The site and characteristics of iodide binding to Arthromyces ramosus peroxidase were examined by X-ray crystallographic analysis, $^1\text{H}$ and $^{127}\text{I}$ NMR, and kinetic studies. X-ray analysis of an A. ramosus peroxidase crystal soaked in a KI solution at pH 5.5 showed that a single iodide ion is located at the entrance of the access channel to the distal side of the heme and lies between the two peptide segments, Phe$^{90}$-Pro$^{91}$-Ala$^{92}$ and Ser$^{151}$-Leu$^{152}$-Ile$^{153}$, 12.8 Å from the heme iron. The distances between the iodide ion and heme peripheral metal groups were all more than 10 Å. The findings agree with the results obtained with $^1\text{H}$ NMR in which the chemical shift and intensity of the methyl groups in the hyperfine shift region of A. ramosus peroxidase were hardly affected by the addition of iodide, unlike the case of horseradish peroxidase. Moreover, $^{127}\text{I}$ NMR and steady-state kinetics showed that the binding of iodide depends on protonation of an amino acid residue with a pK$_a$ of about 5.3, which presumably is the distal histidine (His$^{90}$), 7.8 Å away from the iodide ion. The mechanism of electron transfer from the iodide ion to the heme iron is discussed on the basis of these findings.

Peroxidases (EC 1.11.1.7; donor, H$_2$O$_2$ oxidoreductase) are a family of heme-containing enzymes which catalyze the oxidation of a number of organic and inorganic substrates with hydrogen peroxide (1, 2). The oxidation reaction for organic substrates generally occurs by two sequential one-electron transfer reactions through the formation of intermediate compounds (compound I and compound II). In contrast, the oxidation of inorganic compounds (such as iodide or thiocyanide) is mediated by one two-electron transfer to compound I (3–6). The mechanisms of these electron transfer reactions have yet to be determined. Investigation of the mechanism is necessary in relation to the roles of peroxidases such as thyroid peroxidase, lactoperoxidase, chloroperoxidase, myeloperoxidase, and eosinophil peroxidase that function in hormone synthesis, bactericidal activity, and phagocytosis (1, 2).

Although kinetic studies of the reactions between compound I and halides showed characteristics of second order reactions, various evidence suggested that before the process of electron transfer, halide ions bind to the protein portion of compound I at a particular site in the vicinity of the prosthetic group. Because it is very difficult to study the binding to compound I that causes the reaction, the binding of these ions to peroxidases in the resting state was examined by spectrophotometric (4, 7, 8), kinetic (9, 10), fluorometric (11), $^{127}\text{I}$ NMR (12, 13), $^{15}\text{N}$ NMR (14–17), $^{13}\text{C}$ NMR (17), $^1\text{H}$ NMR (18–19), and optical difference spectroscopy (20–22) techniques. The actual site of the binding of these ions to the enzymes and the mechanism of electron transfer from these ions to the heme irons have yet to be clarified. This is partly due to lack of knowledge about the fine three-dimensional structures of enzymes such as HRP and lactoperoxidase that are used in studies on the binding of halide ions.

Recently, in addition to the peroxidases of plants and animals, many peroxidases have been isolated from fungi and bacteria, and their physicochemical properties are characterized (23). ARP, one such enzyme, is secreted from the hypomycete Arthromyces ramosus (Fungi Imperfecti). Its three-dimensional structure was determined recently by Kunishima et al. (24). It is almost identical in amino acid sequence to the Coprinus cinereus peroxidase which has been characterized by kinetic, chemical, spectroscopic, and NMR methods (25–29). These findings prompted us to attempt to determine the iodide-binding site by x-ray crystallographic analysis. In addition, the binding of iodide ions to ARP was examined by $^1\text{H}$ NMR, $^{127}\text{I}$ NMR, and kinetic techniques to compare with those obtained previously for HRP and lactoperoxidase.

