A new ferredoxin, called FdIII, has been isolated and purified from the photosynthetic bacterium *Rhodobacter capsulatus*. Its complete amino acid sequence has been determined. The FdIII polypeptide consists of 100 residues, including 9 cysteines and has a calculated molecular mass of 10,688 Da, which was confirmed by electrospray mass spectrometry. In its native form, FdIII is a homodimer as deduced from molecular sieve chromatography and non-denaturing polyacrylamide gel electrophoresis, as well as cross-linking experiments. The dimeric ferredoxin was found to contain 15.2 ± 0.6 iron atoms and 13 ± 1 inorganic sulfur atoms, consistent with the presence of four [4Fe-4S] clusters/molecule. The UV visible absorption spectrum of oxidized FdIII exhibited maxima at 282 and at 386 nm and a shoulder near 314 nm. FdIII was fully reduced by excess dithionite at pH 8.0 or photochemically using 5-deazaflavin. Anaerobic oxidative titration of reduced FdIII with thionin indicated that each FdIII monomer exchanges two electrons. Exposure of FdIII to air resulted in a rapid and irreversible oxidative denaturation of the Fe-S clusters. The EPR spectrum of fully reduced FdIII showed a broad signal with an average g value of 1.94 that integrated to about two spins/monomer. EPR analysis of partially reduced FdIII (~20% reduction) revealed a complex set of signals which was interpreted as being the resulting sum of the contribution of two distinct paramagnetic centers. Based on its biochemical and spectroscopic properties, it is concluded that FdIII is a dimeric ferredoxin containing four [4Fe-4S] clusters. The synthesis of FdIII occurs only under growth conditions allowing the derepression of *nif* genes. Results of *in vitro* electron transfer assays indicate that FdIII cannot serve as an electron donor to nitrogenase.

Bacterial ferredoxins form a group of electron-carrier proteins involved in a variety of metabolic functions, such as fermentation of organic compounds, reduction of N₂, CO₂, and sulfate. They generally consist of a single polypeptide, ranging in size between 6 and 15 kDa and contain one or two Fe-S clusters/monomer. Although a few [2Fe-2S] ferredoxins have been described, most bacterial ferredoxins were found to contain cubane-like [4Fe-4S] or [3Fe-4S] clusters (Bruschi and Guerlesquin, 1988). The three-dimensional structure has been described for representative monocluster ferredoxins, the [4Fe-4S] ferredoxin of *Bacillus thuringiensis* (Fukuyama et al., 1988) and the [3Fe-4S] ferredoxin II of *Desulfovibrio gigas* (Kissing et al., 1991) as well as for dicluster ferredoxins including the 2[4Fe-4S] ferredoxin of *Peptococcus aerogenes* (Adman et al., 1973) and the 7Fe ferredoxin from *Azotobacter vinelandii* which contains one [3Fe-4S] and one [4Fe-4S] cluster (Stout et al., 1988; Stout, 1989). In spite of apparent differences in size, amino acid sequence, number, and type of cluster, these ferredoxins of known structure appear to be similar with respect to their polypeptide chain folding (Fukuyama et al., 1988; Stout, 1989; Kissing et al., 1991).

Structural similarities among bacterial ferredoxins were also anticipated from comparison of known amino acid sequences. The alignment of more than 30 sequences highlighted some common features including a conserved cysteine motif, C-X-X-C-X-C-(X)_n-C-P, the last and more distant cysteine being almost always followed by a proline (Fitch and Bruschi, 1988). The cysteines in the motif serve as ligands for a [4Fe-4S] cluster.

In the photosynthetic bacterium *Rhodobacter capsulatus*, we have reported the existence of four different ferredoxins (Jouanneau et al., 1990a, 1991). Three of these ferredoxins, designated FdI, FdIII, and FdIV, have already been biochemically and genetically characterized. FdI is a 2[4Fe-4S] ferredoxin of 64 amino acids, which has sequence similarities with other known ferredoxins from photosynthetic bacteria (Hallenbeck et al., 1982a; Schatt et al., 1998). FdII was shown to contain one [3Fe-4S] and one [4Fe-4S] cluster linked to a polypeptide chain of 111 residues, and closely resembles the 7Fe ferredoxin of *A. vinelandii* (Jouanneau et al., 1990b; Duport et al., 1990). FdIV has been expressed in *Escherichia coli* and appeared as a [2Fe-2S] ferredoxin similar to those found in plant and cyanobacteria (Grabau et al., 1991). FdI and FdIV are encoded by contiguous genes located within a large *nif*-specific region of the chromosome (Schatt et al., 1989; Klipp, 1990). The implication of these two ferredoxins in nitrogen fixation has therefore been proposed (Jouanneau et al., 1991; Saeki et al., 1991). On the other hand, FdIII, which was shown to be constitutively expressed in *R. capsulatus* (Duport et al., 1992) is likely to play an essential role in this bacterium since its structural gene could not be inactivated without impairing cell viability (Duport, 1991; Saeki et al., 1991).

In this report, we describe the purification, and some mo-
Characterization of R. capsulatus Ferredoxin III

Analytical Methods

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was used to estimate the molecular weight of FdIII according to the procedure described by Hedrick and Smith (1968). Bovine serum albumin in dimeric (132,000) and monomeric (96,000) forms, ovalbumin (45,000), carbonic anhydrase (29,000), and spinach ferredoxin (11,500) served as markers. Electrophoretic mobility of FdIII and protein markers was measured in gels ranging from 7 to 15% polyacrylamide (2% increments). Retardation coefficient (slope of the log plot of the electrophoretic mobility versus percent polyacrylamide) and molecular weights were linearly correlated through a log-log plot (Bryan, 1977).

