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Proton and Iodine-127 Nuclear Magnetic Resonance Studies on the Binding of Iodide by Lactoperoxidase†

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Received March 18, 1987; Revised Manuscript Received May 29, 1987

ABSTRACT: Interaction of an iodide ion with lactoperoxidase was studied by the use of ^{1}H NMR, ^{127}I NMR, and optical difference spectrum techniques. ^{1}H NMR spectra demonstrated that a major broad hyperfine-shifted signal at about 60 ppm, which is ascribed to the heme peripheral methyl protons, was shifted toward high field by adding KI, indicating the binding of iodide to the active site of the enzyme; the dissociation constant was estimated to be 38 mM at pH 6.1. The binding was further detected by ^{127}I NMR, showing no competition with cyanide. Both ^{1}H NMR and ^{127}I NMR revealed that the binding of iodide to the enzyme is facilitated by the protonation of an ionizable group with a pK_a value of 6.0–6.8, which is presumably the distal histidyl residue. Optical difference spectra showed that the binding of an aromatic donor molecule to the enzyme is slightly but distinctly affected by adding KI. On the basis of these results, it was suggested that an iodide ion binds to lactoperoxidase outside the heme crevice but at the position close enough to interact with the distal histidyl residue which possibly mediates electron transport in the iodide oxidation reaction.

Peroxidases (EC 1.11.1.7, donor:H_2O_2 oxidoreductase) are a family of heme-containing enzymes which catalyze the oxidation of many organic and inorganic compounds with hydrogen peroxide (Saunders et al., 1961). Of many inorganic compounds, iodide is the most attractive substrate, because it plays an important role in thyroid hormone biosynthesis and bactericidal reaction catalyzed by thyroid peroxidase and lactoperoxidase, respectively (Morrison & Schonbaum, 1976; Nunez & Pommier, 1982). However, the mechanism of oxidation of inorganic compounds by peroxidases has been less sufficiently studied so far (Dunford & Stillman, 1976), as compared with the oxidation of aromatic donor molecules catalyzed by peroxidases, especially by horseradish peroxidase.

It was reported that the oxidation of iodide with hydrogen peroxide catalyzed by thyroid peroxidase and lactoperoxidase occurs in the manner of two-electron transfer, in contrast to one-electron transfer for usual aromatic donor molecules (Magnusson et al., 1984; Ohtaki et al., 1981). The mechanism of the two-electron-transfer reaction, however, is still obscure although several views were presented. In order to elucidate the mechanism, studies on the interaction of iodide with these enzymes are needed.

Horseradish peroxidase is also known to catalyze the oxidation of iodide with hydrogen peroxide in a manner of two-electron transfer only under acidic conditions (Björkstén, 1970). The binding of iodide to horseradish peroxidase was studied by spectrophotometric (Björkstén, 1970), kinetic (Pommier et al., 1973), fluorometric (Ugarova et al., 1981), ^{127}I NMR (Sakurada et al., 1985), and ^{1}H NMR (Sakurada et al., 1987) techniques. The ^{1}H NMR studies disclosed that

† This work was supported in part by a grant-in-aid from the Ministry of Education, Science, and Culture of Japan (62580107).
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0006-2960/87/0426-6478$01.50/0

an iodide ion associates with the enzyme in the close vicinity of the heme peripheral 1- and 8-methyl groups at the place roughly in equal distance.

In the present study, the interaction of an iodide ion with lactoperoxidase was investigated by the use of ^{127}I NMR, ^{1}H NMR, and optical difference spectrum techniques. On the basis of the results, the mechanism of iodide oxidation catalyzed by the enzymes will be discussed, being compared with that of horseradish peroxidase.

Experimental Procedures

Materials. Horseradish peroxidase was purchased as a lyophilized, salt-free powder from Toyobo Co., Ohtsu, Japan. Lactoperoxidase was purified from raw skim milk according to Rombauts et al. (1967) with slight modifications. The RZ values for horseradish peroxidase (A_{403}/A_{280}) and lactoperoxidase (A_{412}/A_{280}) at pH 7.0 were 3.0 and 0.80, respectively. The concentration of the enzymes was determined spectrophotometrically by using molar extinction coefficients of 1.02×10^5 cm^{-1} M^{-1} at 403 nm for horseradish peroxidase (Aibara et al., 1982) and 1.12×10^5 cm^{-1} M^{-1} at 412 nm for lactoperoxidase (Carlström, 1969). Deuterium oxide (>99.85%) was purchased from Commissariat a l'Energie Atomique, Saclay, France. All other reagents were of analytical grade.

