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Biochemical Characterization of NfsA, the *Escherichia coli* Major Nitroreductase Exhibiting a High Amino Acid Sequence Homology to Frp, a *Vibrio harveyi* Flavin Oxidoreductase

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We identified the *nfsA* gene, encoding the major oxygen-insensitive nitroreductase in *Escherichia coli*, and determined its position on the *E. coli* map to be 19 min. We also purified its gene product, NfsA, to homogeneity. It was suggested that NfsA is a nonglobular protein with a molecular weight of 26,799 and is associated tightly with a flavin mononucleotide. Its amino acid sequence is highly similar to that of Frp, a flavin oxidoreductase from *Vibrio harveyi* (B. Lei, M. Liu, S. Huang, and S.-C. Tu, J. Bacteriol. 176:3552–3558, 1994), an observation supporting the notion that *E. coli* nitroreductase and luminescent-bacterium flavin reductase families are intimately related in evolution. Although no appreciable sequence similarity was detected between two *E. coli* nitroreductases, NfsA and NfsB, NfsA exhibited a low level of the flavin reductase activity and a broad electron acceptor specificity similar to those of NfsB. NfsA reduced nitrofurazone by a ping-pong Bi-Bi mechanism possibly to generate a two-electron transfer product.

The oxygen-insensitive nitroreductase activity in *Escherichia coli* consists of one major and two minor components (5). The major component is an NADPH-linked enzyme encoded by *nfsA*, while minor components, encoded by *nfsB* and an unidentified gene (5, 19), can use both NADH and NADPH as electron donors. We have cloned and mapped *nfsB* and analyzed biochemical properties of the purified gene product (NfsB) (29). Our analysis suggested that NfsB is similar in sequence and many biochemical properties to FRase I, the major flavin reductase in *Vibrio fischeri* (31). NfsB was also found to have a low level of flavin reductase activity (29). Furthermore, a single amino acid substitution of Phe-124 of NfsB changed NfsB into an FRase I-like flavin reductase whose activity was three times higher than that of the authentic FRase I (28). Thus, it is reasonable to assume that genes coding for *V. fischeri* FRase I and *E. coli* NfsB nitroreductase are derivatives of a common progenitor gene. Since luminescent bacteria contain several species of flavin reductase (9, 30, 31) and nitroreductase in *E. coli* forms a family consisting of functionally redundant members (5), as with the NfsB-FRase I pair (29, 31), other nitroreductase members in *E. coli* might have their counterparts in the flavin reductase family of luminescent bacteria.

To clarify the evolutionary and biochemical relationships between flavin reductases of luminescent bacteria and *E. coli* nitroreductases, gene cloning and biochemical characterization of *E. coli* nitroreductases other than NfsB may be necessary. Here, we identified the *nfsA* gene, encoding the major nitroreductase in *E. coli*, and characterized its gene product, NfsA. Our results showed NfsA nitroreductase to be a flavoprotein associated tightly with flavin mononucleotide (FMN) and to be the ortholog in *E. coli* of Frp, a flavin oxidoreductase from *Vibrio harveyi* (11, 12, 18), an observation supporting a close evolutionary relation between *E. coli* nitroreductase and luminescent-bacterium flavin reductase families. Although little or no similarity in sequence was detected between NfsA (or Frp) and NfsB (or FRase I), their electron acceptor specificities and reaction modes were similar to each other.

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MATERIALS AND METHODS

Materials. Restriction enzymes and DNA-modifying enzymes were obtained from Nippon Gene (Toyama, Japan) and Toyobo (Osaka, Japan). [α-^{32}P]dCTP (3,000 Ci/ml) was purchased from ICN Biomedicals (Costa Mesa, Calif.). Lysozyme was from Seikagaku (Tokyo, Japan). FMN, riboflavin, nitrofurazone, methyl 4-nitrobenzoate, 4-nitroacetophenone, 4-nitrobenzoate, 4-nitrotoluene, 4-nitrophenol, 4-nitroaniline, menadione, 1,4-benzoquinone, methylene blue, potassium ferricyanide, 2,6-dichloroindophenol sodium, Ponceau SX, Bordeaux S, tartrazine, orange II, and dicumarol were purchased from Wako Pure Chemical (Osaka, Japan). Flavin adenine dinucleotide was a product of Boehringer Mannheim (Indianapolis, Ind.). NADH and NADPH were from Oriental Yeast (Osaka, Japan). Lumiflavin, nitrofurantoin, 4-nitrobenzene methyl sulfonate, and RNase A were purchased from Sigma Chemical (St. Louis, Mo.), and dimethyl pimelimidate (DMP) was purchased from Pierce Chemical (Rockford, Ill.). All these and other chemicals were of the highest grade commercially available.

E. coli AJ212 (higher-level nitrofurantoin-resistant mutant [15]) and JM83 *ara* Δ(*lac-proAB*) F$^-$ *rpsL*(ϕ80*lacZ* Δ*M15*)] (20) and pAJ102 plasmid (15) were used for overexpression.

Overexpression of the *nfsA* gene in *E. coli* cells and preparation of cell extracts. An aliquot (0.25 ml) of overnight culture of *E. coli* AJ212 with pAJ102 was added to 10 ml of Luria-Bertani broth (24) supplemented with 0.1 mg of ampicillin per ml, and 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 (31).

