

# Cloning and Expression of the Gene Encoding Flavodoxin from *Desulfovibrio vulgaris* (Miyazaki F)<sup>1</sup>

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The gene encoding a flavodoxin of *Desulfovibrio vulgaris* (Miyazaki F) was cloned, and overexpressed in *Escherichia coli*. A 1.6-kbp DNA fragment, isolated from *D. vulgaris* (Miyazaki F) by double digestion with *Sal*I and *Eco*RI, contained the flavodoxin gene and its regulatory region. An expression system for the flavodoxin gene under control of the T7 promoter was constructed in *E. coli*. The purified protein was soluble and exhibited a characteristic visible absorption spectrum. HPLC analysis of the recombinant flavodoxin revealed the presence of an identical FMN to that found in the native *D. vulgaris* flavodoxin, and its dissociation constant with FMN was determined to be 0.38 nM. *In vitro* H<sub>2</sub> reduction analysis indicated that the recombinant flavodoxin is active, and its redox potential was determined to be  $E_1 = -434$  and  $E_2 = -151$  mV using methyl viologen and 2-hydroxy-1,4-naphthoquinone, respectively. Its redox behavior was also examined with the recombinant flavodoxin adsorbed onto a graphite electrode. The mutant, A16E, was also produced, which revealed the feature of a conserved Glu residue at the surface of the molecule.

**Key words:** *Desulfovibrio vulgaris*, expression, flavodoxin, FMN-binding, site-directed mutagenesis.

Flavodoxins, a group of small flavoproteins of molecular masses of 15–23 kDa, have been isolated from a variety of microorganisms, in which they are thought to function as electron-transfer proteins in various metabolic pathways (1). They contain a single molecule of non covalent-bound FMN as their prosthetic group, and they exhibit a very negative reduction potential for the semiquinone/hydroquinone couple of the bound FMN cofactor. This negative reduction places these proteins in the potential range of ferredoxins, for which they can substitute *in vitro* and *in vivo* (2, 3). The redox properties of bound FMN in flavodoxins differ from those of protein-free FMN, in that the semiquinone state is stable.

Three-dimensional structures have been determined for

<sup>1</sup> The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D88439.

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Abbreviations: ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; UV/VIS, ultraviolet/visible; BPG, basal-plane pyrolytic graphite; EPG, edge-plane pyrolytic graphite; DMPC, L- $\alpha$ -phosphatidylcholine dimyristoyl; CV, cyclic voltammogram; DPV, differential-pulse voltammogram.

the flavodoxins from *Clostridium beijerinckii* MP (4) and *Chondrus crispus* (5) by X-ray crystallography, and for the *Megasphaera elsdenii* flavodoxin by two-dimensional NMR spectroscopy (6). The structure of the flavodoxin from *Desulfovibrio vulgaris* (Hildenborough) has been well studied in its three redox states by X-ray crystallography or NMR spectroscopy (7–10). The data have shown a multitude of interactions between the flavin and the apo-protein, and have suggested mechanisms by which the apoprotein might modulate the redox properties of the flavin.

Sulfate-reducing bacteria contain many redox proteins, and flavodoxins have been isolated from six strains and their amino acid sequences have been determined (11–15). Much is known about many redox proteins of *D. vulgaris* (Miyazaki F) (16, 17), but little has been reported on the flavodoxin from this strain, and even its primary structure has not been determined yet (18). On the other hand, another flavoprotein, an FMN-binding protein, has been isolated only from this strain, and it has been found to consist of 122 amino acids (19). The semiquinone state of the FMN-binding protein is unstable, and the redox potential between the oxidized state and the fully reduced state has been estimated to be  $-325$  mV. So, the FMN-binding protein appears to be different from other flavodoxins from

sulfate-reducing bacteria in terms of redox properties, but it is still unknown whether or not the FMN-binding protein is the flavodoxin of *D. vulgaris* (Miyazaki F).

We have also carried out a series of genetic studies on the redox proteins of *D. vulgaris* (Miyazaki F) and the previously cloned cytochrome  $c_3$  gene (20), the FMN-binding protein gene (19), the cytochrome  $c$ -553 and cytochrome  $c$  oxidase-like protein genes (21), and the rubredoxin and desulfoferrodoxin genes (22). In the present paper, we report the molecular cloning and sequencing of the gene encoding the flavodoxin of *D. vulgaris* (Miyazaki F), its overproduction in *Escherichia coli*, and some of its characteristics as a holoprotein. A preliminary account of this work was presented previously (23).

