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ANALYSIS OF THE CONDITIONS CAUSING THE OSCILLATORY OXIDATION OF REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE BY HORSERADISH PEROXIDASE

ISAO YAMAZAKI AND KEN-NOSUKE YOKOTA
Biophysics Division, Research Institute of Applied Electricity, Hokkaido University, Sapporo (Japan)

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SUMMARY

The oscillatory consumption of O_2 can be observed when O_2 is introduced continuously into a solution of NADH and horseradish peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7). A peroxidase derivative, Compound III, fulfils quite an unusual role in the NADH oxidation and the enzyme oscillates between ferriperoxidase and Compound III in the same oscillatory cycle of oxygen consumption. This oscillatory reaction is greatly influenced by pH, NADH concentration and the rate of introduction of O_2. The elementary conditions which make the NADH oxidation rate oscillate have been analyzed and a possible mechanism of switch-on and switch-off in this oscillatory reaction is discussed.

INTRODUCTION

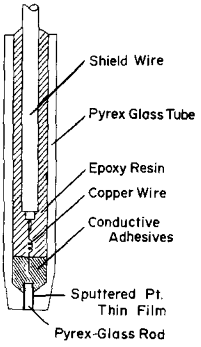
It is widely recognized that periodic phenomena are common in biological systems and the nature of the oscillator driving such phenomena has engaged the attention of many physiologists and biochemists. Recently clear oscillations at the level of enzymic reactions have been reported in the glycolytic system[1-6] and in the peroxidase system[7]. In particular, the oscillations in the level of reduced pyridine nucleotides have been extensively investigated in the glycolytic system. These studies have indicated that a major site for the oscillations is the enzyme phosphofructokinase and that waveforms, period and amplitude are under control to some degree[2]. Recently, sustained oscillations have been observed in a cell-free extract of *Saccharomyces carlsbergensis* supplemented with trehalose[5,6]. However, these oscillations in the glycolytic system have so far been observed in the cell-free extract but not in a pure enzyme system.

The oscillations of the peroxidase reaction, on the other hand, involve a single enzyme and the oscillatory mechanism is related to the delicate function of the peroxidase. Of the numerous peroxidase substrates, reduced pyridine nucleotide is the only known hydrogen donor which undergoes the oscillatory oxidation. The present paper will describe an analysis of the elementary conditions which make the NADH oxidation rate oscillate.

MATERIALS AND METHODS

Horseradish peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) was isolated from wild horseradish according to PAUL's method[8]. Peroxidase II (normal type or high-spin type)[9,10] was separated on a carboxymethyl-cellulose column into 2 major fractions, non-adsorbed (A) and adsorbed (C). Fraction C (according to PAUL's nomenclature[8]) was easily crystallized from $(NH_4)_2SO_4$ solution and was suitable for the oscillatory reactions. The ratio of $A_{403\ \mathrm{m}\mu}/A_{278\ \mathrm{m}\mu}$ for this enzyme was 3.4. The molar concentration of the enzyme was calculated by assuming that the value for $A_{403\ \mathrm{m}\mu}$ is 107.7 $\mathrm{mM}^{-1}\cdot\mathrm{cm}^{-1}$ (ref. 11). NADH was obtained from Boehringer and Soehne.

A Hitachi recording spectrophotometer was used and reactions were carried out in a wide cell, 1 cm × 2 cm × 6 cm (light-path 1 cm) at 25°. In the oscillatory reactions, a mixture of N_2 and O_2 gas was bubbled at a constant rate into the reaction solution (6 ml) through a capillary. A sputtered platinum electrode was immersed in the reaction solution near the surface and the O_2 concentration was measured polarographically[12]. The platinum electrode used, which was kindly supplied by Prof. M. MOCHIZUKI of this Institute, is shown in Fig. 1. Continuous gas bubbling did not disturb either the spectrophotometric or the polarographic estimations.



