Molecular Cloning and Sequence Analysis of the Structural Gene of Ferredoxin I from the Photosynthetic Bacterium *Rhodobacter capsulatus*

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The structural gene (*fdxN*) encoding ferredoxin I (FdI) in the photosynthetic bacterium *Rhodobacter capsulatus* was isolated from a cosmid library by using an oligonucleotide probe corresponding to the N-terminal amino acid sequence of FdI. The nucleotide sequences of the gene and of the 3'- and 5'-flanking regions were determined. The gene *fdxN* codes for a polypeptide of 64 amino acids having a calculated molecular weight of 6,728. Amino acid sequencing of the N- and C-terminal ends of FdI allowed the determination of 86% of the primary structure and confirmed that FdI is the *fdxN* gene product. Sequence comparisons indicate that FdI shares common structural features with ferredoxins containing two [4Fe-4S] clusters, including eight conserved cysteines. Maximal homology was found with a ferredoxin from *Rhodospirillum rubrum*. Northern (RNA) hybridization using a 158-base-pair DNA fragment internal to the *fdxN* coding region revealed the existence of two mRNA transcripts of approximately 330 and 750 nucleotides. Neither of those transcripts was present under *nif*-repressing growth conditions. The 5' end of the smaller transcript was mapped by S1 nuclease protection and primer extension experiments. On the basis of Southern hybridization experiments, by using probes homologous to *fdxN*, *nifE*, and a fragment complementing a *nif* point mutation, *fdxN* was localized inside a cluster of *nif* genes.

Ferredoxins are small iron-sulfur proteins that function as electron carriers in a variety of metabolic processes, including photosynthesis, nitrogen fixation, and fermentative metabolism (8, 9). Most bacterial ferredoxins are single polypeptides ranging from 6,000 to 13,000 daltons (Da) and containing one or two [4Fe-4S] or [3Fe-xS] ($x = 3$ or 4) cluster(s). Despite apparent diversity among these proteins, bacterial ferredoxins share common structural properties, such as the spacing of cysteinyl residues in the peptide chain that serve as ligands for the iron-sulfur clusters (8, 18).

In the photosynthetic bacteria, the primary structures of a few ferredoxins from representative species have been described (21, 22, 31, 33, 50, 51). Comparisons of amino acid sequences indicated that these ferredoxins belong to a distinctive phylogenetic group (16, 17). Some species were found to contain several ferredoxins, each of which may have a distinctive specificity (33, 55, 58). Ferredoxins from *Rhodospirillum rubrum* and *Rhodobacter capsulatus* were shown to transfer electrons to nitrogenase in *in vitro* experiments using an artificial reductant (20, 55, 57).

Two ferredoxins have been isolated from *R. capsulatus* and partially characterized (20, 55). Ferredoxin I (FdI), first isolated by Hallenbeck et al. (20), was four to five times more abundant than ferredoxin II in cells grown under *nif*-derepressing growth conditions (55) (our unpublished results). FdI has an apparent molecular weight of 12,000. It contains seven to eight iron and inorganic sulfur atoms, consistent with the existence of two [4Fe-4S] clusters (20, 55).

In this report, we describe the isolation and nucleotide sequence of the gene coding for FdI. The deduced primary structure, confirmed by amino acid sequencing of 86% of the FdI polypeptide, has features typical of 2[4Fe-4S] ferredox-

ins and additional ones specific to ferredoxins from other photosynthetic bacteria. A remarkable deviation from the conserved pattern CXXCXXCXXCXXCP was noticed in the C-terminal part of FdI, where a methionine was found in place of a proline. Transcription analysis revealed that FdI gene expression was repressed by NH_4^+ . In addition, the FdI gene was localized within a *nif* coding region. These data suggest that FdI is required for nitrogen fixation.

