Interaction of Aromatic Donor Molecules with Lactoperoxidase Probed by Optical Difference Spectra†

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ABSTRACT: On the basis of optical difference spectra, lactoperoxidase (LPO) was shown to form a 1:1 complex with aromatic donor molecules: resorcinol, hydroquinone, phenol, *p*-cresol, guaiacol, aniline, and benzohydroxamic acid. As compared with horseradish peroxidase (HRP), the values of the dissociation constant, K_d, of LPO–donor complexes were found to be 4–720-fold larger and were not greatly changed in the presence of KCN and by changes in pH in the range 4–9. The apparent enthalpy and entropy of the binding reactions were found to be −13 kJ mol^{-1} and −29 J mol^{-1} K^{-1}, respectively, somewhat smaller (in absolute value) than the corresponding values of HRP. The difference spectra of LPO–donor complexes resembled each other, in contrast to the case of HRP, and the bindings of the donors to LPO occurred in a competitive fashion between the donors. Incubation of LPO with phenylhydrazine and hydrogen peroxide markedly depressed donor binding, the intensity of the Soret band, and the catalytic activity, probably as the result of formation of *meso*-phenyl derivatives of the heme. These findings suggest that the binding of aromatic donors to LPO occurs at a specific site, probably near the heme edge, where the electron transfer in the peroxidase reaction may take place.

Lactoperoxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) (LPO)[1] is a hemoprotein enzyme found in bovine milk, saliva, and tears (Paul & Ohlsson, 1985). Since the enzyme is secreted as a soluble protein, the purification and even crystallization were carried out in the early 1940s (Theorell & Åkeson, 1943). However, crystals available so far are not adequate for use in X-ray studies. The primary structure of the enzyme is not known either, although it was suggested to be a single polypeptide chain with M_r about 77 500. The prosthetic group was found to be unusually firmly bound to the protein. Sievers (1979) reported the prosthetic group obtained after Pronase digestion as having the properties of ferriprotoporphyrin IX. Recently, Nicholl et al. (1987) showed heme as being an iron-porphyrin thiol by reductive cleavage with mercaptoethanol in 8 M urea, suggesting the use of peripheral thiol of the heme to form a disulfide bond with cysteinyl residue of the protein.

LPO catalyzes the oxidation of various inorganic and organic compounds (the second substrate) by hydrogen peroxide (the first substrate) via intermediates, as in the case of plant peroxidases (Morrison & Schonbaum, 1976). Although experimental data of these enzymes and their intermediates obtained by many physicochemical methods are being accumulated, insufficient information is available concerning the mechanism of reaction between the intermediates and the second substrates. To help solve this problem, it is necessary to know where and how donors bind to LPO intermediates during the reaction.

Recently NMR and computer modeling studies disclosed that aromatic donor molecules bind to HRP near the heme peripheral 8-CH_3, Arg-183, Tyr-185, and Leu-237 of the protein moiety (Sakurada et al., 1986; Thanabal et al., 1987). It is difficult, however, to explore where these substrates bind to LPO, since NMR peaks of the enzyme are broad and not identifiable due to difficulty in reconstruction of the heme with the apoprotein, unlike the case of HRP.

On the other hand, useful information was obtained from studies of the interaction of peroxidase with donors using the optical difference method as reported for HRP (Schonbaum, 1973; Schejter et al., 1976; Paul & Ohlsson, 1978; Sievers, 1985). Although it is known that the optical absorption of LPO is slightly affected by binding of some substrates such as BHA (Kimura & Yamazaki, 1979), cysteine (Pommier & Cahnmann, 1979), and some phenols (Yajima et al., 1982; Razumas et al., 1985; Sakurada et al., 1987), the affinities are usually very low and the binding constants could not be obtained as exactly as in the case of HRP. Moreover, no information is available for the binding of donors to chemically modified LPO. In the present study, the interaction of substrates with LPO was examined by observing optical difference spectra under various conditions and after modifications of some residues of LPO.

Experimental Procedures

LPO was purified from raw skim milk according to the method of Rombauts et al. (1967) with slight modifications. The ratio of A_{412}/A_{280}, Reinheitzahl (RZ), of the preparation was above 0.80 at pH 7.0. The concentration of LPO was calculated by using ϵ_{412} = 112 cm^{-1} mM^{-1} (Carlström, 1969). HRP (isozyme B + C) was kindly provided by Prof. Y. Morita. The RZ (A_{403}/A_{280}) of the preparation was about 2.5 at pH 7.0. The concentration of HRP was determined by using ϵ_{403} = 102 cm^{-1} mM^{-1} (Aibara et al., 1982). Other chemicals were of analytical grade and quantified from their weights.

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[1] Abbreviations: BHA, benzohydroxamic acid; HRP, horseradish peroxidase; $HRPCN^-$, HRP–cyanide complex; LPO, lactoperoxidase; $LPOCN^-$, LPO–cyanide complex; TNM, tetranitromethane.