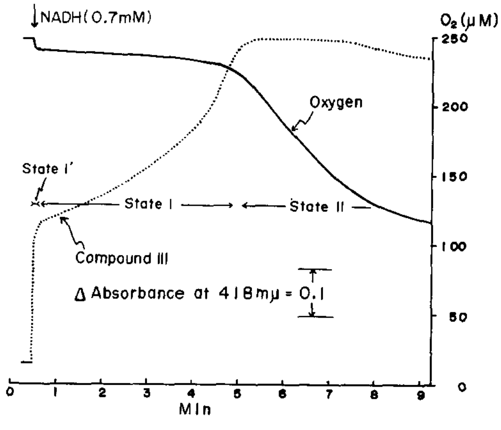


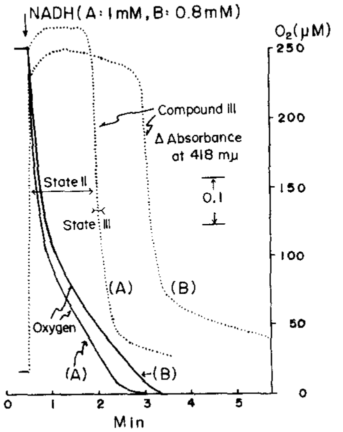
Fig. 1. A cross section of the sputtered platinum electrode.

Fig. 2. Relationship between Compound III formation and NADH oxidation in the closed reaction system. NADH (final concn. 0.7 mM) was added to the air-saturated solution of 10 μM peroxidase in 0.1 M sodium acetate (pH 5.8). Absorbance at 418 mμ and O_2 concentration were measured simultaneously. The increase in absorbance at 418 mμ was identified to be due to the formation of Compound III by scanning in the visible wavelength region. States I', I and II were clearly observable under these conditions, but not State III.

RESULTS

When NADH is added to the aerobic solution of peroxidase at pH 5.8, 2 distinct phases of Compound III formation can usually be observed; first, an instantaneous but partial formation (State I′), and second, a slow but complete formation (State I). The former rapid reaction is found to depend upon a trace amount of H_2O_2 (the final concentration may be a few μM in the experiment shown in Fig. 2) which accumulates in the NADH solution[13]. It should be noticed here that the amount of O_2 consumed during this rapid reaction is comparable to the amount of Compound III formed. The initial rapid formation of Compound III is followed by slow complete formation. During this slow formation of Compound III, the oxidation of NADH is largely inhibited, as can be seen in Fig. 2 by the very slow decrease in O_2 concentration. The rapid oxygen consumption (State II) commences at a point when almost all of the peroxidase has been converted to Compound III. At this pH, however, NADH oxidation is not completed within a reasonable time for reasons which will be discussed later.

At pH 5.0 the features of NADH oxidation are slightly different from those at pH 5.8. When 0.8 mM NADH is added to the aerobic solution of peroxidase (Fig. 3), the formation of Compound III is almost completed during the rapid phase and oxygen consumption takes place without an observable time lag. Whether the slow phase of Compound III formation is separated depends not only upon the pH and the amount of NADH added but also on the amount of H_2O_2 accumulated in NADH solution. A few μM H_2O_2 may be sufficient to convert the enzyme completely to Compound III in State I′ under the conditions shown in Fig. 3. A characteristic feature of this reaction may be a sudden decomposition of Compound III when the O_2 concentration drops below a certain level. In the presence of excess NADH the reaction solution becomes anaerobic and the enzyme is found to consist of a mixture of the ferric and ferrous forms[13]. The proportion of ferrous form increases with de-



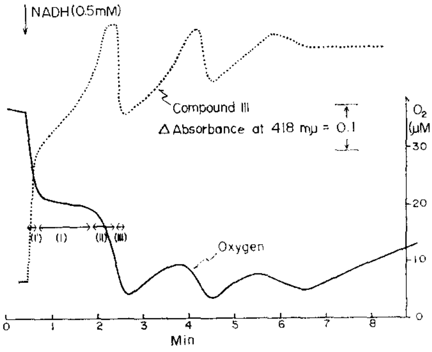


Fig. 3. Relationship between Compound III disappearance and NADH oxidation in the closed reaction system. NADH was added to the air-saturated solution of 10 μM peroxidase in 0.1 M sodium acetate (pH 5.0). States I′ and I were indistinguishable from each other but States II and III were clearly observable under these conditions.

