One- and Two-electron Oxidations of Tyrosine, Monoiodotyrosine, and Diiodotyrosine Catalyzed by Hog Thyroid Peroxidase*

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Stopped flow experiments were carried out with purified hog thyroid peroxidase ($A_{412\text{nm}}/A_{280\text{nm}} = 0.42$). In the steady state of oxidations of L- and D-tyrosines, N-acetytyrosinamide, and monoiodotyrosine, thyroid peroxidase existed in the form of Compound I, the primary catalytic intermediate of peroxidase in its reaction with H$_2$O$_2$. Kinetic results led us to conclude that thyroid peroxidase catalyzes two-electron oxidations of these molecules. In the steady state of oxidation of diiodotyrosine, on the other hand, the enzyme was found in the form of Compound II at pH 7.4, but in the form of Compound I at pH 5.5. The result implies that the mechanism of diiodotyrosine oxidation varied from a one-electron to a two-electron type as the pH decreased.

The selection of mechanisms of oxidation appears to be peculiar to thyroid peroxidase; horseradish peroxidase and lactoperoxidase catalyzed only one-electron oxidations of these five donor molecules. Rate constants for rate-limiting steps in the reactions of these donor molecules with the three peroxidases were measured by overall kinetic and stopped flow kinetic methods.

Thyroid peroxidase is involved in the biosynthesis of thyroid hormone (1–4). The primary reaction catalyzed by the enzyme is iodination of the tyrosyl residues in thyroglobulin. Thyroid peroxidase does catalyze the conversion of diiodotyrosine to thyroxine (5–7). Oxidative coupling reactions of diiodotyrosine and other tyrosine derivatives have also been studied with other peroxidases (5, 8–13). Horseradish peroxidase and lactoperoxidase have been used to characterize the specificity of thyroid peroxidase in the coupling reaction. Although the catalytic properties vary slightly among peroxidases (9–11, 13), these differences do not seem essential for characterizing the nature of thyroid peroxidase.

From stopped flow experiments with detergent-solubilized (14) and purified (15) thyroid peroxidase preparations, we have shown that thyroid peroxidase, unlike lactoperoxidase, catalyzes the oxidation of L-tyrosine by way of two-electron transfer. This finding provides the first clear evidence for two-electron oxidation of organic molecules by the peroxidase systems. Organic molecules such as phenol derivatives are believed to be oxidized to their free radical forms in the presence of peroxidase and H$_2$O$_2$ (16, 17). Therefore, it seems very important to determine whether thyroid peroxidase exhibits a unique catalytic property in the oxidation of monoiodotyrosine and diiodotyrosine as well as L-tyrosine. We have carried out kinetic experiments with purified hog thyroid peroxidase and the results are reported in this paper.

MATERIALS AND METHODS

Hog thyroid peroxidase used in this experiment was prepared as described previously (15). The ratio of $A_{412\text{nm}}$ to $A_{280\text{nm}}$ of our enzyme preparation was 0.42, and its concentration was tentatively calculated by using a value of 114 for $c_{280}$ at 413 nm, which is used for lactoperoxidase (18). Horseradish peroxidase used was isoenzyme C ($A_{412\text{nm}}/A_{280\text{nm}} = 3.4$), and lactoperoxidase ($A_{412\text{nm}}/A_{280\text{nm}} = 9.90$) was donated by Dr. S. Nakamura, Hirosaki University (Hirosaki, Japan) and Dr. S. Kimura, Queen's University (Kingston, Canada).

Stopped flow and other kinetic measurements were performed as described previously (14, 15). The reactions were carried out in 0.1 M sodium acetate (pH 5.5–5.5), potassium phosphate (pH 6.5–8.0), and Tris-HCl (pH 8.5–9.0), at 20 °C.

RESULTS

In general, peroxidase reactions are formulated as follows (16, 19, 20).

$$\text{Peroxidase} + H_2O_2 \xrightarrow{k_1} \text{Compound I} \quad (1)$$
$$\text{Compound I} + AH_2 \xrightarrow{k_2} \text{Compound II} + AH \quad (2)$$
$$\text{Compound II} + AH_2 \xrightarrow{k_3} \text{peroxidase} + AH \quad (3)$$
$$2AH \rightarrow AH + AH \quad (4)$$

(4) $k_3$ $> 10k_1$, (19). When the electron donor (AH$_2$) is an organic molecule, it is believed to be oxidized by way of one-electron transfer (17). If peroxidase is present as Compound II in the steady state of the reaction, Reaction 3 is rate-limiting, and the oxidation of AH$_2$ occurs by way of one-electron transfer. This conclusion can be derived from the analysis of stopped flow traces at two wavelengths isosbestic between any pair of the three enzyme forms involved in the catalytic cycles. Such kinetic results on the oxidations of L- and D-tyrosines, N-acetytyrosinamide, monoiodotyrosine, and diiodotyrosine, in the presence of horseradish peroxidase and lactoperoxidase (Fig. 1), led us to conclude that these reactions occurred by way of one-electron transfer. When Reaction 2 is relatively slow, Compound I must be seen transiently before the formation of Compound II. This was observed during the oxidation of diiodotyrosine by horseradish peroxidase and H$_2$O$_2$ (Fig. 2A). Fig. 2 shows that the catalytic intermediate of horseradish peroxidase and lactoperoxidase in the steady state of diiodotyrosine oxidation was Compound II. The results shown in Figs. 1 and 2 are in accord with the accepted mechanism (Reactions 1 to 3).

