Complex Formation between *Vibrio harveyi* Luciferase and Monomeric NADPH:FMN Oxidoreductase†

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ABSTRACT: A direct transfer of the reduced flavin mononucleotide ($FMNH_2$) cofactor of *Vibrio harveyi* NADPH:FMN oxidoreductase (FRP) to luciferase for the coupled bioluminescence reaction has been indicated by recent kinetic studies [Lei, B., and Tu, S.-C. (1998) *Biochemistry 37*, 14623−14629; Jeffers, C., and Tu, S.-C. (2001) *Biochemistry 40*, 1749−1754]. For such a mechanism, a complex formation of luciferase with FRP is essential, but until now, no evidence for such a complex has been reported. In this work, FRP was labeled at 1:1 molar ratio with the fluorophore eosin. The labeled enzyme was about 30% active in either the reductase single-enzyme or the luciferase-coupled assay. The labeled FRP in either the holo- or apoenzyme form was similar to the native FRP in undergoing a monomer−dimer equilibrium. By measuring the steady-state fluorescence anisotropy of eosin-labeled FRP, it was shown that luciferase formed a complex at 1:1 molar ratio with the monomer of either the apoenzyme or the holoenzyme form of FRP with K_d values of 7 and 11 μM, respectively. Neither the holo- nor the apoenzyme of the labeled FRP in the dimeric form was effective in complexing with luciferase. At maximal in vivo bioluminescence, the *V. harveyi* cellular contents of luciferase and FRP were estimated to be 172 and 3 μM, respectively. The vast majority of FRP would be trapped in the luciferase/FRP complex. Plausible physiological significance of such a finding is discussed.

NAD(P)H:FMN oxidoreductases (flavin reductases) catalyze the formation of reduced flavin via the following reaction:

$$F + NAD(P)H + H^+ \rightarrow FH_2 + NAD(P)^+ \qquad (1)$$

where F is the oxidized flavin substrate and FH_2 is the reduced flavin product. This reduced flavin is necessary for a number of biological processes including release of iron from ferrisiderophores (*1*, *2*), reduction of methemoglobin (*3*, *4*), oxygen activation (*5*), fossil fuel desulfurization (*6*−*8*), and biosynthesis of actinorhodin (*9*) and pristanamycin IIA (*10*). Other enzymes that require reduced flavin include ribonucleotide reductase (*11*, *12*), chorismate synthase (*13*), *Escherichia coli* 4-hydroxyphenyl-acetate 3-hydroxylase (*14*−*16*), EDTA monooxygenase (*17*, *18*), *Chelatobacter heintzii* nitrilotriacetate monooxygenase (*19*, *20*), *Rhodococcus* pyrole-2-carboxylate monooxygenase (*21*), and the light-emitting reaction of luminous bacterial luciferase (*22*−*24*). These reduced flavin-requiring enzymes and processes have recently been reviewed (*25*).

Although a growing number of enzymes are now known to require exogenously supplied reduced flavin for their activities, the processes by which these enzymes acquire their reduced flavin remained unexplored for quite a long while. Free reduced flavins are highly labile under aerobic conditions due to an autocatalytic cycle of nonenzymatic oxidation (*26*−*28*). This would make free diffusion of reduced flavin to acceptor enzymes, if operative, a very inefficient pathway of reduced flavin transfer in vivo. Hence, some forms of direct channeling of reduced flavin from donor to acceptor enzymes have long been suspected to exist. In recent years, we have undertaken a series of studies to delineate the reduced flavin transfer mechanisms using flavin reductase/luciferase couples from luminous bacteria as a model system.

Luciferase from luminous bacteria produces a greenish-blue light via the oxidation of reduced riboflavin 5′-phosphate ($FMNH_2$)[1] and a long-chain aliphatic aldehyde:

$$RCHO + FMNH_2 + O_2 \rightarrow RCOOH + FMN + H_2O + light \qquad (2)$$

Bacterial luciferase does not contain any flavin cofactor and lacks the ability to generate the required reduced flavin

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[1] Abbreviations: $FMNH_2$, reduced riboflavin 5′-phosphate; FMN, oxidized riboflavin 5′-phosphate; FRP, reduced nicotinamide adenine dinucleotide phosphate- (NADPH-) preferring flavin reductase P; FRD, reduced nicotinamide adenine dinucleotide- (NADH-) preferring flavin reductase D; FRG, reduced nicotinamide adenine dinucleotide- (NADH-) and reduced nicotinamide adenine dinucleotide phosphate- (NADPH-) utilizing general flavin reductase; EI, eosin-5-iodoacetamide; FRP−EI, flavin reductase P−eosin-5-iodoacetamide conjugate.

