Crystal Structures of Cyanide- and Triiodide-bound Forms of Arthromyces ramosus Peroxidase at Different pH Values

PERTURBATIONS OF ACTIVE SITE RESIDUES AND THEIR IMPLICATION IN ENZYME CATALYSIS*

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The structures of the cyanide and triiodide complexes of Arthromyces ramosus peroxidase (ARP) at different pH values were investigated by x-ray crystallography in order to examine the behavior of the invariant residues of arginine (Arg-52) and distal histidine (His-56) during the enzyme reaction as well as to provide the structural basis of the active site of peroxidase. The models of the cyanide complexes at pH 7.5, 5.0, and 4.0, respectively, were refined to the R-factors of 17.8, 17.8, and 18.5% using 7.0–1.6-Å resolution data, and those of the triiodide complexes at pH 6.5 and 5.0 refined to 16.9 and 16.8% using 7.0–1.9-Å resolution data.

The structures of the cyanide complexes at pH 7.5, 5.0, and 4.0 are identical within experimental error. Cyanide ion bound to the heme in the bent conformation rather than in the tilt conformation. Upon cyanide ion binding, the N atom of His-56 moved toward the ion by rotation of the imidazole ring around the C-N bond, but there was little conformational change in the remaining residues. The distance between the N atom of His-56 and the nitrogen atom of the cyanide suggests the presence of a hydrogen bond between them in the pH range investigated. In the triiodide complexes, one of the two triiodides bound to ARP was located at the distal side of the heme. When triiodide bound to ARP, unlike the re-arrangement of the distal arginine of cytochrome c peroxidase that occurs on formation of the fluoride complex or compound I, the side chain of Arg-52 moved little. The conformation of the side chain of His-56, however, changed markedly. Conformational flexibility of His-56 appears to be a requisite for proton translocation from one oxygen atom to the other of HOO- by acid-base catalysis to produce compound I. The iron atom in each cyanide complex (low-spin ferric) is located in the heme plane, whereas in each triiodide complex (high-spin ferric) the iron atom is displaced from the plane about 0.2 Å toward the proximal side.

Peroxidases (donor: H2O2 oxidoreductase (EC 1.11.1.7)) are a class of heme proteins which oxidize a wide variety of organic and inorganic compounds by the use of hydrogen peroxide. In addition to their physiological importance, they have attracted attention because of useful applications which include assays of biological substances (Allain et al., 1974; Akimoto et al., 1990) and large scale use for delignification and the bleaching of dyes (Pedersen and Carlsen, 1994). The reaction generally consists of the following steps (Reactions I–IV).

**REACTION STEPS I–IV**

1. Peroxidase (Fe(III)) + H2O2 \( \rightarrow \) compound I (Fe(VI)) + H2O
2. Compound I (Fe(VI)) + AH₂ \( \rightarrow \) compound II (Fe(VI)) + AH\·
3. Compound II (Fe(VI)) + AH\· \( \rightarrow \) peroxidase (Fe(III)) + AH\·
4. 2AH\· \( \rightarrow \) A₂H₂ or A + AH\₂

Since the crystal structure of the yeast cytochrome c peroxidase (CCP), a typical class I peroxidase (Welinder, 1992), was first determined at 1.7-Å resolution (Finzel et al., 1984), the mechanism of compound I formation, the first step in the above equations, has been proposed for CCP. The mechanism of compound I formation suggested is that the distal histidine and arginine concertedly stabilize charge separation and facilitate proton transfer from one oxygen to the other of the peroxide as well as heterolytic cleavage of its O-O bond (Finzel et al., 1984). To confirm the validity of this mechanism, mutant CCP proteins have been prepared, and their three-dimensional structures and spectroscopic and kinetic properties extensively studied. For example, Erman et al. (1993) reported that the distal histidine is critical for the rapid formation of compound I of CCP, and Vitello et al. (1993) reported that arginine is responsible for substrate binding in the heme pocket and for stabilizing compound I.

