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Isolation and Characterization of an Fe_8-S_8 Ferredoxin (Ferredoxin II) from *Clostridium thermoaceticum*

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A second ferredoxin protein was isolated from the thermophilic anaerobic bacterium *Clostridium thermoaceticum* and termed ferredoxin II. This ferredoxin was found to contain 7.9 ± 0.3 iron atoms and 7.4 ± 0.4 acid-labile sulfur atoms per mol of protein. Extrusion studies of the iron-sulfur centers showed the presence of two [Fe_4-S_4] centers per mol of protein and accounted for all of the iron present. The absorption spectrum was characterized by maxima at 390 nm (ε_{390} = 30,400 M^{-1} cm^{-1}) and 280 nm (ε_{280} = 41,400 M^{-1} cm^{-1}) and by a shoulder at 300 nm. The ratio of the absorbance of the pure protein at 390 nm to the absorbance at 280 nm was 0.74. Electron paramagnetic resonance data showed a weak signal in the oxidized state, and the reduced ferredoxin exhibited a spectrum typical of [Fe_4-S_4] clusters. Double integration of the reduced spectra showed that two electrons were necessary for the complete reduction of ferredoxin II. Amino acid analysis indicated a total of 57 residues, including 8 cysteines, 1 methionine, 1 histidine, and 1 arginine, and a molecular weight of 6,748 for the native protein. The ferredoxin is stable under anaerobic conditions for 60 min at 70°C. The average oxidation-reduction potential for the two [Fe_4-S_4] centers was measured as −365 mV.

Ferredoxins are ubiquitous, electron-carrying proteins which have been reported in virtually all known systems (14, 18). In bacteria, a large number of quite homologous ferredoxins containing two [Fe_4-S_4] clusters have been characterized and classified previously as clostridial or clostridial-like ferredoxins (18). However, in 1977, an unusual ferredoxin was isolated from the thermophilic bacterium *Clostridium thermoaceticum* by Yang et al. (17). This ferredoxin was distinguished by containing a single [Fe_4-S_4] cluster, 63 rather than 54 to 56 amino acid residues (17), and limited sequence homology with other clostridial ferredoxins (J. I. Elliott, S.-S. Yang, L. G. Ljungdahl, J. Travis, and C. F. Reilly, Biochemistry, in press). Since a number of bacterial systems have been shown to contain more than one distinct ferredoxin, *C. thermoaceticum* was examined more closely for additional ferredoxins. The work presented here describes the purification and characterization of a second ferredoxin, termed ferredoxin II (Fd II), which has similar properties when compared with other clostridial ferredoxins and, therefore, large differences compared with the ferredoxin isolated by Yang et al. (Fd I) (17).

MATERIALS AND METHODS

C. thermoaceticum DSM521 was grown at 58°C under 100% CO_2 according to the procedure described by Yang et al. (17). *N,N'*-Dimethyl-*p*-phenylenediamine, bathophenanthroline, thioglycolic acid, and benzyl viologen were obtained from Sigma Chemical Co., St. Louis, Mo. Both hexamethylphosphoramide, obtained from Aldrich Chemical Co., Milwaukee, Wis., and benzenethiol, obtained from Eastman Kodak Co., Rochester, N.Y., were distilled before use and stored under nitrogen. HCl for protein hydrolysates was purchased from Alfa Products, Danvers, Md. All other chemicals were of reagent grade and were used without further purification.

Sedimentation equilibrium and sedimentation velocity measurements were made with a Beckman model E analytical ultracentrifuge equipped with absorption optics. Analytical polyacrylamide gel electrophoresis was performed at pH 8.9 according to Brewer and Ashworth (1). Amino acid analyses were done on samples hydrolyzed at 110°C in 6 N HCl in vacuo with a Beckman model 119CL automatic amino acid analyzer. Tryptophan was measured by hydrolysis in the presence of 4% thioglycolic acid (9). Cysteine was measured as *S*-carboxymethylated cysteine according to the procedure of Crestfield et al. (3). Acid-labile sulfide was determined by the procedure of Chen and Mortenson (2) by using *N,N'*-dimethyl-*p*-phenylenediamine and $FeCl_3$ after incubation of the protein in 1% zinc acetate (wt/vol) in distilled water for 2 h. Sodium sulfide standards were prepared fresh and standardized by iodometry. Extrusion of the iron-sulfur centers was done according to Mortenson and Gillum (11) with hexamethylphosphoramide and benzenethiol, the resulting complex having an ε_{458} of 17,200 M^{-1} cm^{-1}. Iron was measured by means of bathophenanthroline and mercaptoacetate (4). Protein concentrations for comparison with the sulfide and

iron measurements were obtained by amino acid analysis of acid-hydrolyzed Fd II containing norleucine as an internal standard.

