Oxidation of Phenolic Compounds by Lactoperoxidase. Evidence for the Presence of a Low-Potential Compound II during Catalytic Turnover†

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ABSTRACT: The lactoperoxidase (LPO)-catalyzed oxidation of *p*-phenols by hydrogen peroxide has been studied. The behavior of the enzyme differs from that of other peroxidases in this reaction. In particular, LPO shows several catalytic intermediates during the catalytic cycle because of its capability to delocalize an oxidizing equivalent on a protein amino acid residue. In the phenol oxidation the enzyme Compound I species, containing an iron−oxo and a protein radical, uses the iron−oxo group at acidic pH and the protein radical in neutral or basic medium. Kinetic and spectroscopic studies indicate that the ionization state of an amino acid residue with pK_a 5.8 ± 0.2, probably the distal histidine, controls the enzyme intermediate forms at different pH. LPO undergoes inactivation during the oxidation of phenols. The inactivation is reversible and depends on the easy formation of Compound III even at low oxidant concentration. The inactivation is due to the substrate redox potential since the best substrate is that with lowest redox potential, while the worst substrate has the highest potential. This strongly indicates that Compound II, formed during catalytic turnover, has a low redox potential, making easier its oxidation by hydrogen peroxide to Compound III. The dependence of LPO activity on the phenols redox potential suggests that the protein radical where an oxidizing equivalent can be localized is a tyrosyl residue.

Heme peroxidases are a class of enzymes that efficiently catalyze the oxidation of a large number of organic and inorganic compounds by using hydrogen peroxide or alkyl hydroperoxides as oxidants (Dunford & Stillman, 1976; Morrison & Schonbaum, 1979). In the classical peroxidase reaction, the enzyme uses the two oxidative equivalents from hydrogen peroxide in a two-step one-electron oxidation of two molecules of substrate (Ortiz de Montellano, 1992; Anni & Yonetani, 1992). During the catalytic cycle the enzyme forms two intermediates, indicated as Compound I and Compound II (Dunford & Stillman, 1976). In Compound I the two oxidative equivalents are stored as the Fe^{IV}═O species and a π-cation radical localized on the porphyrin ring or a radical on an amino acid side chain of the protein, depending on the enzyme. Compound I is a strong oxidant and oxidizes a substrate molecule to give a radical product and the enzyme Compound II. This intermediate is a Fe^{IV}═O species one oxidative equivalent above the resting (Fe^{III}) state of the enzyme. It oxidizes a second molecule of the substrate to a radical, restoring the enzyme in its resting state. If during turnover a large excess of the oxidizing agent, hydrogen peroxide, is present, another enzyme intermediate, Compound III, can be formed. Compound III, formally an iron(II)−dioxygen or an iron(III)−superoxide adduct (Yamazaki et al., 1973; Yamazaki, 1974; Xie & Dolphin, 1994), is three oxidative equivalents above the enzyme resting state. Since it is much less reactive, the accumulation of Compound III during turnover reduces the activity of the enzyme.

Lactoperoxidase (LPO)[1] is a constituent of mammalian milk, saliva, and tears. It is involved in the bacterial defense through the oxidation of thiocyanate ions to the antibacterial species hypothiocyanate ($OSCN^-$) and higher oxyacids (HO_2-SCN and/or HO_3SCN) in the presence of peroxide (Pruitt et al., 1985). The enzyme is a glycoprotein with a molecular weight of approximately 78 000 Da, 10% of which is carbohydrate (Carlstrom, 1969; Sievers, 1980; Ferrari et al., 1995). The protein sequence of the main active fraction from cow's milk is known (Cals et al., 1991), but the X-ray crystal structure of the enzyme is not known. Lactoperoxidase possesses a heme group that is very tightly bound to the polypeptide chain. Early work, based on the difficult extraction of the heme and on pronase digestion of LPO, suggested that the protein contained a protoheme IX group deeply buried in a crevice of the protein molecule (Sievert, 1979, 1980). More recent work, based on reductive cleavage with mercaptoethanol in 8 M urea, suggested that the prosthetic group is protoheme IX with a mercaptomethyl group replacing one of the porphyrin methyl groups and covalently linked to the protein through a disulfide bridge (Nichol et al., 1987). This finding agrees with the odd number of half-cystines found in the protein sequence (Cals et al., 1991). Very recently it has been reported that the LPO heme group contains two hydroxylated methyl groups linked with ester bonds to the apoprotein (Rae & Goff, 1996).

An important feature that distinguishes LPO from other peroxidases is the behavior of the enzymatic intermediates. LPO reacts with hydrogen peroxide to give rise initially to Compound I, CompI(IV,$P^{\bullet+}$), a ferryl porphyrin radical

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[1] Abbreviations: LPO, lactoperoxidase; HRP, horseradish peroxidase; CCP, cytochrome *c* peroxidase; CC, cytochrome *c*; CPO, chloroperoxidase; CompI(IV,$P^{\bullet+}$), ferryl porphyrin radical cation form of Compound I; CompI(IV,$R^{\bullet}$), ferryl protein radical form of Compound I; CompII(IV,RH) ferryl form of Compound II; CompII(III,$R^{\bullet}$), protein radical form of Compound II; DPV, differential pulse voltammetry; E_p, polarographycally determined one-electron oxidation potential; NMR, nuclear magnetic resonance.

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Table 1: Polarographic Peak Potential of Phenolic Substrates and Kinetic Parameters for Their LPO-Catalyzed Oxidation at pH 5.0

n	substrate HO-C_6H_4-R (R)	E_p (mV) *vs* Ag/AgCl/KCl satd	$\Delta\epsilon^a$ (M^{-1} cm^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
1	-$CH(CH_3)_2$	760	2300	1500 ± 130	560 ± 30
2	-CH_3	770	2350	1700 ± 50	570 ± 30
3	-CH_2CH_2COOH	810	1950	950 ± 30	410 ± 20
4	-$CH_2CH_2NH_2$	830	1460	100 ± 10	3.4 ± 0.2
5	-CH_2COOCH_3	870	1900	820 ± 30	260 ± 20
6	L-$CH_2CH(NH_2)COOH$	900	1350	60 ± 40	1.8 ± 0.2
7	D-$CH_2CH(NH_2)COOH$	900	1350	65 ± 40	2.0 ± 0.2
8	-CH_2NH_2	950	2000	55 ± 5	3.0 ± 0.1
9	-CH_2COOH	1060	1890	200 ± 20	77 ± 5

[a] Differential molar extinction coefficient at 300 nm in the formation of the dimeric products of the enzymatic oxidation.

cation. CompI(IV,$P^{\bullet+}$) decays quickly to form a species, analogue to the CCP Compound ES, that contains an oxidizing equivalent on the polypeptide chain (Kimura & Yamazaki, 1979; Hu & Kincaid, 1991; Taurog et al., 1994), which will be identified here as CompI(IV,$R^{\bullet}$). The latter compound reacts with ferrocyanide at acidic pH using the iron−oxo oxidizing equivalent and at neutral or basic pH the amino acid radical oxidizing equivalent (Courtin et al., 1982; Courtin et al., 1984).