The three-dimensional structures of a lignin peroxidase from Phanerochaete chrysosporium (LiP), a peroxidase from Arthromyces ramosus (ARP) and a manganese peroxidase from P. chrysosporium (all class II peroxidases) have been, respectively, determined at 2.0-, 1.9-, and 2.06-Å resolutions (Edwards et al., 1993; Piontek et al., 1993; Pouls et al., 1993; Kunishima et al., 1994; Sundaramoorthy et al., 1994). Although the histidine and arginine residues at the distal side of the heme are conserved in ARP and LiP, structural comparison

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1 The abbreviations used are: CCP, yeast cytochrome c peroxidase; ARP, Arthromyces ramosus peroxidase; LiP, lignin peroxidase.

2 The crystal structure of a peroxidase from Coprinus cinereus, very similar to ARP in the amino acid sequence (Baunsgaard et al., 1993; Sawai-Hatanaka et al., 1995), at 2.6-Å resolution was reported by Petersen et al. (1994). Although the modes of molecular packing differ, probably because of the difference in the precipitants used for crystallization, the molecular structures appear identical.
Structures of CN⁻ and I₃⁻ Complexes of ARP

TABLE I
Conditions and results of the intensity measurements

| pH 7.5  | pH 5.0  | pH 4.0  | pH 6.5  | pH 5.0  |
|--------|--------|--------|--------|--------|
| Oscillation angle (°) | 2.7-5.2 | 2.7-5.2 | 2.7-5.2 | 4.2-5.2 | 4.2-5.2 |
| Overlap (%) | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Coupling constant (°/mm) | 0.5-1.8 | 0.5-1.8 | 0.5-1.8 | 1.5-1.8 | 1.5-1.8 |
| Exposure time (s) | 4.7-10.4 | 5.4-10.4 | 2.7-5.2 | 10.4-18.8 | 8.4-10.4 |
| Number of IPs | 22 | 22 | 22 | 20 | 20 |
| Resolution limit (Å) | 1.6 | 1.6 | 1.6 | 1.9 | 1.9 |
| Measured reflections | 163,223 | 163,962 | 158,600 | 77,736 | 78,184 |
| Independent reflections | 37,405 | 37,662 | 37,412 | 20,924 | 20,830 |
| Completeness (%) | 84.3 | 85.0 | 84.5 | 78.2 | 77.8 |
| Rmerge (%) | 6.30 | 6.26 | 7.11 | 5.73 | 6.20 |

* Oscillation angles and coupling constants were varied so as to minimize the overlap of diffractions.
* After scaling and averaging the intensities of the equivalent reflections, reflections with F < 2σ(F) were rejected.
* Rmerge = Σhkl[Σi(Fihkl) - Σi(Fihkl)]/Σi(Fihkl)

of these peroxidases with CCP has shown that the conformations of the distal histidine residue (His-56 in ARP) relative to the heme differ (Kunishima et al., 1994). In addition, preliminary structural refinement of the triiodide complex of ARP, used for phase determination by the isomorphous replacement method (Kunishima et al., 1994), has shown that the behavior of the distal arginine (Arg-52 in ARP) differs from that observed in CCP when it forms a complex with fluoride (Edwards and Poulos, 1990) or when it is converted to compound I (Fulop et al., 1994). Unexpectedly preliminary ligand binding experiment showed that most ligands bound hardly to the heme besides triiodide and cyanide. Because the behavior of these residues may be affected by the pH, we examined how the structure of ARP changes when it binds ligand at different pH values.

Heme proteins that form complexes with ligands have been investigated extensively to elucidate the nature of the heme iron and structure-property relationships, such as the spin state versus the displacement of the iron atom from the heme plane (Perutz, 1970). For peroxidases, although the cyanide bound forms of CCP, LiP, horseradish peroxidase, and Coprinus cinereus peroxidase have been characterized by NMR (Sturtevant and Erman, 1991; deRopp et al., 1991; Banci et al., 1991; Thalabalet et al., 1988; Veitch et al., 1994), crystallographic studies of these forms are lacking; only the structures of the inhibitor-bound forms of CCP are known (Edwards and Poulos, 1990). In addition, different modes of ligation to the heme have been reported; e.g. in CCP cyanide binds to the heme iron in a tilted configuration (Edwards and Poulos, 1990), whereas in metmyoglobin it binds to its heme iron in a bent configuration (Neya et al., 1993). This difference might be ascribed to the protein environment of the heme, but it is likely that for some heme proteins unambiguous results could not be obtained because of the lack of detailed three-dimensional structures. In particular, in the case that ligand sizes are small, a high resolution x-ray crystal structure is required to detect any subtle structural change and to define the mode of ligand binding.

