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OXYGEN DEPENDENT AND INDEPENDENT STEPS IN LUCIFERASE-$FMNH_2$ OXIDATION

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Upon reaction of luciferase-$FMNH_2$ with oxygen a complex series of absorbance changes occur, leading to the formation of a stable ($t_{\frac{1}{2}}$ about 35 min at 2°) dihydroflavin peroxyluciferase intermediate. Observed at 380, 445, or 600 nm, there is first a rapid absorbance increase which is oxygen-concentration dependent ($k \approx 10^6\ M^{-1}s^{-1}$). Following this there are two oxygen independent steps, first a slow absorbance decrease ($k = 4.3\ s^{-1}$) and then an even slower increase ($k = 0.55\ s^{-1}$). The dihydroflavin peroxide is not expected to have absorption at 600 nm and is thus postulated to be in equilibrium with some flavin species which does absorb in the red.

Luciferase mediates the oxidation by molecular O_2 of reduced flavin mononucleotide to yield FMN and H_2O_2, a reaction which is unusual because of its slowness. Whereas the non-catalyzed reaction is extremely rapid (1) the luciferase reaction takes many seconds (2), involving an enzyme intermediate (designated II) which has been postulated to be the 4a-peroxyflavin (3).

$$\underset{(I)}{E\text{-}FMNH_2} + O_2 \longrightarrow \underset{(II)}{E\text{-}FMNH\text{-}4aOOH} \longrightarrow FMN + H_2O_2 + E$$

As shown in Figure 1, II is characterized spectrally by a single major absorption band in the near u.v. centered at about 372 nm (3,4). Recent studies of the autoxidation in other solvent systems indicate that at least some of the intermediates may in fact be common to the luciferase and the non-enzymatic pathways (5).

Although the first step in oxidation, namely the reaction of the luciferase-$FMNH_2$ complex (I) with oxygen, was known to be fast, it seemed likely that some of the intermediate stages and the influence of oxygen concentration on the kinetics could be measured. Using stopped-flow techniques at 2° this was indeed found to be possible. Based on such

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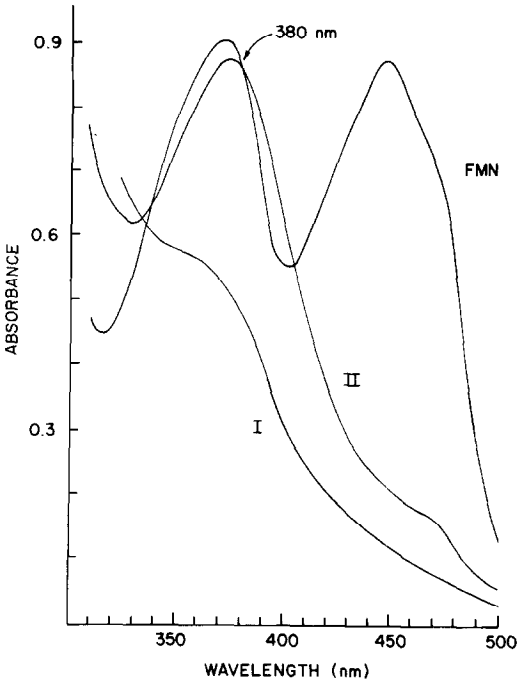

Fig. 1. Absorption spectra of luciferase-flavin intermediates I ($E\text{-}FMNH_2$) and II (E-FMNH-OOH) as well as the product, FMN.

experiments it is shown in this paper that there are transient species subsequent to an initial oxygen concentration-dependent step. Of particular interest is the discovery of a long wavelength (∿ 600 nm) absorbance associated both with the intermediates and with the long-lived luciferase-peroxyflavin.

MATERIALS AND METHODS

Luciferase was purified to homogeneity as previously described (6,7) from Beneckea harveyi strain MAV, #392 (8) and an aldehyde mutant designated M-17 derived therefrom (9). All of the experiments presented were done with luciferase from the mutant M-17 because it lacks photoexcitable luciferase (10,11), a species possessing a chromophore which might interfere spectrally. However, all experiments have been repeated with similar results using wild type B. harveyi luciferase, and also with purified luciferase from another species, Photobacterium fischeri (ATCC #7744). FMN was obtained in pure form by enzymatically hydrolyzing FAD (Sigma) followed by chromatographic purification on DEAE-cellulose (12). $FMNH_2$ was prepared by photoreduction, using a deoxygenated mixture of FMN (300 μM) and EDTA (10 mM) in 0.35 M phosphate buffer, pH 7. After reduction this was mixed anaerobically with deoxygenated luciferase in the same buffer, forming the $FMNH_2$-luciferase complex. Final concentrations in the syringe (3 ml volume) before mixing in the stopped-flow were: luciferase 90 μM; $FMNH_2$, 30 μM; and EDTA 1 mM. Greater than 98% of the $FMNH_2$ can be estimated to be protein-bound at