Since the heme structure and the characteristics of LPO intermediates differ from those of other peroxidases such as HRP, CCP, and CPO, that all contain ferriprotoporphyrin IX, it is of some interest to investigate how these differences affect the reactivity of the enzyme. We report here the enzymatic oxidation of different *p*-substituted phenols by LPO, using hydrogen peroxide as oxidant. These compounds are typical substrates for classical peroxidases (Dunford & Stillman, 1976; Dunford & Adeniran, 1986; Frew & Jones, 1984; Sakurada et al., 1990), and, as shown by our previous studies on HRP, CCP, and CPO (Casella et al., 1991, 1994, 1996), they behave as good structural probes, since it is possible to introduce charges, steric bulk, or stereocenters in the molecule by changing the group *para* to the hydroxyl group. In addition, of particular importance for this work, the nature of the substituent allows to modulate the redox potential of the substrate.

MATERIALS AND METHODS

Materials. Catalase and HRP were obtained from Sigma Chemical Co. as a purified powder and as a freeze-dried powder (RZ = 3.2 at pH 7), respectively, and were used as received. Bovine LPO was purified according to the method of Ferrari et al. (1995) (RZ = 0.85). The concentration of LPO solutions was determined optically by using $\epsilon_{412} = 114$ mM^{-1} cm^{-1}. Methyl (*p*-hydroxyphenyl)acetate was obtained from the corresponding acid upon esterification with methyl alcohol. *p*-Hydroxyphenyl methyl amine was prepared by catalytic hydrogenation of *p*-cyanophenol over Pd/C using acetic acid as solvent and 3 atm of H_2 pressure for 3 h. The other phenolic substrates studied were commercially available. Other reagents were of the highest grade and used as received. Buffers were prepared using deionized, double distilled water. The hydrogen peroxide solutions were prepared by dilution of a 30% (v/v) solution and the exact oxidant concentration was determined by iodimetric titration.

Kinetic Experiments. The kinetic experiments were followed spectrophotometrically with a HP8452A diode array spectrophotometer apparatus using a magnetically stirred, thermostated, 1-cm path length cell or a thermostated stopped-flow apparatus (Applied Photophysics, model RS-1000), dead time 1 ms, using 1-cm or 0.2-cm path length. The temperature during the measurements was kept at 20 ± 0.1 °C, and the pH was controlled by acetate or phosphate buffers. Also the pH of the substrate solutions was adjusted to the desired value. The solutions of the substrates containing ester groups were prepared daily to prevent hydrolysis. The catalytic reactions were followed through the increase of absorbance at 300 nm due to the formation of phenolic dimers in the initial phase of the reaction. The conversion from ΔA/s data into reaction rate in M/s units was done using the difference in extinction coefficient between the products and the substrate. The values of $\Delta\epsilon$ at 300 nm in acetate buffer pH 5.0 for different *p*-substituted phenols were determined using HRP as catalyst, according to the procedure previously published (Casella et al., 1994, 1996), and are reported in Table 1.

Determination of the Catalytic Constants. The enzymatic reactions were performed in acetate buffer pH 5.0. In order to reduce the effect of noise in the reading of absorbance it was found convenient to monitor the difference between the absorbance near 300 nm and that at a wavelength where the absorption remains negligible during the assay (500 nm). In all the experiments the concentration of hydrogen peroxide was 0.5 mM. The following substrate and enzyme concentrations were used: 0−130 mM *p*-cresol, 13 nM LPO; 0−50 mM tyramine, 13 nM LPO; 0−2.3 mM 2-(*p*-hydroxyphenyl)-propane, 13 nM LPO; 0−88 mM *p*-hydroxyphenylpropionic acid, 13 nM LPO; 0−22 mM methyl (*p*-hydroxyphenyl)-acetate, 13 nM LPO; 0−2.5 mM L- and D-tyrosine, 80 nM LPO; 0−100 mM *p*-hydroxyphenyl acetic acid, 13 nM LPO; 0−40 mM *p*-hydroxyphenyl methyl amine, 60 nM LPO. In all cases the reaction rate was obtained from the slope in the initial part of the trace at 300 nm.

Oxidation of p-Cresol at Different pH. The LPO-catalyzed oxidation of *p*-cresol was studied in the pH range from 4.0 to 8.5. The concentrations of the reactants were as follows: 13 nM LPO, 0.5 mM hydrogen peroxide, and 10 mM *p*-cresol. As before, the rate was determined from the slope in the initial part of the trace at 300 nm. The λ_{max} of the enzyme Soret band in the initial phase of the reaction was also observed. To convert the data from ΔA/s to M/s the following $\Delta\epsilon$ values between the dimers and the reactant were used: pH 5, 2290 M^{-1} cm^{-1}; pH 6, 2300 M^{-1} cm^{-1}; pH 7, 2450 M^{-1} cm^{-1}; pH 8, 3350 M^{-1} cm^{-1} (for other pH values the $\Delta\epsilon$ values were interpolated).

Enzyme Intermediates. The spectrum of LPO CompI-(IV,$P^{\bullet+}$) can be recorded using the stopped-flow apparatus by addition of 1 equiv of hydrogen peroxide to the enzyme (∼2 μM) in acetate buffer, pH 5, at low temperature (∼10 °C); its Soret band has $\lambda_{max} = 416$ nm. In a few seconds

the enzyme is transformed into a species two oxidizing equivalents above the native enzyme and containing an amino acid radical, CompI(IV,R•), characterized by $\lambda_{max} = 430$ nm (Courtin et al., 1984). The Compound II intermediate, in the form CompII(IV,RH), can be easily detected at room temperature. Addition of a small excess H_2O_2 (3−5-fold) to LPO (∼5 μM) in the presence of an equimolar amount of a reducing species, such as *p*-cresol, at pH 7 enables to observe the spectrum of CompII(IV,RH) ($\lambda_{max} = 430$ nm). The spectrum of CompII(III,R•) ($\lambda_{max} = 412$ nm) is obtained if the same experiment is performed at pH 4−5. When the concentration of the oxidant is raised to 100 mM the spectrum observed is that of Compound III ($\lambda_{max} = 424$ nm). To obtain Compound III free from the excess H_2O_2, LPO (3 μM) is incubated at pH 5 in 100 mM acetate buffer, with a large excess of hydrogen peroxide (100 mM). Catalase (13 nM) is added next, and the solution is left to incubate for 20 min. This period is sufficient for catalase to dismutate all hydrogen peroxide while lactoperoxidase remains in the Compound III state. The consumption of H_2O_2 can be checked by adding a few crystals of potassium iodide that, in the presence of LPO and H_2O_2, give rise to I_3^- (Jenzer et al., 1986). The positions of the Soret bands (λ_{max}) for the various enzymatic species found here agree very well with those reported in the literature (Jenzer et al., 1986; Courtin et al.,1982).

