Reactions of Purified Hog Thyroid Peroxidase with H₂O₂, Tyrosine, and Methylmercaptoimidazole (Goitrogen) in Comparison with Bovine Lactoperoxidase*

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Stopped flow experiments were carried out with purified hog thyroid peroxidase (A₄₃₆ nm/A₃₄₈ nm = 0.42). It reacted with H₂O₂ to form Compound I with a rate constant of 7.8 × 10⁶ M⁻¹ s⁻¹. Compound I was reduced to Compound II by endogenous donor with a half-life of 0.36 s. Compound I was reduced by tyrosine directly to the ferric enzyme with a rate constant of 7.5 × 10⁶ M⁻¹ s⁻¹. Tyrosine could also reduce Compound II to the ferric enzyme with a rate constant of 4.3 × 10⁶ M⁻¹ s⁻¹. Methylmercaptoimidazole accelerated the conversion of Compound I to Compound II and reacted with Compound II to form an inactivated form, which was discernible spectrophotometrically.

The reactions of thyroid peroxidase with methylmercaptoimidazole quite resembled those of lactoperoxidase, but occurred at higher speeds. The absorption spectra of thyroid peroxidase were similar to those of lactoperoxidase and intestinal peroxidase, but obviously different from those of metmyoglobin, horseradish peroxidase, and chloroperoxidase. Similarity and dissimilarity between thyroid peroxidase and lactoperoxidase are discussed.

Because of the difficulty in obtaining a sufficient amount of purified thyroid peroxidase, its catalytic mechanism has been studied mainly from the overall kinetic data. In the previous paper (1), we have analyzed kinetic intermediates of detergent-solubilized thyroid peroxidase by the stopped flow method. Although the purity of the enzyme was very low in comparison with the most purified enzyme obtained with proteolytic procedures (2), our transparent preparation could give quantitative information for kinetic parameters involved in its iodinating reactions (1).

Bovine lactoperoxidase has a high catalytic activity of iodination and mechanism of its iodination has been studied in many laboratories (3–6). Recently, the inhibitory effect of thioureylen antithyroid compounds on the peroxidase-catalyzed iodination has been investigated in detail with bovine lactoperoxidase (7, 8). The inactivated complex of lactoperoxidase is clearly demonstrated spectrophotometrically, while the effect of thioureylen compounds on the reactions of thyroid peroxidase has been studied only by measuring the overall enzyme activity (9–16).

It is beyond doubt that there are some similarities between the reactions catalyzed by thyroid peroxidase and lactoperoxidase. It seemed to us of particular importance to prepare a sufficient amount of purified thyroid peroxidase to compare catalytic features of these two enzymes on the same analytical basis. Recently, we obtained such an enzyme preparation from hog thyroid microsomes after solubilization, first by detergent treatment and then by a supplementary use of trypsin. Using this enzyme preparation, we could confirm some similarities and dissimilarities between hog thyroid peroxidase and bovine lactoperoxidase.

