Characterization of Hog Thyroid Peroxidase*

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Several fundamental properties of purified hog thyroid peroxidase ($A_{413}$/$A_{480} = 0.55$) were investigated in comparison with bovine lactoperoxidase. The $M_r$ of thyroid peroxidase was 71,000. The prosthetic group of thyroid peroxidase was identified spectrophotometrically as protoheme IX after the enzyme was hydrolyzed with Pronase.

Optical spectra of oxidized and reduced thyroid peroxidases and their complexes with azide and cyanide were very similar to lactoperoxidase, except that lactoperoxidase had two reduced forms with the Soret band either at 446 or 435 nm, and thyroid peroxidase lacked a reduced form having the 446-nm band. From comparison of their pyridine hemochrome spectra, $e_{435}$ at 413 nm of thyroid peroxidase was estimated to be 114, being the same as that of lactoperoxidase.

The cyanide inhibition for the reaction of thyroid peroxidase was competitive with hydrogen peroxide and the inhibition constant was in rough accord with the dissociation constant of its cyanide complex measured from spectrophotometric titration. Azide inhibited the reaction with an inhibition constant which was about one one-thousandth of the dissociation constant for its spectrally discernible complex. The azide inhibition was not competitive with hydrogen peroxide and decreased as the reaction proceeded. Aminotriazole inhibited the reaction strongly, and the inhibition was augmented during the reaction. These inhibition patterns of azide and aminotriazole were more or less observed in the reaction of lactoperoxidase, but not in the case of horseradish peroxidase. Characteristics of animal peroxidases are discussed.

The existence of a peroxidase in the thyroid was confirmed by Dempsey (1) from histochemical experiments, and subsequently many workers have reported purification and characterization of the enzyme (2-13). It is beyond doubt that the enzyme is a hemoprotein, but many fundamental properties are not yet clear. For instance, its reported molecular weight ranges from 45,000 to 104,000 (2, 4-8, 10-12), and its spectral data are also different as shown in Table I. Even the simple question of whether the spectral properties of thyroid peroxidase resemble those of horseradish peroxidase or lactoperoxidase has not yet been answered (2, 6-9).

The purity of some hemoproteins has been conveniently expressed as the ratio of absorbance of the Soret band to that at 280 nm. The highest ratio so far reported for thyroid peroxidase is 0.54 (11) and 0.55 (12). Spectral data on such purified preparations, however, are quite few probably because of the difficulty in getting it in sufficient quantity for the measurements. We have carried out a series of kinetic experiments with purified thyroid peroxidase (14-17). From a rough comparison we suggested that thyroid peroxidase resembled lactoperoxidase (15). In order to determine the concentration of thyroid peroxidase we used a tentative value of 114 for $e_{435}$ at 413 nm (15-17), which had been reported for lactoperoxidase (18, 19).

The optical spectra of intestine (20-22) and uterus (23, 24) peroxidases were reported to be similar to those of lactoperoxidase. Recently, it was also shown in several laboratories (25-27) that the spectra of eosinophil peroxidase are similar to those of lactoperoxidase. Therefore, we thought it very important to investigate some fundamental properties of thyroid peroxidase in comparison with lactoperoxidase. Some results obtained with lactoperoxidase are described in the Miniprint.1

MATERIALS AND METHODS

Hog thyroid peroxidase used in this experiment was prepared from 20 kg of hog thyroid glands according to the method described previously (15). The ratio of $A_{413}$ to $A_{280}$ of our enzyme preparation was 0.55 (Fig. 2), being highest among the preparations so far used in our laboratory. We did not perform further purification and obtained enough of the enzyme for spectral measurements. Bovine lactoperoxidase with a ratio $A_{413}$/$A_{280}$ of 0.92 was used (43). Pronase E (100 x $10^6$ units/g) was obtained from Kaken Chemicals Co. (Tokyo), trypsin (Type 1) from Sigma, and aminotriazole (3-amino-1,2,4-triazole) from Nakarai (Kyoto).