#### EXPERIMENTAL PROCEDURES

**Bacterial Strains and Materials**—*E. coli* strain JM109, *recA* 1,  $\Delta$ (*lac-proAB*), *end A* 1, *gyrA96*, *thi*-1, *hadR* 17, *relA* 1, *supE* 44[F'*traD36*, *proAB*<sup>+</sup>, *lacI*<sup>a</sup>  $\Delta$ M15], was used for cloning, and strain BL21(DE3), F<sup>-</sup>, *ompT* [*lon*]-*hsdS<sub>B</sub>* (rB<sup>-</sup>mB<sup>-</sup>; an *E. coli* B strain) with DE3, a  $\lambda$  prophage carrying the T7 RNA polymerase gene, was used for expression of the flavodoxin gene. *D. vulgaris* (Miyazaki F) was grown (24) and used for DNA isolation. Restriction endonucleases and DNA-modifying enzymes were purchased from Nippon Gene and Takara Shuzo, respectively. [ $\gamma$ -<sup>32</sup>P]ATP (185 TBq/mmol) was obtained from ICN. All other chemicals were of analytical grade for biochemical use.

**Cloning and Sequencing**—Genomic DNA isolated from *D. vulgaris* (Miyazaki F) was prepared by the method of Saito and Miura (25). We performed PCR using genomic DNA as the template. Two primers were designed for this reaction according to the conserved regions of the published amino acid sequences of flavodoxins from other sulfate-reducing bacteria because the amino acid sequence of that from *D. vulgaris* (Miyazaki F) was unknown (see Fig. 3). The nucleotide sequences were as follows: primer-1 was a 23 mer, and its nucleotide sequence was 5'-GGCTCCACC-ACCGGCAACACGGA-3', corresponding to the amino acid sequence of <sup>9</sup>G-S-T-T-G-N-T-E. Primer-2 was a 20 mer, and its nucleotide sequence was 5'-GTCCACCGCGCCG-AGAAGT-3', which is the complementary sequence of the corresponding amino acid sequence of <sup>100</sup>Y-F-C-G-A-V-D. These sequences were based on the codon usage of previously known genes in the same strain. The PCR mixture was phosphorylated with T4 polynucleotide kinase and then ligated with pUC18 previously cut with *Sma*I. We extracted the plasmids from several *E. coli* JM109 cells transformed with this reaction mixture and determined the nucleotide sequence of the inserted DNA. One of the nucleotide-derived amino acid sequences was similar to the amino acid sequence of another flavodoxin from sulfate-reducing bacteria. We then synthesized a 30-mer probe DNA, with the sequence of 5'-GTGTAGCTGCTGTCGCCGAG-CCGAAGCAG-3', which is the complementary sequence of the corresponding amino acid sequence of <sup>89</sup>A-C-F-G-C-G-D-S-S-Y-T. We carried out Southern hybridization with this labeled oligonucleotide at 65°C and detected a band hybridizing to a ca. 1.6-kilobase pair *Sal*I-*Eco*RI fragment by use of a BAS1000, FUJIX (data not shown). So we digested the genomic DNA with *Sal*I and *Eco*RI, and the

digest was separated into several fractions on an agarose gel according to size. The separated fragments were ligated into the *Sal*I-*Eco*RI site of pUC18, and *E. coli* JM109 was transformed with the resulting ligation mixture. One such transformant was found by means of the colony hybridization method to harbor a plasmid carrying the ca. 1.6-kbp *Sal*I-*Eco*RI fragment of *D. vulgaris* (Miyazaki F) DNA, which was named pSE1600. The nucleotide sequence of the inserted fragment was determined by sequencing of its restriction fragments which were cloned into the multicloning site of pUC18, and a deletion mutant was obtained by use of exonuclease III and Mung Bean nuclease. We used the dideoxy chain termination method (26), for which we used a DNA sequencer (A.L.F. II; Pharmacia LKB).

**High-Level Expression of Flavodoxin**—We attempted to construct a high level expression system in *E. coli* using pUT7. Expression vector pUT7 has a T7 promoter and a high copy number (27). Plasmid pUT7 was digested with *Bam*HI, and then blunt-ended and digested with *Nco*I. Another plasmid, pSE1600, was digested with *Eco*RI and blunt-ended and then cut with *Nco*I, because *Nco*I consists of a region of the initiation codon of the flavodoxin gene and *Eco*RI downstream of the gene (see Fig. 1). The resultant plasmid was designated as pUTFLV.

**Site-Directed Mutagenesis**—Position 16 of this flavodoxin is Ala, whereas Glu is conserved at position 16 in all other flavodoxins from sulfate-reducing bacteria. It is located near the FMN-binding region, so it is thought to be important in the binding of FMN. Site-directed mutagenesis was carried out using the modified method of Inouye (28), and we used an 18-mer deoxyoligonucleotide, of which the nucleotide sequence was 5'-CAACACCGAATGGGTCCG-3'. We obtained mutant plasmid pSE1700, and its insertion was ligated to pUT7 by the same procedure as used for pUTFLV. Finally, mutant plasmid pUTFLV2 was obtained, which encoded the A16E mutant flavodoxin.

