The Peroxidase-NADH Biochemical Oscillator. 2. Examination of the Roles of Hydrogen Peroxide and Superoxide

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The peroxidase-NADH oscillator examined here initially consists of a well-mixed aqueous solution of native horseradish peroxidase, reduced β-nicotinamide adenine dinucleotide (NADH), methylene blue (MB^+), and dissolved oxygen combined in a semi-batch reactor under a set of standard conditions. Hydrogen peroxide and superoxide have been implicated as important chemical intermediates. A comprehensive model which includes such intermediates and all initial chemical species has appeared elsewhere. To experimentally explore the role of hydrogen peroxide in the oscillator, H_2O_2 was substituted for MB^+ as an initial ingredient. This substitution allows relatively small, quasi-sinusoidal oscillations sensitive to the oxygen mass transport constant, and predicted earlier in a theoretical model. The oscillations become much larger when MB^+ is added, suggesting that MB^+ might serve as a chemical mediator between the small oscillations seen when H_2O_2 is substituted for MB^+, and the relatively large oscillations observed when MB^+ is present. Catalase and superoxide dismutase are used as enzymatic scavengers for H_2O_2 and $O_2^{\bullet-}$, respectively. The enzymes are added individually to a working oscillator at oxygen minima and maxima to examine the roles and approximate the concentrations of H_2O_2 and $O_2^{\bullet-}$. For the enzyme addition experiments, a perturbation model for oxygen behavior is proposed and applied to the interpretation of experimental data. Two methods of analysis for the addition of the enzyme probes indicate a higher concentration of H_2O_2 and $O_2^{\bullet-}$ at oxygen maxima than at minima. Comparison of experimental and simulated data indicate that the relatively simple model presented here is a reasonable, yet apparently incomplete, representation of oxygen dynamics for the addition of scavenger enzymes to this oscillator.

Introduction

The in vitro aerobic oxidation of reduced β-nicotinamide adenine dinucleotide (NADH) catalyzed by the enzyme horseradish peroxidase (HRP) is a single-enzyme system which allows direct observation of cyclic behavior under specific conditions. The system consists of common biochemicals and serves as an in vitro analogue for the development of methods and principles to directly observe and study the nonlinear dynamics of cyclic, biochemical reactions. This study specifically explores the roles of hydrogen peroxide (H_2O_2) and superoxide, $O_2^{\bullet-}$, both long suspected as important chemical intermediates in the nonlinear dynamics of the oscillator.

The peroxidase-NADH oscillator investigated here is an open system consisting of four chemical species combined in a batch reactor.[1,2] The well-mixed, aqueous solution initially consists of native horseradish peroxidase (Per^{3+}) and methylene blue (MB^+), to which NADH and oxygen are added at constant rates. Specific conditions result in oscillations in the concentration of numerous species over the course of several hours. The peroxidase-catalyzed oxidation of NADH

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

is a dominant reaction.

A significant number of papers on this oscillator have appeared which report a variety of nonlinear behaviors, including sustained or prolonged oscillations,[1-3] bistability,[4] chaos,[5-7] and a period-doubling approach to chaos.[8] Many models which include these features have been proposed for the peroxidase-NADH oscillator.[9-13]

A comprehensive, experimentally based, chemical model was proposed to explain the simple oscillatory behavior.[14] This standardized model, termed the Urbanalator, includes all initial chemical species and several intermediates including H_2O_2 and $O_2^{\bullet-}$. The oscillatory behavior in the Urbanalator is based on the decrease of native enzyme during the oxygen increase, where the enzyme is held in a stable, upper oxidation state. Enzyme recycling is triggered by depletion of the native species and occurs near the onset of oxygen decrease via the intermediate $O_2^{\bullet-}$. The experiments described here actually preceded the development of the Urbanalator, and are partially responsible for its formation.

Previous research[1] focused on establishing a well-defined, controlled, and characterized experimental system to investigate the oscillator. The standard conditions used for all of the following experiments appear in Table 1 of ref 2. MB^+ is necessary for prolonged oscillations under these conditions, but 2,4-dichlorophenol, used by many other groups, is not needed.[1] Systematic control of the present semi-batch system allows oscillations to be maintained for over 5 h, rendering the use of a continuous-flow, stirred tank reactor, its required chemical additions,[15] and its possible complications[2] unnecessary in these studies.