The reactions and spectrophotometric experiments were carried out in 0.1 M potassium phosphate buffer, pH 7.4, at 20 °C (17), unless otherwise noted.

RESULTS

With the use of high-performance liquid chromatography gel filtration, a value of 71,000 ± 1,000 for the $M_r$ of hog thyroid peroxidase was obtained (Fig. 1). Other trace proteins were not detected under the experimental conditions. This molecular weight was comparable with that measured by Danner and Morrison (8) using disc gel electrophoresis in the presence of sodium dodecyl sulfate. We found, however, that the gel electrophoresis of our preparations gave complex pattern.

1 Portions of this paper (including part of "Results," Tables I and II, and Figs. 1-5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 94M-1210, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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**Fig. 1.** *M,* determination of hog thyroid peroxidase. Thyroid peroxidase (TPO) and marker proteins were subjected to gel filtration TSK G3000SW (7.5 × 600 mm) + GSWP (7.5 × 75 mm) equilibrated with 0.1 M phosphate buffer (pH 7.5). The flow rate was 0.5 ml/min. Abbreviations: GO, glucose oxidase (160,000); G6PD, glucose-6-phosphate dehydrogenase (104,000); BSA, bovine serum albumin (68,000); OA, ovalbumin (45,000); HRP, horseradish peroxidase C (40,000); and CTG, chymotrypsinogen (25,000). *M,* is denoted in parentheses.

**Fig. 2.** Spectra of thyroid peroxidase and its cyanide complexes. The cyanide complex of ferric thyroid peroxidase (---) was formed by the addition of 0.5 mM potassium cyanide to 1.8 μM thyroid peroxidase (—). The dotted spectrum was obtained after the addition of a few crystals of sodium dithionite to the complex. The inset shows a dissociation curve for cyanide complex of ferric thyroid peroxidase.

Cyanide and azide are known as inhibitors for hemoprotein enzymes. From plots of the inhibition degree against the concentration of the inhibitors, the inhibition constant was estimated to be about 9 μM for cyanide (Fig. 3, inset). Upon reduction of the complex with dithionite, a new spectral species was observed. The observed spectrum was less resolved in the visible region as compared with that of lactoperoxidase (see Miniprint). As the visible spectrum differed distinctly from that of the reduced peroxidase, the spectral species was concluded to be an azide complex of the reduced enzyme in thyroid peroxidase as well as in lactoperoxidase.

Azide reacted with thyroid peroxidase to form a complex with a dissociation constant of 1.9 mM (Fig. 3, inset). Upon reduction of the complex with dithionite, a new spectral species was observed. The observed spectrum was less resolved in the visible region as compared with that of lactoperoxidase (see Miniprint). As the visible spectrum differed distinctly from that of the reduced peroxidase, the spectral species was concluded to be an azide complex of the reduced enzyme in thyroid peroxidase as well as in lactoperoxidase.

The cyanide complex of ferric thyroid peroxidase (- - -) was formed by the addition of 0.5 mM potassium cyanide to 1.8 μM thyroid peroxidase (—). The dotted spectrum was obtained after the addition of a few crystals of sodium dithionite to the complex. The inset shows a dissociation curve for cyanide complex of ferric thyroid peroxidase.
between $K_i$ and $K_c$. The result obtained indicated $K_i = K_c$. This was contrary to our expectations and the reason is at present unexplained. On the other hand, the azide inhibition was noncompetitive for hydrogen peroxide (Fig. 5B). Since azide has been shown to coordinate at the heme iron to form a spectrally discernible complex with a dissociation constant of 1.9 mM (Fig. 3), it seemed that the inhibition occurring at several $\mu$M of azide was due to another type of interaction, probably between azide and an amino acid residue involved in the catalytic function of thyroid peroxidase. Anomalies of the azide inhibition were seen in the time course of the reaction. Fig. 6 shows that the azide inhibition decreased as the reaction proceeded. Since the same time course was observed when the reaction was started either by the addition of enzyme or hydrogen peroxide, the apparent lag phase was not ascribable to slow dissociation of the azide complex. The mechanism is not yet clear, but there is a possibility that azide is oxidized by the enzyme system. Lactoperoxidase Compound II was found to be reduced by azide with a second-order rate constant of $10^{-2}$ s$^{-1}$. The oxidation of azide by Compound II of thyroid peroxidase could not be measured due to shortage of the enzyme. An inhibitory pattern similar to that shown in Fig. 6 was also observed in the reaction of lactoperoxidase (see Miniprint) but not in the reaction of horseradish peroxidase. Therefore, the decrease of the azide inhibition during the reaction was not ascribable to an interaction between azide and an oxidation product of guaiacol.