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substrate. As such, it is dependent on a class of flavin reductases to generate the required $FMNH_2$ (*22*−*24*, *29*, *30*). The flavin reductases from luminous bacteria can be classified into three types (*31*): flavin reductase P (FRP), which preferentially utilizes NADPH as an electron donor; flavin reductase D (FRD), which preferentially utilizes the NADH; and the general flavin reductase FRG, which displays no marked pyridine nucleotide preference. Using the luciferase/FRP couple from *Vibrio harveyi* and luciferase/FRG couple from *Vibrio fischeri*, we have obtained kinetic evidence for a direct channeling of the $FMNH_2$ cofactor, rather than the reduced flavin product, of these flavin reductases to luciferase from their respective cell strains. Major supporting evidence includes the finding that the flavin reductase K_m values for NAD(P)H and FMN are significantly lower in the coupled reaction with luciferase than those in the single-enzyme spectrophotometric assay (*23*, *30*, *32*−*34*). Additionally, two recent kinetic studies have shown that both FRG and FRP, which act via a ping-pong kinetic mechanism in their single-enzyme spectrophotometric assays, exhibit a sequential kinetic pattern in their luciferase-coupled reactions (*32*, *33*). Furthermore, kinetic measurements with flavin reductases having 2S-FMN as a cofactor also indicate the transfer of the reduced flavin cofactor and not the product to luciferase (*32*, *33*).

The formation of a functional donor/acceptor enzyme complex is mandatory for direct metabolite channeling between two enzymes, regardless of the details of the transfer mechanisms. In the case of bacterial flavin reductase/luciferase systems, *V. harveyi* luciferase immobilized onto cyanogen bromide-treated Sepharose appeared to bind the FRD activity in a crude sample and exhibited an enhanced bioluminescence activity in the coupled reaction (*35*). However, since no significant complex formation was detected for soluble forms of FRD and luciferase, the binding mentioned above may have been induced by the immobilization treatment (*36*). Direct physical evidence for complex formation in solution between any species of bacterial flavin reductase and luciferase has never been documented. We describe here fluorescence anisotropy studies of eosin-labeled FRP, which unambiguously showed that FRP indeed forms a complex with luciferase. Interestingly, the complex is formed between luciferase and the monomeric form of FRP as opposed to the dimeric form that was seen in the crystal structure (*37*). Furthermore, on the basis of cellular contents of FRP and luciferase determined in this work, implications of this complex formation in vivo are discussed.

EXPERIMENTAL PROCEDURES

Enzyme Preparation and Assays. Both luciferase and FRP were purified from recombinant strains as described previously (*31*). For FRP, the single-enzyme assay was based on rates of decrease in A_{340} accompanying the oxidation of NADPH and the luciferase-coupled assay was based on measurements of the bioluminescence peak intensities in reactions in the presence of excess luciferase; details of both assay conditions were described previously (*32*).

Labeling of FRP. An aliquot (1 mL) of a 5 mg/mL stock of FRP was added to 4 mL of an 8 M urea solution. Eosin-5-iodoacetamide (P-29, Molecular Probes, Eugene, OR) stocks were made in ethanol or dimethyl sulfoxide. The labeling agent was added in a 1.2:1 molar ratio to FRP, and the sample was incubated at room temperature, in the dark for 3 h. The sample was then renaturated by rapidly adding 250 mL of 50 mM phosphate buffer, pH 7.0 (FMN was added for holoenzyme preparation but omitted for apoenzyme preparation), while stirring (*38*). The renaturated sample was then concentrated in an Amicon 8050 stirred cell, with a poly(ethersulfone) membrane with a cutoff at 10 000 Da. The sample was then centrifuged at 10 000 rpm for 15 min, to remove any precipitated protein and debris, and dialyzed into 50 mM phosphate buffer, pH 7.0.