SDS-PAGE was performed in a SE200 small vertical slab-gel unit (Hoefer Scientific Instruments) following a procedure adapted from Schagger et al. (1985). Gels were calibrated using two commercial kits of molecular weight markers ranging in size from 84,000 to 14,400 (Pharmacia) and from 18,549 to 2,512 (Merek). Western blotting was performed according to the method of Towbin et al. (1979) as previously described (Jouanneau et al., 1992) using polyclonal anti-FdIII antibodies raised in a white rabbit. Isoelectric point was determined using the first dimension of the O’Farrell’s system (O’Farrell, 1975). For cross-linking experiments, samples of the indicated purified proteins (4 μM) were incubated with diimidoesters (DMS or DMA, 4 mM) in the presence of 0.05 M Tris-HCl, pH 7.5, before being analyzed by slab gel electrophoresis.

To prepare apoferrredoxin, purified FdIII was precipitated in 5% trichloroacetic acid and then dissolved in 0.1 M Tris-HCl, pH 8.5, 6 M guanidine hydrochloride, 1 mM EDTA. The apoferrredoxin was reduced and S-carboxymethylated with iodoacetic acid as described by Allen (1981). For mass spectrometric determination, S-carboxymethyl-FdIII (cm-FdIII) was purified by reverse-phase HPLC on a 4.6 × 100-mm Aquagel column (Brownlee). cm-FdIII was eluted with a 15-min gradient from 0 to 50% acetonitrile in 0.1% trifluoroacetic acid, and the protein was concentrated to 0.1 mM by freeze-drying. Mass spectrum of cm-FdIII was recorded on a Finnigan TSQ 700 mass spectrometer equipped with an electrospray source.

Amino Acids and Peptides Analyses

cm-FdIII (18–20 nmol) was digested with either 1/50 (w/w) of endoproteinase Lys-C or 1/150 (w/w) of trypsin (both crushed from Boehringer Mannheim) for 2 h at 37 °C in 50 mM ammonium bicarbonate, pH 8.5. An equivalent amount of protease was then added, and the digestion was allowed to proceed for another 2 h. Reaction was stopped by acidification to pH below 4.0 using concentrated trifluoroacetic acid. The apoferrredoxin was also cleaved with trypsin ( Worthington Diagnostics) overnight at 37 °C in 0.1 mM ammonium bicarbonate, pH 8.0. The tryptophan-containing peptide, identified by its UV absorption spectrum, was purified by HPLC (see below) and further digested with α-chymotrypsin. Two peptides derived from this digestion, TC1 and TC2, were purified and characterized by amino acid analysis and sequencing.

Peptide mixtures were analyzed by reverse-phase HPLC on either a 3.9 × 300-mm µBondapak C18 column (Waters) at room temperature or a 4.6 × 150-mm Eclipse column (Alltech) at 40 °C. Columns were run with 0.1% trifluoroacetic acid in water and developed with acetonitrile using a HPLC gradient system operated through a Baseline M810 Software (Waters). Amino acid analyses were performed according to the Picotag method (Waters) after acidic hydrolysis of samples in 6 N HCl vapor for 20 h at 106 °C. Amino acid sequence was performed on an Applied Biosystems sequencer (model 477A), equipped with an on-line phenylthiohydantoin analyzer (model 120A). Sequence analyses and secondary structure predictions were performed using the DNASTAR software. For determination of COOH-terminal amino acids, HPLC-purified cm-FdIII was subjected to digestion with carboxypeptidase Y (Boehringer Mannheim) in 50 mM sodium acetate, pH 5.5. Aliquots of the digest were taken at 1, 2, 5, and 20 min and analyzed for amino acids. The COOH-terminal sequence was deduced from the order of apparition of the amino acids in the digest.

Materials and Methods

Bacteria

R. capsulatus strain B10 was grown in 20-liter glass carboys illuminated by ten 120 watt lamps and maintained at ~30 °C. Excess heat was evacuated through a glass cold-finger plunging into the culture and connected to a Colora FK3 cooling unit (Colora Messtechnik GmbH). Cells were grown on a mineral salts medium (Weaver et al., 1976; Hillmer and Gest, 1977) with 2.5 mM glutamate or NH4 as limiting nutrient, harvested with a Sharples continuous flow centrifuge and stored at -70 °C. Alternatively, strain RcM1, which bears the early steps of the purification procedure described above. After the first DEAE-Chromatography, the supernatant was applied to a 1.6 X 50 cm DEAE-cellulose column (Whatman DE52), using two multichannel peristaltic pumps, delivering ~250 ml/h/channel. The columns were successively washed with two bed volumes of 25 mM Tris-HCl, pH 7.5 (later referred as Tris-HCl), and five bed volumes of buffered 0.17 M NaCl, FdIII, as well as FdII, and nitrogenase components, was then loaded with buffered 0.4 M NaCl. This fraction was diluted 4-fold with Tris-HCl and applied to a 2.5 X 10-cm DEAE-cellulose column. A brown band containing the proteins of interest did bind to the column and was eluted with 0.5 M NaCl in Tris-HCl as two 30-ml fractions.

Gel Filtration—Ferredoxins and nitrogenase components were then resolved in five brown fractions by gel filtration on a 5 X 95-cm Ultragel AcA44 column equilibrated with 0.4 M NaCl in Tris-HCl. FdIII was collected at an elution volume of ~1.2 liters, diluted 3-fold in Tris-HCl, and concentrated on a small DEAE-cellulose column. FdIII was eluted as a 2-3-ml fraction in buffered 0.4 M NaCl.

Hydroxylapatite Chromatography—The concentrated ferredoxin fraction was then applied to a 1.6 X 15-cm hydroxylapatite column (HA-ultrogel) equilibrated in Tris-HCl. The column was washed with 50 ml of 5 mM potassium phosphate, pH 7.5, and then developed with a 200-ml linear gradient of phosphate from 0 to 50 mM.

