Biophysical Chemistry 40 (1991) 189–195
Elsevier Science Publishers B.V., Amsterdam

BIOCHE 01568

A new model for oscillations in the peroxidase–oxidase reaction

Stéphane Alexandre * and H. Brian Dunford **

Department of Chemistry, University of Alberta Edmonton, Alberta T6G 2G2, Canada

Received 28 August 1990
Revised manuscript received 27 December 1990
Accepted 3 January 1991

Peroxidase–oxidase reaction; Enzymatic oscillations; Normal mode analysis; Computer simulation

The NADH oxidation catalyzed by horseradish peroxidase is well known to produce oscillations in an open system with diffusion of oxygen. We propose here a model based on a cycle between two oxidation states of the enzyme. The two reactions of the cycle are auto-catalytic and produce a free radical intermediate, but one consumes oxygen. Because of the enzyme cycle, this model is reduced to three independent species. The model can be studied with normal mode stability analysis. The results of this analysis predict that in some cases the steady state should be unstable with an oscillatory behavior. Then the numerical simulations show that sustained oscillations are exhibited by our model. The shape of these oscillations are very similar to those obtained experimentally (I. Yamazaki and K. Yokota, Mol. Cell Biol., 2 (1973) 39).

1. Introduction

Peroxidases are well known to catalyze the reduction of a peroxide (usually but not necessarily hydrogen peroxide) in the presence of reducing species such as phenols, aromatic amines and iodid. In some cases, peroxidases are also able to catalyze the oxidation of some substrates (such as NADH, DHF, isobutyraldehyde and indoleacetic acid) by oxygen without addition of a peroxide. In general this happens when the auto-oxidation of the substrate produces a peroxide or a peroxyacid [1]. In 1965 Yamazaki et al. [2] reported the first observation of damped oscillations during the oxidation of NADH catalyzed by horseradish peroxidase in an open system through which a gaseous mixture of oxygen and nitrogen was continuously bubbled. In a slightly different open system in which the oxygen concentration in the gas phase was maintained constant by blowing a gas mixture of nitrogen and oxygen, Degn observed bistability during this reaction [3]. Using a NADPH regenerating system involving glucose-6-phosphate and glucose-6-phosphate dehydrogenase, and by adding methylene blue and 2,4-dichlorophenol, sustained oscillations were observed during this reaction catalyzed either with lactoperoxidase [4] or horseradish peroxidase (HRP) [5]. In the same kind of system, but by adding NADH instead of using a NADPH regenerating system, Olsen and Degn showed that the decrease in HRP concentration led to chaos [6]. Recently, this latter system was found to produce bistability between an oscillatory and a stable state [7].

In order to explain these oscillations, Yokota and Yamazaki studied both experimentally and theoretically the oxidation of NADH by oxygen catalyzed by HRP in a closed system. They analyzed the different phases of this reaction and numerical simulations of their detailed mechanism was shown to fit the experimental results [8].

* Current address: Fritz-Haber-Institut der Max-Planck-Gesellschaft, Faradayweg 4–6, D1000 Berlin, Germany.
** To whom correspondence should be addressed.

0301-4622/91/$03.50

This system also has been studied intensively from a theoretical point of view. Fed'kina et al. [9], studied the conditions to obtain sustained oscillations in an open system using the Yamazaki mechanism [8], but adding a termination reaction for NAD radicals. They assumed that some highly reactive compounds were in a steady state (these are defined as fast variables), that changes in O_2 concentration exhibited a quasi-stationary behavior, and that NADH concentration was constant. Taking into account that almost all of the reactions known to occur during the oxidation of NADH are catalyzed by HRP, Aguda and Clarke used a stoichiometric network analysis to find a detailed model which was able to exhibit damped oscillations and bistability [10]. Aguda and Larter developed this model and predicted that bistability between a stable steady state and sustained oscillations should be obtained [11] as was found experimentally later [7].

