JOURNAL OF BACTERIOLOGY, June 1994, p. 3536–3543
0021-9193/94/$04.00+0

Vol. 176, No. 12

Identification of the Gene Encoding the Major NAD(P)H-Flavin Oxidoreductase of the Bioluminescent Bacterium *Vibrio fischeri* ATCC 7744

SHUHEI ZENNO,[1,2]* KAORU SAIGO,[2] HIROMASA KANOH,[1] AND SATOSHI INOUYE[1]

Yokohama Research Center, Chisso Corporation, Kanazawa-ku, Yokohama 236,[1] and Department of Biophysics and Biochemistry, School of Science, University of Tokyo, Bunkyo-ku, Tokyo 113,[2] Japan

Received 23 August 1993/Accepted 23 March 1994

The gene encoding the major NAD(P)H-flavin oxidoreductase (flavin reductase) of the luminous bacterium *Vibrio fischeri* ATCC 7744 was isolated by using synthetic oligonucleotide probes corresponding to the N-terminal amino acid sequence of the enzyme. Nucleotide sequence analysis suggested that the major flavin reductase of *V. fischeri* consisted of 218 amino acids and had a calculated molecular weight of 24,562. Cloned flavin reductase expressed in *Escherichia coli* was purified virtually to homogeneity, and its basic biochemical properties were examined. As in the major flavin reductase in crude extracts of *V. fischeri*, cloned flavin reductase showed broad substrate specificity and served well as a catalyst to supply reduced flavin mononucleotide ($FMNH_2$) to the bioluminescence reaction. The major flavin reductase of *V. fischeri* not only showed significant similarity in amino acid sequence to oxygen-insensitive NAD(P)H nitroreductases of *Salmonella typhimurium*, *Enterobacter cloacae*, and *E. coli* but also was associated with a low level of nitroreductase activity. The major flavin reductase of *V. fischeri* and the nitroreductases of members of the family *Enterobacteriaceae* would thus appear closely related in evolution and form a novel protein family.

NAD(P)H-flavin oxidoreductase (EC 1.6.8) (hereafter called flavin reductase) catalyzes the reduction of flavins, including riboflavin, flavin mononucleotide (FMN), and/or flavin adenine dinucleotide (FAD), at the expense of reduced pyridine nucleotides [NAD(P)H]. In luminous bacteria, this enzyme plays an important role in supplying reduced FMN ($FMNH_2$) to the luminescence reaction (22, 36). The luminescence reaction involves a heterodimeric enzyme luciferase which catalyzes the oxidation of $FMNH_2$ and long-chain aliphatic aldehyde (R-CHO) by molecular oxygen with the emission of blue-green light according to the following coupled reaction:

$$FMN + NAD(P)H + H^+ \rightarrow FMNH_2 + NAD(P)^+$$

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

Many flavin reductases have been enzymatically identified in various organisms ranging from bacteria to humans (12, 16, 21, 34, 35, 43, 60), including luminous bacteria such as *Vibrio fischeri* (12, 20, 23, 26, 32, 43, 53), *Vibrio harveyi* (8, 15, 18, 26, 27, 32, 39, 40, 43), *Photobacterium phosphoreum* (32, 43), *Photobacterium leiognathi* (43), and *Photorhabdus luminescens* (47). Their molecular weights, substrate specificities, and reaction mechanisms vary considerably from enzyme to enzyme (12, 22, 27, 40, 53, 56), thus suggesting that flavin reductases are not necessarily related in sequence or evolution, although no amino acid sequences of flavin reductases have been reported except for those of *Escherichia coli* flavin reductase (Fre [50]) and a cholate-inducible flavin reductase from *Eubacterium* sp. (BaiH [17]). Fre is a component of a complex multiprotein system showing ribonucleotide reductase activity in *E. coli* and is similar in sequence to flavin-NAD(P)H-binding domains of many flavin-associated proteins (1, 61). BaiH is similar in amino acid sequence to NADH oxidase from *Thermoanaerobium brockii* and includes a putative FAD-binding domain having considerable sequence homology with those of the disulfide reductases (55).

The luminescence system (*lux* system), which has been most characterized in *V. fischeri*, contains regulatory genes (*luxR* and *-I*) and genes encoding luciferase (*luxA* and *-B*) and fatty acid reductase (*luxC*, *-D*, and *-E*) (36). *V. fischeri* flavin reductase has been partially purified by two groups, who have determined molecular parameters of the enzyme that differ somewhat (12, 43). So far, no flavin reductase gene has been isolated from *V. fischeri*. However, *luxG* possibly encodes flavin reductase, since the LuxG protein is highly similar in amino acid sequence to *E. coli* Fre (1, 61).

As a first step to clarify the molecular nature of flavin reductase from luminous bacteria, *V. fischeri* genes encoding polypeptides with NAD(P)H-flavin reductase activity were cloned. Two genes, unrelated in sequence, were identified (this work and reference 62). One, the subject of this paper, coded for the major *V. fischeri* flavin reductase (FRase I), representing more than 90% of the total flavin reductase activity. The second gene cloned is the counterpart of *E. coli fre* in *V. fischeri* (62). As described elsewhere (62), *V. fischeri fre* is intimately related in evolution to *luxG*, but *fre* and *luxG* code for two different groups of flavin-binding proteins. We have no data showing that the *V. fischeri* LuxG protein is associated with flavin reductase activity.

The molecular weight of FRase I was estimated to be 26,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), whereas that obtained by gel filtration was 41,000. The FRase I gene exhibited no appreciable homology in amino acid sequence to *E. coli* Fre (50) or *Eubacterium* BaiH (17). FRase I appeared to constitute a novel family of proteins including the oxygen-insensitive NAD(P)H nitroreductases of members of the family *Enterobacteriaceae* (*Salmonella typhimurium* [58], *Enterobacter cloacae* [6], and *E. coli*

* Corresponding author. Mailing address: Department of Biophysics and Biochemistry, School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Phone: 81-3-3812-2111, ext. 4404. Fax: 81-3-5684-2394.

[2]). FRase I of *V. fischeri* expressed in *E. coli* showed the highest activity when NADH and FMN, respectively, were used as an electron donor and acceptor. The cloned FRase I stimulated the bioluminescence reaction in vitro but was also associated with a low level of nitroreductase activity.

MATERIALS AND METHODS

Enzymes and chemicals. Partially purified NAD(P)H-flavin oxidoreductase and luciferase from *V. fischeri* (formerly *Photobacterium fischeri*) were purchased from Boehringer Mannheim (Indianapolis, Ind.). Restriction enzymes and DNA-modifying enzymes were obtained from Nippon Gene (Toyama, Japan) and Takara Shuzo (Kyoto, Japan). [γ-^{32}P]ATP (3,000 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), and [α-^{35}S]dATP (1,000 Ci/mmol) were purchased from Du Pont, NEN (Boston, Mass.). Oligonucleotides were synthesized by the phosphoamidite method with an Applied Biosystems (Foster City, Calif.) model 380B DNA synthesizer. Isopropyl-β-D-thiogalactopyranoside (IPTG), nitrofurazone, FMN sodium salt, FAD disodium salt, and riboflavin were purchased from Wako Pure Chemical (Osaka, Japan). Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, D-glucose-6-phosphate disodium salt, NADH sodium salt, $NADP^+$, and NADPH sodium salt were from Oriental Yeast (Osaka, Japan). Decanal and Ponceau S were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All of these and other chemicals were of the highest grade commercially available.

