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STUDY OF PEROXIDASE MECHANISMS BY PULSE RADIOLYSIS

I. SPECTRA OF HORSERADISH PEROXIDASE TRANSIENTS*

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SUMMARY

1. The basic principles for application of pulse radiolysis to the study of peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) mechanisms are discussed and illustrated on the horseradish peroxidase–ascorbic acid–H_2O_2 system.

2. The onset and course of the entire enzymatic cycle is shown in an ascorbic acid–horseradish peroxidase solution after H_2O_2 is formed during a 10-μs electron pulse.

3. Spectral changes with time in the range between 310 and 480 nm show the characteristic absorption maximum of Compound II (418 nm).

4. A transient with an absorption maximum at 355 nm has been observed in the presence and absence of ascorbate.

5. At low ascorbic acid concentration the enzyme disappears at pH 4 by reaction with H_2O_2, with a rate constant $k_1 = 1.19 \cdot 10^7\ M^{-1} \cdot s^{-1}$.

INTRODUCTION

Enzyme systems like the peroxidases (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) can be conveniently studied by pulse radiolysis as will be illustrated on the horseradish peroxidase–H_2O_2–ascorbic acid (AH_2) system.

When aqueous solutions are pulse irradiated with high energy electrons, the situation at the end of the pulse can be described by the following equation:

$$H_2O \text{ —}\sim\sim\rightarrow e^-_{aq},\ H_3O^+,\ H,\ OH,\ H_2O_2,\ H_2 \qquad (I)$$

The radicals and molecular products are homogeneously distributed throughout the irradiated solution, and while the radicals disappear very rapidly (in microseconds or less) either by interaction with themselves or an added scavenger, the molecular product H_2O_2 remains. Hence pulse radiolysis affords a method of instant addition of

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H_2O_2 (substrate) so that the enzymatic reaction can be followed a very short time afterwards.

To protect the enzyme from the destructive attack of the primary radicals, it is necessary to add an efficient radical scavenger. The scavenger should be preferentially a natural hydrogen donor for the given peroxidase, since the radical formed would then not be alien to the system, but hopefully be the same as would be formed during true enzymatic reaction. Hence a situation can be created in which a short time after the pulse the system consists of the enzyme (E), the substrate (H_2O_2), the hydrogen donor (AH_2) and the radical ($AH\cdot$). Ascorbic acid was chosen for the present study because it fulfills all the basic requirements discussed above.

METHODS

Chemicals

L-Ascorbic acid, reagent grade (Fisher Scientific Co.) was used without further purification. Peroxidase Horseradish, RZ 2.9, 3000 units/mg (Nutritional Biochemicals Corp.) was used without further purification. All solutions were prepared in triply distilled water, and bubbled with nitrous oxide to remove all dissolved oxygen and saturate them with the gas.

Apparatus

The electron pulse generator (2 MeV Van de Graaff), optics and monitoring equipment have been described in earlier publications[1,2]. The optical path length in the present experiments was 6.1 cm. The monochromator was set for 2 nm resolution.

RESULTS

It has been well established that the ascorbic acid radical formed by pulse radiolysis is the same as the radical generated by enzymatic catalysis or chemical dismutation of the parent compounds[3–10].

When an ascorbate solution saturated with nitrous oxide is pulse irradiated, the overall mechanism for radical formation and decay above pH 3.5, can be described by reactions (I), (II), (III) and (IV) (ref. 4):

$$N_2O + e^-_{aq} + H_2O \rightarrow N_2 + OH + OH^- \quad (II)$$
$$AH_2 + OH \rightarrow AH\cdot + H_2O \quad (III)$$
$$AH\cdot + AH\cdot \rightarrow AH_2 + A \quad (IV)$$

where AH_2 stands for ascorbate, $AH\cdot$ for ascorbic acid free radical and A for dehydroascorbate. Under high dose conditions the generated ascorbic acid radical can itself become an effective radical scavenger for OH radicals:

$$AH\cdot + OH \rightarrow A + H_2O \quad (V)$$

The scavenging efficiency of this system was tested in the present investigation in terms of the protection it offers horseradish peroxidase against radiation damage. When horseradish peroxidase in solution is exposed to high energy ionizing radiation, it becomes deactivated as shown (Fig. 1) by a series of experiments in which samples of a 1.5 μM enzyme solution saturated with nitrous oxide were exposed to 50-μs pulses of 1.9-MeV electrons. The total energy input per sample was varied up to about 100

