On the Molecular Mechanism of Lactoperoxidase-catalyzed H2O2 Metabolism and Irreversible Enzyme Inactivation*

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Lactoperoxidase-catalyzed H2O2 metabolism proceeds through one of three different pathways, depending on the nature and the concentration of the second substrate as an e-donor and/or on pH conditions. In the lactoperoxidase (LPO)-H2O2 system, at low H2O2 concentrations and/or alkaline conditions the peroxidatic cycle involves ferric LPO → compound I → compound II → ferric LPO conversion, whereas high H2O2 concentrations and/or acidic conditions favor the ferric LPO → compound I → compound II → compound III → ferrous LPO → ferric LPO pathway. The compound III/ferroperoxidase states are associated with irreversible enzyme inactivation by cleavage of the heme moiety and liberation of iron. It is likely that either singlet oxygen or superoxide and hydroxyl radicals are involved in the attack on heme iron, because inactivation correlates with oxygen production and can be decreased to a certain degree by scavengers such as ethanol, 1-propanol, 2-propanol, or mannitol. In the LPO-H2O2-1 system, the enzyme may also be inactivated by I2 generated in the course of enzymatic I- oxidation (i.e., during ferric LPO → compound I → ferric LPO cycles).

The peroxidatic cycle and the intermediate enzyme compounds (Reactions 1–3) were originally described for horseradish peroxidase, a plant heme enzyme from the roots of Armoracia lapathifolia Gilib. (A. rusticana G.M.Sch., Cochlearia Armoracia L.) of the family Cruciferae (reviewed in Ref. 1). Reactions 1–3 demonstrate that compound (Cpd) I is 2 eq and compound II 1 eq more oxidized than the resting enzyme.

\[
\text{peroxidase} + \text{H}_2\text{O}_2 \rightarrow \text{Cpd I} + \text{OH}^- \tag{1}
\]

\[
\text{Cpd I} + \text{AH} \rightarrow \text{Cpd II} + \text{A}^- \tag{2}
\]

\[
\text{Cpd II} + \text{AH} \rightarrow \text{peroxidase} + \text{A}^- + \text{H}_2\text{O}^+ \tag{3}
\]

where AH is the second substrate which acts as an e-donor in the mechanism.

Various suggestions have been made on the molecular mechanism and the structures of the catalytic intermediate compounds I and II (reviewed in Refs. 1–4). Horseradish peroxidase is a well studied example for peroxidases because of its stability, its ease of isolation in large amounts, and its availability from commercial suppliers. Newer models for horseradish peroxidase compounds I and II are based either on experimental data (including spectra of electron nuclear double resonance (5, 6), electron paramagnetic resonance, Mössbauer (7–10), high-field proton nuclear magnetic resonance (11–13), resonance Raman (14–19), and x-ray absorption experiments (20, 21)) or on quantum mechanical calculations (including extended Hückel calculations (22–25), unrestricted Hartree-Fock studies at the INDO (intermediate neglect of differential overlap) (26) and \textit{ab initio} (27), and Xa multiple scattering calculations (28)). Although the results of experiments and those of calculations on various model compounds do not always correlate, they all support a low-spin ferryl moiety (FeO3+) for compounds I and II.

The resting enzyme contains a pentacoordinated high-spin heme at neutral pH and a six-coordinated low-spin heme at alkaline pH (15). Compound I has been assigned almost unanimously with a 2Aδs cation radical state (5–12) or a mixture of 2Aδs and 2Aδd electronic states (13). It has an oxyferryl center (Fe(IV)=O) with an axial symmetry. A significant mixing (spin coupling) between the FeO (S = 1) and the porphyrin spin (S = ½) systems, however, complicates the pure 2Aδs cation radical state (28). The iron-oxygen bonding of compound II is still under discussion. It has been suggested that this enzyme intermediate contains a Fe(IV)=O structure like compound I, but has no radical state any more (16, 18). Alternatively, due to the uptake of a proton and an electron during the transformation from compound I (Reaction 2), compound II has also been proposed to contain a Fe(IV)=OH structure (29). The presence of an iron-oxygen single bond is supported by x-ray absorption studies, which show a longer iron-oxygen distance than found in compounds I and III (20, 21). This data seems to be more reliable than the results from resonance Raman experiments which suffer from potential photoreduction on laser irradiation at certain wavelengths (457.9 and 406.7 nm) resulting in a mixture of ferric and ferrous peroxidase (19, 30).