Experimental Section

The experimental system employed here was previously characterized and described in detail. Unless noted otherwise, the standard conditions listed in Table 1 of ref 2, and as previously discussed,[1] were used in all oscillatory experiments. The system consists of an aqueous, buffered solution (0.1 M

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sodium acetate, pH 5.10) of HRP and MB^+ to which NADH is added at a constant, pulseless rate through a capillary from a pressurized reservoir. A mixture of oxygen and nitrogen is blown rapidly down onto the well-stirred solution. Water evaporation is offset by the influx of NADH solution. Dissolved oxygen is monitored using a Clark-type microelectrode. Oxygen data were acquired at intervals of 10 s. The oscillatory mixture is contained in a thermostated quartz cuvette.

Reagents. The horseradish peroxidase and MB^+ used here were described elsewhere, along with other details.[1-2] Catalase and superoxide dismutase were obtained from Sigma Chemical (St. Louis, MO), used as received, and not assayed. Catalase (E.C. 1.11.1.6; 58 000 Sigma units/mg; Sigma C-100; from bovine liver; MW = 250 000 g/mol) was received as a suspension in water. It was diluted to 147 μM, pH 7.00, 0.01 M sodium acetate (NaAc), and subsequently, 306 μL was added to the working oscillator to achieve 9 μM (130 500 units/mL). From its appearance after experiments, some catalase apparently remained suspended and not completely dissolved in the oscillatory mixture. Superoxide dismutase (SOD; E.C. 1.15.1.1; 4200 Sigma units/mg; Sigma S-2515; from bovine erythrocytes; MW = 31 200 g/mol) was received as the lyophilized powder and diluted to 24.2 μM, pH 7.00, 0.01 M NaAc, and 103 μL were subsequently added to the working oscillator to achieve 0.5 μM (66 units/mL).

The various oxidation states of HRP: the native enzyme, and compounds II, I, and III will be designated Per^{3+}, Per^{4+}, Per^{5+}, and Per^{6+}, respectively, throughout this paper.

Enzyme Additions. Additions of catalase or SOD to the oscillator cuvette were made with a digital pipet using a micropipet tip (Research Products, Mt. Prospect, IL). In the enzyme addition experiments, an aliquot of an equal volume of buffer was deoxygenated in the same manner as the respective enzyme solution and injected into the oscillatory mixture after the oxygen level reached a steady state (Figures 2 and 3). Deoxygenation of the catalase aliquot was hindered by its surfactant quality resulting in somewhat high oxygen levels in blanks. Superoxide dismutase did not exhibit such a problem, and nitrogen-purged blanks for SOD apparently contained very little oxygen.

Hydrogen Peroxide Standardization. Hydrogen peroxide stock solution (30%, about 8.8 M; Fisher, Fair Lawn, NJ) was assayed with Ce^{4+} to determine its concentration prior to use.[16] The titration reaction is

$$H_2O_2 + 2Ce^{4+} \rightarrow 2Ce^{3+} + O_2 + 2H^+ \qquad (2)$$

Ammonium cerium(IV) nitrate ($(NH_4)_2Ce(NO_3)_6$, Aldrich, Milwaukee, WI) was dried 6 h at 85 °C. A solution of approximately 0.2 M Ce^{4+} (known to ±0.01 mM) in 1 M sulfuric acid was prepared from the $(NH_4)_2Ce(NO_3)_6$ and used as the primary standard. The 30% H_2O_2 stock solution was diluted to about 0.1 M with 1 M H_2SO_4, and an aliquot was titrated with the Ce^{4+} solution using 5 drops of 6 mM ferroin (in water; Aldrich) as an indicator. The endpoint is from red-orange to faint blue. An identical aliquot of water was also run as a titration blank.

Results and Discussion

Substitution of H_2O_2 for MB^+. It was suggested that MB^+ mediates the oxidation of NADH by oxygen to yield H_2O_2.[17] To test the hypothesis that MB^+ might serve as an indirect source of H_2O_2 in the oscillator, 0.3 μM H_2O_2 was substituted for MB^+ in the initial mixture, and the results are presented in Figure 1. Because H_2O_2 is unstable, a slight molar excess with respect to MB^+ was used to ensure a final concentration of at least 0.2 μM H_2O_2 in the reaction cuvette. This strategy presumes a 1:1 stoichiometric correspondence between H_2O_2 and MB^+ in the chemical mechanism of the oscillator, for which evidence is entirely lacking. The appropriateness of this ratio or other ratios was not examined here. In Figure 1, only a few severely damped oscillations were observed when $k_{-m} = 0.140$ or 0.160 min^{-1}. Prolonged, but very small oscillations were seen for about 85 min when $k_{-m} = 0.150$ min^{-1}. The effect is sensitive to k_{-m} and when $k_{-m} = 0.150$ min^{-1} (a range of 0.147−0.152 in four replicates) the oscillatory effect seems optimized.

