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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*[1]

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***nfsB*, encoding a minor oxygen-insensitive nitroreductase, was isolated by PCR using primers corresponding to two amino acid sequences conserved among the major flavin reductase from *Vibrio fischeri* and classical nitroreductases from *Salmonella typhimurium* and *Enterobacter cloacae*. The gene product, NfsB, was purified to homogeneity from extracts of *Escherichia coli* cells overexpressing it. *nfsB* was found to be situated at 13 min on the *E. coli* map. Biochemical analysis indicated NfsB to be a polypeptide having a calculated molecular weight of 23,904, capable of forming a homodimer and associated tightly with FMN as a prosthetic group. Although it exhibited a lower affinity to the NfsB apoenzyme than FMN, FAD could serve as an effective substitute for FMN. It was also shown that NfsB has a broad electron acceptor specificity and is associated with a low level of the NAD(P)H-flavin oxidoreductase. The NfsB catalysis obeys the ping pong Bi-Bi mechanism. The K_m value for NADH varied depending on the second substrate used.**

Key words: *Escherichia coli*, flavin reductase, flavoenzyme, *nfsB*, nitroreductase.

How enzymes have acquired various catalytic functions during evolution is one of the central questions of enzymology. A newly identified FRase I family (*1*) may provide a good system for this type of study. It consists of two apparently different enzyme groups, flavin reductase [FRase I from *Vibrio fischeri* (*1*)] and nitroreductase [classical nitroreductases from *Salmonella typhimurium* (*2*) and *Enterobacter cloacae* (*3*)], although these two enzyme groups exhibit a significant amino acid sequence homology (*1*).

In luminous bacteria, *V. fischeri*, FRase I and Fre, a minor flavin reductase (*4*), are presumed to play an essential role in supplying reduced FMN ($FMNH_2$) for the luminescence reaction (*1, 4*). In *Escherichia coli*, which lacks the luminescence reaction, Fre has been shown to represent most of the activity of flavin reductase and to be a member of the ribonucleotide reductase multiprotein system (*5, 6*). Little or no sequence homology has been detected between FRase I and Fre (*1, 4*). Moreover, many flavin reductases have been identified enzymatically in various organisms ranging from bacteria to humans, and their molecular weights, substrate specificities and reaction mechanism have been shown to vary from enzyme to enzyme (*1, 4*). Thus, it might be speculated that flavin reductases have been generated by convergence in evolution.

In *E. coli*, two different types of nitroreductases (NRases), oxygen-insensitive (type I) and oxygen-sensitive (type II), have been recognized (*7, 8*). Classical NRases of *S. typhimurium* and *E. cloacae* may belong to type I (*9, 10*). In *E. coli*, the type I nitroreductase activity consists of one major and two minor components (*11*). The major component is an NADPH-linked enzyme, while the minor ones can use both NADH and NADPH as electron donors. Although some mutants lacking type I nitroreductases have been identified (*12*), the precise chromosomal locations of these mutations have not yet been determined.

For further clarification of the functional and evolutional relationship between flavin reductase and nitroreductase families, we isolated the *E. coli* gene encoding a minor type I nitroreductase encoded by *nfsB* (*11, 12*). The *nfsB* gene product was purified to homogeneity and its biochemical properties were compared with those of purified FRase I. Apart from the flavin reductase activity, NfsB and FRase I were very similar to each other in various biochemical properties, including the ability to form a homodimer, tight association with FMN, and broad electron acceptor specificity. Furthermore, in a separate experiment, we showed that a single amino acid substitution can convert NfsB to an FRase I-like flavin reductase which is 3 times as active as

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Abbreviations: NRase, nitroreductase; nitrofurazone, 5-nitro-2-furaldehyde semicarbazone; DCIP, 2,6-dichloroindophenol; DMP, dimethyl pimelimidate.

the authentic FRase I (*13*). Based on these results, possible evolutional and functional relationships between FRase I and NfsB are discussed.

MATERIALS AND METHODS

Enzymes and Chemicals—Restriction enzymes and DNA modifying enzymes were obtained from Nippon Gene (Toyama), Takara Shuzo (Kyoto), and Toyobo (Osaka). [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from Du Pont NEN (Boston, MA) or ICN Biomedicals (Costa Mesa, CA). Oligonucleotides were synthesized by the phosphoamidite method using a Biosearch Cyclone DNA synthesizer. The following reagents were purchased from Wako Pure Chemical (Osaka): FMN sodium salt, riboflavin, nitrofurazone (5-nitro-2-furaldehyde semicarbazone), dicumarol, methyl 4-nitrobenzoate, 4-nitroacetophenone, 4-nitrobenzoate, 4-nitrotoluene, 4-nitrophenol, 4-nitroaniline, menadione, 1,4-benzoquinone, methylene blue, potassium ferricyanide, horse heart cytochrome *c*, 2,6-dichloroindophenol (DCIP) sodium, Ponceau SX, Bordeaux S, Tartrazine, Orange II, 5-bromo-4-chloro-3-indolyl-β-D-galactoside, ampicillin sodium, proteinase K, and lysozyme. FAD sodium salt was obtained from Boehringer Mannheim (Indianapolis), while NADH sodium salt and NADPH sodium salt were from Oriental Yeast (Osaka). Lumiflavin, nitrofurantoin, 4-nitrobenzene methyl sulfonate, RNase A, and DNase I were obtained from Sigma Chemical (St. Louis, MO). Dimethyl pimelimidate (DMP) was purchased from Pierce Chemical (Rockford, IL). All these and other chemicals were of the highest grade commercially available.

Bacterial Strains and Plasmids—*E. coli* C600 (*hsdR thi-1 thr-1 leuB6 lacY1 tonA21 supE44*) (*14, 15*), D1210 [F$^-$ *hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL*(Str^r) *xyl-5 metl-1 sup44* λ^- *lacI*^{q} *lacY*$^+$] (*16*), and JM83 [*ara* Δ(*lac-proAB*) F$^-$ *rpsL* (ϕ*80lacZ* Δ*M15*)] (*17*) were used for cloning and expression of cloned fragments. Bacteria were cultured in Luria-Bertani (LB) medium (*18*) at 30-37°C. pUC13 (*19*) and pUC118 (*20*) were used as cloning or expression vectors.

