

Purification and Properties of a *nif*-Specific Flavodoxin from the Photosynthetic Bacterium *Rhodobacter capsulatus*

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A flavodoxin was isolated from iron-sufficient, nitrogen-limited cultures of the photosynthetic bacterium *Rhodobacter capsulatus*. Its molecular properties, molecular weight, UV-visible absorption spectrum, and amino acid composition suggest that it is similar to the *nif*-specific flavodoxin, NifF, of *Klebsiella pneumoniae*. The results of immunoblotting showed that *R. capsulatus* flavodoxin is *nif* specific, since it is absent from ammonia-replete cultures and is not synthesized by the mutant strain J61, which lacks a *nif*-specific regulator (NifR1). Growth of cultures under iron-deficient conditions causes a small amount of flavodoxin to be synthesized under ammonia-replete conditions and increases its synthesis under N₂-fixing conditions, suggesting that its synthesis is under a dual system of control with respect to iron and fixed nitrogen availability. Here we show that flavodoxin, when supplemented with catalytic amounts of methyl viologen, is capable of efficiently reducing nitrogenase in an illuminated chloroplast system. Thus, this *nif*-specific flavodoxin is a potential *in vivo* electron carrier to nitrogenase; however, its role in the nitrogen fixation process remains to be established.

Nitrogenase (N₂ase), the key enzyme of biological nitrogen fixation, requires an anaerobic environment, Mg-ATP, and a low-potential reductant. Previously, both flavodoxins and ferredoxins, low-molecular-weight electron carriers, have been shown to be able to transfer electrons to nitrogenase *in vitro*. However, the physiological pathway of electron transfer to nitrogenase has been firmly demonstrated in only one diazotroph, *Klebsiella pneumoniae*, in which it has been established that the protein products of two genes, *nifF* (which encodes a flavodoxin) and *nifJ* (encoding pyruvate:flavodoxin oxidoreductase), constitute a specific electron transport system from pyruvate to nitrogenase (7, 14, 25, 33). The respective genes, which are located within the *nif* gene cluster, are expressed only under nitrogen-fixing conditions (3), and strains mutated in these genes are phenotypically Nif⁻ (26, 34). The electron pathways to nitrogenase in other organisms are much less clear. For example, in *Azotobacter vinelandii*, strains mutated both in a flavodoxin encoded by a gene homologous to *nifF* and ferredoxin I (encoded by *fdxA*) are nevertheless capable of diazotrophic growth (23).

The situation appears to be even more complex in the photosynthetic bacteria. In *Rhodobacter capsulatus*, two soluble ferredoxins (FdI and FdII) have been isolated from nitrogen-fixing cultures and shown to be capable of reducing nitrogenase *in vitro* (12, 38). FdI appears to be *nif* specific in that (i) it is absent under *nif*-repressing conditions and in a strain (J61) mutated in the *nif*-specific regulator *nifR1* (11, 38), (ii) its gene (*fdxN*) is located within one of the three clusters of *nif* genes (28, 30), (iii) the corresponding mRNA is only found under *nif*-derepressing conditions (30), and (iv) *lacZ*-*fdxN* fusions are derepressed 100-fold under N₂-fixing conditions (35). Thus, in *R. capsulatus* FdI is one candidate for the *in vivo* reductant of N₂ase. However, DNA sequence analyses within *nif* region I have identified other low-potential electron carriers that could potentially donate electrons to N₂ase, including

