Oscillations and Complex Dynamics in the Peroxidase−Oxidase Reaction Induced by Naturally Occurring Aromatic Substrates

Ursula Kummer,[†] Marcus J. B. Hauser,[‡] Klaus Wegmann,[†] Lars F. Olsen,[*,‡] and Gerold Baier[†]

Contribution from the Institute for Plant Biochemistry, University of Tübingen, Correnstrasse 41, D-72076 Tübingen, Germany, and Physical Biochemistry Group, Institute of Biochemistry, Odense University, Forskerparken 10, DK-5230 Odense M, Denmark

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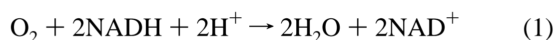
Abstract: The effect of various phenols and aromatic amines on the dynamics of the peroxidase-catalyzed aerobic oxidation of NADH was investigated. We demonstrate that several aromatic compounds may substitute for 2,4-dichlorophenol in inducing sustained oscillations and complex dynamics in the reaction. These aromatic compounds can also act as substrates in the classical peroxidase reaction. Since some of the aromatic compounds studied are naturally occurring substrates for peroxidases, we conjecture that oscillations and complex dynamics may well occur in the intact plant.

Introduction

Complex dynamics have become increasingly important in biochemical and physiological contexts in recent years.[1] An example is the oxidation of NADH by molecular oxygen:



$$O_2 + 2NADH + 2H^+ \rightarrow 2H_2O + 2NAD^+ \quad (1)$$

catalyzed by peroxidase, a reaction which is also known to occur *in vivo*.[2−4] In *in vitro* experiments where the substrates O_2 and NADH are continuously supplied to the reaction mixture, the concentrations of O_2 and NADH in the reaction mixture may exhibit simple and complex periodic oscillations,[5] as well as quasiperiodicity,[6,7] and chaos.[7−11] The type of dynamics displayed by the reaction is highly dependent on the experimental conditions, e.g. the pH of the solution. Previous experiments have shown different dynamic behaviors at pH $\approx$6 compared to at pH $\approx$5, in both semibatch[9,11,12] and continuous-flow stirred tank reactors.[7,10] The most commonly used enzyme for the study of oscillations and complex dynamics in reaction 1 is peroxidase from horseradish. However, peroxidases from other sources such as soybean, milk, and fungi have also been shown to catalyze oscillatory behavior.[13−15]

So far a condition for observing complex dynamics in the peroxidase−oxidase reaction is that 2,4-dichlorophenol and methylene blue are both added to the reaction mixture.[5] In the absence of either one of these modifiers only damped or simple periodic oscillations are obtained.[14,16] However, the precise actions of 2,4-dichlorophenol and methylene blue are not known. It was established long ago that methylene blue inhibits reaction (1),[13,17] and that it seems to protect the enzyme from being destroyed by free radicals.[17] These observations are consistent with recent experiments,[18] indicating that methylene blue may accept electrons from NAD•, which is a key intermediate of the reaction.[16,19]

The role of 2,4-dichlorophenol is even less understood. Addition of 2,4-dichlorophenol increases the overall reaction rate as well as the decomposition of oxyferrous peroxidase (compound III) when both O_2 and NADH are present.[17,20] However, the precise mechanism by which this is happening has yet to be resolved. As a consequence, most of the detailed models of the peroxidase−oxidase reaction do not incorporate 2,4-dichlorophenol directly into the mechanism. In spite of the omission of 2,4-dichlorophenol and methylene blue, some of these models reproduce quite well many of the dynamic behaviors observed experimentally.[11,16,21−24]

In order to elucidate the role of 2,4-dichlorophenol in oscillations and complex dynamics of the peroxidase−oxidase reaction we have conducted a series of experiments where 2,4-dichlorophenol is replaced by other phenols or amino-substituted aromatic compounds. In the present article we report that several of these aromatic compounds promote sustained oscillations and complex dynamics. In contrast to 2,4-dichlorophe-

* Corresponding author, e-mail address: lfo@dou.dk.
† University of Tubingen.
‡ Odense University.
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nol, some of the aromatic compounds studied, e.g. 4-hydroxybenzoic acid, occur naturally in plants.[25] We, therefore, conjecture that oscillations and complex dynamics may also be found in the intact plant.