Cloning and Mapping of the E. coli nfsB Gene, and Construction of the Plasmid Expressing nfsB—Mixed oligonucleotide primers were made on the basis of the amino acid sequences conserved between *V. fischeri* FRase I (*1*), *S. typhimurium* NRase (*2*), and *E. cloacae* NRase (*3*) (see Fig. 2): NR1, 5′-CA[AG]CC[TCAG][TGG][CA]A-[TCAG]TT[TC]AT[TCA]GT; NR2, 5′-A[AG][AGTC]-[GC][ATC][AGTC]GC[AG]TT[AG][TA]A[AG]TC-[TC]TC. PCR was carried out using *E. coli* C600 genomic DNA as a template and oligonucleotides NR1 and NR2 as primers essentially as described by Saiki *et al.* (*21*): 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 60 s, and extension at 72°C for 90 s with a Perkin-Elmer thermal cycler. The amplified DNA was cloned into pUC13 and subjected to sequence analysis. Lambda clones 21A9S and 2D12 were selected from the genomic DNA library of Kohara *et al.* (*22*; Takara Shuzo) with a PCR-amplified cloned DNA fragment as a probe. A 6.0-kb *Hin*dIII fragment of 21A9S was inserted into the *Hin*dIII site of pUC13 to construct pNR1. pNR1 DNA was digested with *Pst*I and then self-ligated to produce pNR2. The *Nru*I-*Pst*I fragment (1.2 kb) from pNR2 was inserted into the *Sma*I-*Pst*I site of pUC118 to obtain pNR3 (see Fig. 1A).

Purification of NfsB—E. coli JM83 cells were transformed with pNR3 by the polyethylene glycol method (*23*). JM83 containing pNR3 was precultured overnight at 30°C. A 120 ml aliquot of preculture was added to 3 liters of LB broth supplemented with ampicillin (0.1 mg/ml) and foam suppressant (0.01%) and cultivated at 37°C for 6 h as described previously (*24*). Cells were harvested by centrifugation and the pellet suspended in 60 ml of 20 mM Tris-HCl buffer (pH 7.0) was subjected to a 10-min sonication using a Branson model 250 sonifier (Danbury, CT). After centrifugation at 12,000 × *g* at 4°C for 30 min, the supernatant fluid was obtained. This was used as the starting cell extract for subsequent purification. The cell extract was dialyzed against 20 mM Tris-HCl (pH 7.0) at 4°C. The dialyzate (65 ml) was then loaded onto a Q Sepharose FF (Pharmacia) anion-exchange column (2.6 × 10 cm) equilibrated with 20 mM Tris-HCl (pH 7.0), washed with 300 ml of 20 mM Tris-HCl (pH 7.0) (flow rate: 1 ml/min) and eluted with a 0-400 mM linear gradient of KCl in 20 mM Tris-HCl (pH 7.0). An aliquot (less than 100 μl) of each fraction (2 ml) was subjected to FMN reductase assay and protein assay, then pooled peak fractions were subjected to gel-filtration on Superose 12 (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.0), with 150 mM NaCl (flow rate: 0.2 ml/min). The gel-filtration step was repeated once more and active fractions were pooled, dialyzed against 50 mM Tris-HCl (pH 7.0) and stored at −20°C for further analysis. All chromatographic procedures were performed at 4°C. If necessary, purified NfsB was treated with 50 mM Tris-HCl (pH 7.0) with 1 mM FMN at 4°C.

Cell extracts were also prepared from *E. coli* D1210 with pNR3, induced by IPTG, as described previously (*1*).

Preparation of NfsB Apoenzyme and Reconstruction of NfsB-Flavin Complexes—Preparation of apoenzyme was performed essentially as described by Strittmatter (*25*). One milliliter of 3 M KBr was added to 1 ml of purified NfsB (3.2 mg/ml) in 100 mM Tris-HCl (pH 7.0) on an ice bath. Five milliliters of saturated ammonium sulfate (pH 1.5) was added to the mixture followed by centrifugation at 12,000 × *g* for 10 min. The precipitate was dissolved in 2.0 ml of 100 mM Tris-HCl buffer (pH 9.0) with 1.5 M KBr and stood on ice for 30 min, followed by dialysis against 350 ml of 0.1 M Tris-HCl (pH 7.0) buffer for 46 h. After centrifugation at 12,000 × *g* for 20 min, the resultant supernatant fluid was used as the apoenzyme solution. Reconstruction of the holoenzyme was carried out essentially as described by Bryant and DeLuca (*10*). One millimolar FMN or FAD and 3.1 mg/ml NfsB apoenzyme were incubated in 50 mM Tris-HCl (pH 7.0) at 10°C for 69 h. Excess flavin was removed by gel filtration. All procedures were carried out in a cold room (4°C), unless noted otherwise. FRase I apoenzyme was prepared and its holoenzymes were reconstructed similarly.

Cross-Linking of NfsB Nitroreductase with DMP—NfsB (1 mg) was cross-linked with 2 mg/ml DMP in 500 μl of 0.2 M triethanolamine-HCl buffer (pH 8.1) for 2 h at 23°C (*26*). The reactions were initiated by the addition of DMP and terminated by the addition of an equal volume of 1 M glycine (pH 8.5). Reaction products were concentrated using a Centricon-10 concentrator (Amicon) and analyzed by SDS-PAGE (see below).

Protein Analysis—Protein concentration was determined

by using bovine plasma γ-globulin as a standard with Bio-Rad protein assay kit (*27*). SDS-PAGE analysis was carried out in 15% (w/v) gel (1 mm thickness) at 25 mA for 2 h (*28*), and the gel was stained with Coomassie Brilliant Blue R-250 (Fluka Chemie, Buchs, Switzerland). Adsorption on CM cellulose (Serva Feinbiochemica, Heidelberg, Germany) was carried out essentially as described by Bryant *et al.* (*11*). Purified NfsB, NADH-nitrofurazone reductase (2 ml of 1.5 μmol/min/ml) was applied to a CM cellulose cation-exchange column (1×4 cm) equilibrated with 5 mM citrate buffer (pH 5.8) and eluted with a 0-400 mM stepwise gradient of NaCl. Absorbance spectra were measured at 23°C with a model U-3210 Hitachi recording spectrophotometer (Tokyo). The extinction coefficient of the protein-bound FMN at 445 nm was determined essentially as described by Ohnishi *et al.* (*29*) using an extinction coefficient of FMN at 445 nm ($\varepsilon = 12{,}500\ M^{-1}\cdot cm^{-1}$).