Second DEAE-Cellulose Chromatography—The ferredoxin containing fraction was then adsorbed to a 1.6 X 25-cm DEAE-cellulose column which was developed with a 500-ml linear gradient of NaCl from 0.17 M to 0.4 M. FdIII was diluted 2-fold in Tris-HCl, concentrated on a small DEAE-cellulose column, and eluted as a 2-3-ml fraction in buffered 0.4 M NaCl.

Reversed-Phase HPLC—Ferredoxin was purified by reverse-phase HPLC on a 4.6 X 150-mm Econosphere C8 column (Alltech) at 40 °C.

Purification Procedure

All steps of purification were carried out under O2-free argon, and dithionite (2 mM) was added to buffers unless otherwise stated.

Cell extract was prepared from ~400 g of packed cells (equivalent to 120 liters of culture) thawed in 800 ml of 0.1 M Tris-HCl, pH 8.0, containing 15 mM EDTA. Cells were treated with lysozyme (0.3 mg/ml) at 30 °C for 15 min, then ruptured by sonication. The crude extract was transferred into 250-ml screw-capped bottles and centrifuged at 40,000 x g for 45 min at 4 °C. Other Proteins

The abbreviations used are: Rcl and Rc2, nitrogenase component enzymes from Boehringer Mannheim) in 50 mM sodium acetate, pH 5.5. Aliquots of the digest were taken at 1, 2, 5, and 20 min and analyzed for amino acids. The COOH-terminal sequence was deduced from the order of apparition of the amino acids in the digest.

Peptide mixtures were analyzed by reverse-phase HPLC on either a 3.9 X 300-mm µBondapak C18 column (Waters) at room temperature or a 4.6 X 150-mm Eclipse column (Alltech) at 40 °C. Columns were run with 0.1% trifluoroacetic acid in water and developed with acetonitrile using a HPLC gradient system operated through a Baseline M810 Software (Waters). Amino acid analyses were performed according to the Picotag method (Waters) after acidic hydrolysis of samples in 6 N HCl vapor for 20 h at 106 °C. Amino acid sequence was performed on an Applied Biosystems sequencer (model 477A), equipped with an on-line phenylthiohydantoin analyzer (model 120A). Sequence analyses and secondary structure predictions were performed using the DNASTAR software. For determination of COOH-terminal amino acids, HPLC-purified cm-FdIII was subjected to digestion with carboxypeptidase Y (Boehringer Mannheim) in 50 mM sodium acetate, pH 5.5. Aliquots of the digest were taken at 1, 2, 5, and 20 min and analyzed for amino acids. The COOH-terminal sequence was deduced from the order of apparition of the amino acids in the digest.

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Spectroscopic Analyses

UV visible absorption spectra were recorded using a diode-array spectrophotometer (Hewlett-Packard, model HP8452A). Redox titrations and transfer of samples into EPR tubes were carried out in an anaerobic glove box (Jacomex, France) under argon, containing less than 4 parts/million of O₂. Spectrophotometric measurement from a cuvette inside the glove box was made possible by connecting the cuvette holder to the HP8452 A spectrophotometer outside the glove box through optic fibers and adapters (Spectrofip system, Photometrics, France). FdIII was reduced either chemically by excess dithionite or photochemically by light-activated 5-deazaflavin. In the latter case, ferredoxin was made 25-50 μM in 10 mM Tris-HCl, pH 8.0, containing 25 mM sodium oxalate and 10-25 μM 5-deazaflavin. The ferredoxin sample placed in a 0.2-cm light-path quartz cuvette was exposed to the light of a 150-watt slide projector introduced into the glove box. Spectrophotometric changes were recorded after about 2 min of equilibration.

EPR spectra were recorded on a Varian E109 spectrometer. Samples were maintained at the indicated low temperatures with a helium flow cryostat (Oxford Instrument, ESR 900). Spins were quantified after double integration of the signals using a 200 μM Cu(II)-EDTA standard solution for calibration.

Biological Assays

NADP reduction tests were carried out as previously described (Hallenbeck et al., 1982a) using illuminated spinach chloroplast fragments as a source of reductant.

Ferredoxin-dependent nitrogenase assays were performed with either illuminated chloroplasts or photoactivated 5-deazaflavin as reductants. In the former case, stoppered-reaction vials (7.2 ml) contained chloroplasts, equivalent to 84 pg of chlorophyll a, 8 mM sodium ascorbate, 50 μM 2,6-dichlorophenol-indophenol, 15 μM dichlorophenyl-dimethylethylamine, 100 μM of an ATP-generating system (Burgess et al., 1980), and ferredoxin in a total volume of 470 μl. The gas-phase consisted of one-tenth acetylene in argon. The reaction was started by injecting 30 μl of a nitrogenase preparation consisting of purified component 1 and component 2 mixed at a molar ratio of 1:8, and stopped after 20 min of incubation at 30 °C in the light with 25 μl of 25% trichloroacetic acid. When 5-deazaflavin was used as reductant, vials contained 25 mM sodium oxalate, 130 μM 5-deazaflavin, 100 μl of ATP-generating system, and ferredoxin in a total volume of 470 μl. Other assay conditions were as described above, except that the flasks were illuminated from below using a 150-watt slide projector. Ethylene produced was measured by gas chromatography (Jouanneau et al., 1983).