Materials and Methods

NADH (grade II) and horseradish peroxidase (330 U/mg, RZ 3.0) were purchased from Boehringer Mannheim; lactoperoxidase from bovine milk (92 U/mg, RZ 0.82), 4-aminobenzoic acid, and *p*-cresol were purchased from Sigma. 2,3,4,6-Tetrachlorophenol was obtained from TCI, aniline and methylene blue from Merck, benzoic acid from Riedel-de Haën, and 2,4-dichlorophenol from Aldrich. All other aromatic compounds were kindly supplied by the Institute of Chemistry, Odense University.

Experiments were performed in a thermostated 21.7 × 21.7 × 40 mm quartz cuvette fitted with an oxygen electrode (Radiometer, Copenhagen) and placed in a Zeiss Specord S10 diode array spectrophotometer. The signals from the oxygen electrode and the spectrophotometer were sampled every 2 s and stored on a computer for later analysis. The data from the spectrophotometer contained the absorbance changes in the region 350−600 nm with a resolution of 1 nm. All enzymatic intermediates and NADH (ferrous and ferric peroxidase, compounds I, II, and III) have distinct absorption spectra in this region of visible light. We are therefore able to measure their temporal evolution by spectral deconvolution.[18] Since UV light may facilitate the decomposition of the enzyme only the tungsten lamp of the spectrophotometer was switched on during measurements in order to minimize such decomposition.

The experiments were performed in a semibatch reactor. The stirred sample had a volume of 10 mL and contained 0.1 M Na−acetate buffer (pH 5.1) or 0.1 M Na−phosphate buffer (pH 6.3), 1.2−1.45 μM peroxidase, 0.1 μM methylene blue, and the aromatic compound in different concentrations. A 0.1 M solution of NADH in distilled water was infused at a flow rate of 45−65 μL/h through a capillary tube connected to a high-precision syringe pump (Harvard Apparatus, Model 22). Oxygen was supplied to the reaction mixture from a 10-mL gas volume above the liquid containing a 1.05% (v/v) oxygen/nitrogen mixture as described in our previous papers.[11,14] The oxygen transfer constant between the gas and the liquid phase was measured as $6.0 \pm 0.2 \times 10^{-3}\ s^{-1}$ at a stirring rate of 1050 rpm. Some experiments were performed in another setup with a sample size of 7 mL and an oxygen transfer constant of $4.2 \pm 0.2 \times 10^{-3}\ s^{-1}$ while the rate of supply of NADH was 25−40 μL/h. Other conditions were as given above.

The reactor containing the solution of enzyme, methylene blue, and aromatic compound in acetate or phosphate buffer was thermostated at 28 °C and equilibrated with pure nitrogen before the start of an experiment. Experiments were typically started by adding NADH at a flow rate of 60 μL/h. As the absorbance at 360 nm, which is mainly due to NADH, reached an OD of about 0.8−1.0, the composition of the gas stream was switched from pure N_2 to the O_2−N_2 mixture. The NADH flow rate was then adjusted such that the NADH concentration oscillated around a constant level. Then the dynamics corresponding to this particular average NADH concentration was recorded for 2000 to 8000 s. By choosing different flow rates we were able to stabilize the average NADH concentration on different levels, associated with different types of dynamics as described in the following section. A low flow rate yields a low average NADH concentration whereas a high flow rate results in a high average concentration of NADH.