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Table I: Difference Spectrum Characterization and Apparent Dissociation Constants of LPO–Donor and HRP–Donor Complexes

enzyme	donor	spectrum of complex (nm) max[b]	min[b]	max	K_d (mM)	$\Delta\epsilon_{peak-trough}$ (mM^{-1} cm^{-1})	type[a]
LPO	hydroquinone (3)[c]	N	407	427	56.6 ± 9.0[d]	13.5 ± 5.1	IV
	phenol (4)	N	408	425	205 ± 59	13.0 ± 3.2	IV
	p-cresol (3)	N	408	425	161 ± 32	21.9 ± 2.0	IV
	aniline (3)	N	407	424	79[e]		IV
	resorcinol (5)	N	407	427	57.8 ± 24.9	14.0 ± 4.1	IV
	guaiacol (3)	N	411	430	190[e]		IV
	BHA (2)	N	406	427	7.9	8.3	IV
HRP	hydroquinone (4)	373	406	420	6.5 ± 0.8	3.9 ± 0.1	I
	phenol (5)	378	403	420	9.4 ± 1.6	4.4 ± 1.2	I
	p-cresol (3)	378	404	423	3.5 ± 0.4	4.9 ± 0.2	I
	aniline (3)	378	404	424	19.0 ± 1.6	6.1 ± 0.2	I
	p-toluidine (3)	276	405	424	9.9 ± 0.5	4.8 ± 0.4	I
	resorcinol (3)	N	N	411	6.7 ± 0.8	3.0 ± 0.2	II
	guaiacol (3)	N	N	408	10.6 ± 3.5	4.1 ± 1.8	II
	BHA (2)	N	376	409	0.011	49	III

[a] Types I, II, III, and IV indicate the difference spectra with characteristics shown in parts A, B, and C of Figure 2 and Figure 1, respectively (see text). [b] N indicates that no peak or no trough was observed in the regions. [c] Number in parentheses = number of experiments. [d] Mean ± SE. [e] Roughly estimated since the plots of 1/[S] vs [E]/ΔA gave slightly curved lines and extrapolated to [E]/$\Delta A \rightarrow$ O.

The difference spectrum of the enzyme–donor complex versus the enzyme was recorded by using a Hitachi 557 computerized double-wavelength double-beam spectrophotometer and black wall cuvettes (light path, 10 mm; width, 5 mm). Initially, both the sample and reference cuvettes were filled with 1.20 mL of the enzyme solution, followed by base-line tracing. Then a small volume (usually 5–20 μL) of the donor solution of an appropriate concentration was successively added to the sample cuvette, with concomitant addition of the same volume of buffer into the reference cuvette. After each addition, the contents of both cuvettes were stirred well with plastic rods. From the resulting difference spectrum curves, the apparent dissociation constants, K_d, were obtained by using

$$\frac{1}{[S]} = \frac{[E]}{K_d}\frac{\Delta\epsilon}{\Delta A} - \frac{1}{K_d} \quad (1)$$

where ΔA is the absorbance of peak minus trough or peak minus zero, [S] and [E] are the concentrations of substrate and enzyme, and $\Delta\epsilon$ is the difference in molar absorptivity (Paul & Ohlsson, 1978). However, when the absorption of donor itself could not be disregarded, the absorption of donor was subtracted from ΔA. The difference spectra were usually recorded at room temperature (about 25 °C) in an air-conditioned room. For the experiments on the effect of temperature on the dissociation, however, the temperature of the cuvettes was controlled with cell holders and circulating water.

Chemical modification of LPO was performed by use of phenylhydrazine or tetranitromethane (TNM). The former is a class of suicide substrate (Walsh, 1982; Ator & Ortiz de Montellano, 1987a,b), and the latter is the specific nitration reagent for tyrosyl residues (Riordan et al., 1966; Itagaki & Hosoya, 1978). For modification of LPO with phenylhydrazine, 50 μL of 4 mM hydrogen peroxide was added to 2 mL of 30 μM LPO plus 1 mM phenylhydrazine in 0.1 M phosphate buffer (pH 7.4) five times, with intervals of 5 min between each addition. The reaction mixture was passed through a PD-10 (Pharmacia) column to remove small molecules. A control experiment was performed by use of guaiacol instead of phenylhydrazine. For the nitration of tyrosyl residues in LPO, 32 μM LPO in 0.1 M Tris-HCl (pH 8.0) plus 0.1 M NaCl was incubated with up to a 57-fold excess of TNM at 28 °C, and the number of tyrosyl residues modified was determined as described (Itagaki & Hosoya, 1978).

Assay for peroxidase activity was carried out by the method described previously (Hosoya & Morrison, 1967) using guaiacol as the second substrate. The reaction product was followed by recording absorbance at 470 nm with a Hitachi Model 200 spectrophotometer. Protein was determined by the method of Bradford (1976) using native LPO as the standard.