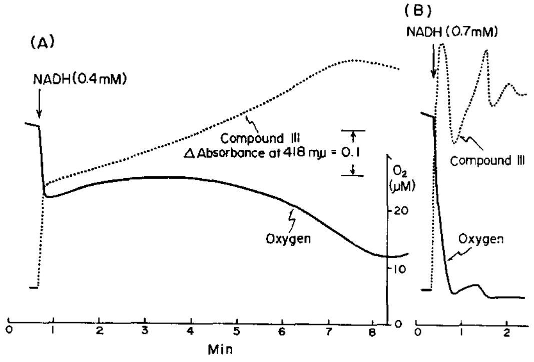
Fig. 4. Typical oscillatory reaction. NADH (final concn. 0.5 mM) was added to the solution of 10 μM peroxidase and 0.1 M sodium acetate (pH 5.0), with 10 ml 3% O_2 bubbled per min. The steady oscillatory reaction was composed of 3 phases; inductive (State I), active (State II) and terminating (State III). State I′ was not involved in the steady oscillatory reaction but appeared only after NADH was added to the aerobic solution of peroxidase.

creasing pH and also with increasing amounts of NADH added. The lifetime of Compound III is related to the velocity of NADH oxidation as can be seen in Fig. 3.

These phenomena of formation and decomposition of Compound III constitute the essential mechanism for the oscillatory oxidation of NADH by peroxidase. Compound III is not a typical active intermediate but rather a kind of regulatory intermediate. The function of Compound III as a regulator in the NADH oxidation may be illustrated by 2 characteristic phenomena. First, NADH oxidation is retarded during the formation of Compound III with rapid oxidation (this commencing after the formation of Compound III is almost complete) and, second, Compound III decomposes immediately after the O_2 concentration drops below a certain limit.

These kinetics of the NADH oxidation in the closed system suggest the possible oscillation of the reaction when it is carried out in an open system, for example, with a continuous supply of O_2. In the experiment of Fig. 4, a mixture of N_2 and O_2 (about 3% O_2) is bubbled into the reaction solution through a capillary. The addition of NADH to this aerobic solution of peroxidase results in an appearance of Compound III with the consumption of O_2. The formation of Compound III is again divided into 2 separate phases, State I′ and State I. The latter phase of Compound III formation follows a curve similar to an exponential one and leads the reaction to State II, where the enzyme exists mainly in the form of Compound III. The rate of oxygen consumption then becomes greater than the O_2 supply and the O_2 concentration of the solution decreases. State II lasts until the O_2 level drops below a certain limit when the rapid decomposition of Compound III occurs (State III). Under the conditions where O_2 is supplied continuously, the accumulation of Compound III is again observed as the O_2 concentration increases and the reaction sequence repeats itself, starting with State I. The oscillatory oxidation of NADH will stop when the NADH concentration of the solution drops below a certain limit. By spectrophotometric analysis, about 0.2 mM NADH is found to remain unoxidized at the end of the oscillatory reaction under the experimental conditions of Fig. 4.