Thermal stability of Fd II was followed by decreases in absorption at 390 nm of a 0.25-mg/ml protein solution in 0.2 M Tris-hydrochloride, pH 7.4. Samples were prepared in stoppered cuvettes and made anaerobic by repeated evacuation and flushing with oxygen-free argon or nitrogen.

Oxidation-reduction potentials of Fd II were measured based on the procedure of Yoch and Arnon (19). Samples of Fd II in 0.2 M Tris-hydrochloride, pH 7.1, were prepared having an absorbance at 390 nm (A_{390}) of 1.0 (33 μM protein, 66 μM iron-sulfur clusters). This solution was made to approximately 60 μM benzyl viologen, placed in cuvettes sealed with rubber septa, and bubbled with oxygen-free nitrogen for 15 min. After an initial absorption spectrum was obtained, 10 μl of 100 mM sodium dithionite was added to yield fully reduced ferredoxin. A second absorption spectrum was obtained, and initial measurements were made at 625 and 443 nm. A small volume of air was syringed into the sample and bubbled for 3 to 5 min with the nitrogen gas. Absorptions at the wavelengths indicated were recorded if a stable reading was observed for 1 to 2 min. The changes at 625 nm were direct measurements of the oxidation state of the benzyl viologen, for absorption changes of the oxidized and reduced Fd II were negligible at this wavelength. The changes at 443 nm were a measurement of the oxidation of Fd II and were corrected for the absorption contributed by the benzyl viologen. Samples totally reoxidized returned to ≥95% of their original absorption. Samples partially reduced with dithionite yielded oxidation-reduction potentials virtually identical to those obtained by reoxidizing the fully reduced system. Calculations were done based on 298 K and $n = 1$, so that E_o' (volts) = $-0.359 + 25.7 \times 10^{-3} \ln [(Fd_r)(BV_o)]/[(Fd_o)(BV_r)]$, where 0.359 V is the oxidation-reduction potential for benzyl viologen (BV) (10) and the subscripts r and o indicate the reduced and oxidized states, respectively.

Electron paramagnetic resonance (EPR) measurements were performed with a Varian E-109 spectrometer. The EPR spectra were directly transmitted via a Hewlett-Packard HP-85F minicomputer for storage of data and double integration of spectra. Additional EPR and experimental conditions are given in the legend to Fig. 2. The spectra were obtained under nonsaturating microwave power conditions.

Fd II has been purified by two methods from *C. thermoaceticum* (Table 1). The first procedure utilized the 45% ammonium sulfate supernatant obtained in our laboratory during the anaerobic purification of formate dehydrogenase (16). This supernatant was obtained as follows, all steps being performed anaerobically. The cell paste (180 g) was suspended in 3 volumes of anaerobic buffer (50 mM triethylamine-maleate [pH 7.5] containing 0.2 mM methyl viologen, 10 mM sodium azide, 10 mM sodium formate, and 2 mM sodium dithionite), broken by using a French pressure cell, and centrifuged at 57,000 × *g* for 60 min. The supernatant was heated to 67°C, incubated for 30 min, and then centrifuged at 31,000 × *g* for 30 min. The supernatant was made to 45% ammonium sulfate and centrifuged as before, and the pellet was retained for further purification of formate dehydrogenase. The resulting supernatant was utilized for the purification of Fd II, the remaining steps being performed aerobically at 0 to 5°C.

(i) Ammonium sulfate precipitation. The 45% ammonium sulfate supernatant was made to 60% saturation by the addition of solid ammonium sulfate. After centrifugation at 13,000 × *g* for 15 min, the supernatant was removed, and the solution was made to 100% saturation in ammonium sulfate. This material was centrifuged as before, and the pellet was dissolved in 200 ml of distilled water.