Assay and purification of NfsA. Reductase activity of NfsA was assayed under reaction conditions described below. The reaction temperature used was 23°C for convenience of measuring the initial velocity. A typical reaction mixture (3.0 ml) contained 50 mM Tris-HCl buffer (pH 7.0), 0.1 mM NADPH (or NADH), a 0.1 mM concentration of a given electron acceptor, and a suitable amount of enzyme. Under a given condition, 0.2 to 3.2, 0.5 to 8.1, and 18 to 270 μg NfsA

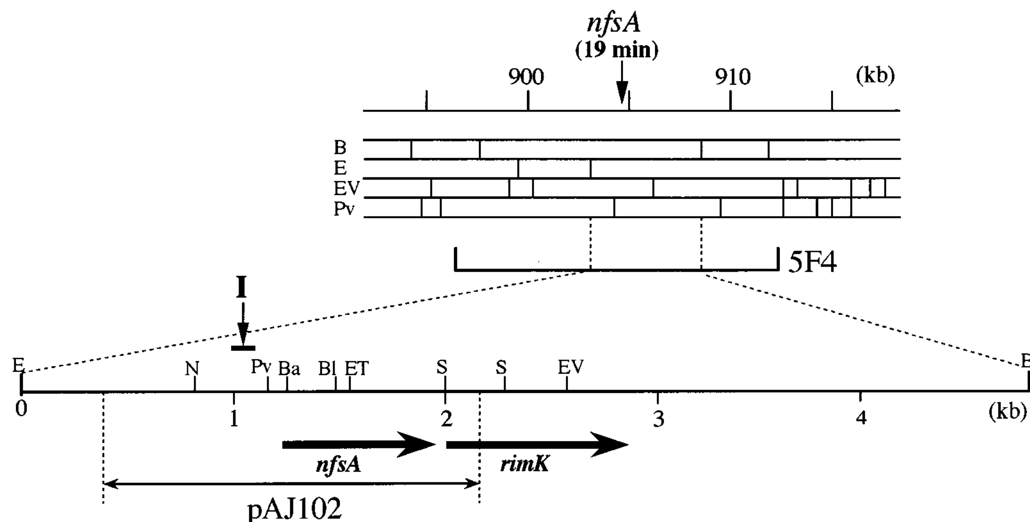

FIG. 1. Restriction map of the *nfsA* locus. The upper half shows a portion of the map of Kohara et al. (14); the position of *thr* at 0 min on the genetic map is taken as 0 kb. The vertical arrow labeled *nfsA* indicates the deduced locus of the *nfsA* gene. The U-shaped line labeled 5F4 shows the size and location of a lambda clone 5F4, a part of which is identical in sequence to the pAJ102 insert. Thick horizontal arrows show sizes and orientations of *nfsA* and *rimK* genes (this work and reference 13). The bar associated with vertical arrow I indicates the insertion site of Tn*1000*, inactivating *nfsA* (15). B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; Pv, *Pvu*II; N, *Nae*I; Ba, *Bal*I; Bl, *Bgl*II; ET, *Eco*T22I; S, *Sma*I.

gave a linear response when nitrofurazone, menadione, and FMN, respectively, were used as electron acceptors. The reaction was initiated by the addition of NADPH (or NADH). Initial velocity was determined by monitoring the change in the amounts of either products or substrates every 6 s in the first 2 min, during which the enzyme reaction was linear. The following molar absorption coefficients were used: 12,960 M^{-1} cm^{-1} (nitrofurazone at 400 nm), 1,000 M^{-1} cm^{-1} (ferricyanide at 420 nm), 20,600 M^{-1} cm^{-1} (2,6-dichloroindophenol at 600 nm), 31,200 M^{-1} cm^{-1} (tartrazine at 422 nm), 19,600 M^{-1} cm^{-1} (Ponceau SX at 500 nm), 26,500 M^{-1} cm^{-1} (Bordeaux S at 522 nm), 13,800 M^{-1} cm^{-1} (orange II at 484 nm), and 6,220 M^{-1} cm^{-1} (NADPH [or NADH] at 340 nm).

Purification of NfsA was carried out as follows. *E. coli* JM83 was transformed with pAJ102 by the polyethylene glycol method (7). Two hundred fifty milliliters of an overnight culture was added to 5 liters of Luria-Bertani broth containing 0.1 mg of ampicillin per ml and 0.01% foam suppressant (Disfoam CE457 from Nippon Oil and Fats, Tokyo, Japan) and cultivated at 37°C for 6 h with aeration as described previously (10). Cells were harvested by centrifugation, suspended in 200 ml of 20 mM Tris-HCl buffer (pH 7.0), and treated with a 20-min sonication at 4°C with 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. The dialysate, which corresponds to the cell extracts in Table 2, was applied to a Q Sepharose FF (Pharmacia) anion-exchange column (2.6 by 10 cm) equilibrated with 20 mM Tris-HCl (pH 7.0), washed with 200 ml of the starting buffer (flow rate: 4 ml/min), and eluted with a 0 to 1 M linear gradient of KCl in 20 mM Tris-HCl (pH 7.0). Peak fractions of NADPH-nitrofurazone reductase activity eluted around 0.3 M KCl (44 ml) were pooled, and 7.8 g of ammonium sulfate was added. The resultant mixture was loaded onto two phenyl-Sepharose 6FF (low-substitution type; Pharmacia) hydrophobic interaction columns (1 by 9 cm) equilibrated with 20 mM Tris-HCl (pH 7.0) containing 15% (wt/vol) ammonium sulfate. Each column was washed with 30 ml of the starting buffer and eluted with a 15 to 0% linear gradient of ammonium sulfate in 20 mM Tris-HCl (pH 7.0) at a flow rate of 2 ml/min. Fractions with enzyme activity were pooled and subjected to phenyl-Sepharose chromatography once more after adjustment of the ammonium sulfate concentration (final: 15%). Pooled peak fractions were dialyzed against 40 mM sodium phosphate (pH 6.0) at 4°C. The dialysate was applied to a Blue Sepharose CL-6B (Pharmacia) affinity chromatography column (size, 1 by 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 NfsA was eluted with 20 ml of 1 mM NADPH at a flow rate of 1 min/ml. The Blue Sepharose chromatography step was repeated once more. After concentration of pooled peak fractions using a Centricon-10 concentrator (Amicon), NfsA was purified to homogeneity by Superose 12 (Pharmacia) gel filtration in 50 mM Tris-HCl (pH 7.0) with 150 mM NaCl (elution rate: 0.2 ml/min). Purified NfsA was dialyzed against 50 mM Tris-HCl (pH 7.0) and stored at −20°C for further analysis. All chromatographic procedures were carried out at 4°C. Except for the enzyme preparation used for cofactor analysis (see below), purified NfsA was treated with 50 mM Tris-HCl (pH 7.0) containing 1 mM FMN at 4°C for 48 h before dialysis.