**Purification of the Expressed Protein**—*E. coli* BL21-(DE3) cells transformed with pUTFLV were cultured for 9 h at 37°C in 1.7 ml LB containing 50  $\mu$ g/ml ampicillin. Eight flasks containing 250 ml of this medium were incubated with 1.7 ml of the culture overnight with shaking at 37°C. Cells were harvested by centrifugation at 6,000 rpm for 15 min. The cell pellet was suspended in 10 mM Tris-HCl (pH 8.0) and sonicated using a Model 201M sonicator (KUBOTA) at 9,000 Hz and 200 W for 10 min. After the suspension had been centrifuged at 35,000 rpm for 2 h at 4°C, the gray supernatant was dialyzed against distilled water overnight at 4°C, and the resulting yellow solution was loaded onto a DEAE-cellulose (DE52) column (2.0  $\times$  9.0 cm) equilibrated with 10 mM Tris-HCl (pH 8.0). The column was washed with 100 ml of the buffer and then developed with a gradient of 0 to 300 mM NaCl in 10 mM Tris-HCl (pH 8.0). Flavodoxin-containing fractions were identified from their optical spectra. The colored fractions were collected and diluted twice, and then loaded onto the DE52 column again under the same conditions. The eluted colored solution ( $A_{268}:A_{448} < 4.5$ ) was dialyzed against distilled water and then lyophilized. Gel-filtration on a Superose HR-12 column (1.0  $\times$  30.0 cm) was carried out using a Pharmacia FPLC system. The eluent was 200 mM NaCl/10 mM Tris-HCl (pH 8.0) and the flow rate was 0.4 ml/min. The purified recombinant flavodoxin was then eluted for 35 min. SDS-PAGE was carried out according to

the method of Laemmli (29) with a gel concentration of 15%.

**Spectroscopic Analysis and N-Terminal Amino Acid Sequencing**—Absorption spectra were recorded at room temperature with a Hitachi 320 spectrophotometer. For the amino acid sequence analysis, we carried out an additional separation. The protein purified by gel filtration was dissolved in distilled water, and then subjected to reverse phase HPLC (RESOURCE-RPC: Pharmacia, 3 ml) with a linear gradient of acetonitrile in 1% TFA at the flow rate of 2 ml/min using a Shimadzu HPLC system. The purified peptide was then analyzed using an Applied Biosystems model 476A protein sequenator.

**Determination of Oxidation/Reduction Potentials and In Vitro Activity**—The redox potentials for the flavodoxin semiquinone/fully reduced flavodoxin ( $E_1$ ) and oxidized flavodoxin/flavodoxin semiquinone ( $E_2$ ) couple were determined by means of equilibrium reactions and spectrophotometric measurements (30) with mixtures of flavodoxin, methyl viologen and 2-hydroxy-1,4-naphthoquinone, respectively. The redox potential,  $E_n$ , for the system at equilibrium was calculated with the Nernst equation,

$$E_n = E_{m,7}(\text{dye}) + \frac{RT}{nF} \ln \frac{[\text{oxidized dye}]}{[\text{reduced dye}]}$$

where  $R$  is the gas constant,  $T$  the absolute temperature,  $F$  the Faraday, and  $n$  the number of electrochemical equivalents. A solution of flavodoxin and a dye in 50 mM sodium phosphate buffer, pH 7, and 3 mM EDTA in a closed all-glass cuvette was made anaerobic by repeated cycles of evacuation and flushing with oxygen-free nitrogen. For the determination of  $E_1$ , we monitored the absorbance at 600 and 730 nm. Methyl viologen in the oxidized state absorbs at neither 600 nor 730 nm, where flavodoxin in the fully reduced state, and the semiquinone state does not absorb at 730 nm.  $\epsilon_{600}$  of flavodoxin in the semiquinone state and the fully reduced state used was at 4,870 and 900  $\text{M}^{-1} \cdot \text{cm}^{-1}$ , respectively. For the determination of  $E_2$ , preliminary experiments showed an isosbestic point between the oxidized and semiquinone states at 506 nm ( $\epsilon_{506} = 3,160 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ). Dye reduction was measured at 620 nm ( $\epsilon_{620} = 4,020 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ), where neither the oxidized flavodoxin nor the dye absorb. For the determination of  $E_1$ , an amount of a dithionite solution was added to the dye along with the flavodoxin in the semiquinone state, all of which were photo-reduced using a 150 W slide projector (31). For  $E_2$ , the dye and oxidized flavodoxin solution mixture was photo-reduced by the same procedure as for  $E_1$ , and the reduction of the dye and the flavodoxin was determined after each period of photo irradiation.

The reduction for the  $\text{H}_2$  assay was carried out under anaerobic conditions. The reaction mixture, with a total volume of 1 ml, contained hydrogenase, cytochrome  $c_3$  and 20  $\mu\text{M}$  flavodoxin in 30 mM phosphate buffer (pH 7.0).