So far, compound III has been of less interest due to the fact that it does not take part in the peroxidatic cycle. Recently, however, it has been identified as an oxymhe adduct from its diamagnetism and its Mössbauer parameters (9), confirming its formation from native (ferric) peroxidase with superoxide anion O2-, from reduced (ferrous) peroxidase with O2, or from compound II with excess H2O2 (reviewed in Ref. 1). X-ray absorption studies (21) have demonstrated an increase in charge density on iron of both compound III and the reduced enzyme, compared to a decrease in compounds I and II, relative to the resting enzyme. This suggests a ferrous state for compound III and, therefore, favors the ferroperoxidase-oxygen assignment for compound III with the dioxygen now bound to the iron.

Work on lactoperoxidase (LPO), a bovine milk peroxidase performed in our laboratory so far (31–33) has revealed com-
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parable catalytic behaviors for LPO and horseradish peroxidase (reviewed for horseradish peroxidase in Ref. 1). In addition, similarities between LPO and thyroid peroxidase have been reported and ferritoporphyrin IX (protoheme IX) has been identified as a common prosthetic group to all three peroxidases (34–38). Consequently, we have used LPO as a model enzyme in all experiments. The link between the peroxidatic cycles in the LPO-$H_2O_2$ system (Reactions 1–3), peroxidase-catalyzed I$^-$ oxidation in the LPO-$H_2O_2$I$^-$ system (Reactions 4 and 5), and irreversible enzyme inactivation in both systems (31–33) still remains to be demonstrated.

**Experimental Procedures**

**Materials**—All chemicals were of analytical grade and purchased from Merck (Darmstadt, Federal Republic of Germany) or Fluka (Buchs, Switzerland) except for hydrogen peroxide ($H_2O_2$, obtained from Siegfried, Zofingen, Switzerland) and LPO (EC 1.11.1.7, 70 units/mg solid, ratio $A_{243}/A_{280} = 0.85$, tLPO,412 nm = 112,300 M$^{-1}$ cm$^{-1}$ (38), obtained from Sigma).

**UV-visible Spectroscopy of LPO**—Spectral properties of LPO upon addition of $H_2O_2$ or $Na_2S_2O_4$ in 0.05 M phosphate buffer at a range of pH were monitored by recording UV-visible absorption in the Soret region (350–450 nm) on a UVIKON model 810 spectrophotometer combined with a UVIKON 21 recorder using serial overlay in 0.5- or 1-min intervals, or on a Hewlett-Packard 8451A diode array spectrophotometer in 0.2- or 0.5-s cycles. Due to its molar absorption coefficient (cf. “Materials”) LPO was used at a concentration of 3 $\mu$M.

**Determination of the $H_2O_2$ Concentration at Various Time Intervals in the LPO-$H_2O_2$ System**—While recording the spectral changes of LPO on addition of $H_2O_2$ as described above, aliquots were taken from the cuvette and immediately introduced into a quartz cuvette containing a solution of 0.1 M I$^-$ and an excess of LPO (50 nM) in 0.05 M phosphate buffer, pH 7.35, to ensure rapid and quantitative utilization of $H_2O_2$ for generation of I$^+$. The absorption of I$^+$ was measured at 353 nm in a Beckman model 25 spectrophotometer, and its concentration was calculated using $A_{353 nm} = 26,900$ M$^{-1}$ cm$^{-1}$ (31). From the stoichiometric ratio of 1 mol of I$^+$ generated per mol of $H_2O_2$, and taking into consideration the dilution of the aliquot, the time course of disappearance of $H_2O_2$ could be reconstructed.

**Kinetics of I$^-$ Oxidation in the LPO-$H_2O_2$I$^-$ System**—Time courses of I$^-$ oxidation with $H_2O_2$ catalyzed by LPO at a concentration of 1 and 2 mM in 0.05 M phosphate buffer at a range of pH were conducted. The assays were monitored by recording I$^+$ absorption on a UVIKON 21 recorder combined with a UVIKON model 810 spectrophotometer (I$^+$ assay), taking into account the equilibrium $I_2^+ + I^- \rightleftharpoons I_3^+$ (1) and the molar absorption coefficient of I$^+$ (see above) for calculations of total I$^-$ oxidized (I$^+$ + I$^-$) (31).