MATERIALS AND METHODS

Materials. Restriction enzymes were from Boehringer GmbH (Mannheim, Federal Republic of Germany) or from Bethesda Research Laboratories, Inc., Gaithersburg, Md. T4 DNA ligase, DNase-free RNase, and nuclease S1 were obtained from Boehringer. T4 polynucleotide kinase was from New England BioLabs, Inc., Beverly, Mass. Avian myeloblastosis virus reverse transcriptase was from Appligene, Illkirch, France. The Sequenase DNA sequencing kit was from United States Biochemical Corp., Cleveland, Ohio. The pUC sequencing kit and the randomly primed DNA kit were from Boehringer. [α - ^{35}S]dATP (37 TBq/mmol), [γ - ^{32}P]ATP (222 TBq/mmol), [α - ^{32}P]dCTP (111 TBq/mmol), Hybond-N membranes, and Hyperfilm for autoradiography were purchased from Amersham France, Les Ulis, France. The Gene Clean kit was from BIO 101 Inc., La Jolla, Calif. TPCK-trypsin, trypsin treated with L-1(tosylamido)2-phenylethyl-chloromethylketone, was obtained from Worthington Diagnostics, Freehold, N.J. 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) was purchased from Clontech Laboratories Inc., Palo Alto, Calif. Ultrogel AcA44 and Sephadex G-50 were from Reactifs IBF, Ville-neuve-la-Garenne, France, and Pharmacia SA, Les Ulis, France, respectively. DEAE-cellulose (DE52) was from Whatman, Inc., Clifton, N.J.

Bacterial strains and cultures. Bacterial strains and plas-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or reference
<i>E. coli</i>		
TB1	$\Delta(lac-pro) strA ara thi hsdR$ $\phi 80 lacZ\Delta M15$	GIBCO BRL
HB101	$F^- recA proA ara xyl mtl$ $lacY galK rpsL supE hsdS$	7
<i>R. capsulatus</i> B10	Wild type	29
Plasmids		
pUC18	Plasmid vector, Ap ^r	56
pHC79	Cosmid vector, Ap ^r Tc ^r	23
pSUP202	Tc ^r Cm ^r Ap ^r <i>mob</i>	45
pWRK300	pSUP202 (3-kb <i>EcoRI</i> insert)	35
pES2	Ap ^r (pHC79, 40-kb <i>Bam</i> HI insert)	This work
pES2.1	Ap ^r (pUC18, 3.8-kb <i>EcoRI</i> insert)	This work
pES2.2	Ap ^r (pUC18, 0.4-kb <i>Hind</i> II insert)	This work

mids used in this work are listed in Table 1. *R. capsulatus* B10 was grown as described previously (20). *Escherichia coli* TB1 and HB101 were grown aerobically at 37°C in tryptone-yeast extract-NaCl (TYN) medium (Bethesda Research Laboratories) and LB medium (28), respectively. When needed, streptomycin and ampicillin were added to growth media at 100 µg/ml each.

Synthesis of oligodeoxynucleotides. Oligonucleotides were synthesized by using phosphoramidite chemistry with an Applied Biosystems synthesizer (model 380A). Oligomers, up to 20 nucleotides in size, were used without further purification. The 27-mer oligonucleotide was purified by reverse-phase high-performance liquid chromatography (HPLC) on a µBondapak C18 column (3.9 by 300 mm; Waters Associates, Inc.). The column was run at 1 ml/min with a linear gradient of 14 to 30% acetonitrile in 0.1 M triethylamine-acetate buffer, pH 7.0; the 27-mer oligonucleotide was eluted at 17 min. Oligonucleotides were end labeled with [γ^{32} -P]ATP by using T4 polynucleotide kinase.

Screening of the genomic library. The gene bank was made of 40-kilobase (kb) *Bam*HI fragments cloned into the cosmid vector pHC79 carried by *E. coli* HB101 (27). Colonies were grown on nylon Hybond-N filters placed on an LB plate for 12 h. They were then incubated overnight to LB plates containing 170 µg of chloramphenicol per ml for plasmid amplification. Colony blotting was carried out as follows. Filters were prehybridized for 3 h at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate)-2× Denhardt solution (12)-0.1% sodium dodecyl sulfate (SDS)-0.1 mg of salmon sperm DNA per ml and then hybridized for 16 h at 65°C in the same solution but lacking salmon sperm DNA and containing the 32 P-labeled 27-mer oligonucleotide. Filters were washed twice in 6× SSC-0.1% SDS for 10 min at room temperature and then for 1 h at 65°C.

Southern blot analysis. Total genomic and plasmid DNA was isolated by established techniques. After electrophoresis on agarose gels, fragments were transferred onto nylon Hybond-N filters. Hybridization conditions were the same as those used for the screening of the genomic library. The temperature of hybridization (*t*) was determined by using the following formula: $t(^{\circ}\text{C}) = 2(A+T) + 4(G+C) - 5^{\circ}\text{C}$, where A, T, G, and C indicate the number of adenine, thymine,

guanine, and cytosine nucleotides, respectively (48). Large fragments of DNA were labeled with [α^{32} P]dCTP by nick translation (41). Hybridization was done in 6× SSC-2× Denhardt solution-0.1% SDS at 65°C. Filters were washed in 6× SSC-0.1% SDS. Autoradiograms were obtained by overnight exposure at -70°C to Amersham Hyperfilm.