Electron transport from or toward C. pasteurianum hydrogenase was tested in two ways. Hydrogenase-mediated ferredoxin reduction was followed spectrophotometrically in a 0.2-cm light path quartz cuvette equipped with a rubber septum. Ferredoxin was made 30 μM in 0.4 ml of 50 mM H₂-saturated Tris-HCl buffer, pH 8.5, C. pasteurianum hydrogenase (3.84 units; 1 unit refers to 1 pmol of H₂ produced/min at 30 °C, pH 8.0, in the presence of SDS (e401 = 0.300 M⁻¹ cm⁻¹)) was injected with a gas-tight syringe, and the extent of ferredoxin reduction was recorded by following the decrease of the chromophore absorption in the range 300-600 nm. Ferredoxin-dependent hydrogen evolution was assayed in 4 ml Wheaton vials fitted with rubber stoppers. Vials were sparged with argon, then filled to 0.49 ml with a mixture containing 10 mM sodium dithionite, 1 mg/ml bovine serum albumin, 50 mM Tris-HCl, and ferredoxin. Vials were incubated at 30 °C with shaking. The reaction was started by injecting 10 μl of C. pasteurianum hydrogenase and stopped after 30 min with 50 μl of 50% trichloroacetic acid. H₂ produced was estimated by gas chromatography as previously described (Kelley et al., 1977).

Other Assays

Proteins were assayed by Lowry’s method (Lowry et al., 1951) and, in the case of nitrogenase components, by the microbiuret method (Goa, 1953). Bovine serum albumin was used as protein standard. FdIII concentration was routinely estimated spectrophotometrically using ε₄₀₀ = 88.0 M⁻¹ cm⁻¹ and in some cases, by quantitative amino acid analysis. The Lowry assay overestimated FdIII concentration by a factor of 1.5. The iron and inorganic sulfide content were determined according to published procedures (Moulis and Meyer, 1982; Chen and Mortenson, 1977, respectively).

RESULTS

Purification and Molecular Properties—Nitrogenase as well as ferredoxins could be isolated together as a fraction, brown in color, from R. capsulatus cell-extracts by chromatography on DEAE-cellulose (see “Materials and Methods”). Upon subsequent gel filtration chromatography, five brown protein bands were separated including the two nitrogenase components and two previously characterized ferredoxins, FdI and FdII. A fifth colored protein, eluting with an apparent molecular weight of about 25,000, was further purified. A combination of chromatographic steps on hydroxylapatite and DEAE-cellulose yielded a preparation virtually homogeneous as judged from polyacrylamide gel electrophoresis (PAGE) in the presence of SDS (Fig. 1, lane b). The purified protein displayed general properties common to all ferredoxins (see below) and was therefore designated ferredoxin III (FdIII).

Following the purification procedure described under “Materials and Methods,” an average of 2.5 mg of FdIII could be obtained from 400 g of packed cells of R. capsulatus B10 (Table I). When a mutant deleted in the nitrogenase structural genes (strain RcM1) was used instead of B10, the yield of recovered FdIII was about twice as high. Interestingly, the amount of FdI obtained from this mutant was also found to...

Table I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein</th>
<th>Mg of protein recovered from*</th>
<th>Strain B10</th>
<th>Strain RcM1</th>
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<tr>
<td>Gel filtration on Ultrigel</td>
<td>RcI</td>
<td>388</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>AcA44 (step 2)</td>
<td>RcI</td>
<td>170</td>
<td>ND</td>
<td></td>
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<tr>
<td></td>
<td>FdII</td>
<td>19.5</td>
<td>35.6</td>
<td></td>
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<tr>
<td></td>
<td>FdIII</td>
<td>5.3</td>
<td>10.2</td>
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<tr>
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<td>2.52</td>
<td>5.65</td>
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<tr>
<td></td>
<td>DEAE-cellulose (steps 3 + 4)</td>
<td>FdI</td>
<td>2.52</td>
<td>5.65</td>
</tr>
</tbody>
</table>

* Proteins were purified from 390 and 375 g of packed bacteria for strains B10 and RcM1, respectively. The amounts of FdI and FdIII were determined from the absorbance at 400 nm using the specific extinction coefficient of each ferredoxin at this wavelength (ε₄₀₀ = 34.0 and 18.0 M⁻¹ cm⁻¹ for reduced FdIII and FdI, respectively).

<sup>a</sup> ND, not detected.
be consistently higher compared to the wild-type strain (Table I).

The molecular weight of the FdIII polypeptide was estimated to be about 11,000 by SDS-PAGE (Fig. 1). Upon gel filtration chromatography, native FdIII behaved like a protein with a molecular weight of 25,000 on Ultrogel AcA44 or of 39,000 on Sephacryl-S100HR. These results, suggesting that FdIII could be di- or trimeric, should be taken with caution since small highly charged polypeptides often behave atypically upon gel filtration chromatography. We therefore used another approach similar to that initially described by Hedrick and Smith (1968) which consisted of comparing the electrophoretic mobility of the ferredoxin in non-denaturating polyacrylamide gels of different concentrations with the mobility of proteins used as molecular weight markers. The size of FdIII, estimated by this technique, was 27,000 confirming the oligomeric nature of the ferredoxin.

The subunit structure of FdIII was then studied using imidoester cross-linking reagents. Two proteins of R. capsulatus having well established subunit structures, namely nitrogenase component 2 (Rc2), which is a homodimer (Hallenbeck et al., 1982b), and ferredoxin II, previously found to be monomeric (Jouanneau et al., 1990b), were subjected to cross-linking under identical conditions (Fig. 1). DMS generated cross-links between the two subunits of Rc2, as evidenced by the size of the product seen upon SDS-PAGE (Fig. 1, lanes m and n). A doublet is actually observed at 67,000 and 75,000 that may correspond to two different types of cross-links between the Rc2 subunits. DMS had no apparent effect on the mobility of FdIII, consistent with the monomeric nature of that ferredoxin. A slight difference of migration occurred in lane k (DMS-treated FdIII) compared to lane j (control), but this was not reproducible and probably arose from an electrophoretic artifact of this particular track. On the other hand, DMS did affect the migration pattern of FdIII since an additional band of M, = 23,000 was clearly visible upon SDS-PAGE (Fig. 1, lanes c–e). No band of higher molecular weight was detected. DMA was much less efficient in cross-linking FdIII as the upper band (M, = 23,000) was barely detectable (Fig. 1, lanes h and i). These experiments provide direct evidence that FdIII is a dimeric ferredoxin.