NMR Measurements. ^{127}I NMR measurements were made on a Bruker CXP-300 FT NMR spectrometer at 60.034 MHz in a 10-mm sample tube with internal D_2O for the frequency lock. Typical spectra consisted of 1000–10000 transients using 8K data points over a 50-kHz spectral window in a quadrature detection mode. Other experimental conditions were essentially the same as described previously (Shimizu & Hatano, 1985).

^{1}H NMR was recorded at 270 MHz on Bruker WH-270 and JEOL GX-270 spectrometers at 23 °C. Samples for the ^{1}H NMR measurements were lyophilized directly inside 5-mm NMR tubes with excess D_2O and dissolved with 0.4 mL of D_2O. Typical spectra were obtained by the accumulation of 20000 transients at 8000 data points over a 60-kHz bandwidth (Sakurada et al., 1987). Proton chemical shifts were referred to the proton signal of trace HDO.

pH and pD values were measured in the NMR tubes using Radiometer pHM-26 with an Ingold pH electrode calibrated with standard aqueous (H_2O) buffer, disregarding the isotope effect. The adjustment of pH values in solutions was performed by adding potassium hydroxide and phosphoric acid.

Measurement of Rate of Iodide Oxidation. The reaction was measured by following I_3^- spectrophotometrically at 350 nm under the conditions described (Hosoya et al., 1962). The pH range of the medium used was 5.0–8.0, using 33 mM potassium phosphate and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer. The extinction coefficient of triiodide was almost constant in the pH range, and the values described previously (Hosoya, 1963) were used throughout this study.

Difference Optical Spectra. Difference optical spectra (enzyme/donor vs. enzyme) were obtained with a Hitachi 557 double-beam computerized spectrophotometer, as described previously (Sakurada et al., 1986), and the K_d value was calculated as described by Paul and Ohlsson (1978). The substrate and enzyme were dissolved in 0.1 M phosphate buffer, pH 7.4, or buffer containing 0.1 M KI.

Results

Interaction of Iodide with Lactoperoxidase Probed by ^{127}I NMR. Figure 1A and Figure 1B show ^{127}I NMR spectra of a KI solution (50 mM, pH 6.1) in the absence or presence of

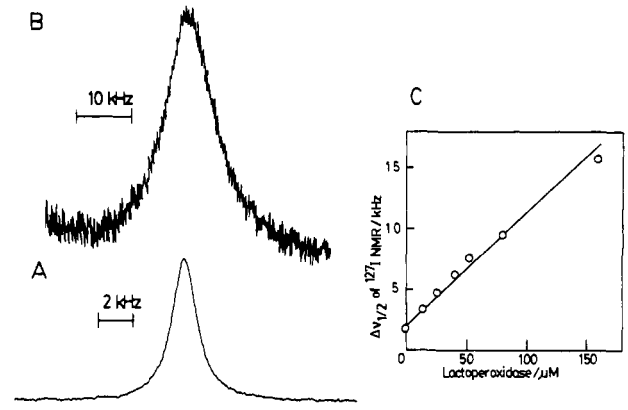

FIGURE 1: ^{127}I NMR spectra of (A) KI (50 mM) at pH 6.1 and (B) KI (50 mM) plus lactoperoxidase (80 μM) at pH 6.1. The $\Delta\nu_{1/2}$ values of (A) and (B) were 1.8 and 10.0 kHz, respectively. Temperature was kept at 290 ± 0.5 K. The inset (C) shows the $\Delta\nu_{1/2}$ change of 50 mM KI by adding lactoperoxidase.

