Mechanism-Based Inactivation of Lactoperoxidase and Thyroid Peroxidase by Resorcinol Derivatives†

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ABSTRACT: Humans are exposed to resorcinol derivatives in the environment through ground water, foods, food additives, drugs, and hair dyes. Epidemiological studies have linked human exposure to phenolic compounds with the thyroid disorder, goiter. The results presented here demonstrate the suicide (mechanism-based) inactivation of thyroid peroxidase (TPO) and the closely related lactoperoxidase (LPO) by resorcinol derivatives. The evidence for this mechanism includes irreversible, hydrogen peroxide-dependent loss of enzymatic activity by kinetics consistent with a suicide mechanism, concomitant with changes in the visible spectrum of the prosthetic heme group and covalent binding of resorcinol (ca. 10 mol/mol of lactoperoxidase inactivated). The inactivation was specific for thyroid peroxidase and lactoperoxidase since the activity of horseradish peroxidase, myeloperoxidase, chloroperoxidase, or the pseudoperoxidase, metmyoglobin, was unaffected by incubation with resorcinol. The enzymatic oxidation of resorcinol by lactoperoxidase was linked to inactivation since the same products were observed spectrally, albeit at a much lower level, as were observed with horseradish peroxidase. The results are consistent with thyroid peroxidase- and lactoperoxidase-catalyzed oxidation of resorcinol derivatives to reactive radical species that covalently bind to amino acid residues unique to these two enzymes. The oxidation of thyroid peroxidase and lactoperoxidase by hydrogen peroxide produces catalytic intermediates containing unpaired electron density on amino acid residues similar to that seen with cytochrome *c* peroxidase. These results provide an explanation for the potency of resorcinol derivatives in the inhibition of LPO and TPO and the goitrogenic responses observed in humans and animals. The widespread occurrence of resorcinol derivatives in the environment suggests that exposure to these compounds may cause thyroid dysfunction in humans.

Resorcinol and a diverse array of other phenolic compounds suppress thyroid hormone synthesis (Cooksey *et al.*, 1985). Humans are exposed to resorcinol and its derivatives in the environment through ground water (Gaitan, 1986), food (Klopfenstein *et al.*, 1983; Gaitan *et al.*, 1989), food additives (Iyengar *et al.*, 1991), drugs (Harvey, 1975), and hair dyes, and there is epidemiological and experimental evidence that demonstrates a positive correlation between goiter in humans and the ingestion of phenolic compounds (Cooksey *et al.*, 1985; Lindsay *et al.*, 1989).

Although the effects of phenolic compounds on the thyroid gland and thyroid hormone synthesis were demonstrated almost 50 years ago, the exact mechanism by which they bring about these changes is obscure. Fawcett and Kirkwood (1953) and Doniach and Fraser (1950) concluded that resorcinol competes with tyrosine for iodination by TPO,[1] which results in diminished amounts of iodotyrosines and the concomitant formation of iodinated resorcinols. However, Woeber and Ingbar (1965) showed that depressed iodide ion uptake and decreased iodotyrosine formation were due to the inhibition of TPO rather than competitive iodination of resorcinol and its derivatives as the mechanism for antithyroid activity. TPO catalyzes the oxidation of iodide ion and the coupling of iodotyrosine residues on thyroglobulin in the synthesis of thyroid hormones (Taurog, 1991).

LPO has been used extensively as a model peroxidase in studies involving *in vitro* iodination and coupling reactions (Lamas *et al.*, 1986; Ohtaki *et al.*, 1982), since it is readily available and shares several similarities with TPO in catalyzing the above reactions. In previous work, we studied the different mechanisms for inhibition of LPO- and TPO-catalyzed reactions by antithyroid chemicals found in the environment (Doerge, 1988; Doerge & Niemczura, 1989; Doerge & Takazawa, 1990; Doerge *et al.*, 1993). In this paper, we present evidence for mechanism-based (suicide) inactivation of LPO and TPO by resorcinol and its derivatives.

MATERIALS AND METHODS

Catechol, guaiacol, hexylresorcinol, hydroquinone, hydrogen peroxide, orcinol, phenol, potassium iodide, pyrogallol, tyrosine, and all enzymes except TPO were obtained from Sigma Chemical Co. Phloroglucinol was obtained from Aldrich Chemical Co. TPO was prepared as described previously (Doerge & Takazawa, 1990). Uniformly ring labeled [^{14}C]-resorcinol (55 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). 2-Iodoresorcinol, 2,6-diiodoresorcinol 2,4,6-triiodophloroglucinol (Weil, 1976), and 4,6-diiodoresorcinol (Nicolet & Samprey, 1927) were synthesized and purified by the methods indicated. All other chemicals used were of analytical grade from commercial sources. Hydrogen peroxide concentration was determined by iodometric titration (Kolthoff *et al.*, 1969), and required dilutions were made daily. Enzyme concentration was determined spectrophotometrically on the basis of Soret absorbance. Kinetic measurements and second-derivative spectral scans were performed using a Hewlett-Packard 8452A

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[1] Abbreviations: CcP, cytochrome *c* peroxidase; CPO, chloroperoxidase; HRP, horseradish peroxidase; LPO, lactoperoxidase; MIT, 3-iodotyrosine; MPO, myeloperoxidase; Mb, myoglobin; PGHS, prostaglandin H synthase; res, resorcinol; TPO, thyroid peroxidase.

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diode array spectrophotometer with an attached Hi-Tech SFA-11 rapid kinetics accessory equipped with an external constant temperature circulating bath (±0.1 °C).

