REACTION OF DETERGENT-SOLUBILIZED THYROID PEROXIDASE WITH HYDROGEN PEROXIDE

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1. Introduction

Thyroid peroxidase is a membrane-bound enzyme that catalyzes the biosynthesis of thyroid hormones. The enzyme has been partially purified after solubilization by proteolysis, mostly combined with detergent treatment [1–11]. Spectral properties have been reported on such enzyme preparations but little on the enzyme solubilized by detergent treatment only. Although thyroid peroxidase is believed to react with hydrogen peroxide to form catalytic intermediates such as compounds I and II, their existence has not yet been detected by spectrophotometric methods.

Here we report some spectral properties of detergent-solubilized hog thyroid peroxidase and show spectrophotometric evidence which indicates the formation of compound II of the enzyme.

2. Materials and methods

Sodium deoxycholate, ADP–Sepharose and Sepharose 6B were obtained from Sigma Co., Concanavalin (con) A–Sepharose from Pharmacia Fine Chemicals, α-methyl-D-mannoside from Nakarai Kagaku and PM 10 membranes for diafiltration from Amicon. NADH-cytochrome b_5 reductase (EC 1.6.2.2), which was purified according to [12], was kindly donated from Drs K. Mihara and S. Tajima, Institute for Protein Research, Osaka University.

The hog thyroid microsomes were prepared by a modification of the procedure in [13]. The enzyme was solubilized with deoxycholate by modifications of the procedure in [14] and partially purified on the basis of the procedure in [15]. The microsomes were suspended (5 mg protein/ml) in 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM KI and 0.4% sodium deoxycholate at 4°C for 30 min. After centrifugation at 140 000 × *g* for 1 h, the supernatant was dialyzed against 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM KI. The fraction between 25–60% ammonium sulfate saturation was dissolved in and dialyzed against the same buffer solution. After NADPH-cytochrome *c* reductase (EC 1.6.99.2) was removed by filtration through an ADP–Sepharose column (0.7 × 2 cm) [16], the filtrate was chromatographed on a Sepharose 6B column (3 × 90 cm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM KI and 0.4% Triton X-100. The fraction that contained peroxidase activity was applied to a con A–Sepharose column (2 × 20 cm), equilibrated with 100 mM Tris–HCl buffer (pH 8.9) containing 0.1 mM KI and 0.4% sodium deoxycholate. After being washed with the buffer solution containing KI and deoxycholate, the Sepharose column was transfered to a beaker and suspended in the same solution except that α-methyl-D-mannoside was added (100 mg/ml). After being stirred overnight at 4°C, the suspension was packed in a tube and the enzyme was eluted from the column (preparation A).

The enzyme preparation (B) used for the reaction of thyroid peroxidase with hydrogen peroxide, was obtained according to the above procedure except for lack of Sepharose 6B chromatography and was washed with 10 mM Tris–HCl buffer (pH 7.5) by the diafiltration technique with a PM 10 membrane until free iodide was considered to be 0.1 nM from the calculation of dilution effect.

Reactions were carried out at 20°C in 0.1 M

potassium phosphate buffer (pH 7.5). Spectrophotometers used were Union Giken SM 401 and Shimadzu UV-300 instruments.

3. Results and discussion

3.1. *Spectral properties*

The Soret spectra of our detergent-solubilized thyroid peroxidase resembled those of the purified enzymes solubilized by the proteolytic procedures [2,5,6,8], except that our reduced enzyme had a shoulder at ~430 nm in addition to an absorption maximum at 421 nm (fig.1A). This spectral pattern might be interpreted in terms of a mixture of high-spin and low-spin forms in the reduced state. The shoulder was reflected in a sharp peak at 430 nm in the reduced–oxidized difference spectrum (fig.1B). The purified enzymes so far obtained by the proteolytic procedures exhibit an absorption maximum at 420–427 nm in the reduced state [2,5,6,8]. For the present, it cannot be decided whether the shoulder is inherent to the native ferrous thyroid peroxidase or is due to the contamination with other hemoproteins. Figure 1B also shows that cytochrome b_5 was present in our preparation at a few percent on the molar ratio to thyroid peroxidase.

3.2. *Reaction with hydrogen peroxide*

It has been reported [3,17] that the Soret absorption band of thyroid peroxidase rapidly disappears upon the addition of 5 mM hydrogen peroxide. A similar phenomenon was observed when 35 μM hydrogen peroxide was added to our enzyme preparation (B). Figure 2 clearly shows that the heme destruction was preceded by the formation of a new intermediate giving a difference spectrum with a peak at 430 nm and a trough at 407 nm. Further addition of 450 μM hydrogen peroxide immediately gave an intense trough at 411 nm, which indicated the complete loss of the Soret absorption band of thyroid peroxidase. The intermediate with a peak at 430 nm and a trough at 407 nm appeared when 3.5 μM hydrogen peroxide was added to the enzyme preparation (fig.2B). In this case, in contrast to the data of fig.2A, a marked spectral recovery occurred within 4 min after the addition of hydrogen peroxide and the loss of the Soret band was smaller. The recovery of the original spectrum was fast and nearly complete when 5 μM KI was added 45 s after the addition of hydrogen peroxide (fig.2C). Time courses of A_{430} are shown in fig.3. The spectral change due to the intermediate formation could be observed when hydrogen peroxide was >1 μM.

The intermediate spectrum was assigned to compound II of thyroid peroxidase for the following two reasons.