Determination of Labeling Stoichiometry. Protein concentrations were determined by the bicinchoninic acid assay (*39*). Bound fluorophore concentrations were determined by measuring the absorbance peak at 519 nm, and molar concentration was determined by use of the published extinction coefficient of 100 000 M^{-1} cm^{-1} (*40*). Labeling stoichiometry was determined to be at a molar ratio of 1:1.

Reduction of FMN Cofactor. One milliliter of 50 mM phosphate buffer, pH 7.0, was pipetted into a 1-mL sidearm anaerobic cuvette, with a gastight Hamilton syringe attachment. The buffer was made anaerobic by multiple rounds of vacuum evacuation, followed by nitrogen purging. FRP, in a 40 μL aliquot, was then added to a concentration of 200 μg/mL and an absorption spectrum was taken between 400 and 600 nm. NADPH, in a 20 μL aliquot, was then added to a concentration of ~100 μM and the spectrum was taken immediately. The reduction of the FMN cofactor was monitored by the decrease in the intensity of the absorption peak centered at 450 nm.

Equilibrium Analytical Ultracentrifugation. Equilibrium analytical ultracentrifugation was performed in a Beckman XL-A analytical ultracentrifuge with a six-channel equilibrium cell. Absorbance was monitored at 519 nm. Data were analyzed with the software package supplied by Beckman Corp. $K_{a(abs)}$ was converted to $K_{d(conc)}$ by the following formula:

$$K_{d(conc)} = \left[K_{a(abs)}\frac{(s\epsilon l)^{n-1}}{n}\right]^{-1} \quad (3)$$

where n = the stoichiometry of the association, ϵ = the extinction coefficient of the fluorophore, l is the path length of the cuvette in centimeters, and s is the labeling stoichiometry.

Steady-State Fluorescence Anisotropy. Fluorescence anisotropy measurements were performed on an SLM Aminco 48000 spectrofluorometer in the T-optics configuration. Samples were placed in a 3-mL fluorescence cuvette with constant stirring. Luciferase was added to the cuvette from a 50 mg/mL stock to various concentrations, with the total additions never exceeding 5% of the total volume of the cuvette. The labeled FRP species was excited at 519 nm, and the emission was measured with a 550 nm cutoff filter in front of both emission photomultiplier tubes. Around 10 measurements were taken for each luciferase titration point. Eosin was chosen to avoid interference from FMN and protein fluorescence. Binding curves were fit to the standard, single-site, hyperbolic binding equation:

$$\Delta r = \frac{B_{max} + [\text{luciferase}]}{K_d[\text{luciferase}]} \quad (4)$$

Table 1: Comparison of FRP and FRP−EI with Respect to Activities and Subunit Dissociation

sample	single-enzyme V_{max}[a] [μmol min^{-1} mg^{-1} (%)]	coupled V_{max}[b] [q s^{-1} mg^{-1} (%)]	subunit dissociation K_d (μM)
FRP	67.9 (100)	2.9×10^{13} (100)	1.8[c]
apoFRP			3.3[c]
FRP−EI	21.7 (32)	7.6×10^{12} (26)	1.4
apoFRP−EI			2.9

[a] Single-enzyme activity measurements were initiated by addition of 20 μL of a 5 mg/mL stock of NADPH into a 1 mL quartz absorbance cuvette containing enzyme and 50 μM FMN. [b] Luciferase-coupled reactions were initiated by addition of 10 μL of a 5 mg/mL NADPH stock into 1 mL of 50 mM P_i, pH 7.0, containing 0.5 nM FRP, 10 μL of a 2 mg/mL luciferase stock, and 10 μL of 0.02% decanal. [c] The K_d values for native FRP are taken from Liu et al. (*38*).

The B_{max} value derived from the above fit was used to determine the fractional saturation value (Y), by dividing Δr by B_{max}, with Δr representing the anisotropy of an individual point at a particular luciferase concentration.

