Biosci. Biotechnol. Biochem., **62** (10), 1978–1987, 1998

Purification and Characterization of NfrA1, a *Bacillus subtilis* Nitro/flavin Reductase Capable of Interacting with the Bacterial Luciferase

Shuhei ZENNO,[1,†] Toshiro KOBORI,[2] Masaru TANOKURA,[2] and Kaoru SAIGO[1]

[1]*Department of Biophysics and Biochemistry, Graduate School of Science, and*
[2]*Biotechnology Research Center, University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan*

Received May 11, 1998

***ipa-43d* is a hypothetical gene identified by the *Bacillus subtilis* genome project (Mol. Microbiol. 10, 371–384 1993; Nature 390, 249–256 1997). The *ipa-43d* protein overexpressed in *E. coli* was purified to homogeneity and its properties were analyzed biochemically. The *ipa-43d* protein was found to be tightly associated with FMN and to be capable of reducing both nitrofurazone and FMN effectively. Although the *ipa-43d* protein catalysis obeys the ping-pong Bi–Bi mechanism, catalysis mode was changed to the sequential mechanism upon coupling with the bioluminescent reaction. Database search showed that *B. subtilis* possessed four genes (*ipa-44d*, *ytmO*, *yddN*, and *yvbT*), encoding proteins similar in amino acid sequence to LuxA and LuxB of *Photobacterium fischeri*, and, in particular, *ipa-44d* is immediately adjacent to the *ipa-43d* gene on the chromosome.**

Key words: *Bacillus subtilis*; nitroreductase; flavin reductase; flavoprotein; protein evolution

The oxygen-insensitive nitroreductase activity in *Escherichia coli* consists of three functionally redundant components, NfsA, NfsB, and NfsC.[1] In previous experiments, *nfsA*[2,3] and *nfsB*[4,5] were cloned and their gene products, NfsA and NfsB, were characterized biochemically.[5,6] NfsA and NfsB form homodimers tightly associated with two FMN cofactors and respectively share a considerable amino acid sequence identity with Frp[7] and FRase I,[8] flavin reductases in bioluminescent bacteria, which are distantly related in amino acid sequence to each other.[6] These flavin reductases can reduce FMN efficiently[7,8] and are implicated to be involved in the bioluminescent reaction.[7–9] Although the tertiary structures of NfsA and NfsB are not yet known, those of Frp and FRaseI have recently been clarified: both Frp and FRaseI are homodimers of interlocking subunits, with the FMN cofactor bound in the dimer interface.

Under physiological conditions, no or little FMN reductase activity was found in wild-type *E. coli* enzymes (NfsA[6]; NfsB[5]). Single amino acid substitutions that affect the tertiary structure of the active center, however, resulted in the conversion of NfsA and NfsB to FMN reductases as active as or more active than authentic FMN reductases such as Frp and FRase I.[12,13] For example, in NfsB, any of ten single amino acid substitutions of Phe-124, a key amino acid in the putative active center, resulted in a nitroreductase/FMN reductase conversion.[12] It may be reasonable to assume that progenitors of the NfsA/Frp and NfsB/FRase I pairs lost FMN reductase activity during evolution in *E. coli* cells or acquired FMN reductase activity during evolution in bioluminescent bacteria, most possibly by altering physical or chemical environments in the vicinity of the active center or the bound cofactor FMN.

To gain a further insight into the evolutionary or molecular mechanisms of divergence of the nitroreductase/flavin reductase family, we did gene searching and biochemical characterization of enzymes similar to NfsA and Frp in bacteria other than *E. coli* and bioluminescent bacteria. Here, we show that *ipa-43d*, a hypothetical gene identified by the *Bacillus subtilis* genome project,[14,15] encodes an NADPH-linked oxidoreductase similar in amino acid sequence to *E. coli* NfsA and *Vibrio harveyi* Frp. The *ipa-43d* protein was overexpressed in *E. coli* cells, purified to homogeneity and analyzed biochemically. The *ipa-43d* protein was associated with both FMN reductase and nitroreductase activities. In addition, the *ipa-43d* protein was found to be capable of interacting with a bacterial luciferase to change its reaction mode from the ping-pong Bi–Bi mechanism to the sequential mechanism.

Materials and Methods

Materials. Sources for all enzymes and chemicals used in this paper have been described elsewhere.[5,6,8] *E. coli* strain, JM83 (*ara Δ(lac-proAB) F⁻ rpsL(f80lacZ ΔM15)*[16] was used for cloning and overexpression of cloned fragments. *B. subtilis* ISW 1214 (*hsrM1 leuA8 metB5*) was purchased from Takara Shuzo (Kyoto, Japan). *Vibrio harveyi* ATCC 33843 was obtained from the American Type Culture Collection. *E. coli* and *B. subtilis* were cultured in Luria-Bertani (LB) medium[17] at 30–37°C, while *V. harveyi* was cultured in Photobacterium medium (Difco Laboratories) at 26°C. Plasmid pIPA43 is a pSU2719[18] derivative which the 8.6-kb *Sal*I/*Bgl*II fragment of λ sacT that contains *ipa-43d* gene is inserted into the *Sal*I/*Bam*HI site of pSU2719.

† To whom correspondence should be addressed: *Shuhei ZENNO, Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.* Tel: 81-3-3812-2111 ext. 4407; Fax: 81-3-5684-2394; E-mail: saigo@biochem00.biochem.s.u-tokyo.ac.jp

Abbreviation: LB, Luria-Bertani

The pIPA43 DNA was digested with *Sal*I/*Eco*RV, filled in, and self-ligated to produce pIPA-43d44d. The pIPA-43d44d DNA was digested with *Eco*RI and then self-ligated to produce the expression plasmid pIPA-43d (see Fig. 1c). After PCR amplification[19] of the genomic DNA of *V. harveyi* with the oligonucleotides (5′ TTGAATTCCCAATAAATGCCGTTATGGTGA and 5′ TTTCTAGATGTGGGTTCAACAAAGTGATGG) based on *frp*-flanking sequence reported by Lei *et al.*,[7] amplified DNA was digested with *Eco*RI/*Xba*I and was cloned into the *Eco*RI/*Xba*I site of pUC118[20] to produce the expression plasmid pFRP. The pFRP insert was confirmed by sequence analysis.

Overexpression of ipa-43d in E. coli cells and preparation of cell extracts. E. coli JM83 was transformed with pIPA-43d by the polyethlene glycol method.[21] An aliquot (0.25 ml) of an overnight culture at 30°C was added to 10 ml LB broth[17] with 0.03 mg chloramphenicol ml^{-1}; the culture was incubated with shaking at 37°C for 6 h. Cells were harvested by centrifugation, and cell extracts were prepared as described previously.[8] *B. subtilis* cell extracts were prepared similarly.

