Response of the Peroxidase-Oxidase Oscillator to Light Is Controlled by MB^+−NADH Photochemistry

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Although the peroxidase-oxidase (PO) oscillator has been widely studied [Scheeline et al. *Chem. Rev.* **1997**, *97*, 739],[1] the continued lack of quantitative agreement between experiment and theory suggests that the molecular components and reactions essential for oscillation dynamics are not fully understood. Particularly, the role of photochemical reactions has been largely overlooked even though the photosensitizer methylene blue (MB^+) has been routinely added to PO reaction mixes by investigators since the first demonstration of sustained oscillations [Nakamura et al. *Nature* **1969**, *222*, 794].[2] We reasoned that the presence of MB^+ in the PO reaction should make oscillations sensitive to visible light exposure. We tested this possibility and observed that both the frequency and the amplitude of O_2 oscillations obtained with a free-running periodic PO oscillator were suppressed in a rapid and reversible manner when exposed to visible light. The effect occurred at illumination wavelengths between 600 and 700 nm, was greatest at 670 nm, and was consistent with the absorbance spectrum of MB^+. Measurements of the rate of NADH oxidation by MB^+ during illumination with red light showed a dose dependence consistent with the response curve observed for periodic PO oscillations. We concluded that PO oscillations were influenced by light through a photochemical effect on MB^+, with the photosensitive reaction being the oxidation of NADH by MB^+. Given these results, photoinduced suppression effects may be a common experimental bias in PO experiments where broadband spectroscopic illumination has been used. Future laboratory work involving the PO oscillator will need to control for MB^+-dependent photochemical effects and theoretical modeling efforts should account for photochemistry involving MB^+ and NADH.

Introduction

The peroxidase-oxidase (PO) oscillatory reaction serves as a laboratory model for complex biochemical reaction networks found in biological systems.[3] It contains basic features for which there are counterparts in biological systems including the enzymatic nature of the reaction, where NADH is the principle electron donor, and the nonlinear, nonequilibrium character of the reaction network, where autocatalysis and feedback-regulation lead to bistability,[4] periodic oscillations,[2] and chaos.[5] The PO oscillator's usefulness as a laboratory model for more complex systems is complemented by its amenability to simulation on the computer.[6−9] This is possible because many of the elementary reaction steps are known and many of the reaction rate constants have been measured.[1] Qualititative models have achieved success in simulating many of the oscillation patterns and bifurcation sequences observed experimentally. However, efforts to extend these models to the point of quantitative prediction have failed, possibly because of either imprecise estimates of reaction rate constants or an incomplete description of the reaction network.[10] Given that the role of photochemistry in the PO reaction network remains mostly unstudied, the goal of this paper was to examine the dependence of the PO oscillator on MB^+−NADH photochemistry. The results show that the PO oscillator is strongly influenced by red light through an action on reactions involving MB^+ and NADH suggesting that quantitative modeling efforts should incorporate such processes in the future.

Our earliest attempts to observe PO oscillations revealed that oscillator dynamics were highly sensitive to illumination by the fluorescent lighting in the laboratory. Shielding of the reaction from light resulted in large complex oscillation patterns that were severalfold greater in amplitude and periodicity than the oscillations observed under laboratory light conditions (Figure 1). The presence of photochemistry in the PO system was the most reasonable explanation for this behavior. A number of reaction components are known to absorb visible light and were initially considered as candidate photomodifiers of the PO oscillator. The first possibility was a photochemical effect involving one or more of the enzyme species because the various oxidized forms of horseradish peroxidase are known to absorb most strongly below 500 nm. However, photochemical effects on the enzyme at visible wavelengths and room-level light intensities have never been reported. The second possibility involved the photodecomposition of NADH into radical products, which might influence the activity of the peroxidase enzyme.[11,12] Yet, this effect may not fully explain all photochemical behavior[13] and, as will be evident below, can lead to underestimation of the importance of visible light in modifying reaction dynamics. The third and most probable explanation was based upon the photochemistry of MB^+, which has an absorbance peak between 650 and 670 nm and is a known photooxidizer of NADH.[14,15] The central role of NADH as an electron