Results

Optical Characterization of LPO- and HRP–Donor Complexes. Although it was previously reported by many investigators that a number of phenol and aniline derivatives and BHA cause slight spectral changes in Soret and visible regions by binding to HRP (Schonbaum, 1973; Schejter et al., 1976; Paul & Ohlsson, 1978), observation of binding of donors to LPO by difference spectra was not feasible. In fact, Schonbaum (1973) reported that BHA scarcely altered the Soret band by LPO, in contrast to a great spectral change in the case of HRP. When high concentrations of BHA were used, the donor was found to show a slight but distinct effect on the Soret band of LPO by its binding to the enzyme (Kimura & Yamazaki, 1979). Subsequently, we (Yajima et al., 1982; Sakurada et al., 1987) and others (Razumas et al., 1985) also found that some phenol and aniline derivatives cause a slight spectral change in the Soret band of LPO when high concentrations were used.

In the present study, we intended to obtain dissociation constants of the equilibria between LPO and various donors by carefully measuring the difference spectra in comparison with those of HRP and donors. Figure 1 shows the difference spectra of the LPO–resorcinol complex (panel A), the LPO–hydroquinone complex (panel B), and the LPO–BHA complex (panel C). From these spectra, the values of K_d and $\Delta\epsilon$ were calculated by using eq 1; the results are presented in Table I. In addition to these donors, the values of LPO–phenol and guaiacol or aniline complexes are also presented in the table, although the latter two values are less reliable (see footnote e of Table I). It is noteworthy that the difference spectra of these complexes seemed to form one category, the maxima and minima of the curves being at 424–427 and 407–408 nm, respectively, and the isosbestic points being approximately 418 and 390 nm (364 nm in the case of BHA).

In the case of HRP, however, it is known that most aromatic donor molecules are divided into two groups with respect to the spectral change (Paul & Ohlsson, 1978). One group is characterized to cause a hypsochromic shift of absorption and some reduction of the maximum absorbance, i.e., phenol, *p*-cresol, hydroquinone, aniline, etc. The other group causes

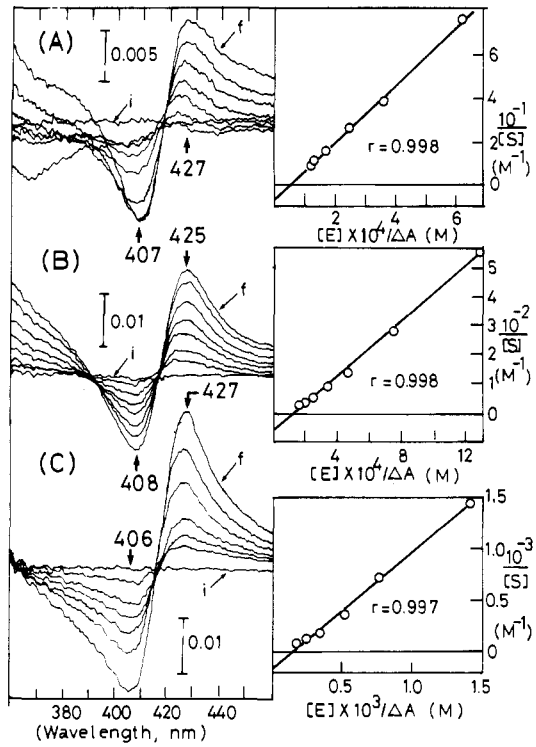

FIGURE 1: Difference spectra of (A) LPO–*p*-cresol complex versus LPO, (B) LPO–resorcinol complex versus LPO, and (C) LPO–BHA complex versus LPO and the analysis of K_d and $\Delta\epsilon$. The difference spectra were obtained as described under Experimental Procedures, and the data were analyzed by using eq 1 to obtain K_d and $\Delta\epsilon$ (right panels). The concentrations of peroxidase at the initial (i) and final (f) stages in both cuvettes and the concentration of donor at the final (f) stage in the sample cuvette were as follows: (A) LPO, 2.89 μM (i) and 2.48 μM (f), *p*-cresol, 62.7 mM (f); (B) LPO, 6.48 μM (i) and 5.56 μM (f), resorcinol, 29.0 mM (f); (C) LPO, 13.5 μM (i) and 13.0 μM (f), BHA, 4.13 mM (f).