DNA subcloning procedure. DNA restriction fragments were electrophoresed and then extracted from low-melting-temperature agarose by using a Gene Clean kit. DNA fragments were ligated, by using T4 DNA ligase, to the pUC18 vector previously linearized and dephosphorylated. Clones harboring recombinant plasmids were selected on TYN medium containing ampicillin and screened for the presence of *R. capsulatus* DNA fragments on plates containing X-gal.

DNA sequencing. DNA sequencing was performed by the dideoxy-chain-termination method (42) after alkaline treatment of double-stranded DNA (11). Sequencing reactions using either the Klenow enzyme with deoxy-7-deazaguanosine or Sequenase (49) were performed as recommended by the manufacturer of the sequencing kits; primers were synthetic oligonucleotides.

DNA transcription analyses. RNA was prepared from 100 ml of photosynthetically grown cultures containing either limiting concentrations of glutamate (5 mM) or excess ammonia (15 mM NH₄⁺). Cultures were quickly cooled in an ice-ethanol bath and harvested at 4°C by centrifugation for 20 min at 12,000 rpm (13,000 × *g*). Cells were lysed in 1% lithium dodecyl sulfate-10 mM EDTA at 65°C. RNA was isolated after several extractions with phenol-chloroform (1:1, by volume) at 65°C. Contaminating DNA was digested for 10 min at 37°C with 1 unit of RNase-free DNase per µg of crude RNA preparation.

For Northern (RNA) blot analysis, total RNA was electrophoresed on a 1.5% agarose gel containing 2.2 M formaldehyde (28). RNA was transferred by vacuum blotting to a Hybond nylon membrane by using a Vacugene apparatus (Pharmacia). Filters were successively prehybridized and hybridized in 6× SSC-5× Denhardt solution-0.5% SDS by using the procedure described for Southern hybridization. A radioactive probe was used (2×10^6 cpm/ml); it was obtained by random-primed DNA labeling of a 158-base-pair (bp) *Nco*I-*Hind*II fragment of pES2.1 by using [α^{32} -P]dCTP. Filters were washed twice in 2× SSC-0.1% SDS and twice in 1× SSC-0.1% SDS at 65°C and exposed to Amersham Hyperfilm at -70°C.

Nuclease S1 transcript analysis was performed as described previously (1). Total RNA was hybridized overnight at 52°C with a 0.4-kb *Hind*II fragment of pES2.1, end labeled with T4 polynucleotide kinase. Single strands were digested with S1 nuclease for 30 min at 37°C. Aliquots of the digest were analyzed on urea-polyacrylamide gels as for DNA sequencing. Restriction fragments used as probes in these experiments were purified by HPLC on a Gen-Pak FAX column (4.6 by 100 mm; Waters) run in 25 mM Tris hydrochloride (Tris-HCl) (pH 8.0)-10 mM EDTA and by using an appropriate gradient of NaCl (47).

Primer extension mapping was performed as described by Golden et al. (19).

Purification of FdI. *R. capsulatus* FdI was purified by a procedure similar to that described by Hallenbeck et al. (20), with the following changes. After the first DEAE-cellulose chromatography, the ferredoxin fraction was applied to a column of Ultrogel AcA44 (2.6 by 110 cm) that had been equilibrated in 50 mM Tris-HCl (pH 7.5)-0.4 M NaCl and was developed at a flow rate of 15 ml/h. The eluate was

TABLE 2. Amino acid compositions of tryptic peptides T1 and T2 isolated from FdI

Amino acid(s) ^a	mol/mol of peptide ^b	
	T1	T2
Aspartate + asparagine	3	3 + 4
Glutamate + glutamine	2	3 + 1
CM-cysteine	3	5
Serine	1	0
Glycine	1	2
Histidine	0	1
Threonine	2	3
Alanine	2	4
Proline	4	2
Tyrosine	0	1
Valine	1	2
Methionine	0	1
Isoleucine	2	2
Leucine	1	1
Lysine	1	1

^a CM-cysteine, S-carboxymethylated cysteine.^b The amino acid content was calculated on the basis of one lysine per peptide. For peptide T2, the amino acid content was derived from the peptide sequence.

found within 200 bp upstream of this promoterlike sequence. Downstream of the TAA stop codon of *fdxN*, there are two inverted repeats (nucleotides 220 to 238 and nucleotides 305 to 331 in Fig. 3) that may function as a transcription terminator signal. The free energy of formation of the corresponding mRNA structures would be -4.25 and -3.52 kJ/mol, respectively (53).