Overproduction and purification of NfrA1. Three hundred ml of an overnight culture of *E. coli* JM83 with pIPA-43d at 30°C was added to 6 l of LB broth containing 30 μg chloramphenicol l^{-1} and 0.01% (v/v) foam suppressant Disfoam CE457 (Nippon Oil and Fats), and cultivated at 37°C for 6 h with aeration as described previously.[22] Cells were harvested by centrifugation, suspended in 250 ml of 20 mM Tris/HCl buffer (pH 7.0), and treated with a 20-min sonication at 4°C using a Branson model 250 sonifier. Treated suspensions were centrifuged at 12,000 g at 4°C for 1 h, and the resultant supernatant fluid was dialyzed against 20 mM Tris/HCl (pH 7.0) at 4°C. Then, the dialysate was put on a Q Sepharose FF (Pharmacia) anion-exchange column (2.6 × 10 cm) equilibrated with 20 mM Tris/HCl (pH 7.0), washed with 200 ml of the starting buffer (flow rate: 4 ml min^{-1}), and eluted with a 0–500 mM linear gradient of KCl in 20 mM Tris/HCl (pH 7.0) (flow rate: 2 ml min^{-1}). Peak fractions of NADPH-nitrofurazone reductase activity (see below) eluted around 300 mM KCl (40 ml) were pooled. Pooled peak fractions were dialyzed against 40 mM sodium phosphate (pH 6.0) at 4°C. The dialysate was put on a Blue Sepharose CL-6B (Pharmacia) affinity chromatography column (size: 1 × 8 cm) equilibrated with 40 mM sodium phosphate buffer (pH 6.0). The column was washed with 30 ml of 40 mM phosphate buffer (pH 6.0) and NfrA1 was eluted with 20 ml of 0.5 mM NADPH at a flow rate of 1 min ml^{-1}. The Blue Sepharose chromatography step was repeated once more. Purified NfrA1 was dialyzed against 50 mM Tris/HCl (pH 7.0) and stored at 4°C for further analysis. All chromatographic procedures were done at 4°C. Except for the enzyme preparation used for co-factor analysis (see below), purified NfrA1 was treated with 50 mM Tris/HCl (pH 7.0) containing 1 mM FMN at 4°C for 48 h before dialysis. *V. harveyi* Frp was overproduced and purified similarly.

Molecular mass mesurement of NfrA1. The molecular size of native NfrA1 was assayed with Superose 12 (Pharmacia) gel-filtration in 50 mM Tris/HCl (pH 7.0) containing 150 mM NaCl (elution rate: 0.2 ml min^{-1}), while that of denatured NfrA1 was estimated by SDS-PAGE. SDS-PAGE analysis was done essentially as described by Laemmli[23] under reducing conditions with 15% (w/v) gel (1-mm thickness) at 30 mA for 1.5 h. The gel was stained with Coomassie Brilliant Blue R-250 (Fluka Chemie).

Enzyme assay. Reductase activities were assayed with the spectrophotometric method as described previously.[5,6] A typical reaction mixture (3.0 ml) contained 50 mM Tris/HCl buffer (pH 7.0), 0.1 mM NADPH (or NADH), 0.1 mM of a given electron acceptor, and a suitable amount of enzyme. The reaction was started by the addition of NADPH (or NADH), and the initial rate was measured at 23°C with a model U-3210 Hitachi recording spectrophotometer. While the bioluminescence reaction coupled with FMN reductase was done by the method of Lei & Tu.[9] The mixture, containing 0.1–0.8 μM FMN, 0.0002% (v/v) decanal, 50 mM potassium phosphate (pH 7.0), 0.1 mg *Photobacterium fischeri* luciferase ml^{-1}, and a suitable amount of purified reductase (30 ng NfrA1 ml^{-1} or 380 ng NfsA ml^{-1}), was preincubated at 23°C for 3 min. One hundred microliters of 0.08–0.8 μM NADPH was than added to 0.1 ml of the mixture to start the reaction. Luminescence was measured at 23°C for 2 min with a Laboscience (Tokyo, Japan) lumiphotometer, model TD 4000 and the values were presented in relative light units (rlu).

Other techniques. Protein was mesured by a dye-binding method[24] with bovine plasma γ-globulin (Bio-Rad) as a standard. TLC was done to characterize the NfrA1-bound flavin[5,6] and the amount of NfrA1-bound FMN was mesured as described by Ohnishi *et al.*[25] Nucleotides were sequenced using a *Bca*Best dideoxy sequencing kit (Takara Shuzo).[26] Nucleotide and amino acid sequences, hydrophobicity,[27] and secondary structures[28] were analyzed using DNASIS software (Hitachi Software Engineering). A protein sequence homology search was done under nr-aa and nr-nt databases with the FASTA system.[29] Other molecular techniques are described in Sambrook *et al.*[17]

Results and Discussion

Identification of NfrA1, a B. subtilis protein similar in amino acid sequence to Frp and NfsA

During the course of the database search, the product of a hypothetical gene *ipa-43d* identified by the *B. subtilis* genome project[14,15] (Fig. 1(a)) was found to be very similar in amino acid sequence to Frp, a flavin reductase in *V. harveyi* and NfsA, the major oxygen-insensitive nitroreductase in *E. coli* (Fig. 1(b)). The putative *ipa-43d* gene product (Ipa-43d) consists of 249 amino acid residues and its molecular weight is 28,320. Except for two insertions 4–6 amino acid long, the Ipa-43d protein showed 41–42% amino acid identity to Frp or NfsA.

