Differential Transfers of Reduced Flavin Cofactor and Product by Bacterial Flavin Reductase to Luciferase†

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ABSTRACT: It is believed that the reduced FMN substrate required by luciferase from luminous bacteria is provided in vivo by NAD(P)H−FMN oxidoreductases (flavin reductases). Our earlier kinetic study indicates a direct flavin cofactor transfer from *Vibrio harveyi* NADPH-preferring flavin reductase P (FRP_H) to the luciferase (L_H) from the same bacterium in the in vitro coupled luminescence reaction. Kinetic studies were carried out in this work to characterize coupled luminescence reactions using FRP_H and the *Vibrio fischeri* NAD(P)H-utilizing flavin reductase G (FRG_F) in combination with L_H or luciferase from *V. fischeri* (L_F). Comparisons of K_m values of reductases for flavin and pyridine nucleotide substrates in single-enzyme and luciferase-coupled assays indicate a direct transfer of reduced flavin, in contrast to free diffusion, from reductase to luciferase by all enzyme couples tested. Kinetic mechanisms were determined for the FRG_F-L_F and FRP_H-L_F coupled reactions. For these two and the FRG_F-L_H coupled reactions, patterns of FMN inhibition and effects of replacement of the FMN cofactor of FRP_H and FRG_F by 2-thioFMN were also characterized. Similar to the FRP_H-L_H couple, direct cofactor transfer was detected for FRG_F-L_F and FRP_H-L_F. In contrast, despite the structural similarities between FRG_F and FRP_H and between L_F and L_H, direct flavin product transfer was observed for the FRG_F-L_H couple. The mechanism of reduced flavin transfer appears to be delicately controlled by both flavin reductase and luciferase in the couple rather than unilaterally by either enzyme species.

Bacterial luciferase catalyzes the oxidation of reduced riboflavin 5′-phosphate ($FMNH_2$)[1] and a long-chain aliphatic aldehyde (RCHO), yielding FMN, fatty acid (RCOOH), water, and greenish blue light (eq 1). In comparison with most known flavin-dependent monooxygenases (or hydroxylases), bacterial luciferase is unusual in its lack of any tightly bound flavin cofactor, inability to reduce flavin, and, hence, requirement of $FMNH_2$ as a substrate. The required $FMNH_2$ is believed to be supplied in vivo by NAD(P)H−FMN oxidoreductases (flavin reductases or FRs) which catalyze the reduction of flavin by NAD(P)H (eq 2).

$$RCHO + FMNH_2 + O_2 \rightarrow RCOOH + FMN + H_2O + \text{light} \quad (1)$$

$$FMN + NAD(P)H + H^+ \rightarrow FMNH_2 + NAD(P)^+ \quad (2)$$

A number of new flavin-dependent hydroxylases have been identified in more recent years, each also relying on reduced flavin as a substrate and often existing as a complex with flavin reductase. These reduced flavin-acceptor hydroxylases include *Streptomyces viridifaciens* isobutylamine *N*-hydroxylase (*1, 2*), two monooxygenases from *Rhodococcus* active in fossil fuel desulfurization (*3*−*5*), a monooxygenase from *Streptomyces coelicolor* for the biosynthesis of actinorhodin (*6, 7*), *Escherichia coli* 4-hydroxyphenylacetate 3-hydroxylase (*8*−*10*), bacterial EDTA monooxygenase (*11, 12*), *Chelatobacter heintzii* nitrilotriacetate monooxygenase (*13, 14*), *Rhodococcus* pyrrole-2-carboxylate monooxygenase (*15*), and pristinamycin IIA synthase from *Streptomyces pristinaespiralis* (*16*). In addition, bacterial and plant chorismate synthases are also dependent on reduced flavin for activity (*17, 18*). Aside from these reduced flavin-requiring enzymes, flavin reductases have also been shown or implicated to activate processes such as iron release from ferrisiderophores (*19, 20*) and superoxide radical formation (*21*).