At the beginning of this study, a different cloning strategy led us to the discovery of another open reading frame (ORF2) presenting a partial homology with *fdxN*. Two probes, SII and SIII (Fig. 1), each consisting of a mixture of tetradecanucleotides, corresponding to the N-terminal sequence of FdI were used to clone homologous DNA from *R. capsulatus*. A 1.8-kb *XhoI* fragment was isolated that hybridized to both probes (43). Sequence analysis of the fragment led to the identification of ORF2, which contains a sequence of 14 nucleotides identical to those found between positions +7 and +20 in Fig. 3. ORF2 presented another stretch of 12 nucleotides homologous to the sequence +34 to +45 of *fdxN*, with only two mismatches. However, the homology was restricted to the two short regions of 14 and 12 nucleotides present in both ORF2 and the 5'-OH part of *fdxN* (data not shown).

The *fdxN* gene was localized, and its direction of transcription was determined on the 3.8-kb *EcoRI* insert of pES2.1. A *PvuI* site was identified from the nucleotide sequence (position 70 to 75, Fig. 3) that is unique in the *EcoRI* insert and divides it into two fragments of 2.0 and 1.8 kb. A unique *HindIII* site was also found in the insert and localized within the 2.0-kb *PvuI-EcoRI* fragment (Fig. 2). The *fdxN* gene could be oriented relative to these two single restriction sites by using probe SI, which hybridizes upstream of the *PvuI* site. Hybridization analysis of the insert digested by *PvuI* with probe SI indicated that the beginning of *fdxN* was situated on the 1.8-kb *EcoRI-PvuI* fragment. We therefore conclude that *fdxN* is transcribed towards the single *HindIII* site of the 3.8-kb insert, as illustrated in Fig. 2.

Localization of *fdxN* on the chromosome of *R. capsulatus*. Southern hybridization between probe SI and chromosomal DNA digested by *EcoRI*, *BamHI*, and *HindIII* revealed three fragments of 3.8, 5.6, and 11.3 kb, respectively (Fig. 4).

FIG. 4. Mapping of *fdxN* next to a 3.55-kb *EcoRI* fragment complementing a *nif* mutation. (Upper panel) Hybridization of *R. capsulatus* total DNA with probe SI (lanes 2 to 4) and with the ³²P-labeled 3.55-kb *EcoRI* probe (lanes 5 to 7). DNA was digested with *EcoRI* (lanes 2 and 5), *BamHI* (lanes 3 and 6), or *HindIII* (lanes 4 and 7) and electrophoresed in 0.7% agarose gel prior to hybridization with either probes. Lane 1 is ³²P-labeled lambda DNA digested with *HindIII*, used as a molecular weight standard. (Lower panel) Partial map of the region of pES2 containing *fdxN*. Positions of the *BamHI* (B), *EcoRI* (E), and *HindIII* (H) restriction sites were deduced from hybridization experiments using probe SI (see text).

The same restriction fragments were detected in the insert of pES2 (shown partially in Fig. 5). SI probing of a double digest of pES2 with *BamHI* and *HindIII* indicated that *fdxN* was carried on a 4.3-kb fragment. Considering that the

FIG. 5. Mapping of *fdxN* next to a 3.0-kb *EcoRI* fragment containing *nifE*. (Upper panel) Plasmid pES2 was digested with *EcoRI* (lanes 1 and 3) and *HindIII* (lanes 2 and 4) and analyzed by hybridization with either an *fdxN*-specific 3.8-kb *EcoRI* probe (lanes 1 and 2) or a *nifE*-specific 3.0-kb *EcoRI* probe (lanes 3 and 4). (Lower panel) Restriction map of the region of pES2 surrounding *fdxN*. Restriction sites were deduced from hybridization experiments of pES2 digests using probes specific for *nifE*, *fdxN*, and a 3.55-kb *EcoRI nif*-specific fragment (see text and Fig. 4).

3.8-kb *EcoRI* fragment contains a single *HindIII* site and no *BamHI* site (Fig. 2), we could deduce from the hybridization data the positions of the 3.8-kb *EcoRI*, 5.6-kb *BamHI*, and 11.3-kb *HindIII* fragments detected by probe SI relative to each other, as illustrated in Fig. 4.

We noticed that the size and the arrangement of these fragments were identical to those found in the restriction map of a *nif* region of *R. capsulatus* already described by Avtges et al. (3) and by Klipp et al. (25). According to this map, we predicted that the 3.8-kb *EcoRI* fragment carrying *fdxN* would be flanked by a 3.0-kb *EcoRI* fragment containing *nifE* on one side and a 3.55-kb *EcoRI* on the other (25) (Fig. 4).

