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A time-dependent bacterial bioluminescence emission spectrum in an *in vitro* single turnover system: Energy transfer alone cannot account for the yellow emission of *Vibrio fischeri* Y-1

(bacterial luminescence/bacterial luciferase/enzyme mechanism)

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ABSTRACT **Yellow fluorescent protein (YFP), which has a bound FMN, was isolated from the marine bacterium *Vibrio fischeri* strain Y-1b. Its presence in a luciferase [alkanal monooxygenase (FMN-linked); alkanal, reduced-FMN:oxygen oxidoreductase (1-hydroxylating, luminescing), EC 1.14.14.3] reaction mixture causes a striking color change, and an increase in bioluminescence intensity, as well as a faster rate of intensity decay, so that the quantum yield is not changed. The emission spectrum shows two distinct color bands, one at 490 nm attributed to the unaltered emission of the luciferase system, the other peaking in the yellow around 540 nm due to YFP emission. The kinetics of the two color bands differ, so the spectrum changes with time. The yellow emission reaches its initial maximum intensity later than the blue, and then both blue and yellow emissions decay exponentially with nearly the same pseudo-first-order rate constants, linearly dependent on [YFP] (from 0.01 sec^{-1} with no YFP to a maximum of ≈0.1 sec^{-1} at 4°C) but exhibiting a saturation behavior. The data can be interpreted by assuming the interaction of YFP with the peroxyhemiacetal intermediate in the luciferase reaction to form an unstable new complex whose breakdown gives the yellow emitter in its excited state. This simple model fits well the data at [YFP] < 15 μM. The results indicate that a single primary excited state cannot be responsible for the blue and the yellow emissions.**

In contrast to most species of bioluminescent bacteria, which emit blue light, a strain (Y-1) of *Vibrio fischeri* emitting yellow light was isolated some years ago (1); a shoulder at 490 nm in the emission spectrum (see Fig. 1*A*) corresponds to the peak of bioluminescence of wild-type *V. fischeri*. The remarkable large spectral shift involved (from λ_{max} = 490 to λ_{max} = 540 nm) was found to be due to the presence, in these bacteria, of a yellow fluorescent protein (YFP) (2), in which the emitting chromophore was identified as FMN (3–5).

Bacterial bioluminescence accompanies the oxidation of $FMNH_2$ and a long-chain aldehyde by molecular oxygen, catalyzed by the enzyme luciferase [alkanal monooxygenase (FMN-linked); alkanal, reduced-FMN:oxygen oxidoreductase (1-hydroxylating, luminescing), EC 1.14.14.3] (6). Luciferase extracted from the Y-1 strain does not appear to differ significantly from normal blue-emitting *V. fischeri* luciferase. It has similar molecular weight and subunit structure and a similarly slow catalytic cycle; a single enzymatic turnover takes ≈100 sec at 4°C. In addition, the light emitted in the reaction of $FMNH_2$ catalyzed by Y-1 luciferase peaks at the characteristic 490 nm (see Fig. 1*B*; ref. 2). However, the presence of YFP in the reaction mixture—but not its chromophore FMN or the apoprotein alone—brings about the yellow emission (see Fig. 1*B*).** The relative magnitude of the bands at 490 and 540 nm was found to depend strongly on the concentration of YFP and temperature (2), suggesting a reversible association between luciferase and YFP.

It has been proposed that the color shift can be attributed to energy transfer (1, 4, 5). For example, the excited chromophore of the luciferase reaction, which is responsible for the blue emission and has been identified as 4*a*-hydroxyflavin (in *Vibrio harveyi*; ref. 7), would donate its energy to YFP, thereby generating excited YFP, which would then emit its yellow fluorescence. Alternatively, it has been suggested that the excited states of the two chromophores could be populated independently (6).

The kinetic and spectral results reported here are not compatible with a mechanism of color shift based exclusively on energy transfer from a single primary excited species. We found that YFP significantly alters the kinetics of the reaction and thus must participate in the reaction at one or several points prior to the formation of the excited state primary emitter. Consistent with our results, Daubner and Baldwin (8) have observed that adding YFP to a luciferase–reductase coupled assay also speeds up the light emission.

