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# The Transfer of Reduced Flavin Mononucleotide from LuxG Oxidoreductase to Luciferase Occurs via Free Diffusion

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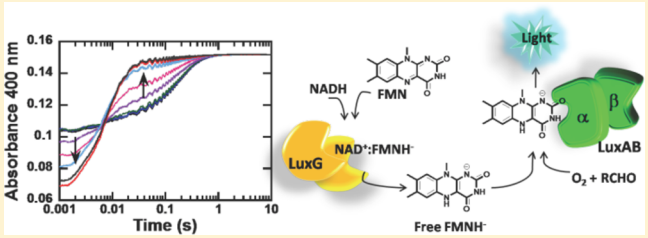
Supporting Information

**ABSTRACT:** Bacterial luciferase (LuxAB) is a two-component flavin mononucleotide (FMN)-dependent monooxygenase that catalyzes the oxidation of reduced FMN ($FMNH^-$) and a long-chain aliphatic aldehyde by molecular oxygen to generate oxidized FMN, the corresponding aliphatic carboxylic acid, and concomitant emission of light. The LuxAB reaction requires a flavin reductase to generate $FMNH^-$ to serve as a luciferin in its reaction. However, $FMNH^-$ is unstable and can react with oxygen to generate $H_2O_2$, so that it is important to transfer it efficiently to LuxAB. Recently, LuxG has been identified as a NADH:FMN oxidoreductase that supplies $FMNH^-$ to luciferase *in vivo*. In this report, the mode of transfer of $FMNH^-$ between LuxG from *Photobacterium leiognathi* TH1 and LuxABs from both *P. leiognathi* TH1 and *Vibrio campbellii* (*Pl*LuxAB and *Vc*LuxAB, respectively) was investigated using single-mixing and double-mixing stopped-flow spectrophotometry. The oxygenase component of *p*-hydroxyphenylacetate hydroxylase (C2) from *Acinetobacter baumannii*, which has no structural similarity to LuxAB, was used to measure the kinetics of release of $FMNH^-$ from LuxG. With all $FMNH^-$ acceptors used ($C_2$, *Pl*LuxAB, and *Vc*LuxAB), the kinetics of FMN reduction on LuxG were the same, showing that LuxG releases $FMNH^-$ with a rate constant of 4.5−6 $s^{-1}$. Our data showed that the kinetics of binding of $FMNH^-$ to *Pl*LuxAB and *Vc*LuxAB and the subsequent reactions with oxygen were the same with either free $FMNH^-$ or $FMNH^-$ generated *in situ* by LuxG. These results strongly suggest that no complexes between LuxG and the various species are necessary to transfer $FMNH^-$ to the acceptors. The kinetics of the overall reactions and the individual rate constants correlate well with a free diffusion model for the transfer of $FMNH^-$ from LuxG to either LuxAB.




Efficient transfer of unstable metabolites between protein components is critical to biological systems, so that side reactions that lead to wasteful and possible deleterious outcomes can be minimized. For example, in aerobes, the transfer of reduced flavin or its reducing equivalents to its partners in multicomponent redox enzyme systems needs to be well-coordinated to prevent the reduced flavin ($FMNH^-$) from generating oxidized flavin and $H_2O_2$ or other reactive oxygen species. Thus, with cytochrome P450 enzymes, it is believed that protein−protein interactions mediate efficient electron transfer from the flavin cytochrome P450 reductase to the heme of the oxygenase.[1] In many two-component flavin-dependent monooxygenases, including bacterial luciferase,[2,3] which is the topic of this work, efficient transfer of $FMNH^-$ from the reductase component to its oxygenase is also fundamentally important.

Bacterial luciferase (LuxAB) is a flavin-dependent monooxygenase that catalyzes the oxidation by molecular oxygen of long-chain aldehydes and $FMNH^-$ to generate corresponding carboxylic acids, oxidized flavin, and visible light.[4−6] The emission of light from the luciferase system is frequently used in diagnostic or detection tools such as gene reporters.[7,8] In the LuxAB system, $FMNH^-$ is supplied *in vivo* by an NAD(P)H-FMN oxidoreductase;[9] thus, according to van Berkel et al.,[10] luciferase is a member of the flavin-dependent monooxygenase class C group. Because $FMNH^-$ is an unstable species, the formation of $FMNH^-$ and the transfer between the reductase and the oxygenase in luciferase *in vivo* must be well-coordinated to prevent its wasteful oxidation. This topic is being investigated by many research groups, but the findings are not unanimous.[11,12]

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The transfer of $FMNH^-$ between reductases and their oxygenases can be achieved via protein−protein interactions or by free diffusion. The mechanism of transferring $FMNH^-$ from the oxidoreductase to the LuxAB systems from *Vibrio harveyi* and *Vibrio fischeri* has been proposed to occur through protein−protein interactions.[12] Tu and co-workers have shown that the emission of light from LuxAB can be transferred via bioluminescent resonance energy transfer (BRET) to a yellow fluorescence protein (YFP) attached to the flavin reductase (FRP), suggesting that protein−protein interactions between FRP and LuxAB exist.[13] Additionally, fluorescence anisotropy measurements of eosin-labeled FRP mixed with *V. harveyi* LuxAB imply the formation of a complex in these systems.[14] In addition to the LuxAB, on the basis of affinity chromatography, chemical cross-linking,[15] and steady-state kinetics analysis,[16] the two-component alkanesulfonate monooxygenase from *Escherichia coli* has been proposed to use a protein-mediated $FMNH^-$-transfer mechanism. These findings are elaborated further in the Discussion.

On the contrary, pull-down experiments with flavin reductase, FRE (from *E. coli*), FRP (from *V. harveyi*), and LuxAB (from *V. harveyi*), gave no evidence of stable complex formation between LuxAB and reductase.[14,17] Campbell et al. proposed that *in vivo*, free diffusion is the mechanism for the transfer of $FMNH^-$ to *V. harveyi* LuxAB.[17] Their proposal is consistent with the fact that *in vitro*, LuxAB can accept $FMNH^-$ generated by flavin oxidoreductases that are not from native luminous bacteria[3,6,17] and *in vivo*, when the luciferase gene is expressed in nonluminous organisms it can emit the bioluminescence by using $FMNH^-$ generated by host reductases.[18−20] Apart from the LuxAB systems, kinetic experiments with other two-component flavin-dependent monooxygenases such as the *Acinetobacter baumannii p*-hydroxyphenylacetate hydroxylase (HPAH), which has a $C_1$ reductase and a $C_2$ oxygenase,[21−23] and the ActVA (oxygenase)−ActVB (reductase) system from *Streptomyces coelicolor*[24] clearly show that no complex is required for efficient transfer of $FMNH^-$ from the reductases to the oxygenases; the oxygenase components are able to bind free $FMNH^-$ very rapidly before it can react with oxygen. Consistent with this conclusion, gel filtration experiments with HPAH could not demonstrate the presence of any protein−protein complexes.[22,23] It was also found that the rate constant for the transfer of $FMNH^-$ from $C_1$ to $C_2$ is nearly identical to that for reduction of cytochrome *c* by $C_1$−$FMNH^-$, because both reaction rates are limited by the rate of dissociation of $FMNH^-$ from $C_1$, which is controlled by a conformational change in the autoinhibitory domain of $C_1$.[22,23] Moreover, the presence of $C_2$ did not alter the rate of cytochrome *c* reduction by $C_1$−$FMNH^-$, again implying that no complex of $C_1$ and $C_2$ was formed during the transfer of $FMNH^-$.[22] Similar findings were also made in analogous experiments with the ActVA−ActVB system when $C_2$ was used as an $FMNH^-$ acceptor instead of the ActVA oxygenase.[24] Interestingly, a combination of diffusion and protein−protein complex formation mechanisms was found to apply to styrene monooxygenase[25] and to HPAH from *Pseudomonas aeruginosa*,[26] making it clear that transfer of reduced flavin can occur by a variety of mechanisms.