Aminotriazole was once found to inactivate catalase in vivo systems (32) and later was found to inactivate lactoperoxidase (33, 34) as well as catalase (35, 36) in vitro systems where hydrogen peroxide was supplied continuously. The latter type of inhibition occurred effectively in the case of thyroid peroxi-
oxidase. Fig. 7 shows that the aminotriazole inhibition increased during the reaction, suggesting that the reaction was inhibited through a reaction of aminotriazole with a reaction intermediate or a substrate complex of thyroid peroxidase. A similar inhibition pattern was observed in the reaction of lactoperoxidase although about a 10-fold concentration of aminotriazole was needed for the same extent of inhibition (see Miniprint).

The Soret difference spectra occurring from the reaction of thyroid peroxidase with hydrogen peroxide had been given by stopped-flow spectrophotometry at varying wavelengths (14, 15). Here we recorded for the first time absolute spectra of hydrogen peroxide compounds of thyroid peroxidase in the Soret and visible regions by the addition of 20 μM H2O2 (Fig. 8A). The reaction product we observed was Compound II but not Compound I because of the fast conversion of Compound I to Compound II (15). A further addition of 200 μM hydrogen peroxide to Compound II resulted in the formation of oxyperoxidase (Compound III), which could be identified from the typical visible bands at 551 and 590 nm. The visible spectra of Compound II and the oxyform of thyroid peroxidase were similar to the respective spectra of lactoperoxidase and intestinal peroxidase (37). As compared with lactoperoxidase and other peroxidases, thyroid peroxidase was relatively unstable in the presence of excess hydrogen peroxide (10), decomposing to a compound having a very weak absorption band in the Soret region (Fig. 8B).

Upon reduction with dithionite, lactoperoxidase (18, 38) and intestinal peroxidase (39) both gave at least two spectral species, the ratio of which depended on pH and incubation time. Such complexity might be the case with reduced thyroid peroxidase. The Soret absorption band of reduced thyroid peroxidase so far reported is scattered over a range from 415 to 427 nm, as shown in Table I. The Soret absorption band of the CO complex was more intensified when the enzyme was reduced with dithionite after the enzyme solution was bubbled with CO (Fig. 9).

FIG. 8. Spectra of Compounds II and III of thyroid peroxidase (A) and degradation of the peroxidase heme (B). A, compound II (-----) was formed by the addition of 20 μM H2O2 to 1.7 μM thyroid peroxidase (----). Compound III (-----) was formed by the addition of 200 μM H2O2 to Compound II. Spectra were recorded from 700 nm at a speed of 150 nm/min, immediately after H2O2 was added. B, the scan was repeated from 700 nm at indicated times (min) after 200 μM H2O2 was added to Compound II. The dotted line was recorded after 1 mM potassium cyanide was added finally.

of our present preparation was located invariably at 433 nm, although the ratio of absorbance of the ferrous to the ferric enzymes varied in a range between 0.9 and 1.2 with time after reduction and with the kind of preparation. In some experiments with less purified preparations, the absorption peak of the ferrous enzyme gradually shifted from 433 nm to shorter wavelengths. It is also true that less-purified preparations tend to have a Soret peak at a shorter wavelength than the inherent one because absorbance due to contamination is apt to increase with the decrease in wavelength. Fig. 9 shows results on a purified preparation giving the highest relative absorbance at 433 nm. When CO was bubbled in the presence of excess dithionite, the spectrum changed to that of a CO complex of the reduced enzyme. The absorbance of the Soret band of the CO complex was more intensified when the enzyme was reduced with dithionite after the enzyme solution was bubbled with CO (Fig. 9).

