The Role of Naturally Occurring Phenols in Inducing Oscillations in the Peroxidase−Oxidase Reaction

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ABSTRACT: The influence of a series of naturally occurring phenolic compounds on the dynamics of the horseradish peroxidase-catalyzed oxidation of NADH by oxygen (the peroxidase−oxidase reaction) was investigated. Various types of dynamic behaviors are induced in the peroxidase−oxidase reaction upon addition of low concentrations of different phenols. The identity of the particular phenol determines the type of dynamics shown (inhibition of the reaction, monotonic reaction, damped oscillations, sustained oscillations, and complex oscillations). The kind of behavior that a given phenolic compound is able to induce is governed by the reduction potentials for the formation of the phenoxyl radical. The phenolic compounds are shown not to be consumed during the reaction. Mechanistic considerations are made on the basis of the reduction potentials of the different reactants present in the peroxidase−oxidase system. We interpret the role of the phenols as electron mediators of the electron transfer between some enzyme intermediates or O_2^- and NADH.

The classical reaction catalyzed by peroxidases is the oxidation of a wide range of hydrogen donors, YH_2, by hydrogen peroxide:

$$H_2O_2 + 2YH_2 \rightarrow 2H_2O + 2YH^{\bullet} \quad (1)$$

Reaction 1 involves the native ferric peroxidase (Per^{3+})[1] and the two enzyme intermediates, compound I (Per^{5+}) and compound II (Per^{4+}) (*1*). The generally accepted catalytic cycle for the peroxidase reaction is

$$Per^{3+} + H_2O_2 \rightarrow Per^{5+} + H_2O \quad (2)$$

$$Per^{5+} + YH_2 \rightarrow Per^{4+} + YH^{\bullet} \quad (3)$$

$$Per^{4+} + YH_2 \rightarrow Per^{3+} + YH^{\bullet} + H_2O \quad (4)$$

However, horseradish peroxidase as well as several peroxidases from other sources is also known to catalyze reactions where molecular oxygen replaces hydrogen peroxide as the oxidant (*1*):

$$O_2 + 2YH_2 \rightarrow 2\,H_2O + 2\,Y \quad (5)$$

As for the latter reaction, which is usually referred to as the peroxidase−oxidase (PO) reaction, only a few electron donors (YH_2) are known. These include reduced nicotinamide adenine dinucleotide (NADH), dihydroxyfumaric acid, indole-3-acetic acid, and triose reductone (*1*), as well as veratryl alcohol (*2*). In addition to reactions 2−4, the PO system also comprises the following enzymatic reactions:

$$Per^{3+} + O_2^- \rightarrow Per^{6+} \quad (6)$$

$$Per^{6+} + YH^{\bullet} \rightarrow Per^{5+} + Y + H^+ \quad (7)$$

$$Per^{3+} + YH^{\bullet} \rightarrow Per^{2+} + Y + H^+ \quad (8)$$

$$Per^{2+} + O_2 \rightarrow Per^{6+} \quad (9)$$

plus a few nonenzymatic reactions (*3*). Thus, in the PO reaction (reaction 5), the enzyme is present in five oxidation states: ferrous peroxidase (Per^{2+}), ferric peroxidase (Per^{3+}), compound I (Per^{5+}), compound II (Per^{4+}), and compound III (Per^{6+}) (*1*).

An interesting feature of the PO reaction is its dynamics. When supplied continuously with NADH and O_2, reaction 5 will display damped oscillations (*4*−*6*). Additions of small amounts of methylene blue and certain phenolic or amino-substituted aromatic compounds enable the PO system to perform sustained oscillations and complex dynamics (*7*−*10*). The latter include chaotic oscillations, which can arise through different pathways, according to the pH of the reaction medium (*9*, *11*−*13*). In fact, the PO system has become a model system for the investigation of oscillatory and complex behavior in biochemical reaction systems.

Sustained oscillations and chaotic behavior were first observed when 2,4-dichlorophenol was used as the cofactor (*7*, *12*−*15*). 2,4-Dichlorophenol therefore rapidly became the standard aromatic compound used in studies of the reaction's dynamics. The choice of 2,4-dichlorophenol as

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[1] Abbreviations: E_0', one electron reduction potential; $E_{1/2}$, half-wave potential; MIMS, membrane inlet mass spectrometry; NADH, reduced β-nicotinamide adenine dinucleotide; NHE, normal hydrogen electrode; Per^{2+}, ferrous peroxidase; Per^{3+}, ferric peroxidase (native state); Per^{4+}, peroxidase compound II; Per^{5+}, peroxidase compound I; Per^{6+}, oxyferrous peroxidase, peroxidase compound III; PhOH, phenolic compound; $PhO^{\bullet}$, phenoxyl radical; PO, peroxidase−oxidase; SCE, saturated calomel electrode.

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the activator introduces a nonphysiological compound into the reaction system. Thus, the PO reaction with 2,4-dichlorophenol has frequently been regarded as a somewhat artificial system without direct physiological relevance. Recently, however, it was shown that other phenolic compounds and aromatic amines may substitute for 2,4-dichlorophenol in inducing sustained oscillations and complex dynamics (*10, 16*). It was found that only aromatic compounds which also act as substrates (YH_2) in reaction 1 can induce oscillations in reaction 5. The precise mechanism by which aromatic compounds participate in the PO reaction is not yet known. It is not very likely that these substances are consumed during the reaction, since only relatively small amounts are needed to induce oscillations which last for many hours (*10, 17*).

Methylene blue is the second cofactor used in the PO reaction system. Like the phenolic compounds, it is a prerequisite for obtaining complex dynamics in reaction 5, but its role in the reaction mechanism is not yet resolved. Methylene blue is thought to act as an inhibitor of peroxidase activity (*7, 17*), and it has recently been shown to regulate the amount of ferrous peroxidase (Per^{2+}) formed during the reaction (*18*).

Studies of the oscillating PO reaction are usually performed using the enzyme from horseradish roots. However, enzymes obtained from various other sources, like bovine milk, soybean, and *Coprinus cinereus* (*Arthromyces ramosus*), were recently shown to catalyze oscillatory oxidation of NADH (*19, 20*). The reaction conditions necessary for observing oscillatory dynamics vary slightly according to the provenance of the enzyme employed. For example, when peroxidase from *C. cinereus* was used, addition of methylene blue was not required to obtain sustained oscillations (*19*).

The PO reaction is known to occur in vivo in plants using NADH derived from the oxidation of malate (*21*−*23*). Since some of the aromatic compounds that induce oscillations occur naturally in plant cells, it was suggested that oscillatory behavior could also occur in the PO reaction in vivo (*10*). Preliminary reports on experiments with horseradish cell extracts show that these extracts can display damped oscillations when supplied with NADH and oxygen (*16*), thus indicating a possibility of oscillatory dynamics also in the intact cell. However, the function of such behavior in an intact plant can only be a matter of speculation at this point.

Oscillations and several of the complex dynamic behaviors have been reproduced by mathematical models of the PO reaction (*6, 11, 24*−*28*). All of these detailed models are derived from a scheme originally proposed by Yokota and Yamazaki (*29*). The models are mainly based on known elementary reaction steps with rate constants measured within a reasonable accuracy (*3*). Even in the most recent of these detailed models methylene blue and phenolic compounds are generally not incorporated directly. Instead, they appear indirectly in some of the reaction steps as rate constants.

To shed more light on the reactions involving phenolic compounds and whether an oscillating PO reaction is possible in an intact organism, we present new studies of the effects of aromatic compounds on oscillations and complex dynamics in the PO reaction using phenolic compounds which occur naturally in plant or animal cells.

MATERIALS AND METHODS

Materials. NADH (grade II) and horseradish peroxidase (RZ 3.2) were purchased from Boehringer Mannheim. The specific activity of the enzyme was 250 units/(mg of lyophilizate) (guaiacol, H_2O_2 at pH 7, 25 °C). We determined corresponding activities of 176 units/(mg of lyophilizate) in 0.1 M sodium phosphate, pH 6.3, and 128 units/(mg of lyophilizate) in 0.1 M sodium-acetate buffer at pH 5.1. The enzyme concentration in solution was determined as the absorption at 403 nm using an extinction coefficient of 1.0×10^5 M^{-1} cm^{-1}. 4-Methylphenol (*p*-cresol), 4-hydroxycinnamic acid, 4-hydroxy-3-methoxycinnamic acid (ferulic acid), and 3-methoxy-D,L-tyrosine were purchased from Sigma. 2-Hydroxycinnamic acid and 3-(4-hydroxyphenyl)propionic acid were obtained from Fluka, and methylene blue was from Merck. Benzoic acid was obtained from Riedel-de Haën, and 2,4-dichlorophenol, L-tyrosine, 4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 4-hydroxy-3-methoxycinnamyl alcohol (coniferyl alcohol), and 7-hydroxy-6-methoxycoumarin (scopoletin) were from Aldrich. All other aromatic compounds were kindly made available by the Institute of Chemistry, Odense University. Chart 1 gives an overview over the chemical structures of the investigated phenolic compounds. Stock solutions of the phenols in 99.9% ethanol were prepared biweekly. L-Tyrosine and 3-methoxy-D,L-tyrosine were prepared as aqueous solutions.