Recently, in *Photobacterium leiognathi* TH1, LuxG, the flavin reductase that is encoded in the same operon as its LuxAB (*Pl*LuxAB), has been shown to be the major flavin reductase supplying $FMNH^-$ for its LuxAB.[9] The availability of LuxG offers the possibility of investigating the functionally relevant *in vivo* mode of transfer of $FMNH^-$ between LuxAB and the reductase. Currently, there is no published evidence to distinguish whether the transfer of $FMNH^-$ from LuxG to *Pl*LuxAB occurs by protein−protein interactions or by simple diffusion. Gel filtration experiments did not detect any stable complexes between LuxG and *Pl*LuxAB.[9] LuxG is a homodimeric enzyme. The anaerobic reaction of LuxG with NADH involves half-sites reactivity, with the first flavin being reduced at a rate of 68 $s^{-1}$ and the second at a rate of 2.8 $s^{-1}$.[27] Here, we investigated the mode of transfer of $FMNH^-$ from LuxG to LuxAB by using LuxG to generate $FMNH^-$ as a substrate for the reactions with $O_2$ of both LuxABs (*Pl*LuxAB and *Vc*LuxAB) and $C_2$. The results show that free diffusion of $FMNH^-$ to either $C_2$ or the LuxAB enzymes is sufficient for efficient overall catalysis.

## MATERIALS AND METHODS

**Reagents.** NADH and flavin adenine dinucleotide (FAD) were purchased from Sigma-Aldrich. FMN was obtained by conversion of FAD to FMN using snake venom from *Crotalus adamanteus* using the method described by Sucharitakul et al.[21] In brief, a mixture containing FAD and snake venom was incubated overnight and then loaded onto a C18 Sep-Pak cartridge (Waters), which was pre-equilibrated with 20 mM potassium phosphate (pH 7.0). The cartridge was washed with 10 mM potassium phosphate (pH 7.0) before FMN was eluted with water. The solution of purified FMN was then freeze-dried and stored at −20 °C until it was used. All operations were conducted in the dark.

Concentrations of the following compounds were determined using the known absorption coefficients at pH 7.0: $\varepsilon_{340}$ = 6220 $M^{-1}$ $cm^{-1}$ for NADH; $\varepsilon_{450}$ = 12200 $M^{-1}$ $cm^{-1}$ for FMN; and $\varepsilon_{277}$ = 1550 $M^{-1}$ $cm^{-1}$ for *p*-hydroxyphenyl acetate (HPA). $C_2$ used in this study was expressed and prepared as described by Thotsaporn et al.[28] The concentration of $C_2$ was estimated from the absorption coefficient value ($\varepsilon_{280}$) of 56700 $M^{-1}$ $cm^{-1}$ in 30 mM MOPS buffer (pH 7.0). LuxG and LuxAB from *P. leiognathi* TH1 were prepared according to the method described by Nijvipakul et al.[9] The LuxG and *Pl*LuxAB concentrations were calculated using absorption coefficients ($\varepsilon_{280}$) of 41700 $M^{-1}$ $cm^{-1}$ for LuxG and 74400 $M^{-1}$ $cm^{-1}$ for *Pl*LuxAB in 50 mM Tris-HCl buffer and 10% (v/v) glycerol (pH 8.0). LuxAB from *V. campbellii* was prepared as previously described by Suadee et al.,[6] and the concentration was estimated according to the absorption coefficient ($\varepsilon_{280}$) of 79600 $mM^{-1}$ $cm^{-1}$ in 30 mM MOPS buffer (pH 7.0).

**Rapid Reaction Experiments.** Reactions were studied using stopped-flow spectrophotometers (TgK Scientific) in both single-mixing and double-mixing modes. The flow system was made anaerobic when it was rinsed with anaerobic buffer and incubated overnight with a solution of 400 $\mu$M protocatechuic acid (PCA) and 0.1 unit/mL protocatechuate dioxygenase (PCD) in potassium phosphate (pH 7.0).[29] Prior to the experiment, the instrument was rinsed thoroughly with 50 mM Tris-HCl (pH 8.0) containing 10% glycerol, and this solution was made anaerobic by being equilibrated with $N_2$ gas that had been passed through an oxygen removal column (Labclear).[27] Unless otherwise specified, all enzyme and reactant solutions were in 50 mM Tris-HCl (pH 8.0) containing 10% glycerol, and stopped-flow experiments were performed at 4 °C. This buffer system was the best that we could find for stabilizing LuxG in its active form.


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The binding of $FMNH^-$ to *Pl*LuxAB and *Vc*LuxAB was studied by comparing reactions of $O_2$ with $FMNH^-$ that had been premixed with LuxAB with those that had not been premixed. For the premixed experiments, 32 $\mu$M FMN in the presence of LuxAB (224 $\mu$M for *Pl*LuxAB or 320 $\mu$M for *Vc*LuxAB) was first reduced stoichiometrically with aliquots of ~10 mM sodium dithionite [in 100 mM potassium phosphate (pH 8.0)]. Reduction of FMN was monitored by a UV−visible spectrophotometer inside a glovebox (Belle Technology). The resulting $FMNH^-$ was transferred into a glass tonometer before being removed from the glovebox.[30] The anaerobic solution containing LuxAB and $FMNH^-$ in the first syringe was mixed with an oxygen-containing buffer from the other syringe of the stopped-flow instrument, and the absorbance changes were monitored at 380 and 450 nm. For the non-premixed experiments, an anaerobic solution of 32 $\mu$M $FMNH^-$ prepared as described above, contained in a glass tonometer, was loaded into the first syringe and mixed with an aerobic solution containing LuxAB (224 $\mu$M *Pl*LuxAB or 320 $\mu$M *Vc*LuxAB) in the second syringe of the stopped-flow instrument, and the reactions were monitored at 380 and 450 nm. All concentrations are given as before mixing.

Double-mixing stopped-flow experiments were conducted by mixing LuxG (80 $\mu$M) and NADH (800 $\mu$M) with 64 $\mu$M FMN in the first mix to generate the $FMNH^-$ [all solutions were in 50 mM Tris-HCl (pH 8.0) containing 10% glycerol and 1 mM DTT]. After various aging times (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 s), this solution was then mixed with aerobic solutions containing either 80 $\mu$M $O_2$ to form the $C_2$−C4a-hydroperoxyflavin species or 224 $\mu$M *Pl*LuxAB or 320 $\mu$M *Vc*LuxAB to generate the respective LuxAB−C4a-(hydro)-peroxyflavin species. To minimize interference from the absorbance of NADH, the kinetic traces were monitored at 400 nm to measure the C4a-(hydro)peroxyflavin species instead of at 380 nm. All concentrations for the double-mixing experiments are reported as concentrations before mixing in the stopped-flow instrument.

**Data Analysis.** Analyses of observed traces from double-mixing stopped-flow experiments were conducted by fitting data to exponential equations using Kinetic Studio (Hi-Tech Scientific, Salisbury, U.K.) or Program A (developed by C. J. Chiu, R. Chung, J. Dinverno, and D. P. Ballou at the University of Michigan). The apparent rate constants ($k_{obs}$) for the reduction of LuxG-bound FMN in the presence of LuxAB or $O_2$ were determined from plots of the absorbance changes observed at 400 nm versus the age times selected before the second mix (0.05−2 s) using Marquardt−Levenberg nonlinear fitting algorithms included in KaleidaGraph (Synergy Software).

