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STUDY OF PEROXIDASE MECHANISMS BY PULSE RADIOLYSIS

II. REACTION OF HORSERADISH PEROXIDASE COMPOUND I WITH O_2^-

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SUMMARY

1. Sodium formate has no effect upon the absorption spectra and the rates of reaction of horseradish peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) and its derivative Compound I. It is a good radiation protector for horseradish peroxidase in aerated aqueous solutions.

2. The rate of formation of Compound I has been determined in a fast kinetics spectrophotometer at 335 nm. During the conversion of horseradish peroxidase to Compound I, no other intermediates are detectable in the range from 300 to 450 nm.

3. Radiation generated superoxide radicals (O_2^-), reduce Compound I to Compound II with a rate constant of $1.60 \cdot 10^6 \pm 0.08 \cdot 10^6$ $M^{-1} \cdot s^{-1}$.

4. A three-dimensional computer presentation (absorbance vs wavelength vs time) shows spectral changes observed after pulse irradiation of an air-saturated formate solution containing horseradish peroxidase.

5. Studies with both pulse radiolysis and γ-ray irradiation indicate that Compound II does not react with O_2^-.

INTRODUCTION

The present study is an extension of an earlier report [1] on the application of pulse radiolysis to the study of peroxidase mechanisms. The accepted mechanism [2,3] for the horseradish peroxidase-catalyzed decomposition of H_2O_2 in the presence of a suitable hydrogen donor (AH_2) is described by the following equations:

$$\text{Enzyme} + H_2O_2 \rightarrow \text{Compound I} \quad (1)$$

$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + AH\cdot \quad (2)$$

$$\text{Compound II} + AH_2 \rightarrow \text{Enzyme} + AH\cdot \quad (3)$$

$$AH\cdot + AH\cdot \rightarrow AH_2 + A \quad (4)$$

Pulse radiolysis is particularly suited for the study of reactions between the enzyme or its compounds and the superoxide radical, since the latter can be easily generated in relatively high concentrations with little interferring compounds. A system most often used for the study of superoxide radicals is an oxygen-containing aqueous solution of sodium formate [4–6]:

$$H_2O \xrightarrow{O_2,\ \mathrm{HCOONa}} O_2^-,\ H_2O_2,\ H_2 \qquad (1)$$

In the case of horseradish peroxidase, however, there are still difficulties in determining the absolute rate constant for the reaction of free enzyme with O_2^-, since H_2O_2, which reacts much more rapidly with the enzyme, is always produced as a molecular product during radiolysis. For this reason the present investigation is centered on the study of the interaction of O_2^- with Compound I.

METHODS

Peroxidase Horseradish R.Z. 3.2, 3324 units/mg, was purchased from Biochemicals Corp.

Durrum stopped-flow spectrophotometer; 2-cm optical light path; volume ratio 1:1; dead time approximately 0.5 ms. The electron pulse generator (2 MeV Van de Graaff), optics and monitoring equipment have been described in earlier publications [7]. The recording equipment consisted of a Transient Recorder Model 802 Biomation, a dual beam oscilloscope Type 555 Tektronix Inc., and a computer interface.

RESULTS

Preliminary experiments without radiation were carried out to determine possible effects of formate upon the compounds of interest and to establish if formate could act as a hydrogen donor. The spectra of horseradish peroxidase and Compound I were found to be identical in presence and absence of 0.1 M sodium formate and are given in Fig. 1. Corresponding rate constant determinations for Reaction 1 under pseudo first-order conditions for disappearance of horseradish peroxidase (395 nm) and formation of Compound I (335 nm) were carried out in a Durrum fast kinetics spectrophotometer at pH 4 and 7 in the presence and absence of sodium formate. Sodium formate had no effect on the rate of formation of Compound I under these conditions. The observed rate constant $k_1 = 1.2 \cdot 10^7 \pm 0.1 \cdot 10^7\ M^{-1} \cdot s^{-1}$, is in close agreement with earlier reports [2–3].

The reaction of Compound I with formate can be neglected in these studies because the observed pseudo first-order changes in absorbance at 427 nm indicated a half-life of the order of 100–150 min in 0.05 M formate solution. Since the rate studies of interest (O_2^- + Compound I) are completed in a matter of seconds or less, they are not affected by the slow changes caused by formate.