**Amino Acid Sequence Determination**—The FdIII polypeptide is composed of 100 amino acids and has a calculated molecular weight of 10,688. The amino acid composition determined after hydrolysis of the ferredoxin in 6 N HCl was in close agreement with that deduced from the sequence (Table II). The primary structure of the FdIII polypeptide as well as the sequencing strategy is presented in Fig. 2. The NH2-terminal sequence of carboxymethylated FdIII was determined by automated Edman degradation up to the 44th residue with uncertainties only at position 41 and 43. Further determination of the FdIII sequence was deduced from the analysis of the peptides generated by specific cleavage of FdIII using trypsin, chymotrypsin, and lysyl endopeptidase. The latter enzyme yielded six peptides L1 to L6, the index number indicating the position in the sequence. Peptides L1 and L2 contained 19 and 5 residues, respectively, and had amino acid compositions that exactly matched the already known NH2-terminal part of FdIII up to Lys54 (Table II and Fig. 2). Peptide L3 was found by sequence determination to span the Gly27-Lys30 region. This peptide is shorter by 2 residues (Cys6 and Ile6) than expected if one assumes that the protease cut at lysyl residues Lys24 and Lys50. It is therefore suggested that cleavage could also occur at the Ile6-Gly27 peptide bond. Peptide L4, encompassing the Val34-Lys50 region, displayed a characteristic UV absorbance spectrum revealing the presence of 1 tryptophanyl residue which passed undetected by regular amino acid analysis. Partial sequence determination of peptide L4 pointed out an overlap with the known NH2-terminal of the protein, and allowed to resolve the FdIII sequence up to the 60th residue. Peptide L5 (Lys50-Ser60) was completely sequenced, except for the last residue, thus providing the COOH terminus of FdIII. Peptide L6 was 1 lysyl residue shorter than L5, but both peptides were otherwise identical. Carboxypeptidase Y analysis of FdIII revealed the three COOH-terminal amino acids in the following order: Ala-Leu-Ser-COOH. At this stage, only a region of 18 residues (position 51–68) remained undetermined. Because the Trp26-Asp90 bond appeared to be resistant to chymotrypsin, digestion of FdIII

**Table II**

<table>
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<th>Amino acid</th>
<th>Residues/peptide</th>
<th>FdIII</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
<th>TC1</th>
<th>TC2</th>
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<td>4.5 (4)</td>
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<td>1.0 (1)</td>
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<td>2.1 (4)</td>
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<tr>
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<td>2.2 (2)</td>
<td>1.8</td>
<td>2.1 (4)</td>
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</table>

* Mean of 3 amino acid analyses after 18, 30, or 48 h of acid hydrolysis.

**TABLE II**

**Amino acid compositions of Cm-FdIII and of peptide derivatives obtained by digestion with lysyl endopeptidase (L1-L6) and by sequential cleavage with trypsin and chymotrypsin (TC1, and TC2)**

Values are from amino acid analysis after 20 h hydrolysis in 6N HCl vapor or from sequence determinations (in parenthesis).
Characterization of *R. capsulatus* Ferredoxin III

by this enzyme failed to provide peptides suitable for sequenc-
ing the unresolved region. On the other hand, peptides in-
cluding the region of interest could be obtained after two
subsequent digestion steps as follows. FdIII was first diges-
ted by trypsin, and the peptide containing the single trypth-
aphan of the ferredoxin sequence, as identified by its absorb-
ance, was purified. This peptide, named T7, was subse-
dently diges-
ted by α-chymotrypsin, yielding five peptides. Three short
peptides were identified as Asp-Val-Met, Ser-Leu-His-Gly,
and an heptapeptide corresponding to Asp98-Gly44 in the FdIII
sequence. The other two peptides, named TCI and TC2, differ-
yed by 4 residues (Table II) and were found by sequence
analysis to share 24 overlapping residues spanning the Leu48-
Lys58 region. Sequence determination of these peptides al-
lowed completion of the elucidation of the FdIII sequence.

Confirmation of the primary structure was obtained through
direct estimation of the FdIII polypeptide mass by electrospr-
ay ionization mass spectrometry (Fenn et al., 1989). The value
obtained for carboxymethylated FdIII is 11,210 ± 2 Da (Fig. 3), in good agreement with the calculated mass deduced
from the amino acid sequence 10,688 Da, incremented of nine
acetyl moieties (522 Da). Like other ferredoxins, FdIII con-
tains more acidic than basic residues resulting in an isoelectric
point of 5.1 (Table III), a value slightly higher than that
predicted by sequence analysis (calculated pI 4.64). Eight of
the 9 cysteines of the FdIII polypeptide appear properly
spaced to serve as ligands for two [4Fe-4S] clusters (Fig. 2).

Spectroscopic Properties—Dithionite-reduced FdIII sponta-
naneously oxidized upon removal of the dithionite on a Sep-
hadex G-25 column because of exposure to oxygen traces likely
present in the argon-saturated buffer. Such a preparation,
referred to as the oxidized form, yielded the absorption spec-
trum shown in Fig. 4. The spectrum is typical of a ferredoxin
containing [XFe-4S] clusters (X = 3 or 4), with maxima at
388 and 282 nm and a pronounced shoulder around 314 nm.
The ratio of absorbance A388/A282 was close to 0.67 for our
best preparations. Upon exposure to oxygen, the chromophore
absorption rapidly decayed indicating that the ferredoxin was
irreversibly inactivated through oxidative denaturation of the
Fe-S clusters. From the first-order kinetics of the absorption
decay, the half-life of FdIII in air was estimated to be 1.5 h.
Due to its high sensitivity to oxygen, FdIII had to be purified
under strictly anaerobic conditions with dithionite present in
buffers. Omission of dithionite resulted in a much lower
purification yield.