Enzyme Assay—Enzyme activities were determined at 23°C using the following extinction coefficients ($M^{-1}\cdot cm^{-1}$): 12,960, nitrofurazone at 400 nm; 1,000, ferricyanide at 420 nm; 20,600, DCIP at 600 nm; 18,500, cytochrome *c* at 550 nm; 31,200, Tartrazine at 422 nm; 19,600, Ponceau SX at 500 nm; 26,500, Bordeaux S at 522 nm; 13,800, Orange II at 484 nm; 6,220, NADH or NADPH. A typical reaction mixture (3.0 ml) contained 50 mM Tris-HCl buffer (pH 7.0), 0.1 mM NADH (or NADPH), 0.1 mM of a given electron acceptor (in the case of cytochrome *c*, 1 mg/ml), and a suitable amount of enzyme. The reaction was initiated by the addition of NADH or NADPH, and the initial rate was measured with a model U-3210 Hitachi recording spectrophotometer.

Flavin Analysis—The enzyme-bound flavin was liberated by denaturation of the purified enzyme with heat treatment at 70°C for 20 min followed by centrifugation at 10,000×*g* for 20 min. The supernatant was analyzed by thin-layer chromatography (TLC) on silica gel (0.25 mm thickness) plates (Merck, Darmstadt, Germany) using a solvent system of 1-butanol-acetic acid-water (4:1:1, by volume) and the developed plates were visualized under UV light at 366 nm.

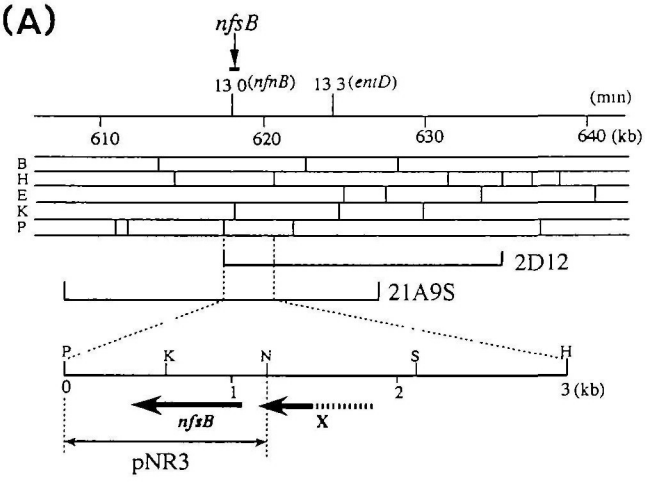
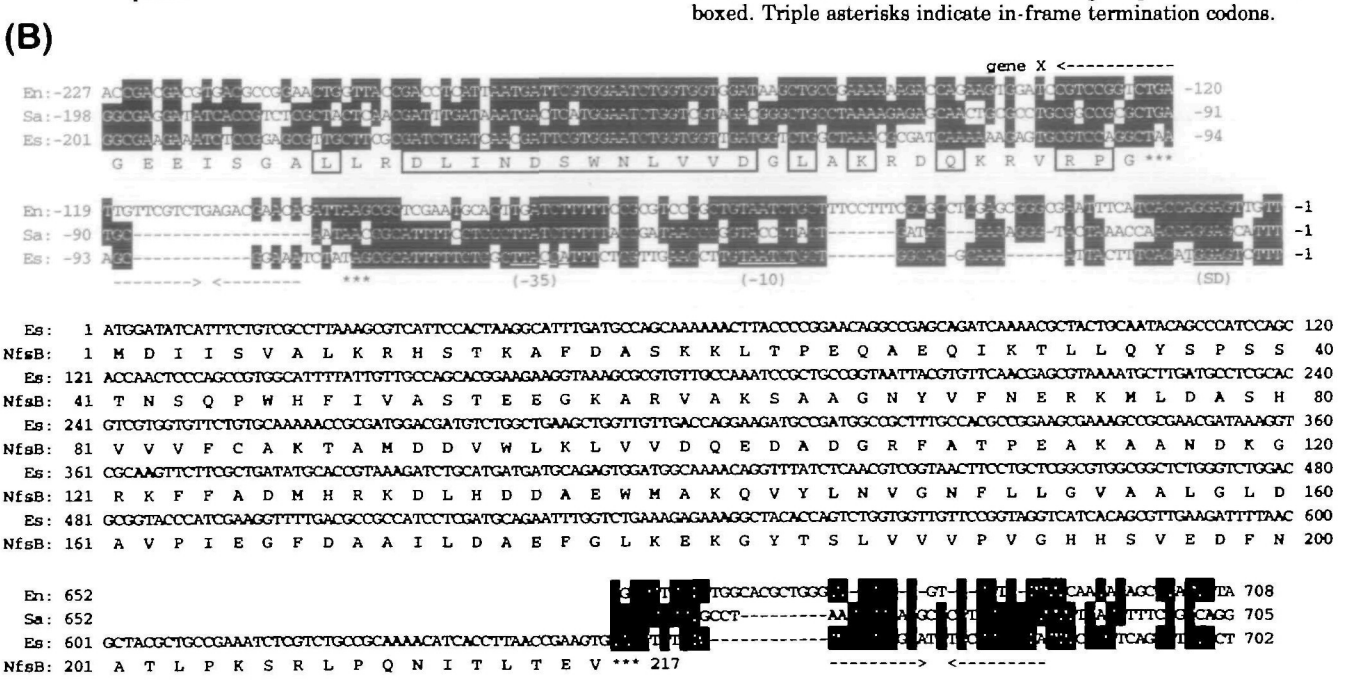