Other techniques. Protein was determined by the dye-binding method (2) with bovine plasma gamma globulin (Bio-Rad, Hercules, Calif.) as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 15% (wt/vol) gel as described by Laemmli (17). The gel was stained with Coomassie brilliant blue R-250 (Fluka Chemie, Buchs, Switzerland). DMP cross-linking was carried out as described previously (3, 29). Spectrophotometric measurements were carried out at 23°C with a model U-3210 recording spectrophotometer (Hitachi, Tokyo, Japan). The amount of NfsA-bound FMN was determined as described by Ohnishi et al. (21). Thin-layer chromatography was carried out to characterize the NfsA-bound flavin (29). The nucleotide sequence was determined with a *Bca*Best dideoxy sequencing kit (Takara Shuzo) (27). Nucleotide and amino acid sequences, hydrophobicity, and secondary structures were analyzed with DNASIS software (Hitachi Software Engineering, Yokohama, Japan). Other molecular techniques are described by Sambrook et al. (24).

Nucleotide sequence accession number. The DDBJ/EMBL/GenBank accession number for the complete nucleotide sequence of the *E. coli nfsA* gene is D38308.

RESULTS AND DISCUSSION

Identification and nucleotide sequence analysis of the *E. coli nfsA* gene. A plasmid, pAJ102, was originally isolated by Kumar and Jayaraman (Fig. 1) (15), who suggested that the pAJ102 insert, a derivative of a DNA fragment at 19 min on the *E. coli* map (1, 14), encodes NfsB nitroreductase. However, our recent findings (29) are apparently inconsistent with this notion. The *nfsB* gene is situated at 13 min on the *E. coli* map and is unrelated in sequence to the insert of pAJ102, thus suggesting that a nitroreductase other than NfsB is encoded by

TABLE 1. NADPH-dependent nitrofurazone reductase activities found in the extracts of *E. coli* cells with pAJ102 and purified NfsA

Pyridine nucleotide	Enzyme source	Activity (nmol/min/mg of protein)
NADH	AJ212[a]	9
	AJ212(pAJ102)[a]	8
	Purified NfsA	375
NADPH	AJ212[a]	28
	AJ212(pAJ102)[a]	619
	Purified NfsA	72,800

[a] Cell extracts were prepared as described previously (31).

(A)