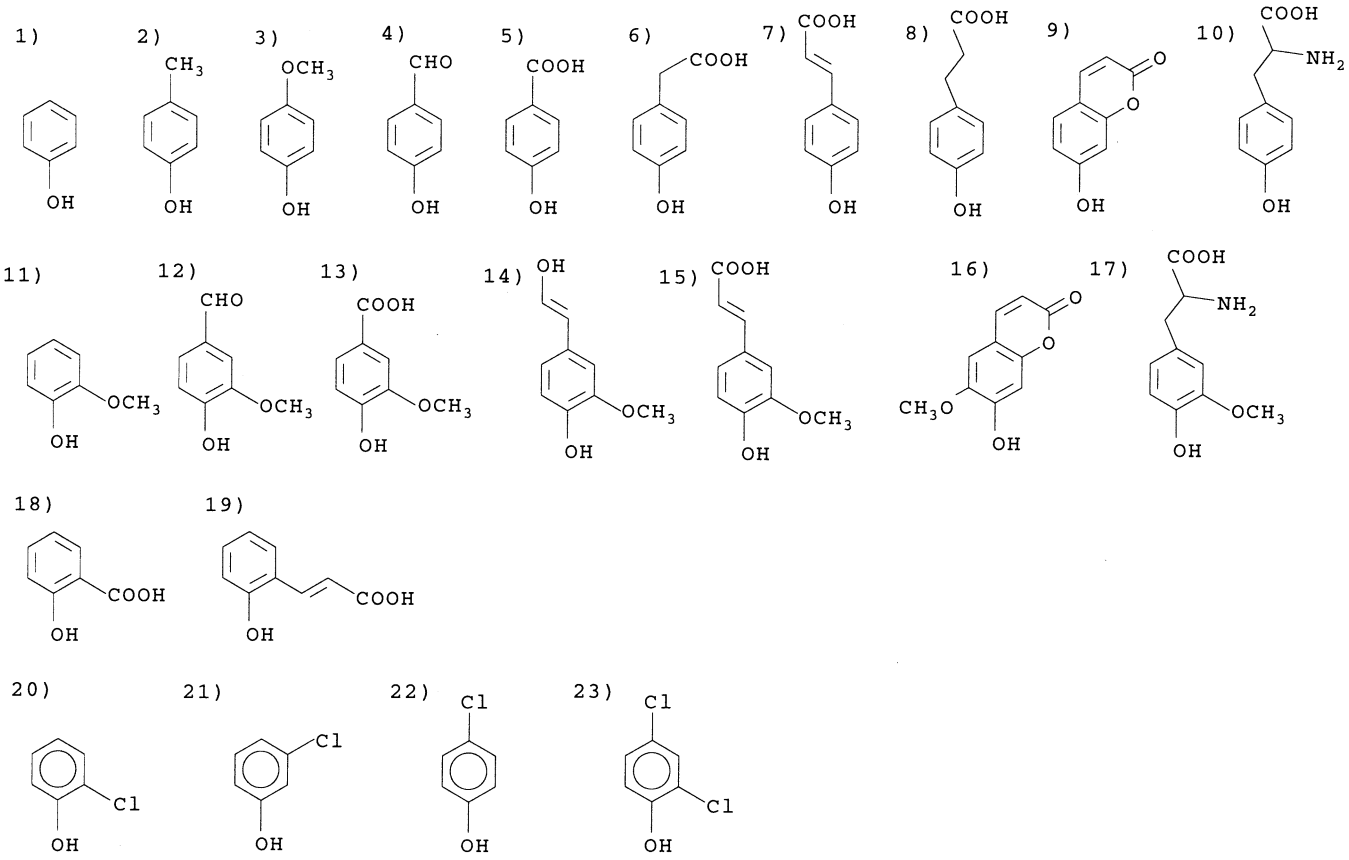
Dynamic Measurements. Experiments were performed in $21.7 \times 21.7 \times 42$ mm quartz cuvette fitted with an oxygen electrode (Radiometer, Copenhagen). The cuvette was mounted in a thermostatic jacket and placed in a Zeiss Specord S10 diode array spectrophotometer. The signals from the oxygen electrode 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. Because UV light may facilitate the decomposition of the enzyme and NADH, only the tungsten lamp of the spectrophotometer was switched on during measurements in order to minimize such decomposition.

The experiments were performed at 28.0 ± 0.1 °C in a semibatch reactor. The stirred sample had a volume of 10 mL and contained 0.1 M sodium acetate buffer (pH 5.1) or 0.1 M sodium phosphate buffer (pH 6.3), 1.2−1.5 μM horseradish 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^{-1} through a capillary tube connected to a high precision syringe pump (Harvard Apparatus, model 22). Oxygen was supplied to the reaction mixture from an approximately 10 mL gas volume above the liquid containing a moistured 1.05% (v/v) oxygen/nitrogen mixture as described in our previous articles (*9, 11, 19*). The rate of diffusion of oxygen into the liquid v_{O2} is given by the equation

$$V_{O_2} = K([O_2]_{eq} - [O_2]) \qquad (10)$$

where K is a constant, $[O_2]$ is the oxygen concentration in the liquid, and $[O_2]_{eq}$ is the oxygen concentration at equilibrium. The magnitude of the oxygen transfer constant K

Chart 1: Phenolic Compounds Investigated[a]



[a] (**1**) Phenol, (**2**) 4-methylphenol (*p*-cresol), (**3**) 4-methoxyphenol (4-hydroxyanisole), (**4**) 4-hydroxybenzaldehyde, (**5**) 4-hydroxybenzoic acid, (**6**) 4-hydroxyphenylacetic acid, (**7**) 3-(4-hydroxyphenyl)propenoic acid (4-hydroxycinnamic acid, *p*-coumaric acid), (**8**) 3-(4-hydroxyphenyl)propionic acid, (**9**) 7-hydroxycoumarin (umbelliferone), (**10**) 3-(4-hydroxyphenyl)-L-alanine (L-tyrosine), (**11**) 2-methoxyphenol (guaiacol), (**12**) 4-hydroxy-3-methoxybenzaldehyde (vanillin), (**13**) 4-hydroxy-3-methoxybenzoic acid (vanillic acid), (**14**) 4-hydroxy-3-methoxycinnamyl alcohol (coniferyl alcohol), (**15**) 4-hydroxy-3-methoxycinnamic acid (ferulic acid), (**16**) 7-hydroxy-6-methoxycoumarin (scopoletin), (**17**) 4-hydroxy-3-methoxyphenyl-D,L-alanine (3-methoxy-D,L-tyrosine, D,L-3-*O*-methyl-DOPA), (**18**) 2-hydroxybenzoic acid (salicylic acid), (**19**) 3-(2-hydroxyphenyl)propenoic acid (2-hydroxycinnamic acid, *o*-coumaric acid), (**20**) 2-chlorophenol, (**21**) 3-chlorophenol, (**22**) 4-chlorophenol, and (**23**) 2,4-dichlorophenol.

depends on the surface area of the solution and hence also on the stirring rate. In our setup we measured K as $(6.0 \pm 0.2) \times 10^{-3}$ s^{-1} at a stirring rate of 1050 rpm.

The reactor containing the solution of enzyme, methylene blue and aromatic compound in acetate or phosphate buffer was 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^{-1}. As the absorbance at 360 nm, which is mainly due to NADH, reached an OD of about 0.9−1.1 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 mean NADH concentration was recorded for 2000 to 8000 s. By infusing NADH at different flow rates, the mean NADH concentration settled on different asymptotic levels. We have previously shown (*9*−*11*) that different asymptotic levels of NADH are associated with different types of dynamics in the presence of 2,4-dichlorophenol and certain other phenols and aromatic amines. At low flow rates, which yield low asymptotic levels of NADH, only simple periodic oscillations are observed. However, at high flow rates, which yield high asymptotic levels of NADH, complex periodic oscillations and chaos may also be observed provided that certain phenols are present as cofactors as described in the following section.

To investigate the fate of the phenolic compounds during the PO reaction, mass spectrometry experiments were performed using 2,4-dichlorophenol as the aromatic modifier. To this purpose, the PO reaction was run in the semibatch reactor (located in the spectrophotometer). After running for 2 h, a 2.0 mL sample of the reaction mixture was transferred to a membrane inlet mass spectrometer (MIMS) (*30*). The probes were placed in the 2.0 mL stirred sample cell mounted on a Balzers QMS 420 quadrupole mass spectrometer. The only separation between the sample cell (whose temperature was maintained at 70 °C) and the high-vacuum part of the mass spectrometer consists of a silicone membrane. This hydrophobic membrane is permeable to volatile organic compounds present in the sample mixture. A steady flow of volatile compounds through the silicone membrane was attained approximately 3 min after transferring the sample to the mass spectrometer. After this initial period the mass spectrum of the sample was recorded. Details of the membrane inlet mass spectrometer are reported in ref 31.

