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Forum Review

Reduced Flavin: Donor and Acceptor Enzymes and Mechanisms of Channeling

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ABSTRACT

Although mechanisms of metabolite channeling have been extensively studied, the nature of reduced flavin transfer from donor to acceptor enzymes remains essentially unexplored. In this review, identities and properties of reduced flavin-producing enzymes (namely flavin reductases) and reduced flavin-requiring processes and enzymes are summarized. By using flavin reductase–luciferase enzyme couples from luminous bacteria, two types of reduced flavin channeling were observed involving the differential transfers of the reduced flavin cofactor and the reduced flavin product of reductase to luciferase. The exact mode of transfer is controlled by the specific makeup of the constituent enzymes within the reductase–luciferase couple. The plausible physiological significance of the monomer–dimer equilibrium of the NADPH-specific flavin reductase from *Vibrio harveyi* is also discussed. Antioxid. Redox Signal. 3, 881–897.

INTRODUCTION

CONSIDERABLE RESEARCH ACTIVITIES have long been directed toward the elucidation of mechanisms of metabolite channeling from a donor enzyme/active site to an acceptor enzyme/active site. This review aims at calling attention to a new aspect of metabolite channeling involving reduced flavin as the metabolite in question. Since the first discovery of flavin cofactors in 1930s, a large number and diverse types of flavoproteins have been identified and characterized. Many of them involve the formation of two-electron-reduced flavin as an obligatory intermediate. Upon the completion of each catalytic cycle, the reduced flavin is oxidized to regenerate the original oxidized flavin cofactor. These flavoproteins do not involve the transfer of reduced flavin molecule itself from one enzyme (or active site) to another enzyme (or active site), and are not the subjects for discussion herein. Instead, this review focuses on two other classes of enzymes, with one class producing reduced flavin as a product and the second class requiring reduced flavin as a substrate or in some other way for the expression of the activity. In addition, one particular reduced flavin donor–acceptor enzyme system, namely the flavin reductase–luciferase couples from luminous bacteria, will be discussed in more detail with respect to structures, functions, and mechanisms of reduced flavin transfer.

ENZYMES THAT GENERATE REDUCED FLAVIN AS A PRODUCT

Flavin reductases

Reduced flavin is generated as a product by NAD(P)H-flavin oxidoreductases or flavin re-

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ductases (FRs), which are generally regarded as enzymes capable of catalyzing the following reaction (Eq. 1):

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

where F stands for the flavin substrate and FH_2 refers to the reduced flavin product. More specifically, in comparison with other electron acceptors for NAD(P)H oxidation, flavin reductases must be able to efficiently utilize oxidized flavin as an active, preferred, or even exclusive substrate. In old literature, the term "diaphorase" refers indiscriminately to any enzyme capable of catalyzing the reduction of artificial electron acceptors (such as dyes, ferricyanide, and quinones) by NAD(P)H. Therefore, the term "diaphorase" does not have any physiological meaning and is rarely mentioned in modern biochemical literature. Some, but not all, diaphorases are active in using flavin as an electron acceptor and could be classified as flavin reductases. However, in general, diaphorases should not be construed as the equivalent of flavin reductases. Lastly, the possibility exists that certain flavin reductases may be associated with other enzymatic activities and, hence, could be designated by alternative names.

Proposed classification/nomenclature

We have proposed that NADPH- and NADH-preferring flavin reductases be named FRP and FRD, respectively, and the general flavin reductase that utilizes NADH and NADPH with similar efficiencies be designated FRG (52). If the substrate specificity of a particular flavin reductase toward NAD(P)H is affected by the flavin cosubstrate, the pyridine nucleotide specificity should be specified in conjunction with the identity of the flavin. Flavin reductases often form a complex with their corresponding reduced flavin-acceptor enzymes and can be co-isolated (see below). Whenever possible, the NAD(P)H specificity of a flavin reductase should be classified using a flavin that is also the preferred substrate for the functionally linked flavin-acceptor enzyme. This will help with the rationalization of NAD(P)H preferences in terms of the functional roles of reduced flavin donor–acceptor enzyme complexes. We believe that the FR(P,D,G) classification is useful from an operational or functional point of view.

Drs. Vincent Nivière and Marc Fontecave and colleagues have proposed that flavin reductases be divided into a nonflavoprotein Class I and a flavoprotein Class II (61, 62). These two classes also show differences in kinetic schemes, sequences and/or protein folds. However, the term "FRase I" has been used extensively by others for a flavoprotein reductase from *Vibrio fischeri* (see below). To avoid confusion, Drs. Nivière and Fontecave have kindly agreed to reclassify the flavoprotein and nonflavoprotein reductases as Class I and Class II, respectively (personal communication).

The pyridine nucleotide specificity- and flavin content-based classifications can be specified individually (*e.g.*, FRD, FRI, etc.) or in conjunction (*e.g.*, FRD-I, FRG-II, etc.). The term "FR" can be used for flavin reductase in general and, in particular, for any flavin reductase that has not been characterized with respect to NAD(P)H specificity or flavin cofactor content. The sequences and crystal structures are known for some flavin reductases and undoubtedly will become available for additional reductases. It will be desirable to revisit the subject of classification in the near future on the basis of sequence and/or protein fold.

Known flavin reductases

A number of known flavin reductases are included in Table 1. Most of the known flavin reductases are bacterial enzymes, but some are from mammalian sources. Reductases shown in Table 1 consist of members from each of the three categories of FRP, FRD, and FRG. Whereas some of these reductases are nonflavoprotein, others have tightly bound flavin mononucleotide (FMN) or, less frequently, flavin adenine dinucleotide (FAD). Moreover, the pyridine nucleotide specificity has no direct relationship to whether flavin is required as a cofactor. The size of flavin reductase monomer is generally small, ranging from 13 to 34.5 kDa molecular mass with most of them within the 21–28 kDa range. Thus far, seven flavin reductases are known to exist as dimers, with the

Table 1. General Properties of Flavin Reductases and Functional Links*

Source	Proposed (other trivial) name	Monomer molecular mass (kDa)	Subunit structure†	Flavin cofactor	NAD(P)H specificity	Functionally linked process or enzyme	References
Vibrio harveyi	FRP-I	26.3	2M ⇆ D	FMN	NADPH	Luciferase	(22, 35, 52, 55)
Vibrio harveyi	FRD	26.7			NADH	Luciferase	(22, 35)
Vibrio harveyi, *Photobacterium phosphoreum*	FRG				NAD(P)H	Luciferase	(44, 88)
Vibrio fischeri	FRG-I (FRase I)	24.6	2M ⇆ D	FMN	NAD(P)H	Luciferase	(33, 37, 95)
Escherichia coli	FRII‡ (Fre)	26.2	M	None	NAD(P)H or NADH‡	Ribonucleotide reductase activation, superoxide generation, reductive release of iron	(9, 16, 21)
Escherichia coli	FRD-II	18.6	D	None	NADH	4-Hydroxyphenylacetate 3-hydroxylase	(20, 67, 92)
Streptomyces viridifaciens	FRP-I	21.3	≥D	FAD	NADPH	Isobutylamine hydroxylase	(64, 65)
Streptomyces coelicolor	FRD-II	18.3	2M ⇆ D	None	NADH	Monooxygenase required for actinorhodin biosynthesis	(41, 42)
Streptomyces pristinaespiralis	FRD-I	28.0		FMN	NADH	Pristinamycin II_A synthase	(3, 76)
Rhodococcus sp. IGTS8	FRD-II	25.0		None	NADH	Two monooxygenases for fossil fuel desulfurization	(24, 49, 63)
Rhodococcus sp.	FRD	18.7			NADH	Pyrrole-2-carboxylate	(2)
Chelatobacter heintzii	FRD-I	34.5	D	FMN	NADH	Nitrilotriacetate monooxygenase	(84, 91)
Bacterium isolates BNC1, DSM 9103§	FRD or FRD-I§	25	D	Flavin§	NADH	EDTA monooxygenase	(66, 89)
Pseudomonas aeruginosa	FRD	27.5			NADH	Reductive release of iron	(25)
Bacillus subtilis	FRP	13			NADPH	Chorismate synthase	(15, 26)
Bovine erythrocyte, liver	FRP	22.0			NADPH	Methemoglobin reduction	(68, 69)
Human erythrocyte	FRP-II	22.1		None	NADPH	Methemoglobin reduction	(6, 93)

*Blank spaces are for cases where the information in question is not available.

†M for monomer and D for dimer.

‡This reductase utilizes NADH and NADPH with similar efficiencies when riboflavin is the cosubstrate, but preferentially utilizes NADH with FMN and, particularly, FAD as the electron acceptor.

§The FRD from bacterium isolate DSM 9103 contains a bound chromophore showing a typical flavin type absorption spectrum.

monomer–dimer equilibria characterized in aqueous solution for three of these species. As will be elaborated later for a flavin reductase–luciferase system, such an equilibrium appears to be physiologically significant.

Three better characterized flavin reductases

The FRP (Class I) from *Vibrio harveyi* (formerly *Beneckea harveyi*), FRase I (Class FRG-I) from *Vibrio fischeri*, and Fre (Class FRII) from *Escherichia coli* are the three flavin reductases that have been characterized extensively to date.