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CTGTTGCATTAATTGCTAAAAGCTATAACTGTTAAACACAATACAGTGAAAAGTTTTAGACTGAAGGCTCACTTTGCAGAGGGAAGCGTATGCGCGCGATCGGTAAATTGCCTAAAGGCG -241
                                                                                                            (-35)
TGTTGATACTGGAATTTATCGGAATGATGCTACTGGCGGTGGCGCTGCTGTCGGTAAGCGACTCCCTGTCGCTGCCTGAGCCATTTTCTCGGCCAGAAGTGCAGATTCTGATGATTTTTC -121
          (-10)
TCGGTGTTTTGCTCATGCTTCCCGCTGCGGTGGTGGTTATTCTTCAGGTGGCAAAACGTCTTGCCCCACAGCTGATGAACCGTCCACCGCAATATTCACGTTCAGAAAGAGAAAAAGATA   -1
                                                                           ***                                  (SD)
ATGACGCCAACCATTGAACTTATTTGTGGCCATCGCTCCATTCGCCATTTCACTGATGAACCCATTTCCGAAGCGCAGCGTGAGGCGATTATTAACAGCGCCCGTGCGACGTCCAGTTCC  120
 M  T  P  T  I  E  L  I  C  G  H  R  S  I  R  H  F  T  D  E  P  I  S  E  A  Q  R  E  A  I  I  N  S  A  R  A  T  S  S  S   40
AGTTTTTTGCAGTGCAGTAGCATTATTCGCATTACCGACAAAGCGTTACGTGAAGAACTGGTGACGCTGACCGGCGGGCAAAAACACGTAGCGCAAGCGGCGGAGTTCTGGGTGTTCTGT  240
 S  F  L  Q  C  S  S  I  I  R  I  T  D  K  A  L  R  E  E  L  V  T  L  T  G  G  Q  K  H  V  A  Q  A  A  E  F  W  V  F  C   80
GCCGACTTTAACCGCCATTTACAGATCTGTCCGGATGCTCAGCTCGGCCTGGCGGAACAACTGTTGCTCGGTGTCGTTGATACGGCAATGATGGCGCAGAATGCATTAATCGCAGCGGAA  360
 A  D  F  N  R  H  L  Q  I  C  P  D  A  Q  L  G  L  A  E  Q  L  L  L  G  V  V  D  T  A  M  M  A  Q  N  A  L  I  A  A  E  120
TCGCTGGGATTGGGCGGGGTATATATCGGCGGCCTGCGCAATAATATTGAAGCGGTGACGAAACTGCTTAAATTACCGCAGCATGTTCTGCCGCTGTTTGGGCTGTGCCTTGGCTGGCCT  480
 S  L  G  L  G  G  V  Y  I  G  G  L  R  N  N  I  E  A  V  T  K  L  L  K  L  P  Q  H  V  L  P  L  F  G  L  C  L  G  W  P  160
GCGGATAATCCGGATCTTAAGCCGCGTTTACCGGCCTCCATTTTGGTGCATGAAAACAGCTATCAACCGCTGGATAAAGGCGCACTGGCGCAGTATGACGAGCAACTGGCGGAATATTAC  600
 A  D  N  P  D  L  K  P  R  L  P  A  S  I  L  V  H  E  N  S  Y  Q  P  L  D  K  G  A  L  A  Q  Y  D  E  Q  L  A  E  Y  Y  200
CTCACCCGTGGCAGCAATAATCGCCGGGATACCTGGAGCGATCATATCCGCCGAACAATCATTAAAGAAAGCCGCCCATTTATTCTGGATTATTTGCACAAACAGGGTTGGGCGACGCGC  720
 L  T  R  G  S  N  N  R  R  D  T  W  S  D  H  I  R  R  T  I  I  K  E  S  R  P  F  I  L  D  Y  L  H  K  Q  G  W  A  T  R  240
TAAAACCGCCACGTCGATGTATGATACGCGGGCTTTTGACCAGGTCTGACAGAGAGGTGCAGGGTGAAAATTGCCATATTGTCCCGGGATGGAACGCTCTATTCGTGTAAGCGGCTGGCG  840
***     ----->          <-----                                  ***        (SD)         M  E  R  S  I  R  V  S  G  W  R   11
                                                                                   rimK -------->
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(B)

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NfsA:  1 MTPTIELICGHRSIRHFTDEPISEAQREAIINSARATSSS  40
 Frp:  1 MNNTIETILAHRSIRKFTAVPITDEQRQTIIQAGLAASSS  40

NfsA: 41 SFLQCSSIIRITDKALREELVTLTGGQKHVAQAAEFWVFC  80
 Frp: 41 SMLQVVSIVRVTDSEKRNELAQFAGNQAYVESAAEFLVFC  80

NfsA: 81 ADFNRHLQICPDAQLGLAEQLLLGVVDTAMMAQNALIAAE 120
 Frp: 81 IDYQRHATINPDVQADFTELTLIGAVDSGIMAQNCLLAAE 120

NfsA:121 SLGLGGVYIGGLRNNIEAVTKLLKLPQHVLPLFGLCLGWP 160
 Frp:121 SMGLGGVYIGGLRNSAAQVDELLGLPENSAVLFGMCLGHP 160

NfsA:161 ADNPDLKPRLPASILVHENSYQPLDKGALAQYDEQLAEYY 200
 Frp:161 DQNPEVKPRLPAHVVVHENQYQELNLDDIQSYDQTMQAYY 200

NfsA:201 LTRGSNNRRDTWSDHIRRTIIKESRPFILDYLHKQGWATR 240
 Frp:201 ASRTSNQKLSTWSQEVTGKLAGESRPHILPYLNSKGLAKR 240
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(C)

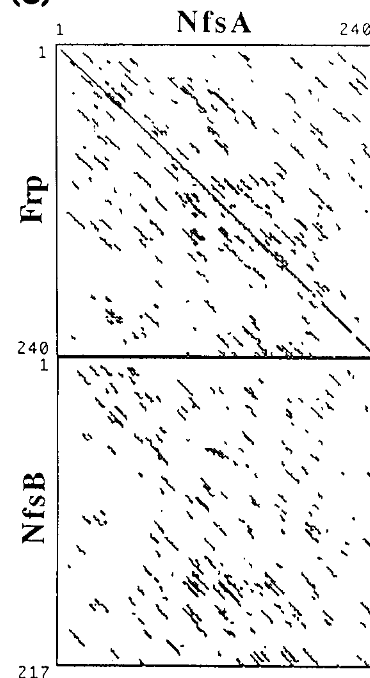


FIG. 2. Sequence analysis of *nfsA* and its product. (A) Nucleotide sequence of *nfsA* and the deduced amino acid sequence of its putative protein product. The −10 region (23), −35 region (23), and Shine-Dalgarno sequence (SD) (26) are underlined. A pair of arrows in the 3′ untranslated region indicates the location of a stem-loop structure, a possible transcription terminator (23). Asterisks indicate in-frame termination codons. The horizontal arrow labeled *rimK* indicates a part of the *rimK* gene (13). (B) Amino acid sequence comparison between *E. coli* NfsA nitroreductase (this work) and *V. harveyi* Frp flavin reductase (18). Identical 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, T, and G; N, D, E, and Q; H, R, and K; M, L, I, and V; and F, Y, and W (25). (C) Homology plots between NfsA and Frp or NfsB.

the pAJ102 insert. To determine which nitroreductase is encoded by the pAJ102 insert, the pyridine nucleotide specificity of the nitrofurazone reaction was examined, since previous experiments (5) indicated that the major nitroreductase is NADPH linked, while both NADH and NADPH serve as effective electron donors for other nitroreductases. As shown in Table 1, nitrofurazone reductase activity was detected only when NADPH was used as an electron donor. The purified pAJ102 gene product was also much more active with NADPH (see below). Thus, the *E. coli* nitroreductase gene cloned in pAJ102 was concluded to be *nfsA*.

