MOLECULAR IODINE AS AN OBLIGATE INTERMEDIATE IN THE IODINATION OF TYROSINE BY CHLOROPEROXIDASE[1,2]

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SUMMARY. Previous studies on the peroxidase-catalyzed iodination of tyrosine have been complicated by non-enzymatic reactions between iodine and tyrosine. The low pH optimum for chloroperoxidase allowed tyrosine iodination to be studied without this complication. Chloroperoxidase catalyzes the iodination reaction in two distinct stages. First, iodide ion is peroxidized to form molecular iodine. Secondly, the enzyme catalyzes a reaction between iodine, hydrogen peroxide, and tyrosine. This second reaction has an absolute requirement for hydrogen peroxide, and is inhibited by the presence of iodide ion.

The iodination of tyrosine residues is an integral step in the synthesis of the hormone thyroxine in the mammalian thyroid gland. Since it is generally believed that a thyroid peroxidase is involved in the iodination reaction, several peroxidases have been studied with respect to their ability to catalyze the iodination of tyrosine. Peroxidases, in general, are able to catalyze the peroxidation of iodide ion to form molecular iodine. Molecular iodine in turn serves as an excellent iodinating agent (non-enzymatic) for tyrosine at pH 5 and above where most peroxidases are active. Thus it is difficult to

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[1] This is the sixth paper of a series dealing with chloroperoxidase. The preceding paper in this series is listed as reference (7).

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implicate peroxidases in tyrosine iodination other than for the formation of molecular iodine. This type of non-specific iodination by the enzymatically generated molecular species would not appear to be a good step in thyroxine biosynthesis since enzymatic control would be lost with the formation of the molecular species. The low pH optimum of chloroperoxidase has permitted a study of tyrosine iodination to be made without complications from the non-enzymatic reaction between molecular iodine and tyrosine. The results show that chloroperoxidase first catalyzes the formation of molecular iodine, and then catalyzes a peroxidatic reaction between molecular iodine and tyrosine.

METHODS AND MATERIALS. Chloroperoxidase was isolated from the growth medium of Caldariomyces fumago and purified as described by Morris and Hager (1966). Enzyme samples used in these experiments had a purity index of 1.0 (A_{403}:A_{280}), indicating an enzyme purity of about 75%. Iodine solutions were prepared by dissolving resublimed iodine in glass distilled water. The concentration of triiodide ion was followed spectrophotometrically by the absorbance change at 350 mμ (Hosoya, 1963). The iodination of tyrosine was followed both by a fluorescence assay and spectrophotometrically. An Aminco-Bowman spectrophotofluorometer was used to monitor the iodination of tyrosine continuously. The excitation wavelength was 275 mμ and the emission wavelength was 303 mμ. The spectrophotometric measurements are based on the fact that tyrosine, 3-iodotyrosine, and 3,5-diiodotyrosine all have different absorption spectra. Edelhoch (1962) has also used these spectral changes as a measure of tyrosine iodination.

RESULTS AND DISCUSSION. Previous papers have indicated that chloroperoxidase can catalyze the peroxidation of iodide ion to iodine (Hager, et al., 1966), and also the iodination of tyrosine (Hager, et al.,

1966; Taurog and Howells, 1966). The iodination of tyrosine can be conveniently followed by a fluorometric assay. Tyrosine, when irradiated at 275 mμ, fluoresces strongly at 303 mμ (Teale and Weber, 1957), while the mono- and dihalogenated derivatives have negligible fluorescence. The relative fluorescence yields of tyrosine, 3-iodotyrosine, and 3,5-diiodotyrosine are 184, 1.6, and 1 respectively. Thus, the fluorescence yield is directly related to the amount of uniodinated tyrosine present. Curve B, Figure 1, shows the change in fluorescence as measured during the progress of the iodination reaction. This curve shows an initial sharp drop in fluo-