a 2Fe plant-type ferredoxin, FdIV (9, 27); another bacterial-type ferredoxin, FdIII (24) (distinct from FdI and FdII); and a putative flavodoxin (16). Here we describe the isolation and characterization of a flavodoxin distinct from that previously suggested from DNA sequence analysis. This flavodoxin is shown to be similar to NifF from *K. pneumoniae*, to be *nif* specific, and to be capable of efficiently reducing N₂ase *in vitro*. Therefore, the complexity of potential electron donors to N₂ase in *R. capsulatus* is increasingly large.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *R. capsulatus* used were SB1003, a spontaneous rifampin-resistant derivative of the wild-type strain, and the Nif⁻ strain J61 (37). Batch cultures were grown photoheterotrophically on defined RCV base medium (15), containing either 1.5 mM NH₄⁺ (N-limited conditions) or 15 mM NH₄⁺ (NH₄⁺ excess) or sparged with N₂ (10 ml/min). For iron-deficient conditions, RCV base medium, with iron omitted, was prepared with deionized water from a Millipore reverse osmosis water purification system (conductivity, <10⁻⁷ mho). For the large-scale purification of flavodoxin, the cells were grown in 80-liter batches under N-limited (1.5 mM NH₄⁺) conditions with excess iron, collected by flow centrifugation, and stored in liquid nitrogen.

Purification of flavodoxin. The initial purification steps (until preparative polyacrylamide gel electrophoresis [PAGE]) were carried out under anaerobic conditions for the simultaneous purification of nitrogenase components and ferredoxins essentially as previously described (13).

(i) Preparation of cell extract. The cells (~300 g [wet weight]) were thawed in 600 ml of 0.1 M Tris-HCl buffer (pH 7.4), sonicated five to seven times (1-min periods, 4°C, maximal power) with a Biosonik III (Bronwill Scientific, Rochester, N.Y.), and centrifuged (100,000 × g, 1 h, 10°C).

(ii) DEAE-cellulose chromatography. The supernatant was applied to a DEAE-cellulose column (DE52; 8 by 2.5 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.4, hereafter

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designated as buffer). After being washed with buffer, the bulk of the MoFe protein was eluted with 0.16 M NaCl in buffer, and then flavodoxin was eluted with 0.22 M NaCl in buffer together with Fe protein, the remaining MoFe protein, and FdI.

(iii) **Sephadex G-100 gel filtration.** Portions (20 ml) of the 0.22 M NaCl eluate were chromatographed on a Sephadex G-100 column (70 by 2.5 cm) equilibrated and developed with 0.4 M NaCl in buffer (0.5 ml/min). Flavodoxin-containing fractions, which eluted between Fe protein and FdI, were collected and then concentrated and desalted by ultrafiltration on an Amicon cell with a YM2 membrane.

(iv) **Preparative PAGE.** The concentrated G-100 fractions were further purified by native PAGE (15%). The yellow-greenish flavodoxin band was excised from the gel, eluted by incubation with two changes of buffer, and concentrated on a small DEAE-cellulose column with elution with 0.4 M NaCl in buffer.

(v) **Anion-exchange high-pressure liquid chromatography (HPLC).** The sample from the previous step was diluted three times with buffer and chromatographed on a Varian 5000 liquid chromatograph equipped with a Protein-Pak DEAE 5PW column (Waters) equilibrated with 0.1 M NaCl in buffer. The column was developed (flow rate, 1 ml/min) by a linear NaCl gradient (0.1 to 0.5 M) in buffer over 20 min. Elution was monitored at 450 nm, and fractions were checked by sodium dodecyl sulfate (SDS)-PAGE (15%) for homogeneity. Homogeneous fractions were combined, diluted three times with buffer, and concentrated on the same column by stepwise elution with 0.5 M NaCl in buffer. Purified flavodoxin was stored frozen at -80°C .

Analytical methods. Absorption spectra were recorded at 23°C on a Hewlett-Packard 8452A spectrophotometer. The molecular weight of flavodoxin was determined by SDS-PAGE, using the discontinuous system described by Laemmli (21). Protein bands were visualized by staining with Coomassie R-250. Pharmacia low-molecular-weight markers were used as standards. Amino acid analysis of *R. capsulatus* flavodoxin was performed at the Sheldon Biotechnology Center, McGill University, Montreal, Quebec, Canada. The activity of flavodoxin as an electron mediator was analyzed in a reconstituted system containing chloroplast fragments as a reductant and *R. capsulatus* nitrogenase (11). *R. capsulatus* nitrogenase proteins and FdI were isolated by the procedure of Hallenbeck et al. (12, 13). Protein concentrations were determined by the method of Bradford (4) by using bovine serum albumin as a standard.