The complete nucleotide sequence of *nfsA* is shown in Fig. 2A. The putative initiation codon was found at positions 1 to 3, and in-frame termination codons were found at positions −45 to −43 and 721 to 723, indicating that *nfsA* is capable of encoding a polypeptide of 240 amino acid residues with a

TABLE 2. Purification yield of NfsA from *E. coli* JM83 cells with pAJ102

Step	Vol (ml)	Total protein (mg)	Total activity (μmol/min)	Sp act (μmol/min/mg of protein)	Yield (%)	Purification factor
Cell extracts	232	4,802	2,413	0.5	100	1
Q Sepharose FF	44	1,408	2,447	1.7	101	3
Phenyl-Sepharose 6FF	38	442	2,415	5.5	100	11
Blue Sepharose CL-6B	24	33.8	1,601	47.3	66	95
Superose 12	13	23.0	1,592	69.2	66	138

calculated molecular weight of 26,799. The putative initiation codon was preceded by sequences similar to the Shine-Dalgarno sequence (26) and −10 and −35 promoter consensus sequences (23). In a previous experiment (15), the insertion of transposon Tn*1000* at or near the upstream regulatory sequence (Fig. 1) was shown to result in inactivation of *nfsA*. The DNA sequence following the coding region contained an imperfect inverted repeat, possibly a terminal signal for transcription. The most striking feature of the deduced amino acid sequence of the *nfsA* gene product (NfsA) is that it is highly similar to that of Frp, an NADPH-flavin reductase in *V. harveyi*. (18) (Fig. 2B). Fifty-one percent of the total amino acids were invariant between NfsA and Frp. Hydrophobicity profiles (16) and predicted secondary structures (6) were also similar to each other (data not shown). Thus, the NfsA and Frp pair is the second example supporting an intimate relationship between oxygen-insensitive nitroreductases in *E. coli* and flavin reductases in luminescent bacteria. However, to our surprise, no appreciable sequence similarity could be detected between the pair consisting of NfsB and FRase I (29, 31) and the NfsA-Frp pair (Fig. 2C).

The nucleotide sequence analysis also showed the 3′-flanking sequence of *nfsA* to include an incomplete open reading frame, starting from nucleotide position 809 (Fig. 2A). A SWISS-PROT database search showed it to correspond to *rimK*, a gene responsible for the modification of ribosomal protein S6 (13) and situated at 19 min on the Kohara map (14), making it possible to map *nfsA* precisely as shown in Fig. 1A. The map position of *nfsA* thus obtained is consistent with the genetic data obtained by McCalla et al. (19), since they suggested that (i) the order of loci in the region relevant to *nfsA* is *lac* (8 min), *nfsB* (13 min) (29), *galK* (17 min), and *nfsA* and (ii) *nfsA* is situated in the vicinity of *galK*.

Purification of NfsA having FMN as a prosthetic group. By Q Sepharose, phenyl-Sepharose, Blue Sepharose, and Superose 12 column chromatography, NfsA was purified to homogeneity from extracts of JM83 harboring pAJ102 (Table 2 and Fig. 3A; see Materials and Methods for details). Although purified NfsA was eluted at the position corresponding to a 47-kDa protein in Superose 12 gel filtration (data not shown), the molecular weight of NfsA estimated by SDS-PAGE was 26,000 (Fig. 3A). To determine whether NfsA is a homodimer or a nonglobular protein, purified NfsA was subjected to DMP cross-linking and the reaction products were analyzed by SDS-PAGE. As shown in Fig. 3B, in contrast to dimerizable NfsB (see lane 5), no band corresponding in size to the putative dimer could be detected even after 9-h treatment (lanes 2 and 3), possibly suggesting that NfsA is a nonglobular protein.

As with NfsB (29), a concentrated solution of NfsA was yellow and this coloration was resistant to dialysis (data not shown). The absorption spectrum in the visible-light region of NfsA purified but not exposed to Tris-HCl buffer containing 1 mM FMN (see Materials and Methods) was identical to that of FMN but was shifted about 5 nm toward the long wavelength (Fig. 4A). The virtually exclusive association of NfsA with FMN was demonstrated by thin-layer chromatography (Fig. 4B). Together, these results indicate that NfsA is a flavoprotein tightly associated with FMN. On the basis of spectroscopy and protein weight analysis, the molar ratio between FMN and the