Radiation studies

All radiation experiments were carried out in air-saturated 0.05 M sodium formate solutions at pH 7.2 and some at pH 8.1. At these pH values the equilibrium, $HO_2 \rightleftharpoons O_2^- + H^+$ ($pK = 4.8$), is far to the right and complications from the HO_2

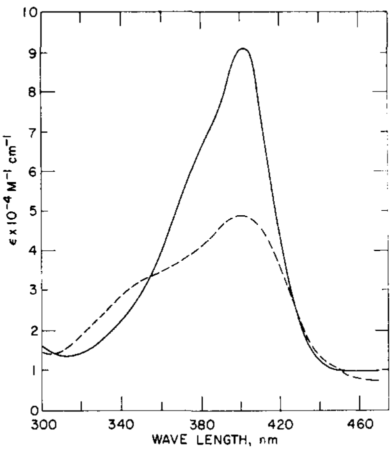

Fig. 1. Spectra of horseradish peroxidase (solid line) and Compound I (broken line) at pH 7 and 23.5 °C. These spectra were identical in the presence and absence of 0.05 M sodium formate.

radical are minimized. The amounts of O_2^- and H_2O_2 (molecular yield) formed by radiation were calculated from ferrous dosimetry calibrations using $G(Fe^{3+}) = 15.5$, $G(O_2^-) = 6.45$ and $G(H_2O_2) = 0.72$. In pulse experiments the concentration of O_2^- was also determined by direct obsrvation of absorbance at 250 nm, using a molar extinction coefficient of 1950 $M^{-1} \cdot cm^{-1}$ [4].

While formate has no effect upon the enzyme under the experimental conditions it does protect the protein against radiation damage. Exposure of horseradish peroxidase in air-saturated 0.5 M formate solutions to radiation doses of up to 180 krad had no effect on its enzymatic activity. Since all experiments described in this report were confined to doses below 10 krad/pulse, radiation damage to the enzyme was excluded as an interfering factor.

A spectral analysis of the reaction product(s) resulting from the interaction of O_2^- with horseradish peroxidase or its derivative Compound I was carried out in the visible spectrum (450–700 nm), since the anticipated products Compound II (absorption maxima at 527 and 555 nm) [8] and/or Compound III (absorption maxima at 546 and 583 nm) [8] have quite different spectra in this region compared to the Soret band, where they are almost indistinguishable (maximum at 418 and 416 nm, respectively) [8].

In studying absorbance changes at several selected wavelengths (396, 411, 427, 527, 555 and 583 nm), preliminary pulse experiments have shown that most radiation induced reactions reach completion in less than one second, and that following this short time period only a negligible change (5–7 %) in absorbance was observed over the next 10–15 min.

In view of the relative stability of the irradiated solutions, a series of ^{60}Co γ-ray studies were carried out with a 7.8 krad/min source in which a 1-min exposure produced $5.2 \cdot 10^{-5}$ M O_2^- and an initial yield of H_2O_2 equal to $5.8 \cdot 10^{-6}$ M. Since these samples could be transferred to the Cary 14 spectrophotometer within 10 s of the termination of irradiation and scanned rapidly (30 s), changes during the scanning due to the slow reactions following irradiation are estimated not to have introduced

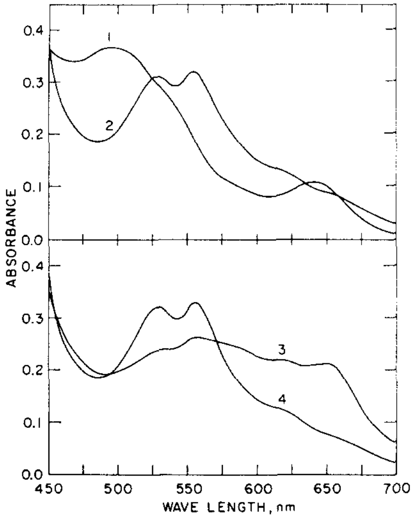

Fig. 2. Spectrum 1. Horseradish peroxidase ($6.7 \cdot 10^{-6}$ M) in air-saturated 0.05 M sodium formate, (pH 7.2). Spectrum 2. Horseradish peroxidase ($6.7 \cdot 10^{-6}$ M) in air-saturated 0.05 M sodium formate (pH 7.2) irradiated with 7.8 krad of ^{60}Co γ-rays. The dose produced $5.2 \cdot 10^{-5}$ M O_2^- and an initial H_2O_2 concentration of $5.8 \cdot 10^{-6}$ M in the solution. Spectrum 3. Absorbance spectrum of an air-saturated 0.05 M sodium formate solution containing $6.7 \cdot 10^{-6}$ M horseradish peroxidase and $1.6 \cdot 10^{-5}$ M H_2O_2, pH 7.2. The spectrum was taken 20 s after mixing. Spectrum 4. Same system as in Spectrum 3 was irradiated with 7.8 krad of ^{60}Co γ-rays.

an error in excess of 2%. The results of these experiments are shown in Fig. 2, where Spectrum 1 shows unreacted horseradish peroxidase before irradiation and Spectrum 2 shows the same solution after 1-min irradiation in the ^{60}Co source. Spectrum 3 shows Compound I scanned 20 s after reacting horseradish peroxidase (final concentration $6.7 \cdot 10^{-6}$ M) with H_2O_2 (final concentration $1.6 \cdot 10^{-5}$ M) in a volume ratio of 1:1. Spectrum 3 did not change for about 3 min after mixing. When the latter solution was irradiated for one minute in the ^{60}Co source, Spectrum 4 was obtained.