Enzyme Assays. Tyrosine iodination was used to quantify the activities of LPO and TPO because of the high reproducibility and linearity of the reaction. Peroxidase (2–4 nM) was incubated with tyrosine (150 μM) and iodide ion (150 μM) in 0.1 M phosphate (pH 7.0) at 25.0 ± 0.1 °C. After 2 min, the reaction was initiated by the addition of hydrogen peroxide (100 μM), and the absorbance at 289 nm due to MIT was followed spectrophotometrically. Initial rates were determined from the linear portion of the progress curve (0–1 min). Guaiacol oxidation was used to measure the activities of CPO, HRP, ferric Mb, and MPO. Peroxidase (1.0–2.5 nM) was incubated with guaiacol (33 mM) in 100 mM phosphate buffer (pH 7.4) at 25.0 ± 0.1 °C. The reaction was started by the addition of hydrogen peroxide (100 μM), and initial rates were determined spectrophotometrically at 470 nm during the linear portion of the curve (0–15 s).

Inactivation of LPO/TPO by Resorcinol Derivatives. LPO (1.0 μM) was incubated with a resorcinol derivative (0.25–0.50 mM) at 25 °C in 100 mM phosphate buffer (pH 7.4) in the presence and absence of H_2O_2 (200 μM) in a total volume of 125 μL. After 2 min of incubation, an aliquot (10 μL) was withdrawn from the reaction mixture and diluted 1000-fold, and the enzyme activity was measured as described above. The residual amount of resorcinol derivative following dilution produced negligible inhibition of peroxidase activity. Incubations with TPO contained 100 nM enzyme, resorcinol derivative (25–50 μM), and H_2O_2 (20 μM). Measurements were made at least in duplicate. Enzyme inactivation experiments were carried out as above in the presence of resorcinol (8.0 μM/250 μM) and iodide (0.1 mM/5.0 mM) or pyrogallol (8–100 μM) in order to determine the protecting ability of alternate substrates.

A rapid method for separation of small molecules from enzymes with minimal dilution effects was chromatocentrifugation. Centrifugal columns (12 × 40 mm) containing Bio-Gel P-6 (Bio-Rad Co., Richmond, CA) were prepared and equilibrated with 100 mM phosphate buffer (pH 7.4). Before LPO samples were applied, columns were equilibrated in buffer and centrifuged at 200*g* for 5 min to remove buffer and minimize dilution. This process was repeated three times. Protein samples (0.2 mL, 16% of the bed volume) were applied to the column and centrifuged at 200*g* for 2 min. The effluent was collected and diluted to known volume, and enzyme activity was measured by the tyrosine iodination procedure described above. Recovery of untreated LPO, monitored by activity and protein content, was 90–96%.

The time course for inactivation of LPO by resorcinol derivatives was determined by incubating LPO (1.0–8.0 μM) in the presence of H_2O_2 (200 μM) and inhibitor. The ratio of [LPO]/[inhibitor] was kept constant throughout each experiment (0.125, 0.083, 0.062, 0.05, 0.05, and 0.04 for resorcinol, hexylresorcinol, phloroglucinol, orcinol, 2-iodoresorcinol, and 4,6-diiodoresorcinol, respectively). The ratio was made as small as possible, although reactions with half-times of less than ca. 30 s were not amenable to study using these methods. However, the kinetic treatment used to analyze the data is completely general with respect to the ratio used (Waley, 1985). H_2O_2 was added to the incubation mixture containing LPO and inhibitor to initiate the inactivation at 25 °C in 100 mM phosphate buffer (pH 7.4); at 10-s intervals an aliquot was withdrawn and diluted 1000-fold, and the remaining enzyme activity was measured. Inactivation half-times ($t_{1/2}$) were determined from the time course plots for inactivation. Inactivation by ethanol (final concentration 2% (v/v)), the solvent for phenolic compounds, and H_2O_2 was found to be insignificant (less than 5%) during the observation period at the concentrations used.

Inactivation of LPO by resorcinol at various pH values (4.2–7.4) was determined by incubating LPO (1.0 μM) with resorcinol (8.0 μM) and H_2O_2 (0.2 mM) at 25 °C in 100 mM acetate (pH 4.2 or 5.2) or phosphate (pH 6.0, 7.0, and 7.4). At various times, aliquots were withdrawn to determine the residual activity. The initial rate of inactivation (k_{obs}) was determined at each pH value from the initial slope of residual activity vs incubation time. The substrate activity of guaiacol was also determined over this pH range.

Partition Ratio Estimation. LPO (1 μM) was incubated with resorcinol (250 μM) at varying concentrations of H_2O_2 (0–100 μM) in 100 mM phosphate buffer (pH 7.4) at 25 °C for 2 min, aliquots (10 μL) were diluted, and the remaining enzyme activity was determined. Decreases in absorbance at 412 nm and other spectral changes were monitored by second-derivative UV–vis spectrophotometry.

Covalent Binding of [^{14}C]Resorcinol. LPO (1.0 μM) was incubated with [^{14}C]resorcinol (250 μM), and the reaction was initiated by the addition of H_2O_2 (0–200 μM) at 25 °C. After 2 min, the reaction was quenched by adding catalase (500 units), and the reaction mixture was transferred to dialysis tubing (molecular weight cutoff = 12000–14000) and dialyzed extensively against 100 mM phosphate buffer (pH 7.4) containing 0.02% thimerosal. After 48 h of dialysis with an additional change of buffer, the contents of the dialysis tubing were subjected to enzyme assay (tyrosine iodination activity) and liquid scintillation counting. The covalent binding of resorcinol was quantified from the amount of [^{14}C]resorcinol bound to LPO using the known specific activity. Control experiments were performed by incubating LPO with [^{14}C]-resorcinol in the absence of hydrogen peroxide. Chromato-centrifugation (see above) gave results comparable to extensive dialysis, so that all data were pooled. Protein content was determined by the Lowry method. Assays were also carried out as described above in the presence of iodide (0.1 or 5.0 mM) or pyrogallol (0.25 or 1.0 mM).