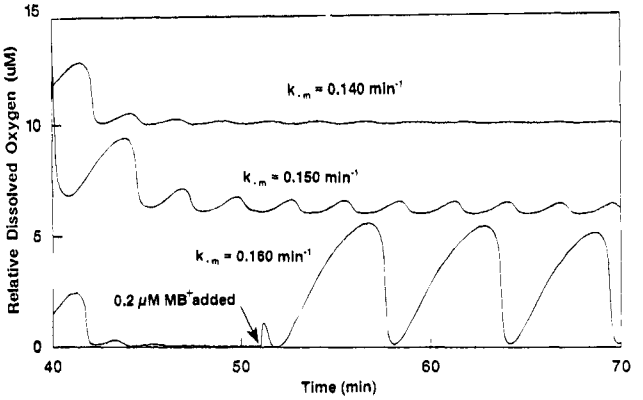

Figure 1. Oxygen data for H_2O_2 initially substituted for MB^+ and addition of MB^+. Initially, 0.3 μM H_2O_2 was substituted for 0.2 μM MB^+. Data were obtained under standard conditions without illumination and at the oxygen mass-transport constants shown. The top two traces are shifted upward for clarity of presentation. MB^+ was added only in the last run as designated.

The existence of relatively small, sinusoidal-type oscillations were predicted under certain conditions in a theoretical model of the peroxidase−NADH system that did not include MB^+.[18] It was suggested that interaction of these small oscillations with a second type of higher amplitude oscillation may explain much of the observed behavior in the peroxidase oscillator. In a previous study performed under otherwise identical conditions (ref 2, Figure 4), with both MB^+ and H_2O_2 initially omitted from the oscillatory mixture, quasi-sinusoidal oscillations were also seen. Larger amplitude oscillations appeared upon the addition of MB^+. Those data along with the data in Figure 1 suggest that MB^+ may be the chemical intermediary which could allow these two oscillatory regimes to interact, and perhaps be the source of some of the exotic dynamic behavior reported by others.[4-8] Figure 1 suggests the possibility of involvement of H_2O_2 in oscillations and that MB^+ may enhance or alter the function of H_2O_2.

The lower trace in Figure 1 demonstrates that the H_2O_2 substituted for MB^+ does not interfere with oscillations induced by the subsequent addition of MB^+. The fact that substitution of MB^+ with H_2O_2 can yield small, but prolonged, oscillations suggests that MB^+ and H_2O_2 may have similar or related roles in the oscillator and perhaps that MB^+ may be linked to H_2O_2 production, as mentioned earlier. However, these results indicate that H_2O_2, at least at the submicromolar concentrations used here, does not function as an exact substitute for MB^+. Data in Figure 1 were taken in darkness and the effect of illumination[2] was not pursued.

Catalase and Superoxide Dismutase as Probes for H_2O_2 and Superoxide. Hydrogen peroxide and superoxide have long been suspected as important intermediate species in the peroxidase−NADH oscillator. In the working oscillator, H_2O_2 likely originates from the disproportionation reaction of superoxide, $O_2^{\bullet -}$, and H^+ (see reaction 6 below).[19] Superoxide