these concentrations (13,14). Rapid mixing of equal volumes from two syringes was achieved at 2°C in the Gibson stopped-flow spectrophotometer (Durrum Inst. Co.), which had a dead time of two milliseconds. Absorbance changes during the course of the reaction were monitored at 380, 445, and 600 nm employing Corning filters # 7-14, 7-59 and 2-73, respectively, placed between the cuvette and the photomultiplier in order to exclude any possible contributions from the bioluminescence emission. Data was photographed from records taken on a Tektronix 564 storage oscilloscope.

RESULTS AND DISCUSSION

Fig. 2 illustrates the absorbance changes observed at 2°C in the stopped flow apparatus at 380, 445, and 600 nm upon mixing the luciferase-$FMNH_2$ complex (intermediate I) with an air-equilibrated (at 20°) buffer, 0.35 M phosphate, pH 7. Under these conditions the product of this reaction, intermediate II, has a half-life of about 35 min, such that its decay is not significant within the time scales shown. Furthermore, intermediate II and the product of its decay, namely FMN, are isosbestic at 380 nm (Fig. 1), so that the decay of II would not be observed at this wavelength even over longer time intervals. At each wavelength a relatively rapid initial absorbance occurs (designated phase A), followed by an absorbance decrease (phase B) and a final increase (phase C).

The velocity of the initial rapid absorbance increase (phase A) was found to vary directly with O_2 concentration over the range 60-600 μM (Fig. 3), with a second order rate constant of 8.7×10^5 M s^{-1} (Fig. 4). From the known rate constant for the decay of light intensity with decanal under these conditions, a K_m for O_2 of about 10^{-8} can be estimated.

The velocities of the absorbance changes designated as phases B and C are both independent of oxygen concentration over the same concentration range at all these wave lengths. This is illustrated for phase B at 600 nm and for phase C at 380 nm at two of the several oxygen concentrations examined (Fig. 5). Thus, subsequent to the bimolecular reaction of $FMNH_2$ with O_2, the initial flavin-oxygen adduct undergoes O_2-independent relaxations to yield a relatively long-lived product, II. A scheme to accommodate

these observations is as follows:

$$E + FMNH_2 \rightleftharpoons I \xrightarrow[(A)]{O_2} I_A \xrightarrow[(B)]{} I_B \xrightarrow[(C)]{} II \rightarrow\rightarrow E + FMN + H_2O_2$$

$$II \uparrow\downarrow II\text{-}600$$

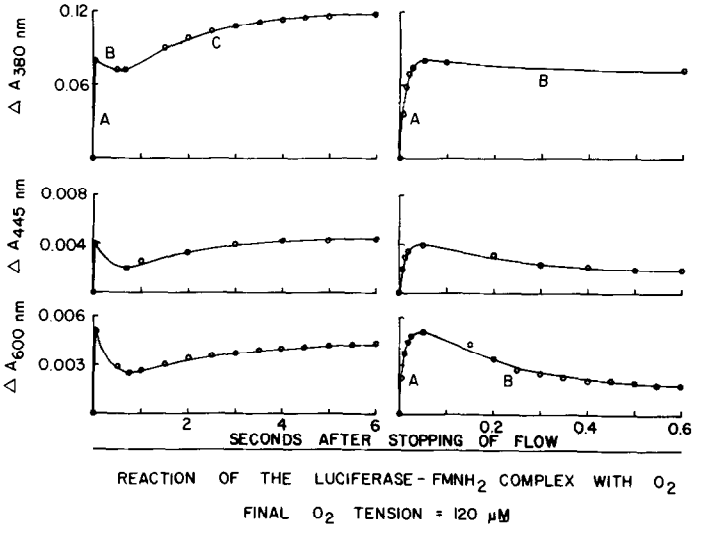

Fig. 2. Transient absorbance changes subsequent to rapid mixing of luciferase-$FMNH_2$ (I) (30 μM) with buffer containing oxygen (240 μM). Recordings made at three wavelengths and on two different time scales are shown. The three distinctive phases are designated A, B, and C.

