A Bacterial Luciferase Reaction with a Negative Temperature Coefficient Attributable to Protein−Protein Interaction†

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ABSTRACT: A yellow fluorescent protein (YFP) present in a strain of bioluminescent bacteria is shown here not only to modify the color and intensity of the emission, as already known and attributed to the interaction of YFP with a luciferase intermediate, but also remarkably to confer a negative temperature dependence to the *in vitro* system. The *in vitro* bioluminescence decay rate is actually independent of temperature in the range 5−25 °C, at ~1 μM YFP concentration. Several hypotheses are considered to explain this effect, based either on inactivation of YFP itself at higher temperatures or on its binding equilibrium with the luciferase intermediate. The first hypothesis is favored. Fluorescence anisotropy measurements show that YFP loses its chromophore at higher temperatures, but this alone cannot account for the negative temperature dependence. Gel chromatography shows the existence of an inactive YFP dimer, and the formation of more dimer at higher temperatures cannot be ruled out but is unlikely in our experimental conditions. Conformational changes may contribute to YFP inactivation. To our knowledge, there is no prior example of an enzymatic reaction in which the rate is slower at higher temperatures, within a physiological range.

The bacterial bioluminescence reaction is catalyzed by bacterial luciferase, a unique enzyme with no known homologs. The reaction involves the mixed function oxidation of reduced flavin mononucleotide and a long chain aldehyde and gives the corresponding acid and FMN with emission of blue-green light (λ_{max} ~ 490 nm) (Tu & Mager, 1995; Meighen, 1994; Baldwin & Ziegler, 1992; Lee et al., 1991). The bioluminescence quantum yield is between 0.1 and 0.3.

The reaction proceeds via several enzyme intermediates (Scheme 1), starting with the 4a-peroxyflavin followed by the peroxyhemiacetal (E·FOOA), which breaks down to generate the 4a-hydroxy FMN in an electronically excited state, the emitter (Kurfürst et al., 1984). Luciferase is an unusual enzyme in that it slows down the reaction it catalyzes; the autooxidation of $FMNH_2$ is 20 times faster than the enzymatic rate (Gibson & Hastings, 1962; Hastings & Gibson, 1963). It is this slow rate that has allowed for the isolation and characterization of some of the enzyme intermediates (Hastings et al., 1973). In the so-called "single turnover assay" used in the present work, the rise and decay of emission intensity are followed subsequent to the addition of $FMNH_2$ to a solution of enzyme and aldehyde.

Some years ago, a strain (Y-1) of the bacterium *Vibrio fischeri* was discovered to emit yellow light (λ_{max} ~ 540 nm; Ruby & Nealson, 1977). This striking spectral shift of ~50 nm was shown to be due to the presence in Y-1 cells of an accessory "yellow fluorescence protein" (YFP)[1] in which the chromophore is FMN (Macheroux et al., 1987; Daubner et al., 1987). The spectrum of the bioluminescence matches the fluorescence maximum of purified YFP, red-shifted from that of free FMN (λ_{max} ~ 525 nm). However, Y-1 cells emit yellow light only at low temperatures; the peak of emission shifts reversibly to the blue at higher temperatures (Cho et al., 1989).

YFP causes similar spectral shifts *in vitro*. At a low concentration of YFP, two emission bands can be readily resolved, one peaking at 490 nm, emitted by the normal emitter of the bacterial reaction (the 4a-hydroxy FMN intermediate, E·F* in Scheme 1), the other at 540 nm, emitted by YFP. The ratio of intensities in these two bands is an index of the effect of YFP under the conditions of the experiment.

Although the ecological niche where this Y-1 bacterium thrives has not been determined, and no Y-1 strains serving as symbionts have thus far been identified, it has been tacitly assumed that it is the color of the light that is of selective advantage. Another role of YFP may be equally or even more important from an evolutionary point of view; *in vitro* at 4 °C and high YFP concentration, the intensity of emission is increased by up to 10-fold as a result of an increase in the turnover rate of reaction (Eckstein et al., 1990). These data led to the conclusion that YFP interacts with a reaction intermediate, most likely the postulated peroxyhemiacetal, E·FOOA, shortening its lifetime and concomitantly shifting the emission maximum. Scheme 2 was proposed to account for the kinetics and for the fact that in the single turnover assay YFP shifts the color of the emission and increases its decay rate even when added after the reaction has started and the light emission is already decaying.

We report here an unusual dependence of rate and emission spectrum on temperature. YFP is without effect at 25 °C but recovers its effectiveness when the temperature is lowered. With decanal and 4−10 μM YFP, the decay of light emission (turnover rate) is actually faster at 4 °C than at 25 °C. This negative temperature dependence is in contrast to the situation with luciferase alone (Scheme 1),

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[1] Abbreviations: YFP, yellow fluorescent protein; FMN, flavin mononucleotide; NADH, reduced nicotinamide adenine dinucleotide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; EFOOA, luciferase peroxyhemiacetal; EF*, excited state of luciferase 4a-hydroxy FMN.