It has long been known that reduced flavin can be rapidly autooxidized (*22*−*24*), rendering free diffusion inefficient for in vivo intermolecular transfer. Moreover, autoxidation of reduced flavin forms cytotoxic products. Therefore, specific channeling of reduced flavin is expected to exist in

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[1] Abbreviations: $FMNH_2$, reduced riboflavin 5′-phosphate; FRP, NADPH-preferring flavin reductase; FRD, NADH-preferring flavin reductase; FRG, general flavin reductase which utilizes NADH and NADPH with similar efficiencies; L, luciferase; enzymes from *Vibrio harveyi* and *Vibrio fischeri* are identified by subscripts H and F, respectively; $FRP_{S,H}$, holoenzyme obtained from FRP_H apoenzyme and 2-thioFMN; $FRG_{S,F}$, holoenzyme obtained from FRG_F apoenzyme and 2-thioFMN; F and FH_2, oxidized and reduced flavins, respectively, that bind to or dissociate from the reductase cofactor site; f and fH_2, oxidized flavin substrate and reduced flavin product, respectively, of reductase; N and NH, $NAD(P)^+$ and NAD(P)H, respectively; q, quantum.

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vivo for at least some donor−acceptor couples. In recent years, we have chosen the flavin reductase−luciferase couple as a model for a series of studies on the molecular nature of reduced flavin transfer. To our knowledge, the mechanisms of intermolecular transfer of reduced flavin have not been delineated for any other biological systems.

We have proposed to classify three types of flavin reductases from luminous bacteria as the NADPH-preferring flavin reductase P (FRP), the NADH-preferring flavin reductase D (FRD), and the general flavin reductase G (FRG), which utilizes NADH and NADPH with similar efficiencies (*25*). Earlier kinetic studies using *Vibrio harveyi* FRP (FRP_H) and FRD (FRD_H) (*26*) and the *Photobacterium fischeri* (reclassified as *Vibrio fischeri*) FRG (FRG_F) (*27*) suggest a direct transfer of $FMNH_2$ from these flavin reductases to luciferase. Our recent study on FRP_H and the *V. harveyi* luciferase (L_H) provides several additional lines of evidence for a direct transfer of $FMNH_2$ between these two enzymes (*28*). The FRP_H has an FMN cofactor (*25*). Interestingly, we found that FRP_H directly transfers its $FMNH_2$ cofactor rather than the $FMNH_2$ product to L_H for the coupled luminescence reaction (*28*). FRG_F, or FRase I (*29*), also has an FMN cofactor (*30*) and is structurally (*31*) related to FRP_H (*32*). In this work, reduced flavin transfer in the FRG_F−L_F, FRG_F−L_H, and FRP_H−L_F couples was elucidated by a series of kinetic and inhibition studies. The flavin analogue 2-thioFMN was also used as a mechanistic probe in additional studies. Two distinct mechanisms of reduced flavin transfer were revealed with one pathway for the direct cofactor transfer and the second pathway for the direct product transfer. Moreover, both the constituent reductase and luciferase in the enzyme couple are important to the control of the transfer mechanism.

EXPERIMENTAL PROCEDURES

Materials. NADPH, NADH, FMN, and decanal were all from Sigma. Stock solutions of decanal were prepared in ethanol. Methods detailed previously were followed for the purification of FMN (*33*) and for the synthesis and purification of 2-thioFMN (*34*). All phosphate buffers were at pH 7.0 and consisted of mole fractions of 0.39 sodium monobase and 0.61 potassium dibase. Purities of enzymes were determined on the basis of patterns of sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

Luciferases. The expression of the cloned *V. harveyi* luciferase L_H and the purification to >95% purity were as described previously (*35*). The pVf plasmid harboring the *luxAB* genes of the *V. fischeri* luciferase L_F was expressed in *Escherichia coli* JM107 (*36*). Cells were grown in Luria−Bertani media with 100 μg/mL amplicillin at room temperature for 48 h, and were harvested by centrifugation. The expressed *V. fischeri* luciferase L_F was purified to >95% homogeneity following the previously detailed procedures (*28*).

Flavin Reductases. The cloned *V. harveyi* FRP was expressed and purified as described previously (*25*). The gene encoding the *V. fischeri* FRG was cloned and expressed in *E. coli* JM109 (*28*). The soluble phase of the crude lysate of *E. coli* JM109 cells was obtained (*25*), and loaded onto a DEAE-Sepharose column preequilibrated and eluted with 50 mM phosphate. When the A_{280} of the eluate dropped to <0.02, the eluting buffer was changed to 300 mM phosphate. Active fractions were pooled, and ammonium sulfate was added to 0.8 M with the pH adjusted to 7.0. The sample was then loaded onto a phenyl Sepharose column, preequilibrated with 0.8 M ammonium sulfate in water, pH 7.0. The column was eluted with the same medium and, when the A_{280} of eluate reached baseline, with 500 mM ammonium sulfate in 50 mM phosphate buffer. Ammonium sulfate was added to the pooled active fractions to 70% saturation, and the sample was centrifuged after 30 min of standing. The pellet was resuspended in 50 mM phosphate, and dialyzed against several changes of the same buffer over ~24 h. The FRG_F so obtained was >90% in purity.