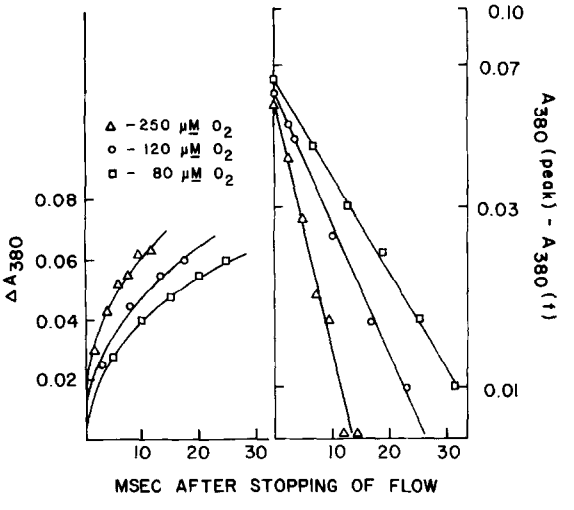

Fig. 3. Effect of oxygen concentration on the initial rapid absorbance change (Phase A) at 380 nm, plotted (left) directly and (right) on a semi-logarithmic scale plotting the difference between the peak absorbance change at about 40 msec and that at time t. Mixing as in figure 2, varying the concentration of dissolved oxygen. Values are those after mixing.

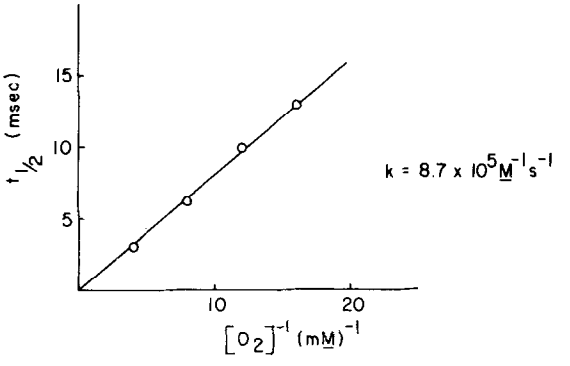

Fig. 4. Effect of oxygen concentration on the half-time of the increase in absorbance at 380 nm (Phase A).

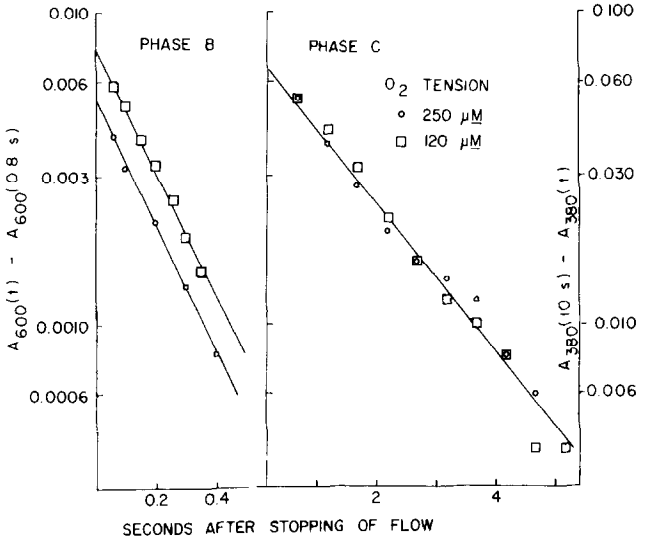

Fig. 5. These experiments illustrate the lack of effect of oxygen concentration on the kinetics of phase B and C. Data taken from experiments similar to those in Figure 2. Similar results were obtained at other oxygen concentrations.

However, there are other equally suitable alternative schemes. For example, it is not apparent whether these relaxations (B and C) occur sequentially or in parallel; we observed the rate of decay of intermediate II to FMN and H_2O_2 to be the same whether the reaction was monitored by following the increase in absorbance at 445 nm (15) or the decrease in absorbance at 600 nm. Since it seems unlikely that the 4a-flavin hydroperoxide would

itself possess any long-wavelength absorbance, it may be assumed that it is in equilibrium with some flavin species which does absorb there: a flavin radical, either free or spin-paired with some other radical, a flavin triplet, a charge-transfer complex, or some substituted flavin (16). In our experiments no signals were observed in the ESR spectrophotometer which could be associated with the luciferase flavin intermediates after addition of oxygen, suggesting that free radicals and triplets should be eliminated from consideration.

The nature of the initial adduct of reduced flavin and O_2 is also of interest. Hamilton (17) suggests that one mechanism to account for the fact that the reaction of triplet O_2 with singlet $FMNH_2$ yields singlet product would first involve the formation of a pair of doublets from the reactants (giving flavin and superoxide radicals) with subsequent spin inversion and radical combination to form singlet product. It is possible that the initial very rapid absorbance change observed upon reaction of I with O_2 reflects the formation of such a pair of doublets or radicals.

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