Peroxidase-Mediated Transformation of Resorcinol. HRP (50 nM) or LPO (100 nM) was incubated with resorcinol (1.0 mM), and the reaction was started by the addition of H_2O_2 (200 μM). The solutions were mixed using a stopped-flow apparatus (mixing time ca. 50 ms) at 10 °C in 0.1 M phosphate buffer (pH 7.4), and the oxidation of resorcinol was monitored spectrophotometrically by repetitive scanning (280–600 nm) at 3-s intervals. No reaction was observed on this time scale in the absence of peroxidase. HPLC using scanning UV detection (220–350 nm) or fixed-wavelength detection (260, 280, 320 nm) using an acetonitrile/water gradient (10–90% acetonitrile) failed to detect any resorcinol oxidation product.

The possible formation of iodinated resorcinol derivatives in the presence of iodide ion, H_2O_2, and LPO was investigated using HPLC with scanning UV detection and ^{1}H NMR. The incubation of resorcinol for these determinations was the same: LPO (100 nM) was incubated at room temperature with resorcinol (100 μM) and iodide ion (200 μM), and H_2O_2 was added to initiate the reaction. Control experiments were conducted (a) in the absence of H_2O_2, (b) in the absence of iodide ion, and (c) in the absence of LPO. After 3 min, the small molecules were separated from LPO by ultrafiltration. For the HPLC determination, 10-μL aliquots of the filtrate were injected onto a NovaPak C18 radial compression

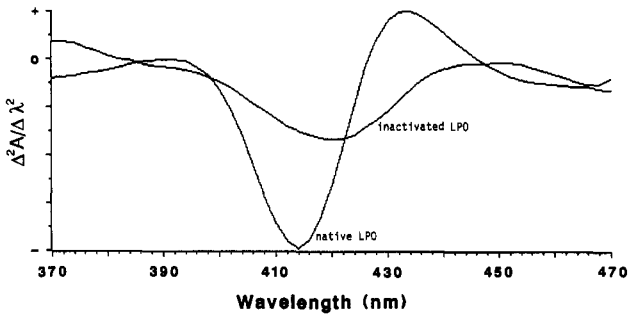


FIGURE 1: Second-derivative Soret spectrum of LPO and inactivated LPO. LPO samples (1 μM) were prepared and spectra recorded as described in Materials and Methods for native LPO and RES-inactivated LPO.

cartridge (Waters Associates, Milford, MA) using 25% acetonitrile/water (v/v) as the mobile phase, and the UV spectrum (220–350 nm) was obtained using a SpectraFocus rapid-scanning detector (SpectraPhysics, San Jose, CA). The retention times for possible iodination products were determined by comparison with chromatograms and UV spectra for authentic standards synthesized by the methods of Weil (1976). Retention times were 3.03, 6.18, and 8.14 min for 2-iodoresorcinol, 2,4-diiodoresorcinol, and 4,6-diiodoresorcinol, respectively.

For ^1H NMR determinations, the filtrate (5 mL) was evaporated to dryness using a Savant SpeedVac concentrator, and D_2O was added to remove exchangeable protons. This process was repeated once and then analyzed at 500 MHz using a Bruker AM500 spectrometer. Resonance positions were estimated using the residual HOD peak as reference (4.67 ppm). The resonances reported by Weil (1976) for several iodoresorcinol derivatives were used to monitor the potential formation of products.

RESULTS

Incubation of resorcinol and its derivatives with LPO or TPO in the presence of hydrogen peroxide resulted in the time-dependent loss of enzymatic activity. The inactivation was irreversible because activity could not be restored by dialysis or chromatocentrifugation. The data in Table 1 show that the inactivation of LPO by resorcinol has an absolute requirement for hydrogen peroxide. Irreversible changes in the second-derivative visible spectrum of LPO were observed concomitant with hydrogen peroxide-dependent loss of enzymatic activity (see Figure 1). The maximal change was a ca. 60% decrease in the Soret absorbance and a blue shift of 12 nm. Additional changes were observed at higher wavelengths (500–600 nm). These spectral changes were unaffected by chromatocentrifugation, dialysis, or the addition of ferrous sulfate. The data in Table 1 also indicate that iodide ion at 0.1 mM did not completely protect the enzyme from inactivation in the presence of hydrogen peroxide. In fact, a modest enhancement of LPO inactivation was observed in the presence of 0.1 mM. However, higher concentrations of iodide ion (5 mM) diminished the amount of LPO inactivation observed. HPLC and ^1H NMR analysis of the products of the incubation mixtures did not reveal any iodination of resorcinol (HPLC detection limit ca. 1 μM or 0.4% conversion for 2-iodoresorcinol, 2,4-diiodoresorcinol, and 4,6-diiodoresorcinol). LPO inactivation by resorcinol was blocked by the addition of pyrogallol in a concentration-dependent fashion, and high concentrations gave complete protection.