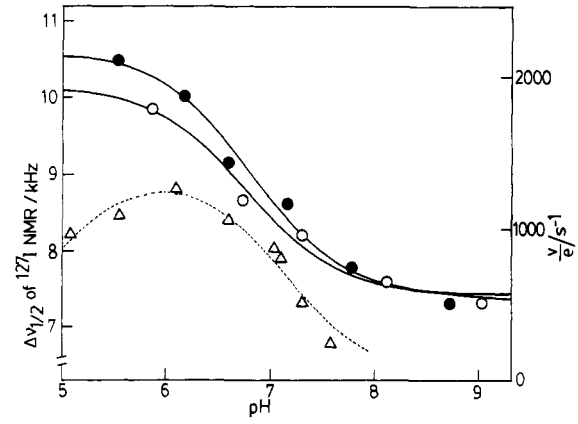

FIGURE 2: ^{127}I NMR line width of KI plus lactoperoxidase in the presence (○) and absence (●) of KCN and enzymatic activity as functions of pH. The solutions for NMR experiments consisted of KI (50 mM) plus lactoperoxidase (80 μM) (●) and of KI (50 mM) plus lactoperoxidase (80 μM) plus KCN (5 mM) (○) in 20 mM citrate/20 mM phosphate buffer. The catalytic activity of lactoperoxidase for iodide oxidation by H_2O_2 (△) was measured under the conditions described under Experimental Procedures. The dashed line was obtained theoretically by using a nonlinear least-squares method; the pK_a values of dissociable groups estimated were 4.8 and 7.1.

lactoperoxidase (80 μM), respectively: the broad ^{127}I NMR signal of I^- ($\Delta\nu_{1/2}$ = 1.8 kHz) was further broadened to 10.0 kHz by adding the enzyme. The $\Delta\nu_{1/2}$ of the I^-/enzyme solution changed linearly with the concentration of the enzyme as shown in Figure 1C.

The effect of pH on the line width of the I^-/enzyme solution was examined at the pH region above 5.5, since the enzyme tended to aggregate below pH 5.5. As shown by the closed circles of Figure 2, deprotonation of an ionizing group with pK_a = 6.8 ± 0.1 (mean ± SD) reduced the line-width broadening by the enzyme until a constant value (7.5 kHz) was obtained above pH 8.5. No pH effect on $\Delta\nu_{1/2}$ of I^- was observed in the absence of the enzyme (Sakurada et al., 1985). On the other hand, the pH effect on enzyme activity (triangles in Figure 2) revealed, on the basis of computer simulation, contribution of two amino acid residues with pK_a = 4.8 ± 0.2 and pK_a = 7.1 ± 0.1, the latter of which is very similar to the pK_a = 6.8 mentioned above. Thus, it is suggested that there are two types of I^- binding to lactoperoxidase: (1) a nonspecific interaction between I^- and the enzyme at a pH above 8.5 with a $\Delta\nu_{1/2}$ of 7.5 kHz and (2) a specific interaction between I^- and the enzyme at a pH below 8 which increases with de-

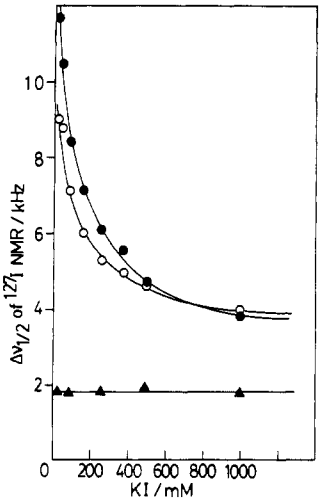

FIGURE 3: $\Delta\nu_{1/2}$ values of ^{127}I NMR as functions of KI concentration. The solution for NMR experiments consisted of KI (50 mM) at pH 6.9 (▲), KI (50 mM) plus horseradish peroxidase (80 μM) at pH 4.0 (○), and KI (50 mM) plus lactoperoxidase (80 μM) at pH 6.2 (●).

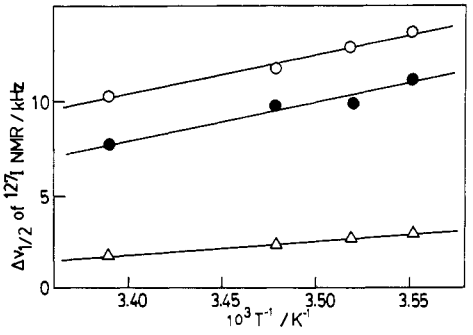

FIGURE 4: ^{127}I NMR line width of KI, KI plus horseradish peroxidase, and KI plus lactoperoxidase as a function of temperature. The solution for NMR experiments consisted of KI (50 mM) at pH 6.9 (△), KI (50 mM) plus horseradish peroxidase (80 μM) at pH 4.0 (●), and KI (50 mM) plus lactoperoxidase (80 μM) at pH 6.0 (○).

creasing pH (pK_a = 6.8–7.1). Figure 2 (open circles) also shows that the addition of KCN causes little effect on the line broadening throughout the pH range used, suggesting that cyanide binding to the heme iron does not interfere with the I^-/enzyme interaction.

