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Characterization of Ferredoxin, Flavodoxin, and Rubredoxin from *Clostridium formicoaceticum* Grown in Media with High and Low Iron Contents

STEVE W. RAGSDALE AND LARS G. LJUNGDAHL*

Department of Biochemistry, University of Georgia, Athens, Georgia 30602

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Ferredoxin, flavodoxin, and rubredoxin were purified to homogeneity from *Clostridium formicoaceticum* and characterized. Variation of the iron concentration of the growth medium caused substantial changes in the concentrations of ferredoxin and flavodoxin but not of rubredoxin. The ferredoxin has a molecular weight of 6,000 and is a four iron-four sulfur protein with eight cysteine residues. The spectrum is similar to that of other ferredoxins. The molar extinction coefficients are 22.6×10^3 and 17.6×10^3 at 280 and 390 nm, respectively. From 100 g wet weight of cells grown with 3.6 μM iron and with 40 μM iron, 5 and 20 mg of ferredoxin were isolated, respectively. The molecular weight of rubredoxin is 5,800 and it contains one iron and four cysteines. The UV-visible absorption spectrum is dissimilar to those of other rubredoxins in that the 373 nm absorption peak is quite symmetric, lacking the characteristic 350-nm shoulder found in other rubredoxins. The flavodoxin is a 14,500-molecular-weight protein which contains 1 mol of flavin mononucleotide per mol of protein. It forms a stable, blue semiquinone upon light irradiation in the presence of EDTA or during enzymatic reduction. When cells were grown in low-iron medium, flavodoxin constituted at least 2% of the soluble cell protein; however, it was not detected in extracts of cells grown in high-iron medium. The rubredoxin and ferredoxin expressed during growth in low-iron and high-iron media are identical as judged by iron, inorganic sulfide, and amino acid analysis, as well as light absorption spectroscopy.

Ferredoxin, flavodoxin, and rubredoxin are low-molecular-weight electron-carrier proteins which are important in anaerobic metabolism. In the metabolism of the acetogenic bacteria, ferredoxin is an electron acceptor for numerous low-potential oxidation-reduction reactions, including pyruvate:ferredoxin oxidoreductase (9, 38, 44), hydrogenase (8; Pezacka and Wood, Arch. Microbiol., in press), NADH:ferredoxin oxidoreductase (38, 44), and CO dehydrogenase (6, 17, 34). Flavodoxin has been shown to substitute for ferredoxin in many redox reactions, but at a slower rate of electron transfer (20, 21). Rubredoxin has been found to substitute for ferredoxin in a few oxidation-reduction reactions, but usually at a very low rate, since the redox potential of rubredoxin is approximately 400 mV higher than that of ferredoxin. Recently, rubredoxin was found to be a superior electron acceptor in the CO dehydrogenase reaction in acetogenic bacteria (33, 34). The properties of rubredoxin have been reviewed (10).

We studied these three electron carriers in *Clostridium formicoaceticum* since flavodoxin has not been isolated from acetogenic bacteria and ferredoxin (12, 44) and rubredoxin (43) have been purified from only one acetogen, *Clostridium thermoaceticum*. Characterization of the electron carriers involved in the acetate biosynthetic pathway is important, since the acetogens carry out a reduction of CO_2 to acetate in a sequence of reactions of four two-electron transfers from some electron donor (CO, H_2, pyruvate, glucose, etc.) to a pathway involving formate dehydrogenase (42) and tetrahydrofolate enzymes (22).

MATERIALS AND METHODS

Thioglycolic acid, DEAE-Sephadex A50-120, and Sepharose CL-6B-200 were obtained from Sigma Chemical Co. Ultrapure hydrochloric acid was from Alfa Thiokol/Ventrol. Ultrogel AcA chromatography gels were from LKB Instruments and Sephadex gels were from Pharmacia. DEAE-cellulose was from Whatman Ltd.