A variety of phenolic compounds was tested for the capacity to inactivate LPO and TPO, in order to establish the structure–activity relationship. Results are depicted in Figure 2. All of the phenolic compounds listed in Figure 2 inhibited both LPO- and TPO-catalyzed reactions when present in the enzyme assay medium (e.g., tyrosine iodination, iodide

Table 1: Inactivation of LPO Activity and Covalent Binding of Resorcinol under Different Incubation Conditions[a]

incubation conditions	activity (% of control)	[^{14}C]resorcinol bound (mol/mol LPO)
LPO	98.6 ± 3.8	–
LPO + H_2O_2	96.4 ± 2.4	–
LPO + RES	103.1 ± 4.6	0.18 ± 0.06
LPO + RES + I^-	98.5 ± 3.2	0.06 ± 0.02
LPO + RES + H_2O_2	17.3 ± 6.6	8.8 ± 2.0
LPO + RES + I^{-a} + H_2O_2	6.2 ± 3.3	16.7 ± 1.8
LPO + RES + I^{-b} + H_2O_2	44.7 ± 4.5	12.8 ± 1.3
LPO + RES + PYG[c] + H_2O_2	72.1 ± 3.6	2.2 ± 0.8
LPO + RES + PYG[d] + H_2O_2	98.7 ± 4.3	0.8 ± 0.1

[a] LPO (1.0 μM) was incubated at 25 ± 0.1 °C in phosphate buffer (pH 7.4) in the presence of H_2O_2 (200 μM) and unlabeled or [^{14}C]resorcinol (0.25 mM) with iodide ion (a, 0.1 mM; b, 5.0 mM) or pyrogallol (PYG) (c, 0.25 mM; d, 1.0 mM) in the indicated combinations. After a 2-min incubation, aliquots were withdrawn and diluted to 1000–2000-fold, and the enzyme activity was measured as rate of tyrosine iodination. In addition, the [^{14}C]resorcinol bound to LPO following dialysis and chromatocentrifugation was determined. Values are mean ± SD (n = 6) (–, experiment performed in the absence of [^{14}C]resorcinol).

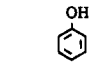
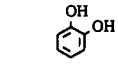
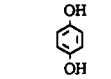
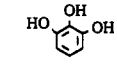
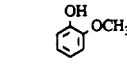
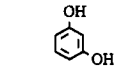
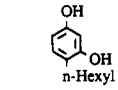
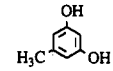
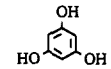
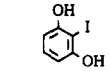
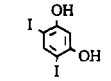
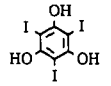
		% LPO Inactivation	% TPO Inactivation
	Phenol	8	12
	Catechol	3	7
	Hydroquinone	6	8
	Pyrogallol	0	15
	Guaiacol	20	17
	Resorcinol	98	97
	Hexyl Resorcinol	95	99
	Orcinol	96	97
	Phloroglucinol	99	98
	2-Iodoresorcinol	92	89
	4,6-Diiodoresorcinol	40	32
	Triiodophloroglucinol	0	2

FIGURE 2: Structure–activity relationship for inactivation of LPO/TPO by phenolic compounds. Incubations with LPO (1.0 μM) were carried out at 25 ± 0.1 °C in phosphate buffer (pH 7.4) in the presence of H_2O_2 (0.2 mM) and phenolic compound (0.2 mM). Incubations with TPO (0.1 μM) contained 25 μM phenolic compound and 20 μM H_2O_2. Other conditions were common to both enzymes. After 2 min of incubation, an aliquot was withdrawn and diluted, and the enzyme activity was determined using the tyrosine iodination assay described in Materials and Methods.

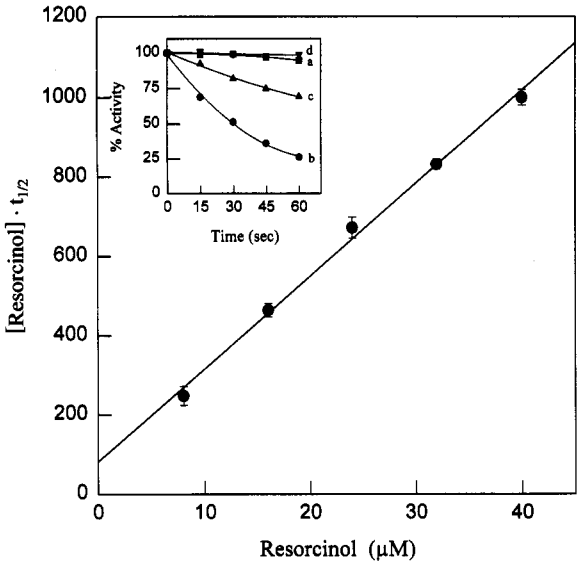


FIGURE 3: Kinetics of inactivation of LPO by resorcinol. The LPO (1.0–5.0 μM) was incubated with varying concentrations of resorcinol (8–40 μM) and H_2O_2 (0.2 mM) at 25 ± 0.1 °C in 0.1 M phosphate buffer (pH 7.4), and the residual activity (see inset) at various time points was determined using the tyrosine iodination assay. The ratio of LPO/resorcinol was kept constant at 0.125. Half-time for inactivation was determined from the time course of inactivation (inset). Ten independent determinations of the half-time were made, and the error bars shown represent standard deviations of the means. Inset: (a) LPO (1 μM) + hydrogen peroxide (0.2 mM); (b) LPO (1 μM) + hydrogen peroxide (0.2 mM) + resorcinol (8 μM); (c) LPO (1 μM) + hydrogen peroxide (0.2 mM) + resorcinol (8 μM) + iodide ion (5 mM); (d) LPO (1 μM) + hydrogen peroxide (0.2 mM) + resorcinol (8 μM) + pyrogallol (250 μM).

oxidation, or guaiacol oxidation; data not shown). However, only those compounds containing the *m*-dihydroxybenzene moiety caused significant enzyme inactivation. Substitution of the resorcinol moiety with iodine, hydroxyl, or alkyl groups did not greatly affect the inactivation, although complete substitution of the ring in 1,3,5-trihydroxy-2,4,6-triiodobenzene precluded inactivation. However, the potency for enzyme inactivation decreased as the number of iodine substituents increased (i.e., 4,6-diiodoresorcinol required a higher concentration than 2-iodoresorcinol for equivalent inactivation).