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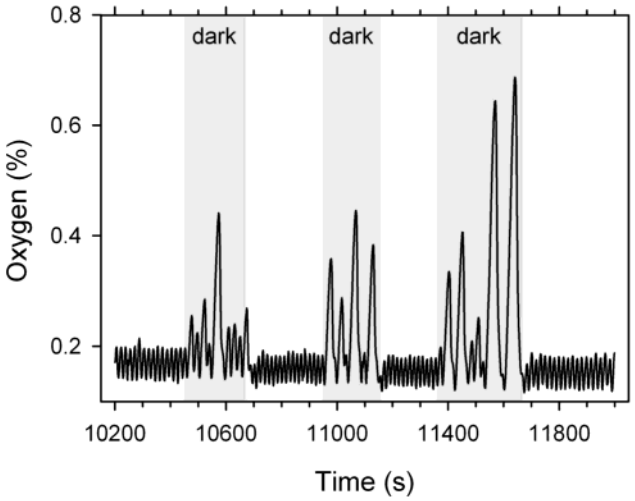

Figure 1. Response of the PO oscillator to laboratory light and darkness. The biochemical conditions for this experiment appear in Table 1A. Measurements represent the percentage saturation of dissolved O_2 in the reaction mixture over time. Shaded bars mark the time points where the laboratory lighting was switched off. Unshaded areas represent intervals where the laboratory fluorescent lighting was switched on. The irradiance of the fluorescent lighting next to the reactor was ~300 μW/cm^2. For this experiment, the reactor was housed inside of a clear acrylic water bath which permitted a significant fraction of the incident light to illuminate the reaction mixture.

donor in the PO reaction[16] suggested that photooxidation of NADH by MB^+ might lead to observable changes in PO oscillator behavior. Our approach was to study a periodic PO oscillator during intervals of light exposure at a variety of wavelengths compared to intervals of darkness. We then measured the near-equilibrium kinetics of NADH oxidation by MB^+ during illumination and darkness. With these two pieces of information, we were able to assess the relevance of MB^+−NADH photochemistry to the PO oscillator.

Materials and Methods

Materials. Peroxidase from horseradish (grade I) and NADH (disodium salt, grade I) were from Boehringer Mannheim. Methylene blue (trihydrate), 4-chlorophenol, 2, 4-dichlorophenol, phosphoric acid, and DTPA were from Sigma. Solutions were prepared on the day of each experiment, except phosphate buffer and phenol solutions (prepared in ethanol) which were made in advance and stored at 4 °C until used.

Reactor. Experiments were conducted in the apparatus which appears in schematic form in Figure 2. It consisted of a 10 × 10 × 45-mm quartz cuvette (NSG Precision Cells; all sides polished) temperature-regulated to 28.0 ± 0.1 °C. The cuvette was isolated from the atmosphere by a cap fitted with a stirrer, an oxygen electrode (Microelectrodes), one gas inflow tube, up to three liquid inflow tubes, and a J-shaped outflow tube. Stirring speed was constant at 1270 ± 1 rpm. In some early experiments, a cylindrical reaction vessel was used.

Illumination. The quartz reactor was illuminated with light carried by a quartz fiber bundle (PTI, London, Canada) from a xenon arc lamp (75 W heat-filtered, PTI; 10-nm band-pass filters, 53800−53990, Oriel) or directly with a current-controlled 656-nm LED (fwhm = 20 nm, Jameco, Belmont, CA). In each case, light was diffused (LSDKITCM-25, POC, Torrance, CA) and collimated before illuminating the reactor. Irradiance was measured with calibrated detectors and a meter (70282 silicon, 70261 thermopile, 70260 meter, Oriel).