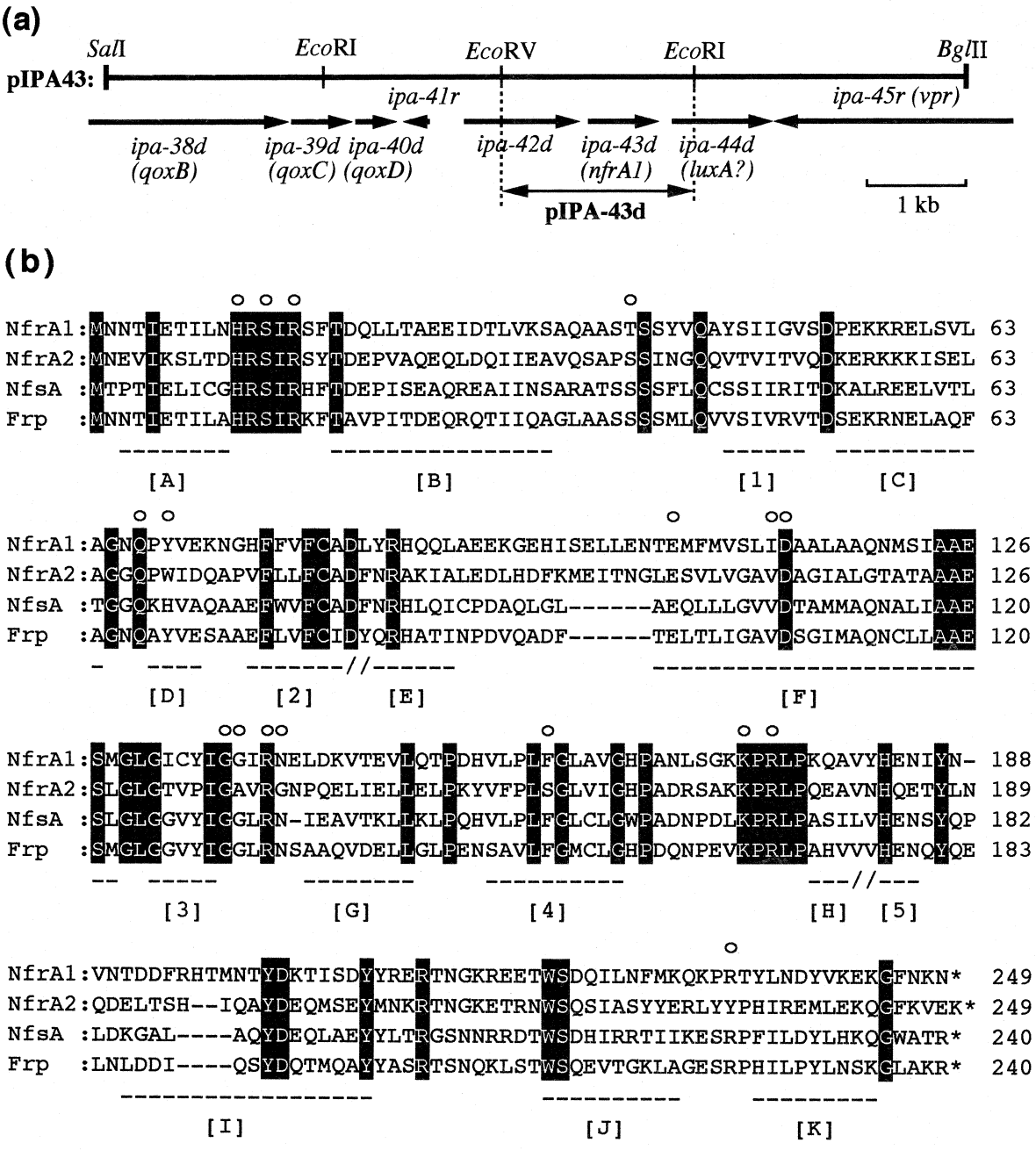

Fig. 1. Physical Map Near the *nfrA1* Locus and the Deduced Amino Acid Sequence of NfrA1 Protein.

(a) Structure of the *B. subtilis* genomic DNA recovered as pIPA43. Horizontal arrows indicate both sizes and locations of genes or hypothetical genes identified by the genome project.[14] Vertical dotted lines show the pIPA-43d insert which includes the *ipa-43d* (*nfrA1*) gene. *ipa-44d*, an immediate neighbor of *nfrA1*, is one of the four *luxAB*-like *B. subtilis* genes (see Fig. 4). (b) Amino acid sequence comparison among NfrA1,[14,15] NfrA2,[15] *E. coli* NfsA[6] and *V. harveyi* Frp.[7] NfrA2 is an NfrA1 cognate found at 37.4°–37.5° in the *B. subtilis* chromosome.[15] Invariant amino acids are indicated by white letters in black boxes. Open circles indicate 17 amino acids surrounding the putative active sites (cofactor FMN loci),[10] Thick bars under the amino acid sequences show secondary α-helices (A-K) and β-sheet structures (1-5) determined by X-ray crystallography.[10] (c) Upstream and downstream sequences flanking the *nfrA1* coding region. Thin underlines labeled (−10), (−35) and (SD), respectively, show −10 region,[31] −35 region[31] and Shine-Dalgarno sequence.[30] A pair of arrows in the 3′-untranslated region indicates the location of a stem-loop structure, a possible transcription terminator.[31] Three consecutive asterisks indicate in-frame termination codons.

Hydrophobicity profiles[27] and predicted secondary structures[28] were also similar to those of Frp and NfsA (data not shown). Since the Ipa-43d protein is associated with flavin reductase and nitroreductase activity (see below), we will hereafter refer to it as *nfrA1* (the *1*st *n*itro/*f*lavin *r*eductase similar in sequence to Nfs*A*; see below for the second gene, *nfrA2*).

The putative initiation codon is preceded by sequences similar to the Shine-Dalgarno sequence[30] and −10 and −35 promoter consensus sequences[31] (Fig. 1(c)). The DNA sequence following the coding region contained an imperfect inverted repeat, possibly a terminal signal for transcription.[31]

NfrA1 is an NADPH-linked nitro/flavin reductase

To decide whether NfrA1 is a flavin reductase or nitroreductase, an expression plasmid (pIPA-43d) was constructed in which the expression of the *nfrA1* gene was under the control of the *lac* promoter-operator system (see Materials and Methods). Two new protein bands, 28 and 24 kDa in size, were detected in the extracts of *E. coli* cells with pIPA-43d (lane 1 in Fig. 2(a)). Subsequent purification (lane 3) showed the 28-kDa band to correspond to NfrA1. As shown in Table 1, both FMN reductase and nitroreductase activities significantly increased when extracts were assayed in the presence of NADPH. Since nitroreductase and FMN reductase activities were always coeluted on column chromatography (see below), NfrA1 is a nitro/flavin reductase, an enzyme capable of reducing both nitrocompounds and flavins such as FMN. That NADPH was much more effective as an electron donor than NADH (Table 1) indicates that NfrA1 is an NADPH-linked enzyme.

Purification of NfrA1 including FMN as a prosthetic group

Using Q Sepharose and Blue Sepharose column chromatography, NfrA1 was purified to homogeneity from the extracts of *E. coli* cells harboring pIPA-43d (Table 2 and Fig. 2(a); see Materials and Methods for details). Elution profiles of Superose 12 (Fig. 2(b)) indicate NfrA1 to be associated not only with FMN reductase activity but also with nitroreductase activity. Although the molecular weight of NfrA1 was estimated and calculated, at about 28,000 by SDS-PAGE (Fig. 2(a)) and