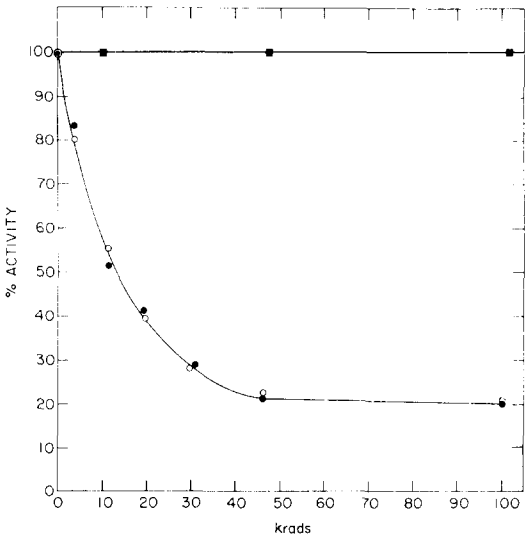

Fig. 1. Percent decrease in horseradish peroxidase activity as a function of total dose (krads) for 1.9-MeV electrons. Solutions contained 1.5 μM horseradish peroxidase in 0.01 M phosphate buffer ●, pH 4.0; ○, pH 7.0; ■, pH 4.0 in presence of 5 mM ascorbate. The solutions were saturated with N_2O.

krads; high doses required the delivery of several pulses per sample. Following the irradiation, the samples were immediately assayed for enzyme activity by the guaiacol method[11]. Corresponding unirradiated controls were assigned 100% activity.

A second series of radiation experiments was carried out with the same enzyme solutions containing 5 mM of ascorbic acid. After irradiation the ascorbic acid was removed by dialysis and the samples were assayed as before for enzymatic activity. The results were compared to controls, which had not been irradiated but were otherwise treated in the same way. Within experimental error no loss in activity could be observed up to 200 krads energy input per sample.

A systematic study of spectral changes as a function of time, following a single 10-μs pulse was carried out with the following three solutions: Solution I (0.43 μM enzyme; 50.0 μM AH_2; 0.71 krad/pulse); Solution II (0.85 μM enzyme; 0.25 mM AH_2; 3.56 krads/pulse); Solution III (0.53 μM enzyme; 10.0 mM AH_2; 3.56 krads/pulse). The solutions were adjusted to pH 4.0 by addition of either NaOH or H_2SO_4, and saturated with N_2O. The pulse experiments were carried out at ambient temperature (23–24 °C). The amounts of H_2O_2 formed were 2.7 $\pm$ 0.2 μM by the 3.56 krads/pulse and 0.54 $\pm$ 0.02 μM by the 0.71 krad/pulse.

Measurements of absorbance changes, were taken in the spectral range (320–480 nm) where horseradish peroxidase and its peroxide addition compounds I and II have maxima with highest molar extinction (Enzyme max. at 403 nm, $\varepsilon = 9.1 \cdot 10^4$, Compound I max. at 410 nm, $\varepsilon = 4.5 \cdot 10^4$; Compound II max. at 418 nm, $\varepsilon = 7.7 \cdot 10^4$)[12]. The spectrum of horseradish peroxidase is not affected by either nitrous oxide or ascorbic acid.

Depending upon the wavelength and scanning time, the observed change in absorbance, Δ(absorbance), which follows the electron pulse, can have either positive

or negative values. In this study these changes are read with respect to a pretrace recorded shortly before the radiation pulse. Actually the oscillograms show change in voltage with time, where the voltage is related to absorbance by the relation ln $V_0/(V_0 - \Delta V) =$ (absorbance). V_0 is the voltage representing 100% transmission before the pulse and ΔV is the difference between trace and pretrace at a given time.