MATERIALS AND METHODS

A number of papers have been reported on the purification procedures of thyroid peroxidase. The procedures might roughly be grouped, according to the solubilization method, into three types including proteolysis (16–18), detergent treatment (19–21), and both of them (22–25). The enzyme used in the present study was prepared according to a modification of the method of Taurog et al. (24). The hog thyroid microsomes prepared by a modification of the method of Hosoya and Morrison (26) were incubated in 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM KI and 0.4% sodium deoxycholate at 4 °C overnight. After centrifugation at 160,000 × g for 1 h, the supernatant was fractionated between 25 to 60% ammonium sulfate saturation. The precipitate was dissolved in and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM KI. The dialyzed enzyme solution was chromatographed on a Sepharose 6B column (2.5 × 100 cm) equilibrated with 20 mM phosphate buffer (pH 8.0) containing 0.1 mM KI and 0.1% sodium deoxycholate. The frction that contained peroxidase activity was concentrated in a small Diaflo ultrafiltration cell with a PM-10 membrane to 10 mg protein/ml. The enzyme solution was then incubated with trypsin (0.1 mg/ml) at 37 °C for 40 min. After addition of trypsin inhibitor (0.2 mg/ml), the enzyme solution was dialyzed against 10 mM phosphate buffer (pH 7.4) containing 0.1 mM KI and applied to a DEAE-cellulose column (1.9 × 25 cm) equilibrated with the same buffer. Linear gradient elution was then started by increasing the KCl concentration up to 0.3 M. The fraction containing peroxidase activity was concentrated with the Diaflo ultrafiltration and applied to a Sephacryl S-300 column (1.9 × 90 cm), equilibrated with 10 mM phosphate buffer (pH 6.8) containing 0.1 mM KI. After the above gel filtration, the enzyme solution was placed on a hydroxylapatite column (1.9 × 10 cm), equilibrated with 10 mM phosphate buffer (pH 6.8) containing 0.1 mM KI. Linear gradient was started from 10 to 200 mM phosphate buffer (pH 6.8) containing 0.1 mM KI. Table I shows the yield and degree of purification of thyroid peroxidase. Thyroid peroxidase activity was assayed according to the method of Hosoya and Morrison (22); one unit of the enzyme activity was defined to oxidize 1 µmol of guaiacol/min. The enzyme was used for the present experiments after removing KI by the Diaflo ultrafiltration. The enzyme concentration was tentatively calculated on the basis of a value of 114 for ε₄₃₆ at 415 nm, which was the same as that used for lactoperoxidase. Bovine lactoperoxidase used was the same as that described pre-
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TABLE I
Activity of thyroid peroxidase at various stages of purification from 5.4 kg of hog thyrogland

<table>
<thead>
<tr>
<th>Stage of purification</th>
<th>Total protein</th>
<th>Total units of activity</th>
<th>Yield</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g x 10^7</td>
<td>%</td>
<td>units/mg protein</td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>15.6</td>
<td>16.4</td>
<td>100</td>
<td>1.05</td>
</tr>
<tr>
<td>0.4% deoxycholate solubilization</td>
<td>12.7</td>
<td>16.1</td>
<td>98.6</td>
<td>1.27</td>
</tr>
<tr>
<td>Sephacryl 6B column</td>
<td>1.75</td>
<td>11.4</td>
<td>69.3</td>
<td>6.50</td>
</tr>
<tr>
<td>DE52 column^a</td>
<td>0.110</td>
<td>4.23</td>
<td>25.8</td>
<td>1.92</td>
</tr>
<tr>
<td>Sephacryl S-300 column</td>
<td>0.0161</td>
<td>1.62</td>
<td>22.1</td>
<td>224</td>
</tr>
<tr>
<td>Hydrolysatite column</td>
<td>0.0065</td>
<td>3.03</td>
<td>18.5</td>
<td>465</td>
</tr>
</tbody>
</table>

^a Trypsin digestion was preceded by this stage without appreciable loss of activity.

The reaction of peroxidase with H2O2 is obligatory in its iodinating activity.

Peroxidase + H2O2 → Compound I

Compound I of animal peroxidases, in general, is short-lived and reduced by endogenous electron donor to Compound II. The half-life was reported to be 0.085 s in our detergent-solubilized thyroid peroxidase (1). Compound I of our purified thyroid peroxidase was more stable and the time course for the formation of Compound I from the ferrie enzyme could be recorded by the stopped flow method. Fig. 2A shows stopped flow traces at varying wavelengths. The formation of Compound I was completed within 60 ms and followed by its reduction to Compound II with a half-life of 0.36 s (see trace a in the inset of Fig. 6). Lines b in Fig. 2A were the traces from 4 to 4.1 s and practically represented the absorbance level for Compound II. Although a small amount of Compound I was formed during the dead time (~2 ms), the difference spectra between reaction times from 2 to 60 ms and from 2 ms to 4 s should nearly correspond to those of ferric minus Compound I and of ferric minus Compound II, respectively.