Bacterial strains and plasmids. Luminous bacterial strains *V. fischeri* ATCC 7744, *Alteromonas hanedai* ATCC 33224, *Vibrio harveyi* ATCC 33843, *Vibrio orientalis* ATCC 33934, and *Photorhabdus luminescens* ATCC 29999 were obtained from American Type Culture Collection. Marine bacteria (*V. fischeri*, *A. hanedai*, *V. harveyi*, and *V. orientalis*) were cultured in *Photobacterium* medium (Difco Laboratories, Detroit, Mich.) at 26°C, while terrestrial bacteria (*P. luminescens*) were cultured in Luria-Bertani medium at 37°C. *E. coli* strains JM109 {*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*ZΔM15*]} (59) and D1210 (10) were used for cloning and expression. D1210 carries *lacI*^{q} and *lacY*$^+$ alleles on the chromosome but otherwise is identical to *E. coli* HB101 [F$^-$ *hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL*(Str^r) *xyl-5 metl-1 sup44* λ^-] (10). Two plasmids, pUC18 (59) and pUC8 (37, 38), were used as cloning and/or expression vectors.

Determination of the N-terminal amino acid sequence. Partially purified flavin reductase (Boehringer Mannheim) was size fractionated by electrophoresis on a 10% Tricine–SDS gel (1-mm thickness; TEFCO, Osaka, Japan) (46) and transferred electrophoretically onto an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) with the Mini-Cell apparatus (TEFCO) at 250 mA for 30 min (52). After transfer, the membrane filter was stained with 0.2% Ponceau S in 1% acetic acid for 1 min. Protein bands on the filter were cut off and subjected to protein sequence analysis with an Applied Biosystems model 470A gas-phase protein sequencer connected to an on-line model 120A phenylthiohydantoin amino acid analyzer (Applied Biosystems) (14).

Cloning, Southern hybridization, and nucleotide sequence analysis. After *V. fischeri* chromosomal DNA was partially digested with *Sau*3AI, 3- to 6-kb fragments fractionated by electrophoresis on an agarose gel were inserted into the *Bam*HI site of pUC18. The resultant plasmids were transformed into *E. coli* JM109. Colony hybridization (45) was carried out with a 1:1 mixture of ^{32}P-labeled FR1 and FR2 (see Fig. 1A) as a probe. Southern blot analysis (45) was performed under a low-stringency condition with a ^{32}P-labeled *Hin*cII-*Stu*I DNA fragment as a probe (see probe A in Fig. 2A). Hybridization was performed in a mixture of 8× SSC (1× SSC is 17 mM sodium citrate and 150 mM NaCl), 0.1% SDS, and 0.1 mg of denatured herring sperm DNA per ml at 50°C for 20 h, and a filter was washed in 6× SSC containing 0.1% SDS at 55°C for 1 h. The nucleotide sequence was determined in two directions by the dideoxy sequencing method by using denatured plasmid templates (24) with a Sequenase DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). Sequence information was analyzed with DNASIS software (Hitachi Software Engineering, Yokohama, Japan) implemented on an NEC-compatible personal computer.

Expression of *V. fischeri* FRase I in *E. coli* and preparation of extracts for enzyme assay and purification. pFR3 DNA was digested with *Stu*I-*Sal*I, filled in, and self-ligated to produce pFR4. The *Hin*cII-*Sal*I fragment (0.9 kb) from pFR4 was inserted into the *Sma*I-*Sal*I site of pUC8 to obtain pFR5, in which the *V. fischeri* FRase I gene having a 5′ extension is under control of the *lac* promoter-operator. Expression plasmid pFR7 was constructed by fill-in ligation of the *Eco*RI site of pFR5 so that D1210 cells harboring pFR7 could overproduce the nonfusion type of the *V. fischeri* FRase I on addition of IPTG. A 250-μl aliquot of overnight culture of D1210 with pFR7 was added to 10 ml of Luria-Bertani broth supplemented with ampicillin (100 μg/ml) and induced at 37°C. A final concentration of 1 mM IPTG was then added to the bacterial culture at 2 h of incubation. After an additional 3-h incubation, cells were harvested by centrifugation and the pellet, suspended in 3.3 ml of extraction buffer (50 mM potassium phosphate [pH 7.0]) containing 1 mM dithiothreitol (DTT) for assay and purification of flavin reductase or Tris-HCl buffer (pH 7.5) containing 1 mM DTT for nitroreductase assay, was treated with a 45-s sonication (Branson [Danbury, Conn.] model 250 sonifier). After centrifugation at 14,000 × *g* at 4°C for 30 min, the supernatant fluid was stored as extracts. *V. fischeri* cell extracts were prepared similarly.

Purification of cloned FRase I. *V. fischeri* FRase I expressed from the cloned gene in *E. coli* was purified as follows. Extracts of D1210 with pFR7 (about 10 ml) were dialyzed at 4°C against 10 mM potassium phosphate (pH 7.0) containing 0.5 mM DTT, loaded onto a Mono Q (Pharmacia LKB) anion-exchange column (0.5 by 5 cm), washed with 60 ml of starting buffer (flow rate, 0.5 ml/min), and eluted with a 50 to 500 mM linear gradient of potassium phosphate (pH 7.0) containing 0.5 mM DTT. An aliquot of each fraction (1.5 ml) was then subjected to flavin reductase assay with FMN and NAD(P)H as substrates (see below). Usually one major peak was observed (Fig. 3A). Pooled peak fractions were concentrated by Centricon 10 (Amicon, Danvers, Mass.) and applied to a Superose 12 (Pharmacia LKB) gel filtration column (1 by 30 cm) preequilibrated in 100 mM potassium phosphate (pH 7.0) with 0.5 mM DTT. The sample was eluted in the same buffer at a flow rate of 0.2 ml/min (fraction volume, 0.3 ml). Peak fractions for FMN reductase activity were pooled and stored at 4°C for further analysis. SDS-PAGE analysis showed the purity of the FRase I thus prepared to be >95% (Fig. 3B [inset]).

Protein analysis. SDS-PAGE analysis was carried out essentially as described by Laemmli (31) under reducing conditions with a 15% polyacrylamide gel (1-mm thickness) at 25 mA for 1.5 h. Protein bands were visualized by staining with Coomassie brilliant blue R-250 (Bio-Rad, Richmond, Calif.). The protein concentration was determined by the dye-binding method of Bradford (4) with bovine gamma globulin as a standard (Bio-Rad).

Enzyme assay. (i) Flavin reductase. Flavin (FMN, FAD, or

riboflavin) reductase activity was determined by measuring the decrease in absorption of NAD(P)H at 340 nm ($\varepsilon = 6.3 \times 10^3 \cdot M^{-1} \cdot cm^{-1}$) in 50 mM potassium phosphate buffer (pH 7.0) (26, 56). The reaction mixture (2.97 ml), containing 0.1 mM flavin and a suitable amount of cell extract or enzyme fraction, was preincubated at 23°C for 5 min. The reaction was initiated by the addition of 30 μl of 10 mM NADH or NADPH. The initial rate of the reaction was measured with a Hitachi recording spectrophotometer (Tokyo, Japan), model U-3210.

