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Vibrio harveyi NADPH-Flavin Oxidoreductase: Cloning, Sequencing and Overexpression of the Gene and Purification and Characterization of the Cloned Enzyme

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NAD(P)H-flavin oxidoreductases (flavin reductases) from luminous bacteria catalyze the reduction of flavin by NAD(P)H and are believed to provide the reduced form of flavin mononucleotide (FMN) for luciferase in the bioluminescence reaction. By using an oligonucleotide probe based on the partial N-terminal amino acid sequence of the *Vibrio harveyi* NADPH-FMN oxidoreductase (flavin reductase P), a recombinant plasmid, pFRP1, was obtained which contained the *frp* gene encoding this enzyme. The DNA sequence of the *frp* gene was determined; the deduced amino acid sequence for flavin reductase P consists of 240 amino acid residues with a molecular weight of 26,312. The *frp* gene was overexpressed, apparently through induction, in *Escherichia coli* JM109 cells harboring pFRP1. The cloned flavin reductase P was purified to homogeneity by following a new and simple procedure involving FMN-agarose chromatography as a key step. The same chromatography material was also highly effective in concentrating diluted flavin reductase P. The purified enzyme is a monomer and is unusual in having a tightly bound FMN cofactor. Distinct from the free FMN, the bound FMN cofactor showed a diminished A_{375} peak and a slightly increased 8-nm red-shifted A_{453} peak and was completely or nearly nonfluorescent. The K_ms for FMN and NADPH and the turnover number of this flavin reductase were determined. In comparison with other flavin reductases and homologous proteins, this flavin reductase P shows a number of distinct features with respect to primary sequence, redox center, and/or kinetic mechanism.

Bacterial luciferase catalyzes the bioluminescent oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde by molecular oxygen to produce flavin mononucleotide (FMN), fatty acid, water, and blue-green light. In such a reaction, one oxygen atom from O_2 is incorporated into the alkyl carboxylic acid product, and the other oxygen atom is recovered in water. Therefore, bacterial luciferase is classified as a monooxygenase. However, luciferase is unique among known flavin-dependent monooxygenases. First, aside from luciferase, no other flavo-monooxygenases are able to catalyze a light-emitting reaction. Second, typical flavo-monooxygenases contain a tightly bound flavin cofactor which can be reduced by an NADH or NADPH substrate through their own catalytic activities. In contrast, bacterial luciferase does not have any tightly associated flavin cofactor and is unable to catalyze the reduction of flavin by NAD(P)H.

In luminous bacteria, the $FMNH_2$ substrate essential for the luciferase reaction is believed to be supplied by NAD(P)H-FMN oxidoreductases (flavin reductases) which catalyze the reduction of flavin by NAD(P)H. Three classes of flavin reductases have been detected in luminous bacteria some years ago. We propose that they can be identified according to the following designations. Flavin reductase D (FRD) exhibits much higher activity with NADH than NADPH (9, 15, 23), flavin reductase P (FRP) is highly specific for NADPH (9, 15), and flavin reductase G (FRG) has a general substrate utilization pattern with similar efficiencies for both NADPH and NADH (5, 34). Both earlier and more recent kinetic analyses indicate that *Vibrio harveyi* FRD (16) and FRP (16, 20, 35) may supply $FMNH_2$ to luciferase through a direct transfer mechanism.

While luciferase has been extensively studied with respect to molecular biology and enzymology, relatively little is known about flavin reductases. One problem which hampers detailed enzymological studies is the low yields of these flavin reductases. In this work, we report the cloning and sequencing of the gene (*frp*) encoding the *V. harveyi* FRP and its overexpression in *Escherichia coli*. An earlier method (15) was modified to obtain FRP from the wild-type *V. harveyi* cells in a homogeneous and highly concentrated form. A new and simple procedure was developed for the purification of the cloned FRP in excellent yields, and the purified enzyme was characterized.

MATERIALS AND METHODS

Bacterial strain and materials. *E. coli* JM109 was obtained from New England Biolabs. Enzymes used for the cloning of the *frp* gene were purchased from New England Biolabs and United States Biochemical Corp. Flavins, $NADP^+$, and NADPH were obtained from Sigma. Oligonucleotide probes were synthesized by using a Biosearch 6800 DNA synthesizer. FMN-agarose was obtained as described previously (8) by using agarose beads (Bio-Gel A-50m; Bio-Rad) activated by bisoxirane (31). $NADP^+$-agarose was prepared according to the method of Lamed et al. (17). All phosphate buffers (P_i [pH 7.0]) consisted of mole fractions of 39% sodium monobase and 61% potassium dibase.