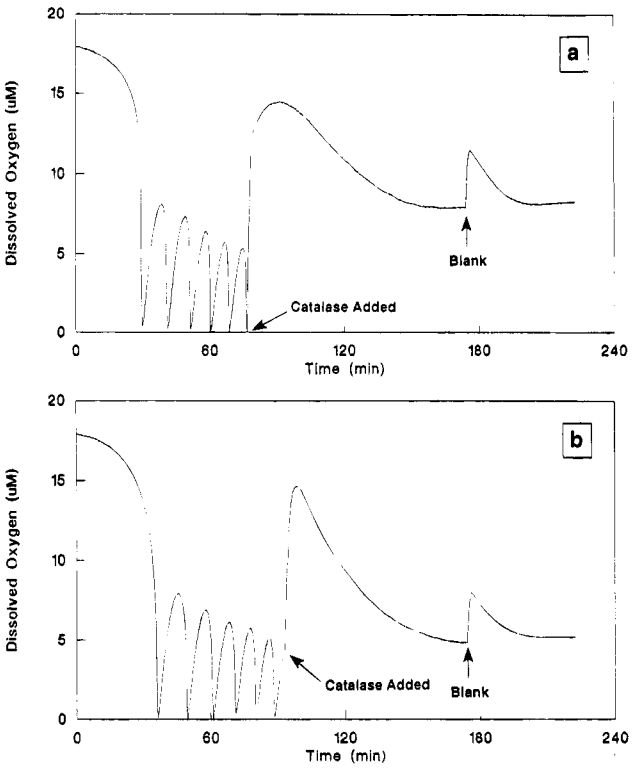

Figure 2. Experimental oxygen data for addition of catalase at a minimum (a) and a maximum (b). 9 μM catalase was added at the oxygen minimum or maximum as shown; when the oxygen level reached a steady state, an equal volume of identically deoxygenated buffer was added. Both runs were initiated under standard conditions (Table 1 in ref 2) without illumination. Data points were acquired at 10 s intervals.

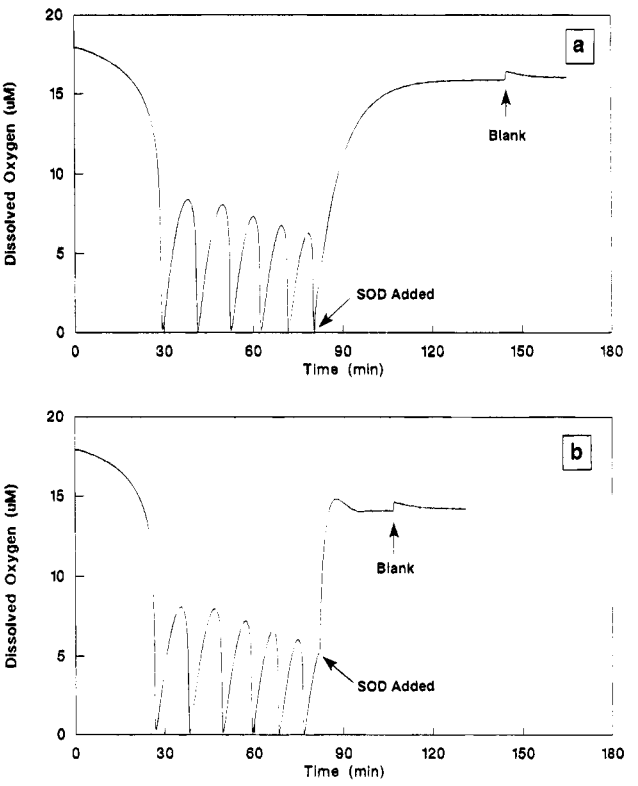

Figure 3. Experimental oxygen data for addition of superoxide dismutase at a minimum (a) and a maximum (b). 0.5 μM superoxide dismutase was added at the oxygen minimum or maximum, as shown, followed by addition of an equal volume of identically deoxygenated buffer. Both runs were initiated under standard conditions (Table 1 in ref 2) without illumination. Data points were acquired every 10 s.

probably originates by reduction of O_2 by NAD$^\bullet$, and NAD$^\bullet$ most likely originates from oxidation of NADH by Per $^{5+}$ and Per^{4+}.[10,20] Both H_2O_2 and $O_2^{\bullet-}$ are thought to oxidize the native enzyme, Per^{3+}, via[21]

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

and[3,22-23]

$$Per^{3+} + O_2^{\bullet-} + 4H^+ \rightarrow Per^{6+} + 2H_2O \quad (4)$$

Presented here is the first direct experimental evidence which strongly suggests that H_2O_2 and $O_2^{\bullet-}$ fill critical roles in oscillatory dynamics, though many chemical models have presumed their involvement.[24] Figures 2 and 3 show the result of addition of the enzyme catalase or superoxide dismutase (SOD) to a working oscillator. Catalase catalyzes the reaction

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad (5)$$

and superoxide dismutase catalyzes

$$2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (6)$$

Both enzymes react rapidly with their respective substrates. The second-order association rate constants are $k = 4 \times 10^7$ M^{-1} s^{-1} for catalase + H_2O_2 ,[25] and $k = 2.3 \times 10^9$ M^{-1} s^{-1} for SOD + $O_2^{\bullet-}$.[26] Consequently, these enzymes can be used as species-selective catalytic scavengers to explore the role of H_2O_2 and $O_2^{\bullet-}$ in the oscillator. SOD reacts with the superoxide anion only,[27] and its catalytic rate constant is independent of pH over the range 4.8-9.5[26] because the protons in reaction 6 do not come from the bulk water.[28] The pK_a for the dissociation of the hydroperoxyl radical, $HO_2^\bullet$, is 4.8,[29] so at pH 5.10 the ratio of $[O_2^{\bullet-}]/[HO_2^\bullet] = 2$. Reaction 6 quickly shifts the equilibrium between the two radical species resulting in the conversion of both.[26] As a consequence, in the computations which follow, $[O_2^{\bullet-}]$ is actually the sum of both species.