Growth of *C. formicoaceticum*. *C. formicoaceticum* ATCC 23439, described by Andreesen et al. (1), was maintained and grown by using the medium of Moore et al. (29) containing 3.6 μM iron as described earlier (2). When high-iron medium was used, the concentration of ferrous ammonium sulfate was 40 μM.

Analytical methods. Protein concentration was determined by the rose bengal dye-binding assay (11), using ovalbumin as standard, or by the extinction coefficients, which were determined as described below. Amino acid analysis was carried out on the homogenous electron carrier proteins by first dialyzing samples for 36 h against double-distilled water and by hydrolyzing in vacuo in ultrapure 6.0 N HCl for 12, 24, 48, and 72 h at 110°C. Tryptophan was determined by acid hydrolysis in 4.0 and 6.0% thioglycolic acid (26), and cysteine was determined as cysteic acid (16). Norleucine was included as internal standard. The partial specific volume was calculated from the amino acid composition (3). The extinction coefficients were calculated by using amino acid analyses to determine the nanomoles of protein relative to the absorbance at a given wavelength. The rose bengal protein assay was standardized for each protein by amino acid analysis.

Sedimentation equilibrium and velocity experiments were performed with a Beckman model E ultracentrifuge equipped with absorption optics. In velocity experiments, the sedimentation coefficient was determined by the moving boundary method (35), using the schlieren peak to detect the position of the boundary as a function of time. Temperature was maintained at 20°C during the velocity and equilibrium experiments. All samples were run in 50 mM potassium phosphate–0.3 M potassium chloride buffer. The sedimentation coefficient was corrected to the condition of water at

* Corresponding author.

TABLE 1. Properties of ferredoxin, rubredoxin, and flavodoxin from *C. formicoaceticum*

Protein	Mol wt determined by:				Partial specific volume[a]	Stokes radius[b]	Iron per mol of protein[c]
	Gel filtration	Sedimentation equilibrium	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis	Amino acid analysis			
Ferredoxin	5,200 ± 500	6,700 ± 200		6,000 ± 100	0.663	9.5 ± 0.4	4.6 ± 0.4
Rubredoxin	5,600 ± 500	5,900 ± 100		5,800 ± 50	0.715	10.0 ± 0.4	1.2 ± 0.2
Flavodoxin	14,000 ± 1,000	16,500	14,500 ± 500	14,000 ± 1,000	0.731	17.4 ± 0.7	ND

20°C. The molecular weights were calculated according to the Svedberg equation (35), assuming a one-component system.

The Stokes radii and molecular weights were determined by using carbonic anhydrase, cytochrome *c*, ovalbumin, chymotrypsinogen A, and RNase A as standards. The correlation coefficient of the line obtained in the standardization was 0.992.

Acid-labile sulfide was analyzed (32) by using sodium sulfide as the standard. Iron was determined with bathophenanthroline (7) and by plasma emission spectroscopy (19). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by the method of Weber et al. (41), and standard alkaline electrophoresis was performed with Tris-glycine (5). Preparative electrophoresis was carried out with an LKB 7900 Uniphor system with pH 8.0 Tris-barbital (14).

Flavodoxin was immobilized to Sepharose CL-6B as described by Mayhew and Strating (28) after cyanogen bromide activation (4).

Purification of flavodoxin, rubredoxin, and ferredoxin from *C. formicoaceticum* cells. Figure 1 gives a summary of the purification procedure. This procedure describes the purification from low-iron-grown cells; however, identical steps were used in the purification of rubredoxin and ferredoxin from high-iron-grown cells.

Crude extract. Frozen cells (205 g) were suspended in 600 ml of 50 mM potassium phosphate buffer, pH 7.2, and extracted as described previously (2). The suspension was centrifuged in a type 35 Beckman rotor at 57,000 × *g* for 90 min. The supernatant (21,000 mg of protein) was used in the purification procedure.