**O$_3$ Measurements in the LPO-$H_2O_2$ and LPO-$H_2O_2$I$^-$ Systems**—Simultaneous measurements of O$_3$, I$^-$, and OH$^-$ (in unbuffered medium) as reaction products according to Reactions 4 and 5, or of O$_3$ and spectral changes of LPO, were performed on an Amino DW-2a TM UV-visible spectrophotometer equipped with a special cuvette mixer. In addition to spectral recordings, O$_3$ concentrations could be measured simultaneously by a Clark electrode (39, 40) fitted to the cuvette from the side. Furthermore, the reaction vessel could be equipped with a thin glass pH electrode through the cover. Reactions were started by adding $H_2O_2$ with a syringe to the N$_2$-saturated medium through narrow slots in the lids of the spectrophotometer and cuvette.

**Statistical Calculations**—Irreversible LPO inactivation as a function of excess $H_2O_2$ and the effects of I$^-$ addition were assessed by regression analysis. Correlation coefficient $r$, intercept $a$, and slope $b$ were calculated for linear ($y = a + bx$), exponential ($y = ae^{bx}$ or In y = ln a + b ln x) regression using a Casio FX-502 P program calculator and the Casio FX-601 P/FX-502 P program library.

**Results**

**LPO-$H_2O_2$ System**—Whereas photometric recordings of compound I require special techniques (e.g., stopped-flow etc.), compounds II and III can easily be detected by addition of a small excess of $H_2O_2$ to LPO (3 $\mu$m) in a buffered medium. Up to a concentration of 50 $\mu$M $H_2O_2$, compound II is the main detectable intermediate at pH 7.33 (Fig. 1A). Beyond this limit of $H_2O_2$, compound III is generated, which has a lifetime ranging from a few minutes up to several hours depending on the excess of $H_2O_2$ (Fig. 1B). The effect of changing pH on compound III formation is discussed elsewhere (42). Absorption maxima ($\lambda_{max}$) of the different LPO states are listed in Table I.

Compound II is directly and completely converted to the resting ferric enzyme, as indicated by an isosbestic point at 421 nm on the descending limb of the original recording of native LPO (Fig. 1A). In contrast, compound III has been shown to be metabolically involved in the reaction leading to irreversible inactivation of LPO (33). The loss of enzyme activity is indicated by the difference between original and final peak heights at 411 nm and by the position of the isosbestic point which no longer coincides with the descending limb of the original spectral recording of native LPO (Fig. 1B). Regression analysis has revealed that the recovery of LPO decreases with excess $H_2O_2$ (Fig. 2). Experimentally determined values closely fit to Equation 6 (correlation coefficient $r$ = 0.9934),

$$y = 100.87 e^{-0.27x} \quad y = \ln 100.87 - 0.27 x$$

where $x$ corresponds to the concentration of $H_2O_2$ (nm) and $y$ to the percentage LPO recovery.

Compound III metabolism is characterized by a red shift (higher wavelengths) prior to reconstitution of the resting enzyme, suggesting the formation of an intermediate compound such as compound II ($\lambda_{max} = 430$ nm) or the reduced ferrous state ($\lambda_{max} = 435$ nm) (Fig. 1, B and C). At the beginning of the red shift, when most of the $H_2O_2$ excess has been consumed, enzyme destruction ceases as suggested by the transient constant absorbance value. The remaining fraction of compound III is then reconverted to the resting ferric enzyme without any further loss, as indicated by the isosbestic point at 420 nm.

If only selected cycles during compound III conversion are plotted, the formation of an intermediate compound with $\lambda_{max}$ between two isosbestic points at 430 and at approximately 447 nm can be demonstrated, suggesting the conversion of compound III to ferrous LPO rather than to compound II (Fig. 1C). In addition, the isosbestic point at 430 nm is clearly different from the one formed at 426 nm during the conversion of compound II to compound III. In the visible range (Fig. 1D) the same assay demonstrates conversion of compound III to a state which absorbs at shorter wavelengths than the isosbestic point at 538 nm and the wavelength minimum around 560–570 nm. This intermediate compound is converted back to native LPO with an isosbestic point at 517 nm.

