Mechanism of Reduced Flavin Transfer from *Vibrio harveyi* NADPH−FMN Oxidoreductase to Luciferase[†]

Benfang Lei[‡] and Shiao-Chun Tu*[,‡,§]

Department of Biology and Biochemistry and Department of Chemistry, University of Houston, Houston, Texas 77204-5934

Received July 31, 1998; Revised Manuscript Received August 20, 1998

ABSTRACT: The mechanisms of reduced flavin transfer in biological systems are poorly understood at the present. The *Vibrio harveyi* NADPH−FMN oxidoreductase (FRP) and the luciferase pair were chosen as a model for the delineation of the reduced flavin transfer mechanism. FRP, which uses FMN as a cofactor to mediate the reduction of the flavin substrate by NADPH, exhibited a ping-pong kinetic pattern with a $K_{m,FMN}$ of 8 μM and a $K_{m,NADPH}$ of 20 μM in a single-enzyme spectrophotometric assay monitoring the NADPH oxidation. However, the kinetic mechanism of FRP was changed to a sequential pattern with a $K_{m,FMN}$ of 0.3 μM and a $K_{m,NADPH}$ of 0.02 μM in a luciferase-coupled assay measuring light emission. In contrast, the *Photobacterium fischeri* NAD(P)H−FMN oxidoreductase FRG showed the same ping-pong mechanism in both the single-enzyme spectrophotometric and the luciferase-coupled assays. Moreover, for the FRP, FMN at concentrations over 2 μM significantly inhibited the coupled reaction in both light intensity and quantum yield, and showed apparent noncompetitive and competitive inhibition patterns against NADPH and luciferase, respectively. No inhibition of the NADPH oxidation was detected under identical conditions. These results are consistent with a scheme that the reduced flavin cofactor of FRP is preferentially utilized by luciferase for light emission, the reduced flavin product generated by the reductase is primarily channeled into a dark oxidation, and luciferase competes against flavin substrate in reacting with the FRP reduced flavin cofactor. An FRP derivative containing 2-thioFMN as the cofactor was also used to further examine the mechanism of flavin transfer. Results again indicate a preferential utilization of the reductase reduced flavin cofactor by luciferase for the bioluminescence reaction.

Enzymes in the NAD(P)H−flavin oxidoreductase (flavin reductase) family catalyze the reduction of flavin at the expense of NAD(P)H. Several species of flavin reductase have been identified in luminous bacteria (*1*−*4*) and are believed to provide reduced riboflavin 5′-phosphate (FMNH$_2$)[1] in vivo as a substrate for bacterial luciferase which catalyzes a luminescent monooxygenation reaction (eqs 1 and 2). Flavin reductases have also been isolated from

$$\mathrm{FMN + NAD(P)H + H^+ \xrightarrow{\text{flavin reductase}} FMNH_2 + NAD(P)^+} \quad (1)$$

$$\mathrm{RCHO + FMNH_2 + O_2 \xrightarrow{\text{luciferase}} RCOOH + FMN + H_2O + light} \quad (2)$$

nonluminous organisms and linked to a variety of biological functions such as the reduction of methemoglobin (*5*, *6*), reductive iron release from siderophores (*7*, *8*), oxygen activation (*9*), activation of ribonucleotide reductase (*10*, *11*) and chorismate synthase (*12*), biosynthesis of the antitumor agent valanimycin (*13*), and desulfurization of fossil fuel (*14*).

Free reduced flavin is subject to rapid autoxidation (*15*). Free diffusion is thus unlikely to be an efficient pathway for reduced flavin transfer in vivo. Hence, specific direct transfers of reduced flavin from flavin reductases to receptor enzymes are expected to exist. However, mechanisms for reduced flavin transfer in biochemical processes have never been well-established.

The luciferase−flavin reductase couple in luminous bacteria provides an excellent system for the delineation of the flavin transfer mechanism. The structure and mechanism of bacterial luciferase have been extensively studied (*16*, *17* and references therein). Properties of several highly purified flavin reductase species from luminous bacteria have been characterized (*3*, *18*−*22*). The formation of FMNH$_2$ by flavin reductases can be directly quantified by following the oxidation of NAD(P)H spectrophotometrically. In addition, luminescence from the flavin reductase−luciferase-coupled assay provides a quantitative and sensitive measure of the FMNH$_2$ transfer from flavin reductase to luciferase. Moreover, using an NADPH-preferring flavin reductase FRP (*3*, *23*), an NADH-preferring flavin reductase FRD (*3*) from *Vibrio harveyi*, and a general NAD(P)H-dependent flavin reductase FRG from *Photobacterium fischeri* (*1*), earlier

[†] Supported by Grant GM25953 from the National Institutes of Health and Grant E-1030 from The Robert A. Welch Foundation.

* Corresponding author: telephone, 713-743-8359; fax, 713-743-8351; e-mail, dtu@uh.edu.

[‡] Department of Biology and Biochemistry.

[§] Department of Chemistry.

[1] Abbreviations: FMNH$_2$, reduced riboflavin 5′-phosphate; FRP, NADPH-preferring flavin reductase P; FRD, NADH-preferring flavin reductase D; FRG, NADH-, and NADPH-utilizing flavin reductase G; FRP$_s$, holoenzyme reconstituted from FRP apoenzyme and 2-thioFMN; q, quantum.

S0006-2960(98)01841-8 CCC: $15.00
Published on Web 09/25/1998

kinetic studies have provided evidence for a direct transfer of $FMNH_2$ from flavin reductase to luciferase.

In this study, the *V. harveyi* luciferase and FRP were targeted for a more thorough and rigorous kinetic analysis of the flavin transfer mechanism. Results indeed indicate the existence of a direct transfer of reduced flavin from FRP to luciferase. Moreover, the purified FRP has a bound FMN cofactor and requires a second flavin molecule as a substrate (*20*). We found that luciferase preferentially utilized the reduced flavin cofactor instead of the reduced flavin product of FRP for luminescence. To our knowledge, this is the first example of a preferential transfer of the reduced flavin cofactor of one enzyme to another enzyme as a substrate.