**Redox Behavior on Bare and Phospholipid-Modified Graphite Electrodes**—We measured the redox behavior on a bare basal-plane pyrolytic graphite (BPG) or an edge-plane pyrolytic graphite (EPG) electrode. Voltammetric measurements were conducted using a cell with a three-electrode configuration under a nitrogen atmosphere at  $22 \pm 2^\circ\text{C}$  in 30 mM phosphate buffer (pH 7.3). An Ag/AgCl electrode in a saturated KCl solution was used as the reference electrode, although all of the potentials cited in

the present paper were referenced to a normal hydrogen electrode. The procedure was that described in detail previously (19) with slight modification. As demonstrated in our previous paper (22), rubredoxin exhibited a fair redox response on a phospholipid-modified graphite electrode. It is expected that a phospholipid film provides a suitable reaction environment for electrode reactions of electron transfer proteins, probably due to the ability of the film to prevent structural changes of the proteins. Thus, the electrochemistry of flavodoxin on a phospholipid-modified BPG electrode was examined. To modify the BPG electrode surface with a phospholipid, L- $\alpha$ -phosphatidylcholine dimyristoyl (DMPC) was used. The procedures will be described in detail in a future paper.

**Identification of the Prosthetic Group, and Determination of Dissociation Constants for the Complexes of Apoflavodoxin with FMN and Riboflavin**—The peptide portion was removed by resolving the purified protein in ice-cold 5% trichloroacetic acid and 0.3 mM EDTA in 0.1 M phosphate buffer (pH 7.0), and centrifugation at 12,000 rpm and  $4^\circ\text{C}$  for 10 min. The solution containing a prosthetic group was then neutralized using diethylether and sodium bicarbonate. To identify the prosthetic group, this solution was loaded onto an HPLC C-8 column (Hiber LiChrosorb RP-8, Cica-MERCK). The eluent was 10% acetonitrile in 0.1% trifluoroacetic acid, and the flow rate was 2 ml/min. This purified FMN solution was also used for the determination of dissociation constants. The dissociation constants ( $K_d$ ) for the binding of FMN and riboflavin to apoflavodoxin were determined by fluorescence titration (32). Microliter quantities of apoflavodoxin were added to 3 ml aliquots of a 0.1  $\mu\text{M}$  flavin solution in 50 mM potassium phosphate (pH 7.0) and 5 mM EDTA at  $25^\circ\text{C}$ . Quenching of the flavin fluorescence occurred within several minutes. The proportion of bound flavin was determined at equilibrium by measuring the extent of quenching.

**Immunoblot Analysis**—To compare the higher structure of the recombinant flavodoxin to that of the FMN-binding protein from the same strain, immunoblot analysis was carried out. Samples were loaded onto a 15% polyacrylamide SDS minislab gel. Blotting onto a nitrocellulose filter was performed electrophoretically, followed by blocking by shaking in 3% (w/v) bovine serum albumin in buffer A containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.5% Tween 20 for 30 min. After a wash with buffer A, the blot was incubated with a 1:1,000 dilution of polyclonal antiserum, raised against the holo-FMN-binding protein purified from *E. coli*, in buffer A for 30 min (19). The blot was then thoroughly washed with buffer A and incubated with 1  $\mu\text{l}$  of alkaline phosphatase-conjugated goat anti-rabbit IgG in buffer A. Following two washes, first with buffer A and then with alkaline phosphatase buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM  $\text{MgCl}_2$ ], the nitrocellulose filter was incubated with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate in 5 ml of alkaline phosphatase buffer. The blot was finally washed with water and dried.

## RESULTS

**Cloning and Nucleotide Sequencing of the Flavodoxin Gene**—The partial restriction map and sequencing strategy used for the determination of the nucleotide sequence of



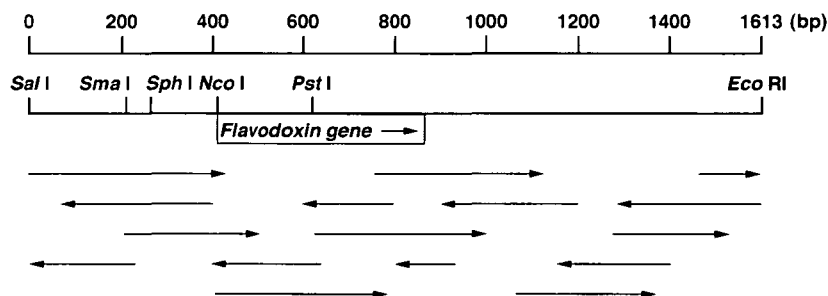
*SalI-EcoRI* are shown in Fig. 1, and the determined nucleotide sequence containing the entire flavodoxin gene is shown in Fig. 2. The open reading frame (ORF) encoding flavodoxin is composed 148 amino acids, including an amino-terminal Met, and is preceded by putative ribosome-binding site (AGGA), which has been observed to comprise nucleotides 404-407 of the *SalI-EcoRI* fragment. The amino acid sequence of the flavodoxin from *D. vulgaris* (Miyazaki F) is homologous to those from other sulfate reducing bacteria, especially that from *D. vulgaris* (Hildenborough), the identity being 67%. However, position 16, which is near the FMN-binding region and located on the surface of the molecule, is Ala, while position 16 in all other flavodoxins from sulfate-reducing bacteria is occupied by Glu (Fig. 3). No other protein gene was found in this cloned fragment.