The inset to Figure 3 shows the kinetics for inactivation of LPO by resorcinol. The inactivation rates showed saturation with respect to resorcinol concentration. The steady-state kinetic treatment of Waley (1985) was used to determine the maximal rate of inactivation (k_i) and the apparent binding constant (K_i, analogous to a Michaelis constant; see Figure 3). In this treatment, half-times are determined experimentally for enzyme inactivation in the presence of inhibitor, such that the ratio of enzyme to inhibitor is constant. Plots, similar to that shown in the inset to Figure 3, were used to determine half-times, which were plotted as shown in Figure 3. The value for k_i is obtained from the slope of the line, and the ordinate intercept/slope gives K_i. These data and those for the other resorcinol derivatives (which showed similar kinetic behavior) are given in Table 2. Substitution of the resorcinol moiety with iodine, hydroxyl, or alkyl groups produced small changes in the kinetic constants.

Figure 4 shows the titration of LPO activity by addition of H_2O_2 in the presence of a high concentration of resorcinol. At low ratios of $[H_2O_2]/[LPO]$, inactivation was linearly related to the equivalents of hydrogen peroxide added and the enzyme was inactivated by more than 80% when the ratio reached to 20. This extrapolation suggests that approximately 20 equiv of hydrogen peroxide, corresponding to the number of

Table 2: Kinetic Constants for the Inactivation of LPO by Resorcinol Derivatives[a]

inhibitor	k_i (min^{-1})	K_i (μM)
resorcinol	1.77	3.48
hexylresorcinol	3.21	7.10
phloroglucinol	5.19	14.14
orcinol	1.73	11.34
2-iodoresorcinol	1.38	9.67
4,6-diiodoresorcinol	1.11	18.03

[a] LPO inactivation kinetics were plotted as shown in Figure 3, and kinetic constants were determined as described in Waley (1985).

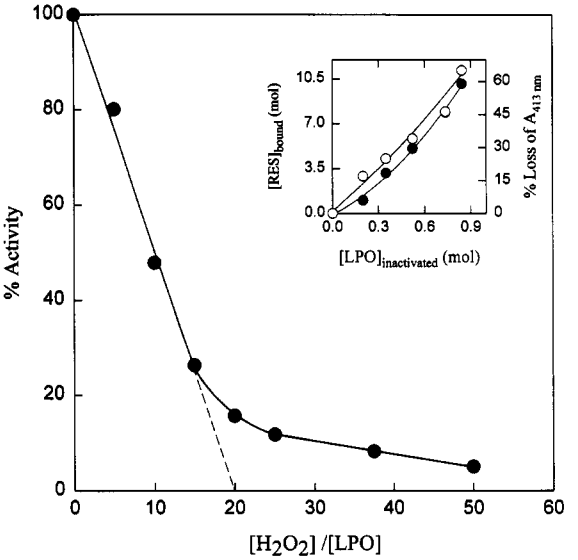


FIGURE 4: Partition ratio estimation. Correlation of covalent binding and the loss of Soret absorbance with the loss of enzyme activity. LPO (1.0 μM) was incubated with resorcinol (0.25 mM) in 0.1 mM phosphate buffer (pH 7.4) at 25 ± 0.1 °C, and the enzyme activity was titrated by the addition of varying but limiting concentrations of H_2O_2 (0–100 μM). After 2 min of incubation, the residual activity was measured by tyrosine iodination. The partition ratio was estimated by extrapolating the linear portion of the enzyme activity curve to the abscissa. Inset: At the above conditions, the decreases in absorbance by second-derivative scanning (○) and covalent binding of [^{14}C]resorcinol (●) were determined and plotted against the loss of enzyme activity.

enzymatic turnover cycles, are required for total inactivation of LPO. This analysis permits estimation of the partition ratio in the absence of other, more direct, data.

Incubation of LPO with [^{14}C]resorcinol resulted in the covalent binding of radioactivity that could not be dissociated by dialysis or chromatocentrifugation (see Table 1). Approximately 10 mol of resorcinol was bound covalently per mole of enzyme inactivated in the presence of excess hydrogen peroxide (see Figure 4, inset), and a strong correlation was seen between the loss of enzyme activity, the covalent binding of [^{14}C]resorcinol to LPO, the decrease in Soret absorbance and the amount of hydrogen peroxide added. The presence of iodide ion (0.1 or 5.0 mM) in the inactivation mixture did not prevent covalent binding, whereas the presence of pyrogallol (≥1.0 mM) prevented the binding almost completely. In fact, the presence of iodide ion caused an increase in [^{14}C]-resorcinol binding even when activity loss was prevented to a large degree (see Table 1). This is similar to the iodide ion-induced enhancement of the binding of benzimidazole-2-thiones to LPO in the absence of inactivation (Doerge *et al.*, 1993). In this case, the formation of reactive species (e.g., sulfenyl iodides) was possible, but the cause for additional resorcinol binding currently is not understood.

