Spectroscopic and Functional Properties of Novel \(2[4\text{Fe-4S}]\) Cluster-containing Ferredoxins from the Green Sulfur Bacterium \textit{Chlorobium tepidum}*

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Two distinct ferredoxins, Fd I and Fd II, were isolated and purified to homogeneity from phototautrophically grown \textit{Chlorobium tepidum}, a moderately thermophilic green sulfur bacterium that assimilates carbon dioxide by the reductive tricarboxylic acid cycle. Both ferredoxins serve a crucial role as electron donors for reductive carboxylation, catalyzed by a key enzyme of this pathway, pyruvate synthase/pyruvate ferredoxin oxidoreductase. The reduction potentials of Fd I and Fd II were determined by cyclic voltammetry to be \(-514\) and \(-584\) mV, respectively, which are more electronegative than any previously studied Fds in which two \([4\text{Fe-4S}]\) clusters display a single transition. Further spectroscopic studies indicated that the CD spectrum of oxidized Fd I closely resembled that of Fd II; however, both spectra appeared to be unique relative to ferredoxins studied previously. Double integration of the EPR signal of the two Fds yielded approximately \(2.0\) spins per molecule, compatible with the idea that \textit{C. tepidum} Fd I and Fd II accept 2 electrons upon reduction. These results suggest that the \textit{C. tepidum} Fd I and Fd II polypeptides each contain two \([4\text{Fe-4S}]\) clusters. \textit{C. tepidum} Fd I and Fd II are novel \([4\text{Fe-4S}]\) Fds, which were shown previously to function as biological electron donors or acceptors for \textit{C. tepidum} pyruvate synthase/pyruvate ferredoxin oxidoreductase (Yoon, K.-S., Hille, R., Hemann, C. F., and Tabita, F. R. (1999) J. Biol. Chem. 274, 29772–29778). Kinetic measurements indicated that Fd I had \(2.3\)-fold higher affinity than Fd II. The results of amino acid sequence alignments, molecular modeling, oxidation-reduction potentials, and spectral properties strongly indicate that the \textit{C. tepidum} Fds are chimeras of both clostridial-type and chromatium-type Fds, suggesting that the two Fds are likely intermediates in the evolutionary development of \([4\text{Fe-4S}]\) clusters compared with the well described clostridial and chromatium types.

The reductive tricarboxylic acid (RTCA)\(^1\) cycle serves as an important pathway to enable diverse organisms to use CO\(_2\) as the sole source of carbon for growth. Despite the importance of this process in nature, many key aspects of the biochemistry and molecular control of this process are unknown. \textit{Chlorobium tepidum} provides a particularly intriguing model system as this anaerobic phototrophic green sulfur bacterium grows rapidly to high densities and is the only known RTCA pathway organism that possesses a potentially useful genetic system (1). By using the enzyme ATP-dependent citrate lyase (2), \textit{C. tepidum} and other RTCA organisms convert citrate to acetyl-CoA and oxaloacetate. The acetyl-CoA produced by this reaction may then be used as a CO\(_2\) acceptor in a unique ferredoxin (Fd)-linked carboxylation reaction catalyzed by the bifunctional enzyme pyruvate synthase (PS)/pyruvate ferredoxin oxidoreductase (PFOR) (Reaction 1). This enzyme is analogous to another key CO\(_2\) fixation enzyme of this pathway, \(\alpha\)-ketoglutarate synthase (KGS/\(\alpha\)-ketoglutarate ferredoxin oxidoreductase (KGOR) (Reaction 2)).

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\begin{align*}
\text{acetyl-CoA} + \text{CO}_2 + \text{H}^+ + \text{Fd}_{\text{red}} & \rightarrow \text{pyruvate} + \text{coenzyme A} + \text{Fd}_{\text{ox}} \\
\text{succinyl-CoA} + \text{CO}_2 + \text{H}^+ + \text{Fd}_{\text{red}} & \rightarrow \alpha\text{-ketoglutarate} + \\
& \quad \text{coenzyme A} + \text{Fd}_{\text{ox}}
\end{align*}
\]

REACTION 1

REACTION 2

Both enzymes require a source of reducing equivalents to function in the CO\(_2\) fixation direction (PS and KGS), and both enzymes transfer reducing equivalents to discrete electron acceptors for decarboxylation (PFOR and KGOR). Recently, we have shown (3) that a novel low potential rubredoxin from \textit{C. tepidum} is a favored electron acceptor for PS/PFOR in the pyruvate oxidation reaction. Moreover, it has been shown unequivocally that the \textit{C. tepidum} enzyme, which is a homodimer of a single polypeptide chain, catalyzes both the PS and PFOR reactions. Rubredoxin, however, was unable to serve as the electron donor for the reductive carboxylation of acetyl-CoA in the PS reaction. Undoubtedly then, a much more electronegative electron carrier is required for this enzymatic reaction, and our initial results (3) indicated that two different ferredoxins isolated from this organism could perform this function. These preliminary results, with purified preparations, are in agreement with studies performed many years ago (4–6) when the