Since the optimum pH of the iodide oxidation reaction catalyzed by lactoperoxidase was found to be 6.2 (Figure 2), $\Delta\nu_{1/2}$ of I^-/lactoperoxidase was measured at pH 6.2 in a wide range of KI concentrations and is presented in Figure 3 together with the data of horseradish peroxidase at pH 4.0 where the rate of the iodide oxidation reaction was highest (data not shown). It is clear that the increase in $\Delta\nu_{1/2}$ of I^- due to binding to lactoperoxidase is more pronounced in the region of lower KI concentration (less than 700 mM) than that for horseradish peroxidase while changes in $\Delta\nu_{1/2}$ in the region of higher KI concentration (more than 700 mM) resembled each other.

The temperature-dependent behaviors of the $\Delta\nu_{1/2}$ values of I^-/lactoperoxidase, I^-/horseradish peroxidase, and I^- only solutions are illustrated in Figure 4. The $\Delta\nu_{1/2}$ value of free I^- was decreased by raising the temperature. Similarly, the $\Delta\nu_{1/2}$ value of the I^-/lactoperoxidase and I^-/horseradish peroxidase solutions was decreased by raising the temperature. The temperature dependences of ^{127}I NMR ($I = {}^5/_2$) of these enzyme solutions are in contrast with those of quadrupolar metal NMR such as ^{25}Mg ($I = {}^5/_2$), ^{43}Ca ($I = {}^7/_2$), or ^{67}Zn ($I = {}^5/_2$) in that the $\Delta\nu_{1/2}$ value of these metal NMR for metal/enzyme solutions was increased by raising the temperature (Shimizu & Hatano, 1982, 1985; Kodaka et al., 1983).

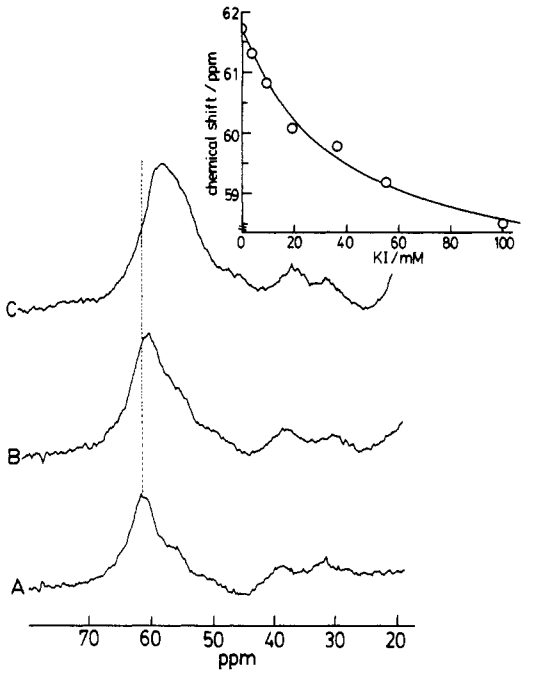

FIGURE 5: ^1H hyperfine-shifted NMR spectra of lactoperoxidase (2 mM) in the presence of 0 (A), 9.1 (B), and 100 mM (C) KI at pH 6.1. These spectra were measured at 296 K, and the chemical shift was referred to the trace HDO signal. The inset shows the change in the chemical shifts of the main peak at about 60 ppm as a function of the concentration of KI. The dissociation constant of iodide from lactoperoxidase was calculated to be 38 mM by using a nonlinear least-squares method from the plots.