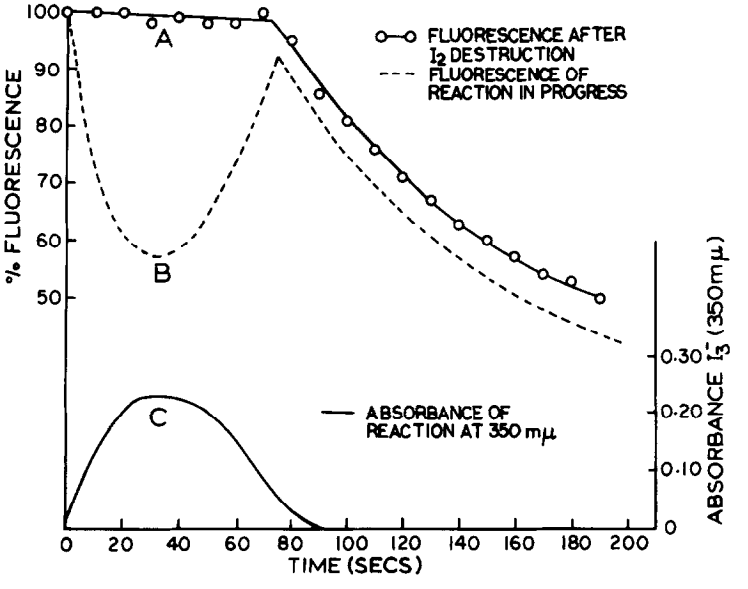

Figure 1. The Iodination of Tyrosine by Chloroperoxidase. The complete reaction mixture contained 300 μmoles of potassium phosphate buffer, pH 2.75, 1 μmole tyrosine, 1.5 μmoles hydrogen peroxide, 0.5 μg chloroperoxidase, and 1 μmole of potassium iodide in a total volume of 3 ml. The reaction was initiated by the addition of chloroperoxidase. Tyrosine fluorescence was measured at 303 mμ after irradiation at 275 mμ. Curve B (----) represents the reaction progress followed by this method. In a similar reaction mixture, the formation of I_3^- was followed in a Gilford spectrophotometer by the absorbance changes at 350 mμ (——, curve C). Curve A (o—o) traces the corrected change in tyrosine fluorescence after the reduction of enzymatically formed iodine to iodide ion. Iodine reduction was accomplished by the addition of 5 μmoles of sodium thiosulfate.

rescence yield, followed by a recovery period, and then a second drop in fluorescence. The apparent initial drop in fluorescence is actually due to the strong absorption of triiodide ion in the ultraviolet, and not to the iodination of tyrosine. The peroxidation of iodide ion to form molecular iodine results in the formation of triiodide ion (I_3^-), since molecular iodine, iodide and triiodide are in facile equilibrium (see equation 1) $I_3^- \rightleftarrows I_2 + I^-$. The ultraviolet absorption of triiodide ion acts as a filter to cause an apparent decrease in tyrosine fluorescence. Curve C measures the appearance and disappearance of triiodide ion as evidenced by absorption changes at 350 mμ. Triiodide ion first increases in concentration, and then decreases as the enzyme continues to oxidize all the remaining iodide ion to iodine. It can be seen by comparing curves B and C that the absorption due to the presence of triiodide ion closely mirrors the initial fluorescence changes.

Curve A of this figure shows the actual loss of tyrosine fluorescence corrected for the interference due to the formation of triiodide ion. In this case, the reaction mixture was scaled up to 60 ml. Three ml aliquots of the reaction mixture were taken at the indicated time intervals, and any iodine present was reduced to iodide by the addition of 5 μmoles of sodium thiosulfate before the fluorescence measurements were made. This treatment was also found sufficient to stop any further iodination of tyrosine. Under these conditions, tyrosine fluorescence could be measured at leisure without the complications due to triiodide ion absorption.

The important point illustrated by this figure is that the iodination of tyrosine begins only after all the iodide ion has been converted to molecular iodine. Spectrophotometric measurements confirm this fact. The spectrum of tyrosine is not affected during the time period that iodide is

being peroxidized to iodine. However, when iodide ion oxidation is complete (as evidenced by the disappearance of triiodide ion absorption), spectral changes indicate the formation of mono- and diiodotyrosine.

Thus, the overall reaction appears to take place in two stages. First, iodide ion is completely oxidized to molecular iodine. Second, chloroperoxidase catalyzes the iodination of tyrosine by molecular iodine. This second step is apparently enzyme catalyzed; otherwise the iodination of tyrosine would begin with the onset of iodine formation, rather than after all the iodide ion had been peroxidized.