Table 1: K_m Values and Kinetic Mechanisms of FRG_F and FRP_H in Single-Enzyme Assays and in Coupled Reactions with L_H and L_F

enzyme(s) used in reductase assay	$K_{m,FMN}$ (μM)	$K_{m,NADPH}$ (μM)	$K_{m,NADH}$ (μM)	kinetic mechanism
FRG_F[a]	220		120	ping-pong
FRG_F−L_F	0.05		3.8	sequential
FRG_F−L_H[b]	4		9	ping-pong
FRP_H[b]	8	20		ping-pong
FRP_H−L_F	0.2	1.1		sequential
FRP_H−L_H[b]	0.3	0.02		sequential

[a] Taken from Tu et al. (*40*). [b] Taken from Lei and Tu (*28*).

Apoenzymes and Reconstituted Holoenzymes of Flavin Reductases. FRP_H (*37*) and FRG_F (*30*) each has an FMN cofactor per monomer. The procedures detailed previously (*37*) were followed for the preparation of the FRP_H apoenzyme and the reconstituted FRP_H and $FRP_{S,H}$ holoenzymes containing FMN and 2-thioFMN, respectively, as a cofactor. Similar procedures (*38*) were also followed for the preparation of the FRG_F apoenzyme and for the reconstituted FRG_F and $FRG_{S,F}$ holoenzymes containing FMN and 2-thioFMN, respectively, as a cofactor.

Enzyme Activity Assays. The activity of FRP_H or $FRP_{S,H}$ was determined by a single-enzyme spectrophotometric assay (*28*) in 1 mL of 50 mM phosphate, containing the designated amounts of FMN and NADPH. The time-dependent decreases in A_{340} associated with the oxidation of NADPH were monitored. An alternative luciferase-coupled assay (*28*) was also used. FRP_H or $FRP_{S,H}$, at 1.2 nM, was assayed in the presence of 1.2−1.5 μM L_H or L_F in 1 mL of 50 mM phosphate containing 10 μM decanal and the designated amounts of NADPH and FMN. Reactions were monitored by following the peak and the time course of the bioluminescence intensity. The light intensity was usually measured in arbitrary light units set on the same scale for each set of experimental measurements. For some measurements, the arbitrary light unit was converted to quanta per second (q/s) using a liquid light standard (*39*) for calibration. The FRG_F and $FRG_{S,F}$ activities were determined by both the single-enzyme spectrophotometric and the luciferase-coupled assays identical to that described above except that NADH was used in place of NADPH.

RESULTS

Steady-State Kinetic Properties of Reductases. The kinetic properties of FRG_F−L_F and FRP_H−L_F coupled reactions were determined in this work. Results are summarized in Table 1 along with those obtained earlier with FRG_F and FRP_H individually and in coupled reactions with L_H. The FRG_F−

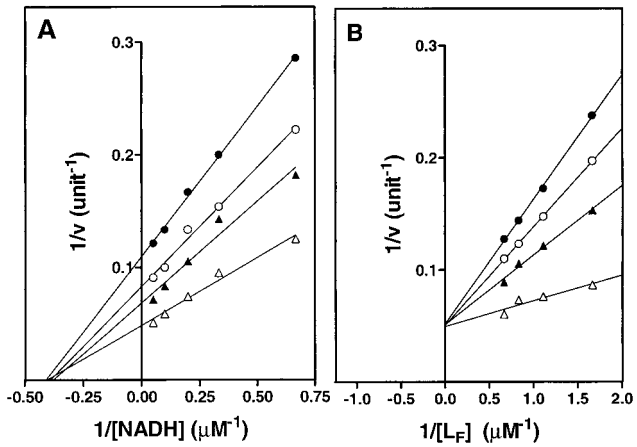


FIGURE 1: Inhibition of the FRG_F-L_F coupled luminescence reaction by FMN. (A) Luminescence intensities (v, in arbitrary units) of the coupled reactions using 1.5 μM L_F and, from the bottom line upward, 2, 4, 6, and 8 μM FMN as an inhibitor are shown as a function of NADH concentration in double reciprocal plots. (B) Luminescence intensities (v, in arbitrary units) of the coupled reactions using 20 μM NADH and, from the bottom line upward, 5, 10, 15, and 20 μM FMN as an inhibitor are shown as a function of L_F concentration in double reciprocal plots.