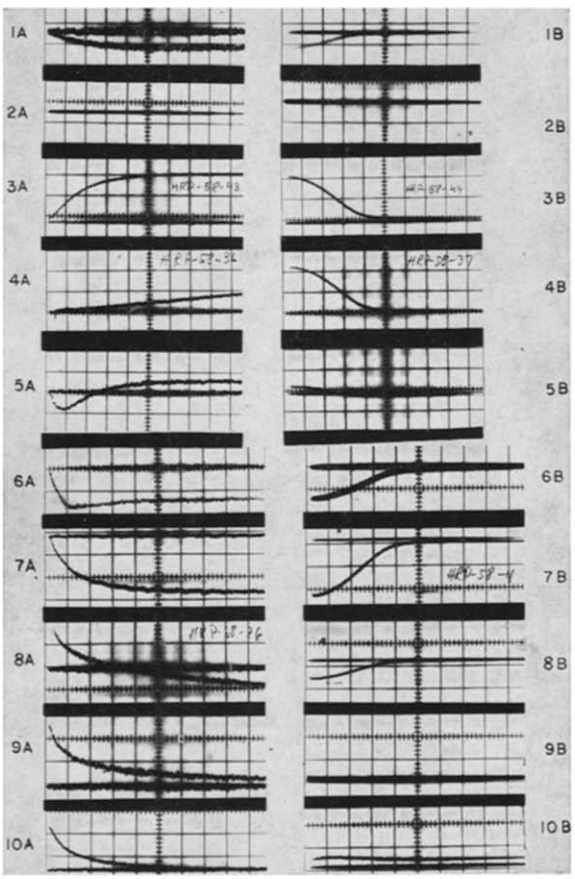

Fig. 2. Oscillograms showing change in voltage with time, after a 10μs pulse of 1.9-MeV electrons deposited 3.56 krads into (a) Solution II (0.85 μM enzyme; 0.25 mM AH_2; N_2O saturated; pH 4), Traces 1A, 1B to 9A, 9B. (b) Solution III (0.53 μM enzyme; 10.0 mM AH_2; N_2O saturated; pH 4), Traces 10A and 10B.

Wavelength (nm)	*Trace No.*	*Scan (s/cm)*	*Trace No.*	*Scan (s/cm)*
470	1A	0.0100	1B	0.5
450	2A	0.0500	2B	0.2
427	3A	0.0100	3B	0.2
420	4A	0.0010	4B	0.5
411	5A	0.0100	5B	0.5
408	6A	0.0100	6B	0.5
403	7A	0.0100	7B	0.5
360	8A	0.0020	8B	0.5
300	9A	0.0005	9B	0.5
360	10A	0.0010	10B	0.2

To illustrate in what detail optical changes with time can be observed, a number of oscillograms are reproduced in Fig. 2. Relatively slow scan times were picked (Solution II), as they show more distinctly the formation and decay of transients (see Discussion) and also have a higher signal-to-noise ratio. Pairs of oscillograms at a given wavelength always show an initial relatively rapid reaction (Series A) and a slower return to the line of the pretrace (Series B). Inspection of the traces (Fig. 2) indicates that:

(1) At 470 nm (1A, 1B), the enzyme is transformed into a less absorbing transient(s).

(2) At 450 nm (2A, 2B), this particular solution shows an isosbestic point, since trace and pretrace coincide.

(3) At 427 nm (3A, 3B), the formation and decay of a strong absorbing transient(s) is observed.

(4) At 420 nm (4A), the slope becomes progressively steeper as time passes suggesting an induction period for the formation of this absorbing transient.

(5) Traces 5A and 6A show dramatically the difference in spectral characteristics between 411 nm and 408 nm. The unusual change in absorbance with time at 411 nm (see also curve for 412 nm in Fig. 3) is due to differences in molar absorbance of the three major components of this system at this wavelength (ε of Compound I $< \varepsilon$ of horseradish peroxidase $< \varepsilon$ of Compound II). These differences in absorbance can be seen both in Fig. 5 (arrow indicates 412 nm) and in Fig. 3 of ref. 14. Hence the initial decrease in absorbance indicates the disappearance of free enzyme with formation of the less absorbing Compound I. The latter upon being converted to Compound II causes an increase in absorbance above the pretrace (pretrace represents absorbance of free enzyme) since it has a higher molar absorbance than free enzyme. Finally, as Compound II is converted to free enzyme absorbance decreases to the level of the pretrace indicating completion of the enzymatic cycle.

It was found that in 10 mM ascorbic acid solutions similar studies require resolution times of the order $<$ 1 ms.

(6) Traces 7A and 7B, indicate that the optical changes at 403 nm are due to the disappearance and reappearance of free enzyme only[13–18], since no visible change in absorbance occurred during the pulse (pretrace and trace coincide at end of pulse).