FRP purifications. For the isolation of FRP from cells of wild-type *V. harveyi* ATCC 33843 (originally obtained as strain MAV or B392 from J. W. Hastings, The Biological Laboratories, Harvard University), initial procedures for luciferase purification (13) were followed through the step of DEAE-

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Sephadex chromatography for the separation of the flavin reductases from luciferase. A pool of FRD and FRP thus obtained (~200 ml, 640 mg of protein) was adjusted to 1.5 M P_i. The sample was then applied to an FMN-agarose column (1.5 by 30 cm) which was preequilibrated and eluted with 1.5 M P_i. When the A_{280} decreased to near the baseline, FRP was recovered by changing the elution buffer to 1.0 M P_i. Under such conditions, FRD was still retained on the column. The enzyme sample was subjected to gel filtration on a Sephadex G-150 column and finally was subjected to $NADP^+$-agarose affinity chromatography according to the method of Jablonski and DeLuca (15). The purified FRP (~30 ml) was concentrated by ~100 fold as follows. The sample was adjusted to 1.6 M P_i by adding solid phosphate and loaded on 0.3 ml of FMN-agarose which was packed in a glass Pasteur pipette and preequilibrated with 1.6 M P_i. The FRP was bound by the matrix material, while the bulk of sample buffer passed through. The bound FRP was then recovered in 0.3 ml by elution with 0.1 M P_i.

A simple method was devised for the purification of FRP expressed in *E. coli*. *E. coli* JM109 cells bearing pFRP1 (an *frp* gene clone) were cultured in five flasks, each containing 1 liter of 2× Luria-Bertani medium with ampicillin (100 mg/liter), at 37°C overnight and then were cultured at room temperature for one more day. The cells were harvested by centrifugation. Fifty grams (wet weight) of wet cells so obtained was sonicated in 750 ml of deionized water, precooled to ~4°C, containing 0.5 mM dithiothreitol (DTT). FRP was first partially purified by DEAE-cellulose batch adsorption and chromatography on a DEAE-Sephadex column (13). The sample was subjected to 75% ammonium sulfate precipitation, dialyzed into 0.1 M P_i, and adjusted to 1.5 M P_i prior to loading on an FMN-agarose column (1.5 by 40 cm), preequilibrated with the same phosphate buffer containing 0.5 mM DTT. Finally, pure FRP was obtained by elution with 1.3 M P_i–0.5 mM DTT.

Enzyme assays. FRP activities were determined at 23°C by monitoring decreases in A_{340} associated with the oxidation of NADPH upon mixing enzyme with 0.16 mM NADPH and 0.05 mM FMN in 1 ml of 50 mM P_i. One unit of enzyme activity is defined as 1 μmol of NADPH oxidized per min ($\varepsilon_{340} = 6.22 \times 10^3\ M^{-1} \cdot cm^{-1}$).

Spectral measurements. Absorption spectra were measured with a Milton Roy Spectronic 3000 unit. Fluorescence emissions were determined with a Perkin-Elmer MPF-44A fluorescence spectrophotometer. This spectrophotometer was also equipped with polarization filters for the measurement of steady-state fluorescence polarizations. Emission spectra were not corrected for wavelength-dependent variations in phototube response or monochromator efficiency.

Determination of the N-terminal amino acid sequence of FRP. The N-terminal sequence of the *V. harveyi* FRP was determined by automated Edman degradation with a gas-phase sequencer (Applied Biosystems 470A) with an on-line PTH amino acid analyzer (Applied Biosystems 120A) (14). These sequence analyses were carried out through a service provided by the Division of Atherosclerosis and Lipoprotein Research, Baylor College of Medicine and the Methodist Hospital, Houston.

Construction of genomic library. A genomic DNA library of *V. harveyi* was constructed as previously described (1). *V. harveyi* genomic DNA was partially digested with *Sau*3AI. Fragments 3 to 6 kb in length were excised from an ethidium bromide-free agarose gel (0.8% agarose) after electrophoresis. The sizes of DNA fragments were determined by comparison with DNA molecular weight standards subjected to electrophoresis in a lane adjacent to the DNA sample. The lane containing the molecular weight standards was cut off and stained with ethidium bromide. A piece of the gel in the sample lane corresponding to the desired size of DNA fragments was cut off, put in a microcentrifuge vial, frozen at −20°C for 2 h, and then thawed at room temperature. The desired DNA was recovered in the supernatant, with a yield of about 50% after centrifugation in a microcentrifuge for 2 min. The DNA so obtained (~0.12 μg) was ligated by T4 DNA ligase for 16 h at 16°C to pUC19 (0.1 μg) previously digested with *Bam*HI and dephosphorylated with alkaline phosphatase. Recombinant DNA was introduced into *E. coli* JM109 (11). Transformed cells were spread on nitrocellulose filters (HATF 085; Millipore) which were placed on top of agar plates prepared in Luria-Bertani medium containing ampicillin (50 mg/liter), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [20 mg/liter]), and 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

Screening of genomic library. The genomic library was screened by hybridization with a ^{32}P-labeled oligonucleotide probe. The oligonucleotide probe (20 pmol) was end labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP (Du Pont) and was purified by passing through a spin column (K1320-1; Clontech). The colonies on the filters were lysed, and the DNA was fixed to the filters (27) and hybridized at 37°C in 30 ml of ^{32}P-labeled probe (5×10^4 cpm/ml) for 18 h according to the method of Ausubel et al. (1). The filters were washed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 30°C for 20 min and exposed to Kodak XAR-5 X-ray film at −80°C for 48 h.