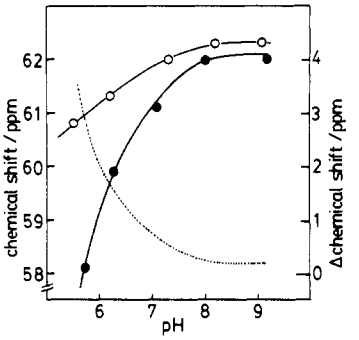

FIGURE 6: Chemical shift changes of the main peak in ^1H hyperfine-shifted NMR spectra at various pHs for lactoperoxidase (2 mM) (○) and lactoperoxidase (2 mM) plus KI (100 mM) (●). The dotted line shows the difference in the chemical shift between lactoperoxidase and lactoperoxidase plus KI.

Interaction of Iodide with Lactoperoxidase Probed by ^1H NMR. The proton NMR spectrum of native lactoperoxidase at pH 6.1 is shown in Figure 5A. The spectrum is almost similar to that reported previously (Morishima & Ogawa, 1982; Goff et al., 1985; Shiro & Morishima, 1986), showing one large, broad, and overlapping signal at about 60 ppm and other small peaks. The origin of these peaks is not assigned because of the difficulty in the heme reconstitution of lactoperoxidase. The major signal caused an upfield shift on adding KI as shown in Figure 5B,C, suggesting the interaction of KI with the periphery of the heme. The value of K_d for binding of iodide to the enzyme is estimated to be 38 ± 5 mM when calculated by the nonlinear least-squares method as shown in the inset of Figure 5.

As shown in Figure 6, the effect of pH on the chemical shift of the major broad signal was observed for both lactoperoxidase and lactoperoxidase/KI, the difference being presented

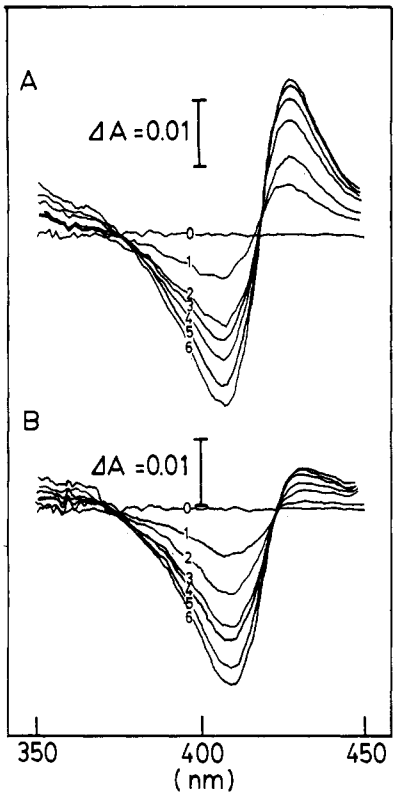

FIGURE 7: Difference optical spectra between lactoperoxidase and lactoperoxidase/resorcinol complex in the absence (A) and presence (B) of iodide (100 mM). The initial condition (0) was 1 mL of 9.7 μM lactoperoxidase in pH 6.0 10 mM phosphate buffer or that containing 100 mM KI. Titration was carried out by adding 30 (1), 60 (2), 90 (3), 120 (4), 150 (5), and 180 (6) μL of a 209 mM resorcinol solution to the sample cell and an equal aliquot of buffer to the reference cell.

as a dotted line in the figure. The difference in the pH effect was increased with decreasing pD, suggesting that the interaction of iodide with the heme periphery is strengthened by protonation of an amino acid residue in the enzyme. The pK_a value of the amino acid residue was estimated to be 6.0 ± 0.3 in D_2O, which may be ascribed to a histidyl residue.

Effect of Iodide on the Association of Aromatic Donor Molecules with Lactoperoxidase. It was previously reported that aromatic donor molecules induce a slight absorption change in the Soret region of horseradish peroxidase by binding to the heme periphery of the enzyme (Paul & Ohlsson, 1978). Our recent studies showed that the binding site of the aromatic donor molecules to horseradish peroxidase is located at a pocket enclosed with the 8-CH_3 of the heme, Tyr-185, and Arg-183 (Sakurada et al., 1986) and that the binding site of iodide is away from the binding site of the aromatic donor molecules so that the dissociation constant of the latter is not affected by iodide (Sakurada et al., 1987). Although it was reported that the interaction of aromatic donor molecules with lactoperoxidase was not detected in the optical difference spectra (Schonbaum, 1973), distinct difference spectra were obtained as shown in Figure 7A using higher concentrations of substrate (resorcinol in this figure): the values of the dissociation constant were found to be 1 order of magnitude higher as compared with the case of horseradish peroxidase. This may be related to the fact that the heme (ferriprotoporphyrin IX) of lactoperoxidase is so firmly buried in the heme crevice with the rigid structure maintained by eight disulfide bonds (Sievers, 1979, 1981): this may cause weaker interaction between the enzyme and the substrate as compared with the case of horseradish peroxidase. In the present study,