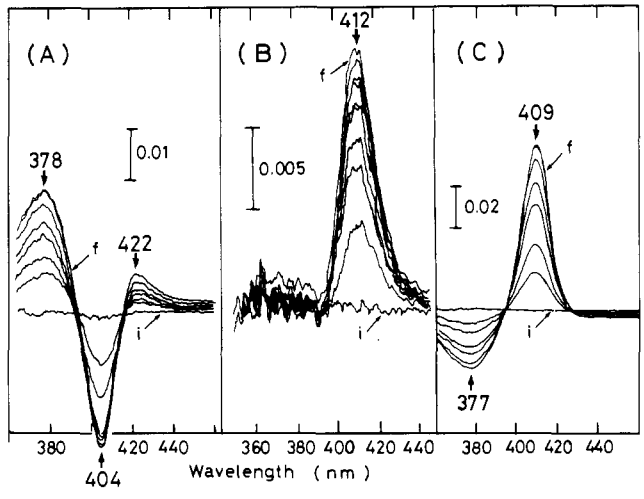

FIGURE 2: Difference spectra of (A) HRP–*p*-cresol versus HRP (type I), (B) HRP–resorcinol versus HRP (type II), and (C) HRP–BHA versus HRP (type III). The concentrations of peroxidase at the initial (i) and final (f) stages in both cuvettes and the concentration of donor at the final stage (f) in the sample cuvette were as follows: (A) HRP, 9.76 μM (i) and 8.36 μM (f), *p*-cresol, 20.0 mM (f); (B) HRP, 8.04 μM (i) and 7.56 μM (f), resorcinol, 2.53 mM (f); (C) HRP, 6.90 μM (i) and 6.27 μM (f), BHA, 3.43 μM (f).

a bathochromic shift of the Soret band and gradual increase in absorption in the visible region (resorcinol, guaiacol, etc.). The typical difference spectra of HRP–donor versus HRP are shown in Figure 2A,B, where *p*-cresol and resorcinol were used as examples. Apart from the donors mentioned above, BHA and its analogues are known to show marked perturbation of

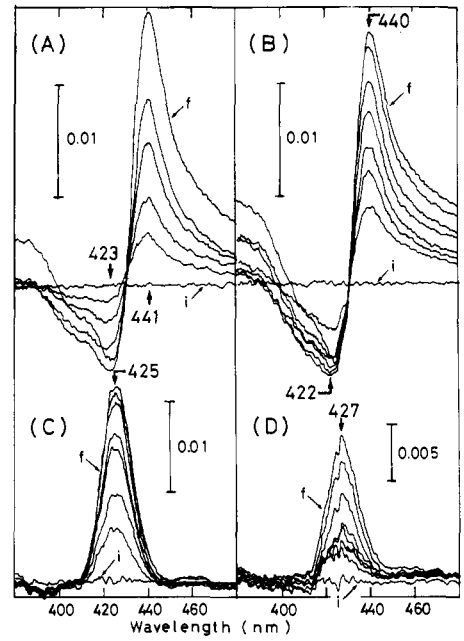

FIGURE 3: Difference spectra of (A) $LPOCN^-$–*p*-cresol versus $LPOCN^-$, (B) $LPOCN^-$–guaiacol versus $LPOCN^-$, (C) $HRPCN^-$–*p*-cresol versus $HRPCN^-$, and (D) $HRPCN^-$–guaiacol versus $HRPCN^-$. The concentrations of peroxidase and KCN at the initial (i) and final (f) stages in both cuvettes and the concentration of donor at the final stages (f) in the sample cuvette were as follows: (A) LPO, 5.70 μM (i) and 5.07 μM (f), KCN, 1.66 mM (i) and 1.59 mM (f), *p*-cresol, 93.0 mM (f); (B) LPO, 9.75 μM (i) and 9.62 μM (f), KCN, 1.63 mM (i) and 1.60 mM (f), guaiacol, 12.4 mM (f); (C) HRP, 10.3 μM (i) and 8.83 μM (f), KCN, 1.63 mM (i) and 1.40 mM (f), *p*-cresol, 20.3 mM (f); (D) HRP, 10.2 μM (i) and 8.74 μM (f), KCN, 1.68 mM (i) and 1.44 mM (f), guaiacol, 14.4 mM (f).

the Soret band of HRP (Schonbaum, 1973). In accordance with Schonbaum's results, the perturbation reached about 10% of original absorption in the Soret band (Figure 2C), in contrast to only 1–4% for the donors mentioned above. These differences are likely due to multiple hydrogen bonds in addition to the usual hydrophobic bonds (Schonbaum, 1973). Taking these facts into consideration, we tentatively designated the difference spectra of hydroquinone, *p*-cresol, etc. type I, those of resorcinol, guaiacol, etc. type II, and that of BHA type III (Figure 2). The difference spectra of LPO–donor complexes will be tentatively labeled type IV, since they are all different from the types of HRP–donor complexes.

Binding of Aromatic Donors to $LPOCN^-$ or $HRPCN^-$. Cyanide is known to bind to the heme iron of HRP and LPO, the dissociation constants being 1.9 μM at pH 6.8 (Araiso & Dunford, 1981) and 42 μM at pH 7.0 (Dolman, et al., 1968), respectively. Thus, we determined the effects of the binding of donors to the enzymes in the presence of 1 mM KCN. As shown in Figure 3A,B the pattern of difference spectra of $LPOCN^-$–donor complexes was similar to those without KCN, except that the peak and trough were red-shifted about 20 nm, due to the absorption of $LPOCN^-$. Thus, these difference spectra were designated type IV′. The difference spectra of $HRPCN^-$ and type II donor complexes were also similar to those of HRP–type II donor complexes, except that the peak and trough red-shifted 20 nm, and therefore it may be called a type II′. For a type I donor, however, the difference spectra of $HRPCN^-$–donor complexes did not show the type I pattern but turned to type II (Figure 3D). It is also noteworthy that the K_d values of these complexes were similar, at least in the order of magnitude, to those obtained in the absence of KCN (compare Table II with Table I). These facts indicate that donors do not bind to the heme iron.