The extinction coefficients given in Table III have been
calculated based on the concentration of ferredoxin which
was determined by quantitative amino acid analysis, using
norleucine as an internal standard. The ε388 of the oxidized
form is close to 60 mM⁻¹·cm⁻¹, a value consistent with the
occurrence of four [4Fe-4S] clusters/dimer. Chemical deter-
minations of the iron and labile sulfur bound to the protein
also indicated that FdIII contained four [4Fe-4S] clusters
(Table III). Reduction of FdIII with excess dithionite at pH
8.0 brought about a bleaching of the chromophore absorp-
tion (Fig. 4) which was rapid within the first minutes and then
slowly reached a steady-state. Upon oxidative titration with
thionin, an estimated 3.4 electrons were exchanged per fer-
redoxin. These results indicate that FdIII can be considered
as a four-electron carrier and are consistent with FdIII being
a four-Fe-S cluster-containing ferredoxin. Since FdIII is a
homodimer, it is expected to contain two equivalent pairs of
Fe-S centers. Reductive titration of these centers was per-
formed at pH 8.0, either by stepwise addition of dithionite, or
by successive photoreduction steps using 5-deazaflavin as a
catalyst. The extent of FdIII reduction was determined by
recording the decrease of the chromophore absorption at 450

---

**Table III**

<table>
<thead>
<tr>
<th>Molecular properties of <em>R. capsulatus</em> FdIII</th>
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<tbody>
<tr>
<td>Molecular weight</td>
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<tr>
<td>By SDS-PAGE</td>
</tr>
<tr>
<td>By SOS-PAGE of cross-linked FdIII</td>
</tr>
<tr>
<td>By mass spectrometry</td>
</tr>
<tr>
<td>Isoelectric point</td>
</tr>
<tr>
<td>Iron-sulfur content</td>
</tr>
<tr>
<td>Fe (mol/mol)</td>
</tr>
<tr>
<td>S (mol/mo)</td>
</tr>
<tr>
<td>Reduced</td>
</tr>
<tr>
<td>Oxidized</td>
</tr>
</tbody>
</table>

* Determined on cm-FdIII.
* Calculated from the amino acid sequence.

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**Fig. 4. UV visible absorbance spectra of *R. capsulatus* FdIII.** Oxidized FdIII (57.5 μM, spectrum a) was obtained by desalting a sample of purified ferredoxin (containing 2 mM dithionite and 0.4 M NaCl) on a 1.5 × 15-cm Sephadex G-25 column preequilibrated in argon-saturated 10 mM Tris-HCl, pH 8.0. Remaining O2 traces re-
sulted in the oxidation of the ferredoxin. Reduced FdIII (56 μM, spectrum b) was prepared by equilibrating the same desalted sample with dithionite (2 mM) for 15 min. Samples were placed in a 0.2-cm light path cuvette inside a glove box maintained under argon. Spectra were recorded with a diode-array spectrophotometer through an optic fiber coupling system (see "Materials and Methods").

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**Fig. 3. Electrospray mass spectrum of cm-FdIII.** The protein was introduced into the ion-source of the spectrometer as a 20 μM aqueous solution in 0.1% trifluoroacetic acid. About 40 pmol of protein was used for the analysis. The mass spectrum is the resulting sum of several 4-s scans recorded in the m/z range 450–2,000 with one unit mass resolution. Peaks correspond to multiprotonated forms of cm-FdIII. The net charge of each ion is indicated above the peaks. The molecular mass of cm-FdIII, 11,210 ± 2 Da, is the average of the values determined for each peak.
Characterization of R. capsulatus Ferredoxin III

The redox potential at each step of the titration was estimated by using methyl viologen as an internal indicator, according to the method described by Yoch and Arnon (1972). The extent of reduction of methyl viologen at each step, calculated from the absorbance at 604 nm, served to calculate the intermediate redox potentials using the Nernst's equation and parameters previously defined by Mayhew (1978) for methyl viologen: $E^\circ = -446$ mV. The observed absorbance changes at 450 nm upon reduction of FdIII could not be correlated with two waves of half-reduction, corresponding to titration of each cluster type. It is therefore suggested that the midpoint redox potentials of each cluster are too close to be distinguished by the technique used. Consistent with this assumption, the EPR analysis described below provides evidence that, upon limited reduction of FdIII (20% extent), both types of cluster appeared to be partially reduced. The values of the midpoint potentials are expected to be near $-440$ mV at pH 8.0, since half-reduction of FdIII was reached at this redox potential.

