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Horseradish Peroxidase-catalyzed Conversion of Iodine to Iodide in Presence of EDTA and H_2O_2*

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Ranajit K. Banerjee, Swapan K. De, Arya K. Bose, and Asoke G. Datta

From the Department of Physiology, Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Calcutta-32, India

EDTA (4 mM) blocks the oxidation of iodide to I_3^- (increase of extinction at 353 nm) by H_2O_2 catalyzed by horseradish peroxidase, which is reversed by the addition of an equimolar concentration of Zn^{2+}. Addition of suboptimal concentration of EDTA (2 mM) not only decreases the rate of forward reaction of I_3^- formation but also causes loss of extinction of the same when I_3^- is generated. The loss of extinction of I_3^- is proportional to the enzyme concentration and is blocked by azide, the inhibitor of the peroxidase. EDTA also causes bleaching of nonenzymatically formed I_3^- (from iodide and H_2O_2) only in the presence of horseradish peroxidase, and the effect is reversed by the equimolar concentration of Zn^{2+}. Both the bleaching of I_3^- by EDTA and reversal of EDTA effect by Zn^{2+} are sensitive to azide. The decrease of extinction of I_3^- (formed by dissolving iodine in KI solution) is dependent on EDTA, H_2O_2, and horseradish peroxidase. Molecular iodine is also bleached but at a slower rate than I_3^-. Evidence is presented to show that this bleaching of I_3^- is due to enzymatic conversion of I_3^- to iodide in presence of EDTA and H_2O_2 and this involves pseudocatalatic degradation of H_2O_2 to O_2.

During our experiments with gastric peroxidase isolated from the mitochondrial fraction of mouse gastric mucosa (1, 2), we observed that triiodide (I_3^-) formation from iodide catalyzed by this enzyme was blocked by EDTA (3). This effect of EDTA was reversed by the addition of an equimolar concentration of Zn^{2+}, whereas Ca^{2+} or Mg^{2+} was less effective. EDTA also blocked the triiodide formation catalyzed by chloroperoxidase, horseradish peroxidase, and lactoperoxidase. This led us to investigate more critically the effect of EDTA on this reaction using horseradish peroxidase as the model enzyme.

In this paper, we are presenting evidence indicating that EDTA not only interfered with the enzymatic formation of I_3^-, but also led to the formation of iodide catalyzed by the same enzyme. Using triiodide as initial reactant, we have further shown that horseradish peroxidase could catalyze the formation of iodide from triiodide only in the presence of EDTA and H_2O_2. This reaction is associated with concomitant pseudocatalatic degradation of H_2O_2 leading to the evolution of O_2.

MATERIALS AND METHODS

Horseradish peroxidase, lactoperoxidase, EGTA,[1] albumin, sodium azide, hemoglobin, and cytochrome *c* were purchased from Sigma. Chloroperoxidase was a generous gift from Prof. L. P. Hager, of the University of Illinois. EDTA, iodine, and potassium iodide (analytical grade) were obtained from Glaxo Laboratories, India. $Na^{131}I$ was purchased from Bhabha Atomic Research Centre, India. All other chemicals used were of reagent grade.

Peroxidase activity was assayed in a Pye-Unicam double beam recording spectrophotometer by monitoring the formation of I_3^- using iodide as electron donor (4). The assay system contained in a final volume of 3 ml the following reagents: 50 mM sodium acetate buffer, pH 5.25, 1.7 mM KI, a suitable amount of the enzyme to get linear assay, and 0.27 mM H_2O_2, which was added last to start the reaction. The formation as well as disappearance of I_3^- were measured by following the change of absorbance at 353 nm. Other additions and experimental conditions have been described in the legends to individual tables or figures. When I_3^- was generated nonenzymatically, the reaction mixture was same as above except that peroxidase was not included and the reaction was allowed to take place at room temperature for 1–2 h until the extinction of I_3^- reached the desired value. Alternatively, I_3^- was prepared by dissolving 5 mg of iodine in 4 ml of 55 mM KI solution before use.

Evolution of O_2 was determined manometrically (5) in a Warburg apparatus. The main compartment of the Warburg flask contained, in a final volume of 3 ml, 0.15 ml of 1 M sodium acetate buffer, pH 5.25, 0.75 ml of I_3^- solution, and 20 μg horseradish peroxidase, whereas the side arm contained 0.6 ml of 0.2 M EDTA and 0.2 ml of 20 mM H_2O_2. In the central well, a fluted blotting paper soaked in 0.2 ml of 15% alkaline pyrogallol was placed, when necessary. The mixture was equilibrated at room temperature (25 °C) for 3 min before starting the reaction by the addition of EDTA and H_2O_2 from the side arm. The evolution of O_2 was followed at an interval of 3 min in the presence and absence of pyrogallol.

