# Redox Potential and Equilibria in the Reductive Half-Reaction of *Vibrio harveyi* NADPH−FMN Oxidoreductase†

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ABSTRACT: *Vibrio harveyi* NADPH:FMN oxidoreductase P ($FRP_{Vh}$) is a homodimeric enzyme having a bound FMN per enzyme monomer. The bound FMN functions as a cofactor of $FRP_{Vh}$ in transferring reducing equivalents from NADPH to a flavin substrate in the absence of *V. harveyi* luciferase but as a substrate for $FRP_{Vh}$ in the luciferase-coupled bioluminescent reaction. As part of an integral plan to elucidate the regulation of functional coupling between $FRP_{Vh}$ and luciferase, this study was carried out to characterize the equilibrium bindings, reductive potential, and the reversibility of the reduction of the bound FMN in the reductive half-reaction of $FRP_{Vh}$. Results indicate that, in addition to NADPH binding, $NADP^+$ also bound to $FRP_{Vh}$ in either the oxidized ($K_d$ 180 $\mu$M) or reduced ($K_d$ 230 $\mu$M) form. By titrations with $NADP^+$ and NADPH and by an isotope exchange experiment, the reduction of the bound FMN by NADPH was found to be readily reversible ($K_{eq} = 0.8$). Hence, the reduction of $FRP_{Vh}$-bound FMN is not the committed step in coupling the NADPH oxidation to bioluminescence. To our knowledge, such an aspect of flavin reductase catalysis has only been clearly established for $FRP_{Vh}$. Although the reductive potentials and some other properties of a R203A variant of $FRP_{Vh}$ and an NADH/NADPH-utilizing flavin reductase from *Vibrio fischeri* are quite similar to that of the wild-type $FRP_{Vh}$, the reversal of the reduction of bound FMN was not detected for either of these two enzymes.

A growing number of two-component flavin-dependent monooxygenases have been shown to function in a wide scope of biochemical processes such as bacterial bioluminescence (*1*, *2*), biosynthesis of the antibiotic agents valanimycin (*3*) and actinorhodin (*4*), and desulfurization of fossil fuel (*5*). More examples and descriptions of the biochemical properties of selected systems can be found in a recent review (*6*). Each two-component monooxygenase system consists of a monofunctional monooxygenase and an NAD(P)H-flavin oxidoreductase (or flavin reductase). The former enzyme catalyzes the hydroxylation of a substrate by molecular oxygen utilizing reduced flavin as a cosubstrate. The required reduced flavin is provided by the corresponding flavin reductase, which catalyzes the reduction of flavin at the expense of NAD(P)H.

Because free reduced flavin is subject to autoxidation (*7*−*9*), it is intriguing how the various monofunctional monooxygenases efficiently acquire their necessary reduced flavin substrate from the flavin reductases. However, mechanisms of reduced flavin transfer from donor to acceptor enzymes, in two-component monooxygenases or any other reduced flavin-requiring systems, have long evaded elucidation. Only recently, a few selected two-component monooxygenases have been subjected to mechanistic investigations with respect to reduced flavin transfer (*4*, *10*, *11*). Among them, the bacterial luciferase−flavin reductase system has been studied considerably more extensively.

Bacterial luciferase catalyzes a reaction in which $FMNH_2$[1] and a long-chain aldehyde are oxidized by molecular oxygen to generate FMN, carboxylic acid, water, and bioluminescence. The required $FMNH_2$ is provided in vivo by flavin reductases, of which the *Vibrio harveyi* NADPH-preferring flavin reductase P ($FRP_{Vh}$) (*10*, *12*−*18*) and the *Vibrio fischeri* NADH/NADPH-utilizing general flavin reductase G ($FRG_{Vf}$) (*10*, *16*, *19*−*23*) have been characterized in considerable detail. Both $FRP_{Vh}$ and $FRG_{Vf}$ are homodimeric flavoenzymes containing a bound FMN per enzyme monomer. Their crystal structures have been determined (*14*, *15*, *22*). Both $FRP_{Vh}$ and $FRG_{Vf}$ undergo a monomer−dimer equilibrium (*13*, *23*). In the case of $FRP_{Vh}$, the monomeric form is the dominant species in vivo and only the monomer binds to luciferase to form a functional complex (*17*, *24*).

The bound FMN in $FRP_{Vh}$ and $FRG_{Vf}$ holoenzymes has dual functionalities. In the absence of bacterial luciferase, the bound FMN functions as a genuine cofactor, and both reductases follow a ping-pong mechanism (*10*, *19*), which involves the sequential events of NAD(P)H binding, reduction of FMN cofactor, $NAD(P)^+$ release, binding and subsequent reduction of a flavin substrate by the reduced

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[1] Abbreviations: $FRP_{Vh}$ and $L_{Vh}$, NADPH-preferring flavin reductase P and luciferase, respectively, from *Vibrio harveyi*; $FRG_{Vf}$ and $L_{Vf}$, NADH/NADPH-utilizing general flavin reductase G and luciferase, respectively, from *Vibrio fischeri*; $FMNH_2$, reduced riboflavin 5′-phosphate.

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cofactor, and, finally, release of the reduced flavin product and regeneration of the original oxidized holoenzyme. The originally bound FMN shuttles between an oxidized and a reduced state but remains bound as a cofactor throughout the catalytic cycle. However, in the presence of their respective luciferase partners, the bound FMN is used by $FRP_{Vh}$ and $FRG_{Vf}$ as a substrate reducible by the NAD(P)H cosubstrate. Both reductases follow a sequential mechanism in generating the reduced flavin product, which is directly transferred to luciferase for the coupled bioluminescence reaction (*10*, *16*), leaving the reductase in an apoenzyme form. The binding of FMN, released from luciferase after the luminescence reaction or made available by other processes, regenerates the $FRP_{Vh}$ and $FRG_{Vf}$ holoenzymes. Moreover, $FRP_{Vh}$ and $FRG_{Vf}$ behave differently in hybrid reductase−luciferase systems (*10*, *16*). $FRP_{Vh}$ follows the same sequential mechanism in reducing and transferring the bound FMN when it is coupled to either the *V. harveyi* luciferase ($L_{Vh}$) or the *V. fischeri* luciferase ($L_{Vf}$). In contrast, the coupled reaction of $FRG_{Vf}$ and *V. harveyi* luciferase ($L_{Vh}$) follows the ping-pong mechanism with the originally bound FMN in the $FRG_{Vf}$ holoenzyme maintaining its functional role as a cofactor. Hence, the mechanism of $FMNH_2$ transfer between flavin reductase and luciferase appears to be rather delicately regulated by both the reduced flavin donor and acceptor enzymes.