^1 The abbreviation used is: MMI, methimercaptoimidazole.
1 was measured as $7.8 \times 10^{8} \text{ M}^{-1} \text{ s}^{-1}$. This value was slightly higher than that reported for our detergent-solubilized thyroid peroxidase (cf. $5.2 \times 10^{8} \text{ M}^{-1} \text{ s}^{-1}$ in Ref. 1). The result indicates that the amount of Compound I formed during the dead time was $\sim 15\%$ of the total enzyme.

It was reported in the previous paper (1) that tyrosine was oxidized by Compound I of thyroid peroxidase by way of a 2-electron transfer while it was oxidized by Compound I of lactoperoxidase by way of a 1-electron transfer. The latter mechanism has been believed to hold true for the reactions of peroxidases with organic compounds (38). Therefore, the 2-electron oxidation of tyrosine by thyroid peroxidase was quite unexpected. At that time we could not exclude the possibility that such an anomalous result was brought about by the insufficient purity of the enzyme. In order to remove ambiguity in this point, we repeated the experiment with our purified thyroid peroxidase. Fig. 3A shows stopped flow traces at varying wavelengths in the reaction containing the enzyme, H$_2$O$_2$, and tyrosine. The reaction consisted apparently of two phases and ended in $\sim 10$ s. The absorbance level of the ferric enzyme was represented by traces b. From the two difference spectra between reaction times from 0.2 to 10 s and from 1 to 10 s (Fig. 3B), it was evident that the enzyme was present mostly as Compound I at a reaction time of 0.2 s and as a mixture of the ferric enzyme and Compound II in the slow phase. The half-life for the decay of Compound II in the slow phase was $\sim 3.2$ s and the second order rate constant for the reduction of Compound II by tyrosine was calculated as $4.3 \times 10^{8} \text{ M}^{-1} \text{ s}^{-1}$. This rate was too slow to explain the observed kinetic traces by assuming that Compound II is an obligatory intermediate in the oxidation of tyrosine by thyroid peroxidase. Therefore, accumulation of Compound I in the fast phase should be explained by assuming that the 2-electron reduction of Compound I by tyrosine is rate-determining.

$$\text{Compound I} + \text{tyrosine} \rightarrow \text{peroxidase} + \text{oxidized tyrosine}$$

According to Chance's equation, $k = x_0/2 \cdot p_{\text{max}} \cdot t_{1/2, \text{ eff}}$ (37), where $x_0$ and $a$ are concentrations of H$_2$O$_2$ and tyrosine, respectively, and $p_{\text{max}}$ and $t_{1/2, \text{ eff}}$ are a maximum concentration (0.73 $\mu$M) and a half-decay time (0.4 s) of Compound I, the second order rate constant for Reaction 2 was measured as 7.5 $\times 10^{8} \text{ M}^{-1} \text{ s}^{-1}$ from a stopped flow trace at 420 nm (Fig. 3A). This value was $\sim 5$-fold higher than that reported previously (1). It was concluded that a small amount of Compound II was formed through the reduction of Compound I by endogenous donor and accumulated because the reduction of Compound II by tyrosine was slow.