Differential Pulse Voltammetry. Polarographic measurements were performed at room temperature in 200 mM acetate buffer, pH 5, using a DPV of 100 mV/s, a pulse amplitude of 50 mV, a glassy carbon work electrode, and an $Ag/AgCl/KCl_{saturated}$ reference electrode. The values of the redox potential measured polarographically correspond to the transformation of the phenols to the corresponding phenoxide radicals. Voltammetric oxidation of phenols causes passivation of the electrode surface that results in rapidly diminishing voltammetric curve response and enlarged peaks. For this reason, the absolute values of the oxidation potentials of the substrates investigated and reported in Table 1 may be affected by the experimental conditions (electrode surface, pH, and concentration of the solutions). However, the differences among the values of the oxidation potentials found for the various substrates are significant because they were obtained exactly in the same experimental conditions.

RESULTS

In the presence of hydrogen peroxide, or alkyl hydroperoxides, peroxidases are able to oxidize *p*-substituted phenols to dimers and, subsequently, to polymerization products. The enzyme in the high oxidation state oxidizes the phenol to a phenoxy radical that evolves independently on the enzyme to give rise to two dimers: the α−α adduct and the Pummerer ketone (Hewson, & Dunford, 1976; Casella et al., 1994, 1996). The reaction can be easily followed spectrophotometrically since the dimers absorb at lower energy than the substrate. The catalytic oxidation of phenols by HRP, CPO, or CCP produces a linear increase of absorbance of the solution with time (Casella et al., 1994, 1996). By following spectrally the enzyme form during turnover it can be seen that these peroxidases are prevalently in the ferryl Compound II state. When analogous experiments are performed with LPO, the observed spectra are different depending on the concentration of hydrogen peroxide and the nature of the phenolic substrate. The major enzymatic

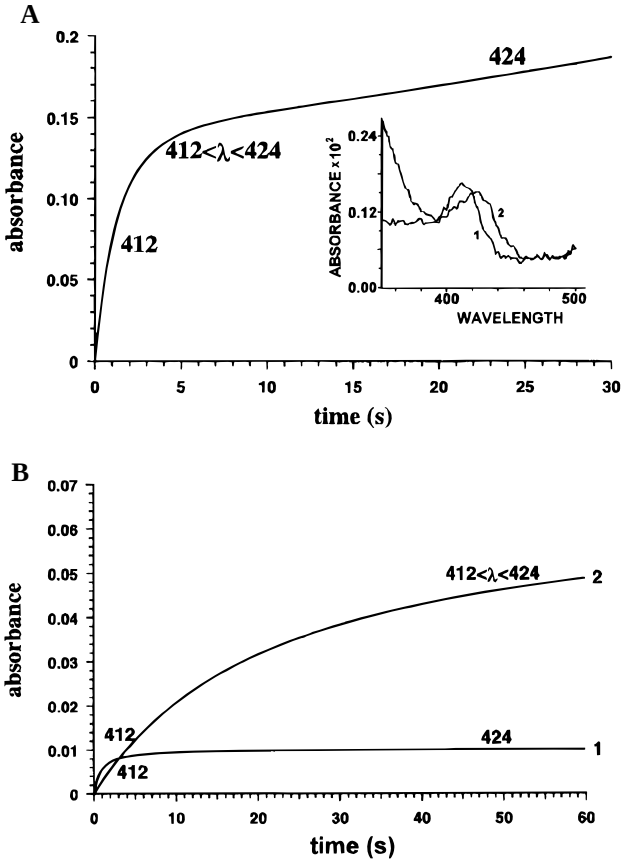


FIGURE 1: Plot of the difference of absorbance (300−500 nm) for the oxidation of *p*-substituted phenols catalyzed by LPO in 200 mM acetate buffer, pH 5.0 at 20 °C. Hydrogen peroxide was added as the last reactant. The numbers near the curves represent the observed enzyme Soret λ_{max} in the different phases of the reactions. A. Reaction of 32.5 mM *p*-cresol, 0.08 μM LPO, and 1.2 mM H_2O_2. The inset represents the enzyme spectra during turnover (1 after 1 s and 2 after 20 s). B. Reaction of 25 mM *p*-hydroxyphenylacetic acid, 0.08 μM LPO, and 1.2 mM H_2O_2 (1) or 0.12 mM H_2O_2 (2).

form present in the various steps of the reaction can be followed by monitoring the enzyme spectrum during turnover.

In the LPO-catalyzed oxidation of *p*-cresol (32.5 mM) at pH 5 in the presence of a low concentration of the oxidant ($[H_2O_2] = 0.12$ mM) the time course of the absorbance at 300 nm in the initial phase of the reaction follows a first-order behavior (data not shown). Moreover, the slope in the initial phase increases linearly with the oxidant concentration in the range around 0.12 mM, indicating that in these conditions the rate-determining step is the reaction of the enzyme with peroxide. From the position of the Soret band during turnover (412 nm) it can be seen that the enzyme is present in solution mostly in the native form. Therefore, at low oxidant concentration, the rate-determining step of the reaction is CompI(IV,P•+) formation. If the experiment is repeated using higher oxidant concentration (1.2 mM) the time course of the absorbance follows a more complicated behavior (Figure 1A). In this figure the position of the Soret λ_{max} of the enzyme form at various times of the reaction is indicated. The shape of the curve indicates that the enzyme is inactivated during the reaction. Extensive dialysis of the enzyme against buffer after the reaction restores the original reactivity, so that the inactivation is reversible. In the plot in Figure 1A three phases can be seen, two of which are linear and contain the enzyme present in a single predominant form, and one intermediate phase characterized by variable

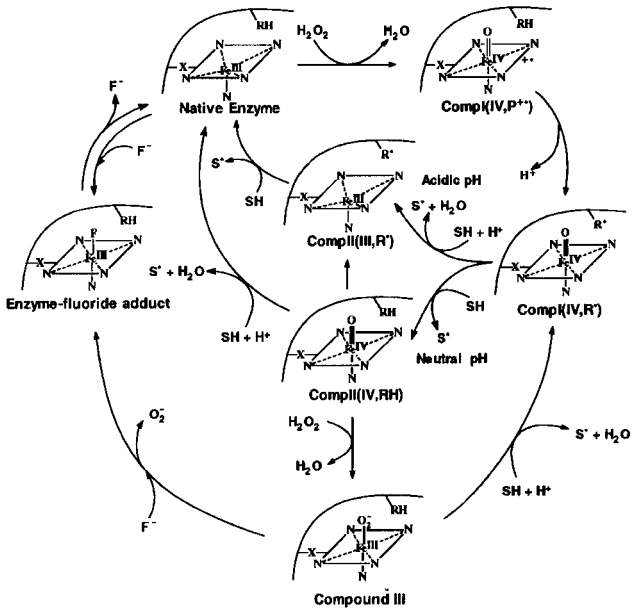


FIGURE 2: Catalytic cycle of LPO in the oxidation of *p*-substituted phenols, including the effect of fluoride. X represents the heme substitution, and RH represents the protein amino acid responsible for the radical species.

slope. In the first linear portion the enzymatic Soret band has λ_{max} 412 nm. This does not correspond to the presence of native enzyme. In fact the kinetic constant for LPO CompI(IV,P$^{\bullet+}$) formation from the resting enzyme is higher than that relative to HRP (Ohlsson et al., 1986). So that formation of CompI(IV,P$^{\bullet+}$) as the slow step of the reaction using this H_2O_2 concentration is unlikely. Moreover, the slope of the initial linear phase is practically unaffected by the change in oxidant concentration in the range around 1.2 mM (data not shown). The 412 nm band is therefore due to the intermediate CompII(III,R$^{\bullet}$) containing the amino acid radical species formed during turnover in acidic conditions. It is likely that during the reaction a small amount of the iron−oxo intermediate CompII(IV,RH) is also formed, but, as it will be shown below, this is more reactive and is not accumulated.