Both $FRP_{Vh}$ and $FRG_{Vf}$ are highly efficient enzymes with turnover rates about 2−3 orders of magnitude faster than their respective luciferase partners. Moreover, the biosynthesis of neither flavin reductase is in concert with the rapid induction of luciferase encoded in the *lux* operon (*1*, *25*, *26*). NAD(P)H is a precious energy source for many biochemical and physiological functions. If NAD(P)H is committed to rapid and irreversible oxidation by flavin reductases without a well-controlled functional coupling with luciferase, significant amounts of NAD(P)H would be wasted in generating $FMNH_2$ for nonproductive autoxidation. Therefore, knowledge on flavin reductases with respect to equilibrium binding of $NAD(P)^+$ and NAD(P)H and reversibility of the reduction of bound FMN is essential to an integral understanding of the regulation of NAD(P)H oxidation for bioluminescence. To date, knowledge along these lines is severely limited or lacking for $FRP_{Vh}$ and $FRG_{Vf}$ in particular and for flavin reductases in two-component monooxygenase systems in general. Such a lack of understanding prompted us to carry out this study to elucidate the redox potential and equilibrium properties of the reductive half-reactions of $FRP_{Vh}$ and, to a more limited scope, $FRG_{Vf}$. We found that, despite many similar properties between $FRP_{Vh}$ and $FRG_{Vf}$ (*6*), the thermodynamic characteristics of their reductive half-reactions have significant differences. These findings, once again, highlight the delicacy in the functional coupling between specific species of flavin reductase and luciferase.

## EXPERIMENTAL PROCEDURES

*Materials*. NADPH, $NADP^+$, $NAD^+$, NADH, FMN, benzyl viologen, phenosafranin, $NAD^+$ kinase (from liver, type IV), and isocitrate dehydrogenase were all from Sigma. [4-$^3$H]$NAD^+$ was obtained from Amersham Biosciences. *V. harveyi* $FRP_{Vh}$ and *V. fischeri* $FRG_{Vf}$ were purified to apparent homogeneity as described previously from *Escherichia coli* harboring pFRP1 (*12*) and pFRG (*10*), respectively. The published methods (*18*) were followed for the construction and purification of the R203A variant of $FRP_{Vh}$, in which the arginine residue of the wild-type enzyme at the 203 position was replaced by an alanine residue. Purity of enzyme samples was judged on the basis of sodium dodecyl sulfate−polyacrylamide gel electrophoresis. All phosphate ($P_i$) buffers were, except noted otherwise, at pH 7.0 and consisted of phosphates at mole fractions of 0.39 sodium monobase and 0.61 potassium dibase.

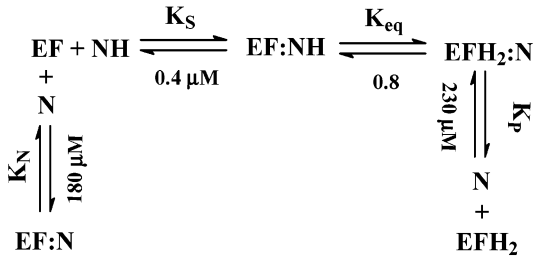
*NADPH and $NADP^+$ Titrations.* One milliliter of the $FRP_{Vh}$ sample in an airtight anaerobic titration cuvette (*27*) was deoxygenated by three cycles of vacuum application and refilling with $N_2$. The enzyme sample under $N_2$ gas was titrated with NADPH and $NADP^+$ by using a microsyringe for the addition of small-volume aliquots of concentrated NADPH and/or $NADP^+$ stock solutions stepwise through the side arm of the cuvette, and the absorption spectrum of the sample at equilibrium was taken after each addition using a Milton Roy 3000 spectrophotometer. When enzyme was titrated with $NADP^+$ in the absence of NADPH, titrations were carried out under aerobic conditions.

*Determination of Reductive Potentials.* Reductive potentials were determined by dithionite titrations performed using an anaerobic titration cuvette in the same way as described above for NADPH titrations. Aliquots of dithionite at appropriate concentrations in the microsyringe were added stepwise into 1 mL of $N_2$-saturated solution containing 2 $\mu$M benzyl viologen and equal molar concentrations of $FRP_{Vh}$ and phenosafranin. Phenosafranin and benzyl viologen were used as an internal standard and a mediator in electron transfer, respectively. The absorption spectrum at equilibrium was taken after each addition, and spectral data were analyzed according to the Nernst equation.

*Synthesis and Purification of [4-$^3$H]$NADP^+$.* In 10 mL of 70 mM phosphate buffer, pH 7.5, containing 5 mM ATP, 5 mM $MgC1_2$, 0.2 mM $NAD^+$, and 250 $\mu$Ci of [4-$^3$H]$NAD^+$ (cpm 99300 for a 4 $\mu$L aliquot) 50 units of $NAD^+$ kinase was added (one unit is defined as the phosphorylation of 1 nmol of $NAD^+$ to $NADP^+$ per minute at pH 7.5 and 37 °C). The solution was incubated for 8 h at room temperature, heated in boiling water for 40 s, cooled on ice, and then loaded onto a DEAE-cellulose column (2 × 22 cm, preequilibrated with 70 mM $P_i$, pH 7.5). The column was washed first with 20 mL of 120 mM $P_i$, pH 7.5, and then with a linear gradient (300 mL total) from 140 to 240 mM $P_i$ (pH 7.5). Fractions were counted, and those containing [4-$^3$H]$NADP^+$ were pooled. The yield was about 14%, with a specific radioactivity of 55 $\mu$Ci/$\mu$mol.

*Test for Conversion of [4-$^3$H]$NADP^+$ to [4-$^3$H]NADPH by $FRP_{Vh}$.* $FRP_{Vh}$ (0.12 $\mu$g) was added to 0.5 mL of $P_i$, pH 7.0, containing 2 mM NADPH and 0.054 $\mu$Ci of [4-$^3$H]-$NADP^+$ (specific radioactivity 55 $\mu$Ci/$\mu$mol). The solution was incubated at room temperature for 1 h. $NADP^+$ (10 mM in 0.01 mL of $P_i$, pH 7.0, used as a marker for spectrophotometric detection at 260 nm) was added and the mixture was loaded onto a DE-52 column (0.5 × 5 cm, preequilibrated with water). The column was sequentially eluted with 1.5 mL of $H_2O$, 5.5 mL of 85 mM $P_i$, pH 7.5, and 300 mM $P_i$, pH 7.5. Fractions were collected at 1 mL each and were counted for radioactivity. A control was run under identical conditions except in the absence of $FRP_{Vh}$.