Reduction of LPO from the ferric to the ferrous form (Fig. 3, 1, 41) by addition of a few grams (approximately 5 mg) of $Na_2S_2O_4$ results in a labile compound with $\lambda_{max} = 444$ nm. This compound is converted within 7 min, in a pH-dependent manner (41), into a more stable compound with $\lambda_{max} = 435$ nm forming an isosbestic point at 439 nm (Fig. 3A). If smaller amounts (approximately 0.1 mg) of $Na_2S_2O_4$ are added, sur-
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FIG. 1. Spectra of compounds II and III of LPO and enzyme inactivation at physiological pH conditions. A, compound II ($\lambda_{\text{max}} = 430$ nm) is formed by addition of 30 $\mu$M H$_2$O$_2$ to 3 $\mu$M LPO ($\lambda_{\text{max}} = 411$ nm). Numbers on top of spectral recordings indicate minutes after addition of H$_2$O$_2$. An isosbestic point between the original spectral recording of native LPO (scan 0) and compound II is depicted at 421 nm. Recovery of enzyme is 100% (scan 60) considering dilution due to H$_2$O$_2$ addition.

B, compound III ($\lambda_{\text{max}} = 423$ nm) is formed 1 min following addition of 500 $\mu$M H$_2$O$_2$ to 3 $\mu$M LPO. The loss of enzyme is indicated by the difference of peak heights between scans 0 and 60 and from the isosbestic point which is no longer positioned on the descending limb of the original spectral recording of native LPO. C, identical with B, however, selecting certain cycles for spectral recordings. Scans 15–20 indicate a red shift and thus conversion to an intermediate compound. Isosbestic points are formed at 430 and around 447 nm. Scans 20–40 show the reconversion of the intermediate compound to native LPO forming an isosbestic point at 421 nm. The difference between the isosbestic points at 426 nm (conversion of compound II to compound III) and at 430 nm (reconversion of compound III to the resting ferric enzyme) suggests conversion of compound III to ferrous LPO rather than to compound II. D, identical with B and C, however, selected spectra recorded in the visible range. A part of compound III (scan 6) is converted to a state which absorbs between 520 and 530 nm and around the absorption minimum at about 570 nm. The spectra of cycles 6, 9, 12, and 15, which form an isosbestic point at 538 nm, can well be assigned to a mixture of compound III and ferrous LPO. This intermediate state is reconverted to the resting ferric enzyme forming an isosbestic point at 517 nm (cycles 20, 23, 28, 33, and 40).

**TABLE I**

Absorption maxima ($\lambda_{\text{max}}$ nm) and absorption coefficients ($\epsilon$, mM$^{-1}$ cm$^{-1}$, in parentheses) of the different LPO compounds at physiological pH

| State                  | $\lambda_{\text{max}}$ nm (\epsilon, mM$^{-1}$ cm$^{-1}$) | Soret  | Visible range |
|------------------------|----------------------------------------------------------|--------|---------------|
| Ferric LPO             | 411 (112.3) 496 (11.7) 540 (9.7) 583 (8.0) 628 (7.0)    | 410    | 562 600 662   |
| Compound I (54)        | 430 (86.4) 535 (13.5) 565 (12.3)                         |        |               |
| Compound II            | 430 (89.0) 549 (17.4) 588 (17.4)                         | 444    | 561 595       |
| Compound III (oxyperoxidase) | 435 (77.8) 526 (16.3) 595 (12.8) | 435 (94.6) 526 (10.0) 595 (17.3) 595 (9.5)  |
| Ferrous LPO unstable   |                                                        | 444    | 561 595       |
| Ferrous LPO stable     |                                                        | 435    | 526 595       |
| Irreversibly inactivated LPO | No peaks above 350 nm                                    |        |               |

Prisingly compound II is produced first (Fig. 3B, cycle 0.5). This is due to a catalytic interaction of LPO with H$_2$O$_2$ originating from a reaction between O$_2$ contained in the air-saturated solution and dithionite (39–41). This intermediate

is very short lived and is rapidly converted into ferrous enzyme and resting LPO (Fig. 3B, cycle 1). Thereafter, ferric LPO steadily decreases in favor of the reduced enzyme and compound III which reach a maximum at cycle 17. Reconversion of the two compounds to the resting ferric LPO is terminated at cycle 90 (Fig. 3C). Recovery (89%) is, unexpectedly, lower than that obtained by substitution of 260 $\mu$M H$_2$O$_2$ for dithionite (94%, Fig. 2). Close inspection of the spectral changes supports the already discussed possibility that native LPO is
reconverted from compound III via the ferrous state.

