Studies of the Biosynthesis of Thyroxine

I. PURIFICATION AND PROPERTIES OF A PARTICULATE IODIDE PEROXIDASE FROM THYROID TISSUE*

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Previously, a number of workers have described preparations of thyroid tissue which catalyze the iodination of free or protein-bound tyrosine (1-7). It was demonstrated that the enzymatic activity was mitochondrial in location, and depending upon the method of preparation or assay, various metals and cofactors were implicated in the reaction. Serif and Kirkwood (1, 2) found that addition of certain metals increased activity, whereas Chaisoff, Taurog, Potter, and Tong (3, 4) reported that flavin mononucleotide and manganous ions stimulated enzymatic catalysis. Alexander (5) suggested that the oxidation of iodide is a peroxidation and found that the addition of hematin to preparations of thyroid tissue stimulated activity (6). In each of these studies, the activity of the enzymes was followed by measuring the production of iodinated tyrosines with radioactive iodine.

The present paper describes the purification and properties of a highly active particulate enzyme system isolated from beef thyroid tissue which catalyzes the peroxidation of iodide to iodine.

EXPERIMENTAL PROCEDURE

Materials and Methods—The amino acids, p-chloromercuri-phenylsulfonic acid, cytochrome c, glucose oxidase (Sigma type III 30,000 to 45,000 e.u. per g), and Trypsin were obtained from the Sigma Chemical Company. Thyroglobulin was obtained from DiCof Laboratories. Trypsin, 1/300 brand, was obtained from Nutritional Biochemicals Corporation. EDTA was obtained from the J. T. Baker Chemical Company, and eosin from Allied Chemical Company.

Thyroglobulin was prepared by the method of Derrien, Michel, and Roche (8). Protein was determined spectrophotometrically by the biuret method (9). Hemin was prepared by the method of Labbe and Nishida (10). Iodine was determined spectrophotometrically at 612 mμ as a starch-iodine complex with a molar absorptivity of 20 X 10³ m⁻¹ cm⁻¹.

Preparation of Enzyme—Beef thyroid was obtained at the slaughterhouse and transported to the laboratory in ice. All subsequent steps were carried out at 0-4°. The glands were cleaned of fat and connective tissue, passed through a meat grinder, and suspended in an equal volume of a solution of 0.155 m KCl, 0.001 m EDTA, and 0.02 m Tris-chloride buffer, pH 6.5. The suspension was homogenized in a Virtis high speed blender for 4 minutes at 7,500 revolutions per minute, and the homogenate was centrifuged for 20 minutes at 1,000 X g. The supernatant was filtered through two layers of cheesecloth and centrifuged at 90,000 X g for 20 minutes in a Spinco model L preparatory ultracentrifuge. The pellet (Residue I) was washed twice by centrifugation with the buffered solution described above and suspended in the same solution. The protein of the suspension was adjusted to 20 mg per ml, and 0.5 mg of deoxycholate per mg of protein was added slowly with stirring. The suspension was centrifuged at 105,000 X g for 30 minutes in the Spinco ultracentrifuge, and the residue was suspended in sufficient solution of 0.155 m KCl, 0.001 m EDTA, and 0.02 m Tris-chloride buffer, pH 8.5, to obtain a protein concentration of 10 mg per ml. Trypsin (0.5 mg per 10 mg of protein) was added, and the mixture was incubated for 30 minutes at 25°, then centrifuged at 105,000 X g for 30 minutes. The pellet was suspended in the pH 8.5 buffered solution described above and represents the enzyme preparation (Residue II) used in the following studies. Preparations of the enzyme stored at -20° for periods of 2 to 4 days retained 70 to 80% of their original activity.

Method of Assay—Assays were carried out in 25-ml Erlenmeyer flasks. The incubation mixture contained 100 μmoles of glucose, 0.5 μmole of potassium phosphate buffer at pH 7.0, 25 μmoles of KI, 100 μg of glucose oxidase, tyrosine or other iodine acceptor, 10 μmoles of sufficient enzyme protein to utilize 5 to 10 μmoles of iodide per hour (usually 0.5 to 10 mg of protein, depending upon the stage of purification), and water to make a final volume of 4.0 ml. The assay mixture was incubated with mild agitation in a Dubnoff shaker for 60 minutes at 38°. The reaction was stopped by deproteinizing with tungstic acid according to the method of Folin and Wu (11). Aliquots of the deproteinized solution were adjusted to pH 4.5, and the inorganic iodide remaining was determined by the method of Fajans (12).