Nucleotide sequencing. The insert containing the *frp* gene was subcloned into M13mp18 and M13mp19 (Pharmacia). The DNA sequence was determined by the dideoxy method (22, 28) with a Sequenase kit from United States Biochemical Corp., [α-^{35}S]dATP from Du Pont, the M13 universal primer, and other synthetic oligonucleotide primers. Sequencing data were analyzed with the software of Devereux et al. (4).

Southern blot analyses. Southern transfers (29) of DNAs from agarose gel to Immobilon-P membrane (Millipore) were performed under alkaline conditions according to the manufacturer's specifications. The conditions for hybridizing with ^{32}P-labeled oligoprobe and washing were the same as those described for the genomic library screening.

Identification of the FRP-bound flavin. A 2-ml solution containing 3 mg of FRP was made to 8 M urea and incubated for 10 min at 23°C. Subsequently, the sample was centrifuged in a spin filter unit with molecular weight cutoff of 10,000 (Millipore). The dissociated flavin was recovered in the filtrate and was separated from urea by chromatography on a DEAE-Sephadex column with elution by 0.2 M P_i. To 1 ml of 0.2 M P_i containing 10 mM EDTA and 50 μM decanal, authentic riboflavin, FMN, FAD, or the flavin isolated from FRP was added to a final concentration of 20 μM. The flavin was reduced by 10 μl of saturating Cu(I) perchlorate in acetonitrile (19), and the solution was injected into 50 mM air-saturated P_i containing luciferase αC106A (a *V. harveyi* luciferase variant in which the cysteine 106 of the α subunit was replaced by an alanine) to initiate the luminescence reactions. The luminescence was measured with a photometer. The αC106A was purified and kindly provided by L. Xi.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence discussed in this paper is U08996.

RESULTS

N-terminal amino acid sequence of FRP and the design of a mixed oligonucleotide probe. The FRP purified from *V. harveyi* as described above showed a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Twenty-three cycles of automatic Edman degradation of the purified FRP revealed a partial N-terminal sequence of Met-Asn-Asn-Thr-Ile-Glu-Thr-Ile-Leu-Ala-His-Gly-Ser-Ile-Ile-Lys-Phe-Thr-Ala-Val-Pro-Ile-Thr. A peptide of the first six amino acid residues, Met-Asn-Asn-Thr-Ile-Glu, was chosen for the design of a mixed oligonucleotide probe. On the basis of several sequences, totaling 5,337 bases, obtained in August 1992 from the GenBank data base, the codon frequencies in *V. harveyi* were calculated with the Genetics Computer Group CODONFREQUENCY program (4). The AUA codon for Ile and the ACA codon for Thr had usage frequencies of 13 and 12%, respectively, and were excluded from the synthesis of the probe to reduce its degeneracy. The probe thus contained 24 of the 48 possible sequences as shown in scheme I.

Scheme I

```
          C
5'-TCAATAGTATTATTCAT-3'
  G G G G
```

Isolation of *frp* clones. A genomic library of *V. harveyi* constructed as described above contained approximately 8,000 colonies. Four positive clones were identified by screening the library with the ^{32}P-labeled mixed oligonucleotide probe shown in scheme I.

The four positive clones were further tested by FRP activity expression and Southern blot analysis. Crude extracts were prepared from *E. coli* host cells cultured from these positive clones and from the same host cells harboring pUC19 as a control. The extracts for three of the four positive clones each showed >1,000-fold-higher levels of FRP activity than that of the control, indicating that the DNA inserts contain the intact *frp* gene and that expression was achieved. In contrast, the lysate for the fourth clone had about the same background activity as that of the control. The three recombinant plasmids expressing FRP activities were further analyzed by Southern blotting. DNA samples from these three plasmids were digested with *Hin*dIII, and the resulting fragments were subject to electrophoresis in agarose gel. The DNA fragments were then transferred to a nitrocellulose filter and hybridized with the same probe used in the library screening. The three recombinant plasmids had inserts 2.4, 3.3, and 6.2 kb in size, and each had only one fragment hybridizing with the probe. All three inserts had a common 0.45-kb fragment which was adjacent to the fragment hybridizing with the probe. The plasmid containing the 3.3-kb insert, designated pFRP1, was chosen for further analyses.

Physical map of pFRP1 and sequence of the *frp* gene. The pFRP1 DNA was digested by individual restriction enzymes or combinations of 12 restriction enzymes which have specific cutting sites within the polylinker region of pUC19. According to sequence analyses and restriction patterns, the direction of the *frp* gene in the insert is opposite that of *lacZ* and Ap genes of pUC19.