We present here detailed structural features of the heme and its environment as deduced from high resolution x-ray analysis of the cyanide and triiodide complexes and show the behavior of the distal histidine and arginine residues when ARP binds to cyanide and triiodide.

EXPERIMENTAL PROCEDURES

Spectrophotometry—The formation of each ARP complex in solution was monitored by ultraviolet-visible absorption using a SHIMADZU UV-3101PC spectrophotometer. For the cyanide complex, the buffer used was 50 mM Tris-HCl, pH 7.5, and the ionic strength of the solution was adjusted to 0.1 M by adding potassium chloride. 200 μM potassium cyanide solution was added stepwise (5 μM/time) to 1 mM of 5.3 μM ARP solution, after which the spectrum was measured by the spectrophotometer.

Complex formation of ARP with triiodide was monitored by adding 3 mM KI₁ solution stepwise to 6 μM ARP solution containing 1 mM KI. The equilibria of triiodide and ARP were examined at pH 6.0, 7.5, and 9.0, the buffers used being 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 ionic strength of the reaction mixture was adjusted to 0.1 M by adding sodium nitrate. 3 mM KI₁ solution was prepared by diluting 30 mM KI₁ stock solution (prepared by adding iodine to about 8 eq molar excess KI1) with 100 mM KI solution.

Preparation of the fluoride complex was attempted by adding sodium fluoride to the ARP solution at pH 5.5. But the absorption spectrum did not change significantly even when a 30 eq molar excess of sodium fluoride was added.

Preparation of the Complex Crystals—ARP was purified and crystallized as described previously (Kunishima et al., 1993). Cyanide-derivative crystals of ARP were prepared by soaking the native ARP crystals for 2 h in mother liquor containing 1 mM KCN, 33% saturated ammonium sulfate. The buffers used were 50 mM Tris-HCl for pH 7.5, 50 mM sodium acetate for pH 5.0, and 50 mM sodium formate for pH 4.0. Treatment with cyanide changed the color of the crystal from brown to red. This reaction appeared to be completed within 1 h.

The triiodide-derivative crystals were prepared by soaking native ARP crystals for 2 h in mother liquor consisting of 33% saturated ammonium sulfate, 20 mM sodium succinate, pH 6.0, and 2 mM KI₁. The derivative crystals were isomorphous with the native ones. For preparation of the derivative, it was essential to lower the pH. The crystal soaked at pH 7.5 showed little change in x-ray diffraction intensity.

Data Collection—Data were collected at 20 °C using synchrotron radiation and the screenless Weissenberg camera for macromolecular crystals (Sakabe, 1991) at the BL6A2 station 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 0.1 Å. A collimator with a diameter of 0.1 mm was used. Each crystal was oscillated around the a-b axis. The radius of the film cassettes, each of which had Fuji Imaging Plates with detection areas of 40 × 20 cm, were 286.5 mm for the data collections of the cyanide complexes and 429.7 mm for those of the triiodide complexes. Data for each complex were obtained from a single crystal. Diffraction data recorded on each imaging plate were read out at 100-μm intervals with a Fuji BA100 then processed with the program systems WEIS (Higashi, 1989) and PROTEIN (Steigemann, 1974). Partial reflections were discarded. The conditions and results of data collection are given in Table I.

Structure Determination—Initially phases were calculated from the model of the native ARP refined at 1.9 Å (Kunishima et al., 1994; PDB code 1ARP). This model was checked with FRODO (Iones, 1978; Roussel and Cambillau, 1989) on an IRIS 4D/35GT computer graphics system. Refinement was done with simulated annealing using the program XPLOR (Brünger et al., 1990; Brünger, 1992).