RESULTS

While studying the peroxidation of iodide by horseradish peroxidase, an interesting observation was made in connection with the effect of EDTA on triiodide formation as shown in Fig. 1. The formation of I_3^- was found to be completely blocked in the presence of 4 mM EDTA, whereas the suboptimal concentration of EDTA (2 mM) not only decreased the initial rate of I_3^- formation by about 50% but it also caused a linear decrease of extinction of I_3^- after attaining the plateau at about the 8th min. Addition of 3 mM N_3^- at that time prevented the loss of the extinction. Similar phenomena were also observed when chloroperoxidase or lactoperoxidase was used instead of horseradish peroxidase. However, the concentration of the enzyme should be so chosen that I_3^- formation may attain equilibrium as early as possible. Fig. 2 indicates the effect of addition of 4 mM EDTA on the enzymatically generated I_3^- (when the reaction tends to saturate) using two different concentrations of horseradish peroxidase. It is clear from the figure that not only the initial rate of I_3^- formation was dependent on horseradish peroxidase concentration, but the initial rate of loss of extinction of I_3^- on addition of EDTA was also proportional to the enzyme concentration. Addition of 3 mM N_3^- prior to the addition of EDTA (not shown) blocked the EDTA effect, indicating that the effect was mediated through the peroxidase. A similar experiment was also

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[1] The abbreviation used is: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

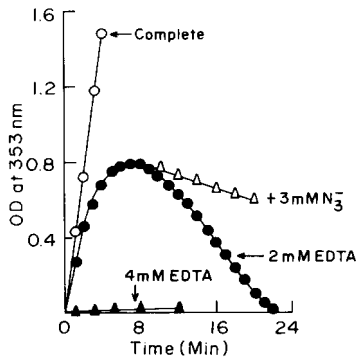

FIG. 1. **Effect of EDTA on horseradish peroxidase-catalyzed I_3^- formation.** Formation of I_3^- was monitored at 353 nm using 2 μg of horseradish peroxidase in the standard assay system described in the text in the presence and absence of EDTA. O, control; ●, +2 mM EDTA; ▲, +4 mM EDTA.

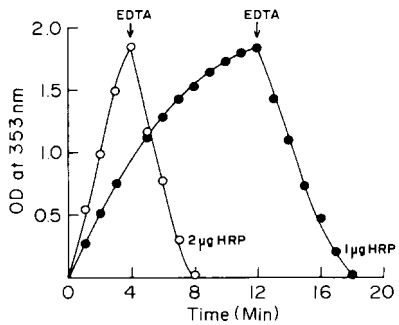

FIG. 2. **Effect of EDTA on the I_3^- formation and disappearance at varying concentration of horseradish peroxidase.** I_3^- was generated by two different concentrations of horseradish peroxidase (HRP), 1 μg (●) and 2 μg (O), respectively, as described in the text. When the extinction reached about 1.88, 4 mM EDTA was added to follow the loss of extinction at 353 nm.

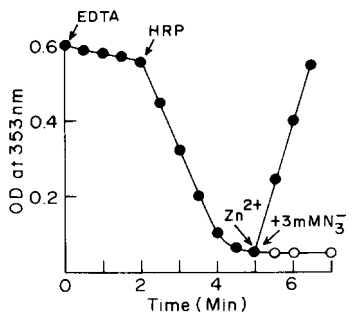

FIG. 3. **Role of peroxidase on the disappearance of I_3^- with EDTA.** I_3^- was produced nonenzymatically for 1–2 h in a reaction mixture containing all the components of peroxidase assay except the enzyme. When the extinction at 353 nm reached around 0.6, 4 mM EDTA was added as indicated by the *arrow*. After 2 min, 2 μg of horseradish peroxidase was added, and the loss of extinction of I_3^- was monitored. After the extinction dropped to 0.05 at 5 min, 4 mM $ZnSO_4$ was added to follow the reappearance of I_3^-. O indicates the effect of Zn^{2+} after addition of 3 mM NaN_3.

carried out with chloroperoxidase (data not shown). However, chloroperoxidase (0.25 μg)-mediated loss of extinction of I_3^- required higher concentration of EDTA (6 mM), and the rate of decrease of extinction was also higher than that of the horseradish peroxidase.