RESULTS

We investigated the dynamics of the PO reaction in the absence and in the presence of several phenolic compounds. We determined the ability of the phenols listed in Chart 1

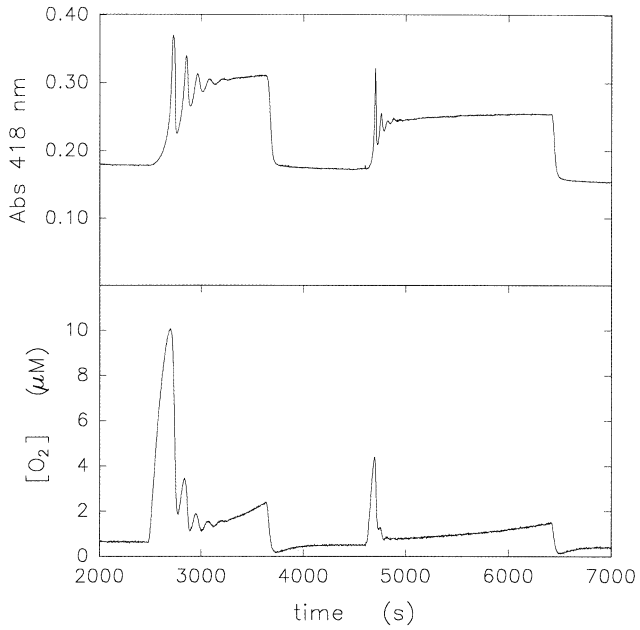


FIGURE 1: Dynamic behavior of the PO reaction in absence of added phenols at pH 5.1, as monitored by the absorbance at 418 nm (absorption maximum of Per^{6+}, top) and the $[O_2]$ in the solution (bottom). The experiment was started with 100% N_2 in the feeding gas stream. At t ≈ 2490 s the composition of the gas stream is switched from 0% to 1.05% (v/v) O_2. This induces damped oscillations in the reaction which approaches a high-O_2 stationary state. At $t \approx 3640$ s the gas stream is switched back to pure N_2 and the reaction goes back to the low-O_2 steady state. At $t \approx 4630$ s the gas stream is again switched from 0% to 1.05% (v/v) O_2, and at $t \approx 6420$ s it is returned to pure N_2.

to induce oscillatory and complex dynamics in the PO reaction at two different pH values, namely, pH 5.1 and 6.3.

First of all, the dynamics of the PO system were studied in the absence of any phenolic cofactor. In this case, the PO reaction shows damped oscillations at pH 5.1 (Figure 1): When the composition of the gas stream is switched from pure N_2 to a O_2/N_2 mixture, the reaction starts to oscillate. With time, however, the oscillations decay in amplitude and increase in frequency, thus asymptotically approaching a stationary state. By contrast, when performed at pH 6.3 and in the absence of phenolic compounds, the PO reaction does not oscillate at all. Instead, we observe a slow, nonoscillatory oxidation of NADH by O_2.

In the presence of phenolic compounds, the PO reaction can exhibit different types of dynamics:

Complete Inhibition of the Reaction (*Labeled "i" in Tables 1−3).* In this case, the oxidation of NADH by oxygen stops upon addition of the phenolic compound. The inhibitory effect can be ascertained by subsequent addition of a phenol known to promote oscillatory dynamics (such as **23** or **5**). If such addition fails to induce reaction 5, the original phenolic compound is said to be inhibitory. An example is shown in Figure 2, where 4-hydroxy-3-methoxycinnamyl alcohol, **14**, is added to the solution, and where subsequent additions of **23** do not reinduce oscillations.

Slow, Nonoscillatory Oxidation of NADH by Oxygen (Labeled "−" in Tables 1−3). Phenolic compounds may stimulate the PO reaction, but cause it to proceed in a nonoscillatory fashion. An example is the time evolution of the PO reaction at pH 5.1 when **15** is added (Figure 3). It is worth noting that, as stated above, at pH 5.1 and in the absence of phenolic cofactors, the PO reaction displays damped oscillations. The presence of the aromatic cofactor causes these oscillations to cease.

Table 1: Effect of Various Naturally Occurring Phenolic Compounds on Oscillations and Complex Dynamics in the PO Reaction at pH 6.3

phenol	concentration (μM)	effect[a]
4-Hydroxyphenyl Derivatives		
(**1**) phenol	60−140	0
(**2**) *p*-cresol	100−140	0
(**3**) 4-methoxyphenol	100	i
(**4**) 4-hydroxybenzaldehyde	10−40	−
(**5**) 4-hydroxybenzoic acid	10−750	++
(**6**) 4-hydroxyphenylacetic acid	10−170	−
(**7**) 4-hydroxycinnamic acid	10−1500	−
(**8**) 3-(4-hydroxyphenyl)propionic acid	10−40	0
(**9**) 7-hydroxycoumarin (umbelifferone)	20−200	++
(**10**) L-tyrosine	5−20	0
4-Hydroxy-3-methoxyphenyl Derivatives		
(**11**) 2-methoxyphenol (guaiacol)	4−60	i
(**12**) 4-hydroxy-3-methoxybenzaldehyde (vanillin)	10−50	++
(**13**) 4-hydroxy-3-methoxybenzoic acid (vanillic acid)	4−100	−
(**14**) 4-hydroxy-3-methoxycinammyl alcohol (coniferyl alcohol)	20−100	i
(**15**) 4-hydroxy-3-methoxycinnamic acid (ferulic acid)	5−50	i
(**16**) 7-hydroxy-6-methoxycoumarin (scopoletin)	5−50	0
(**17**) 3-methoxy-D,L-tyrosine	10−40	−
2-Hydroxyphenyl Derivatives		
(**18**) 2-hydroxybenzoic acid (salicylic acid)	100−300	0
(**19**) 2-hydroxycinnamic acid	5−20	0
Chlorinated Phenols		
(**20**) 2-chlorophenol	20−175	+
(**21**) 3-chlorophenol	10−35	+
(**22**) 4-chlorophenol	10−30	++
(**23**) 2,4-dichlorophenol	10−40	++

[a] "++", complex dynamics; "+", sustained oscillations; "0", damped oscillations; "−", no oscillations; i, inhibition.

Sustained Periodic Oscillatory Dynamics (Labeled "+" in Tables 1−3). Certain phenols, like **1** and **2**, can induce sustained periodic oscillations (Figure 4). The oscillatory dynamics are long-lasting, i.e. they are observed for as long as the substrates NADH and O_2 are supplied to the reaction mixture. For practical purposes, we only include simple periodic oscillations in our definition of sustained oscillations. Sustained oscillations of higher periodicity are considered below.

Complex and Chaotic Dynamics (Labeled "++" in Tables 1−3). Phenolic compounds that induce oscillations of higher periodicity (like **12** in Figure 5) or even chaotic behavior (like **9** in Figure 6) are said to induce complex dynamics. As in the preceding class, the dynamics are long-lasting. To confirm the chaotic nature of a time series, the latter were examined using a series of methods of time series analysis (*32*). We subjected the data shown in Figure 6 to several time series analyses, e.g. attractor reconstruction, Poincaré sections, return maps, and next-amplitude maps. All of these analyses confirmed the chaotic nature of the time series obtained with **9**. In Figure 7, we show the next-amplitude plot obtained by plotting the maximum of the amplitude of an oscillation against the corresponding maximum of the

Table 2: Effect of Phenolic Compounds on Oscillations and Complex Dynamics in the PO Reaction at pH 5.1

phenol	concentration (μM)	effect[a]
4-Hydroxyphenyl Derivatives		
(**1**) phenol	60−140	+
(**2**) *p*-cresol	100−500	+
(**3**) 4-methoxyphenol	100−500	i
(**4**) 4-hydroxybenzaldehyde	10−75	0
(**5**) 4-hydroxybenzoic acid	10−750	++
(**6**) 4-hydroxyphenylacetic acid	10−60	0
(**7**) 4-hydroxycinnamic acid	6−70	0
(**8**) 3-(4-hydroxyphenyl)propionic acid	20−70	0
(**9**) 7-hydroxycoumarin (umbelifferone)	10−250	++
(**10**) L-tyrosine	5−70	0
4-Hydroxy-3-methoxyphenyl Derivatives		
(**11**) 2-methoxyphenol (guaiacol)	10−20	i
(**12**) 4-hydroxy-3-methoxybenzaldehyde (vanillin)	10−50	++
(**13**) 4-hydroxy-3-methoxybenzoic acid (vanillic acid)	10−200	0
(**14**) 4-hydroxy-3-methoxycinammyl alcohol (coniferyl alcohol)	20−80	i
(**15**) 4-hydroxy-3-methoxycinnamic acid (ferulic acid)	1−40	−
(**16**) 7-hydroxy-6-methoxycoumarin (scopoletin)	5−20	0
(**17**) 3-methoxy-D,L-tyrosine	5−60	0
2-Hydroxyphenyl Derivatives		
(**18**) 2-hydroxybenzoic acid (salicylic acid)	100−300	0
(**19**) 2-hydroxycinnamic acid	5−20	0
Chlorinated Phenols		
(**20**) 2-chlorophenol	20−150	+
(**21**) 3-chlorophenol	10−30	+
(**22**) 4-chlorophenol	10−100	++
(**23**) 2,4-dichlorophenol	10−50	++

[a] See Table 1 footnote.