Catalase and SOD were added individually to the oscillatory mixture at either the oxygen minimum or maximum. In a series of trials, increasing amounts of enzyme were added to determine the concentration sufficient to stop oscillations in a single enzyme addition, an amount which was the same for a particular enzyme, regardless of whether it was added at a minimum or maximum. Only data for these stopping amounts (9 μM catalase and 0.5 μM SOD) are shown in Figures 2 and 3. After a steady-state level of oxygen was reached, a buffer blank of equal volume was injected which had been deoxygenated in the same manner for each particular enzyme. The catalase in the stock suspension acts as a surfactant, which made it difficult to exhaustively deoxygenate. Bubbling nitrogen through the catalase suspension caused it to froth, so nitrogen was only blown down onto the liquid. In contrast, the SOD solution was well-behaved and very effectively deoxygenated by nitrogen sparging. Consequently, the blanks are quite different for the two enzymes.

For catalase additions that were less than the determined stopping amount (as low as 1 μM; data not shown), oxygen periods increased 50-100% and amplitudes increased about 10%. For SOD additions less than the stopping amount (as low as 0.1 μM; data not shown), oscillatory periods increased 100-150% and oxygen amplitudes increased 20-30%. Oscillatory damping persisted after addition of catalase or SOD. Note that the steady-state oxygen levels, $[O_2]_{aq,ss}$, are higher when either catalase or SOD are added at an oxygen minimum than at a maximum (see Tables 1 and 2). Furthermore, for SOD

TABLE 1: Conditions and Modeling Results for the Addition of 9 μM Catalase to a Working Oscillator at the Oxygen Minimum or Maximum

	addition	
	minimum	maximum
measured model input parameters		
k_{-m} (min^{-1})	0.143	0.123
$[O_2]_{aq,0}$ (μM)	0	4.50
$[O_2]_{aq,ss}$ (μM)	7.91	4.82
resultant model input parameters		
k_1 (min^{-1})	0.181	0.334
m (min^{-1})	0.324	0.457
model fitting parameters		
R_0 (μM)	30	68
k_2 (min^{-1})	0.2	0.1
result		
$[H_2O_2]$ (μM)	30	68

TABLE 2: Conditions and Modeling Results for the Addition of 0.5 μM SOD to a Working Oscillator at the Oxygen Minimum or Maximum

	addition	
	minimum	maximum
measured model input parameters		
k_{-m} (min^{-1})	0.153	0.135
$[O_2]_{aq,0}$ (μM)	0	5.23
$[O_2]_{aq,ss}$ (μM)	15.87	14.11
resultant model input parameters		
k_1 (min^{-1})	0.0196	0.0362
m (min^{-1})	0.173	0.171
model fitting parameters		
R_0 (μM)	0, (−6)[a]	8.4
k_2 (min^{-1})	0.1	0.7
result		
$[O_2^{\bullet -}]$ (μM)	0, (−6)[a]	8.4

[a] See text for explanation.

additions, the $[O_2]_{aq,ss}$ values are significantly higher than for catalase additions. The addition of 0.5 μM SOD virtually halts oxygen consumption, since the $[O_2]_{aq,ss}$ is 89% of $[O_2]_{aq,\infty}$ when added at a minimum and 79% of $[O_2]_{aq,\infty}$ when added at a maximum.

Modeling the Effect of Catalase or Superoxide Dismutase. No complete, experimentally based model of the peroxidase–NADH oscillator exists, despite much effort.[14,24] In fact, the very criteria of what constitutes a complete model is highly debatable. Consequently, in this first attempt to describe the effect of catalase and SOD, a relatively simple model is proposed to assist in quantitative interpretation of the results of the enzyme addition experiments illustrated in Figures 2 and 3. This initial scheme is far less comprehensive than the Urbanalator model presented elsewhere.[14] The following is suggested to describe oxygen dynamics immediately after the addition of catalase or SOD:

$$O_2(g) \underset{k_{-m}}{\overset{k_m}{\rightleftharpoons}} O_2(aq) \xrightarrow{k_1} \text{products} \quad (7)$$