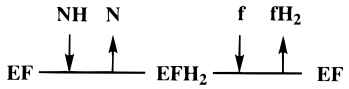
EXPERIMENTAL PROCEDURES

Materials and General Methodology. Bovine serum albumin, FMN, NADH, NADPH, and decanal were from Sigma. Stock decanal solutions were prepared fresh in ethanol. Literature procedures were followed to synthesize and purify 2-thioFMN (*24*) and to purify FMN (*25*). An FRP derivative, FRP_s, was reconstituted from FRP apoenzyme and 2-thioFMN as described previously (*22*). All phosphate (Pi) buffers were at pH 7.0 and consisted of phosphates at mole fractions of 0.39 sodium monobase and 0.61 potassium dibase. Purities of enzyme samples were judged on the basis of sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

Luciferase Purification. The cloned *V. harveyi* luciferase was expressed in *Escherichia coli* JM101 harboring pTH2 (*26*, *27*) and was purified to >95% homogeneity as described previously (*28*). Such a luciferase sample, usually containing low but detectable levels of flavin reductase, was further purified on a DEAE-Sepharose column (2.5 × 40 cm) eluted isocratically with 280 mM Pi. The luciferase so obtained was close to being completely free from flavin reductase; a 1.2 μM luciferase sample contained flavin reductase activities equivalent to ≤5 pM FRP.

Flavin Reductases. V. harveyi FRP was purified to apparent homogeneity as described previously (*20*). *P. fisheri* (formerly as *Vibrio fisheri*) flavin reductase FRG was purified from *E. coli* JM109 harboring the recombinant plasmid pFRG. The pFRG was obtained by cloning the FRG-encoding *frg* gene (*29*) into pUC18 at the *Pst*I and *Eco*RI sites via polymerase chain reaction amplification using *P. fisheri* genomic DNA and primers 5′-CATCATAAGT-T*C*TGCAGACAAGAA-3′ and 5′-CTTTAAATAGAA*T*TC-TACCGTAG-3′. The italicized bases were those mutated to create the *Pst*I and *Eco*RI restriction enzyme sites. *E. coli* JM109 cells harboring pFRG were first cultured in 5 L of 2× Luria-Bertani medium supplemented with 100 mg/L ampicillin at 37 °C to about 1.5 OD_{600}, and then grown at 25 °C for two more days in a BioFlo IIc fermentor (New Brunswick Scientific). Forty grams of wet cell paste so obtained was sonicated for 10 min in 120 mL of ice-cooled 50 mM Pi buffer containing 0.5 mM dithiothreitol. Lysed cells were centrifuged, and the supernatant was directly loaded on a 2.5 × 40 cm DEAE-Sepharose (fast flow, Pharmacia) column pre-equilibrated with 50 mM Pi and isocratically eluted with 0.3 M Pi. The FRG pool so obtained was adjusted to 0.8 M $(NH_4)_2SO_4$ and loaded on a 1 × 20 cm Phenyl-Sepharose (fast flow, high sub, Pharmacia) column pre-equilibrated with 0.8 M $(NH_4)_2SO_4$ in 50 mM Pi. The column was first eluted with 50 mL of the same equilibrium solution and then with 0.5 M $(NH_4)_2SO_4$ in 50 mM Pi to recover FRG. FRG was concentrated by precipitation in 70% ammonium sulfate saturation and dialyzed against 50 mM Pi, 0.5 mM dithiothreitol, overnight. The FRG so obtained was >90% pure.

Scheme 1



Enzyme Assays. Two assay methods were used to determine the activity of FRP at 23 °C. In the single-enzyme spectrophotometric assay, FRP activities were determined in 50 mM Pi containing FMN and NADPH at designated concentrations by monitoring the decrease in A_{340} associated with NADPH oxidation. In the luciferase-coupled assay, reactions were carried out in 50 mM Pi in a 12 × 75 mm glass culture tube (BOREX) and light emission intensities were measured using a calibrated photometer (*30*). Activities of limiting quantities of FRP were determined in the presence of excess luciferase as the steady maximal emission intensity expressed in light units with 1 unit = 4.8×10^8 q·s^{-1}. In some cases, the emission time courses were recorded until the completion of the coupled reactions and total quantum outputs (Q_t) were calculated. Methods detailed previously (*31*) were followed for the analysis of kinetic data with respect to the K_m values and the kinetic mechanism.

RESULTS

Steady-State Kinetic Analyses. The FRP activities were first determined by the single-enzyme spectrophotometric assay as a function of NADPH concentration from 16 to 100 μM. Initial rates of ΔA_{340}/min were determined after adding 3 nM FRP into 1 mL of Pi containing varying amounts of NADPH and a constant level of FMN at 2.6, 5.2, 10.5, 26.3, or 53 μM. A series of parallel lines were obtained from double-reciprocal plots of initial rate versus NADPH concentration. This kinetic pattern, same as that reported earlier (*3*), indicates a ping-pong mechanism for FRP as shown in Scheme 1. According to this scheme, NADPH (NH) first binds to FRP holoenzyme (EF), and then the FMN cofactor is reduced by NADPH. After the release of $NADP^+$ (N) as the first product, the enzyme is left in a reduced form (EFH_2). Subsequently, an exogenously added FMN binds to the flavin substrate (f) site of EFH_2, and a reducing equivalent exchange takes place between the bound $FMNH_2$ cofactor and the FMN substrate. Finally, the $FMNH_2$ product (fH_2) is released and the oxidized FRP is regenerated. The "F" and "f" refer to the flavin bound to or released from the cofactor site and the substrate site, respectively. By using kinetic equations for the ping-pong mechanism (*31*) for analysis, a $K_{m,FMN}$ of 8 μM and a $K_{m,NADPH}$ of 20 μM (Table 1) were obtained.