Results

In the absence of any substituted aromatic compound our experimental system only displays damped oscillations. Addition of certain amounts of some aromatic compounds to the reaction mixture resulted in an increase of the reaction rate and a change in dynamics to sustained periodic oscillations or to complex dynamics (see below). No qualitative differences in the resulting behavior could be observed, if we added the aromatic substrates either before or after the start of an experiment. Table 1 summarizes the effect of some aromatic compounds on the dynamics of the peroxidase−oxidase reaction catalyzed by horseradish peroxidase at pH 5.1 and 6.3. Preliminary experiments showed that the respective aromatic compounds were also able to induce oscillations under otherwise non-oscillatory conditions in reaction 1 catalyzed by lactoperoxidase (sustained oscillations were obtained when using, for instance, 0.7 μM lactoperoxidase (92 U/mg, RZ 0.82), 0.7 μM methylene blue, 5.0 μM 4-chlorophenol, and a NADH inflow rate of 35 μL/h). Aromatic compounds, however, which were unable to induce oscillatory behavior in the reaction catalyzed by horseradish peroxidase behaved in the same way when lactoperoxidase was used.

Table 1. Efficacy of Various Aromatic Compounds to Induce Sustained Oscillations and Complex Dynamics in the Peroxidase−Oxidase Reaction Catalyzed by Horseradish Peroxidase at pH 5.1 and 6.3[a]

aromatic compound	oscillations		complex dynamics	
	pH 5.1	pH 6.3	pH 5.1	pH 6.3
phenol	50 μM	−	−	−
2-chlorophenol	20 μM	20 μM	−	−
4-chlorophenol	20 μM	20 μM	+	+
2,4-dichlorophenol	10 μM	10 μM	+	+
2,3,4,6-tetrachlorophenol	5 μM	5 μM	+	+
2-nitrophenol	−	−	−	−
4-nitrophenol	−	n.d.	−	n.d.
2,4-dinitrophenol	−	n.d.	−	n.d.
p-cresol	200 μM	−	−	−
4-hydroxybenzoic acid	200 μM	200 μM	+	+
aniline	500 μM	500 μM	+	−
4-aminobenzoic acid	1 mM	1 mM	−	n.d.
toluene	−	−	−	−
benzoic acid	−	n.d.	−	n.d.
benzaldehyde	−	n.d.	−	n.d.

[a] The concentrations listed are the approximate minimum concentrations needed to induce sustained oscillations. "+" indicates that the compound also induces complex oscillations whereas "−" indicates that the compound did not induce oscillations or complex dynamics. n.d.: not determined.

From Table 1 it is evident that the presence of a hydroxy or an amino substituent at the aromatic ring is a prerequisite for inducing oscillations. Aromatic compounds which lack such substituents, e.g. toluene and benzoic acid, cannot induce sustained oscillations. As a measure of the efficiency of the various compounds tested, we list (Table 1) the approximate minimum concentration required to induce sustained oscillations. We conclude that aromatic amines are much less efficient compared to phenolic compounds. We further observe that within the group of phenols chlorination increases the efficiency considerably, while substitution with alkyl or carboxyl groups decreases the efficiency. Aromatic compounds with strong electron-withdrawing substituents, such as nitrophenols, will not promote sustained oscillations at all. In fact nitrophenols inhibit the reaction.

We note that several of the aromatic compounds are able to induce sustained oscillations at both pH values studied, although some, e.g. phenol, will induce such oscillations only at pH 5.1. However, we did not find any phenol or aniline derivative that would induce sustained oscillations at pH 6.3, but not at pH 5.1. We were unable to correlate the ability of the aromatic compounds to sustain oscillatory dynamics in the PO reaction with their pK_a values.

Several of the phenolic compounds and aromatic amines also induced complex periodic oscillations and chaotic dynamics. Figure 1 shows an example of a period-adding sequence to chaos at pH 6.3 observed in the presence of 4-hydroxybenzoic acid. The time series represents the absorbance changes at 418 nm, which is due to compound III (oxyferrous peroxidase) as