**EXPERIMENTAL PROCEDURES**

**Materials**—ARP that had been purified by the method of Morita et al. (27) was provided by Dr. T. Amachi. The Reinheit Zahl ($A_{403}/A_{280}$) was 2.63. The concentration of the enzyme was determined spectrophotometrically from the molar extinction coefficient of 405 nm, 1.09 × 10$^3$ cm$^{-1}$·mol$^{-1}$ (30). KI and H$_2$O$_2$ were purchased from Wako (Osaka, Japan). Deuterium oxide (99.85%) was purchased from the Commissariat a l’Energie Atomique, France.

**X-ray Crystallographic Analysis**—The iodide-bound form of the ARP crystal was prepared by soaking the parent ARP crystal, which had been prepared as described previously (24), for 12 h in 20 mM ammonium acetate buffer adjusted to pH 5.5 and containing 33% saturated ammonium sulfate and 30 mM potassium iodide. Diffraction data for the iodide derivative were collected to 2.06 Å resolution at room temperature on an R-AXIS IIC imaging plate area detector. X-rays, generated with a Rigaku rotating anode at 40 kV and 100 mA, were monochro-
aromatized (λ = 1.5418 Å) with graphite. The diffraction data recorded on each imaging plate were read out at 100-μm intervals then processed with PROCESS (32). Intensities of the partial reflections recorded on adjacent two imaging plates were summed to obtain the integrated intensities. The conditions and results of the data collection are summarized in Table I.

Two kinds of difference Fourier syntheses were calculated in order to locate the iodide ion. One was an \( (F_o - F_c) \) synthesis at 2.06 Å resolution, where \( F_o \) is the observed structure factor of the iodide derivative and \( F_c \) the calculated structure factor derived from the atomic parameters of the native crystal. The other was anomalous difference synthesis at 4.0 Å resolution. The phase angles were calculated using the atomic parameters of ARP at pH 4.5 refined at 1.8 Å resolution (33).

The model of the iodide derivative was refined by simulated annealing using the program XPLOR (34). Rearrangement of water molecules and conformational change in the model were checked with the FRODO (35, 36) and IRIS 4D/35GT computer graphics system. When the temperature factors of all the atoms were refined with the occupancy of iodide fixed at 0.3, the temperature factor of the iodide converged to the value of 21.9 Å². The final model contains one iodide ion and 250 water molecules in addition to the protein. The crystallographic \( R \) factor was 16.2% for 19,513 reflections with \( F > 2σ_F \) in the 7.0–2.06 Å resolution range.

**NMR Measurements**—Proton NMR measurements were made with a Bruker AMX-400WB NMR spectrometer at 298 K. Samples dissolved in a deuterated phosphate buffer (100 mM) were measured in an NMR microtub (0.2 ml) with symmetric geometry along the Bo field (37). Typical spectra were obtained by accumulation of 20,000 transients at 32,000 data points over an 80-kHz bandwidth (18). Proton chemical shifts were referred to the proton signal of trace DHO at 4.82 ppm. The \( 1^27I \) NMR was recorded at 80 MHz on the same spectrometer at 298 K equipped with an inverse HX probe. Typical spectra were obtained by 200,000–800,000 transients using 5-degree pulse at 32,000 data points over a 41.6-kHz bandwidth and applied 100-Hz line broadening to the free induction decay prior to the Fourier transform.

**Peroxidase Kinetics**—The rate of oxidation of iodide ion by \( \text{H}_2\text{O}_2 \) was measured at 295 K by following \( I^- \) at 350 nm as described previously (39), except that the total volume of the reaction mixture was reduced to 2.4 ml and the \( \text{H}_2\text{O}_2 \) concentration was 270 μM. The pH range of the medium was 4.0–6.0 in 33 mM acetate buffer. The spectrophotometer used was a Hitachi model UV-3000 spectrophotometer equipped with a thermoregulator made of Peltier units, and the time course was recorded on a floppy disk to instantly obtain the initial rate.

