THE BINDING OF EOSIN Y ON HORSERADISH PEROXIDASE BY SENSITIZED PHOTOOXIDATION

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(*Received for publication May 20, 1981*)

Abstract: Photodynamic inactivation is normally used to study or to modify the important amino acid residues involved in the active site of an enzyme. Horseradish peroxidase shows an exception with its resistance to dye-sensitized photooxidation with a normal low dye concentration. Using high concentration of eosin Y (20 folds), however, it was found possible to bind eosin Y on horseradish peroxidase by photodynamic action. After gel filtration on Sephadex G-150, two major fractions showing the binding of eosin Y on horseradish peroxidase with spectral changes were obtained. A significant shift of absorption peak from 517 nm to 523 nm for eosin Y binding was observed. One fraction shows a reduction of enzymatic activity, whereas, the other fraction exhibits no significant change of enzymatic activity.

INTRODUCTION

Most enzymes are easily inactivated by the illumination with visible light in the presence of an appropriate dye and molecular oxygen. This phenomenon has been known as dye-sensitized photooxidation or termed as photodynamic action (Spikes and MacKnight, 1970). Photodynamic inactivation is normally used to study or to modify the important amino acid residues involved in the active site or in the construction for the conformation of an enzyme (Ray and Koshland, 1961; Hopkins and Spikes, 1969). Under this condition, the concentrations for the enzyme and the dye used are usually in the ratio of about one to five or to ten There are no requirement for the binding between the dye and the enzyme (Spikes and Livingston, 1969).

Recently, it has been reported that (Brandt *et al.*, 1974) on increasing the concentration of dye above that normally used in the dye-sensitized photooxidation led to the binding of dye on several enzymes in addition to photodynamic inactivation. Horseradish peroxidase (HRP) has been well known as one of the exceptional enzymes in that it shows resistance to photodynamic inactivation under normal conditions (Kang and Spikes, 1977). It is therefore, the purpose of this study to investigate whether the increase of dye concentration in the treatment of photodynamic action on HRP would lead to the binding of dye on the enzyme and the photodynamic inactivation of HRP.

MATERIALS AND METHODS

Horseradish peroxidase (E. C. 1. 11. 1. 7.) from Sigma Chemical co., type II, R. Z. 1.37, was purified on CM- and DEAE-cellulose columns to obtain a fraction with R. Z. 3.2 as described before (Kang and Spikes, 1976).

Procedures for photooxidation—For normal photodynamic action, the enzyme to dye ratio is one to ten. For binding study, the dye concentration is increased twenty folds. The reaction

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mixture consists of 20 mg of HRP in 0.9 ml of 0.1 M phosphate buffer pH 7.3 and and 0.1 ml of 1×10^{-4} M eosin Y (EOY) as dye. This solution was stirring in a water-jacket glass vessel which was illuminated by two 250 W reflector lamps from both directions at a distance of 2 cm for 90 min at 25°C. Another set of similar solution was kept in dark as control.

Separation of reaction mixtures—A Sephadex G-25 column (20 cm × 2.3 cm), which was equilibrated with 0.1 M tris-glycine buffer solution, pH 8.2, was used to removed excess EOY from the enzyme molecules. The enzyme containing fraction was then further fractionated with a Sephadex G-150 column (45 cm × 2.3 cm) which was equilibrated with 0.1 M tris-glycine buffer solution, pH 8.2.

Measurement of enzymatic activity—The peroxidase activity was assayed with *o*-dianisidine couple with hydrogen peroxide as substrate as described before (Kang and Spikes, 1977).

The photometric assays and spectral measurement were performed with a Cary Model 14M spectrophotometer.