$$R \xrightarrow{k_2} O_2(aq) \quad (8)$$

In reaction 7, k_m and k_{-m} are the oxygen mass transport constants as defined previously, and k_1 is the pseudo-first-order rate constant of oxygen conversion to its various products, a process assumed to be first order. Reaction 8 is effectively a perturbation step, where R represents either the reactant H_2O_2 or $O_2^{\bullet -}$, which are assumed to be present and catalytically converted to oxygen upon addition of the corresponding enzyme (reaction 5 or 6). This source of oxygen then contributes to the $[O_2]_{aq}$ already present. The rate constant, k_2, for the formation of oxygen from reaction 8 is assumed to be pseudo-first-order in substrate. This process is actually second order for each enzyme (first order in enzyme, first order in substrate),[25,26] but under these particular conditions of probable excess enzyme, both reactions are assumed to be pseudo-first-order. The validity of this assumption was not experimentally investigated here.

An approach to data analysis is presented which uses a new expression for dissolved oxygen derived directly using the oxygen rate law from the proposed model. (A simplified approach is described in the supplemental material). For the addition of catalase or SOD, the rate expression that describes the oxygen dynamics immediately after enzyme addition for the model presented in reactions 7 and 8 is

$$d[O_2]_{aq}/dt = k_m[O_2]_g - (k_{-m} + k_1)[O_2]_{aq} + k_2[R] \quad (9)$$

Laplace transforms[30] provide the easiest method to solve eq 9 for $[O_2]_{aq}$ as a function of time. A straightforward setup, followed by somewhat lengthy algebraic simplification yields

$$[O_2]_{aq} = \alpha + ([O_2]_{aq,o} - \alpha)e^{-mt} + \frac{[R]_0}{1 - m/k_2}(e^{-mt} - e^{-k_2 t}) \quad (10)$$

where

$$\alpha = \frac{K_{eq}[O_2]_g}{1 + k_1/k_{-m}} = \frac{17.9\ \mu M}{1 + k_1/k_{-m}} \quad (11)$$

$$m = k_{-m} + k_1 \quad (12)$$

and where $[O_2]_{aq,0}$ and $[R]_0$ represent $[O_2]_{aq}$ and $[R]$, respectively, at the moment of addition of the competing enzyme. Equation 10 contains a difference between two exponential terms indicating that the $[O_2]_{aq}$ should go through a maximum, which it does in Figures 2 and 3b. The asymptotic behavior of eq 10 as $t \rightarrow \infty$ is easily seen, since all the exponential terms approach zero. Clearly, as $t \rightarrow \infty$, $[O_2]_{aq}$ approaches a steady state, $[O_2]_{aq,ss}$, equal to α. Using α, k_1 is easily found from the steady-state concentration, $[O_2]_{aq,ss}$ as seen toward the end of the traces in Figures 2 and 3. Also, k_{-m} comes from a separate determination at the beginning of each experiment; $K_{eq} = 0.0292$ and $[O_2]_g = 613$ μM, as described earlier.[2] These model input parameters are summarized in Tables 1 and 2. This leaves $[R]_0$ and k_2 to be varied in eq 10 to best fit the model to the experimental data. Inspection of eq 10 shows that $[R]_0$ affects the amplitude of the curve, and k_2 the shape. This is numerically verified by calculation of $[O_2]_{aq}$ from eq 10 using a spreadsheet, and varying $[R]_0$ and k_2.

Several cases of curve fitting results are presented in Figures 4 and 5. For the catalase cases in both parts of Figure 4, the original experimental data are presented along with these same data, minus the blank. The moment of enzyme addition and the moment of blank addition in the experimental data were made to coincide in time. The adjusted traces were then prepared by point-by-point subtraction of the blank. Also shown in Figures 4 and 5 are the simulated data acquired by using the model represented in eq 10 and by varying the values of R_0 and k_2 where the best fit was judged visually. In both catalase cases and in the case of SOD added at an oxygen minimum, the model gave an imperfect fit to the experimental data. The fitting constants are summarized in Tables 1 and 2.