Table II: Characterization of Difference Spectra and Dissociation Constants of LPOCN$^-$–Donor and HRPCN$^-$–Donor Complexes[a]

enzyme	donor	spectrum of complex (nm) min[c]	max	K_d (mM)	$\Delta\epsilon_{peak-trough}$ (mM^{-1} cm^{-1})	type[b]
LPOCN$^-$	hydroquinone	423	441	39.4 ± 5.8[d]	5.5 ± 0.5	IV′
	phenol	425	440	363 ± 56	17.5 ± 2.7	IV′
	p-cresol	423	440	68.4 ± 1.5	12.0 ± 0.3	IV′
	aniline	426	440	178		IV′
	resorcinol	426	440	36.2 ± 3.7	5.0 ± 0.6	IV′
	guaiacol	422	440	43.3 ± 2.9	3.6 ± 0.2	IV′
HRPCN$^-$	hydroquinone	N	425	5.1 ± 0.4	3.3 ± 0.2	II′
	phenol	N	425	8.4 ± 0.5	3.2 ± 0.2	II′
	p-cresol	N	425	5.7 ± 0.9	3.7 ± 0.4	II′
	aniline	N	427	17.1 ± 0.8	4.1 ± 0.1	II′
	p-toluidine	N	425	8.3 ± 0.7	4.5 ± 0.1	II′
	rescorcinol	411	428	7.8 ± 0.5	3.2 ± 0.1	II′
	guaiacol	412	427	11.1 ± 0.6	2.1 ± 0.1	II′

[a] The data were obtained from three experiments, except that of aniline, which is a mean value from two experiments. [b] Types II′ and IV′ indicate the difference spectra with characteristics shown in parts C and A of Figure 3, respectively. [c] N denotes no trough in the region. [d] Mean ± SE.

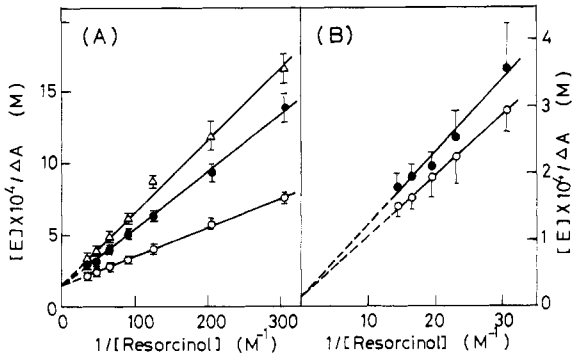

FIGURE 4: Competitive binding of donors to LPO or HRP. (A) Difference spectra of HRP–resorcinol complex versus HRP were observed in the presence of 10 (○), 20 (●), or 30 (△) mM *p*-cresol, and the data were analyzed by using eq 1. Each point is the mean ± SE in 14 experiments. The ranges of concentrations of HRP and resorcinol used were 11.7–13.5 μM and 1.62–29.0 mM, respectively. (B) Difference spectra of LPO–resorcinol complex versus LPO were observed in the presence of 10 (○) or 20 (●) mM *p*-cresol. The ranges of concentrations of LPO and resorcinol used were 8.78–11.1 μM and 31.9–70.5 mM, respectively. Each point is the mean ± SE in three to six experiments.

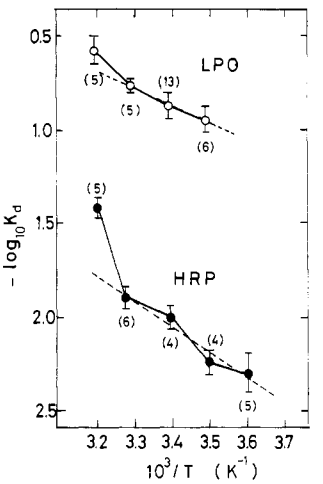

FIGURE 5: Relationship between log K_d and $1/T$. The dissociation constants (K_d) for equilibria between LPO or HRP and resorcinol were determined over the temperature range 0–40 °C. The concentration ranges of enzymes and donors used were as follows: (○) LPO, 5.39–9.91 μM, resorcinol, 25.8–84.3 mM; (●) HRP, 6.96–9.96 μM, resorcinol, 25.8–84.3 mM. The number in parentheses indicates the number of experiments and the bar the SE. Broken lines were obtained by the least-squares method excluding the data at 40 °C.

Competitive Binding of Two Kinds of Donors to the Enzymes. Whether the binding of a type II donor (resorcinol) and a type I donor (*p*-cresol) to HRP competes with each other was examined by using double-reciprocal plots (Figure 4). In these experiments, the differences in absorption at 410 nm were measured in the presence of various concentrations (10, 20, and 30 mM) of *p*-cresol. The plots indicate that these donors bind to HRP competitively, suggesting that these donors bind at identical sites in spite of different difference spectrum patterns.