RESULTS

Fractionation of the reaction mixtures

After illumination, a Sephadex G-25 column was used to remove excess EOY. The fraction containing enzymes was further fractionated with a Sephadex G-150 column. As shown in Fig. 1, the illuminated EOY-HRP mixture was separated into two fractions designated as

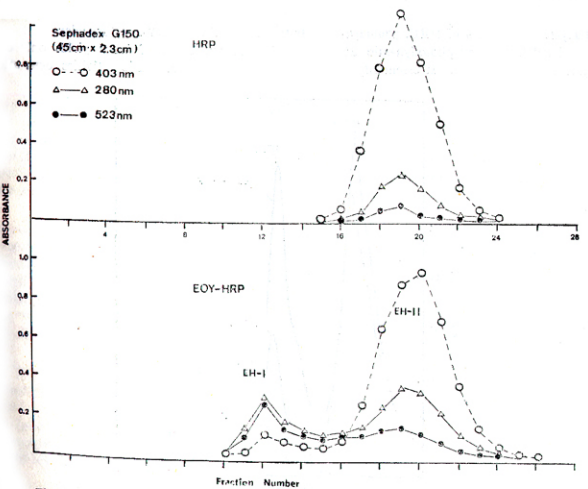

Fig. 1. Fractionation of HRP and EOY-HRP complexes on Sephadex G-150 column.

EH-I and EH-II. The dark control mixture has only one purified HRP fraction with an R. Z. value of 3.5. For a purified HRP usually should have an R. Z. value of over 3.0 which is defined as the ratio of the absorbancy of the enzyme at 403 nm to that at 280 nm (Paul, 1960).

Fraction EH-I exhibits an abnormal lower absorbance at 403 nm than that at 280 nm and at 523 nm. Fraction EH-II also has an R. Z. value of only 2.4.

Spectral properties of the photobinding of EOY on HRP

HRP in aqueous solution has two typical absorption peaks at 497 nm and 640 nm in the red region in addition to Soret band at 403 nm. EOY alone has a sharp absorption peak at 517 nm. Dark control of the HRP and EOY mixture do not show any shift of these absorption peaks except the intensification of 497 nm and 517 nm absorption peaks which is the overlapping of these two peaks as shown in Fig. 2.

On comparing the spectral properties of EH-I against HRP, a remarkable decreasing and broadening of the absorption at 403 nm with a slight increase toward near U. V. region was observed (Fig. 3). Fraction EH-II, on the other hand, shows no major changes at Soret band, but with an increased absorption and shift of the absorption peaks from 497 nm and 517 nm to 523 nm (Fig. 3).

High concentration of HRP and EH-II were used to resolve the absorption changes in the red region. As shown in Fig. 4, the increased absorption peak is actually at 523 nm, neither at 497 nm of HRP nor at 517 nm of EOY. A steep shoulder attributed by 497 nm of HRP was still there. It, therefore, exists a shift of 6 nm from 517 nm of EOY to 523 nm due to its binding on HRP.

Enzymatic activities of HRP, photodynamically-treated HRP and EOY-HRP complex

HRP treated by photodynamic action with low concentration of EOY yields only one purified HRP fraction. It essentially retains all of its original enzymatic activities even after

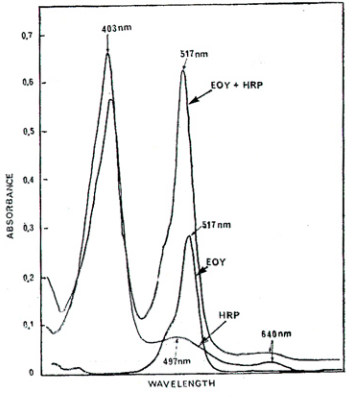

Fig. 2. Absorption spectral properties of HRP, EOY, and HRP mixture.

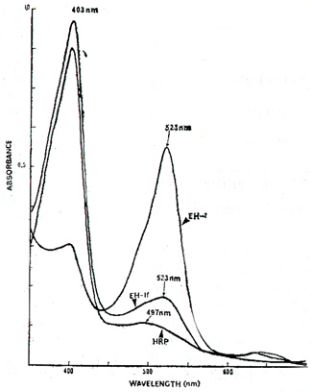

Fig. 3. Comparision on the absorption spectral properties of EH-I and EH-II against HRP.

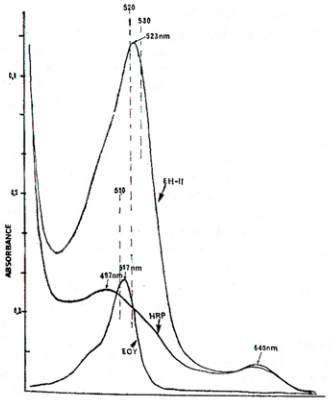

Fig. 4. The red-shift absorption spectra of EH-II by the binding of EOY on HRP.