Spectroscopy. Light from an intensity-stabilized lamp (60000 Series 100 W QTH, Oriel; spectral shaping filters, Schott;

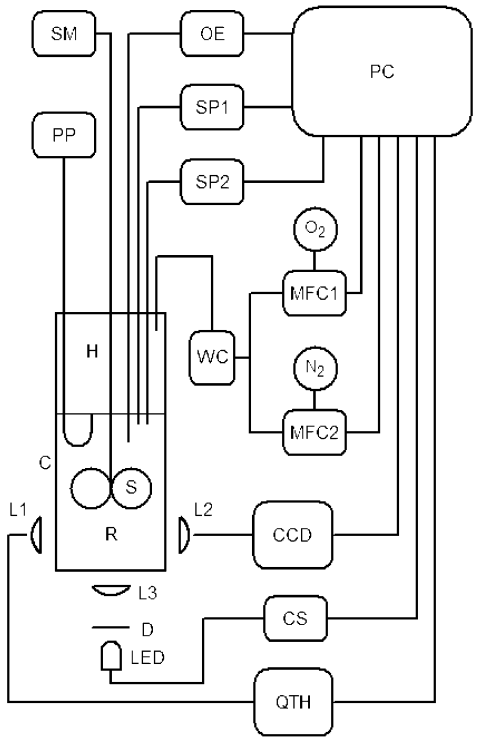

Figure 2. Schematic diagram of the apparatus used for PO oscillation and near-equilibrium experiments. Reactants (R) were contained in a quartz fluorescence cuvette (C) and mixed with a glass or plastic stirrer (S) rotated by a stepper motor, shaft and pulley system (SM). A gas headspace (H) was maintained above the reactants by continuous peristaltic pumping (PP) action on a J-shaped outflow tube to a waste reservoir. High purity O_2 and N_2 gas was bubbled through a 50-cm column of water (WC) before reaching the headspace. The volume flow rate of each gas was metered by a mass flow controller (MFC1 or MFC2) controlled with a personal computer (PC). Liquid reactants were delivered via capillaries by up to three PC-controlled precision syringe pumps (SP1 & SP2; SP3 not shown for clarity). Dissolved-O_2 readings were made with an oxygen electrode (OE) and acquired with the PC. Absorbance spectra from a 10-mm path through the reactants were measured with a CCD spectrometer and captured by a PC at 4 spectra per second. Illumination for spectroscopy was from a stabilized quartz tungsten halogen lamp (QTH). Light was carried to and from the cuvette with quartz fiber optic cables and collimating lenses (L1 & L2). A second illumination channel approached the cuvette from below and consisted of a PC-controlled current source, light emitting diode (LED), holographic diffuser (D), and collimating lens (L3). For some experiments, the LED was replaced with a quartz fiber bundle connected to a xenon lamp with heat and band-pass filters. The cuvette was placed in a water bath temperature-regulated to 28.0 ± 0.1 °C.

custom current supply) was delivered to the reactor by an optical fiber (400 μm quartz, Ocean Optics) terminated with a collimating lens. Transmitted light was collected by a similarly constructed fiber assembly, detected by a CCD spectrometer (390−440 nm, 50 μm slit, 2400 lines/mm, Ocean Optics, 4 spectra/s), converted to an absorbance spectrum **A**, and multiplied with $(\mathbf{E}^T\mathbf{E})^{-1}\mathbf{E}^T$ to yield **C**, a vector containing the reactant concentrations.[17] The matrix **E** was constructed from a spectrum of NADH obtained in distilled−deionized H_2O.

Data Acquisition and Computer Control. An IBM-compatible PC fitted with plug-in instrument cards (AT-AO-6, SC-2040, and AT-MIO-16E-1, National Instruments, Austin TX) was used to control the apparatus. Programming was done with LabView virtual instrumentation software (National Instruments).