Table 3: *Inactivation of Various Peroxidases by Resorcinol*[a]

peroxidase	% activity remaining
LPO	18.2 ± 5.4
TPO	12.9 ± 3.4
MPO	95.1 ± 1.3
CPO	97.3 ● 2.8
HRP	98.6 ● 2.9
metMb	93.5 ± 1.9

[a] Peroxidases (1 μM, except TPO which was 0.1 μM) were incubated with resorcinol (250 μM for LPO and TPO and 500 μM for all others), and hydrogen peroxide was added (200 μM) at 25 °C in phosphate buffer (pH 7.4, 100 mM). After 3 min, the residual guaiacol oxidation activity was determined. The activity determined in the absence of resorcinol was taken as 100%.

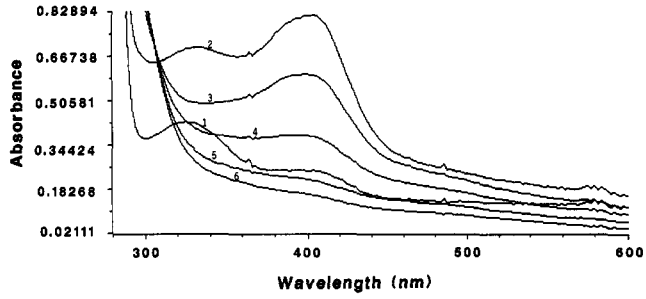


FIGURE 5: Repetitive scans showing the oxidation of resorcinol by HRP. Reaction was carried out in the presence of HRP (0.05 μM), H_2O_2 (0.2 mM), and resorcinol (1.0 mM) at 10 ± 0.1 °C in phosphate buffer (pH 7.4). The reagents were mixed using the stopped-flow apparatus, and scans were recorded immediately after mixing (scan 1 at ca. 50 ms) and at 3-s intervals (scans 2–6).

The substrate activity of resorcinol was investigated using LPO and HRP. HRP activity was unaffected by incubation with resorcinol and hydrogen peroxide under conditions that completely inactivated LPO and TPO (see Table 3). This finding was also observed for other peroxidases (e.g., MPO, CPO, and the pseudoperoxidase, ferric Mb). The addition of hydrogen peroxide to HRP and resorcinol caused rapid changes in the UV–visible spectrum consistent with enzyme-mediated oxidation and coupling of radical products to form colored species (see Figure 5). The scan taken immediately after the addition of hydrogen peroxide (mixing time ca. 20 ms) showed absorption maxima at 330 and 410 nm and a shoulder at 580 nm. In the next 3 s, the absorbance reached a maximum followed by a rapid decrease. The duration of the reaction was 20 s. Incubation of LPO with resorcinol and hydrogen peroxide produced spectral changes very similar to those described for HRP, although the magnitude was much smaller due to the rapid inactivation of LPO that occurred.

It was determined that the resorcinol oxidation products formed by HRP did not affect the activity of LPO. HRP (1 μM) was incubated with resorcinol (250 μM) and hydrogen peroxide (500 μM). After a 30-s or 10-min incubation, catalase was added, and the soluble oxidation products were separated from proteins by ultrafiltration. Addition of the fraction containing small molecules produced no inhibition of LPO activity.

The inactivation of LPO by resorcinol was dependent on pH as shown in Figure 6. The initial rates for inactivation increased as the pH was decreased, even when all of the other conditions were kept constant. The rate of LPO-catalyzed guaiacol oxidation decreased from 0.045 AU/min at pH 8 to 0.015 at pH 4.1 (data not shown).

DISCUSSION

The data presented here are consistent with mechanism-based inactivation of LPO and TPO by resorcinol derivatives.

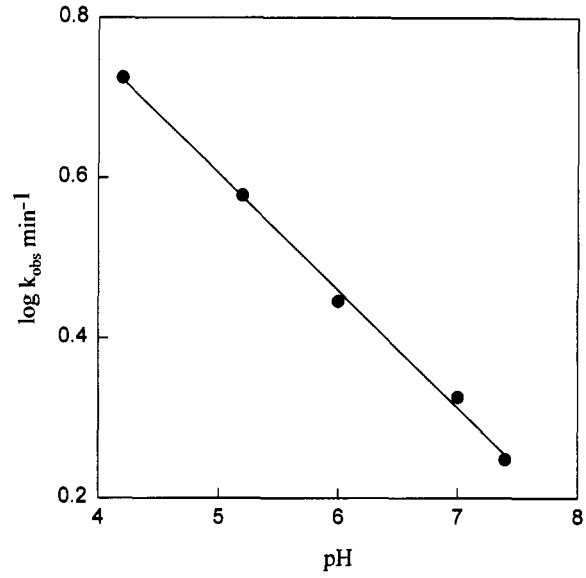


FIGURE 6: Dependence of LPO inactivation by resorcinol on pH. The reaction mixture contained 0.1 M acetate or phosphate buffer at the indicated pH, 1.0 μM LPO, 8.0 μM resorcinol, and 0.2 mM H_2O_2. The incubations were carried out at 25.0 ± 0.1 °C, and LPO activity was measured using the tyrosine iodination assay described in the Materials and Methods section. The initial rate (k_{obs}) of LPO inactivation was determined at each pH from the slope of the residual activity vs incubation time. Data are the averages of at least two experiments each.