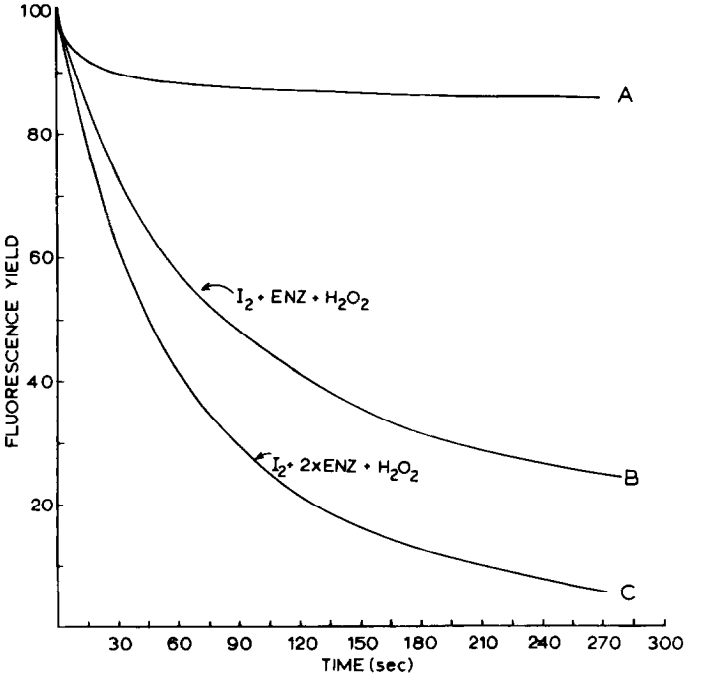

Figure 2. The Enzymatic Iodination of Tyrosine with Iodine. The complete reaction mixture contained 6 mmoles potassium phosphate buffer, pH 2.7, 20 μmoles of tyrosine, 20 μmoles hydrogen peroxide, 10 μmoles iodine, and 10 μg chloroperoxidase in a total volume of 60 ml. The reaction was initiated with the addition of chloroperoxidase, and 2.9 ml aliquots were withdrawn at 15 second intervals, and added to 5 μmoles of sodium thiosulfate in 0.1 ml to stop the reaction. The fluorescence yield of each aliquot was determined in an Aminco-Bowman spectrofluorometer by irradiation at 275 mμ and measuring the fluorescence at 303 mμ. Curve A shows the reaction progress when either hydrogen peroxide, iodine, or chloroperoxidase was left out of the reaction mixture. Curve B represents the total reaction mixture. In curve C the amount of chloroperoxidase was doubled (20 μg).

If this proposed sequence of events is correct, then it should be possible to substitute iodine for iodide ion in the reaction mixture and observe the iodination of tyrosine. This substrate substitution should remove the initial time lag in tyrosine iodination by omitting the first step in the mechanism, the oxidation of iodide ion. This hypothesis was substantiated by experimental observation as shown in Figure 2. Curve B in this figure shows the rapid immediate loss in fluorescence due to tyrosine iodination when hydrogen peroxide, tyrosine, iodine, and chloroperoxidase are all present in the reaction mixture. Leaving out any one of these components causes the reaction to fall to the blank rate (curve A). Thus, chloroperoxidase catalyzes the iodination of tyrosine by molecular iodine in a reaction that requires the presence of hydrogen peroxide. Stoichiometry studies indicate that equations 2 and 3 describe the intermediate formation and utilization of molecular iodine in the overall iodination of tyrosine (equation 4).

$$2\ I^- + H_2O_2 \rightarrow I_2 + 2\ OH^- \qquad (2)$$

$$\underline{I_2 + H_2O_2 + 2 \text{ tyrosine} \rightarrow 2 \text{ monoiodotyrosine} + 2\ H_2O} \qquad (3)$$

Sum:

$$2\ I^- + 2\ H_2O_2 + 2 \text{ tyrosine} \rightarrow 2 \text{ monoiodotyrosine} + 2\ OH^- + 2\ H_2O \qquad (4)$$

Excess iodide ion inhibits tyrosine iodination by diverting the enzyme to exclusive production of molecular iodine. It is interesting to note that, in normal thyroid glands, the addition of iodide ion also transiently inhibits the formation of organically bound iodide (Serif and Kirkwood, 1956). Furthermore, Morris, Eberwein, and Hager (1962) demonstrated that antithyroid agents act as competitive inhibitors in the halogenation reactions catalyzed by chloroperoxidase. These observations indicate that the iodination of tyrosine in the thyroid gland may proceed by a mechanism similar to that shown here for chloroperoxidase.

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