The nature of the thyroid peroxidase heme has caused much discussion. From a spectrophotometric analysis of its alkaline pyridine hemochrome, it was concluded by Taurog and his co-workers (7, 11) that the heme of thyroid peroxidase is not protoheme IX. The conclusion was derived from the difference in the position of α-band of the pyridine hemochrome between protoheme IX and the heme of thyroid peroxidase. Fig. 10A shows absorption spectra of alkaline pyridine hemochrome of our preparation of thyroid peroxidase. The position of α-band was consistent with a value of 562 nm reported by Taurog et al. (7). We were interested in comparing the data with those of lactoperoxidase because similar questions had been directed to the chemical nature of lactoperoxidase heme (40). Using Pronase-treated lactoperoxidase, Carlström (18) and Sievers (40) concluded that the heme of lactoperoxidase was identified as protoheme IX. Similar experiments were carried out with
prothione IX at least when it is freed from the protein by proteolysis. The alkaline pyridine hemochromes of thyroid peroxidase and lactoperoxidase without the Pronase treatment would be a mixed ligand complex with pyridine only on one side of protoporphyrin IX, as suggested by Sievers in lactoperoxidase.

As reported by Sievers (40), the heme was degraded during Pronase hydrolysis and the molar absorption coefficient of thyroid peroxidase could not be measured in comparison with pyridine hemochrome of protoporphyrin IX. Assuming that the molar absorption coefficients of alkaline pyridine hemochromes of lactoperoxidase and thyroid peroxidase are the same, we could compare the values of the two enzymes. The absorption coefficient of \( \alpha \)-band of alkaline pyridine hemochrome of lactoperoxidase was estimated to be 22.2 on the basis that \( \epsilon_{413} \) of lactoperoxidase = 114 (19). Since a thyroid peroxidase solution with absorbance at 413 nm = 0.153 gave alkaline pyridine hemochrome having absorbance at 562 nm = 0.030, \( \epsilon_{413} \) of thyroid peroxidase was estimated to be 114, the same value as that of lactoperoxidase.

**DISCUSSION**

From the present work we conclude that thyroid peroxidase is not identical but is very similar to lactoperoxidase. Since spectral similarities have been observed between lactoperoxidase and other animal peroxidases of intestine (20–22), uterus (23, 24), and eosinophil granule (25–27), it can be said that there is a group of animal peroxidases that resemble each other in spectral properties (Table II). The spectral similarities will be summarized as follows. 1) These peroxidases exhibit a Soret band at 412–415 nm and a 4-band oxidized visible spectrum. 2) Their cyanide complexes exhibit a Soret band at 429–430 nm and a visible band at 553–555 nm. 3) The \( \alpha \)-band of their pyridine hemochrome is situated at a wavelength slightly higher than the corresponding peak derived from protoporphyrin IX. 4) The main visible band of the reduced peroxides is situated at 563–565 nm. In these respects, these animal peroxidases differ from myeloperoxidase as well as typical high-spin protoporphyrin hemoproteins, such as hemoglobin, myoglobin, and plant peroxidases.

