The Peroxidase–Oxidase Oscillator and Its Constituent Chemistries

Alexander Scheeline,* Dean L. Olson, Erik P. Williksen, and Gregg A. Horras

Department of Chemistry, University of Illinois, 600 S. Mathews St., Urbana, Illinois 61801

Margaret L. Klein and Raima Larter*

Department of Chemistry, Indiana University—Purdue University at Indianapolis, 402 N. Blackford St., Indianapolis, Indiana 46202

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Contents

I. Introduction

In recent years, there has been an explosion of interest and controversy in the oscillatory reaction of NADH with O_2 catalyzed by horseradish peroxidase or other peroxidases. Since Yokota and Yamazaki first reported oscillations in a mixture of horseradish peroxidase, NADH, and oxygen in 1965,[1] the literature has gradually accumulated on the chemistry and dynamics of this reaction. As late as 1978, there were only two research groups studying the oscillatory dynamics of this widely important reaction: Yamazaki's group in Tokyo, and Degn and his co-workers at Odense, Denmark. Now, an international community has investigated both the chemistry and nonlinear dynamics of this system. The peroxidase–oxidase (PO) oscillator remains the only single enzyme system to exhibit oscillations *in vitro* in homogeneous, stirred solution. Not only periodic behavior, but also a wide variety of nonlinear behaviors have been observed: bistability, birhythmicity, complex oscillations, quasi-periodicity, as well as period-adding and period-doubling to chaos. An example of the temporal behavior of $[O_2]$ in a sequence of experiments and simulations demonstrating period-doubling in this system is shown in Figure 1.

A number of approaches have been taken to understanding both the detailed chemistry and phase space topology of the oscillations. Proposals for models of both the pure oscillations and chaos have broadened in scope. The purpose of this paper is to review the biochemistry and chemistry of peroxidase, concentrating on those aspects relevant to periodic and chaotic oscillations. We emphasize critical evaluation of proposed mechanisms and putative rate constants, identifying and correcting some inconsistencies in the literature. We also identify outstanding problems in the field (some of which our research groups are studying). Dynamical systems theory and chaos are not reviewed; these may be found elsewhere.[2−6]

A. General Background

Horseradish peroxidase, HRP, from the roots of the horseradish plant (genus *Armoracia*), is a cell wall-bound enzyme. It consists of a ferric heme group, 308 amino acid residues, two Ca^{2+}, and eight neutral carbohydrate side chains.[7] The amino acid sequence has been determined by Welinder.[7] The total molecular weight is between 40 000 and 44 000.[7,8] The X-ray crystal structure has been determined but at low resolution[9] and, more recently, at 2 Å resolution, both by X-ray diffraction and NMR.[10,11]

Up to 40 isozymes of HRP have been detected, but for many little is known about their relative activities.[7] Major isozymes have been divided into groups. Shannon, Kay, and Lew separated seven isozymes into two groups based on activity, pH, and other catalytic properties.[12] Three A isozymes were found to be catalytic in acidic solution; while isozymes B, C, D, and E were active at neutral pH.[13] Yamazaki, Tamura, and Nakajima included a third category for a basic peroxidase isolated as a ferric–cyanogenic complex.[7,14,15] The currently common classification is in three groups. Isozymes A1 through A3 are acidic enzymes; isozymes B1 through B3, C1, and C2 are neutral,[16] while D and E1 through E6 are basic.[17] In much of the early literature, the enzyme prepara-

* Authors to whom correspondence should be addressed.

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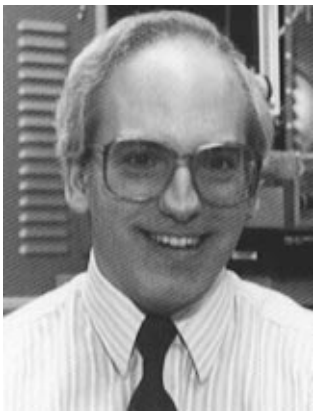
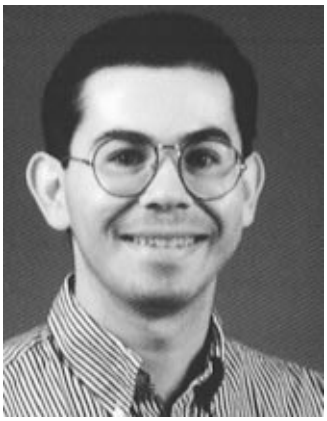


Alexander Scheeline is associate professor of chemistry at the University of Illinois at Urbana—Champaign. He received his B.S. in chemistry at Michigan State University in 1974 and his Ph.D., also in chemistry, at the University of Wisconsin—Madison in 1978. Following a year at the National Institute for Standards and Technology as a National Research Council Postdoctoral Fellow, he became assistant professor of chemistry at the University of Iowa before moving to Illinois in 1981. In 1990–91, he was a program officer in the Analytical and Surface Chemistry Program at the National Science Foundation.

Dean L. Olson received his B.S. in chemistry from the University of Minnesota in 1979, his M.S. in environmental chemistry from the University of North Carolina in 1983, and his Ph.D. in chemistry from the University of Illinois at Urbana—Champaign in 1994. He is currently a postdoctoral fellow at Illinois, focusing on development of NMR for detection and identification of picomolar amounts of neuropeptides separated chromatographically or electrophoretically.



Erik P. Williksen is a senior at the University of Illinois at Urbana—Champaign. He was a Ronald E. McNair summer research fellow when critically evaluating rate constants for inclusion in this paper.

Gregory A. Horras is a graduate student in the Medical Scholars program at the University of Illinois at Urbana—Champaign. He received his B.S. in chemistry at the University of Northern Iowa in 1991. Following additional training at Harvard Medical School and the University of Iowa, he has pursued a Ph.D. in chemistry at Illinois and recently commenced training for an M.D.

Margaret L. Klein received her B.S. in biochemistry from Purdue University, West Lafayette, in 1978. For a number of years, she worked at Great Lakes Chemical and in the Toxicology Lab at Wishard Hospital in Indianapolis. In 1992, she received an M.S. in chemistry from IUPUI. This review is based in part on her thesis.

Raima Larter has been a member of the faculty at IUPUI since 1981 and has had an active research career in the area of nonlinear dynamics, with special emphasis on biological aspects of oscillatory and chaotic behavior. In recent years, her area of interest has expanded beyond the study of enzyme kinetics to include, in part, bringing to bear the tools of nonlinear dynamics to the study of dynamical diseases such as epilepsy.

tion was assumed to consist of just one isozyme, or it was assumed that the activities of the major isozymes are nearly the same and that the lesser forms are noninterfering. This may account for some of the conflicting findings among some studies. Some later investigations use commercial preparations of A or C isozymes.[7] One study of HRP-C and HRP-A_2 found differences in reactivity between the two.[13] The use of site-directed mutagenesis to vary enzyme

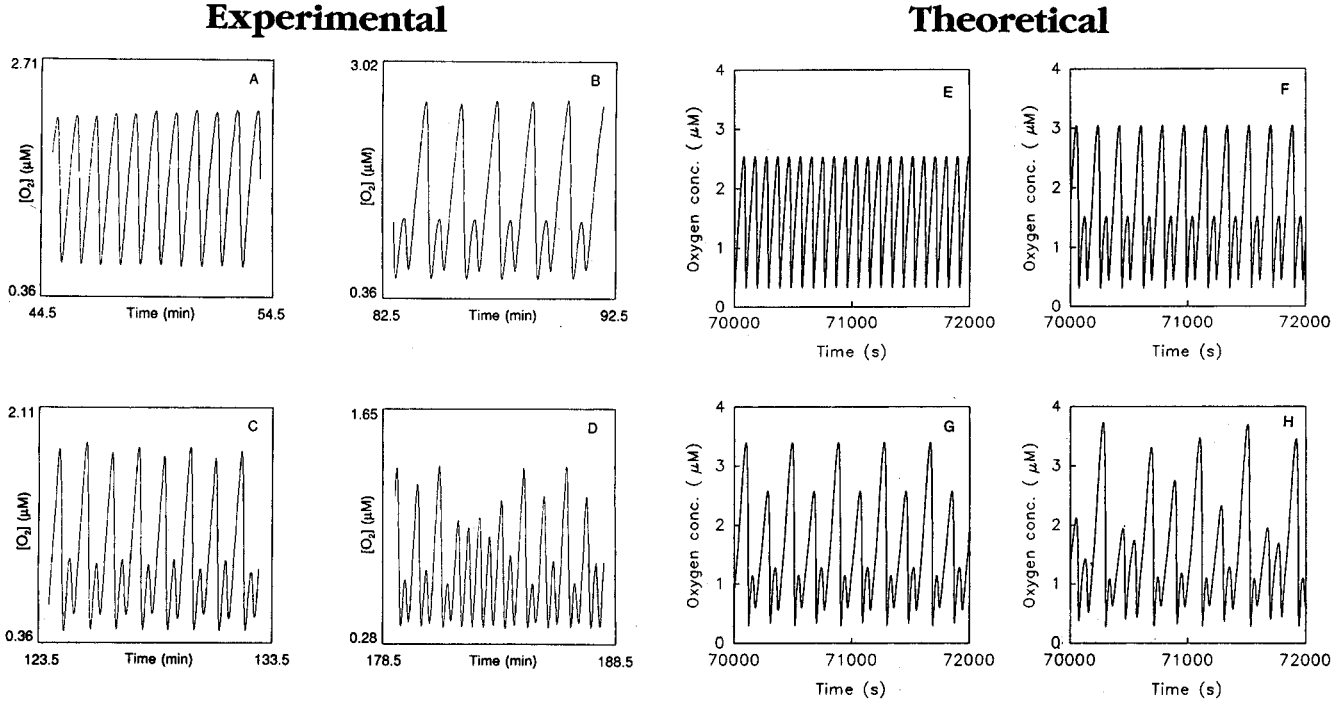


Figure 1. Example of dynamical behavior of the PO oscillator: (i) Experimental, periodic (a), period 2 (b), period 4 (c), and chaos (d). From ref 109. (ii) Theoretical, from ref 84 e−h analogous to a−d.

properties is providing increasing means for studying mechanistic differences among both natural and artificial isozymes.[10] While the glycan side chains can be important in differentiating isozymes, progress in understanding their structure and effects is less advanced than understanding of amino acid residue effects.

Peroxidase is usually known for its role as a hydrogen peroxide scavenger.[18] It also has been found to take part in two processes in the lignification of cell walls in higher plants. First, under aerobic conditions, peroxidase produces H_2O_2 by oxidation of NADH. Secondly, it initializes the polymerization of 4-hydroxylcinnamyl alcohols to form lignin.[19] Lignin is deposited within crevices in laminated layers of cellulose to give the cells rigidity and resistance to decay.[20,21] The initial step, the oxidation of NADH by peroxidase, has been extensively studied and modeled, primarily using HRP. Other plant studies have focused on the peanut, barley, and tobacco plants. Mechanisms similar to that in horseradish were found in these plants, as well as in studies of some trees.[22−24] Details not only of lignification but also of other roles of peroxidases in plant biosynthesis have been thoroughly reviewed.[25]

The free-radical chemistry of peroxidase is important in mammalian systems as well.[26] Myeloperoxidase has been implicated in the generation of hypochlorite (by reaction with superoxide radicals, in turn generated by NADPH oxidase) which helps destroy bacterial walls during antisepsis,[27] and the role of lactoperoxidase in milk is believed to be similar. Peroxidase in the head of sperm is thought to catalyze pellicle formation on the outside of eggs, sealing each egg against further fertilization immediately following sperm penetration. In all of these cases, explosive growth in free radical concentration is essential for carrying out a biological function. Such dynamics were well studied for two decades.[28] Radical dynamics are important in formation of arterial plaques,[29] so that oscillatory dynamics may provide a means of regulating growth of these undesirable structures (although clearly much more than just radical-initiated polymerization is involved). What is currently unclear is the biological importance, if any, of slow modulation of radical dynamics typical in an oscillatory reaction. Are such oscillations autonomous, or are they driven by and synchronized with other biological clocks? Entrainment of coupled mechanical oscillators to the same frequency, followed by phase locking, is well known.[30] Phase-locking in glycolysis has been observed when the concentration of glucose is sinusoidally varied.[31] Phase-locking between the peroxidase system and any *in vivo* chemical oscillation has not been demonstrated (nor, so far as we can determine, sought).

Peroxidase is frequently used for chemical, biological, and medical diagnostics. Neural axons provide conduits for peroxidase, so that observing the consumption of some reactant for which peroxidase is a substrate, or monitoring product formation from such reactions, provides a means for tracing axons and, thus, biological neural network connectivity.[32] Peroxidase is also a chemiluminescence catalyst, aiding detection of reducing agents in flowing streams.[33] Peroxidase's wide pH window of activity (from 2 to 10) makes it a robust system for diagnostic use. Surprisingly, only a relatively narrow window from pH 4.8 through 7.0 has been explored in characterizing its oscillatory dynamics. 90% of the pre-1995 literature uses a pH close to 5.1.

There are five redox forms of the peroxidase enzyme known to be catalytically active: the native form and four intermediates. In early work they were identified by color changes occurring during the reaction.[34−36] In 1960, using EPR spectrometry,

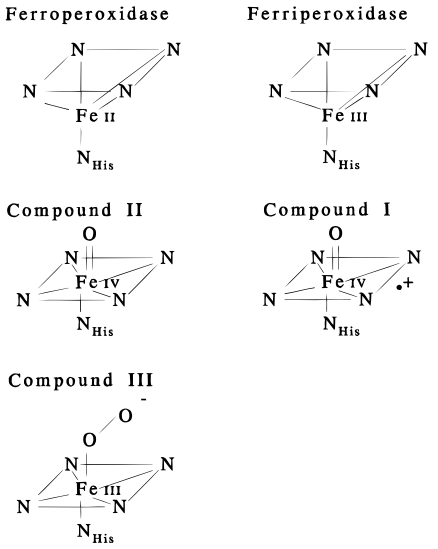


Figure 2. Heme iron and oxygen structures of main redox states of peroxidase. Adapted from Dawson (ref 41). Glycoprotein structure not shown, and details of heme ring also omitted to emphasize Fe/oxygen structure, importance of distal histidine in reactivity, and free-radical character of coI.

