# On the Mechanism of the Pseudocatalatic Degradation of Hydrogen Peroxide by Lactoperoxidase/Iodide

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Hydrogen peroxide is catalytically disproportionated by lactoperoxidase in the presence of iodide ions, $K_m$ = 55 μM in 100 mM sodium phosphate, pH 7.00, 25 °C. Products formed are water and molecular oxygen. The reaction is competitively inhibited by hydrogen sulfite, $K_i$ = 0.24 mM in 100 mM sodium phosphate, pH 7.00, 25 °C.
The stoichiometry of the reaction is identical with the corresponding catalase reaction but the mechanism differs. A mechanistic model for lactoperoxidase-iodide dismutation of hydrogen peroxide is discussed.

Lactoperoxidase (LP; EC 1.11.1.7), molecular weight 78500, consists of a single peptide chain[1,2] of unknown sequence carrying 10% carbohydrates and one heme group. The tertiary structure is maintained by eight disulfide bridges.[1,2] It seems now well established that the prosthetic group is protoheme noncovalently bound[3,4] in a narrow hydrophobic crevice.[5,6] More information about LP structure is collected in a recent review.[7] Chance demonstrated[8,9] that LP gives the same intermediates, with the same oxidation states during the peroxidatic cycle as the plant peroxidases do.

$$\text{E} + \text{H}_2\text{O}_2 \xrightarrow{k_1} \text{cpd I} + \text{H}_2\text{O}_2$$

$$\text{cpd I} + \text{donor} \xrightarrow{k_2} \text{cpd II} + \text{products}$$

$$\text{cpd II} + \text{donor} \xrightarrow{k_3} \text{E} + \text{products}.$$

Cpd I is formally pentavalent since it is reduced by two equivalents to the native state. Cpd II is reduced by one equivalent to the native state. The three steps separately cover several steps. However, contrary to this finding, Courtin *et al.* showed that cpd II of LP is also two oxidizing equivalents above the native enzyme, and postulated a difference in the localization of the second equivalent.[10] The substrate profile differs slightly from that of plant peroxidases. LP kinetics have recently been reviewed.[11] (Cpd I and cpd II are in the generally accepted simplified model of peroxidase activity by Keilin, Thorell, Chance and George).

LP has, besides the peroxidase function, the ability to catalytically disproportionate dithionite ions[12] and, together with $Br^-$ or $I^-$ in a pseudocatalytic or catalatic way, decompose $H_2O_2$. The stoichiometry of the decomposition of $H_2O_2$ by LP/$Br^-$ or LP/$I^-$ is the same as when $H_2O_2$ is decomposed by catalase, but the mechanism seems to differ. Klebanoff and Hamon showed that LP and MP together with $H_2O_2$ and halides also have an antibacterial[13,14] and cytotoxic effect.[15] Direct evidence for the involvement of singlet oxygen ($^1O_2^*$) has come from Allen *et al.*[16,17] Evidence for the formation of $^1O_2^*$ from the system LP/$H_2O_2$/$Br^-$ or $I^-$ has been obtained by IR[18] and chemiluminescence,[19] and by the oxidation of a singlet oxygen trap, 2,5-diphenylfuran.[19] It seems now well established that $Cl^-$, $I^-$ and $Br^-$ are oxidized to $Cl^+$, $I^+$ and $Br^+$, respectively, by peroxidase cpd I in a 2-electron transfer.[20–22] Different forms of oxidized iodine bound to enzyme during the pseudo-catalatic degradation of $H_2O_2$ have been pseudocatalatic degradation of $H_2O_2$ have been zyme-$I^+$ complex[22,24] and an enzyme-$OI^-$ complex.[25,26] Reaction models have been proposed,[24,26] but results in this study together with previous publications point to a different mechanistic model for the pseudocatalatic degradation of $H_2O_2$ by LP/$I^-$.

## Material and methods

Lactoperoxidase, $\varepsilon_{412} = 112.3\ mM^{-1} \cdot cm^{-1}$,[27] was prepared from cow's milk,[28] the DEAE-cellulose chromatography step being excluded; $A_{412}/A_{280} \geqq 0.93$. Catalase, $\varepsilon_{406} = 115\ mM^{-1} \cdot cm^{-1}$,[29] from bovine liver (Sigma, St. Louis, Cat. No. C-100) was twice chromatographed on a Sephacryl®-300 (Pharmacia, Sweden) column, diameter 26 mm, length 900 mm, in 50 mM sodium phosphate pH 7.00 at coldroom temperature. About half of the starting material was excluded by this procedure.