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Chance's equation (21). In contrast, the intermediate of L- and D-tyrosines, N-acetyltyrosinamide, and monoiodotyrosine was not Compound 11 in the reaction of thyroid peroxidase with L-tyrosine as Compound I (15). Kinetic traces for D-tyrosine stopped flow experiments were carried out in the presence of peroxidase, electron donor (concentrations are indicated on the figure), 10 μM H2O2, at pH 7.4. Base lines (---) and second traces from 10 to 20 s (-----). A, stopped flow traces on 1 μM horseradish peroxidase at 431.5 nm (---) and at 410 nm (-----). B, stopped flow traces on 0.75 μM lactoperoxidase at 432 nm (---) and at 422.5 nm (-----). The increase in absorbance (-----) indicates the formation of Compound II and the decrease in absorbance (---) the formation of Compound I. The kinetic traces on D-tyrosine resembled those on L-tyrosine and were omitted in this figure.

The reaction catalyzed by thyroid peroxidase was not simple. The kinetic traces shown in Fig. 3 were grouped into two patterns. There was no essential difference among the three peroxidases in the mechanism of diiodotyrosine oxidation at pH 7.4. The enzyme intermediate observed in the steady state of diiodotyrosine oxidation was apparently Compound II, and the rate constant for Reaction 3 could be measured directly from comparison of Figs. 3 and 4. From the shapes of the two difference spectra shown in Fig. 4B, the enzyme intermediate appearing during the oxidation of diiodotyrosine was found to be Compound II when the reaction was carried out at pH 7.4, but was Compound I at pH 5.5. The intermediate was Compound I in the oxidation of monoiodotyrosine at either pH 7.4 or 5.5 (Fig. 4A).

The rate constant for Reaction 3 could be measured directly from the plot in the inset of Fig. 3 to be 2.0 × 10^5 M^-1 s^-1 according to Chance's equation (21). In contrast, the intermediate of thyroid peroxidase appearing in the steady state of oxidations of L- and D-tyrosines, N-acetyltyrosinamide, and monoiodotyrosine was not Compound II (Fig. 3). We have identified the intermediate in the reaction of thyroid peroxidase with L-tyrosine as Compound I (15). Kinetic traces for D-tyrosine resembled those for L-tyrosine, and were omitted in Fig. 3.
from the reaction of Compound II with electron donors. Kinetic traces for monooiodotyrosine and diiodotyrosine are shown in Fig. 5. Similar experiments had been carried out with detergent-solubilized hog thyroid peroxidase (22). In this experiment, care was taken to start the reaction after the amount of H$_2$O$_2$ became negligibly small in Compound II solutions. Since Compound II was slowly reduced back to the ferric enzyme at the expense of endogenous donor, the reaction was started by the addition of an electron donor at a time when a small percentage of the peroxidase was present as the ferric enzyme (upper traces in Fig. 5). The observed first order rate constant was proportional to the concentration of added electron donor (Fig. 5, lower portion), and the second order rate constants thus calculated are listed in Table I, together with data on L- and D-tyrosines, N-acetyltyrosinamide, and monoiodotyrosine.

Despite the difference in the mechanism of oxidations of L- and D-tyrosines, N-acetyltyrosinamide, and monoiodotyrosine among thyroid peroxidase and the other peroxidases, no apparent difference could be detected in the difference spectra of their oxidation products. In Fig. 6, the spectral data on L-tyrosine and monoiodotyrosine are shown. By following the absorbance changes, the rates of oxidations of the electron donors were measured. Under experimental conditions in which Reaction 1 was not rate-limiting, the rate must be proportional to the donor concentration. Fig. 7 shows an example in which diiodotyrosine was used as the electron donor. From these overall kinetic experiments, the rate constant for Reaction 2' or 3' could be measured, and the results are shown in Table II. Here, Reaction 2' is formulated as follows.