(ii) Nitroreductase. Oxygen-insensitive nitroreductase activity was determined by measuring the decrease in absorption of nitrofurazone at 375 nm ($\varepsilon = 1.5 \times 10^4 \cdot M^{-1} \cdot cm^{-1}$) as described by Watanabe et al. (57). The reaction mixture (3 ml) contained an NADPH-generating system consisting of a final concentration of 6.7 mM glucose-6-phosphate, 1.33 U of glucose-6-phosphate dehydrogenase per ml, 40 μM $NADP^+$, 50 mM Tris-HCl (pH 7.5), and 1 mM DTT. Prior to the addition of a 750-μl aliquot of 0.2 mM nitrofurazone dissolved in water, the reaction mixture was preincubated with cell extract or enzyme fraction at 37°C for 5 min. The oxidation rate of nitrofurazone was calculated with the initial rate.

(iii) Bioluminescence reaction. The bioluminescence reaction coupled with flavin reductase was carried out according to the method of Tu and Hastings (54). The mixture, containing 0.25% (wt/vol) bovine serum albumin (fraction V), 0.05 mM FMN, 0.001% (vol/vol) decanal, 0.005% (wt/vol) Triton X-100, 55 mM potassium phosphate (pH 7.0), 10 μg of bacterial luciferase per ml, and a suitable amount of cell extract, was preincubated at 25°C for 5 min. One hundred microliters of 0.5 mM NADH was then added to 100 μl of the mixture to start the reaction. Luminescence was measured at 25°C for 2 min with a Laboscience (Tokyo, Japan) lumiphotometer, model TD 4000.

Nucleotide sequence accession number. The DDBJ, EMBL, and GenBank accession number for the nucleotide sequence of the FRase I gene of *V. fischeri* is D17743.

RESULTS AND DISCUSSION

Identification of putative flavin reductase protein in a commercial preparation. A preparation rich in *V. fischeri* flavin reductase was obtained commercially and used as a starting material for identifying the gene encoding the enzyme. SDS-PAGE indicated that the commercial preparation of *V. fischeri* flavin reductase contained three major proteins with sizes of 26, 36, and 66 kDa, along with several minor components (data not shown). To address the question of whether one of the three major proteins is associated with flavin reductase activity, each protein was removed from the polyacrylamide gel, the N-terminal amino acid sequence was determined, and a sequence homology search was made with the Swiss-Prot data base (release 12). The 66-kDa protein was found to be bovine serum albumin exogenously added to the preparation for stabilization of flavin reductase activity. The 36-kDa protein was highly similar in sequence to *S. typhimurium* (7) and *E. coli* (7, 30, 33) *O*-acetylserine (thiol)-lyase A, which catalyzes the reaction in which L-cysteine is synthesized from *O*-acetyl-L-serine and sulfide (7, 33). Sequence homology between the 36-kDa protein of *V. fischeri* and *O*-acetylserine (thiol)-lyase A from *S. typhimurium* or *E. coli* was 71 to 74% (22 to 23 of 31 residues) (Fig. 1B), suggesting that the 36-kDa protein is the *O*-acetylserine (thiol)-lyase A of *V. fischeri*. No protein with a significant sequence homology to 26-kDa protein could be found, although, as described below, a more recent release from the Swiss-Prot data base (release 24) showed the 26-kDa protein to have appreciable amino acid sequence homology

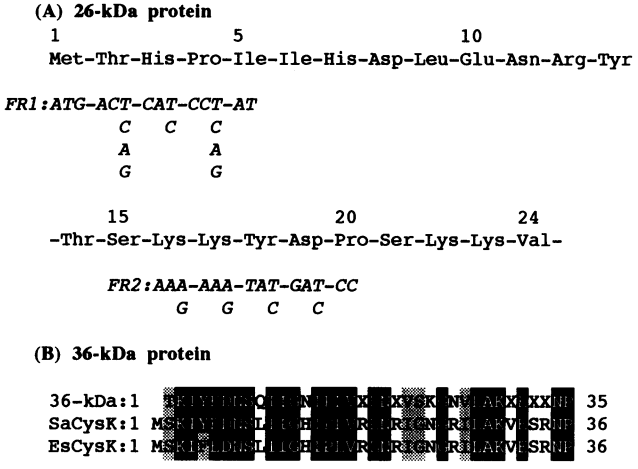


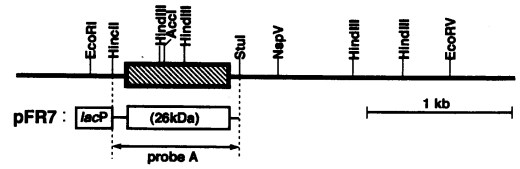
FIG. 1. N-terminal amino acid sequence of 26-kDa protein (A) and N-terminal amino acid sequence homology between the 36-kDa protein and *O*-acetylserine (thiol)-lyase A of *S. typhimurium* and *E. coli* (B). (A) FR1 and FR2, two oligonucleotides used for screening. Their nucleotide sequences are shown below the corresponding amino acid sequences. (B) Lines labeled 36-kDa, SaCysK, and EsCysK, respectively, show N-terminal sequences of the 36-kDa protein of *V. fischeri* and *O*-acetylserine (thiol)-lyase A from *S. typhimurium* (7) and *E. coli* (7, 30, 33). X represents an amino acid residue which could not be identified.

with *E. cloacae* and *S. typhimurium* oxygen-insensitive NAD(P)H nitroreductases (6, 58) and *Thermus thermophilus* NADH oxidase (41).

The commercial preparation of *V. fischeri* flavin reductase was size fractionated by gel filtration with Superose 12. Not only FMN but also FAD and riboflavin reducing activities were found to occur closely in parallel with the distribution of the 26-kDa protein (data not shown). *E. coli* cells possessing the cloned gene encoding the 26-kDa protein expressed a high degree of flavin reductase activity (see below). Thus, the 26-kDa protein quite likely is the *V. fischeri* flavin reductase.

Cloning of the gene encoding the 26-kDa protein. Oligonucleotides FR1 and FR2 were prepared on the basis of the N-terminal amino acid sequence of the 26-kDa protein (Fig. 1A) and were used as probes for screening a genomic DNA library of *V. fischeri*. Five positive clones, containing inserts of 3.0 to 5.5 kb, were isolated from about 10^4 clones. By using FR1 as a primer, partial nucleotide sequences of the inserts of these five clones were determined. All showed identical sequences, and thus an arbitrarily chosen clone (pFR3 [Fig. 2A]) was used for subsequent study. The complete nucleotide sequence of the open reading frame encoding the 26-kDa protein, along with its 5′ and 3′ flanking sequences, is shown in Fig. 2B.