(7) The high absorbance at the end of the pulse in Oscillogram 8A, indicates that ascorbic acid radicals had been formed during the pulse. At this wavelength ascorbic acid radical has one of its maxima[4]. Since the trace drops in time below the pretrace, it is quite apparent that changes in enzyme absorbance also take place. The slow return of the trace (8B) to the pretrace, represents regeneration of free enzyme.

(8) Oscillogram 9A, shows at 300 nm a very rapid second-order decay of ascorbic acid radical. The rate constant computed from this trace, was $k = 7.6 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ as compared to $k = 8.0 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ reported earlier at pH 4 in the absence of enzyme[4]. That the enzymatic reaction does not interfere at this wavelength can be best seen from Oscillogram 9B, where pretrace and retrace are superimposed.

(9) Oscillograms 10A and 10B were taken with a 10 mM ascorbic acid solution (for experimental detail see caption of Fig. 2). As can be seen in 10A, the trace returns to the pretrace in about 5–6 ms, to be followed by a slow build-up (approx. 0.2 s, 10B) and decay. Comparison with 8B, indicates that these subtle differences will have to be resolved by kinetic analysis of the system over a wide ascorbic acid range.

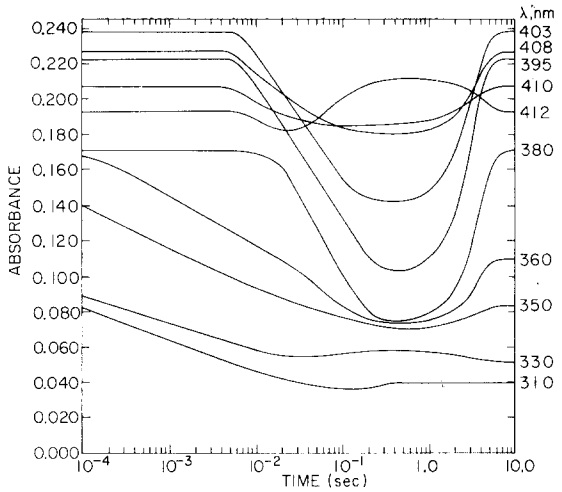

Fig. 3. Change in absorbance with time at selected wavelengths in Solution I (0.43 μM enzyme; 50.0 μM AH_2; 0.71 krad/pulse) after irradiation for 10 μs with 1.9-MeV electrons.

The entire course of the enzymatic reaction following an electron pulse is most clearly illustrated with the data obtained from Solution I (Figs 3 and 4). The curves in these figures, show change in absorbance with time at different wavelengths. Each curve represents the combined results of three pulse experiments determined under identical conditions, but recorded by oscilloscope with different scan times. The combined overlapping scan times give a time range from 10^{-4} to 10 s. The absorbance at a given wavelength and time, is equal to $(\text{absorbance})_0 \pm \Delta(\text{absorbance})$ where $(\text{absorbance})_0$ is the absorbance of free enzyme before the pulse and Δ(absorbance) is the computed change in absorbance (difference between trace and pretrace on oscillogram) at a given time. All Δ(absorbance) values were corrected for deviations (approx. 2%) in energy input per pulse.

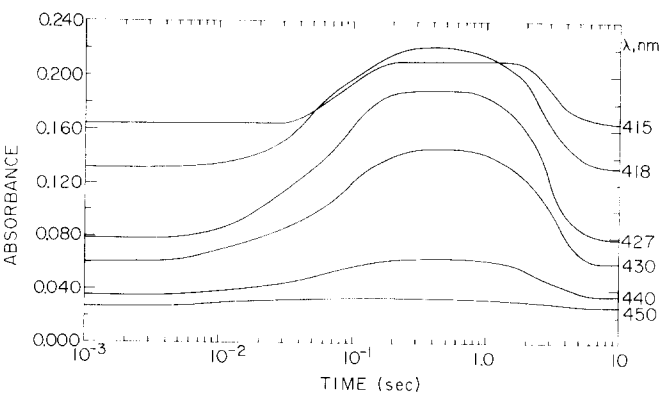

Fig. 4. Change in absorbance with time at selected wavelengths in Solution I (0.43 μM enzyme, 50.0 μM AH_2; 0.71 krad/pulse) after irradiation for 10 μs with 1.9-MeV electrons.