Table 3: Comparison of Half-Wave Potentials $E_{1/2}$ for the Oxidation of Phenols to Phenoxyl Radicals (Reaction 11) and the Dynamics of the Peroxidase−Oxidase Reaction at pH 5.1 and 6.3 (Symbols i, −, 0, +, and ++ are as defined in Table 1)

compound	dynamics at pH 5.1	dynamics at pH 6.3	$E_{1/2}$ (mV vs NHE)	ref
Phenols				
(**3**) 4-methoxyphenol	i	i	648[b,c]	34
(**11**) 2-methoxyphenol	i	i	698[b,c]	34
(**7**) 4-hydroxycinnamic acid	0	−	780[d]	38
(**13**) vanillic acid	0	−	781[e]	
(**2**) *p*-cresol	+	0	785[b,c]	34
(**20**) 2-chlorophenol	+	+	867[b,c]	34
(**1**) phenol	+	0	875[b,c]	34
(**22**) 4-chlorophenol	++	++	895[b,c]	34
(**23**) 2,4-dichlorophenol	++	++	902[b,f]	35
(**12**) vanillin	++	++	930[b,g]	36
(**5**) 4-hydroxybenzoic acid	++	++	958[b,c]	34
(**21**) 3-chlorophenol	+	+	977[b,c]	34
(**18**) 2-hydroxybenzoic acid	0	0	1087[b,c]	34
4-nitrophenol	i[a]	nd[a]	1166[b,c]	34
Aromatic Amines				
aniline	++[a]	+[a]	869[b,c]	34
4-aminobenzoic acid	+[a]	+[a]	956[b,c]	34

[a] Data from ref 10; nd, not determined. [b] Calculated from data obtained vs SCE, using E_o' (vs NH) = E_o' (vs SCE) + 242 mV. [c] Conditions: pH 5.6, 24 °C 50:50 H_2O/2-propanol. [d] Conditions: pH 8.5 at 22 ± 2 °C, aqueous solution. [e] Calculated from data and after formula given in ref 34. [f] Conditions: in H_2O with 0.14 M LiCl as supporting electrolyte, 25 °C. [g] From Figure 9 of ref 36. Conditions: pH 5.8, 50:50 H_2O/2-propanol.

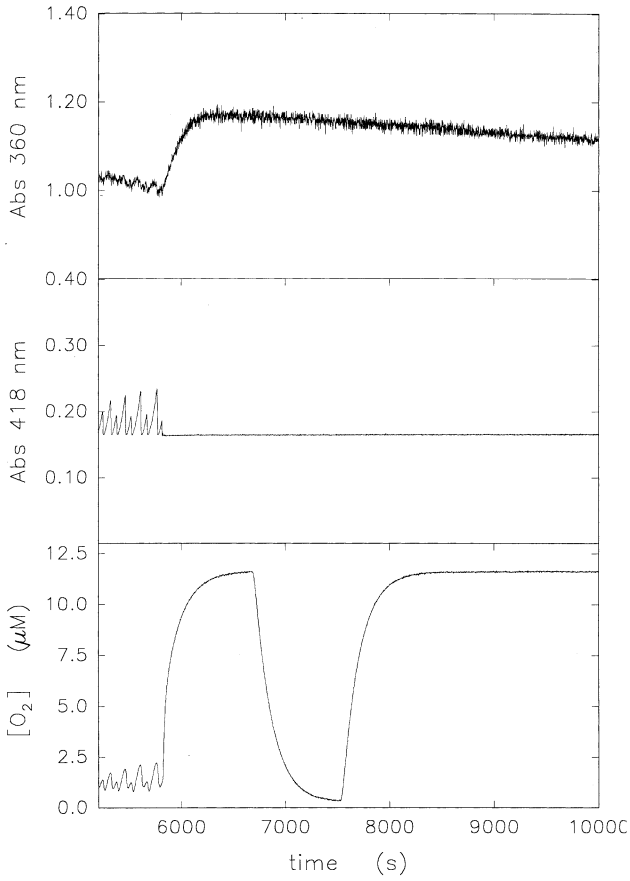


FIGURE 2: Inhibitory effect caused by coniferyl alcohol (**14**), as followed by the absorption at 360 nm (corresponding to NADH, top), 418 nm (Per^{6+}, middle), and the by the oxygen electrode (bottom). Sustained oscillations of period 2 are observed at $t \leq$ 5800 s. The period 2 oscillations were induced by the presence of 25 μM 2,4-dichlorophenol (**23**) in the reaction mixture. At $t = 5800$ s (**14**) was added to yield a 20 μM solution, and oscillatory dynamics as well as the PO reaction were inhibited. At $t = 6700$ s the gas stream is switched to 0% (v/v) O_2. At $t = 7550$ s the gas stream is reset to 1.05% (v/v) O_2, and additional **23** added (yielding a solution that is 30 μM in **23**). Changes in the concentrations of **23** and O_2 did not lead to a restart of the PO reaction.

preceding oscillation. The next-amplitude plot defines a one-humped map, which is characteristic for chaotic time series. Similar next-amplitude maps have been obtained with **23** (*9*), **5** (*10*), and **12** (*16*) present as cofactors in the PO reaction.

Damped Oscillations (Labeled "0" in Tables 1−3). Addition of certain phenolic compounds, like **7** and **8**, induce damped oscillations (Figure 8). Here, oscillatory dynamics arises as the composition of the gas stream is changed from pure N_2 to an O_2/N_2 mixture. The oscillations gradually dwindle in amplitude and increase in period as time progresses. Eventually the system settles on a stationary state. Damped oscillations thus represent a hybrid between a nonoscillatory reaction and sustained oscillations. The degree of damping varies considerably with the nature of the aromatic compound and with the experimental conditions. In the case of scopoletin (**16**) we observe damped oscillations which last considerably longer than the corresponding damped oscillations observed in the absence of any aromatic cofactor (compare Figures 1 and 8). However, certain other phenols (e.g. **10** and **17**) cause a damping which is as strong as, or even stronger than, that observed with the enzyme

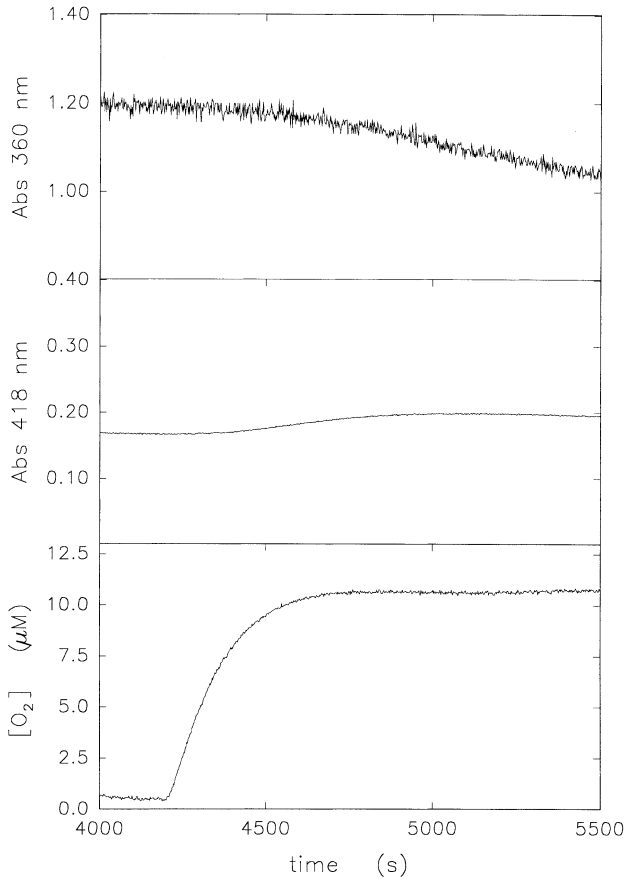


FIGURE 3: Nonoscillatory dynamics of the PO reaction. Ferulic acid (**15**) was added to the reaction mixture (pH 5.1) to yield a 5 μM solution of **15**. The dynamics were followed at 360 nm (corresponding to NADH, top), 418 nm (Per^{6+}, middle), and by the oxygen electrode (bottom). At $t = 4200$ the gas stream was switched from 0 to 1.05% (v/v) O_2. After switching to aerobic conditions, the absorbance at 360 nm (i.e. NADH) began to decrease monotonically, indicating a nonoscillatory progress of the PO reaction.

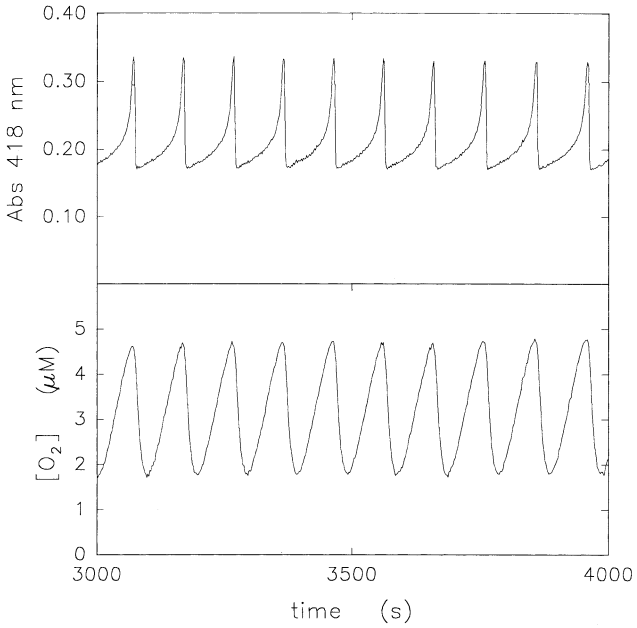


FIGURE 4: Sustained periodic oscillations obtained when umbelliferone (**9**) was added as to yield a 200 μM solution, at pH 5.1. The dynamics were followed at 418 nm (Per^{6+}, top) and the by the oxygen electrode (bottom). Sustained oscillations were observed for as long as the substrates NADH and O_2 were added to the reactor.