As is apparent, Spectra 2 and 4 are identical showing the characteristic peaks of Compound II, which suggests that the predominant reaction sequence under radiation conditions is the interaction between enzyme and H_2O_2 yielding Compound I, which in turn is reduced by the superoxide radical to Compound II.

To confirm the observed reduction of Compound I to Compound II by O_2^- in the ^{60}Co γ-ray studies, pulse experiments were designed to measure the corresponding rate constants and to give a detailed account of the accompanying absorbance changes in the visible spectrum.

The study of the spectral changes occurring shortly after the radiation pulse, were carried out in such a fashion that all variables were held constant (concentrations of enzyme, formate, O_2^-, O_2, and H_2O_2; energy input per pulse; pulse length; pH) except the wavelength. By repeating pulse experiments at 5-nm intervals between 450 and 700 nm, the total spectral change occurring in this region was mapped as a function of time. The results are illustrated in Fig. 3a and 3b. The three-dimensional display of absorbance vs wavelength vs log time, was obtained by using a special computer subroutine (Pure Joy). Fig. 3a, shows the view where, for all practical

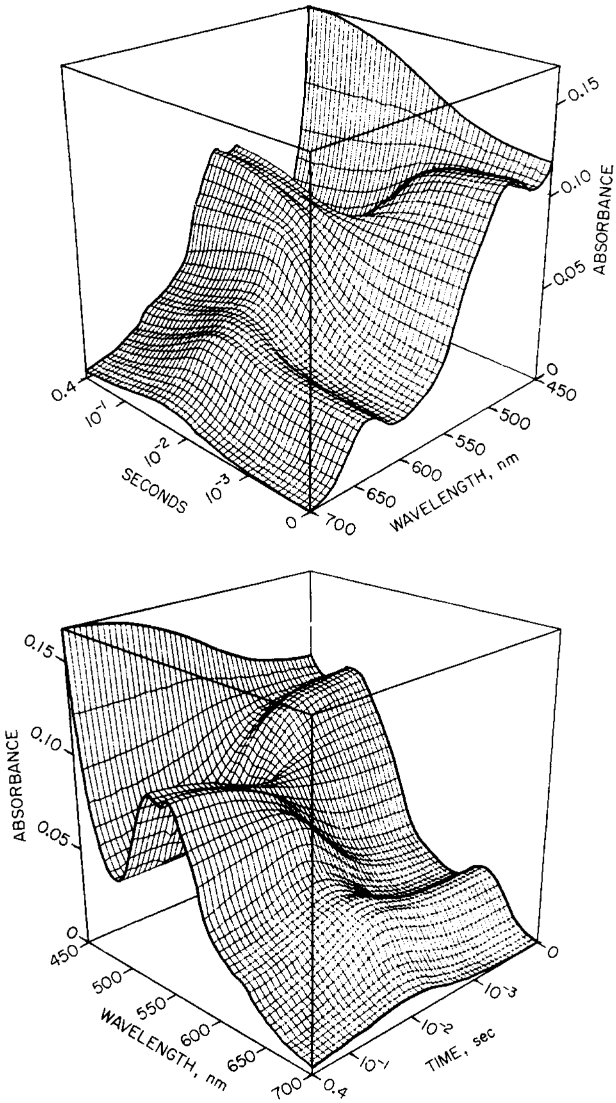

Fig. 3a, b. Three dimensional-computer presentation of spectral changes observed after pulse irradiation of an air-saturated 0.05 M sodium formate solution (pH 7.2) containing $1.8 \cdot 10^{-6}$ M horseradish peroxidase. The 12-μs pulse of 1.9-MeV electrons produced $5.2 \cdot 10^{-6}$ M H_2O_2 (initial yield) and $4.7 \cdot 10^{-5}$ M O_2^-. The optical light path was 6.1 cm. The monochromator was set for 2 nm resolution. The spectrum (4b) at 0.4 s shows the characteristic maxima of Compound II at 527 and 555 nm.