The *V. harveyi* FRP was first detected and isolated more than two decades ago, and was found to be highly specific for NADPH (22, 34, 35). The gene encoding this reductase was later cloned, sequenced, and overexpressed (52). The expressed enzyme has been purified to apparent homogeneity and found to contain one FMN cofactor per 26-kDa monomer (52). Apoenzyme of FRP was prepared in a form suitable for reconstitution with FMN (K_d = 0.2 μM, 23°C) or 2-thioFMN (K_d = 0.3 μM, 23°C) to form active holoenzymes (55). The 1.8-Å crystal structure of this reductase has been reported (73), providing the first high-resolution structure for a flavin reductase. The crystalline FRP assumes an interlocking homodimeric form with each subunit comprising two domains and containing one bound FMN. The first and major domain consists of a four-stranded antiparallel β-sheet flanked by helices on either side. The second and smaller domain reaches out from one subunit to embrace the major domain of the other subunit. The entire *si* face of the FMN cofactor isoalloxazine ring is buried, thus necessitating the specific transfer of the pro-R hydrogen at the C-4 position of NADPH (83) to the *re* face of the FMN cofactor. The dimeric FRP crystal structure has 9,352 Å^2 of intersubunit contact surface (73), the largest among known dimeric proteins with similar sizes. Interestingly, *V. harveyi* FRP undergoes a monomer–dimer equilibrium with a K_d of 1.8 μM at 4°C (55). The crystal structure of *V. harveyi* FRP in complex with the inhibitor NAD^+ has also been determined (75). The bound NAD^+ is the most folded in comparison with that bound to any other proteins with known crystal structures. On the basis of such a structure, we hypothesized that NADPH/$NADP^+$ shuttle between an open and a closed form during *V. harveyi* FRP catalysis, with the open form of NADPH active in hydride transfer and the closed form of $NADP^+$ ready for release (75). The *V. harveyi* FRP has a nine-residue (A_{201}-S-R-T-S-N-G-K-L_{209}) loop that was not resolved in the crystal structure. Recently, we found that the Arg^{203} residue within this loop is critical for NADPH recognition and binding (87). The sequence of FRP (52) is highly similar to that of the major nitroreductase NfsA from *Escherichia coli* (51% identity) (96) and *Bacillus subtilis* (41% identity) (100). Both nitroreductases contain a tightly bound FMN and preferentially utilize NADPH. The NfsA from *B. subtilis* reduces both nitrofurazone and FMN effectively (100), whereas the substitution of the Glu^{99} residue by glycine converts the *E. coli* NfsA into a flavin reductase (99). Recently, the crystal structure of the *E. coli* NfsA has been solved at 1.7-Å resolution (44), showing considerable similarity to the structure of *V. harveyi* FRP. The peptide segment corresponding to the disordered loop in FRP was structurally resolved for NfsA.

The *Vibrio fischeri* FRase I/FRG-I, effective in using NADH or NADPH as an electron donor, has earlier been partially characterized (11, 82). The gene for this reductase was cloned, sequenced, and successfully expressed (95). Similar to the *V. harveyi* FRP, the *V. fischeri* FRase I/FRG-I is an FMN-containing flavoprotein (33) specific for the transfer of the pro-R hydrogen at the C-4 of NAD(P)H to the FMN cofactor (33). The crystal structure of this reductase has also been determined (45), showing a dimeric structure with one FMN cofactor per monomer. The overall folding of the *V. fischeri* FRase I/FRG-I is similar to that of the *V. harveyi* FRP and the NADH oxidase (NOX) from *Thermus thermophilus* (30) despite a relatively low 10–21% sequence identity. These three enzymes represent a new class of flavoprotein fold. However, it has been noted (45, 73, 75) that FRase I/FRG-I and NOX are substantially more homologous to each other than to FRP. One major structural difference is that the FRP disordered A_{201}–L_{209} loop, which contains the Arg^{203} critical to the specific recognition of

NADPH, is not present in the NADH/NADPH-indiscriminating FRase I/FRG-I or NOX. The minor nitroreductase NfsB of *E. coli*, which contains an FMN cofactor and utilizes both NADH and NADPH effectively, has been shown to resemble the *V. fischeri* FRase I/FRG-I in sequence but has a low flavin reductase activity (98). A substitution of the Phe^{124} residue by serine, however, converts the NfsB to a flavin reductase even more active than FRase I/FRG-I (97).

The *E. coli* Fre/FRII, originally isolated as a component of an enzyme complex involved in the activation of ribonucleotide reductase (16), represents a different class of flavin reductases that do not contain any flavin cofactor. The gene for this reductase has been cloned, sequenced, and overexpressed (13, 71). The Fre/FRII is a 26.2-kDa monomer capable of using riboflavin as the best substrate in accepting the pro-R hydrogen from the C-4 position of NADH and NADPH with similar efficiencies (62). However, this reductase shows a moderate and strong preference for NADH as the electron donor with FMN (13) and FAD (62), respectively, as electron acceptors. Consistent with its lack of any redox cofactor, it exhibits a sequential kinetic mechanism with ordered substrate binding of NAD(P)H followed by flavin (13, 62). Interestingly, the reductase also releases the products in a fixed order, but with reduced flavin first followed by $NADP^+$ (13) or with NAD^+ first followed by reduced flavin (62). The isoalloxazine ring of the flavin substrate is critical to the binding by Fre/FRII (13). Both the nicotinamide ring and the adenosine moiety of NADH are important to binding, whereas NADPH binds to the reductase almost exclusively through the nicotinamide ring (62). Although Fre/FRII is not a flavoprotein, several lines of consideration (60) suggest that Fre/FRII may be closely related to a flavoprotein family of which the spinach ferredoxin-$NADP^+$ reductase (FNR) is a structural prototype (5). The Fre/FRII contains a four-residue motif similar to a corresponding sequence in FNR known to interact with flavin (5). It has been shown that the Ser^{49} residue within this motif of Fre/FRII is indeed critical to flavin binding and catalytic activity (60). Mechanistic similarities also exist between Fre/FRII and FNR in that two charge transfer complex intermediates detected in the Fre/FRII reaction are similar to those observed for FNR (61). Recently, the crystal structure of Fre/FRII has been determined (32). Although the sequence similarities are low, the crystal structure of Fre/FRII provides direct evidence that it is structurally similar to the ferredoxin reductase family of flavoproteins. A major difference, however, can be noted; the AMP moiety of FAD is important to the flavin binding by the FNR family flavoproteins, but Fre/FRII does not have any AMP binding site (32). Fre/FRII-like flavin reductases have also been found in luminous bacteria *Photorhabdus luminescens*, *Vibrio fischeri*, *Vibrio harveyi*, and *Vibrio orientalis* (94). The Fre/FRII-like reductase in *V. fischeri* is a minor reductase species and is different from the major FRase I/FRG-I (94). Thus far, none of these Fre/FRII-like reductases in luminous bacteria have been well characterized.

BIOLOGICAL FUNCTIONS AND ENZYMES THAT REQUIRE REDUCED FLAVIN

At the present, the true physiological functions of free reduced flavin in biological processes have not been well established. However, some earlier and a number of more recent reports have indicated that reduced flavin produced by flavin reductases is effective in activating certain biological functions and is required as a substrate by acceptor enzymes. Examples of these flavin reductase-linked biological processes or enzymes are included in Table 1 along with their corresponding flavin reductases.

Reduced flavin as a reductant

The first type of molecular action by reduced flavin is simply for it to act as an effective reductant or electron-transfer mediator (18). In most cases, ferric complexes or iron proteins can be reduced by flavin reductases via their reduced flavin product. In microbial systems, evidence has been obtained to indicate that flavin reductase is effective in reductive activa-

tion of the iron center of ribonucleotide reductase (10, 16, 17), and in reducing ferrisiderophores for iron release (9, 25). Flavin reductases from mammalian erythrocytes and other tissues have also been shown to efficiently reduce methemoglobin (6, 68, 69, 93). In another mode of action, the *E. coli* Fre/FRII has been shown to stimulate superoxide radical production by supplying reduced flavin (21). The mechanistic roles of flavin reductases in these processes, especially under *in vivo* conditions, are still poorly understood. The fact that free NAD(P)H is not effective and flavin reductase is required for the activation of these biological functions suggests that either reduced flavin is a preferential, or possibly specific, reductant or flavin reductase can interact with the reduced flavin acceptor for a more efficient transfer of the reducing equivalent. However, it remains to be established whether or not these activities of flavin reductases mentioned above are physiologically significant.

Reduced flavin as a substrate for monofunctional flavin-dependent monooxygenases

Flavin-dependent monooxygenases (or hydroxylases) can be divided into a "bifunctional flavohydroxylase" group and a "monofunctional flavin-dependent hydroxylase" group. In earlier years, the vast majority of known flavin-dependent hydroxylases belonged to the bifunctional flavohydroxylase group, which can catalyze the following overall reaction (Eq. 2):

$$SH + NAD(P)H + H^+ + O_2 \rightarrow S\text{-}OH + NAD(P)^+ + H_2O \quad (2)$$

where SH is a substrate and S-OH is the hydroxylated product. A key feature of this class of enzymes is that they contain a tightly bound flavin cofactor and, hence, are genuine flavoproteins. The term "bifunctional" refers to the fact that a single hydroxylase species can catalyze the reduction of the tightly bound flavin cofactor by NAD(P)H in the reductive half-reaction and the subsequent oxidative half-reaction to generate oxygenated flavin intermediates (1) and, finally, the hydroxylated product. In some cases, such as salicylate hydroxylase, the substrate is decarboxylated as well as hydroxylated. No separate flavin reductase is needed by these bifunctional flavohydroxylases.

In contrast, the monofunctional flavin-dependent hydroxylases have no bound flavin cofactor and cannot reduce flavin by NAD(P)H. Instead, they require reduced flavin (FH_2) as a substrate in catalyzing the following reaction (Eq. 3):

$$SH + FH_2 + O_2 \rightarrow S\text{-}OH + F + H_2O \quad (3)$$

The required FH_2 must be acquired from an external source, converted to a flavin-4a-hydroperoxide intermediate for substrate hydroxylation and, finally, released as an oxidized flavin product. Different from the bifunctional flavohydroxylases, the monofunctional flavin-dependent hydroxylases can only catalyze the oxidative half-reaction. The essential reduced flavin substrate for these hydroxylases is believed to be supplied *in vivo* by flavin reductases. These monofunctional hydroxylases are not canonical flavoproteins for they do not contain any bound flavin cofactor. However, they are flavin-dependent on the basis of their absolute requirement for the reduced flavin substrate. The term "two-component monooxygenase (or hydroxylase)" is often used in recent publications. It should be noted that, although the enzyme complex comprising a monofunctional monooxygenase and a functionally (or structurally) linked flavin reductase can be appropriately designated as a "two-component monooxygenase," this term should not be used to specify the monooxygenase component itself within the "two-component" enzyme complex.