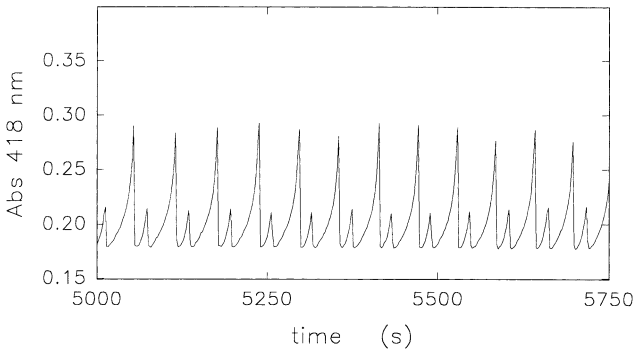


FIGURE 5: Sustained oscillations of period 2, i.e., a large-amplitude oscillation followed by a small-amplitude oscillation. Vanillin (**12**) was added to yield a 40 μM solution, at pH 5.1. The dynamics were followed at 418 nm (corresponding to Per^{6+}). These oscillations were observed for as long as the substrates NADH and O_2 were added to the reactor.

alone. Some phenolic compounds, like **8**, **10**, **16**, **18**, and **19**, induced damped oscillations at both pH values, i.e. also under conditions where the reaction does not oscillate at all in the absence of any cofactor (pH 6.3).

The influences of the phenolic compounds listed in Chart 1 on the dynamics of the PO reaction at pH 5.1 and 6.3 are listed in Tables 1 and 2. A comparison of the effects induced at the two pH values shows that the occurrence of nonlinear dynamic behavior is favored at pH 5.1 compared to at pH 6.3. While **1** and **2** support sustained oscillations at pH 5.1, they only induce damped oscillations at pH 6.3. Analogously, **4**, **6**, **7**, **13**, and **17**, which are able to induce damped oscillations at pH 5.1, only support nonoscillatory reaction at pH 6.3. In the presence of **15** the PO reaction proceeded slowly without oscillations at pH 5.1 whereas inhibition was observed at pH 6.3. During our experimental studies we did not encounter a single phenolic compound that was able to promote a type of dynamics at pH 6.3 which is more complex than that observed at pH 5.1.

An important question concerns the properties of the aromatic compounds that are responsible for determining which kind of nonlinear dynamics will be induced in the PO reaction. As reported previously (*10*), the aromatic compound has to carry either an aromatic hydroxy or an aromatic amino substituent in order to become a cofactor in the PO reaction. However, the mere presence of a phenolic hydroxyl group alone does not lead to complex or chaotic behavior, as seen from Tables 1 and 2. Instead, different phenolic compounds are associated with different degrees of dynamical complexity.

We examined the phenolic compounds of Chart 1 with respect to additional structural features which may be responsible for inducing certain types of dynamic behaviors. These compounds can be divided into four categories, according to their substitution pattern: 4-hydroxyphenyl derivatives, 2-hydroxyphenyl derivatives, 4-hydroxy-3-methoxyphenol derivatives, and chlorinated phenols. While compounds from the first three groups occur naturally as physiological substrates in plants or animals, this is not the case with chlorophenols. The classification according to the different substitution patterns at the aromatic ring, however, does not reveal any information about further structural

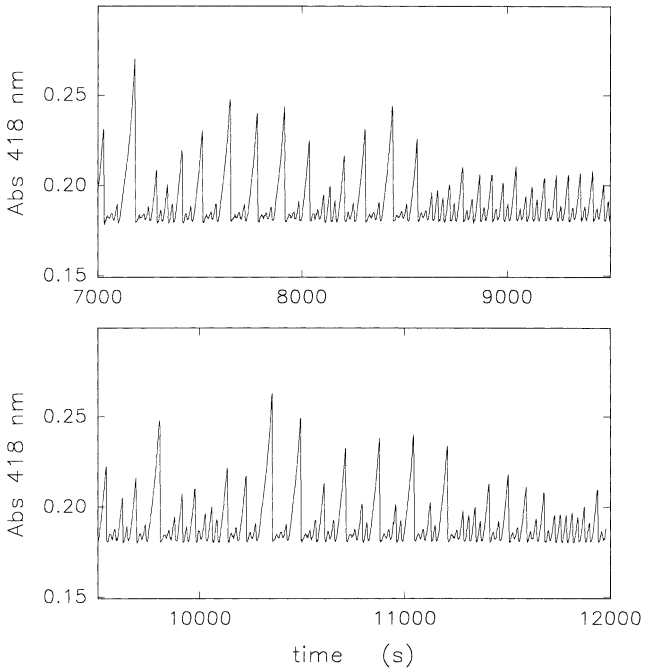


FIGURE 6: Chaotic oscillations. Umbelliferone (**9**) was added as to yield a 200 μM solution, at pH 6.3. The dynamics were followed at 418 nm (corresponding to Per^{6+}). Oscillations were observed as long as the substrates NADH and O_2 were added to the reactor.

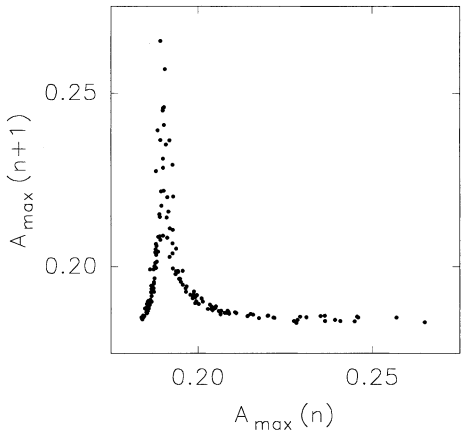


FIGURE 7: Next-amplitude plot constructed from the chaotic state shown in Figure 6, as monitored at 418 nm (corresponding to the Per^{6+}). Here we plotted the absorbance maximum of one oscillation, A_{max} $(n + 1)$ against the absorbance maximum of the preceding oscillation A_{max} (n).

prerequisites for inducing nonlinear dynamic behavior. On the contrary, different kinds of dynamics can be realized within a group of aromates with a common substitution pattern. Thus, we conclude that there is no correlation between the type of dynamics induced and the substitution pattern of the aromatic substrates.

We performed a few studies using 2-hydroxyphenyl derivatives in order to investigate the influence of ortho substitution. Here, only damped oscillations (for **18** and **19**) or complete inhibition of reaction 5 (for **11**) were observed. By contrast, the dynamics caused by the corresponding para-substituted analogues may be much more complex: while **18** displays only damped oscillations, its para-substituted analogue **4** is able to induce chaos. These differences in reactivity might be due to intramolecular interaction between the substituents in ortho position. To test whether meta-

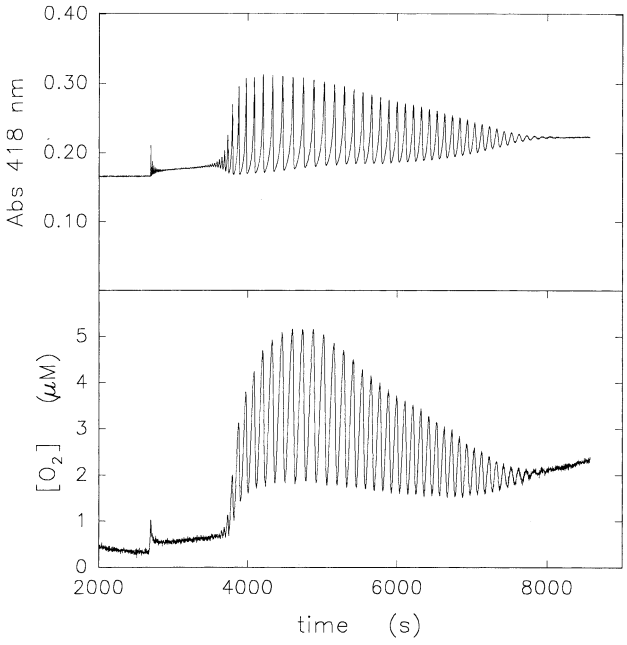


FIGURE 8: Damped oscillations in the presence of 10 μM scopoletin (**16**) at pH 5.1. The dynamics were followed at 418 nm (Per^{6+}, top), and the by the oxygen electrode (bottom). At $t = 2700$ s, the gas stream was switched from 0 to 1.05% (v/v) O_2 content. Damped oscillations arose after a lag time of ~1000 s. Although the oscillations eventually damped out, they lasted for over 3000 s (compare Figure 1).

substituted phenols are also able to induce oscillations, we used 3-chlorophenol. As seen from Tables 1 and 2, all three monochlorophenols (**20**−**22**) induce oscillatory dynamics in the PO reaction.