Scheme 1




## RESULTS

*Equilibrium Scheme in the Reductive Half-Reaction.* Initial experiments showed that the addition of excess NADPH into $FRP_{Vh}$ only partially bleached the absorbance of its bound FMN in the absence of $O_2$ and exogenous FMN. Furthermore, the bleaching was partially reversed by the addition of $NADP^+$. These findings led us to propose Scheme 1 to account for the reaction steps involved in the reductive half-reaction of $FRP_{Vh}$. Values of various constants are based on determinations made in this study.

Following this scheme, NADPH (NH) binds to oxidized $FRP_{Vh}$ holoenzyme (EF) to form an EF:NH complex. The bound FMN (F) is reduced by NH in EF:NH to generate the reduced enzyme ($EFH_2$) which remains in complex with $NADP^+$ ($EFH_2$:N). The release of $NADP^+$ (N) from $EFH_2$:N yields the free reduced enzyme ($EFH_2$). $NADP^+$ is also a competitive inhibitor against NADPH binding. $K_N$, $K_S$, and $K_P$ are the dissociation constants for complexes EF:N, EF:NH, and $EFH_2$:N, respectively, and the equilibrium constant of the conversion of EF:NH to $EFH_2$:N is defined as $K_{eq} = [EFH_2:N]/[EF:NH]$. This scheme was then subjected to experimental tests as described below.

*Aerobic $NADP^+$ Titration.* Oxidized $FRP_{Vh}$ holoenzyme EF was found capable of binding $NADP^+$, resulting in substantial changes in its absorption spectrum. When $FRP_{Vh}$ was titrated with $NADP^+$ from 0.1 to 0.98 mM, the difference spectra of ($FRP_{Vh}$ + $NADP^+$) − $FRP_{Vh}$ showed two peaks at 392 and 506 nm, two partially overlapped troughs between these two peaks, and two well-defined isosbestic points at 405 and 467 nm (Figure 1A). Changes of $\Delta A_{506}$ signals were analyzed according to eq 1 following the method of Wu and Hammes (*28*)

$$K_N = \frac{[(EF)_o - P][(N)_o - nP]}{P} \quad (1)$$

where $n$ and $K_N$ are the number of $NADP^+$ binding sites per $FRP_{Vh}$ monomer and the dissociation constant for EF:N, respectively; $(EF)_o$ and $(N)_o$ are the total molar concentrations of oxidized $FRP_{Vh}$ and $NADP^+$, respectively; and $P$ is the equilibrium concentration of EF:N. The term $P$ at different $(N)_o$ was calculated using the equation $P = (\Delta A/\Delta A_{max}) \times (EF)_o$. $\Delta A_{max}$ was $\Delta A_{506}$ when FRP is saturated with N and was determined to be 0.149 by double reciprocal plotting of $\Delta A_{506}$ and [$NADP^+$]. Equation 1 can be rearranged to eq 2

$$\frac{(N)_o}{P} = \frac{K_N}{(EF)_o - P} + n \quad (2)$$

(*28*). Consistent with eq 2, a linear relationship was obtained in plotting $(N)_o/P$ against $1/[(E)_o - P]$ (Figure 1B). On the basis of values of the intercept on the ordinate and the slope, $FRP_{Vh}$ was found to bind 0.99 $NADP^+$ per enzyme monomer with $K_N = 1.8 \times 10^{-4}$ M.

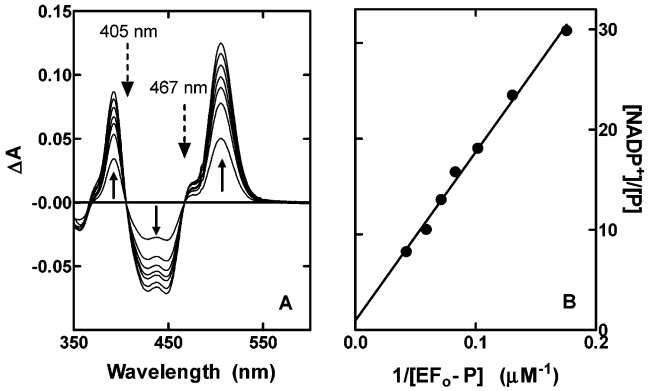



FIGURE 1: Titration of $FRP_{Vh}$ with $NADP^+$. (A) A 1 mL solution containing 46 $\mu$M $FRP_{Vh}$ in 50 mM $P_i$ was titrated with $NADP^+$ (at total $NADP^+$ concentrations of 0.1, 0.2, 0.3, 0.39, 0.49, 0.69, and 0.98 mM) at 23 °C. The spectrum of the sample was recorded before $NADP^+$ addition and when the binding reached equilibrium after each addition. The difference spectra ($FRP_{Vh}$ + $NADP^+$) − $FRP_{Vh}$ are presented as a function of $NADP^+$ concentration with the solid line arrows indicating the direction of absorbance change with increasing amounts of $NADP^+$. Two well-defined isosbestic points (shown as $\Delta A = 0$) are indicated by the dashed arrows. (B) $\Delta A_{506}$ was analyzed according to eq 2, allowing the calculation of the dissociation constant and number of $NADP^+$ binding sites from the slope and intercept, respectively.

*Anaerobic $NADP^+$ Titration.* In the presence of saturating NADPH, [EF] and [EF:N] in Scheme 1 are negligible. Therefore, eq 3 describes the relationship between [N] and

$$\frac{[E_{red}]}{[E_{ox}]} = \frac{[EFH_2:N] + [EFH_2]}{[EF:NH]} = K_{eq} + \frac{K_{eq}K_p}{[N]} \quad (3)$$

the ratio of total reduced $FRP_{Vh}$ over total oxidized FRP, i.e., $[E_{red}]/[E_{ox}]$, at equilibrium. To test eq 3, $FRP_{Vh}$ was titrated with $NADP^+$ in the presence of a high concentration of NADPH, and the spectrum was recorded before and after the addition of NADPH and each addition of $NADP^+$. The absorption peak of the oxidized $FRP_{Vh}$ at 453 nm was bleached by about 87% upon the reduction of $FRP_{Vh}$ by saturating NADPH without any addition of $NADP^+$. The bleaching was gradually but partially reversed when increasing levels of $NADP^+$ were added (Figure 2A). When increases in $A_{453}$ were plotted as a function of the $NADP^+$ concentration, a hyperbolic curve was obtained reaching about 55% of the original $A_{453}$ before bleaching by NADPH (not shown). Such absorbance increases due to the $NADP^+$ additions were not reversed by further addition of NADPH, indicating that NADPH was indeed saturating. In principle, the EF species would partition between EF:NH and EF:N in the presence of NADPH and $NADP^+$. A complete recovery of the oxidized $FRP_{Vh}$ should occur when a sufficiently high level of $NADP^+$ was added to out-compete NADPH in this partitioning. However, additions of $NADP^+$ only led to a partial recovery of the oxidized reductase (Figure 2A). This is because the level of NADPH originally added (0.3 mM) was 750-fold of the $K_S$ whereas the highest level of $NADP^+$ added (2.1 mM) was only 11.7-fold of $K_N$. Hence the partitioning between EF:NH and EF:N strongly favors the former under our experimental conditions. There-