In order to understand more about the role of peroxidase on the disappearance of I_3^- by EDTA, I_3^- was prepared nonenzymatically using iodide and H_2O_2, and the effect of peroxidase was studied. Fig. 3 shows that the extinction of I_3^- did not change significantly on addition of 4 mM EDTA but addition of horseradish peroxidase (2 μg) caused a sharp decrease of extinction of I_3^-. At 5 min, when the extinction reached a value of 0.05, addition of 4 mM $ZnSO_4$ (equimolar to EDTA) to scavenge the EDTA led to the reappearance of I_3^- to the original level. This indicated that horseradish peroxidase was still active and could catalyze the formation of I_3^- from the residual iodide and H_2O_2 present in the system as well as the iodide formed (shown in later experiment) during the loss of extinction of I_3^-. Chloroperoxidase (2 μg) under an identical condition (data not shown) catalyzed the same reaction but at a relatively higher rate than horseradish peroxidase. However, lactoperoxidase (0.5 μg) also possesses the same property (data not shown), but requires as high as 8 mM EDTA to catalyze the bleaching of the I_3^-. N_3^- in all the cases could block the reaction when added prior to the addition of peroxidase. When horseradish peroxidase was added before the addition of EDTA, there was no loss of extinction; on the other hand, I_3^- formation increased further due to enzymatic oxidation of iodide present in the system (data not shown).

It may be presumed that the loss of extinction of I_3^- may be due to an adduct of I_3^- with the EDTA in the presence of the enzyme and H_2O_2. If it were so, the loss of extinction of I_3^- on addition of EDTA as well as reappearance of I_3^- after the addition of Zn^{2+} would have been instantaneous. On the contrary, the results show that both the above-mentioned phenomena were time-dependent peroxidation reactions. The alternate possibility is the backward reaction resulting in the conversion of I_3^- to I^- with EDTA in the presence of the peroxidase. However, requirement of H_2O_2 for the bleaching of I_3^- color rules out a true back reaction. The disappearance of I_2 after the addition of EDTA could be further demonstrated by starch solution. Addition of starch solution to the incubation system before the addition of EDTA showed an absorbance of 2.0 at 660 nm, whereas upon addition of the starch solution a few minutes after the addition of EDTA, when I_3^- extinction went down nearly zero, absorbance at 660 nm showed only 0.2. In order to check whether the bleached product contained I^-, the experiment was carried out with the limiting amount of iodide. In Fig. 4, *curve a*, 1 μmol of I^- (instead of 5 μmol) was allowed to be peroxidized to I_3^- with horseradish peroxidase until there was no further increase of extinction, even after the addition of extra amounts of the

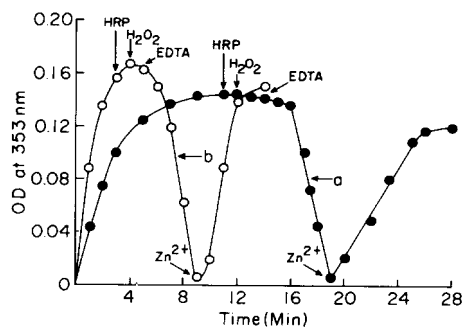

FIG. 4. **Effect of varying horseradish peroxidase concentrations on the rate of I_3^- formation, I_3^- disappearance with EDTA, and reappearance of I_3^- on addition of Zn^{2+} using a limiting amount of iodide.** In *curve a*, I_3^- was generated in the reaction mixture containing all the ingredients for peroxidase assay in the concentrations as mentioned in the text except that horseradish peroxidase used was 3.5 μg and the concentration of KI was 0.33 mM instead of 1.7 mM. EDTA (4 mM) was added when no further oxidation of iodide was observed on further addition of horseradish peroxidase (1 μg) and H_2O_2 (0.4 μmol) as indicated by the *arrows*. 4 mM $ZnSO_4$ was added when EDTA-induced loss of extinction reached around 0.025. In *curve b*, the same experiment was repeated on initial addition of 7.0 μg of horseradish peroxidase.

enzyme and H_2O_2. Addition of 4 mM EDTA at this point brought down the extinction to 0.02, and subsequent addition of an equimolar amount of Zn^{2+} caused about 80% recovery of I_3^-, which could be completely prevented by the addition of N_3^- prior to the addition of Zn^{2+} (data not shown). This indicates that I_3^- was converted to I^- after the addition of EDTA, and I^- could not be reconverted to I_3^- as long as EDTA was present in the system. I^- was peroxidized to I_3^- only after removal of EDTA by Zn^{2+}. The entire process of initial I_3^- formation, bleaching with EDTA, and subsequent I_3^- formation on addition of Zn^{2+} were dependent on the enzyme concentration. When 7 μg of horseradish peroxidase (*curve b*) was used instead of 3.5 μg as in *curve a*, all three rates were found to be increased.