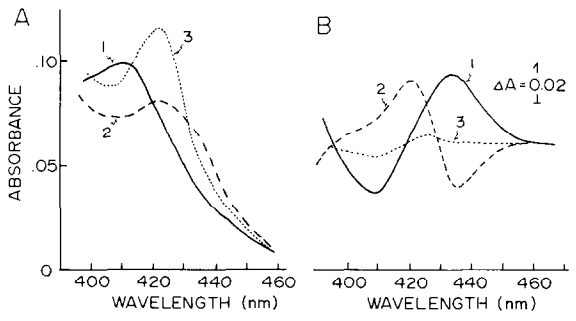

Fig.1. Spectral properties of the purified hog thyroid peroxidase: 1.28 mg protein (prep. A)/ml 0.1 M potassium phosphate (pH 7.5). (A) Absorption spectra: 1, thyroid peroxidase; 2, a few crystals of sodium dithionite were added to 1; 3, CO was bubbled into 2. (B) Difference spectra: 1, Reduced (dithionite)-oxidized; 2, reduced (dithionite)-CO-reduced; 3, reduced (5 min after the addition of 100 μM NADH and 1 unit NADH-cytochrome b_5 reductase)-oxidized.

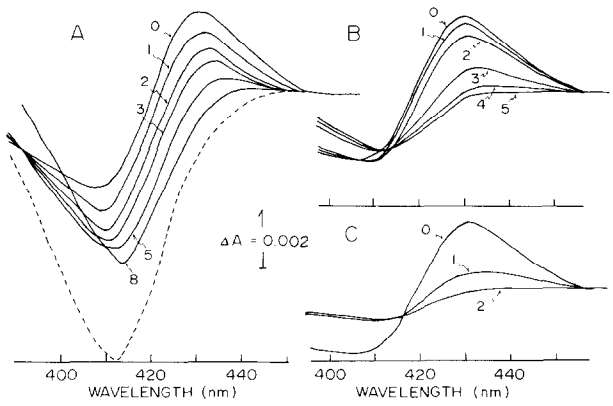

Fig.2. Difference spectra induced by the addition of H_2O_2 to thyroid peroxidase: 1.84 mg protein (prep. B)/ml. (A) Spectra were scanned from 370–460 nm in 40 s, at 0, 1, 2, 3, 5 and 8 min after the addition of 35 μM H_2O_2. The times are indicated in the figure. The regular noise was manually averaged out. The broken line was recorded after a further addition of 450 μM H_2O_2. (B) The same as in (A) except that H_2O_2 was 3.5 μM. (C) The same as in (B) except that 5 μM KI was added 45 s after the addition of 3.5 μM H_2O_2.

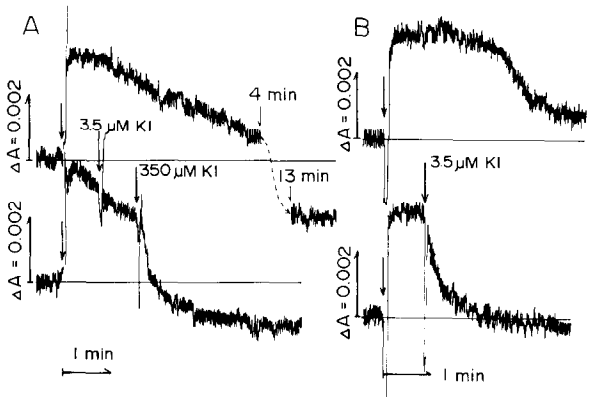

Fig.3. Dual-beam record of the ΔA_{430} induced by the addition of H_2O_2 to thyroid peroxidase. The reference wavelength was 470 nm. The experimental conditions were the same as described for fig.2. (A) 35 μM H_2O_2 was added at the first arrow. KI was added at 3.5 and 350 μM at the second and the third arrows, respectively. In the upper diagram the trace between 4–13 min was omitted. (B) H_2O_2 3.5 μM, and KI 3.5 μM, were added at the first and the second arrows, respectively.

(1) The difference spectrum with a peak at 430 nm and a trough at 407 nm was similar in shape to the known difference spectra of compound II of other peroxidases, though the peak and trough positions differ from peroxidase to peroxidase. For instance, the peak and the trough wavelengths are 423 and 397 nm for horseradish peroxidase C, 437 and 411 nm for bovine lactoperoxidase, and 441 and 411 nm for hog intestinal peroxidase, respectively. These values were estimated from data in [18,19].

(2) The data on the time course for the intermediate formation were in accord with the following mechanism [20,21]:

$$\text{Peroxidase} + H_2O_2 \xrightarrow{k_1} \text{Compound I} \quad (1)$$

$$\text{Compound I} + \text{(endogenous) AH} \xrightarrow{k_2} \text{Compound II} + \text{A} \quad (2)$$

$$\text{Compound II} + \text{(endogenous) AH} \xrightarrow{k_3} \text{peroxidase} + \text{A} \quad (3)$$

Similar phenomena have been reported in the case of bovine lactoperoxidase and hog intestinal peroxidase. Spontaneous conversion from compound I to compound II occurs at half-life times of 80 ms for hog intestinal peroxidase [19] and of 200 ms for bovine lactoperoxidase [19,22]. The conversion from compound II to the ferric enzyme has been observed in the cases of these enzymes, the half-life time varying from minutes to hours depending on the experimental conditions [23].

From the overall kinetic experiments in [5] obtained values of 1.1×10^7 M^{-1} s^{-1} for k_1 and of 1.3×10^5 M^{-1} s^{-1} for k_3 when the exogenous donor was iodide in the reaction of thyroid peroxidase. The latter value was roughly in accord with the data shown in fig.3. Ascorbate was found to serve as the electron donor in reaction 3, but ~100 μM ascorbate was needed to cause the same spectral change as seen in fig.2C. It should be noted here that these results do not necessarily imply the formation of iodide radicals in the reaction of thyroid peroxidase under physiological conditions. The possibility that iodide is oxidized to I^+ or IOH cannot be excluded, as has been discussed in the reaction of horseradish peroxidase [24–26].

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