Assuming a catalatic degradation of H$_2$O$_2$ (33, 43), 1 mol of O$_2$ should be produced from 2 mol of H$_2$O$_2$ consumed. This is confirmed by measurements of H$_2$O$_2$ consumption and O$_2$ generation (Fig. 4). The results correspond very closely to the theoretical stoichiometric ratio up to a concentration of about 500 mM H$_2$O$_2$. Beyond this limit some O$_2$ is lost probably due to oversaturation of the medium and generation of O$_2$ bubbles (39, 40).

H$_2$O$_2$ consumption (Fig. 4, top) and O$_2$ production (Fig. 4, bottom) consist of two phases. The initial fast a-phase occurs before compound III formation, i.e., during compound II cycles, while the slow b-phase corresponds to the compound III state (first arrows on each curve of Fig. 4 indicate the maximal amount of compound III formed). O$_2$ production is terminated soon after the beginning of the red shift and during the reconversion of ferroperoxidase to the resting ferric enzyme (second and third arrows, respectively, on each curve of Fig. 4).

LPO-H$_2$O$_2$-I$^-$ and Related Systems—Addition of small amounts of a superior e$^-$ donor than H$_2$O$_2$, e.g., I$^-$ (5–30 mM), to fixed concentrations of LPO (3 μM) and H$_2$O$_2$ (10 mM) accelerates the metabolism of H$_2$O$_2$. In parallel with the consequently faster restitution of native LPO there is a positive correlation between increased recovery of native LPO and an increase in ln[I$^-$] in the incubation medium (Fig. 5 and Equation 7, correlation coefficient r = 0.97),

$$y = -41.77 + 30.56 \ln x$$

(7)

where x corresponds to the concentration of I$^-$ (μM) and y to the percentage of LPO recovery.

Above 30 μM I$^-$ the system displays oscillations (1) which are presently under investigation in our laboratories. An accelerated turnover and an increase in enzyme recovery can also be obtained with addition of small amounts of ethanol, 1-propanol, 2-propanol, or mannitol (mM concentrations) instead of I$^-$. However, a OH$^-$-producing system forces the enzyme to remain in the compound III state until no absorption in the Soret region is left. Such a OH$^-$-producing system arises as a result of the interaction of EDTA (2–50 mM) with the iron released during irreversible inactivation of LPO. The EDTA-iron complex so formed reacts with the H$_2$O$_2$ present in the incubation mixture in excess, producing free OH$^-$ (33, 34). Addition of alcohols to this EDTA-containing system is not able to free the enzyme from compound III and only accelerates this inactivation process (results not shown).

I$^-$ oxidation and O$_2$ generation as a function of variable concentrations of LPO (1 or 2 mM) and substrate have been measured simultaneously (Fig. 6). Whereas the time course of net ln[I$^-$]/I$^-$ production is the result of I$^-$ oxidation (Reaction 4) and simultaneously occurring I$_2$ reduction (Equation 5, pseudo-catalatic H$_2$O$_2$ degradation (31)), O$_2$, when generated,
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**FIG. 4.** Consumption of H$_2$O$_2$, generation of O$_2$, and formation of intermediate compounds at physiological pH conditions. Top, degradation of H$_2$O$_2$; bottom, generation of O$_2$. Arrows on top of each curve indicate from left to right: most of the enzyme is in the state of compound III (first arrow); beginning of the red shift with formation of ferrous LPO (second arrow), and beginning of reconversion of ferrous LDO to the resting ferric state (third arrow). H$_2$O$_2$ degradation and O$_2$ generation are characterized by a fast α-phase and a slow β-phase. O$_2$ measurement of the 1 mM H$_2$O$_2$ sample does not include the part which is lost due to oversaturation of the medium and generation of O$_2$ bubbles.

**FIG. 5.** Recovery of LPO at pH 7.33 in the presence of I$^-$. ▲, experimentally determined values; ----, theoretical regression analysis. Increasing amounts of I$^-$ (5–30 μM) added to an incubation medium containing LPO (3 μM) and H$_2$O$_2$ (10 nM) partially protects the enzyme from rapid inactivation. LPO recovery can be described by a logarithmic function, y = -41.77 + 36.56 ln x, where x = I$^-$ concentration (μM) and y = percent of LPO recovery. Correlation coefficient, 0.97.

is not subsequently consumed in the reactions discussed in Fig. 6.

