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THE OSCILLATING PEROXIDASE-OXIDASE REACTION IN AN OPEN SYSTEM

ANALYSIS OF THE REACTION MECHANISM

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Summary

1. The oscillations in the peroxidase-oxidase reaction in an open system with NADH as the hydrogen donor are caused by the reaction starting and stopping at critical concentrations of the substrates O_2 and NADH. The existence of such critical concentrations is typical of branched chain reactions.

2. The critical concentrations of O_2 and NADH that determine the initiation of the reaction are mutually dependent.

3. The branching reactions that determine these critical concentrations involve compounds I and II.

4. Superoxide may be involved in the branching reactions by reacting with NADH and ferriperoxidase. At pH 5.1 the rate constant for the latter reaction is determined as $1.5 \cdot 10^5\ M^{-1} \cdot s^{-1}$, whereas for the former reaction only an upper limit for the rate constant of $3.5 \cdot 10^4\ M^{-1} \cdot s^{-1}$ could be estimated. These relatively low rate constants suggest that alternative branching reactions may also be involved.

Introduction

The peroxidase-oxidase reaction

$$2YH_2 + O_2 \rightarrow 2Y + 2H_2O \quad (1)$$

has a number of peculiar properties including autocatalysis and substrate inhibition by oxygen. When the reaction is taking place in an open system the first of these properties may give rise to damped and sustained oscillations [1—7] and chaos [8] whereas the substrate inhibition causes bistability [3,7,9].

Although it is widely agreed that the peroxidase-oxidase reaction is a free radical branched chain reaction there is still some uncertainty about its mechanism, the main problem being the role of superoxide. The peroxidase-oxidase reaction is inhibited by superoxide dismutase [10,11] and this has been taken

as evidence for superoxide being involved in the branching reactions. It has been suggested that the oxidation of NADH by superoxide is involved in the chain branching. The rate constant of this reaction determined by Land and Swallow [12] at pH 8.6 is too small to propose that the reaction should be included in the reaction mechanism of the peroxidase-oxidase reaction. However Patriarca et al. [13] have suggested that the oxidation of NADH by superoxide may be faster at low pH (i.e. pH < 6) than at high pH. The reaction may also occur at an appreciable speed if NADH is enzyme bound as found with lactate dehydrogenase [14] but so far there is no evidence for a peroxidase · NADH complex.

The formation of compound III (oxyperoxidase) from ferriperoxidase and superoxide has also been suggested as part of the branching reactions. Whereas at neutral pH this reaction was shown to occur with myelo peroxidase [15], Bielski et al. [16] using horseradish peroxidase were unable to detect the formation of compound III from ferriperoxidase and superoxide generated by pulse radiolysis.

The experiments described here were carried out to try to establish that the oscillations in the peroxidase-oxidase reaction in the open system are caused by an autocatalytic reaction mechanism determining critical concentrations of the substrates, NADH and O_2. Secondly an attempt has been made to elucidate the individual reaction steps which may be involved in the autocatalysis. In particular an attempt was made to decide whether superoxide is involved in the reaction mechanism.

Methods and Materials

Experiments with the oscillating peroxidase-oxidase reaction in an open system were conducted in a dual-wavelength spectrophotometer (Hitachi-Perkin Elmer 356) using a 5 ml hexagonal glass cuvette (optical path length 16 mm) fitted with a constant speed stirrer and thermostated at 28°C. O_2 was introduced by diffusion from a gas phase in which the oxygen content was regulated by mixing N_2 and air using a digital gas mixer. NADH was pumped into the reaction mixture through a capillary by an infusion pump operating at very low speeds with a high precision (Harvard apparatus Co. model 971). Oxygen was measured with a Clark type electrode (Radiometer, Copenhagen) and NADH was measured spectrophotometrically at 380 nm using 400 nm as reference wavelength. A more complete description of the system has appeared previously [6,7].

The reactions of superoxide with NADH and ferric peroxidase were carried out in a double beam spectrophotometer (Cary 118C) fitted with a stirrer for efficient mixing. Superoxide was formed electrolytically in 0.1 M tetra-*n*-butyl ammonium bromide as described by McCord and Fridovich [17] and Odajima and Yamazaki [15]. The superoxide content of the electrolysis cell measured by the catalase method [15] reached a constant value of 10 mM after approximately 140 min of electrolysis time with a current of 6 mA. The presence of O_2^- in the solution was confirmed by testing its ability to reduce cytochrome c^{3+} [17].

Horseradish peroxidase (RZ 1.0), xanthine oxidase and NADH were pur-

chased from Boehringer. Methylene blue, 2,4-dichlorophenol and xanthine were obtained from Merck. 2,4-Dichlorophenol was dissolved in 96% ethanol before use.