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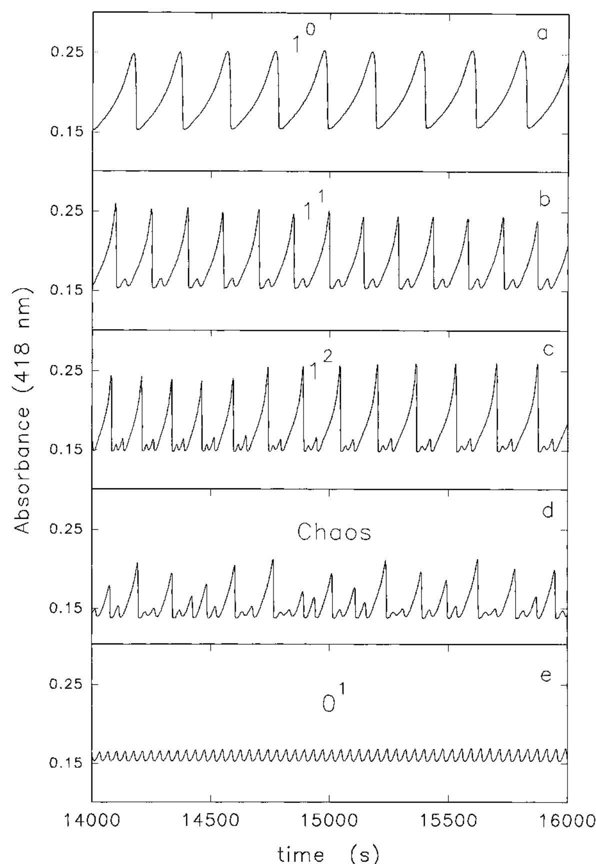

Figure 1. Period-adding sequence in the peroxidase−oxidase reaction in the presence of 4-hydroxybenzoic acid. The time-series shown were monitored at 418 nm, corresponding to the absorption maximum of compound III. Experimental conditions: 1.3 μM horseradish peroxidase, 750 μM 4-hydroxybenzoic acid, pH 6.3; other conditions as in the Materials and Methods section. Dynamic behavior in response to increase in the average concentration of NADH, from a to e: (a) periodic oscillations of periodicity 1^0 at $[NADH]_{av}$ = 73.0 μM, (b) mixed mode oscillations of periodicity 1^1 at $[NADH]_{av}$ = 91.6 μM, (c) 1^2 oscillations at $[NADH]_{av}$ = 94.0 μM, (d) chaotic state at $[NADH]_{av}$ = 95.6 μM, and (e) 0^1 oscillations at $[NADH]_{av}$ = 97.8 μM.

confirmed by deconvolution of the spectrum (350−600 nm). The changes in dynamics observed in Figure 1a−e were obtained by raising the average concentration of NADH in steps. At low concentrations of NADH (Figure 1a) we observe simple periodic oscillations. At slightly higher NADH concentrations (Figure 1b) this behavior changes to a pattern where one large-amplitude oscillation alternates with one small-amplitude oscillation. Such an oscillation is referred to as a 1^1 oscillation. Generally, such mixed-mode oscillations composed of L large-amplitude oscillations and S small-amplitude oscillations are referred to as displaying a L^S periodicity. A further increase in the NADH level causes the disappearance of the 1^1 oscillations and the appearance of a new type of dynamics where one large-amplitude oscillation alternates with two small-amplitude oscillations (Figure 1c), i.e. a 1^2 periodicity. Further increases in the average NADH concentration result in emergence of chaotic oscillations (Figure 1d). At even higher concentrations of NADH we again observe periodic oscillations, this time with a much reduced amplitude. The first periodic state to appear is one composed of two small-amplitude oscillations with different amplitudes (0^2, not shown). Then a simple periodic state consisting of oscillations with a single small amplitude is observed (0^1, Figure 1e). This is followed by a non-oscillatory steady state (not shown). The sequence of periodic states which precede the chaotic state is called a *period-adding* sequence ($1^0 \rightarrow 1^1 \rightarrow 1^2 \rightarrow 1^3 \rightarrow ... \rightarrow$ chaos), and this sequence is one of the well-established routes to chaotic dynamics described theoretically.[26] The chaotic oscillations shown in Figure 1d could be further analyzed by constructing a next-amplitude map, i.e. a plot where each maximum of the oscillations is plotted against the maximum of the preceding oscillation. The plot is shown in Figure 2. If such a plot defines a random scatter of points the corresponding time series is essentially random. On the other hand, if the plot shows a well-defined functional relationship between consecutive amplitudes the corresponding dynamics is chaotic. The latter is indeed the case here.