**RESULTS**

**Iodide-binding Site and Its Environment**—The ARP crystal soaked in KI solution was isomorphous with the native crystal and showed no significant change in conformation on the binding of iodide. The \( (F_o - F_c) \) and anomalous difference maps are shown in Fig. 1, and the peak heights that appear in these maps are listed in Table II. The \( (F_o - F_c) \) map showed only one significant peak per asymmetric unit. Its height was greater than 15σ, whereas the second highest peak was less than 6σ. The anomalous difference map of the derivative crystal showed four significant peaks per asymmetric unit. The highest peak appeared at the iron site in the heme group, and the second highest peak appeared at exactly the same site as in the \( (F_o - F_c) \) map. The third and fourth largest peaks were at the sites of the two presumed calcium sites (24). These findings strongly suggest that the iodide binds to ARP at a single site. The occupancy of the present iodide derivatives is estimated to be about 30% based on the peak height at this site in the anomalous difference map. The anomalous difference map also confirmed the two calcium sites proposed from circumstantial evidence.

A close-up view of the environment of the iodide-binding site is shown in Fig. 2. The binding site is between the two peptide segments, Phe90-Pro91-Ala92 and Ser151-Leu152-Ile153, which respectively continue to helices C and E (24) and form the upper rim of the access channel to the heme distal side. The site is near the surface side of Ile153 and, at its opposite side (inside of ARP), there is the imidazole ring of the distal histidine (His94). The coordinate of the bound iodine atom and the distances from the iodine atom to a few adjacent amino acid residues are listed in Table III.

### Table I

**Conditions and results of intensity measurement**

| Condition | Value |
|-----------|-------|
| Wavelength (Å) | 1.5418 |
| Oscillation angle (degrees) | 1.5 |
| Overlap (degrees) | 0.0 |
| Exposure time (min) | 15 |
| Number of imaging plates | 60 |
| Resolution limit (Å) | 2.06 |
| Measured reflections | 125,031 |
| Independent reflections | 20,178 |
| Completeness (%) | 95.3 |
| \( R_{merge} \) (%) | 6.2 |

\( R_{merge} = \sum \frac{|\langle I(hkl) \rangle| - |\langle I(hkl) \rangle|}{\sum I(hkl)} \)

2 Except for iodine and iron the crystal contained no element with a Δ\( \rho \) value greater than that of calcium. Because neither of the wavelengths of the absorption edges of iodine and calcium is within an adjustable range, even if synchrotron radiation is used, it is impossible to determine exactly the species of these atoms using the x-ray diffraction alone.

**FIG. 1.** Electron densities of the \( (F_o - F_c) \) (A) and anomalous difference (B) Fourier maps for the iodide derivative superimposed on the Co model of ARP. The density for the \( (F_o - F_c) \) map is drawn at 6σ and that for anomalous difference map at 2.5σ.
Interaction of Iodide with ARP Probed by $^{127}$I NMR and $^1$H NMR—Typical $^{127}$I NMR spectra are shown in Fig. 3A. An aqueous KI solution (100 mM, pH 6.25) had a very broad $^{127}$I NMR signal with a $\Delta\nu_{\text{1/2}}$ value of approximately 2.0 kHz (upper panel). The addition of ARP to the KI solution further broadened the signal (lower panel). The $\Delta\nu_{\text{1/2}}$ values of the I/ARP complex solution varied linearly with the enzyme concentration but leveled off at about 300 $\mu$M (Fig. 3B). pH had a slight effect on the $\Delta\nu_{\text{1/2}}$ of I/ARP, a distinct increase in the line width of the I/ARP occurring with a decrease in pH (Fig. 3C, solid circles). This suggests that protonation of an ionizable group with $pK_a$ of $<5.5$ has a role in iodide binding, but the exact $pK_a$ value could not be obtained because the enzyme tended to become insoluble in the acidic region.