Results

The sawtooth oscillations of O_2 and NADH concentrations in the peroxidase-oxidase reaction with continuous supplies of O_2 and NADH are shown in Fig. 1. To ensure that the onset of the reaction is not a time dependent phenomenon similar experiments were done with different infusion rates of NADH. These experiments showed that the infusion rate of NADH had no effect on the critical concentration of NADH at which the reaction starts. The same conclusion can be drawn from experiments where the flow of NADH is stopped before the critical concentration is reached (Fig. 2). Once started, however, the reaction starts again at levels of NADH below the initial switch-on concentration. The explanation for this is that the critical concentration is highly influenced by the presence of intermediates of the reaction. Initially the concentrations of reaction intermediates will be close to zero. The situation is quite different after the reaction has been switched on and off, since one of the intermediates of the reaction, H_2O_2, has a relatively long lifetime and may not have disappeared until the reaction is initiated again.

In the model of the peroxidase-oxidase reaction described previously [6,7] the initiation of the reaction is determined by the following reactions

$$A + B + X \xrightarrow{k_1} 2X$$

$$X \xrightarrow{k_2} P$$

in which A is O_2, B is NADH and X is some intermediate catalyzing its own formation (autocatalysis). According to this model the reaction is initiated whenever $d[X]/dt > 0$, i.e.

$$k_1[A][B][X] > k_2[X]$$

when [A] is constant and [B] continuously varying the reaction will start when [B] becomes greater than its critical concentration defined as

$$[B]_{crit} = \frac{k_2}{k_1[A]} \tag{2}$$

To investigate the effect of the initial concentration of O_2 on the critical concentration of NADH a series of experiments were performed in which the liquid was equilibrated with different amounts of O_2 in the gas phase. A plot of the experimentally determined value of $[NADH]_{crit}$ against the reciprocal oxygen concentration shows that the actual relation between $[NADH]_{crit}$ and $[O_2]$ can be formulated as

$$[NADH]_{crit} = \frac{\alpha}{[O_2]} + \beta \tag{3}$$

where α and β are constants.

It is usually assumed that the enzyme intermediates, compound I and com-

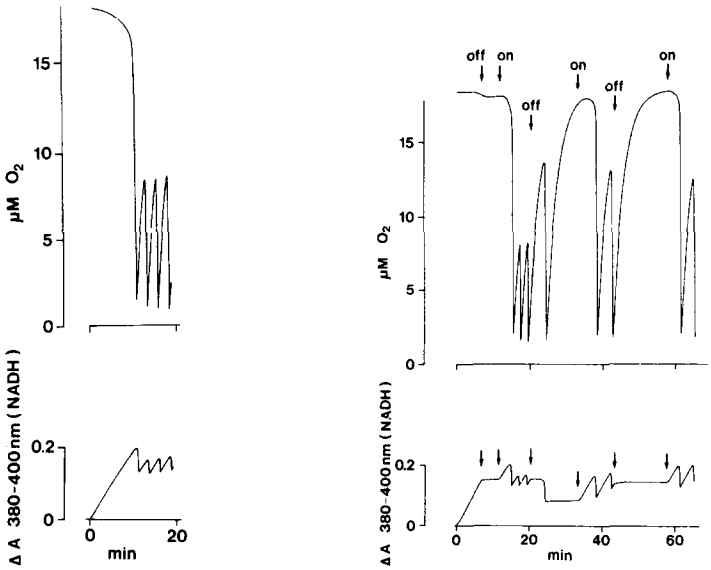

Fig. 1. Sawtooth oscillations in the peroxidase-oxidase reaction in an open system. Experimental conditions: 1.1 μM peroxidase, 10 μM 2,4-dichlorophenol, 0.1 μM methylene blue in 0.1 M acetate buffer pH 5.1. The solution is in contact with a gas phase containing O_2 (1.65% by volume). 0.2 M NADH is infused at a rate of 12.5 μl/h. Temperature 28° C.

Fig. 2. Effect of stopping the NADH infusion at various times. Experimental conditions as in Fig. 1. The flow of NADH was turned off and on as indicated by the arrows.

pound II, involved in the reduction of H_2O_2 by different hydrogen donors are part of the branching reactions in the peroxidase-oxidase reaction. In order to investigate this point experiments similar to that in Fig. 1 were performed with addition of small amounts of hydroquinone. Hydroquinone is an excellent hydrogen donor in the peroxidase reaction with H_2O_2 but cannot serve as a hydrogen donor in the peroxidase-oxidase reaction when O_2 acts as the electron acceptor. Compared to NADH, hydroquinone reacts rapidly with compounds I and II but the free radicals of hydroquinone produced by this reaction cannot cause chain branching. Therefore hydroquinone might be expected to have a chain terminating effect in the peroxidase-oxidase reaction and cause an increase in the critical concentration of NADH. A plot of $[NADH]_{crit}$ against the amount of hydroquinone added shows that the critical concentration increases linearly with the concentration of hydroquinone.