In the case of LPO (Figure 4B), such competition was also observed, although the use of high concentrations of donors made it less clear.

Effect of pH and Temperature on the Dissociation Constant. When the effect of pH on the dissociation constant of the HRP–resorcinol complex was examined, little change was found over the pH range 4.0–11.0. The dissociation constant of the LPO–resorcinol complex was examined in the pH range 4–9 and also found to be almost constant in the pH region.

The effect of temperature on the binding of resorcinol to enzymes was examined over the range 10–40 or 0–40 °C for LPO or HRP, respectively. As shown in Figure 5, both van't Hoff plots gave slightly curved lines, suggesting that $\Delta H°$ and $\Delta S°$ of the binding are somewhat dependent on temperature. However, at least below 30 °C, approximately linear relationships were found between log K_d and $1/T$ in both enzymes (Figure 5). From straight lines, the values of $\Delta G°$, $\Delta H°$, and $\Delta S°$ were calculated to be −11.4 kJ mol^{-1}, −25 kJ mol^{-1}, and −47 J mol^{-1} K^{-1} for HRP and −4.7 kJ mol^{-1}, −13 kJ mol^{-1}, and −29 J mol^{-1} K^{-1} for LPO. The values for HRP(B+C) are in good agreement with those obtained previously for HRP(C2) by Paul and Ohlsson (1978). As discussed by them, such negative entropy and a large negative enthalpy are characteristic of these enzymes, suggesting a specific donor–enzyme interaction. The smaller values (in absolute value) in LPO indicate that binding of donor to LPO is looser than that to HRP.

Figure 5 shows that the K_d value tends to increase at high temperature. This may be explained by assuming that fluctuation in structure induced by high temperature facilitates the binding of donors to the pocket near the heme peripheral 8-CH_3 (Sakurada et al., 1986). Deviation at 40 °C of log K_d appears less prominent in the case of LPO, suggesting more resistance for the structural disturbance.

Binding of BHA to Phenylhydrazine-Treated LPO. Phenylhydrazine is known to be a kind of suicide substrate for peroxidase. Recently, Ator and co-workers (Ator & Ortiz de Montellano, 1987a; Ator et al., 1987b) reported that incu-

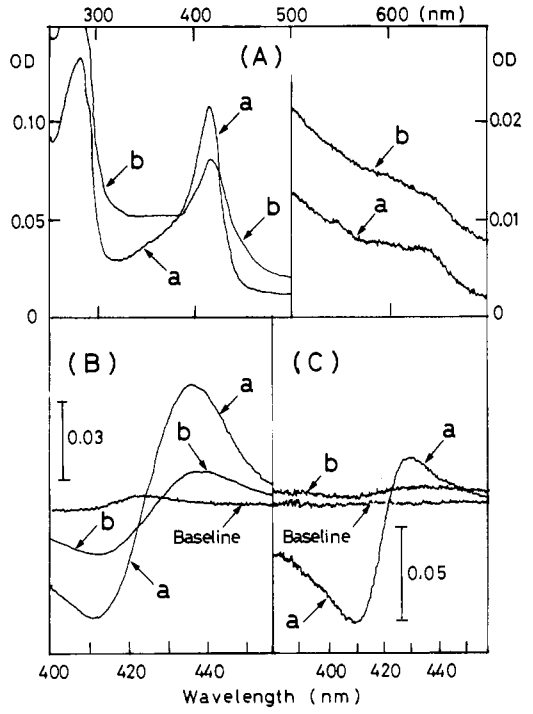

FIGURE 6: Optical absorption spectrum of phenylhydrazine-modified LPO and its reaction with hydrogen peroxide or BHA. (A) Absorption spectra of native LPO (curve a) and phenylhydrazine-treated LPO (curve b). Concentrations of enzymes were 1 μM. The treatment of LPO with phenylhydrazine and hydrogen peroxide was performed as described under Experimental Procedures. The cuvettes used were 10 mm in length and 10 mm in width. (B) Reaction of native LPO and phenylhydrazine-treated LPO with hydrogen peroxide. After sample and reference cuvettes were filled with 2 mL of 1 μM native LPO or phenylhydrazine-treated LPO, 5 μL of 1.6 mM hydrogen peroxide was added to the former. Difference spectrum was observed within 1 min. (C) Binding of BHA by native LPO (curve a) or phenylhydrazine-treated LPO (curve b). After sample cuvette and reference cuvette were filled with 1 mL of native LPO (6.25 μM) or modified LPO (6.25 μM), 0.25 mL of 200 mM BHA was added to the sample cuvette and 0.2 mL of the buffer to the reference cuvette. The cuvettes used in (B) and (C) were 10 mm in length and 4 mm in width.

bation of HRP with phenylhydrazine and hydrogen peroxide markedly depressed both the catalytic activity and the intensity of the Soret band, forming *meso*-phenyl derivatives of the heme.