The first methionine codon, ATG, was found at nucleotides 1 to 3, and in-frame termination codons were found at −66 to −64 and 655 to 657 (Fig. 2B), indicating that the cloned fragment has a long open reading frame capable of encoding a polypeptide with a length of 218 amino acid residues and a calculated molecular weight of 24,562. As underlined in Fig. 2B, this presumed polypeptide was identical in N-terminal amino acid sequence to the 26-kDa protein, suggesting that the open reading frame identified here is the coding region for the 26-kDa protein. The initiation codon was preceded by a sequence (-AGTAAGGT-) quite similar to the Shine-Dalgarno consensus sequence (-AAGGAGGT-) for *E. coli* (49). A



(B)

```
                                                    (-35)
TGTCACATATGGCAAATTAAATATTGAGTATGCCTTGCTTGTTGACATCATAAG    -55
          (-10)                                      ***
TTGTGCAGACAAGAATGTCTGTGGATTAAAATTTCACAAGTAAGGTTTATTATT    -1
                                                  (SD)
ATGACGCATCCAATTATTCATGATCTTGAAAATCGTTATACATCAAAAAAATAT     54
M  T  H  P  I  I  H  D  L  E  N  R  Y  T  S  K  K  Y   18
GACCCATCAAAGAAAGTATCTCAAGAAGATTTAGCGGTTTTGCTTGAGGCTCTG   108
D  P  S  K  K  V  S  Q  E  D  L  A  V  L  L  E  A  L   36
CGTTTATCTGCTTCTTCAATTAATTCACAGCCTTGGAAATTCATTGTTATTGAA   162
R  L  S  A  S  S  I  N  S  Q  P  W  K  F  I  V  I  E   54
TCCGATGCAGCGAAGCAAGGTATGCATGATTCGTTTGCAAATATGCATCAGTTT   216
S  D  A  A  K  Q  G  M  H  D  S  F  A  N  M  H  Q  F   72
AATCAACCTCACATCAAAGCGTGTTCTCATGTGATTTTATTTGCAAATAAGCTT   270
N  Q  P  H  I  K  A  C  S  H  V  I  L  F  A  N  K  L   90
TCGTATACACGAGATGATTATGATGTGGTTTTATCTAAAGCGGTTGCTGACAAG   324
S  Y  T  R  D  D  Y  D  V  V  L  S  K  A  V  A  D  K  108
CGTATTACTGAAGAGCAAAAAGAAGCTGCTTTTGCTTCGTTTAAGTTTGTAGAA   378
R  I  T  E  E  Q  K  E  A  A  F  A  S  F  K  F  V  E  126
TTGAACTGTGATGAAAATGGTGAGCATAAAGCATGGACTAAGCCTCAAGCTTAT   432
L  N  C  D  E  N  G  E  H  K  A  W  T  K  P  Q  A  Y  144
TTAGCTCTTGGTAATGCTCTGCATACATTAGCTAGACTGAACATTGACTCAACA   486
L  A  L  G  N  A  L  H  T  L  A  R  L  N  I  D  S  T  162
ACAATGGAAGGCATTGATCCTGAATTATTGAGTGAAATTTTTGCTGATGAATTA   540
T  M  E  G  I  D  P  E  L  L  S  E  I  F  A  D  E  L  180
AAAGGGTATGAATGTCATGTTGCTTTAGCCATTGGTTATCATCATCCAAGCGAA   594
K  G  Y  E  C  H  V  A  L  A  I  G  Y  H  H  P  S  E  198
GATTATAATGCCTCTTTGCCTAAGTCTCGTAAGGCATTTGAAGCAGTAATTACC   648
D  Y  N  A  S  L  P  K  S  R  K  A  F  E  A  V  I  T  216
ATCCTTTAGATTCTTAATGTTTGAGATGAAGAAAAGCCAGCGATTTAGCTGTGC   702
I  L  ***
TTTGTTTGTGCAAAAATGTTCCTAATGGCGTATTACTACGGTAGGAAGTCTATT   756
TAAAGTTTCTTTTACTCTTTGGTATTAATTGTCAATTACGCGGAAATCATTATC   810
TAACTAGGCCT
```

FIG. 2. Genomic structure (A) and nucleotide sequence (B) of the gene coding for the 26-kDa protein of *V. fischeri*. (A) Restriction map of the DNA insert in pFR3. The hatched box indicates the coding region for the 26-kDa protein. The structure of an expression plasmid, pFR7, is schematically shown below the map. *lac*P, *lac* promoter-operator sequence; probe A, a 0.9-kb *Hin*cII-*Stu*I fragment used as a hybridization probe. (B) Putative −10 and −35 sequences (44) and the Shine-Dalgarno (SD) sequence (49) are underlined, while asterisks show the positions of in-frame termination codons. Paired horizontal arrows show the locations and sizes of inverted repeats. The deduced amino acid sequence of the 26-kDa protein is shown below the nucleotide sequence. The amino acid sequence of the N-terminal region determined by direct protein sequencing is underlined.

putative promoter region, consisting of a −35 sequence (-TT GACA-) starting at nucleotide −67 and a −10 sequence (-AAGAAT-) starting at −44, was identified on the basis of homology with the consensus promoter sequence of *E. coli* (44). The upstream regulatory region also included an inverted repeat at nucleotides −49 to −34. Palindromic sequences of this type may possibly interact with modulators for gene expression (25). The DNA sequence following the coding region contained a stem-loop structure, possibly a terminal signal for transcription (44).

By using a 0.9-kb *Hin*cII-*Stu*I fragment (Fig. 2A) as a probe, genomic DNAs of *A. hanedai*, *V. harveyi*, *V. orientalis*, and *P. luminescens* were analyzed by Southern blotting under less-stringent conditions. Nucleotide sequences similar to the 26-kDa protein-encoding gene were present in all luminous bacteria examined (data not shown). With PCR, *E. coli* (nonluminescent bacterium) was shown to contain a putative homolog of the 26-kDa protein-encoding gene (63 [see EsFR in Fig. 4]).

Significant increment in flavin reductase activity in *E. coli* cells expressing the 26-kDa protein-encoding gene of *V. fischeri*. To confirm whether the cloned open reading frame encodes the flavin reductase of *V. fischeri*, an expression plasmid (pFR7) was constructed in which the expression of the 26-kDa protein-encoding gene was controlled by the *lac* promoter-operator system (Fig. 2A). A new protein band, identical in size to the authentic 26-kDa protein, was found to be present in the soluble fraction of *E. coli* cells having pFR7 on IPTG induction (data not shown).

TABLE 1. Flavin reductase and nitroreductase activities in cell extracts

Pyridine nucleotide	Sample[a]	Flavin reductase activity (μmol/min/mg of protein) of:			Nitroreductase activity (nmol/min/mg of protein) of nitrofurazone
		FMN	FAD	Riboflavin	
NADPH	D1210	0.02	0.01	0.01	6.8
	D1210(pFR7)	4.43	2.84	0.87	40.9
	ATCC 7744	0.60	0.37	0.45	8.8
	BM enzyme	2.32	1.59	0.54	43.4
NADH	D1210	0.04	0.04	0.01	
	D1210(pFR7)	11.81	7.41	2.70	
	ATCC 7744	1.26	0.95	0.84	
	BM enzyme	6.02	4.04	1.57	

[a] D1210, D1210(pFR7), and ATCC 7744 are crude extracts. BM enzyme corresponds to a commercial flavin reductase preparation from Boehringer Mannheim.

As shown in Table 1, a significant increment in flavin reductase activity was noted in *E. coli* (D1210) cells harboring pFR7. The level of flavin reductase activity was highest when FMN and NADH were used as an electron acceptor and donor, respectively.

The bioluminescence reaction was also extensively stimulated on coupling with bacterial luciferase and *E. coli* extracts having overexpressed *V. fischeri* flavin reductase (IPTG-induced extracts of D1210 with pFR7). Differences in initial slope and steady-state level indicated a >100-fold stimulation in the bioluminescence reaction compared with the background level (without the expression plasmid pFR7). Thus, it is concluded that the cloned 26-kDa protein is a flavin reductase of *V. fischeri*, which may serve as a good supplier of $FMNH_2$ to the bioluminescence system in vitro.