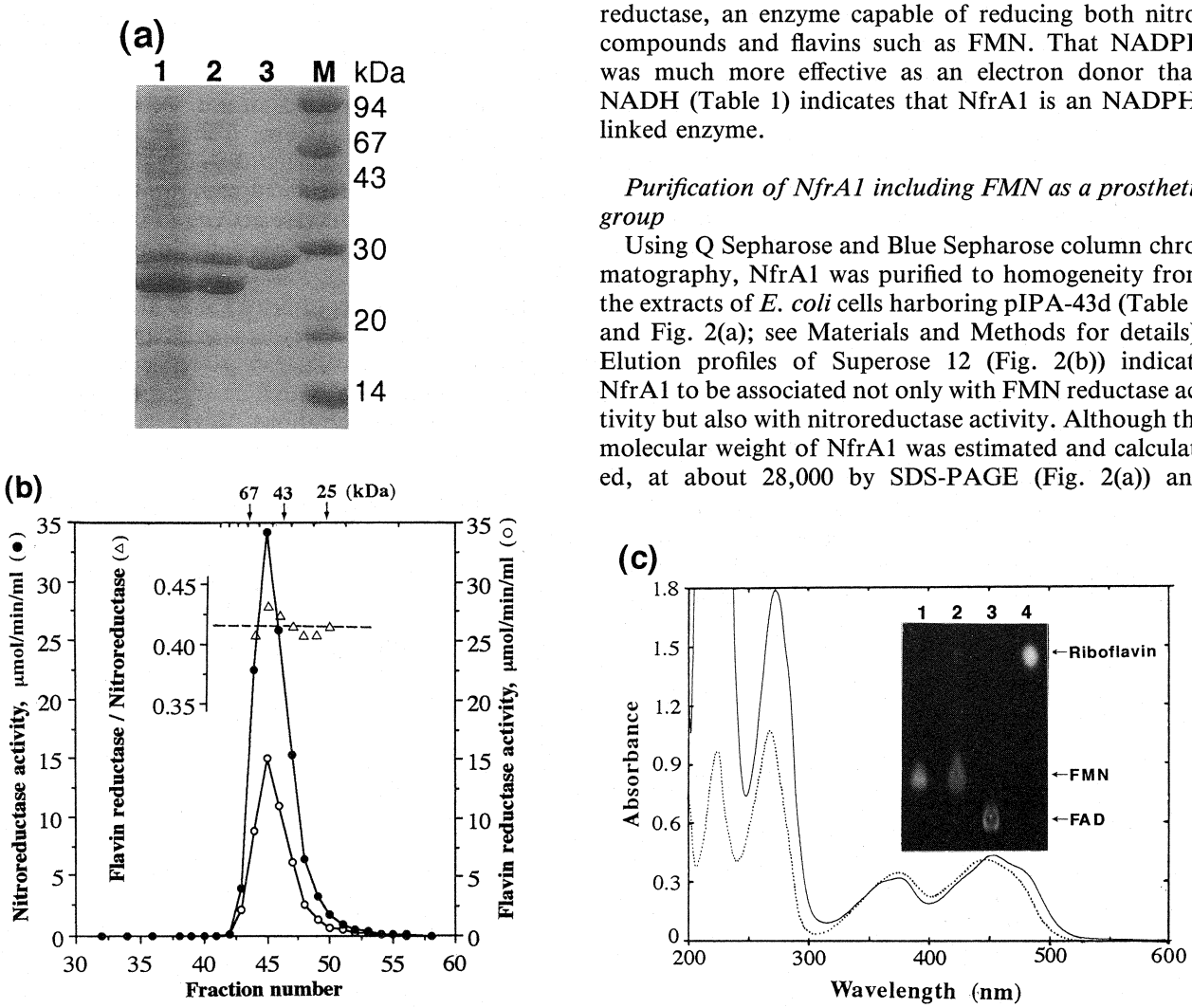

Fig. 2. Purification of NfrA1 Protein with FMN Reductase and Nitrofurazone Reductase Activities.

(a) SDS-PAGE patterns of NfrA1 preparations. Lanes: 1, crude cell extracts of JM83 with pIPA-43d (163 μg); 2, pooled peak fractions of the first cycle of Q Sepharose FF chromatography (40 μg); 3, pooled peak fractions of Blue Sepharose CL-6B chromatography (25 μg); M, protein size markers (Pharmacia). Molecular sizes are shown in the right margin. (b) Superose 12 elution profiles of purified NfrA1. FMN reductase and nitrofurazone reductase activities are coeluted. Note the ratio between FMN reductase activity and nitrofurazone reductase activity is constant throughout peak fractions. Molecular sizes are shown in the upper margin. The molecular size of NfrA1 can be estimated at 53 kDa. (c) Absorption spectra of purified NfrA1 holoenzyme and free-FMN. After unbound flavin in enzyme solution was removed by gel filtration, the absorption spectrum of NfrA1 was measured. Solid and dotted lines, respectively, show spectra of 1.86 mg/ml (66 mM) of NfrA1 and 35 mM free FMN in 50 mM Tris/HCl buffer (pH 7.0) containing 150 mM NaCl. The NfrA1 spectrum has peaks at 451, 374, 273, and 218 nm, while peaks of free FMN are 448, 375, 268, and 224 nm. Inset: TLC patterns of NfrA1-bound and authentic flavins. Lane 1, flavins released from NfrA1; lane 2, authentic FMN; lane 3, authentic FAD; lane 4, authentic riboflavin. Arrows show positions of riboflavin, FMN and FAD.

nucleotide sequencing (see Fig. 1(b)), NfrA1 protein and activity were eluted at the position corresponding to 53-kDa protein in Superose 12 gel filtration (Fig. 2(b)). As shown below, amino acids forming and/or surrounding the active center of Frp are highly conserved in NfrA1. The FMN cofactors in Frp have been shown to be situated in the pockets formed at the interface of two protein subunits,[10] and NfrA1 is tightly associated with FMN (see below). Thus, it is strongly suggested that, as with active Frp,[10] active NfrA1 is a homodimer.

A concentrated solution of purified NfrA1 was yellow, and this coloration was resistant to dialysis (data not shown). The absorption spectrum in the visible-light region of purified NfrA1 was identical to that of FMN but was shifted about 5 nm toward the long wavelengths (Fig. 2(c)). Almost exclusive association of NfrA1 with FMN was demonstrated by thin-layer chromatography (Fig. 2(c) inset). Together, these results indicate that NfrA1 is a flavoprotein tightly associated with FMN. Based on spectroscopy and protein weight analysis, the molar ratio between bound FMN (cofactor FMN) and protein was estimated at 0.96, thus suggesting that 1 mol of FMN binds to 1 mol of NfrA1 subunit. Note that two different FMN molecules are involved in the case of FMN reduction: FMN tightly associated with the enzyme as a prosthetic group and FMN serving as a substrate.

NfrA1 reduces nitrofurazone and FMN according to the ping-pong Bi-Bi mechanism

Previous experiments showed that FMN reduction by Frp,[32] and the first step of nitrofurazone reduction by NfsA[6] obey the ping-pong Bi-Bi mechanism. Thus, FMN and nitrofurazone reduction by NfrA1 were analyzed using double-reciprocal plots of initial velocity versus concentrations of NADPH, FMN or nitrofurazone. As partly shown in Fig. 3(a) and 3(b) parallel lines were observed in all cases, suggesting that both FMN reduction and nitrofurazone reduction obey the ping-pong Bi-Bi mechanism (Fig. 3(e)). K_m, V_{max} and V_{max}/K_m values are shown in Table 3.

V. harveyi Frp is associated with nitroreductase activity as strong as E. coli NfsA and NfrA1

A previous experiment[6] showed no or little FMN reductase activity associated with NfsA, the major *E. coli* nitroreductase (see also Table 4). Jablonski and DeLuca[33] isolated Frp from *V. harveyi* as a major NADPH-FMN reductase. In this study, we showed both nitroreductase and FMN reductase activities are associated with NfrA1. These findings, however, do not necessarily mean that NfrA1 is an intermediate in substrate specificity between NfsA and Frp, since Jablonski and DeLuca[32] did not examine whether Frp is capable of reducing nitro-compounds or not. To clarify this point, we purified *V. harveyi* Frp, from *E. coli* cells overexpressing it, to homogeneity (data not shown) and examined whether it is associated with nitroreductase activity. Frp had nitroreductase activity (Table 4), indicating that Frp may also be a nitro/flavin reductase as with NfrA1.