Determination of in Vivo Concentration of FRP. V. harveyi cells were grown in seawater medium as described elsewhere (*41*), to a density of 1.3 OD at 600 nm. This stage of growth corresponded to maximal in vivo bioluminescence. A 10-mL aliquot of cells was then taken and centrifuged at 5000 rpm for 15 min. Cells were then resuspended in 10 mL of chilled dH_2O containing 1 g of Tris base for lysis. This suspension was allowed to sit at 4 °C for 1 h, under constant stirring. The lysate was then spun at 15 000 rpm for 15 min. The pH was adjusted to 7.0, and the supernatant and pellet were then assayed for FRP activities in the presence of saturating NADPH (~150 μM). Greater than 90% activity in the supernatant was taken as evidence of nearly complete lysis. Intracellular concentration was calculated from published specific activity (*31*), and *V. harveyi* cell volume was determined by use of cell dimensions derived from electron microscopy (*42*).

RESULTS

Labeling of FRP. FRP monomer contains three cysteine residues, each of which was considered as a potential target for sulfhydryl labeling. Initial labeling attempts on native FRP failed to yield a conjugated product, prompting us to assume that the sulfhydryls were not sufficiently exposed to solvent and therefore inaccessible for labeling. FRP was subsequently labeled after denaturation as described and the stoichiometry of labeling was determined to be 1:1. The labeled enzyme was shown to retain 32% and 26% of the single-enzyme and luciferase-coupled activities, respectively (Table 1). In comparison with $A_{453} = 0.096$ for the native FRP at 200 μg/mL, the labeled enzyme at the same concentration was found to have an A_{453} of 0.108. Therefore, the FRP−EI sample fully retained the bound FMN cofactor. Moreover, the FMN cofactor of both the native FRP and FRP−EI were found to be completely reduced upon anaerobic addition of saturating levels of either NADH or NADPH.

Equilibrium Analytical Ultracentrifugation. To investigate whether the labeling affected the dimerization of FRP, we utilized equilibrium analytical ultracentrifugation. Both the holoenzyme (Figure 1) and the apoenzyme (Figure 2) forms of FRP−EI were found to undergo a monomer−dimer

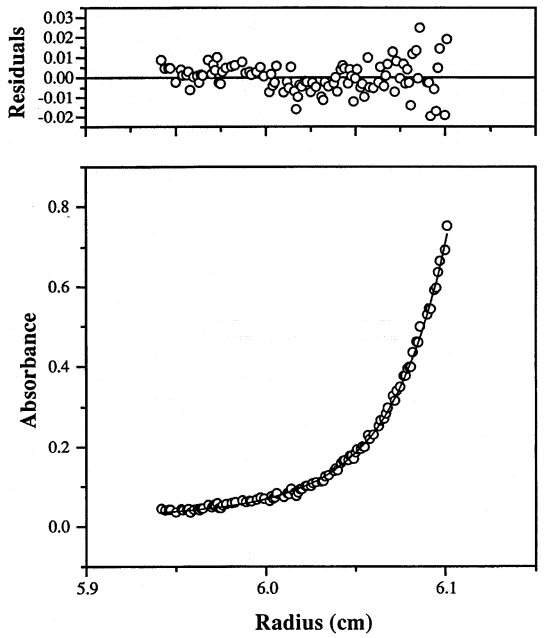


FIGURE 1: Equilibrium ultracentrifugation of the holoenzyme form of FRP−EI. FRP−EI (initial $A_{519} = 0.5$) was subjected to centrifugation in 50 mM P_i buffer, pH 7.0, at 27 000 and 30 000 rpm and 4 °C until reaching equilibrium. (○) Experimental data; (—) theoretical fit of the monomer−dimer equilibrium to eq 3 with a K_d of 1.4 μM. The distribution of residuals is shown in the upper panel.