## RESULTS

**Effect of *Pl*LuxAB on LuxG Reduction.** If protein−protein interactions are involved in the transfer of $FMNH^-$ from LuxG to *Pl*LuxAB, it might be expected that the presence of *Pl*LuxAB would affect the reduction of FMN by LuxG. To test this notion, the reaction of NADH and FMN as catalyzed by LuxG was monitored in the absence and presence of *Pl*LuxAB by stopped-flow spectrophotometry at 450 nm for FMN reduction and at 600 nm for the formation of pyridine nucleotide−flavin charge-transfer intermediates (Figure 1). Reduction of FMN in the presence of *Pl*LuxAB (Figure 1A, top trace) showed biphasic kinetics, similar to the kinetics of LuxG reduction in the absence of *Pl*LuxAB (bottom trace). The

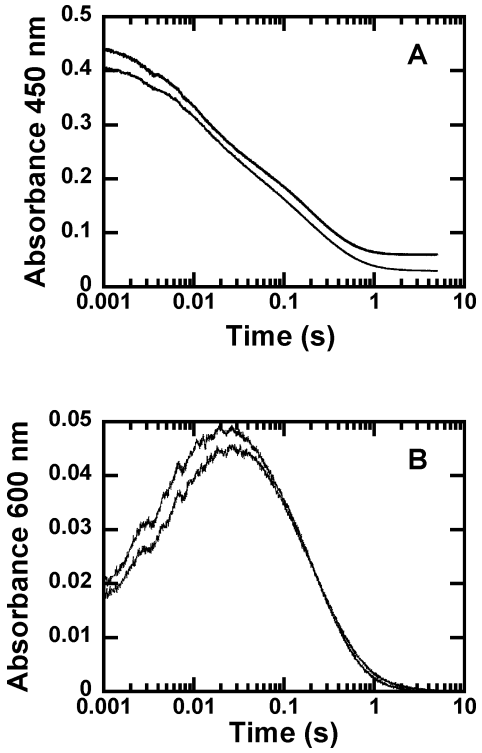


**Figure 1.** Kinetic traces for the reduction of FMN bound to LuxG. An anaerobic solution of 100 $\mu$M LuxG and 600 $\mu$M NADH in the absence (bottom trace) or presence (top trace) of 224 $\mu$M *Pl*LuxAB was mixed with 80 $\mu$M FMN in the stopped-flow spectrophotometer. The absorbance during the reactions was monitored at 450 (A) and 600 nm (B). All concentrations are given as before mixing. The data were analyzed as two phases with observed rate constants of 78 ± 4 and 5.0 ± 0.6 $s^{-1}$ for the first and second phases, respectively.

first phase accounted for 50% of the absorbance decrease at 450 nm with a rate constant of 78 ± 4 $s^{-1}$, while the second phase (also 50% of the total absorbance decrease at 450 nm) proceeded at a rate of 5.0 ± 0.6 $s^{-1}$. The presence of *Pl*LuxAB also did not alter the kinetics of charge-transfer complex formation and decay in the LuxG reaction; the reduction of LuxG in the presence of *Pl*LuxAB as monitored at 600 nm showed two phases (Figure 1B), similar to the reaction of LuxG alone (bottom trace). The first phase showed an absorbance increase due to formation of the $FMNH^-$−$NAD^+$ charge-transfer complex,[27] and this was concurrent with the reduction of FMN as observed at 450 nm. The second phase, the loss of the charge-transfer spectrum, correlated with the slower phase of FMN reduction. Thus, in the presence of *Pl*LuxAB, the LuxG-bound FMN is reduced according to a half-sites reactivity model as previously described for the reaction in its absence;[27] in this model, flavin reduction occurs on one subunit at a time, and the reduction of the second subunit is limited by the release of reduced $FMNH^-$ (and, probably, the $NAD^+$) from the first subunit of LuxG.

**Reaction of $O_2$ with *Pl*LuxAB either Premixed or Not Premixed with $FMNH^-$.** During the luciferase reaction, *Pl*LuxAB binds $FMNH^-$ before reacting with $O_2$ to form the C4a-peroxyflavin intermediate (*Pl*LuxAB−$FMNHOO^-$), a process that can be monitored by absorbance at 380 nm (Scheme 1). When an anaerobic solution containing $FMNH^-$ and *Pl*LuxAB (*Pl*LuxAB−$FMNH^-$) is mixed with aerobic buffer in the stopped-flow spectrophotometer (premixed experiment),


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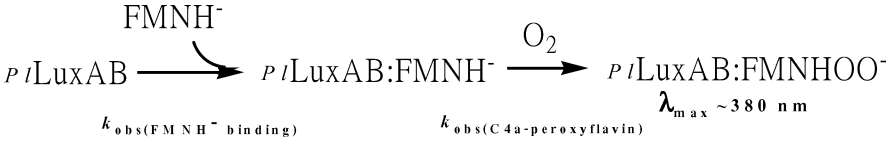
**Scheme 1. Free FMNH⁻ Binds to *Pl*LuxAB before Reacting with Oxygen To Form the C4a-Peroxyflavin**




an increase in absorbance at 380 nm occurs that is due to formation of the C4a-peroxyflavin. If an anaerobic solution containing $FMNH^-$ is mixed with an aerobic solution of *Pl*LuxAB (non-premixed experiment), the observed reaction as monitored at 380 nm includes both the binding of the free $FMNH^-$ and the subsequent reaction with oxygen to form C4a-peroxyflavin. Using data from these two experiments and eq 1 allowed estimation of the apparent rate constant of binding of $FMNH^-$ to *Pl*LuxAB. The $k_{obs(overall)}$ was obtained from the non-premixed experiment, while $k_{obs(C4a\text{-}peroxyflavin\ formation)}$ was from the premixed experiment (see Scheme 1).

$$1/k_{obs(overall)} = 1/k_{obs(FMNH^- binding)} + 1/k_{obs(C4a\text{-}peroxyflavin\ formation)} \quad (1)$$

In the non-premixed experiment, in which an anaerobic solution of 32 $\mu$M $FMNH^-$ was mixed with a solution of 224 $\mu$M *Pl*LuxAB containing 0.26 mM $O_2$, three phases were observed (concentrations before mixing). The first phase (0.01−0.6 s) showed an increase in absorbance at 380 nm with a rate of 1.9 ± 0.1 $s^{-1}$ (solid line, Figure 2A) without significant formation of FMN, as monitored at 450 nm (dashed line, Figure 2A), indicating that this phase is due to the formation of the C4a-peroxyflavin. The second phase (0.6−3 s) was accompanied by an increase in absorbance at 450 nm (accounting for ~30% of the total change) (dashed line, Figure 2A). The kinetics of this phase correlated with the kinetics of the reaction of $O_2$ with free $FMNH^-$ to produce FMN (trace with empty circles). Therefore, the second phase is likely due to a small fraction of free $FMNH^-$ that was not bound at the concentration of LuxAB used (*Pl*LuxAB:$FMNH^-$ ratio of 7:1) reacting with $O_2$. When the same experiment was conducted with higher *Pl*LuxAB:$FMNH^-$ ratios (10:1 and 20:1), the amplitudes for this phase at 450 nm were smaller while the amplitudes of the previous phase at 380 nm were larger (Supporting Information). The third phase showed a slow small increase in the absorbance at 380 nm that was synchronized with a larger increase in absorbance at 450 nm. This phase can be attributed to elimination of $H_2O_2$ from the C4a-peroxyflavin that had been formed during the first phase to form FMN. There is very little extinction change at 380 nm in this conversion.