Luciferase from luminous bacteria catalyzes the reaction (Eq. 4):

$$R\text{-}CHO + FMNH_2 + O_2 \rightarrow R\text{-}COOH + FMN + H_2O + light \quad (4)$$

where R-CHO is a long-chain aliphatic aldehyde and R-COOH is the carboxylic acid product. For a long period of time, bacterial luciferase is the only well characterized flavin-dependent monofunctional hydroxylase. A substantial body of work has been done on this enzyme, and a number of reviews are available in the literature. Only some selected

features of this enzyme are briefly discussed herein. All known bacterial luciferases, mostly from marine sources, are $\alpha\beta$ dimers. The reaction mechanism has been extensively elucidated, especially by Hastings and colleagues (see 29 and 81 and references therein). The turnover rate of luciferase is extremely slow, ~20 min^{-1} at 23°C using decanal as a substrate. Therefore, long-lived intermediates are expected to exist. Indeed, two species of oxygenated flavin intermediates, namely the FMN-4a-hydroperoxide (27, 28, 78, 79, 85) and the FMN-4a-hydroxide (46, 47, 58), have been isolated and characterized. The crystal structures of *V. harveyi* luciferase have been determined for the $\alpha\beta$ heterodimer (14) and the β_2 homodimer (74, 77). The native $\alpha\beta$ dimer of luciferase is highly active in luminescence (29), but about four orders of magnitude lower activities have also been detected with individual subunits (86). The active species have been shown to be the α monomer and β_2 dimer (7). Interestingly, same as the native $\alpha\beta$ luciferase (51), the luciferase α and β_2 also each has a single active site for the binding of an $FMNH_2$ and an aldehyde as substrates and an aldehyde inhibitor site that overlaps with the $FMNH_2$ site (7). The exact locations of these specific binding sites are still a matter of speculation. The αHis^{44} and αHis^{45} are known to be critical to luciferase activity (90). Interestingly, the αHis^{45} is essential to the formation of the FMN-4a-hydroperoxide intermediate (53), whereas the αHis^{44} is a catalytic base functioning at a step after the formation of the FMN-4a-hydroperoxide intermediate (31).

Other members of the monofunctional flavin-dependent hydroxylase class have been discovered over the last decade. Included in Table 1 are, in addition to luciferase, 4-hydroxyphenylacetate 3-hydroxylase (67, 92), isobutylamine hydroxylase (65), pyrrole-2-carboxylate monooxygenase (2), nitrilotriacetate monooxygenase (84, 91), EDTA monooxygenase (66, 89), pristinamycin IIA synthase (3, 76), a monooxygenase required for actinorhodin biosynthesis (42), and two monooxygenases for fossil fuel desulfurization (24, 49, 63). The pristinamycin II_A synthase is actually an $FMNH_2$-requiring hydroxylase capable of catalyzing the hydroxylation of pristinamycin II_B and subsequent dehydration of the hydroxypristinamycin II_B intermediate to form the final product pristinamycin II_A (76).

Regarding the flavin substrate specificity, the 4-hydroxyphenylacetate 3-hydroxylase (67, 92) and pyrrole-2-carboxylate monooxygenase (2) preferentially use $FADH_2$, and the isobutylamine hydroxylase can utilize both $FMNH_2$ and $FADH_2$ (65). In contrast, $FMNH_2$ is required by EDTA monooxygenase (66, 89), nitrilotriacetate monooxygenase (84, 91), pristinamycin II_A synthase (76), two monooxygenases (*i.e.*, Dsz C and Dsz A) for dibenzothiophene desulfurization (24, 49, 63), and bacterial luciferase (29).

Several unusual features of monofunctional hydroxylases can be identified. First, among all known bifunctional and monofunctional flavin-dependent hydroxylases, bacterial luciferase is the only one that catalyzes a light-emitting reaction. Second, the sulfide/sulfoxide monooxygenase (Dsz C) from *Rhodococcus* sp. IGTS8 (49) and the EDTA monooxygenase (89) each catalyze two consecutive monooxygenation reactions. Third, although Eq. 3 is consistent with the reactions catalyzed by most monofunctional hydroxylases, the enzyme Dsz A from *Rhodococcus* sp. IGTS8 requires two NADH molecules per cycle of catalysis. A mechanism has been proposed to account for such a reaction stoichiometry with the Dsz A still functioning as a monooxygenase (63).

Although definitive proofs remain to be established, probably flavin reductases are functionally linked to monofunctional hydroxylases *in vivo*. A strong supporting evidence is that several species of the monofunctional hydroxylases are known to form structural complexes with their corresponding flavin reductases. These include nitrilotriacetate monooxygenase (84), EDTA monooxygenase (89), pyrrole-2-carboxylate monooxygenase (2), and, as will be described later, bacterial luciferase.

Other functions of reduced flavin

The chorismate synthase, a nonhydroxylase enzyme, catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate to chorismate in the aromatic amino acid biosynthesis pathway (15, 26). The reaction does not involve any overall change in the redox states. However, $FMNH_2$ is essential to the activity of cho-

rismate synthase (4, 56). A mechanism has been proposed (56) in which the reduced flavin first transfers one electron to the substrate, giving rise to a radical pair of flavin semiquinone and a dephosphorylated allylic radical derived from the substrate. In subsequent steps, a homolytic C-H bond cleavage of the allylic radical occurs, leading to a one-electron back transfer to the flavin semiquinone to form the chorismate product and regenerate $FMNH_2$. In such a mechanism, $FMNH_2$ has a "hidden" redox role, transforming from a dihydroflavin to a semiquinone and back to a dihydroflavin. There are also strong indications that chorismate synthase is functionally coupled to the flavin reductase. In fact, the fungal chorismate synthases from *Neurospora crasa* and *Saccharomyces cerevisiae* are bifunctional enzymes each containing intrinsic reductase and chorismate synthase activities (43). For the monofunctional chorismate synthase, it was found that the synthase from *Bacillus subtilis* exists together with a flavin reductase and a 3-dehydroquinate synthase as a trimeric enzyme complex (15).

Similar to bacterial chorismate synthase, the catalytic activity of *E. coli* DNA photolyase also involves the initial transfer of an electron from an excited state $FADH_2$ to the thymine dimer to form a flavin semiquinone and a radical anion intermediate (39, 40, 70). After additional chemical transformation, a subsequent step of electron transfer from the radical anion to the flavin semiquinone results in the formation of two thymine monomers and regeneration of the ground state $FADH_2$. The isolated photolyase contains a neutral FAD semiquinone that can be easily converted to $FADH_2$ by dithionite or photochemically. However, it is unclear how DNA photolyase acquires the $FADH_2$ *in vivo* or whether any flavin reductase is involved in such a process. Chorismate synthase- and DNA photolyase-like enzymes and other new reduced flavin-requiring enzymes are likely to be discovered in the future.

MECHANISMS OF REDUCED FLAVIN TRANSFER

Many biological functions and enzymes are dependent on reduced flavin supply for activities. However, the mechanisms of reduced flavin transfer *in vitro* or *in vivo* remain essentially unexplored at the present for any donor–acceptor system except the flavin reductase–bacterial luciferase couple. Information revealed by studies on the latter system is summarized below.

Direct channeling of reduced flavin from reductase to luciferase

In general, there are at least three possible mechanisms for the acceptor protein or enzyme to acquire reduced flavin: (a) free diffusion of reduced flavin to acceptor, (b) reduction of acceptor-bound oxidized flavin by an external reductant including free or bound reduced flavin, and (c) direct channeling of reduced flavin from a donor to an acceptor. Conceivably, all three mechanisms could be operational *in vitro* or *in vivo*. However, free diffusion cannot be an efficient way for reduced flavin transfer. Reduced flavin is sensitive to rapid autooxidation following an autocatalytic process (12, 23, 57):

$$FH_2 + O_2 \rightarrow F + H_2O_2 \text{ (slow)} \quad (5)$$

$$FH_2 + F \rightarrow 2\ FH^{\cdot} \quad (6)$$

$$FH^{\cdot} + O_2 \rightarrow F + O_2^{-\cdot} + H^+ \quad (7)$$

$$FH^{\cdot} + O_2^{\cdot -} \rightarrow F + H_2O_2 \quad (8)$$

Initially, the reduced flavin (FH_2) is oxidized directly but slowly by oxygen to generate oxidized flavin (F) and hydrogen peroxide (Eq. 5), accounting for only a few percent of the total oxidized flavin formation. As soon as some oxidized flavin is formed, a one-electron transfer between FH_2 and F leads to the formation of two molecules of flavin semiquinone ($FH^{\cdot}$) (Eq. 6). One flavin semiquinone then reacts with oxygen to generate F and superoxide radical (Eq. 7). A second molecule of the flavin semiquinone subsequently reacts with the superoxide radical to form F and H_2O_2 (Eq. 8). In an air-saturated aqueous solution, $FMNH_2$ has a half-life of only ~25 ms. Autooxidation of reduced flavin will not only waste otherwise useful reducing equivalents, but will also generate harmful oxygen species. Although bacterial luciferase can utilize exogenously added free

$FMNH_2$ for the luminescence reaction *in vitro*, free diffusion is not likely to be either an efficient or a major way to deliver $FMNH_2$ to luciferase *in vivo*.

In connection with the second possible mechanism, the resting state of bacterial luciferase binds $FMNH_2$ tightly, but does not bind oxidized FMN appreciably. However, the oxidized FMN product formed in each cycle of catalysis is released slowly from luciferase due to a slow decomposition of the ternary enzyme-product complex of luciferase, FMN, and fatty acid (54). It is conceivable that the luciferase-bound FMN product can be reduced by external reductant (reduced flavin included) coming into contact with the bound FMN. The luciferase–flavin complex is then reduced and recharged for the next cycle of catalysis. At present, results of reductase–luciferase coupled reaction *in vitro* are inconsistent with such a mechanism (see below), but no sufficient information is available to rule in or out this mechanistic possibility *in vivo*.