Inflow and Outflow of Reactants. Delivery of reactants was with up to three capillaries (Figure 1b) connected by polyethylene tubing to water-cooled (20 °C) syringes (10-ml gastight,

TABLE 1: Experimental Conditions

	A − Figure 1	B − Figures 3a and 4	C − Figure 3b	D − Figure 5
reactor	batch	CSTR	semibatch	semibatch
syringe #1	not applicable	0.458 mg/mLHRP 2.4-U G6P-DH in buffer 33.3 μL/min	48-mM NADH in distilled−deionized water 24 μL/h	48-mM NADH in distilled−deionized water 20−22 μL/h
syringe #2	not applicable	55-mM G6P 1-mM NAD^+ 33.3 μL/min	not applicable	not applicable
syringe #3	not applicable	buffer only 33.3 μL/min	not applicable	not applicable
initial conditions	8.3 μM HRP 1.2 mM NAD^+ 2 U/mL G6P-DH	infuse equal amount from syringe 1, 2, and 3	2.9-μM HRP	buffer only
buffer	0.1 μM MB^+ 50 μM 2,4-DCP 0.1 M phosphate pH 6.30	0.1 μM MB^+ 20 μM 2,4-DCP 0.1 M phosphate 100 μM DTPA pH 6.30	0.1 μM MB^+ 90 μM 4-CP 0.1 M phosphate 10 mM DTPA pH 6.30	0.1 μM MB^+ 0.1 M phosphate 10 mM DTPA pH 6.30
gas	10% O_2:N_2 100 mL/min	0.8% O_2:N_2 50 mL/min	1.1% O_2:N_2 40 mL/min	0.52% O_2:N_2 40 mL/min
reactor	27 mm diameter cylindrical container 8.0 mL volume	10 × 10 × 45 mm quartz cuvette 2.2 mL volume	10 × 10 × 45 mm quartz cuvette 2.0 mL volume	10 × 10 × 45 mm quartz cuvette 1.9 mL volume

SGE, Austin, TX) loaded onto syringe pumps (PHD2000 or 22, Harvard Apparatus). In semibatch reactor experiments,[5] a 0.5-mL gastight syringe (Hamilton) was used. Where necessary, the volume of reactants was held steady by peristaltic aspiration (Minipuls3, Gilson) on the J-shaped outflow tube. The headspace above the reactants was purged with a humidified mixture of O_2 and N_2 gas (each gas had a 99.998% purity specification, Altair, CA) regulated by two mass flow controllers (1259C MKS Instruments, Andover, MA). The O_2 transfer rate at the gas−liquid interface was approximately -4×10^{-3} s^{-1}.

PO Oscillation Conditions. Peroxidase-oxidase oscillations were obtained with batch, semibatch, or open-flow conditions[18] as described in Table 1. In some experiments, NADH was supplied to the reaction by direct infusion with a syringe pump (Figure 3b). In others, a NADH recycling system was used (Figures 3a and 4). Qualitatively similar waveshapes and periodicities were obtained with each delivery method. The quantitative results reported in Figure 3b were obtained using the following method. The cuvette was filled with 2.0 mL of buffer solution (see Table 1, column C). A small aliquot of HRP (prepared in buffer) was added resulting in a final concentration of 2.9 μM in the cuvette. A gastight syringe filled with 48 mM NADH (distilled−deionized water) delivered its contents at a constant flow rate of 24 μL/min. Gassing was with a humidified mixture of 1.1% O_2:N_2 at a total volume flow rate of 40 mL/min. The cuvette was illuminated with light from the xenon source (~10 μW/cm^2) or light from the LED (≤50 μW/cm^2).

Near-Equilibrium Measurements. The rate of NADH disappearance was observed with absorbance spectroscopy on 1.9 mL, 0.1 M phosphate-buffered solution (pH 6.30, 10-mM DTPA) containing 0.1 μM MB^+. The reactants were stirred continuously and gassed at 40 mL/min with a 0.52% O_2:N_2 mixture. One thousand seconds into each experiment, NADH was added by a small aliquot of concentrated stock solution (≤40 μL) to bring the final concentration in the reactor to 900 μM. After an additional 700 s (the final 200 s of which was designated D1), the red LED was turned on and the irradiance was stepped every 200 s in the sequence 1, 3, 15, and 50 μW/cm^2 followed by an interval of darkness (D2). The NADH readings were analyzed by a linear regression on the final 150 s of each 200-s interval, and each slope value was interpreted as an estimate of NADH oxidation by MB^+. Dark-control experiments were done in an identical manner, except the LED was never turned on. Dark-control experiments provided a measure of the systematic variation in NADH disappearance.