Three fragments were obtained by digestions of the 3.3-kb insert with *Hin*dIII and *Eco*RI and were each inserted into M13mp18 and M13mp19 and sequenced with the M13 universal primer. Partial sequences of these fragments were determined to identify the location of the *frp* gene. The DNA sequence encoding the known N-terminal amino acid sequence of FRP was found to start at base 210 upstream of one *Hin*dIII site in the insert. The sequence of a 1.0-kb region containing the entire *frp* gene was determined in both directions with synthetic primers; the DNA sequence so obtained is shown in Fig. 1.

```
                                                                    .
GTT TAC GCT CCC AAT AAA TGC CGT TAT GGT GAA GAT TCA GCC AAA TAG AAC CAC TCT TCA

                                                                    .
GGA AGC CAG AAC ATC ATG AAC AAT ACG ATT GAA ACC ATT CTT GCT CAT CGC TCT ATC CGA
                    Met Asn Asn Thr Ile Glu Thr Ile Leu Ala His Arg Ser Ile Arg
                    1   .   .   .   .   .   .   .   .   .   .   .   .   .   .
AAA TTC ACC GCA GTT CCT ATT ACT GAT GAA CAA AGA CAA ACC ATC ATT CAA GCA GGT TTA
Lys Phe Thr Ala Val Pro Ile Thr Asp Glu Gln Arg Gln Thr Ile Ile Gln Ala Gly Leu

                                                                    .
GCT GCG TCT TCT TCT AGT ATG CTT CAA GTC GTC TCA ATC GTT CGA GTG ACT GAC TCT GAA
Ala Ala Ser Ser Ser Ser Met Leu Gln Val Val Ser Ile Val Arg Val Thr Asp Ser Glu

                                                                    .
AAG CGT AAC GAA TTG GCT CAA TTT GCT GGT AAC CAA GCT TAT GTT GAA AGT GCG GCT GAG
Lys Arg Asn Glu Leu Ala Gln Phe Ala Gly Asn Gln Ala Tyr Val Glu Ser Ala Ala Glu

                                                                    .
TTC TTA GTG TTT TGT ATT GAT TAT CAG CGC CAT GCA ACC ATC AAT CCT GAT GTA CAG GCA
Phe Leu Val Phe Cys Ile Asp Tyr Gln Arg His Ala Thr Ile Asn Pro Asp Val Gln Ala

                                                                    .
GAC TTT ACA GAA CTA ACT CTG ATT GGA GCA GTA GAT TCT GGA ATC ATG GCA CAA AAC TGC
Asp Phe Thr Glu Leu Thr Leu Ile Gly Ala Val Asp Ser Gly Ile Met Ala Gln Asn Cys

                                                                    .
TTG CTT GCA GCC GAG TCT ATG GGA TTA GGT GGC GTA TAT ATT GGA GGA CTA AGG AAT AGC
Leu Leu Ala Ala Glu Ser Met Gly Leu Gly Gly Val Tyr Ile Gly Gly Leu Arg Asn Ser

                                                                    .
GCA GCT CAA GTT GAT GAG CTA TTG GGC TTA CCG GAA AAT AGC GCG GTG TTG TTT GGT ATG
Ala Ala Gln Val Asp Glu Leu Leu Gly Leu Pro Glu Asn Ser Ala Val Leu Phe Gly Met

                                                                    .
TGC TTA GGG CAT CCC GAT CAA AAT CCC GAA GTA AAG CCA CGC CTA CCT GCA CAT GTG GTT
Cys Leu Gly His Pro Asp Gln Asn Pro Glu Val Lys Pro Arg Leu Pro Ala His Val Val

                                                                    .
GTT CAT GAA AAT CAA TAC CAA GAG CTA AAT TTA GAT GAT ATT CAG AGC TAC GAT CAA ACT
Val His Glu Asn Gln Tyr Gln Glu Leu Asn Leu Asp Asp Ile Gln Ser Tyr Asp Gln Thr

                                                                    .
ATG CAA GCG TAT TAT GCG AGC CGT ACA AGC AAT CAA AAA CTG AGT ACA TGG TCG CAA GAA
Met Gln Ala Tyr Tyr Ala Ser Arg Thr Ser Asn Gln Lys Leu Ser Thr Trp Ser Gln Glu

                                                                    .
GTC ACT GGG AAG CTT GCT GGT GAG TCG CGA CCT CAT ATT CTG CCG TAC TTG AAC AGT AAG
Val Thr Gly Lys Leu Ala Gly Glu Ser Arg Pro His Ile Leu Pro Tyr Leu Asn Ser Lys

                                                                    .
GGG CTA GCA AAA CGC TAA TAT CAT TGA AAT GAT GGT TTG TTG TAT GAA ATC GTT CAT CAA
Gly Leu Ala Lys Arg  *
                .   240 .   .   .   .   .   .   .   .   .   .   .   .   .   .
ACC ATC ACT TTG TTG AAC CCA CAT CAT ATT TTG ACC ATA CGC TGC CAA CTT TCT GGC AAA

                                                                    .
ACC ATA ACA AAG CGC TAT TGA CTC AGA ACA AAA AAC ACG ACA TAC ATC ACA TTT TAA AAC

                                                                    .
AAA GCA ATC ACT TTG TTG AAC CCA CAT CA
```

FIG. 1. Nucleotide sequence of the *V. harveyi frp* gene and deduced amino acid sequence. The first and last amino acid residues are numbered. The stop codon is marked by an asterisk.

The sequence contains an open reading frame starting at position 76 and ending at position 795 with a TAA stop codon. This open reading frame encodes a polypeptide with a length of 240 amino acid residues and a molecular weight of 26,312. The deduced amino acid sequence for the first 23 residues at the N terminus matches that determined by Edman degradation of FRP, except for two residues. The DNA sequence revealed Arg at both positions 12 and 15, but the protein sequencing showed Gly-12 and Ile-15. This minor discrepancy is believed to result from the lower degree of accuracy of the protein sequencing technique.