Regarding the direct channeling mechanism, evidence was made available more than two decades ago to suggest that $FMNH_2$ can be directly transferred from reductases to luciferase. It can be reasoned that the K_m values of reductase for the flavin and pyridine nucleotide substrates should be the same in both the reductase single-enzyme assay and the reductase–luciferase coupled assay if the latter uses truly excess amounts of luciferase and luciferase receives $FMNH_2$ by free diffusion. On the other hand, knowing that the turnover rate of luciferase is markedly slower than those of reductases, the overall efficiency of a reductase–luciferase functional complex will be limited by the much slower luciferase. Therefore, FMN and NAD(P)H at levels much lower than the $K_{m,FMN}$ and $K_{m,NAD(P)H}$ of reductase in the single-enzyme assay would be sufficient for 50% maximal bioluminescence emission in the coupled assay involving a luciferase–reductase functional complex and direct channeling of $FMNH_2$ within the complex. The same argument can be made in comparing the substrate levels optimal for reductase in the single-enzyme and coupled assays. For simplicity, the subscripts F and H will be used to refer to *V. fischeri* and *V. harveyi*, respectively, as the bacterial sources of luciferase (L) and flavin reductase, and the FRase I/FGR-I from *V. fischeri* will be abbreviated as FRG_F. Duane and Hastings (11) found that the optimal concentration of FMN for FRG_F activity in a coupled assay containing excess L_F was nearly 100 times lower than that in the FRG_F single-enzyme spectrophotometric assay. Jablonski and DeLuca (35) also observed that the K_m values for FMN and reduced pyridine nucleotide for FRD_H and FRP_H determined by the L_H-coupled assays were substantially lower than those in the respective single-enzyme assays (Table 2). These findings were interpreted to indicate a direct transfer of $FMNH_2$ from reductase to luciferase in a functional complex. Similar studies were carried out more recently for FRP_H and FRG_F in single-enzyme and coupled assays using various reductase–luciferase combinations (Table 2). In all cases, substantially lower K_m values were observed in coupled assays

TABLE 2. KINETIC PROPERTIES OF FRG_F, FRD_H, AND FRP_H INDIVIDUALLY AND IN COUPLED REACTIONS WITH L_H AND L_F

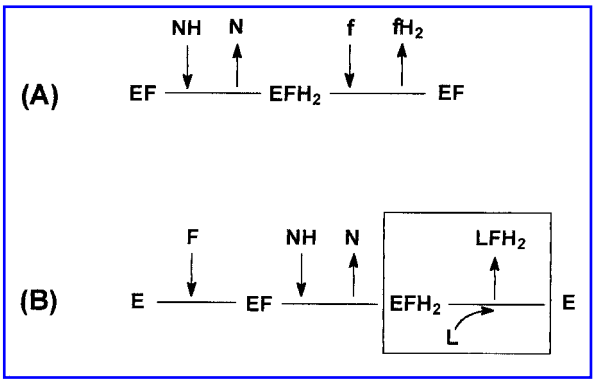
Enzyme system	$K_{m,FMN}$ (μM)	$K_{m,NADH}$ (μM)	$K_{m,NADPH}$ (μM)	*Kinetic mechanism*	*Reference*
FRP_H	14		40	Ping-pong	(35)
	8		20	Ping-pong	(50)
FRP_H-L_H	2.5		1.6		(35)
	0.3		0.02	Sequential	(50)
FRP_H-L_F	0.2		1.1	Sequential	(37)
FRD_H	1.0	47		Sequential	(35)
FRD_H-L_H	0.35	5.0			(35)
FRG_FF	73	80			(11)
	220	120		Ping-pong	(82)
FRG_F-L_F	0.05	3.8		Sequential	(37)
FRG_F-L_H	4	9		Ping-pong	(50)

than in reductase single-enzyme assays. Therefore, a direct transfer of $FMNH_2$ was indicated not only for FRP_H-L_H and FRG_F-L_F, but also for the FRG_F-L_H and FRP_H-L_F mixed couples.

Direct transfer of reduced flavin cofactor of reductase to luciferase

The mechanisms of reduced flavin transfer from reductase to luciferase have been examined by using L_H and L_F in various combinations with FRP_H and FRG_F. The FRP_H-L_H couple was first examined in detail, and the results led to the conclusion that FRP_H directly transfers its reduced flavin cofactor rather than the reduced flavin product to L_H as the major pathway for the coupled luminescence reaction (50). The supporting evidence is derived from three lines of studies.

Change of kinetic mechanism. FRP_H exhibits a ping-pong mechanism in the single-enzyme assay (Table 2). As FRP_H has a bound FMN cofactor, the observed ping-pong mechanism can be accounted for by Scheme 1A. For Scheme 1A and other schemes: E is the reductase apoenzyme; N and NH are, respectively, the oxidized and reduced pyridine nucleotide; F and FH_2 are, respectively, the oxidized and reduced flavin cofactor; f and fH_2 are, respectively, the oxidized flavin substrate and reduced flavin product. Interestingly, a sequential mechanism was observed for FRP_H in the L_H-coupled luminescence reaction (50). This is interpreted to indicate a direct transfer of reductase reduced flavin cofactor to luciferase (Scheme 1B). Initially, the NADPH binds to the oxidized FRP holoenzyme (EF). The FMN cofactor is then reduced and $NADP^+$ is released as a product. The $FMNH_2$ cofactor of the reduced reductase (EFH_2) is then directly transferred to luciferase for the bioluminescence reaction, and the reductase is left as an apoenzyme. The binding of the exogenously added flavin to the apo-reductase regenerates the original oxidized holoenzyme. When rearranged to have the apoenzyme as the starting enzyme species, FRP_H now follows a sequential mechanism in the L_H-coupled reaction (Scheme 1B). FRG_F also follows a ping-pong mechanism in the single-enzyme assay (82). The kinetic mechanisms of FRP_H and FRG_F were found (37) to switch over to the sequential pattern in the FRP_H-L_F and FRG_F-L_F coupled reactions (Table 2). Therefore, the same Scheme 1B can also be followed to support a direct transfer of reductase reduced flavin cofactor to luciferase in the FRP_H-L_F and FRG_F-L_F couples.



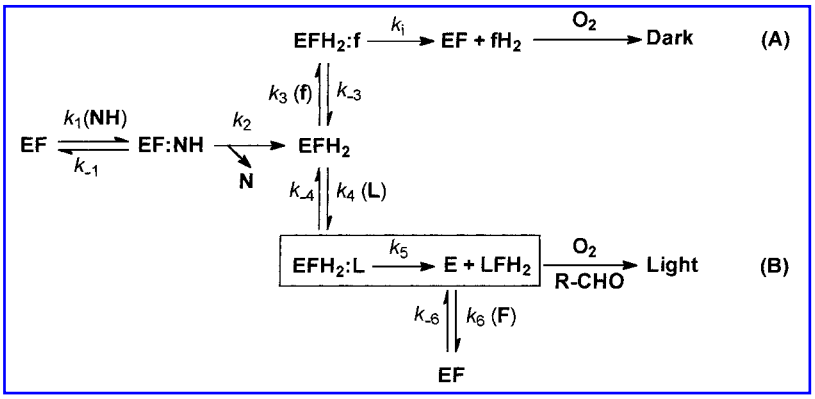
SCHEME. 1. (**A**) Ping-pong mechanism of FRP_H and FRG_F in the respective single-enzyme reactions. (**B**) Sequential mechanism for the FRP_H-L_H, FRP_H-L_F, and FRG_F-L_F coupled reactions with steps of the direct transfer of reductase reduced flavin cofactor to luciferase highlighted by the enclosing box. (Adapted from Lei B and Tu S-C. *Biochemistry* 37: 14623–14629, 1998.)

Inhibition of coupled luminescence reaction by flavin. Scheme 2 was proposed for further kinetic analyses of the FRP_H-L_H coupled reaction (50). Scheme 2B is a more detailed version of Scheme 1B based on a direct transfer of reductase reduced flavin cofactor to luciferase (highlighted by the enclosing box). In addition, Scheme 2A shows that exogenously added flavin (f) is expected to compete against luciferase in forming a complex with the reduced reductase (EFH_2) to generate the fH_2 product, which undergoes a rapid and dark autooxidation. Following Scheme 2, kinetic rate (Eqs. 9 and 10) can be obtained on the basis of the original analyses by Lei and Tu (50):

$$\frac{1}{\nu} = \frac{C_1 + C_2[L] + C_3[f]}{C_4[L]V_{max}} + \frac{C_5[L] + C_6[f]}{C_4[L]V_{max}} \frac{1}{[NH]} \quad (9)$$

$$\frac{1}{\nu} = \frac{C_5 + C_2[NH]}{C_4[NH]V_{max}} + \frac{C_5[NH] + \{C_6 + C_3[NH]\}[f]}{C_4[NH]V_{max}} \frac{1}{[L]} \quad (10)$$

In both equations, ν and V_{max} are, respectively, the observed and the maximal intensi-



SCHEME 2. Light and dark pathways in the FRP_H-L_H, FRP_H-L_F, and FRG_F-L_F coupled reactions. Steps of the direct transfer of reductase reduced flavin cofactor to luciferase are highlighted by the enclosing box. (Adapted from Lei B and Tu S-C. *Biochemistry* 37: 14623–14629, 1998.)

ties of the coupled luminescence reaction, and C_1 through C_6 are constants (50). Equation 9 is arranged in a form for the analysis of the inhibitory effect of FMN against varying concentrations of NADPH at a constant level of luciferase. In double-reciprocal plots of $1/V$ versus 1/[NH], [f] appears in both the (ordinate) intercept term and the slope term. Hence, high concentrations of exogenously added FMN should be a noncompetitive (*i.e.*, including the conventional noncompetitive and the mixed) inhibitor against NADPH. On the other hand, Eq. 10 is shown for the examination of the FMN inhibition against varying concentrations of luciferase using a constant amount of NADPH. Only the slope term of the $1/\nu$ versus 1/[L] double reciprocal plot is affected by [f]. Therefore, FMN should be a competitive inhibitor against luciferase. Experimental results indicated that, indeed, FMN at concentrations higher than the $K_{m,FMN}$ in the coupled reaction was a noncompetitive inhibitor against NADPH and a competitive inhibitor against luciferase in the FRP_H-L_H coupled reaction (50).