Initially for each cyanide complex, the solvent molecules around the...
heme were omitted, and the model was refined by XPLOR using the diffraction data with $F > 2\sigma_F$ in the 7.0–1.6 Å resolution range. A $2F_o - F_c$ map clearly located the cyanide ion at the sixth coordination position of the heme iron. When the positions of the peaks in the $2F_o - F_c$ and $F_o - F_c$ maps satisfied the geometries of the hydrogen bond with the protein and/or another water molecule, they were included in the subsequent refinement. The final refinement was done including the solvent molecules as well as the cyanide ion.

For the triiodide complexes, a difference Fourier map with the coefficient of $(F_{I_3} - F_p) \exp(\pi \alpha_x)$ at 2.2 Å resolution located two clearly resolved triiodide ions near the heme, where $F_{I_3}$ and $F_p$ are the structure factors of the triiodide complex and the native crystal, respectively. A $F_o - F_c$ difference Fourier using the refined derivative phases $(\alpha_x)$ was then used to position the water molecules. Final refinement used the diffraction data with $F > 2\sigma_F$ in the 7.0–1.9 Å resolution range. Results are given in Table II. The mean coordinate error for each model is estimated to be about 0.2 Å (Luzatti, 1952).

### RESULTS

**Formation of the Complexes**—Fig. 1 compares the absorption spectra of the cyanide and triiodide complexes with that of the native ARP. Cyanide binding to ARP results in a rapid spectral shift in $\lambda_{max}$ from 405 to 423 nm with an isosbestic point at 414 nm. The spectral change was completed when a 3.7 eq molar excess of cyanide to ARP was added. Other spectral changes seen on cyanide binding are the disappearance of the peaks at 505 and 645 nm and appearance of peaks at 364 and 550 nm. There was little pH dependence of the spectra in the range between pH 9.0 and 5.5, which is consistent with the findings for C. cinereus peroxidase (Lukat et al., 1989). The spectra clearly show that treatment with cyanide converts the electronic state of the heme iron from Fe(III) high-spin to Fe(III) low-spin (Morita et al., 1988).

### Table II

|                  | Cyanide complex | Triodide complex |
|------------------|-----------------|------------------|
|                  | pH 7.5          | pH 5.0          | pH 4.0          | pH 6.5          | pH 5.0          |
| Resolution range (Å) | 7.0–1.6        | 7.0–1.6        | 7.0–1.6        | 7.0–1.9        | 7.0–1.9        |
| Number of atoms refined$^a$ | 2,846 (308)    | 2,840 (302)    | 2,827 (289)    | 2,829 (284)    | 2,856 (311)    |
| Number of reflections ($F > 2\sigma_F$) | 36,860       | 37,120         | 36,865         | 20,379         | 20,282         |
| Average temperature factors ($A^2$)$^b$ | 16.3 (14.7)   | 17.0 (15.4)    | 16.8 (15.3)    | 18.0 (16.6)    | 19.3 (17.6)    |
| R-factor (%)      | 17.8            | 17.8            | 18.5           | 16.9           | 16.8           |
| Root-mean-square deviations from ideal values |                  |                  |                |                |                |
| Bond distances (Å) | 0.018           | 0.018           | 0.019          | 0.019          | 0.018          |
| Angle distances (Å) | 0.051          | 0.050           | 0.052          | 0.052          | 0.049          |
| Planar groups (Å) | 0.023           | 0.024           | 0.026          | 0.027          | 0.026          |
| Chiral volumes ($A^3$) | 0.133         | 0.131           | 0.133          | 0.130          | 0.133          |
| Torsion angles of $\phi$ (deg.) | 3.1            | 3.2             | 3.2            | 3.1            | 2.9            |

$^a$ Numerals in parentheses are the numbers of water molecules.

$^b$ Individual temperature factors were refined after XPLOR refinement. The values in parentheses are for the atoms of the protein and the heme group.