DE23 cellulose. The supernatant of the ultracentrifugation step was applied to a DE23 cellulose column (5.5 by 38 cm) which was washed with 50 mM potassium phosphate, pH 7.2. The top layer of this column containing the electron carriers was removed with a spatula and placed on a fresh bed of DE23 cellulose equilibrated with Tris-hydrochloride (0.2 M, pH 7.6). The column was washed with 0.25 M Tris-

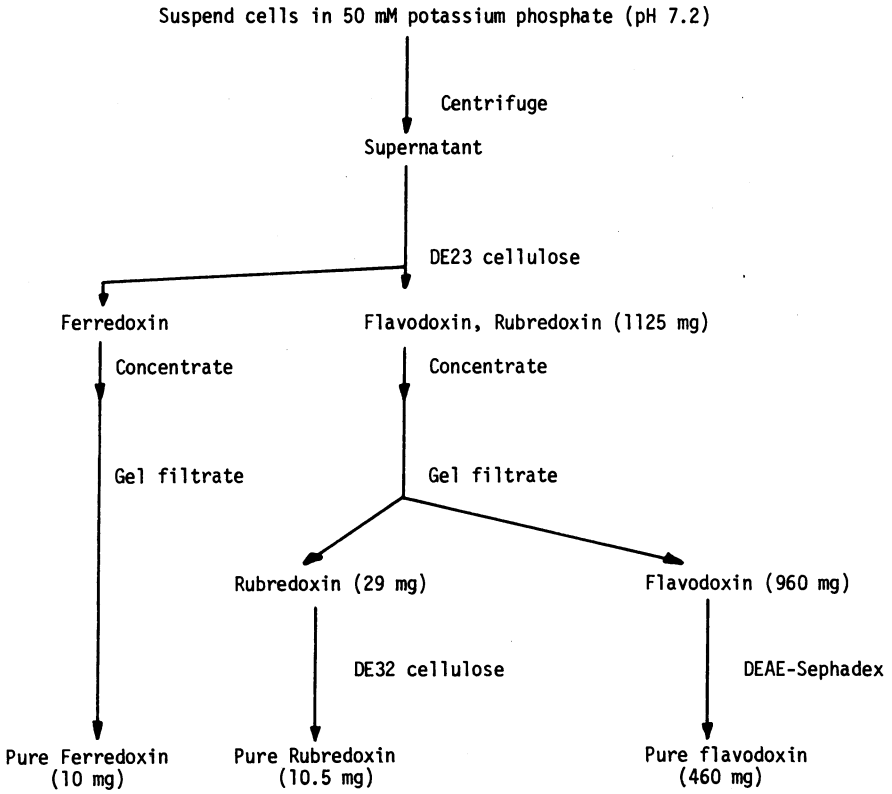

FIG. 1. Purification of rubredoxin, flavodoxin, and ferredoxin from 205 g of *C. formicoaceticum* cells grown in low-iron medium. The final yields of ferredoxin and flavodoxin were dependent upon the concentration of iron in the medium. See the text for details.

TABLE 1. (Continued)

Sulfur per mol of protein	Sedimentation coefficient ($S_{20,w}$)	Molar extinction coefficient (liter mol^{-1} cm^{-1}) at 280 nm	390 nm	490 nm	272 nm	373 nm	444 nm
4.3 ± 0.4	ND[d]	22,600	17,600				
0	ND	18,200		7,300		9,200	
0	2.1	ND			38,600	6,500	7,700

[a] Determined from amino acid analysis.
[b] Determined from gel filtration.
[c] Average of iron determinations by the Doeg-Ziegler method and by plasma emission spectroscopy, using the amino acid analysis for the determination of the protein concentration.
[d] ND, Not determined.

hydrochloride extensively before the flavodoxin and rubredoxin were eluted together with 0.3 M Tris-hydrochloride. The 2-liter eluate was concentrated to 50 ml by using a DE32 cellulose column (2.6 by 20 cm) and then to 15 ml with an Amicon UM-2 ultrafiltration membrane. This concentrate contained 1,125 mg of protein when cells grown in low-iron medium were used. Ferredoxin was eluted from the first DE23 column after the elution of flavodoxin and rubredoxin with 0.5 M Tris-hydrochloride.