Other theoretical work focussed on the similarity of the observed oscillations with abstract theory which predicts such behavior [12]. The first abstract model was based upon four independent species: O_2, NADH and two free radical intermediates designated X and Y [13]. The nature of the species X and Y is discussed below. This model involved two feedback loops. The first loop is the auto-catalytic generation of X and the second one is a feedback loop on X involving Y as an intermediate. This model has been found to exhibit sustained oscillations [13] as well as complex oscillations [13,14] or in some cases chaos [15,16]. Olsen modified this model by removing oxygen from the first loop and by producing 3X instead of 2X in the second loop [17]. This later model predicted chaos for a larger range of parameters [18]. However, because of the removal of oxygen from the first loop, the later model [17] is less in agreement than the earlier one with the detailed mechanism [11].

We now turn our attention to the nature of X and Y. Aguda et al., assigned X to NAD• and Y to oxyperoxidase (compound III) [18]. In the earlier model of Olsen and Degn X and Y were both believed to be free radical intermediates and not an oxidized form of the enzyme [13]. Also, if Y is oxyperoxidase, to be in agreement with experiment [8], it should be formed when oxygen is at high concentration. In fact, in all of the abstract models Y is consumed during the auto-catalytic step involving oxygen in the second loop [13,17,18]. Lastly, if Y corresponds to oxyperoxidase another form of the enzyme should be involved in the model, since almost all of the native HRP is converted to oxyperoxidase during the oscillation [8].

We present here a theoretical model involving two oxidation states of the peroxidase. These two forms of the enzyme are likely the native peroxidase and oxyperoxidase because of their importance during the oscillations [8,11]. The experimental analysis of the peroxidase–oxidase reaction showed that the formation of oxyperoxidase and its decomposition are auto-catalytic [8]. Therefore, our model is based on two auto-catalytic reactions which generate a cycle between the two oxidation states. We assume that NADH concentration is constant and consider four intermediate species, P, X, Y and A, three of which are independent. P is native enzyme, X is a free radical intermediate (likely NAD•), Y is oxyperoxidase and A oxygen. We analyzed this model by using the normal mode stability analysis in order to find the conditions to obtain oscillations. We present also the result of numerical simulations which show that our model is able to exhibit sustained oscillations.

2. The model

Our model involves the following chemical steps:

$$P + A + X \xrightarrow{k_1} Y + 2X \tag{1}$$

$$Y + X \xrightarrow{k_2} P + 2X \tag{2}$$

$$B \xrightarrow{k_{3i}} X \tag{3}$$

$$X \xrightarrow{k_4} \text{Product} \tag{4}$$

$$A_0 \overset{k_t}{\rightleftharpoons} A \tag{5}$$

In reaction (3), B is a species present at constant concentration so we define the product $k_{3i}[B]$ as

k_3. The products of reaction 4 are assumed to play no further role in the reactions.

The first two steps (reactions 1 and 2) are auto-catalytic and create a cycle between the two oxidation states of the enzyme P and Y. This can be justified after the detailed mechanism proposed in order to explain the peroxidase-oxidase reaction [8]. From this mechanism, it appears that the formation of oxyperoxidase from the native enzyme is auto-catalytic and involves the radical NAD•. In the same way, the decomposition of oxyperoxidase back to the native form is also auto-catalytic involving NAD•.

Step 3 represents the initial formation of the free radical X by the usual cycle of peroxidase. In the experiments, this initial formation occurs because of the hydrogen peroxide which is always present in the solution of NADH [8]. In order to simplify the model the rate of this step is assumed to be constant. This simplification is justified by our results as we will see later.

The free radical is consumed during the auto-decomposition step 4. The last step 5 corresponds to the exchange of oxygen between the solution and the gas phase (k_t being the transfer rate constant and A_0 the equilibrium concentration of A in absence of reactions).