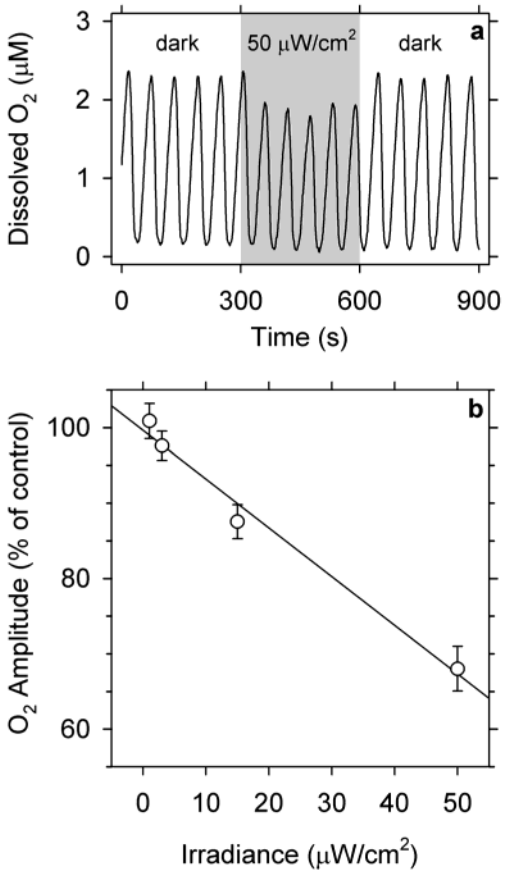

Figure 3. Red light suppression of PO oscillations. a. A typical recording of dissolved O_2 saturation in the CSTR during PO oscillations. The reaction mixture was exposed to light from a light-emitting diode (656-nm peak, 20-nm fwhm) at an irradiance level of 50 μW/cm^2 during the segment from 300 to 600 s (shaded area). Before and after red light exposure, the PO reaction was kept in darkness. Oscillations were obtained using conditions in Table 1B. b. Summary plot from six experiments similar to Figure 3a, except conditions in Table 1C were used. Plot shows the average oscillation amplitude during red light exposure relative to the oscillation amplitude observed in darkness before and after exposure (open symbols). Error bars are one s.e.m, and a first-order linear regression is shown by the solid line.

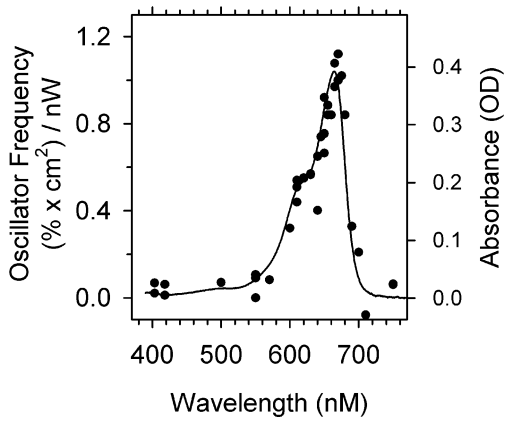

Figure 4. Action spectrum obtained from the oscillating peroxidase-oxidase reaction. Relative change in oscillator frequency due to illumination at wavelengths across the visible spectrum from 400 to 750 nm (left axis). Relative changes were normalized to the irradiance measured at the reactor during each experiment, which accounted for slight variations in lamp output between the different wavelengths. Each symbol represents an individual determination of light influence as described in Figure 3a. The solid line represents the absorbance spectrum of 4 μM MB^+ prepared in phosphate buffer (right axis in units of optical density).

On five separate days, five light and five dark-control experiments were performed. On each day the slope estimates from each like-treated segment were pooled. The values in Figure 5b represent mean ± s.e.m. based on the five between-day estimates.