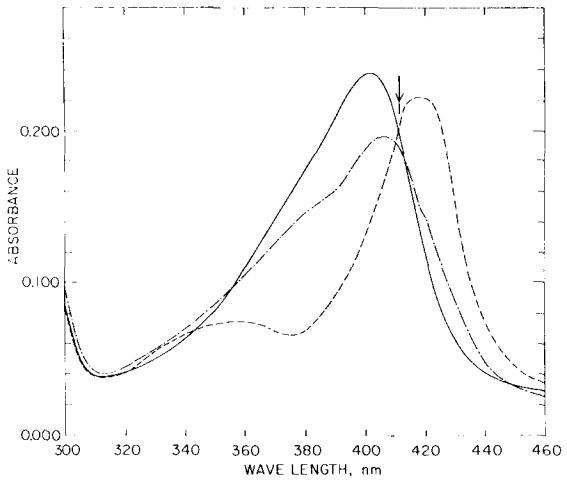

Fig. 5. Absorption spectra of horseradish peroxidase and transients observed in Solution I (0.43 μM enzyme; 50.0 μM AH_2; 0.71 krad/pulse) following a 10-μs pulse of 1.9-MeV electrons. ———, spectrum of horseradish peroxidase before and 10 s after the pulse; — · —, spectrum of transients (70% free enzyme; 14% Compound I; 15% Compound II) 30 ms after termination of pulse; ---------, spectrum of transients (Compound II > 90%) 0.5 s after termination of pulse. (Arrow 412 nm.)

An alternate presentation of data is shown in Figs 5, 6, and 7, where absorbance at selected times is plotted as a function of wavelength, for Solutions I, II and III.

Experiments on the activating effect of ascorbic acid on the rate of disappearance of free enzyme, has been extended to concentrations of $9.5 \cdot 10^{-2}$ M. Changes in absorbance at 395 nm with time followed strictly pseudo first-order kinetics (Table I).

TABLE I

OBSERVED FIRST-ORDER DECREASE IN ABSORBANCE AT 395 nm UPON ADDITION OF 2.7 $\pm$ 0.2 μM H_2O_2 BY PULSE RADIOLYSIS TO HORSERADISH PEROXIDASE SOLUTIONS (1 μM) CONTAINING VARIABLE AMOUNTS OF ASCORBIC ACID (pH 4, 23–24 °C)

Ascorbic acid (M)	$t_{\frac{1}{2}}$ *(s)*	k *(s^{-1})*
$3.80 \cdot 10^{-5}$	$2.17 \cdot 10^{-2}$	32.0
$1.00 \cdot 10^{-4}$	$1.88 \cdot 10^{-2}$	37.0
$5.00 \cdot 10^{-4}$	$1.38 \cdot 10^{-2}$	50.0
$1.00 \cdot 10^{-3}$	$9.70 \cdot 10^{-3}$	71.0
$5.00 \cdot 10^{-3}$	$3.00 \cdot 10^{-3}$	230.0
$1.00 \cdot 10^{-2}$	$2.10 \cdot 10^{-3}$	320.0
$2.00 \cdot 10^{-2}$	$1.50 \cdot 10^{-3}$	460.0
$5.00 \cdot 10^{-2}$	$1.30 \cdot 10^{-3}$	530.0
$9.50 \cdot 10^{-2}$	$1.30 \cdot 10^{-3}$	530.0

DISCUSSION

The interaction of horseradish peroxidase with H_2O_2 in the presence of ascorbic acid, can be described by the following set of equations:

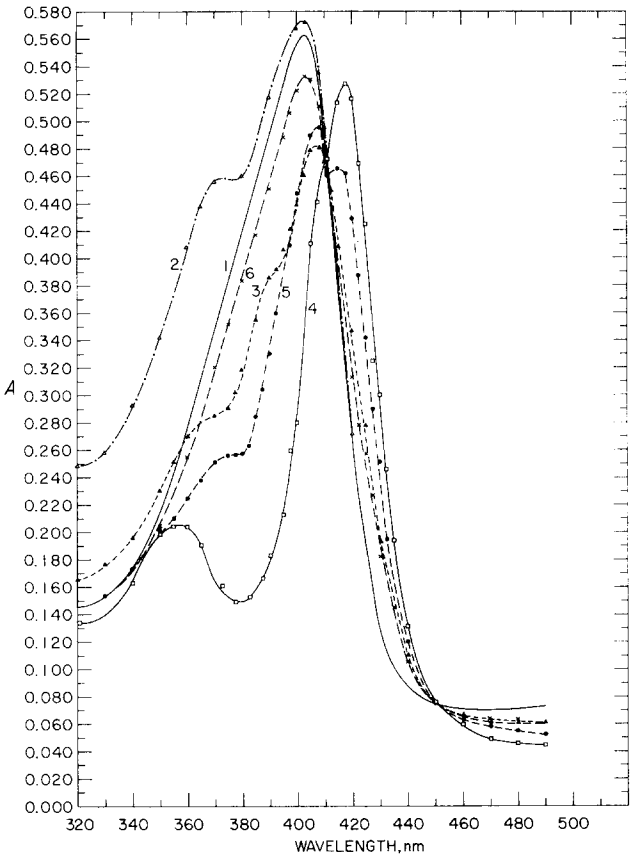

Fig. 6. Spectra observed at different times in Solution II (0.85 μM enzyme; 0.25 mM AH_2; 3.56 krads/pulse) after it had been pulse irradiated for 10 μs with 1.9-MeV electrons. Curve 1, spectrum of horseradish peroxidase before pulse; 2, spectrum at the end of the 10 μs pulse; 3, spectrum 3 ms after pulse; 4, spectrum 48 ms after pulse; 5, spectrum 1 s after pulse; 6, spectrum 1.5 s after pulse.

Enzyme	+ H_2O_2	$\rightleftharpoons$ Compound I			(VI)
Compound I	+ AH_2	$\rightarrow$ Compound II	+ AH·		(VII)
Compound II	+ AH_2	$\rightarrow$ Enzyme	+ AH·		(VIII)
AH·	+	$\rightarrow$ AH·	+ AH_2		(IV)

While the sequence of these reactions and the corresponding rate constants were determined by Chance[13–18], inclusion of the ascorbic acid free radical into the classic mechanism is credited to Yamazaki and co-workers[5–7], who observed the radical in this system by electron spin resonance.

In pioneering with flow techniques, Chance[13–18] demonstrated that the complete enzymatic cycle could be followed spectrophotometrically, if the concentration of ascorbic acid was kept below $2 \cdot 10^{-4}$ M. This upper limit of ascorbic acid concentration was fixed by the mixing time (1–2 ms) of the flow apparatus. The present study reports on how the time resolution problem can be overcome by application of pulse radiolysis, which operates with ease in the microsecond range.

As is apparent from Figs 3 and 4, absorbance changes due to enzymatic

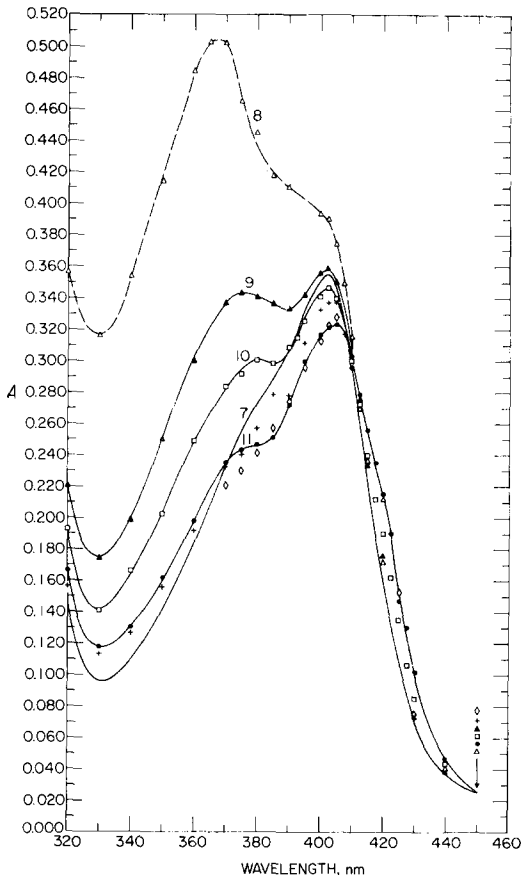

Fig. 7. Spectra observed at different times in Solution III (0.53 μM enzyme; 10.0 mM AH_2; 3.56 krads/pulse) after it had been pulse irradiated for 10 μs with 1.9-MeV electrons. Curve 7, spectrum of horseradish peroxidase before pulse; 8, spectrum at the end of the 10 μs pulse; 9, spectrum 1 ms after pulse; 10, spectrum 2 ms after pulse; 11, spectrum 8 ms after pulse; ◊, spectrum 20 ms after pulse; +, spectrum 100 ms after pulse.