The same tests were extended to the FRP_H-L_F and FRG_F-L_F coupled reactions. Similar to the FRP_H-L_H system, high concentrations of FMN inhibited both the FRP_H-L_F and the FRG_F-L_F coupled reactions noncompetitively against reduced pyridine nucleotide and competitively against L_F (37).

2-ThioFMN as a mechanistic probe. Apoenzymes of FRP_H and FRG_F can bind 2-thioFMN as a cofactor to produce active holoenzymes $FRP_{S,H}$ (55) and $FRG_{S,F}$ (72), respectively. It is also known that 2-thioFMNH_2 is an active substrate for luciferase, but the luminescence quantum yield is markedly lower than that obtainable with $FMNH_2$ (59, 79). These properties make 2-thioFMN an invaluable mechanistic probe for distinguishing the direct transfer of the reduced flavin cofactor from the reduced flavin product of reductase to luciferase. FMN was used as a common substrate for various coupled reactions using reductases containing either FMN or 2-thioFMN as a cofactor. The rates of NAD(P)H oxidation by the reductases in these coupled reactions were measured spectrophotometrically in duplicate samples. The observed initial luminescence intensities were normalized to the same rate of NAD(P)H oxidation (hence the same rate of $FMNH_2$ product formation). The normalized initial luminescence intensities for the $FRP_{S,H}$-L_H and FRP_H-L_H coupled reactions should be the same if the reductase directly transfers the reduced flavin product (*i.e.*, $FMNH_2$) to luciferase for luminescence. On the other hand, FRP_H and $FRP_{S,H}$ will generate $FMNH_2$ and 2-thioFMNH_2 cofactor, respectively, at the onset of the coupled reaction. Therefore, the normalized initial light intensity of the $FRP_{S,H}$-L_H coupled reaction should be markedly lower than that of the FRP_H-L_H coupled reaction if the reductase reduced flavin cofactor is directly transferred to luciferase for luminescence. In an experimental test, the normalized initial light intensity of the $FRP_{S,H}$-L_H couple was only 6% of that for the FRP_H-L_H couple (Table 3). This provides strong support for the direct transfer

Table 3. Relative Initial Coupled Luminescence Intensity Using FMN as a Substrate for Different Reductase–Luciferase Coupled Reactions

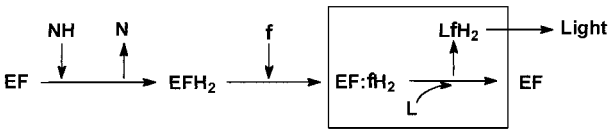
	*Relative light intensity**	*Reference*
$(FRP_{S,H}\text{-}L_H)/(FRP_H\text{-}L_H)$	6.0×10^{-2}	(50)
$(FRP_{S,H}\text{-}L_F)/(FRP_H\text{-}L_F)$	4.9×10^{-4}	(37)
$(FRG_{S,F}\text{-}L_F)/(FRG_F\text{-}L_F)$	7.2×10^{-2}	(37)
$(FRG_{S,F}\text{-}L_H)/(FRG_F\text{-}L_H)$	1.8	(37)

*For all coupled reactions, the initial light intensities measured at 5 s after the addition of the flavin reductase to initiate the reaction were normalized to correspond to a constant level of NAD(P)H oxidation rate.

of the reduced flavin cofactor of FRP_H to L_H. Similar tests were also carried out, and much lower initial light intensities were obtained for $FRP_{S,H}$-L_F in comparison with FRP_H-L_F and for $FRG_{S,F}$-L_F in comparison with FRG_F-L_F (Table 3). Therefore, a direct transfer of the reductase reduced flavin cofactor to luciferase as the major pathway is also shown for FRP_H-L_F and FRG_F-L_F.

Direct transfer of reduced flavin product of reductase to luciferase

A quite different pattern of reduced flavin transfer was detected with the FRG_F-L_H couple. A direct channeling of $FMNH_2$ from FRG_F to L_H was indicated by the much lower $K_{m,FMN}$ and $K_{m,NADH}$ in the coupled assay in comparison with those determined in the FRG_F single-enzyme assay (Table 2). However, the ping-pong mechanism of FRG_F in the single-enzyme assay remained unchanged in the L_H-coupled assay (Table 2), indicating a direct transfer of the reductase reduced flavin product to luciferase in the FRG_F-L_H coupled reaction (Scheme 3). On the basis of this scheme, exogenously added flavin is not expected to have any inhibitory effect on the light intensity of the coupled reaction. Indeed, no inhibition of the luminescence activity of the FRG_F-L_H coupled reaction was detected using FMN up to 120 μM (37). The 2-thioFMN probe was also used to test the nature of reduced flavin transfer in the FRG_F-L_H couple (37). By using FMN as a common substrate, the normalized initial luminescence intensity of the $FRG_{S,F}$-L_H couple was similar to that of the FRG_F-L_H couple (Table 3). Therefore, all available experimental results support the conclusion that FRG_F directly transfers the reduced flavin product to L_H as a major pathway in the coupled reaction.



SCHEME 3. Ping-pong mechanism of the FRG_F-L_H coupled reaction. Steps of the direct transfer of reductase reduced flavin product to luciferase are highlighted by the enclosing box.

Additional remarks

FRP_H and FRG_F are similar with respect to crystal structure (45, 73), protein size and sequence (52, 95), FMN cofactor content (33, 52), and monomer–dimer equilibrium (36, 55). The *V. harveyi* and *V. fischeri* luciferases are also similar in sequence and subunit structure (8, 19, 38). Despite these similarities, our results indicate two types of reduced flavin transfer mechanisms. A direct transfer of the reductase reduced flavin cofactor to luciferase occurs within FRP_H-L_H, FRP_H-L_F, and FRG_F-L_F whereas FRG_F transfers its reduced flavin product to L_H for the coupled luminescence reaction. The mechanism of reduced flavin transfer appears not to be unilaterally controlled by either the reductase or luciferase, but is dictated by the specific makeup of the constituent enzymes in the reductase–luciferase couple.

COMPLEX OF REDUCTASE AND LUCIFERASE

Regardless of the detailed mechanism, a direct channeling of reduced flavin from a donor to an acceptor requires the formation of the donor–acceptor complex, either steady or transient. *V. harveyi* luciferase immobilized onto cyanogen bromide-treated Sepharose appeared to bind the FRD_H activity in a crude sample, and exhibited an enhanced bioluminescence activity in the coupled reaction (80). However, direct physical evidence for complex formation in solution between any species of bacterial flavin reductase and luciferase has never been reported until recently for the *V. harveyi* FRP and

luciferase by fluorescence anisotropy measurements (36). The FRP_H apoenzyme was labeled at 1:1 molar ratio by the fluorescent probe eosin, and active holoenzyme was reconstituted from the labeled apoenzyme and FMN. Both the labeled reductase holoenzyme and apoenzyme undergo a monomer–dimer equilibrium with respective K_d values of 1.3 and 2.0 μM (36) similar to the 1.8 μM K_d of the native FRP_H (55). By using low concentrations (*e.g.*, 0.13 μM) of the labeled FRP_H apoenzyme or holoenzyme to ensure the near-complete dissociation of the reductase, titration with *V. harveyi* luciferase led to marked increases in the fluorescence anisotropy of the reductase-bound eosin, indicating a complex formation between luciferase and reductase. The K_d values for the complex formation with luciferase were found to be 7 and 11 μM for the labeled apoenzyme and holoenzyme, respectively. In contrast, when the dimeric form was the predominant species at a high concentration (*e.g.*, 13 μM) of the labeled reductase apoenzyme or holoenzyme, titration with luciferase led to no detection of complex formation by fluorescence anisotropy measurements. All the kinetic data discussed earlier for the direct transfer of $FMNH_2$ from FRP_H to L_H were obtained with the FRP_H existing in the monomeric form, which is active in binding to luciferase.

The finding that only monomeric FRP_H forms a complex with L_H is likely to be physiologically relevant. By measuring the cellular volume and the enzyme contents in cells grown to maximal luminescence intensity, the intracellular concentrations of *Vibrio harveyi* FRP and luciferase were estimated to be 3 and 170 μM, respectively (36). The $\sim$3 μM *in vivo* concentration of FRP_H is close to the 1.8 μM K_d of FRP_H monomer–dimer equilibrium. Moreover, luciferase at $\sim$170 μM is at a saturating level for complex formation with monomeric FRP_H. The conversion of existing FRP_H monomers to complexes with luciferase will trigger further dissociation of any remaining FRP_H dimers, resulting in essentially a complete dissociation of FRP_H and complex formation with luciferase. Thus, the *in vivo* luminescence appears to be regulated by the limiting cellular content of FRP_H. As FRP_H is essentially all in complex with luciferase, nonproductive oxidation of NADPH *in vivo* by free FRP_H monomers or dimers can be kept at a minimum.

At the present, the field of structural and mechanistic delineation of reduced flavin transfer is still in its infancy. In addition to the reductase–luciferase system from luminous bacteria, a number of other reduced flavin donor–acceptor enzyme couples have already been identified, and undoubtedly more will be discovered in the near future. This author believes that a significant and rapid advancement of this young field in the coming years can be optimistically anticipated.

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ABBREVIATIONS

E and EF, apoenzyme and holoenzyme, respectively, of flavin reductase; F, as subscript, *Vibrio fischeri*; f and fH_2, oxidized and reduced flavins, respectively, that bind to or dissociate from the flavin reductase substrate (or product) site; F and FH_2, oxidized and reduced flavins, respectively, that bind to or dissociate from the flavin reductase cofactor site; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FNR, ferredoxin-$NADP^+$ reductase; FR, flavin reductase; FRD, NADH-preferring flavin reductase; FRG, general flavin reductase that utilizes NADH and NADPH with similar efficiencies; $FRG_{S,F}$, FRG_F holoenzyme containing 2-thioFMN as the flavin cofactor; FRP, NADPH-preferring flavin reductase; $FRP_{S,H}$, FRP_H holoenzyme containing 2-thiolFMN as the flavin cofactor; H, as subscript, *Vibrio harveyi*, L, luciferase; N and NH, $NAD(P)^+$ and NAD(P)H, respectively; NOX, NADH oxidase.