Neither SOD data set was adjusted for the relatively small oxygen blanks. In the case of SOD addition at a minimum

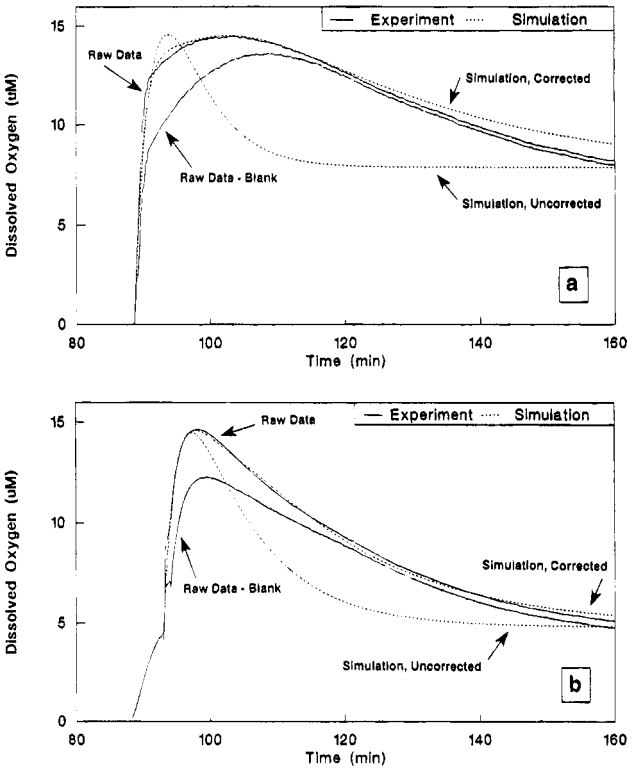

Figure 4. Simulated oxygen data for the addition of catalase at a minimum (a) and a maximum (b). The solid lines represent the raw experimental data and the raw data with the blank subtracted, as indicated. The dashed lines are simulated data computed at intervals of 10 s with and without a model correction factor (see supplemental material), as shown. The kinetic model and the corresponding parameters used to generate the simulated results are described in the text. See Table 1.

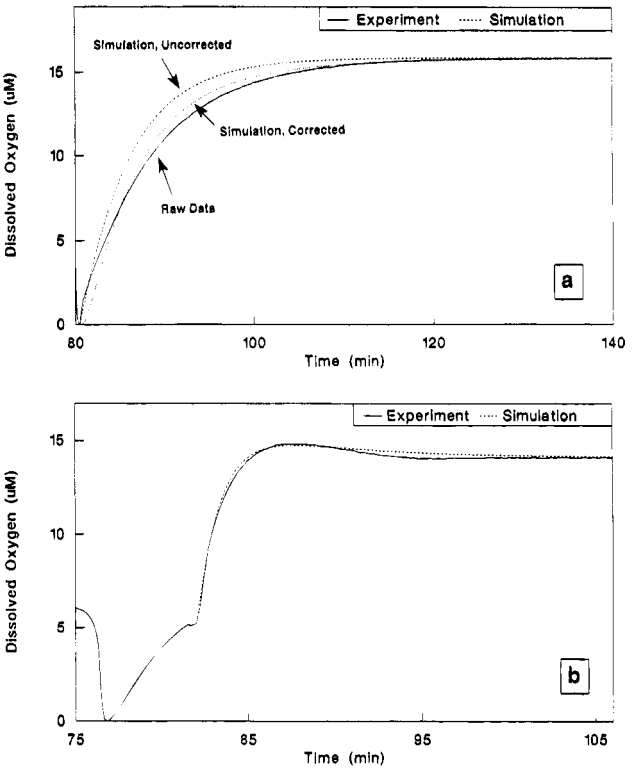

Figure 5. Simulated oxygen data for the addition of superoxide dismutase at a minimum (a) and a maximum (b). The solid line represents experimental data. The other lines depict simulated data with and without a model correction factor (see supplemental material), as indicated, and are computed at intervals of 10 s. The kinetic model and corresponding parameters used to generate the simulated results are described in the text. See Table 2.

(Figure 5a), the model gave a reasonable fit, and use of a correction factor improved the fit somewhat (see supplemental material). The best fit to the SOD data at a minimum was achieved using the model without the correction factor, and by setting $R_0 = -6$ μM. This nearly perfect fit is not shown because it virtually overlays the original data. The interpretation of a negative R_0 is that $O_2^{\bullet-}$ initially reacts to consume oxygen in some unknown, direct or indirect reaction before it is catalytically transformed into oxygen by SOD. In the case of SOD added at a maximum, the model gave a good fit to the experimental data without use of a correction factor as shown in Figure 5b.