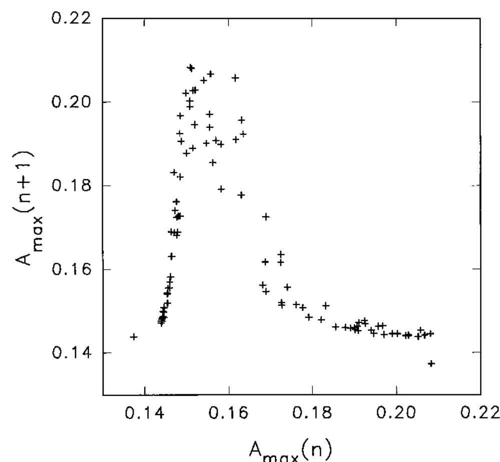

Figure 2. Next-amplitude map constructed from the chaotic state shown in Figure 1d, which was monitored at 418 nm, corresponding to the absorption maximum of compound III. Here we plot the absorbance maximum of one oscillation, A_{max} $(n + 1)$, against the absorbance maximum of the preceding oscillation, A_{max} (n).

Repeating the above-mentioned experiments at pH 5.1 yields a different route to chaos, namely the so-called *period-doubling* route[27] (data not shown). Here the sequence of periodic states begins with a simple periodic state where all oscillations have the same amplitude. When the average NADH concentration is increased, this state transforms into a state composed of oscillations with two different amplitudes, and later into a state composed of oscillations with four different amplitudes, etc..., and finally into a chaotic state. A change in routes to chaos with changing pH was recently obtained with 2,4-dichlorophenol as the activator.[12] The same effect on complex dynamics was observed here with 4-hydroxybenzoic acid, with 4-chlorophenol, and with 2,3,4,6-tetrachlorophenol, i.e. a period-doubling route to chaos at pH 5.1 and a period-adding route to chaos at pH 6.3.

Discussion

Phenolic compounds and aromatic amines are known to accelerate various peroxidase-catalyzed reactions including the peroxidase−oxidase reaction.[2−4,17,28−30] According to our results this property seems to correlate with the ability to induce oscillatory behavior. In principle four possible hypotheses could account for the acceleration effect: (i) interaction of the aromatic compound with only non-enzymatic intermediates; (ii) stoichiometric oxidation of the aromatic compound by the enzyme and interaction of some intermediates with the peroxidase−oxidase reaction; (iii) changes of the enzymatic properties following binding of the aromatic compound; and (iv) enzymatic formation

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of an aromatic radical and subsequent recovery of the aromatic compound by reaction of the radical with NADH to yield $NAD^•$.

All of the investigated aromatic compounds which induce oscillatory behavior are also substrates in the classical peroxidase cycle:

$$Per^{3+} + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (2)$$

$$\text{compound I} + ArH \rightarrow \text{compound II} + Ar^• \quad (3)$$

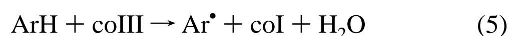
$$\text{compound II} + ArH \rightarrow Per^{3+} + Ar^• + H_2O \quad (4)$$

where Per^{3+} denotes ferric peroxidase. Therefore, hypothesis (i) appears unlikely. This is further supported by the finding that nitrophenols, which cannot act as substrates for this reaction, also do not facilitate oscillations. However, it is not clear why nitrophenols inhibit the peroxidase−oxidase reaction itself. Other aromatic compounds, such as toluene, which also cannot act as substrates for reactions 3 and 4 and do not induce oscillations, have no effect on the rate of the peroxidase−oxidase reaction.