For the premixed reaction in which an anaerobic solution containing 32 $\mu$M $FMNH^-$ and 224 $\mu$M *Pl*LuxAB (*Pl*LuxAB−$FMNH^-$) was mixed with buffer containing 0.26 mM $O_2$, the reaction resulted in three phases but the kinetics and amplitudes of absorbance changes were different from those in the non-premixed experiment. We also noted a change in the starting absorbance for the reaction (at 0.002 s), suggesting that part of the reaction occurred during the mixing dead time of the stopped-flow instrument (Figure 2B). The first phase (0.002−0.2 s) is an absorbance increase at 380 nm with an observed rate constant of 17 ± 1 $s^{-1}$. This phase was likely due to formation of the C4a-peroxyflavin because there was no obvious change in absorbance at 450 nm (dashed line, Figure 2B). The second phase showed simultaneous increases in absorbance at 380 and 450 nm with kinetics similar to those for the reaction of $O_2$ with unbound $FMNH^-$ (trace with empty circles, Figure 2A). It accounted for ~35% of the total change at 450 nm. We conclude that at this concentration of *Pl*LuxAB, 65% of the $FMNH^-$ was bound. The third phase was due to the elimination of $H_2O_2$ from the C4a-peroxyflavin that had been formed during the dead time and first phase. This phase accounted for ~65% of the total amplitude change at 450 nm. Thus, in the premixed experiment, the kinetics of C4a-peroxyflavin formation in the *Pl*LuxAB reaction are complicated and likely involve two steps, one occurring during the dead time and another one occurring at a rate of 17 ± 1 $s^{-1}$.

These results suggest that the *Pl*LuxAB−$FMNH^-$ complex reacts with oxygen to form the C4a-peroxyflavin with a rate constant of ≥17 ± 1 $s^{-1}$. Using eq 1 and the value determined for the formation of the C4a-peroxyflavin in the non-premixed experiment (1.9 ± 0.1 $s^{-1}$), the observed rate constant for

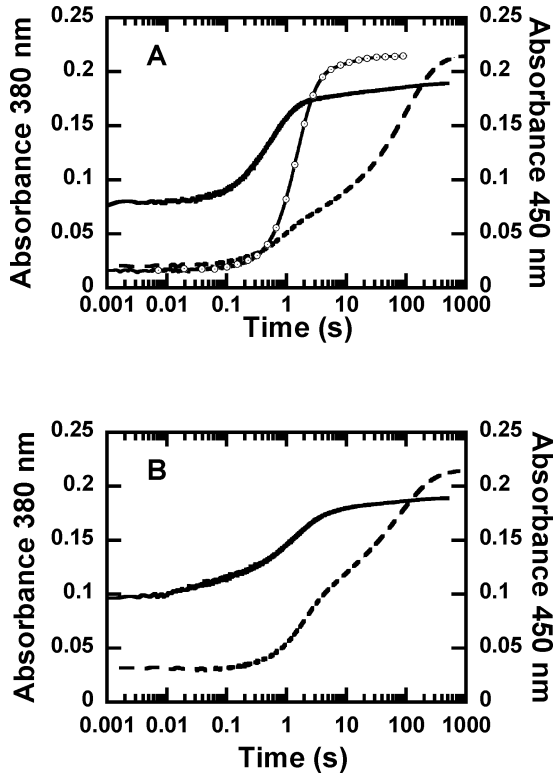



**Figure 2.** Reactions of $O_2$ with *Pl*LuxAB either premixed or non-premixed with $FMNH^-$. (A) For the non-premixed reaction, an anaerobic solution of 32 $\mu$M $FMNH^-$ was mixed with a solution containing 224 $\mu$M *Pl*LuxAB and 0.26 mM $O_2$. (B) For the premixed reaction, an anaerobic solution containing 224 $\mu$M *Pl*LuxAB and 32 $\mu$M $FMNH^-$ was mixed with a solution containing 0.26 mM $O_2$. Solid and dashed lines are kinetic traces monitored at 380 and 450 nm, respectively. The trace with empty circles represents the control reaction of mixing 32 $\mu$M $FMNH^-$ with 0.26 mM $O_2$ as monitored at 450 nm. All concentrations are given as before mixing.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binding of $FMNH^-$ to *Pl*LuxAB under these conditions is estimated to be ~1.9−2.1 $s^{-1}$, which is equivalent to a second-order rate constant of ~1.8 × $10^4$ $M^{-1}$ $s^{-1}$.

**Mechanism of Transfer of $FMNH^-$ from LuxG to LuxAB.** We investigated whether transfer of $FMNH^-$ between LuxG and LuxAB involves protein−protein interactions or occurs via free diffusion. We used double-mixing stopped-flow techniques to first reduce LuxG-bound flavin by NADH and then, at various intervals, mixed this solution with the oxygenase component of HPAH from *A. baumanii* ($C_2$) to rapidly trap the $FMNH^-$ as it is released from LuxG. $C_2$ binds free $FMNH^-$ very rapidly (≥$10^7$ $M^{-1}$ $s^{-1}$), and the resulting $C_2$−$FMNH^-$ complex reacts with oxygen with a rate constant ~1.1 × $10^6$ $M^{-1}$ $s^{-1}$ to form a relatively stable C4a-hydroperoxyflavin.[31] Thus, in the presence of 0.13 mM oxygen used in these experiments, any $FMNH^-$ released from LuxG would form the $C_2$−C4a-hydroperoxyflavin species with an effective rate constant of 143 $s^{-1}$,[31] which is considerably faster than the observed rate of formation of the C4a-peroxyflavin of the *Pl*LuxAB−$FMNH^-$ complex [≥17 ± 1 $s^{-1}$ (Figure 2B)]. The structure of $C_2$[32] is not related to that of LuxAB,[33] so that it is unlikely to have efficient interactions with LuxG. We therefore presume that $FMNH^-$ from LuxG is transferred to $C_2$ by simple diffusion. It was also confirmed that $C_2$ has no effect on the reduction of LuxG-bound FMN by NADH (data not shown).

The LuxG−NADH complex was mixed anaerobically with FMN in the first mix, and after various aging times (0.05−5 s), this solution was mixed with a solution containing $C_2$ and $O_2$. The reactions were monitored at 400 nm (instead of the maximal absorbance of the C4a intermediate at 380 nm) to minimize interference from the absorbance of NADH (Figure 3A). The absorbance observed immediately after the second mix decreased with an increasing aging time because NADH progressively reduced the LuxG-bound FMN during the aging period. $FMNH^-$ released from LuxG during the aging period rapidly bound to $C_2$ and reacted with oxygen to form the C4a-hydroperoxyflavin, as indicated by the increase in absorbance at 400 nm during the first 0.03 s after the second mix. The absorbance observed right after mixing can be correlated with the amount of $FMNH^-$ present, while the amplitude of the rapid increase in absorbance at 400 nm (in the first 30 ms) corresponds to the amount of $FMNH^-$ that had been released from LuxG at the time of the second mix and therefore was available to react with $C_2$ and form the C4a-hydroperoxyflavin. A plot of the absorbance amplitude changes at 400 nm (in the first 0.03 s after the second mix) versus the various aging times used yielded an apparent rate constant of 4.6 ± 0.6 $s^{-1}$ (inset in Figure 3A). This apparent rate constant results from a combination of the reduction of FMN, the release of $FMNH^-$ from LuxG, and the binding of $FMNH^-$ to $C_2$.