### Fig. 1

Absorption spectra of the native ARP (solid line), the cyanide (dot-dash line), and triiodide (dashed line) complexes. The spectrum of the native ARP is identical to that of C. cinereus peroxidase (Morita et al., 1988).
the side chain, indicative of the presence of several conformers. Neither the pH value nor the species of the bound ion appeared to correlate with a favorable conformer of this side chain. It is likely that the side chain of Met-243 is flexible.

Binding Mode of Cyanide—The $(2F_o - F_c)$ map of the cyanide complex at pH 5.0 is shown in Fig. 2. The maps at pH 7.5 and 4.0 were very similar to the map at pH 5.0. The electron density corresponding to cyanide is clearly visible at the sixth coordination position of the heme iron. The geometries around the heme iron are summarized in Table III. The hemes in the cyanide complexes are, as in the native and triiodide complexes, distorted into a saddle shape, with pyrroles I and II tilted toward the proximal side and pyrroles II and IV tilted toward the distal side.

The heme in a protein binds to a cyanide with the C-N bond being often tilted from the normal of the heme plane, where two ways of binding are possible: tilt and bent conformations (Deatherage et al., 1976). As deviation from the spherical shape of the electron density is small due to the short C-N distance of the cyanide (1.15 Å), it was difficult to define its orientation precisely even by 1.6-Å resolution analysis. From Table III there appears to be no clear correlation between the bent angles and pH values. The cyanide is tilted slightly from the normal of the heme plane (the mean angle of the Fe-C(CN) vector to the heme plane is 87°), and the bending of Fe-C-N is significant (the mean angle of Fe-C-N is 159°). Therefore the bent configuration is more likely for the cyanide complex of ARP.

Interaction between Cyanide and His-56—A structural comparison between the native ARP and the cyanide complex is shown in Fig. 3. Upon cyanide binding, a small but notable structural change occurred at the distal histidine (His-56); the imidazole ring rotated around the C α-Cγ bond by 12° so that the Nα atom was close to the cyanide. The CN-Nα(His-56) distances in the complexes range from 2.75 to 2.80 Å (Table III), and the other geometries are excellent for hydrogen bonding. The fact that there is sufficient space for the cyanide to take the other orientation without rearrangement of the protein strongly suggests that the cyanide nitrogen and Nα atom of His-56 are close together due to an attracting force. Therefore it is most likely that the cyanide is hydrogen bonded to the Nα of His-56 at all the pH values investigated. The crystal structure of the cyanide-bound CCP at pH 6 indicated a hydrogen bond between the cyanide and the Nα of the distal histidine (His-52) but it was not defined whether cyanide or His-52 was the donor of the bond (Edwards and Poulos, 1990).

NMR studies on cyanide-bound horseradish peroxidase, CCP, LiP, and C. cinererus peroxidase enabled assignment of the signal of the hydrogen atom bonded to each Nα of the distal histidine in the pH range of 7 and 6 (Thanaabad et al., 1988; Satterlee and Erman, 1991; de Ropp et al., 1991; Veitch et al., 1994). Our present crystallographic and NMR results show that it is reasonable that His-56 is the hydrogen donor in the hydrogen bond in the cyanide-bound ARP, which is why its Nα is protonated in the pH range investigated. The Nα atom of His-56 must be protonated because the hydrogen bond network of Nα (His-56)−C=O (Asn-93) and NH2 (Asn-93)−C=O (Glu-87) is retained in the cyanide complexes. The hydrogen bond between the Nα atom of His-56 and the cyanide even at pH 7.5 is noteworthy because the Nα atom of the distal histidine is usually deprotonated at pH 7.5, the pKa value of the free histidine being about 6. We conclude that the pKa of the hydrogen-bonded network of CN−H−His-56 in the cyanide complex is much higher than that of the distal histidine in the native enzyme. Upon cyanide binding, a few of the water molecules are rearranged to form a hydrogen bond network, the cyanide...
pushing Wat-674 away by 1.6 Å and Wat-676 by 1.8 Å (Fig. 3).