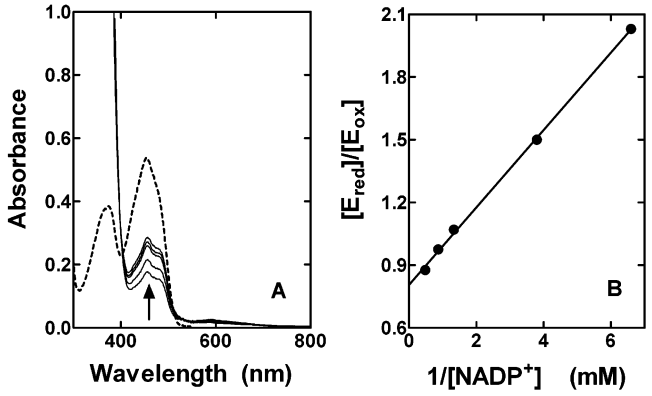



FIGURE 2: Anaerobic titration of $FRP_{Vh}$ with $NADP^+$ in the presence of NADPH. (A) $FRP_{Vh}$, at 40 $\mu$M, in 1 mL of $N_2$-saturated 50 mM $P_i$ containing 0.3 mM NADPH was titrated anaerobically at 23 °C with 0.15−2.1 mM $NADP^+$. Spectra were taken after stepwise titrations when equilibria were achieved. The arrow shows the direction of spectrum change with increasing amount of $NADP^+$. The spectrum of $FRP_{Vh}$ without NADPH or $NADP^+$ is also presented (dashed line) as a reference. (B) $[E_{red}]/[E_{ox}]$, concentration ratio of total reduced over total oxidized $FRP_{Vh}$, is plotted against the reciprocal of $[NADP^+]$ using the data from panel A.

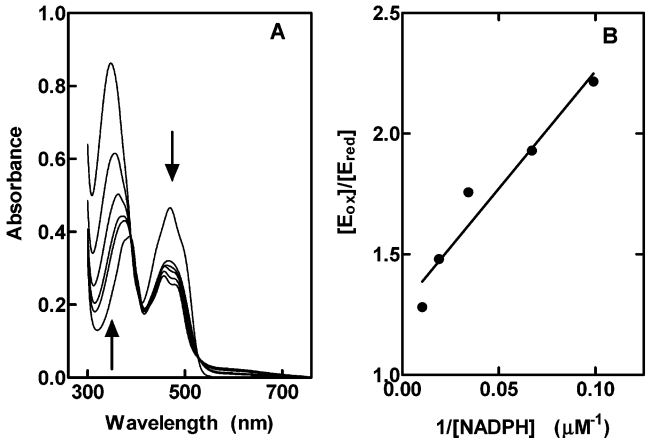



FIGURE 3: Anaerobic titration of $FRP_{Vh}$ with NADPH in the presence of $NADP^+$. (A) Absorption spectra of $FRP_{Vh}$ after stepwise addition of NADPH into 1 mL of $N_2$-saturated $P_i$ containing 40 $\mu$M $FRP_{Vh}$ and 3 mM $NADP^+$ at 23 °C under anaerobic conditions. The total concentrations of NADPH immediately after addition were, in the direction of arrows, 0, 60, 80, 100, 140, and 200 $\mu$M. (B) Plots of $[E_{ox}]/[E_{red}]$ versus the reciprocal of free NADPH concentration using data derived from panel A.

fore, the partial recoveries of oxidized $FRP_{Vh}$ upon $NADP^+$ additions were primarily a consequence of the reversion of $EFH_2$ to $EFH_2$:N and the subsequent equilibrium between $EFH_2$:N and EF:NH.

It should be noted that low levels of absorption in the 540−700 nm range were detectable after $NADP^+$ addition (Figure 2A). Species contributing to such absorption could be flavin semiquinone (*29*) or the charge complex of EF:NH and/or $EFH_2$:N, similar to those observed for *E. coli* flavin reductase Fre (*30*). However, the amounts of long-wavelength-absorbing species were quite low. Hence, for simplicity, these species are not included in Scheme 1. Accordingly, absorbance signals at 453 nm (Figure 2A) provide a measure of the total oxidized enzyme ($E_{ox}$) and total reduced enzyme ($E_{red} = E_{total} - E_{ox}$). The total $NADP^+$ concentration was determined from the level of added $NADP^+$ and the amount of NADPH oxidation. Data shown in Figure 2A can then be analyzed according to eq 3 as shown in Figure 2B. As expected, a linear relationship was observed between the ratio of $[E_{red}]/[E_{ox}]$ and the reciprocal of $NADP^+$ concentration. On the basis of the intercept on the ordinate and the slope, $K_{eq}$ and $K_P$ were determined to be 0.8 and $2.3 \times 10^{-4}$ M, respectively.

The possible formation of an $EFH_2$:NH complex between NADPH and reduced enzyme E:$FH_2$ was considered. However, the recovery of oxidized flavin absorbance upon the addition of $NADP^+$ was not reversed by further addition of NADPH (Figure 2A), suggesting that the $EFH_2$:NH complex formation was negligible in competition with the $EFH_2$:N formation under our experimental conditions. Hence, $EFH_2$:NH is not included in Scheme 1.