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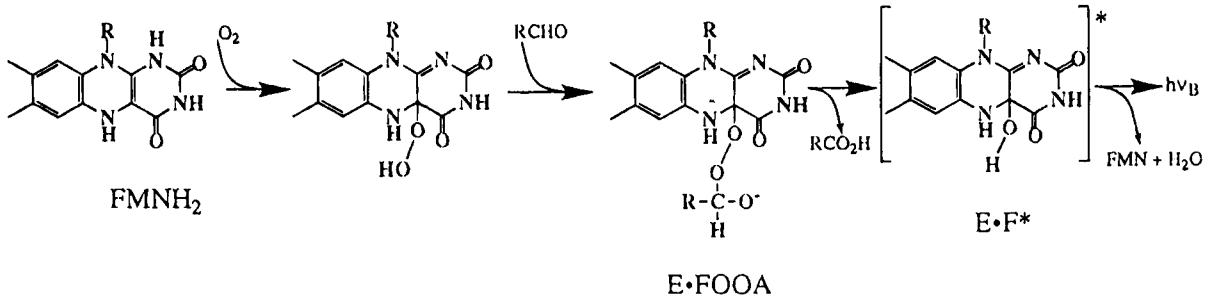
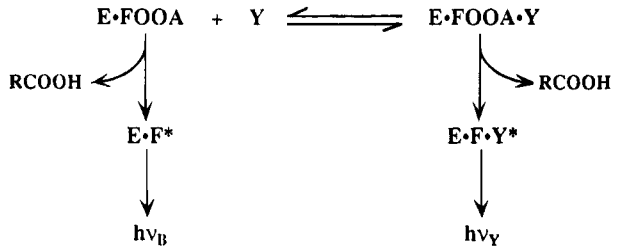
Scheme 1. Reactants, Products, and Enzyme Intermediates in the Reaction of Bacterial Luciferase



Scheme 2. Reaction of Luciferase Peroxyhemiacetal (E•FOOA) with YFP (Y) To Form the Protein−Protein Complex E•FOOA•Y[a]



[a] E•F* and E•F•Y* represent, respectively, the excited states of the blue and yellow emitting intermediates; $h\nu_B$ and $h\nu_Y$ represent, respectively, the emitted blue and yellow photons.

where the rate constant of the light-emitting reaction increases with temperature in the expected Arrhenius fashion. This system is an example of a "temperature-compensated" rate process in an enzymatic system. Indeed, at a relatively low concentration of YFP (~1 or 2 μM), the rate is essentially independent of temperature over the range 4−25 °C.

To explain these results based on Scheme 2, several alternatives can be considered.

(1) YFP may be inactive at higher temperatures as a result of (a) a shift in a dimerization equilibrium, YFP's activity being associated with either the monomer or the dimer only; (b) the loss of its FMN chromophore; or (c) a reversible conformational change.

(2) The complexation of YFP and the peroxyhemiacetal may be reversible, with higher temperature favoring dissociation, hence the blue pathway.

The present study was undertaken to assess the validity of each of these hypotheses. Chromatography, fluorescence spectra, and anisotropy measurements were used to characterize YFP and to determine its integrity as a function of temperature and dilution, with the goal of explaining the kinetic data obtained at different temperatures.

EXPERIMENTAL PROCEDURES

Chemicals. Riboflavin 5′-phosphate (FMN) and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Sigma (St. Louis, MO), and dithiothreitol (DTT) was obtained from Gibco (Gaithersburg, MD). The chromatographic materials were purchased from Pharmacia (Uppsala, Sweden). Pharmacia protein standards (Blue Dextran, ovalbumin, ribonuclease, albumin, and chymotrypsinogen A) were used for the calibration of the gel filtration column. Bio-Rad (Hercules, CA) prestained protein standards (phosphorylase B, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme) were used for identification in SDS gel electrophoresis. (2,2-Diphenylpropyl)amine, decanal, and EDC were obtained from Aldrich (Milwaukee, WI) and used without further purification. EDTA and other chemicals were obtained from Fisher (Pittsburgh, PA) or Mallincrodt (St. Louis, MO).

Cultures and Growth of V. fischeri Y-1. Cells of a bright single colony of Y-1 isolated on a sea water complete agar medium (3 g of yeast extract, 5 g of bactotryptone, 2 mL of glycerol, and 15 g of agar per liter of 80% sea water) were grown in a liquid sodium chloride complete medium (Nealson, 1978) at 17 °C with shaking (100 rpm). Growth was monitored by measurement of the optical density of the culture at 660 nm in a UVICON-820 (Kontron) spectrophotometer, and bioluminescence was measured in a photometer (Mitchell & Hastings, 1971). The cells were harvested by centrifugation between late-log and stationary phase (A_{660} ~ 1.8−3.5).

Purification and Analysis of Proteins. The lysis of 54 g of the frozen and thawed cells was performed by stirring the cells at 4 °C for 1 h with lysis buffer (5 mL of 10 mM phosphate buffer, pH 7.0, 10 mM EDTA, and 1 mM DTT) per gram wet weight of cells. The lysate was centrifuged for 15 min at 15000*g* to remove insoluble matter and the supernatant subjected to ammonium sulfate fractionation (Hastings et al., 1978). The protein precipitating between 40 and 80% saturation of ammonium sulfate was redissolved in lysis buffer with a lower concentration of EDTA (1 mM) and chromatographed on a 2.5 × 30 cm DEAE-Sephacel ion-exchange column, eluting with a phosphate gradient (0.01 to 1.5 M) to give purified YFP (~95%) and luciferase rich fractions (Karatani & Hastings, 1993). The pooled YFP fractions were concentrated by ultrafiltration in an Amicon apparatus equipped with a PM 10 filter. The yield was ~12.5 mg of YFP.

High-purity YFP was obtained by gel filtration of the above sample on a 2.5 × 80 cm G 75 superfine column with a 0.01 M phosphate buffer containing 0.1 mM EDTA and 1 mM DTT as eluant at 4 °C. The most abundant of the several fluorescent peaks (Karatani & Hastings, 1993) was used.

Crude FMN reductase for the multiple turnover assay was prepared by the pooling of fractions from ion-exchange chromatography which showed reductase activity and concentration of this solution (Hastings et al., 1978, 1985).