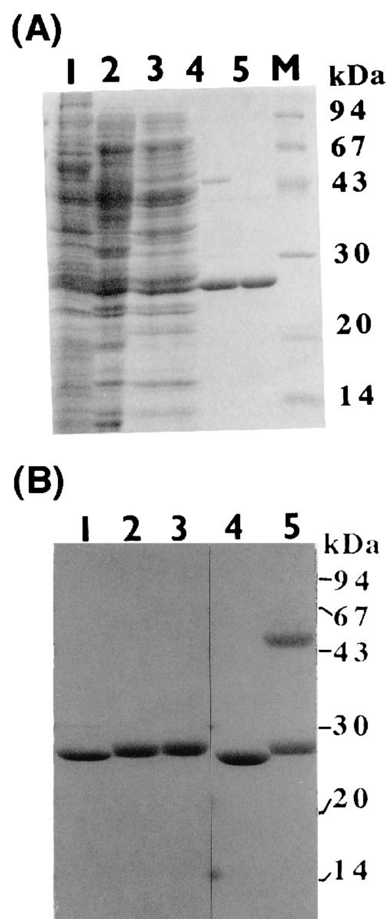


FIG. 3. Purification of NfsA. (A) SDS-PAGE patterns of NfsA preparations. Lanes: 1, crude cell extracts (supernatant of sonicates; protein applied = 207 μg); 2, pooled peak fractions of the first cycle of Q Sepharose FF chromatography (160 μg); 3, pooled peak fractions of phenyl-Sepharose 6FF chromatography (116 μg); 4, pooled peak fractions of Blue Sepharose CL-6B chromatography (14 μg); 5, pooled peak fractions of the final Superose 12 chromatography (9 μg); M, size markers. (B) Sensitivity to DMP cross-linking. Purified NfsA was subjected to DMP cross-linking, which was carried out in 50 μl of 0.2 M triethanolamine-HCl buffer (pH 8.1) for 2 or 9 h, as described by Zenno et al. (29). Lanes: 1, untreated NfsA; 2, NfsA treated with DMP for 2 h; 3, NfsA treated for 9 h; 4, untreated NfsB; 5, NfsB treated for 2 h. Size markers are phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (25 kDa), trypsin inhibitor (20 kDa), and α-lactoalbumin (14 kDa).

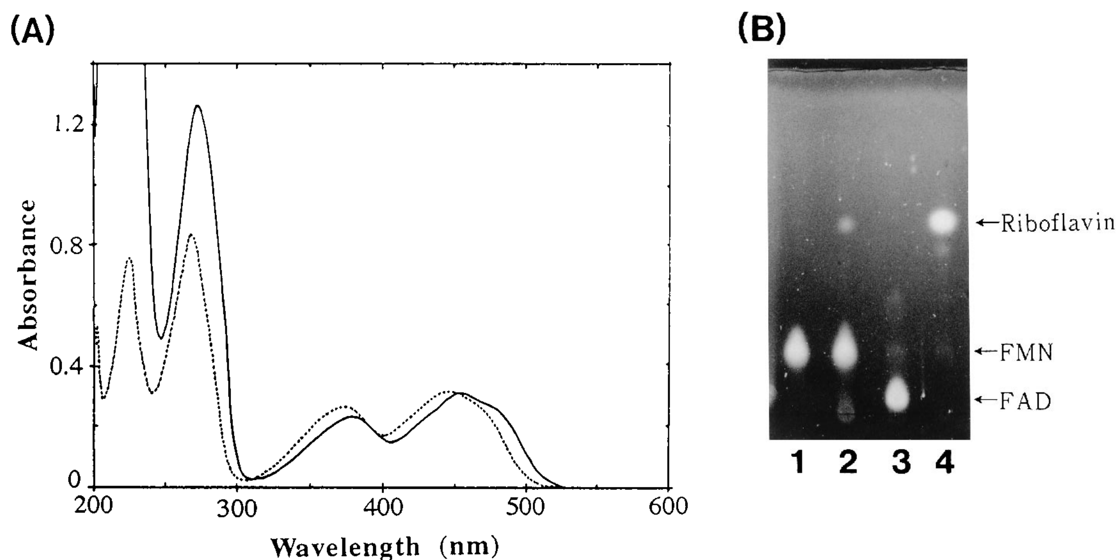
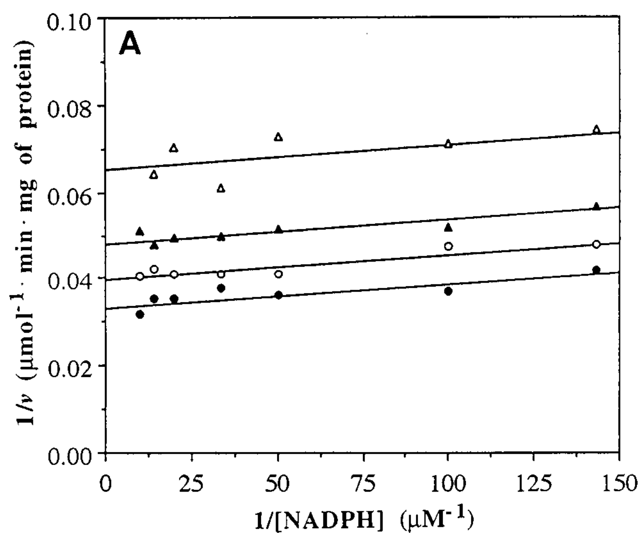
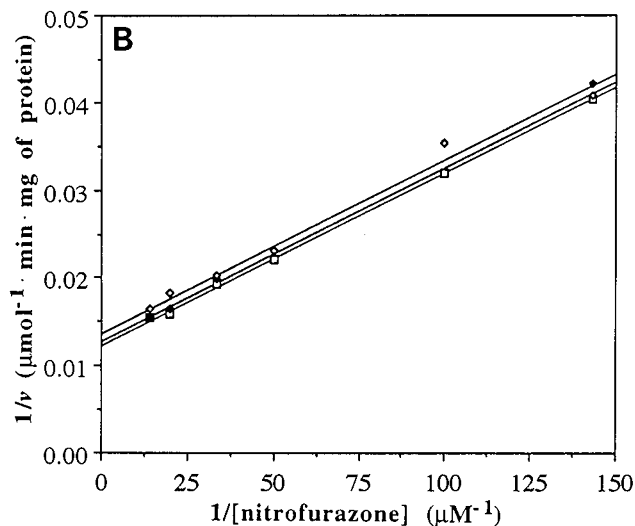