When the reaction was monitored at 450 nm to directly follow the flavin reduction (Figure 3B), a rate constant of ~6.7 ± 0.1 $s^{-1}$ was observed for all traces acquired after each delay time. This value is similar to that for reduction of the second subunit flavin in LuxG (5.0 $s^{-1}$) as shown in Figure 1A. The slight discrepancy between these two values could be due to residual effects of the first phase of reduction influencing the evaluation of the second phase of reduction in the double-mixing experiment. Given the similarity of the values for reduction of the second flavin and release of $FMNH^-$ from LuxG, we conclude that the release of $FMNH^-$ is largely controlled by reduction of the second FMN. After complete

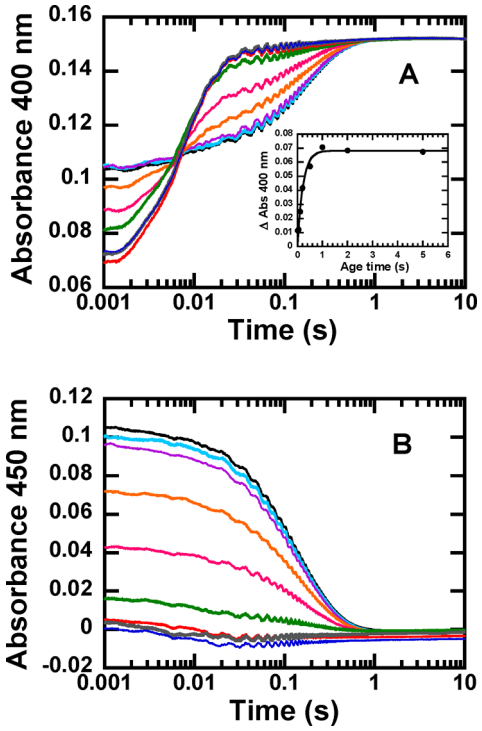


**Figure 3.** Double-mixing experiment with LuxG and $C_2$. A solution of 80 μM LuxG and 800 μM NADH was first mixed with 64 μM FMN under anaerobic conditions. The aging time was varied [0.01 (black), 0.02 (cyan), 0.05 (purple), 0.1 (orange), 0.2 (pink), 0.5 (green), 1 (red), 2 (gray), and 5 s (blue)] before the sample was mixed with a solution of 80 μM $C_2$ and 0.26 mM $O_2$ in the second mix. The reactions were monitored at (A) 400 and (B) 450 nm. The inset of panel A is a plot of the amplitudes of changes in absorbance at 400 nm occurring in the first 0.03 s vs the aging times preceding the second mix. The apparent rate constant from the plot was calculated to be 4.6 ± 0.6 $s^{-1}$.

reduction on LuxG, it appears that all $FMNH^-$ dissociates from LuxG because (i) no spectral charge transfer due to $FMNH^-$:$NAD^+$ bound to LuxG could be detected and (ii) the amount of C4a-hydroperoxyflavin formed on $C_2$ is comparable to the amount of total FMN in the reaction.

This conclusion was further substantiated by analysis of the traces in Figure 3A for the formation of the C4a-hydroperoxyflavin. At shorter aging times (0.01−0.2 s), the formation of C4a-hydroperoxyflavin occurs with an observed rate constant of 5.3 ± 0.3 $s^{-1}$. At longer aging times (>1 s), there is a single phase with an observed rate constant of 134 ± 8 $s^{-1}$, which is comparable to the rate constant (143 $s^{-1}$) of C4a-hydroperoxyflavin formation previously reported using this concentration of $O_2$.[31] At intermediate aging times, two phases can be observed, one of 134 $s^{-1}$ and the second of 5.3 $s^{-1}$. When the aging time was short, the whole reaction was limited by the rate of release of free $FMNH^-$ from LuxG [correlating with the reduction of the second subunit of LuxG (~5.0 $s^{-1}$)] (Scheme 2). With longer aging times, all of the $FMNH^-$ is released from LuxG so that it can be bound by $C_2$. Thus, the process of dissociation of $FMNH^-$ from LuxG no longer limits the overall rate of C4a-peroxyflavin formation. The observed rate constant for the entire process at short aging times is due to the combined reactions of reduction of FMN, release of $FMNH^-$ from LuxG, binding of $FMNH^-$ to $C_2$, and the reaction of $C_2$:$FMNH^-$ and $O_2$ and can be analyzed with eq 2. Because the


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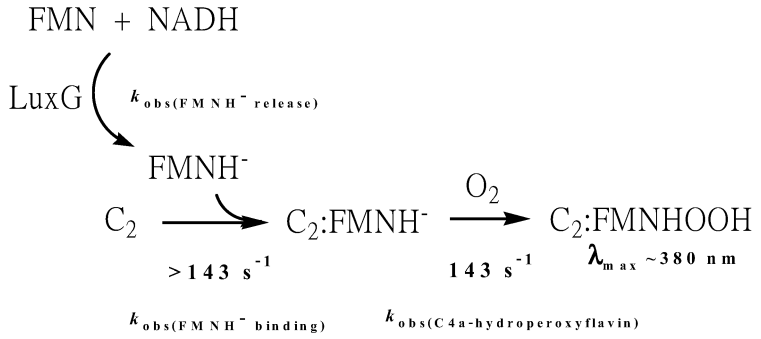
**Scheme 2. Release of FMNH$^-$ from LuxG and Its Transfer to $C_2$ via a Free Diffusion Model**




observed rate constant of the overall process is 5.3 s$^{-1}$ (from Figure 3A at short aging times), the rate of binding of free FMNH$^-$ to $C_2$ is >143 s$^{-1}$, and the rate of C4a-peroxyflavin formation is 143 s$^{-1}$, the overall process must be limited by release of FMNH$^-$ from LuxG (~5 s$^{-1}$).

$$1/k_{obs(overall)} = 1/k_{obs(FMNH^- release)} + 1/k_{obs(FMNH^- binding)} + 1/k_{obs(C4a\text{-}hydroperoxyflavin)} \quad (2)$$

When the same type of experiment was conducted with *Pl*LuxAB in place of $C_2$ (Figure 4), as observed in Figure 3A, the dead-time absorbance at 400 nm decreased as the aging time was increased because of the progress of FMN reduction on LuxG, and the absorbance change at 400 nm during the first phase of the reaction due to formation of the *Pl*LuxAB−C4a-peroxyflavin complex (0.01−4 s) increased with longer aging times. A plot of the amplitude of the absorbance change at 400 nm versus the aging time yielded an observed rate constant of 8.5 ± 1 s$^{-1}$ (inset of Figure 4), which is essentially the same as that seen in the analogous $C_2$ experiment (inset of Figure 3A). Again, the process represented in this plot reflects the release of FMNH$^-$ from LuxG. We also noted that an initial lag phase of absorbance at 400 nm prior to formation of C4a-peroxyflavin was observed for LuxAB but not in the $C_2$ system. The lag phase is likely caused by slow binding of FMNH$^-$ to *Pl*LuxAB.