To test this hypothesis, hybridization experiments were performed on total DNA and pES2 by using probes specific to *nifE*, *fdxN*, and the 3.55-kb *EcoRI* fragment mentioned above. The 3.55-kb *EcoRI* fragment was made available to us by J. C. Willison, who had shown that this fragment complemented a *nif* point mutation (*nif-32*), and mapped next to the 3.8-kb *EcoRI* fragment in the *nif* region described in references 3 and 25 (54; T. Gastineau and J. C. Willison, unpublished data). The 3.55-kb *EcoRI* fragment was ³²P labeled by nick translation and used to probe total DNA digested by *EcoRI*, *BamHI*, and *HindIII*. The hybridization pattern is compared with that obtained with probe SI in Fig. 4. Both probes detected an 11.3-kb *HindIII* fragment and a 5.6-kb *BamHI* fragment. Hybridization with pES2 digests gave essentially the same results and provided evidence that the two probes hybridized to the same *BamHI* and *HindIII* restriction fragments (data not shown). These results are consistent with the 3.8-kb *EcoRI* fragment carrying *fdxN* being contiguous with the 3.55-kb *EcoRI* fragment, as shown in Fig. 4.

The 3.0-kb *EcoRI* insert of pWKR300 carrying the *nifE* gene of *R. capsulatus* (35) was also used to probe pES2. The 3.0-kb fragment was ³²P labeled by nick translation and hybridized with *BamHI* and *HindIII* digests of pES2. The result is compared with that obtained by probing the same pES2 digests with the *fdxN*-containing 3.8-kb *EcoRI* fragment (Fig. 5). A 6.8-kb *HindIII* fragment was detected by the two probes, consistent with *fdxN* and *nifE* being located on two adjacent *EcoRI* fragments, as shown in Fig. 5. As anticipated from the partial map of pES2, the 11.3-kb *HindIII* fragment containing *fdxN* was also detected by the 3.8-kb *EcoRI* probe (Fig. 5). Total DNA probed under identical conditions yielded the same hybridization pattern as pES2 (data not shown). This indicates that, during the construction of the cosmid library, no rearrangement of DNA fragments had taken place in the 40-kb insert of pES2, at least in the region studied. From these results and from those obtained with the labeled 3.55-kb *EcoRI* fragment and probe SI, a map of the region surrounding *fdxN* was deduced (Fig. 5). This map coincides with that previously published (3, 25), confirming the location of *fdxN* inside a *nif* region.

Transcription analysis of *fdxN*. Total RNA from *R. capsulatus* was subjected to Northern blot analysis by using as a probe a 158-bp *HindII-NcoI* internal fragment of *fdxN* isolated from pES2.2. Plasmid pES2.2 itself was obtained by subcloning a 0.4-kb *HindII* fragment from pES2.1 in pUC18 (Table 1). In cells derepressed for *nif* gene expression (growth with limiting concentrations of glutamate), two transcripts were found, comprising approximately 330 and 750 nucleotides (Fig. 6). Densitometric scanning of the autoradiogram indicated that the 330-base transcript was about five to six times more abundant than the larger transcript. Considering the size of *fdxN*, the major transcript

FIG. 6. Northern hybridization analysis of *fdxN*. Total RNA (20 μ g) from *R. capsulatus* grown on either 5 mM glutamate (lane 1) or 15 mM NH_4^+ (lane 2) was subjected to Northern blot analysis. A 158-bp internal fragment of *fdxN* labeled by random priming to 2×10^6 cpm/ml was used as a probe. RNA molecular size markers are indicated on the left in kilobases.

of 330 nucleotides is probably monocistronic, FdI being the only translation product. RNA from NH_4^+ -grown cells gave no detectable hybridizing signal, indicating that *fdxN* transcription is repressed by ammonia. Hence, regulation of *fdxN* resembles that of *nif* genes.