In order to investigate whether I^- was really produced as a reaction product after the addition of EDTA, the entire reaction as in Fig. 4, *curve a*, was allowed to proceed in the presence of $^{131}I^-$. The reaction mixtures at zero time (before the addition of H_2O_2), at steady state (before the addition of EDTA), and when the extinction dropped almost to zero after the addition of EDTA, were extracted with benzene, which dissolves iodine keeping the free iodide in the aqueous layer. The benzene and aqueous layers were counted for radioactivity, and the results are shown in Table I. It is clear from the table that at zero time almost all the counts of the reaction mixture remained as iodide, since it could not be extracted by benzene. At the steady state before the addition of EDTA, the counts were almost evenly distributed in the aqueous as well as in the benzene layer, suggesting 50% conversion of iodide to iodine. After the addition of EDTA, when the I_3^- extinction dropped to zero, almost all the counts could be recovered as iodide in the aqueous layer, indicating quantitative reconversion to iodide from iodine. Finally, addition of Zn^{2+} re-established the distribution of radioactivity as was observed before the addition of EDTA, *i.e.* about 50% iodide and 50% iodine. Chromatography and autoradiography of the aqueous layer indicated the presence of only one spot having the same R_F value as iodide. No other radioactive spot could be detected on the autoradiogram when an aliquot of aqueous layer was chromatographed on Whatmann No. 3MM paper using a butanol/ethanol/2 N NH_3 system (5:1:2) as developer.

In order to study the requirement of H_2O_2, EDTA, and the enzyme in the peroxidase-catalyzed iodide formation from I_3^-, the investigations were carried out in I_3^- prepared chemically by dissolving iodine in potassium iodide solution. Fig. 5

TABLE I

Distribution of iodine and iodide before and after the addition of EDTA

The experimental condition is exactly the same as in *curve a*, Fig. 4, except that $Na^{131}I$ (150,000 cpm) was added along with KI. The entire reaction mixture at desired time was mixed with an equal volume of benzene and vortexed, and both the organic and aqueous phase were counted for radioactivity. An aliquot of the aqueous phase (0.3 ml) was used for chromatography and autoradiography.

System	Organic phase I_2	Aqueous phase I^-	Iodide spot	Other spots
		cpm		
1. Reaction mixture at zero time	1,176	137,002	134,440	Nil
2. Reaction mixture before EDTA addition	60,219	62,019	57,024	Nil
3. Reaction mixture after EDTA addition	5,121	133,836	132,480	Nil
4. Reaction mixture after Zn^{2+} addition	55,782	70,317	66,650	Nil

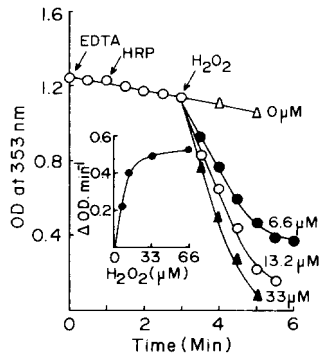

FIG. 5. **Requirement of H_2O_2 for horseradish peroxidase-catalyzed loss of extinction of I_3^- with EDTA.** I_3^- was prepared by dissolving iodine in KI solution, and 75 μl of this solution were added to the cuvette containing 150 μmol of acetate buffer, pH 5.25, in a final volume of 3 ml so as to get an extinction around 1.3 at 353 nm. EDTA was then added to the cuvette, placed inside the spectrophotometers, to get a final concentration of 4 mM, as indicated by the *arrow*, followed by the addition of 2 μg of horseradish peroxidase. The fall of extinction was monitored after the addition of H_2O_2 at a final concentration of 0 (△), 6.6 (●), 13.2 (○), and 33 μM (▲). The *inset* represents the rate as a function of varying concentrations of H_2O_2.