Binding Mode of Triiodide—Fig. 4a shows the (F_{obs} - F_{native}) \exp(\alpha_{native}) difference electron density map superimposed on the entire ARP model and demonstrates that all the significant changes are confined to the active site. Two triiodides bound to one molecule of ARP; one near the entrance of the substrate access channel (external site), the other to the active site (internal site). The external site is a mixture of two individual sites and two of the four iodine atom positions coincide. This explains why least-squares refinement of the heavy atom parameters in the isomorphous replacement method indicated that the occupancy of one of the three iodine atoms was low (Kunishima et al., 1994). The electron density of triiodide was clearly divided to each iodine atom (Fig. 4b). The minus peak near the imidazole ring of His-56 apparently shows the conformational change of His-56 caused by the binding of triiodide. Structural refinement of each triiodide complex, in which the occupancy and temperature factors of the triiodide ions were refined alternately using XPLOR at the final stage, suggests that the triiodide is fully bound to the heme.

The geometry of the triiodide bound heme is given in Table IV. For convenience, iodine atoms of the internal site are named I_A, I_B, and I_C in the order of their proximity to the heme iron. I_A is placed above the heme iron, and the triiodide is nearly parallel to the heme plane. The interaction between the heme iron and the triiodide appears to be weak as judged by the little spectral change on triiodide binding.

Movement of Active Site Residues on Triiodide Binding—Fig. 5 shows the superposition of the active site residues of the triiodide complex on those of native ARP. The side chain of Arg-52 moved marginally toward I_B on triiodide binding, the maximum positional shift being 0.19 Å at N_{a}. The hydrogen bonding scheme was unchanged. The side chain of Arg-52 forms hydrogen bonds with two amino acids (Asn-93, Gly-94) and one buried water molecule (Wat-394).

In contrast to Arg-52 in ARP, a small but significant conformational change of His-56 was induced by triiodide binding. The imidazole ring of His-56 rotated around the C_{6}-C_{11} and C_{11}-C_{12} bonds by 8 and 4°, respectively, resulting in the maximum positional shift of 0.73 Å at N_{a}. This movement must be due to the large van der Waals radius of the iodine atom; the distance between I_A in the triiodide complex and N_{a} (His-56) is only 2.85 Å, less than the sum of their van der Waals radii, if the conformation of His-56 is unchanged.

The orientation of the imidazole ring of the distal histidine in ARP differs from the orientations in CCP and LiP (Table V). They are constrained by the hydrogen bond between the N_{a} atom and the O=C of asparagine (Asn-93 in ARP) (Kunishima et al., 1994). The rotations caused by triiodide binding occurred in a direction such that the conformation of His-56 in ARP was close to the conformations in CCP and LiP, but their amounts were not large compared to the difference between the torsion angles in these peroxidases.

Discussion

Heme Geometries—Generally the iron of ferric heme proteins in the low-spin state lies in the heme plane, whereas in the high-spin state it deviates from the plane toward the proximal side (Perutz, 1970; Kuriyan et al., 1986; Quillin et al., 1993; Neya et al., 1993); but, in the ligand-bound forms of CCP this correlation is lacking (Edwards and Poulos, 1993). The present crystallographic results (Tables III and IV) show that the heme iron is located on the heme plane in the cyanide complex (deviation 0.04 Å) but is displaced from that plane (0.17 Å) toward the proximal side in the triiodide complex. Moreover in the native ARP at pH 5.5, where the heme iron is hexacoordinated and in the high-spin state, the iron atom is displaced from the heme plane by 0.18 Å. Kunishima et al. (1994) reported that the heme of ARP at pH 7.5 was hexacoordinated, the 6th position of the heme being occupied by a water molecule. Recently the 6th ligand, however, was characterized as ammonia derived from ammonium sulfate used as the precipitant in crystallization, and the absorption spectrum indicated that ARP at pH 7.5 had a low-spin component to some extent. The displacement of the iron atom from the plane at pH 7.5 is 0.07 Å. Therefore in the case of ARP the correlation between the spin-state and the displacement of the iron atom from the heme plane is clearly seen.