Tandem gel filtration. The rubredoxin-flavodoxin concentrate from the DE23 step was applied to a tandem AcA 54-AcA 44 column setup (both 2.6 by 85 cm). When run in low-salt buffers, flavodoxin seemed to aggregate, so 0.2 M KCl was included in the 50 mM Tris buffer. Under these conditions, flavodoxin eluted in a tight band. The flavodoxin solution contained 960 mg of protein, whereas the rubredoxin eluate had 29 mg of protein. Rubredoxin eluted well-separated from flavodoxin.

The ferredoxin from DE32 was applied to the same gel filtration setup and run in the same buffer system. The resulting ferredoxin was pure as judged by the absorption ratio (absorbance at 390 nm $[A_{390}]/A_{280}$), and by sedimentation velocity and equilibrium experiments.

DE32 cellulose. Rubredoxin from the tandem column was adsorbed to a 5-ml DE32 cellulose column equilibrated with Tris-hydrochloride (0.1 M), and a 200-ml linear gradient from 0.2 to 0.5 M Tris-hydrochloride was run. The red, rubredoxin-containing fraction was concentrated on a small DE32 cellulose column from 75 to 2.5 ml. The yield of rubredoxin was 10.5 mg with an absorption ratio (A_{280}/A_{490}) between 2.35 and 2.50.

The rubredoxin from this step was judged to be pure by sodium dodecyl sulfate and alkaline electrophoresis, sedimentation velocity, and absorbance ratio. In sedimentation equilibrium experiments the ln of the concentration versus r^2 plot was linear, and the molecular weights calculated with both 490- and 280-nm scans were equivalent, which also indicates homogeneity.

DEAE-Sephadex chromatography and preparative electrophoresis. Final purification of flavodoxin could be accomplished by either DEAE-Sephadex chromatography or preparative electrophoresis. Flavodoxin from the tandem column was absorbed to a DEAE-Sephadex column (2.6 by 22 cm) in 50 mm Tris-hydrochloride (pH 7.6) and eluted with a 2-liter gradient of 0 to 0.25 M sodium chloride in 0.1 M Tris-hydrochloride. The yield of flavodoxin was 460 mg with an absorption ratio (A_{272}/A_{373}:A_{444}/A_{373}) of 6.36:1.28. Preparative electrophoresis, described above, yielded a protein with the same characteristics as that obtained in the DEAE-Sephadex step. It was essential to use the pH 8.0 Tris-barbital system in preparative electrophoresis since the Tris-glycine pH 8.9 system caused loss of the flavodoxin cofactor, flavin mononucleotide. Dissociation of flavin mononucleotide from the protein is reversible, however, as shown below. The protein from this step was homogenous as judged by alkaline and sodium dodecyl sulfate electrophoresis and sedimentation equilibrium and velocity experiments.

RESULTS

Effect of iron on the relative amounts of electron carriers. When grown in medium containing only 3.6 μM iron salts, flavodoxin is a major component of the soluble cell protein. Approximately 2% of the soluble cell protein (230 mg per 100 g wet weight of cells) was recovered as homogenous flavodoxin. Under these iron-poor conditions, approximately 5 mg of ferredoxin could be recovered per 100 g wet weight of cells. When the medium was supplemented with 40 μM of iron, the amount of ferredoxin that could be recovered as the homogenous protein increased four- to fivefold. Flavodoxin was undetectable in extracts of high iron-grown cells. Rubredoxin amounted to 3 to 5 mg per 100 g of cells, and the amount was unaffected by addition of supplemental iron to the growth medium.

Properties of electron transfer proteins from *C. formicoaceticum*. The properties of the electron transfer proteins are summarized in Table 1. The amino acid compositions, iron and inorganic sulfur contents, and molecular weights of ferredoxin, rubredoxin, and flavodoxin were the same regardless of whether the cells were grown in high- or low-iron medium. Only one type of ferredoxin or rubredoxin was seen during chromatography in the purification procedures for high- or low-iron-derived proteins, which is in contrast to results with *C. thermoaceticum*, which contains two ferredoxins (12, 44) and two rubredoxins (43).