*NADPH Titration*. The spectra were measured under anaerobic conditions after each addition of a small aliquot of NADPH into $N_2$-saturated $FRP_{Vh}$ containing a constant level of 3 mM $NADP^+$. Higher levels of $FRP_{Vh}$ reduction (indicated by decreasing $A_{450}$) were observed at increasing levels of NADPH addition (Figure 3A). Since the $NADP^+$ level was 13 times the $K_P$, free fully reduced $FRP_{Vh}$ ($EFH_2$) should not exist in any significant amounts. Therefore, the data in Figure 3A can be analyzed according to eq 4 derived

$$\frac{[E_{ox}]}{[E_{red}]} = \frac{[EF] + [EF:N] + [EF:NH]}{[EFH_2:N]} = \frac{1}{K_{eq}} + \frac{K_S(K_N + [N])}{K_N K_{eq}[NH]} \quad (4)$$

from Scheme 1. The absorbance at 467 nm, an isosbestic point in the conversion between EF and EF:N (Figure 1A), was used to calculate the total amount of the oxidized $FRP_{Vh}$ species assuming that EF:NH has a similar absorptivity at this wavelength. Free NADPH concentrations were determined from the observed $A_{340}$ after corrections for $A_{340}$ contributed by $FRP_{Vh}$ and the enzyme-bound NADPH. The concentration of bound NADPH was determined from the relationship $[EF:NH] = [EFH_2:N]/K_{eq} = [E_{red}]/K_{eq}$. As predicted by eq 4, $[E_{ox}]/E_{red})$ versus 1/[NADPH] showed a linear line (Figure 3B), allowing the determination of $K_{eq} = 0.8$ and, based on $K_N$ determined from Figure 1, $K_S = 0.4$ $\mu$M. The value of $K_{eq}$ determined from this NADPH titration correlated well with that determined from the anaerobic $NADP^+$ titration shown in Figure 2.

*Reductive Potential*. The reductive potential of $FRP_{Vh}$ was determined by spectral titration with dithionite using the phenosafranin redox couple as a standard. The absorption peaks of oxidized $FRP_{Vh}$ and phenosafranin (PS) at 453 and 520 nm, respectively, can be completely bleached upon full reduction by dithionite. After the addition of a given amount of dithionite, an equilibrium state was reached for both redox pairs of $FRP_{Vh,ox}/FRP_{Vh,red}$ and $PS_{ox}/PS_{red}$. Spectral measurements of the sample at various equilibrium states after the addition of increasing amounts of dithionite are shown in Figure 4A. At each equilibrium state, the concentration ratio of oxidized over reduced phenosafranin ($[PS_{ox}]/[PS_{red}]$) was determined from the ratio of $A_{540}/\Delta A_{540}$, where $A_{540}$ is the observed absorbance at equilibrium state and $\Delta A_{540}$ is the original absorbance minus $A_{540}$. $FRP_{Vh}$ had no contribution

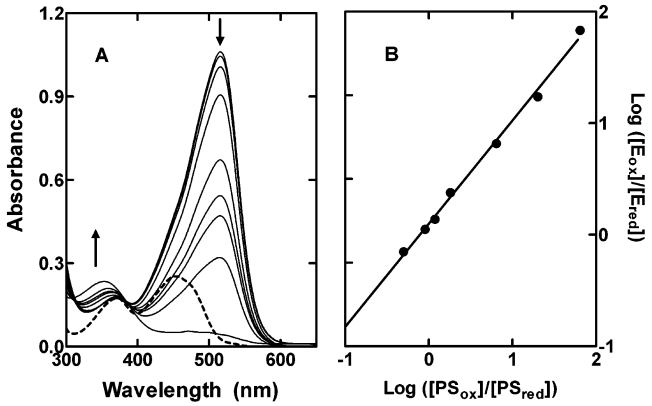



FIGURE 4: Determination of the reductive potential of $FRP_{Vh}$. Aliquots of dithionite were added into 1 mL of 50 mM $P_i$ containing 20 $\mu$M phenosafranin, 2 $\mu$M benzyl viologen, and 20 $\mu$M $FRP_{Vh}$. (A) Changes of the sample spectra with increasing amounts of dithionite addition are shown in the direction of the arrows. The spectrum of $FRP_{Vh}$ before dithionite addition is shown as the dashed line. (B) At each equilibrium state after the addition of dithionite, the ratio of oxidized over reduced phenosafranin ($[PS_{ox}]/[PS_{red}]$) was determined from the ratio of $A_{540}/\Delta A_{540}$, where $A_{540}$ is the observed absorbance at equilibrium state and $\Delta A_{540}$ is the original absorbance minus $A_{540}$. The ratio of oxidized over reduced $FRP_{Vh}$ ($[E_{ox}]/[E_{red}]$) was similarly determined from $A_{465}/\Delta A_{465}$, where all absorbance readings at 465 nm were corrected for the contributions from oxidized phenosafranin. The log−log plot of $[E_{ox}]/[E_{red}]$ versus $[PS_{ox}]/[PS_{red}]$ is presented.

to observed $A_{540}$. The concentration ratio of oxidized over reduced $FRP_{Vh}$ ($[E_{ox}]/[E_{red}]$) was similarly determined from $A_{465}/\Delta A_{465}$ where all absorbance readings at 465 nm were corrected for the contributions from oxidized phenosafranin. These data can be analyzed following the rearranged Nernst equation:

$$\log([E_{ox}]/[E_{red}]) = (\Delta E^{\circ}_{PS} - \Delta E^{\circ}_{FRP})\frac{2F}{2.303RT} + \log([PS_{ox}]/[PS_{red}]) \quad (5)$$

The log−log plot of $[E_{ox}]/[E_{red}]$ against $[PS_{ox}]/[PS_{red}]$ using data from Figure 4A gives a linear line (Figure 4B) with a slope of 0.95 and an intercept of 0.098. Accordingly, the standard reductive potential of $FRP_{Vh}$ was calculated to be −255 mV using the intercept and −252 mV for $\Delta E^{\circ}_{PS}$.

For an accurate determination of the standard reductive potential of $FRP_{Vh}$ by the method summarized above, there should be no significant binding of phenosafranin by $FRP_{Vh}$ so that the known $\Delta E^{\circ}_{PS}$ of −252 mV for free phenosafranin can be applied to eq 5. This critical requirement was confirmed by equilibrium dialysis measurements. $FRP_{Vh}$ (0.5 mL, 55 $\mu$M) was put into a Slide-A-Lyzer cassette with 10000 MWCO (Pierce) and dialyzed against 500 mL of 100 $\mu$M phenosafranin in 50 mM $P_i$ for 24 h. The phenosafranin level in the $FRP_{Vh}$-containing sample was spectrally determined to be 101 $\mu$M in comparison with 100 $\mu$M in the outside solution. Apparently $FRP_{Vh}$ did not bind phenosafranin significantly under our experimental conditions.