purposes, zero time is the end of the 12-μs electron pulse before the enzyme reactions set in. Hence the observed absorbance at time zero shows the spectrum of unreacted enzyme. As is apparent, changes in absorbance are not noticeable until the ms range (10^{-3}–10^{-2} s) is reached. Fig. 3b, shows a cross-cut at 0.4 s with the two characteristic peaks of Compound II at 527 and 555 nm respectively. The location of these peaks can be seen with greater precision in the two-dimensional presentation in Fig. 4,

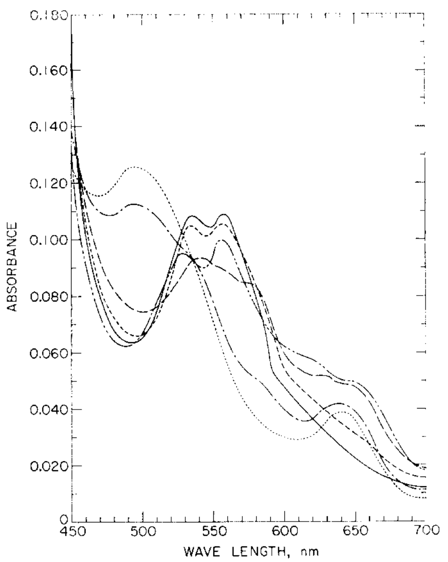

Fig. 4. Spectral changes observed upon pulse irradiation of an air-saturated 0.05 M sodium formate solution, pH 7.2, containing $1.8 \cdot 10^{-6}$ M horseradish peroxidase. A 12-μs pulse of 1.9-MeV electrons produced $5.2 \cdot 10^{-6}$ M H_2O_2 and $4.7 \cdot 10^{-5}$ M of superoxide radicals. Curves: · · · · · ·, before the pulse; ·-·-·-·-·-·-, 2 ms after the pulse; — — —, 12 ms after the pulse; --------, 70 ms after the pulse; ———, 400 ms after the pulse; -·-·-·-·-·-, 5 min after the pulse. The last curve was recorded on a Cary 14 spectrophotometer.

which shows the absorption spectra of free enzyme before pulsing and at various selected times after irradiation. Although the absorption curve at 12 ms after irradiation shows a small shoulder at 580 nm, it never develops into a definitive spectrum of Compound III indicating that the latter is present at best only as a trace in comparison to Compound II. Subsequent variation of the ratio of O_2^-/enzyme up to 160:1 did not affect either the spectral or kinetic results. In all cases the predominant species observed after termination of the reaction was Compound II.

When solutions of Compound I ($1.4 \cdot 10^{-6}$ M prepared by mixing enzyme and H_2O_2) were pulsed twice in succession ($4.8 \cdot 10^{-6}$ M O_2^- per pulse), the pulses being spaced 1, 2 or 4 s apart, no significant change in the absorption spectrum was observed following the first pulse. These experiments suggest that Compound II, which is always formed by the first pulse, does not react with O_2^- produced in the subsequent pulses, even when the radical was produced at a rate of 160 μmoles/pulse.

Rate studies by pulse radiolysis

The rate of reaction between the radiation generated superoxide radical and Compound I was followed spectrophotometrically at 410 nm (isosbestic point for free enzyme and Compound II) and 427 nm (isosbestic point for free enzyme and Compound I), thus the absorbance change at 410 nm represents the disappearance of Compound I and that at 427 nm represents the formation of Compound II.

The experiments were carried out in such a way, that the enzyme and H_2O_2 peroxide, both in air-saturated 0.05 M sodium formate, were rapidly mixed and transferred to the radiation cell to be pulse irradiated within one minute of mixing time. A small excess of H_2O_2 was used to assure that Reaction 1 had gone rapidly to completion. The system irradiated consisted of Compound I and the excess of H_2O_2. The rate measurements for Reaction 5 were determined under pseudo-first-order conditions and are given in Table I. The average value for the second-order rate constant is $k_5 = 1.60 \cdot 10^6 \pm 0.08 \cdot 10^6\ M^{-1} \cdot s^{-1}$. The good agreement between the rate of disappearance of Compound I (at 410 nm) and the rate of appearance of Compound II (at 427 nm) indicate that no other significant reactions were taking place in this system. When similar experiments were carried out at pH 8.1, the same k_5 value (within experimental error) was obtained indicating that the reacting species is O_2^- and not HO_2.

