BACTERIAL BIOLUMINESCENCE: ABSORPTION AND FLUORESCENCE CHARACTERISTICS AND COMPOSITION OF REACTION PRODUCTS OF REDUCED FLAVIN MONONUCLEOTIDE WITH LUCIFERASE AND OXYGEN

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Summary: The initial reaction products of $FMNH_2$, oxygen and bacterial luciferase are a flavoprotein, free FMN and hydrogen peroxide. This flavoprotein then adds a mole of oxygen to give a product which either reacts with a long-chain aldehyde to give bioluminescence, or in the absence of aldehyde decays to free enzyme, free FMN and hydrogen peroxide.

Quantum yield measurements and chemical identification have recently established the *in vitro* reaction of bacterial bioluminescence: (1-3)

$$2FMNH_2 + 2O_2 + RCHO \xrightarrow{E} 2FMN + H_2O_2 + H_2O + RCOOH$$

This is a luciferase (E) catalyzed oxidation of $FMNH_2$ and a long-chain (N>8) aliphatic aldehyde resulting in the emission of light.

If the addition of aldehyde to the reaction is delayed after the other components, the intensity of the light emission obtained diminishes in proportion to the delay. This has been interpreted as the formation of a long-lived intermediate which either reacts with aldehyde to give light or if aldehyde is absent decays away in a first order manner (4). This intermediate is stabilized at low temperature, the half-life being about seven minutes at 5°C with the MAV luciferase. We have taken advantage of this fact to characterize the initial products of the reaction of oxygen with $FMNH_2$ on the enzyme by determining the rates of utilization of oxygen and appearance of FMN and H_2O_2.

Two steps in the reaction of oxygen with $FMNH_2$ and enzyme may be discerned in the formation of the initial products, the first much faster than the second. The $FMNH_2$ is completely oxidized in the first step by only one-half equivalent of oxygen to form one-half equivalent H_2O_2. The enzyme is

apparently reduced in this step since one-half of the $FMNH_2$ injected is found as free FMN, and the other half bound to form a flavoprotein. This flavoprotein adds oxygen in the second slower step to form an oxygenated intermediate which can break down ($t_{\frac{1}{2}}$ 7 min) to form free enzyme, FMN and H_2O_2.

EXPERIMENTAL

Luciferase from the bacterium type "MAV" (5) was prepared by the method previously reported for the purification of *Photobacterium fischeri* luciferase (1) with the additional step of elution from DEAE-Sephadex by a linear gradient. The luciferase appeared homogenous by the normal criteria of disc electrophoresis and velocity sedimentation. FMN (86% nominal purity) was purchased from Fluka AG, and further purified by DEAE-cellulose chromatography. All other chemicals were of the best available reagent grade.

All spectra and reaction rates were measured at 5°C. Absorption spectra were determined on a Cary 14 spectrophotometer and fluorescence on an instrument previously described (6). Fluorescence polarization was determined by the use of polaroid filters with this instrument. Fluorescence lifetimes were determined with a TRW nanosecond lifetime instrument.

Oxygen consumption was followed by a Clark oxygen electrode (Rank Brothers, Cambridge, England) calibrated as previously described (1). Hydrogen peroxide was determined by measuring the difference in oxygen consumption in the presence of catalase (1).

RESULTS

When $FMNH_2$ is added to luciferase in air-saturated solution the autooxidation competes with the reaction with enzyme:

$$FMNH_2 \begin{cases} \xrightarrow{E} E\text{-}FMNH_2 \longrightarrow \text{light} \\ \xrightarrow{O_2} FMN + H_2O_2 \end{cases}$$

Since the autooxidation is fast (7) and the overall rate of light emission

far slower (4), luciferase must be present in a high concentration to outcompete oxygen for $FMNH_2$. A concentration of 40 μM (3 mg/ml) has been found sufficient for this since the quantum yield of bioluminescence with respect to $FMNH_2$, $Q_B(FMNH_2)$, is observed to reach a constant value of 3% in the complete *in vitro* reaction at this concentration and above (2). This maximum $Q_B(FMNH_2)$ is unchanged from room temperature to 0°C.

The decay rate of the long-lived intermediate formed when luciferase reacts with oxygen and $FMNH_2$ was determined by injecting 4.0 or 8.0 nmoles of $FMNH_2$ (0.1 cc) into an air-saturated solution of luciferase (40 μM, 1 cc) followed at a known time interval by injection of decanal (0.1 cc, aqueous, 400 μM). The relative quantum yield $Q_B(FMNH_2)$ was found to decrease logarithmically with the time interval with a $t_{\frac{1}{2}}$ of seven minutes.