Because P and Y are cycling species, the sum of their concentrations remains constant and equal to the total enzyme concentration [E]:

$$[P] + [Y] = [E] \tag{6}$$

The system may be reduced to three independent species (Y, A, X) and is governed by the following three kinetic equations:

$$\frac{d[X]}{dt} = k_1([E] - [Y])[A][X] + k_2[Y][X] + k_3 - k_4[X] \tag{7}$$

$$\frac{d[Y]}{dt} = k_1([E] - [Y])[A][X] - k_2[Y][X] \tag{8}$$

$$\frac{d[A]}{dt} = -k_1([E] - [Y])[A][X] + k_t([A]_0 - [A]) \tag{9}$$

3. Steady state

By setting the differential eqs. (7) to (9) to zero, one can find the values of the steady state concentrations of A and X:

$$[A]_s = \frac{k_2[Y]_s}{k_1([E] - [Y]_s)} \tag{10}$$

$$[X]_s = \frac{k_3}{k_4 - 2k_2[Y]_s} \tag{11a}$$

or

$$[X]_s = \frac{k_t([A]_0 - [A]_s)}{k_2[Y]_s} \tag{11b}$$

From eq. (9) and the values of the steady state for A and X (eqs. 10 and 11) the steady state value for Y is then given by the quadratic equation (eq. 12):

$$\alpha_2[Y]_s^2 - \alpha_1[Y]_s + \alpha_0 = 0 \tag{12a}$$

with

$$\alpha_0 = k_1k_4k_t[E][A]_0 \tag{12b}$$

$$\alpha_1 = k_1k_2(2k_t[A]_0 + k_3)[E] + k_4k_t(k_1[A]_0 + k_2) \tag{12c}$$

and

$$\alpha_2 = k_2\{k_1(2k_t[A]_0 + k_3) + 2k_2k_t\} \tag{12d}$$

Because all the coefficients (α_0, α_1, α_2) of the quadratic equation (eq. 12a) are positive, the two roots are positive. However, some conditions on the steady state value for Y can be deduced from the fact that the steady state values for A and X must be positive. From eq. (10)

$$[Y]_s < [E] \tag{13a}$$

and from eq. (11a)

$$[Y]_s < \frac{k_4}{2k_2} \tag{13b}$$

In addition, because $[A]_s$ must be smaller than $[A]_0$ (see eq. 11b) a third condition can be found on $[Y]_s$ from eq. (10):

$$[Y]_s < \frac{k_1[E][A]_0}{k_2 + k_1[A]_0} \tag{13c}$$

Because of these constraints (eqs. 13a–c) it can be shown that if k_3 is not equal to zero (eqs. 12c, d) only the smaller of the two roots is a valid steady state.

4. Stability analysis

The stability analysis of this steady state can be carried out after evaluating the Jacobian matrix for the steady state (eq. 14) [18].

$$L = \begin{pmatrix} 2k_2[Y]_s - k_4 & -k_1[A]_s[X]_s + 2k_2[X]_s & k_1([E] - [Y]_s)[X]_s \\ 0 & -k_1[A]_s[X]_s - k_2[X]_s & k_1([E] - [Y]_s)[X]_s \\ -k_2[Y]_s & k_1[A]_s[X]_s & -k_1([E] - [Y]_s)[X]_s - k_t \end{pmatrix} \tag{14}$$

The eigenvalues (ω) of this matrix are then given by the characteristic equation [18,19]:

$$\omega^3 - T\omega^2 + \delta\omega - \Delta = 0 \tag{15}$$

in which T is the trace of the matrix, δ the sum of the principal minor of rank 2 and Δ the determinant.