To create an autocatalytic reaction sequence from the reactions known or proposed to be involved in the peroxidase-oxidase reaction one could focus on the formation and disappearence of O_2^-, which is usually assumed to be essential for the reaction to take place. O_2^- is formed by the reaction [12]

$$NAD^{\cdot} + O_2 \xrightarrow{k_4} NAD^+ + O_2^- \qquad (4)$$

and it may be consumed by one or more of the following reactions

$$H^+ + O_2^- + NADH \xrightarrow{k_5} H_2O_2 + NAD^{\cdot} \qquad (5)$$

$$O_2^- + \text{ferriperoxidase} \xrightarrow{k_6} \text{compound III (oxyperoxidase)} \quad (6)$$

$$2H^+ + 2O_2^- \xrightarrow{k_7} H_2O_2 + O_2 \quad (7)$$

Reactions 5 and 6 can be viewed as chain branching steps since H_2O_2 can combine with ferriperoxidase giving compound I that again will produce two new NAD· radicals. Compound III can be reduced to compound I by accepting one electron. Reaction 7 will be neither a chain terminating nor a chain branching step since only two NAD· radicals will be formed from two superoxide radicals.

Reaction 6 can be studied by the help of the xanthine oxidase reaction [17] in which O_2^- is formed as a byproduct in the oxidation of xanthine by O_2

$$O_2 + \text{xanthine} \rightarrow \text{uric acid} + H_2O_2 + O_2^- \quad (8)$$

Fig. 3 shows the spectrum of peroxidase after the addition of xanthine oxidase to a solution containing ferriperoxidase and xanthine. The spectrum shows the formation of compound III, as indicated by the peaks at 542 nm and 578 nm [18] which can be ascribed to the reaction of ferriperoxidase and superoxide. (The addition of an equivalent amount of H_2O_2 in place of xanthine results in the formation of compound II.) The spectrum of ferriperoxidase was not altered by the addition of xanthine oxidase alone. In order to study the kinetics of reaction 6, compound III formation was recorded at 452 nm (the isosbestic point of ferriperoxidase, compound I and compound II [19]). The initial rate of formation of compound III was measured with different amounts of peroxidase in the solution. According to the theory of Sawada and Yamazaki [20], k_6 can be determined from the following equation

$$\frac{v_8}{v_6} = 1 + \frac{k_7}{k_6^2} \frac{v_6}{[\text{per} \cdot \text{Fe(III)}]^2} \quad (9)$$

where v_8 is the rate of the xanthine oxidase catalyzed superoxide formation, v_6 is the initial rate of compound III formation and [per · Fe(III)] denotes the initial concentration of ferriperoxidase. v_8 is easily obtained by extrapolation of v_6 to infinite concentrations of ferriperoxidase. In Fig. 4, v_8/v_6 is plotted against $v_6/[\text{per} \cdot \text{Fe(III)}]^2$. From the slope of the plot a value of $k_6 = 1.5 \cdot 10^5\ M^{-1} \cdot s^{-1}$ is obtained using $k_7 = 2.0 \cdot 10^7\ M^{-1} \cdot s^{-1}$ [21]. This value of k_6 is considerably lower than the values reported earlier [19,20] that are in the range $1.9 \cdot 10^6$— $3.5 \cdot 10^7\ M^{-1} \cdot s^{-1}$.

The xanthine oxidase assay could not be used to estimate the value of k_5 since it could be shown that xanthine oxidase catalyzed the oxidation of NADH by O_2 in the absence of xanthine at pH 5.1 (The product of the reduction of O_2 is H_2O_2 rather than water indicating that the oxidation of NADH is not caused by contamination of xanthine oxidase by peroxidase.) Instead, reaction 5 was studied using superoxide generated electrolytically. The result of adding superoxide to a solution of NADH at pH 5.1 is shown in Fig. 5. The change in the absorbance at 340 nm can be ascribed to the effect of dilution. It is therefore concluded that reaction 5 must be rather slow. The exact value of k_5 cannot be determined from these experiments. However, assuming that the NAD· radicals formed by reaction 5 are dismutated to NAD^+ and NADH rather