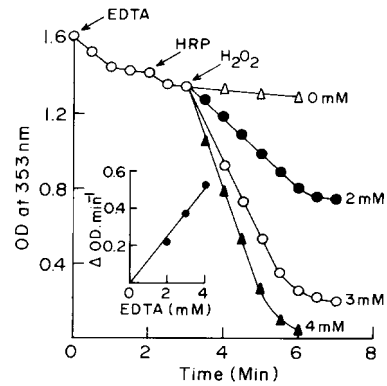

FIG. 6. **Requirement of EDTA for horseradish peroxidase-catalyzed disappearance of I_3^- in presence of H_2O_2.** The protocol was the same as in Fig. 5 except that EDTA at varying concentrations was added before the addition of horseradish peroxidase (2 μg). The reaction was started by the addition of 66 μM H_2O_2. ●, 2 mM EDTA; ○, 3 mM EDTA; ▲, 4 mM EDTA. The *inset* represents the rate at varying concentrations of EDTA.

shows the effect of varied concentration of H_2O_2 on the horseradish peroxidase-catalyzed disappearance of I_3^-. The *inset* shows that the rate of disappearance of I_3^- was proportional to the H_2O_2 concentration up to 13.2 μM, after which the rate falls. The rate of loss of extinction was insignificant if H_2O_2 was either omitted or added (33 μM) after the addition of 3 mM N_3^- (data not shown). The requirement of EDTA in the above reaction is shown in Fig. 6. In the absence of EDTA, I_3^- did not disappear to a significant extent after the addition of only horseradish peroxidase and H_2O_2, indicating absolute requirement of EDTA for the reaction. The loss of I_3^- was proportional to the EDTA concentration as shown in the *inset* of the figure. With 4 mM EDTA, when the extinction dropped nearly to zero, addition of an equimolar concentration of Zn^{2+} reversed the reaction (data not shown) by converting iodide back to I_3^-. Fig. 7 shows the dependence of the rate of the reaction on the enzyme concentration. Not only did horseradish peroxidase show this effect with EDTA, but other peroxidases also showed bleaching of I_3^- in the presence of EDTA and H_2O_2. Among the various peroxidases tested, chloroperoxidase had the highest activity, followed by horseradish

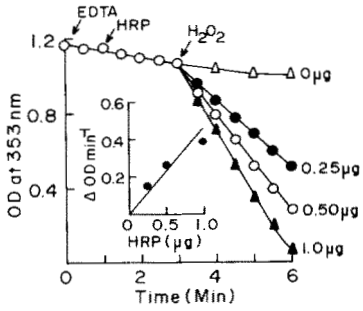

FIG. 7. **Requirement of enzyme for disappearance of I_3^- with EDTA and H_2O_2.** The protocol was the same as in Fig. 5 except that varying concentrations of horseradish peroxidase were added after the addition of 4 mM EDTA to the I_3^- solution. The reaction was started by adding H_2O_2 (66 μM). △, 0 μg; ●, 0.25 μg; ○, 0.50 μg; ▲, 1 μg of horseradish peroxidase. The *inset* is the rate of the reaction as a function of the enzyme.

TABLE II

Relative efficiency of different hemoproteins on the disappearance rate of I_3^-

I_3^- was prepared by dissolving iodine in KI solution as described in the text. The loss of extinction at 353 nm was monitored after adding 4 mM EDTA and 0.25 μg of chloroperoxidase, 1 μg of horseradish peroxidase, 0.2 μg of lactoperoxidase, 2 μg of cytochrome *c*, or 5 μg of albumin. The reaction was started by the addition of 66 μM H_2O_2.

	Loss of extinction of I_3^-
	$\Delta A\ min^{-1}\ mg^{-1}$
Chloroperoxidase	1824
Horseradish peroxidase	311
Lactoperoxidase	115
Hemoglobin	75
Cytochrome *c*	28.5
Albumin	3.6

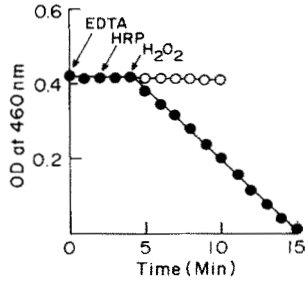

FIG. 8. **Role of horseradish peroxidase on the loss of extinction of iodine with EDTA and H_2O_2.** An aliquot of a saturated aqueous solution of iodine was taken in a cuvette containing 50 mM acetate buffer, pH 5.25, to get an extinction around 0.45. EDTA (4 mM) was then added followed by horseradish peroxidase (2 μg). The reaction was started by the addition of 132 μM H_2O_2 and the loss of extinction of I_2 was followed at 460 nm. ○ indicates the complete system without EDTA.