Kinetic parameters of NfrA1, Frp and NfsA are summarized in Table 3. Enzyme specificity at limiting concentrations of the substrate is reflected by V_{max}/K_m, while V_{max} may serve as an index for substrate specificity at saturating concentrations. In general, V_{max} values of

Table 1. Nitro/Flavin Reductase Activity in the Extracts of *E. coli* Cells with pIPA-43d

Pyridine nucleotide	Enzyme source	Reductase activity (nmol min^{-1} mg^{-1} of protein) on:	
		nitrofurazone	FMN
NADPH	*E. coli* JM83	24	15
	JM83 (pIPA-43d)	2260	749
	Purified NfrA1	53000	22000
	B. subtilis ISW 1214	28	63
NADH	*E. coli* JM83	6	25
	JM83 (pIPA-43d)	39	10
	Purified NfrA1	220	110
	B. subtilis ISW 1214	7	24

Table 3. Kinetic Parameters of NfrA1, Frp amd NfsA

Enzyme	Substrate	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1} of protein)	V_{max}/K_m
NfrA1	NADPH*	0.85	61	72
	NADPH=	3.9	10	2.6
	nitrofurazone‖	16.3	97	5.9
	FMN‖	4.7	9	2.0
Frp	NADPH*	12.4	145	12
	NADPH=	7.9	25	3.1
	nitrofurazone‖	19.5	142	7.3
	FMN‖	25.8	23	0.9
NfsA+	NADPH*	11.0	85	7.7
	NADPH=	0.75	1.1	1.5
	nitrofurazone‖	5.5	45	8.1
	FMN‖	2.5	1.1	0.4

* K_ms obtained when nitrofurazone was used as an electron acceptor.
= K_ms obtained when FMN was used as an electron acceptor.
‖ K_ms obtained when NADPH was used as an electron donor.
\+ Zennon *et al.*[6]

Table 2. Purification of NfrA1 from JM83 (pIPA-43d) Extracts

	Volume (ml)	Total protein (mg)	Total activity (μmol min^{-1})	Specific activity (μmol min^{-1} mg^{-1} of protein)	Yield (%)	Purification factor
Crude extracts	287	6228	14092	2.3	100	1
Q Sepharose	40	1584	12725	8.0	90	3
Blue Sepharose CL-6B	14	142	7450	52.5	53	23

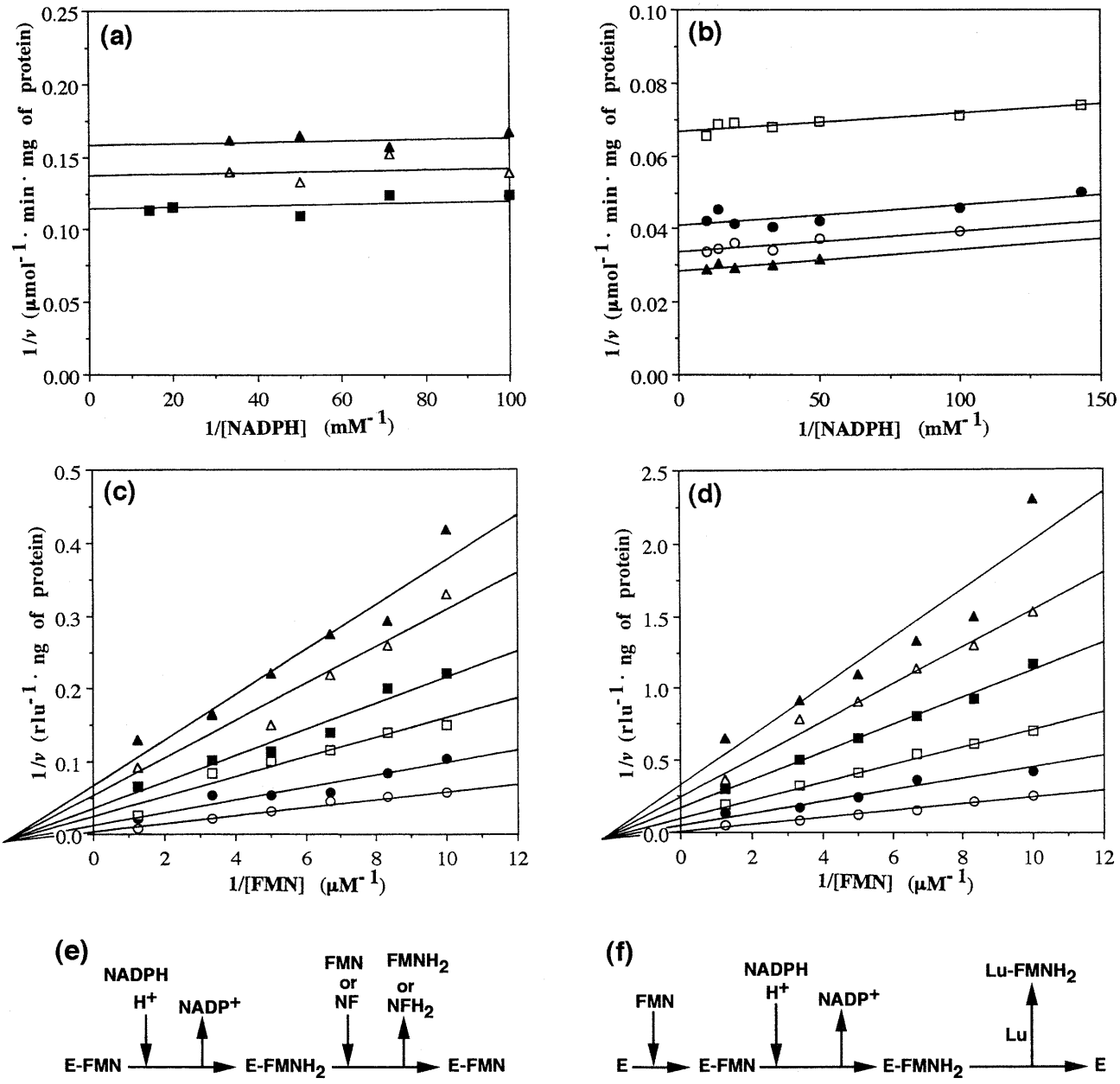

Fig. 3. Lineweaver-Burk Plots and Deduced Mechanisms of NfrA1 Catalysis.

(a) Double-reciprocal plots of initial velocity versus NADPH concentration of FMN reductase activity in the absence of the bacterial luminescent system. Constant concentrations of FMN are: ▲, 10 μM; △, 20 μM; ■, 50 μM. (b) Double-reciprocal plots of NADPH-nitrofurazone reductase activity. Constant concentrations of nitrofurazone are: □, 3 μM; ●, 5 μM; ○, 7 μM; ▲, 10 μM. (c, d) Double-reciprocal plots of FMN reductase activity in the presence of the bacterial luminescent system. Luciferase-coupled assay was done as follows. 1.3 μM *P. fischeri* luciferase, nitroreductase (0.11 nM NfrA1 or 1.4 nM NfsA) and 13 μM decanal were mixed with FMN at given concentrations and 0.04–0.4 μM NADPH. NADPH concentrations are, from top line to bottom, 0.04 μM (▲), 0.05 μM (△), 0.08 μM (■), 0.1 μM (□), 0.16 μM (●) and 0.4 μM (○). As a nitroreductase, NfrA1 (c) and NfsA (d) were used. (e) In the absence of the bacterial luminescent reaction system, the catalytic mode of NfrA1 is the ping-pong Bi-Bi mechanism, in which NfrA1 tightly associated with cofactor FMN serves as a holoenzyme. (f) In the presence of the bacterial luminescent reaction system, the reaction mode of NfrA1 is the sequential mechanism, in which the reduced form of the FMN cofactor is presumed to be used as a substrate for bacterial luciferase. E, NfrA1 not associated with cofactor FMN. E-FMN and E-$FMNH_2$, respectively, show NfrA1 tightly associated with the oxidized and reduced forms of FMN. Nitrofurazone and its reduced form, respectively, are shown by NF and NFH_2. Lu and Lu-$FMNH_2$ show luciferase and luciferase-$FMNH_2$ complex, respectively.