Chromatography—Samples to be chromatographed were handled in the same manner as above, but instead of being deproteinized with tungstic acid, the reaction mixture was adjusted to pH 2.0 with H₂SO₄, and then extracted three times with equal volumes of 1-butanol saturated with water which was 50% saturated with Na₂S₂O₃. The combined butanol extracts were evaporated to dryness, and the residue was taken up in 0.5 ml of 2 N NH₄OH. Aliquots of 25 μl were applied to Whatman No. 1 filter paper for ascending chromatography in two systems:

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1-butanol-acetic acid-H2O, 4:1:1; and 1-butanol-2 NH4OH,
1:1. Tyrosine and thyrionin compounds were detected by
reaction with diazotized sulfanilic acid (13).

RESULTS

Purification and Distribution of Enzyme System—In a prelimi-
nary report (14), we have described the recovery of activity
during purification of the enzyme system. The final enzyme

<table>
<thead>
<tr>
<th>Tissue tested</th>
<th>µ moles/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residue II (2 mg of protein)</td>
<td>12.5</td>
</tr>
<tr>
<td>Residue II boiled 5 minutes (2 mg of protein)</td>
<td>0</td>
</tr>
<tr>
<td>Beef heart mitochondria (5 mg of protein)</td>
<td>0</td>
</tr>
<tr>
<td>Rat liver mitochondria (5 mg of protein)</td>
<td>0</td>
</tr>
<tr>
<td>Rat liver microsomes (5 mg of protein)</td>
<td>0</td>
</tr>
<tr>
<td>DPNH oxidase (beef heart) (5 mg of protein)</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome c oxidase (beef heart) (5 mg of protein)</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome c (purified) (10 mg)</td>
<td>4.8</td>
</tr>
<tr>
<td>Cytochrome c (purified) (5 mg)</td>
<td>4.3</td>
</tr>
<tr>
<td>Cytochrome c (boiled 5 minutes) (10 mg)</td>
<td>4.5</td>
</tr>
</tbody>
</table>

FIG. 1. Utilization of iodide as a function of time. Assays were
performed as described in “Experimental Procedure” with 2 mg of
enzyme protein in each assay. Incubation was stopped at 15,
30, 60, and 120 minutes.

Fig. 2. Utilization of iodide as a function of the amount of
enzyme protein used. Assays were performed in the standard
manner for 1 hour as described in the text. The amount of enzyme
protein used was 0.5, 1.0, 2.0, and 3.0 mg.

preparation represents 20% of the total units of activity in the
crude particulate fraction (Residue I). The method of assay
was not sufficiently sensitive to detect activity in the crude
thyroid homogenates and in the cellular and nuclear debris
separated by low speed centrifugation. The activity of the un-
washed residue at 90,000 × g and the final product varied from
preparation to preparation. The most active preparations
catalyzed the peroxidation of over 10 μmoles of iodide per mg of
protein per hour, but most preparations catalyzed the peroxida-
tion of approximately 5 to 6 μmoles of iodide per mg of protein
per hour. The activity of unwashed residue at 90,000 × g varied
depending upon the volume of buffer used in preparation, since
washing served to remove inactive protein. When the enzyme
was prepared in unbuffered KCl or in potassium phosphate
buffer instead of the KCl-EDTA-Tris solution described in
“Experimental Procedure,” the activity of the washed residue at
90,000 × g was not appreciably affected; but preparations of the
trypsin residues were consistently less active and varied widely
between preparations. Solutions of polyvinylpyrrolidone and
glucose both proved to be unsatisfactory and yielded particles
of consistently low activity.