REFERENCES

1. Ballou DP. Flavoprotein monooxygenases. In: *Flavins and Flavoproteins*, edited by Bray RC, Engel PC, and Mayhew SG. Berlin: Walter de Gruyter, 1984, pp. 605–618.
2. Becker D, Schrader T, and Andreesen JR. Two-component flavin-dependent pyrrole-2–carboxylate monooxygenase from *Rhodococcus* sp. *Eur J Biochem* 249: 739–747, 1997.
3. Blanc V, Lagneaux D, Didier P, Gil P, Lacroix P, and Crouzet J. Cloning and analysis of structural genes from *Streptomyces pristinaespiralis* encoding enzymes involved in the conversion of pristinamycin IIB to pristinamycin IIA (PIIA): PIIA synthase and NADH:riboflavin 5′-phosphate oxidoreductase. *J Bacteriol* 177: 5206–5214, 1995.
4. Bornemann S, Ramjee MN, Lowe DJ, Thorneley RNF, Coggins JR, Abell C, Balasubramanian S, Hawkes TR, Nichols WW, and Davies GM. Studies on *Escherichia coli* chorismate synthase. In: *Flavins and Flavoproteins*, edited by Yagi K. Berlin: Walter de Gruyter, 1994, pp. 843–846.
5. Bruns CM and Karplus PA. Refined crystal structure of spinach ferredoxin reductase at 1.7 Å resolution: oxidized, reduced and 2′-phospho-5′-AMP bound states. *J Mol Biol* 247: 125–145, 1995.
6. Chikuba K, Yubisui T, Shirabe K, and Takeshita M. Cloning and nucleotide sequence of a cDNA of the human erythrocyte NADPH-flavin reductase. *Biochem Biophys Res Commun* 198: 1170–1176, 1994.
7. Choi H, Tang C-K, and Tu S-C. Catalytically active forms of the individual subunits of *Vibrio harveyi* luciferase and their kinetic and binding properties. *J Biol Chem* 270: 16813–16819, 1995.
8. Cohn DH, Mileham AJ, Simon MI, Nealson KH, Rausch SK, Bonam D, and Baldwin TO. Nucleotide sequence of the *luxA* gene of *Vibrio harveyi* and the complete amino acid sequence of the α subunit of bacterial luciferase. *J Biol Chem* 260: 6139–6146, 1985.
9. Coves J and Fontecave M. Reduction and mobilization of iron by a NAD(P)H:flavin oxidoreductase from *Escherichia coli. Eur J Biochem* 211: 635–641, 1993.
10. Coves J, Delon B, Climent I, Sjoberg BM, and Fontecave M. Enzymic and chemical reduction of the iron center of the *Escherichia coli* ribonucleotide reductase protein R2. The role of the C-terminus. *Eur J Biochem* 233: 357–363, 1995.
11. Duane W and Hastings JW. Flavin mononucleotide reductase of luminous bacteria. *Mol Cell Biochem* 6: 53–64, 1975.
12. Eberlein G and Bruice TC. 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, 1983.
13. Fieschi F, Nivière V, Frier C, Décout J-L, and Fontecave M. The mechanism and substrate specificity of the NADPH:flavin oxidoreductase from *Escherichia coli. J Biol Chem* 270: 30392–30400, 1995.
14. Fisher AJ, Thompson TB, Thoden JB, Baldwin TO, and Rayment I. The 1.5-Å resolution crystal structure of bacterial luciferase in low salt conditions. *J Biol Chem* 271: 21956–21968, 1996.
15. Fitzpatrick T, Amrhein N, and Macheroux P. Analysis of a trimeric complex involving chorismate synthase from *Bacillus subtilis*. In: *Flavins and Flavoproteins 1999*, edited by Ghisla S, Kroneck P, Macheroux P, and Sund H. Berlin: Agency for Scientific Publications, 1999, pp. 749–752.
16. Fontecave M, Eliasson R, and Reichard P. NAD(P)H:flavin oxidoreductase of *Escherichia coli*. A ferric iron reductase participating in the generation of the free radical of ribonucleotide reductase. *J Biol Chem* 262: 12325–12331, 1987.
17. Fontecave M, Eliasson R, and Reichard P. Enzymatic regulation of the radical content of the small subunit of *Escherichia coli* ribonucleotide reductase involving reduction of its redox centers. *J Biol Chem* 264: 9164–9170, 1989.
18. Fontecave M, Coves J, and Pierre JL. Ferric reductases or flavin reductases? *Biometals* 7: 3–8, 1994.
19. Foran DR and Brown WM. Nucleotide sequence of the *lux*A and *lux*B genes of the bioluminescent marine bacterium *Vibrio fischeri. Nucleic Acids Res* 16: 777, 1988.
20. Galán B, Díaz E, Prieto MA, and García JL. Functional analysis of the small component of the 4-hydroxyphenylacetate 3-monooxygenase of *Escherichia coli* W: a prototype of a new flavin:NAD(P)H reductase subfamily. *J Bacteriol* 182: 627–636, 2000.
21. Gaudu P, Touati D, Niviere V, and Fontecave M. The NAD(P)H:flavin oxidoreductase from *Escherichia coli* as a source of superoxide radicals. *J Biol Chem* 269: 8182–8188, 1994.
22. Gerlo E and Charlier J. Identification of NADH-specific and NADPH-specific FMN reductases in *Beneckea harveyi. Eur J Biochem* 57: 461–467, 1975.
23. Gibson QH and Hastings JW. The oxidation of reduced flavin mononucleotide by molecular oxgen. *Biochem* J 83: 368–377, 1962.
24. Gray KA, Pogrebinsky OS, Mrachko GT, Xi L, Monticello DJ, and Squires CH. Molecular mechanisms of biocatalytic desulfurization of fossil fuels. *Nat Biotechnol* 4: 1705–1709, 1996.
25. Hallé F, and Meyer JM. Iron release from ferrisiderophores. A multi-step mechanism involving a NADH/FMN oxidoreductase and a chemical reduction by $FMNH_2$. *Eur J Biochem* 209: 621–627, 1992.
26. Hasan N and Nester EW. Purification and characterization of NADPH-dependent flavin reductase. An enzyme required for the activation of chorismate synthase in *Bacillus subtilis. J Biol Chem* 253: 4987–4992, 1978.
27. Hastings JW and Balny C. The oxygenated bacterial luciferase-flavin intermediate. Reaction products via the light and dark pathways. *J Biol Chem* 250: 7288–7293, 1975.
28. Hastings JW, Balny C, LePeuch C, and Douzou P. Spectral properties of an oxygenated luciferase-flavin intermediate isolated by low-temperature chro-

matography. *Proc Natl Acad Sci U S A* 70: 3468–3472, 1973.
29. Hastings JW, Potrikus CJ, Gupta SC, Kurfürst M, and Makemson JC. Biochemistry and physiology of bioluminescent bacteria. *Adv Microb Physiol* 26: 235–291, 1985.
30. Hecht HJ, Erdmann H, Park HJ, Sprinzl M, and Schmid RD. Crystal structure of NADH oxidase from *Thermus thermophilus*. *Nat Struct Biol* 2: 1109–1114, 1995.
31. Huang S and Tu S-C. Identification and characterization of a catalytic base in bacterial luciferase by chemical rescue of a dark mutant. *Biochemistry* 36: 14609–14615, 1997.
32. Ingelman M, Ramaswamy S, Nivière V, Fontecave M, and Eklund H. Crystal structure of NAD(P)H:flavin oxidoreductase from *Escherichia coli*. *Biochemistry* 38: 7040–7049, 1999.
33. Inouye S. NAD(P)H-flavin oxidoreductase from the bioluminescent bacterium, *Vibrio fischeri* ATCC 7744, is a flavoprotein. *FEBS Lett* 347: 163–168, 1994.
34. Jablonski E and DeLuca M. Purification and properties of the NADH and NADPH specific FMN oxidoreductases from *Beneckea harveyi*. *Biochemistry* 16: 2932–2936, 1977.
35. Jablonski E and DeLuca M. Studies of the control of luminescence in *Beneckea harveyi*: properties of the NADH and NADPH:FMN oxidoreductases. *Biochemistry* 17: 672–678, 1978.
36. Jeffers CE. *Identification and Characterization of Interactions Between NAD(P)H:FMN Oxidoredoctase and Luciferase*. Ph.D. thesis, University of Houston, 2000.
37. Jeffers CE and Tu S-C. Differential transfers of reduced flavin cofactor and product by bacterial flavin reductase to luciferase. *Biochemistry* 40: 1749–1754, 2001.
38. Johnston TC, Thompson RB, and Baldwin TO. Nucleotide sequence of the *lux*B gene of *Vibrio harveyi* and the complete amino acid sequence of the β subunit of bacterial luciferase. *J Biol Chem* 261: 4805–4811, 1986.
39. Jorns MS. The catalytic mechanism of DNA photolyase. In: *Flavins and Flavoproteins*, edited by Edmondson DE and McCormick DB. Berlin: Walter de Gruyter, 1987, pp. 233–246.
40. Jorns MS. Role of flavoproteins in light-dependent reactions. In: *Flavins and Flavoproteins*, edited by Stevenson KJ, Massey V, and Williams CH Jr. Calgary, Alberta: University of Calgary Press, 1997, pp. 103–112.
41. Kendrew SG, Harding SE, Hopwood DA, and Marsh EN. Identification of a flavin:NADH oxidoreductase involved in the biosynthesis of actinorhodin. Purification and characterization of the recombinant enzyme. *J Biol Chem* 270: 17339–17343, 1995.
42. Kendrew SG, Hopwood DA, and Marsh EN. Identification of a monooxygenase from *Streptomyces coelicolor* A3(2) involved in biosynthesis of actinorhodin: purification and characterization of the recombinant enzyme. *J Bacteriol* 179: 4305–4310, 1997.
43. Kitzing K, Amrhein N, and Macheroux P. Overexpression of the bifunctional chorismate synthase of *Neurospora crassa*. In: *Flavins and Flavoproteins*, edited by Ghisla S, Kroneck P, Macheroux P, and Sund H. Berlin: Agency for Scientific Publications, 1999, pp. 203–206.
44. Kobori T, Sasaki H, Lee WC, Zenno S, Saigo K, Murphy KEP, and Tanokura M. Structure and site-directed mutagenesis of a flavoprotein from *Escherichia coli* that reduces nitrocompounds. Alteration of pyridine nucleotide binding by a single amino acid substitution. *J Biol Chem* 276: 2816–2823, 2001.
45. Koike H, Sasaki H, Kobori T, Zenno S, Saigo K, Murphy ME, Adman ET, and Tanokura M. 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, 1998.
46. Kurfürst M, Ghisla S, and Hastings JW. Characterization and postulated structure of the primary emitter in the bacterial luciferase reaction. *Proc Natl Acad Sci U S A* 81: 2990–2994, 1984.
47. Kurfürst M, Macheroux P, Ghisla S, and Hastings JW. Isolation and characterization of the transient, luciferase-bound flavin-4a-hydroxide in the bacterial luciferase reaction. *Biochim Biophys Acta* 924: 104–110, 1987.
48. Lavi JT, Raunio RP, and Stahlberg TH. Affinity purification of bacterial luciferase and NAD(P)H:FMN oxidoreductases by FMN-Sepharose for analytical applications. *J Biolumin Chemilumin* 5: 187–192, 1990.
49. Lei B and Tu S-C. Gene overexpression, purification, and identification of a desulfurization enzyme from *Rhodococcus* sp. strain IGTS8 as a sulfide/sulfoxide monooxygenase. *J Bacteriol* 178: 5699–5705, 1996.
50. Lei B and Tu S-C. Mechanism of reduced flavin transfer from *Vibrio harveyi* NADPH-FMN oxidoreductase to luciferase. *Biochemistry* 37: 14623–14629, 1998.
51. Lei B, Cho KW, and Tu S-C. Mechanism of aldehyde inhibition of *Vibrio harveyi* luciferase. Identification of two aldehyde sites and relationship between aldehyde and flavin binding. *J Biol Chem* 269: 5612–5618, 1994.
52. Lei B, Liu M, Huang S, and Tu S-C. *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, 1994.
53. Li H, Ortego BC, Maillard KI, Willson RC, and Tu S-C. Effects of mutations of the αHis45 residue of *Vibrio harveyi* luciferase on the yield and reactivity of the flavin peroxide intermediate. *Biochemistry* 38: 4409–4415, 1999.
54. Li Z and Meighen EA. The turnover of bacterial luciferase is limited by a slow decomposition of the ternary enzyme-product complex of luciferase, FMN, and fatty acid. *J Biol Chem* 269: 6640–6644, 1994.