peroxidase and lactoperoxidase (Table II). Other hemoproteins like hemoglobin and cytochrome *c* had very little activity, and albumin had practically no effect. Fig. 8 demonstrates a similar experiment with aqueous solution of iodine instead of I_3^-. Addition of EDTA or H_2O_2 in the presence or absence of horseradish peroxidase did not catalyze any loss of extinction of iodine at 460 nm until all three components were present. However, the rate of horseradish peroxidase-catalyzed loss of extinction of iodine (A_{18} change min^{-1} mg^{-1}) was found to be much lower than that of the I_3^- (A_{311} change min^{-1} mg^{-1}).

Assuming that the bleaching of I_3^- under the above conditions involves a catalatic degradation of H_2O_2, O_2 should be the other product besides iodide. Alkaline pyrogallol is known to absorb oxygen quantitatively and for this reaction, evolution of oxygen, if any, was looked for in the presence and absence of alkaline pyrogallol in Warburg's apparatus. Fig. 9 shows the evolution of O_2 as a function of time under identical conditions where I_3^- lost its extinction on addition of EDTA, horseradish peroxidase, and H_2O_2. The figure also shows that O_2 evolution was significantly low in the absence of any one of the three components, indicating dependency on horseradish peroxidase, EDTA, and H_2O_2.

DISCUSSION

From the foregoing experiments, it appears that EDTA has two significant effects on horseradish peroxidase-catalyzed peroxidation of iodide. It does not only block the conversion of I^- to I_3^- but also bleaches the extinction of I_3^-, generated enzymatically by peroxidation reaction or prepared nonenzymatically. The first effect has been shown in Fig. 1 using 4 mM EDTA, whereas the second effect is evident from Figs. 2–7. Both the effects of EDTA could be observed in Fig. 1, where suboptimal concentration (2 mM) of EDTA was used. Evidence has also been presented showing that both these effects are dependent on the presence of peroxidase. However, all the peroxidases are not equally active in bleaching the extinction of I_3^-, as our results show that chloroperoxidase is more active than horseradish peroxidase and lactoperoxidase. Hemoglobin or cytochrome *c* also catalyzes the reaction but at a much slower rate, possibly due to their pseudoperoxidase activity. Albumin does not possess the property, indicating that the intact heme part bound to the protein is necessary for the reaction. Sensitivity to azide and requirement of H_2O_2 are also indicative of the specific involvement of the peroxidase in the reaction. The reaction is specific to EDTA, as similar compounds such as EGTA, Tris, or triethylamine cannot catalyze the reaction. EDTA effect is observed only when iodide is used as an electron donor and not with any other electron donor like *O*-dianisidine. This indicates that the complete block of forward reaction of I_3^- formation by 4 mM EDTA is not due to inactivation of the enzyme.

In order to get an idea about the nature of the reaction product formed after the interaction of I_3^- with EDTA, we observed no development of color on addition of starch, indicating the absence of iodine as a product. Thus it was thought that the loss of extinction of I_3^- may be due to the formation of a horseradish peroxidase-EDTA-I_3^- complex. However, if it were so, the formation of I_3^- would have been instantaneous after the addition of Zn^{2+} to scavenge the EDTA from the complex. On the contrary, the regeneration of I_3^- due to addition of Zn^{2+} was a rate reaction and sensitive to azide, indicating that I_3^- is generated enzymatically from iodide produced by the bleaching of I_3^- with EDTA. The loss of extinction of I_3^- by EDTA is comparable to what is observed

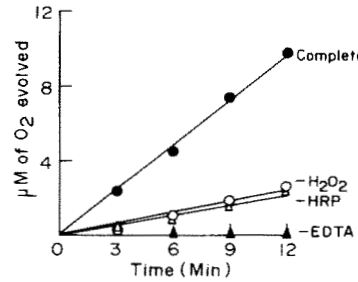

FIG. 9. **Oxygen evolution from H_2O_2 during horseradish peroxidase-catalyzed reduction of iodine in the presence of EDTA.** Oxygen evolution was monitored in a Warburg apparatus using the assay system as described in the text. ●, complete system; ○, complete minus H_2O_2; △, complete minus horseradish peroxidase; ▲, complete minus EDTA.

on addition of tyrosine, where the decrease of extinction of I_3^- is due to formation of iodotyrosine. The possibility of iodination of EDTA is, however, remote. If EDTA were iodinated, addition of Zn^{2+} could not release iodide from the iodinated compound. These perhaps help to postulate an alternate possibility that the loss of extinction of I_3^- in the presence of EDTA, H_2O_2, and horseradish peroxidase is due to conversion to iodide. Chromatography of the aqueous phase after benzene extraction of the reaction mixture containing a limiting amount of iodide (^{131}I) clearly shows that the product of the bleaching reaction is nothing but iodide.