From the position of the Soret band (λ_{max} 424 nm) in the second linear step, it can be seen that the inactivation is due to the continuing formation and accumulation of Compound III, which is a much less reactive catalytic intermediate. The slope of the curve in this phase is much smaller than that in the first phase but is not zero. In fact, Compound III reacts with the substrate, albeit slowly, forming the active CompI-(IV,P$^{\bullet+}$), which supports the reaction. The slope in this phase is a combination of different steps. In fact, the reaction of Compound III with the substrate forms CompI(IV,P$^{\bullet+}$) which activates the normal cycle of the enzyme involving CompII-(IV,RH), CompII(III,R$^{\bullet}$), and the resting state. In the period between the two linear phases the continuous change in the slope is due to a change in the composition of enzymatic species in solution. Since there is accumulation of Compound III, the Soret band moves from 412 to 424 nm.

The catalytic behavior is in accord with the enzymatic cycle represented in Figure 2. It differs from those of classical peroxidases such as HRP for the greater importance assumed by the formation of Compound III and for the possibility to have two intermediates with an oxidative equivalent above the native enzyme in the LPO−H_2O_2 system. In order to confirm the cycle in Figure 2 the same experiment as that reported in Figure 1A was performed, but the order of introduction of the reagents was changed. In particular, the substrate *p*-cresol was introduced as the last reactant. In this way, the enzyme in the presence of hydrogen peroxide forms Compound III and then, introducing the substrate, the conditions become similar to those of the second phase of the experiment represented in Figure 1A. In fact, in this experiment a straight line with a slope similar to, but slightly smaller than, that in the second phase of the previous experiment was obtained. The smaller slope observed is due to the fact that the enzyme was reacted with a large excess of oxidant, and this causes some degradation of the protein. In order to circumvent this problem, the Compound III species was pre-formed and the excess hydrogen peroxide was immediately removed using catalase. The experiment in Figure 1A was then repeated, but the enzyme was introduced in the Compound III state. A straight line with slope very similar to that of the Figure 1A (second phase) was obtained, confirming that the inactivation of LPO is due to the formation of Compound III.

In the attempt to reduce the formation of the Fe(III)−O_2^- species in the LPO catalytic cycle, the use of *tert*-butyl hydroperoxide as oxidant was attempted, since this may be less efficient than H_2O_2 in the formation of Compound III. However, *t*-BuOOH reduces also the formation rate of CompI(IV,P$^{\bullet+}$) (Ohlsson et al., 1986). Thus, when the concentration of oxidant is low enough to neglect the formation of Compound III, the rate-determining step becomes the first step of the cycle, i.e., formation of CompI-(IV,P$^{\bullet+}$).

A different way to prevent the accumulation of the low reactive Compound III during turnover is to accelerate the conversion process to other enzymatic intermediates. Halogen ions are known to bind to LPO (Sievers, 1980; Modi et al., 1989a; Sakurada et al., 1987), but among these we cannot use Br^- or I^-, since they are oxidized by the LPO−H_2O_2 system (Doerge, 1986). Bromide and iodide do not bind directly to the iron center (Modi et al., 1989a; Sakurada et al., 1987), while fluoride binds to iron(III) giving rise to a high-spin adduct (Sievers, 1979). This observation suggests that fluoride can be active in the displacement of superoxide from the iron(III)−O_2^- adduct, thus restoring the native enzyme, and chloride would do the same if it could bind to the iron center. A series of experiments was performed by preparing the hydrogen peroxide-free Compound III by the catalase method described in the experimental section and observing the formation of the native enzyme in the presence of the following reagents: (1) *p*-cresol (32.5 mM); (2) chloride (25 mM); (3) fluoride (25 mM); (4) *p*-cresol (32.5 mM) and chloride (25 mM); (5) *p*-cresol (32.5 mM) and fluoride (25 mM). Chloride alone does not effect the decomposition of the Fe(III)−O_2^- species. In addition, when added together with *p*-cresol it did not detectable alter the time course of resting enzyme formation. Furthermore, chloride did not alter the course of the reaction in experiments such as those represented in Figure 1. This indicates that Cl^- does not bind to the LPO iron center. By contrast, native enzyme formation is strongly accelerated by fluoride, both with or without *p*-cresol, for the capability of fluoride to bind directly to the iron. The LPO−F^- complex obtained in the decomposition of Compound III is in the high-spin state and has an optical spectrum similar to that of the native enzyme (λ_{max} 412 nm). Thus, it is possible to prevent the accumulation of Compound III during the kinetic experiments using

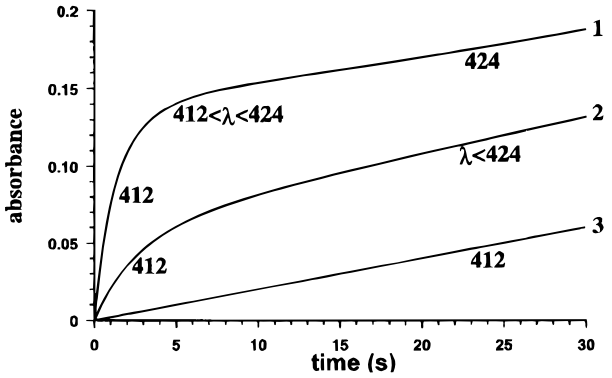


FIGURE 3: Plot of the difference of absorbance (300−500 nm) for the oxidation of *p*-cresol (32.5 mM) by H_2O_2 (1.2 mM) catalyzed by LPO (0.08 μM) in 200 mM acetate buffer, pH 5.0 at 20 °C in the presence of different amounts of fluoride. The numbers near the curves represent the observed enzyme Soret λ_{max} in the different phases of the reactions. (1) $[F^-] = 0$ mM; (2) $[F^-] = 1$ mM; (3) $[F^-] = 25$ mM.

fluoride as decomposition agent. Figure 3 shows the effect of fluoride in the time course of the oxidation of *p*-cresol with H_2O_2 catalyzed by LPO, together with the indication of the enzyme Soret band during the different phases of the reaction. The halogen ion affects the reaction in a concentration dependent manner. The presence of fluoride modifies the kinetic mechanism as shown in Figure 2. One effect of fluoride is to reduce the slope of the first phase of the reaction because, on binding to LPO, it reduces the concentration of free enzyme. The effect of fluoride on the second phase is to increase the slope because it reduces the lifetime of the low reactive Compound III species. Moreover, above 25 mM fluoride there is no appreciable accumulation of Compound III. Also, using fluoride it is not possible to simplify the cycle, because when the concentration of fluoride is raised to prevent the accumulation of Compound III the halogen binds to LPO inhibiting the formation of CompI(IV,$P^{\bullet+}$). Thus the spectrum of the LPO−F^- adduct is observed.