There is, however, some confusion in the above spectral classification. In general the reduced animal peroxidases are unstable and their Soret band varies with experimental conditions (18, 39) and enzyme preparations (25–27). It seems that there are at least two spectral species having the Soret and visible regions (18, 39). This conversion becomes faster at acidic pH and the partial proteolysis of the enzyme makes the A form unstable. The most important problem to be solved will be why the A form does not appear in the reduced thyroid peroxidase. It might be explained simply by assuming that our enzyme preparation is slightly modified through partial digestion with trypsin (15) without changes in the spectral and catalytic properties of the ferric enzyme. At the moment, we cannot exclude the possibility that the lack of the A form is an inherent nature of thyroid peroxidase. At any rate, the difference between the A and B forms is probably ascribed to the arrangement of distal groups in the reduced peroxides, because the diversity in spectral data disappears when cyanide or CO is coordinated to the heme iron of the reduced enzymes.

Besides the spectral properties, the following properties also appear to be characteristics of the animal peroxidases represented by lactoperoxidase. 1) These peroxidases consist...
of basic protein and are purified mostly by cation-exchange chromotography. This is the case with myeloperoxidase. 2) The heme is tightly bound to the protein and is hardly freed by the extract with organic solvent at acidic pH. This is also the case with myeloperoxidase. Morell and Clezy (41) suggested that an ester linkage is present between the heme and protein in lactoperoxidase. 3) These peroxidases have relatively high affinity for protein in lactoperoxidase.

**Peroxidase isoenzymes are known to have high affinity for protein in lactoperoxidase.**

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The inhibition pattern (Fig. 7) suggests that amidinozole inhibition progresses during the reaction, although it is confirmed only with peroxidases from milk, thyroid, and intestine. The inhibition pattern (Fig. 7) suggests that amidinozole reacts with peroxidase Compound I or II to inactive the enzyme. The irreversible inhibition of lactoperoxidase and thyroid peroxidase caused by methyl mercaptomidazoles has been ascribed to its reaction with Compound II of the enzymes (15, 43). This type of inhibition is of particular interest from the physiological point of view, and its generalization should be confirmed by a systematic study.

**REFERENCES**

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This paper continued that thyroid peroxidase resembles lactoperoxidase in many respects. Here, we supplement the lactoperoxidase data which are related to our conclusion but not reported so far.

The spectra of azide complexes of lactoperoxidase were similar to those of thyroid peroxidase and the resolution was better as shown in Fig. 1. Particularly in the visible spectrum of the azide complex of reduced lactoperoxidase. The reaction of lactoperoxidase was completely inhibited by azide of a concentration which gave no change in the absorption spectrum of the enzyme. At certain concentrations of azide a lag phase was observed in the reaction of lactoperoxidase with guaiacol. The inhibition of lactoperoxidase by azide was irreversible and the inhibition by azide of a concentration which inhibited the lactoperoxidase completely was increased in contrast, the inhibition by azide of a concentration which inhibited the lactoperoxidase at a certain concentration of azide was increased. The reaction of lactoperoxidase was completely inhibited by azide of a concentration which gave no change in the absorption spectrum of the enzyme. At certain concentrations of azide a lag phase was observed in the reaction of lactoperoxidase with guaiacol. The inhibition of lactoperoxidase by azide was irreversible and the inhibition by azide of a concentration which inhibited the lactoperoxidase completely was increased in contrast, the inhibition by azide of a concentration which inhibited the lactoperoxidase at a certain concentration of azide was increased.

The spectra of azide complexes of lactoperoxidase resembled the lactoperoxidase spectra after the enzyme had been reduced with dithionite. The spectra of azide complexes of lactoperoxidase were similar to those of thyroid peroxidase. The reaction of lactoperoxidase was completely inhibited by azide of a concentration which gave no change in the absorption spectrum of the enzyme. At certain concentrations of azide a lag phase was observed in the reaction of lactoperoxidase with guaiacol. The inhibition of lactoperoxidase by azide was irreversible and the inhibition by azide of a concentration which inhibited the lactoperoxidase completely was increased in contrast, the inhibition by azide of a concentration which inhibited the lactoperoxidase at a certain concentration of azide was increased. The reaction of lactoperoxidase was completely inhibited by azide of a concentration which gave no change in the absorption spectrum of the enzyme. At certain concentrations of azide a lag phase was observed in the reaction of lactoperoxidase with guaiacol. The inhibition of lactoperoxidase by azide was irreversible and the inhibition by azide of a concentration which inhibited the lactoperoxidase completely was increased in contrast, the inhibition by azide of a concentration which inhibited the lactoperoxidase at a certain concentration of azide was increased.