Yamazaki, Mason, and Piette[37] were the first to find direct evidence of free-radical intermediates produced in the peroxidase reaction. The structure of these intermediates are not as well known as for the native enzyme, but their reactive properties have been extensively studied.[2] In its native form the iron in the enzyme's heme group is in the +3 oxidation state; hence, this form of the enzyme will be referred to here as Per^{3+}. In much of the literature, it is known as ferriperoxidase. The removal of two electrons from Per^{3+} yields a free-radical form called Compound I (coI, cpI, or Per^{5+}). A single electron reduction of coI produces Compound II (coII, cpII, or Per^{4+}). Another important form is oxyperoxidase, Compound III (coIII, cpIII, or Per^{6+}), where a molecule of oxygen is bound to the enzyme as an axial ligand of iron. A ferrous form of peroxidase has also been identified (Per^{2+}).[12,19,38] Parallels to all these forms have been seen in human myeloperoxidase.[39,40] Skeletal structures in the vicinity of the heme site are shown in Figure 2.[41] The distal histidine is a distinctive feature of peroxidases, differentiating them from cytochromes, myoglobin, and hemoglobin. Oxygen binds *trans* to the histidine, with reduced forms having the iron below the plane of the heme while oxidized forms are more planar.

Other forms of peroxidase have been proposed as intermediates in a suicide pathway.[42] At pH 6.3, hydrogen peroxide can destroy peroxidase. CoI can react with hydrogen peroxide to decompose to a P-670 species. The intermediate leading to P-670 can also decompose (with loss of O) to ferriperoxidase or (with loss of superoxide radical) coII. While the rate of formation of the suicidal intermediate is not reported, roughly 450:1 of the intermediate returns to reactive enzymatic forms rather that decomposing to P-670. Since two H_2O_2 molecules are required for formation of the suicidal intermediate, decomposition can be minimized by working in a minimally oxidizing environment.

B. Free Radicals and Single Electron Oxidations

Free-radical chemistry, when not needed to initiate polymerization, is generally regarded as destructive in biological systems. Thus, peroxidase's role in scavenging radicals or in generating radicals which in turn can be scavengers, is critical to long-term survival and thriving of plants stressed by exogenous radicals.[43] In addition to enzymatic reactions, some reactions induced by ionizing radiation and photochemical processes, oxygen and hydrogen peroxide chemistry, and some aging processes can generate endogenous radicals.[44] Tabulations of rate constants for many systems involving $H^{\bullet}$, $OH^{\bullet}$, $O_2^{\bullet -}$, and other simple radicals are available.[45] Perhaps simpler to use is an on-line database with comprehensive radical reaction rate constants.[46]

Many radical intermediates are produced by single electron oxidation−reductions mediated by enzymes. Of particular interest here is the reduction of oxygen to superoxide or hydroperoxide radical (the distinction being a function of pH). Either a subsequent electron transfer or an initial two-electron reduction leads to formation of H_2O_2. These and other oxygen reactions play an important role in the PO reaction. Transition metals also participate in the electron transfers. The heme iron and ring in peroxidase accommodates electrons as the peroxidase reaction progresses, producing the various radical intermediates. A significant reason that the PO oscillator mechanism is still not completely understood is that a complete listing of important radicals, their reactions, and rate constants, is not available.

C. A Cellular Model

Experiments with intact cell wall extracts have revealed a general biological mechanism, referred to here as a cellular model, for the peroxidase-catalyzed production of the H_2O_2 necessary for lignification.[19,47] Other experiments have contributed details or alternative explanations for some of these reactions. These will be discussed later in the context of a biochemical model. One cellular model, proposed by Gross, Janse, and Elstner,[48] is illustrated in Figure 3. For this mechanism, catalytic amounts of Mn^{2+} and a monophenol are required.[19] The sum of the reactions of this model is nominally

$$2\text{malate} + 2O_2 \rightarrow 2\text{oxaloacetate} + 2H_2O_2$$

The system employs two enzymes: malate dehydrogenase (MDH) and peroxidase. MDH is irreversibly bound to the cell wall and is involved in a reaction that shuttles reduction equivalents into the cell wall from the cytoplasm, while oxaloacetate is removed, maintaining equilibrium. Within the cell wall MDH mediates the oxidation of malate, reducing NAD^+ to NADH, and thus supplying NADH for the production of hydrogen peroxide.[47] From this point, there are two pathways producing H_2O_2: one involving peroxidase and one peroxidase-free, possibly involving a NADH−MDH complex. Both are required in order to produce enough H_2O_2 for lignification to occur. Since several reactions depend upon the presence of H^+, an acidic medium is assumed, although the actual pH of the reaction environment is unknown.

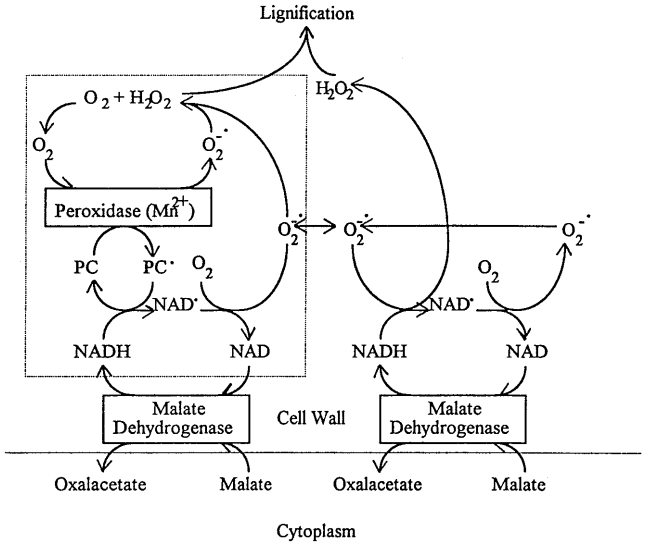


Figure 3. Reaction network diagram of the cellular model. Cell wall-bound malate dehydrogenase catalyzes the two-electron reduction of NAD^+ to NADH with malate as reductant (returning oxaloacetate to the Krebs cycle). NADH, various phenols, and oxygen, in reactions catalyzed by Mn^{2+} and peroxidase, produce H_2O_2 and phenolate radicals which initiate and sustain lignin formation. The dotted line encloses the portion of the cellular model represented in the biochemical model. The diagram is based on Gross *et al.* (ref 47).

NADH is oxidized in both paths. The pathways are coupled by the superoxide radical $O_2^{\bullet-}$ which is produced and consumed in both.[19]

In the peroxidase-dependent path, the enzyme oxidizes a monophenol (PC) by one electron/molecule. Catalytic amounts of Mn^{2+} are present, and oxygen is required. The monophenol radical then oxidizes NADH to regain an electron producing the free radical NAD•. NAD• is oxidized by oxygen, again by one electron, generating NAD^+. Both the oxidation of the monophenol and the oxidation of NAD• produce superoxide radical $O_2^{\bullet-}$. The $O_2^{\bullet-}$ radicals combine with available protons to produce H_2O_2 and O_2, with the oxygen feeding back into the process. The superoxide radical is also available to the nonenzymatic path and may oxidize NADH to NAD• while producing H_2O_2. In this path, the second single electron oxidation uses O_2, also generating $O_2^{\bullet-}$ and NAD^+. NAD^+ from both paths feed back into the MDH cycle. The H_2O_2 produced by each path is used in the peroxidase-mediated production of phenoxy radicals which polymerize to form lignin.

There have been several studies of the role of phenols in this reaction and the effectiveness of various monophenols including those used as substrates in lignification. It was proposed that coniferyl alcohol both stimulates the production of H_2O_2 and, hence, lignification and acts as a substrate in polymerization leading to lignification.[47,48] Both roles may not take place simultaneously. The concentration of coniferyl alcohol corresponding to optimal polymerization has been found to inhibit H_2O_2 production.[48] This perhaps suggests a switching/regulating mechanism. Nevertheless, how "optimized" the polymerization process needs to be for efficient biological activity is an open question.

D. Peroxidase Oscillator Phenomenology

When some peroxidases (particularly horseradish peroxidase, bovine lactoperoxidase, *Arthromyces* peroxidase, and *Coprinus* peroxidase[49]) are used as catalysts for the reaction of a reducing agent such as NADH, NADPH, dihydroxyfumarate, or indole-3-acetic acid[50] with oxygen, the species concentrations do not evolve to a steady state. Rather, oxygen concentration increases above the steady state value, a burst of radicals rapidly consumes the oxygen, and the cycle repeats. Chemiluminescence has been observed synchronously with oscillations for some of these substrates.[50,51] Oxygen is continuously introduced, and NADH is either continuously added or else maintained at nearly constant concentration by continually reducing NAD^+ (either introduced directly or produced by the PO reaction) back to NADH using glucose 6-phosphate and glucose-6-phosphate dehydrogenase.[52] Typically, methylene blue and a phenol are added as modifiers. This reaction has received much attention, since variation of either the concentration of the phenol, the concentration of the enzyme, or the mean concentration of NADH have shown interesting dynamical behavior such as periodic oscillations, chaotic oscillations, and both period doubling and period adding routes to chaos. The dynamical richness of the system has proven attractive for nonlinear dynamical studies, and in recent years the correspondence between models and experiments for peroxidase oscillations has approached that of the best understood inorganic oscillatory systems. Still unclear is whether these dynamical instabilities have *in vivo* equivalents, although there is tantalizing evidence that oscillations involving oxygen and NADH can occur following brief oxygen starvation in some bacterial species (although such oscillations may be due to perturbations to glycolysis or other processes).[53] A major reason that a systematic search for such *in vivo* behavior has not been feasible is that a comprehensive set of rate constants to allow extrapolation of current understanding to *in vivo* conditions has not been available. While the rest of this review explains what is understood of peroxidase oscillatory chemistry, it may be that the tabulation of rate constants in Table 1 is of greatest utility, since we believe it contains the most accurate relevant rate constants from the past 40 years of literature. An HTML version of the table, updated as appropriate, will be maintained on the Internet, linked to A. Scheeline's home page. (Accessible via http://www.chem.uiuc.edu/scheeline/gsche2.htm.)

II. Biochemical Model

Many reactions have been proposed from equilibrium and kinetic studies to account for steps in cellular models, in particular the steps in the peroxidase formation of hydrogen peroxide beginning at the point NADH (substrate) enters the paths. In Figure 3, the dotted line encloses that part of the cellular model included in the biochemical model. Some of the nonenzymatic steps in this model could also occur in the peroxidase-independent reaction cycle in the cellular model. The net reaction of the biochemical model is nominally[18]

Table 1. Elementary Reactions in the Biochemical Model: Recommended Values[a]