In order to assess the effect of the substrate structure in the reaction, the phenols reported in Table 1 were used. Among those not carrying an amino group, the more interesting behavior is exhibited by *p*-hydroxyphenylacetic acid, since it is a poor substrate in the reaction with LPO. Figure 1B shows the time course of the reaction with this acid. From the curves shown it can be seen that, at the same oxidant concentration as with *p*-cresol, the amount of products formed is much lower. Moreover, the enzyme inactivation due to the formation of Compound III completely stops the reaction and this occurs even at low oxidant concentration. The inactivation is reversible, since upon dialysis the enzyme recovers full activity. From Figure 1B it can be seen that formation of the phenolic dimers is higher when the oxidant concentration is low because in these conditions the rate of formation of Compound III is slower.

Upon addition of *p*-cresol to the reaction between LPO and *p*-hydroxyphenylacetic acid when the inactivated phase has been reached, the formation of *p*-cresol dimers is observed, even though at a slower rate with respect to that obtained in the second phase of the experiment shown in Figure 1A. This latter finding indicates that the low reactive *p*-hydroxyphenylacetic acid may compete with *p*-cresol in the binding to LPO. To confirm this, the experiment shown in Figure 1A was repeated in the presence of increasing quantities of *p*-hydroxyphenylacetic acid. The shape of the curve was found to be the same as in the absence of the acid while the reaction (in terms of slope of the time course curve and product formed at a given time) was depressed (data not shown). Considering the structural similarity between the two phenols and knowing that LPO has a rather closed active site, with the heme buried inside the protein structure (Sievers, 1979, 1980) the inhibition can be due to a competition for the same binding site. Thus, the difference in reactivity between the two substrates does not seem to be due to a different mode of interaction with the enzyme.

The *p*-substituted phenols used as substrates in the LPO-catalyzed peroxidase reaction can be divided into two groups depending on the absence (compounds **1**−**3**, **5**, and **9** in Table 1) or presence (**4** and **6**−**8**) of an amino group. All of the compounds carrying this group are poor substrates for LPO, but in general, the reactivity of all of the substrates seems to depend strongly on their redox potential. To assess this point the kinetic parameters for the enzymatic oxidation of *p*-substituted phenols were determined using the Michaelis−Menten approach (Segel, 1975). The experiments where performed at pH 5 and using high oxidant concentration. In these conditions the reaction between the CompII(III,$R^{\bullet}$) species and the substrate can be considered as the rate-determining step of the reaction. Since at high H_2O_2 concentration the formation of Compound III is fast, in order to minimize the problem of enzyme inactivation, the initial rate of the reaction was obtained considering the absorbance changes within a few seconds after the introduction of the oxidant. This reduces the precision of the kinetic measurements, so that the parameters k_{cat}/K_M and particularly k_{cat} have a significant degree of uncertainty. For some substrates (particularly L- and D-tyrosine) the evaluation of k_{cat} is also complicated by the limited solubility in aqueous buffer. However, the differences among the values of the kinetic parameters for different phenols are much larger than the uncertainty in the measurements. Table 1 reports the parameters k_{cat}/K_M and k_{cat} together with the polarographically determined redox potential of the *p*-substituted phenols. From these data it can be seen that in both groups of phenolic compounds, devoid of or carrying the amino group, the best substrate has the lowest redox potential, while the worst substrate has the highest redox potential. As shown in Figure 4 the log (k_{cat}) of enzymatic oxidation of phenols **1**−**3**, **5**, and **9** is linearly correlated with their one-electron oxidation potential. A similar correlation appears to exist for the series of substrates carrying the amino substituents, although this is less reliable due to the limited number of data and the high uncertainty in the k_{cat} values for the two tyrosines.

Oxidation of p-Cresol at Different pH. An interesting feature of LPO is that the one-electron reduction of CompI(IV,$P^{\bullet+}$) (or CompI(IV,$R^{\bullet}$)) gives rise to a mixture of intermediates depending on the pH. Studies of Courtin et al. (1982, 1984) have shown that the reduction of CompI(IV,$R^{\bullet}$) (referred to in the original paper as "Compound II") with ferrocyanide produces the species $PFe^{III}-R^{\bullet}$ (CompII(III,$R^{\bullet}$) in the present paper) when the reaction is carried out at pH 4.2, while at pH 7 or higher the predominant species formed upon reduction is the iron−oxo species CompII(IV,RH). It was therefore of interest to investigate how the enzymatic activity of LPO in the phenol oxidation is affected by changes in the pH of the solution. These

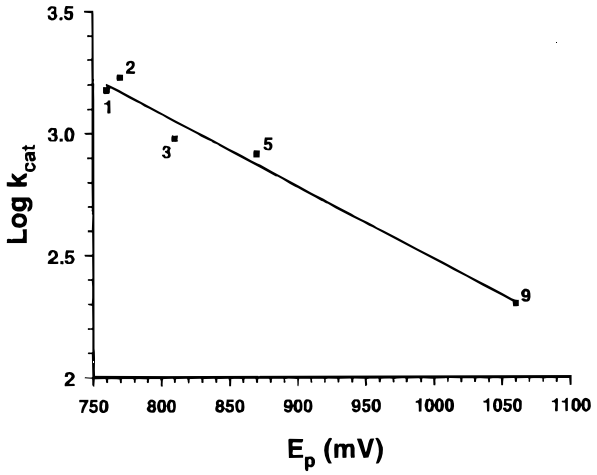


FIGURE 4: Dependence of LPO activity on the substrate peak potential. The value of k_{cat} is in s^{-1} and that of E_p in mV *vs* saturated calomel electrode. Numbers refer to the phenolic substrates reported in Table 1.