Increasing LPO from 1 to 2 nM at fixed H$_2$O$_2$ (500 μM) and I$^-$ (10 nM) concentrations (Fig. 6A) accelerates the reaction rates of I$^-$ oxidation and O$_2$ generation. Simultaneously, net production of I$_2$/I$_3$ increases, but no change in the final O$_2$ concentration can be observed.

When LPO (2 nM) and I$^-$ (10 mM) are kept constant and H$_2$O$_2$ is raised from 100 μM up to 500 μM (Fig. 6B), I$_2$/I$_3$ and O$_2$ generation rates increase. An excess of H$_2$O$_2$ leads to irreversible inactivation of LPO is previously reported (33).

This in turn leads to a higher nonproductive H$_2$O$_2$ consumption and O$_2$ generation due to the now prevailing I$^-$ reduction, whereas the amount of I$_2$/I$_3$ is decreased as a result of this I$^-$ withdrawal.

If LPO (2 nM) and H$_2$O$_2$ (500 μM) are kept constant and the concentration of I$^-$ is raised from 1 mM up to 100 mM (Fig. 6C), both the rate and the amount of I$_2$/I$_3$ production increase. I$^-$ reduction is suppressed at high I$^-$ concentrations due to a shift in the equilibrium between I$_2$ and I$^-$. Therefore, rate and amount of O$_2$ generation are significantly diminished. I$^-$ oxidation and O$_2$ production at fixed amounts of LPO (2 nM), H$_2$O$_2$ (300 μM), and I$^-$ (10 mM) are strongly influenced by changes of pH (Fig. 6D). Whereas net I$_2$/I$_3$ generation is drastically reduced upon an increase of pH from 6.6 to 7.6, amount and rate of O$_2$ generation are favored at alkaline pH conditions according to Reactions 4 and 5. No enzymatic I$^-$ oxidation occurs upon addition of 50 μM H$_2$O$_2$ to a nonbuffered solution containing LPO (1 nM) and I$^-$ (10 mM) below pH 4.0 and above pH 10.2. Between these limits of pH, catalysis begins at rates which are comparable to those in buffered media, but the reaction breaks down independent of the H$_2$O$_2$ concentration after having produced a certain amount of OH$^-$. The pH at which the reaction breaks down depends on the concentration of I$^-$ as I$^-$ increases, so does the amount of OH$^-$ formed, resulting in a shift of pH as predicted by Reactions 4 and 5 and by the shift in the equilibrium between I$_2$ and I$^-$. (see “Experimental Proce-
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**Discussion**

Peroxidation of \( \text{H}_2\text{O}_2 \) by LPO can occur through one of three cycles depending on the nature of the second substrate as an \( e^- \) donor (Reactions 1–3, Fig. 7): (i) (ferric) LPO → compound I → LPO (48); (ii) LPO → compound I → compound II (see the introduction); and (iii) LPO → compound I → compound II → compound III → ferrous LPO → LPO (this work). The latter pathway occurs only when \( \text{H}_2\text{O}_2 \) in excess is added to LPO. The degree of irreversible enzyme inactivation as a result of activation of this pathway depends in turn on the relationship of \( \text{H}_2\text{O}_2 \) excess and pH ((33, 42); Fig. 1, B–D; Fig. 2). Under alkaline conditions compound III can barely be detected, whereas at an acidic pH this state is more stable and detectable.

Compound I formation, i.e. the initial reaction of LPO with \( \text{H}_2\text{O}_2 \), includes an acid-base catalysis of amino acid residues close to the active site on the enzyme (e.g. Asp-43 and Arg-38 in the case of horseradish peroxidase (44)), with the release of \( \text{OH}^- \) and an \( e^- \) transfer from the porphyrin ring to iron (44–47). Restitution of the resting ferric enzyme can be achieved by either a single \( 2e^- \) transfer (e.g. from \( \text{I}^- \) (48) or by two \( 1e^- \) transfers via compound II (Reactions 1–3). In the absence of \( \text{I}^- \) and at low \( \text{H}_2\text{O}_2 \) concentrations compound II is the main detectable intermediate, suggesting a \( 1e^- \) transfer from compound I to compound II (Fig. 1A). The \( e^- \) could either be donated from the apoprotein or from \( \text{H}_2\text{O}_2 \). In the latter case superoxide radicals (\( \text{HO}_2^- \)) are generated as intermediates of the first \( 1e^- \) transfer and \( \text{O}_2^- \) as final product (Fig. 7). Apart from \( \text{O}_2^- \), \( \text{HO}^- \) is also released on the recombination of compound II to LPO (29), originating from an oxygen atom and a \( \text{H}^+ \) from the first step of the cycle plus two \( \text{H}^+ \) from the pathway compound I → compound II → LPO. In this way, \( \text{HO}^- \) from the initial phase is neutralized, and thus the same reaction performed in water instead of buffer solution does not break down as occurs in the presence of \( \text{I}^- \). These reactions are in line with mechanisms already proposed in the literature (21, 29, 45, 49).

