range. Refinement results are shown in Table 1B. The mean coordinate error is estimated to be about 0.2 Å from a Luzatti plot [25]. The atomic parameters will be deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY.

3. Results and discussion

3.1. Absorption spectroscopy

When ammonium sulfate was present, the spectrum at a high pH differed from that of the high-spin type of native enzyme, whereas there was no detectable spectral change in the absence of ammonium sulfate. The spectrum at pH 9.0 in the presence of ammonium sulfate showed peaks at 414 and 541 nm and a shoulder at 360 nm, similar to the spectrum of the low-spin type heme iron. Isosbestic points between the spectra of the high-spin and the low-spin states of ARP were at 407, 471, 526, and 605 nm. This indicates that the high- and low-spin states are in equilibrium. Furthermore, the spectrum of unbuffered ARP solution to which free ammonia had been added was identical to that of the ARP solution in the presence of ammonium sulfate at pH 9.0 (data not shown). These observations suggest that the ammonia derived from the ammonium sulfate binds to the heme iron forming a low-spin complex at a high pH, whereas the resting ARP is in the high-spin state. The spectrum of the ARP solution in the presence of ammonium sulfate at pH 7.5 suggests that ammonia binds partially to ARP in the crystal. This property of ARP differs from that of HRP. The spectra of HRP solution was unchanged when ammonium sulfate was added (data not shown).

3.2. Coordination to the heme iron

The environment of the distal side of the heme in ARP at pH 4.5 is shown in Fig. 2. The conformation of the protein mole-

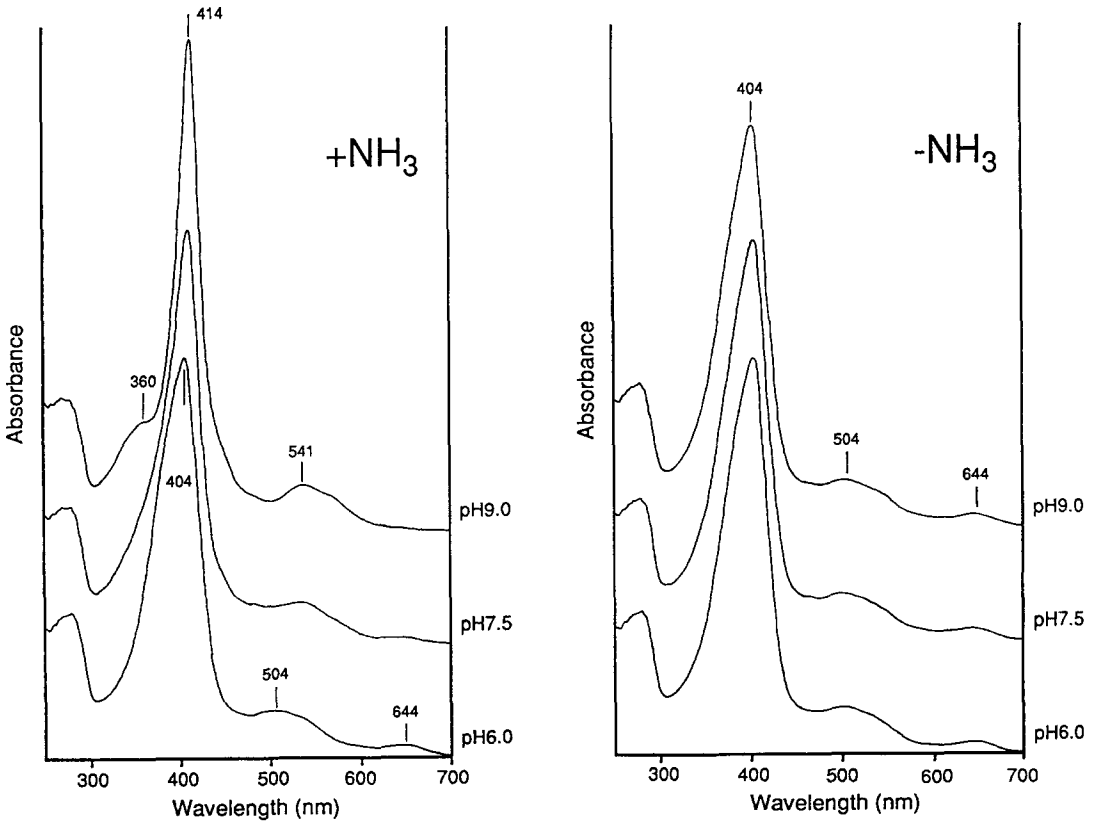

Fig. 1. Absorption spectra of ARP at different pH values. Left: in the presence of 1.4 M ammonium sulfate. Right: in its absence.