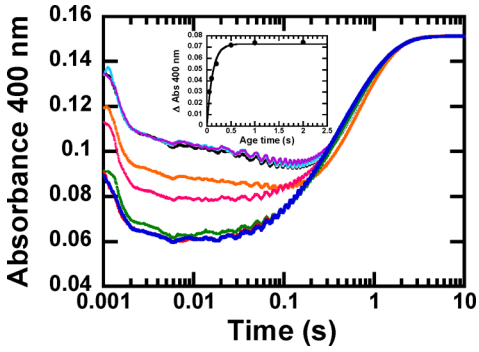



**Figure 4.** Double-mixing experiment with LuxG and *Pl*LuxAB. A solution of 80 μM LuxG and 800 μM NADH was first mixed with 64 μM FMN under anaerobic conditions. The aging time was varied [0.01 (black), 0.02 (cyan), 0.05 (purple), 0.1 (orange), 0.2 (pink), 0.5 (green), 1 (red) and 2 s (gray)] before a solution of 224 μM *Pl*LuxAB and 0.26 mM $O_2$ was introduced into the second mix. All concentrations given are before mixing. The inset is a plot of amplitude changes from 0.01 to 4 s in the absorbance at 400 nm vs aging time. The apparent rate constant of the plot was calculated to be 8.5 ± 1 s$^{-1}$.

For $C_2$, the enzyme binds FMNH$^-$ very rapidly; thus, a lag was not visible.

The rate constant of formation of the *Pl*LuxAB−C4a-peroxyflavin complex (for all aging times) in Figure 4 was analyzed to be 1.6 ± 0.2 s$^{-1}$, which is the same (within error) as the rate constant of C4a-peroxyflavin formation when free FMNH$^-$ was mixed with an aerobic buffer containing *Pl*LuxAB [the non-premixed setup (Figure 2A)]. Clearly, the use of LuxG to generate FMNH$^-$ *in situ* for reaction with *Pl*LuxAB does not increase the rate of formation of the *Pl*LuxAB−C4a-peroxyflavin complex. With the reaction in Figure 4 being limited by the rate of binding of FMNH$^-$ to *Pl*LuxAB [1.9−2.1 s$^{-1}$ (Figure 2)], these results are consistent with the transfer of FMNH$^-$ from LuxG to *Pl*LuxAB occurring via a free diffusion model. According to this model for LuxG-*Pl*LuxAB, using eq 2 and the rate constants of all steps involved [release of FMNH$^-$ by LuxG (5.0 s$^{-1}$), binding of FMNH$^-$ to *Pl*LuxAB (2.1 s$^{-1}$), and formation of C4a-peroxyflavin (>17.3 s$^{-1}$)], the overall $k_{obs}$ of formation of the *Pl*LuxAB−C4a-peroxyflavin complex in this experiment can be calculated to be 1.4 s$^{-1}$, compared to the observed rate constant of 1.6 ± 0.2 s$^{-1}$ in Figure 4.

We also performed an experiment similar to that in Figure 4 but using LuxAB from *V. campbellii* (*Vc*LuxAB), a LuxAB with a sequence that is more than 55% identical to that of *Pl*LuxAB.[6] Premixed and non-premixed experiments like those illustrated in Figure 2 were used to determine the rate constants for binding of FMNH$^-$ to *Vc*LuxAB and for the formation of the C4a-peroxyflavin of *Vc*LuxAB from prebound FMNH$^-$. At 0.13 mM $O_2$ (concentration after mixing), the preincubated *Vc*LuxAB and FMNH$^-$ reacted with $O_2$ to form the C4a-peroxyflavin at a rate of 219 ± 14 s$^{-1}$ (Figure 5), while in the non-premixed experiment, the formation of C4a-peroxyflavin was much slower (6.0 ± 0.2 s$^{-1}$). Under these conditions, the observed rate constant for the binding of free FMNH$^-$ to *Vc*LuxAB was calculated using eq 1 to be ~6.2 s$^{-1}$ (equivalent to a second-order rate constant of ~3.9 × 10$^4$ M$^{-1}$ s$^{-1}$), which is somewhat faster than its binding to *Pl*LuxAB (1.9−2.1 s$^{-1}$) but is comparable to the rate of release of FMNH$^-$ from LuxG (5.0 s$^{-1}$).

When the double-mixing reaction was conducted with the LuxG−*Vc*LuxAB complex (Figure 6) and analyzed according to the methods used for *Pl*LuxAB, the overall results were similar to those depicted in Figure 4. A plot of the amplitude change in absorbance at 400 nm (0.02−2 s) after the second mix versus aging time is comparable to the inset plots of Figures 3A and 4 and yielded a rate constant of 4.5 ± 0.4 s$^{-1}$ for the release of FMNH$^-$ from LuxG (inset of Figure 6). The observed rate constant for formation of C4a-peroxyflavin was calculated to be 3.1 ± 0.3 s$^{-1}$ at shorter aging times (0.01−0.2 s) but was 5.1 ±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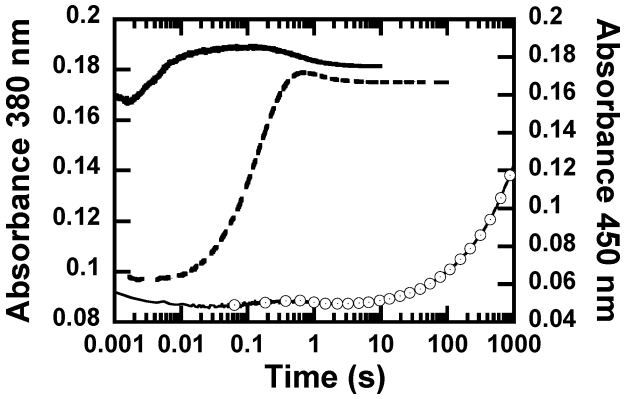


**Figure 5.** Reaction of $O_2$ with *Vc*LuxAB premixed and non-premixed with $FMNH^-$. For the premixed reaction (—), an anaerobic solution of 320 $\mu$M *Vc*LuxAB and 32 $\mu$M $FMNH^-$ was mixed with the same buffer containing 0.26 mM $O_2$. For the non-premixed reaction (---), an anaerobic solution of 32 $\mu$M $FMNH^-$ was mixed with a solution of 320 $\mu$M *Vc*LuxAB containing 0.26 mM $O_2$ in the same buffer. All concentrations are given as before mixing. The trace with empty circles was recorded for the premixed reaction monitored at 450 nm. It indicates the formation of oxidized FMN. Because of the rapid reaction of the *Vc*LuxAB–$FMNH^-$ complex with oxygen in the premixed experiment, most of the C4a-peroxyflavin ($A_{380}$ trace) forms during the dead time of the stopped-flow instrument.

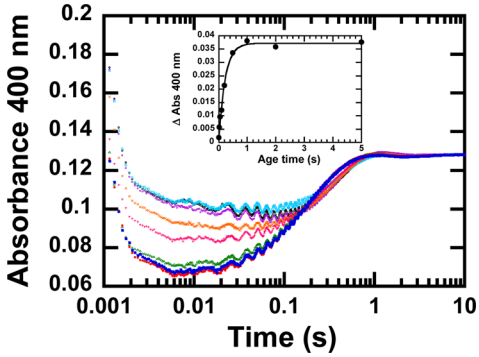


**Figure 6.** Double-mixing stopped-flow experiments with LuxG and *Vc*LuxAB. A solution of 80 $\mu$M LuxG and 800 $\mu$M NADH was first mixed with 64 $\mu$M FMN under anaerobic conditions. The aging times were varied [0.01 (black), 0.02 (cyan), 0.05 (purple), 0.1 (orange), 0.2 (pink), 0.5 (green), 1 (red) and 2 s (blue)] before a solution of 320 $\mu$M *Vc*LuxAB and 0.26 mM $O_2$ was introduced into the second mix. All concentrations given are before mixing. The inset is a plot of amplitude changes that had occurred at the time of the second mix vs aging time. The apparent rate constant of the plot was calculated to be 4.5 ± 0.4 $s^{-1}$.