Compound I + AH$_2$ → peroxidase + A

### Table I

| Electron donor        | pH   | Rate constant | M$^{-1}$s$^{-1}$ |
|-----------------------|------|---------------|------------------|
| L-Tyrosine            | 7.4  | 9.0 x 10$^4$  |                  |
| D-Tyrosine            | 7.4  | 2.6 x 10$^2$  |                  |
| N-Acetyltyrosinamide  | 7.4  | 9.0 x 10$^9$  |                  |
| Monooiodotyrosine     | 7.4  | 1.8 x 10$^4$  |                  |
| Diiodotyrosine        | 7.4  | 1.0 x 10$^{-1}$ x 1.7 x 10$^9$ |     |
|                       | 5.5  | 1.5 x 10$^4$  |                  |

* From analysis on a side reaction appearing in stopped flow traces, a value of 1.6 x 10$^3$ M$^{-1}$ s$^{-1}$ had been given for detergent-solubilized hog thyroid peroxidase (14).

* The larger value was obtained by adding diiodotyrosine at a time when the Compound II concentration was decreased to about one-half. The larger value seemed accurate, though its precise estimation became difficult.
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**TABLE II**

| Electron donor     | Rate constants \( (10^8 \text{ M}^{-1} \text{s}^{-1}) \) for rate-limiting reactions at pH 7.4 |
|--------------------|-----------------------------------------------------------------------------------------------|
|                    | Overall | Stopped flow | Overall | Stopped flow | Overall | Stopped flow |
| L-Tyrosine         | 1.0 (0.753) \(^a\) | 1.1 (2.0) \(^b\) | 6.3 (10.3) \(^a\) | 9.8 | 10 \(^c\) | 57 \(^d\) |
| D-Tyrosine         | 0.4 (1.55) \(^a\) | 0.7 | 5.3 (6.89) \(^a\) | 8.3 | 13 \(^c\) | 65 \(^c\) |
| N-Acetyltyrosinamide | 8.3 | 17.0 | 15.0 | 15.0 | 38 \(^c\) | 124 |
| Monoiodotyrosine   | 1.7 | 1.6 | 11.2 | 14.0 | 22 \(^c\) | 37 \(^c\) |
| Diiodotyrosine     | 2.7 | 2.2 (1.7) \(^b\) | 1.7 | 2.1 | 17 | 37 |

\(^a\) Values in parentheses were given at pH 8.2 and 25 \(^\circ\)C by Bayse et al. \((27)\).

\(^b\) Values in parentheses were given at 25 \(^\circ\)C by Ralston and Dunford \((44)\).

\(^c\) Values should be for \(k_i^t\) and others for \(k_i\) (see the text).

\(^d\) A value of 75 was obtained in our previous experiment \((15)\).

\(^e\) Data on D-tyrosine are not shown in figures, but the reaction patterns were similar to those for L-tyrosine.

\(^f\) Only this value is measured at pH 5.5.

![Fig. 8. The pH activity curves (A) on thyroid peroxidase-catalyzed oxidations of L-tyrosine (Tyr), monoiodotyrosine (MIT), and diiodotyrosine (DIT). The reaction mixtures contained 50 \(\mu\text{M} \text{ H}_2\text{O}_2, 0.5 \text{ mM Tyr, MIT, or DIT, and 0.04 \text{ mM thyroid peroxidase at pH 7.4. The reactions were followed at 310 nm for Tyr and at 340 nm for MIT and DIT. In B, the pH dependence of } \Delta \text{abs}_{340} \text{ used for the calculation is shown.}]

In Table II, rate constants estimated from the stopped flow traces (Figs. 1 and 3) according to Chance's equation (21) are also listed. The rate constant would be for Reaction 3 when the enzyme intermediate observed was Compound II, and for Reaction 2' when the intermediate was Compound I. The \(k_i^t\) value thus obtained would deviate from its true value as the concentration of electron donor decreases, because the reduction of Compound I to Compound II by endogenous donor becomes predominant. The biphasic decay of Compound I in the reaction with monoiodotyrosine (Fig. 3) might be explained by this effect, and the rate constant calculated from the trace would be a tentative value.

The pH dependence of thyroid peroxidase activity has been measured for oxidations of guaiacol \((23, 24)\) and iodide \((24, 25)\), and for iodination \((9, 10, 25)\) and coupling \((10)\). The measurement of the rates of oxidations of tyrosine and their derivatives at varying pH value was not easy because of the pH dependence of absorbance \((26, 27)\). Fig. 8A shows pH activity curves for thyroid peroxidase when the electron donor was L-tyrosine, monoiodotyrosine, and diiodotyrosine. The rates of oxidation were estimated by using data shown in Fig. 8B. There was a remarkable difference in the optimal pH between monoiodotyrosine and diiodotyrosine. The difference would be ascribable to the fact that the phenolic group is deprotonated more easily in diiodotyrosine than in monoiodotyrosine.