Compound III can be formed by excess \( \text{H}_2\text{O}_2 \) reacting with compound II (Figs. 1B and 7) and leading to a ferrylperoxi-

dase-hydrogen peroxide adduct, LPO[Fe(IV)–\( \text{H}_2\text{O}_2 \)]. Since there is an isosbestic point at 426 nm, the formation pathway via compound II dominates the alternative direct production of compound III starting from native LPO and \( \text{HO}_2^- \) and leading to a ferriperoxidase-superoxide adduct, LPO[Fe(III)–\( \text{HO}_2^- \)]. Two sets of data would suggest molecular rearrangements of LPO[Fe(IV)–\( \text{H}_2\text{O}_2 \), resulting in the isoelectric ferriperoxidase-oxygen compound, LPO[Fe(II)–\( \text{O}_2 \), as an intermediate. First, such an intermediate has been identified by its diamagnetism, Mössbauer, and x-ray absorption spectra (see the introduction). Second, from the results of this study, the isosbestic point displayed at 430 nm (Fig. 1C) between compound III and the following intermediate state clearly differs from the one at 426 nm during the conversion from compound II to compound III. This intermediate state then releases \( \text{O}_2 \) (Figs. 4 and 6) and displays the easily overlooked ferrous state in the spectrum before it is reconverted to the resting ferric enzyme (Fig. 1, C and D). These pathways are in line with the observed stoichiometry of one \( \text{O}_2 \) generated per two \( \text{H}_2\text{O}_2 \) consumed (Fig. 4, taking into account the loss of \( \text{O}_2 \) bubbles when the solution is saturated).

Irreversible LPO inactivation depends on the presence of excess \( \text{H}_2\text{O}_2 \) (Figs. 1B, 2, and 6B). In the absence of \( \text{I}^- \), this occurs during compound III formation and before establishment of isosbestic points, which indicate conversion to another state without any further loss of enzyme (Fig. 1, B–D (33)). LPO is not able to escape from inactivation by supply of superoxide dismutase, but reconversion of compound III/ferriperoxidase to native LPO is gradually slowed down indicating that \( \text{HO}_2^- \) is not the inactivating species (42). This correlates with the absence of inactivation during LPO → compound I → compound II → LPO cycles (see above and Fig. 1A).

It is reasonable to compare irreversible inactivation of LPO, which includes cleavage of the heme moiety and liberation of iron (33), with the oxygenation of hemin at one of its \( \alpha \)-methene bridges and subsequent ring opening to form verdoglin and biliverdin as primary steps in heme destruction. \( \text{HO}_2^- \) itself does not attack the autoxidation-sensitive porphyrin plane of hemin (50). However, \( \text{HO}_2^- \) is involved in the Haber-Weiss reaction (Reaction 8, \( \text{pK}_a \text{HO}_2^- = 4.8 \) (50)), which in fact consists of a chain mechanism with \( \text{Fe}^{4+} \), \( \text{OH}^- \), and \( \text{HO}_2^- \) as chain carriers and in which \( \text{HO}_2^- \), and not \( \text{HO}_2^- \), is capable of attacking \( \text{H}_2\text{O}_2 \) (Reactions 9–14 (44); Reaction 10 is the Fenton reaction).

\[ \text{HO}_2^- (or \text{O}_2^-) + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{H}_2\text{O} (or \text{OH}^-) + \text{O}_2 \]  

Initiation: \( \text{Fe}^{4+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}_2^- + \text{H}^+ \)  

Propagation: \( \text{Fe}^{4+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \)  

\[ \text{OH}^- + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{HO}_2^- \]  

Termination: \( \text{HO}_2^- + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{O}_2 + \text{H}^+ \)  

\[ \text{OH}^- + \text{Fe}^{3+} \rightarrow \text{OH}^- + \text{Fe}^{2+} \]  

Oxygenation of the porphyrin ring can be assigned to \( \text{OH}^- \) and/or singlet oxygen (\( \Delta \text{G}_{\text{OOH}} = 0 \)) attack. An oxygenating species has also been detected in the course of destruction of cytochrome \( c \) by excess \( \text{H}_2\text{O}_2 \) (51).