Results

Under conditions of darkness, stable periodic oscillations were obtained with an average O_2 amplitude of 2.24 ± 0.03 μM (mean ± s.d., Figure 3a). When illuminated with the red LED at an irradiace of 50 μW/cm^2, the O_2 amplitude was reduced to 1.91 ± 0.19 μM. Switching off the LED resulted in darkness and a return of the oscillation amplitude to 2.23 ± 0.03 μM. The effect of light was rapid and usually took less than one oscillation period to reach a full response. Because the amplitude of O_2 oscillations was similar before and after exposure to red light, we averaged the amplitudes from both dark periods to obtain a best estimate of the dark control. The relative decrease in oscillator amplitude compared to the dark control was 14.5 ± 1.5%.

Repetition of an experiment similar to the one shown in Figure 3a demonstrated that the average amplitude of PO oscillations was reduced during light exposure by an amount proportional to the irradiance level (Figure 3b). Higher levels of illumination reduced the O_2 amplitude to a greater degree than lower illumination levels as observed by the average relative change in oscillation amplitude between light and dark conditions. A first-order regression on the average relative effect size against irradiance revealed a statistically significant linear correlation ($P < 10^{-6}$). The slope of the line was −0.68 ± 0.06% cm^2/μW with an intercept at 99.8 ± 1.4%. For irradiance levels greater that 50 μW/cm^2, the degree of oscillator suppression continued to increase until oscillations were completely suppressed (data not shown).

Repetition of an experiment similar to the one shown in Figure 3a, but with band-pass-filtered light from a xenon-arc lamp resulted in the action spectrum in Figure 4. Relative effect sizes were normalized to the incident light level in order to account for small variations in irradiance because of adjustment limitations of the light source and optics. The plot revealed that light was most effective at suppressing oscillations at illumination wavelengths near 670 nm. Exposure to visible wavelengths outside the range of 600−700 nm had little to no measurable effect on the PO oscillation frequency.