55. Liu M, Lei B, Ding Q, Lee JC, and Tu S-C. *Vibrio harveyi* NADPH:FMN oxidoreductase: preparation and characterization of the apoenzyme and monomer–dimer equilibrium. *Arch Biochem Biophys* 337: 89–95, 1997.
56. Macheroux P, Bornemann S, and Thorneley RNF. Role of reduced FMN in the catalytic mechanism of chorismate synthase. In: *Flavins and Flavoproteins*, edited by Stevenson KJ, Massey V, and Williams CH Jr. Calgary, Alberta: University of Calgary Press, 1997, pp. 113–122.
57. Massey V, Palmer G, and Ballou D. On the reaction of reduced flavins with molecular oxygen. In: *Oxidases and Related Redox Systems*, edited by King JE, Mason HS, and Morrison M. Baltimore, MD: University Park Press, 1973, pp. 25–43.
58. Matheson IBC, Lee J, and Müller F. Bacterial bioluminescence: spectral study of the emitters in the *in vitro* reaction. *Proc Natl Acad Sci U S A* 78: 948–952, 1981.
59. Mitchell G and Hastings JW. The effect of flavin isomers and analogues upon the color of bacterial bioluminescence. *J Biol Chem* 244: 2572–2576, 1969.
60. Nivière V, Fieschi F, Décout J-L, and Fontecave M. Is the NAD(P)H:flavin oxidoreductase from *Escherichia coli* a member of the ferredoxin-$NADP^+$ reductase family? Evidence for the catalytic role of serine 49 residue. *J Biol Chem* 271: 16656–16661, 1996.
61. Nivière V, Vanoni MA, Zanetti G, and Fontecave M. Reaction of NAD(P)H:flavin oxidoreductase from *Escherichia coli* with NADPH and riboflavin: identification of intermediates. *Biochemistry* 37: 11879–11887, 1998.
62. Nivière V, Fieschi F, Décout J-L, and Fontecave M. The NAD(P)H:flavin oxidoreductase from *Escherichia coli*. Evidence for a new mode of binding for reduced pyridine nucleotides. *J Biol Chem* 274: 18252–18260, 1999.
63. Oldfield C, Pogrebinsky O, Simmonds J, Olson ES, and Kulpa CF. Elucidation of the metabolic pathway for dibenzothiophene desulphurization by *Rhodococcus* sp. strain IGTS8 (ATCC 53968). *Microbiology* 143: 2961–2973, 1997.
64. Parry RJ and Li W. An NADPH:FAD oxidoreductase from the valanimycin producer, *Streptomyces viridifaciens*. Cloning, analysis, and overexpression. *J Biol Chem* 272: 23303–23311, 1997.
65. Parry RJ and Li W. Purification and characterization of isobutylamine *N*-hydroxylase from the valanimycin producer *Streptomyces viridifaciens* MG456-hF10. *Arch Biochem Biophys* 339: 47–54, 1997.
66. Payne JW, Bolton H Jr, Campbell JA, and Xun L. Purification and characterization of EDTA monooxygenase from the EDTA-degrading bacterium BNC1. *J Bacteriol* 180: 3823–3827, 1998.
67. Prieto MA and Garcia JL. Molecular characterization of 4-hydroxyphenylacetate 3-hydroxylase of *Escherichia coli*. A two-protein component enzyme. *J Biol Chem* 269: 22823–22829, 1994.
68. Quandt KS and Hultquist DE. Flavin reductase: sequence of cDNA from bovine liver and tissue distribution. *Proc Natl Acad Sci U S A* 91: 9322–9326, 1994.
69. Quandt KS, Xu F, Chen P, and Hultquist DE. Evidence that the protein components of bovine erythrocyte green heme binding protein and flavin reductase are identical. *Biochem Biophys Res Commun* 178: 315–321, 1991.
70. Sancar GB, Jorns MS, Payne G, Fluke DJ, Rupert CS, and Sancar A. Action mechanism of *Escherichia coli* DNA photolyase. III. Photolysis of the enzyme–substrate complex and the absolute action spectrum. *J Biol Chem* 262: 492–498, 1987.
71. Spyrou G, Haggard-Ljungquist E, Krook M, Jornvall H, Nilsson E, and Reichard P. Characterization of the flavin reductase gene (*fre*) of *Escherichia coli* and construction of a plasmid for overproduction of the enzyme. *J Bacteriol* 173: 3673–3679, 1991.
72. Tang C-K. *Bacterial Bioluminescence: I. Catalytically Active Form of the Beta Subunit of Vibrio harveyi Luciferase and Its Kinetic and Binding Properties. II. Kinetic Studies on the Mechanism of Photobacterium fischeri NAD(P)H:FMN Oxidoreductase and Its Apoenzyme*. Ph.D. thesis, University of Houston, 1997.
73. Tanner JJ, Lei B, Tu S-C, and Krause KL. Flavin reductase P: structure of a dimeric enzyme that reduces flavin. *Biochemistry* 35: 13531–13539, 1996.
74. Tanner JJ, Miller MD, Wilson KS, Tu S-C, and Krause KL. Structure of bacterial luciferase β_2 homodimer: implications for flavin binding. *Biochemistry* 36: 665–672, 1997.
75. Tanner JJ, Tu S-C, Barbour LJ, Barnes CL, and Krause KL. Unusual folded conformation of nicotinamide adenine dinucleotide bound to flavin reductase P. *Protein Sci* 8: 1725–1732, 1999.
76. Thibaut D, Ratet N, Bisch D, Faucher D, Debussche L, and Blanche F. Purification of the two-enzyme system catalyzing the oxidation of the D- proline residue of pristinamycin IIB during the last step of pristinamycin IIA biosynthesis. *J Bacteriol* 177: 5199–5205, 1995.
77. Thoden JB, Holden HM, Fisher AJ, Sinclair JF, Wesenberg G, Baldwin TO, and Rayment I. Structure of the β_2 homodimer of bacterial luciferase from *Vibrio harveyi*: x-ray analysis of a kinetic protein folding trap. *Protein Sci* 6: 13–23, 1997.
78. Tu S-C. Isolation and properties of bacterial luciferase-oxygenated flavin intermediate complexed with long-chain alcohols. *Biochemistry* 18: 5940–5945, 1979.
79. Tu S-C. Isolation and properties of bacterial luciferase intermediates containing different oxygenated flavins. *J Biol Chem* 257: 3719–3725, 1982.
80. Tu S-C and Hastings JW. Physical interaction and activity coupling between two enzymes induced by immobilization of one. *Proc Natl Acad Sci U S A* 77: 249–252, 1980.
81. Tu S-C and Mager HIX. Biochemistry of bacterial bioluminescence. *Photochem Photobiol* 62: 615–624, 1995.
82. Tu S-C, Becvar JE, and Hastings JW. Kinetic studies