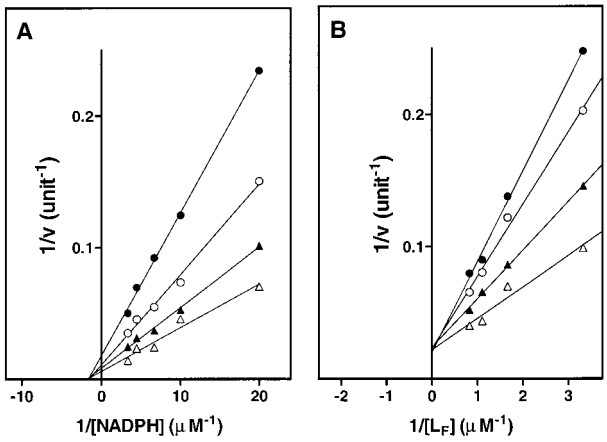


FIGURE 2: Inhibition of the FRP_H-L_F coupled luminescence reaction by FMN. (A) Luminescence intensities (v, in arbitrary units) of the coupled reactions using 1.5 μM L_F and, from the bottom line upward, 5, 10, 15, and 20 μM FMN as an inhibitor are shown as a function of NADPH concentration in double reciprocal plots. (B) Luminescence intensities (v, in arbitrary units) of the coupled reactions using 20 μM NADPH and, from the bottom line upward, 10, 15, 20, and 25 μM FMN as an inhibitor are shown as a function of L_F concentration in double reciprocal plots.

L_F coupled reactions were carried out using four levels of FMN (0.1−0.4 μM) and five levels of NADH (3−40 μM). At the four constant levels of FMN, the double reciprocal plots of the luminescence activity versus NADH concentration showed a series of intersecting lines with a common converging point above the abscissa and to the left of the ordinate. Such a pattern indicates a sequential mechanism contrary to the ping-pong mechanism observed with FRG_F in the single-enzyme assay (*40*). Subsequently, the intercepts on the ordinate and the slopes were plotted against the reciprocals of the FMN concentrations. Each of the two secondary plots showed a linear line, allowing the determination of $K_{m,FMN}$ (0.05 μM) and $K_{m,NADH}$ (3.8 μM). In comparison, much higher values of $K_{m,FMN}$ (220 μM) and $K_{m,NADH}$ (120 μM) were determined by the FRG_F single-enzyme spectroscopic assay (*40*). The FRP_H-L_F coupled reactions were similarly carried out using four levels of FMN (6.25−50 nM) and four levels of NADPH (10−100 nM). The primary double reciprocal plots of luminescence activity versus NADPH concentration at the four constant levels of FMN again showed a series of converging lines, indicating a sequential mechanism contrary to the ping-pong mechanism of FRP_H in the single-enzyme assay (*28*). The same methods described above were used to construct the two secondary plots for the determination of $K_{m,FMN}$ (0.2 μM) and $K_{m,NADPH}$ (1.1 μM). As shown in Table 1, these K_m values were again much lower than the $K_{m,FMN}$ (8 μM) and $K_{m,NADPH}$ (20 μM) of FRP_H in the single-enzyme assay (*28*).

FMN Inhibition of the Coupled Reactions. FMN, at 2−20 μM, was found to inhibit the FRG_F-L_F coupled reaction apparently noncompetitive with NADH (Figure 1A) and competitive with L_F (Figure 1B). Similarly, 5−25 μM FMN also inhibited the FRP_H-L_F coupled reaction apparently noncompetitive with NADPH (Figure 2A) and competitive with L_F (Figure 2B). In contrast, FMN at up to 120 μM was found inactive in inhibiting the FRG_F-L_H coupled reaction.