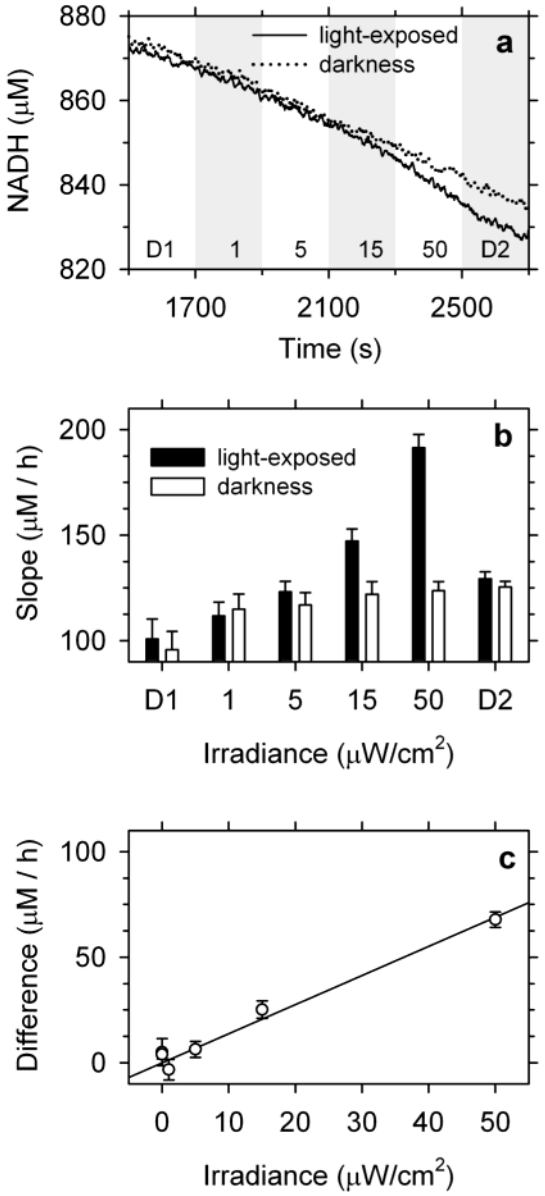

Figure 5. Influence of red light on the disappearance of NADH by MB^+. a. One experiment showing the time course of NADH disappearance during darkness and illumination at four light intensities from a 656-nm LED (solid line). Illumination at each irradiance value (in μW/cm^2) is indicated along the bottom of the plot, and the duration is marked by the white and shaded areas. The dotted line represents a second experiment, which was identical to the first, except the LED remained off for the complete time course. b. Average rate of NADH consumption estimated from 25-paired experiments similar to Figure 5a (solid line). Bars represent the average slope from 25, 150-s intervals exposed to one light intensity (solid) or dark condition (open) as described in part a. Error bars represent s.e.m based on five mean values each calculated from five replicate experiments. c. Difference between the average light-exposed and the average dark control values from part b. The D1 and D2 conditions are plotted at 0 on the abscissa. Error bars represent the s.e.m. from part b added in quadrature.

Exposure of a mixture of MB^+, NADH, and O_2 to the red LED resulted in an increased rate of NADH disappearance (Figure 5a). This was observed as a decrease in the slope of the NADH concentration curve compared to a similar experiment carried out in darkness. Illumination at the highest

irradiance level resulted in the steepest slope value. The rate of NADH disappearance observed during the dark periods at the beginning and end of each experiment were similar. The overall rate of NADH disappearance in darkness appeared to vary systematically, but to a lesser degree than the effect of light (Figure 5a, dotted line).

The pooled results quantitatively verified the qualitative assessment of the time series data in Figure 5a. The average rate of NADH disappearance showed a graded response to illumination intensity with the largest rate of disappearance at the highest irradiance level, an intermediate rate at 15 μW/cm^2, and the lowest rates at the smallest irradiance levels and dark periods (dark bars, Figure 5b). Only a minor variation in NADH disappearance was observed for the corresponding dark-control experiments (open bars, Figure 5b) and was quantitatively smaller than the rate of NADH disappearance for the samples illuminated at 50 μW/cm^2. After subtraction of the systematic change in NADH disappearance, the effect of illumination showed a linear dose relationship (Figure 5c). The rate of NADH consumption at the highest illumination intensity was 68 $\pm$ 4 μM/h compared to 4 $\pm$ 7 μM/h for the pooled dark segments D1 and D2.

Discussion

The results from this study demonstrate that the target reaction for red light in the PO oscillator is the oxidation of NADH by MB^+. This conclusion is based on four lines of experimental evidence: (i) The effect of light on the PO oscillator (Figure 3a) is consistent with a photochemical effect on one or more microscopic rate constants in the PO reaction network. For instance, the suppression of PO oscillations was rapid (<1 period) and proportional to the illumination level (Figure 3b). When the illumination ceased the oscillations rapidly returned to their preexposed periodicity and amplitude. These observations are consistent with an effect on a photochemical reaction that is rapid and reversible compared to the time scale of the O_2 oscillation period. (ii) MB^+ was involved in the response of the PO oscillator to red light based on the measured action spectrum (Figure 4). This was evident from the consistency between the degree of oscillator suppression and the amount of MB^+ absorbance at each wavelength. (iii) In the PO reaction, the only known reaction partner for MB^+ was NADH[1]. (iv) Although it has been recognized for some time that the rate of NADH oxidation by MB^+ is enhanced during red light illumination,[14,15] it was not clear from the literature whether this process occurred at irradiance levels found to affect the PO oscillator. Our direct measurements of NADH disappearance in the presence of MB^+ during red light exposure verified this possibility. The measurements were made in the same experimental setup used to observe PO oscillations, and the degree of NADH disappearance was consistent with the decrease in O_2 amplitude for the same range of illumination intensities. Therefore, the similarity in onset and relative effect size between the light-dependent responses of the MB^+−NADH reaction and the PO oscillator represents an additional level of support for the participation of MB^+−NADH photochemistry in the control of the PO oscillator.

In the presence of NADH and O_2, photoexcited MB^+ is reduced to leucomethylene blue (MBH), and then oxidized to MB^+ in a cyclic reaction where reactive oxygen species and hydrogen peroxide are generated.[19] Our data support the possibility that the concentration of these photochemical byproducts will be higher during exposure to red light compared to darkness and could directly interact with the PO reaction cycles leading to suppression of oscillations. However, additional spectroscopic work will be required to determine which photochemical reaction products are responsible for the red light-induced suppression of PO oscillations.