The range of NADH concentration which causes the oscillatory reaction is very narrow. For example, slightly less NADH can scarcely cause the reaction, as shown



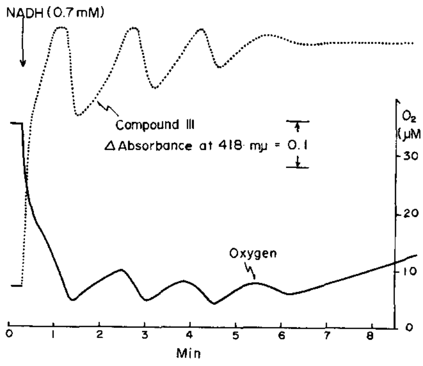
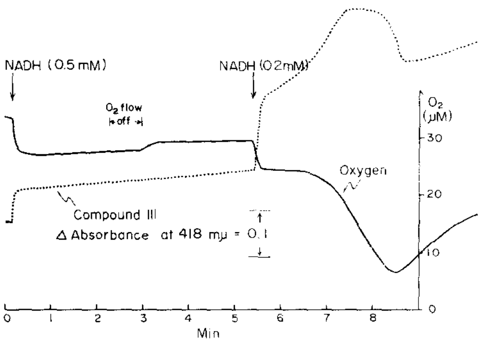


Fig. 5. Slow reaction (A) and damped oscillation (B). Experimental conditions were the same as those in Fig. 4, except for NADH concentrations. The final concentrations of NADH were 0.4 mM (A) and 0.7 mM (B).

Fig. 6. Typical oscillatory reaction. NADH (final concn. 0.7 mM) was added to the solution of 10 μM peroxidase and 0.1 M sodium acetate (pH 5.4) with 10 ml 3% O_2 bubbled per min.

in Fig. 5A. The usual rapid formation of Compound III and consumption of oxygen (State I′) are observed on the addition of NADH, but the State I reaction is much slower in this case. The oxygen-consuming oxidation of NADH gradually occurs after about 70% of the enzyme has been converted to Compound III. The rate of oxygen consumption is not fast enough to lower the O_2 concentration below the limit where State III is initiated and consequently the rapid decomposition of Compound III cannot be observed in this case. The oscillation of the reaction is not observable under these experimental conditions. On the other hand, a slight excess of NADH causes rapid damping of the oscillations. In this type of reaction (Fig. 5B), the level of Compound III approaches 70–80% of the total enzyme after a few cycles and in this case fluctuations in the O_2 concentration do not fully respond to the Compound III oscillation.

The relationship between NADH concentration and oscillation pattern depends greatly upon the pH of the reaction solution. Fig. 6 shows that 0.7 mM NADH causes a stable oscillatory reaction at pH 5.4. At the same pH, 0.5 mM NADH is too low to allow the oscillatory reaction to proceed (Fig. 7). The interruption of O_2 supply for about 30 sec 2 min subsequent to the NADH addition does not result in a decrease of O_2 concentration. This means that no measurable oxidation of NADH occurs during this stage. A further addition of 0.2 mM NADH makes the reaction oscillate, though not completely, as shown in Fig. 7. Fig. 8 shows that 1 mM NADH is too much to keep the reaction oscillating and causes rapid damping.



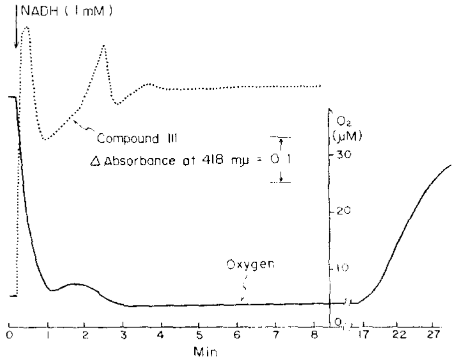
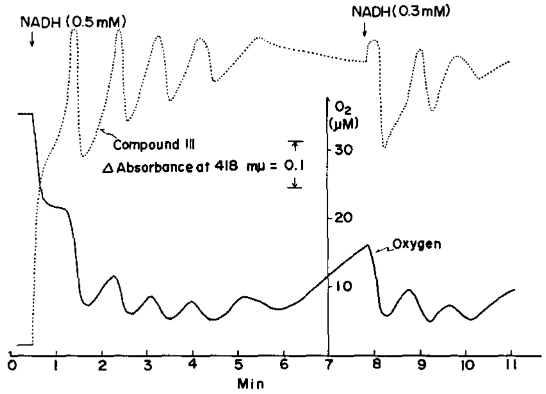


Fig. 7. Transition from a slow reaction to an oscillatory reaction by the further addition of NADH. Only a minor change in State I′ was observed when NADH (final concn. 0.5 mM) was added to the solution of 10 μM peroxidase and 0.1 M sodium acetate (pH 5.4), bubbled with 10 ml 3% O_2 per min. The interruption of O_2 supply did not change the constitution of the reaction solution. The further addition of NADH (final concn. 0.2 mM) converted the reaction into one of an oscillatory type though not completely.