Pure luciferase was obtained by affinity chromatography (Holzman & Baldwin, 1982) of the luciferase fraction from the Sephacel column. The affinity resin was prepared from ECH Sepharose 4B and (2,2-diphenylpropyl)amine using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide as a coupling agent. For gel filtration, 0.5 mL of YFP in 0.01 M phosphate buffer, pH 7, 0.1 mM EDTA, 1 mM DTT, and 50 μM FMN were loaded at 4 °C on a 1.5 × 30 cm G 75

superfine column and eluted with the same buffer, with or without 5 μM FMN at a flow rate of 3 mL/h.

The BCA (Pierce, Rockford, IL) protein assay was used for the quantitative determination of protein, and denaturing polyacrylamide gel electrophoresis was performed according to Laemli (1970), in a MiniProtean apparatus (BioRad).

In Vitro Assays. The single turnover luciferase assay was performed by mixing 20 μL of luciferase, 20 μL of 1 M phosphate buffer, pH 7.0, and 20 μL of 0.01% decanal emulsion in water prepared by sonication, the volume being made up to 200 μL with double-distilled water, and injecting 200 μL of 50 μM $FMNH_2$ solution (prepared by catalytic reduction of FMN with hydrogen over Pt−asbestos), to initiate the reaction.

Fluorescence and Bioluminescence Measurements. Fluorescence emission spectra were recorded with a SPEX FluoroMax temperature-controlled fluorimeter with software for control and data analysis provided by SPEX (Edison, NJ). Bioluminescence emission spectra were recorded with the same instrument with the excitation lamp turned off. Fluorescence lifetimes were determined by the photon-counting method (Wilson et al., 1984).

Fluorescence emission anisotropy of YFP solutions in 50 mM phosphate buffer, pH 7.0, were determined with excitation at 460 nm and emission monitored at 540 nm with a phase-shift fluorimeter (SLM 48000; Champaign, IL). Dissociation constants of YFP were calculated on the following basis. The fraction f_B of bound chromophore FMN was calculated by the following equation (Lakowicz, 1983)

$$f_B = \frac{r - r_F}{(r_B - r)R + r - r_F}$$

where r is the anisotropy of the solution, r_F is the anisotropy of the free chromophore ($r_F = 0.0175$), r_B is the anisotropy of the bound chromophore (taken as the maximum measured value at saturation, $r_B = 0.2645$), and R is the ratio of the quantum yields of bound and free FMN ($R = \sim 1 \pm 30\%$). R was estimated in two ways, from the fluorescence intensities of solutions of known concentrations of pure FMN and pure YFP ($\lambda_{max} \approx 540$ nm) and from the ratio of the fluorescence lifetimes of FMN and YFP. The dissociation constant was calculated as follows (Lakowicz, 1983):

$$K_D = \frac{[\text{FMN}][\text{apo-YFP}]}{[\text{YFP}]} = \frac{[\text{YFP,total}](1 - f_B)^2}{f_B}$$

The slope and intercept of the van't Hoff plot were determined by the least squares method. The enthalpy and entropy values derived from the van't Hoff plot are not affected beyond experimental accuracy by the 30% uncertainty attached to R.

Kinetics. The kinetics of bioluminescence emission decay in the blue and yellow were determined using the "slewing" function of the SPEX Fluoromax instrument operated with the excitation lamp turned off. Data were acquired at 450 nm (I_{450}) and 540 nm (I_{540}) alternating at intervals of 1.5 s. B_{490}, the blue emission intensity (at 490 nm), and Y_{540}, the yellow intensity (at 540 nm), were calculated by correcting for the spectral overlap of these two emissions, as follows (Cho et al., 1989):

$$B_{490} = 2I_{450} \quad \text{and} \quad Y_{540} = I_{540} - I_{450}$$

The yellow to blue ratio was defined as $Y/B = Y_{540}/B_{490}$.

First order rate constants of light emission decay were determined as the slopes of the plots of the logarithm of light intensity versus time, using the data analysis utilities of the SPEX FluoroMax instrument.

The relative spectral areas of the separate blue and yellow emissions were based on the integration of the YFP fluorescence spectrum for the yellow band, and for the blue band on the integration of the bioluminescence spectrum recorded using the multiple turnover assay (Cho et al., 1989) in the absence of YFP. The area under the normalized yellow band was 74% of that of the blue band. The total amount of light emitted was estimated for the complex (yellow and blue) bioluminescence spectrum, as follows:

$$I_{total} = B_{490} + 0.74Y_{540}$$

with both B_{490} and Y_{540} expressed in arbitrary units.

The present preparation of YFP gave consistently higher Y/B ratios at given protein concentrations than the earlier YFP sample (Eckstein et al., 1990), even though the effect of YFP on the reaction rate is in excellent agreement with the previous data. It is clear that subtle and not yet characterized properties of YFP play a role that is not understood.

Measurement of Bioluminescence Intensities in the Presence of KI. Bioluminescence intensities and decay rates were measured in assays containing 1 μM luciferase without or with 2 μM YFP. The final concentration of KI was varied from 0 to 0.25 M by the addition of 4 M KI solution, and the ionic strength was maintained by the addition of the appropriate amount of 4 M KCl solution, for a total concentration of salts of 0.25 M in each experiment.

RESULTS

Gel Filtration of YFP Shows Monomeric and Dimeric Forms. Although it had been reported that YFP is a dimer (Daubner et al., 1987), only a monomeric form was identified in our earlier studies (Karatani & Hastings, 1993). In the present work, we have found both forms, but only the monomer is active.

Upon gel filtration on G75 of purified YFP at 4 °C (see Experimental Procedures), two fractions exhibiting the characteristic YFP fluorescence were always evident, one with an apparent molecular mass of ~24 kDa and the other ~48 kDa (Figure 1A). These two fractions were analyzed on SDS−polyacrylamide gels and found to be identical in size (~22 kDa, data not shown), indicating that the two fractions from the G75 columns correspond to monomeric and dimeric YFPs.