MATERIALS AND METHODS

Bacteria. A *V. fischeri* strain Y-1b with a high yellow/blue ratio *in vivo* (Y/B = 5.5) at 17°C was obtained by selection of a single colony for brightness (9) on seawater complete agar medium (750 ml of seawater, 3 g of yeast extract, 5 g of bactotryptone, 2 ml of glycerol, and 25 g of agar per liter of medium; ref. 10). Bacteria were grown in liquid seawater complete medium at 17°C and harvested by centrifugation at 4°C. The growth of bacteria was followed by measuring the OD at 660 nm. After harvesting, the cell paste was stored at −20°C. The light emission was measured in the photometer of Mitchell and Hastings (11).

Chemicals. Flavin mononucleotide (FMN), EDTA, and dithiothreitol were purchased from Sigma and decanal was from Aldrich. The standard buffer was 30 mM phosphate buffer (pH 7.0) with 1 mM EDTA and 1 mM dithiothreitol. $FMNH_2$ was prepared by reduction of FMN by H_2/Pt asbestos.

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Abbreviation: YFP, yellow fluorescent protein.

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**At high concentrations of free FMN, the bioluminescence spectrum may be slightly distorted due to trivial energy transfer.

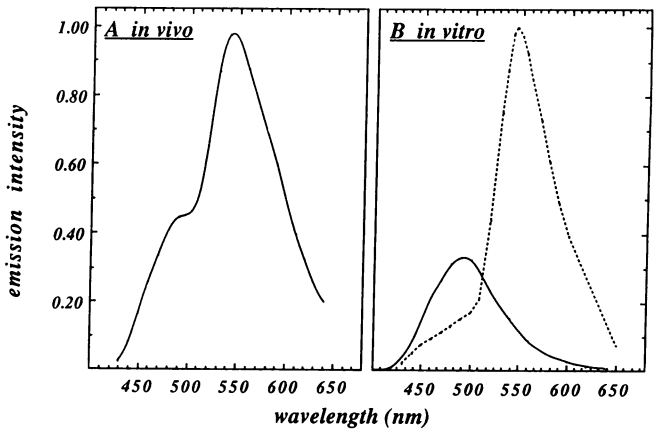


FIG. 1. (*A*) Bioluminescence emission spectrum of *V. fischeri* Y-1 *in vivo* at 17°C. (*B*) Emission spectra of *in vitro* bioluminescence with purified *V. fischeri* Y-1b luciferase in the absence (—) and presence (·····) of purified YFP (final concentration, 15 μM), showing that YFP increases both the intensity integrated over the spectrum and the Y/B ratio from 0 to 6.5 under the conditions of this experiment.

Luciferase and YFP Purification. The preparation and purification of YFP (3) and luciferase (12) were performed as described. Protein concentrations were determined with the Bio-Rad kit using Coomassie blue (13) and ovalbumin as the protein standard; estimates of concentrations were based on molecular weights of 79,000 and 25,000 for luciferase and YFP, respectively.

***In Vitro* Assay.** The assay was performed at 4°C by rapid injection of 50 μl of 50 μM $FMNH_2$ to a solution containing buffer, luciferase (final concentration, ≈1 μM), and 10 μl of decanal (0.1% in ethanol) and preincubated with different amounts (as specified) of purified YFP (total final vol, 160 μl). In the experiments of Fig. 1*B*, 10 μl of dodecamine (0.01% in EtOH) was added to decrease the decay rate of light emission. Its effect has been found to be similar to that of dodecanol (14).

The course of luminescence decay was monitored either in a spectrofluorometer (SPEX Fluorolog 1681 or Perkin–Elmer MPF-44) at 460 and 540 nm or in a photometer modified to accommodate two filters: an interference filter centered at 450 nm (FWHM, 11 nm) and a Corning cut-off filter no. 2-78 transmitting at $\lambda > 580$ nm. The data obtained with the filter photometer were converted to intensities at 460 and 540 nm on the basis of calibration spectra. All measurements were corrected for the spectral response of the equipment.