(ii) DEAE-cellulose chromatography. A column of Whatman DE-32 cellulose (1.5 by 6 cm) was preequilibrated with 0.1 M Tris-hydrochloride, pH 7.1, and washed with 3 column volumes of distilled water before use. The dissolved pellet from step i was applied to the column, forming a dark-brown band, and washed with 0.2 M Tris-hydrochloride, pH 7.1, until the eluant gave an A_{260} of ≤0.2. The ferredoxin was then eluted in a small volume with 0.65 M Tris-hydrochloride, pH 7.1.

(iii) Gel permeation chromatography. The sample from step ii was applied to a Sephadex G-50 superfine column (2.5 by 80 cm) which had been equilibrated with 0.05 M Tris-hydrochloride, pH 7.1, containing 0.1 M KCl and 30% glycerol. The column was developed with the same buffer, and the ferredoxin was eluted at approximately 265 ml as a peak of predominantly pure ferredoxin. Fractions containing Fd II with an A_{390}/A_{280} ratio of 0.72 to 0.74 were pooled.

A second purification procedure was also used to obtain homogeneous Fd II from cell paste. All steps were done aerobically at 0 to 5°C, unless otherwise specified.

(i) Whole-cell wash. A 180-g sample of cell paste, which had been stored frozen, was suspended in 2 volumes of distilled water, the pH being adjusted to 6.5 with NH_4OH, if necessary. After being stirred for 15 min at room temperature (cell suspension reached 5°C), the suspension was centrifuged at 27,000 × *g* for 15 min. The supernatant was retained.

TABLE 1. Purification procedures for Fd II from *C. thermoaceticum*[a]

Step	Total A_{390}	A_{390}/A_{280}	Protein (mg)
Procedure 1			
Crude homogenate	4,500	0.04	
45% $(NH_4)_2SO_4$ precipitation[b]	1,560	0.05	
60 to 100% $(NH_4)_2SO_4$ precipitation	220	0.13	
DEAE-cellulose	45	0.56	
Sephadex G-50	25	0.73	5.5[c]
Procedure 2			
Cell wash	472	0.07	
DEAE-cellulose	90	0.66	
Sephadex G-50	45	0.72	10.0

[a] Based on 180 g of cell paste.

[b] Obtained from the anaerobic purification of formate dehydrogenase (see text) (16).

[c] Based on ε_{390} = 30,400 M^{-1} cm^{-1} and molecular weight = 6,748.

(ii) DEAE-cellulose chromatography. The sample from step i was applied to a column (1 by 10 cm) of Whatman DE-32 cellulose which had been preequilibrated with 0.1 M Tris-hydrochloride, pH 7.1. A very tight, dark-brown band formed at the top of the resin. After the sample was washed with distilled water until the effluent was free of A_{260}, the elution was continued until the water layer completely entered the resin surface. The cellulose material was then removed as a solid core of resin by upward-directed air pressure. The dark band of ferredoxin was removed from the top of the column, suspended in a few milliliters of distilled water, and applied to a column (0.5 by 1.0 cm) of fresh DEAE-cellulose. This column was washed with 0.2 M Tris-hydrochloride, pH 7.1, and then the ferredoxin was eluted with 0.35 M Tris-hydrochloride, pH 7.1. Fractions with A_{390}/A_{280} ratios greater than 0.64 were pooled and either applied directly to a Sephadex G-50 column or lyophilized. Lyophilized samples were redissolved in 40% glycerol before application to the Sephadex G-50 column.

(iii) Gel permeation chromatography. Samples from step ii were treated as described in step iii of purification procedure 1. Fractions containing Fd II with an A_{390}/A_{280} ratio of 0.72 to 0.74 again were pooled.

RESULTS

Purification. Fd II from *C. thermoaceticum* was obtained in a homogeneous form by two procedures as described above and summarized in Table 1. Yields were in the range of 5 to 10 mg from 180 g of cell paste. Yields of Fd I obtained from the same amount of cells are slightly lower, 4 to 5 mg (17). Homogeneity of Fd II was determined by single bands on polyacrylamide disc gel electrophoresis (1), a single peak was determined by sedimentation velocity, and a single species was determined by sedimentation equilibrium. The maximum A_{390}/A_{280} ratio obtained was 0.74.