0.1 $s^{-1}$ at longer aging times (0.5–2 s). The observed overall rate constant at shorter aging times (3.1 $s^{-1}$) could be closely approximated as 2.7 $s^{-1}$ using eq 2 and the rate constants involved for the free diffusion model [reduction of FMN by LuxG (5.0 $s^{-1}$), binding of free $FMNH^-$ to *Vc*LuxAB (6.2 $s^{-1}$), and reaction with $O_2$ to form C4a-peroxyflavin (219 $s^{-1}$); $1/k_{obs(overall)} = 1/5.0 + 1/6.2 + 1/219$]. The increase in the measured rate constant (5.1 ± 0.1 $s^{-1}$) at longer aging times occurs because flavin reduction (5 $s^{-1}$) was complete and no longer limits the overall rate of C4a-peroxyflavin formation. Therefore, a rate constant of ~5 $s^{-1}$ for formation of C4a-peroxyflavin was observed. Figure 7 shows similar calculations for experiments with $C_2$, *Pl*LuxAB, and *Vc*LuxAB. Each of the systems fit the diffusion model for the transfer of $FMNH^-$.

## DISCUSSION

The transient kinetic data reported here clearly suggest that $FMNH^-$ from LuxG is transferred to LuxAB from *P. leiognathi* TH1 or *V. campbellii* via free diffusion. The kinetics of release of $FMNH^-$ from LuxG are not influenced by the presence of LuxAB, even though the concentration of LuxAB employed in this study is much higher than that of LuxG (5-fold higher for *Pl*LuxAB and 8-fold higher for *Vc*LuxAB). Under physiological conditions, because the genes of LuxAB and LuxG are encoded in the same operon, amounts of each protein synthesized are likely similar. The binding of $FMNH^-$ to LuxAB and its subsequent reaction with oxygen are not affected by the presence of LuxG. LuxG has long been proposed to serve as a reductase that generates $FMNH^-$ for LuxAB because the genes for both enzymes are located on the same *lux* operon, similar to other known reductases and oxygenases of two-component monooxygenase systems.[9,25,28,34–37] However, the functional role of LuxG as an *in vivo* reductase in the luminous bacteria has only been shown recently.[9]

*Pl*LuxAB belongs to the class of LuxAB enzymes having faster luminescence decay, whereas most of the reported reaction kinetics are of LuxAB from the slow luminescence decay type,[38] such as those of LuxAB from *V. harveyi*[39,40] and *V. campbellii*.[6] In the absence of aldehyde, an example of C4a-peroxyflavin intermediates of the fast decay type is *Pl*LuxAB, which decays at ~0.01 $s^{-1}$ (Figure 2), whereas the rate of decay of C4a-peroxyflavin of a slower type such as *Vc*LuxAB is 0.002 $s^{-1}$.[6] The rate constant of C4a-peroxyflavin formation from the reaction of the *Pl*LuxAB–$FMNH^-$ complex with oxygen (0.13 mM) is ~17 $s^{-1}$, at 4 °C, which is significantly lower than the value reported for LuxAB from *V. harveyi* (350 $s^{-1}$, at 25 °C)[40] or *V. campbellii* (253 $s^{-1}$, at 4 °C).[6]

The experiments described by Figures 3 and 4 demonstrate the methodology for quantitative analysis of the kinetics of metabolite transfer in multienzyme systems. The data in Figure 1 clearly show that the presence of LuxAB does not influence the reaction kinetics of LuxG upon either flavin reduction or $FMNH^-$ release. The overall kinetics of the reactions of LuxG and various types of $FMNH^-$ acceptors ($C_2$, *Pl*LuxAB, and *Vc*LuxAB) investigated by double-mixing stopped-flow spectrophotometry (Figures 3–6) are consistent with free diffusion models (Figure 7). With each of the oxygenases used, LuxG releases $FMNH^-$ with a rate constant of ~4.5–6 $s^{-1}$. At the shorter aging times before the second mix, the reactions of LuxG and oxygenases were therefore limited by the release of $FMNH^-$ from LuxG. At longer aging times, the reactions were limited by other factors, such as binding of $FMNH^-$ to the oxygenases for *Pl*LuxAB and *Vc*LuxAB. These rates were determined to be the same as those observed in the reaction of free $FMNH^-$ with *Pl*LuxAB (Figure 2) or *Vc*LuxAB (Figure 5).[6]

The transient kinetic methods employed in this study are useful for distinguishing between the modes of metabolite transfer (direct transfer or free diffusion) between protein partners, an issue that has been investigated for several enzymatic systems since the 1990s. Transient kinetic approaches have advantages over steady-state methods in that individual rate constants can be measured and analyzed quantitatively to distinguish between the two modes of $FMNH^-$ transfer. Similar methods were previously used to demonstrate that competent $FMNH^-$ transfer occurs via free diffusion in HPAH ($C_1$–$C_2$) from *A. baumannii*[22] and the


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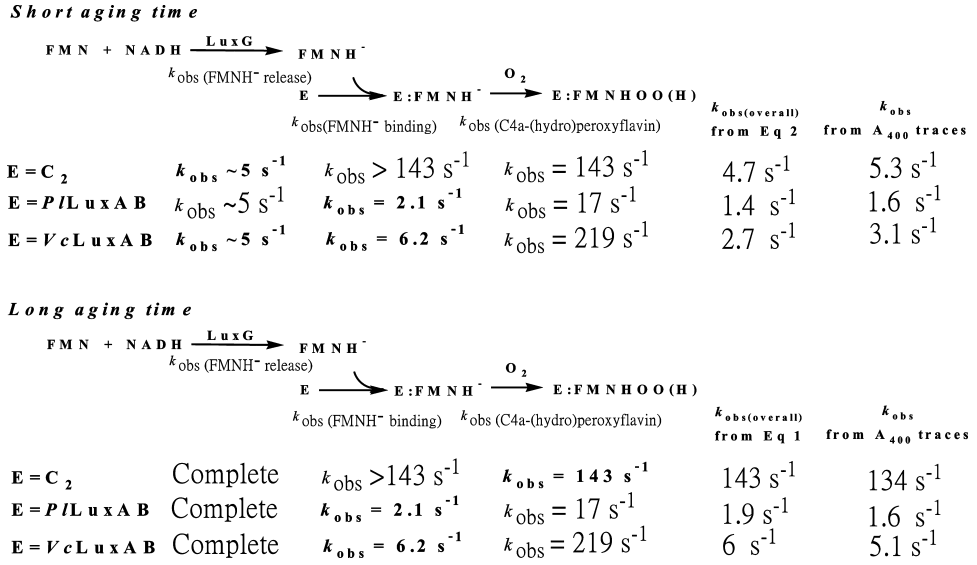


**Figure 7.** Summary of correlations between overall and individual rate constants according to a free diffusion model. Overall rate constants from the calculation according to eq 2 for short aging times and eq 1 for long aging times are compared to the observed rate constants analyzed from $A_{400}$ traces of double-mixing experiments. The $FMNH^-$ generated by LuxG in the first mixing is transferred to acceptor enzymes (E) ($C_2$, *Pl*LuxAB, and *Vc*LuxAB) before reacting with $O_2$ to form C4a-(hydro)peroxyflavin, which can be monitored at 400 nm after the second mixing. At shorter aging times, the formation of C4a-(hydro)peroxyflavin in the LuxG−$C_2$ system is limited by the release of $FMNH^-$ from LuxG (5 $s^{-1}$) (bold letters) because the $FMNH^-$ binding and C4a-(hydro)peroxyflavin formation steps are very fast. Therefore, the overall observed rate constant calculated according to a free diffusion model in eq 2 is similar to the rate constant of $FMNH^-$ release. For the LuxG−*Pl*LuxAB system, the binding of $FMNH^-$ (2.1 $s^{-1}$) to *Pl*LuxAB limits the rate of formation of C4a-(hydro)peroxyflavin. For the LuxG−*Vc*LuxAB reaction, C4a-(hydro)peroxyflavin formation is limited by both the release of $FMNH^-$ from LuxG (5 $s^{-1}$) and the binding of $FMNH^-$ to *Vc*LuxAB (6.2 $s^{-1}$). At longer aging times, the LuxG reaction is complete prior to mixing the second mix with the oxygenase; thus, the overall observed rate constant could be calculated according to eq 1. In general, overall rate constants obtained from calculation vs those from experimental observation agree quite well, evidence that free $FMNH^-$ is transferred from LuxG to acceptor proteins via free diffusion.