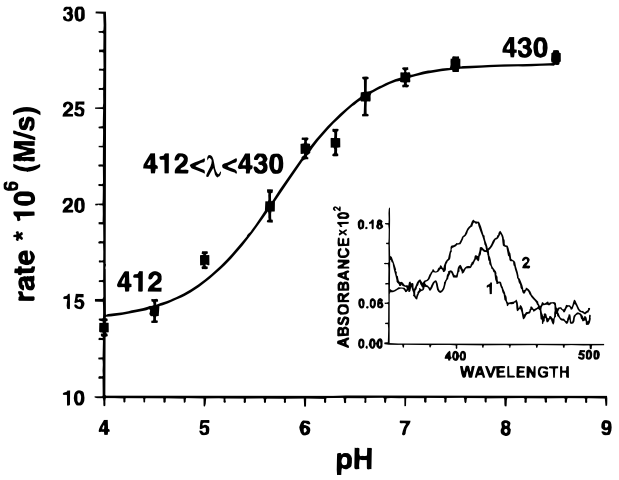


FIGURE 5: Rates of *p*-cresol dimers production at different pHs. The reactions were performed in 200 mM phosphate buffer at 20 °C. The concentrations of the reactants were as follows: 13 nM LPO, 10 mM *p*-cresol, and 0.5 mM H_2O_2. In the inset are shown the spectra of the enzyme after 1 s in the experiments performed at pH 4.5 (spectrum 1) and pH 8.0 (spectrum 2).

experiments were performed using *p*-cresol as substrate since in this case the rate of formation of Compound III is low and, therefore, the inactivation of LPO can be neglected in the pH range studied. The oxidant concentration was kept high in order to increase the velocity of CompI(IV,P$^{\bullet+}$) formation and make the substrate oxidation by either CompII(IV,RH) or CompII(III,R$^{\bullet}$) the rate-determining step. The kinetic experiments were performed in the pH range from 4.0 to 8.5. Since the pK_a of *p*-cresol is ~10 (Critchlow & Dunford, 1972), in this pH range the substrate is essentially in the protonated form and the fraction present as phenolate can be neglected. Moreover, as shown by studies on the oxidation of *p*-cresol catalyzed by HRP (Critchlow & Dunford, 1972) the enzyme catalysis occurs on the non-ionized fraction of the substrate. Figure 5 represents the observed reaction rate at different pH. Above the curve and in the inset the λ_{max} of the enzyme during turnover is shown. At acidic pH the predominant enzymatic form is CompII(III,R$^{\bullet}$), while in basic condition it is CompII(IV,RH). There is correspondence between high enzymatic activity and the fraction of CompII(IV,RH). Thus, the increased reaction rate at basic pH is connected with the increased fraction of CompI(IV,R$^{\bullet}$) transformed by the substrate in the Compound II iron−oxo species, CompII(IV,RH) after reduction of the amino acid radical. Fitting of the data considering that the pH dependent reactivity is associated with the ionization state of a single LPO amino acid residue gives a pK_a of 5.8 ± 0.2.

DISCUSSION

Considerable research effort has been spent in the attempt to understand how the protein structure around the heme affects the hemoprotein function (electron transfer, oxygen transport, or oxidation catalysis) and activity. Much less is known of the effects attributable to a different heme structure. Lactoperoxidase is useful to this end since it contains an iron porphyrin disulfide (from 18-mercaptomethyl-2,7,12-trimethyl-3,8-divinylporphyrin-13,17-dipropionic acid) (Nichol et al., 1987) or an iron porphyrin diester (from 2,12-dihydroxymethyl-7,18-trimethyl-3,8-divinylporphyrin-13,17-dipropionic acid) (Rae & Goff, 1996), while classical peroxidases such as HRP contain iron protoporphyrin IX. Moreover, like CCP, LPO differs from common peroxidases for the capability to use an amino acid radical during catalysis (Kimura & Yamazaki, 1979; Hu & Kincaid, 1991; Taurog et al., 1994). Using HRP, CCP, or CPO as catalyst in the oxidation of *p*-substituted phenolic compounds, under hydrogen peroxide saturation conditions, the enzyme is present during turnover mostly in the Compound II state (Casella et al., 1994, 1996). Instead, using LPO with the same substrates the behavior is different and depends strongly on the reaction conditions. In particular, note the following:

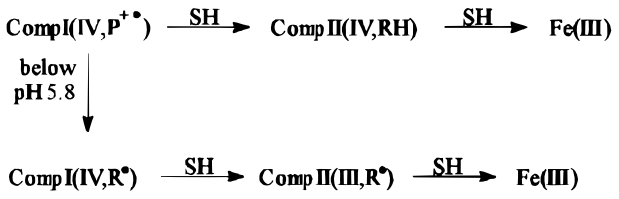
(a) The enzymatic species involved in the oxidation depend on the pH of the solution. In acidic conditions, and neglecting the formation of Compound III, the catalytic cycle is native enzyme → CompI(IV,P$^{\bullet+}$) → CompI(IV,R$^{\bullet}$) → CompII(III,R$^{\bullet}$) → native enzyme; while in basic conditions it is native enzyme → CompI(IV,P$^{\bullet+}$) → CompI(IV,R$^{\bullet}$) → CompII(IV,RH) → native enzyme (in this way it resembles the CCP cycle).

(b) While for the other peroxidases the formation of Compound III during catalysis can be neglected when the oxidant concentration is not too high, for LPO the formation of Compound III, and then inactivation, occurs even at low H_2O_2 concentration. In addition, the velocity of the inactivation process depends strongly on the substrate (with phenols having high redox potential the inactivation is fast).

(c) The oxidation of phenols by LPO depends strongly on the substrate redox potential. In fact, when the phenolic $E°$ is high, the kinetic parameters (k_{cat} and k_{cat}/K_M) are small and there is a fast inactivation of the enzyme to Compound III. Moreover, at pH 5, the enzyme seems to be a very poor catalyst in the oxidation of *p*-substituted phenols bearing an amino group.

The explanation of these differences starts from the catalytic cycle. It is represented in Figure 2 and differs from those of the other peroxidases for the presence of two intermediates bearing a radical delocalized on an amino acid residue. The cycle resembles that of CCP, which delocalizes a radical on a tryptophan residue (Trp191) to form Compound ES (Bosshard et al., 1991; Anni & Yonetani, 1992; Ortiz de Montellano, 1992) but, during turnover, the radical reacts faster than the ferryl group, so that CCP uses only one intermediate having an oxidizing state above the resting enzyme. The reaction of Compound I through the ferryl

Scheme 1



group was observed only using equimolar quantities of reducing agent (not during turnover) and in particular conditions (Coulson et al., 1971; Hahm et al., 1993, Hahm et al., 1994; Liu et al., 1994; Miller et al., 1994). Upon reaction with hydrogen peroxide, LPO forms CompI(IV,P$^{\bullet+}$), which decays to give CompI(IV,R$^{\bullet}$), similar to the CCP Compound ES. The one-electron reduction of CompI(IV,R$^{\bullet}$) by the phenols gives a variable mixture of CompII(IV,RH) and CompII(III,R$^{\bullet}$) depending on pH. The study of LPO enzymatic activity in the oxidation of *p*-substituted phenols, in particular the reaction rates and the predominant enzymatic species during turnover at different pH, can give information on the nature of the amino acid residue controlling the selective reduction of the ferryl species and the radical localized on the apoprotein. Figure 5 shows the observed *p*-cresol oxidation rates *vs* pH. At acidic pH, where the enzyme is present in the initial phase of the reaction mostly in the CompII(III,R$^{\bullet}$) form, the reaction is slower, whereas in basic conditions the enzyme form is CompII(IV,RH) and the reactivity is higher. The different reactivity observed at different pH depends on the relative fraction of CompII(IV,RH) and CompII(III,R$^{\bullet}$) formed from CompI(IV,R$^{\bullet}$). An inflection point at pH 5.8 $\pm$ 0.2 can be seen. The shape of the curve can thus be assigned to the protonation state of an amino acid having pK_a 5.8. This is probably a histidine residue in the active site, presumably the distal histidine. Studies on the binding of aromatic donor molecules (Modi et al., 1989b), iodide (Sakurada et al., 1987), and thiocyanate ions (Modi et al., 1989a) to LPO have shown that these interactions are controlled by an amino acid with a pK_a value of 6.1, 6.0−6.8, and 6.4, respectively. The pK_a value obtained in these binding experiments refers to an amino acid of the enzyme in the native state, while that obtained kinetically refers to CompI(IV,R$^{\bullet}$).