Amino acid analysis. The amino acid analysis of Fd II is presented in Table 2 and compared with that of Fd I. The extensive differences are most obvious for the amino acids valine, tyrosine, phenylalanine, tryptophan, and cysteine, and for the total residue content. Characteristically of ferredoxins, a high number of acidic residues were present. Residues not commonly observed in clostridial and related ferredoxins were histidine, arginine, and methionine. Eight cysteine residues were measured, enough to bind two [Fe_4-S_4] clusters (see below). The calculated molecular weights of the apoprotein and the native protein (assuming two [Fe_4-S_4] clusters) were 6,044 and 6,748, respectively.

Iron and sulfur analyses. Iron and acid-labile sulfide analyses showed 7.9 ± 0.3 atoms of iron and 7.4 ± 0.4 atoms of sulfur per mol of Fd II. Since acid-labile sulfide measurements have been reported to be slightly low when standardized sulfide solutions are used (2), each ferredoxin molecule presumably contained eight atoms of iron and eight of sulfur. The ε_{390} was 30,400 M^{-1} cm^{-1}, consistent with eight atoms of iron if an ε_{390} of 4,000 M^{-1} cm^{-1} per iron atom can be assumed (7). The ε_{280} was 41,100 M^{-1} cm^{-1} (see absorption spectrum, Fig. 1). Extrusion studies on the iron-sulfur centers showed only the presence of [Fe_4-S_4] centers, with more than 95% of the iron content being accounted for by extruded clusters (Fig. 1). The A_{458}/A_{550} ratio was 1.8, which indicates an acceptable extrusion with little or no free iron being present in the sample (11).

Oxidation-reduction potential. The oxidation-reduction potential for Fd II with benzyl viologen as an electron mediator was measured as −365 ± 6 mV over a wide range of percent oxidized Fd II (Table 3). The oxidation-reduction potentials of the two clusters appear identical within the limits of the experimental procedure.

EPR analysis. EPR analyses were performed on the native (oxidized) and the dithionite-reduced forms of Fd II (Fig. 2). A weak signal with a g value of 2.01 was observed for the oxidized species, characteristic of a number of clostridial ferredoxins (13, 15). The spectrum of reduced Fd II was of a typical rhombic low-potential [Fe_4-S_4] center with g values of 2.05, 1.92, and 1.89. Double integration of the reduced spec-

TABLE 2. Amino acid composition of Fd II from *C. thermoaceticum* and its comparison with Fd I

Amino acid	No. of residues in:	
	Fd II[a]	Fd I[b]
Asp	7.1 (7)	9
Thr	3.1 (3)	3
Ser	2.3 (2)	3
Glu	9.8 (10)	9
Pro	3.2 (3)	4
Gly	4.3 (4)	3
Ala	6.2 (6)	4
Val	1.9 (2)	8
Met	1.0 (1)	1
Ile	3.6 (4)	4
Leu	2.8 (3)	3
Tyr	1.1 (1)	0
Phe	0.1 (0)	1
His	1.1 (1)	1
Lys	1.2 (1)	2
Arg	0.9 (1)	1
Trp[c]	0.0 (0)	1
Cm-Cys[d]	7.8 (8)	6

[a] Numbers in parentheses indicate nearest integer value. Fd II has eight iron and eight sulfur atoms per mol.

[b] Data from Yang et al. (17). Fd I has four iron and four sulfur atoms per mol.

[c] From hydrolysis in the presence of 4% thioglycolate (9).

[d] CM-Cys, *S*-Carboxymethylated cysteine (6).