*Reduction of $FRP_{Vh}$ R203A*. $FRP_{Vh}$ R203A has very low affinities for both NADPH and $NADP^+$ but is catalytically active (*18*). As shown in Figure 5, R203A was completely reduced by NADPH in the presence of 0.26 mM $NADP^+$ under anaerobic conditions. This is in sharp contrast with wild-type $FRP_{Vh}$ that had about 50% in oxidized form under

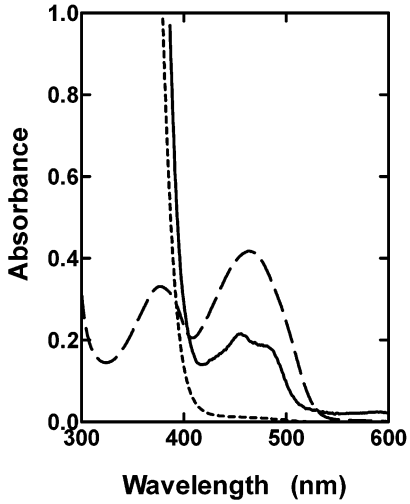



FIGURE 5: Comparison of wild-type and the R203A variant of $FRP_{Vh}$ in their reduction by NADPH in the presence of $NADP^+$. Spectra of 40 $\mu$M wild-type (solid line) and R203A (short dashed line) $FRP_{Vh}$ were taken under anaerobic conditions in the presence of 0.3 mM NADPH and 0.26 mM $NADP^+$. The off-scale absorbance in the region of <400 nm was mainly from NADPH, and the bleaching of the $A_{450}$ peak was due to the reduction of $FRP_{Vh}$ enzymes. The spectrum of R203A (long dashed line) before the addition of $NADP^+$ and NADPH is also presented as a reference.

the same conditions. The reductive potential of $FRP_{Vh}$ R203A was previously determined to be −259 mV (*18*).

*$NAD^+$ Binding by and Reductive Potential of $FRG_{Vf}$*. Addition of $NAD^+$ up to 10 mM did not change the spectrum of $FRG_{Vf}$. $FRG_{Vf}$ remained in a completely reduced state in the presence of 0.3 mM NADH and $NAD^+$ at levels up to 10 mM under anaerobic condition. The reductive potential of $FRG_{Vf}$ was determined to be −215 mV by the dithionite titration similar to that described above for $FRP_{Vh}$.

*Reversibility of FRP Reduction by NADPH*. When $FRP_{Vh}$ was mixed with NADPH and [4-$^3$H]$NADP^+$ without any exogenously added FMN, the enzyme can only carry out the first half of the overall reaction to allow the reduction of bound FMN and the production of $NADP^+$. If the conversion of EF:NH to $EFH_2$:N (Scheme 1) is irreversible, there should be no reduction of $NADP^+$ back to NADPH. However, as shown in Figure 6, the labeled [4-$^3$H]$NADP^+$ in the solution was significantly converted to NADPH in the presence of $FRP_{Vh}$. Clearly, the step of $FRP_{Vh}$ holoenzyme reduction by NADPH is readily reversible.

## DISCUSSION

Although $FRP_{Vh}$ and $FRG_{Vf}$ share a number of similarities with respect to their mechanistic and structural properties (*6*), there are significant differences between these two reductases as well. $FRP_{Vh}$ strongly prefers NADPH as a substrate and has a disordered loop within which the Arg203 residue is critical to NADPH recognition and binding (*18*). On the other hand, $FRG_{Vh}$ does not have this disordered loop and can utilize NADH and NADPH with similar efficiencies. Moreover, in hybrid-coupled reactions of $FRP_{Vh}$−$L_{Vf}$ and $FRG_{Vf}$−$L_{Vh}$, the two reductases follow different mechanisms in reducing their originally bound FMN (*10*, *16*). In this study, the binding of reduced and oxidized pyridine nucleotides and the redox properties have been characterized for $FRP_{Vh}$ and, for some critical comparisons, also for a R203A

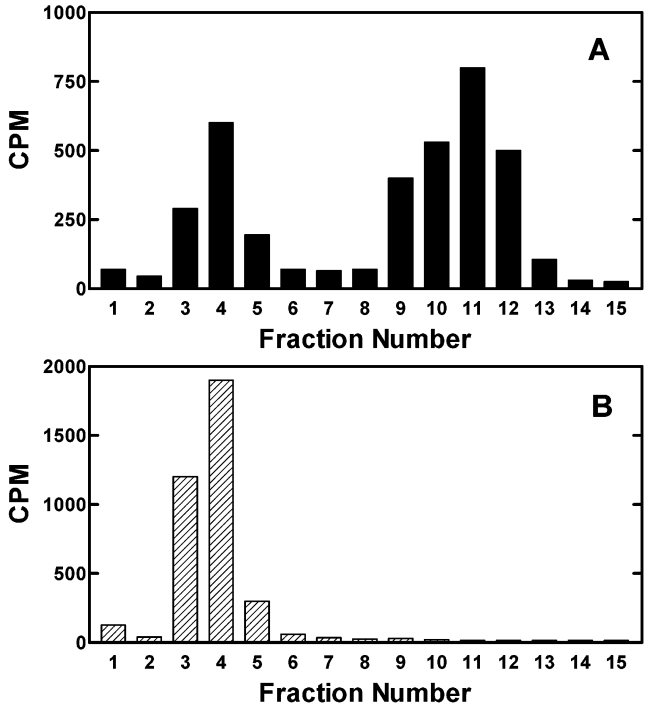



FIGURE 6: Reversibility of $FRP_{Vh}$ reduction by NADPH. (A) A reaction solution containing 0.12 $\mu$g of $FRP_{Vh}$, 2 mM NADPH, and 0.054 $\mu$Ci of [4-$^3$H]$NADP^+$ (specific radioactivity = 55 $\mu$Ci/$\mu$mol) in 0.5 mL of 50 mM $P_i$, pH 7.5, was incubated for 1 h. Unlabeled $NADP^+$ (0. 01 mL of a 10 mM stock) was then added as a marker. The solution was subjected to chromatography on a DE-52 column as described in the text, and 1 mL fractions were collected and counted for tritium. (B) A control run under conditions identical to that described for panel A except without any addition of $FRP_{Vh}$. The peak (at fraction 4) in both panels is associated with $NADP^+$ whereas the second peak (at fraction 11) in panel A is associated with NADPH based on another control run using NADPH only.

variant of $FRP_{Vh}$ and for $FRG_{Vf}$. Again, some significant differences were uncovered.