Determination of Y/B Ratio. Partial spectral overlap of the blue and yellow emission bands must be taken into account in estimating the relative contributions from these two emissions. In the blue luminescence, the intensities at 460 and 540 nm are both equal to 50% of the intensity at $\lambda_{max} = 490$ nm. In the yellow band, which peaks at 540 nm, the intensity at 460 nm is negligible. Therefore, the Y/B ratio was calculated as follows:

$$Y/B = (I_{540} - I_{460})/(2 \times I_{460}),$$

where I_{460} and I_{540} are the intensities measured at 460 and 540 nm.

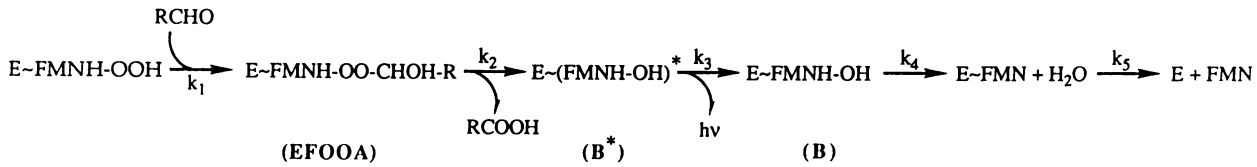
RESULTS AND DISCUSSION

Reactions were initiated at 4°C by injection of catalytically reduced $FMNH_2$ in buffer containing luciferase and saturating decanal (10 μM). Under such conditions, only a single enzymatic turnover occurs; all $FMNH_2$ not bound initially to luciferase is quickly autoxidized by dissolved oxygen and thus effectively removed from the reaction mixture within 1 or 2 sec (15).

With decanal, the luminescence emission reaches its maximum intensity within 1–2 sec and then decays exponentially with a rate constant of $\approx 0.01\ sec^{-1}$ at 4°C. The presence of YFP in a buffered solution of luciferase and aldehyde (and, where appropriate, preincubation for 5 min at 4°C) had the following effects: (*i*) The appearance of a shoulder at 540 nm in the emission spectrum at low concentration of YFP; this becomes the dominant peak at high YFP concentrations (Fig. 1*B*). (*ii*) An increase in the emission intensity integrated over the whole spectrum. (*iii*) Faster and nearly identical first-order rates of luminescence decay in both the blue and yellow (Fig. 2). These rates increase linearly with [YFP] (Fig. 3) but become gradually independent at [YFP] > ≈30 μM (Fig. 3 *Inset*). Similarly, the Y/B ratio increases with [YFP] up to ≈50 μM but actually drops down at very high concentrations (Fig. 3 *Inset*). (*iv*) The peak intensity and its rate of decay increase in parallel, so the reaction's quantum yield is not significantly different in the presence of YFP. (*v*) The initial kinetics of the emission are markedly different in the blue and yellow band, with the peak intensity occurring distinctly later in the yellow than in the blue (Fig. 2). Consequently, the Y/B ratio is time dependent (Fig. 2 *Inset*).

Significantly, when YFP is added after the reaction has already started, the color shift and the increase in intensity and its rate of decay are also observed (Fig. 4). Increases in intensity and decay rate are also observed when the reaction has been initiated with 2-thio-$FMNH_2$. In this case, the emission in the absence of YFP peaks at ≈540 nm (16), therefore at the red edge of the absorption spectrum of YFP (FMN chromophore). Yet, upon the secondary addition of YFP, the intensity increases to a higher value in ≈25 sec and then decays at a faster rate than without YFP.

All of these results lend themselves to a straightforward interpretation. Indeed, the *in vitro* bacterial reaction catalyzed by luciferase is especially suited for kinetic analysis, because a single catalytic step (one enzyme turnover) can be followed (15); the oxidation of a given amount of the substrate $FMNH_2$ bound to luciferase (E) occurs concomitantly with the oxidation of aldehyde, according to Scheme I (7). Note the abbreviations in parentheses below certain intermediates.



Scheme I

In this scheme, k_2 and k_4 are small compared to k_1 and the relative rates of these steps depend on aldehyde chain length. Although the 4*a*-hydroxyflavin intermediate has a lifetime of minutes at 9°C, the lifetime of its singlet excited state (B* here) is expected to be only a few nanoseconds, thus exceedingly short compared to the time scale of the overall reaction, which is measured in minutes.