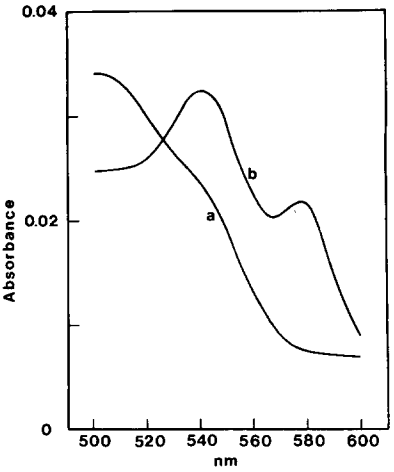

Fig. 3. Formation of compound III from ferriperoxidase and superoxide formed by the xanthine oxidase reaction. (a) Spectrum of 3.2 μM ferriperoxidase in 0.1 M acetate buffer pH 5.1, 50 μM xanthine and 0.1 mM EDTA (b) Spectrum of peroxidase 5 min after the addition of xanthine oxidase to a concentration of 40 ng/ml. Temperature 25°C.

than reacting with oxygen through reaction 4 we are able to estimate an upper limit for the rate constant k_5 by integrating the equations

$$\frac{d[NADH]}{dt} = -\tfrac{1}{2}\, k_5[NADH][O_2^-] \tag{10}$$

$$\frac{d[O_2^-]}{dt} = -\left(k_5[NADH][O_2^-] + k_7[O_2^-]^2\right) \tag{11}$$

The factor $\frac{1}{2}$ in Eqn. 10 refers to the assumption that half of the NAD· radicals formed in reaction 5 are reforming NADH by dismutation.

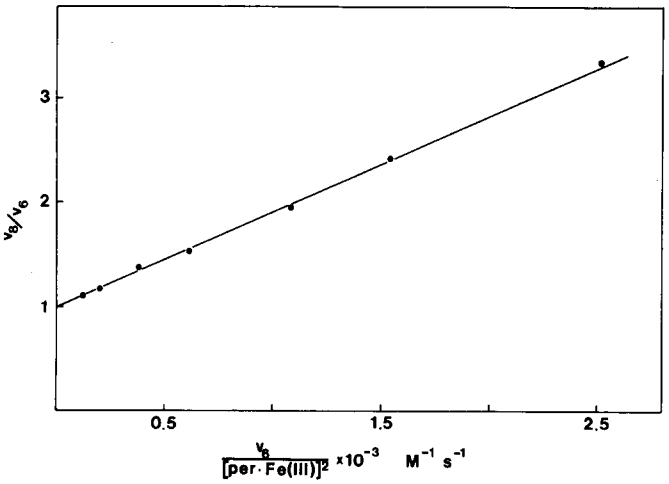

Fig. 4. Plot of v_8/v_6 against $v_6/[per \cdot Fe(III)]^2$. The formation of compound III was measured at 452 nm after the addition of 80 ng/ml of xanthine oxidase to a solution containing 0.1 M acetate buffer pH 5.1, 50 μM xanthine and 0.1 mM EDTA and various amounts of ferriperoxidase. Temperature 25°C.

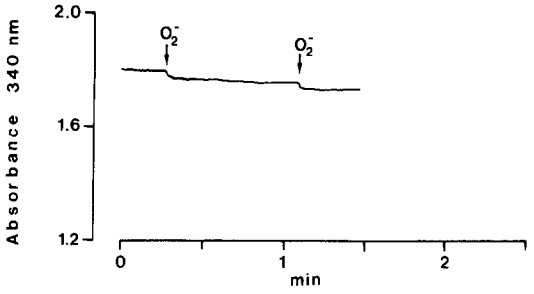

Fig. 5. Oxidation of NADH by superoxide at pH 5.1. To a solution of 0.3 mM NADH was added 160 μM and 160 μM of superoxide respectively at the indicated arrows. Superoxide was formed in 0.1 M tetra-*n*-butyl ammoniumbromide in *N,N*-dimethyl formamide.