Table I: Effect of Iodide and Nitrate on the Binding of Resorcinol to Lactoperoxidase[a]

I^-	NO_3^-	$K_d \times 10^3$ (M)	$\Delta\epsilon_{426-406} \times 10^{-3}$ (cm^{-1} M^{-1})
–	–	71.7 ± 4.6[b,c]	21.0 ± 1.8
+	–	175.7 ± 42.1[b]	20.6 ± 4.4
–	+	107.8 ± 23.2[c]	28.4 ± 5.2

[a] The values of K_d and $\Delta\epsilon_{426-406}$ were obtained from optical difference spectra in the presence (100 mM) and absence of iodide and nitrate at pH 6.0. The mean ± SE was tabulated. [b] $p < 0.05$; the difference of the mean of the K_d value is significant. [c] $p > 0.10$; the difference of the mean of the K_d value is not significant.

an attempt has been made to examine whether or not the optical difference spectra caused by the binding of resorcinol are affected by adding KI. As shown in Figure 7B and Table I, the iodide ion was able to afford an appreciable effect on the optical difference spectra of enzyme/resorcinol vs. enzyme, giving a slightly different dissociation constant. The possibility that the effect was due to high ionic strength can be excluded since the high concentration of sodium nitrate exerted no significant effect (Table I).

Discussion

As in the case of horseradish peroxidase (Sakurada et al., 1985), ^{127}I NMR studies demonstrated that an iodide ion binds to lactoperoxidase in two different manners: one, nonspecific and the other, specific (Figure 2). The former represents pH-independent binding and does not correlate with the specific activity of the enzyme, whereas the latter is controlled by the amino acid residue with $pK_a = 6.8$ and correlates with the activity above pH 6.

The changes of the $\Delta\nu_{1/2}$ of ^{127}I NMR caused by adding the enzyme (Figures 1C and 3) are much larger than those observed for Ca^{2+}/protein or Mg^{2+}/protein solutions (Shimizu & Hatano, 1985), suggesting that the exchange rate of I^- from the enzyme is very fast and/or the binding ability of I^- to the enzyme is not very high. The suggestion was confirmed by the temperature dependences of the $\Delta\nu_{1/2}$ of ^{127}I NMR for the I^-/enzyme solutions in that the $\Delta\nu_{1/2}$ of the I^-/enzyme solution was decreased by raising the temperature (Figure 4). The $\Delta\nu_{1/2}$ values of quadrupolar metal NMR, such as ^{25}Mg ($I = {}^5/_2$), ^{43}Ca ($I = {}^7/_2$), and ^{67}Zn ($I = {}^5/_2$) NMR, for metal/small molecule (ATP, ADP, or imidazole) solutions were increased by raising the temperature, while those for the metal/large molecule (polypeptides, calmodulin, or DNA) solutions were decreased by raising the temperature (Shimizu & Hatano, 1982, 1985; Kodaka et al., 1983). The $\Delta\nu_{1/2}$ values of the metal/small molecule solutions are dominated by a correlation time that describes the reorientation of the entire molecule, whereas those of the metal/large molecule solutions are dominated by chemical exchange (Shimizu & Hatano, 1985). In these respects, the temperature dependences of the ^{127}I NMR for the I^-/enzyme solutions (Figure 4) provide interesting features. ^{127}I NMR of the I^-/enzyme solutions under our experimental conditions is probably in the range of a fast exchange region with $k_{off} > 10^4$ s^{-1}, which is in accordance with the low binding ability ($K_d = 38$ mM) of I^- to the enzyme estimated from ^1H NMR (Figure 5).