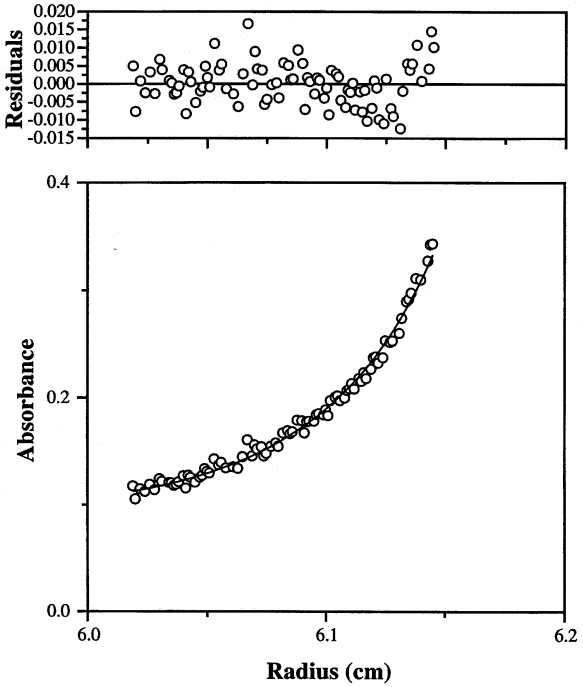


FIGURE 2: Equilibrium ultracentrifugation of the apoenzyme form of FRP−EI. Apo-FRP−EI (initial $A_{519} = 0.3$) was subjected to centrifugation in 50 mM P_i buffer, pH 7.0, at 27 000 and 30 000 rpm and 4 °C until reaching equilibrium. (○) Experimental data; (—) theoretical fit of the monomer−dimer equilibrium to eq 3 with a K_d of 2.9 μM. The distribution of residuals is shown in the upper panel.

equilibrium, exhibiting dimerization K_d values of 1.4 and 2.9 μM, respectively, closely resembling their respective K_d values of the native enzyme (Table 1).

Fluorescence Anisotropy of FRP−EI. Two concentrations of FRP−EI were assayed, 0.13 and 13 μM. On the basis of

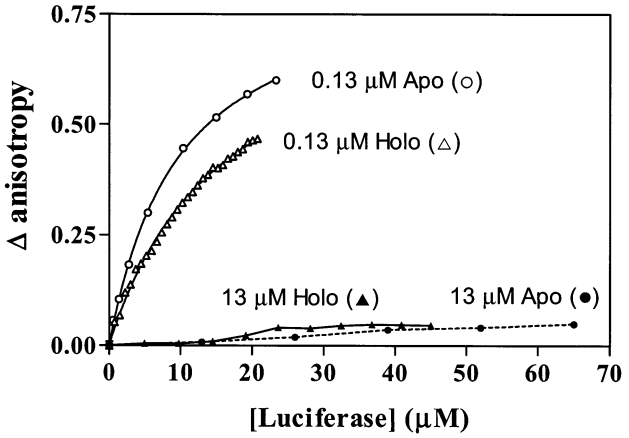


FIGURE 3: Interaction of the of FRP−EI holoenzyme and apoenzyme with luciferase. FRP−EI holoenzyme at a total concentration of 0.13 μM (△) or 13 μM (▲) was titrated with luciferase. Similarly, the FRP−EI apoenzyme at 0.13 μM (○) or 13 μM (●) was also titrated with luciferase. The lines for the 0.13 μM FRP−EI holo- and apoenzyme samples are the theoretical fits to eq 4. In the case of the 13 μM FRP−EI holo- and apoenzyme samples, the lines simply connect the datum points.

Table 2: K_d Values for Luciferase/FRP−EI Complex Formation

enzyme	total concn (μM)	K_d (μM)
FRP−EI	0.13	11
	13	*a*
apoFRP−EI	0.13	7
	13	a

[a] Not detectable.