Fig. 8. Damped oscillation in the presence of 1 mM NADH and 10 μM peroxidase and 0.1 M sodium acetate (pH 5.4) bubbled with 10 ml 3% O_2 per min. The reaction reached the steady state about 4 min after commencement and the O_2 concentration began to increase about 17 min after the reaction had started.

Although the reaction system is very simple, the reaction mechanism is complicated and will be discussed later. A slight change in the experimental conditions will give a change in the results. It is not easy to get a completely reproducible oscillation pattern when the experiments are carried out on different days even if the experimental conditions are thought to be identical. For example, the experiment in Fig. 9 was carried out under almost the same conditions as that of Fig. 4, but gave faster cyclic responses. This may be due mainly to differences in the enzyme preparation used and also in the gas-bubbling conditions. In the experiment of Fig. 9, after the first oscillatory responses ceased, another addition of 0.3 mM NADH caused further oscillations although these were not identical with the first series.



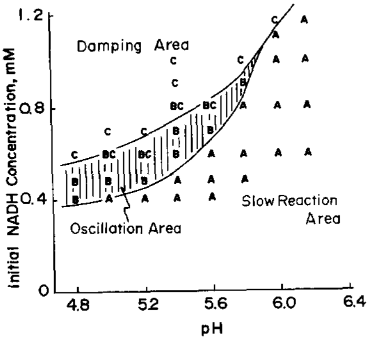
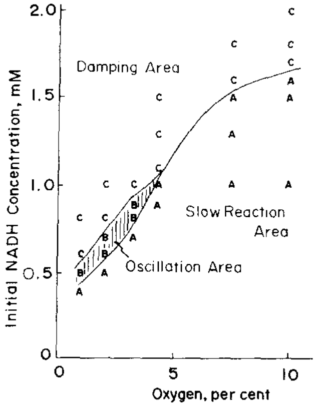


Fig. 9. Typical oscillatory reaction. The experimental conditions were the same as those in Fig. 4 except that a different peroxidase preparation was used. Approx. 0.2 mM NADH remained unoxidized when the first oscillatory responses ceased. The further addition of NADH (final concn. 0.3 mM) caused renewed oscillations.

Fig. 10. Effect of the initial NADH concentration upon the reaction pattern at different pH values: 10 μM peroxidase, 0.1 M sodium acetate (pH 4.8–5.8) or 0.1 M potassium and sodium phosphates (pH 6.0–6.4), bubbled with 10 ml 3% O_2 per min. The reaction pattern was classified into 3 phases: A, slow reaction (Figs. 5A and 7); B, oscillation (Figs. 4, 6 and 9); C, damped oscillation (Figs. 5B and 8).

It is now clear that there is a distinct range of NADH concentrations which produce a stable oscillation and Fig. 10 shows how the oscillation area (designated as B) is dependent upon the pH. Below this level of NADH the oxidation velocity is too slow to give a proper oscillatory reaction. This type of reaction is designated as slow reaction (A) in Fig. 10. Above a certain level of NADH the oxidation is very fast and the oscillations damp rapidly. In the damped reaction (C) the level of Compound III converges to a certain level, usually 20 or 30% below the maximum. As can be seen in Fig. 10 there is no range of NADH concentration which gives proper oscillations above pH 6.0 under these experimental conditions. It should be noticed here that if the oscillation starts from Area B the regular oscillation will always last slightly beyond the line between B and A in Fig. 10.