When LPO was incubated with hydrogen peroxide and phenylhydrazine under similar conditions, the absorption of the Soret band was similarly reduced to about 75% of the control, with a concomitant 95% loss of activity (Figure 6A). As a control experiment, phenylhydrazine was replaced by guaiacol, but no change in absorption of LPO was observed. Although it could not be clarified due to the difficulty of splitting of the heme whether the heme was similarly modified as in the case of HRP, comparable change in the intensity without shift of the Soret band suggests that this was the case.

That the activity loss of the modified LPO mentioned above is not ascribed to inability to react with hydrogen peroxide is clear from the experiments shown in Figure 6B. In these experiments, after LPO or phenylhydrazine-treated LPO in the sample cuvette was incubated with 4 equiv of hydrogen peroxide, the difference spectrum against the control was observed (curves a and b, respectively). Both showed peaks at 436–438 nm and troughs at 412–413 nm, although the optical difference between the peak and trough of curve b was about 35% of that of curve a. The presence of the former difference spectrum cannot be explained by assuming that there was unmodified LPO in the preparation since the activity loss was 95% as described above. Rather, it suggests that phenylhydrazine-treated LPO is more or less as capable of forming compound I as native LPO is.

As previously described, BHA causes a slight spectral change in the Soret band of LPO by binding to the enzyme. Curve a of Figure 6C is the difference spectrum of LPO–BHA versus LPO, showing a peak at 428 nm and a trough at 408 nm. In contrast, BHA did not reveal a distinct difference spectrum for the phenylhydrazine-treated LPO (curve b of Figure 6C), probably indicating no BHA binding.

Binding of Donors to TNM-Treated LPO. When LPO was treated with TNM under the conditions mentioned under Experimental Procedures, it was found that about three tyrosyl residues were nitrated with the reagents without loss of the activity. No change in difference spectrum was observed when the TNM-treated LPO and resorcinol were used compared with the case of native LPO, indicating that the modification of tyrosine with the reagent does not affect the interaction of these donors with LPO (not shown).

Mäkinen and Mäkinen (1982) reported that modification of two tyrosines of LPO with diazotized sulfanilate caused a complete loss of activity. The reason for the discrepancy between their results and ours is not clear. One possibility would be that steric hindrance of modified groups is greater in diazotization than in nitration.

Discussion

Although several aromatic donor molecules bring about a slight spectral change in the optical spectra of LPO by binding to the enzyme, the dissociation constants for the enzyme–donor complexes are usually markedly higher than those of HRP–donor complexes (Table I). This implies that affinities of aromatic donors to LPO are in general lower than to HRP, requiring higher concentrations of donors in the experiments. Thus, the experiments with LPO usually need more careful manipulation. In the present experiments, therefore, the difference spectra were always examined in comparison with corresponding ones for HRP. Our results with HRP were found generally to be in agreement with those of Paul and Ohlsson (1978), supporting the validity of the procedures used here.

In contrast to the appearance of three different types of difference spectra of HRP–donor complexes, the patterns of difference spectra of LPO–donor complexes were similar to each other (type IV in our notation), as shown in Figure 1 and Table I. In addition, the K_d values are greater than those of HRP: 4–46-fold in the case of donors of types I and II and 720-fold in the case of BHA (Table I).

A question arises: where do the donors bind to LPO? In the case of HRP, taking advantage of identified NMR signals (La Mar et al., 1980), we could locate aromatic donors near the heme peripheral 8-CH_3 (Sakurada et al., 1986). As for LPO, however, hyperfine-shifted NMR signals are very broad and have not yet been identified because of difficulty in reversible splitting of the heme. Moreover, the change in NMR spectra by binding of aromatic donors is very small. Thus, it is not possible to locate exactly where the aromatic donors bound to LPO. However, the results obtained here (Table I and II, Figure 6, and the values of $\Delta H°$ and $\Delta S°$ for the binding of donors to LPO) suggest that aromatic donors bind to LPO mainly with a hydrophobic bond at a specific site that is not the heme iron but close to the heme edge and a tyrosine residue of the surrounding protein. Our previous data also indicated that the binding site of aromatic donors is near the iodide binding site, which is close to the distal histidine outside the heme crevice (Sakurada et al., 1987), and that partial

charge transfer from the donor to the heme of LPO may occur (Kitagawa et al., 1983).