Immunoblotting with rabbit anti-*R. capsulatus* flavodoxin or anti-*R. capsulatus* Fe protein was carried out essentially as described earlier (11). For flavodoxin quantitation, the membranes were developed with Amersham ECL chemiluminescence detection reagents (according to the manufacturer's protocol). The X-ray films were scanned, and the appropriate bands were quantitated by using a Molecular Dynamics personal densitometer and purified homogeneous flavodoxin as a standard. Antiserum against flavodoxin was raised by subcutaneous injections of homogeneous preparations in rabbits (8). Antiserum against *R. capsulatus* Fe protein was prepared by using bands excised from SDS-polyacrylamide gels (1). High-speed supernatants were used for the immunoblotting experiments and were prepared as follows. The cells from mid- to late-log-phase cultures were pelleted by centrifugation ($15,000 \times g$, 20 min, 4°C), resuspended in a small volume of 0.1 M Tris-HCl buffer (pH 7.4), and sonicated anaerobically three to five times (20-s periods, 4°C). After unbroken cells and cell debris were removed by centrifugation ($200,000 \times g$, 2 h, 4°C),

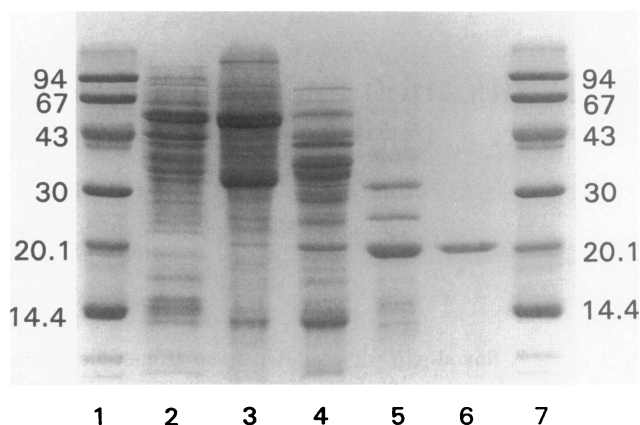


FIG. 1. SDS-PAGE analysis of various fractions during the purification of *R. capsulatus* flavodoxin. Proteins present in the different fractions were separated by SDS-PAGE (15% total acrylamide) and stained with Coomassie brilliant blue. Lanes: 1 and 7, molecular mass standards (sizes given in kilodaltons); 2, high-speed supernatant (34 μg of protein); 3, DEAE-cellulose chromatography, 0.22 M NaCl fraction (30 μg); 4, Sephadex G-100 gel filtration (15 μg); 5, preparative PAGE (4.5 μg); 6, HPLC anion-exchange chromatography (1.2 μg).

the resulting high-speed supernatant was frozen in liquid nitrogen until used for analysis.

RESULTS

Properties of *R. capsulatus* flavodoxin. The purification procedure given in Materials and Methods allowed the preparation of about 1 mg of homogeneous flavodoxin in five steps from 300 g of cells (Fig. 1). This represents a final yield of about 20%. The initial concentration of flavodoxin is low (see below), so it is not visible on Coomassie-stained gels during initial stages of purification (Fig. 1).

We have not estimated the fold purification at each step of the purification. However, it is evident from the protein profiles obtained by SDS-PAGE that preparative electrophoresis was highly effective. The remaining impurities were removed by anion-exchange HPLC, yielding essentially pure flavodoxin (Fig. 1). The advantage of the present procedure is that it also allows the simultaneous purification of nitrogenase components and FdI. The molecular weight of *R. capsulatus* flavodoxin was estimated from SDS gels to be 21,500, placing it in the high-molecular-weight group of flavodoxin. In the oxidized state (as isolated), the optical absorption spectrum exhibited maxima at 274, 370, and 450 nm, with a shoulder at 470 nm (Fig. 2). The absorbance ratio, A_{450}/A_{274} , for homogeneous flavodoxin ranged for various preparations from 0.23 to 0.26. The molar absorptivity coefficient, calculated on the basis of a molecular mass of 21.5 kDa, is $11.7 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 450 nm. Partial reduction of the *R. capsulatus* flavodoxin by the addition of sodium dithionite gave the blue semiquinone form that was characterized by bleaching of the 450-nm maximum and the appearance of a new broad maximum at 600 nm (Fig. 2). Further addition of dithionite produced the colorless, fully reduced form. The amino acid composition of *R. capsulatus* flavodoxin was determined (Table 1). It shows a predominance of acidic residues over basic ones, which corresponds to its ability to bind to DEAE-cellulose.