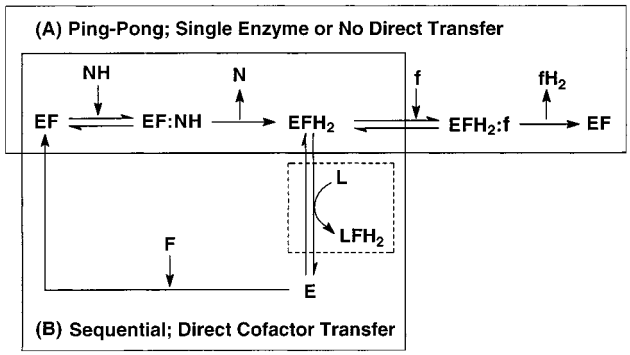
2-ThioFMN as a Mechanistic Probe for the Coupled Reaction. The reconstituted holoenzymes $FRG_{S,F}$ and $FRP_{S,H}$ were both active in the single-enzyme and the luciferase-coupled assays. Coupled reactions were carried out under conditions described for Table 2 using FMN as a common substrate and various combinations of reductase/luciferase. The initial light intensities of the coupled reactions by FRG_F-L_F and $FRG_{S,F}-L_F$ were first measured and normalized to the same rate of NADH oxidation (hence the same rate for $FMNH_2$ product formation) determined spectrophotometrically using duplicate samples. The normalized luminescence intensity of the $FRG_{S,F}-L_F$ couple was close to 2 orders of magnitude lower than that of the FRG_F-L_F couple. In contrast, when FRG_F and $FRG_{S,F}$ were each coupled with L_H, the normalized light intensity of the $FRG_{S,F}-L_H$ couple was similar to that of the FRG_F-L_F couple. FRP_H was also compared with $FRP_{S,H}$ in their L_F coupled reactions. The normalized light intensity of the $FRP_{S,H}-L_F$ coupled reaction was more than 3 orders of magnitude lower than that of the FRP_H-L_F couple. The results of the FRP_H-L_H couple and the $FRP_{S,H}-L_H$ couple reported earlier are also included in Table 2 for comparison.

Table 2: Initial Coupled Luminescence Intensity and NAD(P)H Oxidation Rate Using FMN as a Substrate for Different Reductase−Luciferase Combinations[a]

reductase/luciferase combination	NAD(P)H oxidation[b] (ΔA_{340}/min)	light intensity[c] (q/s)	relative light intensity/NAD(P)H oxidation
FRG_F-L_F	0.10	1.9×10^7	1
$FRG_{S,F}-L_F$	0.06	8.0×10^5	7.2×10^{-2}
FRG_F-L_H	0.10	1.5×10^8	1
$FRG_{S,F}-L_H$	0.06	1.6×10^8	1.8
FRP_H-L_F	0.09	7.4×10^8	1
$FRP_{S,H}-L_F$	0.09	3.6×10^5	4.9×10^{-4}
FRP_H-L_H[d]			1
$FRP_{S,H}-L_H$[d]			6.0×10^{-2}

[a] The coupled reactions utilized 3.5 μM FMN and 225 μM NADH for the FRG-containing samples and 225 μM NADPH for the FRP-containing samples. The reaction was initiated at 23 °C by the addition of the reductase. [b] Measured spectrophotometrically using duplicate samples. [c] Measured at 5 s after the initiation of the reaction. [d] Taken from Lei and Tu (*28*).

Scheme 1



DISCUSSION

The initial phase of this work focused on distinguishing reduced flavin-free diffusion from direct transfer. When switching from the single-enzyme to the coupled assay using excess luciferase, the K_m values of reductase for FMN and NAD(P)H should remain unchanged if luciferase does not form any complex with reductase and receives $FMNH_2$ by free diffusion. On the other hand, the turnover rate of luciferase is markedly slower than those of FRP_H and FRG_F. If luciferase forms a functional complex with reductase and receives $FMNH_2$ through direct transfer, the turnover rate of the complex will be limited by the much slower luciferase. Consequently, 50% maximal activity of the reductase−luciferase couple would be reached at concentrations of FMN and NAD(P)H much lower than the respective K_m values determined in the reductase single-enzyme assays. The K_m values of FRP_H for FMN and NADPH are substantially higher in the single-enzyme assay than those determined by the coupled luminescence assay using excess L_H (*26*, *28*). Markedly higher $K_{m,FMN}$ and $K_{m,NADH}$ have also been found for FRG_F in its single-enzyme assay than those in the FRG_F-L_H coupled assay (*28*). Therefore, both FRP_H-L_H and FRG_F-L_H rely on direct $FMNH_2$ transfer for the coupled luminescence reaction. We now found that the K_m values of FRG_F and FRP_H for FMN and reduced pyridine nucleotide in the L_F coupled assays were all substantially lower than the respective K_m values in the FRG_F and the FRP_H single-enzyme assays (Table 1). Evidently, both FRG_F-L_F and FRP_H-L_F enzyme pairs also directly transferred $FMNH_2$ from the reductase to luciferase in the coupled reaction.