Fluorescence

On addition of $FMNH_2$ to an air saturated solution of luciferase (5°C), a fluorescent product appears within the first 30 seconds which has a fluorescence spectral distribution identical to that of FMN. The fluorescence intensity remains constant for several minutes at 50% of that expected if all of the added $FMNH_2$ were fully oxidized and free in solution. The fluorescence lifetime and polarization of fluorescence were measured to be 6±1 nsec and <2% respectively; these values were indistinguishable from free FMN. From this data it is concluded that one-half the added $FMNH_2$ is now oxidized and free in solution while the rest must be bound to some form of luciferase. Control experiments with FMN and this luciferase show that FMN fluorescence is not quenched in the presence of MAV luciferase.

Absorption

Several minutes after the addition of $FMNH_2$, the optical density at 445 nm is found to be 84% of that expected if all of the $FMNH_2$ were fully oxidized. The total absorption spectra of the initial reaction mixture and of the final products are shown in figure 1. The initial absorption spectrum A ($60 < t < 180$ seconds) has absorption maxima at 445 and 370 nm, the same as for free

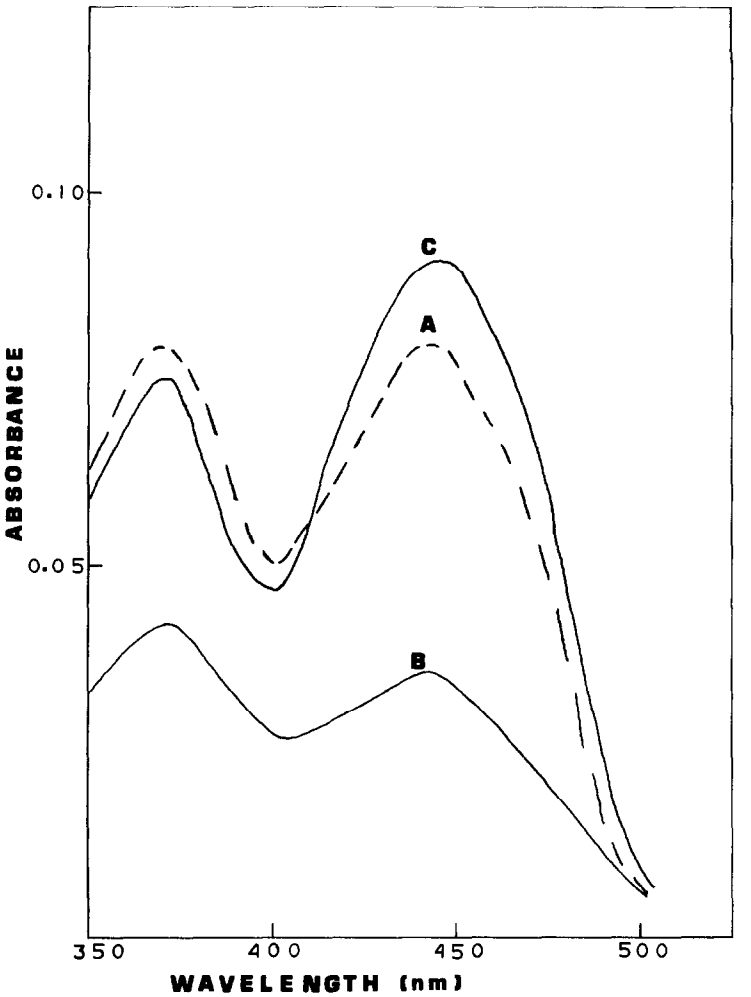

Fig. 1. Absorption spectra of reaction intermediates formed on injection of $FMNH_2$ into a solution of luciferase and oxygen. Curve A is the initial absorption spectrum (60 sec < t < 180 sec). Curve B is the spectrum obtained on subtraction of an FMN spectrum corresponding to one-half the $FMNH_2$ injected from curve A. Curve C is the final product (t >1200 sec.) and corresponds to free FMN.

FMN except that the 370 nm absorption is considerably enhanced and greater than that expected if 100% of the $FMNH_2$ injected were present as free FMN. Since the fluorescence results have shown that half the FMN is in fact free, subtraction of a free FMN spectrum equal to 50% of the total expected for the amount of $FMNH_2$ added from curve A, results in curve B. This spectrum corresponds to the non-fluorescent FMN and can be seen to have an absorption with a strongly enhanced 370 nm maximum which taken together with the absence