TABLE I

OBSERVED PSEUDO FIRST-ORDER DISAPPEARANCE OF COMPOUND I AT 410 nm AND FORMATION OF COMPOUND II AT 427 nm

Compound I was produced by mixing horseradish peroxidase and 1.5 equivalents of H_2O_2. It was then irradiated (12-μs pulse of 1.9 MeV electrons) in 0.05 M sodium formate solution at pH 7.2 and 23.5 °C. The pulse produced $3.0 \cdot 10^{-7}$ M superoxide radicals and $3.3 \cdot 10^{-8}$ M of H_2O_2.

Wave length (nm)	Compound I ($M \times 10^6$)	k_{obs}, (s^{-1})	$k_{calc} \times 10^{-6}$ ($M^{-1} \cdot s^{-1}$)
411	1.38	2.12	1.54
427	1.38	2.16	1.56
411	2.14	3.46	1.62
427	2.14	3.38	1.58
411	3.16	5.32	1.68
427	3.16	5.58	1.77
411	4.15	6.30	1.52
427	4.15	6.42	1.55

DISCUSSION

It has been shown that formate does not interfere with the reactions being investigated but it protects the enzyme from attack by the primary radicals generated in pulse radiolysis.

The results from the stopped-flow experiments show that the rate of formation of Compound I can be determined independently at 335 nm, instead of the traditional 410 nm. They indicate that in the conversion of horseradish peroxidase to Compound I, no other reaction intermediates are observed throughout the range from 300 to 450 nm.

In view of the fact that H_2O_2 is produced as a molecular product in radiolysis and also as an end-product of O_2^- disproportionation, it is almost impossible to determine accurately the rate constant for the reaction of O_2^- with free enzyme in pulse radiolysis, where the initial ratio of O_2^-/H_2O_2 is about 9. The difficulty lies in the inherent magnitude of the rate constants for reactions (1) and (5):

$$\text{enzyme} + H_2O_2 \xrightarrow{k_1 = 1.2 \cdot 10^7\ M^{-1} \cdot s^{-1}} \text{Compound I} \quad (1)$$

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

$$\text{enzyme} \quad + O_2^- \xrightarrow{\qquad\qquad} \text{Compound III} \qquad (6)$$

Reaction 1 had been thoroughly studied in the past. In the present investigation $k_5 = 1.60 \cdot 10^6 \pm 0.08 \cdot 10^6\ M^{-1} \cdot s^{-1}$ was determined under conditions which eliminates interference from Reaction 1. Both spectral and kinetic evidence indicate that O_2^- acts as a reducing agent toward Compound I.

Attempts to determine Reaction 6, which has been extensively studied in biological systems [3], failed under present experimental conditions. Since Compound III was not observed in the present investigation, it is assumed that the rate constant k_6 must be orders of magnitude smaller than k_1.

That O_2^- does not react with Compound II was shown in successive pulse experiments, in which the first pulse converted Compound I to Compound II (Reaction 5), and O_2^- generated in subsequent pulses did not change the absorption spectrum of Compound II. A similar conclusion can be drawn from the ^{60}Co γ-ray studies, where under steady-state conditions $6.7 \cdot 10^{-6}$ M of Compound I had been exposed to a total of $5.2 \cdot 10^{-5}$ M O_2^-. In view of the magnitude of k_5, Compound I was effectively converted to Compound II during the first 10 s of irradiation, hence Compound II was exposed to $4.52 \cdot 10^{-5}$ M of O_2^- during the remaining 50 s of irradiation without detectable change in its spectrum.

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REFERENCES

1 Bielski, B. H. J. (1972) Biochim. Biophys. Acta 289, 57–67
2 Brill, A. S. (1966) in Comprehensive Biochemistry (Florkin, M. and Stotz, E. H., eds), Vol. 14, pp. 447–479, Elsevier, Amsterdam
3 Yamazaki, I., Nakajima, R., Miyoshi, K. and Tamura, M. (1973) in Oxidases and Related Redox Systems (King, T. E., Mason, H. S. and Morrison, M., eds), pp. 407–418, University Park Press, Baltimore
4 Bielski, B. H. J. and Gebicki, J. (1969) in Advances in Radiation Chemistry, Vol. 2, pp. 210–211, Wiley-Interscience, New York
5 Behar, D., Czapski, G., Rabani, J., Dorfman, L. M. and Schwarz, H. A. (1971) J. Phys. Chem. 74, 3209–3213
6 Anbar, M. and Neta, P. (1967) J. Appl. Radiat. Isot. 18, 493–523
7 Chan, P. C. and Bielski, B. H. J. (1973) J. Am. Chem. Soc. 95, 5504–5508
8 Brill, A. S. and Williams, R. J. P. (1961) Biochem. J. 78, 246–253