If the horseradish peroxidase-catalyzed conversion of I_3^- to I^- involves pseudocatalatic degradation of H_2O_2, O_2 should be the other product. Our results clearly show that disappearance of I_3^- is associated with the evolution of O_2 and the latter is dependent on the presence of EDTA, H_2O_2, and horseradish peroxidase during the bleaching reaction. The generation of O_2 from H_2O_2 in this horseradish peroxidase-catalyzed reaction in the presence of EDTA may be termed as pseudocatalatic degradation of H_2O_2, where H_2O_2 is decomposed to O_2 in a catalase-like reaction. It is not a true catalatic reaction where one molecule of H_2O_2 acts as an electron donor and other molecule acts as an electron acceptor. In this pseudocatalatic degradation, O_2 may be formed by a redox reaction between iodinium ion, I^+ or I_2 and H_2O_2 according to the following reaction.

$$I^+ + H_2O_2 \rightarrow O_2 + I^- + 2H^+ \quad (1)$$

$$I_2 + H_2O_2 \rightarrow O_2 + 2I^- + 2H^+ \quad (2)$$

Formation of O_2 in such a redox reaction between H_2O_2 and I_2 or I^+ has been known for a long time (6, 7) and has recently been reported in the peroxidase/H_2O_2/iodide system (8–11).

The basic mechanism of iodide oxidation may be represented as follows.

$$I^- \rightleftharpoons I^+ + 2e \quad (3)$$

$$I^+ + I^- \rightleftharpoons I_2 \quad (4)$$

$$\text{Sum: } 2I^- \rightleftharpoons I_2 + 2e \quad (5)$$

$$I_2 + I^- \rightleftharpoons I_3^- \quad (6)$$

$$K = [I_3^-]/[I_2][I^-]$$

In the process of the redox reaction, H_2O_2 acts as an electron acceptor and iodide as the electron donor which is oxidized to I^+ (iodinium ion), as reported by Alexander (4, 12). In the absence of a suitable acceptor such as tyrosine, I^+ readily reacts with other nucleophils such as I^-, Cl^-, or OH^- to form I_2, ICl, or IOH, respectively. In presence of excess iodide, there is an instantaneous formation of I_3^-. Most of the I_2 remains bound as I_3^-, as the equilibrium constant, K, for $[I_3^-]/[I_2][I^-] = 714$ at 25 °C and hydrolysis of I_2 or polyiodide formation is not significant (13, 14). Equilibrium conditions for the reaction $I_2 + I^- \rightleftharpoons I_3^-$ have been studied extensively resulting in different K values and have recently been well documented by Huwiler and Kohler (11). It is now generally believed that the first step of iodide oxidation (Equation 3) involves two electron transfers catalyzed by peroxidase-H_2O_2 complex known as Compound I (12 15), whereas the subsequent reactions (Equations 4 and 6) are spontaneous transformations, neither of which is enzyme-catalyzed. The redox reaction (Equation 3) is affected by oxidizing or reducing agent, whereas Equations 4 and 6 are association equilibrium and can be shifted by many compounds, as discussed by Huwiler and Kohler (11).

The mechanism of peroxidase-catalyzed iodination reaction and the involvement of iodine free radical (16, 17), iodinium ion (15, 18, 19), or hypoiodite (9, 20) in the process have been proposed. The most extensive studies in this direction have been presented by Hager and co-workers (21, 22) on chloroperoxidase-catalyzed chlorination reaction. They suggested that the possible structure of the halogenating intermediate is an -OCl ligand on the ferric heme of the enzyme. Morrison and Schonbaum (20) proposed the formation of an enzyme-hypoiodous complex (EOI in the reaction between Compound I and iodide). Very recently, Magnusson *et al.* (10) have proposed that peroxidase combines with H_2O_2 to form an activated enzyme, Compound I (represented by EO). Compound I is proposed to be an Fe(IV)-porphyrin π-cation radical (23). This has been supported by Hager and co-workers (21, 22) who reported that Compound I of chloroperoxidase contains a single oxygen atom derived from H_2O_2. Morrison and Schonbaum (20) also used EO to describe Compound I of various peroxidases. According to Magnusson *et al.* (10), EO oxidizes I^- to form an intermediate represented as $[EOI]^-$, where I is present in an oxidation state equivalent to I^+. $[EOI]^-$ may give rise to the ferric form of the peroxidase and IO^-, which is a free anion, rather than the enzyme-bound hypoiodite. The scheme (Ref. 10) may be represented as follows.