Three hypotheses can be made to explain the different LPO behavior at different pH:

(1) At pH above 5.8 CompI(IV,P$^{\bullet+}$) does not decay to give the CompI(IV,R$^{\bullet}$). The ferryl porphyrin cation radical reacts with the substrate forming only CompII(IV,RH) as shown in Scheme 1. The limit of this hypothesis is that the transition CompI(IV,P$^{\bullet+}$) → CompI(IV,R$^{\bullet}$) was observed also at pH above 7 (Courtin et al., 1982, 1984), even though in this experiment the transition was observed using equimolar quantity of H_2O_2 and no substrate. During turnover the situation is different, and the reaction between CompI(IV,P$^{\bullet+}$) and the substrate could be faster than its decay. This can prevent the formation of CompI(IV,R$^{\bullet}$).

(2) The reactivity of the ferryl group of CompI(IV,R$^{\bullet}$) depends strongly on the protonation state of the amino acid (distal histidine) with pK_a 5.8, while the reactivity of the protein radical does not. In fact, the ferryl reaction occurs with uptake of a proton according to reaction 1, while when the radical reacts there is electron transfer without net proton release or uptake from the ionized amino acid (reaction 2):

$$\text{CompI(IV,R}^{\bullet}) + \text{SH} + \text{H}^{+} \rightarrow \text{CompII(III,R}^{\bullet}) + \text{S}^{\bullet} + \text{H}_2\text{O} \quad (1)$$

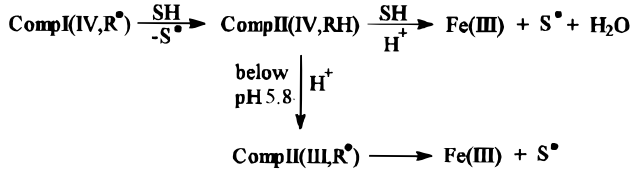
$$\text{CompI(IV,R}^{\bullet}) + \text{SH} \rightarrow \text{CompII(IV,RH)} + \text{S}^{\bullet} \quad (2)$$

At acidic pH (below 5.8), the amino acid is protonated and CompI(IV,R$^{\bullet}$) prefers to react using the ferryl group (reaction 1). CompII(III,R$^{\bullet}$) is then formed. When the amino acid is in the deprotonated form (above pH 5.8), CompI(IV,R$^{\bullet}$) reacts with the substrate using the amino acid radical (reaction 2) and then the ferryl group is maintained in CompII(IV,RH). This hypothesis considers the difference in reactivity between the ferryl or amino acid radical to be small and that, on changing the pH, the reactivity order is inverted. The small difference in reactivity between the ferryl CompII(IV,RH) species and the radical CompII(III,R$^{\bullet}$) species, shown in Figure 5, partially agrees with this observation (even if the presence of two oxidizing equivalents in CompI(IV,R$^{\bullet}$) can alter their individual reactivity). This explanation suffers the problem that if the reactivity of the radical is considered independent of the pH, while that of the ferryl group increases lowering the pH, a lower enzyme activity at basic pH can be expected, while the observed behavior is opposite. This is probably due to the fact that CompII(IV,RH) and CompII(III,R$^{\bullet}$) have lower redox potential with respect to CompI(IV,R$^{\bullet}$) so that the former two species are more sensitive to the redox properties of the substrate. The radical intermediate has lower redox potential than the ferryl intermediate and, therefore, its oxidative capability is reduced. This interpretation agrees with stopped-flow studies on the electron transfer from cytochrome *c* to cytochrome *c* peroxidase Compound I made by Hahm et al. (1994), where it was found that the reduction of the radical is nearly independent of pH from 5 to 8.

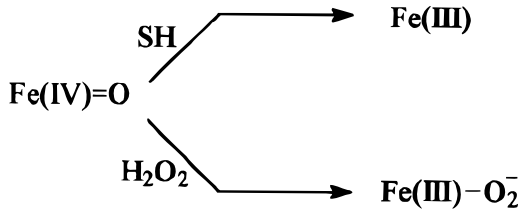
(3) At any pH CompI(IV,R$^{\bullet}$) uses the amino acid radical in the oxidation of the substrate, thus forming CompII(IV,RH). But in acidic conditions (below pH 5.8) the intermediate reacts to transfer the oxidizing equivalent from the iron−oxo to the radical (Scheme 2). In favor of this hypothesis is the observation of Courtin et al. (1984) that on lowering the pH from 7 to 4.2 CompII(IV,RH) is transformed into CompII(III,R$^{\bullet}$). The transformation of the ferryl species in the protein radical indicates that the former is more reactive and accounts for the reduced activity of the enzyme at acidic pH. However, this hypothesis does not explain why, if CompI(IV,R$^{\bullet}$) prefers to react using the protein radical, the ferryl species is more reactive in the reduced intermediate than the radical (but the presence of two oxidative centers in CompI(IV,R$^{\bullet}$) can alter the reactivity of each of them).

Studies on the oxidation of cytochrome *c* using CCP Compound I (Coulson et al., 1971; Hahm et al., 1994) indicate that there are two possible mechanisms to explain the observed CCP reactions, similar to the hypotheses 2 and 3 above. More recent works (Liu et al., 1994; Miller et al., 1994) indicate that the two intermolecular one-electron transfer reactions that reduce the CCP Compound I to the resting enzyme proceed via reduction of the Trp-191 radical by CC at any pH and utilize a single, unique electron transfer pathway. The latter finding could support that also for the LPO−H_2O_2−phenol system the reaction can proceed with a first step in which the radical oxidizing equivalent is used, in accord with hypothesis 3. In the CCP-CC system also the second electron transfer proceeds via the Trp-191 radical,

Scheme 2



Scheme 3



formed upon oxidation of the amino acid by the ferryl group. But the reaction between two redox proteins requires the preliminary formation of a complex and then the electron transfer. Pelletier and Kraut (1992) proposed from the crystal structure of the complex between cytochrome *c* peroxidase and yeast iso-1-cytochrome *c* the presence of an electron-transfer pathway from the heme of cytochrome *c* to the indole group of Trp-191. The presence of such a pathway enables CCP to react by two electron transfers from the radical. In the LPO−phenol system the aromatic substrate can approach the heme and then the reaction using the ferryl oxidizing equivalent can occur. Therefore, with the available data, recognition of the real pathway among the three possibilities presented above is not possible.