ActVA−ActVB system from *S. coelicolor*,[23] and via combined diffusion and complex formation in HPAH from *P. aeruginosa*.[26]

The findings in this report also are consistent with those of Campbell et al.,[17] which indicate that FRE oxidoreductase from *E. coli* does not form a stable complex with *V. harveyi* LuxAB, and with our previous report that LuxG and LuxAB cannot form a protein complex.[9] The observation that LuxAB can efficiently use $FMNH^-$ generated from any of a variety of reductases is also consistent with the conclusion that no complexes are required for the transfer of $FMNH^-$. For example, FRE from *E. coli* can be used to provide $FMNH^-$ for LuxAB *in vitro* to generate light. The level of light intensity from the use of FRE and LuxAB is comparable to that using LuxAB and the FRP reductase from *V. harveyi*. As FRE is derived from *E. coli*, which is a not a luminous organism, it is not likely that FRE would be optimized to form specific protein−protein interactions with LuxAB from *V. harveyi*. Moreover, when the LuxG gene in *P. leiognathi* TH1 was inactivated, a dim phenotype was nevertheless observed.[9] These data indicate that although LuxG is a major flavin reductase supplying $FMNH^-$ to the LuxAB system, LuxAB can also utilize $FMNH^-$ generated by other flavin reductases inside *P. leiognathi* TH1.

On the contrary, Tu and co-workers proposed that the transfer of $FMNH^-$ between the FRP reductase and LuxAB from *V. harveyi* occurs via a direct channeling mechanism. The evidence supporting formation of an FRP reductase−LuxAB complex was obtained from BRET experiments in which the transfer of light emitted from the LuxAB reaction via BRET to the fluorescent YFP-FRP implied that LuxAB and FRP reductase are located close to each other. Further evidence that FRP and LuxAB physically interact consisted of there being alterations in the steady-state kinetics of FRP when they were measured in the presence of LuxAB. However, in this experiment, the reaction of FRP alone was assayed by measuring NAD(P)H consumption, whereas the reaction that included both FRP and LuxAB was monitored by light emission.[14,41] Thus, the measurements were not actually comparable; in the case where light was measured, the process involved several steps: the reduction of the flavin by FRP, the transfer of the reduced flavin to LuxAB, the reaction with oxygen, and the emission of light. In contrast, the reaction with FRP alone required only reduction of the flavin. For the alkanesulfonate monooxygenase (SsuD−SsuE) system, a change in the kinetic mechanism and the kinetic parameters of the SsuE reductase due to the presence of the SsuD oxygenase was interpreted as an indication of the formation of the SsuD−SsuE complex.[16] Substantiating this interpretation, Abdurachim and Ellis described an SsuD−SsuE complex using both affinity chromatography and cross-linking experiments.[15] Thus, for the Ssu system, the data provide stronger support for a direct transfer mechanism.

Although the mechanisms for controlling $FMNH^-$ transfer can vary among various enzyme systems, they must all be successful in minimizing wasteful autoxidation of $FMNH^-$. The transfer of $FMNH^-$ via complex formation should help in preventing $FMNH^-$ reoxidation by minimizing the exposure to oxygen. However, the transfer via free diffusion can also be achieved if the binding of $FMNH^-$ to the monooxygenase is faster than oxidation of free $FMNH^-$ (~0.45 $s^{-1}$ under air saturation at 4 °C and pH 8). For example, $FMNH^-$ binds to $C_2$ with a $k_{on}$ of >$10^7$ $M^{-1}$ $s^{-1}$ and a $K_d$ of ~1.2 $\mu$M.[31,42,43] These


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kinetic and thermodynamic parameters lead to the binding of free $FMNH^-$ to $C_2$ before it can react with $O_2$; therefore, complex formation is not required for this system.[22] The binding of $FMNH^-$ to LuxAB is rather slow compared to that of other oxygenases such as $C_2$. Under these experimental conditions, $FMNH^-$ binds to *Pl*LuxAB and *Vc*LuxAB with observed rate constants of 1.9−2.1 $s^{-1}$ (~1.8 × $10^4$ $M^{-1}$ $s^{-1}$) and 6.2 $s^{-1}$ (~3.9 × $10^4$ $M^{-1}$ $s^{-1}$), respectively. These rates are still greater than the rate of reaction with free $FMNH^-$ and air-saturated oxygen at 4 °C (~0.45 $s^{-1}$) (Figure 2). The actual concentrations of oxygen *in vivo* are likely to be considerably lower than those used here, and this would yield an even slower reaction. Moreover, the reaction of $FMNH^-$ with $O_2$ is autocatalytic, so the initial part of the reaction is quite slow (in the first 20 ms, <5% will be oxidized).[21] The half-sites reactivity of LuxG in which the reduction of FMN in the second subunit cannot proceed until the $FMNH^-$ generated from a first subunit is released may be a fine-tuning feature for controlling the slow release of $FMNH^-$ from LuxG such that it is suitable for the slow binding properties of the *Pl*LuxAB partner. These means of regulating the release of reduced flavin from LuxG might help in preventing wasting of cellular reducing equivalents in addition to avoiding generation of harmful reactive oxygen species.

It should be noted that our data only suggest that the formation of the LuxG−LuxAB protein complex is not necessary and free diffusion serves as an efficient means for the transfer of the $FMNH^-$ between the two proteins. They do not rule out formation of the transient complex of LuxAB and LuxG under different conditions, e.g., at much higher concentrations of LuxG and LuxAB. As *in vivo* concentrations of LuxG and LuxAB are unlikely to greatly exceed the concentrations used in our experiment to a great extent, it is reasonable to propose that direct transfer of reduced flavin via the transient protein complex may not be relevant under physiological conditions.

In conclusion, all of our data are consistent with the transfer of $FMNH^-$ from LuxG to LuxAB being achieved through a free diffusion mechanism. The methodology and results reported here can be used as a basis for other investigations of the transfer of unstable metabolites between multicomponent systems.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

One additional figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

R.T. and W.P. contributed equally to this work.



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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

LuxG, NADH:flavin oxidoreductase from bioluminescent bacteria; LuxAB, bacterial luciferase; HPAH, *p*-hydroxyphenylacetate hydroxylase; HPA, *p*-hydroxyphenylacetate; $C_2$, oxygenase component of HPAH from *A. baumannii*; NADH, reduced nicotinamide adenine dinucleotide; $FMNH^-$, reduced flavin mononucleotide; *Pl*, *P. leiognathi*; *Vc*, *V. campbellii*.

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