$$E + H_2O_2 \xrightarrow{H_2O} EO \rightarrow [EOI]^- \rightarrow E + IO^- \quad (7)$$

I_3^- is then formed in the following two successive steps, neither of which is enzyme-catalyzed.

$$IO^- + I^- + H^+ \rightarrow I_2 + OH^- \quad (8)$$

$$I_2 + I^- \rightleftharpoons I_3^- \quad (9)$$

In the context of this mechanism of peroxidase-catalyzed triiodide formation, our data may now be explained as follows: EDTA blocks the formation of triiodide in the forward reaction as a result of oxidation of IO^- by H_2O_2 to form O_2 and I^- according to the following reaction.

$$IO^- + H_2O_2 \xrightarrow{\text{horseradish peroxidase}} O_2 + H_2O + I^- \quad (10)$$

Equation 10, which is possibly favored in the presence of EDTA by a still unknown mechanism, prevents Equation 8 from occurring, blocking the formation of I_3^- (Equation 9). Thus, the inhibition of I_3^- formation with EDTA is really an apparent inhibition and arises because the product of the first enzyme-catalyzed reaction, namely IO^- (Equation 7) is consumed in a second enzyme-catalyzed step (Equation 10), leading to the formation of O_2 and I^-. This is consistent with our finding that, using a limiting amount of iodide containing ^{131}I in the reaction mixture (Fig. 4), EDTA caused the liberation of ^{131}I (Table I, System 3). In fact, Equation 10 which involves the reduction of hypoiodite by H_2O_2 has been studied by Liebhafsky (6, 24), who reported the following reaction: $HIO + H_2O_2 \rightarrow H^+ + I^- + H_2O + O_2$. This reaction may provide the chemical basis for iodide-dependent pseudocatalatic degradation of H_2O_2 by thyroid peroxidase or lactoperoxidase (9, 11).

Our data also indicate that in the presence of EDTA and H_2O_2, horseradish peroxidase catalyzes the loss of extinction of preformed I_3^- with concomitant formation of O_2. It may be mentioned here that the extinction of I_3^- decreases very slowly either in the presence of EDTA or in the presence of H_2O_2 or by both. This is perhaps the nonenzymatic chemical reaction which becomes very fast on addition of the enzyme. This fast reaction does not occur in the absence of EDTA. It appears that, in presence of EDTA, the conformation of the enzyme may so change that it catalyzes the conversion of I_2 to I^- with concomitant oxidation of H_2O_2 to O_2 as shown in the following

reaction:

$$I_3^- \rightleftharpoons I^- + I_2 \quad (11)$$

$$\text{horseradish peroxidase} \downarrow + H_2O_2$$

$$O_2 + 2I^- + 2H^+ \quad (12)$$

The evolution of O_2 as shown in our data (Fig. 9) indicates that Equation 12 is occurring in our system. It has further been shown that the rate of bleaching with EDTA using only I_2 is much slower than I_3^- (Fig. 8). It appears that I_3^-, being a charged molecule, is a better interacting species than nonpolar iodine.

It is thus possible that, in the presence of EDTA, horseradish peroxidase is catalyzing the formation of iodide from iodine in a redox reaction associated with the pseudocatalatic degradation of H_2O_2 to O_2. This iodide-dependent pseudocatalatic activity of the peroxidases has been extensively studied by Taurog and co-workers (9, 10) using lactoperoxidase and thyroid peroxidase. Although we have shown that horseradish peroxidase also possesses this activity, the higher rate of bleaching of I_3^- with chloroperoxidase in our system indicates that this enzyme may possess pseudocatalatic activity much higher than horseradish peroxidase. In fact Thomas *et al.* (25) have demonstrated that chloroperoxidase possesses significant catalase activity in both the presence and absence of Cl^-. However, the role of EDTA in our system is not clearly understood and it opens up a new field for further investigation.

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