The fact that LPO inactivation can either be decreased by alcohols or \( \text{I}^- \) (Fig. 5) in a concentration-dependent manner or proceeds until the enzyme is fully destroyed as soon as

**Fig. 7.** Pathways in LPO-catalyzed \( \text{H}_2\text{O}_2 \) metabolism. \( \text{I}^- \) reacts with compound I by direct \( 2e^- \) transfer (48). The normal peroxidatic cycle includes ferric LPO → compound I → compound II → ferric LPO (see the introduction). \( \text{H}_2\text{O}_2 \) in excess leads to formation of compound III and recombination to the resting enzyme via the ferrous state (this work). The compound III pathway is combined with irreversible inactivation of LPO.
EDTA is added to the incubation medium (results not shown) supports the view that O₂ may be the most damaging species acting on LPO. On the other hand, ¹O₂ is able to oxidize many diamagnetic bio-organic molecules (33, 50–52). Whereas O₂ production in the LPO-H₂O₂ system can be attributed to the catalytic activity of the enzyme (33, 43), O₂ generation in the PO-H₂O₂-I⁻ system occurs due to chemical I₂ reduction (Figs. 4 and 6). Although there is a positive correlation between O₂ production and irreversible LPO inactivation (Figs. 4 and 6), the involvement of ¹O₂ in the destruction of heme cannot be proven. O₂ measured in our systems (Fig. 6) may be generated as a result of enzyme inactivation but is not necessarily the cause of it. I⁻ present in large amounts, or at least additional ¹O₂ quenchers such as 1- or 3-methylhistidine, is supposed to quench ¹Ag O₂ readily (52), mainly when it is produced in a purely chemical reaction remote from the active site of the enzyme (i.e., in the LPO-H₂O₂-I⁻ system, Fig. 6, A–D). However, irreversible inactivation of LPO occurs nevertheless, except for cases where O₂ production is low (e.g., in the “compound II” way, Fig. 7).

Despite the positive correlation between O₂ production and irreversible enzyme destruction, we have also to consider the possibility that LPO could be inactivated during enzymatic I⁻ oxidation. Incubation of LPO (5 μM) in 1 mM aqueous I₂ and removal of excess I₂ by lyophilization indeed reduces enzyme activity. Excess H₂O₂ could well inactivate LPO via the equilibrium between

\[
\text{LPO} + \text{H}_2\text{O}_2 \rightarrow \text{LPO}{-}\text{H}_2\text{O}_2
\]

and removal of excessive irreversible enzyme destruction, we have also to consider the possibility that LPO could be inactivated during enzymatic I⁻ oxidation. Incubation of LPO (5 μM) in 1 mM aqueous I₂ and removal of excess I₂ by lyophilization indeed reduces enzyme activity. Excess H₂O₂ could well inactivate LPO via the equilibrium between

\[
\text{LPO} + \text{H}_2\text{O}_2 \rightarrow \text{LPO}{-}\text{H}_2\text{O}_2
\]

and removal of excessive

\[
\text{O}_2 + \text{LPO} \rightarrow \text{LPO}{-}\text{O}_2
\]

site of the enzyme

\[
\text{LPO} + \text{O}_2 \rightarrow \text{LPO}{-}\text{O}_2
\]

and 6), the involvement of

\[
\text{H}_2\text{O}_2 + \text{I}^- \rightarrow \text{H}_2\text{O} + \text{I}_2
\]

in the LPO-H₂O₂-I⁻ system, Fig. 6). Thus, more work is required to test fully the proposed models. Experiments including spin trapping are in progress which are intended to reveal either Iₐ or oxygen free radicals as damaging species on the peroxidase. The outcome of this work will provide some hints which may help to explain hitherto unsolved problems with special regard to the pathophysiology of the thyroid gland.

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