The proton NMR of native ARP at pH 5.5 is shown in Fig. 4A. The hyperfine-shifted spectrum in the region of 60–90 ppm is characterized by three peaks (a, b, and c), the size of the last peak being about twice of that of peaks a and b. The spectral pattern is similar to that of C. cinereus peroxidase whose amino acid sequence is almost identical to that of ARP (28, 40, 41). Therefore, it is most likely that peaks a, b, and c arise from protons of heme peripheral methyls at 3, 8, and 1 plus 5, respectively. We showed previously that an iodide ion induces marked changes in both chemical shift and line width of 1- and 8-methyl protons of HRP (18). In the case of ARP, however, these changes were small compared with those of HRP. The $K_d$ value for the binding of iodide is estimated to be 50 mM at pH 5.5 based on the chemical shift and intensity changes (Fig. 4, B and C).

Effect of pH on the Rate of the Oxidation of Iodide—Previous studies on the rate of oxidation of iodide with HRP compound I showed that protonation of an ionizable group with a $pK_a$ value of 4.0 (18) or 4.6 (4) is necessary for the reaction. For comparison, the rate of the iodide oxidation catalyzed by ARP was examined in the acidic region. The Lineweaver-Burk plots in Fig. 5 indicate that the apparent $K_a$ values at pH 4.5, 5.0, 5.5, 5.75, and 6.0, respectively, are 13, 16, 23, 34, and 62 mM and that the $pK_a$ of the ionizable group involved in iodide oxidation is estimated to be 5.3, although slight deviations were found in the low and high concentration regions at pH 6.0 and 4.5.

**DISCUSSION**

The x-ray crystallographic analysis reported here shows that the ARP-iodide complex contains only one iodine atom, which is located at the entrance of the access channel to the distal side of the heme, 12.8 Å away from the heme iron (Fig. 1, Fig. 2, and Table III). To our knowledge, this is the first report on the binding site of the electron donor molecule to peroxidase determined by x-ray crystallographic analysis.

When the ARP crystal was soaked in 2 mM KI solution containing about 15 mM KI, two triiodide ions bound to one ARP molecule, but no iodide ion was found on the enzyme (45). One of the two triiodide-binding sites, the external one, is close to the iodide-binding site, 4.8 Å from the end iodine atom of the triiodide ion of the external site. Because the distance is slightly larger than the sum of the van der Waals radii of the two iodine atoms (4.3 Å), the fact that no iodide ion was found on the surface of ARP molecule in previous experiments is explained not by simple steric hindrance but by the repulsive force produced by two negative ions and by lower affinity of the iodide ion than the triiodide ion.

It is noteworthy that no iodide ion was found in the heme pocket of ARP in either the present or previous (45) experiments. The absence of iodide ion in the latter case is explained by assuming that iodide, even though once bound, was released by the triiodide, which has a higher affinity for the distal side of the heme. The reason for the absence of iodide ion in the heme pocket of ARP, which is large enough to accommodate even the triiodide ion and involves positively charged groups, however, is not clear.

The binding of iodide to ARP in solution was examined by $^{127}$I NMR and $^1$H NMR. $^{127}$I NMR findings showed reversible binding of iodide to ARP with a very fast (less than a millisecond) exchange rate, facilitated by protonation of amino acid residues with a $pK_a$ value of $<5.5$. In contrast, $^1$H NMR showed that the hyperfine-shifted methyl resonance is scarcely affected by the addition of iodide ion. This agrees with the long (>10 Å) distances between the iodide and the heme periphery methyl group found by x-ray analysis. The $^1$H NMR results indicate that the iodide-binding affinity is fairly low ($K_d = 50$ mM at pH 5.5).

Participation of an ionizable group with a $pK_a$ value of about 5.3 was suggested from the steady-state kinetics (Fig. 5). Although the residue was not identified, it is likely to be the distal histidine (His$^{56}$) in view of amino acid residues in the vicinity of the iodide-binding site. It must be noted that chemical modification of the distal histidine (His$^{42}$ corresponding to the His$^{56}$ of ARP) in HRP abolished iodide oxidation activity with-

**TABLE II**

Peak heights in difference Fourier maps

|       | Fe      | Ca(1)  | Ca(2)  | Other highest peak |
|-------|---------|--------|--------|-------------------|
| $F_o - F_e$ | 460     | 187$^a$|        |                   |
| Anomalous difference | 420 692 409 | 286$^b$|        |                   |

$^a$ Rearranged water molecule on the binding of iodide to ARP.