In the case of HRP, NMR and computer modeling indicated that the aromatic donor binds to the enzyme near the heme peripheral 8-CH_3, Tyr-185, and Arg-183 residues, the phenyl ring of the donor being perpendicular to the heme plane (Sakurada et al., 1986). This result and the effect of alkylation of the meso position by alkylhystidine (Ator et al., 1987a,b) lead to the supposition that electron transfer occurs at the heme edge. While cytochrome *c* peroxidase was shown to form a 1:1 complex with cytochrome *c*, the two hemes were shown to be parallel with an edge separation of 16.5 Å (Poulos & Kraut, 1980). Thus, it was suggested that electron transfer from cytochrome *c* to the heme iron of the enzyme takes place through an intrinsic bridge of interaction, ionic interaction and hydrogen bonds connecting the two hemes (Poulos & Kraut, 1980). On the other hand, the heme of LPO is not protoheme itself but has an SH at the heme peripheral 8-CH_3 that is thought to form a S–S bridge with an SH group of the protein (Nicholl et al., 1987); the conformation of the peptide segment in contact with the heme may be changed to a considerable degree. Thus, it is tempting to speculate that the phenyl group of donors interacts with heme to facilitate the electron transfer from donor to heme iron.

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Registry No. BHA, 495-18-1; phenylhydrazine, 100-63-0; cyanide, 57-12-5; hydroquinone, 123-31-9; phenol, 108-95-2; *p*-cresol, 106-44-5; aniline, 62-53-3; resorcinol, 108-46-3; guaiacol, 90-05-1; peroxidase, 9003-99-0.

REFERENCES

Aibara, S., Yamashita, H., Mori, E., Kato, M., & Morita, Y. (1982) *J. Biochem. (Tokyo) 92*, 531–539.

Araiso, T., & Dunford, H. B. (1981) *J. Biol. Chem. 256*, 10099–10104.

Ator, M. A., & Ortiz de Montellano, P. R. (1987a) *J. Biol. Chem. 262*, 1542–1551.

Ator, M. A., David, S. K., & Ortiz de Montellano, P. R. (1987b) *J. Biol. Chem. 262*, 14954–14960.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Carlström, A. (1969) *Acta Chem. Scand. B23*, 203–212.

Dolman, D., Dunford, H. B., Chowdhury, D. M., & Morrison, M. (1968) *Biochemistry 7*, 3991–3996.

Hosoya, T., & Morrison, M. (1967) *J. Biol. Chem. 242*, 2828–2836.

Itagaki, E., & Hosoya, T. (1978) *Endocrinol. Jpn. 25*, 43–53.

Kimura, S., & Yamazaki, I. (1979) *Arch. Biochem. Biophys. 198*, 580–588.

Kitagawa, T., Hashimoto, S., Teraoka, J., Nakamura, S., Yajima, H., & Hosoya, T. (1983) *Biochemistry 22*, 2788–2792.

La Mar, G. N., de Ropp, J. S., Smith, K. M., & Langry, K. C. (1980) *J. Biol. Chem. 255*, 6646–6652.

Mäkinen, K. K., & Mäkinen, P.-L. (1982) *Biochem. Biophys. Res. Commun. 105*, 1402–1407.

Morrison, M., & Schonbaum, G. R. (1978) *Annu. Rev. Biochem. 45*, 861–888.

Nicholl, A. W., Angel, L. A., Moon, T., & Clezy, P. S. (1987) *Biochem. J. 247*, 147–150.

Paul, K.-G., & Ohlsson, P.-I. (1978) *Acta Chem. Scand. B32*, 395–404.

Paul, K.-G., & Ohlsson, P.-I. (1985) in *The Lactoperoxidase System. Chemistry and Biological Significance* (Pruitt, K. M., & Tenovuo, J. O., Eds.) pp 15–29, Marcel Dekker, New York.

Pommier, J., & Cahnmann, H. J. (1979) *J. Biol. Chem. 254*, 3006–3010.

Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 10322–10330.

Razumas, V. I., Vbyunenko, O. N., Venozhinskis, Y. V., & Kukyus, Y. Y. (1985) *Biokhimiya (Moscow) 50*, 1362–1366.

Riordan, J. F., Soklolovsky, M., & Vallee, B. L. (1966) *J. Am. Chem. Soc. 88*, 4104–4105.

Rombauts, W. A., Schroeder, W. A., & Morrison, M. (1967) *Biochemistry 6*, 2965–2977.

Sakurada, J., Takahashi, S., & Hosoya, T. (1986) *J. Biol. Chem. 261*, 9657–9662.

Sakurada, J., Takahashi, S., Shimizu, T., Hatano, M., Nakamura, S., & Hosoya, T. (1987) *Biochemistry 26*, 6478–6483.

Schejter, A., Lanir, A., & Epstein, N. (1976) *Arch. Biochem. Biophys. 174*, 36–44.

Schonbaum, G. R. (1973) *J. Biol. Chem. 248*, 502–511.

Sievers, G. (1979) *Biochim. Biophys. Acta 579*, 181–190.

Sievers, G. (1985) *Protides Biol. Fluids 32*, 129.

Thanabal, V., de Ropp, J. S., & La Mar, G. N. (1987) *J. Am. Chem. Soc. 109*, 265–272.

Theorell, H., & Åkeson, A. (1943) *Ark. Kemi, Mineral. Geol. 17B*, No. 7.

Walsh, C. (1982) *Tetrahedron 38*, 871–909.

Yajima, H., Hosoya, T., & Nakamura, S. (1982) *Seikagaku 54*, 776.