FdIII was also analyzed by EPR spectroscopy. The spectrum of the oxidized form, shown in Fig. 5, appeared to be composed of two weak signals, a broad rhombic resonance with $g$ values at 2.07, 1.91, and 1.87 attributed to a [4Fe-4S] cluster with a $S = 1/2$ spin state and a resonance near $g = 2.00$ which may be indicative of a [3Fe-4S] in the oxidized form. The latter signal accounted for less than 0.01 spin/FdIII monomer and could arise from limited [4Fe-4S] to [3Fe-4S] interconversion. The rhombic signal, which accounted for less than 0.1 spin/FdIII monomer, may be ascribed to a partially reduced [4Fe-4S] center. Using 5-deazaflavin as a photocatalyst, the ferredoxin was then reduced to an extent of approximately 20%, as calculated from the decrease of the chromophore absorption at 450 nm. EPR analysis of FdIII at this stage of reduction revealed a complex spectrum, which resulted from the superimposition of at least two independent rhombic signals (Fig. 5, spectrum b). One signal was similar to that of oxidized FdIII (spectrum a) but with increased intensity, and the other showed distinct features at $g = 2.04$, 1.94, and 1.88. The calculated overall contribution of the signals in spectrum b accounted for approximately 0.5 spin/FdIII monomer. These data suggested that the 20% reduced FdIII contained two partially reduced [4Fe-4S] centers which had no detectable magnetic interaction between each other. Further reduction of FdIII (50 and 75% reduction) promoted the appearance of a broad EPR signal centered at $g = 1.94$ with the concomitant disappearance of the distinct features observed at 20% reduction (data not shown). The spectrum of the fully reduced FdIII is shown in Fig. 5, trace c. Double integration of the signal yielded an estimated value of $-2.0$ spins/FdIII subunit, thus providing additional evidence that the FdIII polypeptide bound two [4Fe-4S] clusters. The broad signal at $g = 1.94$ was optimally observed at temperatures below 10 K, broadened at higher temperatures, and became barely detectable above 40 K. These observations indicated that the EPR signals of fully reduced FdIII had fast relaxation times, comparable to those observed for other ferredoxins containing two [4Fe-4S] clusters. Such features have been interpreted as being a consequence of spin-spin interactions between the clusters (Mathews et al., 1974). However, due to the dimeric nature of FdIII, additional intercluster interactions might also be expected, depending on the relative proximity of these centers in the dimer.

**Electron Transfer to R. capsulatus Nitrogenase and C. pasteurianum Hydrogenase**—The biological activity of various ferredoxins has been measured in *in vitro* assays where either nitrogenase or *C. pasteurianum* hydrogenase served as electron acceptors (Carter et al., 1980; Fitzgerald et al., 1980). When supplied with illuminated plant chloroplasts as a source of strong reductants, ferredoxin I (FdI) from *R. capsulatus* promoted nitrogenase activity in a concentration-dependent fashion (Table IV). 5-Deazaflavin-reduced FdI was also found to act as an electron donor to the nitrogenase complex. On

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**Table IV**

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Nitrogenase activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Electron donor</th>
<th>Hydrogenase activity&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>Electron donor</td>
<td>Hydrogenase activity&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>FdIII</td>
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</table>

<sup>a</sup> Nmole of C<sub>2</sub>H<sub>4</sub> reduced/min/nmol Rc2. Each assay vial contained 0.16 nmoles of Bchl and 1.25 nmoles of Rc2, and a source of reductant, either illuminated chloroplast fragments (I) or photoactivated 5-deazaflavin (II). Control nitrogenase assay, with 10 mM dithionite as a reductant, yielded 13.0 nmoles of C<sub>2</sub>H<sub>4</sub> reduced/min/nmol Rc2.

<sup>b</sup> Milli units. Assays were carried out at pH 8.5 with 10 mM dithionite as reductant and 0.48 unit, pH 8.0, of *C. pasteurianum* hydrogenase.

<sup>c</sup> MV, methyl viologen.
the other hand, no nitrogenase activity was detectable when FdIII was tested under similar conditions with either source of reductants. Since spectrophotometric measurements showed that FdIII was readily reduced by 5-deazaflavin, it is inferred that the lack of FdIII-mediated nitrogenase activity resulted primarily from the failure of reduced FdIII to transfer electrons to nitrogenase.

FdIII, tested over a wide range of concentrations, was also incompetent in the H₂ evolution assay, linking the reducing power of dithionite to C. pasteurianum hydrogenase activity (Table IV). On the other hand, FdI was found to be a suitable mediator and the rate of H₂ evolution appeared to be dependent on FdI concentration. Besides, C. pasteurianum hydrogenase apparently did not serve as an electron donor to FdIII, since no reduction of the ferredoxin occurred upon incubation with the hydrogenase under H₂ at pH 8.0 and 8.5 (data not shown). These results suggest a lack of interaction between FdIII and C. pasteurianum hydrogenase. In addition, FdIII failed to substitute for plant ferredoxin in the photoreduction of NADP by illuminated chloroplasts. The lack of detectable electron transfer activity of FdIII in the various systems tested suggests that this ferredoxin has a relatively high functional specificity in R. capsulatus.

Regulation of the FdIII Biosynthesis—The presence of the FdIII polypeptide in cell extracts of R. capsulatus was examined by Western blot analysis. Bacteria were from cultures grown either in the light by photoheterotrophy or in darkness by fermentation of fructose in the presence of dimethyl sulfide as an accessory oxidant (Yen and Marrs, 1977). The nitrogen source in the cultures was either growth-limiting (N₂ or 2 mM glutamate) or in excess (15 mM NH₄). FdIII was detected in cells grown on N₂ or a limiting source of fixed nitrogen. The synthesis was not dependent on light since cells grown in the dark did not show any FdIII (data not shown). These results suggest a lack of interaction between FdIII and C. pasteurianum hydrogenase. In addition, FdIII failed to substitute for plant ferredoxin in the photoreduction of NADP by illuminated chloroplasts. The lack of detectable electron transfer activity of FdIII in the various systems tested suggests that this ferredoxin has a relatively high functional specificity in R. capsulatus.

Discussion

Ferredoxin III from R. capsulatus, which has been purified and biochemically characterized in this study, is one of the four ferredoxins so far identified in this bacterium (Jouanneau et al., 1991). Its primary structure determined by direct peptide sequencing was found to be identical to that deduced from ORF5, a gene identified by DNA sequence analysis as part of a nif operon (Moreno-Vivian et al., 1989). The latter authors found ORF5 to be preceded by two ATG and predicted the second ATG to be the most probable initiation codon. The protein sequence determined in this work (Fig. 2) validates their prediction and further indicates that the initial methionyl residue was removed upon maturation of the FdIII polypeptide. Since ORF5 has been shown to encode a ferredoxin, namely FdIII, we proposed to rename the gene fdxB (Jouanneau et al., 1990a).