Purification of the cloned 26-kDa flavin reductase and evidence showing it to be FRase I, the major flavin reductase of *V. fischeri*. Duane and Hastings (12) showed by gel filtration that *V. fischeri* contains one major flavin reductase with an estimated size of 43,000. To discriminate this major enzyme from other minor flavin reductases in *V. fischeri*, we call it FRase I hereafter. FRase I was capable of using as electron donors both NADH and NADPH, with the V_{max} with NADH approximately twice that of the V_{max} with NADPH (12, 26, 32). As electron acceptors, FMN was most effective, FAD was considerably effective, and riboflavin was ineffective (12).

To determine whether our 26-kDa flavin reductase of *V. fischeri* corresponds to FRase I, the 26-kDa enzyme expressed in *E. coli* was purified by a combination of anion-exchange chromatography (Mono Q) and gel filtration (Superose 12) and its elution profiles were compared with those of the major flavin reductase (FRase I) in crude *V. fischeri* extracts or a 1:1 mixture of the purified 26-kDa enzyme and partially purified *V. fischeri* FRase I. The purity of the cloned flavin reductase thus purified was >95% (see SDS-PAGE patterns in the inset of Fig. 3B). It was also found that FRase I represents >90% of

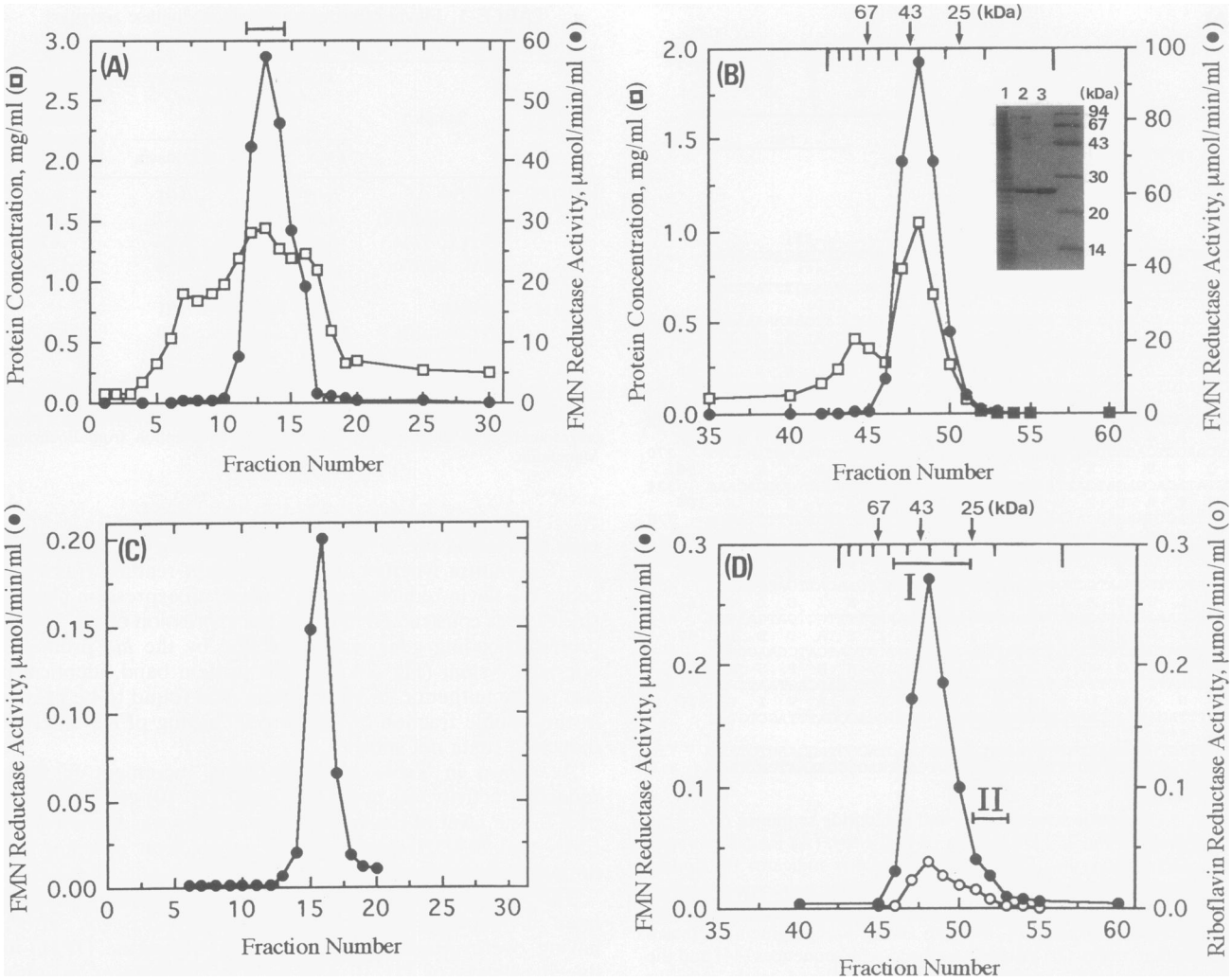


FIG. 3. Chromatographic profiles of FRase I. ●, NADH-FMN reductase activity; ○, NADPH-riboflavin reductase activity; □, protein concentration. (A) Mono Q elution profiles of extracts of D1210 with pFR7. A single activity peak can be detected. Fractions labeled by a bar in the upper margin were pooled and used for further purification with Superose 12. (B) Superose 12 elution profiles of cloned Mono Q-purified FRase I. Locations of molecular size markers (albumin, 67,000; ovalbumin, 43,000; chymotrypsinogen A, 25,000) are shown by vertical arrows in the upper margin. The molecular weight of FRase I can be estimated at 41,000. The inset shows SDS-PAGE patterns. Lanes: 1, crude extracts of D1210 with pFR7; 2, FRase I partially purified by Mono Q (fraction 13 in Fig. 3A); 3, purified FRase I (fraction 48 in Fig. 3B). Locations of molecular size markers (phosphorylase *b*, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,000; α-lactalbumin, 14,000) are shown on the right side. (C) Mono Q elution profiles of a 1:1 mixture of the major flavin reductase partially purified from crude *V. fischeri* extracts and cloned FRase I (26-kDa enzyme) purified from *E. coli* cells (D1210 with pFR7). Note that only a single flavin reductase activity peak can be seen. (D) Superose 12 elution profiles of flavin reductase activity of crude extracts of *V. fischeri*. I and II, respectively, show fractions rich in FRase I and FRase II (a minor flavin reductase). The estimated molecular weight of the peak I enzyme (FRase I) is 41,000. FRase I appeared to represent >90% of the total flavin reductase activity in *V. fischeri* extracts.

the total flavin reductase activity in crude extract (Fig. 3D). As shown in Fig. 3B to D, no appreciable difference in elution profiles of Mono Q and Superose 12 column chromatography was detected between the cloned 26-kDa enzyme and FRase I, strongly suggesting that the cloned 26-kDa flavin reductase is FRase I.