All chemicals used were of analytical grade (Merck, Darmstadt). Water was bidistilled from glass vessels. All experiments were carried out at, if not otherwise stated, 25 ± 0.1 °C in 100 mM sodium phosphate, pH 7.00.

Oxygen evolution was measured polarographically with a Clark oxygen electrode, type YSI 4004 (Yellow Springs Instrument Co, Ohio). The water-jacketed cell with 4 ml measuring volume was made in our departmental workshop. Temperature was controlled by a Julabo S-100 water circulating bath (Julabo, Seelbach). The battery powered device for control of the polarizing voltage, and recorder output was built according to the YSI 4004 oxygen probe instructions.

Ordinary time scale spectrophotometric measurements were carried out on a Beckman DU-7® spectrophotometer and the rapid reactions on a Union Giken (RA-401, RA-415) rapid reaction analyzer. Both instruments were equipped with thermostatically controlled cuvette holder compartments, and routinely checked for wavelength accuracy and absorbance linearity.

$H_2O_2$ concentration was determined enzymatically using horseradish peroxidase, type C, EC 1.11.1.7 (HRPC) and dicarboxidine (γ,γ'-(4,4'-diamino-3,3'-biphenylylenedioxy)-dibutyric acid)[30] as hydrogen donor. In 50 mM sodium phosphate pH 6.90, a stable brown product was formed with $\varepsilon_{440} = 12.2\ mM^{-1} \cdot cm^{-1}$ as determined on the basis of $H_2O_2$.[31]

## Results

With a Clark-type oxygen electrode the formation of $O_2$ from a solution of $H_2O_2$ was measured after addition of LP and $I^-$. The rate of formation of $O_2$ is dependent on the concentration of LP and $I^-$,[26,32,33] and the concentration of the substrate. In this study, the concentration of $I^-$ was chosen to achieve best effect,[32,33] and the concentration of LP to obtain suitable rate of $O_2$ evolution for the electrode system. No oxygen release was observed in the absence of LP or $I^-$ or $H_2O_2$. The reaction occurred equally well under anaerobic conditions. Fig. 1 gives the recorder traces when various concentrations of $H_2O_2$ were catalytically degraded by LP and $I^-$. Final pen deflections are equal to those obtained with catalase and the same concentrations of hydrogen peroxide. From the tangents drawn (Fig. 1) initial velocity rates ($v_o$) were calculated and found to vary with the $H_2O_2$ concentration. A double-reciprocal plot of the initial velocity rate versus substrate concentration resulted in a nonlinear curve (Fig. 2). The corresponding plot from control experiments with beef liver catalase gave a straight line ($r > 0.999$), $K_m = 10.2$ mM. Ogura and Yamazaki[29] reported $K_m = 1.3$ M when they measured the disappearance of $H_2O_2$ in the presence of beef liver catalase.

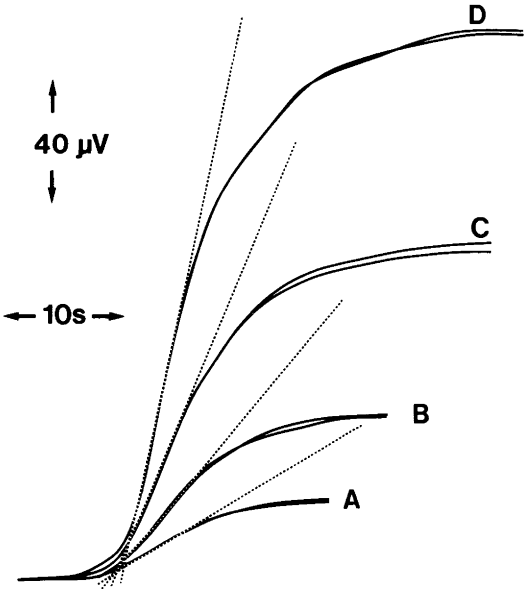


*Fig. 1.* Recorder traces from measurements of $O_2$ evolution with a Clark electrode. 23.7 nM LP, 97.2 μM $I^-$. Initial concentrations of $H_2O_2$ were: A 49.0 μM, B 73.5 μM, C 121.7 μM and D 193.3 μM. Dotted lines are tangents representing initial velocity rates. 100 mM sodium phosphate, pH 7.00, 25 °C.