In this mechanism, peroxidase-mediated oxidation (requiring hydrogen peroxide) of resorcinol produces a reactive species that covalently binds at or near the heme-containing active site to irreversibly block enzymatic activity. The data in Table 1 show that the inactivation is diminished in the presence of alternate substrates (5 mM iodide ion and 0.25 and 1 mM pyrogallol) that compete for the hydrogen peroxide-oxidized enzyme intermediates (compounds I and II). Irreversible changes in the UV–visible spectrum of the prosthetic heme (see Figure 1) correlate well with the degree of enzyme inactivation, as well as the covalent binding of radiolabeled resorcinol (see Figure 4). Under certain conditions, both iodide ion and pyrogallol can act as alternate substrates, but pyrogallol was more effective in protecting against inactivation and covalent binding by resorcinol. This suggests that scavenging of reactive intermediates by pyrogallol may also be involved. The role of iodide ion in enhancing LPO inactivation and [^{14}C]resorcinol binding currently is not understood.

Approximately 10 mol of resorcinol is bound per mole of LPO inactivated. This suggests that although dramatic changes are observed in the visible spectrum of resorcinol-inactivated LPO, the porphyrin ring is not the exclusive target for binding. Previous studies with methimazole and related suicide substrates for LPO and TPO showed that those inhibitors, which also effect dramatic changes in the visible spectrum of LPO, bind to the heme in a 1:1 stoichiometry (Doerge, 1986). This is in contrast with the action of 3-amino-1,2,4-triazole (amitrole), which binds to the protein moiety of LPO in a stoichiometry of ca. 7:1 (Doerge & Niemczura, 1989). This binding correlated with the loss of two histidine and five tyrosine residues in amitrole-inactivated LPO (Chang & Schroeder, 1973). However, the changes in the visible spectrum of resorcinol-inactivated LPO are greater than those for amitrole. At the present time, therefore, it is not possible to state, with certainty, the locus of resorcinol binding in LPO. Preliminary experiments showed similar changes in the heme spectrum of CcP concomitant with resorcinol inactivation, and in this case it could be shown that covalent binding

predominantly occurred to the dissociated apoprotein (D. R. Doerge and R. L. Divi, unpublished results). This experiment is not possible with LPO because of the covalent attachment of the heme to the protein (Nichol *et al.*, 1987).

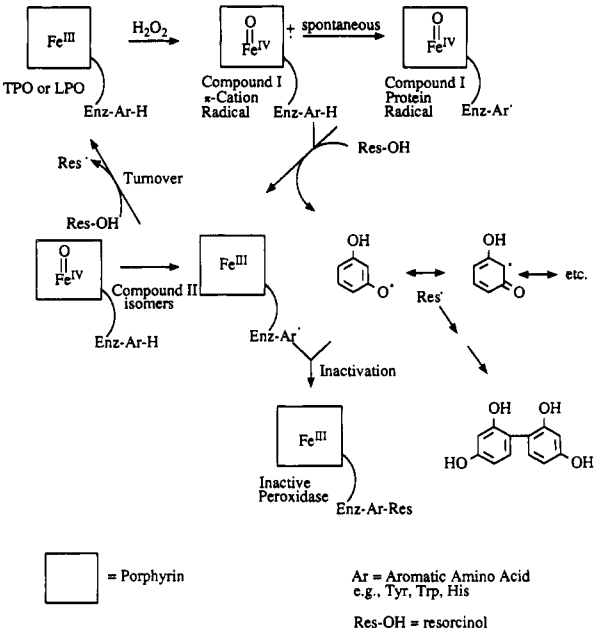
The kinetics of LPO inactivation by resorcinol and its derivatives were also consistent with a suicide mechanism (Waley, 1985). The rapid, time-dependent loss of activity in the presence of both resorcinol and hydrogen peroxide showed saturation kinetics with respect to resorcinol concentration (see Figure 2). Resorcinol and its derivatives were potent (K_i = 3–18 μM), and maximal inactivation rate constants (extrapolated to infinite inhibitor concentration) were similar for all analogs tested (see Table 2). The partition ratio, the ratio between inhibitor turnover and enzyme inactivation rate constants, was estimated to be 20 in the presence of excess resorcinol (see Figure 4). This is greater than those seen for methimazole and model compounds (2.5–5; Doerge, 1986, 1988), but less than that for amitrole (33; Doerge & Niemczura, 1989).

The substrate activity of resorcinol was suggested by the formation of oxidized products formed upon incubation of resorcinol with LPO in the presence of hydrogen peroxide (see Figure 5). The formation of such products concomitant with enzyme inactivation is consistent with a mechanism of suicide inactivation. The substrate activity of resorcinol was confirmed by using HRP, which was found to be insensitive to inactivation by resorcinol (see Table 3). Figure 5 shows the formation and reaction of a primary oxidation product from the action of HRP/hydrogen peroxide on resorcinol. These spectra are consistent with those described for the one-electron oxidation of resorcinol by ferricyanide ion that resulted in the formation of a dimeric coupling product (Bhattacharjee & Mahanti, 1983). It was also determined that incubation of LPO and TPO with resorcinol oxidation products produced in high levels by the incubation of resorcinol with HRP did not cause a loss of activity.