The optimum pH and temperature of purified FRase I were 7.5 and 26°C, respectively. Purified FRase I exhibited a substrate preference similar to but somewhat different from that described by Duane and Hastings (12) (Table 2). Although the molecular mass of FRase I was estimated to be 24.5 to 26 kDa by amino acid sequence and SDS-PAGE, that obtained by gel filtration was 41 kDa, a value essentially identical to that

TABLE 2. K_ms of FRase I

Substrate	K_m (M) of purified FRase I	Duane and Hastings K_m (M)[a]
FMN[b]	3.8×10^{-5}	7.3×10^{-5}
FAD[b]	1.2×10^{-4}	1.4×10^{-4}
NADH[c]	2.7×10^{-4}	0.8×10^{-4}
NADPH[c]	1.8×10^{-4}	4.0×10^{-4}

[a] K_ms obtained by Duane and Hastings (12) with 20% pure FRase I.
[b] K_ms obtained when NADH was used as an electron donor.
[c] K_ms obtained when FMN was used as an electron acceptor.

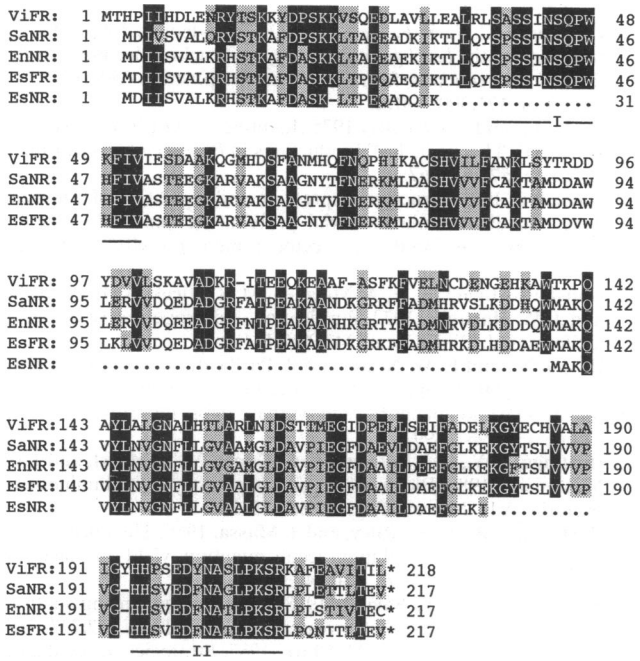


FIG. 4. Amino acid sequence homology among *V. fischeri* FRase I and oxygen-insensitive NAD(P)H nitroreductases of *S. typhimurium*, *E. cloacae*, and *E. coli*. ViFR, *V. fischeri* FRase I (this work); SaNR, *S. typhimurium* nitroreductase (58); EnNR, *E. cloacae* nitroreductase (6); EsFR, *E. coli* counterpart of *V. fischeri* FRase I (63); EsNR, a partial sequence of *E. coli* nitroreductase (2). Identical amino acids are indicated by white letters in black boxes, while similar amino acids are indicated by shaded boxes. Only homology and similarity to ViFR are shown. Amino acids belonging to the same group 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 (48). Gaps were inserted to increase the sequence homology. Underlines show two highly conserved regions (regions I and II).

obtained by Duane and Hastings. It might be suggested that FRase I is a nonglobular protein or a homodimer of the 26-kDa polypeptide.

FRase I and oxygen-insensitive NAD(P)H nitroreductases of *S. typhimurium*, *E. cloacae*, and *E. coli* form a new protein group. By using the Swiss-Prot data base (release 24), amino acid sequence comparisons were made between *V. fischeri* FRase I and the flavin-associated proteins so far identified. *E. coli* Fre (50), *Eubacterium* BaiH (17), rat liver NAD(P)H-quinone reductase (3), spinach ferredoxin-NADP$^+$ reductase (28), glutathione reductase from human erythrocytes (29), human placental aldose reductase (9), cytochrome b_2 from *Saccharomyces cerevisiae* (19), and *Desulfovibrio vulgaris* flavodoxin (13) all failed to show significant homology to *V. fischeri* FRase I. However, as shown in Fig. 4, appreciable sequence homology with oxygen-insensitive NAD(P)H nitroreductases from members of the family *Enterobacteriaceae* (*S. typhimurium* [58], *E. cloacae* [6], and *E. coli* [2]) was noted. Marginal sequence homology with *T. thermophilus* NADH oxidase (41) was also detected. Oxygen-insensitive NAD(P)H nitroreductases are flavoproteins which catalyze the reduction of various nitroaromatic compounds to metabolites that are highly toxic, mutagenic, and/or carcinogenic (5, 11, 51, 57). In particular, in *E. cloacae* nitroreductase, 1 mol of FMN molecule has been shown to bind to 1 mol of the enzyme as a cofactor and either NADH or NADPH has been shown to serve as an electron donor (5). *E. coli* nitroreductase, whose

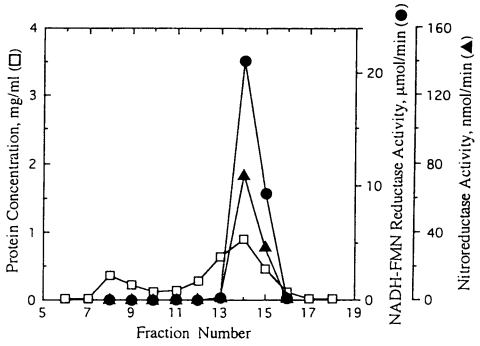


FIG. 5. Intimate association of flavin reductase and nitroreductase activity in extracts of *E. coli* cells expressing *V. fischeri* FRase I. IPTG-induced extracts of D1210 with pFR7 were subjected to gel filtration with Superose 12. Two-tenths of a milliliter of D1210 (pFR7), pretreated with IPTG, was applied to a column (1 by 30 cm) equilibrated with 50 mM Tris-HCl buffer (1 mM DTT, 200 mM NaCl [pH 7.3]). The total protein applied was 2.24 mg. Fractions (1 ml each) were collected at a flow rate of 0.2 ml/min. Each fraction was assayed for nitroreductase activity (▲), flavin reductase activity (●), and protein concentration (□).

partial sequence has been reported (2), was found to be very similar, if not identical, in sequence to the *E. coli* homolog of the 26-kDa protein-encoding gene of *V. fischeri* (63 [compare with EsFR in Fig. 4]). *T. thermophilus* NADH oxidase is a monomeric FAD-containing flavoenzyme which catalyzes the oxidation of NADH by the reduction of oxygen (42). The identity and similarity of the amino acid sequences of *V. fischeri* FRase I and *S. typhimurium* or *E. cloacae* nitroreductases were 32 to 34 and 56%, respectively. The identity and similarity of *S. typhimurium* and *E. cloacae* nitroreductases were 88 and 93%, respectively. *T. thermophilus* NADH oxidase possesses the least homology. Except for several long deletions and/or insertions, homology between *T. thermophilus* NADH oxidase and *V. fischeri* FRase I was estimated to be 22%. There are two regions (regions I and II in Fig. 4) highly conserved in the amino acid sequence. Bryant et al. (6) suggest that the region (residues 153 to 158) including the Gly-X-Gly-X-X-Gly motif is involved in the binding of NAD(P)H in *E. cloacae* nitroreductase. However, our amino acid sequence alignment showed this motif not to be conserved in other members of the family, and thus it may be fortuitous.

These findings together suggest that FRase I of *V. fischeri* may be a member of a novel protein family including oxygen-insensitive NAD(P)H nitroreductases of *S. typhimurium*, *E. cloacae*, and *E. coli*.