no.	reaction	rate constant	pH	ref(s)
2	$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$	2.0×10^7 M^{-1} s^{-1}	5.10	56
2a	$2O_2^{\bullet-} \rightarrow O_2^{2-} + O_2$	<0.3 M^{-1} s^{-1}	any	56
3	$HO_2^{\bullet} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2$	7.6×10^5 M^{-1} s^{-1}	any	56
4	$O_2^{\bullet-} + HO_2^{\bullet} \rightarrow HO_2^- + O_2$	8.7×10^7 M^{-1} s^{-1}	any	56
5	$NADH + O_2 \rightarrow NAD^+ + H_2O_2$	3.0×10^{-6} s^{-1}	5.6[b]	60
6	$NAD^{\bullet} + O_2 \rightarrow O_2^{\bullet-} + NAD^+$	2.0×10^9 M^{-1} s^{-1}	8.60	57
7	$NADH + O_2^{\bullet-} \rightarrow H_2O_2 + NAD^{\bullet}$	$\ll 27$ M^{-1} s^{-1}	8.60	38, 57
7a	$NADH + HO_2^{\bullet} \rightarrow H_2O_2 + NAD^{\bullet}$	1.8×10^5 M^{-1} s^{-1}		146
7b	$NADH + O_2^{\bullet-} \rightarrow HO_2^- + NAD^{\bullet}$	no reaction		146
7c	$NADH + HO_2^{\bullet} \rightarrow H_2O_2 + NAD^{\bullet}$	1.1×10^5 "apparent"	5.25	146
8	$Mn^{2+} + O_2^{\bullet-} \rightarrow Mn^{3+} + H_2O_2$			19, 76, 147
9	$Mn^{3+} + NADH \rightarrow NAD^{\bullet} + H^+ + Mn^{2+}$	not found in literature		
	$Mn^{3+} + O_2^{\bullet-} \rightarrow Mn^{2+} + H_2O_2$	not found in literature		
10	$Per^{3+} + H_2O_2 \rightarrow coI + H_2O$	0.9×10^7 M^{-1} s^{-1}	5.00	34
11	$coI + NADH \rightarrow coII + NAD^{\bullet}$	9.44×10^4 M^{-1} s^{-1}	5.10	79
12	$coII + NADH \rightarrow Per^{3+} + NAD^{\bullet} + H_2O$	5.37×10^4 M^{-1} s^{-1}	5.10	79
13	$coI + O_2^{\bullet-}/HO_2^{\bullet} \rightarrow coII + O_2$	8.37×10^7 M^{-1} s^{-1} [c]	5.10	76
13a	$coI + HO_2^{\bullet} \rightarrow coII + O_2$	2.2×10^8 M^{-1} s^{-1}		76
13b	$coI + O_2^{\bullet-} \rightarrow coII + O_2$	1.6×10^6 M^{-1} s^{-1}		76
14	$coI + NAD^{\bullet} \rightarrow coII + NAD^+$	1.5×10^8 M^{-1} s^{-1}	7.00	79
15	$coII + NAD^{\bullet} \rightarrow Per^{3+} + NAD^+$	conjecture		79
16	$Per^{2+} + H_2O_2 \rightarrow coII$	5×10^4 M^{-1} s^{-1}		38
17	$coI + H_2O_2 \rightarrow Per^{3+} + O_2$	500 M^{-1} s^{-1}		38
18	$coII + H_2O_2 \rightarrow Per^{3+} + HO_2^{\bullet}$	2.1 M^{-1} s^{-1}		38
19	$coI + H_2O_2 \rightarrow coII + HO_2^{\bullet}$	<2		38
		1.7×10^7 M^{-1} s^{-1}	5.1	100
20	$Per^{3+} + O_2^{\bullet-} \rightarrow coIII$	1.9×10^6 M^{-1} s^{-1}	5.5	60
		3.2×10^7 M^{-1} s^{-1}		148
21	$Per^{2+} + O_2 \rightarrow coIII$	5.8×10^4 M^{-1} s^{-1}	7.00	1, 40, 80
21a	$coIII \rightarrow Per^{2+} + O_2$	0.05 s^{-1}		82
22	$coII + H_2O_2 \rightarrow coIII$	16 M^{-1} s^{-1}	*d*	81
23	$coIII \rightarrow Per^{3+} + O_2^{\bullet-}$	slow		1
24	$coIII \rightarrow coII + H_2O_2$	2.0×10^{-3} s^{-1}	5.0	82
25	$coIII + coII \rightarrow 2Per^{3+} + O_2$	3×10^4 M^{-1} s^{-1}		82
26	$coIII + coI \rightarrow Per^{3+} + coII + O_2$	10^4 M^{-1} s^{-1}		82
27	$coIII + Per^{2+} \rightarrow Per^{3+} + coI$	"very fast"	*e*	60, 80
28	$Per^{3+} + NAD^{\bullet} \rightarrow Per^{2+} + NAD^+$	8×10^8 M^{-1} s^{-1}	7.00	79
29	$coIII + NAD^{\bullet} \rightarrow coI + NAD^+$	1.3×10^8 M^{-1} s^{-1}	5.60	60
30	$2NAD^{\bullet} \rightarrow (NAD)_2$	5.6×10^7 M^{-1} s^{-1}	6.40	57, 87
31	$2NAD^{\bullet} + H^+ \rightarrow NADH + NAD^+$	weak evidence		60
33	$(NAD)_2 + H_2O_2 + 2H^+ \rightarrow 2NAD^+ + 2H_2O$	none given		93
34	$MBH + O_2 \rightarrow H_2O_2 + MB^+$	1.62×10^2 M^{-1} s^{-1}	9.0	62
35	$MB^+ + NADH \rightarrow NAD^+ + MBH$	3.57 M^{-1} s^{-1}	9.0	62
37	$coIII + PC \rightarrow coI + PC^{\bullet}$ (various phenols)	2×10^0 to 4.1×10^4 M^{-1} s^{-1}	5.0 to 7.0	82
	$MB^{\bullet-} + O_2^{\bullet-} \rightarrow MB^+ + H_2O_2 + 2OH^-$	2×10^8 M^{-1} s^{-1}	>9	64
	$MBH + O_2 \rightarrow H_2O_2 + MB^{\bullet} + OH^-$	5.5×10^5 M^{-1} s^{-1}	>9	64
	$MBH + O_2 \rightarrow O_2^{\bullet-} + MB^{\bullet} + H^+$	4×10^{-5} M^{-1} s^{-1}	>9	64
	$2MB^{\bullet} \rightarrow MBH + MB^+ + OH^-$	2×10^6 M^{-1} s^{-1}	>9	64
	$MB^{\bullet-} + O_2 \rightarrow MB^+ + O_2^{\bullet-}$	3×10^6 M^{-1} s^{-1}	>9	64
	α-NADH → β-NADH	1.02×10^{-3} s^{-1}	7.0	134
	β-NADH → α-NADH	1.75×10^{-4} s^{-1}	7.0	134
	α-NADH → α-(6HTN)AD	2.1×10^{-4} s^{-1}	7.0	134
	β-NADH → β-(6HTN)AD	0.00276 s^{-1}	5.2	133
	α-(6HTN)AD → c-THNAD	0.0042 s^{-1}	5.2	133

[a] Only the best available rate constant is listed. In many instances, quite different rate constants have been used in specific models cited in this review. For lists of other rate constants, see ref 149. Reactions are intentionally unbalanced and reflect elementary steps consistent with rate constant dimensions. Where H^+ or OH^- are reactants, pH is listed where known. [b] Assuming $[O_2] = 150$ μM, $k_5 = 4.5 \times 10^{-10}$ M^{-1} s^{-1}. [c] Rate constant includes effect of dissociation equilibrium. [d] See[38,78] for $k_{22} = 250$ M^{-1} s^{-1}. Adediran and Lambeir[81] carefully examine pH dependence and so have greater credibility for their rate constant value. [e] Reaction 27 together with reaction 28 is an alternate to reaction 29. Suppose coIII builds up; when O_2 is exhausted, excess NAD produces Per^{2+}, which then disproportionates, giving a result difficult to distinguish from reaction 29.

$$2NADH + O_2 \rightarrow 2NAD^+ + 2H_2O$$

In fact it has not been demonstrated experimentally that exactly this stoichiometry applies under conditions where oscillations occur. $(NAD)_2$ and H_2O_2 are both plausible products whose concentrations at the termination of oscillation have not been reported. Neither would be present in significant quantity at equilibrium, but equilibrium may, in fact, not be obtained on a time-scale commensurate with experiments.

There are several dozen elementary reactions to be considered in the development of a biochemical model of oscillatory dynamics. Reactions discussed in this section which may influence kinetics are listed in Table 1. These reactions are highly interdependent and many are competitive. Some have low rate constants, but even a slow reaction may contribute to the dynamics of the system.[54] Rate constants,

including pH at which each applies where this is known, are also listed in Table 1. We have adopted the convention of showing all proposed elementary reactions so that reactants shown and rate constants have consistent units. This means that protons, hydroxide ions, and water may not appear as reactants even when stoichiometrically required.

Incorporating all of these steps into a reaction scheme makes the reaction diagram very complex and effectively untestable. No existing model attempts to do so. We present here those combinations of fundamental reactions which have been used in an attempt to study the peroxidase−oxidase system, but present other reactions to suggest alternative models for future consideration.

A. Nonenzymatic Oxygen Radical Reactions

As is evident in the cellular model, the reactions involving oxygen species, which take place around the peroxidase reactions, have a profound effect on the character of the system. They can consume resources by competing with the enzymatic reactions, as well as produce resources used by the enzyme. There are two important oxygen radical species present in the reaction system: $O_2^{\bullet -}$ and $HO_2^{\bullet}$,[55] with $HO_2^{\bullet}$ the stronger oxidant. The extent to which they are available to the enzyme reaction depends largely on pH. The following equilibrium has a $pK_a = 4.88$:[13,56,57]

$$HO_2^{\bullet} \rightleftharpoons O_2^{\bullet -} + H^+ \qquad (1)$$

At low pH, $HO_2^{\bullet}$ is the predominate species; its concentration is insignificant above pH 8. Different kinetics between hydroperoxide and superoxide radicals contributes to the pH dependence of the peroxidase system dynamics. Halliwell[19] maintained that slow disproportionation of $O_2^{\bullet -}$ could occur

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

or (2)

$$O_2^{\bullet -} + O_2^{\bullet -} \rightarrow O_2^{2-} + O_2$$

but it was already known[56] that such disproportionation occurred (absent superoxide dismutase) only via reaction with one or more $HO_2^{\bullet}$ species. $HO_2^{\bullet}$ disproportionates according to

$$HO_2^{\bullet} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2 \qquad (3)$$

and this reaction can dominate at low pH.[19,55,56,58] The reaction of the two radicals with one another may be dominant at intermediate pHs[55]

$$HO_2^{\bullet} + O_2^{\bullet -} + H_2O \rightarrow O_2 + H_2O_2 + OH^-$$

or (4)

$$HO_2^{\bullet} + O_2^{\bullet -} \rightarrow O_2 + HO_2^-$$

especially at pH 5.1 at which peroxidase oscillations are typically observed. Other species must compete with disproportionation in order to react with superoxide. *In vivo* the disproportionation reaction is accelerated by superoxide dismutase.

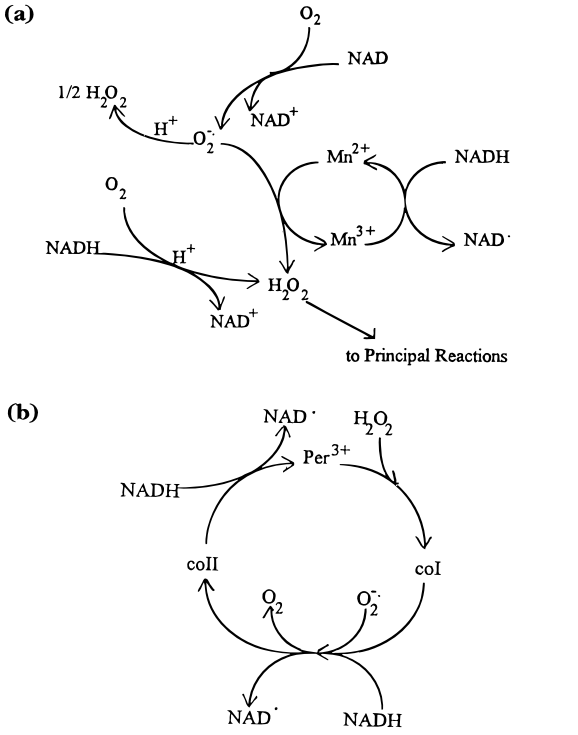


Figure 4. Biochemical model reactions: (a) preliminary reactions and (b) principal reactions. Oxygen and NADH are input. $NAD^{\bullet}$ enters from the principal reactions. The preliminary reactions produce H_2O_2 and $O_2^{\bullet -}$.

B. Preliminary Reactions

Figure 4 show the reactions in the biochemical model which (a) produce the species involved in the catalytic cycle (the preliminary reactions) and (b) the reactions in the catalytic cycle itself (the principal reactions).

H_2O_2 in catalytic amounts is required to initiate oxidation of NADH by peroxidase.[1] One source of H_2O_2 may come from the autooxidation of NADH which occurs spontaneously, but very slowly[58,59]

$$NADH + O_2 + H^+ \rightarrow NAD^+ + H_2O_2 \qquad (5)$$

Even if no H_2O_2 is added initially, the H_2O_2 produced in reaction 5 is enough to initiate the mechanism.[1,60] In typical oscillatory experiments, the reaction modifier methylene blue (MB) can catalyze this reaction at alkaline or neutral pH.[61,62] In all likelihood, free-radical intermediates are of importance as well.[63,64] While the oxygen/reduced MB dynamics have been extensively studied at alkaline pH, NADH + MB reacts sufficiently slowly at acidic pH that rate constants are not adequately known. In fact, unless O_2 is excluded from the solution, changes in [MB] cannot be observed in acidic solution.

Once a small amount of H_2O_2 is present, a second pathway to H_2O_2 production involving $O_2^{\bullet -}$ may begin. Yamazaki and Yokota suggested that the key reaction to generate $O_2^{\bullet -}$ is[1,60]

$$NAD^{\bullet} + O_2 \rightarrow O_2^{\bullet -} + NAD^+ \qquad (6)$$

Several reactions compete for the $O_2^{\bullet -}$. One reaction involves the oxidation of NADH by $O_2^{\bullet -}$ to regenerate

catalytic H_2O_2[60]

$$NADH + O_2^{\bullet -} + H^+ \rightarrow NAD^{\bullet} + H_2O_2 \quad (7)$$

According to Halliwell,[19] reaction 7 is accelerated *in vivo* in the presence of the cofactor, Mn^{2+}. He proposed the following pair of reactions:

$$Mn^{2+} + O_2^{\bullet -} \rightarrow Mn^{3+} + H_2O_2 \quad (8)$$

$$Mn^{3+} + NADH \rightarrow NAD^{\bullet} + H^+ + Mn^{2+} \quad (9)$$

the net reaction being reaction 7. In Halliwell's experiments, Mn^{2+} is introduced in experiments as $MnCl_2$. The optimal concentration of Mn^{2+} was found to be 10^{-5} M at pH 7.4. The requirement for Mn^{2+} is quite specific. Akazawa and Conn[65] tested nine other metal cations: Ni^{2+}, Cd^{2+}, Cu^{2+}, Al^{3+}, Fe^{3+}, Mg^{2+}, Zn^{2+}, and Co^{2+}. The only cation having any effect on the rate of oxidation was Co^{2+}, and kinetic studies showed that overall no significant change in extent of oxidation occurred. Inhibition of the reaction occurred when Cu^{2+} was substituted.[65]

More recent work in some instances is at odds with these conclusions. There is evidence that reactions 8 and 9 may be affected by the presence of various buffers. In aqueous solutions and high buffer concentration, Mn^{2+} is complexed by buffer components. Cabelli and Bielski[66,67] studied reaction 7 where Mn^{2+} is complexed with formate, sulfate, phosphate, or pyrophosphate. In all cases, at low pH reaction 7 apparently proceeds as written above. In the presence of formate, sulfate, and phosphate and at near neutral pH, MnO_2^+ is formed via a detectable transient intermediate. When formate and sulfate are the ligands, the MnO_2^+ disappears to unknown products without forming detectable amounts of Mn^{3+}. For phosphate and pyrophosphate, the MnO_2^+ is oxidized to Mn^{3+}. This step seems to be the rate-limiting step. The differences in kinetics for each ligand and the pH dependence of the kinetics arises from the different pK_a values for the manganese complexes and $O_2^{\bullet -}$ (see reactions 1−4) and the different reactivities of the acid/base conjugate pairs. Thus complexation by buffers would alter the kinetics of the catalysis by Mn^{2+} in the PO reaction. The effect is experimentally manifest *in vitro*, and may or may not accurately reflect the environment in the plant cell wall.[66,67] One may conclude[67] that, in acetate buffer, Mn^{2+} catalytically consumes $O_2^{\bullet -}$, producing H_2O_2 and O_2. In other words, Mn^{2+} is acting as a superoxide dismutase. This is consistent with the effect of Mn^{2+} on the peroxidase−oxidase oscillator.[68]

Complexation between metal ions and either superoxide or NADH may be expected to alter reaction kinetics. Such effects have not been established to effect oscillatory dynamics, but much closely related chemistry does show the consequences of such influence. Both Fe^{3+} and Fe^{2+} can bind to NADH.[69] Al^{3+} and Cu^{2+} both catalyze the oxidation of NADH or model compounds by oxygen.[70−72] We are currently investigating if the presense of trace metals has a significant effect on oscillatory dynamics and, if so, how *in vivo* conditions might differ from common *in vitro* behavior, where pure reactants and deionized water limit the influence of metallic ions.