Fig. 1. **Physical map (A) and nucleotide sequence (B) of the *nfsB* gene.** (A) Scales are expressed in kilobase (kb) coordinates in which the position of *thr* at 0 min on the genetic map is taken as 0 kb. A part of the physical map described by Kohara *et al.* (*22*) is shown. U-shaped bars labeled with 2D12 and 21A9S show the sizes and locations of two *nfsB*-positive clones. Two thick arrows indicate *nfsB* and its putative neighbor, X. B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; N, *Nru*I; S, *Stu*I. (B) The nucleotide sequence of the *E. coli nfsB* gene (Es) is shown together with the nucleotide sequences flanking the NRase genes of *S. typhimurium* (Sa; *2*) and *E. cloacae* (En; *3*). The deduced amino acid sequence of the NfsB protein is shown below the nucleotide sequence. Thin lines labeled (−10), (−35), and (SD), respectively, show the −10 box (*43*), the −35 box (*43*), and the Shine-Dalgarno sequence (*44*). A pair of arrows in the 3′-untranslated region indicates the location of a stem-loop structure, a possible transcription terminator (*43*). Nucleotides invariant among Es, Sa, and En are indicated as white letters in black boxes. The inverted repeat in the 5′ upstream region shows a putative transcription terminator signal for the immediately upstream gene, X, of *E. cloacae*. Amino acids conserved in the X gene product of *E. coli* are boxed. Triple asterisks indicate in-frame termination codons.



Other Cloning Techniques—DNA fragments were labeled with [α-^{32}P]dCTP (3,000 Ci/ml) by random priming (*30*). DNA sequences were determined by using the *Bca*Best dideoxy sequencing kit (Takara Shuzo) (*31, 32*), and DNASIS software (Hitachi Software Engineering, Yokohama). Other techniques have been described by Sambrook *et al.* (*18*).

RESULTS

Identification and Map Position Determination of the nfsB Gene—The *E. coli* counterpart of *V. fischeri* FRase I gene and classical NRase genes from *S. typhimurium* and *E. cloacae* was isolated by PCR followed by screening of a genomic DNA library (Fig. 1A). Nucleotide sequence analysis showed the *E. coli* counterpart to encode a polypeptide of 217 amino acid residues with a calculated molecular weight of 23,904 (Fig. 1B). As suggested previously (*1*), the identity in amino acid sequence to FRase I was 33%, whereas that to the classical NRases was 88–89% (Fig. 2). This finding may suggest that the polypeptide encoded by the *E. coli* counterpart is a nitroreductase, but not a flavin reductase. The sequence similarity can also be extended to *Thermus thermophilus* NADH oxidase (Nox; *33*), a flavoenzyme catalyzing the oxidation of NADH by the reduction of oxygen (*34*) (amino acid sequence identity: 23%), a putative flavin reductase from *Haemophilus influenzae* (*35*) (amino acid sequence identity: 25%) and a putative nitroreductase from *Mycoplasma*-like organism (GeneBank accession number: L22217) (amino acid sequence identity: 18%) (Fig. 2).

Biochemical experiments have suggested that *E. coli* contains three species of oxygen-insensitive nitroreductases, IA, IB1, and IB2 (*11*). The major nitroreductase (IA), encoded by *nfsA*, can use only NADPH as an electron donor, while both NADH and NADPH serve as effective electron donors for IB1 (the *nfsB* gene product) and IB2. IA and IB2 can bind to CM-cellulose at pH 5.8, but IB1 can not. Thus, the fact that both NADH and NADPH are effective electron donors (Table I) indicates that the *E. coli* counterpart of FRase I is not IA, but either IB1 or IB2. The inability to bind to CM-cellulose at pH 5.8 (virtually all activity was recovered in the pass-through fractions) excludes the possibility that the *E. coli* counterpart is IB2. Thus, we conclude that the *E. coli* counterpart of FRase I is IB1, the *nfsB* gene product. We hereafter refer to the *E. coli* counterpart of FRase I and its gene as NfsB and *nfsB*, respectively.

Analysis using the Kohara library indicated that *nfsB* is situated at kilobase coordinate 617–619, or at 13.0 min on the *E. coli* map (*22, 36*) (Fig. 1A). Consistent with this, genetic data of McCalla *et al.* (*12*) show *nfsB* to be situated between *lac* (7 min) and *gal* (18 min) on the *E. coli* linkage map.

Purification of NfsB as a Flavoprotein Dimer—NfsB was purified from cell extracts of *E. coli* JM83 harboring pNR3, a *lac*-operator/promoter-dependent expression plasmid. NfsB was overproduced and eventually represented 20% of the total soluble protein. Cell extracts were applied to a Q Sepharose FF column equilibrated with 20 mM Tris-HCl (pH 7.0) and eluted by a 0–400 mM KCl gradient. Peak fractions were pooled and subjected to Superose 12 gel filtration, and the 27-kDa protein was purified virtually to homogeneity (Fig. 3). As shown in Fig. 3A, purified NfsB is