The minor lag phase common to both lactoperoxidase/$I^-$ (Fig. 1) and catalase reactions orig-

inates from the diffusion of $O_2$ through the thin teflon membrane covering the electrode chamber. Semilogarithmic plots of the end phases resulted in straight lines. Calculated $t_{\frac{1}{2}}$ increased linearily with increased initial concentrations of $H_2O_2$. Corresponding plots from catalase degradation of $H_2O_2$ gave $t_{\frac{1}{2}}$ values independent of the initial concentration of $H_2O_2$.

$HSO_3^-/SO_3^{2-}$ inhibits the LP/$I^-$ catalyzed evolution from $H_2O_2$. On account of the nonlinear double reciprocal Lineweaver-Burk plot both in absence (Fig. 1) and presence of inhibitor, type and degree of inhibition will be discussed.

Formation of cpd I and II from LP (2.37 μM) after addition of a 10-fold excess of $H_2O_2$ was followed by spectral changes in the millisecond and 0.1 second range. Rate of formation of cpd I and the spontaneous transformation to cpd II were in agreement with previously published values.[6,34] The presence of 1.01 mM $HSO_3^-/SO_3^{2-}$ somewhat hampered the formation of cpd I from $1.8 \cdot 10^7 \cdot M^{-1} \cdot s^{-1}$ to $1.2 \cdot 10^7 \cdot M^{-1} \cdot s^{-1}$. No formation of cpd I or II could be detected in the presence of 100 μM $I^-$. Addition of 740 μM $HSO_3^-/SO_3^{2-}$ together with 100 μM $I^-$ resulted in an initial, with time reversible, decrease in Soret absorption indicating formation of cpd I and/or cpd II.

## Discussion

According to Hewson and Hager (Ref. 35 and references therein) chloroperoxidase and catalase can both evolve $O_2$ from $H_2O_2$ via a mechanism in which both oxygen atoms in the evolved $O_2$ molecule emanate from the same molecule of $H_2O_2$. The nonlinear curve (Fig. 2) in the case of LP/$I^-$ is not caused by abnormalities, like the formation of the inactive cpd III in the presence of excess of $H_2O_2$, but might instead mirror a different mechanism as compared to catalase. Dixon and Webb[36] presented theoretically calculated curves in double reciprocal plots when the substrates also act as an activator. The expression

$$\frac{1}{v} = \frac{1}{V} + \frac{K_s}{V}\left(1 + \frac{K_{sa}}{K_s}\right)\frac{1}{s} + \frac{K_{sa}K_s'}{V} \cdot \frac{1}{s^2}$$

will, in general, give curved double reciprocal plot and not a straight line owing to the term involving $s^2$ in the denominator. The term $s^2$ arises

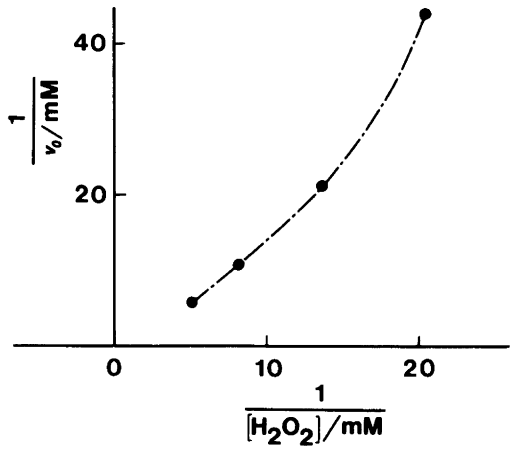


*Fig. 2.* A Lineweaver-Burk double reciprocal plot of the initial rate *vs.* $H_2O_2$ concentration when $H_2O_2$ is decomposed by LP/$I^-$. From Fig. 1.