The competitive binding study shown in Figure 2 indicates that cyanide exerts no appreciable effect on iodide binding by the enzyme, suggesting that I^- may bind to the enzyme at a non-heme binding site.

The specific binding of iodide was further studied by the use of ^1H NMR. KI induced an upfield shift (Figure 5) for the hyperfine-shifted broad strong peak at about 60 ppm which

is ascribed to the heme peripheral methyl protons (Goff et al., 1985; Shiro & Morishima, 1986). The finding indicates that the iodide ion bound to the enzyme is located in the vicinity of the heme methyl protons. The site is, however, not so clearly defined as in the case of horseradish peroxidase, since the hyperfine-shifted proton resonances of four methyl groups are not well separated, and therefore not assigned. The line width of the broad peak showed no appreciable change by the addition of iodide (Figure 5), suggesting that the distance between iodide bound to the enzyme and the heme methyl may be considerably longer than in the case of horseradish peroxidase (Sakurada et al., 1987). Thus, we suppose that the direct contact of iodide to the methyls may be more or less hindered due to a tight packing of polypeptide side chains composing the heme pocket.

The dissociation constant of iodide binding by lactoperoxidase at pH 6.1 was found to be 38 mM on the basis of ^1H NMR spectra (Figure 5). This is distinctly lower than that for horseradish peroxidase at optimum pH (pH 4.0) since the latter was 100 mM (Sakurada et al., 1987). This seems to correspond to the results shown in Figure 3 that the increase in $\Delta\nu_{1/2}$ by binding of iodide was more pronounced at lower concentrations of iodide as compared with the case of horseradish peroxidase. Thus, it is likely that the structure of lactoperoxidase is more suitable for the oxidation of iodide.

The results shown in Figures 2 and 6 indicate that the binding of iodide by lactoperoxidase is facilitated by the protonation of an amino acid residue with a pK_a value of 6.0–6.8. It is of interest to note that previous kinetic (Dolman et al., 1968) and NMR (Shiro & Morishima, 1986) studies suggested the presence of a residue with a pK_a value of 6.3 or 6.0 in the distal region. These facts lead us to the supposition that the residue with $pK_a = 6.0$–6.8 found in the present study may be the distal histidine and that the binding of iodide to the enzyme may occur at the imidazole NH^+ of the histidyl residue or its vicinity, near the entrance of the heme crevice. Recent NMR results on horseradish peroxidase indicated that an iodide ion binds to the enzyme at almost equal distance from the heme peripheral 1- and 8-methyl groups at the distal side of the heme, becoming closer to the distal histidine by protonation of the ionizable group with a pK_a value of 4.0 (Sakurada et al., 1987). Thus, the approach of iodide to the distal histidine may be a prerequisite for the oxidation of iodide catalyzed by peroxidase.

As reported previously (Björkstèn, 1970; Magnusson et al., 1984; Ohtaki et al., 1981), the oxidation of iodide catalyzed by lactoperoxidase or horseradish peroxidase takes place in the manner of two-electron transfer from iodide to the heme iron, in contrast to one-electron transfer in the case of oxidation of most aromatic donor molecules. It is still unclear how the two-electron transfer occurs from iodide bound to the site mentioned above from the heme iron. We suppose that the distal histidine may bind with both I^- and $Fe^{IV}{=}O$ of compound I, forming a route, $I^-{---}H^+{-}N{=}{-}C{=}{-}N{-}H{---}O{=}Fe^{IV}$, since $Fe^{IV}{=}O$ of compound II associates with the NH of the imidazole ring of the distal histidine by hydrogen bonding (Hashimoto et al., 1986). The route formed by conjugated double bonds and hydrogen bonds may facilitate the transport of electrons from iodide to the heme iron although much should be done to prove this. After electron transport, I^+ or subsequently formed HOI (Dunford & Ralston, 1983) will react with another I^- nearby to form I_2 or I_3^-. In the presence of high concentrations of tyrosine and low concentrations of iodide, I^+ or HOI will attack tyrosine which is previously bound to the enzyme (Nunez & Pommier, 1982), resulting in the formation of monoiodotyrosine on the enzyme (Bayes et al., 1972). That the binding site of tyrosine is very close to the iodide binding site is suggested from the results of the optical difference spectrum that an iodide ion interfered with the binding between lactoperoxidase and resorcinol (Figure 7 and Table I). At any rate, it seems that the distal histidine plays an important role in the oxidation of iodide catalyzed by lactoperoxidase.