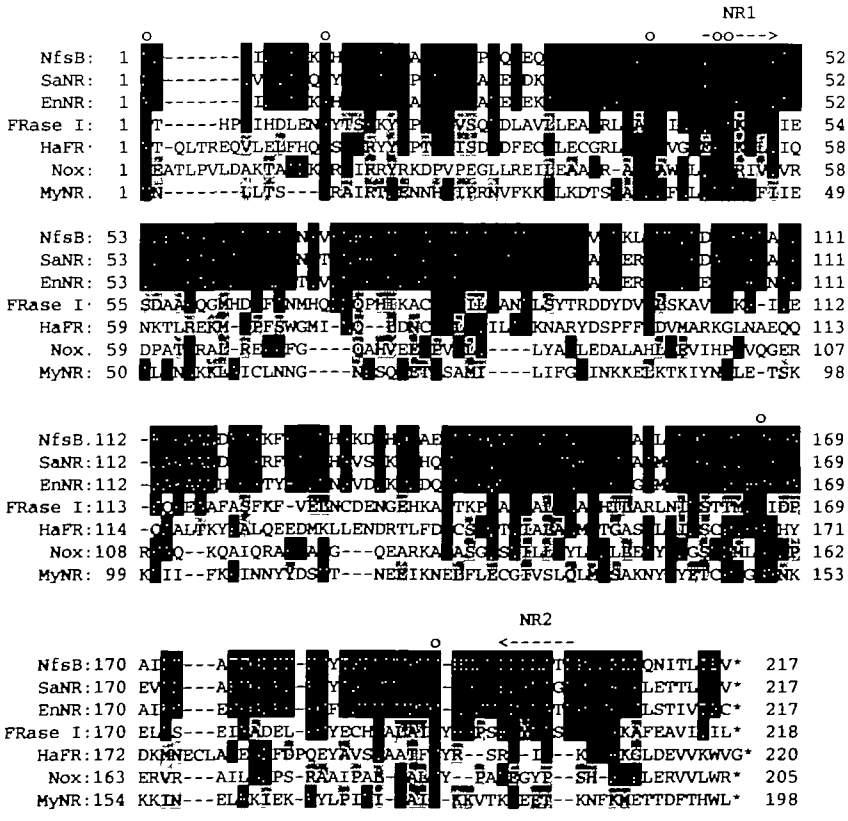


Fig. 2. **Amino acid sequence homology among NfsB and other flavoenzymes.** NfsB, *E. coli* nitroreductase (this work); SaNR, *S. typhimurium* nitroreductase (*2*); EnNR, *E. cloacae* nitroreductase (*3*); FRase I, *V. fischeri* flavin reductase (*1*); HaFR, flavin reductase homolog of *H. influenzae* (*35*); Nox, *T. thermophilus* NADH oxidase (*33*); MyNR, nitroreductase homolog of *Mycoplasma*-like organism (GenBank accession number: L22217). White letters in black boxes, amino acids invariant among NfsB and NRases from *S. typhimurium* and *E. cloacae* nitroreductases. O, amino acid conserved among all members examined. Hatched boxes, amino acids similar to the invariant amino acids. Amino acids belonging to the same groups are as follows: A, S, P, T, and G; N, D, E, and Q; H, R, and K; M, L, I, and V; F, Y, and W (*45*). Gaps (-) were inserted to increase the sequence homology. Horizontal arrows show the amino acid sequences corresponding to PCR primers NR1 and NR2.

associated with a low level of FMN reductase activity: the specific activity as FMN reductase was about 1/15 that as nitroreductase.

Nucleotide sequence analysis along with SDS-PAGE (Fig. 3) indicated the molecular weight of NfsB to be 2.4 or 2.7×10^4, while that estimated by gel filtration was 4.3×10^4 (see Fig. 3A). To determine whether NfsB is a non-globular protein or a homodimer, purified NfsB was subjected to DMP cross-linking (*26*), and the reaction products were analyzed by SDS-PAGE. As shown in Fig. 3B, a new band corresponding in size to the dimer (about 5×10^4) was detected. DMP cross-linked products were also analyzed by gel filtration (Fig. 3B). Enzyme activity and cross-linked dimers were eluted at the same position (see the arrow labeled D). Neither enzyme activity nor a protein peak was detected at the expected monomer position (see the arrow labeled M). In contrast, SDS-PAGE showed both monomer (arrow 1) and dimer (arrow 2) to be recovered in putative dimer fractions of gel filtration at a virtually identical ratio. We interpret these results as suggesting that nearly all the molecules exist in a dimeric form and a small amount of free monomer is present in the preparation examined. These results may suggest that active NfsB is a homodimer.

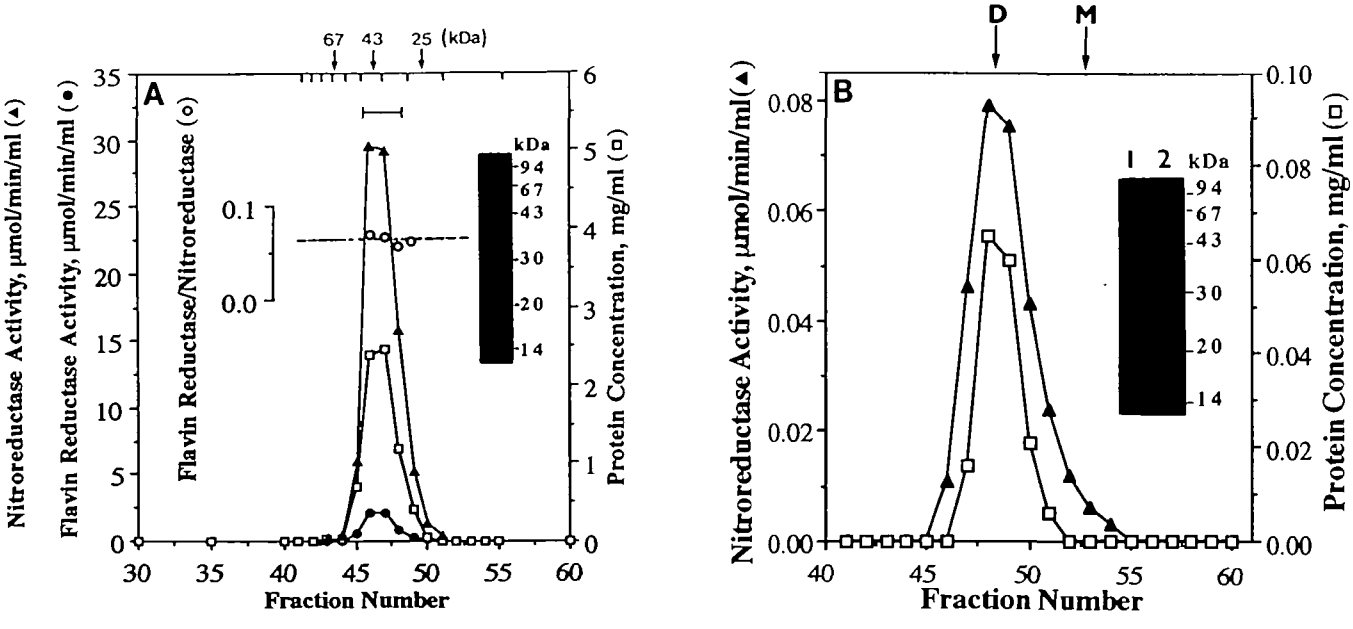
TABLE I. **Flavin reductase and nitroreductase activities in extracts of *E. coli* cells with pNR3.**

Pyridine nucleotide	Extract[a]	Reductase activities (nmol/min/mg of protein) on:	
		FMN	Nitrofurazone
NADH	D1210[b]	52	32
	D1210 (pNR3)	426	5,240
NADPH	D1210[b]	17	116
	D1210 (pNR3)	315	4,400

[a]Cell extracts were prepared as previously described (*1*). [b]de Boer *et al.* (*16*).