**Purification of the Recombinant Flavodoxin**—We purified the recombinant flavodoxin from a cell lysate of *E. coli* BL21(DE3) harboring pUTFLV. Through chromatographic steps on DE52 and Superose HR12, a large amount (25 mg/g, wet cell) of flavodoxin was purified to homogeneity on SDS-PAGE. The molecular weight in the denatured state

was determined to be approximately 19,000 by SDS-PAGE (Fig. 4), which was rather different from the calculated value (15,600) based on the amino acid sequence deduced from the nucleotide sequence. The result of amino acid sequencing of the amino terminus was Ala-Asn-Val-Leu-Ile-Val-Tyr-Gly-Ser-Thr, and Met was not detected. Therefore, the molecular mass of the recombinant flavodoxin appears to be 15,900, which includes one molecule of FMN.

**Identification of the Prosthetic Group**—To identify the prosthetic group, bound to the recombinant flavodoxin, the UV-visible spectrum of the purified holoprotein was measured, as shown in Fig. 5. In the visible region, absorption maxima were observed at 377 and 448 nm, which is characteristic of proteins that bind to flavin derivatives. The prosthetic group extracted with trichloroacetic acid was subjected to reverse phase HPLC on a C-8 column, and the retention time of the obtained prosthetic group was compared with those of the flavin derivatives. As shown in Fig. 6, the retention time of the prosthetic group released from the recombinant flavodoxin was exactly the same as that of FMN. The  $A_{448}:A_{268}$  ratio of the holoprotein was

Fig. 1. Partial restriction map and sequencing strategy for the cloned 1.6-kbp *SalI-EcoRI* fragment. The open reading frame is shown within the box. The arrows indicate the region sequenced on each DNA strand for the restriction fragment cloned into the multicloning site of pUC18, and the deletion mutants obtained on treatment with exonuclease III and mung bean nuclease. Some restriction enzyme sites are also indicated.



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          90      100      110      120      130      140      150      160
CTTTTTTCTTGAAGGATTTTCATTTTCATTACGGTTCCTCAACGATCAACCTCAACGACGCCCCCATGCCACA
-35 region      -10 region
          170      180      190      200      210      220      230      240
TCACTCCACGTATCCAGCCTCCGGCCTTCCCGTCCGTCCTGCAACCCGGGGCGGACGGGGAACGCCACCC
          250      260      270      280      290      300      310
CTGCACGCATGCGGCACGTTCCGCGAGTTGCGCGCATCCCCCTGCTCACCTGCCTATTGGGCCATTGGGTCCACCC
          330      340      350      360      370      380      390      400
ACCAGGGGAACGGTGTAGTCGGGCGCCTGACGGGTGGCCCACTCGAACACCGACACGCTTCACAGACACCATCCACC
          410      420      430      440      450      460      470      480
CGGAGGATTCCCATGGCCAACGTGCTCATCGTCTACGGTTCACCACCGGCAACACCGCCTGGGTGCGCGAAACCGTCGG
SD sequence M A N V L I V Y G S T T G N T A W V A E T V G
          490      500      510      520      530      540      550      560
TCGCGACATCGCGGAAGCGGGCCACAGCGTTGAAATCCGCGACGCGGGCCAGGTAGAGGCCGAAGGACTGTGCGAAGGCC
R D I A E A G H S V E I R D A G Q V E A E G L C E G R
          570      580      590      600      610      620      630      640
GCGACCTCGTGTTCGGCTGCCACCTGGGGCGACGAAATCGAATGCAAGTGCAGGACGACTTCATCCACCTGTACGAA
D L V L F G C S T W G D D E I E L Q D D F I H L Y E
          650      660      670      680      690      700      710      720
TCGCTGGAAGCCACGGGCGGGCAAGGGCCGGCCCTGCTTCGGCTGCGGCGACAGCAGTACACCTACTTCTCGGG
S L E A T G A G K G R A A C F G C G D S S Y T Y F C G
          730      740      750      760      770      780      790      800
CGCGGTGGATGCCATCGAAGAGCCCTGTCCGGCCTTGGCGGGACATCGTGGCAGATTGCTGAAAATCGACGGCGATC
A V D A I E E R L S G L G A D I V A D S L K I D G D P
          810      820      830      840      850      860      870      880
CCCGCACCATGCGCGACGATGTTCCGCTGGGGCGGGCGCGTGGTGACCGCCCTGTAGGGCTGCCCGATTGCGACACC
R T M R D D V S A W A G R V V T A L *

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Fig. 2. Partial nucleotide sequence of the cloned 1.6-kbp fragment. The amino acid sequence deduced from the possible open reading frame is shown with one-letter abbreviations. The putative ribosome-binding site, the -35 and -10 regions are indicated.