The ability of *R. capsulatus* flavodoxin to act as an electron carrier was assessed by using chloroplast fragments and *R. capsulatus* nitrogenase (Fig. 3). Nitrogenase activity in this

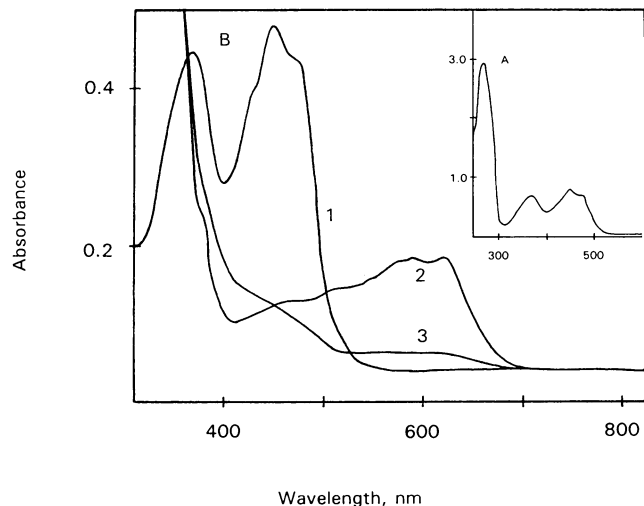


FIG. 2. UV-visible absorption spectra of homogeneous *R. capsulatus* flavodoxin (A) and its changes during dithionite titration (B). Lines: 1, oxidized (before addition of dithionite); 2, semiquinone; 3, fully reduced. Experiments were carried out in a quartz cuvette fitted with a rubber stopper and flushed with argon. The spectra are of 60 (A) and 40 (B) μM flavodoxin solution in 50 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl at room temperature.

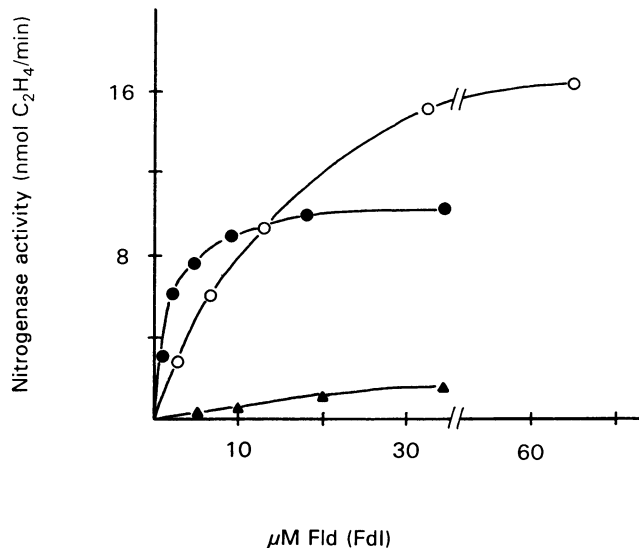


FIG. 3. Activity of *R. capsulatus* nitrogenase with flavodoxin and FdI reduced by illuminated chloroplasts. The reaction mixture (0.2-ml final volume) contained the following: sodium ascorbate, 8 mM; dichlorophenol indophenol, 50 μM; dichloromethyl urea, 20 μM; heated chloroplast fragments (24 μg of chlorophyll); buffer; an ATP-regenerating system; and nitrogenase (11). Flavodoxin (Fld) and FdI concentrations are as indicated. Symbols: ▲, flavodoxin (without methyl viologen); ●, flavodoxin (in the presence of 1 μM methyl viologen); ○, FdI (without methyl viologen).