Table 1. The enzymatic activities of HRP, PDA-HRP and EOY-HRP complex

Reaction Mixtures	Molecular ratio	Photo-illumination*	Gel filtration Sephadex G-150	Specific** Activity
1% HRP solution 2.5×10^{-4} M EOY Phosphate buffer, pH 7.3	1:100	90 min.	One HRP fraction	2239±20(7)
1% HRP solution 5×10^{-5} M EOY Phosphate buffer, pH 7.3	1:200	90 min.	Fraction I Fraction II	829±8(7) 253±12(7)
1% HRP solution 5×10^{-5} M EOY Phosphate buffer, pH 7.3	1:200	dark control	One HRP fraction	22272±17(7)

* The light energy striking the reaction surface is 250 joules•sec^{-1} cm^{-2}.

** One unit of enzymatic speific activity is defined as the $\frac{A460/min}{11.3\times mg\ enzyme/ml\ assay\ mixture}$

90 min of illumination as compared to dark control (Table 1). During the illumination, when the concentration of EOY was increased by 20 folds, two fractions were obtained (Fig. 1). The EH-I fraction shows a great reduction of its enzymatic activity by about only 35% remaining after 90 min of illumination as shown in Table I. The other fraction, EH-II, which has EOY bound on HRP as shown from the spectral properties (Figs. 3 and 4) exhibits no loss of enzymatic activities as compared to dark control and to low EOY concentration in the photodynamically treated HRP. Therefore, it shows the photobinding of EOY on HRP by using high concentration of dye could be accomplished without any loss of the enzymatic activity in the photodynamic action.

DISCUSSION

In this study, the resistance of HRP to low dye concentration of photodynamic action was again re-confirmed as has been previously reported (Kang and Spikes 1977). However, the concentration of dye is increased 20 folds in the illumination system, two fractions, EH-I and EH-II were obtained. By using a millimolar extinction coefficient of 95 mM^{-1} cm^{-1} at 403 nm and 13 mM^{-1} cm^{-1} at 280 nm (Shih *et al.*, 1971), the EH-II consists of about 75.5% of the total HRP in the system and, therefore, EH-I would consists of the other 24.5% of the total system.

EH-I showed an abnormal lower absorption at 403 nm than at 280 nm suggesting a possible aggregation of the heme-containing enzyme, HRP. Since it has been well known the aggregation of porphyrins and porphyrin-containing compounds would have the characteristic reduction and broadening of the absorption peak of Soret band (Falk, 1964) as shown in Fig. 2. EH-I also has a higher absorption peak at 523 nm suggesting that it contained EOY bound on HRP. This aggregation of HRP could possibly due to the EOY as a cross-linker to link HRP together. However, it may still exist the possibility of the aggregation of EOY-bound HRP. It would remain further study to clearify.

EH-II fraction showed an R. Z. value of 2.4 only, exhibiting the presence of EOY in addition to HRP itself. Furthermore, the spectral properties of EH-II showed a significant shift and intensification of absorption peak from 517 nm to 523 nm. The intensification could be due to the overlapping at 497 nm of HRP and 517 nm of EOY. However, the 6 nm of red-shift from 517 nm to 523 nm is the characteristic of EOY binding on polymeric molecules as has been reported by several other investigators (Bellin 1968, MacKnight and Spikes 1972) with concomitant change of color as from orange to pink in this case.

Brandt *et al.* (1974) reported the use of high dye concentration in photodynamic system, not

only would inactivate the enzymatic activities of macromolecules but also could cause the binding of dye on the macromolecules. In this study, 75% of HRP in the high dye concentration of photodynamic system shows the binding of EOY on HRP without any significant loss of enzymatic activities (Table I). This proves as one additional exceptional property of HRP from the other macromolecules (Brandt *et al.*, 1974). The EOY binding site on HRP and its binding mechanism would then require further study.

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