Frp were twice as large as those of NfrA1, suggesting that, at saturating concentrations of subtrates, Frp is an enzyme more effective in both FMN and nitrofurazone reduction than NfrA1. In contrast, comparison of V_{max}/K_m values may indicate that Frp is an NADPH oxidase much less effective than NfrA1 in the presence of excess nitrofurozone.

NfsA is capable of effectively reducing not only nitrocompounds but also quinones, ferricyanide, and flavins lower in molecular weight than FMN,[6] most of which

Table 4. Electron Acceptor Activities of NfrA1, NfsA and Frp

Electron acceptor	Reductase activity (μmol min^{-1} mg^{-1} of protein) of:			
	NfrA1	NfsA*	Frp	Frp$^{=}$
Flavins				
FMN	22	1	20 (87)	(51)
FAD	29	3	19	(51)
Riboflavin	60	7	18	—
Lumiflavin	60	10	16	—
Nitro compounds				
Nitrofurazone	53	73	104 (100)	—
Methyl 4-nitrobenzoate	33	24	57	—
Quinones				
Menadione	62	24	119	(51)
1,4-benzoquinone	99	163	260	—
Miscellaneous				
Tartrazine	0.7	0.8	0.1	—
Methylene blue	15	3	62	(12)
Ferricyanide	205	251	410	(84)
2,6-dichloroindophenol	46	27	98	(69)

Enzyme activity was measured using 50 mM Tris/HCl buffer (pH 7.0), while values in parentheses show activities in 50 mM potassium phosphate buffer (pH 7.0). —, not determined. References: * Zenno *et al.*;[6] = Jablonski and DeLuca.[32]

may not be natural substrates (see Table 4). Thus, we next examined the electron acceptor activity of purified NfrA1 and Frp (Table 4). Both NfrA1 and Frp were found to have a broad electron-acceptor activity spectrum very similar to that of NfsA. Taken together, these results may indicate that NfrA1, Frp and NfsA are members of an identical nitro/flavin reductase family and NfsA has lost an FMN reductase activity in evolution without losing other activities.

One unexpected finding is that Frp FMN reductase activity varies depending on the composition of the buffer used: 50 mM potassium phosphate buffer (pH 7.0), which had been used by Jablonski and DeLuca,[32] gave more than 4-fold the activity with the 50 mM Tris/HCl buffer (pH7.0), we routinely used (see Table 4), suggesting possible involvement of amino groups in interactions between FMN (substrate) and Frp but not those between nitro-compounds and Frp.

Bioluminescent reaction alters catalysis mode of NfrA1

The catalytic mode of Frp has been shown to be changed from the ping-pong Bi–Bi mechanism to the sequential mechanism upon coupling with the bioluminescent reaction.[9] It was postulated that this is due to direct transfer of the reduced form of FMN cofactor from Frp to bacterial luciferase (or LuxA/LuxB complexes) so that Frp apoenzyme and holoenzyme, respectively, may serve as an active enzyme (E) and ES complex in the bacterial luciferase-dependent FMN reduction (see Fig. 3(f)). Putative luciferase/FMN reductase complexes have been reported in the case of *Photorhabdus luminescens*[34] and *V. harveyi*.[35] Thus, we examined the possible effect of the bioluminescent reaction on kinetics of the NfrA1-dependent reduction of FMN. As in the case of Frp,[9] the catalytic mode of NfrA1 was found to be altered from the ping-pong Bi–Bi mechanism to the sequential mechanism upon coupling with the bioluminescent reaction (Fig. 3(c)). As luciferase, we used that prepared from *P. fischeri* (Boehringer Mannheim).

Although much less effective, NfsA, an *E. coli* nitroreductase, could also supply $FMNH_2$ to the bacterial luminescent system with a similar catalytic mode alteration (Fig. 3(d)). NfsA-97-99G, an FMN reductase version of NfsA was much more effective as an $FMNH_2$ supplier than wild-type NfsA.[13] Together, these observations indicate that, although *B. subtilis* is not a luminescent bacterium, a *B. subtilis* enzyme, NfrA1, can supply the reduced form of FMN cofactor to *P. fischeri* luciferase as effectively as does Frp, an authentic bioluminescent bacterial enzyme.

These findings also prompted us to search for *luxAB*-like genes in the *B. subtilis* chromosome.[14,15] We found four putative *luxAB*-like genes (Fig. 4). In particular, one of them, *ipa-44d*, was found to be an immediate neighbor of *ipa-43d* (Fig. 1(a)). Sixteen and twelve percent of Ipa-44d amino acids were conserved in *P. fischeri* LuxA and LuxB, respectively. We tested whether the bioluminescent reaction occurs in *B. subtilis* cells and their extracts in the presence of decanal, FMN and dithionite, but so far no positive signals were obtained. Thus, it may follow that *B. subtilis* LuxA or LuxB homologs can produce no or little visible light upon supplying of $FMNH_2$.

NfrA1 may have an FMN-binding pocket very similar in three-dimensional structure to that in Frp

Searching of the entire *B. subtilis* chromosome showed that the *B. subtilis* chromosome contains the second *nfsA/frp*-type gene (*nfrA2;* see Fig. 1(b)) along with an *nfsB/FRase* I-type gene (*yfkO*) and three *nox*-type genes (*ydgI*, *ydfN*, and *yodC*)[15,39] (data not shown). Amino acid sequence similarity may suggest that NfrA1 and YfkO, respectively, are the orthologs of *E. coli* NfsA and NfsB, respectively. NfrA2 is a member of the NfsA/Frp family but slightly more distant from NfsA and Frp than is NfrA1.

A recent structural analysis of Frp showed that the FMN cofactor is stabilized with electrostatic interactions in the crevice formed in the dimer interface.[10] The FMN hydrogen bonds to side chains of His-11, Ser-13, Arg-15, Tyr-69, Gln-67, Lys-167 and Arg-169 and to the main chain Gly-130 and Gly-131 and Ser-39 of the other subunit. Arg-225 and Arg-135 appear important for the determination of substrate specificity.[10] Our amino acid sequence alignment (Fig. 1(b)) showed that, except for Ser-39 and Val-106, all key 17 amino acids of Frp, which are labeled with open circles in Fig. 1(b), are invariant in NfrA1. In NfrA2, twelve amino acids are conserved and two are replaced with similar amino acids. Together, these results indicate that the FMN-binding pocket or active center of NfrA1 and probably NfrA2 is very similar in structure to that of Frp.[10]