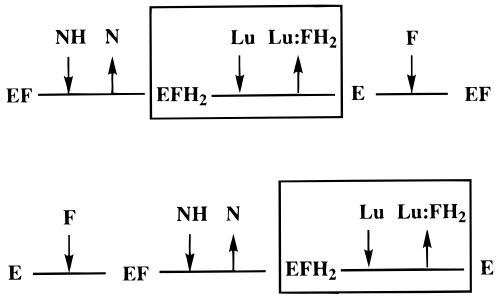
Luciferase-coupled reactions were also carried out by adding 10 μL of NADPH (final concentration 0.01−0.15 μM) into 1 mL of Pi containing 0.5 nM FRP, 1.2 μM luciferase, 10 μM decanal, and a constant level of FMN at 0.1, 0.2, 0.4, or 0.6 μM. Light intensity reached a steady level within a few seconds and lasted for 1 min or longer. A

Table 1: K_m Values and Kinetic Mechanisms of FRP and FRG Determined by Reductase Single-Enzyme Spectrophotometric Assay and Luciferase-Coupled Luminescence Assay

assay method	$K_{m,FMN}$ (μM)	$K_{m,NADPH}$ (μM)	$K_{m,NADH}$ (μM)	kinetic mechanism
FRP spectrophotometric	8	20		ping-pong
FRP−luciferase coupled	0.3	0.02		sequential
FRG spectrophotometric[a]	220		120	ping-pong
FRG−luciferase coupled	4		9	ping-pong

[a] Taken from Tu et al., 1979 (*33*).

Scheme 2



family of converging lines was obtained from double-reciprocal plots of light intensity versus NADPH concentration, allowing the calculation of 0.3 μM for the $K_{m,FMN}$ and 0.02 μM for the $K_{m,NADPH}$ (Table 1). Consistent with earlier findings (*3*), these K_m values revealed by the coupled assay were significantly smaller than the respective $K_{m,FMN}$ and $K_{m,NADPH}$ determined by the spectrophotometric assay. However, results from the coupled assays indicate a sequential mechanism in contrast to the ping-pong mechanism shown by the spectrophotometric assay.

It is known that the *P. fischeri* FRG also has a bound FMN cofactor (*32*) and follows a ping-pong mechanism, determined by the single-enzyme spectrophotometric assay, with a $K_{m,FMN}$ of 220 μM and a $K_{m,NADH}$ of 120 μM (*33*). Steady-state light emissions were measured by the coupled assay by adding NADH (varying from 1.5 to 50 μM) into 1 mL of Pi containing 1.5 nM FRG, 1.2 μM luciferase, 10 μM decanal, and a constant level of FMN at 0.5, 1.0, 2.0, 4.0, 6.0, or 8.0 μM. Interestingly, double-reciprocal plots of light intensity versus NADH concentration at different constant levels of FMN again generated a set of parallel lines. In contrast to the *V. harveyi* FRP, the *P. fisheri* FRG still followed a ping-pong mechanism in the luciferase-coupled assay, with the $K_{m,FMN}$ and $K_{m,NADH}$ determined to be 4 and 9 μM, respectively (Table 1). Therefore, the change of kinetic pattern observed with the *V. harveyi* FRP in two different assays was clearly a genuine property of this reductase.

Hypothesis of FRP−Luciferase Coupling Mechanism. We propose the following minimal scheme to reconcile the apparent sequential mechanism in the FRP−luciferase-coupled reaction. As shown in Scheme 2 upper pathway, the initial events leading to the release of $NADP^+$ and the formation of EFH_2 are the same as those in Scheme 1. We propose that luciferase (Lu) forms a complex with EFH_2, and a direct transfer of the $FMNH_2$ cofactor from reduced FRP to luciferase occurs. This key step is highlighted by the enclosure box. After the release of Lu−FH_2, the reductase is left in the apoenzyme form (E). The exogenously added FMN subsequently binds to the flavin cofactor

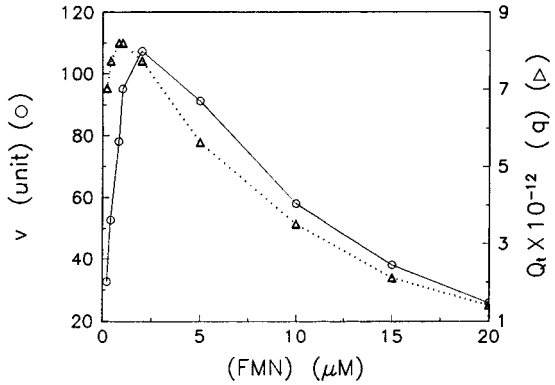


FIGURE 1: Effects of FMN on light intensity and total quantum output in the luciferase−FRP-coupled reaction. Maximum light intensities (○) and total quantum output (△) of the coupled reactions are plotted against FMN concentration. The reactions were initiated by the addition of 10 μL of 10 μM NADPH into 1 mL of Pi, pH 7.0, containing 1.0 nM FRP, 2.4 μM luciferase, 10 μM decanal, and different levels of FMN from 0.2 to 20 μM.

(F) site of the apoenzyme to regenerate the holoenzyme EF. When Scheme 2 is viewed with the reductase apoenzyme as the starting point (Scheme 2 lower pathway), it is clear that the coupled assay would follow an apparent sequential kinetic pattern.

FMN Inhibition of Coupled Reaction. We found that FMN at >2 μM caused significant inhibitions of the luciferase-coupled reaction. As shown in Figure 1, initially light intensity increased at higher FMN concentrations and reached an apparent maximal emission intensity at about 2 μM FMN. However, the luminescence intensity markedly decreased with further increase of FMN concentration. The effect of FMN concentration on the total quanta of light emission was similar to that on the emission intensity except that the maximal total quantum output was reached at about 1 μM FMN. In contrast, no inhibition was observed with FMN on the rate of NADPH oxidation by FRP in the spectrophotometric assay in the absence or presence of luciferase and decanal at concentrations similar to those used in the coupled assay.