Ferredoxin. The average molecular weight of ferredoxin, calculated from gel filtration, amino acid analysis, and sedimentation equilibrium, was 6,000. Amino acid analysis (Table 2) gave eight cysteines and a partial specific volume of 0.663 ml/g. By both plasma emission spectroscopy and the method of Doeg and Ziegler (7), approximately four irons were found per mole of protein. Four acid-labile sulfides were found per mole protein. The molar extinction coefficients were 22,600 at 280 nm and 17,600 at 390 nm. It was essential to determine protein concentration by using amino acid analysis including an internal standard. Use of the rose

TABLE 2. Amino acid analysis of ferredoxin, rubredoxin, and flavodoxin from *C. formicoaceticum*

Amino acid	Ferredoxin	Rubredoxin	Flavodoxin
Asx	8	8	7
Thr	2	2	4
Ser	2	1	3
Glx	5	5	28
Pro	4	6	4
Gly	4	6	21
Ala	8	3	9
Cys	8	4	1
Val	5	6	8
Met	1	1	9
Ile	4	2	3
Leu	0	0	14
Tyr	2	3	2
Phe	0	2	2
His	0	0	0
Lys	1	3	8
Arg	0	0	2
Trp	0	1	1

bengal (11) or Lowry et al. (24) assay gave values for protein concentration that were seriously in error. The purified ferredoxin had an absorbance ratio (A_{390}/A_{280}) of between 0.74 to 0.78 and a spectrum similar to those of other ferredoxins (30).

Rubredoxin. The average molecular weight of rubredoxin was 5,800. As in other rubredoxins, four cysteines were found. The number of irons per mole was approximately 1 to 1.5; however, since the extinction coefficients, absorption ratio (A_{280}/A_{490}), and cysteine content were so similar to those of the one-iron rubredoxin, we feel that nonessential iron must be absorbed to the protein. Plasma emission spectroscopy revealed the presence of 3.0 Ca, 8.6 K, 4.2 Na, and 2.0 P per mol protein, so it is reasonable that nonfunctional iron could also be bound. By absorbing the purified rubredoxin to DEAE-cellulose and then washing with pH 5.5 sodium citrate buffer (0.1 M), lower iron readings (approximately 1.0 mol of Fe per mol of protein) than those found in the nontreated sample were obtained.

The spectrum of rubredoxin was unlike that of other rubredoxins (Fig. 2) in that the 373-nm peak was quite symmetric. Thus, the *C. formicoaceticum* rubredoxin lacks a shoulder at 350 nm that is characteristic of other rubredoxins (10, 23, 43). The same spectrum was obtained from rubredoxins isolated from both high- and low-iron-derived cells.

Rubredoxin from *C. formicoaceticum* contains no histidine or arginine (Table 2), a common characteristic of rubredoxins. Tryptophan also is present in this rubredoxin. The partial specific volume is 0.715 ml/g.

Flavodoxin. The average molecular weight of flavodoxin is 14,500. Extinction coefficients are shown in Table 1. A partial specific volume of 0.730 ml/g was obtained from the amino acid composition (Table 2). The spectrum (Fig. 3) of the stable semiquinone formed by treatment with light in the presence of EDTA (25) or CO plus CO dehydrogenase was very similar to that of the *Megasphaera elsdenii* flavodoxin (27). The cofactor was identified as flavin mononucleotide by coupling the protein to a Sepharose-CL-6B and eluting cofactor with 5% trichloracetic acid plus 0.3 mM EDTA as described by Mayhew et al. (28). Flavin adenine dinucleotide, flavin mononucleotide, and riboflavin were equilibrated with the column, but only flavin mononucleotide would bind to the Sepharose-bound apoprotein.