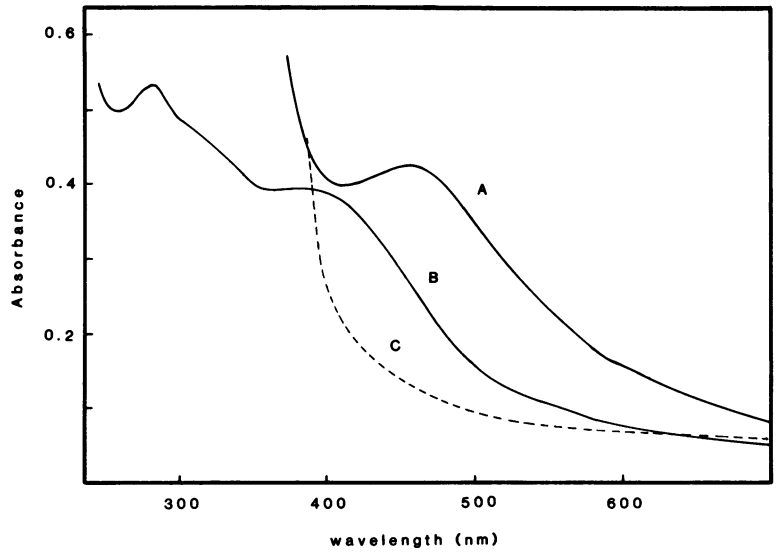

FIG. 1. (A) Absorption spectrum of iron-sulfur centers from Fd II extruded by means of hexamethylphosphoramide and benzenethiol. The final protein concentration was 12.7 μM; the concentration of extruded [Fe_4] clusters was calculated as 25 μM based on $\varepsilon_{458} = 17{,}200\ M^{-1}\ cm^{-1}$ (11). (B) Absorption spectrum of native Fd II, as isolated, with an A_{390}/A_{280} of 0.73. The protein concentration was 13.2 μM in 0.2 M Tris–hydrochloride, pH 7.4. (C) Absorption spectrum of the Fd II sample described in (B) after reduction with sodium dithionite.

trum correlated with the uptake of two electrons for complete reduction of Fd II.

Temperature stability. As measured by the decrease in A_{390}, Fd II was stable for 60 min at 70°C under anaerobic conditions, showing less than a 10% decrease in A_{390}. Similar stability was observed under aerobic conditions at 65°C. At 80°C, the temperature at which Fd I is stable (17), Fd II attained its maximum decrease in A_{390} in approximately 10 min.

Sedimentation studies. Sedimentation velocity experiments with purified Fd II showed a single species with a sedimentation coefficient of 1.7S. Sedimentation equilibrium studies demonstrated a single species with a molecular weight of approximately 7,000. By using the molecular weight of 6,748 determined from amino acid analysis and iron and sulfur measurements, a partial specific volume of 0.63 ml/g was calculated based on the sedimentation equilibrium measurements. This value was similar to those for the ferredoxin from *Clostridium acidiurici* of 0.63 and 0.61 ml/g obtained by density gradient columns (7) and differential sedimentation equilibrium (6), respectively.

DISCUSSION

A second ferredoxin was isolated from the thermophilic bacterium *C. thermoaceticum* and designated Fd II. The amino acid analysis comparison with the ferredoxin isolated by Yang et al. (17) (now designated Fd I) clearly showed the two proteins to be distinct (Table 2). Of particular interest was the total number of residues, for amino acid analysis of Fd II demonstrated 57 residues, a value more typical for clostridial ferredoxins, whereas Fd I has an unusual total of 63 amino acids. Both ferredoxins contained amino acid residues atypical of clostridial ferredoxins (arginine, methionine, and histidine), and Fd I included a tryptophan without precedent in clostridial ferredoxins. The iron and sulfur analyses and the extrusion studies clearly established Fd II as containing two [Fe_4-S_4] clusters, again distinct from the single [Fe_4-S_4] cluster of

TABLE 3. Oxidation-reduction potential measurements of Fd II from *C. thermoaceticum*[a]

$[Fd_r/Fd_o]^b$	$[BV_o/BV_r]^b$	E_o' (V)[c]
14.74	0.06	−0.362
14.29	0.05	−0.368
5.52	0.16	−0.362
2.53	0.29	−0.367
1.83	0.32	−0.373
1.38	0.44	−0.372
0.80	1.16	−0.361
0.57	1.32	−0.366
0.45	2.67	−0.354
0.07	11.35	−0.365

[a] Performed as described in the text.
[b] Subscripts indicate oxidized (*o*) and reduced (*r*) states; BV, Benzyl viologen.
[c] Mean, −0.365 ± 0.006.