The approach to analysis of the data in Figures 2 and 3 using the proposed model does not provide entirely satisfactory results. The method uses a temporal expression for $[O_2]_{aq}$ but does not adequately accommodate the blanks in the case of catalase and returns values for $[H_2O_2]$ and $[O_2^{\bullet-}]$ that either seem rather high or must be explained by an additional reaction. Neither approach includes the concentration of enzyme used. Other complications could render the present model inadequate. The analysis for the addition of catalase ignores the possibility that catalase is inhibited by $O_2^{\bullet-}$ [31] as well as reacting with H_2O_2. Furthermore, the rate constant for the reaction of H_2O_2 with Per^{3+} (which is 1.8×10^7 M^{-1} s^{-1}; [21] see reaction 3) is 3.6 times larger than it is with catalase (5×10^6 M^{-1} s^{-1}).[25] The assumption that the added catalase converts all the H_2O_2 present instantly and completely to products is probably untrue. Instead, the added catalase competes for H_2O_2 with the peroxidase already present. This may explain why a concentration of catalase in excess of the [HRP] was needed to halt oscillations.

In contrast, the rate constant for the reaction of $O_2^{\bullet-}$ with Per^{3+} (1×10^8 M^{-1} s^{-1};[22] see reaction 4) is considerably smaller than with SOD (2.3×10^9 M^{-1} s^{-1};[26] see reaction 6). Recall that, compared to catalase, much smaller molar levels of SOD were needed to stop oscillations. However, SOD can exhibit peroxidative activity in the presence of H_2O_2.[32] It has been noted specifically that when SOD is added to an H_2O_2-producing system as a probe for $O_2^{\bullet-}$, unexpected effects can arise.[22] SOD is also inactivated by H_2O_2,[33] but only slowly at pH 5.[34] Furthermore, SOD can inhibit the aerobic oxidation of NADH by peroxidase if SOD is present from the beginning (i.e., if no H_2O_2 is initially present).[35] Apparently $O_2^{\bullet-}$ initiates aerobic NADH oxidation by peroxidase, but $O_2^{\bullet-}$ is not involved once oxidation has begun.[35] Despite all the complications regarding SOD, recall that the model worked best for SOD added at a maximum.

It was shown elsewhere that additions of 7.2 μM H_2O_2 can, under some conditions, cause the oscillator to switch from an oscillatory mode to a steady state.[36] However, the experimental results presented here are the first which directly implicate H_2O_2 and $O_2^{\bullet-}$ as critically important species present in the peroxidase–NADH oscillator. Clearly, a full explanation of the experimental data in Figures 2 and 3 requires further investigation.[37] A more refined, experimentally based kinetic model, reported elsewhere,[14] has provided an improved qualitative and quantitative interpretation of experimental data pertaining to the roles of H_2O_2 and $O_2^{\bullet-}$ in the oscillator.

Conclusions

The experimental strategy of substituting hydrogen peroxide for methylene blue, and the use of species-selective enzyme probes has revealed a probable connection between the interaction of these two species in the oscillator. Initial substitution of H_2O_2 for MB^+ allows small oscillations sensitive to the

oxygen mass-transport constant. The function of H_2O_2 may be enhanced by MB^+ or have a similar role, but H_2O_2 is not an exact substitute for MB^+, which is essential in achieving relatively large oscillations.[1,2] H_2O_2 or MB^+ could serve as chemical mediators between the large- and small-amplitude oscillations observed here and predicted by a previous model.[18] This interaction may be the source of some of the more complex dynamic behavior reported for this oscillator.[4−8] The exact chemical basis of the peroxidase−NADH oscillations examined here is still not entirely understood, but the Urbanalator model has come closest in proposing a link between MB^+ and H_2O_2.

The addition of catalase and superoxide dismutase to a working oscillator can be used successfully to examine the roles and approximate the concentrations of hydrogen peroxide and superoxide. Using a perturbation model, a method of analysis for the addition of these enzymatic scavengers indicates a higher concentration of H_2O_2 and $O_2^{\bullet-}$ at oxygen maxima than at oxygen minima. The simulated data generated by the model described here do not exactly match the experimental data, probably due to the complications of several possible side reactions. Concentrations of these species are in the micromolar range as computed from the model under varying conditions and assumptions. The addition of SOD was better simulated by the model than was the addition of catalase. The use of scavenger enzymes which are selective for H_2O_2 and $O_2^{\bullet-}$ strongly indicate that these two species both play a key function in oscillator dynamics, which agrees with expectations.

The research reported here will assist in the development of an improved model for the peroxidase−NADH oscillator which is more consistent with experimental conditions and results. An increased understanding of this enzyme-regulated oscillatory reaction could eventually lead to better knowledge about the occurrence and control of cyclic processes in biological systems.

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Supplementary Material Available: Additional material is available on topics indicated in the text (5 pages). To order, see any current masthead page.

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