system with flavodoxin as an electron mediator was significantly lower than that in the presence of FdI (Fig. 3) or FdII (not shown). However, addition of methyl viologen (1 μM) dramatically increased flavodoxin-supported nitrogenase activity with maximal activity only somewhat lower than that with FdI. (The optimal concentration of methyl viologen for this reaction has not been established.) Perhaps more importantly, at low concentrations (less than 10 μM), flavodoxin was more effective as an electron donor than FdI. Methyl viologen alone at 1 μM did not support nitrogenase activity and inhibited (30 to 40%) its activity in the presence of FdI and FdII (not

shown). Therefore, *R. capsulatus* flavodoxin resembles the analogous proteins from *K. pneumoniae* (40) and *A. vinelandii* (41) species in that (i) catalytic concentrations of methyl viologen greatly increase their effectiveness in mediating electron flow from illuminated chloroplasts to nitrogenase and (ii) maximal activity is lower than that in the presence of ferredoxin. Low concentrations of FdI (0.1 to 0.3 μM) also stimulated flavodoxin-supported nitrogenase activity, but this stimulation was appreciably lower than that obtained with methyl viologen (not shown).

TABLE 1. Amino acid composition of *R. capsulatus* flavodoxin

Amino acid	No. of residues
Asx.....	21
Thr.....	8
Ser.....	9
Glx.....	14
Pro.....	8
Gly.....	18
Ala.....	21
Cys.....	ND ^a
Val.....	9
Met.....	2
Ile.....	7
Leu.....	24
Tyr.....	3
Phe.....	16
His ^b	16
Lys.....	10
Arg ^b	16
Trp.....	ND
Total no. of residues.....	202

^a ND, not determined.

^b The values for His and Arg are probably high because of the comigration of unidentified substance in the sample, possibly derived from flavin mononucleotide.

Regulation of flavodoxin synthesis. We investigated several aspects of flavodoxin synthesis by using immunoblotting with antiserum directed against flavodoxin and high-speed supernatant extracts of cells grown photoheterotrophically under different conditions. The results were quantitated by using densitometry. Independently of growth conditions, immunoblotting showed that all the flavodoxin was recovered in the soluble fraction, unlike *K. pneumoniae*, for which, in one case, a portion of flavodoxin has been reported to be membrane associated (34). However, others have reported that all of the flavodoxin of *K. pneumoniae* is found in the soluble fraction (36). Under iron-sufficient conditions, the presence of flavodoxin coincided with that of the Fe protein of nitrogenase, i.e., it was *nif* specific. Thus, ammonia represses the synthesis of both proteins, and both proteins were highly expressed when cultures were grown under N₂-fixing conditions or N-limiting conditions (Fig. 4). Under N-limiting conditions, flavodoxin constituted 0.15% of the protein in the high-speed supernatant. In *K. pneumoniae* flavodoxin has been reported to be present as 0.5% of the total soluble protein (7). It is interesting to note that under N₂-fixing conditions, the total amount of Fe protein was twofold greater than under N-limiting conditions, whereas flavodoxin content actually decreased by 60%. In batch cultures, in vivo N₂ase activity under N₂-fixing conditions