Fig. 2. ($2F_o-F_c$) map of the environment of the distal side of the heme in ARP at pH 4.5. Red stars indicate solvent molecules. The hydrogen bond between the water molecule and N_ε atom of the distal histidine is shown by a dashed line.

cule shows little change, whereas the solvent molecules around the distal side of the heme show marked rearrangement. Electron density, clearly seen at the sixth position of the heme iron at pH 7.5 [7], is absent in the low pH form. The heme iron of ARP at a low pH has typical pentacoordinated geometry. The distance from the heme iron to the nearest solvent molecule is 3.9 Å, too far to form a bond[1]. These results together with the present absorption spectroscopic data indicate that the sixth coordination position of the heme iron is occupied by ammonia when the pH is high. Moreover, the displacement of the iron atom from the heme plane is 0.19 Å at pH 4.5, whereas it is 0.07 Å at pH 7.5, indicative that lowering the pH increases the component of the high-spin state[2]. The structure at pH 4.5 presented here may represent the physiological form of the resting enzyme and is the first example of a peroxidase with a typical pentacoordinated heme iron demonstrated by crystallography. This is consistent with the resonance Raman and NMR studies done on *C. cinereus* peroxidase which showed that at a neutral pH the ferric state is characteristic of a five-coordinated high-spin heme [26,27].

In CcP, LiP, MnP, and ascorbate peroxidase, a water molecule occupies the 6th position of each heme [14–17]. The geometries around that water molecule are compared in Table 2. The molecule lies at a position suitable for hydrogen bonding with the N_ε of the distal histidine. In each peroxidase the distance between N_ε and the water molecule is in the range of hydrogen bonding and the bond approximately bisects the C_δ-N_ε-C_ε angle. Moreover, the water molecule is approximately in the plane of the imidazole ring, which is consistent with the description of Thanki et al. obtained from an analysis of the distributions of water molecules around amino acid residues [28]. In brief, the position of the water molecule on the distal side of the heme in peroxidases appears to be dominated by the orientation of the imidazole ring rather than by interaction with the heme. The different orientation of the imidazole ring in ARP [7,8] causes marked deviation of the water molecule from the 6th position of the heme. Generally in peroxidases the interaction between the water molecule and the heme iron appears to be weak, which is consistent with the spectroscopic evidence for CcP [10,11] and LiP [12,13]. The heme iron of HRP was shown to be pentacoordinated by EPR [29] and by Raman spectroscopy [30], but the detailed crystal structure of HRP has yet to be determined.

Quillin et al. obtained the crystal structures of distal histidine mutants of sperm whale myoglobin in which metmyoglobins with hydrophobic substitutions at residue 64 lack a water mol-

[1]In our attempt to prepare compound I of ARP, a crystal free of ammonium sulfate was obtained by soaking the parent crystal in 25 mM Tris buffer at pH 7.0 that contained saturated sodium sulfate and 3 mM H_2O_2. The structure was refined to $R = 16.4\%$ using 17,721 diffraction data to 2 Å resolution measured as described for the low pH form. ARP was not converted to compound I. There was no water molecule that is close enough to form a bond to the heme iron.

[2]High resolution X-ray analyses of the cyanide and triiodide complexes of ARP clearly showed that displacement of the iron atom from the heme plane is correlated to the spin-state. In the low-spin cyanide complex the iron lies in the heme plane, whereas in the high-spin triiodide complex it deviates from the plane by 0.17 Å [8].

Table 1
Data collection and crystallographic refinement results

(A) Data collection

Number of IPs	26
Resolution limit (Å)	1.8
Measured reflections	171,644
Independent reflections	28,453
Completeness (%)[a]	90.4
R_{merge} (%)[b]	6.98

[a] After scaling and averaging the intensities of the equivalent reflections, those with $F < 2\sigma_F$ were rejected.
[b] $R_{merge} = \Sigma_i \Sigma_h | I(hkl) - <I(hkl)> | / \Sigma_i \Sigma_h <I(hkl)>$

(B) Crystallographic refinement

Number of atoms refined[a]	2,823 (285)
Number of reflections ($F > 2\sigma_F$)	27,884
R-factor (%)	18.8
Root-mean-square deviations from ideal values:	
Bond distances (Å)	0.020
Angle distances (Å)	0.054
Planar groups (Å)	0.032
Chiral volumes (Å^3)	0.133
Torsion angles of ω (°)	3.4

[a] Numerals in parentheses are the number of water molecules.

Table 2
Comparison of the geometries around the water molecule on the distal side of the heme

	ARP		LiP[a]	MnP	CcP[b]
Distance (Å)					
$N_\varepsilon \ldots H_2O$	2.62	2.65	2.67	2.98	3.17
$Fe \ldots H_2O$	3.89	2.73	2.71	2.80	2.65
Angles (°)					
$N_\varepsilon \ldots H_2O \ldots Fe$	128	158	152	161	167
$Fe \ldots H_2O$ to pyrrole N plane[c]	62	81	81	74	74
Displacement (Å) of H_2O from the imidazole ring plane	0.19	0.56	0.54	0.21	0.30

[a] Two molecules in the asymmetric unit.
[b] Taken from PDB (code 1CCP).
[c] The pyrrole N plane was calculated using the coordinates of four pyrrole nitrogen atoms (N_A, N_B, N_C and N_D).

ecule at the 6th coordination position,whereas those with polar residues at this position retain a bound water molecule [31]. The findings for ARP described above may spur characterization of the ligand in the myoglobin mutants because the crystals were obtained in a high concentration of ammonium sulfate at pH 9.0[3].

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