reaction can be easily followed above 380 nm, since above this wavelength absorbance due to ascorbic acid radical becomes negligible. At 403 nm the molar extinction of horseradish peroxidase is 150 times that of the ascorbic acid radical. Since the curves above 380 nm (Figs 3 and 4) show no change in absorbance until about $5 \cdot 10^{-3}$ s after termination of the electron pulse, it indicated that ascorbic acid radical does not react with horseradish peroxidase.

The kinetic properties of the ascorbic acid radical were checked in this system at 310 nm, since at this wavelength the spectrum of horseradish peroxidase does not undergo significant changes during the decay time of the radical. Its rate of decay was similar to the rate reported earlier in the absence of enzyme[4] (see also Results, Oscillogram 9A, Fig. 2).

Formation and disappearance of Compound II (418 nm), can be easily observed (Figs 5 and 6) in solutions of low ascorbic acid concentrations (50–250 μM) on account of its high steady-state concentration (about 90% 0.5 s after the pulse, Fig. 5). A

high steady-state concentration of Compound II is expected in view of preliminary rate measurements ($k_{VII} = 4.5 \cdot 10^5\ M^{-1} \cdot s^{-1}$ and $k_{VIII} = 1.1 \cdot 10^4\ M^{-1} \cdot s^{-1}$ for 50 μM AH_2), which indicate that the ratio of k_{VII}/k_{VIII} is about 40. The numerical values for these rate constants are in close agreement with values reported earlier for buffered solutions[16].

The effect of very high ascorbate concentration (10 mM) upon the steady-state concentration of Compounds II is illustrated in Fig. 7, where absence of spectral detail is in agreement with steady-state kinetics, which predicts a decrease in concentration of intermediates with increasing hydrogen donor concentration.

While Compound II can be observed directly, the presence of Compound I in these experiments can be deduced only from appropriate kinetic measurements at 410 nm, where according to Chance[14] horseradish peroxidase and Compound II are isosbestic. Hence, with the use of spectral information from Fig. 3 of ref. 14, it can be shown that 30 ms after termination of the electron pulse, the concentrations of Compound I was approx. 0.06 μM or 14% of total enzyme present (see Fig. 5).

Preliminary kinetic measurements at 395 nm show that when $2.7 \pm 0.2 \cdot 10^{-6}$ M H_2O_2 are added by pulse radiolysis to an ascorbate solution (38 μM, pH 4) containing horseradish peroxidase (1 μM), the enzyme disappears by reaction with H_2O_2 (VI) with a rate constant $k = 1.19 \cdot 10^7\ M^{-1} \cdot s^{-1}$ which is in good agreement with earlier reports[13–16]. Increasing concentrations of ascorbic acid increase the pseudo first-order change in absorbance at this wavelength (Table I), an effect already reported by Chance[13–16]. As is apparent, the change in the first-order rate constant with ascorbate concentration, follows a sigmoid curve. Since appropriate combinations of kinetic constants can lead to this type of curve, an extensive study of all rate constants over a wide ascorbic acid concentration range has been initiated to establish if the observed effect is kinetic in nature or due to changes involving the structure of the enzyme.

Inspection of Figs 5 and 6, shows the formation of a transient compound which has an absorption maximum at 355–360 nm. Its formation and disappearance in the presence of 10 mM ascorbate is shown in Oscillogram 10B (Fig. 2). Since a similar absorption spectrum was observed in the absence of ascorbate, it can be concluded, that although its absorption maximum coincides with that of the ascorbic acid radical it is not related to it.

The above illustration and guidelines for use of pulse radiolysis in the study of peroxidase mechanisms, may possibly be also applicable to other enzyme systems in which free radicals have been observed or for which substrate molecules or inhibitors could be synthesized by this method on a very short time scale.

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