**DISCUSSION**

Since a typical feature in peroxidase reactions is the formation of radical species of bivalent redox molecules, it has been suggested that free radicals are formed as intermediates in the processes of thyroid hormone biosynthesis, particularly in iodination \((2, 4, 5, 13, 28, 29)\) and in the coupling reaction \((2, 4, 13)\). Björkström \((30)\), however, found that iodide reduced Compound I of horseradish peroxidase directly to the ferric form. The mechanism was confirmed by stopped flow experiments \((31)\). From the analysis of enzyme intermediates during the oxidation of iodide, thyroid peroxidase and lactoperoxidase were found to catalyze a two-electron oxidation of iodide \((14)\). Therefore, the enzymatic iodinating species is not an iodine free radical, but probably an iodinium cation, as suggested by several workers \((2, 12, 14, 25, 32, 33)\). The proposed mechanism would also explain the inhibitory action of anti-thyroid agents and excess iodide on the peroxidase-catalyzed iodination \((34, 35)\).

Now, a similar question is directed to the coupling reaction. We suggest, from data shown in Figs. 3 and 4, that, at physiological pH, diiodotyrosine is oxidized to its free radical by the thyroid peroxidase system. The rate constant for the reduction of Compound II by diiodotyrosine at pH 7.4 is measured by three different methods, as shown in Figs. 3, 5, and 7. The values thus obtained lie between \(1 \times 10^4\) and \(3.7 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) (Tables I and II). The rate constant might be concluded to be about \(2 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\), though further experiments are needed for its accurate estimation.

The reactions of Compound I of thyroid peroxidase with L- and D-tyrosines, N-acetyltyrosinamide, and monoiodotyrosine are rather exceptional. The accumulation of Compound I in the steady state of peroxidase reactions would be interpreted in terms of alternative mechanisms.

\[ \text{AH}_2 \xrightarrow{\text{Compound I}} \text{AH}^- \]

**Case 1**

\[ \text{AH}_2 \xrightarrow{\text{Compound I}} \text{AH}^- \xrightarrow{\text{peroxidase}} \]

When the electron donor is L-tyrosine, the mechanism is in accord with Case 1, because the reduction of Compound II by L-tyrosine is too slow to explain the kinetic data by the Case 2 mechanism \((14)\). Similarly, the Case 2 mechanism can be
Electron transfer mechanism is affected by experimental conditions. As regards the pH dependence, a two-electron mechanism tends to change into a one-electron type at the pH increase. Such reactions are reductions of O₂ and p-benzoquinone by the xanthine oxidase system (40-42) and the oxidation of sulfite by the horseradish peroxidase system (43). The reason might be that the two-electron transfer is feasible only when the supply of proton is not limited at a crucial moment of the electron transfer. The fact that diiodotyrosine deprotonates with a pKₐ value of 6.37 (44) may be related to the pH-dependent change in the mechanism of electron transfer from diiodotyrosine to Compound I of thyroid peroxidase. It has been reported (45, 46) that the reduction of Compound I of horseradish peroxidase to the ferric enzyme is accompanied by an uptake of 2 protons. The apparent two-electron transfer from L-tyrosine and moniodotyrosine to Compound I of thyroid peroxidase is notable as regards the coupling mechanism. The primary product of phenol compounds (ROH) is a phenoxy radical (RO·) in a one-electron mechanism and probably a phenoxy cation (RO⁺) in a two-electron mechanism. The two-electron transfer tends to occur in a specifically bound complex between enzyme and substrate (17). An apparent two-electron oxidation has also been proposed for oxidation of o-dianisidine by Compound I of horseradish peroxidase, although the enzyme intermediate in the steady state is not Compound I itself in this case (47).

According to Josephy et al. (48), however, this reaction may not be a two-electron oxidation.

Several investigators have questioned whether thyroid peroxidase functions specifically and differently in thyroid hormone biosynthesis, as compared with other peroxidases (1, 9, 10, 14, 15, 28, 49-51). Regarding iodination, lactoperoxidase is as active or more active than thyroid peroxidase (9, 49). Nunez (4) has suggested specificity of thyroid peroxidase in the coupling reaction. Michot et al. (52) reported that iodide increases the rate of tyrosine coupling in the presence of thyroid peroxidase, but not of horseradish peroxidase and lactoperoxidase. As shown in Table II, thyroid peroxidase catalyzes the oxidations of tyrosine and diiodotyrosine much faster than others do. Thyroid peroxidase and lactoperoxidase resemble each other in many respects, but a distinct difference between the two enzymes is now found in the mechanism of oxidation. Its physiological implication is most interesting and should be elucidated by further experiments. Since the coupling reaction in vivo occurs in thyroglobulin molecules, the environment of tyrosyl groups would control both iodination and coupling reactions. We are now studying whether the mechanism derived from the kinetic data with free L-tyrosine and iodotyrosines is applicable to the reactions in thyroglobulin.

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