As with FRase I (*37*, our unpublished results), a concentrated solution of NfsB was yellow in color and this coloration was not eliminated by extensive dialysis (data not shown). The absorption spectra of NfsB and FMN were very similar to each other, except for about 5 nm shifts of the absorption maxima towards long or short wavelengths (Fig. 4A). These findings may indicate NfsB to be a flavoprotein. Analysis using TLC showed that more than 90% of bound flavin is represented by FMN, while the remainder is riboflavin or possibly FAD (Fig. 4A), suggesting that NfsB purified from *E. coli* cell extracts is heterogeneous with respect to the bound flavin species. In accordance with this, both reconstructed NfsB-FMN and NfsB-FAD (NfsB associated with FMN and FAD, respectively) were enzymatically active (see below). Our estimation based on spectroscopy and dry weight analysis suggested that 1 mol of flavin molecule binds to 1 mol of the protein monomer unit (data not shown).

Reconstruction of NfsBs Having Either FMN or FAD as a Prosthetic Group—To determine whether the bound flavin is an essential component of the enzyme, flavin-free enzyme (apoenzyme) of more than 99% purity was prepar-



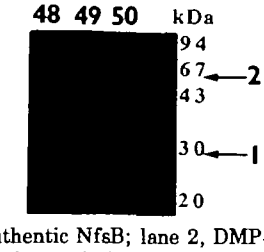


Fig. 3. **Gel filtration patterns of purified cloned NfsB (A) and cross-linked NfsB (B).** ●, NADH-FMN reductase activity; □, protein concentration; ▲, NADH-nitrofurazone reductase activity; ○, ratio of FMN reductase activity to nitrofurazone reductase activity. Protein size markers: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), chymotrypsinogen A (25 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). (A) Superose 12 elution profiles. Locations of molecular size markers are shown by vertical arrows in the upper margin. The inset, an SDS-PAGE patterns of purified NfsB (10 μg). (B) Superose 12 elution profiles of DMP-cross-linked NfsB. Vertical arrows labeled D and M, respectively, show the positions of a putative homodimer mand onomer of NfsB. The inset shows NfsB to be cross-linked by DMP. Lane 1, purified authentic NfsB; lane 2, DMP-cross-linked NfsB. Arrows labeled 1 and 2 in the lower margin show the locations of uncross-linked monomers and cross-linked dimers, respectively.

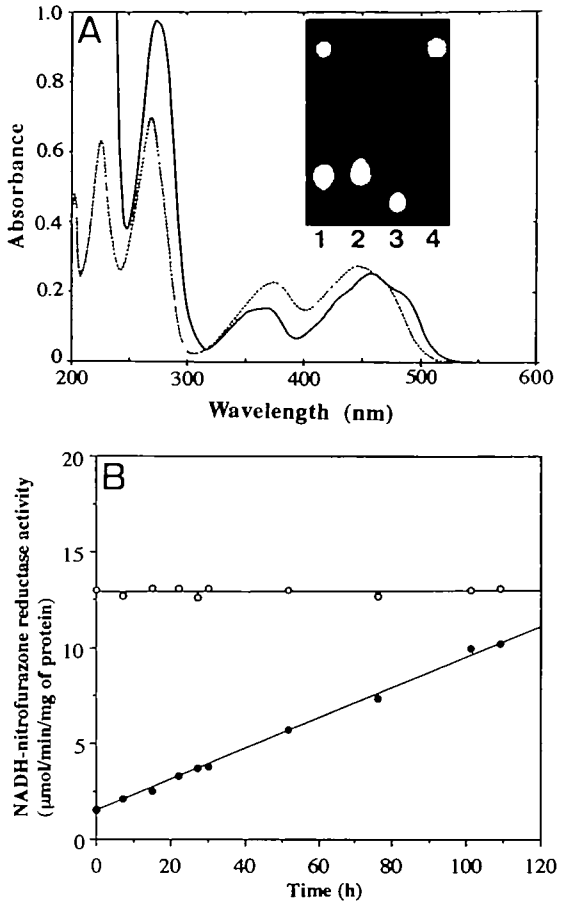


Fig. 4. **Flavin analysis of the purified NfsB (A) and reconstruction of flavin-NfsB complexes (B).** (A) Absorption spectrum of NfsB. The sample consists of 1.14 mg/ml or 48 μM NfsB in 50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl. Purified NfsB, a solid line; authentic FMN, a dotted line. Absorbance maxima in the visible light region are at 370 and 457 nm, while those for free FMN are at 375 and 448 nm. Inset, TLC patterns of flavins. Lanes: 1, flavins from purified NfsB; 2, authentic FMN; 3, authentic FAD; 4, authentic riboflavin. (B) Time course of reconstruction of NfsB-FMN (○) and NfsB-FAD (●). Degrees of reconstruction were measured by following the increment of the NADH-nitrofurazone reductase activity. Reconstruction was carried out at 0°C.

ed (see "MATERIALS AND METHODS"). Table II shows that little or no enzyme reaction proceeds unless either FMN or FAD is added exogenously, suggesting that the bound flavin is essential for the NfsB activity.