FMN Inhibition Pattern. Light emission intensities were measured as a function of NADPH concentration at several constant FMN levels from 5 to 20 μM which are much higher than the $K_{m,FMN}$ determined in the coupled assay. A series of converging lines were obtained from the reciprocal plots of light intensity versus NADPH concentration (Figure 2A), and both the slope and intercept linearly increased with FMN concentration (Figure 2B), suggesting an apparent noncompetitive inhibition[2] of FMN against NADPH.

Following a conventional noncompetitive inhibition in the presence of >2 μM FMN one would anticipate the reduction in the light intensity (v in q· s^{-1}) but not in the total light output (Q_t). The presence of the FMN inhibitor would trap some of the FRP in a form inactive to support the luciferase bioluminescence, but the remaining active FRP should be able to completely utilize the total NADPH for bioluminescence in the coupled reaction. However, contrary to a simple noncompetitive inhibition, high concentrations of FMN were inhibitory to both the luminescence intensity and the total quantum output (Figure 1). Therefore, Scheme 3 is proposed

[2] The noncompetitive inhibition referred to in this report includes the conventional noncompetitive inhibition and the mixed inhibition.

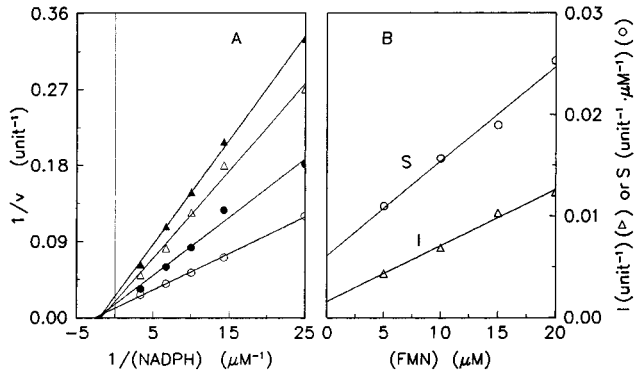
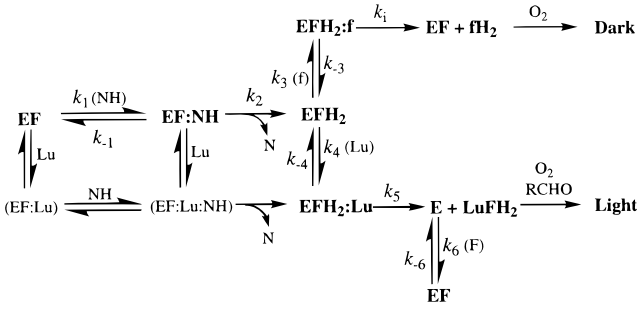


FIGURE 2: Inhibition of FMN against NADPH in the luciferase−FRP-coupled assay. Maximal steady emissions were measured after the initiation of the reaction by the addition of 10 μL of NADPH into 1 mL of 50 mM Pi, pH 7.0, containing 1.2 μM luciferase, 10 μM decanal, 0.5 nM FRP, and different levels of FMN. Final NADPH concentrations were from 0.04 to 0.3 μM. (A) Light intensities versus NADPH concentrations are presented as double-reciprocal plots. FMN concentrations were, from the bottom line upward, 5, 10, 15, and 20 μM. (B) The slopes (○) and the ordinate intercepts (△) in panel A are each plotted as a function of FMN concentration.

Scheme 3



as a minimal kinetic pathway to account for the results shown in Figures 1 and 2.

The two enzyme species shown in parentheses are excluded for consideration for now and will be discussed later. Again, F and f are the flavin species bound to or released from the cofactor site and the substrate site, respectively. It is assumed that luciferase and the FMN substrate (f) compete in reacting with the FRP reduced flavin cofactor (FH_2). This reduced flavin cofactor is directly transferred from FRP to luciferase leading to a light-emitting pathway whereas the reduced flavin product (fH_2) of FRP is released to the medium and is thus primarily channeled into a dark oxidation reaction. It should be noted that the dark reaction (upper pathway in Scheme 3) is identical to the ping-pong pathway in Scheme 1 whereas the light reaction (lower pathway in Scheme 3) is the same as the sequential pathway in Scheme 2.

A steady-state kinetic equation (eq 3) was derived on the

$$v = \frac{V_{max}A[NH]}{B + C[NH]} \tag{3}$$

$$\frac{1}{v} = \frac{C}{AV_{max}} + \frac{B}{AV_{max}}\frac{1}{[NH]} \tag{4}$$

basis of Scheme 3 where $A = k_1k_2k_4(k_i + k_{-3})[Lu]$; $B = k_4k_5(k_{-1} + k_2)(k_i + k_{-3})[Lu] + k_3k_i(k_{-1} + k_2)(k_5 + k_{-4})[F]$; $C = k_1k_2(k_i + k_{-3})(k_5 + k_{-4}) + (k_i + k_{-3})(k_1k_4k_5 + k_1k_2k_4)[Lu] + (k_5 + k_{-4})(k_1k_ik_3 + k_1k_2k_3)[F]$; and $V_{max} = k_5[E_t]$.

The terms $[E_t]$, [Lu], [NH], and [F] are the concentrations of total FRP, luciferase, NADPH, and FMN, respectively. Considering $K_d = 0.2$ μM for FMN binding to FRP apoenzyme (*22*), at [F] $\geq$ 5 μM the term of free E is negligible in deriving eq 3. Furthermore, eq 3 can be rearranged to eq 4 suitable for a double-reciprocal plot of $1/v$ versus 1/[NH]. At a constant concentration of luciferase and several constant levels of FMN, both the slope and the ordinate intercept of such a double-reciprocal plot should assume the general format of $Y = a + b[F]$, where Y is the slope or intercept and a and b are both constants. Consistent with eq 4, the results shown in Figure 2A revealed an apparent noncompetitive inhibition of FMN against NADPH. Furthermore, both the slope and the intercept of the plots determined in Figure 2A increased linearly with increasing concentrations of FMN (Figure 2B).