ACKNOWLEDGMENTS

We thank Professors T. Miyazawa (University of Tokyo) and M. Yamazaki (Chiba University) for the use of NMR spectrometers. We also thank C. Kurokawa for technical assistance.

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Primary Structure of Paim I, an α-Amylase Inhibitor from *Streptomyces corchorushii*, Determined by the Combination of Edman Degradation and Fast Atom Bombardment Mass Spectrometry

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Received December 15, 1986; Revised Manuscript Received April 2, 1987

ABSTRACT: Paim I, a protein α-amylase inhibitor, inhibits animal α-amylases from pig, dog, cow, horse, etc. but has no activity against human salivary and pancreatic amylases. The primary structure of Paim I has been determined by Edman degradation and fast atom bombardment mass spectrometry (FABMS). This protein is a single-chain polypeptide of 73 amino acid residues with a calculated molecular weight from the sequence data of 7415.3 (monoisotopic molecular weight) and 7420.2 (average molecular weight). The sequencing strategy chosen for Paim I consists of four steps. First, the accurate molecular weights of the intact and tetra-S-carboxymethylated Paim I are determined by fast atom bombardment mass spectrometry. Second, the primary fragments generated by *Staphylococcus aureus* V8 protease are isolated by reversed-phase high-performance liquid chromatography. The molecular weights of these subpeptides are determined by FABMS. The peptides that must be sequenced are selected by the molecular weights of these subpeptides and the tetra-S-carboxymethylated Paim I. Third, these subpeptides and the whole protein are sequenced by automated Edman degradation. Finally, the primary structure of tetra-S-carboxymethylated Paim I is confirmed by the combination of tryptic, chymotryptic, and *S. aureus* V8 protease digestion and FABMS. The sequence of Paim I is compared with those of Haim II, Hoe-467A, Z-2685, and AI-3688 because they have different α-amylase inhibition spectra against mammalian α-amylases but belong to a family of related proteins.

Many protein amylase inhibitors, which inhibit mammalian α-amylases specifically but have no activity against plant and microbial α-amylases, are obtained from plants and microbes (Aschauer et al., 1983; Maeda et al., 1983; Marshall & Lauda, 1975; Murao et al., 1981). These phenomena indicate that animal α-amylases have specific site(s) for protein α-amylase inhibitors in addition to binding sites for substrates, i.e., α-1,4-glucan. Therefore, elucidation of the inhibition mechanism of these inhibitors might provide important information on the characteristics of animal α-amylases. These inhibitors should be useful not only for the elucidation of active sites of animal α-amylases but also for the investigation of protein–protein interaction. In addition, the measurement of serum and urine amylase has clinical significance in the diagnosis of diseases. For this purpose, an amylase inhibitor could be used in the determination of activities of amylase isozyme (O'Donnell et al., 1977). Immobilized amylase inhibitors are also effective for purification of amylases (Buonocore et al., 1975; Burrill et al., 1981).

During the screening of amylase inhibitors of microbial origin, we isolated an inhibitor that strongly inhibited pig pancreatic α-amylase. The α-amylase inhibitor was designated Paim I (pig pancreatic α-amylase inhibitor of microbes) (Murao et al., 1983, 1985). Paim I inhibited animal α-amylases from pig, dog, cow, horse, etc. but had no activity against human salivary and pancreatic amylases (Oouchi et al., 1985; Arai et al., 1985a). This is an important characteristic of Paim I. Thus, Paim I might be useful in investigating differences between human and other animal amylases.

This paper describes the spectrum of inhibition of Paim I as compared with Haim II (Arai et al., 1985a,b; Goto et al., 1983, 1985a,b; Murai et al., 1985; Murao et al., 1980a,b), which inhibits human salivary and pancreatic amylases. Knowledge of the exact molecular weight of the protein is very useful in determining the primary structure of the protein. We report molecular weights of Paim I, tetra-S-carboxymethylated Paim I, and endopeptidase fragments of tetra-S-carboxymethylated Paim I as determined by FABMS[1] and the primary

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0006-2960/87/0426-6483$01.50/0