Overexpression of *frp*. Crude lysates prepared from *E. coli* JM109 cells at different growth stages all showed very low levels of the FRP-type activity (Fig. 2). Similar results were obtained from the same cells transformed with pUC19. In contrast, efficient expression of the FRP activity was detected in *E. coli* JM109 cells harboring pFRP1. The expression of FRP activity initially lagged significantly behind the cell growth, but once it occurred, it increased at rates substantially faster than the rate of cell growth (Fig. 2). These findings indicate an apparent induced expression for the cloned *frp* gene. The expression of *frp* was highly efficient, but no significant difference was detected for FRP activities in extracts of cells grown with or without the IPTG inducer of the *lac* promoter. Fifty grams of transformed *E. coli* cells harboring pFRP1 typically contained about 150 mg of FRP, while 400 g of *V. harveyi* cells had a total content of FRP of only about 2.5 mg, indicating 480-fold higher cellular content of FRP in the former cells. Also, on the basis of the total NADPH-dependent flavin reductase activity per gram of cells (wet weight), *E. coli* cells bearing pFRP1 could express *frp* at a level 5,000-fold

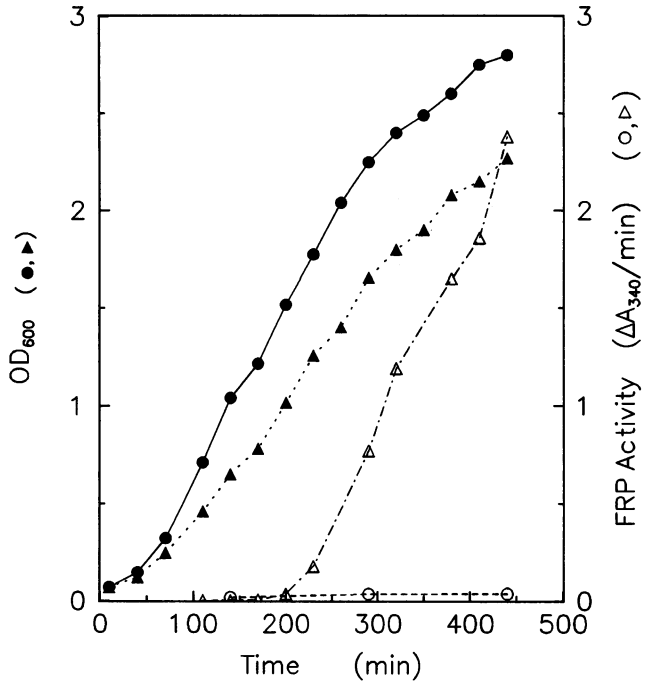

FIG. 2. Expressions of FRP activity in wild-type *E. coli* JM109 cells and those harboring pFRP1. *E. coli* JM109 cells harboring pFRP1 were cultured in Luria-Bertani medium containing ampicillin (100 mg/liter) until reaching an optical density at 600 nm (OD_{600}) of 0.7. Sixty milliliters of this culture was inoculated into 1 liter of the same medium, and cell growth was monitored by measuring changes in OD_{600} (▲). At each time point indicated, 1 ml of culture was withdrawn and the cells were collected by centrifugation. The cells were suspended in 0.2 ml of 50 mM phosphate buffer (pH 7.0) and sonicated at about 4°C. The supernatant was collected after centrifugation, and the FRP activity was determined at 23°C and was presented as the total activity in 0.5 ml of the cell culture (△). As a control, the OD_{600} (●) and FRP activity (○) were also determined similarly for *E. coli* JM109 cells grown in Luria-Bertani medium without ampicillin under otherwise identical conditions. In all cases, cells were grown at 37°C.