When amounts of the peroxidase were used that catalyzed the
disappearance of 10 μmoles of iodide per hour, it was found
necessary that the glucose-glucose oxidase system produce 15
μmoles of H2O2 per hour in order that H2O2 concentration would
not be rate-limiting. Production of H2O2 up to 100 μmoles per
hour was not injurious to the iodide peroxidase system. In the
studies reported here, enough glucose oxidase was provided to
produce 30 μmoles of H2O2 per hour. When the peroxidase
casused the disappearance of 10 or more μmoles of iodide per hour,
the experiment was repeated with less enzyme.

The preparations of enzyme contained small amounts of the
cytochromes associated with the terminal electron transport
systems and, in particular, cytochrome a. DPNH and succinic
oxidase activities were not present. A small amount of cyto-
chrome e oxidase activity was measurable.

A variety of other tissues were tested for iodide peroxidase
activity. Table I shows the absence of such activity in beef
heart and rat liver mitochondria, rat liver microsomes, beef heart
DPNH oxidase, and beef heart cytochrome e oxidase. Purified
cytochrome e catalyzed the disappearance of iodide from the
assay system, but this catalysis did not appear to be enzymatic,
since the rate of reaction was unaffected by heating the cyto-
chorme e at 100° for 5 minutes and was not proportional to the
amount of cytochrome e added to the assay.

Properties of Enzyme—Fig. 1 demonstrates the relationship
between the disappearance of iodide from the incubating solution
and the time of reaction. As shown in the figure, the uptake of
iodide is linear with time within the range of 10 to 12 μmoles of
iodide utilized.

Fig. 2 shows the relationship between the rate of iodide utiliza-
tion and the amount of enzyme protein added to the assay system.
As shown in the figure, the rate of reaction was zero when enzyme
was omitted from the assay. When increasing amounts of
enzyme protein were added, there was a linear increase in the
rate of iodide utilization up to the point where 10 to 12 μmoles
were used per hour.

Fig. 3 illustrates the effect of preincubation at increasing tem-
peratures on the catalytic activity of the enzyme. Before assay,
the enzyme was incubated at a variety of temperatures for 5
minutes. As can be seen, the enzyme maintained its full activity
...
Fig. 3. The effect of preheating on enzymatic activity. Aliquots of enzyme were incubated at the temperature indicated for 5 minutes. The assay was then performed in the standard manner as described in the text; 2 mg of protein were used for each assay.

after being incubated at 60° for 5 minutes, but lost all activity when incubated at 75° for 5 minutes. A number of other peroxidases, e.g. myeloperoxidase, show this tolerance to heat. Preparations of enzyme, when treated 30 minutes in a 10-ke Raytheon sonic oscillator, lost 80 to 90% of their activity. Freezing with Dry-Ice destroyed all activity.

The effect of variations in pH on the enzymatic activity of the preparations is shown in Fig. 4. In these studies, sufficient glucose oxidase was used at each pH to produce 30 μmoles of H₂O₂ per hour. As can be seen, the pH optimum is broad with a maximum at approximately pH 7. Although the lower pH values are below the buffering range of phosphate, there was little change in pH during the assay. The activity was the same over a wide range of ionic strength. When the assay was performed with Tris buffer instead of phosphate, the activity of the enzyme was one-third that found in phosphate buffer.

Table II shows the activity of iodide peroxidase with a variety of iodine acceptors. As can be seen, the enzyme preparation (Residue II) was inactive in the absence of an acceptor, or when diiodotyrosine or phenylalanine was used as acceptor. Monoiodotyrosine, histidine, and a variety of proteins acted as satisfactory acceptors, although the rates of iodide disappearance were not as rapid as with tyrosine. The products of the reaction when tyrosine, monoiodotyrosine, or protein was used as the acceptor were iodinated tyrosines as shown by chromatographic analysis (Table III). If protein was used as the acceptor, the incubation mixture was adjusted to pH 8.5 and digested with pancreatin for 12 hours before chromatography. When H₂O₂, 10 μmoles, was added directly to the enzyme system in the absence of tyrosine, iodine was formed as the final product as determined spectrophotometrically by the formation of the starch-iodine complex.