FRP_H and FRG_F each has two flavin sites: a flavin substrate/product site and an FMN cofactor site (*25*, *30*). In addition to the flavin product, the flavin cofactor of both reductases can dissociate from the holoenzymes (*37*, *38*) and, hence, is transferable. Therefore, the reduced flavin for the direct transfer by FRP_H-L_H, FRG_F-L_H, FRG_F-L_F, and FRP_H-L_F could be either the flavin cofactor or the product of the reductases. For the rest of this work, three lines of studies were carried out to distinguish these two possibilities. First, kinetic mechanisms of reductases in single-enzyme and luciferase-coupled reactions were characterized. We previously observed that a ping-pong mechanism for FRP_H in the single-enzyme assay was converted to a sequential mechanism in the L_H coupled reaction (*28*). The ping-pong mechanism for the single-enzyme reaction is depicted as Scheme 1A. In this and the other scheme, E is the reductase apoenzyme, F and FH_2 are, respectively, the oxidized and reduced flavins that bind to or dissociate from the reductase cofactor site, f and fH_2 are, respectively, the oxidized flavin substrate and reduced flavin product, and NH and N are, respectively, the reduced and oxidized pyridine nucleotides. In addition, Scheme 1B was proposed for the FRP_H-L_H coupled reaction (*28*). After the reduction of the FRP_H holoenzyme, the reduced flavin cofactor is directly transferred to luciferase, and the reductase is left in the apoenzyme form. The step of direct cofactor transfer is highlighted by the dashed-line box. Finally, the flavin added exogenously binds to the apoenzyme to regenerate the reductase holoenzyme. When viewed with the reductase apoenzyme as the starting enzyme species in Scheme 1B, the FRP_H-L_H couple should follow a sequential mechanism as was actually observed.

The second line of study was directed at the examination of the inhibitory effects of exogenously added flavin on the reductase−luciferase coupled reaction. As also illustrated in Scheme 1, exogenously added flavin (shown as f) should compete against luciferase in reacting with the reduced flavin cofactor of reductase to generate the fH_2 product. While the direct cofactor transfer to luciferase leads to efficient luminescence (Scheme 1B), the fH_2 product of reductase is oxidized in a dark pathway (Scheme 1A). Following such a scheme, kinetic equations were obtained (*28*) to predict the nature of FMN inhibition and are shown below in slightly modified forms:

$$\frac{1}{v} = \frac{C_1 + C_2[\mathrm{L}] + C_3[\mathrm{F}]}{C_4[\mathrm{L}]V_{max}} + \frac{C_5[\mathrm{L}] + C_6[\mathrm{F}]}{C_4[\mathrm{L}]V_{max}} \frac{1}{[\mathrm{NH}]} \quad (3)$$

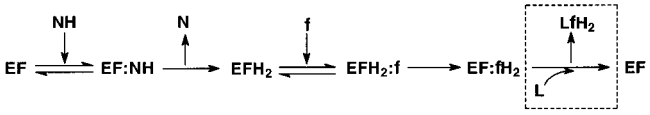
$$\frac{1}{v} = \frac{C_5 + C_2[\mathrm{NH}]}{C_4[\mathrm{NH}]V_{max}} + \frac{C_5[\mathrm{NH}] + \{C_6 + C_3[\mathrm{NH}]\}[\mathrm{F}]}{C_4[\mathrm{NH}]V_{max}} \frac{1}{[\mathrm{L}]} \quad (4)$$

Equation 3 predicts an apparent noncompetitive inhibition by FMN against NADPH at a constant level of luciferase. Equation 4 also predicts an apparent competitive inhibition of FMN against luciferase at a constant level of NADPH. For both equations, C_1 through C_6 are various constant terms (*28*). We found earlier that FMN at high concentrations indeed inhibited the FRP_H-L_H coupled reaction competitively against luciferase and noncompetitively against NADPH (*28*), thus providing strong support to the proposed direct cofactor transfer by FRP_H-L_H.