FIG. 4. Tight association of FMN with NfsA. (A) Absorption spectra of purified NfsA and free FMN. After unbound FMN possibly was removed by gel filtration, the absorption spectrum of NfsA was measured. Solid and dotted lines, respectively, show spectra of NfsA at 0.86 mg/ml (32 μM) and 27 μM FMN in 50 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl. The NfsA spectrum has peaks at 454, 380, 273, and about 230 nm, while peaks of free FMN are 448, 375, 268, and 224 nm. (B) Thin-layer chromatography patterns of the NfsA-bound flavin and authentic flavins. Lane 1, flavins released from NfsA; lane 2, authentic FMN; lane 3, authentic flavin adenine dinucleotide (FAD); lane 4, authentic riboflavin.

NfsA protein was estimated at 0.82, thus suggesting that 1 mol of FMN binds to 1 mol of NfsA.

NfsA reduces nitrofurazone according to the ping-pong Bi-Bi mechanism. Analysis of purified NfsA confirmed that NfsA uses NADPH but not NADH for nitrofurazone reduction (Table 1). Nitrofurazone appears eventually to be reduced to open-chain nitrile (8), suggesting that, as a whole, six electrons are transferred upon nitrofurazone reduction (22). We are not sure whether NfsA is responsible for all these steps. Here, we analyzed only the first reduction step using double-reciprocal plots of initial velocity versus NADPH or nitrofurazone concentrations (Fig. 5A and B). Parallel lines in Fig. 5 suggest that, as with Frp, the catalysis mode of NfsA nitrofurazone reductase is the ping-pong Bi-Bi mechanism (Fig. 5C). Since no anion free radical (one-electron-transfer product) was generated upon nitrofurazone reduction by *E. coli* oxygen-insensitive nitroreductases (22), it may be reasonable to assume that the first step of nitrofurazone reduction is a two-electron-transfer reaction which transforms nitrofurazone with the nitro group to its derivative with the nitro group. K_m values





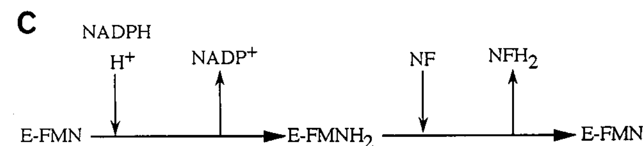


FIG. 5. Lineweaver-Burk plots and the deduced reaction mechanism of NfsB. Double-reciprocal plots of initial velocity versus concentrations of NADPH (A) or nitrofurazone (B). NADPH-nitrofurazone reductase activity was assayed under the condition of constant concentrations of nitrofurazone (A) or NADPH (B). (A) △, 3 μM; ▲, 5 μM; ○, 7 μM; ●, 10 μM; (B) ◇, 50 μM; ◆, 70 μM; □, 100 μM. (C) A deduced catalysis mechanism of NfsA. NfsA is a flavoprotein tightly associated with FMN as a prosthetic group. E-FMN and E-$FMNH_2$, respectively, indicate NfsA-FMN and NfsA-$FMNH_2$ complexes.

TABLE 3. Electron acceptor specificities of NfsA, NfsB, and Frp

Electron acceptor(s)	Reductase activity (μmol/min/mg of protein)[a] of:		
	NfsA	NfsB	Frp
Flavins			
FMN	1	1	51
FMN + dicumarol	—	0.6	43
Flavin adenine dinucleotide	3	1	51
Riboflavin	7	2	—
Lumiflavin	10	41	—
Nitro compounds			
Nitrofurazone	73	13	—
Nitrofurazone + FMN	20	13	—
Nitrofurazone + dicumarol	63	0	—
Methyl 4-nitrobenzoate	24	43	—
Nitrofurantoin	82	21	—
4-Nitrobenzene methyl sulfonate	52	94	—
4-Nitroacetophenone	74	49	—
4-Nitrobenzoate	3	1	—
4-Nitrotoluene	1	0.4	—
4-Nitrophenol	0.5	0.1	—
4-Nitroaniline	0.6	0.1	—
Quinones			
Menadione	24	60	51
Menadione + dicumarol	21	0.2	—
1,4-Benzoquinone	163	251	—
Azo compounds			
Bordeaux S	0.02	0	—
Bordeaux S + FMN	0.02	0	—
Tartrazine	0.8	0	—
Tartrazine + FMN	0.1	0.1	—
Orange II	0	0	—
Orange II + FMN	0	0.2	—
Ponceau SX	0.01	0	—
Ponceau SX + FMN	0	0.04	—
Miscellaneous			
Methylene blue	3	2	12
Ferricyanide	251	387	84
Ferricyanide + FMN	140	452	—
2,6-Dichloroindophenol	27	2	69
2,6-Dichloroindophenol	29	3	—

[a] NfsB results are from the work of Zenno et al. (29). Frp results are from the work of Jablonski and DeLuca (12). Linear reaction ranges for substrates are as follows: 10 to 200 μM, FMN; 50 to 150 μM, nitrofurazone; 10 to 200 μM, menadione. The maximum error range was 5% in all measurements. —, not determined.

for NADPH and nitrofurazone were estimated to be 11.0 and 5.5 μM, respectively, suggesting that the affinity to NADPH of NfsA is somewhat stronger than that of Frp (20 μM [18]).