Additional discussion

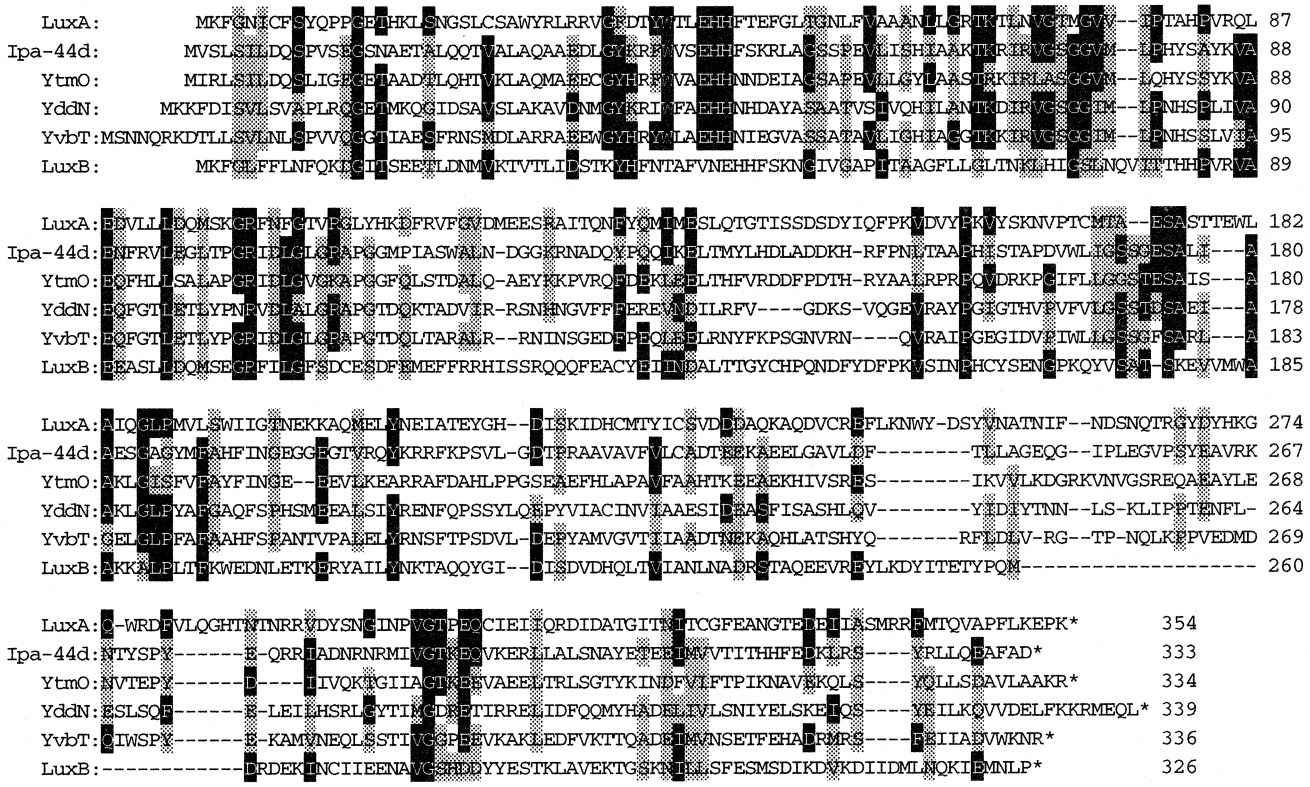

Fig. 4. Comparison of Amino Acid Sequences of Four *B. subtilis* LuxAB-Like Proteins and Authentic *luxA* and *luxB* Gene Products.

LuxA, the α subunit of *P. fischeri* luciferase;[37] Ipa-44d, *B. subtilis* hypothetical protein;[14,15] YtmO, *B. subtilis* hypothetical protein;[15] YddN, *B. subtilis* hypothetical protein;[15] YvbT, *B. subtilis* alkanal monooxygenase homolog;[15] LuxB, the β subunit of *P. fischeri* luciferase.[37] Invariant amino acids are indicated by white letters in black boxes, while similar amino acids are in grey boxes. Amino acid groups are as follows: A, S, P, and G; N, D, E, and Q; H, R, and K; M, L, I, and V; F, Y, and W.[38]

In this work, we have shown that *ipa-43d* encoding an NADPH-linked nitro/flavin reductase that is similar in amino acid sequence and biochemical properties to NfsA nitroreductase and Frp FMN reductase. To our knowledge, NfrA1 is the first non-luminescent-bacterial NfsA/Frp-type enzyme capable of reducing FMN and supplying the reduced form of cofactor FMN to the bacterial luciferase. Since four *luxAB*-like genes were identified in the *B. subtilis* chromosome[15] (see Fig. 4), the presence or absence of *luxAB*-like genes might be critically important for NfsA/Frp family members to acquire or lose FMN reductase activity in evolution.

Although virtually no amino acid sequence identity can be detected between Frp and FRase I,[6] recent crystallographic analyses showed that Frp is similar in overall folding to FRase I.[10,11] In particular, an amino-terminal short region involved in FMN binding appeared to be chemically similar among all members of Frp/NfrA1 and FRase I families (Fig. 5), suggesting that these enzymes may possibly be derivatives of a common progenitor.

```
      Nox:8  LDAKTAALKRRSIRRYR-KDPVPE  30
FRase I:3  HPIIHDLENRYTSKKYDPSKKVSQ  26
    NfsB:1  MDIISVALKRHSTKAFDASKKLTP  24
   NfrA1:2  NNTIETILNHRSIRSFT-DQLLTA  24
   NfrA2:2  NEVIKVLTDHRSIRSYT-DEPVAQ  24
     Frp:2  TPTIETILAHRSIRKFT-AVPITD  24
    NfsA:2  NNTIETICGHRSIRHFT-DEPISE  24
```

Fig. 5. Amino Acid Sequence Similarity between Frp/NfrA1 and FRase I Families.

Bold face letters show chemically similar amino acids. Nox, *Thermus thermophilus* NADH oxidase[36]; FRase I, *P. fischeri* flavin reductase[8]; NfsB, *E. coli* NAD(P)H nitroreductase[5]; NfrA1, *B. subtilis* nitro/flavin reductase[14,15]; NfrA2, *B. subtilis* nitro/flavin reductase homolog[5]; Frp, *V. harveyi* flavin reductase[7]; NfsA, *E. coli* NADPH nitroreductase.[6] FRase I family includes Nox, FRase I and NfsB, while Frp/NfrA1 family, NfrA1, NfrA2, Frp, and NfsA.

Acknowledgments

We thank Dr. Marie-Francoise Hullo for providing the plasmid pIPA43. This work was supported in part by grants from the Ministry of Education, Science, Sports, and Culture of Japan to K.S.

References

1) Bryant, D. W., McCalla, D. R., Leeksma, M., and Laneuville, P., Type I nitroreductases of *Escherichia coli*. *Can. J. Microbiol.*, **27**, 81–86 (1981).
2) Kumar, A. N. and Jayaraman, R., Molecular cloning, characterization and expression of a nitrofuran reductase gene of *Escherichia coli*. *J. Biosci.*, **16**, 145–159 (1991).
3) Chatterjee, P. K. and Sternberg, N. T., A general genetic approach in *Escherichia coli*: for determining the mechanism(s) of action of tumoricidal agents: Application to DMP840, a tumoricidal agent. *Proc. Natl. Acad. Sci. USA*, **92**, 8950–8954 (1995).