In this work, the FRG_F-L_F, FRG_F-L_H, and FRP_H-L_F couples were subjected to the same two lines of investigation as described above. Similar to FRP_H, FRG_F by itself also exhibits a ping-pong mechanism (Scheme 1A) (*40*). However, a sequential mechanism (Scheme 1B) was observed for FRG_F or FRP_H when coupled with L_F (Table 1). These results indicate that direct cofactor transfer was operational for the FRG_F-L_F and FRP_H-L_F coupled reactions. Such a mechanism would predict, according to Scheme 1 and eqs 3 and 4, apparent noncompetitive inhibition against NAD(P)H and competitive inhibition against luciferase by high concentrations of FMN in the FRG_F-L_F and FRP_H-L_F coupled reactions. Results shown in Figure 1 for the FRG_F-L_F couple and in Figure 2 for the FRP_H-L_F couple are in full agreement with such predictions.

In contrast to the FRP_H-L_H, FRP_H-L_F, and FRG_F-L_F couples, FRG_F exhibited the same ping-pong mechanism in

Scheme 2: Ping-Pong; Direct Product Transfer



the single-enzyme and the L_H coupled assays (Table 1). However, on the basis of the markedly lower $K_{m,FMN}$ and $K_{m,NADH}$ in the coupled reaction, $FMNH_2$ is still directly transferred from FRG_F to L_H. These observations (*28*) and considerations led us to suggest direct flavin product transfer (highlighted by the dashed-line box) by FRG_F-L_H for the coupled luminescence reaction (Scheme 2). Moreover, according to Scheme 2 and in contrast to direct cofactor transfer, there is no dark side pathway for $FMNH_2$ oxidation. Hence, exogenously added FMN is not expected to inhibit the FRG_F-L_H coupled reaction against NADH or luciferase. Consistent with such a prediction, no inhibition of the FRG_F-L_H coupled reaction was detected at up to 120 μM FMN.

The third line of study for distinguishing direct cofactor transfer from direct product transfer involves the use of 2-thioFMN as a mechanistic probe. Active holoenzyme $FRP_{S,H}$ can be reconstituted from 2-thioFMN and the FRP_H apoenzyme (*37*). For luciferase, reduced 2-thioFMN is an active substrate but with a bioluminescence quantum yield marked lower than that of the $FMNH_2$ substrate (*41, 42*). When FRP_H and $FRP_{S,H}$ were each coupled with L_H using FMN as a common substrate, the initial light intensity normalized to the same rate of NADPH oxidation (and hence the same rate of $FMNH_2$ product formation) was found to be much higher for FRP_H-L_H than for $FRP_{S,H}-L_H$ (*28*). If the flavin product (i.e., $FMNH_2$) of FRP_H and $FRP_{S,H}$ is directly transferred to L_H, then similar intensities of luminescence should be observed for FRP_H-L_H and $FRP_{S,H}-L_H$. Therefore, the much lower luminescence intensity detected with the $FRP_{S,H}-L_H$ couple strongly supports the conclusion for direct cofactor transfer by FRP_H (or $FRP_{S,H}$)$-L_H$ in the coupled luminescence reaction.

Active holoenzyme $FRG_{S,F}$ can also be obtained from the FRG_F apoenzyme and 2-thioFMN (*38*). Similar to the test described above, the luminescence activities of $FRG_{S,F}$ and $FRP_{S,H}$ were compared with FRG_F and FRP_H, respectively, in L_F coupled reactions. As shown in Table 2, much lower normalized initial luminescence intensities were observed in coupled reactions using $FRG_{S,F}$ in comparison with FRG_F and using $FRP_{S,H}$ in comparison with FRP_H. These findings support further the direct cofactor transfer by FRG_F-L_F and FRP_H-L_F in the coupled luminescence reaction.