NfsA has a broad electron acceptor specificity similar to that of NfsB. NfsB nitroreductase has been shown to exhibit not only a weak FMN reductase activity but also a relatively broad electron acceptor specificity (29) (see Table 3). Thus, we examined the electron acceptor specificity of NfsA using various compounds (Table 3). Values were averages of two independent measurements. It is evident that, as with NfsB, NfsA is associated with a low level of FMN reductase activity. NfsA is capable of reducing nitrofurazone over five times more effectively than does NfsB, while as menadione reductase, NfsA is less effective. While no azoreductase activity has ever been detected in NfsB except for the presence of FMN (Table 3), NfsA is associated with a low level of azoreductase. Interestingly, FMN significantly inhibited the tartrazine azoreductase activity of NfsA.

However, regardless of these variations in the efficiency of reduction, as a whole, the electron acceptor specificity spectrum of NfsA is similar to that of NfsB (Fig. 6A). This positive correlation was highlighted when substrate specificities for 4-substituted nitrobenzene compounds were compared among NfsA, NfsB, and FRase I (Fig. 6B). The logarithm of velocity per milligram of protein was not only proportional to the redox potential of the substrate but also virtually independent of the enzyme species used. It may thus follow that active centers of NfsA, NfsB, and FRase I are similar in structure, despite of the apparent absence of amino acid sequence homology between NfsA and NfsB or FRase I (Fig. 2C). Since in NfsB and FRase I (29) the bound FMN is essential for electron transfer, the above finding may indicate that FMN associated tightly with NfsA to serve as a mediator for electron transfer from NADPH to various electron acceptors.

Nitrofurazone reduction by NfsA is six times more rapid than that by NfsB (Table 3). We have also shown that the nitrofurazone reductase activity of NfsB is amino acid sequence dependent (28). Thus, it is quite feasible that although nitrofurazone is not a natural substrate, its reduction by NfsA

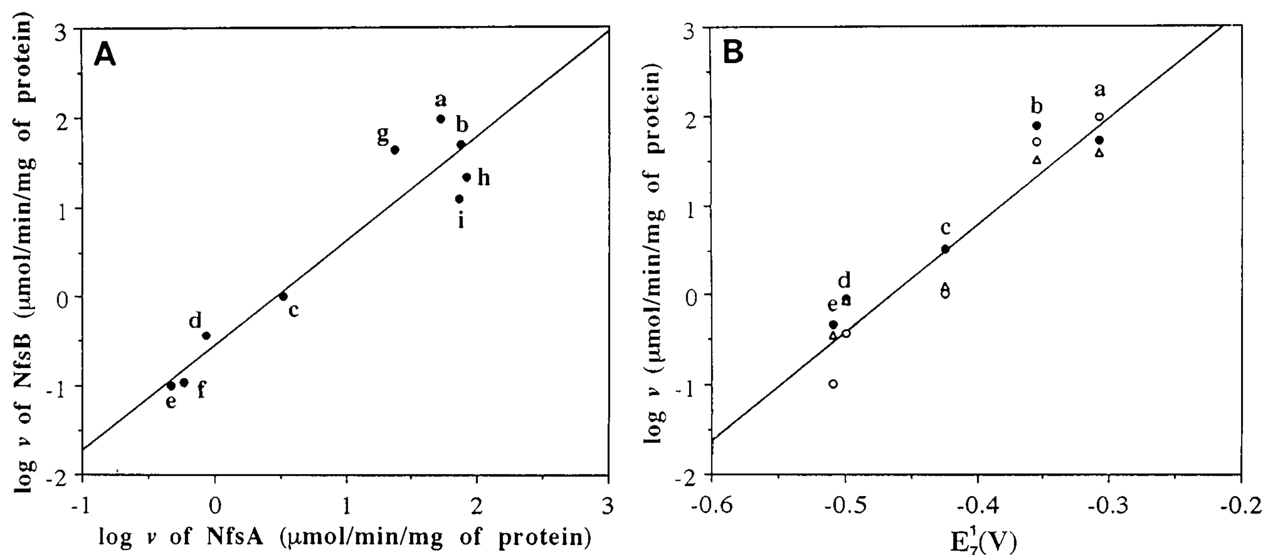


FIG. 6. Positive correlation in substrate spectrum between NfsA and NfsB (A) and the redox potential dependency of the initial velocity (B). a,4-nitrobenzene methyl sulfonate; b, 4-nitroacetophenone; c, 4-nitrobenzoate; d, 4-nitrotoluene; e, 4-nitrophenol; f, 4-nitroaniline; g, methyl 4-nitrobenzoate; h, nitrofurantoin; i, nitrofurazone. Data shown in Table 3 are quadrately plotted. (B) ●, NfsA; ○, NfsB; △, FRase I. One-electron midpoint potentials for nitrobenzene compounds are according to the work of Bryant and DeLuca (4), while initial velocities of FRase I and NfsB are according to the work of Zenno et al. (29).

is an enzymatic reaction. In contrast to NfsB and FRase I (29), NfsA nitroreductase is inhibited by FMN (Table 3), which may indicate a critical role of the protein moiety in binding artificial electron acceptors.

In conclusion, we showed that, as with the NfsB-FRase I pair (29, 31), NfsA, an *E. coli* nitroreductase, and Frp, a luminescent-bacterium flavin reductase (18), are very similar not only in amino acid sequence but also in many biochemical properties, strongly suggesting a close evolutionary relationship between the oxygen-insensitive nitroreductase family in *E. coli* and the flavin reductase family in luminescent bacteria.

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