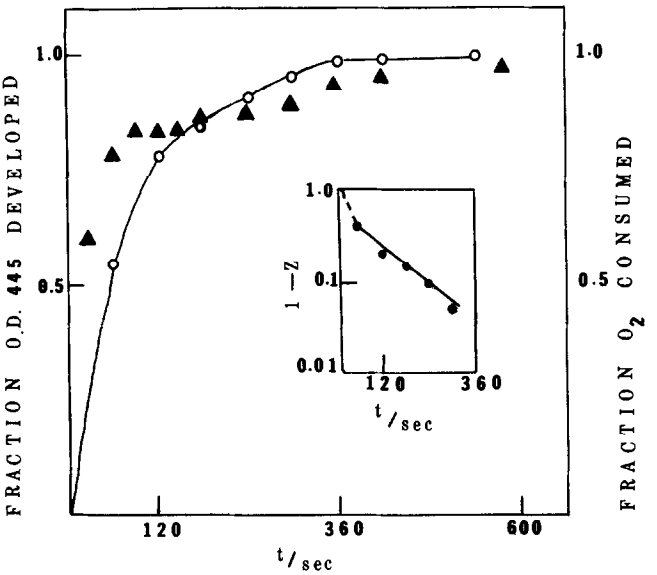

Fig. 2. Rates of oxygen utilization and O.D. 445 appearance on injection of $FMNH_2$ into a solution of luciferase and oxygen. The triangles (▲) represent the appearance of O.D. 445 as a fraction of the final O.D. 445. The open circles represent the O_2 consumed as a fraction of the final O_2 consumption. The semilog plot (insert) shows the psuedo first order reaction of the intermediate with oxygen. The Y axis (1-Z) is one minus the fractional oxygen consumption.

of fluorescence is suggestive of a flavoprotein. These present results are not sufficiently precise to distinguish other characteristics exhibited by many flavoproteins such as a slight red shift of the 445 nm peak.

If the reaction products are allowed to decay by standing for 15 minutes or warming to room temperature, the absorption curve C results. This curve is identical to free FMN with an optical density at 445 nm corresponding exactly to that predicted from the added $FMNH_2$.

Rate of Oxygen Utilization and Appearance of FMN

The response time of the oxygen electrode prevented measurements of the oxygen concentration being made in time intervals less than one minute. The rate of oxygen utilization is compared in Figure 2 to the appearance of optical density at 445 nm. The insert in figure 2 shows that the oxygen utilization is in two steps, the first being quite rapid followed by a second slower psuedo first order uptake of oxygen with a $t_{\frac{1}{2}}$ of 1.3 min. The initial increase

in OD 445 is followed by a slower increase which corresponds to the long-lived intermediate decay ($t_{\frac{1}{2}}$,7 min) and presumably represents the conversion of flavoprotein to free FMN. The first oxygen utilization rate probably corresponds to the rapid OD 445 appearance which is at a rate far slower than for oxygen oxidation of $FMNH_2$ in free solution. The preliminary results indicate that the initial OD 445 production is not first order.

Hydrogen Peroxide

If oxygen utilization is measured in the presence of catalase (0.1 mg/ml) it is found in the first minute 0.5 equivalents of H_2O_2 are formed per $FMNH_2$ added. If the reaction products are allowed to decay (15 min) another 0.5 equivalents of H_2O_2 are produced.

DISCUSSION

These results may be described by the following scheme in which we will designate the two intermediates as X_1 and X_2. The initial step is

$$E + 2FMNH_2 + O_2 \xrightarrow[\text{Fast}]{E} X_1 + H_2O_2 + FMN$$

Two moles of $FMNH_2$ react with oxygen and luciferase to yield one mole of H_2O_2, one mole of free FMN and one mole of enzyme bound FMN. This represents an overall process composed no doubt of a sequence of reactions. The intermediate X_1 then takes up another mole of oxygen at a second slower rate to yield X_2 which in the presence of aldehyde, gives rise to bioluminescence or in its absence, decays in a first order manner with a half-life of 7 minutes, that is:

$$X_1 + O_2 \xrightarrow{(t_{\frac{1}{2}}\ 1.3\ \text{min.})} X_2 \xrightarrow{(t_{\frac{1}{2}}\ 7\ \text{min.})} E + FMN + H_2O_2$$

$$X_2 \xrightarrow{RCHO} E + FMN + H_2O + RCOOH + h\nu$$

It would appear reasonable that X_1 is a flavoprotein of composition H_2E-FMN. Disulfide bond reduction to cysteines would fulfill this role (8) and this is suggested by the observed inhibition of the light reaction by arsenite (4).

Oxygen addition yields X_2 which is probably a sulfur hydroperoxide. A flavin hydroperoxide must be eliminated as the absorption spectra of X_1 and X_2 correspond to fully oxidized free and bound FMN and not to flavin hydroperoxides which have maximum absorptions at 410 or below 380 nm, with little contribution at 445 nm (9,10). The composition of X_2 must be HOOHE-FMN which explains why it can break down to H_2O_2 with release of FMN or react with aldehyde to give bioluminescence.

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