Monomer−dimer conversion appears to be only slowly reversible, even when excess FMN ligand is present in the solution loaded on the gel filtration column. Both monomer and dimer were reloaded and run separately on the same column. The monomer was recovered mostly as a ~24 kDa protein and the dimer mostly as a ~48 kDa species, but some of the other species was always present (Figure 1A). Similar results were obtained regardless of whether the column was equilibrated and run in buffer with or without FMN.

The average specific fluorescence intensity (defined as the fluorescence intensity at 540 nm integrated over all fractions of a given peak divided by the total protein concentration in

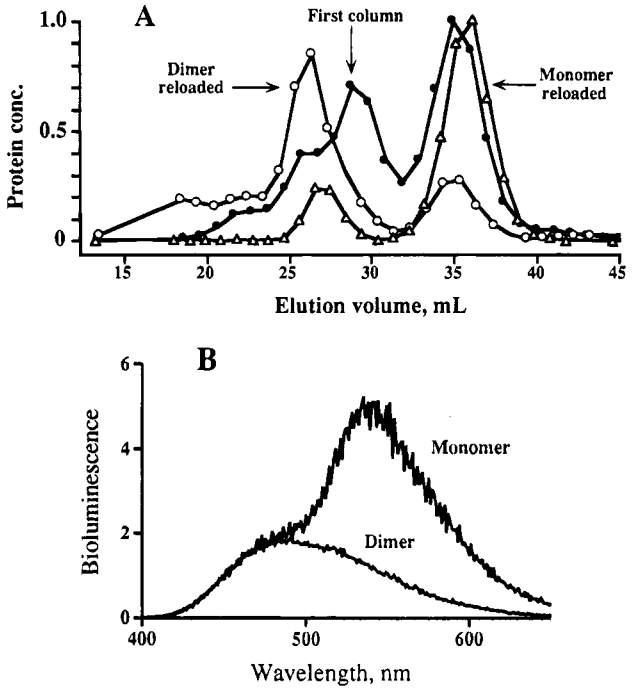


FIGURE 1: Gel filtration chromatography of YFP solutions on a Sephadex G75 column and emission spectra of luciferase reactions in the presence of the monomer and dimer, at 4 °C. (A) YFP-enriched fraction from ion exchange chromatography (solid dots), dimeric YFP reloaded (open circles), and monomeric YFP reloaded (triangles). Protein concentrations in arbitrary units. (B) *In vitro* bioluminescence spectra (in arbitrary units) with monomeric and dimeric YFP fractions from Sephadex chromatography.

the same peak) was lower for the dimer (4.8 units) than for the monomer (9 units). Although these values were not corrected for the dissociation of FMN from YFP (see below), which was evident from the shape of fluorescence peaks of the fractions obtained by gel filtration, it is concluded that the dimer is formed from two molecules of YFP with the loss of one FMN.

Monomeric YFP, recovered after gel filtration, was found to be active in the production of yellow light in the bioluminescence assay, while the dimer was not (Figure 1B) since the emission spectrum peaks at ~490 nm, as with no YFP. With 2.8 μM monomeric YFP in the assay mixture, a *Y/B* ratio of 2.0 was observed in the luminescence emission, while with 0.63 μM dimer, the *Y/B* ratio was less than 0.1, instead of an expected value of 0.45 for the monomer at that concentration. The dimeric form of YFP thus appears to be inactive.

Fluorescence Spectra, Lifetime, and Anisotropy of YFP. The effect of temperature and dilution on the integrity of YFP was first investigated by fluorescence spectroscopy. The emission maximum shifted from 538 to 530 nm when a 1 μM YFP solution was heated from 4 to 20 °C (Figure 2), indicative of the liberation of FMN (the fluorescence λ_{max} of free FMN is 525 nm). Such spectral shifts were reversed upon recooling to 4 °C. Dilution resulted in a similar shift of the emission maximum (data not shown).

To quantify the effects of increased temperature and dilution on the dissociation of FMN from apo-YFP, the fluorescence anisotropy of YFP solutions was measured at different concentrations of YFP up to 6 μM (Figure 3) and temperatures (4−35 °C; YFP was denatured and precipitated above 35 °C). At 15 °C and 5.8 μM YFP, an anisotropy

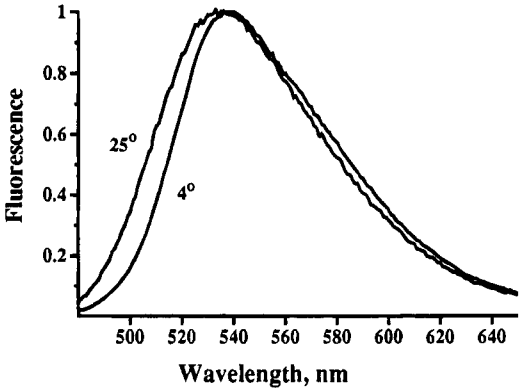


FIGURE 2: Normalized fluorescence emission spectra of 1 μM YFP (λ_{exc}, 460 nm) at 4 and 25 °C. Fluorescence intensity in arbitrary units.

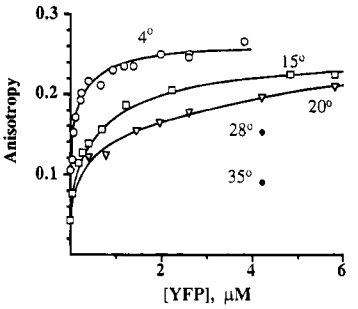


FIGURE 3: Effect of temperature and concentration on the fluorescence anisotropy of YFP.