the dimerization K_d determined by analytical ultracentrifugation, these two concentrations represent a primarily monomeric state and a primarily dimeric state, respectively. The monomeric form of the FRP−EI holoenzyme showed interaction with luciferase, as was evident by increases in anisotropy as a function of luciferase concentration (Figure 3). The K_d value of luciferase/monomeric FRP−EI complex formation was found to be 11 μM (Table 2). With 13 μM FRP−EI holoenzyme, only small increases in anisotropy were observed at higher concentrations of luciferase (Figure 3). The amounts of various species in the sample after luciferase titrations cannot be determined from these anisotropy data but can be estimated if it is assumed that all components are in equilibrium. If significant amounts of FRP dimer/luciferase complex existed, the levels of anisotropy increases at high luciferase concentrations should be much higher than what were actually observed. Hence, our results indicate that the dimeric FRP−EI holoenzyme was ineffective in forming a complex with luciferase. The apoenzyme form of FRP−EI was also assayed for interaction with luciferase following the same experimental design, and similar results were acquired. Data are again shown in Figure 3 and summarized in Table 2. The dimeric apo-FRP−EI also had no significant interaction with luciferase, whereas the monomeric species formed a complex with luciferase with a K_d of 7 μM, not much different from that of the holoenzyme.

Binding Stoichiometry. The data shown in Figure 3 for the titration of 0.13 μM FRP−EI holoenzyme with luciferase were also used for a Hill plot (Figure 4), where Y is the fractional saturation value of FRP−EI as defined earlier. A

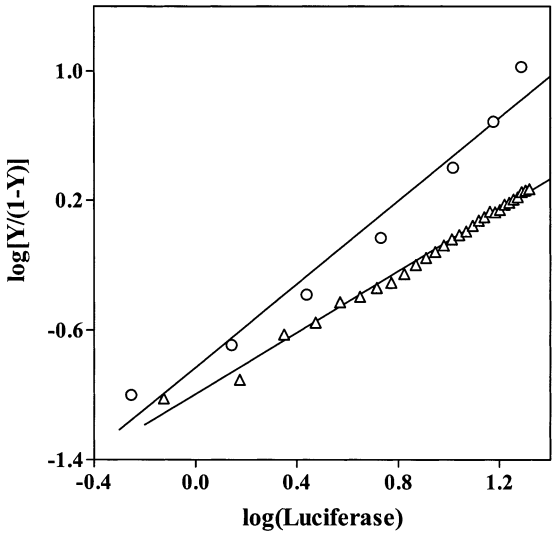


FIGURE 4: Hill plot of the binding of luciferase by FRP−EI holo- and apoenzyme. The results in Figure 3 for the titration of 0.13 μM of FRP−EI holoenzyme (△) and apoenzyme (○) by luciferase are analyzed according to Hill plot. The term Y is the fractional saturation of FRP−EI by luciferase binding.

linear plot was obtained ($R^2 = 0.992$) with a slope (=number of luciferase bound per FRP monomer) of 0.95 ± 0.02. The luciferase binding stoichiometry was similarly determined for the FRP−EI apoenzyme. A linear line ($R^2 = 0.968$) with a slope of 1.28 ± 0.11 was obtained (Figure 4). It appears that one FRP−EI monomer, as either holo- or apoenzyme, formed a complex with one luciferase $\alpha\beta$ dimer. The K_d values of 10 and 7 μM for the binding of luciferase to FRP−EI holo- and apoenzyme, respectively, determined from the Hill plots correlate well with those determined according to eq 4.

Determination of Intracellular FRP and Luciferase Concentrations. The total FRP and luciferase activities in cell lysate were used to calculate the intracellular concentrations of these two enzymes, as described. FRP and luciferase intracellular concentrations were determined to be approximately 3 and 172 μM, respectively. These concentrations are consistent with the results of the same calculations performed with the enzyme yield data from Jablonksi and DeLuca's paper (*43*), which suggests intracellular concentrations of 4 and 213 μM. Consequences of these concentrations will be discussed later in this paper.

DISCUSSION

Fluorescence anisotropy signals are highly sensitive to, among other factors, the state of molecular aggregation. Hence this approach was chosen for the investigation of the complex formation between *V. harveyi* FRP and luciferase. To this end, a procedure was developed for the labeling of FRP with the fluorophore eosin to obtain FRP−EI, with a labeling stoichiometry of one eosin per FRP monomer. Eosin was chosen as the labeling fluorophore due to its high excitation efficiency at wavelength of 519 nm, which lies significantly outside the flavin absorption peaks. Cysteines were targeted for labeling because all three of the cysteines lie at a significant distance from the proposed active site; the α carbon of the nearest cysteine, C156, was measured to be 17 Å from the proposed NADPH binding site (*44*). So

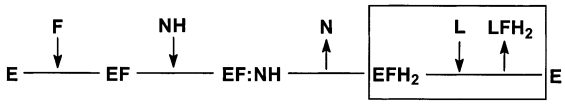
it was hoped that labeling of any of the cysteine residues would not have any detrimental effects on FRP activity. In support of such an expectation, the labeled FRP fully retained the FMN cofactor and was active in both the single-enzyme and the luciferase-coupled assays at levels about 30% those of the native FRP.