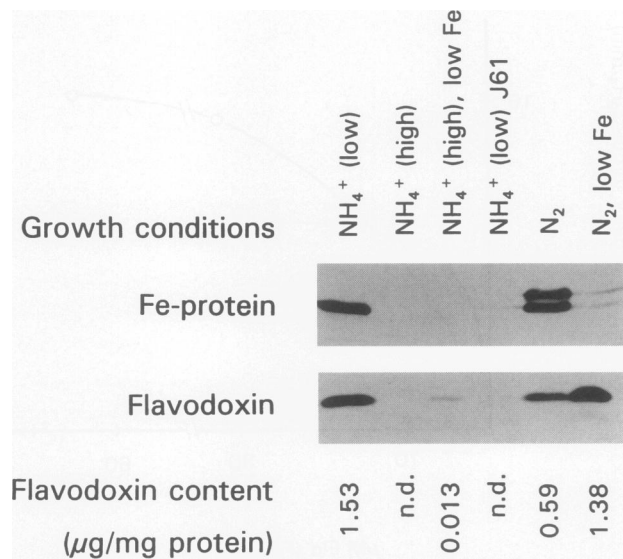


FIG. 4. Immunoblotting analysis of *R. capsulatus* (wild-type and strain J61) cells grown under various conditions. The cells were grown as indicated, and extracts were prepared as described in Materials and Methods. The proteins (18 µg) were separated by SDS-PAGE (15% total acrylamide), transferred to nitrocellulose membranes, and probed with antisera raised against *R. capsulatus* Fe protein or flavodoxin. Flavodoxin content in the samples was quantitated as described in Materials and Methods. The values with standard errors were 1.53 ± 0.18 , 0.013 ± 0.007 , 0.59 ± 0.03 , and 1.38 ± 0.2 µg/mg of protein. n.d., not detected.

is less than under N-limiting conditions (2, 39), and a substantial portion of the Fe protein appears to be modified under these conditions (Fig. 4). Thus, the amount of flavodoxin observed appears to be more closely related to N₂ase activity than to total Fe protein. Further evidence that the synthesis of flavodoxin (under iron-sufficient conditions) is *nif* specific was obtained by examining extracts of strain J61 grown under N-limiting conditions. This strain is mutated in the *nif*-specific regulatory gene *nifR1*, which is unable to derepress the synthesis of a variety of *nif* gene products (37), including Fe protein (Fig. 4) and FdI (11). Likewise, flavodoxin was not synthesized by this strain (Fig. 4).

In many organisms, the synthesis of flavodoxin is induced under iron deprivation conditions, when it presumably functionally replaces ferredoxin. We examined the effect of iron deficiency on flavodoxin synthesis under nitrogen-fixing conditions and in the presence of excess ammonia. Under nitrogen-fixing (iron-deficient) conditions, flavodoxin synthesis was increased more than twofold over iron-sufficient conditions. N₂-fixing cells were relatively sensitive to iron limitation, with no growth occurring after a second transfer to iron-deficient media. Accordingly, cultures subjected to iron deprivation contained four to five times less Fe protein. Thus, the increased synthesis of flavodoxin under these conditions might be directly caused by iron limitation or could be indirectly due to increased nitrogen limitation in cultures with decreased Fe protein content and decreased in vivo nitrogenase activity. Finally, ammonia-replete, iron-deficient cultures showed a low level of flavodoxin synthesis (0.013 µg/mg of protein) which was more than 100-fold lower than that observed under nitrogen-limiting, iron-sufficient conditions.

DISCUSSION

There has been only one previous report on the isolation of a flavodoxin from a photosynthetic bacterium, the purification and characterization of a flavodoxin from ammonia-grown cultures of *Rhodospirillum rubrum* (6). Although the flavodoxins from *R. rubrum* and *R. capsulatus* can both be placed in the group of high-molecular-weight flavodoxins, they differ in several respects. Their oxidized absorption spectra show significant differences in the position of long-wave-length maxima (450 nm for *R. capsulatus* and 460 nm for *R. rubrum*) which may reflect a slightly different arrangement of flavin mononucleotide in the protein. The analogous maxima for the *nif*-specific flavodoxins from *K. pneumoniae* and *A. vinelandii* are found at 454 nm (7, 20, 25). Previously, it was suggested that *R. capsulatus nif* region I contained a sequence encoding a flavodoxin (16). This has since been retracted (GenBank accession number X54054). Moreover, this sequence has subsequently been shown to be part of a larger open reading frame that corresponds to a flavin binding protein of undetermined function (18, 29). Thus, the gene encoding the flavodoxin isolated here is probably outside of this already-sequenced area. Definitive proof will be obtained by cloning and sequence analysis of the flavodoxin gene. This work is currently in progress and will also enable us to determine the similarities of this flavodoxin with others.