By incubating 0.13 mM apoenzyme and either 1 mM FMN or FAD (see "MATERIALS AND METHODS"), NfsB having only FMN or FAD as a prosthetic group was prepared. Figure 4B shows that, under a given condition, binding between FMN and the apoenzyme occurs virtually instantly, while binding of FAD to the apoenzyme is quite slow. In contrast, NfsB-FAD, once formed, is essentially as active as NfsB-FMN (Table II). Steady-state kinetics of NfsB-FMN using NADH as an electron donor and either nitrofurazone (Fig. 5, A and B) or FMN (data not shown) as an electron acceptor suggested that, as with FRase I (*38*, our unpublished results), the NfsB catalysis obeys the ping pong Bi-Bi mechanism (Fig. 5C), although its affinity for NADH was very strong, so that the activity of NfsB nitroreductase was almost independent of the concentration of NADH over 17 μM (Fig. 5B and Table III). The FAD enzyme appeared to have lower affinities for nitrofurazone and FMN than the FMN enzyme (data not shown).

One unexpected finding from kinetic analysis of NfsB is that the K_m value for NADH obtained using FMN as an electron acceptor significantly differs from that obtained using nitrofurazone as an electron acceptor (Table III), possibly as a consequence of hysteresis or a conformational change of the substrate-free enzyme upon substrate binding.

Similarity and Dissimilarity in the Electron Acceptor Specificity—If *V. fischeri* FRase I and *E. coli* NfsB are evolutionarily related to each other, their electron acceptor specificity may be similar to some extent. Thus, we examined the electron acceptor specificity of NfsB and FRase I (Table II). As nitroreductases, NfsB-FMN and NfsB-FAD appeared virtually indistinguishable from FRase I (and its FAD enzyme version). The finding that the addition of FMN had no appreciable effect on the reduction rate of nitrofurazone by either NfsB or FRase I may suggest that (i) the reduction of nitrofurazone is not mediated by the reduced form of free FMN, and (ii) possible binding sites of nitrofurazone and FMN on NfsB or FRase I do not overlap physically. Both FMN- and FAD-associated nitroreductases were inhibited by dicumarol (Table II), an observation showing a striking difference between NfsB or FRase I and NfsA, the major *E. coli* nitroreductase (*39*).

Table II also shows NfsB to be much less effective than FRase I as an FMN reductase. Flavin species other than lumiflavin were poor substrates for NfsB. However, it is worth pointing out here that the specific activity of NfsB for lumiflavin, the smallest flavin so far examined, was similar to or even higher than that of FRase I. These findings might support the notion that NfsB is a kind of defective version of FRase I, which has lost almost all FMN/FAD reductase activity in evolution, but still possesses the active center for the reduction of the isoalloxazine ring of flavin substrates.

According to Table II, NfsB is much more effective than FRase I as far as the reduction of 1,4-benzoquinone, DCIP, or ferricyanide is concerned, suggesting that these activities are also protein-moiety-dependent. In contrast, the reduction of cytochrome *c* and azo compounds appears to be independent of the protein moiety, but dependent on the addition of protein-free FMN, indicating that these reactions may not be enzymatic, but may be due to a direct interaction between the reduced form of free FMN and a given electron acceptor. The low level of a cytochrome *c* reduction activity of NfsB might be due to a possible nonenzymatic interaction between NfsB-bound FMN (a prosthetic group) and cytochrome *c*.

DISCUSSION

In the present paper, we identified the *nfsB* gene, and biochemically characterized its gene product (NfsB), an oxygen-insensitive nitroreductase similar in sequence to *V. fischeri* FRase I. Our results showed that NfsB nitroreductase is a dimeric flavoprotein associated with a low level of FMN/FAD reductase activity. In an independent experiment, Michael *et al.* (*40*) determined the nucleotide sequence of a nitroreductase from *E. coli* B. The sequence

TABLE II. **Electron acceptor specificities of NfsB and FRase I.**

Electron acceptors		Reductase activities (μmol/min/mg of protein)					
		NfsB enzymes			FRase I enzymes		
		NfsB-FMN	NfsB-FAD	Apoenzyme	FRase I-FMN	FRase I-FAD	Apoenzyme
Flavins:	FMN	0.9	1.9	1.6	35	19	14
	FMN+dicumarol	0.6	–	–	0.1	–	–
	FAD	1.1	1.2	0.5	23	12	1.3
	Riboflavin	1.5	2.4	0.01	38	16	0.1
	Lumiflavin	41	56	0.06	25	12	0.1
Nitro compounds:	Nitrofurazone	13	14	0	14	15	0.03
	Nitrofurazone+FMN	13	–	12	12	18	5.8
	Nitrofurazone+dicumarol	0	–	–	0.1	–	–
	Methyl 4-nitrobenzoate	43	48	0.06	24	11	0.03
	Nitrofurantoin	21	–	–	13	–	–
	4-Nitrobenzene methyl sulfonate	94	–	–	37	–	–
	4-Nitroacetophenone	49	–	–	32	–	–
	4-Nitrobenzoate	1.0	–	–	1.2	–	–
	4-Nitrotoluene	0.4	–	–	0.8	–	–
	4-Nitroaniline	0.1	–	–	0.6	–	–
	4-Nitrophenol	0.1	–	–	0.4	–	–
Quinones:	Menadione	60	57	0.04	48	23	0
	Menadione+dicumarol	0.2	–	–	0.3	–	–
	1,4-Benzoquinone	251	201	0.1	47	19	0.2
Azo compounds:	Bordeaux S	0	–	–	0	–	–
	Bordeaux S+FMN	0	–	–	0.1	–	–
	Tartrazine	0	–	–	0	–	–
	Tartrazine+FMN	0.1	–	–	0.05	–	–
	Orange II	0	–	–	0	–	–
	Orange II+FMN	0.2	–	–	0.2	–	–
	Ponceau SX	0	–	–	0	–	–
	Ponceau SX+FMN	0.04	–	–	0.03	–	–
Miscellaneous:	Methylene blue	1.7	4.4	0.1	18	9.0	0.2
	Ferricyanide	387	800	0.2	40	24	0
	Ferricyanide+FMN	452	–	–	83	62	35
	Cytochrome *c*	2.7	0.1	0	0.4	0	0
	Cytochrome *c*+FMN	4.0	2.0	2.1	139	47	16
	DCIP	2.2	1.3	0.03	0.4	0.1	0.07
	DCIP+FMN	3.4	3.5	6.6	0.4	0.5	0.5