Fig. 1. Spectra of azide complexes of lactoperoxidases. The azide complex of reduced lactoperoxidase (---) was formed by the addition of 100 mM sodium azide to 5.5 nM lactoperoxidase (-). The dotted spectrum was obtained by the addition of a few crystals of sodium dithionite to the complex. The absorbance was expanded 2.5-fold in the visible region.

Fig. 2. Effect of azide on the guaiacol oxidation catalyzed by lactoperoxidase (LPO). The reaction mixture contained 24 nM LPO, 1.2 mM guaiacol, 110 pM H_2O and AT (the concentration is denoted in the figure). AT was added after LPO in A and before LPO in B.

Fig. 3. Effect of azide on the guaiacol oxidation catalyzed by lactoperoxidase. The reaction mixture contained 24 nM LPO, 1.2 mM guaiacol, 110 pM H_2O and AT (the concentration is denoted in the figure). AT was added after LPO in A and before LPO in B.

Fig. 4. Time-dependent spectral change of trypsin-treated reduced lactoperoxidase. Lactoperoxidase (6.1 nM) was incubated with 1.3 mg trypsin in 1 ml of 0.1 M potassium phosphate, pH 7.4, for 7 h at 30°C (---). Then, the spectra were recorded at 550 nm (- -), and 55 min after (- - - -).
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Table I
Spectral data reported on thyroid peroxidase

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<tr>
<th>Source</th>
<th>Fe^{3+}</th>
<th>Fe^{2+-}CN</th>
<th>Fe^{2+}</th>
<th>Fe^{2+-}CO</th>
<th>Pyridine hemochrome</th>
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<tr>
<td>Mallof and Sondak (1)</td>
<td>Calf</td>
<td>412</td>
<td>423</td>
<td>418</td>
<td>370</td>
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<td>Yip (2)</td>
<td>Beef</td>
<td>410</td>
<td>415</td>
<td>427</td>
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<td>Pig</td>
<td>420</td>
<td>415</td>
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Table II
Comparison of absorption maxima of some peroxidases

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<th>Fe^{2+}</th>
<th>Fe^{2+-}CO</th>
<th>a-band of pyridine hemochrome</th>
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<td>433,550,565,590</td>
<td>433,536,567,576</td>
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<td>Milk (b)</td>
<td>430,502,587,586</td>
<td>430,555,585</td>
<td>445</td>
<td>435,555,565,590</td>
<td>445,536,567,576</td>
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<tr>
<td>Intestine (c)</td>
<td>414,505,586,640</td>
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<td>450,555,565</td>
<td>435,535,565,590</td>
<td>560,566,571</td>
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<tr>
<td>Uterus (d)</td>
<td>414</td>
<td>453,500,586</td>
<td>445</td>
<td>435,555,565,590</td>
<td>445,536,567,576</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of pronase-pretreatment on the alkaline pyridine hemochrome of lactoperoxidase. The absorbance was expanded 2-fold in the visible region. A, 2.5 μM pyridine and 70 μM NaOH (both were final concentrations) were added to 7.25 μM lactoperoxidase. By this procedure the enzyme was converted to pyridine hemochrome (———) upon the addition of a few crystals of sodium dithionite. B, lactoperoxidase (2.7 μM) was incubated with 20 units pronase in 1.0 ml 10.1 M potassium phosphate, pH 7.4) for 18 h at 38°C. Then, the spectrum was greatly modified (———). Pyridine hemochrome (———) and hemochrome (———) were made as described in A.