Nevertheless, the results of immunoblotting strongly suggest that the flavodoxin isolated here is a *nif* gene product. Its synthesis is repressed by excess ammonia and blocked in a mutant strain (J61) that lacks a functional *nifR1*, a specific regulator of *nif* genes. Similarly, excess ammonia suppresses the synthesis of the *nif*-specific flavodoxin of *A. vinelandii* (19, 20), and NifF of *K. pneumoniae* is absent in Nif⁻ strains (27, 34). In a variety of organisms, flavodoxin synthesis is regulated by the availability of iron, such that under conditions of iron deprivation, when it is thought to replace ferredoxin in cellular metabolic reactions, its synthesis is greatly increased. To our knowledge, the possible regulation of *nif*-specific flavodoxin synthesis with respect to available iron has never been tested. We quantitated the amount of *R. capsulatus* flavodoxin protein present in iron-deprived cultures under various conditions. Iron deprivation increased flavodoxin synthesis (twofold) in nitrogen-fixing cultures. However, it is not obvious if this is due to iron limitation itself or to an increased nitrogen limitation caused by a decrease observed here in active nitrogenase. However, iron-deficient, ammonia-replete cultures showed a low level of flavodoxin synthesis which was absent in iron-replete cultures. This suggests that flavodoxin synthesis is independently regulated by iron availability in addition to its regulation by fixed nitrogen. The molecular details of this regulation could be interesting and remain to be elucidated. Nevertheless, the results presented here clearly demonstrate that this flavodoxin is primarily regulated in response to the absence of fixed nitrogen.

Several pieces of evidence suggest that this flavodoxin serves to transfer electrons to N₂ase in vivo. First, it appears, in terms of its physicochemical properties, to be highly similar to the NifF of *K. pneumoniae*. Second, it was capable of efficiently reducing nitrogenase in vitro. High levels of flavodoxin-dependent nitrogenase activity in an illuminated chloroplast system required catalytic amounts of methyl viologen, as previously shown for *A. vinelandii* and *K. pneumoniae* flavodoxin (40, 41). As previously shown for this nonphysiological system (31, 42), maximal activity with flavodoxin was less than obtained with ferredoxin. However, at low concentrations, (<10 µM) flavodoxin was much more effective than ferredoxin. The appar-

ent K_m for flavodoxin was 1.5 μM , whereas that for ferredoxin was 10 μM . Obviously, the best test of efficiency would be with a naturally reconstituted system.

Even though FdI is effective (at relatively high concentrations) at reducing N_2 ase, it does not appear to be absolutely required for *in vivo* nitrogenase activity, since a transposon insertion mutant is still capable, albeit at lower levels, of carrying out nitrogen fixation *in vivo* (32). FdIII and FdIV do not seem capable of reducing nitrogenase, at least *in vitro* (17). Thus, the flavodoxin isolated here is a real candidate for the physiological reductant of nitrogenase in *R. capsulatus*. How it in turn is reduced is an interesting question that remains to be clarified. There have been no reports of potential reductant-generating pathways in crude extracts of *R. capsulatus*. For *Rhodobacter sphaeroides*, it has been proposed that reductant is generated via an energized membrane (10). Pyruvate-driven nitrogenase reduction, stimulated by the addition of ferredoxins, has been observed by using crude extracts of *R. rubrum* (22), and a pyruvate:ferredoxin oxidoreductase has been isolated and characterized (5). However, it appears to be neither *nif* specific nor required for *in vivo* nitrogen fixation. Preliminary results (not shown) indicate that *R. capsulatus* contains a pyruvate oxidase capable of reducing methyl viologen. Its potential role in reducing flavodoxin and the further molecular characterization of the *nif*-specific flavodoxin of *R. capsulatus* are under investigation. Thus, experiments to isolate the flavodoxin gene and construct specific mutants to test whether or not this flavodoxin is an electron donor to nitrogenase *in vivo* are currently in progress.

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