A comparison of the reactivity of 4-hydroxyphenyl derivatives **4** and **5** with those of the homologous 4-hydroxy-3-methoxyphenyl derivatives **12** and **13** yields further remarkable results. When considering the 4-hydroxyphenyl derivatives, the aldehyde **4** only induces damped oscillations while the carboxylic acid **5** supports complex and chaotic dynamics. In the case of the 3-methoxylated analogues, the opposite situation is encountered, i.e. the aldehyde **12** induces chaotic dynamics while the carboxylic acid **13** only amounts to damped oscillations. Thus, the nature and oxidation state of the substituent in the 1-position does not seem to be the crucial factor in determining the kind of dynamics.

From the observations described above, we conclude that there is no simple relationship between the types of dynamics induced and the structural features of the phenolic compounds. A similar conclusion has been drawn previously by Lee et al. in a study of the effects of phenolic compounds on the peroxidase catalyzed oxidation of indole-3-acetic acid (*33*).

As opposed to the structural and substitution patterns, a very good correlation is found when comparing the types of induced dynamic behavior with the electrochemical potentials for the oxidation of phenols to phenoxyl radicals. This correlation is presented in Table 3, where we also included data for some aromatic compounds studied in a previous article (*10*). By ordering the phenolic compounds according to values of their half-wave potentials ($E_{1/2}$) for the oxidation of phenols to phenoxyl radicals,

$$PhOH \rightarrow PhO^{\bullet} + H^{+} + e^{-} \qquad (11)$$

we obtain a clear and structured picture. Phenols with low oxidation potentials, such as the methoxyphenols **3** and **11** inhibit the PO reaction. As the value of $E_{1/2}$ increases to ~740 mV vs NHE, the phenolic compounds acquire the ability to induce damped oscillations. Phenols whose half-wave potentials fall in the range of approximately 780−880 mV vs NHE are able to induce sustained oscillations. At even higher reduction potentials, i.e. in the range of approximately 890−960 mV vs NHE, the phenolic compounds can give rise to complex and chaotic dynamic behavior. Upon further increase in the value of the half-wave potentials ($E_{1/2}$) the PO reaction successively passes domains where the phenols are able to promote first sustained oscillations, then damped oscillations, and finally a nonoscillatory reaction. Phenolic compounds with very high reduction potentials, like 4-nitrophenol, will again cause an inhibition of the PO reaction. Thus, we observe a layering of dynamic behaviors as a function of the reduction potential of the phenolic compounds with respect to reaction 11. In the core of this "onion"-like structure we find the phenols that promote complex and chaotic dynamics.

The values of the reduction potentials strongly depend on the reaction conditions at which they were determined. The $E_{1/2}$ values compiled in Table 3 were collected from the literature (*34*−*36*) where they were determined in the pH range of 5.6−5.8 in H_2O/2-propanol solutions. The values of $E_{1/2}$ are known to vary with pH. When comparing the electrochemical data (obtained at pH 5.6−5.8) with the dynamics induced in the present experiments by the different phenolic compounds, small discrepancies are unavoidable. This is the case when comparing the dynamics induced by 2-chlorophenol (**22**) with phenol (**1**) at pH 6.3. Here, **22** promotes sustained oscillations, while **1** only induces damped oscillations, despite the fact that the $E_{1/2}$ values are listed as 867 and 875 mV vs NHE, respectively. This discrepancy is possibly due to small differences in the pH dependency of the $E_{1/2}$ value of these two phenolic compounds.

In Table 3 we also list the half-wave potentials for two aromatic amines known to induce oscillatory dynamics in the PO reaction (*10*). Again, we observe a very good agreement between $E_{1/2}$ and their ability to induce dynamic behavior. However, small shifts toward lower values of $E_{1/2}$ are observed. This might be due to the fact that we are comparing the ability of radical formation, i.e. reaction 11, of phenols with that of aromatic amines.

To acquire more insight into the fate of the phenolic compounds in the PO reaction, we conducted a few membrane inlet mass spectrometry (MIMS) experiments. MIMS allows selective and quantitative determination of concentrations of neutral volatile compounds in the reaction solution. Experiments were started in the semibatch reactor at pH 6.3 using 25.0 μM **23** as the phenolic cofactor. We prepared the PO system in three different dynamic regimes by adjusting the levels of NADH in the reaction mixture: (i) at low NADH concentration levels where the PO reaction settles on a steady state which is characterized by high O_2 concentration; (ii) at somewhat higher NADH concentrations where the reaction settles on an oscillatory state of simple periodicity; and (iii) at even higher NADH levels where a steady state of low O_2 (and high NADH) concentration is attained. Two hours after the start of an experiment, a 2.0 mL probe of the reaction mixture was transferred to the membrane inlet mass spectrometer, and analyzed for the concentration of **23**. In all cases, the signals due to **23** showed intensities corresponding to 97.4−101.7% of that of a 25.0 μM standard solution of **23**. This suggests, that within the measurement error, the concentration of 2,4-dichlorophenol remains constant throughout the experiment, independently of the type of dynamics present in the PO reaction.

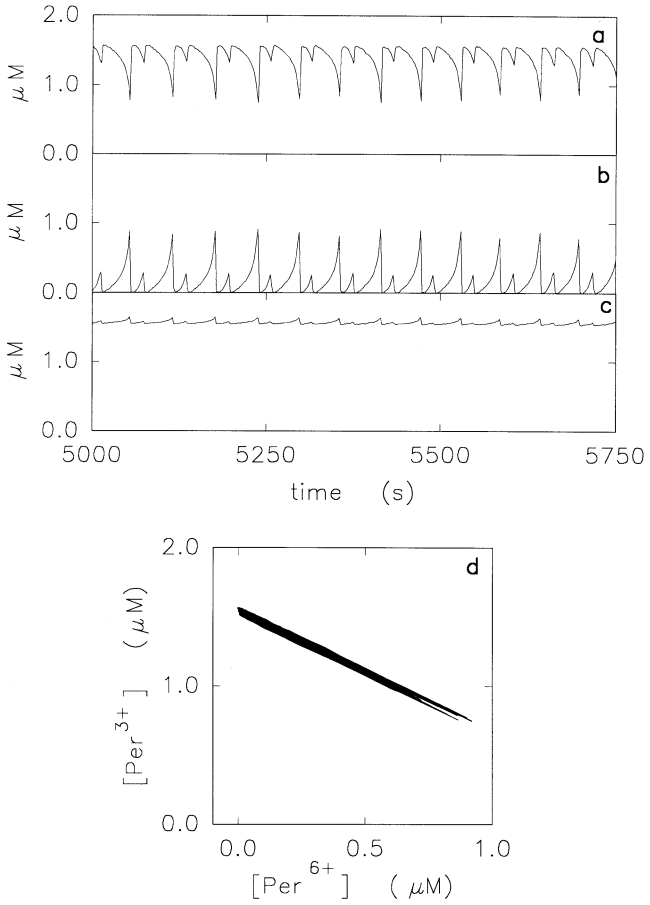


FIGURE 9: Time series and phase plot of the concentrations of Per^{3+} and Per^{6+}. Full spectra corresponding to the period 2 time series shown in Figure 5 were deconvoluted to yield the temporal evolution of the enzyme intermediates. Temporal changes in the concentrations of (a) Per^{3+} and (b) Per^{6+}. (c) Sum of the concentrations of Per^{3+} and Per^{6+}. Deviations from a straight line indicate contributions from the other three enzyme oxidation states. (d) Phase plot of $[Per^{3+}]$ vs $[Per^{6+}]$. The high degree of interconversion between Per^{3+} and Per^{6+} leads to an almost straight line.

Finally, the relative contributions of the five enzyme intermediates (Per^{2+} to Per^{6+}) present in the PO reaction were determined. To this purpose the data belonging to the time series presented in Figure 5 were analyzed. The data consist of a series of spectra from 350 to 600 nm (1 nm resolution), sampled every 2 s. We deconvoluted the temporal evolution of selected wavelengths as described in ref *18* to yield the temporal behavior of the concentrations of NADH, Per^{2+}, Per^{3+}, and Per^{6+}. The enzyme is present almost exclusively in only two oxidation states, namely Per^{3+} and Per^{6+}, as inferred by the sum $[Per^{3+}] + [Per^{6+}]$ (Figure 9). The time series for the concentrations of Per^{3+} and Per^{6+} indicates that these two enzyme intermediates are essentially interconverted. This is further supported by the almost linear phase plot of the concentration of Per^{3+} vs the concentration of Per^{6+} (Figure 9d). Furthermore, we did not find any other