The study of the reaction of Compound I is more complicated in the case of LPO with respect to CCP because with LPO the high oxidative intermediate has a short lifetime. Also, the study of the catalytic cycle is complicated by the fast inactivation of LPO due to the formation of Compound III and the high number of intermediates present during turnover. This number may even increase further if CompI-(IV,$P^{\bullet+}$) could be oxidized at the protein site, to give a species three oxidizing equivalents above the resting state, CompI-(IV,$P^{\bullet+}$,$R^{\bullet}$), as it has been found in the CCP case (Miller et al., 1992).

In order to explain the easy formation of Compound III during the phenol oxidation reaction it is useful to isolate the steps involving CompII(IV,RH) in the catalytic cycle. Once CompII(IV,RH) is formed, it can react in two ways: with the substrate, forming the native enzyme, or with hydrogen peroxide, forming Compound III (Scheme 3).

The enzymes HRP and CPO prefer to react with the substrate, and the reaction with H_2O_2 becomes important only at much higher oxidant concentration. Lactoperoxidase behaves in an opposite way. In fact, the inactivation reaction can be neglected only at very low oxidant concentration, where the formation of the CompI(IV,$P^{\bullet+}$) intermediate is the rate-determining step. At higher H_2O_2 concentration, Compound III is the predominant enzymatic species present in solution. This difference in the LPO behavior can be explained considering the low oxidative reactivity of its CompII(IV,RH) intermediate that reduces its efficiency in the restoration of the resting enzyme and favors the formation of the low reactive Compound III. Studies on the effect of the paramagnetic LPO heme group on the longitudinal and transversal relaxation rates of the nuclei of bound aromatic donor molecules (Modi et al., 1989b), and in particular tyrosine protons (Casella et al., 1991), by NMR have shown that these molecules bind further away from the iron center in LPO (average distance for aromatic protons, ∼11 Å) with respect to HRP (∼8−10 Å). This greater distance, and possibly also a different orientation of the substrate in the active site, could reduce the rate of the electron transfer process in the oxidation of phenolic compounds by the CompII(IV,RH) intermediate. The small H_2O_2 molecule, instead, can easily reach the iron center to give rise to the formation of Compound III. On the other hand, a factor controlling the reactivity of the substrates appears to be their redox potential. Increasing the substrate redox potential leads not only to a decrease in enzyme reactivity (Table 1) but also to faster inactivation of LPO to Compound III (Compare Figure 1A and 1B). This strongly indicates that CompII-(IV,RH) has a relatively low redox potential, which makes difficult the oxidation of phenols having high potential. The low potential of CompII(IV,RH) facilitates its oxidation by hydrogen peroxide to form the Compound III species. This explains the easy formation of the iron(III)$-O_2^-$ intermediates even during turnover with low H_2O_2 concentration.

The presence of an unusual peripheral substitution (Nichol et al., 1987; Rae, & Goff, 1996) within the hemin skeleton, modified by sulfur or oxygen insertion, alters the electronic properties of LPO. It was observed that the different prosthetic group influences the UV/vis spectra of LPO derivatives (the Soret band is red-shifted with respect to other peroxidases) and its NMR spectra (Thanabal & La Mar, 1989). The prosthetic group apparently influences also the redox potential of various enzymatic forms. In particular, the functionalization of the methyl groups of the porphyrin could reduce the CompII(IV,RH) redox potential. Thus, the different catalytic behavior of LPO with respect to, e.g., HRP in the formation of Compound III is probably due to the concomitance of two effects: the unusual structure of the porphyrin and the larger distances (2−3 Å) that the donor molecules can reach from the iron center in the LPO−substrate complexes.

When the oxidation of *p*-substituted phenols occurs in acidic conditions and with an excess of H_2O_2, in the initial phase of the reaction the enzyme is present in the CompII-(III,$R^{\bullet}$) state and the rate-determining step of the reaction is the oxidation of the phenol by the protein radical intermediate. As shown by the data in Table 1 the kinetic parameters k_{cat} and k_{cat}/K_M, obtained at pH 5, are strongly reduced by the presence of an amino group in the substrate. It is likely that with this group protonated the substrates bind to the protein with a disposition unfavorable for electron transfer, thus reducing k_{cat}. At the same time the affinity of these phenols is reduced, thus increasing the K_M value. The low affinity of LPO for positively charged phenols is probably due to the presence of basic amino acids such as arginine and histidine in the active site (Thanabal & La Mar, 1989; Shiro & Morishima, 1986). A similar behavior was observed with HRP and CPO (Casella et al., 1994, 1996) and may reflect common structural features of peroxidases. The data in Table 1 indicate that the kinetic parameters are also strongly affected by the substrate redox potential. Between the two parameters, k_{cat}/K_M is more reliable, but its value is controlled by various factors and not only by the electron transfer rate once the protein−substrate adduct is formed. The parameter k_{cat} is regulated by the electron transfer and the diffusion of the phenoxide radical product from the active site to the bulk solution. With low reactive

substrates the diffusion can be considered fast with respect to the oxidation process at the active site and the parameter k_{cat} is controlled essentially by the redox process. Thus, this is the parameter to be compared with the redox potential. From Table 1 it can be seen that upon increasing E_p the enzymatic activity is depressed.

The linear correlation between the log k_{cat} values and the phenol redox potential indicates that the rate of electron transfer, once the LPO−substrate complex is formed, depends on the difference between the E_p values of the enzyme intermediate CompII(III, R•) and that of the phenol. A small value for this difference can explain the ability of LPO to discriminate among the phenolic substrates, at least those not carrying amino groups. A similar dependence of LPO enzymatic activity on substrate redox potential was found in the oxygenation of organosulfur compounds (Doerge, 1986). It is interesting that the LPO activity is low with substrates having redox potential higher than that of tyrosine. The substrate *p*-hydroxyphenylacetic acid, in particular, exhibits high E_p and low reactivity, which is also accompanied by fast enzyme inactivation. These findings suggest that the amino acid residue of LPO where the radical is localized may have a redox potential close to 900 mV. This residue can thus be a tyrosine residue close to the heme, even though a tryptophan residue cannot be excluded. The importance of a tyrosine residue close to the heme was also seen in the HRP−H_2O_2 system. Miller et al. (1995) have shown that after replacement of Phe-172 with a tyrosine, HRP forms an intermediate bearing a radical on tyrosine and then the enzyme behaves like LPO.

The reason why LPO forms a radical intermediate and a low redox potential Compound II is not known, but it may be linked to its functional activity. The heme group is deeply buried in the interior of the protein and the reaction of large molecules may be hindered by a small access channel, while the natural substrate, the small SCN^- ion, can approach the heme easily. Also the presence of a radical intermediate and a low redox potential Compound II presumably is a way for the enzyme to be not involved in undesired reactions and to save the oxidizing equivalents for the oxidation of thiocyanate. In fact, for the antibacterial activity LPO needs hydrogen peroxide and since the milk is poor in H_2O_2 the enzyme must use, and not lose in several reactions, the oxidative capacity supplied by the bacteria themselves.

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