As an alternative hypothesis, we considered the possibility that P-670, a termination product of the peroxidase cycle with an absorbance maxima between 660 and 670 nm, might act as an alternative target molecule for red light.[20] However, P-670 could not explain our results for the following three reasons: (1) Using spectroscopic methods, we did not observe P-670 during our experiments (data not shown). (2) The rapid and reversible response of the PO oscillator to light (see Figure 3a) was inconsistent with the slow, irreversible kinetics of P-670 shown by others.[9] (3) The red light sensitivity persisted in the absence of enzyme, where the production of P-670 was not possible (Figure 5). Therefore, given the improbability of P-670 as a target molecule and the absence of other red-absorbent molecular species in the reaction mixture, we concluded that MB^+−NADH photochemistry was responsible for the oscillator suppression effects observed in our experiments.

Similar to our findings with red light, suppression of PO oscillations has been reported for illumination wavelengths below 248 nm but at much higher illumination intensities (total power impingent on the sample reported was ~18.5 mW).[13] Based on our findings, it would be interesting to determine whether excitation of MB^+ near its 246-nm absorbance peak enhances the oxidation of NADH in a manner similar to the effect of illumination at or near 670 nm. Alternatively, it has been observed that the peroxidase enzyme can be reduced in a photodependent manner by NADH.[11] Although the enzyme purity and the illumination conditions were not reported in sufficient detail to allow direct comparison to our results, the work does suggest that the photoionization of NADH to reactive radical species might act as an alternative path for photochemical effects in the PO oscillator and possibly explain the findings with high-intensity UV light.

The ability to influence the PO oscillator with red light presents the possibility that artifacts in oscillation patterns might occur during spectroscopic measurements. During our PO oscillator experiments, spectroscopy was avoided in favor of O_2 saturation readings from the electrode. Our near-equilibrium measurements employed a sensitive CCD spectrometer that needed less than 5 μW/cm^2 of broadband light. According to our findings, 5 μW/cm^2 of illumination is expected to have only a minor effect on the rate of NADH disappearance (see Figure 5). Previous PO investigations by others have used broadband light and less sensitive photodiode arrays to collect absorbance spectra from the PO reaction. It is reasonable then to assume that some of these earlier studies exposed the reaction to light of sufficient intensity to bias the PO oscillations toward artificially suppressed or possibly nonoscillating states. This may provide a partial explanation as to why some attempts at reproducing oscillator states between laboratories have failed and why quantitative models of the PO oscillator have yet to be constructed. In future experiments, the levels of red light at the reactor should be reported and if possible minimized to eliminate concern for experimental artifacts.

Red light might be exploited as a tool to probe the dynamical properties of the PO oscillator. Red light could be used to bias the oscillator toward desirable states. Cycling of light levels might be used to test how the PO oscillator responds to a range of well-defined stimuli. This later possibility might enhance the development of theoretical models through an iterative process of model construction and refinement verified by intervening

PO experiments where a series of light stimulus-response tests are used for model assessment. This would provide a great improvement over inflow methods, which are not amenable to fast variations and are subject to pumping and mixing delays.

Conclusions

This work identified the basic action of red light on the oscillating peroxidase-oxidase reaction. The principle target molecule for red light was identified as MB^+, and the data suggest that the oxidation of NADH by photoexcited MB^+ is the reaction linking the basic photochemical effect to the suppression of oscillations in the PO reaction network. The intrinsic light sensitivity of the PO oscillator should lead to at least three important opportunities for future investigation: (1) to explore the detailed connection between MB^+−NADH photochemistry and the reaction mechanisms responsible for PO oscillations, (2) to investigate the dynamical features of the PO reaction with perturbations at time scales not previously attainable with conventional techniques, and (3) to reassess existing theoretical models with the eventual construction of a fully quantitative model of the PO oscillator.

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