Conformation of Arg-52 and Its Functional Role—The distal arginine has been proposed to be responsible for substrate binding and for stabilizing compound I (Vitello et al., 1993).
The present crystallographic studies of the ligand-bound forms of ARP showed that Arg-52 moved little on ligand binding. In contrast, the distal arginine (Arg-48) in CCP moved markedly when it bound to fluoride (Edwards and Poulos, 1990), its side chain by about 2 Å to optimize the hydrogen-bonded interactions with the fluorine atom. In addition, when CCP was converted to compound I, the side chain of Arg-48 moved close to the ferryl oxygen atom to form a hydrogen bond (Fülöp et al., 1994). These results indicate that the side chain of the distal arginine of ARP is more tightly fixed than CCP. In fact, ARP has more hydrogen bonds than CCP for anchoring the side chain. It appears that little movement of Arg-52 occurs during compound I formation in ARP.

Finzel et al. (1984) speculated on the coordination of hydroperoxide to CCP, and on the basis of the structure of the fluoride-bound CCP, Edwards and Poulos (1990) suggested that O1 becomes hydrogen-bonded to both N$_H$ and N$_{as}$H$^+$ of the distal arginine (Arg-48; identical to Arg-52 in ARP) by movement of its side chain during compound I formation. One of the functional roles of the distal arginine is neutralization of the negative charge of fluoride (as well as HOO$^-$), and in the case of CCP, fluoride binding to the heme was achieved by movement of its side chain. We obtained no evidence that ARP forms a complex with fluoride ion. In the case of ARP the assumed position of the fluoride and that of the N$_e$ of Arg-52 are too far apart to interact. A possible explanation for the inability

TABLE IV
Geometry of triiodide-bound heme

|                  | pH 6.5 | pH 5.0 |
|------------------|--------|--------|
| Distances (Å)    |        |        |
| Fe–I$_3$         | 3.25   | 3.29   |
| I$_3$–N$_e$ (His-56) | 3.08   | 3.39   |
| Fe to pyrrole N plane | 0.16   | 0.17   |
| Fe–N$_e$ (His-184) | 2.09   | 2.13   |
| Angles (°)       |        |        |
| Fe–I$_3$–I$_3$   | 108    | 106    |
| I$_3$–Fe to pyrrole N plane | 84     | 86     |
| Dihedral angles (°) |     |        |
| Pyrrole N to pyrrole I | 9      | 5      |
| Pyrrole N to pyrrole II | 13     | 11     |
| Pyrrole N to pyrrole III | 1      | 3      |
| Pyrrole N to pyrrole IV | 9      | 10     |

The Fourier coefficient was $(F_{I3} - F_{native}) \exp(i\alpha_{native})$, where $F_{I3}$, $F_{native}$, and $\alpha_{native}$ respectively, are the observed structure factors of the complex and the native crystals and the phase angle of the native crystal at pH 7.5. An α carbon backbone and the active site residues (Arg-52 and His-56) of ARP are superimposed on the map. The minimum contour level is ±3σ. b, close-up view of the internal triiodide ion. The coefficient was $(F_{c} - F_{I3}) \exp(i\alpha_{I3})$, where $F_{c}$ and $\alpha_{I3}$ were calculated from the atomic parameters of all the atoms except those of the ion.

**FIG. 4. Difference Fourier maps for the triiodide complex.** a, the Fourier coefficient was $(F_{I3} - F_{native}) \exp(i\alpha_{native})$, where $F_{I3}$, $F_{native}$, and $\alpha_{native}$ respectively, are the observed structure factors of the complex and the native crystals and the phase angle of the native crystal at pH 7.5. An α carbon backbone and the active site residues (Arg-52 and His-56) of ARP are superimposed on the map. The minimum contour level is ±3σ. b, close-up view of the internal triiodide ion. The coefficient was $(F_{c} - F_{I3}) \exp(i\alpha_{I3})$, where $F_{c}$ and $\alpha_{I3}$ were calculated from the atomic parameters of all the atoms except those of the ion.
of ARP to form a stable fluoride complex is the lack of interaction, because the side chain of Arg-52 of ARP is fixed.