on the mechanism of bacterial NAD(P)H:flavin oxidoreductase. *Arch Biochem Biophys* 193: 110–116, 1979.
83. Tu S-C, Lei B, Yu Y, and Liu M. Characterization of *Vibrio harveyi* NADPH:FMN oxidoreductase and mechanism of reduced flavin transfer to luciferase. In: *Flavins and Flavoprotein*, edited by Stevenson KJ, Massey V, and Williams CH Jr. Calgary, Alberta: University of Calgary Press, 1997, pp. 357–366.
84. Uetz T, Schneider R, Snozzi M, and Egli T. Purification and characterization of a two-component monooxygenase that hydroxylates nitrilotriacetate from "*Chelatobacter*" strain ATCC 29600. *J Bacteriol* 174: 1179–1188, 1992.
85. Vervoort J, Müller F, O'Kane DJ, Lee J, and Bacher A. Bacterial luciferase: a carbon-13, nitrogen-15, and phosphorus-31 nuclear magnetic resonance investigation. *Biochemistry* 25: 8067–8075, 1986.
86. Waddle J and Baldwin TO. Individual α and β subunits of bacterial luciferase exhibit bioluminescence activity. *Biochem Biophys Res Commun* 178: 1188–1193, 1991.
87. Wang H, Lei B, and Tu S-C. *Vibrio harveyi* NADPH-FMN oxidoreductase Arg203 as a critical residue for NADPH recognition and binding. *Biochemistry* 39: 7813–7819, 2000.
88. Watanabe H and Hastings JW. Specificities and properties of three reduced pyridine nucleotide-flavin mononucleotide reductases coupling to bacterial luciferase. *Mol Cell Biochem* 44: 181–187, 1982.
89. Witschel M, Nagel S, and Egli T. Identification and characterization of the two-enzyme system catalyzing oxidation of EDTA in the EDTA-degrading bacterial strain DSM 9103. *J Bacteriol* 179: 6937–6943, 1997.
90. Xin X, Xi L, and Tu S-C. Functional consequences of site-directed mutation of conserved histidyl residues of the bacterial luciferase α subunit. *Biochemistry* 30: 11255–11262, 1991.
91. Xu Y, Mortimer MW, Fisher TS, Kahn ML, Brockman FJ, and Xun L. Cloning, sequencing, and analysis of a gene cluster from *Chelatobacter heintzii* ATCC 29600 encoding nitrilotriacetate monooxygenase and NADH:flavin mononucleotide oxidoreductase. *J Bacteriol* 179: 1112–1116, 1997.
92. Xun L and Sandvik ER. Characterization of 4-hydroxyphenylacetate 3-hydroxylase (HpaB) of *Escherichia coli* as a reduced flavin adenine dinucleotide-utilizing monooxygenase. *Appl Environ Microbiol* 66: 481–486, 2000.
93. Yubisui T, Matsuki T, Takeshita M, and Yoneyama Y. Characterization of the purified NADPH-flavin reductase of human erythrocytes. *J Biochem* (*Tokyo*) 85: 719–728, 1979.
94. Zenno S and Saigo K. Identification of the genes encoding NAD(P)H-flavin oxidoreductases that are similar in sequence to *Escherichia coli* Fre in four species of luminous bacteria: *Photorhabdus luminescens, Vibrio fischeri, Vibrio harveyi*, and *Vibrio orientalis. J Bacteriol* 176: 3544–3551, 1994.
95. Zenno S, Saigo K, Kanoh H, and Inouye S. 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, 1994.
96. Zenno S, Koike H, Kumar AN, Jayaraman R, Tanokura M, and Saigo K. Biochemical characterization of NfsA, the *Escherichia coli* major nitroreductase exhibiting a high amino acid sequence homology to Frp, a *Vibrio harveyi* flavin oxidoreductase. *J Bacteriol* 178: 4508–4514, 1996.
97. Zenno S, Koike H, Tanokura M, and Saigo K. Conversion of NfsB, a minor *Escherichia coli* nitroreductase, to a flavin reductase similar in biochemical properties to FRase I, the major flavin reductase in *Vibrio fischeri*, by a single amino acid substitution. *J Bacteriol* 178: 4731–4733, 1996.
98. Zenno S, Koike H, Tanokura M, and Saigo K. Gene cloning, purification, and characterization of NfsB, a minor oxygen-insensitive nitroreductase from *Escherichia coli*, similar in biochemical properties to FRase I, the major flavin reductase in *Vibrio fischeri. J Biochem* (*Tokyo*) 120: 736–744, 1996.
99. Zenno S, Kobori T, Tanokura M, and Saigo K. Conversion of NfsA, the major *Escherichia coli* nitroreductase, to a flavin reductase with an activity similar to that of Frp, a flavin reductase in *Vibrio harveyi*, by a single amino acid substitution. *J Bacteriol* 180: 422–425, 1998.
100. Zenno S, Kobori T, Tanokura M, and Saigo K. Purification and characterization of NfsA1, a *Bacillus subtilis* nitro/flavin reductase capable of interacting with the bacterial luciferase. *Biosci Biotechnol Biochem* 62: 1978–1987, 1998.

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1. Jingyuan Xiong, Holly R. Ellis. 2012. Deletional studies to investigate the functional role of a dynamic loop region of alkanesulfonate monooxygenase. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **1824**:7, 898-906. [CrossRef]
2. Hae-Won Chung, Shiao-Chun Tu. 2012. Structure–Function Relationship of Vibrio harveyi NADPH–Flavin Oxidoreductase FRP: Essential Residues Lys167 and Arg15 for NADPH Binding. *Biochemistry* 120606135101004. [CrossRef]
3. Bruce A. Palfey, Claudia A. McDonald. 2010. Control of catalysis in flavin-dependent monooxygenases. *Archives of Biochemistry and Biophysics* **493**:1, 26-36. [CrossRef]
4. Gloria Saab-Rincón , Brenda Valderrama . 2009. Protein Engineering of Redox-Active Enzymes. *Antioxidants & Redox Signaling* **11**:2, 167-192. [Abstract] [Full Text PDF] [Full Text PDF with Links]
5. Navneet Jawanda, Jerry Ebalunode, Alexey Gribenko, James Briggs, J. Ching Lee, Shiao-Chun Tu. 2008. A single-residue mutation destabilizes Vibrio harveyi flavin reductase FRP dimer. *Archives of Biochemistry and Biophysics* **472**:1, 51-57. [CrossRef]
6. Shiao-Chun Tu. 2008. Activity coupling and complex formation between bacterial luciferase and flavin reductases. *Photochemical & Photobiological Sciences* **7**:2, 183. [CrossRef]
7. Zhi-Jie Liu, Huizhong Chen, Neil Shaw, Sherryll L. Hopper, Lirong Chen, Siwei Chen, Carl E. Cerniglia, Bi-Cheng Wang. 2007. Crystal structure of an aerobic FMN-dependent azoreductase (AzoA) from Enterococcus faecalis. *Archives of Biochemistry and Biophysics* **463**:1, 68-77. [CrossRef]
8. Michiko E. Taga, Nicholas A. Larsen, Annaleise R. Howard-Jones, Christopher T. Walsh, Graham C. Walker. 2007. BluB cannibalizes flavin to form the lower ligand of vitamin B12. *Nature* **446**:7134, 449-453. [CrossRef]
9. E. V. Vetrova, N. S. Kudryasheva, V. A. Kratasyuk. 2007. Redox compounds influence on the NAD(P)H:FMN?oxidoreductase?luciferase bioluminescent system. *Photochemical & Photobiological Sciences* **6**:1, 35. [CrossRef]
10. Xi Li, Shiao-Chun Tu. 2006. Activity coupling of Vibrio harveyi luciferase and flavin reductase (FRP): Oxygen as a probe. *Archives of Biochemistry and Biophysics* **454**:1, 26-31. [CrossRef]
11. Lei Xu, Dongfang Zhang, Wanmeng Mu, Willem J.H. van Berkel, Zhaofeng Luo. 2006. Reversible resolution of flavin and pterin cofactors of His-tagged Escherichia coli DNA photolyase. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **1764**:9, 1454-1461. [CrossRef]
12. Nadezhda S. Kudryasheva. 2006. Bioluminescence and exogenous compounds: Physico-chemical basis for bioluminescent assay. *Journal of Photochemistry and Photobiology B: Biology* **83**:1, 77-86. [CrossRef]
13. Claudia Guerrero-Barajas, Jim A. Field. 2005. Enhancement of anaerobic carbon tetrachloride biotransformation in methanogenic sludge with redox active vitamins. *Biodegradation* **16**:3, 215-228. [CrossRef]
14. Toshiki Furuya, Shusuke Takahashi, Yuichiro Iwasaki, Yoshitaka Ishii, Kuniki Kino, Kohtaro Kirimura. 2005. Gene cloning and characterization of Mycobacterium phlei flavin reductase involved in dibenzothiophene desulfurization. *Journal of Bioscience and Bioengineering* **99**:6, 577-585. [CrossRef]
15. E. V. Vetrova, N. S. Kudryasheva, A. J. W. G. Visser, A. van Hoek. 2005. Characteristics of endogenous flavin fluorescence ofPhotobacterium leiognathi luciferase andVibrio fischeri NAD(P)H:FMN-oxidoreductase. *Luminescence* **20**:3, 205-209. [CrossRef]
16. Claudia Guerrero-Barajas, Jim A. Field. 2005. Riboflavin- and cobalamin-mediated biodegradation of chloroform in a methanogenic consortium. *Biotechnology and Bioengineering* **89**:5, 539-550. [CrossRef]
17. Toshiki Furuya, Shusuke Takahashi, Yoshitaka Ishii, Kuniki Kino, Kohtaro Kirimura. 2004. Cloning of a gene encoding flavin reductase coupling with dibenzothiophene monooxygenase through

coexpression screening using indigo production as selective indication. *Biochemical and Biophysical Research Communications* **313**:3, 570-575. [CrossRef]

18. 2003. Trend of Most Cited Papers (2001-2002) in ARS. *Antioxidants & Redox Signaling* **5**:6, 813-815. [Citation] [Full Text PDF] [Full Text PDF with Links]
19. Kevin A Gray, Gregory T Mrachko, Charles H Squires. 2003. Biodesulfurization of fossil fuels. *Current Opinion in Microbiology* **6**:3, 229-235. [CrossRef]
20. John C. Low, Shiao-Chun Tu. 2003. Energy Transfer Evidence for In Vitro and In Vivo Complexes of Vibrio harveyi Flavin Reductase P and Luciferase ¶. *Photochemistry and Photobiology* **77**:4, 446-452. [CrossRef]
21. John C. Low, Shiao-Chun Tu. 2003. Energy Transfer Evidence for In Vitro and In Vivo Complexes of Vibrio harveyi Flavin Reductase P and Luciferase¶. *Photochemistry and Photobiology* **77**:4, 446. [CrossRef]