The range of NADH concentration for the stable oscillation is also affected by the rate of O_2 supply into the reaction solution; this is shown in Fig. 11. Here, the rate of O_2 supply is controlled by changing the O_2 content of the gas mixture at a constant flow rate (20 ml/min). In Fig. 11, slow reaction (A) means that the NADH oxidation in State II is not fast enough to lower the O_2 concentration below the limit where the rapid decomposition of Compound III is initiated.



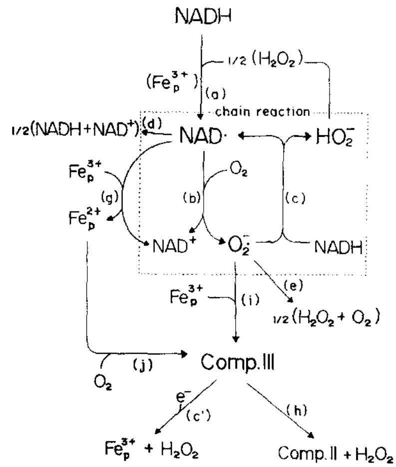


Fig. 11. Effect of the initial NADH concentration upon the reaction pattern at different rates of O_2 supply: 10 μM peroxidase, 0.1 M sodium acetate (pH 5.2). The rate of O_2 supply was controlled by changing the O_2 content of the gas mixture at a constant flow rate (20 ml/min). In this case the term slow reaction is not an absolute definition of the rate of NADH oxidation as described in the text.

Fig. 12. The mechanism of the aerobic oxidation of reduced pyridine nucleotides by peroxidase. The main reactions are an enzymic formation of monodehydro NADH (Reaction a) and a chain reaction (Reactions b and c). Reaction a is a peroxidase cycle and is composed of 3 elementary reactions[13]. In Reaction c', maximum gain will be obtained when Compound III accepts an electron from NADH but the gain will decrease when it accepts an electron from ferroperoxidase or the NAD radical. The thermodynamic consideration of these elementary reactions has been elaborated elsewhere[20].

DISCUSSION

The oscillation of NADH oxidation rate by peroxidase was predicted after analyzing the mechanism of the reaction which has been reported elsewhere[7,13]. The remarkable feature of the reaction is an accumulation of peroxidase Compound III which is a kind of regulator. The characteristics of the accumulation of the regulatory intermediate results in the ability of the enzyme to turn itself on and off and in this manner to produce an oscillation in NADH oxidation rate when the reaction is carried out in the open system.

The mechanism of this oscillatory oxidation of NADH is essentially the same as that of one cycle of the reaction which occurs in the closed system. This mechanism is shown in Fig. 12 (ref. 13). As the NAD free radical is thought to be an active intermediate in the chain reaction shown in Fig. 12, the gain of the active intermediate during one cycle of the reaction will be discussed. The NADH free radical is, in fact, originally produced by a peroxidatic reaction (Reaction a) at the expense of a trace amount of H_2O_2 accumulated in the NADH solution. The first possible reaction of the NAD free radical is Reaction g (with ferriperoxidase), Reaction d (dismutation) or Reaction b (with O_2). Reaction d consumes the 2 active intermediates and in this case the gain will be zero. Reaction b gives an O_2^- radical which may then react in 3 different ways: these are Reaction i (with ferriperoxidase), Reaction c (with NADH) and Reaction e (dismutation). Reactions b and c constitute a complete chain reaction

TABLE I

THE GAIN OF THE NAD FREE RADICAL OBTAINED BY ONE CYCLE OF THE CHAIN REACTION

Reaction paths		*Nature of the reaction*	*Gain*
1st step	*2nd step*		
(d) $NAD\cdot \xrightarrow{H^+} \frac{1}{2}(NAD^+ + NADH)$		Dismutation of NAD·	0
(b) $NAD\cdot + O_2 \rightarrow NAD^+ + O_2^-$	(i) $O_2^- + Fe_p{}^{3+} \rightarrow Fe_p{}^{3+}O_2^-$	Compound III formation	0*
	(e) $O_2^- \xrightarrow{H^+} \frac{1}{2}(O_2 + H_2O_2)$	Dismutation of O_2^-	1
	(c) $O_2^- + NADH \rightarrow HO_2^- + NAD\cdot$	Complete chain reaction	3
(g) $NAD\cdot + Fe_p{}^{3+} \rightarrow NAD^+ + Fe_p{}^{2+}$	(j) $Fe_p{}^{2+} + O_2 \rightarrow Fe_p{}^{3+}O_2^-$	Compound III formation *via* ferroperoxidase	