	* ! *## ** ****! *	# #	# * * * ## ** *
1.	MANVLIVYGS TTGNTAWVAE TVGRDIAEAG HSVEIRDAGO VEAEGLCEGR DLVLFGCSTW		
2.	MPKALIVYGS TTGNTTEYTAE TIARELADAG YEVDSDRAAS VEAGGLFEGF DLVLLGCSTW		
3.	MSKSLIVYGS TTGNTETAEE YVAEAFENKE IDVELKNVTD VSVADLNGNY DIVLFGCSTW		
4.	MSKVLIVPGS STGNTESIAQ KLEELIAAGG HEVTLNNAAD ASAENLADGY DAVLFGCSAW		
5.	MPKALIVYGS TTGNTTEGVAE AIAKTLNSEG METTVVNVAD VTAPGLAEGY DVVLLGCSTW		
6.	MGKALVVFGS TTGNTETVAE VVAKVLEESG MAVDLKNATK VKAAGLAEGY DLVVFGCSTW		
7.	MSKVLILFGS STGNTESIAQ KLEELVAAGG HEVTLNNAE ASADNLADGY DAVLMGCSTW		
	* ** * * * # ! * * * #**** #### #**		
1.	<sup>61</sup> GDDEIELQDD FIHLYESLEA TGAGKGRAAC FCGDSSYTY FCGAVDAIEE RLSGLGADIV		
2.	GDDSIELQDD FIPLFDSLEE TGAQGRKAC FCGDSSYTY FCGAVDAIEE KLKNLGAEIV		
3.	GEEIEELQDD FIPLYDSLEN ADLKGKQVSV FCGDSDYTY FCGAVDAIEE KLEKMGAVVI		
4.	GMEDELMQDD FLSLFEFNR IGLAGRKVAE FASGDQYEH FCGAVPAIEE RAKELGATII		
5.	GDDEIELQED FVPLYEDLDR AGLKDKKQV FCGDSSYTY FCGAVDVIEK KAEELGATLV		
6.	GDDEIELQED FIPLYDDLGA AGLGGRKQV FCGDSSYTH FCGAVDAIAE KAASLGAKVI		
7.	GMEDELMQDD FAPLFDEMEN MGLKGKQLAA FASGDMEYEH YCGAVPAIEE KAYLGLAEVI		
	*## * # #		
1.	<sup>121</sup> ADSLKIDGDP RTMRDDVSAW AGRVVTAL		
2.	QDGLRIDGDP RAARDDIVGW AHDVRGAI		
3.	GDSLKIDGDP --ERDEIVGW GSGIADKI		
4.	AEGLKMEGDA SNDPEAVASF AEDVLKNL		
5.	ASSLKIDGEP --DSAEVLDW AREVLRV		
6.	DLPLKIDGAP --DTAEARDW AKEVLRSA		
7.	PEGLKIEGDA SSDPDAVSFA AEDVLK		

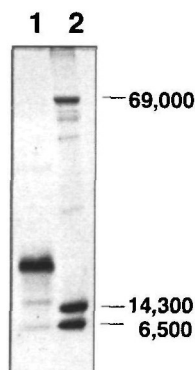


Fig. 4. SDS-PAGE of the purified recombinant flavodoxin. Lane 1, recombinant flavodoxin; lane 2, molecular mass marker mixture.

estimated to be 0.234, so it should be proper that flavodoxin expressed in *E. coli* as a holoprotein binds to FMN as a prosthetic group in a molar ratio of 1.

**Electrochemical and FMN-Binding Properties of the Flavodoxin and Its Mutant (A16E)**—We confirmed that the recombinant flavodoxin was reduced by  $H_2$  with hydrogenase and cytochrome  $c_3$  *in vitro*. The redox potentials and dissociation constants with FMN, and the riboflavin of the recombinant and mutant flavodoxin are summarized in Table I. These data indicate that these flavodoxins have basically the same characters as to prosthetic group-binding and redox potentials, and are almost the same as that from *D. vulgaris* (Hildenborough) (30).

**Redox Behavior with a Graphite Electrode of the Wild-Type Flavodoxin**—CV revealed a pair of anodic and cathodic peaks using either a BPG or EPG electrode at all the pH values, although the response was much smaller than with the flavodoxin-adsorbed EPG electrode. The formal potential of flavodoxin ( $E^{\circ}$ ) at pH 7.3 was determined to be  $-477 \pm 3$  mV from the midpoint potential between the anodic and cathodic peak potentials as well as the peak potentials of a differential-pulse voltammogram (DPV) and

Fig. 3. Comparison of the amino acid sequences of flavodoxins from *Desulfotribrio*. Lane 1, *D. vulgaris* (Miyazaki F) (this work); lane 2, *D. vulgaris* (Hildenborough) (36, 37); lane 3, *D. salexigens* (12); lane 4, *D. desulfuricans* [Essex 6] (13); lane 5, *D. gigas* (ATCC19364) (14); lane 6, *D. gigas* (ATCC-29494) (14); lane 7, *D. desulfuricans* (ATCC-27774) (15). \* means all identical; # means identical except for one strain; ! means identical except for *D. vulgaris* (Miyazaki F); - indicates a gap. The amino acid sequences of the flavodoxins from *D. vulgaris* (Miyazaki F) and *D. vulgaris* (Hildenborough) are 66.9% identical to each other.