DISCUSSION

The concentration of iron in the growth medium significantly affects the relative concentrations of ferredoxin and flavodoxin, but not of rubredoxin, in cells of *C. formicoaceticum*. Similar findings have been reported for *M. elsdenii* (27) and *Clostridium pasteurianum* (20). With *C. pasteurianum*, the maximal amount of ferredoxin is formed by bacteria grown in media containing 40 μM of iron salts (20). From 100 g of *C. formicoaceticum* cells grown in 40 μM iron medium we isolated over 20 mg of ferredoxin but found no evidence for a flavodoxin. In contrast, from the same amount of cells grown with 3.6 μM iron in the medium, only about 5 mg of ferredoxin was isolated, whereas, flavodoxin constituted almost 2% of the soluble protein.

It is apparent that ferredoxin is replaced by flavodoxin in cells grown in low-iron media. In addition, Schönheit et al. (36) have shown that degradation of ferredoxin occurs in *C. pasteurianum* during periods of iron deprivation and it is utilized as a source of iron. Therefore, it is interesting that under low-iron conditions *C. formicoaceticum* and *M. elsdenii* (27) produce significant amounts of rubredoxin. This may indicate an important role for rubredoxin in these bacteria. However, the only postulated role so far for rubredoxin in anaerobic bacteria involves CO dehydrogenase, since rubredoxin is the most active acceptor of CO dehydrogenase electrons in the acetogenic bacteria (33, 34).

The molecular weights and other properties of ferredoxin, flavodoxin, and rubredoxin from *C. formicoaceticum* were similar to those reported for those proteins from other bacteria. By using amino acid analysis to determine the amount of protein and plasma emission spectroscopy as well as a colorimetric method to determine iron, we found that ferredoxin contained only four Fe and four S per mole of protein. The extinction coefficient, using the same absolute method for determination of protein concentration, agreed with the conclusion that the *C. formicoaceticum* ferredoxin

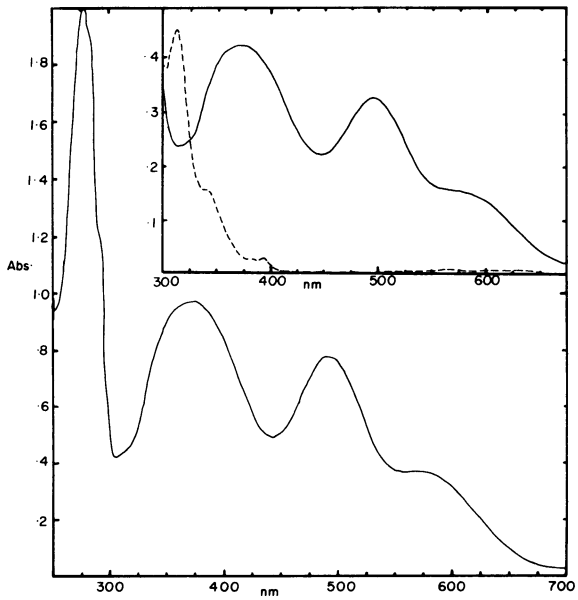

FIG. 2. Light absorption spectrum of *C. formicoaceticum* rubredoxin, 0.64 mg/ml in 50 mM Tris-hydrochloride, pH 7.6. Inset shows rubredoxin, 0.25 mg/ml, oxidized (———) and enzymatically reduced with *C. thermoaceticum* CO dehydrogenase (----) by bubbling with CO for 5 min before addition of enzyme.