The luminescence activity of the $FRG_{S,F}-L_H$ couple was also compared with that of the $FRG_{S,F}-L_H$ couple. As shown in Table 2, the normalized initial light intensity of the $FRG_{S,F}-L_H$ coupled reaction was similar to that of the FRG_F-L_H coupled reaction, in sharp contrast to the patterns exhibited by the FRP_H-L_H, FRP_H-L_F, and FRG_F-L_F couples. Therefore, the direct product transfer proposed for the FRG_F-L_H couple is further substantiated.

In addition to free diffusion and direct transfer of flavin, another mechanistic possibility is the direct reduction of luciferase-bound FMN by the reductase-bound reduced flavin through electron transfer rather than flavin exchange. For the FRG_F-L_H couple, this possible mechanism is only a minor variation of the direct product transfer shown in Scheme 2. This new mechanism differs from Scheme 2 in that luciferase first forms a complex with FMN and this complex (Lf) binds to and reacts with the reduced reductase (EFH_2) to produce the LfH_2 for luminescence. The flavin cofactor (F or FH_2) remains bound to the reductase during the reaction, and the fH_2 product utilized by luciferase for luminescence is formed when bound to the FRG_F-L_H couple. Therefore, the new version is still a direct product transfer mechanism. For the FRP_H-L_H, FRG_F-L_F, and FRP_H-L_F couples, the possibility for direct electron exchange rather than flavin exchange is inconsistent with two observations. First, $FRG_{S,F}$ and $FRP_{S,H}$ are both quite active in single-enzyme assays. If electrons are directly transferred to the luciferase−FMN complex from reduced reductase, then the luminescence activities of $FRP_{S,H}-L_H$, $FRG_{S,F}-L_F$, and $FRP_{S,H}-L_F$ in coupled reactions should be similar to the respective activities [using exogenously added FMN as a common substrate and normalized to the same rate of NAD(P)H oxidation] of FRP_H-L_H, FRG_F-L_F, and FRP_H-L_F. In contrast, the observed light activities of the former three enzyme couples were marked lower than those of the latter three enzyme couples. Next, the direct electron exchange mechanism requires that the flavin cofactor of reductase remains bound and unexchanged during the coupled reaction. Under such conditions, the kinetic mechanisms of FRP_H-L_H, FRG_F-L_F, and FRP_H-L_F in coupled reactions should remain the ping-pong type in contrast to the sequential mechanisms as observed. It should be noted, however, our experiments involve measurements of the light intensities near the onset of the coupled reactions. Luciferase was reported to assume a different but catalytically active conformation after a single cycle of catalysis (*43*). It is uncertain at present whether this altered form of luciferase would behave differently with respect to the mechanism of flavin or electron transfer in coupled reactions under turnover conditions.

FRP_H and FRG_F share some significant structural similarities, including the general crystal structure (*29, 32*), protein size and sequence (*25, 44*), and FMN cofactor (*25, 30*). The luciferases from *V. harveyi* and *V. fischeri* are also closely related in sequence and subunit structure (*45*−*47*). Therefore, it is highly interesting that our results indicate direct cofactor transfer by FRP_H-L_H (*28*), FRP_H-L_F, and FRG_F-L_F and a different mechanism of direct product transfer by FRG_F-L_H. If the mechanism of flavin transfer is dictated unilaterally by either the reductase or the luciferase within the FRP_H-L_H, FRP_H-L_F, FRG_F-L_F, and FRG_F-L_H couples, then one type of transfer would be expected for two enzyme couples and the second type of mechanism would be expected for the other two couples. Since three couples exercised one type of transfer and the fourth couple followed a different mechanism, the reduced flavin transfer appears to be controlled by the specific makeup of the constituent enzymes in the reductase−luciferase couple. Our studies used not only the FRP_H-L_H and FRG_F-L_F couples but also the mixed FRP_H-L_F and FRG_F-L_H couples in which the two constituent enzymes are from different organisms. No direct physiological significance is claimed for the latter two mixed enzyme couples. However, the inclusion of all four reductase−luciferase couples in this work is necessary for the identification of the specific roles of individual reductase

and luciferase species in the regulation of the transfer mechanism. Although an increasing number of other reduced flavin donor−acceptor enzyme couples have been identified in recent years, their mechanisms of reduced flavin transfer have, thus far, not been explored. The delicate regulation of the reduced flavin transfer mechanisms of the reductase−luciferase systems studied in this work may serve as an interesting reference for the other reduced flavin donor−acceptor enzyme couples.

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