The mechanism by which antithyroid compounds block thyroid peroxidase has been studied in a number of laboratories (7-15, 38-41). Recently, it was reported that the antithyroid and other thiolic compounds cause irreversible changes in the Soret spectra of the lactoperoxidase-H$_2$O$_2$ complex (7, 8, 42). The spectral changes were reasonably related with the inactivation of lactoperoxidase. The effect of MMI on the fast phase of reaction of lactoperoxidase with H$_2$O$_2$ was studied spectrophotometrically by the stopped flow method at varying wavelengths. From the difference spectra between reaction times from 2 to 15 ms and from 2 to 500 ms (Fig. 4A), it was found that accumulation of Compound I was very small during the conversion from the ferric enzyme to Compound II in the presence of 100 $\mu$M MMI. The result indicates that the conversion from Compound I to Compound II was markedly accelerated by MMI. In the presence of 2.5 $\mu$M MMI, although Compound I could be detected in the early phase of the reaction (Fig. 4B), its conversion to Compound II was also accelerated. The effect of MMI concentration on the conversion from Compound I to Compound II was measured at 430 nm, an isosbestic point between the ferric enzyme and Compound I of bovine lactoperoxidase (Fig. 4C). The observed first order rate constant was nearly proportional to the MMI concentration below 25 $\mu$M and the second order rate constant for the reaction between Compound I of lactoperoxidase and MMI was measured approximately as $5 \times 10^{8} \text{ M}^{-1} \text{ s}^{-1}$.

Compounds II of lactoperoxidase was then converted to an MMI complex in a similar manner as reported with other thioureylene and thiol compounds (7, 8, 42). The results suggested that the Soret spectra of final products vary with the kind of such compounds. Fig. 5 shows time-dependent spectral changes during the formation of MMI complex. The Soret spectrum recorded immediately after the addition of MMI was measured approximately as $5 \times 10^{8} \text{ M}^{-1} \text{ s}^{-1}$. The results differed from that of Compound I, the Soret spectra of final products varied with the kind of such compounds. Fig. 5 shows time-dependent spectral changes during the formation of MMI complex. The Soret spectrum recorded immediately after the addition of MMI resembled that of Compound II, but the visible spectrum differed from that of Compound II. Although the reaction of Compound II with MMI appeared to be not so simple, the half-reaction time was $\sim 10$ s in the presence of 20 $\mu$M MMI (inset of Fig. 5). The formation of MMI complex was completely inhibited by the addition of iodide in slight molar excess with respect to MMI.

A similar spectral species was also formed in the reaction of

![Fig. 3. Spectral changes in thyroid peroxidase during the oxidation of tyrosine. A, stopped flow traces at varying wavelengths (indicated on the figure) in the presence of 300 $\mu$M tyrosine, 11 $\mu$M H$_2$O$_2$ and 1 $\mu$M enzyme. B, difference spectra between 0.2 and 10 s (O) and between 1 and 10 s (S). Traces a and b denote time intervals from 0 to 10 s and from 10 to 20 s, respectively.](image-url)

![Fig. 4. Reduction of lactoperoxidase Compound I by MMI. A and B, difference spectra of intermediates of the enzyme in the reaction of 6.8 $\mu$M enzyme with 10 $\mu$M H$_2$O$_2$ and 100 (A) or 2.5 (B) $\mu$M MMI. Difference spectra were obtained from stopped flow traces between reaction times from 2 to 15 ms (C) and from 2 to 500 ms (S) for A and from 2 to 25 ms (C) and from 2 to 500 ms (S) for B, C, stopped flow traces at varying concentrations of MMI: 0 (a), 1.25 (b), 2.5 (c), 25 (d), 25 (e), and 100 $\mu$M (f) in the presence of 1 $\mu$M enzyme and 11 $\mu$M H$_2$O$_2$. The base line represented the absorbance level of Compound II.](image-url)
FIG. 5. Formation of MMI complex of lactoperoxidase. Spectral changes after 20 μM H₂O₂ was added to a mixture of 2.9 μM enzyme and 10 μM MMI. The spectra were recorded immediately (—) and 9 min (•••) after the addition of H₂O₂ from 500 to 700 nm toward the shorter wavelengths at a speed of 120 nm/min. The spectrum remained almost unchanged from 9 to 20 min. The inset shows stopped flow traces at 412 and 430 nm for the reaction of 0.9 μM enzyme with 11 μM H₂O₂ plus 20 μM MMI.