For the steady state to be unstable, at least one eigenvalue (or its real part in the case of a complex) must be positive. Taking into account the constraints (eq. 13a–c) on the steady state in the evaluation of the matrix coefficients, it can be demonstrated that T and Δ are always negative while δ is always positive. Therefore if the three roots of eq. (15) are real numbers, one is negative while the other two are of the same sign. In the same way if two roots are complex conjugates, the third one is real and negative. Therefore only the sign of two roots are important, and this sign is positive only if the following necessary condition is valid [20].

$$T\delta - \Delta > 0 \tag{16}$$

Further information given by the eigenvalues is the manner of evolution from the steady state after introduction of a small perturbation. If ω is real, the stability analysis predicts a non-oscillatory evolution while if ω is complex an oscillatory evolution is predicted. To obtain complex eigenvalues the following condition must occur [20]:

$$4\delta^3 - T^2\delta^2 + 4T^3\Delta + 27\Delta^2 - 18T\delta\Delta > 0 \tag{17}$$

Therefore to predict the behavior of this system after calculating the matrix coefficients, we only need to evaluate the sign of the two conditions (16) and (17). Taking these considerations into account, we have plotted the stability diagrams of the systems by varying two of the following parameters (kinetic constants, k_1 to k_4; transfer rate constant, k_t; total concentration of enzyme [E] or equilibrium concentration of the species A $[A]_0$). These diagrams show that if the eigenvalues are real, their sign is always negative while if two eigenvalues are complex the sign of the real part can be either negative or positive. Therefore: (1) if the eigenvalues are real, the steady state is stable; (2) if two eigenvalues are complex with their real parts negative, the steady state is stable with an oscillatory evolution; and (3) if two eigenvalues are complex with positive real parts, the steady state is unstable with an oscillatory evolution. Because the steady state is unique we can expect in the second case damped oscillations and in the third one sustained oscillations.

We found that in order to obtain oscillatory behavior of the system, there is a lower limit for the kinetic constants k_1 and k_2 as well as the concentrations of $[A]_0$ and [E]. On the other hand there is an upper limit for the kinetic constant k_3 and the transfer rate constant k_t. However in the case of k_3, its value must not be negligible compared to $2k_t[A]_0$. Finally we found that the value of the fourth kinetic constant k_4 is more crucial than the other parameters for the occurrence of oscillations. Figures 1(a–c) show some characteristic trends: a large domain in which damped oscillations (DO) may occur; the two complex eigenvalues for DO have a negative real part. Contained within DO is a small region in which sustained oscillations (SO) may occur; the complex eigenvalues for SO have a positive real part. Figure 1(d) shows clearly the importance of the k_4 value on the complex eigenvalue domain, and the