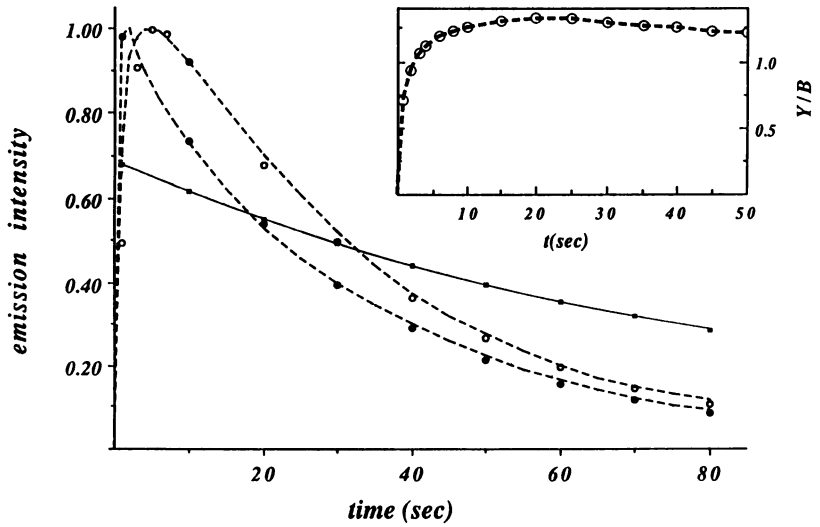
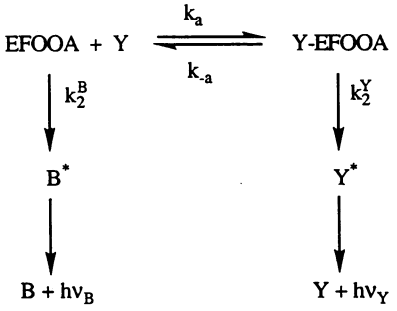


FIG. 2. Luminescence intensity of *in vitro* reactions at 4°C as a function of time. —, 450 nm with no YFP; -----, 450 nm and 540 nm, both with 5 μM YFP. The traces with YFP were normalized at peak intensities. The points (■, no YFP; ●, with YFP at 450 nm; ○, with YFP at 540 nm) are calculated on the basis of Scheme II with the following values for the rate constants: $k_a = 4000\ sec^{-1}\cdot M^{-1}$; $k_2^B = 0.011\ sec^{-1}$; $k_2^Y = 0.55\ sec^{-1}$. The calculated curves with YFP are normalized at I_{max}. (*Inset*) Y/B ratio versus time in the same experiment.

Because of the short lifetime of B*, energy transfer to Y cannot be a sufficient explanation for our observations. Although energy transfer could lead to spectral changes, it could not alter the reaction kinetics. Indeed, since B* decays with a rate constant of the order of $10^8\ sec^{-1}$, it is clear that energy transfer from this species to Y could not be responsible for the changes in the initial kinetics, which take place on a time scale of seconds. Consequently, YFP must interact with one of the reaction intermediates prior to formation of the excited state. The key intermediate is most likely the peroxyhemiacetal EFOOA (Scheme I), since the effects of YFP are manifest even when YFP is added secondarily, after all the peroxyhemiacetal has been generated and has started to break down (Fig. 4).

The increase in decay rates of blue and yellow light, together with the slow onset of yellow emission (observations *iii* and *v* above), suggest that YFP binds to the luciferase-bound peroxyhemiacetal and thus shortens its lifetime. We propose that the concentration of a new intermediate, Y-EFOOA, builds up; its breakdown generates the yellow emitter Y* directly. This simple hypothesis is represented by Scheme II. In this scheme, all modes of decay of EFOOA and of Y-EFOOA are lumped together as k_2^B and k_2^Y; other modes of decay of these two intermediates would only affect the quantum yield, not the kinetics.