Using the observation that the concentration of NADH is almost the same after the addition of O_2^- the following expression can be obtained from the integration

$$\frac{\Delta[\mathrm{NADH}]}{[\mathrm{NADH}]} = \frac{k_5}{2k_7} \ln \frac{\frac{k_5}{k_7}[\mathrm{NADH}]}{[\mathrm{O}_2^-]_0 + \frac{k_5}{k_7}[\mathrm{NADH}]} \tag{12}$$

where Δ[NADH] refers to the amount of NADH oxidized and $[O_2^-]_0$ refers to the amount of superoxide initially present in the solution. From the absorption change in Fig. 5 a maximal value of Δ[NADH]/[NADH] of 0.005 can be estimated which means that k_5 is less than $3.5 \cdot 10^4\ M^{-1} \cdot s^{-1}$ using $k_7 = 2.0 \cdot 10^7\ M^{-1} \cdot s^{-1}$ [21]. However if a considerable amount, say 99%, of the NAD· radicals formed by reaction 5 is reacting with oxygen to form superoxide and NAD^+ through reaction 4 the value of k_5 may be as low as $8.5 \cdot 10^3\ M^{-1} \cdot s^{-1}$. Using the p$K$ = 4.88 of superoxide [21] and the limit of $k_5 < 3.5 \cdot 10^4\ M^{-1} \cdot s^{-1}$ and assuming that the active species of superoxide is the acid form ($HO_2^·$) we are able to calculate a limiting value of $k_5 < 18\ M^{-1} \cdot s^{-1}$ at pH 8.6. This value is compatible with the estimate of Land and Swallow [12] that $k_5 \ll 27\ M^{-1} \cdot s^{-1}$ at this pH.

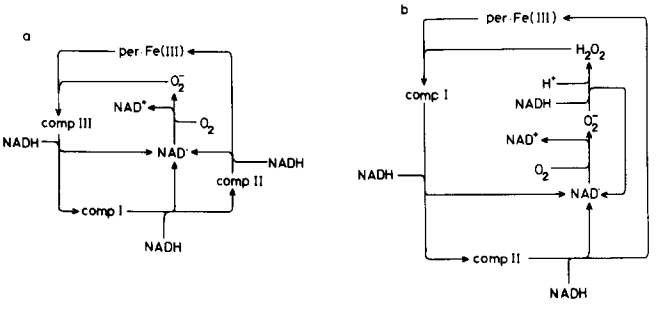

Fig. 6. Tentative reaction mechanism for the peroxidase-oxidase reaction. The reaction mechanism is supposed to contain both schemes (a) and (b) (see text).

Discussion

The present experiments have confirmed the suggestion made earlier [6,7] that the oscillations in the peroxidase-oxidase reacton are caused by the autocatalytic nature of the reaction determining threshold concentrations of the substrates O_2 and NADH that switch the reaction on and off. Little can be said about the branching steps in the reaction but the effect of hydroquinone on the switch-on concentration of NADH indicates that compounds I and II are involved. This finding is consistent with earlier suggestions. Compound I can be formed by two alternative pathways as shown in Eqns. 13 and 14

$$H_2O_2 + \text{ferriperoxidase} \rightarrow \text{compound I} \tag{13}$$

$$\text{compound III} + e^- \rightarrow \text{compound I} \tag{14}$$

The former is part of an autocatalytic reaction sequence since compound I causes the formation of 2 NAD· radicals which in turn produce 2 molecules of H_2O_2 plus 2 NAD· radicals through reactions 4 and 5. The latter reaction may also be part of an autocatalytic pathway if the electron is donated by NADH. The two autocatalytic reaction pathways are shown in Fig. 6. It has been shown previously [6,7] that two coupled autocatalytic reactions are necessary to explain the oscillations of the peroxidase-oxidase reaction in the open system. The two autocatalytic schemes in Fig. 6 have a common pathway (from compound I through compound II to ferriperoxidase) resulting in coupling between them. The schemes in Fig. 6 are therefore compatible with our previously described model [6,7].

The rate constants of reactions 5 and 6 measured here are small compared to the rate constant of the dismutation of superoxide at pH 5.1 (reaction 7)). In order to account for the relatively high rates of oxygen consumption observed (exceeding 10^{-6} M/s) fairly high concentrations of superoxide (of the order of 10^{-7}—10^{-6} M) should be found. Since the rate of reaction 7 increases with the square of the superoxide concentration whereas the rates of reactions 5 and 6 only increases linearly with the concentration of O_2^-, this implies that reactions 5 and 6 should compete strongly with the dismutation of superoxide. As the dismutation of superoxide is neither a branching step nor a termination step this would result in a reduction of the branching and hence a low acceleration of the overall reaction. This is not a in agreement with the observation that under the conditions where the reaction oscillates the acceleration is high. We can therefore not exclude the possibility that branching reactions other than 5 and 6 are involved in the peroxidase-oxidase reaction although the exact nature of these remains unknown.

The inhibition of the peroxidase-oxidase reaction with NADH as the hydrogen donor by superoxide dismutase has normally been taken as evidence for the participation of superoxide in the reaction [10,11,19]. However caution is needed in drawing such conclusions in a reaction where other free radicals are involved, since to the author's knowledge there is at present only little information about the specificity of superoxide dismutase.

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