Other peroxidases tested were also insensitive to inactivation by resorcinol derivatives (see Table 3). The activity of CPO, MPO, and the pseudoperoxidase, ferric Mb, was unaffected by preincubation under conditions that produced significant inactivation of LPO and TPO. In all cases, resorcinol did *inhibit* the peroxidase-mediated oxidation of guaiacol, suggesting alternate substrate activity and, therefore, no restriction for entry into the active site. These findings, and the well-established radical mechanism for phenolic oxidation by peroxidases (Sakurada *et al.*, 1990), suggest that formation of the resorcinol radical at the active sites of LPO and TPO led to covalent binding to targets unique to these two peroxidases.

LPO and TPO differ from the classical peroxidase mechanism based on HRP in that the catalytic mechanism involves spontaneous isomerization of the initially formed oxoferryl π-cation radical form of compound I to an oxoferryl protein radical form of the compound analogous to CcP compound ES (Erman *et al.*, 1989). As is the case for CcP compound ES, the protein radical form of LPO (and TPO) is stable (Deme *et al.*, 1985). It has been proposed that aromatic amino acid residues in LPO/TPO are involved by analogy with CcP (tryptophan 191; Erman *et al.*, 1989), or a tyrosine residue in ferryl myoglobin (Davies, 1990) or PGHS (Smith *et al.*, 1992). We propose that the translocation of oxidizing equivalents onto aromatic amino acid residues makes this the target for covalent binding of resorcinol radicals at the active sites of LPO and TPO (see Scheme 1). The protein radical form of ferryl myoglobin (Davies, 1990) and that inferred for

Scheme 1: Proposed Mechanism for Inactivation of TPO/LPO by Resorcinol



MPO (Harrison *et al.*, 1980) are not inactivated by resorcinol (see Table 3). This suggests that specific structural features of LPO and TPO confer sensitivity to resorcinol and other inactivating species.

Scheme 1 shows the reactions proposed: hydrogen peroxide converts LPO/TPO into the π-cation radical compound I, which can react directly with resorcinol or undergo spontaneous isomerization to the protein radical form of compound I, depending on the relative rates for the two reactions. Oxidation of resorcinol by either form of compound I puts the resorcinol radical and compound II, the one-electron-oxidized form, together in the active site. Deme *et al.* (1985) showed that the oxoferryl and ferric/protein radical forms of compound II exist. Furthermore, the data of Deme *et al.* (1985) are consistent with the reduction of the oxoferryl heme in both compound I forms at high and low pH. At pH 4.2, 1 equiv of ferrocyanide ion completely eliminated the absorbance due to the oxoferryl heme at 430 nm. At pH 7.4, only partial reduction occurred (ca. 25%). Deme *et al.* (1985) also showed that the oxoferryl form of compound II could be converted to the protein radical form by lowering the pH. The reverse reaction, i.e., conversion of the protein radical to the oxoferryl form by raising the pH, did not occur. These observations are consistent with the presence of both forms of compound II at neutral pH (see Scheme 1). We propose that the juxtaposition of the radical pair leads to the coupling of resorcinol and the amino acid residues containing unpaired electron spin density. This results in the covalent binding, irreversible inactivation, and spectral shifts observed. The dramatic changes observed in the visible spectrum suggest that radical addition both to amino acid residues and to the heme could occur.

The mechanism proposed in Scheme 1 predicts that conditions which favor the ferric/radical form of compound II should enhance inactivation by resorcinol. Resorcinol-mediated inactivation (Figure 6) increases as pH decreases, and this is not related to a decreased substrate ability since the rate of guaiacol oxidation (a prototype phenolic substrate) actually decreased over this pH range. Furthermore, the pK_{a1} value for resorcinol is 9.81, suggesting that changes in its ionization do not affect the reaction in this pH range. This suggests that it is not an increase in substrate activity that is

responsible for the enhancement of resorcinol-mediated inactivation at low pH.

It was previously noted that resorcinol and phloroglucinol were much more potent inhibitors of LPO and TPO activity than other mono- and dihydroxybenzene derivatives (Taurog *et al.*, 1992). This is consistent with the structure–inactivation relationship shown in Figure 2. These data emphasize the requirement for the *m*-dihydroxybenzene moiety with at least one unsubstituted carbon center on the ring. It should be noted that other dihydric phenols (e.g., catechol and hydroquinone), as well as monophenolics, all *inhibit* peroxidase-catalyzed reactions by acting as alternate substrates. This is consistent with the substrate activity of the dihydric phenols with HRP/hydrogen peroxide that correlates with the respective oxidation potential (Sakurada *et al.*, 1990). The resorcinol radical differs from the analogous species derived from catechol and hydroquinone in that stabilization of the radical center by interaction with the molecular orbitals on the other oxygen is not possible due to the meta, rather than ortho or para, orientation. The resulting destabilization makes the resorcinol radical a more reactive intermediate and more likely to react at the enzyme active site with an appropriate partner. The low-level, but consistent, inactivation seen with all phenols is consistent with this hypothesis.

This study clarifies the potent inhibitory action of resorcinol on LPO and TPO, as well as the goitrogenic response in humans and laboratory animals from exposure to environmental compounds containing the resorcinol moiety. The significance of the widespread presence of resorcinol derivatives in the environment is underscored by the resorcinol moiety contained in many flavonoids, important natural products present at high levels in many foods of plant origin (Pierpoint, 1986; Singleton, 1981). The importance of dietary flavonoids (e.g., myricetin, catechin, and quercetin) in the etiology of environmental thyroid disease is suggested by the resistance of endemic goiter to the universal iodination of salt in some parts of India. In these areas, the principal foodstuffs are grains that contain high levels of flavonoids found to inhibit TPO and other thyroid-specific processes important to biosynthesis of thyroid hormones (R. L. Divi, unpublished results).

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