Conformational Flexibility of His-56 and the Mechanism of Compound I Formation—The distal histidine has been proposed to catalyze proton translocation from one oxygen to the other of the hydrogen peroxide by acid-base catalysis. The mechanism of compound I formation proposed for ARP is shown schematically in Fig. 6. Important questions arise immediately about the behavior of the active site residues of ARP in enzyme catalysis. 1) How does the substrate bind to the active site of ARP in which the conformation of the side chain of Arg-52 is fixed at the site observed in the native enzyme? 2) Does the imidazole of His-56 have favorable geometries for proton translocation? In order to gain insight into enzyme catalysis, we examined the structures of the substrate-enzyme complex and transitional complex using computer graphics.

The proposed model of the H$_2$O$_2$-enzyme complex is shown in Fig. 7. Hydrogen peroxide would displace two water molecules in the active site; Wat-674 which is the closest one to the heme iron and is hydrogen-bonded to the N$_e$ of His-56, and Wat-675 which is hydrogen-bonded to the N$_o$ of Arg-52 and Wat-674. It is possible to place hydrogen peroxide on the distal side of the heme in such a way that one oxygen atom is on the heme iron and the other is hydrogen bonded to Arg-52 without conformational change in the Arg-52 side chain. If the H-O-O-H torsion angle in hydrogen peroxide is assumed to be orthogonal, a stable conformer, the hydrogen bond of N$_e$-H (Arg-52)–O1 will direct the O2-H bond toward the N$_o$ of His-56 because the N$_o$ of Arg-52 bears hydrogen and is a donor of the hydrogen bond. Such geometry of the hydrogen peroxide relative to the active site of ARP appears to be favorable for the subsequent proton abstraction from O2 to the N$_o$ of His-56.

Directing the N$_e$-H of His-56 toward O1 for proton translocation is achieved by rotations around the C$_{\alpha}$C$_{\beta}$ and C$_{\beta}$C$_{\gamma}$ bonds. The directions of these rotations are the same as when triiodide binds to ARP, but the amount is larger. If these bonds were rotated so as to place H-N$_e$ (His-56) on the O1 atom with the remaining residues unchanged, the geometries of the hydrogen bond between N$_o$ of His-56 and O$_d$ of Asn-93 would become unfavorable. To achieve a tetrahedral arrangement around O1, i.e. to interact the O1 and His-56-N$_e$, which is favorable for the subsequent proton translocation from His-56$^H$ to O1, another type of additional movement such as the shift of hydroperoxide and/or the dynamic behavior of the protein molecule should be considered. We could not build a reasonable model of the transitional complex II for ARP assuming that the conformation of the distal histidine is retained as in the native enzyme.

In conclusion, the structures of the native and triiodide-bound form of ARP suggest that the mechanism of compound I formation of ARP is similar to that of CCP as proposed by Finzel et al. (1984). The behavior of the active site residues during compound I formation for ARP differs, however, from that for CCP. Our computer modeling study suggests that Arg-52 maintains the conformation of hydrogen peroxide favorable for compound I formation without side chain movement and that

| TABLE V | Conformation of distal histidine |
|---------|---------------------------------|
|         | N-C$_{\alpha}$C$_{\beta}$-C$_{\gamma}$ | C$_{\beta}$C$_{\gamma}$-C$_{\delta}$ |
| ARP     | Native (pH 7.5) | −67 | 5 |
| CN      | (pH 7.5)        | −68 | 16 |
| CN      | (pH 5.0)        | −69 | 17 |
| CN      | (pH 4.0)        | −68 | 17 |
| I$_3$   | (pH 6.5)        | −71 | 17 |
| I$_3$   | (pH 5.0)        | −76 | 9  |
| Lip     | Native (pH 4.5) | −70$^a$ | 65$^a$ |
| CCP    | Native (ICCP)   | −90 | 48 |
| CCP    | Ligand (2CYP)   | −86 | 37 |

$^a$ Mean value of two crystallographic independent molecules.
conformational flexibility of His-56 is necessary for proton translocation from one oxygen atom to the other of the hydroperoxide.

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