The same Scheme 3 is also applicable to the analysis of the inhibitory effect of FMN on the total quantum output of the coupled reaction. When the coupled reactions were carried out at a constant starting concentration of NADPH and several levels of FMN at 5−20 μM until the exhaustion of NADPH, the quantum yield (ϕ; defined as quantum per NADPH molecule consumed) can be expressed following Scheme 3 as the ratio of the rate of NADPH oxidation by the sequential pathway over the total rate of NADPH oxidation through both the sequential and ping-pong pathways (eq 5).

$$\phi = \phi_{max}\frac{k_4(k_i + k_{-3})[Lu]}{k_4(k_i + k_{-3})[Lu] + k_3(k_5 + k_{-4})[F]} \tag{5}$$

The term ϕ_{max} is the maximal quantum yield in the absence of any dark oxidation of NADPH. Equation 5 predicts that ϕ would decrease at increasing concentrations of FMN. Therefore, the observation that high levels of FMN inhibited the total quantum output of the coupled reaction (Figure 1) can also be accounted for.

Luciferase Titration. The coupled reactions were carried out at varying concentrations of luciferase, a constant starting level of NADPH, and several constant concentrations of FMN. For the analysis of data, eq 4 was rearranged to eq 6

$$\frac{1}{v} = \frac{C_1}{V_{max}} + \frac{C_2 + C_3[F]}{V_{max}}\frac{1}{[Lu]} \tag{6}$$

where C_1, C_2, and C_3 are all constants. This would predict a saturation curve upon titration with luciferase and an apparent competitive inhibition of FMN against luciferase in measuring the light intensity of the coupled reaction. Both predictions were confirmed by experimental results shown in Figure 3, parts A and B. Similar analyses were also made using the results of quantum yield (based on NADPH consumed) measurements. Equation 5 was rearranged to eq 7 which also predicts a saturation curve upon titration with

$$\frac{1}{\phi} = \frac{1}{\phi_{max}} + \frac{k_3(k_5 + k_{-4})[F]}{\phi_{max}k_4(k_i + k_{-3})}\frac{1}{[Lu]} \tag{7}$$

luciferase and an apparent competitive inhibition of FMN against luciferase. Again, both predictions were confirmed by experimental results shown in Figure 3, parts A and C.

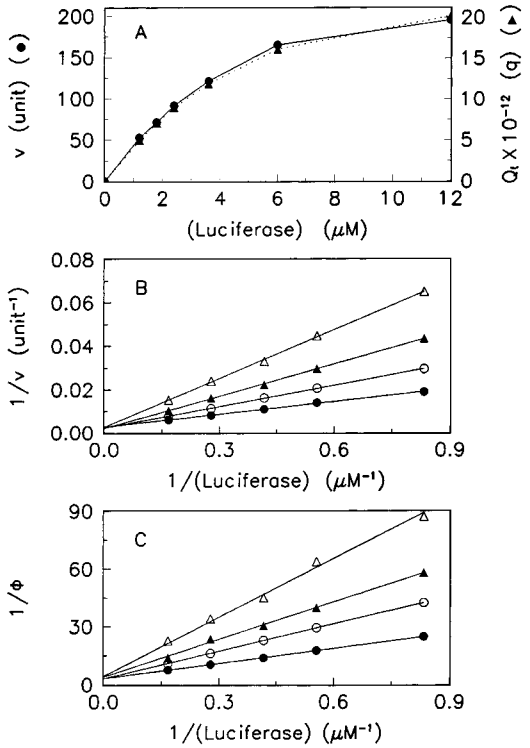


FIGURE 3: Effects of luciferase on light intensity and quantum yield in the luciferase−FRP-coupled assay. (A) Ten microliters of 20 μM NADPH was added into 1 mL of Pi, pH 7.0, containing 2 mg of bovine serum albumin, 80 μM decanal, 1 nM FRP, 5 μM FMN, and luciferase at indicated concentrations to initiate the coupled reaction. The maximum light intensity and total quantum output are plotted as functions of luciferase concentration. (B) The same experiment described for panel A was repeated using different levels of FMN. Results are shown as double-reciprocal plots of maximum light intensity versus luciferase concentration. (C) Data were obtained from the same experiments described for panel B. Results are shown as double-reciprocal plots of quantum yield (i.e., number of quanta per molecule of NADPH oxidized) versus luciferase concentration. For both panels B and C, the FMN concentrations were, from the bottom line upward, 5, 10, 15, and 20 μM.

FRP_s as Mechanistic Probe for the Luciferase−FRP Coupling. Both the native FRP and the FRP_s (reconstituted from the FRP apoenzyme and 2-thioFMN) can utilize either FMN or 2-thioFMN as an active substrate (*22*). In turn, luciferase can use both $FMNH_2$ and 2-thio$FMNH_2$ as a substrate for bioluminescence (*34*). However, the 0.16 quantum yield for the former flavin is much higher than the 0.003 quantum yield for the latter flavin (*35*). In this study, coupled reactions were carried out by adding FRP or FRP_s to 2 mL of Pi containing NADPH, luciferase, decanal, and FMN or 2-thioFMN. Reaction rates were determined by following the time-dependent decreases in A_{340} and by measuring luminescence intensity about 5 s after the initiation of the reaction. Four samples were tested with the reductase/flavin substrate combinations of FRP/FMN, FRP_s/FMN, FRP/2-thioFMN, and FRP_s/2-thioFMN. Under the conditions described in Table 2, the FRP_s/FMN and FRP/2-thioFMN samples showed similar NADPH oxidation rates and luminescence intensities whereas the FRP_s/2-thioFMN sample exhibited low activities by both measurements and the FRP/FMN sample showed a low NADPH oxidation rate but a very high luminescence intensity. The ΔA_{340}/min is a measure of the rate of NADPH oxidation and, hence, reduced flavin formation. Table 2 also shows a comparison of the four samples in terms of relative luminescence intensity normalized to the same rate of NADPH oxidation (or reduced flavin formation). The FRP/FMN sample had the highest normalized light intensity/NADPH oxidation ratio whereas 17-, 12.5-, and 330-fold reductions were observed for the FRP_s/FMN, FRP/2-thioFMN, and FRP_s/2-thioFMN samples, respectively.