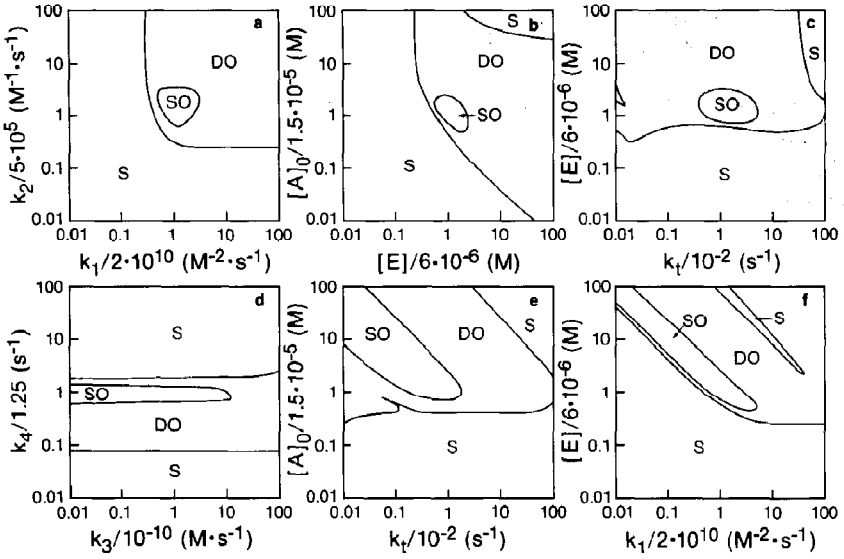

Fig. 1. Stability diagram drawn on a logarithmic scale by varying two parameters of the system, one at a time, and by evaluating whether the eigenvalues are complex or real. Three different domains can be obtained. A stable domain, S, which has three negative real eigenvalues; a sustained oscillation region, SO; and a damped oscillation region, DO. The three eigenvalues for SO and DO are: one negative and real and two conjugate complex values with real parts negative (DO) or positive (SO). All these diagrams have been drawn using the following parameter values: $k_1 = 2\times10^{10}\ M^{-2}\ s^{-1}$; $k_2 = 5\times10^5\ M^{-1}\ s^{-1}$; $k_3 = 10^{-10}\ M\ s^{-1}$; $k_4 = 1.25\ s^{-1}$; $k_t = 10^{-2}\ s^{-1}$; $[A]_0 = 1.5\times10^{-5}\ M$; $[E] = 6\times10^{-6}\ M$. Therefore these values are used for evaluation of the central point.

fact that the system is unstable for a large range of k_3 values. Finally in Figs. 1(e) and (f) large domain is developed in which sustained oscillations can be expected. Figures 1(e) and (f) show the same kind of phenomenon: the sustained oscillation (SO) domain being arranged along the diagonal of the diagram. In order to remain within SO a diminution of the transfer rate constant k_t may require an increase of the equilibrium concentration $[A]_0$. Also a diminution of the kinetic constant k_1 may require an increase of the enzyme concentration [E]. This can be explained by the influence of $k_t[A]_0$ or $k_1[E]$ in the steady state equations and in the matrix coefficients.

5. Simulations

We have performed numerical simulations using eqs. (7) to (9) directly in the computer program which are in agreement with the results of the stability analysis. In the domain in which the eigenvalues are real and negative, we verified that the steady state was stable without oscillatory behavior. When two eigenvalues were complex with a negative real part, we obtained (as expected) damped oscillations. Finally in the last domain in which two eigenvalues are complex with a positive real part (so that the steady state is

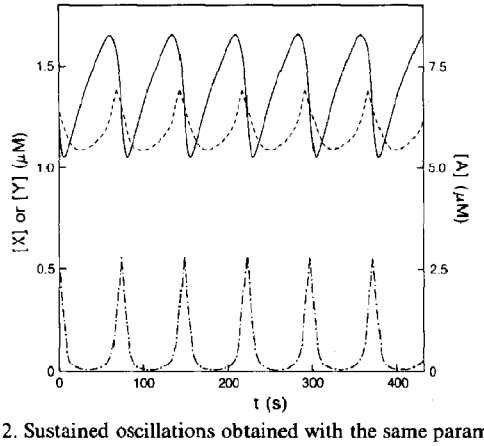

Fig. 2. Sustained oscillations obtained with the same parameter values as in Fig. 1. A(———); Y(------); X(·–·–·).

unstable) we obtained sustained oscillations. Figure 2 shows an example of these sustained oscillations.

An analysis of the effect of the various parameters show that the amplitude and period of the sustained oscillations are maximum for their values which correspond to the center of the SO domain on the stability diagrams (Fig. 1a–c). In the case of k_4, a small increase or decrease of its value in the SO domain leads to a fast decrease of the period and amplitude. In the case of values of the parameters k_1, k_2, k_t, [E] and $[A]_0$ the same kind of change does not change drastically the amplitude and period of oscillations. However, when their values are close to the one of the DO domain, the amplitude and period decrease leading to damped oscillations in the DO domain. In the case of k_3, if its value is taken within the SO domain near the DO domain, the amplitude and period decrease. However, if the value of k_3 used for drawing Fig. 2 is divided by 10,000, the sustained oscillations remain unaltered. Therefore, the simplification we made on the third step (reaction 3) is justified. If the kinetics of this step were more complex, it would lead to a decrease of its rate with time, which as we see does not change the oscillations.