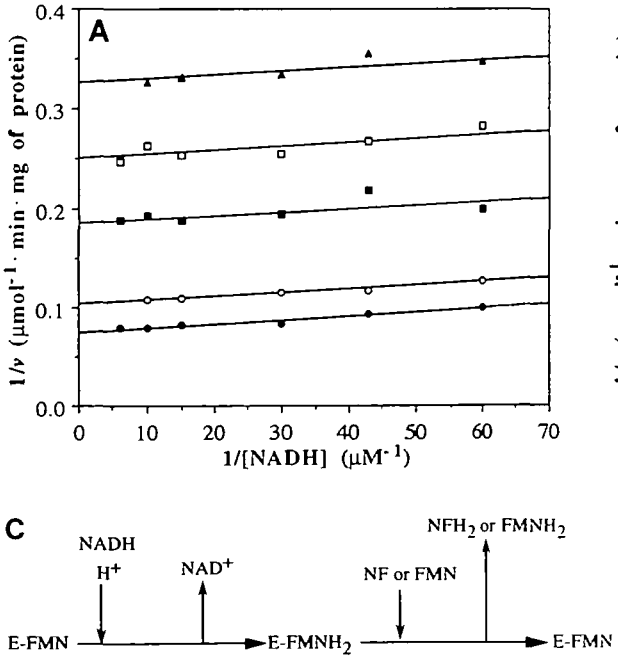


Fig. 5. **Double-reciprocal plots of initial velocity *versus* NADH or nitrofurazone concentration.** Constant concentrations of nitrofurazone (A) and NADH (B) are: ●, 100 μM; ○, 67 μM; ■, 33 μM; □, 23 μM; ▲, 17 μM. (A, B) NADH-nitrofurazone reductase activities are plotted as a function of the inverse of [NADH] (A) or [nitrofurazone] (B). (C) Proposed catalytic mechanism of NfsB. Nitrofurazone and its reduced form, respectively, are shown by NR and NRH_2. E-FMN or E-$FMNH_2$ indicates the NfsB-FMN or NfsB-$FMNH_2$ complex, respectively.

TABLE III. **Kinetic parameters of NfsB.**

Substrate	K_m (μM)	V_{max} (μmol/min/mg of protein)
NADH[a]	22	43
NADH[b]	5.4	1.3
Nitrofurazone[c]	153	32
FMN[c]	29	1.1

Michaelis constant (K_m) and maximal velocity (V_{max}) were determined based on the data shown in Fig. 5. [a] K_m and V_{max} values obtained using nitrofurazone as an electron acceptor. [b] K_m and V_{max} values obtained using FMN as an electron acceptor. [c] K_m and V_{max} values obtained using NADH as an electron donor.

was found to be identical to that of *E. coli* K12 NfsB, determined in the present work.

Reaction Mechanisms of NfsB and FRase I—Our results showed NfsB purified from *E. coli* cells to be tightly associated with FMN, although *in vitro* reconstruction experiments suggested that FAD can also serve as an effective substitute for FMN (see Fig. 4). Steady-state kinetics suggested that the presence of the bound FMN is mandatory for the enzyme reaction (see Table III) and the nitroreductase and flavin reductase reactions both follow the ping pong Bi-Bi mechanism (see Fig. 5). A similar situation holds for FRase I. Using partially purified FRase I, Tu *et al.* (*38*) showed the catalysis by FRase I to proceed *via* the ping pong Bi-Bi mechanism. We confirmed this using purified FRase I (unpublished results). Data in Table II also show the association between FRase I and FMN to be essential for the reduction of riboflavin and lumiflavin. Thus, in the case of FMN reduction by NfsB and FRase I, two FMN molecules must be involved: one as a coenzyme or a prosthetic group tightly associated with the apoenzyme, and the other as a substrate. In this sense, the model proposed recently by Inouye (*37*) is not acceptable. On the basis of maleimide inhibition experiments, Tu *et al.* (*38*) suggested that electrons from NAD(P)H are first transferred to a putative S-S bond of the protein moiety to generate two sulfhydryl (SH) groups. Then, the second substrate (electron acceptor), such as FMN, is reduced by the hydrogen of the SH group to generate $FMNH_2$. However, our sequence alignment (see Fig. 2) showed no cysteine residue invariant between FRase I and NfsB. In addition, neither flavin-free NfsB (NfsB apoenzyme) nor FRase I apoenzyme exhibited any significant reductase activity in the absence of FMN (see Table II). Thus, we presume that, as with most flavoproteins (*41*), the FMN bound tightly as a prosthetic group to the NfsB apoenzyme or FRase I apoenzyme serves as the mediator of electron flow from NAD(P)H to flavin or nitro compound substrates. Our unpublished data (Koike, H., Zenno, S., Nishino, T., Saigo, K., and Tanokura, M.) suggested that two electrons were transferred from NADH to bound-FMN. Therefore, the finding that double reciprocal plots of the initial velocity *versus* NADH or nitrofurazone concentration are linear (see Fig. 5, A and B) may suggest that the first step of nitrofurazone reduction is a 2-electron-transfer reaction, and, hence, either two subunits or active sites of NfsB are enzymatically independent of each other, or one of the two subunits or active sites is inactivated. A recent structural analysis of Nox (*T. thermophilus* NADH oxidase) (*42*), having a 23% homology to NfsB, showed that two bound FMN molecules are distantly situated and hence may not interact with each other. The above reaction mode also suggests that the first intermediate of the nitrofurazone reduction by NfsB is its nitroso derivative.

Evolution of NfsB and FRase I—We examined the electron acceptor activities of 23 compounds. As shown in Table II, except for large flavin molecules, 1,4-benzoquinone and ferricyanide, NfsB, and FRase I showed very similar spectra of electron acceptor specificities. This finding seems to be consistent with the notion that the genes encoding NfsB and FRase I are evolutionary descendants of a common progenitor. In fact, we recently succeeded in converting NfsB nitroreductase to an FRase-I-like flavin reductase (*13*). Our data showed that removal or single amino acid substitutions of Phe-124 resulted in a 100-fold increment of flavin reductase activity (3 times as active as the authentic FRase I) without marked loss of the nitroreductase activity.

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