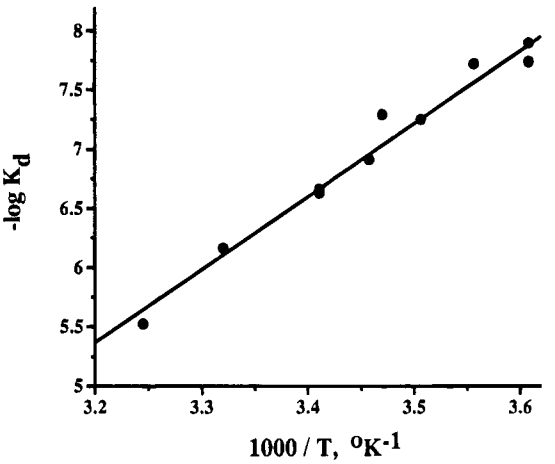


FIGURE 4: van't Hoff analysis of dissociation constants of YFP.

value of 0.224 was measured, while at the same temperature but at a very low YFP concentration (26 nM), the anisotropy was much lower (r = 0.0424), in fact close to the value for free FMN (r = 0.0175). This indicates a drastic change of size of the fluorophore with dilution.

An increase of temperature also resulted in smaller anisotropy values, and the effect was reversible. Upon changing the temperature from 4 to 20 °C and then again to 4 °C, the anisotropy of a 2 μM YFP solution varied from 0.2303 to 0.1634 and then back to 0.2309. The equilibrium constant, K_d, for the dissociation of YFP to FMN and apo-YFP was calculated from the anisotropy data at different temperatures (see Experimental Procedures). A van't Hoff plot of the logarithm of K_d (Figure 4) was then used to determine the standard free enthalpy ($\Delta H°$ = 29 kcal/mol) and entropy ($\Delta S°$ = 68 eu) of the dissociation.

Table 1: Fluorescence Lifetimes of YFP Solutions at Different Temperatures and Concentrations

concentration (μM)			
YFP	luciferase	temp (°C)	lifetime (ns)
0.3	0	4	5.5
1	0	4	6.4
4	0	4	7.0
4	1	4	6.9
1	0	20	5.5
FMN	1 μM	20	4.4

In keeping with the anisotropy results, the fluorescence lifetime of YFP also changes upon dilution from 6.9 ns at 4 μM to 5.5 ns at 0.3 μM, at 4 °C (Table 1); τ_F of FMN dissolved in water is 4.4 ns (Karatani & Hastings, 1993). In addition, dissociation of FMN from YFP was directly verified by ultrafiltration of a YFP sample on a 10 000 molecular weight cutoff Centricon filter. FMN was identified in the filtrate by its fluorescence emission spectrum ($\lambda_{max} \sim 525$ nm), identical to that of authentic FMN.

Although the gel filtration experiments gave clear evidence for YFP dimerization, the expected changes in fluorescence anisotropy due to dimerization would be too small to detect in the concentration range investigated (up to 6 μM). Complexation between YFP and luciferase should have been detectable, however, but at 4 °C and either 0.4 or 4.0 μM YFP, the presence of up to 1.8 μM luciferase had no significant effect on the anisotropy of YFP (data not shown) or on the fluorescence lifetime (Table 1).

Effect of Iodide on Luminescence Intensity and Its Rate of Decay, with and without YFP. Potassium iodide affects the bioluminescence intensity (I) and its rate of decay (k_d) differently in the presence or absence of YFP (Figure 5). These effects and their qualitative interpretation are as follows.

With no YFP, iodide *increases* k_d (Figure 5A), most likely by decomposing the peroxyhemiacetal or other peroxy intermediates, and it decreases the bioluminescence intensity. The Stern–Volmer plot of I_0/I (Figure 5B) is not linear because iodide quenches both by reacting with the peroxyhemiacetal (as above) and therefore preventing the formation of the excited emitter (4a-hydroxy FMN) and by direct collisional quenching of this emitter.

In the presence of 2 μM YFP, iodide actually decreases the rate of yellow and blue bioluminescence decay (which at [KI] = 0 is ~2 times larger than with no YFP; see below, Figure 7A). This is attributed to a competition between I^- and YFP for reaction with the peroxyhemiacetal at the active site. As the concentration of iodide increases, it gradually displaces YFP; at [KI] = 0.25 M, k_d is entirely dictated by the concentration of iodide and is therefore the same with or without YFP.

Whereas iodide decreases the values of k_d by the same factor in the blue and yellow spectral bands, the relative intensities in these two bands respond differently to iodide. Iodide is considerably more effective at quenching the yellow emission, because the FMN chromophore of YFP either has a longer fluorescence lifetime than the 4a-hydroxy FMN or is more exposed to I^-, or both.

Effect of YFP Concentration and Temperature on in Vitro Bioluminescence. The initial bioluminescence intensity with decanal and 2.3 μM luciferase at 4 °C was measured as a function of YFP concentration in $FMNH_2$-initiated assays. As previously reported (Eckstein et al., 1990), with increasing YFP concentration, the initial intensity at 540 nm increased while that at 450 nm decreased (Figure 6A). But since the increase in the yellow was larger than the decrease in the blue, the initial overall intensity increased, as did the *Y/B* ratio, which depends on the concentrations of both YFP and

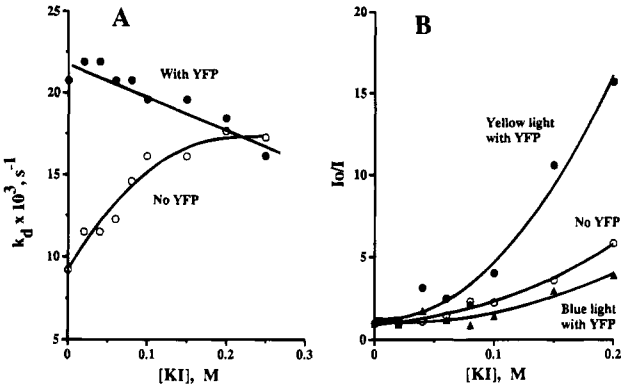


FIGURE 5: Effects of potassium iodide on the luciferase (1 μM) reaction with and without 2 μM YFP at 4 °C. (A) Effect on the rate constant of bioluminescence decay. The open circles are the average of three experiments, and the solid dots are the average of the rates of decay of blue and yellow light. (B) Effect on the bioluminescence intensity.