S1 nuclease protection and primer extension experiments were performed to determine the 5' end of the major transcript of *fdxN* (Fig. 7). For S1 nuclease mapping, a 0.4-kb *HindII* fragment (pES2.2 insert) extending from inside *fdxN* to 285 nucleotides upstream of the initial ATG codon was used as a probe. The *HindII* fragment was ³²P labeled with T4 polynucleotide kinase, denatured to single strands by heating for 3 min at 95°C, and annealed to RNA extracted from glutamate-grown cells. Following S1 nuclease digestion, analysis of protected fragments on a sequencing gel revealed a major band migrating at the level of an A in the noncoding strand (Fig. 7 [left]) and corresponding to a T at position -22 of the coding strand (Fig. 3). Fainter bands were detected above, which may correspond to slightly longer transcripts (up to 16 bases longer) or alternatively may arise from incomplete nuclease digestion. The latter possibility is less likely since these upper bands were still visible when experiments were done with twice as much nuclease. Full protection of the probe was also apparent (data not shown), suggesting the presence of a transcript extending from a start point further upstream. The 5' end of the *fdxN* transcript was also mapped by primer extension by using an oligonucleotide complementary to the coding strand at about 80 nucleotides downstream of the ATG initiation codon. DNA strands generated by reverse transcriptase were analyzed on a sequencing gel next to a DNA ladder obtained by using the same oligonucleotide as a primer for sequencing (Fig. 7 [right]). Several bands detected in the lower part of the gel probably result from incomplete transcription of the mRNA. The longest extended fragments have their 5' end mapping in the same region of the nucleotide sequence as that identified by S1 protection experiments. The most intense band revealed in this region corresponds to an A in the coding strand (position -24). This A is situated two nucleotides upstream of a T identified as a possible 5' end by S1 mapping. Fainter bands above may correspond to longer transcripts, the longest one extending 14 nucleotides upstream at the level of a T (A on the coding strand at position -38). A signal at the level of the same T was also detected in the S1 protection experiment, suggesting that the *fdxN* transcript has a 5' end at this position.

Peptide sequence analysis. The *fdxN* gene is capable of encoding a protein of 64 amino acids. The N-terminal part of the deduced polypeptide exactly matched that of the N-

FIG. 7. Mapping of the 5' end of the *fdxN* transcript. For the S1 nuclease protection experiment (left panel), a 0.4-kb *Hind*III fragment 5' end labeled by T4 polynucleotide kinase was hybridized to 75 μ g of total RNA from cells grown with limiting concentrations of glutamate. After nuclease S1 digestion, a sample was analyzed on a DNA sequencing gel (lane S1). On the same gel, a DNA sequencing ladder of the complementary DNA strand was generated starting from the 3'-OH end of the 0.4-kb *Hind*III fragment located in *fdxN*. Sequencing of the complementary strand was performed by the chain termination method and initiated from the following primer: 5'-(157)GACGCATTGCGGCAGGTC(139)-3'. Primer extension was generated from the following oligonucleotide: 5'-GGTGTGCGCGTTGATCAC-3' (position 114 to 97 in Fig. 3). The oligonucleotide was 32 P labeled by T4 polynucleotide kinase and annealed to 50 μ g of RNA overnight at 50°C. Extension was performed in the presence of 20 units of avian myeloblastosis virus reverse transcriptase for 1 h at 42°C. The DNA strand generated was analyzed on a sequencing gel (lane Pe) next to a DNA ladder of the complementary strand sequenced from the same primer (right panel). On the left, arrows point to nucleotides corresponding to a signal in the S1 nuclease protection (S1) or primer extension (Pe) experiment.

terminal FdI protein obtained by Edman degradation (Fig. 1), if we assume that the initial methionine is processed during maturation of the gene product. The molecular weight of the *fdxN* gene product was calculated to be 6,728. That value is half that obtained by gel filtration with the isolated FdI protein (20, 55). To resolve the discrepancy between these two determinations, a peptide analysis of FdI was undertaken. CM-FdI was prepared and subjected to proteolytic digestion by trypsin. The trypsin digest was subjected to reverse-phase HPLC. Only two peptides, T1 and T2, were detected. Their amino acid compositions are given in Table 2. Peptide T1 is likely to be part of the N-terminal region of FdI since it contains a serine and since the only serine of the protein is present in that region (Fig. 1); on the other hand, peptide T2 has no serine. The amino acid content of peptide T1 corresponded to the sequence extending from Ile-4 to Lys-27 in Fig. 3, except for one cysteine. The apparent lack of one cysteine in the amino acid determination of T1 is likely due to loss of S-carboxymethylated cysteine during

FIG. 8. Comparison of ferredoxin amino acid sequences from (a) *Clostridium pasteurianum* (52) and photosynthetic bacteria; (b) *Rhodospirillum rubrum* (31); (c) *Chromatium vinosum* (21, 32); (d) *Chlorobium limicola* II (51); (e) *Chlorobium limicola* I (50); (f) *Chlorobium thiosulfatophilum* (22); (g) *Rhodopseudomonas palustris* (33); (h) *R. capsulatus* (this work). The sequences are divided into two parts that may have originated from an ancestral gene duplication. Gaps (-) were inserted in order to optimize alignment of the ferredoxin sequences and to show the correspondence between the two parts of the proteins.

acid hydrolysis of the peptide. The peptide T2, containing 37 amino acids, was entirely sequenced. Its structure was found to be identical to the C-terminal region of the *fdxN* gene product extending from Lys-28 to Ala-64 (Fig. 3). Hence, FdI appears to consist of only three tryptic peptides—T1, T2, and the small N-terminal tripeptide Ala-Met-Lys—that we failed to detect by HPLC, probably because of its small size. These results confirmed that FdI is indeed the *fdxN* gene product.