4) Michael, N. P., Brehm, J. K., Anlezark, G. M. and Minton, N. P., Physical characterization of the *Escherichia coli* B gene encoding nitroreductase and its over-expression in *Escherichia coli* K12. *FEMS Microbiol. Lett.*, **124**, 195–202 (1994).
5) 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.*, **120**, 736–744 (1996).
6) Zenno, S., Koike, H., Kumar, A. J., 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).
7) 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).
8) Zenno, S., Saigo, K., Kanoh, H., and Inouye, S., Identification of the gene encoding the major NAD(P)H-flavin oxidoreductase of the bioluminescent bacteria *Vibrio fischeri* ATCC 7744. *J. Bacteriol.*, **176**, 3536–3543 (1994).
9) Lei, B. and Tu, S.-C., Characterization of the *Vibrio harveyi* FMN:NADH oxidoreductase expressed in *Escherichia coli*. In "Flavins and Flavoproteins 1993", ed. Yagi, K., Walter de Gruyter & Co., Berlin, pp. 847–850 (1994).
10) Tanner, J. J., Lei, B., Tu, S.-C., and Krause, K. L., Flavin reductase P: Structure of a dimeric enzyme that reduces flavin. *Biochemistry*, **35**, 13531–13539 (1996).
11) Koike, H., Sasaki, H., Kobori, T., Zenno, S., Saigo, K., Murphy, M. E. P., Adman, E.T., and Tanokura, M., 1.8 A 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).
12) 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).
13) Zenno, S., Kobori, T., Tanokura, M., and Saigo, K., Conversion of NfsA, the major *Escherichia coli* nitroreductase, to a flavin reductase having 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).
14) Glaser, P., Kunst, F., Arnaud, M., Coudart, M.-P., Gonzales, W., Hullo, M.-F., Ionescu, M., Lubochinsky, B., Marcelino, L., Moszer, I., Presecan, E., Santana, M., Schneider, E., Schweizer, J., Vertes, A., Rapoport, G., and Danchin, A., *Bacillus subtilis* genome project: cloning and sequencing of the 97 kb region from 325° to 333°. *Mol. Microbiol.*, **10**, 371–384 (1993).
15) Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S.-K., Codani, J.-J., Connerton, I. F., Cummings, N. J., Daniel, R. A., Denizot, F., Devine, K. M., Dusterhoft, A., Ehrlich, S. D., Emmerson, P. T., Entian, K. D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Fuma, S., Galizzi, A., Galleron, N., Ghim, S.-Y., Glaser, P., Goffeau, A., Golightly, E.J., Grandi, G., Guiseppi, G., Guy, B. J., Haga, K., Haiech, J., Harwood, C. R., Henaut, A., Hilbert, H., Holsappel, S., Hosono, S., Hullo, M.-F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., Klaerr-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, P., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S.-M., Levine, A., Liu, H., Masuda, S., Mauel, C., Medigue, C., Medina, N., Mellado, R. P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., ORelly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S.-H., Parro, V., Pohl, T. M., Portetelle, D., Porwollik, S., Prescott, A. M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Roche, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S. J., Serror, P., Shin, B.-S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T., Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A., Tanaka, T., Terpstra, P., Tognoni, A., Tosato, V., Uchiyama, S., Vandenbol, M., Vannier, F., Vassarotti, A., Viari, A., Wambutt, R., Wedler, E., Wedler, H., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K., Yasumoto, K., Yata, K., Yoshida, K., Yoshikawa, H.-F., Zumstein, E., Yoshikawa, H., and Danchin, A., The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature*, **390**, 249–256 (1997).
16) Messing, J. and Vieira, J., A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene*, **19**, 269–276 (1982).
17) Sambrook, J., Fritsch, E.F., and Maniatis, T., "Molecular Cloning: a Laboratory Manual". 2nd edn. Cold Spring Harbor Laboratory, New York, (1989).
18) Martinez, E., Bartolome, B., and de la Cruz, F., pACYC184-derived cloning vectors containing the multiple cloning site and *lacZ*• reporter gene of pUC8/9 and pUC18/19 plasmids. *Gene*, **68**, 159–162 (1988).
19) Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A., Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487–491 (1988).
20) Vieira, J. and Messing, J., Production of single-stranded plasmid DNA. *Methods Enzymol.*, **153**, 3–11 (1987).
21) Chung, C. T., Niemela, S. L., and Miller, R. H., One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. USA*, **86**, 2172–2175 (1989).
22) Inouye, S., Zenno, S., Sakaki, Y., and Tsuji, F. I., High-level expression and purification of apoaequorin. *Protein Expr. Purif.*, **2**, 122–126 (1991).
23) Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685 (1970).
24) Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
25) Ohnishi, K., Niimura, Y., Yokoyama, K., Hidaka, M., Masaki, H., Uchimura, T., Suzuki, H., Uozumi, T., Kozaki, M., Komagata, K., and Nishino, T., Purification and analysis of a flavoprotein functional as NADH oxidase from *Amphibacillus xylanus* overexpressed in *Escherichia coli*. *J. Biol. Chem.*, **269**, 31418–31423 (1994).
26) Uemori, T., Ishino, Y., Fujita, K., Asada, K, and Kato, I., Cloning of the DNA polymerase gene of *Bacillus caldotenax* and characterization of the gene product. *J. Biochem.*, **113**, 401–410 (1993).
27) Kyte, J. and Doolittle, R. F., A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.*, **157**, 105–132 (1982).
28) Chou, P. Y. and Fasman, G. D., Empirical predictions of protein conformation. *Annu. Rev. Biochem.*, **47**, 25–76 (1978).
29) Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Liipman, D. L., Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403–410 (1990).
30) Shine, J. and Dalgarno, L., The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proc. Natl. Acad. Sci. USA*, **71**, 1342–1346 (1974).
31) Rosenberg, M. & Court, D., Regulatory sequences involved in the promotion and termination of RNA transcription. *Annu. Rev. Genet.*, **13**, 319–353 (1979).
32) 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).
33) Jablonski, E. and DeLuca, M., Purification and properties of the NADH and NADPH specific FMN oxidoreductases from

Beneckea harveyi. Biochemistry, **16**, 2932–2936 (1977).
34) Schmidt, T. M., Kopecky, K., and Nealson, K. H., Bioluminescence of the insect pathogen *Xenorhabdus luminescens. Appl. Environ. Microbiol.*, **55**, 2607–2612 (1989).
35) Tu, S.-C. and Hastings, J. W., Physical interaction and activity coupling between two enzymes induced by immobilization of one. *Proc. Natl. Acad. Sci. USA*, **77**, 249–252 (1980).
36) Park, H.-J., Kreutzer, R., Reiser, C. O. A., and Sprinzl, M., Molecular cloning and nucleotide sequence of the gene encoding a H_2O_2-forming NADH oxidase from the extreme thermophilic *Thermus thermophilus* HB8 and its expression in *Escherichia coli. Eur. J. Biochem.*, **205**, 875–879 (1992).
37) Foran, D. R., and Brown, W. N., Nucleotide sequence of the LuxA and LuxB genes of the bioluminescent marine bacterium *Vibrio fischeri. Nucleic Acids Res.*, **16**, 777–777 (1988).
38) Schwartz, R. M., and Dayhoff, M. O., Matrices for detecting distant relationships. In "Atlas of Protein Sequence and Structure", vol. 5, supplement 3, ed. Dayhoff, M. O. National Biochemical Research Foundation, Washington, D.C., pp. 353–358 (1978).