Table 2: Comparison of NADPH Oxidation Rate and Coupled Luminescence Intensity Using Different Reductase/Flavin Substrate Combinations[a]

reductase/flavin substrate	NADPH oxidation[b] (ΔA_{340}/min)	light intensity[c] ($10^{-11} \times$ q·s^{-1})	relative (light intensity/NADPH oxidation)
FRP/FMN	0.08	144	1
FRP_s/FMN	0.10	10	6×10^{-2}
FRP/2-thioFMN	0.11	15	8×10^{-2}
FRP_s/2-thioFMN	0.04	0.2	3×10^{-3}

[a] Final reaction solution contained 0.08 μM FRP or FRP_s, 2.4 μM luciferase, 10 μM decanal, 1.7 μM 2-thioFMN or 3.5 μM FMN, and 225 μM NADPH in 50 mM Pi, pH 7.0. The reaction was initiated at 23 °C by the addition of reductase. [b] Calculated from the linear time courses of changes in A_{340} observed from the onset to 5 min for the FRP/FMN and FRP_s/2-thioFMN samples and to about 2 min for the FRP_s/FMN and FRP/2-thioFMN samples. The latter two samples showed a gradual upward curvature after 2 min. [c] Measured at about 5 s after the addition of the reductase. The measured intensities represented the maximal levels for the FRP/FMN, FRP/2-thioFMN, and FRP_s/2-thioFMN samples, but the FRP_s/FMN sample showed a slow increase to 62×10^{11} q·s^{-1} over 3 min.

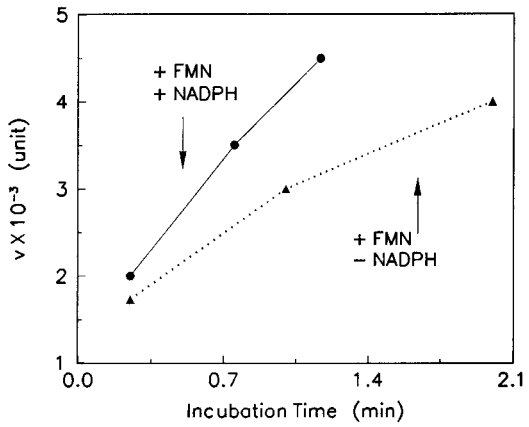
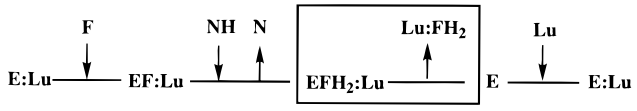


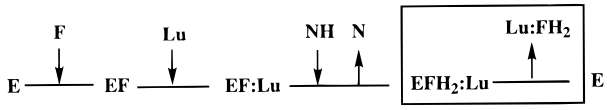
FIGURE 4: Effect of preincubation of FRP_s with FMN in the absence or presence of NADPH on the maximum luminescence intensity in the luciferase-coupled reaction. FRP_s (0.08 μM) was first incubated with 10 μM decanal, 3.5 μM FMN, and 225 μM NADPH in 1 mL of 50 mM Pi, pH 7.0. Luciferase (10 μL of a 0.24 mM stock) was added to initiate the light emission reaction after different times of the preincubation. The peak light intensities are plotted against the incubation times (●). The experiment was repeated under identical conditions except that FRP_s was first incubated with FMN and decanal in the absence of NADPH and the same amount of NADPH was added together with luciferase after different times of the incubation to initiate the luminescence reaction. The peak light intensities are plotted against the incubation times (▲).

In another series of experiments, a limiting quantity of FRP_s was incubated with FMN, NADPH, and decanal, and then excess luciferase was added after different times to initiate the coupled luminescence reaction. Higher initial peak light intensities were observed after longer incubation times (solid line in Figure 4). The experiment was repeated except that FRP_s was first incubated with FMN and decanal and then a luciferase−NADPH mixture was added after different times to initiate the luminescence. Qualitatively the same results were obtained (dotted line in Figure 4), but

Scheme 4



Scheme 5



the levels of luminescence intensity and the rate of luminescence activity increase during the incubation were both lower than those observed when FRP_s was incubated with both FMN and NADPH.

DISCUSSION

The K_m values for FMN and NAD(P)H determined for flavin reductases in the luciferase-coupled luminescence assay have been found to be significantly lower than those measured in the reductase single-enzyme spectrophotometric assay (*1*, *3*, *23*). These findings were interpreted to indicate the formation of a luciferase−reductase functional complex on the basis of the following consideration. If the reduced flavin product from reductase reaches luciferase by free diffusion and a true excess of luciferase is used for the coupled assay, the K_m for FMN and NAD(P)H determined for the reductase should be the same as those determined by the reductase spectrophotometric assay. However, the luciferase turnover rate is substantially slower than those of flavin reductases. If a functional complex is formed between flavin reductase and luciferase for a direct transfer of reduced flavin, then 50% maximal luminescence activity would be reached in the coupled assay at FMN and NADPH concentrations much lower than the respective K_m levels determined by the single-enzyme spectrophotometric assay.