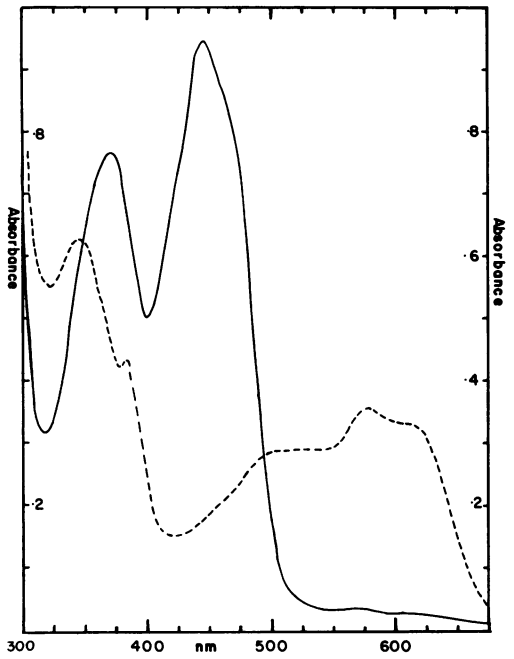

FIG. 3. Light absorption spectrum of *C. formicoaceticum* flavodoxin, 1.80 mg/ml, in 50 mM Tris-hydrochloride, pH 7.6. Oxidized flavodoxin (———) was enzymatically reduced with *C. thermoaceticum* CO dehydrogenase (----) by bubbling with CO for 5 min before addition of enzyme.

is a four-iron ferredoxin, like that of *C. thermoaceticum* ferredoxin I (44) and ferredoxins from *Desulfovibrio gigas* (39), *Desulfovibrio desulfuricans* (45), *Bacillus stearothermophilus* (31), *Spirochaeta stenostrepa* (18), and *Bacillus polymyxa* (37). However, the *C. formicoaceticum* ferredoxin contains eight cysteine residues, whereas *B. stearothermophilus* (15), Spirochaeta stenostrepa (18), and *B. polymyxa* (37) ferredoxins contain four and *C. thermoaceticum* (13), *D. gigas* (39), and *D. desulfuricans* (45) ferredoxins have six cysteine residues. Since all ferredoxins so far isolated which contain eight cysteines also contain eight Fe and eight S, it seems that the *C. formicoaceticum* ferredoxin should be convertible into an eight Fe-eight S protein. Even though the purification procedure utilized in preparation of the ferredoxin was quick and rather mild, it is possible that a cluster could have dissociated from the protein. Sequence analysis should explain the difference between this and other eight cysteine-containing ferredoxins.

The *C. formicoaceticum* rubredoxin light absorption peaks and the ratio of the peak at 490 nm to that at 280 nm were similar to those found for other purified rubredoxins. However, the *C. formicoaceticum* we found lacked a shoulder at approximately 350 nm that has been characteristic of other rubredoxins (10, 23, 43). In addition, the electron paramagnetic resonance spectrum (not shown here) of the *C. formicoaceticum* rubredoxin showed only the g = 4.3 resonance, and we did not detect the g = 9.4 resonance seen in other rubredoxins. This g = 9.4 signal is quite temperature sensitive and is always weaker than the g = 4.3 resonance, however, and electron paramagnetic resonance studies at various temperatures have not yet been carried out. We have not yet found the reasons for these spectral differences, since the iron and amino acid analyses were so similar to those of other rubredoxins.

Flavodoxin from *C. formicoaceticum* was similar to other flavodoxins (21, 27). When cells were grown in a medium containing 3.6 μM iron salts, it was a major component of the cell and constituted at least 2% of the soluble cell protein. The flavodoxin from *C. formicoaceticum* was used in resonance coherent and anti-Stokes Raman scattering spectroscopy and was found to resemble the *M. elsdenii* flavodoxin, in contrast to the *Desulfovibrio* and *Azotobacter vinelandii* flavodoxins (40).

Recently, ferredoxin, rubredoxin, and flavodoxin from *C. formicoaceticum* have been used as electron acceptors for *C. thermoaceticum* (33), *C. formicoaceticum*, and *Acetobacterium woodii* carbon monoxide dehydrogenases (34). We found in all cases that rubredoxin is the best natural electron acceptor, followed by ferredoxin and the flavodoxin. We postulate that rubredoxin, therefore, is the primary natural electron carrier for CO dehydrogenase in acetogenic bacteria.

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