The FdIII primary structure is characterized by the occurrence of two groups of 4 cysteine residues showing the spacing pattern, C-X-C-X-C-X-C-X-C, typical for ferredoxins containing two [4Fe-4S] clusters. Consistent with the likely presence of two FeS-binding sites in the FdIII sequence, biochemical and spectroscopic evidence presented herein strongly supported the conclusion that each FdIII polypeptide bound two [4Fe-4S] clusters. When compared with other dicluster ferredoxins, the FdIII amino acid sequence displayed several unique features. In the cysteine group nearest to the NH₂ terminus, the 4th cysteine is not followed by a proline, like in most ferredoxins, but by a glycine (Gly⁷⁰). Proline replacement in a similar position has been observed in only three out of more than 50 sequences of bacterial ferredoxins reported in the data banks. These include Desulfovibrio africanus FdIII (Boivier-Lapierre et al., 1987) and D. vulgaris Miyazaki Fd (Okawara et al., 1986), and R. capsulatus FdI (Schatt et al., 1989). More importantly, the FdIII polypeptide is almost twice as long as most [4Fe-4S] ferredoxins due to an extended NH₂ terminus and to the occurrence of a 43-residue long sequence between the 2 groups of cysteines. Comparable sequence features have only been reported for two related archaeabacterial ferredoxins isolated from Thermoplasma acidophilum (Wakabayashi et al., 1983) and Sulfolobus acidocaldarius (Minami et al., 1985), respectively. However, these two ferredoxins have no obvious sequence similarity with FdIII. In other dicluster ferredoxins, the region between the 2 groups of cysteines is usually 18 residues long, and the folding of the polypeptide chain encompassing this region and the cluster-binding sites has been found to be very similar in P. aerogenes Fd and A. vinelandii FdI, representative of 8Fe and 7Fe ferredoxins, respectively (Adman et al., 1974; Stout, 1989). In this respect, the FdIII polypeptide could be expected to have a distinctive chain folding.

Based on secondary structure predictions, the FdIII polypeptide would consist of 53% α-helix, a high score compared to other ferredoxins, which generally have little secondary structures. Interestingly, the region delimited by the 2 groups of cysteines (Gly³⁶-Ala⁷⁸) would be essentially α-helical (Asp⁴⁸-Val⁵⁰ and Thr⁶⁵-Ala⁷⁶) with a small β turn in the middle (Ala⁵¹-Glu⁵⁶). Consistent with the dimeric nature of FdIII, it is tempting to speculate that the putative α-helical regions might be involved in the association of the FdIII subunits and/or the stabilization of the dimer.

As previously pointed out (Moreno-Vivian et al., 1989), FdIII resembles the NifJ protein, a nif-specific oxidoreductase of Klebsiella pneumoniae, in that both proteins possess two 4-cysteine motifs ([4Fe-4S]-binding sites) separated by 43 residues. Although the NifJ polypeptide is much bigger than FdIII (120 kDa; Shah et al., 1983; Arnold et al., 1988), it is also a dimer and the 43-residue long region specified above is predicted to be mostly α-helical, like the corresponding region of FdIII (prediction based on our sequence analysis).

Ferredoxins are mostly monomeric proteins although a few cases of oligomeric forms have been reported, mainly in Desulfovibrio species (Bruschi et al., 1976; Hatchikian et al., 1979; Guerlesquin et al., 1980). The Desulfovibrio ferredoxins just mentioned consist of a single small subunit (M, ~6000) accommodating a single 3Fe or 4Fe cluster (Kissinger et al., 1991). D. gigas FdI and FdII, which have been shown to represent two oligomeric forms of the same basic subunit (a trimer and a tetramer, respectively), have distinct functions in the phosphorolytic reaction and in sulfate reduction, respectively (Bruschi et al., 1976). This example illustrates the physiological significance of oligomerization in the ferredoxins from Desulfovibrio. As for ferredoxins from other sources, except for the [2Fe-2S] ferredoxin from C. pasteurianum which was shown to be dimeric (Meyer et al., 1984, 1986), none has been unambiguously demonstrated as forming oligomers in the native form. In this respect, proposed oligomeric forms based on a single criterion (such as the relative mobility upon gel filtration) should be regarded with caution, since ferredoxins often have atypical electrophoretic and chromatographic mobilities, and some of them, particularly those O₂-labile, have a tendency to form aggregates upon oxidative
Characterization of R. capsulatus Ferredoxin III

The function of FdIII is probably associated with nitrogen fixation because, on the one hand, its biosynthesis appeared to be regulated according to the availability of fixed nitrogen (this study) and on the other hand, its structural gene was found to be part of a nif operon (Moreno-Vivian et al., 1989).

FdIII has been found to be non-essential for nitrogen fixation as deduced from interposon-mutagenesis experiments. A role for this ferredoxin in the biosynthesis of FeMo-cofactor of nitrogenase was suggested, based on the observation that fdxB (ORF5) is cotranscribed with the nifE, nifN, nifX, and nifQ genes known to participate in this process (Moreno-Vivian et al., 1989). In addition, electron transfer assays indicated that, contrary to Fd, FdIII did not serve as an electron donor to the nitrogenase complex (Table III). Since FdIII was also incompetent in electron transfer reactions often used to assay ferredoxins, it is inferred that this ferredoxin may have a relatively specific function, perhaps correlated with its dimeric structure. In this respect, we cannot rule out the possibility that FdIII catalyzes some as yet unknown enzymatic reaction.

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REFERENCES


Characterization of R. capsulatus Ferredoxin III