We have shown that monomeric $FRP_{Vh}$ can form a complex with luciferase that is functionally active in vitro and in vivo (*17*, *24*). This satisfies a critical requirement for a direct metabolite channeling system. However, $FRP_{Vh}$ is constitutively synthesized (*26*) whereas the biosynthesis of $L_{Vh}$ is subjected to the induction of the *lux* operon (*1*). During the life cycle of *V. harveyi*, periods must exist in which part or even most of the $FRP_{Vh}$ molecules are not functionally coupled to luciferase. Moreover, $FRP_{Vh}$ has a $k_{cat}$ of 4170 $min^{-1}$ (*18*), much faster than the 2−20 $min^{-1}$ $k_{cat}$ (depending upon the aldehyde substrate) for luciferase (*31*). If $FRP_{Vh}$ is allowed to rapidly and irreversibly convert NADPH to $NADP^+$ for the formation of nonproductive $FMNH_2$, not only precious NADPH would be wasted but also toxic oxygen species would be generated from autoxidation of reduced flavin. This study demonstrated for the first time that, in addition to forming a structural and functional complex with luciferase (*17*, *24*), the utilization of NADPH by $FRP_{Vh}$ is sensitive to other factors for regulation.

First and foremost, the step of reduction of the bound FMN is readily reversible as indicated by the finding of $K_{eq}$ = $[EFH_2:N]/[EF:NH]$ = 0.8 on the basis of results of $NADP^+$ titration in the presence of saturating NADPH (Figure 2) and NADPH titration in the presence of saturating $NADP^+$ (Figure 3). In another independent test, [4-$^3$H]$NADP^+$ was found to be converted back to [4-$^3$H]NADPH in the reductive half-reaction of $FRP_{Vh}$ (Figure 6). Therefore, the reversibility of reduction of the bound FMN by NADPH has been clearly established for $FRP_{Vh}$. The physiological implication of this finding is that the reduction of the bound FMN of $FRP_{Vh}$ by NADPH is not the committed step in NADPH oxidation. It should be noted that, whenever $FRP_{Vh}$ is not completely trapped by $L_{Vh}$ in complex formation, the reversibility of the reduction of $FRP_{Vh}$-bound FMN would decrease but not prevent the formation of reduced flavin that is uncoupled from luciferase bioluminescence. The reduced flavin so generated by $FRP_{Vh}$ would be a wasteful product if the sole function of $FRP_{Vh}$ in vivo is to provide $FMNH_2$ for luciferase. However, in metabolite channeling, a completely leak-free coupling of a donor enzyme with one acceptor enzyme may not be desirable if the metabolite generated by the former is needed by other enzymes or processes. In this connection, the possibility remains that the reduced flavin generated by $FRP_{Vh}$ may have other acceptor enzymes. There is yet another level of regulation shared by $FRP_{Vh}$ and $FRG_{Vf}$ to reduce wasteful oxidation of NAD(P)H. The $K_{m,NADPH}$ of $FRP_{Vh}$ and the $K_{m,NADH}$ of $FRG_{Vf}$ are 0.02 and 9 $\mu$M, respectively, when coupled to luciferase but are markedly elevated to 20 and 120 $\mu$M, respectively, when free from coupling with luciferase (*10*). This would decrease the abilities of both reductases, when they are not functionally coupled to luciferase, to compete with other NAD(P)H utilizing enzymes in the cells.

The reversibility of reduction of the reductase-bound FMN is quite sensitive to perturbation. When R203A is compared with the wild-type $FRP_{Vh}$, this mutation results in a marked elevation of $K_m$ for NADPH but only small changes in its standard reductive potential, $K_d$ for FMN cofactor binding, $K_m$ for flavin substrate, and $k_{cat}$ (*18*). However, in sharp contrast to wild-type $FRP_{Vh}$, R203A was fully reduced by NADPH in the presence of 0.26 mM $NADP^+$ (Figure 5), showing no detectable degree of reversal of the reduction of FMN cofactor. $FRG_{Vf}$ also has a standard reductive potential similar to that of $FRP_{Vh}$. However, different from $FRP_{Vh}$, full reduction of $FRG_{Vf}$ was obtained at 0.3 mM NADH, and no reversal of the reduction of the bound FMN was observed by the addition of $NAD^+$ up to 10 mM. The reversibility of $FRP_{Vh}$ in NADPH oxidation is further in contrast to the irreversibility of reduction of flavin substrate by NADPH catalyzed by the *E. coli* flavin reductase Fre, which does not utilize flavin as a cofactor and follows a sequential mechanism (*30*). To date, the particular aspect of reversibility of flavin reduction/NAD(P)H oxidation has, to our knowledge, only been demonstrated for $FRP_{Vh}$.

In addition to NADPH binding, $NADP^+$ also binds to oxidized and reduced $FRP_{Vh}$ with dissociation constants of 180 $\mu$M (Figure 1) and 230 $\mu$M (Figure 2), respectively. As shown in Scheme 1, the $NADP^+$ binding to $EFH_2$ would favor the reversal of $NADP^+$ to NADPH, and $NADP^+$ binding to EF would trap $FRP_{Vh}$ in a form inactive in oxidizing NADPH. Qualitatively, these properties of $FRP_{Vh}$ allow another level of regulation of NADPH oxidation by this reductase. Because no information is available on the $NADP^+$ level at different stages of the life cycle of luminous bacteria, the physiological significance of such a regulation is uncertain at the present.