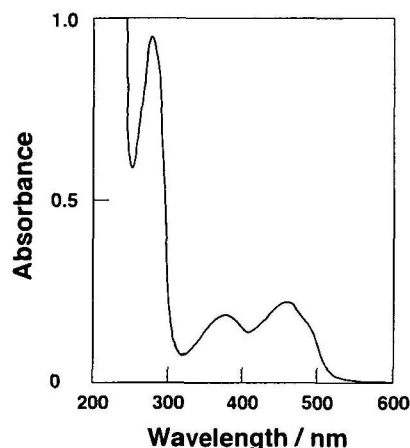


Fig. 5. Ultraviolet and visible spectra of the purified flavodoxin expressed in *E. coli* transformed with pUTFLV. The absorption spectrum was recorded at room temperature using a Hitachi 320 spectrophotometer. The spectrum represents the recombinant oxidized flavodoxin of *D. vulgaris* (Miyazaki F), and is identical to that of that from *D. vulgaris* (Hildenborough) (37).

an ac voltammogram. The plot of  $E^{\circ}$  as a function of pH could be fitted to three straight lines (23). The slopes were  $-58$  mV/pH at  $pH < 8.5$  and  $pH > 10$ , and  $-29$  mV/pH at  $8.5 < pH < 10$ . Therefore, the redox reaction of flavodoxin adsorbed on the EPG electrode is a two-proton transfer process at  $pH < 8.5$  and  $pH > 10$ , while it is a two-electron one-proton transfer process at  $8.5 < pH < 10$ . Extrapolation to pH 7.0 gave  $E_{m,7}(EPG) = -435$  mV, which is almost same as the  $E_1$  of flavodoxin in the solution phase.

**Electrochemistry of the Wild-Type Flavodoxin on a Phospholipid-Modified BPG Electrode**—CV showed two pairs of anodic and cathodic peaks. The peak charge of the CV wave of the pc1-pa1 couple was approximately 1/5 that of the CV wave of the pc2-pa2 couple. From the  $v$ -dependence of the peak potentials, the  $E^{\circ}$  obtained for the two pairs from the CV responses were  $E^{\circ}_{p1} = -318$  mV and  $E^{\circ}_{p2} = -136$  mV. Both  $E^{\circ}_{p1}$  and  $E^{\circ}_{p2}$  were more positive than  $E_{m,7}(EPG)$ . The plot of the peak potentials against pH in the range of  $5.2 < pH < 9.2$  was a straight line with a slope of  $-59$  mV/pH, indicating that the redox reaction is a two-electron two-proton process.

**Immunoblot Analysis**—The recombinant flavodoxin reacted with a polyclonal antibody against the FMN-binding



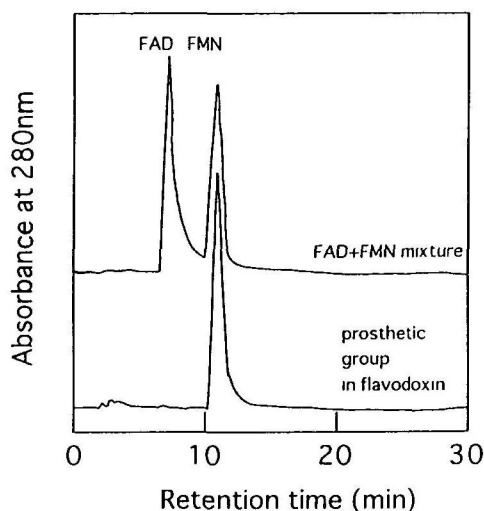


Fig. 6. Identification of the prosthetic group by HPLC. The extracted prosthetic group was subjected to reverse-phase HPLC on a C8 column (Hiber LiChrosorb RP-8) with 10% acetonitrile in 0.1% trichloroacetic acid at the flow rate of 2 ml/min.

TABLE I. The dissociation constants of apoprotein with FMN and riboflavin, and the redox potentials of the recombinant and mutant (A16E) flavodoxins.  $K_d$  stands for the dissociation constant, and  $E_1$  and  $E_2$  stand for the redox potentials between hydroquinone and semiquinone, and semiquinone and the fully oxidized state, respectively.

		Wild type	A16E
$K_d$ with FMN	(nM)	0.38	0.36
$K_d$ with riboflavin	(mM)	2.13	2.16
$E_1$	(mV)	-434	-425
$E_2$	(mV)	-151	-136

protein 100 times less than the recombinant FMN-binding protein (see Fig. 7). This suggests that the higher structures of the flavodoxin and FMN-binding protein are not similar enough for the FMN-binding protein antibody to crossreact.