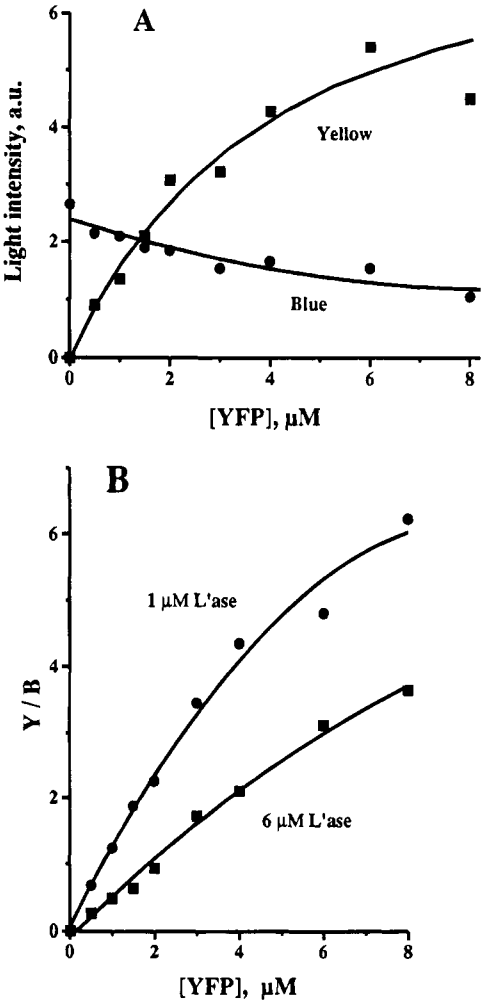


FIGURE 6: Effect of YFP concentration on (A) the initial blue (λ_{490}) and yellow (λ_{540}) bioluminescence intensity with 1 μM luciferase and (B) the *Y/B* ratio at two concentrations of luciferase. All measurements at 4 °C.

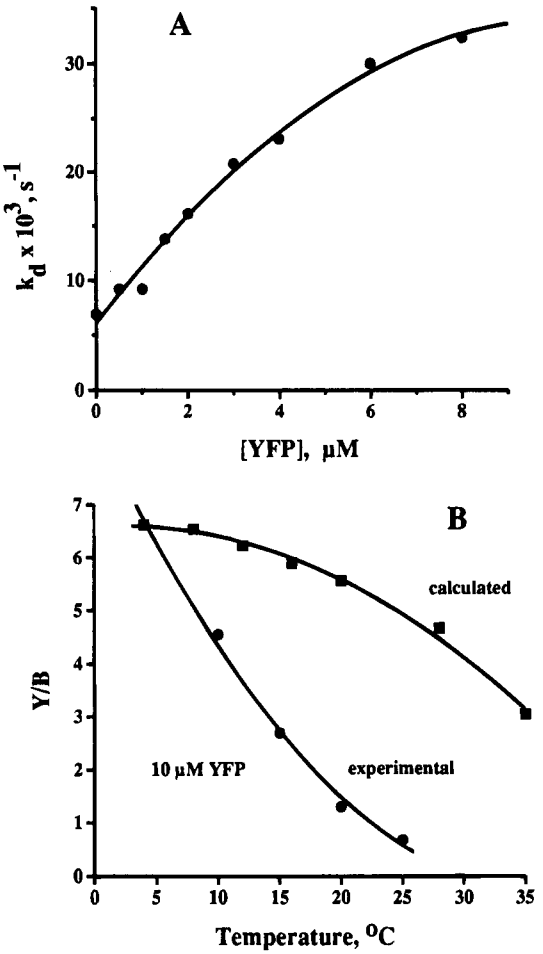


FIGURE 7: (A) Effect of YFP concentration on the rate constant of bioluminescence decay at 4 °C (blue and yellow emissions decay at the same rate). (B) Effect of temperature on the *Y/B* ratio of bioluminescence, with 10 μM YFP and 1 μM luciferase. Plotted on the same graph is a curve of the relationship of *Y/B* and temperature calculated on the basis of K_d.