$^b$ Observed at the Cys$^{93}$-Cys$^{129}$ disulfide bridge.
Moreover, we earlier found that, with respect to lactoperoxidase, protonation of an ionizable group with a $K_a$ value of 6.0–6.8, probably the distal histidyl residue, is essential for iodide oxidation (13).

The location of the iodide ion described here is for ARP at the resting state, not for ARP compound I. However, it is unlikely that the binding sites differ, because the surface structure of ARP may not be influenced by the formation of the oxyferryl group, as seen from the ligation of cyanide in ARP (45) and the formation of compound I in cytochrome $c$ peroxidase (47) and catalase (48). The first step of iodide oxidation therefore would be electron transfer from the iodide-binding site reported here to the imidazolium group of the distal histidine. As shown in Fig. 2, direct binding of iodide to the imidazolium of His$^{56}$ is prevented by several amino acid residues, the distance being about 8 Å. Long range electron transport from the iodide to the imidazole therefore would occur, as in the cytochrome $c$ peroxidase and cytochrome $c$ system (49, 50).

The next step would be electron transfer from the imidazole to the oxyferryl group of compound I. The accumulated evidence indicates that a hydrogen bond is formed between the imidazole of the distal histidine (His$^{42}$) and the oxyferryl group

| Coordinate of the iodide ion and its distance from the adjacent amino acid residues, heme iron, and peripheral methyl groups |
|---------------------------------------------------------------|
| Coordinate of the iodide ion                                  |
| $x = 8.05$, $y = 24.49$, $z = 36.75$ |
| Distance (Å) of the iodide ion from $C_\beta$ of Phe$^{70}$ |
| 3.8 |
| $C_5$ of Pro$^{32}$                                           |
| 3.8 |
| N of Ser$^{131}$                                              |
| 4.0 |
| N of Ala$^{92}$                                               |
| 4.3 |
| Fe of Heme                                                   |
| 12.8 |
| C of Heme 1-methyl                                           |
| 10.4 |
| C of Heme 3-methyl                                           |
| 13.9 |
| C of Heme 5-methyl                                           |
| 16.8 |
| C of Heme 8-methyl                                           |
| 10.8 |

FIG. 3. $^{127}$I NMR spectra of the KI solution. A, the spectra of KI (100 mM) were obtained in the absence (upper panel) and presence (0.38 mM, lower panel) of ARP. B, the $\Delta$H$_{1/2}$ change as a function of the ARP concentration. [KI] = 100 mM. C, the $\Delta$H$_{1/2}$ change as a function of pH. [ARP] = 0.38 mM, [KI] = 100 mM.

FIG. 4. Hyperfine-shifted portions of the $^1$H NMR spectra of ARP in the presence and absence of iodide. A, the enzyme concentration was 1.22 mM at pH 5.5, and the concentrations of potassium iodide were 0 (upper curve) and 100 mM (lower curve). Changes in chemical shift and intensity by the addition of iodide are shown in B and C, respectively. An arbitrary unit is used for intensity. The curves in the figures were drawn based using nonlinear least squares fitting calculation with a common value at $K_d = 50$ mM.

FIG. 5. Lineweaver-Burk plots of the rate of oxidation of iodide by $H_2O_2$ as catalyzed by ARP. The reaction rate was determined as described under “Experimental Procedures” in 1.5–2.5 mM enzyme at pH 4.5 (○), 5.0 (●), 5.5 (△), 5.75 (▲), and 6.0 (□).
of HRP compound II (51–57). This also may be the case for ARP, because a hydrogen bond between FeCN$^-$ and the imidazole of His$^{56}$ is expected (45). The hydrogen bond may play an important role in the proton tunneling mechanism in the electron transfer from the imidazole to the oxyferryl group (58, 59). Further theoretical treatment of electron transfer will be made on the basis of the information on the spatial relationship reported here.

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