## REFERENCES


1. Hastings, J. W., Potrikus, C. J., Gupta, S. C., Kurfürst, M., and Makemson, J. C. (1985) Biochemistry and physiology of bioluminescent bacteria, *Adv. Microb. Physiol. 26*, 235−291.
2. Tu, S.-C. (2003) Bacterial Bioluminescence: Biochemistry, in *CRC Handbook of Organic Photochemistry and Photobiology* (Horspool, W. M., and Lenci, F., Eds.) pp 136.1−136.17, CRC Press, Boca Raton, FL.
3. Parry, R. J., and Li, W. (1997) Purification and characterization of isobutylamine N-hydroxylase from the valanimycin producer *Streptomyces viridifaciens* MG456-hF10, *Arch. Biochem. Biophys. 339*, 47−54.
4. Valton, J., Filisetti, L., Fontecave, M., and Nivière, V. (2004) A two-component flavin-dependent monooxygenase involved in actinorhodin biosynthesis in *Streptomyces coelicolor*, *J. Biol. Chem. 279*, 44362−44369.
5. Lei, B., and Tu, S.-C. (1996) Gene overexpression, purification, and identification of a desulfurization enzyme from *Rhodococcus* sp. strain IGTS8 as a sulfide/sulfoxide monooxygenase, *J. Bacteriol. 178*, 5699−5705.
6. Tu, S.-C. (2001) Reduced flavin: Donor and acceptor enzymes and mechanisms of channeling, *Antioxid. Redox Signaling 3*, 881−897.
7. Gibson, Q. H., and Hastings, J. W. (1962) The oxidation of reduced flavin mononucleotide by molecular oxygen, *Biochem. J. 83*, 368−377.
8. Massey, V., Palmer, G., and Ballou, D. (1973) On the reaction of reduced flavins with molecular oxygen, in *Oxidases and Related Redox Systems* (King, J. E., Mason, H. S., and Morrison, M., Eds.) Vol. 1, pp 25−43, University Park Press, Baltimore, MD.
9. Eberlein, G., and Bruice, T. C. (1983) The chemistry of a 1,5-diblocked flavin. 2. Proton and electron-transfer steps in the reaction of dihydroflavins with oxygen, *J. Am. Chem. Soc. 105*, 6685−6697.
10. Lei, B., and Tu, S.-C. (1998) Mechanism of reduced flavin transfer from *Vibrio harveyi* NADPH−FMN oxidoreductase to luciferase, *Biochemistry 37*, 14623−14629.
11. Louie, T. M., Xie, X. S., and Xun, L. (2003) Coordinated production and utilization of $FADH_2$ by NAD(P)H-flavin oxidoreductase and 4-hydroxyphenylacetate 3-monooxygenase, *Biochemistry 42*, 7509−7517.
12. Lei, B., Liu, M., Huang, S., and Tu, S.-C. (1994) *Vibrio harveyi* NADPH-flavin oxidoreductase: cloning, sequencing and overexpression of the gene and purification and characterization of the cloned enzyme, *J. Bacteriol. 176*, 3552−3558.
13. Liu, M., Lei, B., Ding, Q., Lee, J. C., and Tu, S.-C. (1997) *Vibrio harveyi* NADPH:FMN oxidoreductase: preparation and characterization of the apoenzyme and monomer−dimer equilibrium, *Arch. Biochem. Biophys. 337*, 89−95.
14. Tanner, J. J., Lei, B., Tu, S.-C., and Krause, K. L. (1996) Flavin reductase P: structure of a dimeric enzyme that reduces flavin, *Biochemistry 35*, 13531−13539.
15. Tanner, J. J., Tu, S.-C., Barbour, L. J., Barnes, C. L., and Krause, K. L. (1999) Unusual folded conformation of nicotinamide adenine dinucleotide bound to flavin reductase P, *Protein Sci. 8*, 1725−1732.
16. Jeffers, C. E., and Tu, S.-C. (2001) Differential transfers of reduced flavin cofactor and product by bacterial flavin reductase to luciferase, *Biochemistry 40*, 1749−1754.
17. Jeffers, C. E., Nichols, J. C., and Tu, S.-C. (2003) Complex formation between *Vibrio harveyi* luciferase and monomeric NADPH:FMN oxidoreductase, *Biochemistry 42*, 529−534.
18. Wang, H., Lei, B., and Tu, S.-C. (2000) *Vibrio harveyi* NADPH−FMN oxidoreductase Arg203 as a critical residue for NADPH recognition and binding, *Biochemistry 39*, 7813−7819.
19. Tu, S.-C., Becvar, J. E., and Hastings, J. W. (1979) Kinetic studies on the mechanism of bacterial NAD(P)H:flavin oxidoreductase, *Arch. Biochem. Biophys. 193*, 110−116.
20. Zenno, S., Saigo, K., Kanoh, H., and Inouye, S. (1994) Identification of the gene encoding the major NAD(P)H-flavin oxidoreductase of the bioluminescent bacterium *Vibrio fischeri* ATCC 7744, *J. Bacteriol. 176*, 3536−3543.
21. Inouye, S. (1994) NAD(P)H-flavin oxidoreductase from the bioluminescent bacterium, *Vibrio fischeri* ATCC 7744, is a flavoprotein, *FEBS Lett. 347*, 163−168.
22. Koike, H., Sasaki, H., Kobori, T., Zenno, S., Saigo, K., Murphy, M. E., Adman, E. T., and Tanokura, M. (1998) 1.8 Å crystal structure of the major NAD(P)H:FMN oxidoreductase of a bioluminescent bacterium, *Vibrio fischeri*: overall structure, cofactor and substrate-analog binding, and comparison with related flavoproteins, *J. Mol. Biol. 280*, 259−273.
23. Tang, C. K., Jeffers, C. E., Nichols, J. C., and Tu, S.-C. (2001) Flavin specificity and subunit interaction of *Vibrio fischeri* general NAD(P)H-flavin oxidoreductase FRG/FRase I, *Arch. Biochem. Biophys. 392*, 110−116.
24. Low, J. C., and Tu, S.-C. (2003) Energy transfer evidence for *in vitro* and *in vivo* complexes of *Vibrio harveyi* flavin reductase P and luciferase, *Photochem. Photobiol. 77*, 446−452.
25. Duane, W., and Hastings, J. W. (1975) Flavin mononucleotide reductase of luminous bacteria, *Mol. Cell. Biochem. 6*, 53−64.
26. Jablonski, E., and DeLuca, M. (1978) Studies of the control of luminescence in *Beneckea harveyi*: properties of the NADH and NADPH:FMN oxidoreductases, *Biochemistry 17*, 672−678.
27. Williams, C. H., Jr., Arscott, L. D., Matthews, R. G., Thorpe, C., and Wilkinson, K. D. (1979) Methodology employed for anaerobic spectrophotometric titrations and for computer-assisted data analysis, *Methods Enzymol. 62*, 185−198.
28. Wu, C.-W., and Hammes, G. G. (1973) Relaxation spectra of aspartate transcarbamylase. Interaction of the native enzyme with an adenosine 5′-triphosphate analog, *Biochemistry 12*, 1400−1408.
29. Massey, V., and Palmer, G. (1966) On the existence of spectrally distinct classes of flavoprotein semiquinones. A new method for the quantitative production of flavoprotein semiquinones, *Biochemistry 5*, 3181−3189.
30. Nivière, V., Vanoni, M. A., Zanetti, G., and Fontecave, M. (1998) Reaction of NAD(P)H:flavin oxidoreductase from *Escherichia coli* with NADPH and riboflavin: Identification of intermediates, *Biochemistry 37*, 11879−11887.
31. Tu, S.-C. (1979) Isolation and properties of bacterial luciferase-oxygenated flavin intermediate complexed with long-chain alcohols, *Biochemistry 18*, 5940−5945.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