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STUDY OF PEROXIDASE MECHANISMS BY PULSE RADIOLYSIS*

III. THE RATE OF REACTION OF O_2^- AND HO_2 RADICALS WITH HORSERADISH PEROXIDASE COMPOUND I

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SUMMARY

Horseradish peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) Compound I forms when the enzyme reacts with hydrogen peroxide. Rates of reaction of O_2^- and HO_2 radicals with Compound I were measured in the pH range 3.8 to 8.8. The rate constants were $1.6 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ for O_2^- and $2.2 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ for HO_2.

INTRODUCTION

The superoxide radical O_2^- is the conjugated base of the HO_2 radical:

$$HO_2 \rightleftharpoons O_2^- + H^+ \qquad (1,-1)$$

The pK_{HO_2} of this equilibrium is 4.8 [1]. These radicals can be generated in the absence of interfering transients by pulse radiolysis of formate solutions containing molecular oxygen [2]:

$$H_2O \xrightarrow{O_2,\ HCOONa} O_2^-,\ H_2O_2,\ H_2 \qquad (2)$$

Previous studies have shown that formate protects the enzyme from radiation damage and does not affect its activity [3].

Horseradish peroxidase Compound I forms rapidly when the free enzyme reacts with hydrogen peroxide [4]:

$$\text{Enzyme} + H_2O_2 \xrightarrow{k_3 = 1.2 \cdot 10^7\ M^{-1} \cdot s^{-1}} \text{Compound I} \qquad (3)$$

In the presence of a slight excess of hydrogen peroxide the product is stable for several minutes, so that the following reactions can be studied in isolation by pulse radiolysis:

$$\text{Compound I} + HO_2 \xrightarrow{k_4 = 2.2 \cdot 10^8\ M^{-1} \cdot s^{-1}} \text{Compound II} + O_2 + H^+ \qquad (4)$$

$$\text{Compound I} + O_2^- \xrightarrow{k_5 = 1.6 \cdot 10^6\ M^{-1} \cdot s^{-1}} \text{Compound II} + O_2 \qquad (5)$$

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METHODS, RESULTS AND DISCUSSION

Horseradish peroxidase (R.Z. 3.2; 3324 units/mg, Nutritional Biochemicals Corp.) was dissolved in air-saturated triple-distilled water. Compound I was prepared by mixing 0.2 ml of the enzyme solution with 5.0 ml of a hydrogen peroxide solution containing formate, phosphate, and molecular oxygen. The final concentrations are recorded in figure captions. The pH of the solutions was adjusted by addition of either phosphoric acid or sodium hydroxide. Within one minute of mixing, the solutions were pulsed with 2 MeV electrons in a Van de Graaff accelerator, as described previously [5]. Pulses of 10 μs duration were used, which produced a total of 0.6 μM of HO_2 and O_2^-.

The rate of reduction of Compound I to Compound II by the radicals was measured at 427 nm, the isosbestic point of the free enzyme and Compound I. A recorded trace of the absorption change following a pulse is illustrated in Fig. 1. The semilogarithmic plot of the results shows good agreement with pseudo first-order

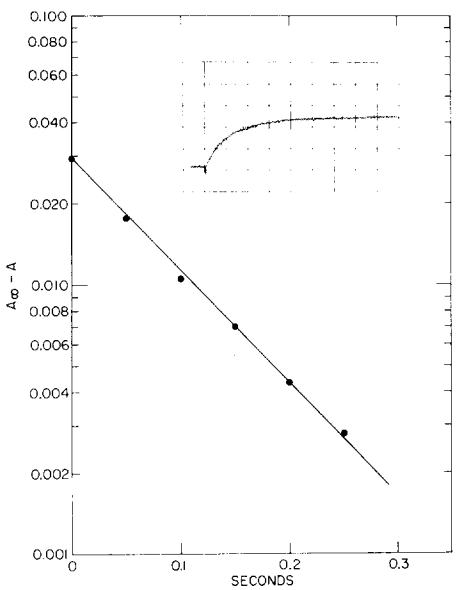

Fig. 1. Illustration of Compound I reduction (ΔCompound I = $2.5 \cdot 10^{-7}$ M) by O_2^- ($2.7 \cdot 10^{-7}$ M/7 μs pulse) under pseudo first-order conditions at 427 nm (6.1 cm optical light path), pH 8, 21 °C. The solution contained 5.75 μM Compound I; 15 μM H_2O_2; 50 mM phosphate; 50 mM sodium formate and 0.25 mM of molecular oxygen. Insert: Trace recorded at 0.04 V/cm versus 0.1 s/cm.

kinetics. Applicability of pseudo first-order conditions to these experiments was established over a wide concentration range of Compound I in the previous study [3].

The rate of disappearance of Compound I depends on the concentrations of HO_2 and O_2^-. These are pH dependent, through the equilibrium (1,-1). The overall mechanism of this process is therefore described by Eqns 1,-1, 4 and 5, which lead to equation

$$k_{obs} = [A]\left[\frac{k_4 + k_5 X}{1 + X}\right] \tag{6}$$

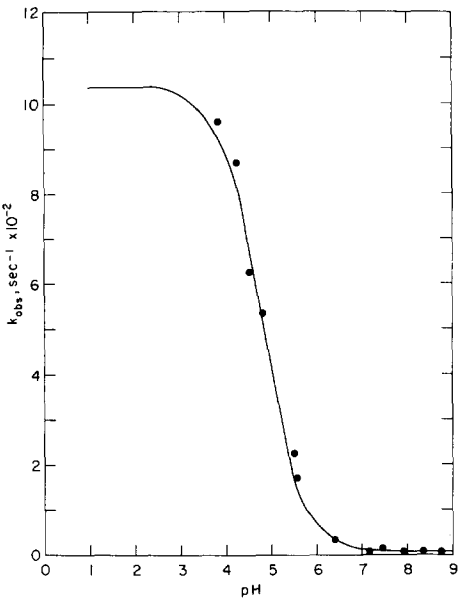

Fig. 2. Effect of pH on k_{obs} for the reduction of Compound I by HO_2 and O_2^-. The solid line was calculated with Eqn 6. All solutions contained 4.72 μM Compound I; 15 μM H_2O_2; 50 mM phosphate; 50 mM sodium formate and 0.25 mM of molecular oxygen; 21 °C.

where $[A]$ is the concentration of Compound I, and X is the ratio $K_{HO_2}/[H^+]$. In the plateau region above pH 7, where the contribution of HO_2 is insignificant, the rate constant k_5 is $1.6 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. Rapid increase of k_{obs} below neutrality indicated that HO_2 reacts more rapidly than O_2^- with Compound I. It was not possible to obtain k_4 directly from experiments, because below pH 3.8 the results were scattered, rendering the measurements of rates unreliable. The probable explanation for this lies in a change in the properties of the peroxidase under acidic conditions. However, k_4 can be evaluated by substituting experimentally determined k_{obs} values at pH 3.8–4.8 into Eqn 6. The solid line, which shows good agreement with the experimental results, was calculated with an average value for k_4 of $2.2 \cdot 10^8 \pm 0.2 \cdot 10^8$ $M^{-1} \cdot s^{-1}$.

The results of these experiments indicate that even under conditions approximating physiological state, the perhydroxyl radical makes a significant contribution to the rate of reduction of Compound I.

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