DISCUSSION

The predicted primary structure of the polypeptide deduced from the nucleotide sequence of *fdxN* was found to be identical to the amino acid sequence of FdI determined by Edman degradation (55 amino acids out of 64 were identified by protein sequencing). These results demonstrate unambiguously that FdI is the *fdxN* gene product. The calculated molecular weight of the apoferridoxin was 6,728, a value about half that of the holoprotein previously determined by gel filtration (20, 55). This discrepancy may reflect an atypical behavior of FdI on gel filtration or a tendency of the protein to form dimers.

FdI contains nine cysteines, eight of which may accommodate two [4Fe-4S] clusters. In previous reports, the properties of the UV-visible absorption spectrum and chemical determinations of iron and sulfur contents suggested that FdI contained two [4Fe-4S] clusters (20, 55). However, the conclusions concerning the molecular content of Fe and S atoms might have been erroneous if based on a molecular weight of 12,000. Recently, we reestimated the Fe and S content of a homogeneous preparation of FdI (A_{385}/A_{280} ratio, 0.81). When related to the amino acid content of FdI, the number of Fe and S atoms per monomer of 6,728 was 8.0 ± 0.2 and 7.0 ± 0.5 , respectively, consistent with the presence of two [4Fe-4S] clusters.

A comparison of the amino acid sequence of FdI from *R. capsulatus* with that of the 2[4Fe-4S] ferredoxins from *Clostridium pasteurianum* and from six photosynthetic bacteria is given in Fig. 8. The selected ferredoxins contain two sets of four cysteines that are presumably ligands for two [4Fe-4S] clusters. These two sets of cysteines show a conserved spacing pattern and are located in two homolo-

gous domains of the primary structure. It has been proposed that the two homologous halves of those ferredoxins derived from a common ancestor by gene duplication (14, 15). Comparison of the ferredoxins half by half, as presented in Fig. 8, indicates that the C-terminal sequence of FdI from *R. capsulatus* displays an insertion of six amino acids in the middle of the typical cysteine motif CXXCXXCXXCP. A similar insertion is noticeable in the ferredoxins from other photosynthetic bacteria, except in that from *Rhodospirillum rubrum* (Fig. 8). The ferredoxin isolated from *Rhodopseudomonas palustris* proved to be the one most similar to *R. capsulatus* FdI, with 60% identical amino acids.

FdI exhibits only 13 of the 16 invariant amino acids observed in other ferredoxin structures (Fig. 8) because of the replacement of three conserved residues at positions 18, 53, and 55. The replacement of a proline by a methionine at position 55 appears to be rather unusual. The proline belongs to the conserved sequence CXXCX_nCXXCPCP ($n = 2$ to 8; Fig. 8) encountered in almost all bacterial ferredoxins believed to contain [4Fe-4S] cluster(s) (8). To date, such a proline replacement has been reported for only two ferredoxins isolated from two *Desulfovibrio* species, namely *D. africanus* Benghazi (6) and *D. vulgaris* Miyazaki (39). In these ferredoxins, a glutamic acid residue of the C-terminal half replaces the proline and the cysteine next to it is probably one ligand of a [3Fe-xS] cluster (6). Another distinctive property of FdI is its relatively low content of aromatic amino acids; it contains a single tyrosine, no phenylalanine, and no tryptophan. From this observation, one can expect FdI to have a relatively high A_{385}/A_{280} ratio compared with other 2[4Fe-4S] ferredoxins. The previously reported values for the A_{385}/A_{280} ratio of FdI were 0.74 (20) and 0.78 (55). Recently, we have obtained an FdI preparation with an A_{385}/A_{280} ratio of 0.81, which is close to that found for the 2[4Fe-4S] ferredoxin of *C. pasteurianum* (37).