Hypothesis (ii) assumes the stoichiometric oxidation products of the added aromatic compounds as the active species. The effect of the various aromatic compounds on the reaction is permanent. This would not be the case if the concentration of the aromatic compound decreased significantly in the course of the reaction, unless a reaction product is the only component responsible for the long-lasting effect. In the latter case, however, all active oxidation products have to act precisely the same way upon the enzyme. Additionally, the conversion from the aromatic substrates to their reactive intermediates must occur instantaneously for all active aromatic compounds studied, since no induction period could be observed upon addition of aromatic substrates. Taking these requirements into account, we consider hypothesis (ii) to be unlikely. Furthermore, it was observed that the peroxidase−oxidase reaction with NADH as a substrate explicitly inhibits stoichiometric oxidation of phenolic compounds.[4]

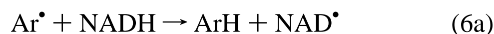
As for hypothesis (iii), it has been shown that phenolic compounds and aromatic amines bind directly to peroxidase,[30] and that phenols bind more strongly than aromatic amines. The dissociation constant for such binding is on the order of >10 mM for both phenols and amines. On the other hand, the concentrations of phenols and aromatic amines required to induce sustained oscillations are at least an order of magnitude lower than the dissociation constants.

A recycling of the aromatic compound via enzyme-catalyzed formation of the aromatic radical (hypothesis (iv)) could occur either through reactions 3 and 4 or through the reaction between the aromatic compound and compound III (coIII):



$$ArH + coIII \rightarrow Ar^• + coI + H_2O \quad (5)$$

The resulting free radical $Ar^•$ could then react with either NADH or the $NAD^•$ radical:



$$Ar^• + NADH \rightarrow ArH + NAD^• \quad (6a)$$

$$Ar^• + NAD^• + H^+ \rightarrow ArH + NAD^+ \quad (6b)$$

Compound III is an intermediate which occurs in the peroxidase−oxidase reaction, but not in the classical peroxidase cycle (reactions 2−4). Due to the stability of compound III, its decomposition has been proposed to be rate limiting for the peroxidase−oxidase reaction.[13,19] Phenolic compounds and aromatic amines stimulate the decomposition of compound III.[17,20] In a recent study of a detailed mechanism of the peroxidase−oxidase reaction it was shown that a period-doubling route to chaos could be induced by increasing the rate constant of the reaction:[24]

$$NAD^• + coIII \rightarrow NAD^+ + coI \quad (7)$$

A similar period-doubling route to chaos was observed experimentally when the concentration of 2,4-dichlorophenol was increased in steps.[9] These observations are consistent with a direct reaction between the aromatic compound and compound III as explained above. However, previous studies have shown that in the absence of NADH the breakdown of compound III is not accelerated by phenols.[31,32]

The recycling of the aromatic compound could explain its permanent effect. Furthermore, the relative stability of the respective free aromatic radicals seems to correlate with the efficiency of a given aromatic compound to induce oscillatory behavior. Assuming that the unpaired electron in the aromatic radical $Ar^•$ is delocalized through the aromatic π-electron system, a higher degree of chlorination of the aromatic ring provides increasing stability to the radical due to resonance of the substituents with the ring. When the possibility of such a stabilization through increased delocalization is not possible, we observe a decrease in the efficiency in inducing oscillations (compare 4-hydroxybenzoic acid to phenol and 4-aminobenzoic acid to aniline in Table 1). We are currently studying new models of the peroxidase−oxidase reaction which include the reactions discussed above.

Several of the phenols used in the present study occur naturally in plants; e.g. phenolic derivatives of benzoic acids are widespread in higher plants. 4-Aminobenzoic acid is a precursor in the synthesis of folic acid, and 4-hydroxybenzoic acid acts as a cross-linking agent of the polysaccharide components of the cell wall.[25,33] Peroxidases are often linked to the cell wall where they catalyze a peroxidase−oxidase reaction using NADH derived from the oxidation of malate.[2−4] In the present paper we have shown that the peroxidase−oxidase reaction supplemented with natural phenolic compounds and aromatic amines will show oscillatory and complex dynamics *in vitro* at a broad range of experimental conditions. We therefore conclude that such behavior may also occur *in vivo*. However, specific predictions for parameter values required to induce oscillations in a horseradish root are not yet possible. This would require knowledge about the local concentrations of peroxidase and aromatic compounds within the cells of a root. Such information is not yet available. However, as part of our efforts to obtain oscillations in *in vivo* systems, we are commencing investigations using homogenized cell-wall suspensions from horseradish roots.

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