* Compound III slowly autodecomposes probably according to Eqn. 2. This reaction is very slow compared with the reactions listed in this table but it gives a maximum gain (3) whenever it occurs.

which is outlined by a dotted line in Fig. 12. At the end of one cycle of the reaction it gives 1 mole of NAD free radical and 1 mole of H_2O_2.

As H_2O_2 gives 2 moles of NAD free radical through Reaction a (refs. 14,15) the gain in this reaction path is 3. When dismutation of O_2^- (Reaction e) occurs, it gives 0.5 mole H_2O_2 and the gain is 1. These data are listed in Table I. An intermediate, Compound III, is invariably accumulated in the NADH oxidation, probably by Reactions g and j or Reactions b and i (Fig. 12). Because of the sluggish activity of Compound III toward NADH and the slow autodecomposition, it may be reasonable to assume that when the reactions of NAD free radical terminate in the formation of Compound III the gain is nearly zero; details of this will be discussed later. The approximate value of the gain may now be calculated by the following equation:

$$\text{Gain of the chain reaction} = \frac{v_b}{(v_b + v_d + v_g)} \times \frac{(3v_c + v_e)}{(v_c + v_e + v_i)} \qquad (1)$$

where v_b, v_c, v_d, v_e, v_i and v_g are the reaction velocities of the respective reactions of b, c, d, e, i and g shown in Fig. 12. At a particular pH, this gain will be influenced by the concentrations of O_2, NADH and ferriperoxidase. An increase in O_2 concentration results in an increase in the gain by increasing v_b, the rate of Reaction b. An increase in the NADH concentration is, however, very effective in increasing the gain by increasing Reaction c, as can be judged from Eqn. 1. A remarkable feature of this reaction system seems to be an inhibitory action by peroxidase itself. In the presence of excess peroxidase v_i predominates over v_c and v_e because O_2^- has a high affinity towards ferriperoxidase[14]. The gain in this case is close to zero. Under these conditions, a trace amount of H_2O_2 produces approximately stoichiometric amounts of Compound III through Reactions a, b and i. The ratio of Compound III formed to added H_2O_2

depends greatly upon the concentration of NADH and the pH of the reaction solution, because NADH competes with ferriperoxidase for O_2^- and Reaction c becomes favorable as the pH decreases. The formation of Compound III (State I) follows somewhat of an exponential type of curve, as shown in Figs. 2 and 4. This can be explained by assuming that the gain increases as the ferriperoxidase decreases. This exponential increase of Compound III seems to represent very peculiar kinetics; it is important for the initiation of the oscillatory reactions (switch-on mechanism).

Rapid oxidation of NADH always occurs when the level of Compound III approaches its maximum (State II). During this period of the reaction the gain of the chain reaction may be kept at a maximum. Compound III is found to react with many hydrogen donors[16] but the reaction between Compound III and NADH seems to be very sluggish although no direct experimental evidence has been obtained on this point[13]. Compound III, however, autodecomposes with a half-time of about 2 min which depends to some extent on the pH (ref. 17). This reaction might be formulated as follows,

$$\mathrm{Fe_p}^{3+}\mathrm{O_2}^- \text{ (Compound III)} + \mathrm{H_3O^+} \rightarrow \mathrm{Fe_p}^{3+}\,\mathrm{OH} \text{ (Compound II)} + \mathrm{H_2O_2} \qquad (2)$$

(Fe_p^{3+} stands for ferriperoxidase.)