Scheme II

In fact, at low concentrations of YFP (<15 μM), our results can be well accounted for by assuming that, on the time scale of a turnover, the formation of the complex Y-EFOOA is irreversible—i.e., $k_{-a} = 0$. Scheme II is then simply a set of consecutive reactions, which predict that the blue emission (I_B) will decay exponentially (Eq. 1), whereas the time curve of the yellow emission (I_Y) can be fitted to the difference of two exponentials (Eq. 2). The rate parameters are functions of all the rate constants of the scheme as well as of the concentration of YFP.

$$I_B \propto k_2^B[\text{EFOOA}] \propto \exp(-\lambda_1 t), \quad [1]$$

$$I_Y \propto k_2^Y[\text{Y-EFOOA}] \propto \exp(-\lambda_1 t) - \exp(-\lambda_2 t). \quad [2]$$

Since blue and yellow emissions decay at practically the same rate (after t_{max}; observation *iii* above), and this rate is faster than in the absence of YFP, YFP destabilizes the peroxyhemiacetal—i.e., $k_2^Y > (k_2^B + k_a[Y])$. In this case, $\lambda_1 = k_2^B + k_a[Y]$ and $\lambda_2 = k_2^Y$. Figs. 2 and 3 show that such a simple scheme can indeed satisfactorily simulate the luminescence decay curves as well as the dependence of these rates on the concentration of YFP.

At higher concentrations of YFP, however, Scheme II is no longer a good model of our results. Indeed, if irreversible complexation is assumed as above, then Scheme II predicts that the rate of decay of the blue light should keep on increasing linearly with the concentration of YFP, while the yellow rate of decay should reach a maximum value when $k_2^Y \leq (k_2^B + k_a[Y])$ and remain constant thereafter. In fact, we observed that both blue and yellow intensity decay rates become independent of YFP at high concentrations.

On the other hand, if complexation is reversible ($k_{-a} \neq 0$), the system represented by the coupled reactions of Scheme II would reach equilibrium and therefore show saturation at high concentrations of YFP, as indeed observed. In this case, the model predicts that the blue light no longer decays as a single exponential but as a sum of two exponentials (Eq. 3), with the same final rates of decay of both blue and yellow light.

$$I_B \propto \exp(-\lambda_1 t) + \exp(-\lambda_2 t). \quad [3]$$

In fact, we see no indication of any deviation from a single exponential decay of the blue emission at any concentration of YFP. It might be argued that a small contribution of a second exponential component of very low amplitude might not be detected. However, if such were the case, k_{-a} would have to be so small compared to k_a as to cause only a very small curvature in the plots of the rates of intensity decay vs.

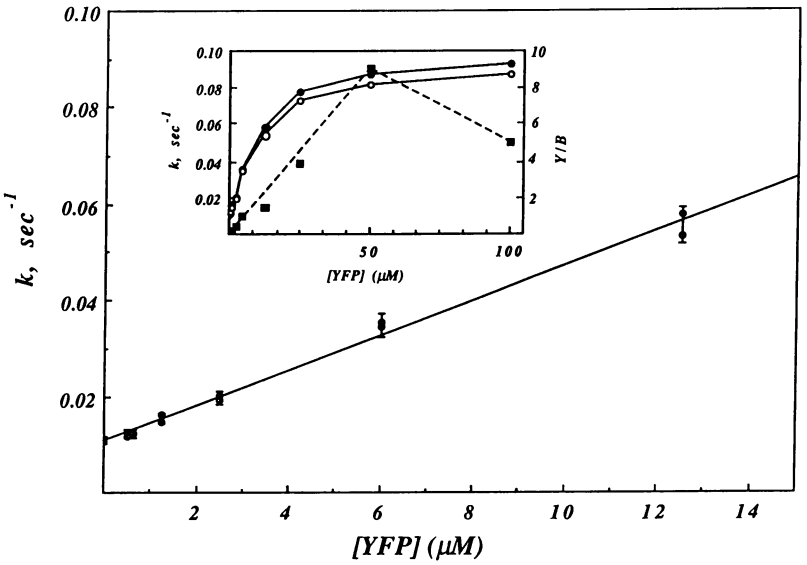


FIG. 3. Decay rates of luminescence intensity at 4°C as a function of YFP concentration. The reaction was monitored at 450 nm (●) and 540 nm (○). The solid line is calculated on the basis of Scheme II with the same values of rate constants as in Fig. 2. (*Inset*) Same, but over a larger range of YFP concentration. Here the lines connect the experimental points. *Inset* also shows Y/B ratio (■) versus YFP concentration.