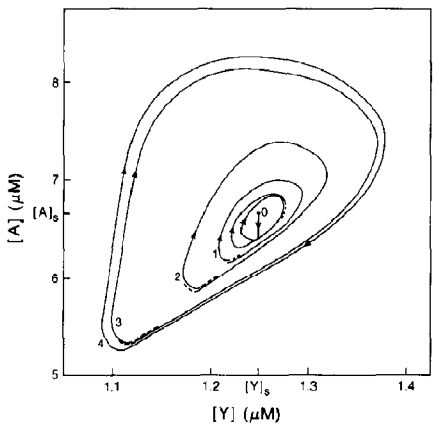

Fig. 3. Evolution of the limit cycle when starting with the steady state and after putting $k_t = 0\ s^{-1}$ for 2 s at $t = 100$ s. Initial concentrations: [A] = 6.576 μM; [Y] = 1.249 μM; [X] = 0.1369 μM. Parameters as in Fig. 1. Only parts of the evolution are shown here at the following starting times: (0) $t = 100$ s; (1) $t = 1300$ s; (2) $t = 3000$ s; (3) $t = 6000$ s. (4) $t = 10000$ s.

Depending upon the initial conditions a large variation can occur in the time required to reach the limit cycle (sustained oscillations). For example, we performed a simulation starting at the steady state conditions (Fig. 3). In order to perturb the system away from the steady state and to obtain a faster response of the system, we put the transfer rate constant equal to $0\ s^{-1}$ for 2 s at $t = 100$ s. Then the oscillations start. At the beginning the limit cycle is very small but increases slowly with time. After more than 8000 s the final limit cycle is then obtained. Following the same behavior as the concentration maxima, the period increases with time starting at 63.5 s for the first period and ending at 76.5 s when the final limit cycle is reached (Fig. 3).

6. Discussion

In order to explain the oscillations in the peroxidase–oxidase reaction with NADH, we built a model based mainly on a cycle between two oxidation states of the peroxidase. The normal mode stability analysis of this model showed clearly the importance of the kinetic constant of the fourth step which corresponds to the termination reaction of a free radical intermediate species X (reaction 4). The connection between the transfer rate constant (k_t) and the equilibrium concentration of A, $[A]_0$, was clearly demonstrated (Fig. 2e). If k_t is less than some maximum value and $[A]_0$ more than some minimum value, a decrease of k_t and an increase of $[A]_0$ of the same order will keep the input flux of the species A constant and will not affect the result of the stability analysis. A similar relationship was found between the kinetic constant of the first step (reaction 1) and the total concentration of enzyme E when their values are bigger than some minimum value (Fig. 2F).

Using the results of the stability analysis, numerical simulations confirmed the results of this analysis. We have shown that our model is able to exhibit damped and sustained oscillations. From a qualitative point of view the shape of the oscillations for species A and Y are very similar to the experimental ones for oxygen and oxyperoxidase

[5]. However, we have not found with the model chaos or complex oscillations as in the experiment. Because only one steady state is predicted, bistability cannot be found with our model.

To the best of our knowledge, chaos or complex oscillations was observed experimentally only when running the oxidation of NADH by HRP in the presence of methylene blue and dichloro-1,4-phenol (DCP) [5,6]. On the same hand, from a theoretical point of view, the detailed model involving all the known reactions for this reaction system but which did not take into account methylene blue or DCP, was never found to exhibit these phenomena [11]. Methylene blue in this reaction was assumed to be a catalyst [13]. However, up to now its exact role has not been elucidated.

Recently, the oxidation of sulfide ion by methylene blue has been found to exhibit sustained oscillation in a continuous stirred tank reactor (CSTR) [21]. The mechanism of these oscillations has been studied and it was suggested that the oxidation of the reduced form of methylene blue is indirect, auto-catalytic and involves methylene blue radical as well as superoxide radicals [22,23]. Actually, the oxidation of NADH by methylene blue has been investigated under anaerobic or aerobic conditions in a closed system [24]. This reaction was found to be of first order in respect to methylene blue and to exhibit a saturation kinetic behavior in respect to NADH. If the re-oxidation of methylene blue is auto-catalytic [15,16], oscillations would be expected during the oxidation of NADH by methylene blue in an open system with diffusion of oxygen. In any case, this reaction should be taken into account in a realistic mechanism for the oscillations of the peroxidase–oxidase reaction with NADH.

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