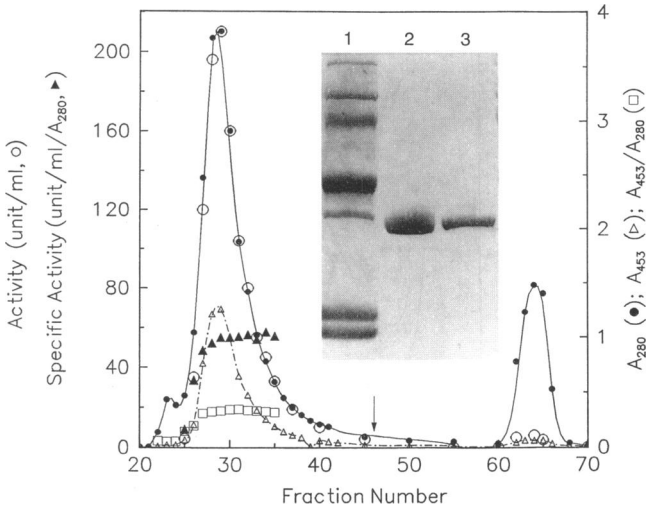

FIG. 3. Purification of FRP by FMN-agarose chromatography. A 25-ml partially purified enzyme sample (200 mg of protein) in 1.5 M P_i–0.5 mM DTT was applied to an FMN-agarose column (1.5 by 40 cm), preequilibrated with the same buffer and eluted with 1.3 M P_i–0.5 mM DTT. The elution buffer was changed to 0.1 M P_i at the fraction indicated by an arrow, and 5-ml fractions were collected throughout. All phosphate buffers were adjusted to pH 7.0. Symbols: ○, activity in units per milliliter; ▲, specific activity in units per milliliter/A_{280}; ●, A_{280}; △, A_{453}; □, A_{453}/A_{280}. (Inset) SDS-PAGE patterns for FRP purified from *E. coli* JM109 harboring pFRP1 (lane 2) and wild-type *V. harveyi* (lane 3). Lane 1 was for protein standards consisting of, from top to bottom, the myosin H chain (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

higher than that of the FRP-type activity of *E. coli* cells harboring pUC19.

Purification of FRP. A new and simple procedure was developed for the purification of FRP from *E. coli* host cells. This method involves initial partial purifications by batch adsorption and DEAE-Sephadex chromatography and the final treatment by FMN-agarose column chromatography. Results for a typical preparative run are summarized in Table 1, and the profile for the final FMN-agarose chromatography is shown in Fig. 3. With elution at 1.3 M phosphate (pH 7.0), FRP activities comigrated with a yellow color band and were both recovered under the same major peak. Most of the fractions under this peak showed a constant specific FRP activity and a constant A_{453}/A_{280} ratio (Fig. 3). The overall yields of the purification of the cloned FRP were excellent (Table 1). A significantly more laborious method reported earlier (15) was also followed, with minor modifications, to obtain FRP from *V. harveyi*. On the basis of SDS-PAGE, the wild-type and cloned FRP samples obtained as described above were both homogeneous and showed an identical molecular weight of 28,000 (Fig. 3, inset).

TABLE 1. Purification of the FRP expressed in *E. coli*[a]

Purification step	Vol (ml)	A_{280}	Total activity (U)[b]	Sp act (U/A_{280}/ml)	Yield (%)
Lysate	750	54	8,200	0.2	100
Batch adsorption	950	2.6	12,340	5.0	150
DEAE-Sephadex	210	1.45	11,140	36.6	136
FMN-agarose	130[c]	1.02[c]	7,500	56.6[d]	91

[a] From 50 g of cells (wet weight).
[b] One unit is defined as 1 μmol of NADPH oxidized per min.
[c] Value corresponded to a total amount of 85 mg of purified FRP.
[d] Value corresponded to 88.4 U/mg.

FMN-agarose chromatography was also highly effective in concentrating diluted FRP samples. As described in Materials and Methods, >30 ml of a diluted FRP sample was first adjusted to 1.6 M P_i (pH 7.0) and then applied to a small FMN-agarose column. The enzyme can be recovered in 0.3 ml, at an ~100-fold high protein concentration, by elution with 0.1 M P_i.

Characterization of FRP and the bound cofactor. By steady-state kinetic analysis of initial velocities of the cloned FRP at various levels of substrates, the K_ms for NADPH and FMN were found to be 20 and 7 μM, respectively, and the specific activity was found to be 56.6 $\mu mol \cdot min^{-1} \cdot A_{280}^{-1} \cdot ml^{-1}$ or 88 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. The turnover number was 2,160/min, essentially identical to that (2,040/min) reported for the wild-type FRP (15).

By chromatography on a Sephadex G-150 column, equilibrated and eluted with 0.1 M P_i, a molecular mass standard

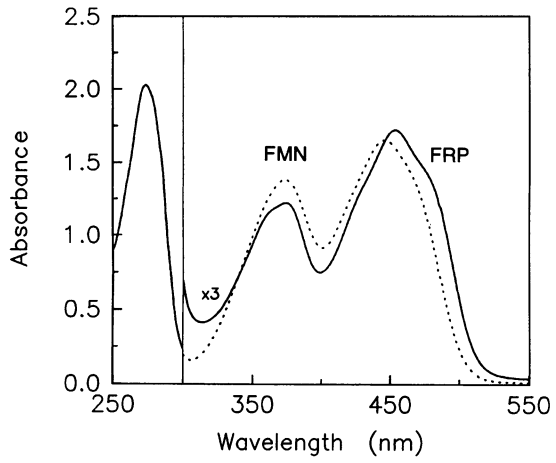

FIG. 4. Absorption spectrum of oxidized FRP expressed in *E. coli*. The spectrum of an apparently homogeneous enzyme (45 μM in 50 mM P_i [pH 7.0]) was measured in a 1-cm path cuvette (solid line). For comparison, the spectrum of free FMN at the same concentration is also shown (dotted line). In both cases, A_{300} to A_{550} values were magnified threefold for presentation.

curve was constructed with bovine serum albumin (68 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa) as size standards. On the basis of gel filtration under identical conditions, the molecular weight of the native form of the cloned FRP was found to be 33,000. In comparison with monomeric molecular weights of 26,312 according to the deduced amino acid sequence and 28,000 on the basis of SDS-PAGE, the gel filtration results indicate a monomeric form for FRP. For these three methods, only the molecular weight determination by gel filtration can be affected by the shape of the native protein. The somewhat larger monomeric molecular weight revealed by molecular sieve under nondenaturing conditions is possibly a result of an asymmetric shape of FRP.