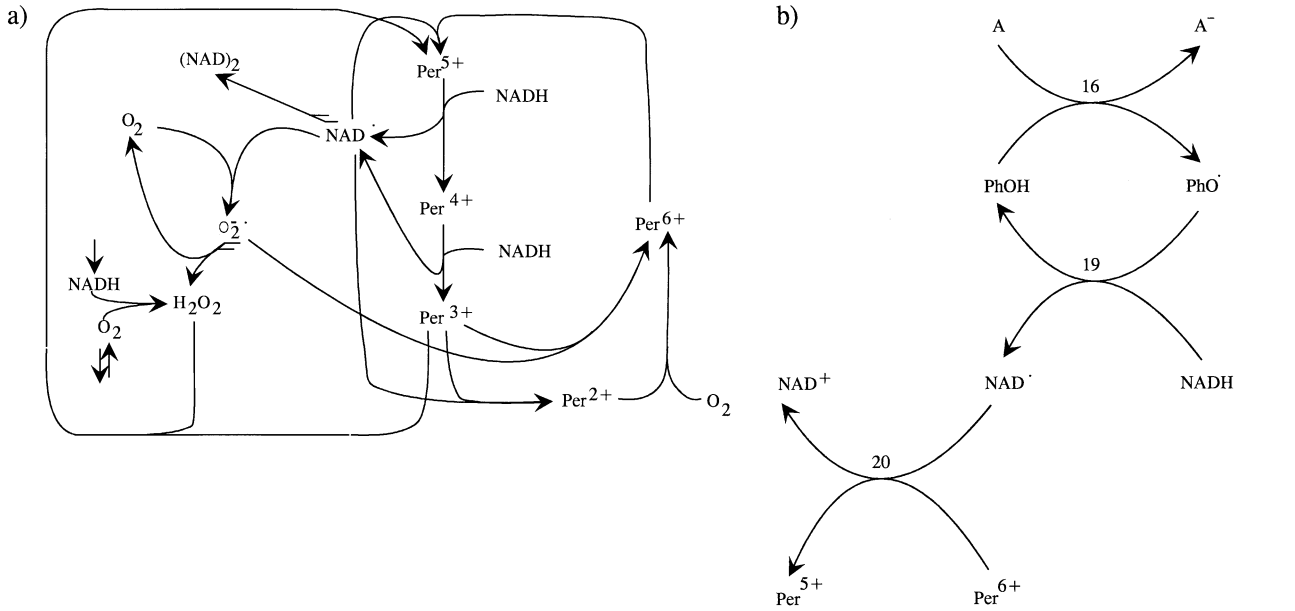


FIGURE 10: Reaction scheme for the peroxidase−oxidase reaction: (a) reaction mechanism as proposed by Bronnikova et al. (*26*). Like most other current reaction schemes it does not include the chemistry of phenolic compounds. For clarity the arrows symbolizing the flow of O_2 and NADH into and out of the semibatch reactor are indicated only once. (b) Proposed modification to the scheme in (a) to include the chemistry of phenolic cofactors. The modification is based on the present experiments. The numbers in the scheme refer to the reactions presented in the text. The component labeled A in reaction 16 may be any of the components Per^{5+}, Per^{4+}, or O_2^{-}.

spectrophotometric evidence for accumulation of Per^{5+} and Per^{4+} during the conversion of Per^{6+} to Per^{3+}.

DISCUSSION

In the present article we have demonstrated a correlation between the ability of phenolic compounds to induce different types of dynamic behavior in the PO reaction and the electrochemical potentials for the phenoxyl radical formation (reaction 11). By contrast, structural features of the investigated phenolic compounds were found not to have any significant influence on the dynamics. It is noteworthy that, recently, the reactivities of substituted indole-3-acetic acids toward Per^{5+} were also shown to be crucially dependent on the reduction potentials (*37*). Furthermore, the parameter governing the enhancement of peroxidase-induced chemiluminescence of luminol was recently found to be the reduction potentials rather than structural effects (*38*).

The remarkable correlation between the observed dynamic behavior and the reduction potentials of the phenols underlines the importance of single-electron-transfer reactions in the PO reaction. An interesting aspect is the observation of a layering of dynamic behaviors as a function of the half-wave potentials $E_{1/2}$ of the phenoxyl radical formation. Here, phenolic compounds which are able to induce the most complex types of dynamics (i.e. chaotic behavior) form the core of this "onion"-like structure. The more the values of $E_{1/2}$ diverge from those of the central region, the less complex are the dynamics that the aromatic compounds can induce.

A series of detailed chemical reaction schemes (*6*, *24*−*27*) has been developed to account for the dynamics of the peroxidase−oxidase reaction. Figure 10a shows a recently proposed reaction scheme (*26*), which involves the five enzyme intermediates Per^{2+}−Per^{6+}, NADH, O_2, $NAD^{\bullet}$, O_2^{-}, and H_2O_2. Computer simulations of this reaction scheme, using experimentally determined rate constants, have shown very good similarity to the experimental observations of simple and complex periodic oscillations as well as chaotic behavior (*9*, *11*, *20*, *26*). However, it is to be noted that this scheme, like the majority of other mechanistic models of the peroxidase−oxidase reaction, does not involve any phenolic compounds.

In the following we should address the question of which intermediates in the PO reaction may act as oxidizing agents for the phenoxyl radical formation (reaction 11). We focus on the reduction potentials of the different components present in the PO system, to determine the possible reaction partners for the phenolic compounds. Once they are assessed, the mechanistic role of the phenolic compounds will be discussed. On the basis of the electrochemical considerations, we suggest that phenols act as electron mediators, a picture that is consistent with the results of the MIMS experiments.

Electrochemical data for phenolic compounds are abundant in the literature. However, these data have been determined under a wide range of different experimental conditions, making direct comparisons difficult. In Table 3, we therefore restricted ourselves to present data obtained under similar experimental conditions (hence, not all the compounds listed in Tables 1 and 2 can be listed in Table 3). The data present in Table 3 were measured in the interval pH 5.6 to 5.8, and recalculated with respect to NHE (where necessary). The exact determination of the reduction potentials at different conditions can be obtained by the equation

$$E = E_0 + \frac{RT}{ZF} \ln \frac{[\text{ox}]}{[\text{red}]} \qquad (12)$$

where R is the gas constant, F the Faraday constant, T the temperature, z the number of electrons transferred, and [ox] and [red] the oxidized and the reduced side of the reaction, respectively.

Table 4: One-Electron Reduction Potentials E_0' of Intermediates Present in the Enzymatic Reactions

reaction	E_0' (mV vs NHE)	pH	ref
$Per^{6+} + e^- \rightarrow Per^{5+}$	−[a]		
$Per^{5+} + e^- \rightarrow Per^{4+}$	898 ± 3[b]	7.0	40
	879[c]	7.0	41
	941[d]	6.53	42
	948[d]	6.34	42
	965[d]	6.06	42
$Per^{4+} + e^- \rightarrow Per^{3+}$	869 ± 2[b]	7.0	40
	903[c]	7.0	41
	959 ± 4[d]	6.53	42
	968[d]	6.34	42
	992 ± 2[d]	6.06	42
$Per^{3+} + e^- \rightarrow Per^{2+}$	−258[e]	7.20	39
	−220[e]	6.37	39
	−198[e]	5.93	39
$NAD^+ + e^- \rightarrow NAD^\bullet$	−922 ± 8[f]	7.0	52
$NAD^\bullet + H^+ + e^- \rightarrow NADH$	282[f]	7.0	52
$O_2 + e^- \rightarrow O_2^-$	−330[g]	7.2	54
	−330	7.0	3, 51
$O_2^- + 2\,H^+ + e^- \rightarrow H_2O_2$	940 ± 20[h]	7.0	50
	890	7.0	3, 51

[a] To our knowledge not determined. [b] 25 °C, spectroelectrochemical measurement vs $IrCl_6^{2-}/IrCl_6^{3-}$. [c] 15 °C, spectroelectrochemical measurement vs $IrCl_6^{2-}/IrCl_6^{3-}$. [d] 20 °C, spectroelectrochemical measurement vs $IrCl_6^{2-}/IrCl_6^{3-}$, peroxidase isoenzyme C. [e] 25 °C, reductive titration with $Na_2S_2O_4$ for isoenzymes C and B. [f] By pulse radiolysis and electron transfer between equilibria with 1,1′-butano-4,4′-dimethyl-2,2′-bipyridylium dibromide. [g] By pulse radiolysis. [h] Calculated value.

In the PO reaction, the enzyme is present in five oxidation states, forming four redox couples, separated by single-electron transfers. Spectroelectrochemical data (Table 4) are available in the literature for three of these redox couples, namely Per^{5+}/Per^{4+}, Per^{4+}/Per^{3+}, and Per^{3+}/Per^{2+}. To our knowledge, data for the redox couple Per^{6+}/Per^{5+} have not yet been reported.

Inspection of the data in Table 4 reveals negative values for the one-electron potential of the Per^{3+}/Per^{2+} redox couple (*39*). However, to act as the oxidizing agent for the phenoxyl radical formation (reaction 11), a redox couple must possess higher reduction potentials than those of the phenoxyl radical/phenol couple ($E_{1/2} \sim 640-960$ mV for the phenols used in the present study, see Table 3). Thus, the reduction of Per^{3+} to Per^{2+} has a far too negative reduction potential in order to act as the oxidizing partner in reaction 11.

The reported one-electron reduction potentials E_0' for the Per^{5+}/Per^{4+} and Per^{4+}/Per^{3+} couples are quite similar (*40*−*42*). The pH dependence of these one-electron reduction potentials has been studied by Hayashi and Yamazaki, who reported $E_0'(Per^{5+}/Per^{4+}) = 948$ mV and $E_0'(Per^{4+}/Per^{3+}) = 968$ mV at pH 6.34 (*42*). The enzyme couples Per^{5+}/Per^{4+} and Per^{4+}/Per^{3+} are thus able to oxidize phenolic compounds with reduction potentials $E_{1/2} < 950$ mV to their respective phenoxyl radicals. It is noteworthy, that phenols with half-wave potentials in the region 770 mV $\leq E_{1/2} \leq$ 960 mV induce complex and chaotic dynamics (Table 3). However, as the values of the half-wave potentials $E_{1/2}$ of the phenols approach 960 mV, it becomes more difficult for the enzyme couples Per^{5+}/Per^{4+} and Per^{4+}/Per^{3+} to oxidize the phenols. These observations suggest that the ease by which the phenols are oxidized to phenoxyl radicals could become one of the parameters governing the dynamics of the PO reaction.