Although this is a slow reaction it gives a maximum gain (3) whenever it occurs. Compound II reacts with NADH and produces 1 mole of NAD free radical while H_2O_2 produces 2 moles of NAD free radical. It is very likely that this type of reaction is slowly proceeding after Compound III accumulates.

One more remarkable mechanism in this reaction is the rapid decomposition of Compound III (State III) when the O_2 level falls below a certain level. This is a very important mechanism for switching-off the NADH oxidation. As can be seen in the mechanism (Fig. 12) the decrease in the O_2 level results in the accumulation of strong reductants such as the NAD free radical and perhaps also ferroperoxidase. The direct demonstration of the reaction between Compound III and NAD free radical is difficult but ferroperoxidase is found to react very rapidly with Compound III (refs. 17,18). This type of decomposition of Compound III may be of the reductive decomposition type which has been discussed elsewhere[17].

$$\mathrm{Fe_p}^{3+}\mathrm{O_2}^- \text{ (Compound III)} + e^- + \mathrm{H^+} \rightarrow \mathrm{Fe_p}^{3+} + \mathrm{O_2H^-} \qquad (3)$$

The H_2O_2 formed in this reaction might be used to produce another molecule of NAD free radical and accelerate the Reaction 3. The reduction induced by H_2O_2 has been reported in the peroxidase system[19].

The steady oscillatory reaction is thus composed of 3 phases: inductive (State I), active (State II) and terminating (State III). Typical features of these phases are listed in Table II. State I′ is not involved in the steady oscillatory reaction but appears only after NADH is added to the aerobic solution of peroxidase. The rapid formation of Compound III in State I′ is dependent on the pH, the NADH concentration and the amount of H_2O_2 accumulated in the NADH solution. It is eliminated if the NADH solution is preincubated with catalase[13].

The gain in the chain reaction is an important value which is closely related to the oscillation pattern. A suitable magnitude is necessary to cause a good oscillating reaction. The rate of Reaction c has considerable influence on the gain and Reaction c becomes faster as the pH decreases and the NADH concentration increases. Thus,

TABLE II

REACTION PHASES IN THE OSCILLATORY OXIDATION OF NADH

State	*Characteristics of the reaction*	*Dominant reaction (Fig. 12)*	*Gain of the chain reaction*	*Oxidation velocity*
I′	H_2O_2-initiated accumulation of Compound III	a, b, i	approx. 0	fast
I	Exponential increase of Compound III (switch-on)	(h), a, b, i	0—1	slow
II	Steady and rapid oxidation of NADH	(h), (a), b, c,	1—3	fast
III	Reductive decomposition of Compound III (switch-off)	a,c′*	(3—1) → 0	fast → slow

* The switch-off step may not be simple and the detailed mechanism will be discussed in a forthcoming paper.

when the pH decreases the NADH concentration should decrease in order to maintain the rate of Reaction c constant and to make the reaction oscillate in a regular way. This relationship is shown in Fig. 10. When Reaction c is very fast the overall gain will become greater than unity before the formation of Compound III is complete. In the presence of sufficient NADH and O_2, rapid turns of the chain reaction give the O_2^- radical which causes the complete conversion of ferriperoxidase to Compound III. However, in the second or third cycle of the oscillations shown in Figs. 5B and 8, the O_2 decreases below a limiting concentration before Compound III reaches its maximum value and Compound III begins to decompose. After a few cyclic responses Compound III converges to a certain level, where the rate of NADH oxidation balances the rate of O_2 introduction. This might be a main reason for the rapid damping of the oscillations. This type of rapid damping can be avoided to some degree by increasing the rate of O_2 introduction as shown in Fig. 11. When Reaction c is too slow, NADH oxidation can hardly be observed.

Mn^{2+} and phenols have been known to be activators for the NADH oxidation catalyzed by peroxidase. The effect of these activators upon the oscillation pattern is interesting and is now under investigation.

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