The FRP activity comigrated with a yellow color band during both DEAE-Sephadex and FMN-agarose chromatographies, indicating that FRP has a tightly bound chromophore. After dissociation from FRP by treatment with 8 M urea, the yellow color was easily bleached by reduction with dithionite or Cu(I)-EDTA, as expected for flavins. The identity of the tightly bound flavin in FRP was determined by comparison with authentic flavins in terms of their light emission activities in the luciferase reaction as described in Materials and Methods. The light intensities in the luciferase reaction obtained with reduced riboflavin and FAD were 0.3 and 5%, respectively, of that exhibited by the $FMNH_2$, whereas the flavin from FRP was ≥85% as active as the $FMNH_2$ standard. Therefore, the tightly bound flavin cofactor in FRP is identified as FMN.

The absorption spectrum of a purified FRP was measured first in the absence of any denaturant and then in the presence of 8 M urea for the dissociation of flavin. Using the known ε_{450} for the free FMN (corrected for change caused by the presence of urea) for calibration, a value of ε_{453} = 12,800 $M^{-1} \cdot cm^{-1}$ was determined for the purified FRP. The protein concentration of the same enzyme sample was determined by the Lowry method (21) with bovine serum albumin as a standard, revealing a content of 0.96 bound FMN per monomer of FRP on the basis of a molecular weight of 26,312. The purified FRP showed two peaks in the visible region at A_{375} and A_{453} and a third peak in the UV range at A_{273} (Fig. 4). The former two absorption peaks are typical for flavins. However, distinct from the free FMN, the bound FMN cofactor showed a lower degree of absorptivity for the A_{375} peak and a slightly increased A_{453} peak. The latter peak is also red-shifted by 8 nm from the corresponding peak of free FMN.

The *frp* gene sequence indicates that there is a single tryptophan residue in FRP. This is consistent with the finding that the purified FRP showed a peak at A_{273} derived primarily from the flavin (Fig. 4) instead of the usual peak at A_{280} for most proteins. The purified FRP is characterized by the following spectral properties: A_{453}/A_{273} = 0.28, A_{453}/A_{375} = 0.32, and A_{273} = 0.58 $mg \cdot ml^{-1}$.

Upon excitation at 375 nm, weak fluorescence was detected for a 6.25-μM FRP sample in 0.05 M P_i with an uncorrected emission maximum at 520 nm. The intensity of this emission was 2.5% of that from a free FMN sample at the same concentration. An enzyme sample twice as concentrated gave only a slight change rather than a twofold increase in fluorescence intensity. The emission spectrum of the FRP sample and that of free FMN were identical in shape and in steady-state polarization. These findings, taken together, strongly indicate that the bound FMN was completely or nearly nonfluorescent and that the very weak fluorescence signals observed with the diluted FRP samples were from the trace amounts of dissociated FMN.

DISCUSSION

The *lux* operon in all luminous bacteria characterized thus far contains a minimum of five structural genes arranged in the order *CDABE* (2). The *luxA* and *luxB* genes encode the α and β subunits of luciferase, respectively, and the *luxCDE* sequences specify the components of the aldehyde synthetase complex. There is another common gene, *luxG*, in marine luminous species (2, 32). The LuxG protein sequence deduced from *V. harveyi luxG* shares 38% identity with the *E. coli* flavin reductase (FRE) sequence deduced from the *fre* gene (30). Therefore, it was suggested that *luxG* encoded a flavin reductase for the production of $FMNH_2$ substrate for luciferase (37). However, a link between the LuxG protein and the flavin reductase activity has, thus far, not been demonstrated. The *frp* gene we cloned exhibits only 18.7% identity with *luxG* with respect to the deduced amino acid sequences and lacks significant similarities with other *lux* genes of *V. harveyi*. Clearly, *frp* and *luxG* are different genes in *V. harveyi*.

In *V. harveyi*, the synthesis of luciferase is under the regulation of autoinduction, whereas the synthesis of FRP was reported to be constitutive (16). Therefore, the expression of *frp* is most likely not under the regulation of the *lux* operon. Interestingly, the *frp* in *E. coli* harboring pFRP1 was apparently expressed through induction (Fig. 2). In comparison with the cellular contents of FRP in *V. harveyi* and the FRP-type activities in the control *E. coli* host cells, this apparent induction of *frp* in pFRP1 was quite efficient. Sequencing results indicate that the *frp* in pFRP1 was expressed in a direction opposite that of *lacZ*. Moreover, the expression of *frp* in *E. coli* cells harboring pFRP1 was about the same with or without the IPTG inducer for the *lac* promoter. Apparently, the *frp* gene was under the control of a different promoter, possibly one encoded by the *V. harveyi* DNA insert. The regulatory mechanism of the expression of *frp* in pFRP1 remains to be elucidated.