FIG. 6. Reactions of thyroid peroxidase with MMI. The spectrum was recorded from 700 nm immediately after 10 μM H₂O₂ was added to a mixture of 0.7 μM enzyme and 10 μM MMI. No intermediate spectra were observed in the Soret region when the wavelength was scanned at a speed of 120 nm/min from 500 nm. The inset shows stopped flow traces at varying concentrations of MMI; 0 (a), 3.75 (b), and 20 (c) μM in the presence of 0.8 μM enzyme and 11 μM H₂O₂. The base line denotes traces from 10 to 11 s and corresponds to the absorbance level of compound II for a, but probably of a mixture of compound II and MMI complex for b and c.

thyroid peroxidase with MMI plus H₂O₂ (Fig. 6). In this case, the formation was so fast that no distinct intermediate phase could be seen at an ordinary wavelength-scanning rate. The inset in Fig. 6 shows that MMI greatly accelerated the formation of compound II from compound I. It could be concluded from the results shown in Figs. 4 to 6 that MMI reacted similarly with the two peroxidases, but faster with thyroid peroxidase than with lactoperoxidase. This conclusion was confirmed by measuring the peroxidase activity at several incubation times after the enzymes were mixed with both MMI and H₂O₂ (Fig. 7). When the enzyme activity was measured by the addition of H₂O₂, no appreciable inhibition of MMI was detected for the oxidation of iodide as reported already (7, 12-14). The inactivation started after the enzyme was incubated with both MMI and H₂O₂. Similar results have been reported in the reaction of thyroid peroxidase with propylthiouracil (12). Fig. 7 indicates that thyroid peroxidase was inactivated much faster than lactoperoxidase. Here, the catalytic reaction was started by the addition of iodide as an electron donor (43, 44). The activity appeared to be partially recovered by the addition of iodide in the early phase of inactivation. The degree of recovery of the activity decreased as the incubation proceeded.

FIG. 7. Effect of the preincubation time with MMI plus H₂O₂ on the inactivation of peroxidases. The peroxidase activity was measured by the addition of 2 mM KI at varying incubation times indicated by arrows after the enzymes were mixed with 20 μM H₂O₂ and 5 μM MMI. A, 36 nm lactoperoxidase; B, 30 nm thyroid peroxidase.

DISCUSSION

Evidently, the three animal peroxidases in Table II resemble each other in their spectral properties and differ from typical protoporphyrin IX-containing proteins. The difference spectra of ferric minus compound I and ferric minus compound II are also alike between these three peroxidases (compare with Refs. 1 and 27). Moreover, it is of interest to note that problems concerning the chemical nature of prosthetic groups of these peroxidases are not definitely settled for similar reasons. Quite a number of experiments have been carried out to define the nature of their heme main by measuring the pyridine hemochromogen spectra after alkalization or proteolysis of the proteins, or extract of the heme with organic solvents, particularly in lactoperoxidase (29, 35, 43) and thyroid peroxidase (2, 18, 22-25, 46). The results are very similar between the two peroxidases and might be summarized as follows: their prosthetic groups are possibly ferriprotoporphyrin IX, but there are anomalies in its binding to the proteins. Similar conclusion was recently obtained with hog intestinal peroxidase from the resonance Raman study (47). One more similarity between these three peroxidases would be instability of their reduced form (30, 45, 48). It appears that ferrous high-spin forms primarily derived from these ferric enzymes are slowly converted to low-spin forms at neutral pH (30, 47).