C. Principal Reactions

The main reaction cycle of the peroxidase-oxidase reactions are generally agreed to be reactions 10−12[1,38,60,73,74] (see Figure 4b). The reactants, $O_2^{\bullet -}$ and H_2O_2 are produced by the preliminary reactions as shown in Figure 4a. The principal reactions involve these two species in a catalytic cycle in which the enzyme converts two NADH molecules to two $NAD^{\bullet}$ free radicals.

$$Per^{3+} + H_2O_2 \rightarrow coI + H_2O \quad (10)$$

$$coI + NADH \rightarrow coII + NAD^{\bullet} \quad (11)$$

$$coII + NADH \rightarrow Per^{3+} + NAD^{\bullet} + H_2O \quad (12)$$

Since the preliminary reactions consume one $NAD^{\bullet}$ for each one produced, the total biochemical model (preliminary plus principal reactions) yields a net gain of two $NAD^{\bullet}$. Thus the overall process corresponding to these reactions is autocatalytic in $NAD^{\bullet}$. This autocatalysis is important in some of the models of oscillations.[18,60] The overall reaction corresponding to steps 10−12 has Per^{3+} being regenerated after cycling through the intermediates coI and coII. pH dependence of reaction 10 has been precisely examined by Dolman *et al.*[75] The rate constant above pH 5.5 plateaus at 1.9×10^7 M^{-1} s^{-1}. Rate constants for reactions 11 and 12 over the pH range 4.5−10 have been reported by Kashem and Dunford.[74]

In an additional reaction, coI can compete for the $O_2^{\bullet -}$ to form coII:[76]

$$coI + O_2^{\bullet -} \rightarrow coII + O_2 \quad (13)$$

$NAD^{\bullet}$ is also reactive with coI and coII. In the former case, experiments have been performed at pH 7 in phosphate buffer:

$$coI + NAD^{\bullet} \rightarrow coII + NAD^+ \quad (14)$$

The corresponding reaction with coII

$$coII + NAD^{\bullet} \rightarrow Per^{3+} + NAD^+ \quad (15)$$

cannot be studied experimentally due to the reactivity of coII in acidic solution. One can conjecture that the rate of reaction 15 should be similar to reaction 14 since the rate of reaction 12 is similar to the rate of reaction 11.

Per^{2+} can divert H_2O_2 from the principal reactions to form coII.[38]

$$Per^{2+} + H_2O_2 \rightarrow coII \quad (16)$$

It is unlikely that this reaction is important in oscillatory dynamics since the reaction of Per^{2+} with oxygen proceeds to coIII with a similar rate constant (reaction 20), and oxygen concentration substantially exceeds H_2O_2 concentration continuously in the reaction.

Omitted from all extant models of the peroxidase oscillator are reactions of hydrogen peroxide with coI

and coII:[38]

$$\text{coI} + H_2O_2 \rightarrow \text{Per}^{3+} + O_2 \quad (17)$$

$$\text{coII} + H_2O_2 \rightarrow \text{Per}^{3+} + HO_2^{\bullet} \quad (18)$$

$$\text{coI} + H_2O_2 \rightarrow \text{coII} + HO_2^{\bullet} \quad (19)$$

Considering that the relevant experiments have been performed in phosphate buffer at pH 7 and 5 C rather than acetate buffer at pH 5.1 and 25 °C, it is difficult to estimate to what extent reactions 17−19 compete with more commonly included reactions.

D. Compound III Reactions

The coIII reactions have a regulatory effect on the oxidation of NADH through the inactivation of Per^{3+} by oxygen. In the presence of an excess of oxygen vs NADH, most of the peroxidase is found as coIII,[60] formed via

$$\text{Per}^{3+} + O_2^{\bullet-} \rightarrow \text{coIII} \quad (20)$$

The rate of reaction 20 increases with decreasing pH. This can be explained by the predominance of $HO_2^{\bullet}$ radical at low pH. Per^{3+} has been found to be more reactive with $HO_2^{\bullet}$ than with $O_2^{\bullet-}$, although the rate constant for this reaction has not been determined.[77] There are two other reactions that can produce coIII, starting with Per^{2+} and coII:[38,78−81]

$$\text{Per}^{2+} + O_2 \rightarrow \text{coIII} \quad (21)$$

and

$$\text{coII} + H_2O_2 \rightarrow \text{coIII} \quad (22)$$

Compound III is relatively inert in the peroxidase−oxidase reaction.[60] However, several reactions can return coIII to the active enzyme. When the oxygen concentration is low, i.e. the oxygen in the reaction solution is depleted, coIII decays very slowly by reaction 23, but faster than it dissociates by the reverse of reaction 22,[1,80] as shown in reaction 24. Reaction 23 is accelerated by Per^{2+},[77] as earlier suggested in a mechanistically more defensible way (reaction 25) by Tamura and Yamazaki.[82] Of comparable importance is the reaction of coIII and coI (reaction 26):

$$\text{coIII} \rightarrow \text{Per}^{3+} + O_2^{\bullet-} \quad (23)$$

$$\text{coIII} \rightarrow \text{coII} + H_2O_2 \quad (24)$$

$$\text{coIII} + \text{coII} \rightarrow 2\text{Per}^{3+} + O_2 \quad (25)$$

$$\text{coIII} + \text{coI} \rightarrow \text{Per}^{3+} + \text{coII} + O_2 \quad (26)$$

Yamazaki and Yokota proposed another pathway from coIII to active enzyme[60]

$$\text{coIII} + \text{Per}^{2+} \rightarrow \text{Per}^{3+} + \text{coI} \quad (27)$$

where the Per^{2+} originated in the reaction[79]

$$\text{Per}^{3+} + \text{NAD}^{\bullet} \rightarrow \text{Per}^{2+} + \text{NAD}^{+} \quad (28)$$

The net reaction is

$$\text{coIII} + \text{NAD}^{\bullet} \rightarrow \text{coI} + \text{NAD}^{+} \quad (29)$$

Interestingly, Yokota credits reaction 29 to Wittenberg,[80] who makes no mention whatsoever of nicotinamide adenine dinucleotide! It has been suggested that reaction 21 could compete with reaction 27 to consume Per^{2+}.[38,80]

Reaction 28 is a point of some controversy. Kobayashi[79] states the reaction goes faster at lower pH than at the neutral pH at which he reports a rate constant. In an early citation, Fed'kina *et al.* suggested the reaction has a lower rate constant than did Kobayashi.[83] Some models leave the reaction out entirely, maintaining that reaction 20 completely subsumes the combination of reactions 21 and 28. Part of the problem until recently has been that evidence for the presence of Per^{2+} under oscillatory conditions using HRP has been lacking, although it had been detected when some other peroxidases are used.[49,84] Indeed, Bronnikova *et al.*[84] include reactions 21 and 28 explicitly, getting excellent agreement between experimentally observed oscillation phase space plots and those of models. Recently, Hauser and Olsen[85] have found definitive spectroscopic evidence for the presence of Per^{2+} in HRP-catalyzed PO oscillations (particularly at pH > 6), removing most of the remaining doubts about its participation in the reaction mechanism. Simulation also indicates the importance of its inclusion in explaining some observed dynamics such as the parameter values at which period doubling occurs.

E. Reactions of NAD• and OH•

$NADH^{\bullet-}$ (NADH reduced by one electron) is a strong acid, with pK_a of −3.5.[86] If formed, it quickly dissociates (rate constant $\sim 10^7$ s^{-1}) to $NAD^{\bullet} + H^+$. In addition to reactions 6, 28, and 29, $NAD^{\bullet}$ reacts with another $NAD^{\bullet}$ by dimerizing:[57,60,87−89]

$$2\text{NAD}^{\bullet} \rightarrow (\text{NAD})_2 \quad (30)$$

Disproportionation may also occur

$$2\text{NAD}^{\bullet} + H^+ \rightarrow \text{NAD}^{+} + \text{NADH} \quad (31)$$

although to a much lesser extent, regenerating NADH.[57] This is generally not considered significant in the overall reaction scheme.[90] In either case, these reactions limit the amount of $NAD^{\bullet}$ as an active intermediate and terminate the free radical explosion induced by the principal reactions. Reaction 30 has also been considered a terminating reaction for $NAD^{\bullet}$.[91] However, Avigliano and co-workers have found that the dimerization of $NAD^{\bullet}$ can be reversed by photoinduced oxidation.[92]

$$(\text{NAD})_2 + O_2 + 2H^+ + h\nu \rightarrow 2\text{NAD}^{+} + 2H_2O \quad (32)$$

If $(NAD)_2$ is only partially oxidized or O_2 only partially reduced, one obtains intermediates such as $NAD^{\bullet}$ or H_2O_2. In fact, in a 1985 paper,[93] it appeared that $(NAD)_2$ could even be a substrate for coI. Only

qualitative demonstrations of this were given. In the absence of rate constants, reaction 32 has not been included in Figure 4.

Yokota and Yamazaki[60] include an additional reaction for $NAD^{\bullet}$ in which this species combines with H_2O_2 to form hydroxyl radical:

$$NAD^{\bullet} + H_2O_2 \rightarrow NAD^{+} + H_2O + OH^{\bullet} \quad (33)$$

Considering that $OH^{\bullet}$ is quite reactive and may be involved in deactivating HRP, it is surprising that no chemically realistic models since 1977 have included its production much less its reactive dynamics. We have indirect evidence (work in progress) that excessive amounts of oxygen may damp oscillations by attacking HRP, converting it to P-670 or other forms not typically appearing in discussions of enzyme function. One must be cautious in interpreting damping of oscillations as nonlinear phenomena (exponential decay to a steady state, for example[94]) when in fact they may simply be chemistry at work (consumption of a reactant[95]). Characterization of the role of hydroxyl radical must await trapping experiments which can selectively identify $OH^{\bullet}$ in the presence of $NAD^{\bullet}$, $HO_2^{\bullet}$, and perhaps $PhO^{\bullet}$ species. Such technology exists.[96]

F. Role of Methylene Blue

Methylene Blue has been claimed to "stabilize" oscillations in these experiments. Its role has been said to be regulatory in nature and to accelerate the oxidation of NADH through the following reactions:[61,62,97]

$$MBH + O_2 \rightarrow H_2O_2 + MB^{+} \quad (34)$$

$$MB^{+} + NADH \rightarrow NAD^{+} + MBH \quad (35)$$

There is some uncertainty about whether these reactions are important in oscillations since the experiments in which reactions 34 and 35 were observed were carried out at a higher oxygen flow rate than in the oscillation experiments.[62,94] Hauser[63,64] studied the detailed kinetics of the reaction of MB^{+} with O_2 and $O_2^{\bullet-}$ at alkaline pH. Indeed, ALL measurements of MBH and MB^{+} reaction kinetics have been made at pHs higher than those employed in the PO oscillator. MB's reactions have proven too slow or too oxygen-sensitive to study at acidic pH (or, at least, proceed by paths giving only weak spectral changes). Recently, by keeping O_2 uptake at a sufficiently low value, stable, undamped PO oscillations have been maintained without added MB (G. Horras, unpublished observations). Further complicating the picture is evidence by Olson and Scheeline[98,99] that addition of MB actually slows oxygen consumption, contradicting the prediction of reactions 34 and 35. The role of MB in the PO reaction remains unclear.

G. Role of 2,4-Dichlorophenol

A general reaction by which active enzyme can be regenerated from the inactive coIII is

$$coIII + e^{-} \rightarrow coI \quad (36)$$

where the electron could be donated by a variety of species.[81,100] For example, in the presence of certain phenols (referred to as PC in Figure 3), such as *p*-cresol, 4-hydroxycinnamyl acid, and, most effectively, 2,4-dichlorophenol, the rate of decomposition of coIII increases. The presence of a phenol appears to increase the rate of oxidation of NADH.[19,60] Also, the stimulatory effects of both cofactors (PC and Mn^{2+}) are additive, indicating separate mechanisms of action.[19] The proposed mechanism by which PC acts to decompose coIII is

$$coIII + PC \rightarrow coI + PC^{\bullet} \quad (37)$$

where coI is then free to react with NADH, thus accelerating its oxidation.[82] This reaction is enhanced by the addition of H_2O_2. The fate of $PC^{\bullet}$ is unknown in experiment; possibly it dimerizes. However, in the cell wall where PC might represent phenols naturally found there (such as 4-hydroxycinnamyl alcohol), $PC^{\bullet}$ can serve as a monomer for the polymerization of lignin. In addition, there is evidence that these phenols may enhance NADH oxidation in some other, unspecified way.[19] Recently, Kummer *et al.*[49] suggested that PC could bind to peroxidase, acting either as an allosteric rate accelerator or otherwise altering enzyme structure to speed reversion of coIII to coI. Structural demonstration of such binding has not been reported, however.

There is an alternative view. Its relevance to oscillatory behavior is equally speculative. Phenols are known to act as reductants of coI and coII in a manner entirely analogous to NADH:

$$coI + PC \rightarrow coII + PC^{\bullet} \quad (38)$$

$$coII + PC \rightarrow Per^{3+} + PC^{\bullet} \quad (39)$$

In contrast to the mechanisms which assert phenols interact directly with coIII, one may expect radical exchange with NAD:

$$PC^{\bullet} + NADH \rightarrow PC + NAD^{\bullet} \quad (40)$$

followed by reaction of $NAD^{\bullet}$ with coIII as in reaction 29.