No known protein sequences were found to share truly high degrees of identity with the amino acid sequence of the *V. harveyi* FRP by searching the currently available peptide sequence data bases (Nonredundant PDB, Swiss-Prot, PIR, SPUpdate, GenPept, and GPUpdate) through the BLAST

TABLE 2. Identities and similarities of amino acid sequences among flavin reductases and related proteins[a]

Enzyme or protein	% of identity or similarity with:			
	FRP	FRE	LuxG	NOX
FRP		17.9	18.7	30.6
FRE	41.5 (7)		39.3	20.8
LuxG	43.8 (9)	62.0 (2)		12.7
NOX	53.8 (8)	49.5 (10)	40.4 (8)	

[a] FRP and the LuxG protein are from *V. harveyi*, FRE is the flavin reductase from *E. coli*, and NOX is from *T. thermophilus*. Percentages of amino acid identities (upper right half) and similarities (lower left half) were determined with the Genetics Computer Group GAP program (4). Numbers in parentheses are the number of gaps in the sequence alignments.

network service. The NADH oxidase (NOX) from *Thermus thermophilus* (24) is the protein most closely related to the *V. harveyi* FRP, sharing 30.6% identity and 53.8% similarity in amino acid sequences. Other known proteins show much lower levels of identity or similarity. *T. thermophilus* NOX catalyzes the oxidation of NADH by oxygen to form NAD^+ and H_2O_2. The activities of NOX require exogenously added flavin (25). Therefore, *T. thermophilus* NOX may possibly be a flavin reductase active in producing first a reduced flavin product and producing subsequently H_2O_2 through oxidation of the reduced flavin by molecular oxygen.

A comparison of *V. harveyi* FRP with *V. harveyi* LuxG, FRE, and *T. thermophilus* NOX was made with respect to protein sequence similarities and identities by using the Genetics Computer Group GAP program (4) (Table 2). Apparently, FRP and NOX are more closely related to each other, whereas LuxG is more homologous to FRE. Within each of these two subgroups, approximately 30 to 40% identities and 50 to 60% similarities were observed. Much lower levels of identity or similarity were detected between these two subgroups. The alignment of the four sequences by the Genetics Computer Group PILEUP program (4) shows only two conserved residues.

Flavin reductases have also been detected in other luminous bacteria (*Vibrio fischeri* [5] and *Photobacterium phosphoreum* [18]), nonluminous microorganisms (*Bacillus subtilis* [12], *E. coli* [6], *Eubacterium* sp. [7], and *Pseudomonas aeruginosa* [10]), and human (36) and bovine (26) erythrocytes. With the exceptions of flavin reductases from *Eubacterium* sp. and *B. subtilis*, most known flavin reductases have similar monomeric sizes ranging from ~20 to 30 kDa. While flavin serves as an effective electron acceptor for all of these flavin reductases, some of them are able to use other electron acceptors as well. The involvement of certain flavin reductases in the reduction and release of iron has also been noted (3, 10). Compared with these flavin reductases and *T. thermophilus* NOX, *V. harveyi* FRP shows some unique properties. In addition to *V. harveyi* FRP, only the *Eubacterium* sp. flavin reductase and the *T. thermophilus* NOX are known to have a flavin cofactor. However, the cofactor for both of these latter two enzymes is FAD (7, 25). Moreover, the *Eubacterium* sp. flavin reductase also requires an iron-sulfur center (7). Kinetics-wise, only *V. harveyi* FRP (16, 35) and *V. fischeri* FRG (33) have thus far been shown to exhibit a ping-pong mechanism. However, the latter does not involve an intrinsic flavin as a cofactor in catalysis (33) as *V. harveyi* FRP does.

A new and simple purification scheme has been developed for the cloned FRP, involving FMN-agarose chromatography as a key step. Although the operational principle of FMN-agarose chromatography is not fully understood, it is believed to involve, at least in part, hydrophobic binding of FRP to the matrix. In accord with this interpretation, the binding of FRP to the FMN-agarose matrix was found to be quite sensitive to the phosphate buffer concentration, with tight bindings favored by higher phosphate levels (e.g., ≥1.5 M). As shown in Fig. 3, a slight change of the phosphate buffer from 1.5 M to 1.3 M substantially facilitated the elution of FRP, allowing efficient recovery of FRP and its separation from impurities. This also provides a very useful means for the concentration of FRP samples by adjusting a diluted enzyme solution to a high phosphate concentration (e.g., 1.6 M), applying it to a small FMN-agarose column, and recovering the enzyme by elution with a small volume of a low-phosphate-concentration buffer (e.g., 0.1 M).

The purified FRP has interesting spectral characteristics. Significant absorption spectrum perturbations of the FMN cofactor occurred upon binding to the cloned FRP (Fig. 4). These spectral changes are indicative of substantial interactions between the flavin isoalloxazine and the protein. In accord with this conclusion, the binding of the FMN cofactor to FRP was associated with a complete or nearly complete quenching of the flavin fluorescence. Moreover, the *V. harveyi* FRP has a single tryptophan residue and thus has an unusual peak at A_{273} rather than the usual A_{280} maximum for most proteins. For practical purposes, we found that the A_{273}/mg of protein and the A_{453}/A_{273} or A_{453}/A_{280} ratio are very useful indices for characterizing the purities of FRP samples.

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