Further studies showed that although the activity of the iodide peroxidase was not inhibited by preincubation with concentrations of iodine as high as 5 mm for 15 minutes at 0°, glucose oxidase activity was 50% inhibited by 20 μM levels of iodine. Therefore, it would appear that the presence of an acceptor such as tyrosine is necessary in the peroxidase assay system to remove iodine and thus protect the glucose oxidase.

The following studies were performed to determine whether the reaction involving the iodination of tyrosine by iodine was enzymatic in nature. First, the nonenzymatic rate of iodination of tyrosine was studied under the conditions of our assay. In this experiment, 0.125 mmole of potassium phosphate buffer, pH 7.0, 2.0 mg of soluble starch, and varying amounts of iodine were placed in a cuvette with sufficient water to give a final volume of 0.90 ml. Tyrosine, 0.01 ml, was added, and the rate of disappearance of the starch-iodine complex was measured at
Inhibitor | 0.1 mM inhibitor in assay | Enzyme preincubated with 1.0 mM inhibitor* |
--- | --- | --- |
KCN | % | % |
CuCl₂ | 20 | 85 |
CoCl₂ | 10 | 10 |
Propylthiouracil | 30 | 30 |

*The amount of inhibitor carried into the assay resulted in an assay concentration of 0.1 mM.

With iodine concentrations between 10 and 50 μM and a final tyrosine concentration of 2.5 mM (the same tyrosine concentration as in the iodide peroxidase assay), the nonenzymatic disappearance of iodine was in excess of 30 amoles per hour and, therefore, would not represent a rate-limiting step in the peroxidase assay, since the preparations of peroxidase catalyzed the formation of only 5 to 10 amoles of iodine per hour. When the iodine concentration was below 10 μM, the reaction did not proceed nonenzymatically. In the following experiments, an attempt was made to supply iodine to the thyroid particles in very low concentrations. Therefore, the enzyme preparation (5 mg of protein) to be tested plus 5 mg of bovine serum albumin (to act as a nondialyzable tyrosine source) were dialyzed against KI solutions so that very small amounts of iodine were present in the bag. The suspension in the bag was then diazotized against dilute solutions of iodine so that very small amounts of iodine were present in the bag. The iodine, which is only slightly soluble in water, was dissolved in KI so that the ratio was 100 moles of I⁻ per mole of I₂. Varying amounts of the KI-I₂ solution were placed in 10 ml of 0.05 M potassium phosphate buffer, pH 7.5, to act as the dialysis solution. After 1 hour the iodine remaining in the dialysis fluid was reduced with Na₂S₂O₃. The protein solution was adjusted to pH 8.5 with KOH and digested for 12 hours with pancreatin. The resulting solution was chromatographed, and the products were identified after diazotization as described in "Experimental Procedure." When the initial iodine concentration in the dialysis fluid was between 0.1 and 10 μM, no iodinated tyrosines were formed in the presence or absence of the purified peroxidase (Residue II). However, when Residue I (the crude particulate fraction) was added to the dialysis bag instead of Residue II, monoiodotyrosine and diiodotyrosine were formed. Preliminary heating of Residue I at 100°C for 3 minutes completely inhibited the formation of the iodotyrosines.

A variety of substances have been reported by other investigators (1-6) either to activate or to inhibit the activity of thyroid tissue preparations. Cu⁺², Co⁺³, CN⁻, and propylthiouracil (1 mM in the assay system) inhibited the peroxidation of iodide by the peroxidase (Residue II) approximately 80% as measured by iodide disappearance. In other experiments, the enzyme (Residue II) was preincubated with the inhibitors at 1 mM concentration for 10 minutes at room temperature. An amount of enzyme was then assayed so that the concentration of inhibitor in the assay mixture was 0.1 mM. Table IV compares the percentage of inhibition of enzymatic activity by the inhibitors at 0.1 mM concentration in the assay system with and without preincubation of the peroxidase with the inhibitor. As can be seen, the inhibition caused by propylthiouracil, Cu⁺², and Co⁺³ was slight and the same with and without preincubation. On the other hand, CN⁻ produced much more inhibition when preincubated with the enzyme at a concentration of 1 mM. Since none of the inhibitors affected the uptake of oxygen by glucose oxidase in the concentration used, it would appear that CN⁻ affected the peroxidase directly. The mode of inhibition by Cu⁺², Co⁺³, and propylthiouracil cannot be explained by these studies.