Other possibilities for the role of phenols exist. Rate constants for reaction of compounds I and II with many phenols have been tabulated.[101−105] These can range from 10^3 to 10^8 M^{-1} s^{-1}. $OH^{\bullet}$ reacts at a diffusion-limited rate ($k \sim 2 \times 10^9$ to 1.4×10^{10} M^{-1} s^{-1} [103,106]) with phenol. At least some substrates (such as dihydroxyfumarate) generate $OH^{\bullet}$ when reducing coIII.[59,107] A suggestion that DCP (in the presense of Mn^{2+}) could generate hydroperoxide radical has languished, uncited, for many years.[59]

It is thus not surprising that the mechanism by which 2,4-dichlorophenol (DCP) influences the dynamics of the peroxidase oscillator is for practical purposes unknown. There are various assertions that its role is due to acceleration of reaction 29, but since Halliwell's work in 1978,[19] the literature is long on rationalization and short on hard data. Even recent work which purported to show that network topology forced one to conclude that DCP's influence

was in the commonly asserted path listed rate constants without justification (even at the level of describing whether they were freshly determined, obtained from the literature, or simply chosen to provide a model with desirable characteristics).[108] Nevertheless, Olsen's assertion that period doubling behavior for his 1983 model (described in the following section) can be obtained by correlating the value for his k_3 with [DCP],[109] the finding in realistic models that such period doubling can come from correlating the rate constant for reaction 29 with [DCP], and the demonstration by Bensen[110] that the realistic models can be reduced to Olsen's model, with reaction 29 correlating with the Olsen model step 3 all would indicate that there is some way in which DCP influences the rate of coIII decomposition. The precise nature of this influence remains uncertain.

III. Abstract Models

A variety of models have been published, based on reactions of a limited number (usually four) of species, the behavior of which appears to mimic the dynamics of NADH and O_2 in the PO reaction. These models have lent themselves to detailed studies of the phase space behavior of the peroxidase system (to the extent that the models reflect PO chemistry). Much of this study has been based upon the early work of Olsen and Degn[18] who proposed a four-variable model composed of two coupled autocatalytic cycles. Further computer simulations and theoretical explanations of the behavior of this model were given by Degn, Olsen, and Perram,[111] commonly known as the DOP model. This was succeeded by a variant in 1983 whose dynamics bore an uncanny resemblance to experimental data.[112] It has only been recently that chemically detailed models have been shown to be quite analogous to this "Olsen 1983" model.

These abstract models involve molecular oxygen (A), NADH (B), and two key species, the free radical intermediates X and Y. The enzyme in its various forms and additional species such as DCP and MB do not appear explicitly in either the DOP or Olsen 1983 model. The enzyme, however, is considered to play an *implicit* role through the values of the first and third rate constants. The 1983 model is

$$\begin{aligned} \mathrm{B} + \mathrm{X} &\xrightarrow{k_1} 2\mathrm{X} \\ 2\mathrm{X} &\xrightarrow{k_2} 2\mathrm{Y} \\ \mathrm{A} + \mathrm{B} + \mathrm{Y} &\xrightarrow{k_3} 3\mathrm{X} \\ \mathrm{X} &\xrightarrow{k_4} \mathrm{P} \\ \mathrm{Y} &\xrightarrow{k_5} \mathrm{Q} \\ \mathrm{X}_0 &\xrightarrow{k_6'} \mathrm{X} \\ \mathrm{A}_0 &\underset{k_{-7}}{\overset{k_7'}{\rightleftharpoons}} \mathrm{A} \\ \mathrm{B}_0 &\xrightarrow{k_8'} \mathrm{B} \end{aligned} \tag{41}$$

The first three reactions in this model comprise the two autocatalytic cycles. The termination reactions, steps 4 and 5, are necessary to remove radicals, allowing steady states to exist. The remaining steps in the mechanism involve an initiation reaction (step 6) for one of the free radical species, a reversible reaction (step 7) which models the equilibration of gaseous oxygen with dissolved oxygen, and the slow perfusion of NADH into the reactor vessel (step 8). Experimentally, the PO reaction has typically been carried out in reactors which are not true CSTRs but, rather, open systems involving only inflow (not outflow) of the reactants. This conserves enzyme, although one must take care that solution volume (balanced between inflows and evaporation) is constant and that products (P and Q in model 41, NAD^+ and possibly other species in experiments) do not influence dynamics. Within these constraints, model 41 is consistent with the typical experimental configuration.

Simulation studies with the four-variable models have been carried out by numerically solving (using either a Runge–Kutta or a Gear-type algorithm[113]) the associated system of ordinary differential equations (ODEs). The ODEs for the 1983 model are

$$\begin{aligned} \mathrm{d}A/\mathrm{d}t &= -k_3\mathrm{ABY} + k_7 - k_{-7}\mathrm{A} \\ \mathrm{d}B/\mathrm{d}t &= -k_1\mathrm{BX} - k_3\mathrm{ABY} + k_8 \\ \mathrm{d}X/\mathrm{d}t &= k_1\mathrm{BX} - 2k_2\mathrm{X}^2 + 3k_3\mathrm{ABY} - k_4\mathrm{X} + k_6 \\ \mathrm{d}Y/\mathrm{d}t &= -k_3\mathrm{ABY} + 2k_2\mathrm{X}^2 - k_5\mathrm{Y} \end{aligned} \tag{42}$$

where $k_6 = k_6'\mathrm{X}_0$, $k_7 = k_7'\mathrm{A}_0$, and $k_8 = k_8'\mathrm{B}_0$.

Numerical solutions of both this and the DOP model equations reproduce both the simple as well as the complex and chaotic behavior seen experimentally in the PO reaction. For the parameter values used in these simulations, the chaotic oscillations computed from the 1983 model, eqs 42, seem to be qualitatively and quantitatively more similar to the chaos seen experimentally than do those from the DOP model. In addition, the more detailed models discussed in the following section can, in some cases, be directly related to features of the 1983 model.

IV. Chemically Realistic Models

A number of more detailed models of the peroxidase reaction have been proposed and investigated over the years. Several different models have been found capable of explaining the existence and origin of oscillatory behavior, both regular and chaotic. All of these models have a common "skeleton" of steps shown by the solid lines in Figure 5. Note that only four of the five known enzyme–substrate complex intermediates appear in this skeleton mechanism. The fifth, Per^{2+}, is present in some of the models through the steps shown with dotted lines; it is interesting that inclusion of the Per^{2+} species in this way always seems to allow for chaotic behavior, whereas without these steps only regular, periodic dynamics are observed. The intermediate $NAD^\bullet$ is critical in all the proposed schemes primarily because it is involved in at least one autocatalytic cycle, i.e. a cycle in which the consumption of one $NAD^\bullet$ radical results in the production of more than one radical.

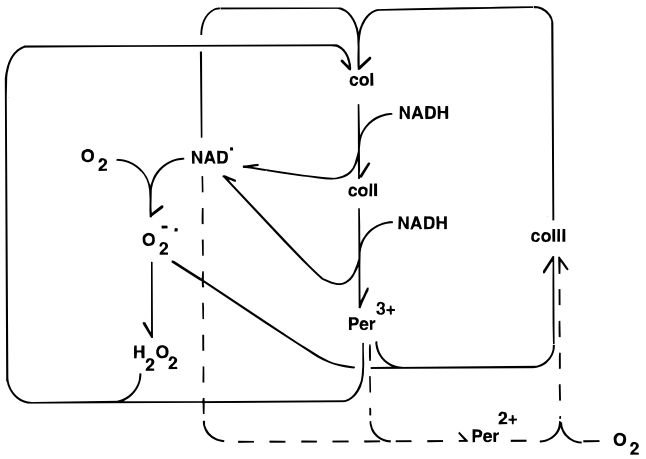


Figure 5. Skeleton mechanism for chemically realistic models. Solid lines lead to simple oscillations only; inclusion of Per^{2+} may allow for more complicated dynamics.

The differences between the mechanisms to be described below involve the specific nature of the step converting coIII to coI as well as the inclusion of additional reactions. A more detailed review of the older models has been previously published by one of us[6] and the reader is referred to this earlier source for more information.

In 1977, Yokota and Yamazaki proposed a detailed model for the PO reaction, henceforth called the YY model.[60] The bimolecular reaction between coIII and NAD• is considered to be the dominant decomposition pathway for coIII in the YY mechanism, even though a second pathway involving the production of coII and H_2O_2 from coIII is included. Simulation studies with the YY mechanism by Yokota and Yamazaki reproduced the four phases in the coIII kinetics they had observed experimentally in a closed system: initial burst, induction, steady state, and abrupt decomposition. These studies did not reveal any oscillatory behavior in this model, however. Subsequent investigations by Aguda and Larter[91] showed several mathematical inconsistencies in the rate equations used by Yokota and Yamazaki in their simulations; this may be a partial explanation for the absence of oscillatory behavior in the YY model. However, the YY mechanism models a closed system since it does not allow for input of reactants or a termination pathway for the autocatalytic species; as such, it would not be expected to yield sustained oscillations. A year later, Olsen reported further on reaction networks consistent with several aspects of observed oscillatory behavior.[100]

A model proposed by Fed'kina, Ataullakhanov, and Bronnikova in 1984[114] is simpler than the YY model but adds a unimolecular termination reaction for the NAD• radicals; this addition corrects a problem with the YY model which caused the concentration of NAD• to increase monotonically throughout the YY simulation. Simulations with this model, which has come to be known as the FAB mechanism, were carried out by applying certain simplifying assumptions; these assumptions were, in fact, so broad in scope that the eight-variable FAB mechanism was reduced to a two-variable approximate mechanism. The assumptions involved taking the other six species to be at steady or quasi-steady state. The two-variable approximation does show oscillatory behavior and has other features which are partially consistent with experimental observations. Quantitative comparison with experiment is not possible, however, because of the sweeping assumptions made in the reduction of the FAB mechanism.

A model identical to the FAB model in all respects *except* that the NAD• termination reaction is taken to be bimolecular was proposed by Aguda and Clarke.[115] This model, known as model A, exhibits bistability and simple periodic behavior[116] but is apparently not capable of producing complex oscillations or chaos. Aguda and Larter[91] proposed a modification of model A in which a reaction involving oxygen could serve to "force" the nonautocatalytic feedback loop. This model is referred to as model C (for "chaos") and includes the fifth, and last, enzyme species, Per^{2+} by including the steps indicated by a dashed line in Figure 5. An additional difference between models A and C is the inclusion of a step for the flow of NADH into the system; NADH, therefore, becomes a time-dependent variable with this change. Simulations with model C[117] revealed the existence of simple oscillations followed by a period doubling sequence of bifurcations which occurred when the total enzyme concentration was varied. This sequence of period doublings resulted in chaos, with a next-amplitude map that was similar to that seen experimentally.

A recent flurry of papers reporting new models[84,95,108] and new experimental observations[85] have served to focus attention on a subset of the steps comprising the so-called "detailed models" described above. It has often been assumed that only a subset of the long list of reactions initially included by Yokota and Yamazaki are essential for complex behavior (i.e. oscillations and chaos) but determining which subset to retain has taken many years and many experimental investigations to ascertain. It seems that a consensus is finally emerging regarding the essential subset of steps which lead to *oscillatory* behavior in the PO reaction; determining the source of *chaotic* behavior remains more elusive, however. The possibilities have begun to narrow considerably, but there are still some disagreements as to the roles which different steps play in the origin of chaotic behavior in the PO reaction network.

The Urbanalator (see Figure 6), one of the more recently proposed models, was created by considering only steps which had been directly observed experimentally during the course of oscillations and not inferred from other studies. It is similar to model A,[116,117] but differs in several steps. Model A's essential feature of a double autocatalytic cycle is retained in the Urbanalator as well as in two recent modifications, also depicted in Figure 6. Two species, DCP and methylene blue (MB), have not often been included in any of the detailed models because so little is known about their role in producing oscillations but attempts are made to do so in these recent modeling efforts. Both species were reported, early on, to be necessary for stabilizing oscillatory behavior, but oscillations have recently been observed when both DCP[95] and MB[49] are absent. However, chaotic behavior and associated complex periodic oscillations

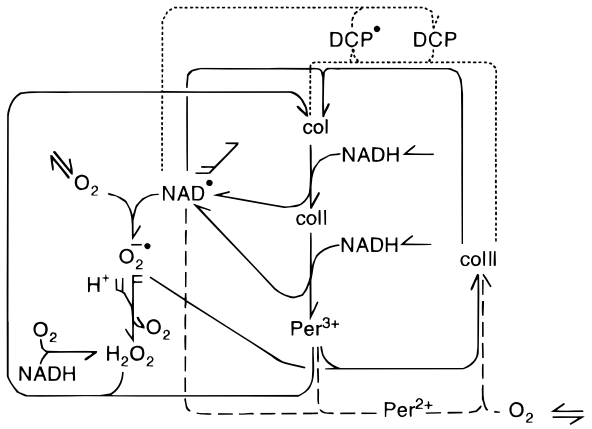


Figure 6. Recent chemically detailed models: (solid line) "the Urbanalator", ref 95; (dashed line) addition of Per^{2+} by Odense group, ref 84; (dotted line) suggested mechanism of DCP modulation of dynamics, ref 119; (double tails on arrows indicative of second-order reactants, i.e. dimerization of NAD• is second order in NAD•.

have not, as yet, been observed in the absence of these two species, so their role in the origin of chaos is still unclear.

The Urbanalator and model A differ in three important ways: (1) the Urbanalator includes the direct conversion of superoxide radical into hydrogen peroxide via H^+, i.e. $2H^+ + 2O_2^{\bullet-} \rightarrow O_2 + H_2O_2$; (2) the Urbanalator also includes the direct oxidation of NADH by molecular oxygen, $NADH + O_2 + H^+ \rightarrow NAD^+ + H_2O_2$, a step which was not included in *any* of the previously suggested models (including model A) but which may be very important, particularly in the presence of MB which catalyzes this reaction;[62] and (3) a reaction in model A, $NADH + O_2^{\bullet-} \rightarrow H_2O_2 + NAD^{\bullet}$, is eliminated from the Urbanalator since reaction 6, consumption of NAD• to form superoxide or hydroperoxide radical, is nearly diffusion limited. The inclusion of NAD• formation from superoxide in models of the PO reaction can be traced to an early suggestion of Yokota and Yamazaki[1] (1965) who, while not reporting direct experimental evidence of its existence, reiterated its importance[60] in 1977 by stating that it "*appears to be an essential reaction in propagation of the chain reaction*". The success of the Urbanalator and its derivatives in reproducing experimental observations of oscillation makes this reasoning less compelling.