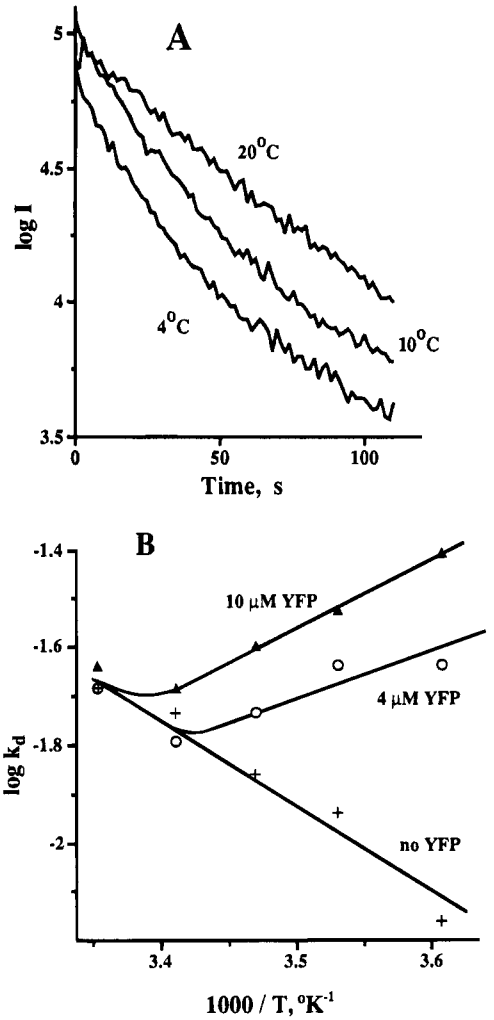


FIGURE 8: (A) Semilogarithmic plots of light intensity decay at three temperatures in reactions with 10 μM YFP and 1 μM luciferase. (B) Temperature dependence of log k_d (the rate constant of bioluminescence decay) with 1 μM luciferase and no YFP and with 4 and 10 μM YFP.

luciferase (Figure 6B). Increasing the YFP concentration also increased the rate constant of light intensity decay (i.e. the enzymatic turnover rate, Figure 7A), by the same factor in both colors, such that the quantum yield of bioluminescence was not significantly changed. All these effects of YFP are very sensitive to temperature and practically disappear above ~20 °C, as indicated by the *Y/B* ratio (Figure 7B, experimental curve).

Most strikingly, temperature has opposite effects on the rate of intensity decay with and without YFP. While k_d increases with temperature in assays with luciferase alone, as expected, it decreases in the presence of YFP (Figure 8A). The apparent activation energy of the luciferase reaction without YFP is ~10 kcal/mol, while in the presence of 4 to 10 μM YFP, it has negative values (Figure 8B). In this range of YFP concentrations, the peak intensity also decreases when the temperature increases, since it reflects the turnover rate.

DISCUSSION

Monomeric and Dimeric Forms of YFP. Active YFP was shown by gel filtration to be a monomeric protein of ~24 kDa, as previously reported (Macheroux et al., 1987; Karatani & Hastings, 1993). However, a dimeric protein of 48 kDa having a fluorescence emission spectrum identical to that of monomeric YFP was also found, which appears inactive in causing yellow bioluminescence. The dimeric and monomeric protein fractions gave identical bands on denaturating gels. The specific fluorescence emission intensities of the two forms suggest that the dimer is formed by the interaction of one apo-YFP molecule with one holo-YFP:

$$\text{YFP} + \text{apoYFP} \rightleftharpoons \text{YFP}-\text{apoYFP}$$

Alternatively, two holo-YFPs might associate with the release of one FMN.

The inactivation of YFP via its dimerization should increase with concentration, which is not consistent with the results reported here (Figure 6B). However, earlier experiments had shown a decrease in *Y/B* at very high YFP concentrations, which had been tentatively interpreted as resulting from YFP inactivation via dimerization (Eckstein et al., 1990). It is likely that crude and partially purified preparations contain some monomer and some dimer, probably in variable amounts, and thus may differ in their specific activities. Although Daubner et al. (1987) found YFP to be a homodimer, they did not explicitly report whether they had tested the dimer for activity and did not specify the gel filtration conditions under which no monomer was found. In a recent study in our laboratory (Karatani & Hastings,

1993), no dimer was found, but two active monomeric forms of YFP were observed, almost identical with respect to activity, fluorescence spectrum, lifetime, and relative quantum yield. In our experiments, their respective amounts appear to depend on growth conditions.

We have at present no information on the effect of temperature on the state of YFP association and therefore cannot definitely rule out its dimerization as the explanation of YFP's ineffectiveness at higher temperatures (point 1a in the introduction section). It is well-known that, depending on the protein, an increase in temperature can promote association or dissociation (Weber, 1992). Our gel filtration experiments suggest, however, that the equilibrium between monomeric and dimeric forms of YFP is only slowly reversible at 4 °C and may therefore not have been reached during the incubation period prior to the initiation of the luciferase reaction in the experiments of Figure 8. If this was the case, a change in dimerization status of YFP could not account for the results. Further experiments will be necessary to reach a firm conclusion.

Holo- and Apo-YFP. Fluorescence spectra and anisotropy data both indicate that FMN can reversibly dissociate from YFP. Dilution and an increase in temperature result in a shift of the fluorescence emission maximum of YFP from ~540 nm toward 525 nm, the emission maximum of free FMN, and in a large decrease in anisotropy (Figures 2 and 3). At 15 °C and very low concentration (0.026 μM), the anisotropy of a YFP solution was found to be nearly equal to that of free FMN, indicating that FMN dissociates from YFP almost quantitatively at high dilution, which is confirmed by ultrafiltration. One can calculate from the Perrin equation that dissociation of the dimer to the monomer could not account for the large change in anisotropy with dilution nor for the final value at very low concentration.

The dissociation constant of YFP at 4 °C, pH 7, in 50 mM phosphate buffer is 1×10^{-8} M. It is interesting that the thermochemical parameters of the dissociation, i.e. $\Delta H° = 29$ kcal mol^{-1} and $\Delta S° = 68$ eu, are nearly identical with those reported for the lumazine protein [$\Delta H° = 28.0$ kcal mol^{-1} and $\Delta S° = 67$ eu, Visser and Lee (1980)], a blue-emitting accessory protein from other bacterial species having a significant sequence similarity with YFP (O'Kane et al., 1991).