We again observed much lower $K_{m,FMN}$ and $K_{m,NAD(P)H}$ for the *V. harveyi* FRP and *P. fischeri* FRG by the luciferase-coupled assay than those by the reductase spectrophotometric assay (Table 1 and ref *33*). However, in contrast to the *P. fischeri* FRG, the *V. harveyi* FRP showed a change from the ping-pong mechanism in the single-enzyme assay to a sequential mechanism in the coupled assay (Table 1). Scheme 2 is proposed to account for such an unexpected observation. A key feature of this scheme, highlighted by the enclosure box, is that the reduced flavin cofactor of FRP is preferentially utilized by luciferase for luminescence. When luciferase is added at a constant and excess level, the utilization of FMN and NADPH by the FRP apoenzyme would follow a sequential mechanism as shown in Scheme 2 lower pathway. As will be detailed later, another important feature of this scheme is that luciferase competes against the FMN substrate in reacting with the FRP reduced flavin cofactor. It should be emphasized that, as long as these two key features are maintained, the change from the ping-pong to the sequential mechanism in the coupled assay does not confine the binding of luciferase to only the reduced form of FRP as shown in Scheme 2. In fact, luciferase could bind to either the FRP apoenzyme (Scheme 4) or the oxidized holoenzyme (Scheme 5) and still shows a change from the ping-pong to the sequential mechanism in the coupled assay. This work is aimed at elucidating the mechanism of $FMNH_2$ transfer from FRP to luciferase rather than the exact nature of the FRP−luciferase complex.

Another important and unexpected finding was that FMN at >2 μM significantly inhibited not only the intensity (i.e., the rate) but also the total quantum output of the luminescence reaction. The inhibition of the total quantum output by FMN requires the existence of an additional dark pathway for the oxidation of $FMNH_2$ formed at the expense of NADPH. Scheme 3 was developed to account for all of the observations described above. According to the main part of Scheme 3 (including all enzyme species except the two species in parentheses), luciferase competes against the flavin substrate (f) in reacting with the reduced FRP (EFH_2). This scheme forms a basis for the predictions of an apparent noncompetitive inhibition of coupled reaction luminescence intensity by FMN against NADPH (eq 4), decreasing total quantum output at increasing levels of FMN (eq 5), and competitive inhibitions of the luminescence intensity (eq 6) and quantum yield (eq 7) by FMN against luciferase. All of these predictions are fully consistent with the results shown in Figures 1−3.

Scheme 3 can be expanded to include the two enzyme species in parentheses for a more general consideration of the reductase−luciferase complex formation. For those experiments in which the inhibitory effects of FMN were demonstrated, the FMN concentrations used were much higher than the K_d of FMN binding to the cofactor site of FRP apoenzyme. Consequently, the possible bindings of luciferase to oxidized FRP (EF), FRP−NADPH complex (EF−NH), and reduced FRP (EFH_2) are all included in the expanded Scheme 3 but the luciferase binding to FRP apoenzyme is not. Kinetic analyses of this expanded Scheme 3 also predict an apparent noncompetitive inhibition of the light intensity by FMN against NADPH and an apparent competitive inhibition by FMN against luciferase. Again, regardless of the detail events in the binding of luciferase to FRP, our results fully support the conclusions that the reduced flavin product (fH_2) generated by FRP is not efficient in supporting luciferase luminescence, that luciferase preferentially utilizes the reductase reduced flavin cofactor (FH_2) for the light emission, and that luciferase competes with the flavin substrate in reacting with the FRP reduced flavin cofactor.

The existence of separate flavin cofactor and substrate sites on FRP is critical to the Schemes 2 and 3. This is supported by the following findings. The isolated FRP holoenzyme, which has a bound FMN cofactor (*20*), can utilize not only flavin but also other electron acceptors as a substrate (*3*). We have also shown that neither riboflavin nor FAD shows any appreciable binding to FRP apoenzyme but that both flavins are good substrates for the FMN-bound FRP holoenzyme (*22*).

2-ThioFMN and FRP_s were used for further tests of Scheme 3. Table 2 shows the relative light intensity normalized to the same NADPH oxidation (or reduced flavin formation) rate for different reductase/flavin samples. If the reduced flavin product (fH_2) formed by reductase is preferentially utilized by luciferase, then FRP/FMN and FRP_s/FMN should exhibit the same normalized luminescence intensity. However, the latter was markedly less efficient in light emission. For FRP_s/FMN, the initial form of reduced flavin cofactor was 2-thio$FMNH_2$ which has a much lower quantum yield with luciferase than $FMNH_2$ (*34*, *35*). Therefore, our

results indicate that the reduced flavin cofactor of the reductase was preferentially transferred to luciferase for the light reaction. Also, the initial forms of reduced flavin cofactor should be $FMNH_2$ and 2-thio$FMNH_2$ for FRP/2-thioFMN and FRP_s/2-thioFMN, respectively. Indeed, the former showed a significantly higher normalized luminescence intensity than the latter (Table 2).

The FRP_s/FMN sample, as described in the Table 2 legend, showed a gradual increase of light intensity over the initial 3 min. In addition, when FRP_s was preincubated with FMN in the presence or absence of NADPH and the coupled luminescence was subsequently initiated, higher light intensities were observed after longer periods of preincubation (Figure 4). No lag period in the ΔA_{340} time course was ever detected in any of the spectroscopic assays for FRP or FRP_s in the absence or presence of luciferase. Therefore, increases in the luminescence intensity shown in Figure 4 cannot be due to slow binding of the FMN substrate to the reductase. The FRP_s concentration (0.08 μM) used in these experiments was significantly lower than the 0.3 μM K_d for the 2-thioFMN binding to FRP apoenzyme (*22*). A gradual replacement of the 2-thioFMN cofactor by the exogenous FMN, added at 3.5 μM and having a K_d of 0.2 μM (*22*), would occur. Therefore, the observed time-dependent increases in luminescence intensity (Figure 4) further support the preferential utilization of FRP reduced flavin cofactor by luciferase for luminescence. Moreover, with FMN as a cosubstrate, FRP_s has a slightly faster rate of NADPH oxidation than FRP (Table 2). If the $FMNH_2$ product formed by FRP or FRP_s is preferentially utilized by luciferase for the coupled luminescence reaction, the replacement of the 2-thioFMN cofactor on FRP_s by FMN during the preincubation should result in a small gradual decrease in the luminescence activity, a prediction contradicted by our observation. Furthermore, the increase in the peak luminescence intensity was faster when FRP_s was preincubated with FMN and NADPH (a condition allowing turnover and formation of the reduced flavin cofactor by the reductase) than with just FMN (Figure 4). Apparently, the reduced flavin cofactor was easier to dissociate from the reductase than the oxidized cofactor, thus providing further support for an efficient utilization of the reduced flavin cofactor of the reductase by luciferase for luminescence.