The second of the new steps introduced in the Urbanalator, the oxidation of NADH by molecular oxygen, is catalyzed by methylene blue (MB), although this has only been studied at pH 8−9, much higher than the normal pH 5.1 used in experimental studies of the PO reaction. Olson *et al.* suggested[95] a catalytic role for the species MB^+ involving the intermediate MBH, but did not consider this cycle explicitly in simulations with the Urbanalator. Ross and co-workers[108] have studied the role of the additional steps involving MB using stoichiometric network analysis[118] and concluded that no essential change in the stability properties occurs whether MB is explicitly included or not. Bronnikova *et al.*[84] also did not include MB explicitly in their simulations, all of which supports the assumption that this species can be taken to be proportional to the rate constant of this step.

Simulations with the Urbanalator revealed the existence of simple oscillations when experimentally realistic rate constants were used. No evidence of complex oscillations or chaos was reported for this model. To study the origin of chaotic behavior, Bronnikova *et al.* suggested the addition of the same two steps to the Urbanalator which were used to transform model A into model C. Bronnikova *et al.*[84] found that adding these steps to the Urbanalator produced a model which exhibited complex oscillations and chaos, arising via a period-doubling route and described by a chaotic attractor that bears a remarkable resemblance to that observed experimentally by Geest *et al.*,[109] as shown in Figure 1. Even more recently, additional modifications were made by Hemkin and Larter[119] to include 2,4-dichlorophenol specifically and to study the effect of varying the pH. These latter studies indicate that the range of conditions over which complex behavior is observed widens at higher pHs than normally used in experimental studies of the oscillations. The role of DCP may or may not be as indicated in Figure 6; while the simulations agree qualitatively with experiment in some regards, some quantitative disagreement still remains.

V. Critical Comments and Connection between Models and Experiments

There is no shortage of confusion in the literature concerning the mechanisms of action of peroxidase, although clarifying data continue to accumulate.[40] Part of this is due to the difficulties in obtaining accurate experimental rate constants. Some modeling studies feature random mixing of order-of-magnitude estimated rate constants with those found from experiments or interpretation of models. At times it is difficult to derive unique meanings for dynamics demonstrated by models. Additional difficulty comes from the complex nature of the system, where species with weak signatures to many instrumental methods have a substantial effect on reaction rates. Species which may not be directly detected in any experiment (for example, ferroperoxidase in HRP oscillator data published prior to 1996) may nevertheless be present in sufficient quantity to be important in the dynamics. Species which are generally regarded as radical sources may, under some circumstances, act as radical scavengers,[120−122] going against chemical intuition and further complicating a comparison of models with experimental data. A reaction by reaction critique of some of the rate constants extracted from the literature is available from the authors as a .DBF file posted on the Internet. Recommended values appear in Table 1.

A. Critique of Experiments

Much care is required to carry out experiments in a definitive manner on the PO system. A number of factors which have not been universally dealt with adequately are discussed in the paragraphs which follow.

Whether one characterizes all reactants or not, all that are present may influence the course of reaction. Almost all measurements use enzyme as-received

from commercial sources. Boehringer-Mannheim has been the primary source for many of the experiments; supposedly HRP from others sources fails to give oscillations, but it is unknown if this is due to impurities in the other sources or in the Boehringer-Mannheim material. Perhaps isozyme distribution is a factor. Apparently Boehringer's stock product is at least 90% isozyme C, which would suggest either that isozyme C uniquely supports oscillations or that conditions under which this isozyme supports oscillations differ significantly from those for other isozymes.

Species known to be present but presumed to be inert may in fact be reactive. Peyton *et al.*[123] have shown that acetate buffer is reactive with superoxide radical. Sychev[124,125] notes that ethanol is a radical scavenger, so that the ethanol concomitant with DCP may be a reactant, not a spectator. Ethanol modulation of the activity of catalase, which is structurally similar to peroxidase, has been seen by Davison and co-workers.[126]

pH influences many of the rate constants. Only recently have oscillations at pH other than 5.1 been studied in any detail. It has been with such experiments that the important role of Per^{2+} has become clear. Experiments at higher pHs have revealed new types of complex dynamics, further complicating the situation and indicating that experimentally measured rate constants need to be found for a range of pH values.

Reactors and other apparatus components are reactive, yet excluded from all quantitative explanations in the literature. One thesis details the effects of unpickled (surface not passivated by oxidation in nitric acid) stainless steel, various polymers, and Pyrex glass in contrast to quartz.[127] Others have related similar experiences as orally transmitted conventional wisdom. Only one experiment has demonstrated that it is unlikely that Fe^{3+}, corroded from stainless steel, is the culprit in causing observed effects.[69] Other suggestions remain untested and must be regarded as merely speculative.

Temperature affects all reactions; however, its effect on the PO oscillator system has not been systematically studied. Most experiments have been carried out in thermostated reactors, but different labs do not always use the same temperature (although 20−30 °C is the typical range).

As with most work involving oscillating reactions, there is an implicit assumption in all work done on the PO reaction that the solution is homogeneous or, at least, that mixing is fast compared to all chemistry. By analogy to careful work on the effects of stirring on the Belousov−Zhabotinskii reaction by Dutt and Menzinger[128,129] and Schneider *et al.*,[130] it is likely that there are regimes where heterogeneity influences observed dynamics. Assorted cell geometries including hexagonal, square, and round, with stirring centered and off-center have been reported. No measurements of reactant spatial distribution have appeared.

Experiments last for hours, even days. It is presumed that only reactions involving oscillations affect reagents, but this is not true. NADH, particularly, is subject to slow degradation by hydrolysis and anomerization.[131−134] This oversight is not unique to PO oscillator research. Less than 1% of papers employing NADH as a reactant cite these papers or account for the hydration and cyclization of NADH. Measured rate constants for anomerization, addition of water across the double bond β to the pyridinium nitrogen, and cyclization of NADH are given in the rate constant table, Table 1. A caveat is that data were not obtained from deaerated solutions (P. Brown, personal communication, March, 1996), so some oxygen chemistry may have occurred without being taken into account. We have preliminary indications that oxygen, dissolved in solution in equilibrium with air, can accelerate NADH decomposition by as much as 30%. It is premature to suggest a cause for this observation. In any event, while the tacit presumption in our concern here is that β-NADH is the primary electrochemically active dinucleotide species, with other, related species possibly behaving differently, there are no literature data to our knowledge which address the issue. Both the geometric selectivity of enzymes and the importance of conjugation in effecting molecular redox behavior seed this concern.

It is often difficult to describe the exact sequence of parameters leading to a given dynamical state. Particularly when a specific condition, accessible only over a narrow range of parameters is sought, various flow rates may have been "tweaked" to arrive at a given asymptotic state. In addition, irreproducibility in reactant flow rates may make it impossible to know experimental conditions precisely. While chaotic systems are sensitive to initial conditions, it is not the case that all irreproducibility is due to this particular sensitivity. While Aguda *et al.*[135] demonstrated how short-term excursions in concentration can shift asymptotic dynamics, the whole issue of how reaction initiation sequences and reactant concentration choices have biased our view of this system has not been addressed. Ross's work with step perturbations[108,118] at least potentially may clarify some of this ambiguity.

Effects of trace impurities, including heavy metal ions, Ca^{2+}, Mg^{2+}, and minute amounts of impurity enzymes (including most importantly catalase and superoxide dismutase) have generally received little attention. Our experiments with Mn^{2+} have not been published,[68] as it appeared that the results could be explained as Mn^{2+} interacting with superoxide and H_2O_2, with the peroxidase system providing a reservoir of reactants which interacted with Mn^{2+} linearly. This would only change steady-state concentration levels, not overall dynamics. Fe^{3+} has been even less directly studied.[69] Considering that weak interactions with multiple species probably defines the actual biological situation, this is an area worthy of greater study.

What previously unidentified species are critical to the mechanism? The recent work in Olsen's lab has established a role for Per^{2+} most clearly. What about NAD^+, $(NAD)_2$, isozymes, the various P-6*xx* compounds (P-670 for example), and assorted enzyme forms bearing the scars of free radical oxidation? What proof is there that the stoichiometry of the oscillator is even $O_2 + 2NADH + 2H^+ \rightarrow 2H_2O +$

$2NAD^+$? Much "obvious", simple work remains to be done.

B. Critique of Theories

Some of the qualitatively most successful theories have been chemically nonspecific. Most particularly, Olsen's 1983[112] model using species A, B, X, and Y, with A ~ [O_2], B ~ [NADH], X and Y unspecified, had the best qualitative match between theory and experiment available for over a decade. Olsen speculated that one rate constant (k_1) was proportional to [peroxidase], and another (k_3) was proportional to [DCP],[136] but these assignments were not based on any rigorous fitting of model results to experiment. Also the chemical identity of X and Y were speculative; possibilities include X = $NAD^•$ and Y = coIII. Even though justification was lacking then, in recent work, built upon the Olson, Williksen, and Scheeline model,[95] it has almost been possible to establish mechanistic relationships with experimental variables for all steps in the Olsen 1983 model.[110] One can also show that the Bronnikova modification of the Olson *et al.*[84] model (which includes Per^{2+}) can be reduced to the Olsen 1983 model, at least during the time when oxygen concentration is increasing. We are close to the point where abstract models and chemically detailed models can be mapped onto each other. So even though the early abstract models were more speculative than one might have liked, they are turning out to be quite close to the probable mechanism of this reaction.

Some modeling studies have used rate constants blatantly at odds with experimental reality. In fact, one of our own early attempts with model C produced oscillations only with rate constants far from experimental values. Similarly, the recent Ross model[108] requires the rate of $NADH + H_2O_2 \rightarrow NAD^+ + H_2O$ to be rapid when in fact, experimentally, no reaction occurs (at least in the absence of a catalyst).[137] The model also hypothesizes reactions between DCP and other species without any experimental basis other than the need to tightly interconnect portions of the reaction network to fit with the Jacobian experimentally measured for the system. Again, as in the early work with model C, oscillatory and chaotic behavior appears when rate constant values that are not experimentally supported are used. Building on such models, or testing them experimentally, is difficult.

Many of the so-called chemically realistic models are incomplete, in that they account for only a subset of species known to be present in the experimental system. It is one thing to demonstrate that a species is irrelevant after including it in a model (perhaps because it is in steady state with some other species, and so can effectively be modeled kinetically as being that other species). It is quite something else to simply ignore it. All models which leave out MB or DCP when the experimental systems include these species are subject to this limitation; however, as discussed earlier, accurately including these species in the models requires more detailed experimental information.

The abstract models explicitly attempt to account for observed system nonlinear dynamics; chemically realistic models typically are assembled in the hope that such nonlinearities will be manifest, but avoid forcing the issue. Any model which cannot be related to the dynamical features of experimental data, no matter how logical on a chemical or biochemical basis, is suspect. Yokota and Yamazaki's earliest models could not avoid this problem, as they appeared before the field of nonlinear dynamics had been developed.

The need to fit experimental measurements to model parameters has, at times, led to misconstruing of experimental variables. For example, oxygen transfer between gas and liquid phases depends on a rate term with

$$d[O_{2,l}]/dt = k_f[O_{2,g}] - k_r[O_{2,l}]$$

In some theories, k_f and k_r were taken as being identical. In fact, $k_f/k_r = k_H$, the Henry's Law constant for the given solution temperature and ionic strength.[138] Only if one takes [$O_{2,g}$] as a fittable constant can one get away with setting the two k's equal, and since both oxygen concentrations are measurable, such fitting is unjustifiable. Similarly, DCP is typically injected into the reaction in ethanol solution. While ethanol alone does not cause a noticeable perturbation to the reaction, it may still influence dynamics during a radical burst initiated by other constituents of the oscillator.

Lastly, models have to take into account the limitations of experiments, so that one does not overfit data. Just as models idealize details of speciation, so experiments are subject to noise, finite sensor response time, blanks, and interferences. In deriving models from data, one ignores such measurement limitations at great peril.

VI. Future Directions

Presumably with the substantial effort being made to understand the PO oscillator, the essentials of the mechanism involving HRP-derived peroxidase will soon be clear. Hints at subsequent useful research directions are already appearing. Mammalian, bacterial, and fungal enzymes have recently come under more extensive study, and differences in rates of various fundamental steps are noted compared to HRP-catalyzed oscillations.[49] This may be due, in part, to the multiple associated subunits of many mammalian peroxidases; plant peroxidases tend to be monomers. While photochemistry has been known to be important in this system for many years,[98,139] models which include the effects of photoexcitation and photogeneration of radicals have not been experimentally tested. Fed'kina *et al.*[139] have predicted that modulation of illumination of the PO system should be able to drive it into chaos without requiring DCP or other additional reactant. This may be a specific case of use of parameter modulation to control reaction dynamics. While Schneider *et al.*[140−142] have succeeded in controlling the PO system using active feedback, neither passive control nor arbitrary driving of the system into and out of chaos has been achieved. Various approaches in the literature may well give rise to such control.[143−145]

Finally, complex oscillations are likely to be a general behavior in biology. There are indications,

Table A-1. Hauser and Olsen Molar Absorptivities[150]

	ϵ'_{360}	ϵ'_{403}	ϵ'_{418}	ϵ'_{439}
NADH	4.3×10^3	50	0	0
Per^{2+}	3.8×10^4	5.1×10^4	6.6×10^4	8.9×10^4
Per^{3+}	4.3×10^4	1.0×10^5	5.4×10^4	1.3×10^4
coI	3.5×10^4	5.4×10^4	3.9×10^4	1.5×10^4
coIII	2.7×10^4	6.5×10^5	1.06×10^5	2.9×10^4

Table A-2. Olson, Williksen, and Scheeline Molar Absorptivities[95]

	ϵ'_{360}	ϵ'_{402}	ϵ'_{418}
NADH	7.8×10^3	~0	0.00
Per^{2+}	ignored	ignored	ignored
Per^{3+}	ignored	8.19×10^4	4.58×10^4
coIII	ignored	5.01×10^5	8.77×10^4

for example, that catalase may also display oscillatory behavior under some conditions.[126] Yet, the peroxidase system is the most thoroughly studied oscillatory enzyme system (exceeding in detail and volume the work done on glycolysis). One may anticipate extension of the approaches described here to other radical-generating enzyme systems, and also to allosterically regulated enzymes, which are far more numerous and subtle in their dynamics than the rather blatant radical chain oscillators of which the peroxidase system is archetypal.