from the idea that the enzyme, after activation, reacts with another substrate molecule. If this plot is modified to $1/\sqrt{v_o}$ vs. 1/s, a straight line is obtained. In the case of LP/$I^-$, the plot $1/\sqrt{v_o}$ vs. $1/[H_2O_2]_{init}$ does not give straight lines. If LP in the presence of $I^-$, contrary to catalase, evolves $O_2$ via a mechanism in which the oxygen atoms in the evolved $O_2$ molecule emanate from different molecules of $H_2O_2$ then the plot should be $1/\sqrt{v_o}$ vs. $1/[H_2O_2]^2_{init}$ providing that one of the $H_2O_2$ molecules activates the enzyme. A $1/\sqrt{v_o}$ vs. $1/[H_2O_2]^2_{init}$ plot of the results in Fig. 1 is given in Fig. 3. The $K_{m.app}$ value of 55 μM calculated from

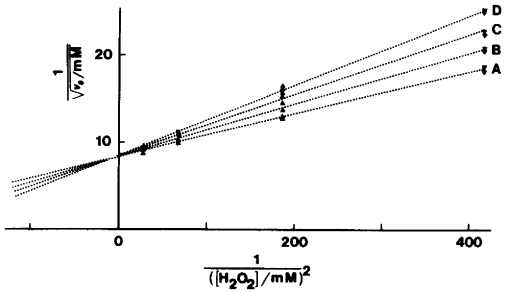


*Fig. 3.* A Lineweaver-Burk double reciprocal plot of the $HSO_3^{1-}$ inhibition of the rate of $O_2$ evolution from $H_2O_2$. The plot was made according to the discussion Cf. text. 23.7 nM LP, 97.2 μM $I^-$. Initial concentrations of $H_2O_2$ 49–193 μM. Inhibitor concentrations: A0, B 25 μM, C 50 μM and D 75 μM giving $K_m$ 55 μM, 64 μM and 70 μM, respectively. 100 mM sodium phosphate, pH 7.00, 25 °C.

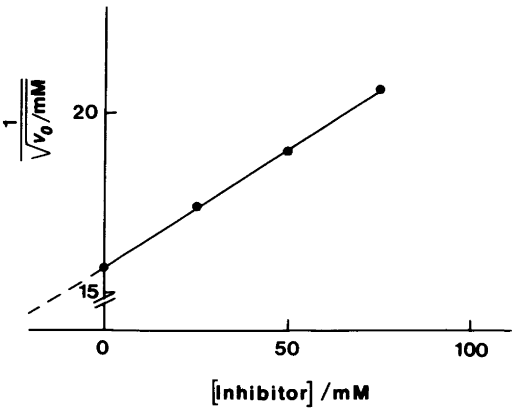


*Fig. 4.* Plot of the inhibitory effect by $HSO_3^-$. Reciprocal rate taken at 58 μM $H_2O_2$ in Fig. 3. $K_i$ 238 μM. Correlation coefficient 0.999.

the straight line ($r \geqq 0.999$) is about 200 times lower than our $K_{m,app}$ for the corresponding reaction with beef liver catalase (cf. Results). $HSO_3^-/SO_3^{2-}$ acts as a competitive inhibitor when LP transmutes $S_2O_4^{2-}$ to $S_2O_3^{2-}$ and $HSO_3^-/SO_3^{2-}$.[12] The $O_2$ evolution from $H_2O_2$ catalyzed by the LP/$I^-$ system is also competitively inhibited by $HSO_3^-/SO_3^{2-}$. The inhibitor effect plotted according to the above gave $K_{m,app}$ values of 55 μM, 60 μM, 64 μM and 70 μM for 0 μM, 0.25 μM, 50 μM and 75 μM $HSO_3^-/SO_3^{2-}$, respectively. An inhibitor plot (Fig. 4) gave $K_i$ = 0.24 mM.

A difference between catalase and LP/$I^-$ degradation of $H_2O_2$ can also be seen in the rate of the end phase of the reaction curves. Contrary to the true catalase reactions, $t_{\frac{1}{2}}$ varied with the initial concentration of $H_2O_2$ in the case of LP/$I^-$. It has been proposed and shown that cpd I of LP and thyroid peroxidase, EC 1.11.1.7 (TP) oxidize $Br^-$ in a 2-electron step to give $OBr^-$ [18,19,25,37] and $I^-$ to give $OI^-$.[23,26] Increased initial [$H_2O_2$] at maintained [$I^-$] would result in initially high concentration of $I^+$ or $OI^-$ leaving less $H_2O_2$ for the second step, and a decrease in the rate of $O_2$ evolution at the end phase.