The findings that FRP−EI had one fluorophore attached per FRP monomer and the labeled enzyme retained 30% of the native enzyme activity should be considered further. These findings could be interpreted in three ways. First, the labeling can be assumed to be isotropic with about 33% yield for the tagging of each of the three cysteines. In this scenario, the labeling of either of two of the three cysteines yielded inactive species, whereas the labeling of the third cysteine produced a fully active enzyme. Only one-third of the labeled enzyme sample is active but all labeled enzyme molecules are positive in fluorescence anisotropy measurements. Such an enzyme preparation would not be desirable for the intended study on complex formation with luciferase. We found that, similar to the native FRP, the FMN cofactor in the FRP−EI sample was fully reduced in <3 s of manual mixing with saturating levels of NADH or NADPH. This finding clearly ruled out the first possibility of labeling, for which only $^1/_3$ of the enzyme cofactor could be reduced by NAD(P)H. The second possibility is that the enzyme was again labeled with equal 33% probabilities at each of the three cysteines, and all three labeled enzyme species were about 30% as active as the native enzyme. And the third possibility is that one specific cysteine was 100% labeled whereas the other two cysteines remained untagged. Moreover, the specifically labeled enzyme was about 30% as active as the native enzyme. Our results cannot distinguish the second from the third labeling possibility. However, both labeling possibilities would yield partially but uniformly active enzyme samples, which are quite suitable for the work described in this report.

Similar to the native FRP, the holo- and apoenzyme forms of FRP−EI also underwent a monomer−dimer equilibrium with K_d values of 1.4 and 2.9 μM, respectively (Figures 1 and 2; Table 1). FRP−EI samples at 0.13 and 13 μM were each titrated with increasing concentrations of luciferase, and fluorescence anisotropy signals were followed to monitor any complex formation between luciferase and either the monomeric or the dimeric form of the reductase. Interestingly, for the FRP−EI holoenzyme, the monomeric but not the dimeric reductase was effective in forming a complex with luciferase (Figure 3). Similar results were also obtained for the apoenzyme of FRP−EI (Figure 3). In both cases, it appears that one monomeric FRP was bound to one $\alpha\beta$ luciferase. The possibility that the binding of luciferase with FRP could be induced by the eosin fluorophore cannot be completely dismissed. However, this appears to be unlikely on the basis that the eosin-labeled FRP dimer was not nearly as efficient as the labeled FRP monomer in forming a complex with luciferase.

This laboratory had made attempts in the past to detect complex formation between FRP and luciferase by molecular sieve chromatography and light scattering techniques. However, no significant complex formation was observed. High concentrations of both FRP and luciferase were used in these earlier attempts in the hope of favoring complex formation. In view of the present findings, these earlier negative experiences are most likely consequences of using FRP in its dimeric form.

Scheme 1



For direct metabolite channeling from a donor to an acceptor enzyme, formation of a complex between the two functionally linked enzymes, either transient or stable, is a mandatory requirement. It should be emphasized that this study documents the first direct physical evidence for the detection of complex formation between FRP and luciferase in solution. Therefore, our present findings provide an important support to the direct transfer of the $FMNH_2$ cofactor from FRP to luciferase proposed recently on the basis of our kinetic studies (*32*, *33*). Key features of our proposed direct transfer mechanism are shown in Scheme 1 (in which E is the FRP apoenzyme; EF is FRP holoenzyme; L is luciferase; F and FH_2 are oxidized and reduced FMN, respectively; and N and NH are $NADP^+$ and NADPH, respectively). Unlike the ping-pong mechanism of FRP in its single-enzyme reaction, the luciferase−FRP coupled reaction involves the direct transfer of the FMN cofactor of FRP, after its reduction by NADPH, to luciferase, leaving FRP in an apoenzyme form. The subsequent binding of oxidized FMN regenerates the original FRP holoenzyme. When viewed with the apoenzyme of FRP as the starting point, the kinetic mechanism follows a sequential pattern. Because apoenzyme of FRP is an integral part of the $FMNH_2$ direct transfer mechanism, we have also examined the ability of FRP−EI apoenzyme to form a complex with luciferase. Once again, the monomeric rather than the dimeric form of the FRP−EI apoenzyme was effective in binding to luciferase. Therefore, E and EF in Scheme 1 can be specifically identified as the monomeric reductase species.