VII. Appendix. Useful Constants for Characterizing Reactants Involved in the Peroxidase–Oxidase Reaction

A. Absorption Spectroscopy

Qualitative spectra for the various redox states of HRP can be found in Yamazaki and Yokota.[38,60] Molar absorptivities at a set of wavelengths useful for quantification of the various redox states are quoted from Hauser and Olsen[150] (Table A-1). All units are M^{-1} cm^{-1}, pH 6.3.

We have previously reported[95,98,99] different values for some molar absorptivities at pH 5.1 (Table A-2). These values are based on a presumed molecular weight of 42 100 Dalton, and presume no impurities in the peroxidase as received from Boehringer-Mannheim. Considering that the preponderance of literature values as cited in[95] give ϵ_{402} for Per^{3+} of $\sim 1.0 \times 10^5$ M^{-1} cm^{-1}, and that all these apparent absorptivities are below both Olsen and Hauser and also below a preponderance of other literature by a mean of 23%, it is likely that impurities in the enzyme led to an overestimation of the amount taken, and thus an underestimation of the molar absorptivities. A reliable literature value[151] gives ϵ_{340}(NADH) (the absorption maximum) of 6.22×10^3, which is more nearly consistent with the Hauser–Olsen result.

Additional useful absorptivities and wavelengths are given in Table A-3.

B. Electrochemistry and Equilibrium

pK_a values for common components of the PO system at or near 25 °C are given in Table A-4.

Table A-3. Additional Molar Absorptivities

substance	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})	ref(s)
2,4-dichlorophenol	524	2.05×10^4	105
methylene blue	668	6.4×10^4	152
$NAD^\bullet$	250	1.9×10^4	57, 153, 154
	400	2.5×10^3	
$(NAD)_2$	250	3.7×10^4	57, 153, 154
	340	6.5×10^3	

Table A4

substance	pK_a	ref
$NADH^{\bullet+}$	−3.50	86
$HO_2^\bullet$	4.88	56
MB	3.80	155
DCP	7.89	156

Table A-5. Electrochemical Data for Half-Reactions Associated with the PO Oscillator[a]

half-reaction	E_0 or E'_0, V	pH (for E'_0)	ref(s)
$NAD^+ + 2e \rightarrow NADH + H^+$	−0.3113	7.0 (μ = 0.1 M)	157
$NAD^+ + e \rightarrow NAD^\bullet$	−0.922		158
	−0.94		159
$NAD^\bullet + e \rightarrow NADH$	0.28	0	158
	0.30		159
$NADH^{\bullet+} + e \rightarrow NADH + H^+$	0.76	7.0	160, 161
$MB^+ + 2e \rightarrow MBH$	0.10	5.1	162
$O_2 + e \rightarrow O_2^{\bullet-}$	−0.33	7.0	163
$O_2 + 2e + 2H^+ \rightarrow H_2O_2$	0.281	7.0	163
$O_2 + 3e + 3H^+ \rightarrow H_2O + OH^\bullet$	0.31	7.0	163
$O_2 + 4e + 4H^+ \rightarrow 2H_2O$	0.815	7.0	163
$O_2^{\bullet-} + e + 2H^+ \rightarrow H_2O_2$	0.89	7.0	163
$O_2^{\bullet-} + 2e + 3H^+ \rightarrow H_2O + OH^\bullet$	0.64	7.0	163
$O_2^{\bullet-} + 3e + 4H^+ \rightarrow 2H_2O$	0.09	7.0	163
$H_2O_2 + e + H^+ \rightarrow H_2O + OH^\bullet$	0.38	7.0	163
$H_2O_2 + 2e + 2H^+ \rightarrow H_2O$	1.349	7.0	163
$OH^\bullet + e + H^+ \rightarrow H_2O$	2.31	7.0	163
$2,4\text{-DCP}^\bullet + e + H^+ \rightarrow 2,4\text{-DCP}$	0.66[b]		164
$Per^{5+} + e + H^+ \rightarrow Per^{4+}$	0.88		165
$Per^{4+} + e + H^+ \rightarrow Per^{3+}$	0.90		165

[a] E_0 shown if pH unspecified, E'_0 for specific pH. Ionic strength m specified where known. [b] Half-wave potential vs SCE, not E_0 vs SHE.

E_0 values (or E'_0 where pH is specified in the literature) for half-reactions important in the PO system are tabulated in Table A-5.

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Note Added in Proof

Rate constants for the interaction of Mn(II) with H_2O_2 and superoxide are now available.[166]

IX. References

(1) Yokota, K.; Yamazaki, I. *Biochim. Biophys. Acta* **1965**, *105*, 301.
(2) Ott, E. *Chaos in Dynamical Systems*; Cambridge University Press: Cambridge, UK, 1993.
(3) Holden, A. V. *Chaos*; Princeton University Press: Princeton, NJ, 1986.
(4) Scott, S. K. *Chemical Chaos*; Oxford University Press: Oxford, UK, 1991.
(5) Goldbeter, A. *Biochemical Oscillations and Cellular Rhythms*; Cambridge University Press: Cambridge, UK, 1996.

(6) Larter, R.; Olsen, L. F.; Steinmetz, C. G.; Geest, T. In *Chaos in Chemistry and Biochemistry*; Field, R. J., Györgyi, L., Eds.; World Scientific Pub.: London, 1993; Chapter 7.
(7) Welinder, K. G. *Eur. J. Biochem.* **1979**, *96*, 483.
(8) Dunford, H. B. In *Peroxidases in Chemistry and Biology*; Everse, J., Grisham, M. B., Everse, K. E., Eds.; CRC Press: Cleveland, 1991; Vol. II.
(9) Chang, C. S.; Yamazaki, I.; Sinclair, R.; Khalid, S.; Powers, L. *Biochemistry* **1993**, *32*, 923.
(10) Veitch, N. C.; Gilfoyle, D. J.; White, C. G.; Smith, A. T. In *Plant Peroxidases: Biochemistry and Physiology*; Obinger, C., Burner, U., Ebermann, R., Penel, C., Greppin, H., Eds.; University of Geneva: Geneva, 1996; p 1.
(11) Schuller, D. J.; Ban, N.; van Huystee, R. B.; McPherson, A.; Poulos, T. L. *Structure* **1996**, *4*, 311.
(12) Shannon, L. M.; Kay, E.; Lew, J. Y. *J. Biol. Chem.* **1966**, *241*, 2166.
(13) Shimizu, N.; Kobayashi, K.; Hayashi, K. *Biochim. Biophys. Acta* **1989**, *995*, 133.
(14) Yamazaki, I.; Tamura, M.; Nakajima, R. *Mol. Cell. Biochem.* **1981**, *40*, 143.
(15) Yamazaki, I.; Nakajima, R. In *Molecular and Physiological Aspects of Plant Peroxidases*; Greppin, H., Penel, C., Gaspar, Th., Eds.; University of Geneva: Geneva, 1986; p 71.
(16) Aibara, S.; Yamashita, H.; Mori, E.; Kato, M.; Morita, Y. *J. Biochem.* **1982**, *92*, 531.
(17) Aibara, S.; Kobayashi, T.; Morita, Y. *J. Biochem.* **1981**, *90*, 489.
(18) Olsen, L. F.; Degn, H. *Biochim. Biophys. Acta* **1978**, *523*, 321.
(19) Halliwell, B. *Planta* **1978**, *140*, 81.
(20) Raven, P. H.; Evert, R. F.; Curtis, H. *Biology of Plants*, 3rd ed.; Worth: New York, 1981.
(21) Wallace, R. A.; King, J. L.; Sanders, G. P. *Biology: The Science of Life*, 2nd ed.; Scott, Foresman, and Co.: Glenview, 1986.
(22) Mader, M.; Amberg-Fisher, V. *Plant Physiol.* **1982**, *70*, 1128.
(23) Van Huystee, R. B.; Lobarzewski, J. *Plant Sci. Lett.* **1982**, *27*, 59.
(24) Harkin, J. M.; Obst, J. R. *Science* **1973**, *180*, 296.
(25) Campa, A. In *Peroxidases in Chemistry and Biology*; Everse, J., Grisham, M. B., Everse, K. E., Eds.; CRC Press: Boca Raton, 1991; Vol. 2.
(26) Halliwell, B.; Gotteridge, J. M. C. In *Methods in Enzymology*; Parker, L., Glaser, A. N., Eds.; **1990**, *186*, 1.
(27) Cross, A. R. In *The Molecular Basis of Oxidative Damage by Leukocytes*; Jesaitis, A. J., Dratz, E. A., Eds.; CRC Press: Boca Raton, FL, 1992; p 37.
(28) Sbarra, A. J., Strauss, R. R., Eds. *The Respiratory Burst and Its Physiological Significance*; Plenum Press: New York, 1988.
(29) Das, K. C.; Misra, H. P. *J. Biol. Chem.* **1992**, *267*, 19172.
(30) van der Pol, B.; van der Mark, J. *Nature* **1927**, *120*, 363.
(31) Markas, M.; Kuschmitz, D.; Hess, B. *FEBS Lett.* **1984**, *172*, 235.
(32) Peters, A.; Palay, S. L.; Webster, H. de F. *The Fine Structure of the Nervous System: Neurons and Their Supporting Cells*, 3rd ed.; Oxford Univ. Press: New York, 1991.
(33) Hool, K.; Nieman, T. A. *Anal. Chem.* **1988**, *60*, 834.
(34) Chance, B. *Arch. Biochem.* **1949**, *22*, 224.
(35) George, P. *Biochem. J.* **1953**, *54*, 267.
(36) Chance, B.; Powers, L.; Ching, Y.; Poulos, T.; Schonbaum, G. R.; Yamazaki, I.; Paul, K. G. *Arch. Biochem. Biophys.* **1984**, *235*, 596.
(37) Yamazaki, I.; Mason, H. S.; Piette, L. *J. Biol. Chem.* **1960**, *235*, 2444.
(38) Yamazaki, I.; Yokota, K. *Mol. Cell. Biochem.* **1973**, *2*, 39.
(39) Kettle, A. J.; Winterbourn, C. C. *Biochem. J.* **1988**, *252*, 529.
(40) Kettle, A. J.; Winterbourn, C. C. In *Plant Peroxidases: Biochemistry and Physiology*; Obinger, C., Burner, U., Ebermann, R., Penel, C., Greppin, H., Eds.; University of Geneva: Geneva, 1996; p 134.
(41) Dawson, J. H. *Science* **1988**, *240*, 433.
(42) Arnao, M. B.; Acosta, M.; del Rio, J. A.; Varon, R.; Garcia-Canovas, F. *Biochim. Biophys. Acta* **1990**, *1041*, 43.
(43) Kayanoki, Y.; Fujii, J.; Islam, K. N.; Suzuki, K.; Kawata, S.; Matsuzawa, Y.; Taniguchi, N. *J. Biochem.* **1996**, *119*, 817.
(44) Pryor, W. A. *Free Radicals in Biology*; Academic Press: New York, 1976; Vol. I.
(45) Buxton, G. V.; Greenstock, C. L.; Helman, W. P.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1988**, *17*, 513.
(46) Helman, W. P.; Ross, A. B. *RCDC Databases*, Radiation Chemistry Data Center, University of Notre Dame, Notre Dame, IN. rcdc@rcdvax.rad.nd.edu.
(47) Gross, G. G.; Janse, C.; Elstner, E. F. *Planta* **1977**, *136*, 271.
(48) Mader, M.; Fussl, R. *Plant Physiol.* **1982**, *70*, 1132.
(49) Kummer, U.; Valeur, K. R.; Baier, G.; Wegmann, K. L.; Olsen, L. F. *Biochim. Biophys. Acta* **1996**, *1289*, 397.
(50) Degn, H. In *Biological and Biochemical Oscillators*; Chance, B., Pye, E. K., Ghosh, A. K., Hess, B., Eds.; Academic Press: New York, 1973; p 97.
(51) Watanabe, N.; Inaba, H. *Photochem. Photobiol.* **1993**, *57*, 570.
(52) Yamazaki, I.; Yokota, K.-N. In *Biological and Biochemical Oscillators*; Chance, B., Pye, E. K., Ghosh, A. K., Hess, B., Eds.; Academic Press: New York, 1973; p 109.
(53) Harrison, D. E. F. In *Biological and Biochemical Oscillators*; Chance, B., Pye, E. K., Ghosh, A. K., Hess, B., Eds.; Academic Press: New York, 1973; p 399.
(54) Clarke, B. L. In *Adv. Chem. Phys.*, *43*; Prigogine, I., Ross, J., Eds.; Academic Press: New York, 1980; p 1.
(55) Bielski, B. H. J.; Schwarz, H. A. *J. Phys. Chem.* **1968**, *72*, 3836.
(56) Behar, D.; Czapski, G.; Rabani, J.; Dorfman, L. M.; Schwarz, H. A. *J. Phys. Chem.* **1970**, *74*, 3209.
(57) Land, E. J.; Swallow, A. J. *Biochim. Biophys. Acta* **1971**, *234*, 34.
(58) Bielski, B. H. J.; Allen, A. O. *J. Phys. Chem.* **1977**, *81*, 1048.
(59) Bernofsky, C.; Wanda, S.-Y. C. *J. Biol. Chem.* **1982**, *257*, 6809.
(60) Yokota, K.; Yamazaki, I. *Biochemistry* **1977**, *16*, 1913.
(61) Williams, D. C.; Seitz, W. R. *Anal. Chem.* **1976**, *48*, 1478.
(62) Sevcik, P.; Dunford, H. B. *J. Phys. Chem.* **1991**, *95*, 2411.
(63) Hauser, M. J. B. Untersuchungen zum Reaktionsmechanismus des "Methylenblau-Oszillators" (Study of Reaction Mechanisms of "Methylene Blue Oscillators"). Diploma thesis, Universitats Würzburg, 1989.
(64) Resch, P.; Munster, A. F.; Schneider, F. W. *J. Phys. Chem.* **1991**, *95*, 6270.
(65) Akazawa, T.; Conn, E. E. *J. Biol. Chem.* **1958**, *232*, 403.
(66) Cabelli, D. E.; Bielski, B. H. J. *J. Phys. Chem.* **1984**, *88*, 3111.
(67) Cabelli, D. E.; Bielski, B. H. J. *J. Phys. Chem.* **1984**, *88*, 6291.
(68) Lymburner, J. Effects of Manganese on the Peroxidase-NADH Biochemical Oscillator. B.S. thesis, University of Illinois at Urbana-Champaign, December, 1994.
(69) Lvovich, V.; Scheeline, A. *Arch. Biochem. Biophys.* **1995**, *320*, 1.
(70) Kong, S.; Liochev, S.; Fridovich, I. *Free Rad. Biol. Med.* **1992**, *13*, 79.
(71) Adler, A. J.; Caruso, C.; Berlyne, G. M. *Nephron* **1995**, *69*, 34.
(72) Park, J. W.; Yun, S. H.; Park, K. K. *Bull. Korean Chem. Soc.* **1988**, *9*, 298.
(73) Halliwell, B.; de Rycker, J. *Photochem. Photobiol.* **1978**, *28*, 757.
(74) Kashem, M. A.; Dunford, H. B. *Biochem. Cell Biol.* **1986**, *64*, 323.
(75) Dolman, D.; Newell, G. A.; Thurlow, M. D.; Dunford, H. B. *Can. J. Biochem.* **1975**, *53*, 495.
(76) Bielski, B. H. J.; Gebicki, J. M. *Biochim. Biophys. Acta* **1974**, *364*, 233.
(77) Rotilio, G.; Falcioni, G.; Fioretti, E.; Brunori, M. *Biochem. J.* **1975**, *145*, 405.
(78) Yamazaki, I.; Piette, L. H. *Biochim. Biophys Acta* **1963**, *77*, 47.
(79) Kobayashi, K.; Hayashi, K.; Swallow, A. J. *Biochemistry* **1990**, *29*, 2080.
(80) Wittenberg, J. B.; Noble, R. W.; Wittenberg, B. A.; Antonini, E.; Brunori, M.; Wyman, J. *J. Biol. Chem.* **1967**, *242*, 626.
(81) Adediran, S. A.; Lambeir, A.-M. *Eur. J. Biochem.* **1989**, *186*, 571.
(82) Tamura, M.; Yamazaki, I. *J. Biochem.* **1972**, *71*, 311.
(83) Fed'kina, V. R.; Ataullakhonov, F. I.; Bronnikova, T. V.; Balabaev, N. K. *Studia Biophys.* **1978**, *72*, 195.
(84) Bronnikova, T. V.; Fed'kina, V. R.; Schaeffer, W. M.; Olsen, L. F. *J. Phys. Chem.* **1995**, *99*, 9309.
(85) Hauser, M. J. B.; Olsen, L. F. *J. Chem. Soc., Faraday Trans.* **1996**, *92*, 2857.
(86) Carlson, B. W.; Miller, L. L.; Neta, P.; Grodkowski, J. *J. Am. Chem. Soc.* **1984**, *106*, 7233.
(87) Samec, Z.; Bresnahan, W. T.; Elving, P. J. *J. Electroanal. Chem.* **1982**, *133*, 1.
(88) Blankespoor, R. L.; Miller, L. L. *J. Electroanal. Chem.* **1984**, *171*, 231.
(89) Engstrom, R. C.; Weber, M.; Wunder, D. J.; Burgess, R.; Winquist, S. *Anal. Chem.* **1986**, *58*, 844.
(90) Ataullakhanov, F. I.; Zhabotinskii, A. M. *Biophysica* **1975**, *20*, 596.
(91) Aguda, B. D.; Larter, R. *J. Am. Chem. Soc.* **1990**, *112*, 2167.
(92) Avigliano, L.; Carelli, V.; Casini, A.; Finazzi-Agro, A.; Liberatore, F. *Biochim. Biophys. Acta* **1983**, *723*, 372.
(93) Avigliano, L.; Carelli, V.; Casini, A.; Finazzi-Agro, A.; Liberatore, F. *Biochem. J.* **1985**, *226*, 391.
(94) Thompson, D. R.; Larter, R. *Chaos* **1995**, *5*, 448.
(95) Olson, D. L.; Williksen, E. P.; Scheeline, A. *J. Am. Chem. Soc.* **1995**, *117*, 2.
(96) Janzen, E. G.; Zhang, Y.-K.; Arimura, M. *J. Org. Chem.* **1995**, *60*, 5434.
(97) Rastogi, R. P.; Srinivas, G.; Das, M. *Ind. J. Chem.* **1994**, *33A*, 750.
(98) Olson, D. L.; Scheeline, A. *J. Phys. Chem.* **1995**, *99*, 1204.
(99) Olson, D. L.; Scheeline, A. *J. Phys. Chem.* **1995**, *99*, 1212.
(100) Olsen, L. F. *Biochim. Biophys. Acta* **1978**, *527*, 212.
(101) Dunford, H. B.; Adeniran, A. J. *Arch. Biochem. Biophys.* **1986**, *251*, 536.
(102) Young, M.; Steelink, C. *Phytochemistry* **1973**, *12*, 2851.
(103) Oturan, M. A.; Pinson, J. *J. Phys. Chem.* **1995**, *99*, 13948.
(104) Sakurada, J.; Sekiguchi, R.; Sato, K.; Hosoya, T. *Biochemistry* **1990**, *29*, 4093.
(105) Metelitza, D. I.; Litvinchuk, A. V.; Savenkova, M. I. *J. Mol. Catal.* **1991**, *67*, 401.
(106) Courteix, A.; Bergel, A. *Enzyme Microb. Technol.* **1995**, *17*, 1094.