No detectable spectral changes could be registered in the 'rapid scanner' during the reaction; thus the oxidation of $I^-$ by LP cpd I must take place at a much higher rate than the formation of LP cpd I. By comparison, TP cpd I oxidizes $I^-$ at a rate of $2.1 \times 10^7 \cdot M^{-1} \cdot s^{-1}$, which is almost an order of magnitude higher than the rate of formation of TP cpd I.[22] $HSO_3^-/SO_3^{2-}$ hampers the formation of LP cpd I very little but is oxidized by LP cpd I at a lower rate than $I^-$ [38] and in that way competitively inhibits the reaction, resulting in detectable LP cpd I and/or LP cpd II.

Dunford and Ralston[23] proposed that HRP(ferric) forms a complex with $OI^-$, a complex which reacts very fast with $I^-$ to give $I_2$ and $OH^-$ as products, not $O_2$. Magnusson *et al.*[26] postulated a LP(ferric) · $OI^-$ complex that reacts with $H_2O_2$ and gives $O_2$ at [$I^-$] < 100 μM. Huwiler and Kohler[24] proposed that $I^+$ of $I_2$ ($I^+ + I^-$) reacts nonenzymatically with $H_2O_2$[39] and in that way, evolves $O_2$. According to Liebhafsky and Wu[39] $OI^-$ (and $OBr^-$) reacts with $H_2O_2$ and gives $O_2$, $I^-$ (or $Br^-$) and $H_2O$. No appearance of spectral changes opposes a tight ferric enzyme-$OI^-$ complex, and the kinetic pattern (cf. above) points to an enzymatic second reaction. Thus, the pseudocatalatic ability must be found in the structural difference at or near active site(s) between LP and HRP.

Piatt and O'Brien found, by a trapping technique, evidence for singlet oxygen formation in the system LP/$Br^-$/$H_2O_2$ and postulated that all oxygen is produced via the excited state.[19] Singlet oxygen may be the initial reaction product when $OBr^-$ reacts with $H_2O_2$.[19,39] In preliminary experiments, Piatt and O'Brien found indications of an excited carbonyl group.[19] Similar findings are reported from the system myeloperoxidase/$Cl^-$/$H_2O_2$.[40] A study of LP by means of magnetic circular dichroism and electron spin resonance spectroscopy indicated a carboxyl group as the distal ligand of the heme group at room temperature.[41] A p$K_a$ value of about 3.5 estimated from its effect on $S_2O_4^{2-}$,[12] the rate of spontaneous transfer from the first to the second reduced form,[12] the rate of

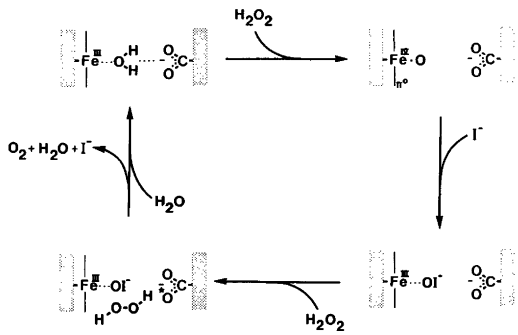


*Fig. 5.* A proposed model for the pseudocatalatic degradation of $H_2O_2$ to $O_2$ and $H_2O$.

reduction of LP with $S_2O_4^{2-}$,[42] and optical titrations[43] support the existence of a carboxyl group at or close to the active site. Chemical modification of the carboxyl group results in almost complete loss of peroxidase activity[12] and a 10-fold decrease in transfer time from the first to the second reduced form of LP.[12]

Results in this and other studies are, in accordance with the discussion, summarized in a proposed hypothetical model (Fig. 5) for the pseudocatalatic degradation of $H_2O_2$. The model is greatly simplified; every step involves series of events. The preliminary products formed after the reaction with the second $H_2O_2$ molecule are probably HI, $OH^-$ and $^1O_2^*$ which, in part, is consistent with previous findings.[26]

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