Scheme 1 also provides a basis for addressing additional intriguing questions. For example, it would be informative to compare the rate of oxidation of free $FMNH_2$ with that of the FRP-bound $FMNH_2$ cofactor when FRP is and is not in a complex with luciferase. In addition, FRP binds the FMN cofactor with a K_d of 0.2 μM (*38*), and the K_d for $FMNH_2$ binding by luciferase is about 0.8 μM (*45*). It would also be important to determine the binding affinity for the $FMNH_2$ cofactor by the free FRP and, more relevant to Scheme 1, the K_d values of the luciferase-bound FRP for both the oxidized and reduced flavin cofactor and the $FMNH_2$ binding K_d by luciferase while in complex with FRP.

The present finding that monomeric but not dimeric reductase was effective in complexing with luciferase also provides important insights into the regulation of bacterial bioluminescence in vivo. We obtained a K_d of 1.8 μM for FRP monomer−dimer equilibrium and a K_d of 11 μM for the complex formation of luciferase with monomeric FRP−EI. We also estimated that when *V. harveyi* cells were grown to exhibit maximal in vivo bioluminescence, their cellular content of FRP holoenzyme was about 3 μM (a level quite sensitive to perturbations of the monomer−dimer equilibrium) and that of luciferase was about 172 μM (which is sufficiently high to trap most monomeric FRP in complex formation with luciferase). FRP apoenzyme was not detect-

able. As an approximation, it is assumed that the native FRP holoenzyme behaves similarly to FRP−EI in complex formation with luciferase and there are no significant protein complex formations other than the dimerization of FRP and the binding between luciferase and FRP. With total concentrations of 172 and 3 μM for luciferase and FRP, there would be 0.19 μM free monomeric FRP, 0.003 μM FRP dimer, and 2.81 μM luciferase/FRP complex at equilibrium. Approximately 94% of the total FRP would be in complex with luciferase. This is important to energy conservation by *V. harveyi* cells. We have recently shown that the $FMNH_2$ product generated by free FRP was far less efficient in supporting the luciferase bioluminescence reaction than the $FMNH_2$ directly channeled from FRP to luciferase within a functional complex of these two enzymes (*32*). If most FRP molecules were not in complex with luciferase, considerable amounts of NADPH would be consumed by free FRP to generate $FMNH_2$ which could not be efficiently utilized by luciferase for bioluminescence but could be autooxidized to form H_2O_2 and, subsequently, other toxic oxygen species.

Another plausible physiological significance of trapping most FRP in a complex with luciferase can also be rationalized. At 23 °C, FRP has a turnover rate of 2160/min (*31*), which is 340 times faster than that of luciferase (6.3/min) with the physiological tetradecanal as a substrate (*46*). By forming a functional complex with the much slower luciferase, FMN and NADPH at levels much lower than the K_m of the free FRP for these two substrates would be sufficient for 50% maximal bioluminescence emission by the luciferase/FRP complex. Results obtained with purified FRP and luciferase in the in vitro coupled bioluminescence reaction fully confirmed such an expected kinetic pattern; the K_m values for FMN and NADPH were reduced from 8 and 20 μM, respectively, in the FRP single-enzyme reaction to 0.3 and 0.02 μM, respectively, in the luciferase-coupled reaction (*32*). Therefore, complex formation of nearly all FRP with luciferase would enable the *V. harveyi* cells to be highly efficient in bioluminescence at relatively low levels of FMN and, particularly, NADPH.

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