(107) Dordick, J. S.; Klibanov, A. M.; Marletta, M. A. *Biochemistry* **1986**, *25*, 2946.
(108) Hung, Y.-F.; Schreiber, I.; Ross, J. *J. Phys. Chem.* **1995**, *99*, 1980.
(109) Geest, T.; Steinmetz, C. G.; Larter, R.; Olsen, L. F. *J. Phys. Chem.* **1992**, *96*, 5678.
(110) Bensen, D. S.; Scheeline, A. *J. Phys. Chem.* **1996**, *100*, 18911.
(111) Degn, H.; Olsen, L. F.; Perram, J. W. *Ann. N. Y. Acad. Sci.* **1979**, *316*, 623.
(112) Olsen, L. F. *Phys. Lett.* **1983**, *94A*, 454.
(113) Gear, C. W. *Numerical Initial Value Problems in Ordinary Differential Equations*; Prentice-Hall: Englewood Cliffs, 1971.
(114) Fed'kina, V. R.; Ataullakhanov, F. I.; Bronnikova, T. V. *Biophys. Chem.* **1984**, *19*, 259.
(115) Aguda, B. D.; Clarke, B. L. *J. Chem. Phys.* **1987**, *87*, 3461.
(116) Aguda, B. D.; Larter, R.; Clarke, B. L. *J. Chem. Phys.* **1989**, *90*, 4168.
(117) Aguda, B. D.; Larter, R. *J. Am. Chem. Soc.* **1991**, *113*, 7913.
(118) Hung, Y.-F.; Ross, J. *J. Phys. Chem.* **1995**, *99*, 1974.
(119) Larter, R.; Hemkin, S. *J. Phys. Chem.* **1996**, *100*, 18924.
(120) Krylov, S. N.; Aguda, B. D.; Ljubimova, M. L. *Biophys. Chem.* **1995**, *53*, 213.
(121) Krylov, S. N.; Dunford, H. B. *J. Phys. Chem.* **1996**, *100*, 913.
(122) Krylov, S. N.; Dunford, H. B. In *Plant Peroxidases: Biochemistry and Physiology*; Obinger, C., Burner, U., Ebermann, R., Penel, C., Greppin, H., Eds.; University of Geneva: Geneva, 1996; p 59.
(123) Peyton, G. R.; Glaze, W. H. *Environ. Sci. Technol.* **1988**, *22*, 761.
(124) Sychev, A. Ya.; Skurlatov, Yu. I.; Isak, V. G.; Khiem, M. K. *Russ. J. Phys. Chem.* **1978**, *52*, 1685.
(125) Sychev, A. Ya.; Skurlatov, Yu. I.; Isak, V. G.; Khiem, M. K. *Russ. J. Phys. Chem.* **1978**, *52*, 1686.
(126) Davison, A. J.; Kettle, A. J.; Fatur, D. J. *J. Biol. Chem.* **1986**, *261*, 1193.
(127) Olson, D. L. Experimental and Theoretical Studies of the Peroxidase-NADH Biochemical Oscillator: an Enzyme-Mediated Chemical Switch. Ph.D. thesis, Univ. of Illinois, August, 1994; Dissertation Abstracts no. 9512502.
(128) Menzinger, M.; Dutt, A. K. *J. Phys. Chem.* **1990**, *94*, 4510.
(129) Dutt, A. K.; Menzinger, M. *J. Phys. Chem.* **1992**, *96*, 8447.
(130) Hauser, M. J. B.; Lebender, D.; Schneider, F. W. *J. Phys. Chem.* **1992**, *96*, 9332.
(131) Oppenheimer, N. J.; Kaplan, N. O. *Biochemistry* **1974**, *13*, 4675.
(132) Oppenheimer, N. J. In *Pyridine Nucleotide Coenzymes, Part A*; Dolphin, D., Avramovic, O., Poulson, R., Eds.; Wiley: New York, 1987; p 324.
(133) Miksic, J. R.; Brown, P. R. *Biochemistry* **1978**, *17*, 2234.
(134) Klemm, A.; Steiner, T.; Cumme, G. A.; Horn, A. *Anal. Biochem.* **1993**, *212*, 375.
(135) Aguda, B. D.; Hofmann Frisch, L.-L.; Olsen, L. F. *J. Am. Chem. Soc.* **1990**, *112*, 6652.
(136) Geest, T.; Olsen, L. F.; Steinmetz, C. G.; Larter, R.; Schaffer, W. M. *J. Phys. Chem.* **1993**, *97*, 8431.
(137) Schellenberg, K. A.; Hellerman, L. *J. Biol. Chem.* **1958**, *231*, 547.
(138) Olson, D. L.; Scheeline, A. *Anal. Chim. Acta* **1993**, *283*, 703.
(139) Fed'kina, V. R.; Ataullakhanov, F. I.; Bronnikova, T. V. *Theor. and Exper. Chem.* **1988**, *24*, 165.
(140) Forster, A.; Hauck, T.; Schneider, F. W. *J. Phys. Chem.* **1994**, *98*, 184.
(141) Lekebusch, A.; Forster, A.; Schneider, F. W. *J. Phys. Chem.* **1995**, *99*, 681.
(142) Forster, A.; Merget, M.; Schneider, F. W. *J. Phys. Chem.* **1996**, *100*, 4442.
(143) Petrov, V.; Gaspar, V.; Masere, J.; Showalter, K. *Nature* **1993**, *361*, 240.
(144) Luscher, E.; Hubler, A. *Helv. Phys. Acta* **1989**, *62*, 544.
(145) Mettin, R.; Lauterborn, W.; Hubler, A.; Scheeline, A. *Phys. Rev. E* **1995**, *51*, 4065.
(146) Nadezhdin, A.; Dunford, H. B. *J. Phys. Chem.* **1979**, *83*, 1957.
(147) Cheng, F. S.; Christian, G. D. *Anal. Chem.* **1977**, *49*, 1785.
(148) Sawada, Y.; Yamazaki, I. *Biochim. Biophys. Acta* **1973**, *327*, 257.
(149) Klein, M. L. Computational Studies of the Peroxidase Reaction. M.S. thesis. Indiana University—Purdue University at Indianapolis, 1992.
(150) Hauser, M. J. B.; Olsen, L. F. In *Plant Peroxidases: Biochemistry and Physiology*; Obinger, C., Burner, U., Ebermann, R., Penel, C., Greppin, H., Eds.; University of Geneva: Geneva, 1996; p 82.
(151) Rizzo, A.; Pande, A.; Luisi, P. L. In *Pyridine Nucleotide Coenzymes, Part A*; Dolphin, D., Avramovic, O., Poulson, R., Eds.; John Wiley: New York, 1987; p 102.
(152) Resch, P.; Field, R. J.; Schneider, F. W.; Burger, M. *J. Phys. Chem.* **1989**, *93*, 8181.
(153) Land, E. J.; Swallow, A. J. *Biochim. Biophys. Acta* **1968**, *162*, 327.
(154) Swallow, A. J. *Photochem. Photobiol.* **1968**, *7*, 683.
(155) Perrin, D. D. *Dissociation Constants of Organic Bases in Aqueous Solution*; Butterworths: London, 1965.
(156) Kortum, G.; Vogel, W.; Andrussow, K. *Dissociation Constants of Organic Acids in Aqueous Solution*; Butterworths: London, 1961.
(157) Miller, S. L.; Smith-Magowan, D. *J. Phys. Chem. Ref. Data* **1990**, *19*, 1049.
(158) Anderson, R. F. *Biochim. Biophys. Acta* **1980**, *590*, 277.
(159) Farrington, J. A.; Land, E. J.; Swallow, A. J. *Biochim. Biophys. Acta* **1980**, *590*, 273.
(160) Matsue, T.; Suda, M.; Uchida, I.; Kato, T.; Akiba, U.; Osa, T. *J. Electroanal. Chem. Interfacial Electrochem.* **1987**, 234, 163.
(161) Miller, L. L.; Valentine, J. R. *J. Am. Chem. Soc.* **1988**, *110*, 3982.
(162) Commission on Analytical Reactions and Applications. *Pure Appl. Chem.* **1978**, *50*, 488.
(163) *Oxygen Complexes and Oxygen Activation by Transition Metals*; Martell, A. E., Sawyer, D. T., Eds.; Plenum Press: New York, 1988; p 133.
(164) *CRC Handbook Series in Organic Electrochemistry*; CRC Press: Boca Raton, 1977; p 1.
(165) Yamazaki, I. In *Plant Peroxidases: Biochemistry and Physiology*; Obinger, C., Burner, U., Ebermann, R., Penel, C., Greppin, H., Eds.; University of Geneva: Geneva, 1996; p 34.
(166) Jacobsen, F.; Holcman, J.; Schested, K. *J. Phys. Chem. A* **1997**, *101*, 1324.

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