FRase I is associated with a low level of nitroreductase activity. Remarkable homology in the primary structures of FRase I of *V. fischeri* and *Enterobacteriaceae* oxygen-insensitive NAD(P)H nitroreductases may suggest that the *V. fischeri* enzyme catalyzes the reduction of nitro compounds, and this is shown to be so in Table 1. Oxygen-insensitive nitroreductase activity was detected in both a commercial preparation of *V. fischeri* FRase I and IPTG-induced extracts of D1210 with pFR7. Intimate associations of flavin reductase and nitroreductase activities were also demonstrated by Superose 12 column chromatography (Fig. 5). More than 98% of the total flavin reductase and nitroreductase activities were recovered in two fractions, 14 and 15. The 26-kDa FRase I protein was also detected only in fractions 14 and 15 (data not shown). The specific activity of the flavin reductase (about 40% pure) was estimated to be 23 μmol/min/mg of protein, while that of the

nitroreductase was 80 nmol/min/mg of protein. *V. fischeri* FRase I showed limited activity of oxygen-insensitive NADPH nitroreductase when nitrofurazone was used as a substrate.

In conclusion, we succeeded in cloning the gene which codes for the major flavin reductase in *V. fischeri*. Nucleotide sequence analysis showed that the major flavin reductase in *V. fischeri* forms a novel protein family including oxygen-insensitive NAD(P)H nitroreductases from members of the family *Enterobacteriaceae*.

ACKNOWLEDGMENTS

We are grateful to S. Shimasaki for aid in determination of amino acid sequences.

This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan to K.S.

REFERENCES

1. **Andrews, S. C., D. Shipley, J. N. Keen, J. B. C. Findlay, P. M. Harrison, and J. R. Guest.** 1992. The haemoglobin-like protein (HMP) of *Escherichia coli* has ferrisiderophore reductase activity and its C-terminal domain shares homology with ferredoxin $NADP^+$ reductases. FEBS Lett. **302:**247–252.
2. **Anlezark, G. M., R. G. Melton, R. F. Sherwood, B. Coles, F. Friedlos, and R. J. Knox.** 1992. The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954). I. Purification and properties of a nitroreductase enzyme from *Escherichia coli*—a potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). Biochem. Pharmacol. **44:**2289–2295.
3. **Bayney, R. M., J. A. Rodkey, C. D. Bennett, A. Y. H. Lu, and C. B. Pickett.** 1987. Rat liver NAD(P)H:quinone reductase nucleotide sequence analysis of a quinone reductase cDNA clone and prediction of the amino acid sequence of the corresponding protein. J. Biol. Chem. **262:**572–575.
4. **Bradford, M. M.** 1976. 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.
5. **Bryant, C., and M. DeLuca.** 1991. Purification and characterization of an oxygen-insensitive NAD(P)H nitroreductase from *Enterobacter cloacae*. J. Biol. Chem. **266:**4119–4125.
6. **Bryant, C., L. Hubbard, and W. D. McElroy.** 1991. Cloning, nucleotide sequence, and expression of the nitroreductase gene from *Enterobacter cloacae*. J. Biol. Chem. **266:**4126–4130.
7. **Byrne, C. R., R. S. Monroe, K. A. Ward, and N. M. Kredich.** 1988. DNA sequences of the *cysK* regions of *Salmonella typhimurium* and *Escherichia coli* and linkage of the *cysK* regions to *ptsH*. J. Bacteriol. **170:**3150–3157.
8. **Charlier, J., and E. Gerlo.** 1975. Evidence for specific NADH- and NADPH-FMN reductases in bacterial bioluminescence. Arch. Int. Physiol. Biochim. **83:**354–356.
9. **Chung, S., and J. LaMendola.** 1989. Cloning and sequence determination of human placental aldose reductase gene. J. Biol. Chem. **264:**14775–14777.
10. **de Boer, H. A., L. J. Comstock, and M. Vasser.** 1983. The *tac* promoter: a functional hybrid derived from the *trp* and *lac* promoters. Proc. Natl. Acad. Sci. USA **80:**21–25.
11. **Doi, T., H. Yoshimura, and K. Tatsumi.** 1983. Properties of nitrofuran from *Escherichia coli* B/r. Chem. Pharm. Bull. **31:**1105–1107.
12. **Duane, W., and J. W. Hastings.** 1975. Flavin mononucleotide reductase of luminous bacteria. Mol. Cell. Biochem. **6:**53–64.
13. **Dubourdieu, M., and J. L. Fox.** 1977. Amino acid sequence of *Desulfovibrio vulgaris* flavodoxin. J. Biol. Chem. **252:**1453–1463.
14. **Esch, F.** 1984. Polypeptide microsequence analysis with the commercially available gas-phase sequencer. Anal. Biochem. **136:**39–47.
15. **Fisher, J., R. Spencer, and C. Walsh.** 1976. Enzyme-catalyzed redox reactions with the flavin analogues 5-deazariboflavin, 5-deazariboflavin 5′-phosphate, and 5-deazariboflavin 5′-diphosphate, 5′→5′-adenosine ester. Biochemistry **15:**1054–1064.
16. **Fontecave, M., R. Eliasson, and P. Reichard.** 1987. NAD(P)H: flavin oxidoreductase of *Escherichia coli*: a ferric iron reductase participating in the generation of the free radical of ribonucleotide reductase. J. Biol. Chem. **262:**12325–12331.
17. **Franklund, C. V., S. F. Baron, and P. B. Hylemon.** 1993. Characterization of the *baiH* gene encoding a bile acid-inducible NADH: flavin oxidoreductase from *Eubacterium* sp. strain VPI 12708. J. Bacteriol. **175:**3002–3012.
18. **Gerlo, E., and J. Charlier.** 1975. Identification of NADH-specific and NADPH-specific FMN reductases in *Beneckea harveyi*. Eur. J. Biochem. **57:**461–467.
19. **Guiard, B.** 1985. Structure, expression and regulation of a nuclear gene encoding a mitochondrial protein: the yeast *L*(+)-lactate cytochrome *c* oxidoreductase (cytochrome b_2). EMBO J. **4:**3265–3272.
20. **Gunsalus-Miguel, A., E. A. Meighen, M. Z. Nicoli, K. H. Nealson, and J. W. Hastings.** 1972. Purification and properties of bacterial luciferases. J. Biol. Chem. **247:**398–404.
21. **Hasan, N., and E. W. Nester.** 1978. Purification and characterization of NADPH-dependent flavin reductase: an enzyme required for the activation of chorismate synthase in *Bacillus subtilis*. J. Biol. Chem. **253:**4987–4992.
22. **Hastings, J. W., C. J. Potrikus, S. C. Gupta, M. Kurfurst, and J. C. Makemson.** 1985. Biochemistry and physiology of bioluminescent bacteria. Adv. Microb. Physiol. **26:**235–291.
23. **Hastings, J. W., W. H. Riley, and J. Massa.** 1965. The purification, properties, and chemiluminescent quantum yield of bacterial luciferase. J. Biol. Chem. **240:**1473–1481.
24. **Hattori, M., and Y. Sakaki.** 1988. Dideoxy sequencing method using denatured plasmid templates. Anal. Biochem. **152:**232–238.
25. **Hoopes, B. C., and W. R. McClure.** 1987. Strategies in regulation of transcription initiation, p. 1231–1240. *In* F. C. Neidhart, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
26. **Jablonski, E., and M. DeLuca.** 1977. Purification and properties of the NADH and NADPH specific FMN oxidoreductases from *Beneckea harveyi*. Biochemistry **16:**2932–2936.
27. **Jablonski, E., and M. DeLuca.** 1978. Studies of the control of luminescence in *Beneckea harveyi*: properties of the NADH and NADPH:FMN oxidoreductases. Biochemistry **17:**672–678.
28. **Karplus, P. A., K. A. Walsh, and J. R. Herriott.** 1984. Amino acid sequence of spinach ferredoxin:$NADP^+$ oxidoreductase. Biochemistry **23:**6576–6583.
29. **Krauth-Siegel, R. L., R. Blatterspiel, M. Saleh, E. Schiltz, R. H. Schirmer, and R. Untucht-Grau.** 1982. Glutathione reductase from human erythrocytes: the sequences of the NADPH domain and of the interface domain. Eur. J. Biochem. **121:**259–267.
30. **Kumagai, H., H. Suzuki, H. Shigematsu, and T. Tochikura.** 1989. S-carboxymethylcysteine synthase from *Escherichia coli*. Agric. Biol. Chem. **53:**2481–2487.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
32. **Lavi, J. T., R. P. Raunio, and T. H. Stahlberg.** 1990. Affinity purification of bacterial luciferase and NAD(P)H:FMN oxidoreductases by FMN-sepharose for analytical applications. J. Biolumin. Chemilumin. **5:**187–192.
33. **Levy, S., and A. Danchin.** 1988. Phylogeny of metabolic pathways: *O*-acetylserine sulphydrylase A is homologous to the tryptophan synthase beta subunit. Mol. Microbiol. **2:**777–783.
34. **Lipsky, R. H., and P. B. Hylemon.** 1980. Characterization of a NADH:flavin oxidoreductase induced by cholic acid in a 7α-dehydroxylating intestinal *Eubacterium* species. Biochim. Biophys. Acta **612:**328–336.
35. **Lo, H.-S., and R. E. Reeves.** 1980. Purification and properties of NADPH:flavin oxidoreductase from *Entamoeba histolytica*. Mol. Biochem. Parasitol. **2:**23–30.
36. **Meighen, E. A.** 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. **55:**123–142.
37. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
38. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene **19:**269–276.