Other metals tested, Mn⁺², Mg⁺², Fe⁺³, and Cu⁺³, had no effect on the enzyme at 1 mM concentrations. Molybdenum, either as (NH₄)₂MoO₄ or MoO₃, catalyzed the peroxidation of iodide nonenzymatically under the conditions of the assay. The products of the reaction were identified by chromatographic techniques as mono- and diiodotyrosine. Further studies of the reaction are in progress. Hematin, 0.1 to 1 mM, added to the assay or preincubated with the enzyme, had no effect on enzymatic activity. p-Chloromercuriphenylsulfonic acid, EDTA, and KClO₄, had no effect upon the enzyme at 1 mM concentrations.

**DISCUSSION**

Preparations of thyroid tissue which produce iodinated tyrosines in the presence of iodide ions and tyrosine have been described by a number of investigators (1-7). Assays for all of the enzyme preparations depended upon the production of small amounts of radioactive iodine-labeled iodotyrosines. The enzyme described in this report is particulate in nature and is several times more active than the most active preparations described previously (1-7). The preparation studied by Cunningham and Kirkwood (2) did not precipitate at 185,000 × g and did not appear to be particulate in nature. This preparation had an activity of about 10 to 30 μmole of iodide utilized per mg of protein per hour. Taurog et al. (3, 4) were not primarily concerned with purification, and the thyroid homogenates and particles that they studied had the same range of activity. The crude homogenates of salivary gland and thyroid studied by Alexander (5) had activities as high as 0.1 μmole of iodide utilized per mg of protein per hour. The thyroid peroxidase later described by Alexander (6) had a much lower activity, about 0.01 to 0.015 μmole of iodide utilized per mg of protein per hour. The soluble enzyme described by De Groot and Davis (7) is much more active than the others. Their most active preparation utilized 2.3 μmole of iodide per mg of protein per hour. The most active preparation made by the method described in these studies catalyzed the peroxidation of 20 μmole of iodide per mg of protein per hour. Most preparations catalyzed the peroxidation of 5 to 6 μmole of iodide per mg of protein per hour. As reported previously (14), H₂O₂ is necessary for enzymatic activity at all stages of purification. As the enzyme is purified, there is an increasing need for tyrosine to act as an iodine acceptor in order to protect the glucose-glucose oxidase system (and, therefore, H₂O₂ production) from inhibition by iodine. Under the conditions of the assay, the formation of iodinated tyrosines from iodine and tyrosine progresses nonenzymatically at a rate sufficiently rapid not to be rate-limiting. When the purified peroxidase (Residue II) was dialyzed against low concentrations of iodine, it did not catalyze the production of iodinated tyrosines, indicating that the production of mono- and diiodotyrosine by...
this system was nonenzymatic in nature. However, under the
same conditions of low iodine concentration, the earlier and less
pure particulate thyroid fraction (Residue I) did catalyze the
production of iodinated tyrosines, demonstrating that a particu-
late enzyme is present in thyroid tissue which catalyzes the
iodination of tyrosine.

SUMMARY

A highly active particulate enzyme was isolated from beef
thyroid tissue which catalyzed the peroxidation of iodide. The
enzymatic reaction was studied under conditions which made the
peroxidation of iodide rate-limiting, and the enzyme described
was several times more active than previous preparations.
Under the conditions of the assay, tyrosine was iodinated rapidly
nonenzymatically. However, with very low concentrations of
iodine, less pure particulate preparations of thyroid tissue were
able to iodinate tyrosine enzymatically. The purified peroxidase
did not appear to be inhibited by 1 mM propylthiouracil, disodium
ethlenetetraacetate p-chloromercuriphenylsulfonic acid, potassium
perecholate, and a variety of metals, but was strongly in-
hibited by 1 mM cyanide.

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