Mechanisms for reduced flavin transfer in biological systems are poorly understood in general. The preferential utilization of the reduced flavin cofactor of FRP by luciferase shown by this study is an interesting and surprising finding. However, the reduced flavin transfer mechanism proposed for the *V. harveyi* FRP−luciferase couple does not preclude other mechanisms for reduced flavin transfer to luciferase. In fact, the results described for the FRG−luciferase couple support a direct transfer of the reduced flavin product from FRG to luciferase. Clearly, the mode of reduced flavin transfer by different donor−acceptor pairs should not be generalized at the present. Moreover, luciferase may not rely on just one type of mechanism to acquire reduced flavin in vivo. There are at least three plausible mechanisms: free diffusion of $FMNH_2$ to luciferase, reduction of luciferase-bound FMN by exogenous reductant(s) including free reduced flavin, and a direct transfer from flavin reductase to luciferase within a functional complex. This work is confined to the investigation of the third transfer mechanism and, primarily, with respect to the *V. harveyi* luciferase-FRP couple.

REFERENCES

1. Duane, W., and Hastings, J. W. (1975) *Mol. Cell. Biochem. 6*, 53−64.
2. Gerlo, E., and Charlier, J. (1975) *Eur. J. Biochem. 57*, 461−467.
3. Jablonski, E., and DeLuca, M. (1978) *Biochemistry 17*, 672−678.
4. Watanabe, H., and Hastings, J. W. (1982) *Mol. Cell. Biochem. 44*, 181−187.
5. Quandt, K. S., Xu, F., Chen, P., and Hultquist, D. E. (1991) *Biochem. Biophys. Res. Commun. 178*, 315−321.
6. Chikuba, K., Yubisui, T., Shirabe, K., and Takeshita, M. (1994) *Biochem. Biophys. Res. Commun. 198*, 1170−1176.
7. Hallé, F., and Meyer, J. (1992) *Eur. J. Biochem. 209*, 621−627.
8. Covès, J., and Fontecave, M. (1993) *Eur. J. Biochem. 211*, 635−641.
9. Gaudu, P., Touati, D., Nivière, V., and Fontecave, M. (1994) *J. Biol. Chem. 269*, 8182−8188.
10. Fontecave, M., Eliasson, R., and Reichard, P. (1987) *J. Biol. Chem. 262*, 12325−12331.
11. Covès, J., Nivière, V., Eschenbrenner, M., and Fontecave, M. (1993) *J. Biol. Chem. 268*, 18604−18609.
12. Hasan, N., and Nestor, E. W. (1978) *J. Biol. Chem. 253*, 4987−4992.
13. Parry, R. J., and Li, W. (1997) *Arch. Biochem. Biophys. 339*, 47−54.
14. Lei, B., and Tu, S.-C. (1996) *J. Bacteriol. 178*, 5699−5705.
15. Gibson, Q. H., and Hastings, J. W. (1962) *Biochem. J. 83*, 368−377.
16. Fisher, A. J., Thompson, T. B., Thoden, J. B., Baldwin, T. O., and Rayment, I. (1996) *J. Biol. Chem. 271*, 21956−21968.
17. Tu, S.-C., and Mager, H. I. X. (1995) *Photochem. Photobiol. 62*, 615−624.
18. Michaliszyn, G. A., Wing, S. S., and Meighen, E. A. (1977) *J. Biol. Chem. 252*, 7495−7499.
19. Inouye, S., and Nakamura, H. (1994) *Biochem. Biophys. Res. Commun. 205*, 275−281.
20. Lei, B., Liu, M., Huang, S., and Tu, S.-C. (1994) *J. Bacteriol. 176*, 3552−3558.
21. Tanner, J. J., Lei, B., Tu, S.-C., and Krause, K. L. (1996) *Biochemistry 35*, 13531−13539.
22. Liu, M., Lei, B., Ding, Q., Lee, J. C., and Tu, S.-C. (1997) *Arch. Biochem. Biophys. 337*, 89−95.
23. Tu, S.-C., Lei, B., Yu, Y., and Liu, M. (1997) in *Flavins and Flavoproteins* (Stevenson, K. J., Massey, V., and Williams, C. H., Jr., Eds.) pp 357−366, University of Calgary Press, Calgary, Alberta, Canada.
24. Föry, W., and Hemmerich, P. (1967) *Helv. Chim. Acta 50*, 1766−1774.
25. Massey, V., and Swoboda, B. E. P. (1963) *Biochem. Z. 338*, 474−484.
26. Belas, R., Mileham, A., Cohn, D., Hilmen, M., Simon, M., and Silverman, M. (1982) *Science 218*, 791−793.
27. Xi, L., Cho, K.-W., Herndon, M. E., and Tu, S.-C. (1990) *J. Biol. Chem. 265*, 4200−4203.
28. Lei, B., Cho, K.-W., and Tu, S.-C. (1994) *J. Biol. Chem. 269*, 5612−5618.
29. Zenno, S., Saigo, K., Kanoh, H., and Inouye, S. (1994) *J. Bacteriol. 176*, 3536−3543.
30. Hastings, J. W., and Weber, G. (1963) *J. Opt. Soc. Am. 53*, 1410−1415.
31. Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104−137.
32. Inouye, S. (1994) *FEBS Lett. 347*, 163−168.
33. Tu, S.-C., Becvar, J. E., and Hastings, J. W. (1979) *Arch. Biochem. Biophys. 193*, 110−116.
34. Mitchell, G., and Hastings, J. W. (1969) *J. Biol. Chem. 244*, 2572−2576.
35. Tu, S.-C. (1982) *J. Biol. Chem. 257*, 3719−3725.

BI981841+