#### DISCUSSION

In this study, we isolated the gene encoding a flavodoxin from *D. vulgaris* (Miyazaki F) by means of PCR. This is the first report of determination of the amino acid sequence of the flavodoxin from *D. vulgaris* (Miyazaki F). Our results indicate that the sulfate-reducing bacteria commonly contain flavodoxin with highly homologous amino acid sequences, and that they have some conserved sequences around the FMN-binding region. However, the redox and FMN-binding properties of the A16E mutant indicated that the conserved Glu-16 residue is not particularly important, although it is located near the FMN-binding region and is on the surface of the molecule. The A16E mutant may interact with hydrogenase in a different manner from the wild type. Recently, the electrostatic effects of surface Glu or Asp residues on redox potentials of the flavodoxin from *D. vulgaris* (Hildenborough) were reported, and the redox potential between semiquinone and hydroquinone was correlated with the number of acid-to-amide substitutions (32). However, the redox properties (both  $E_1$  and  $E_2$ ) of the

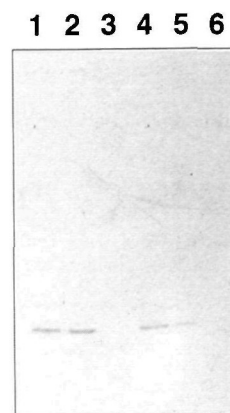


Fig. 7. Immunoblot analysis of the recombinant flavodoxin and FMN-binding protein. Lane 1, purified FMN-binding protein, 8 pmol; lane 2, purified FMN-binding protein, 4 pmol; lane 3, purified FMN-binding protein, 0.8 pmol; lane 4, purified flavodoxin, 800 pmol; lane 5, purified flavodoxin, 400 pmol; lane 6, purified flavodoxin, 80 pmol.

A16E mutant appear to be similar to those of the wild-type flavodoxin. The relationship between the number of acidic amino acid residues and the redox properties may not be so simple.

It is known that free FMN adsorbed on an EPG electrode exhibits two pairs of anodic and cathodic peaks under the same conditions (Sagara, T. and Takaki, S., unpublished data). Therefore, the observed voltammetric response with a bare graphite electrode could be attributable to the redox response of FMN bound to the peptide, *i.e.* flavodoxin. Taken together with the appearance of the single peak in the voltammograms, the semiquinone state of the flavodoxin adsorbed on a bare EPG electrode appears to be unstable, as opposed to the flavodoxin in the solution phase. In the case of free FMN, CV with a phospholipid-modified BPG electrode gives rise to a sigmoidal-shaped wave, even at a low  $v$  (Sagara, T., Takaki, S., Honda, M., Ezoe, K., and Nakashima, N., unpublished data). Because the CV wave of flavodoxin on the phospholipid-modified BPG electrode at a low  $v$  was peak-shaped, the CV response obtained was not due to free FMN released from flavodoxin. Although two pairs of CV responses were observed, it would not be correct to equate  $E'_{p1}$  and  $E'_{p2}$  to  $E_1$  and  $E_2$ , respectively. The appearance of the two pairs may rather indicate that two states of flavodoxin coexisted in the phospholipid film. It is note worthy that the redox activity of the flavodoxin adsorbed on the EPG electrode was stable for a week, although a slight decrease in the peak current over time was observed. Therefore, it may be promising for electro-engineering as a bio-tip when recombinant DNA techniques are being used.

All flavodoxins have a typical structure, that is, a five-stranded parallel  $\beta$ -sheet flanked on either side by a pair of  $\alpha$ -helices. FMN lies mostly below the surface of the molecule, and the isoalloxazine ring of FMN appears to be planar, and is buried between two segments of the polypeptide chain and the side groups, Trp-60 and Tyr-98 (7). Tyr-98 is especially important in modulating the redox potential by means of site-directed mutagenesis (33). These two amino acids are also conserved in the flavodoxin

from *D. vulgaris* (Miyazaki F), which is thought to have a structure similar to those of other flavodoxins. However, immunoblot analysis clearly indicated that flavodoxin is a different molecule from the FMN-binding protein in the higher structure, although they both have a similar amino acid sequence region (Thr-Trp-Asn or Gly). Gly-61 of flavodoxin has been reported to be important in stabilizing the semiquinone state (34), while the Trp residue in this region is important for FMN-binding in the case of the FMN-binding protein ( $K_d$  with FMN of the wild-type FMN-binding protein is 0.43 nM, unpublished data). We are now studying the structures of the FMN-binding protein and flavodoxin in detail by NMR, and preliminary data have indicated that they are not similar to each other in the interaction between FMN and the peptide chain (35). Although both the FMN-binding protein and flavodoxin have almost the same molecular weight and bind to FMN with almost the same dissociation constant from the same strain of *D. vulgaris*, the FMN-binding protein is a different molecule from flavodoxin based on the amino acid sequence, higher structure, and redox properties.

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