On the basis of the dissociation constant at 15 °C (K_d = 0.1 μM; Figure 4), one calculates that, upon a 4 μM YFP solution being heated from 4 to 15 °C, only about 15% FMN will dissociate from YFP, whereas the *Y/B* ratio is reduced by more than a factor of 3 by the same change of temperature (Figure 7B). To account for the decrease in yellow intensity, K_d would need to be 50 times larger, well outside the limits of error. Therefore, chromophore dissociation (point 1b in the introduction section) is insufficient for explaining either the changes in *Y/B* ratio related to temperature and dilution or the negative temperature coefficient of the reaction.

Reversibility of the Interaction of YFP with the Peroxyhemiacetal. If 4 μM YFP accelerates the rate of the reaction by a factor of ~4 at 4 °C, why is the same YFP concentration without effect at 28 °C, where 70% of YFP is still undissociated? A possible explanation (point 2 in the introduction section) is that the binding of YFP to the luciferase intermediate is temperature dependent and reversible. Reversibility of this process had previously been ruled out on the basis of the reaction kinetics; Scheme 1 predicts that reversibility should cause the blue emission to decay as the sum of two exponentials (Eckstein et al., 1990), which was not observed within the accuracy of the measurements. However, the model evidently oversimplifies the reaction, by assuming, for example, that one can consider an "initial concentration" of peroxyhemiacetal and follow its decay with time.

An apparent negative activation energy is usually considered to be indicative of a complex mechanism (unless it results from the trivial removal of a reactant, as in heat denaturation of an enzyme, for example, or a temperature dependent process of inactivation of YFP, as discussed above). The textbook example is the gas phase reaction of 2NO and O_2 to give $2NO_2$. Its mechanism is still debated (McKee, 1994), but the consensus is that it involves a pre-equilibrium, the reversible association either of 2NO followed by reaction of this complex with O_2 or of NO with O_2 followed by reaction of that complex with another NO. In the case of YFP, one could similarly propose that the dissociation of the YFP–peroxyhemiacetal complex (abbreviated here as E·FOOA·Y as in Scheme 2) controls the reaction.

$$\text{E·FOOA} + \text{Y} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{E·FOOA·Y} \xrightarrow{k_3} \text{E·F·Y*}$$

At low temperatures, the reaction would proceed forward to generate the yellow emitter, whereas at high temperatures the back reaction would become dominant; hence, YFP would lose its effects on both kinetics and color. Attractive as this hypothesis is, it is energetically unlikely (besides being incompatible with the reaction kinetics, as pointed out earlier; Eckstein et al., 1990). If one considers (Figure 8B) that, at 4 °C and 10 μM YFP, the rate is 6-fold higher than it is without YFP, while these two rates are the same at 25 °C, one calculates that the apparent negative activation energy of the YFP reaction is

$$E_a = E_a^{(k_1)} + E_a^{(k_3)} - E_a^{(k_2)} \simeq -16 \text{ kcal}$$

(where $E_a^{(k_1)}$, etc. are the activation energies of the forward and back processes). Assuming modest values of 5 kcal for $E_a^{(k_1)}$ and $E_a^{(k_3)}$, this would require that $E_a^{(k_2)} \geq 26$ kcal, outside of reasonable possibilities.

What, Then, Could Account for the Negative Temperature Coefficient? At this point, we have no definite answer. If the pre-equilibrium hypothesis discussed above (point 2) appears unlikely, the loss of YFP functional integrity (point 1) seems more credible. We know that a loss of the chromophore does occur, but this is only a partial explanation. We canot rule out YFP dimerization nor exclude a conformational change (point 1c in the intoduction section), as suggested by Leisman and Nealson (1982). The last hypothesis would be the most difficult to prove. The iodide quenching experiments of Figure 5 clearly show that YFP protects peroxy intermediates at the active site from reaction with I^-; indeed, if the qualitative explanation of these results is correct, it takes about 0.25 M KI to successfully compete with 2 μM YFP, so that the rates of bioluminescence decay at that concentration of iodide are the same with or without YFP. This implies a close association of YFP with luciferase-bound intermediates, which is further supported by studies with aldehydes of chain lengths longer and shorter than decanal, as well as with inhibitors (Cho et al., 1989).

The *Y/B* ratio, for example, was found to be very dependent on the aldehyde chain length and therefore on the reactants' geometry at the active site. It seems that subtle and reversible changes in YFP conformation with temperature could affect YFP's ability to reach a critical interaction geometry at the active site.

Conclusions. The interaction of *V. fischeri* Y-1 luciferase with the yellow fluorescent protein creates a complex enzymatic system for light emission. Over the range of ecological temperatures, the reaction rate and hence the emission intensity have a negative temperature coefficient; at a concentration of ~1 μM YFP, the system is actually "temperature-compensated", such that the reaction rate is approximately the same from ~5 to 25 °C. An understanding of the mechanism of this system may bear on effects of temperature on proteins and physiological systems more generally (Somero, 1995). A striking example is the temperature compensation of circadian rhythms, where there is little or no change in their ~24 h period over a physiological temperature range (Pittendrigh, 1954; Ruoff, 1994).

The selective advantage conferred by YFP to the Y-1 bacterial strain may be related not to the color shift it causes (see the introduction section) but to its effect on the rate of the reaction; at 4 °C, the light intensity may be increased by 10-fold by YFP. If this Y-1 bacterium is cultured as a symbiont, as are certain other strains of *V. fischeri* (Nealson & Hastings, 1991), and if its bioluminescence is of selective advantage to the host, then it would be of benefit to the host to minimize energy expenditure for metabolism while maximizing light intensity (Hastings et al., 1987). Indeed, the more general question is, "how are growth and luminescence regulated independently in symbionts"? YFP appears to be an answer; the growth rate of a standing symbiotic bacterial population would be very low at 4 °C, but in the presence of this protein, bioluminescence would be maximal.

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