The reactions of phenols with Per^{5+} and Per^{4+}

$$PhOH + Per^{5+} \rightarrow PhO^\bullet + Per^{4+} \quad (13)$$

$$PhOH + Per^{4+} \rightarrow PhO^\bullet + Per^{3+} + H_2O \quad (14)$$

form the reductive part of the well-known enzymatic cycle of the peroxidase pathway (reaction 1). Since all phenolic compounds investigated in the present study are substrates for the peroxidase reaction, they also participate in the PO reaction, i.e. reactions 13 and 14 also take place during the PO reaction. There exists a correlation between $E_{1/2}$ of the phenoxyl radicals and the rate constants k_{13} and k_{14}: the faster the reaction proceeds, the easier the phenol is oxidized (*43*, *44*). This reflects the fact that reactions 13 and 14 describe direct electron-transfer reactions between enzyme intermediates and phenols (*43*, *44*).

However, the analogous reduction of Per^{6+} by phenolic compounds

$$PhOH + Per^{6+} \rightarrow PhO^\bullet + Per^{5+} \quad (15)$$

has so far only been observed in the presence of an electron donor (YH_2) of the PO reaction, i.e. NADH, indole-3-acetic acid or dihydroxyfumaric acid. In the absence of such electron donors, the phenolic compounds are unable to convert Per^{6+} to Per^{3+}. Dordick et al. demonstrated that **1** did not reduce Per^{6+} after traces of dihydroxyfumaric acid were removed by filtration through a column (*45*). Similarly, Per^{6+} from lignin peroxidase was reported to be stable toward a series of phenolic compounds in the absence of suitable reducing equivalents (*2*) (such as NADH, indole-3-acetic acid, and dihydroxyfumaric acid).

Per^{6+} constitutes an important component in the PO reaction. The reactivity of the phenolic compounds toward Per^{6+} holds a key for estimating their role in the PO reaction. On one hand, the phenols stimulate (or inhibit) the reduction of Per^{6+} in the presence of a known electron donor of the PO reaction (*45*−*49*). On the other hand, phenols alone are unable to reduce Per^{6+} (reaction 15) in a direct reaction. These findings indicate that the phenolic compounds play a role as cofactors or as mediators in the reduction of Per^{6+}.

To act as cofactors or mediators, the phenols must be able to reduce a substance, A, present in the PO system, thus generating the phenoxyl radicals:

$$PhOH + A \rightarrow PhO^\bullet + A^- + H^+ \quad (16)$$

Similar to reactions 13 and 14, the rate of reaction 16 is expected to correlate with the half-wave potentials of the redox couple $PhO^\bullet/PhOH$. A^- cannot be the reducing agent for Per^{6+}, as the A/A^- couple must have a more positive reduction potential than the $PhO^\bullet/PhOH$ couple. The phenoxyl radicals, in turn, may participate in a further one-electron transfer reaction and oxidize another molecule, say BH,

$$PhO^\bullet + BH \rightarrow PhOH + B^\bullet \quad (17)$$

thus recycling the phenolic compounds. The molecule $B^\bullet$ in turn is expected to reduce Per^{6+} to Per^{5+}.

In the next step, we attempt to identify the components of the PO reaction system that are able to fulfill the

requirements set for reactions 16 and 17 to occur. The oxidation of the phenols to the phenoxyl radicals, as described in reaction 16, is achieved by both, Per^{5+} and Per^{4+}, as evidenced by reactions 13 and 14. In addition, the superoxide radical, O_2^- is another possible oxidizing agent for the phenols

$$PhOH + O_2^- + H^+ \rightarrow PhO^\bullet + H_2O_2 \qquad (18)$$

since the reduction potential of the O_2^-/H_2O_2 couple is sufficiently high (*3*, *50*, *51*).

As to the nature of the species BH in reaction 17, one might consider the difference in reactivity when Per^{6+} and phenols are allowed to react (reaction 15) in the presence or absence of NADH. The inability of the phenolic compound alone to reduce Per^{6+} suggests a contribution of NADH in reaction 15. Therefore, we assume that NADH is the species BH of reaction 17, such that

$$PhO^\bullet + NADH \rightarrow PhOH + NAD^\bullet \qquad (19)$$

The $NAD^\bullet$ radical formed in reaction 19 is expected to be sufficiently reactive to reduce Per^{6+} to Per^{5+}. The reduction potential for the $NAD^+/NAD^\bullet$ couple has been determined as $E_0' = -922$ mV at pH 7.0 (*52*) (Table 4). Thus, the oxidation of $NAD^\bullet$ to NAD^+ is associated with a reduction potential which also should support the reaction.

$$NAD^\bullet + Per^{6+} \rightarrow NAD^+ + Per^{5+} \qquad (20)$$

It is worth noting that $NAD^\bullet$ radicals have been proposed as the reducing agents for Per^{6+} (*29*). However, this postulated reaction has not been verified experimentally yet.

Thus, the assumption that the phenolic compounds act as electron mediators leads to the following picture (Figure 10b): the phenolic compounds can be oxidized to the respective phenoxyl radicals by three reaction partners, namely, by Per^{5+}, Per^{4+}, and O_2^-, (reactions 13, 14, and 18). The phenols are regenerated by reaction 19 which forms $NAD^\bullet$ radicals which in turn are able to reduce Per^{6+} to Per^{5+} (reaction 20).

A further important question concerns the fate of the phenols and phenoxyl radicals in the PO reaction. According to our discussion, the role of the phenolic compounds in the PO reactions is to transfer electrons to Per^{5+}, Per^{4+}, and O_2^- from NADH. Effective phenolic electron mediators are expected not to be consumed during the PO reaction. This is indeed what we observed in the MIMS experiments, when 2,4-dichlorophenol (**23**) was used as cofactor. Furthermore, peaks at *m*/*z* ratios corresponding to dimer products could not be detected. Thus, our MIMS experiments rule out any depletion of the phenolic compound during the PO reaction. The phenolic cofactor must therefore always be present either as the phenol or as the phenoxyl radical. The latter is probably reconverted by excess NADH to the original phenolic compound in the time frame of the MIMS experiment (~3 min). We therefore exclude the formation of aromatic dimers in reaction 5. Our results and conclusions are further supported by observations that NADH inhibits peroxidase-catalyzed oxidation of scopoletin (**16**) (*47*, *48*).

The function of the phenolic compounds in the PO reaction (reaction 5) differs considerably from the role of the phenolic compounds in the peroxidase reaction (reaction 1). While phenolic compounds act as electron mediators of the PO reaction, they are typically oxidized to radicals in the peroxidase reaction. These radicals react forming coupling products and polymeric compounds; in the case of **14** the first steps of the lignin formation from the monomers can be observed (*53*). However, dimerization and condensation reactions lead to a depletion of the phenolic compound.

To summarize, we have shown that the type of dynamic behaviors induced in the PO reaction by different phenolic compounds correlate with their reduction potential for the phenoxyl radical formation. Considerations on the basis of the reduction potentials suggest that the phenols play a role as electron mediators between the electron acceptors Per^{5+}, Per^{4+}, and O_2^- and the donor NADH. The phenolic compounds were shown not to be consumed during the PO reaction, thus explaining their long-lasting effects on the dynamics. These results support the conjecture that a recycling of the phenols during the PO reaction was the most likely mechanism, as pointed out in a previous article (*10*). The present findings as summarized in Figure 10b have to be incorporated in any new detailed reaction mechanism, like that shown in Figure 10a, which attempts to model the effect of phenolic compounds in the PO reaction. Research along these lines is currently in progress.

ACKNOWLEDGMENT

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CA REGISTRY NUMBERS (PROVIDED BY THE AUTHORS)

Reduced β-nicotinamide adenine dinucleotide (NADH), disodium salt [606-68-8]; horseradish peroxidase [9003-99-0]; methylene blue [7220-79-3]; phenol [108-95-2]; 4-methylphenol [106-44-5]; 4-methoxyphenol [150-76-5]; 4-hydroxybenzaldehyde [123-08-0]; 4-hydroxybenzoic acid [99-96-7]; 4-hydroxyphenylacetic acid [156-38-7]; 4-hydroxycinnamic acid [501-98-4]; 3-(4-hydroxyphenyl)propionic acid [501-97-3]; umbelliferone [93-35-6]; L-tyrosine [60-18-4]; guaiacol [90-05-1]; vanillin [121-33-5]; vanillic acid [121-34-6]; coniferyl alcohol [458-35-5]; ferulic acid [537-98-4]; scopoletin [92-61-5]; 3-methoxy-D,L-tyrosine [4214-13-5]; salicylic acid [69-72-7]; 2-hydroxycinnamic acid [614-60-8]; 2-chlorophenol [95-57-8]; 3-chlorophenol [108-43-0]; 4-chlorophenol [106-48-9]; 2,4-dichlorophenol [120-83-2].

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