In *R. capsulatus*, two ferredoxins have so far been identified as distinct from FdI (55) (our unpublished data). FdII was apparently synthesized under various growth conditions (55), whereas the third ferredoxin, which we called FdIII, was detected only under *nif*-derepressing conditions (unpublished data). On the basis of our amino acid sequence analysis, it appears that FdIII is encoded by a recently identified open reading frame, ORF5, localized in a *nif* region comprising *nifE*, *nifN*, *nifX*, and *nifQ* (34). When choosing a name for the FdI gene, we took into consideration available information on the structure of the *R. capsulatus* ferredoxins. The term *fdxH* had been given to the gene coding for a heterocyst ferredoxin containing a single [2Fe-2S] cluster in the cyanobacterium *Anabaena* sp. strain PCC 7120 (5). The designation *fdxA* had been proposed for the gene encoding *Azotobacter vinelandii* FdI (36). Since the FdIs from the two bacterial species show relatively weak homology, *fdxA* seemed inappropriate to *R. capsulatus* FdI. Besides, on the basis of our amino acid sequence analysis, *R. capsulatus* FdII is quite similar to *A. vinelandii* FdI (unpublished data). We therefore suggest that the term *fdxA* be reserved for the gene coding for *R. capsulatus* FdII. The term *fdxN* has been proposed to designate genes encoding ferredoxinlike polypeptides that are involved in nitrogen fixation and located next to *nifB* in the N_2 -fixing species *Bradyrhizobium japonicum* (13), *A. vinelandii* (24), and *Rhizobium meliloti* (38). Recently, the *fdxN* gene from *Rhizobium meliloti* was reported to be essential for nitrogen fixation (26). In *R. capsulatus*, there are two functional copies of *nifB* but no ferredoxinlike gene was found in the vicinity of either copy (30). However, two genes coding for ferredoxins were

mapped inside a *nif* gene cluster on the chromosome. The FdI gene and ORF5, coding for FdIII, were located upstream and downstream, respectively, of *nifE* (Fig. 5) (34). Since FdI displays significant homology with *fdxN* gene products (up to 50% homology with *Rhizobium meliloti* FdxN) whereas FdIII does not, we proposed that the FdI structural gene of *R. capsulatus* be called *fdxN*.

FdI was shown to act as an electron donor to nitrogenase in *in vitro* experiments (20, 55), and its synthesis was shown to be optimal under growth conditions allowing derepression of nitrogenase (55). These biochemical data suggested that FdI is involved in N_2 fixation. This hypothesis is now supported by genetic evidence reported in the present study. First, transcription of *fdxN* was apparently turned off when cells were provided with an excess of fixed nitrogen (Fig. 6, lane 2) in a way similar to the regulation of *nif* genes. Second, a consensus sequence specific to *nif* promoters (4) was identified upstream of the *fdxN* coding region (Fig. 3). Third, the structural gene *fdxN* is carried on a 3.8-kb *EcoRI* fragment that was localized inside a *nif* gene cluster (Fig. 4 and 5).

Northern hybridization using a probe internal to *fdxN* revealed the existence of two specific mRNA transcripts of about 330 and 750 nucleotides (Fig. 6). The 5' end of the smaller transcript was mapped in a limited region of 16 bp, starting 22 bp upstream of the initiation codon (Fig. 7). Several bands detected in this region by nuclease S1 protection and primer extension experiments may correspond to multiple origins of transcription. However, no consensus sequence typical of a sigma factor binding site was identified within the right distance from this putative transcription start site. In particular, the sequence identified upstream and presenting remarkable similarities with the NtrA binding site (see Fig. 3) is located 100 bp from the putative origin, a distance much greater than the regular -12/-24-bp spacing observed in *nif* promoters (4). Moreover, no upstream activating sequence specific to *nifA*-activated genes (10) was detected within 250 bp of the site mapped in Fig. 7. Such a sequence was found in front of the *nifH* gene of *R. capsulatus* (40).

Alternatively, the bands observed in the 5'-end-mapping experiment (Fig. 7) may represent mRNA fragments generated by splitting of a longer transcript originating further upstream. The existence of a longer transcript is substantiated by the detection of a 750-nucleotide-long mRNA fragment on a Northern blot (Fig. 6). In the S1 mapping experiments, we observed that some of the probe was fully protected, which suggests the presence of a transcription start point beyond the *HindII* site (position -285 in Fig. 3). On the basis of these data, our working hypothesis is that the 750-nucleotide-long mRNA results from the cotranscription of *fdxN* and a gene located within the preceding 400 bp of DNA. In accordance with this assumption, our preliminary results indicate the existence of a 282-bp open reading frame immediately upstream of *fdxN*.

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