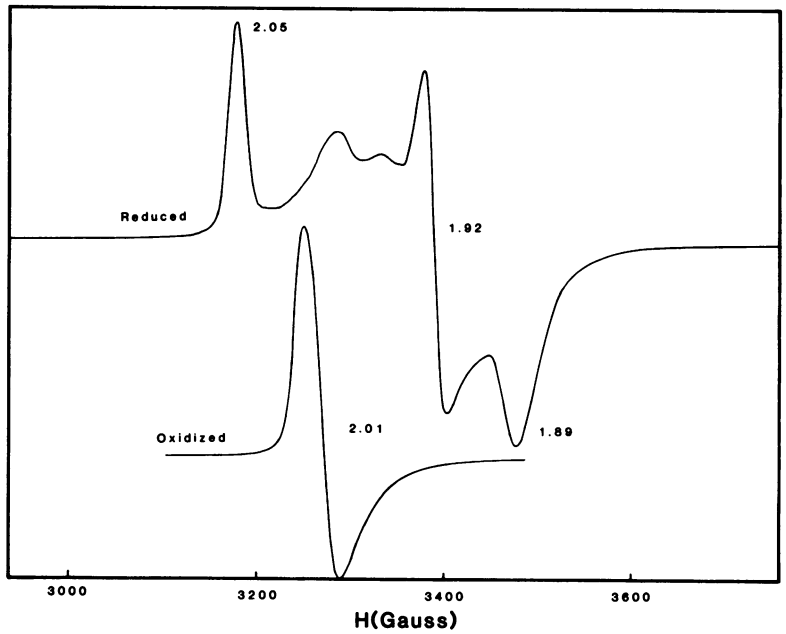

FIG. 2. EPR spectra of Fd II from *C. thermoaceticum* in oxidized (as isolated) and fully reduced states. EPR measurements were made with a Varian E-109 spectrometer operating at a 100-kc modulation; an Air Products ADP-E automatic helium temperature control system was used. EPR conditions: microwave power, 0.1 mW; modulation amplitude, 10 G; scanning rate, 200 G/min; time constant, 0.128 s; temperature, 10 K; frequency, 9.128 GHz. The concentration of Fd II was 7.0 mg/ml (8.3 mM iron) in 1.0 M Tris-hydrochloride, pH 7.4. Relative gains were five times for the reduced protein and one time for the oxidized protein.

Fd I. The oxidation-reduction potentials of the two ferredoxins were very similar: Fd II has a potential of −365 mV (Table 3), and Fd I has a potential of −350 mV (unpublished data). Thus, *C. thermoaceticum* was shown to contain both a clostridial-like Fe_8-S_8 ferredoxin, Fd II, and a unique Fe_4-S_4 ferredoxin, Fd I (17).

During the purification procedure described by Yang et al. (17) for Fd I, a rapid decrease in A_{390} (approximately a 30% decrease in 12 h at 0°C) was observed for partially purified protein solutions. Therefore, to examine *C. thermoaceticum* for the presence of additional ferredoxins, more rapid purification steps, as described above, were developed. Fd II was found to be completely lost early in the purification procedure used for the isolation of Fd I. In its homogeneous form, however, Fd II was significantly more stable than at any earlier stage of purification. It could be stored aerobically for 2 to 3 months in 50% glycerol at −20°C with a 10 to 20% loss in A_{390}. Similar stability was observed for samples stored at 5°C under anaerobic (not reduced) conditions.

EPR analyses of the oxidized and reduced forms of Fd II correlated well with the presence of [Fe_4-S_4] clusters. The reduced spectrum was similar to those obtained from ferredoxins with one [Fe_4-S_4] cluster (13) and from ferredoxins with two [Fe_4-S_4] clusters which are partially reduced (8, 12). The completely reduced spectrum of Fd II (two electrons per protein molecule) was virtually identical to that of the clostridial-like ferredoxin from *Micrococcus lactilyticus* (two [Fe_4-S_4] clusters) when it is 20% reduced. The *M. lactilyticus* ferredoxin, like most Fe_8-S_8 ferredoxins, shows a strongly altered spectrum upon full reduction, apparently from interaction between the clusters (8). At 20% reduction, ferredoxin molecules with only one reduced cluster per molecule will predominate, and the spectrum will be indicative of a noninteracting cluster.

It was interesting to observe that in purification procedure 2, Fd II was washed free from frozen-thawed cells of *C. thermoaceticum*, but Fd I was not. Although both ferredoxins have similar oxidation-reduction potentials, compartmentalization and the ability of Fd II to undergo two one-electron transfers per molecule suggested that these ferredoxins have different cellular roles. Recently, Drake et al. (5) have shown a complex reaction system for the synthesis of acetyl phosphate from methyltetrahydrofolate in *C. thermoaceticum*. This enzyme system was found to require a low-potential ferredoxin, ap-

parently to reduce enzyme-bound CO_2 to formate. Additionally, ferredoxin appeared to be involved in NADP reduction, which was coupled to the CO_2-to-formate reaction of formate dehydrogenase. Whether each of these reactions was associated with a different ferredoxin in vivo will require additional study.

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