39. **Michaliszyn, G. A., S. S. Wing, and E. A. Meighen.** 1977. Purification and properties of a NAD(P)H:flavin oxidoreductase from the luminous bacterium, *Beneckea harveyi*. J. Biol. Chem. **252:** 7495–7499.
40. **Nefsky, B., and M. DeLuca.** 1982. Studies on the NADH and NADPH:riboflavin 5′-phosphate (FMN) oxidoreductases from *Beneckea harveyi*: characterization of the FMN binding sites. Arch. Biochem. Biophys. **216:**10–16.
41. **Park, H.-J., R. Kreutzer, C. O. A. Reiser, and M. Sprinzl.** 1992. 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. (Author's correction, **211:**909, 1993.)
42. **Park, H.-J., C. O. A. Reiser, S. Kondruweit, H. Erdmann, R. D. Schmid, and M. Sprinzl.** 1992. Purification and characterization of a NADH oxidase from the thermophile *Thermus thermophilus* HB8. Eur. J. Biochem. **205:**881–885.
43. **Puget, K., and A. M. Michelson.** 1972. Studies in bioluminescence. VII. Bacterial NADH: flavin mononucleotide oxidoreductase. Biochimie **54:**1197–1204.
44. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13:**319–353.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Schagger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
47. **Schmidt, T. M., K. Kopecky, and K. H. Nealson.** 1989. Bioluminescence of insect pathogen *Xenorhabdus luminescens*. Appl. Environ. Microbiol. **55:**2607–2612.
48. **Schwartz, R. M., and M. O. Dayhoff.** 1978. Matrices for detecting distant relationships, p. 353–358. *In* M. O. Dayhoff (ed.), Atlas of protein sequence and structure, vol. 5, supplement 3. National Biochemical Research Foundation, Washington, D.C.
49. **Shine, J., and L. Dalgarno.** 1974. 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.
50. **Spyrou, G., E. Haggård-Ljungquist, M. Krook, H. Jörnvall, E. Nilsson, and P. Reichard.** 1991. Characterization of the flavin reductase gene (*fre*) of *Escherichia coli* and construction of a plasmid for overproduction of the enzyme. J. Bacteriol. **173:**3673–3679.
51. **Tatsumi, K., T. Doi, H. Yoshimura, H. Koga, and T. Horiuchi.** 1982. Oxygen-insensitive nitrofuran reductases in *Salmonella typhimurium* TA100. J. Pharmacobio-Dyn. **5:**423–429.
52. **Towbin, H. T., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
53. **Tu, S.-C., J. E. Becvar, and J. W. Hastings.** 1979. Kinetic studies on the mechanism of bacterial NAD(P)H:flavin oxidoreductase. Arch. Biochem. Biophys. **193:**110–116.
54. **Tu, S.-C., and J. W. Hastings.** 1980. Physical interaction and activity coupling between two enzymes induced by immobilization of one. Proc. Natl. Acad. Sci. USA **77:**249–252.
55. **Untucht-Grau, R., R. H. Schirmer, and R. L. Krauth-Siegel.** 1981. Glutathione reductase from human erythrocytes. Amino acid sequence of the structurally known FAD-binding domain. Eur. J. Biochem. **253:**4086–4089.
56. **Watanabe, H., and J. W. Hastings.** 1982. Specificities and properties of three reduced pyridine nucleotide-flavin mononucleotide reductases coupling to bacterial luciferase. Mol. Cell. Biochem. **44:**181–187.
57. **Watanabe, M., M. Ishidate, Jr., and T. Nohmi.** 1989. A sensitive method for the detection of mutagenic nitroarenes: construction of nitroreductase-overproducing derivatives of *Salmonella typhimurium* strains TA98 and TA100. Mutat. Res. **216:**211–220.
58. **Watanabe, M., M. Ishidate, Jr., and T. Nohmi.** 1990. Nucleotide sequence of *Salmonella typhimurium* nitroreductase gene. Nucleic Acids Res. **18:**1059.
59. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of M13mp18 and pUC19 vectors. Gene **33:**103–119.
60. **Yubisui, T., T. Matsuki, K. Tanishima, M. Takeshita, and Y. Yoneyama.** 1977. NADPH-flavin reductase in human erythrocytes and the reduction of methemoglobin through flavin by the enzyme. Biochem. Biophys. Res. Commun. **76:**174–182.
61. **Zenno, S., S. Inouye, and K. Saigo.** 1992. Does the *luxG* gene in luminous bacteria code for an NAD(P)H-FMN oxidoreductase? Genetics (Life Sci. Adv.) **11:**85–91.
62. **Zenno, S., and K. Saigo.** Identification of the genes encoding NAD(P)H-flavin oxidoreductases that are similar in sequence to *Escherichia coli* Fre in four species of luminous bacteria: *Photorhabdus luminescens*, *Vibrio fischeri*, *Vibrio harveyi*, and *Vibrio orientalis*. J. Bacteriol. **176:**3544–3551.
63. **Zenno, S., and K. Saigo.** Unpublished data.