[YFP], again a totally inadequate representation of our results (Fig. 3 *Inset*).

At this point, we must therefore conclude that although the simple mechanism of Scheme II, with no or very little back reaction, is compatible with our main observations at low concentrations of YFP, we have no explanation for the behavior at higher concentrations. Some type of inhibitory process might be involved, such, for example, as inactivation of YFP via dimerization. Scheme II is also unable to account for the small (≈5%) but systematic difference between the yellow and blue decay rates.

This phenomenological analysis does not presume the chemical nature of the interaction between YFP and any of the species involved in the reaction. YFP may simply alter the conformation of the luciferase at its active site, thereby destabilizing one or several of the intermediates. As mentioned earlier, one could imagine that within the luciferase–peroxyhemiacetal–YFP complex the "normal" excited emitter (B*) is formed first. Since the spectral overlap between donor and acceptor is not unfavorable, the excited state of Y could then be populated via energy transfer. However, the distinct onset kinetics in the blue and the yellow allow us to conclude that no substantial part of the blue emission observed in the presence of YFP could be derived from such a hypothetical excited B* within the complex. Thus a single primary electronically excited species cannot be responsible for emission at the two different wavelengths. This conclusion is reinforced by the results obtained when the luciferase reaction is initiated with 2-thio-$FMNH_2$. With this flavin, the overlap with the absorption spectrum of YFP is unfavorable, yet the secondary addition of YFP (as in Fig. 4) increases the emission intensity and reaction rate much as it does with $FMNH_2$.

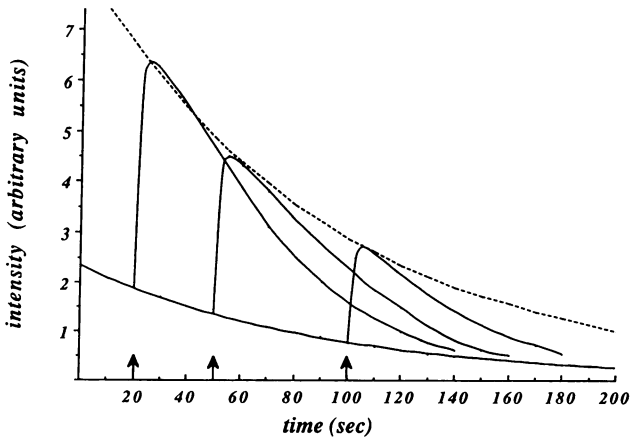


FIG. 4. Effect of delayed addition of YFP on the intensity at 540 nm; three experiments are presented together; YFP (50 μl; final concentration, 3.75 μM) was added secondarily 20, 50, or 100 sec after initiation of the reaction with FMNH, using purified luciferase and decanal. Note that the dashed line through the peak intensities attained with YFP indicates the same rate of decay as in the absence of YFP (bottom line).

The alternative—namely, the direct formation of Y*, as proposed here—brings to mind examples of intermolecularly "activated" chemiluminescence, where a fluorescer catalyzes the decomposition of a peroxide via electron or charge transfer and gets electronically excited in the process (17, 18). The identity of the YFP fluorescence spectrum and the yellow band of the bioluminescence (3) is consistent with such a mechanism.

The major conclusions of the present paper are that YFP must form a reactive complex with at least one intermediate, the peroxyhemiacetal, and that energy transfer alone cannot account for the effect of YFP in the *in vitro* bioluminescence of Y-1. Whether this conclusion has validity in other bioluminescent systems that exhibit color shifts (19) needs to be considered. A case in point is the effect of the blue fluorescent protein, with a pteridine (lumazine) prosthetic group acting as emitter, in the luciferase reaction of a different species of luminous bacteria (20, 21). The effect of the lumazine protein on the kinetics of the reaction is suggestive of a mechanism similar to the one outlined here.

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