The remarkable difference between lactoperoxidase and thyroid peroxidase is present in the mechanism of reaction between compound I and tyrosine. The reduction of compound I by tyrosine occurs by way of a 1-electron transfer in lactoperoxidase and by way of a 2-electron transfer in thyroid peroxidase according to the definition of Yamazaki (36). Since various phenol compounds seem to be oxidized by peroxidase systems by way of a 1-electron transfer, the 2-electron reduction of thyroid peroxidase Compound I by tyrosine may be exceptional. It is suggested that one requirement for the 2-electron transfer mechanism in the enzymatic oxido-reductive reaction is tight interaction between the enzyme and substrate (49). If there is such specific interaction between tyrosine and thyroid peroxidase, it must be very important from the physiological point of view. But, it should be noted that Compound I of thyroid peroxidase oxidizes exclusively iodide first when
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FIG. 8. Comparison of reaction scheme between thyroid peroxidase and lactoperoxidase. Values in parentheses denote apparent first order rate constants (s⁻¹) in the presence of 10 μM H₂O₂, 500 μM tyrosine, 12 μM I⁻, or 20 μM MMI. The rate of spontaneous conversion from Compound I to Compound II depends on the purity of peroxidases. Second order rate constants (M⁻¹ s⁻¹) used for calculation: for thyroid peroxidase (TPO): 7.8 × 10⁴ for TPO + H₂O₂, 2.1 × 10¹⁰ for Compound I + I⁻ (Ref. 1), 7.5 × 10⁶ for Compound I + Tyr, −2 × 10⁶ for Compound I + I² (Ref. 1), and 4.3 × 10⁷ for Compound II + Tyr. For lactoperoxidase (LPO): 1.2 × 10⁶ for LPO + H₂O₂ (Ref. 27), >1.1 × 10⁶ for Compound I + Tyr (based on the fact that no Compound I accumulates in the oxidation of Tyr) (Ref. 1), 5 × 10⁶ for Compound I + MMI, 60 for Compound II + I⁻ (Ref. 5), and 1.1 × 10⁸ for Compound II + Tyr (Ref. 1). Reactions of MMI with Compound I and Compound II occur at least 2- and 4-fold faster, respectively, in TPO than in LPO (Figs. 4-6). It is not clear whether the formation of MMI complexes obeys second order kinetics.

The mechanism of action of thioureylen antithyroid compounds has been proposed by several workers (7-9, 11-15, 39, 40). As suggested by Taurog (12), there are at least two modes of action of MMI. First, MMI inhibits iodination by competing with tyrosyl for an oxidized form of iodine on the enzyme, the model reaction being studied with chloroperoxidase (40). Second, MMI irreversibly inactivates thyroid peroxidase (11, 12, 14, 15). However, the mechanism of inactivation being demonstrated spectrophotometrically with lactoperoxidase (7, 8, 42). From these and our previous (1) and present data, we are led to propose reaction schemes of thyroid peroxidase and lactoperoxidase as shown in Fig. 8. MMI competes with tyrosine and iodide for the oxidized form of iodine and also for Compounds I and II. The latter competition might be important, particularly in the mechanism of inactivation of these peroxidases by MMI. MMI is metabolized by the thyroid peroxidase system possibly in three ways. 1) MMI is oxidized by the oxidized form of iodine (12, 14, 52). 2) MMI reacts with Compound II of thyroid peroxidase to form primarily an adduct. It appears that the reaction resembles that of lactoperoxidase with MMI and its free radical form though the reaction may not proceed catalytically. Its relation with the oxidation of diiodotriiodothyronine by peroxidase systems (55) is unknown.

If one assumes that tyrosine and iodide are both oxidized by way of a 2-electron transfer in the thyroid peroxidase reaction, involvement of free radicals in the iodination of tyrosine (s₅, s₇) can be excluded. Various derivatives have been proposed for the oxidized form of iodine (13, 58). For the oxidized iodine species, a simple form, I⁺, is used in Fig. 8, as reported by several workers (1, 59-62). If the iodination of tyrosine occurs through the reaction of tyrosine with the I⁺ species, the specific 2-electron oxidation of tyrosine by thyroid peroxidase Compound I might be related to the coupling reaction. Nunez et al. (57, 63) have suggested unique function of thyroid peroxidase in the coupling reaction. This is an interesting problem to be solved on the molecular level.

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