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1 The abbreviations used are: RTCA, reductive tricarboxylic acid; PS, pyruvate synthase; PFOR, pyruvate ferredoxin oxidoreductase; KGS, \(\alpha\)-ketoglutarate synthase; KGOR, \(\alpha\)-ketoglutarate ferredoxin oxidoreductase; Fd, ferredoxin; ThDP, thiamine diphosphate; MNZ, metronidazole; PAGE, polyacrylamide gel electrophoresis.
RTCA pathway was initially discovered in the related organism Chlorobium limicola forma thiosulfatophilum. In these earlier studies, unpurified preparations of both PS and KGS were shown to require reduced ferredoxin. From the above studies, it is reasonable to assume that PS/PFOR and KGS/KGOR each require one or more highly electronegative electron donors to function in CO2 assimilation. Flavodoxin (7, 8) and 8-hydroxy-5-deazaflavin (9), in addition to Fd (10–14), have also been implicated as potential electron donors.

To study further the biochemistry of the RTCA cycle, particularly in C. tepidum, we attempted to isolate and analyze low potential electron carriers from this organism that might function in the RTCA cycle. It is well known that the small protein Fd has one or more iron-sulfur clusters involved in electron transfer. This well studied protein has been isolated from many different organisms, and several years ago two different ferredoxins, Fd I and Fd II, were isolated from the green sulfur bacterium C. limicola. In this earlier study, however, only the amino acid sequences were reported (15), and no biochemical, spectroscopic, or physiological properties were noted. Certainly it is known that Fd may have several specific functions. This protein is particularly required by microbial systems that catalyze electron transfer processes, including CO2 reduction, N2 fixation, sulfite reduction, NAD(P) reduction, photophosphorylation, and the fermentation of organic compounds (16–22).

Plant-type Fds contain [2Fe-2S] active redox centers, whereas bacterial-type Fds have [3Fe-4S] and one or two [4Fe-4S] cubane clusters in their active sites. Each cluster is capable of serving as a one-electron carrier. A single organism may possess numerous Fds, which are distinguished by their unique spectroscopic properties, protein sequences, reduction potentials, and [Fe-S] clusters, among other biochemical and physiological properties (16–22). In bacteria, one or two [4Fe-4S]2+/1+ clusters are commonly known as either general clostridial-type Fds or chromatium-type Fds. The [4Fe-4S]2+/1+ cluster of clostridial-type contains a Cys-Xaa-Cys-Xaa-Xaa-Cys-Pro motif for cluster ligation and has been structurally characterized (16, 17, 23, 32, 33). The chromatium-type Fds possess specific differences in the second [4Fe-4S]2+/1+ cluster as well as having a longer extension at the C terminus. Notably they utilize a Cys-Xaa-Xaa-Cys-Xaa7-Cys-Xaa-Xaa-Cys-Pro motif for cluster ligation, having 5–6 amino acids more than the clostridial-type between the second and third cysteines at the C terminus. The exception is the archaeal ferredoxin from Methanobacterium thermautotrophicum strain AH (27). Monocluster Fds from Pyrococcus furiosus, Thermatoga maritima, and Hydrogenobacter thermophilus were found to act as electron transfer proteins between PFOR and a terminal hydrogenase (24, 34–36). Recently [3Fe-4S][4Fe-4S] or [4Fe-4S] Fds from Sulfolobus sp. strain 7, Chromatium vinosum, Azobacter vinelandii, and Thauera aromatica were characterized and found to exhibit different reduction potentials, corresponding to the two [4Fe-4S] clusters, cluster I and cluster II (25, 29, 32, 37).

The reduction potentials of these chromatium-type Fds ranged from −280 to −486 mV for cluster I and from −530 to −660 mV for cluster II. These properties are very different from the clostridial-type. These proteins thus raise intriguing evolutionary questions and suggest that the C. tepidum Fds are intermediates in the development of important [2Fe-4S]-type Fds, which eventually evolved into the clostridial- and chromatium-types by gene duplication events. Finally, the results of this study present the initial detailed description of the spectroscopic and biochemical properties of these unique Fds from green sulfur bacteria; the possible functional role and biological activity of [2Fe-4S] cluster Fds in autotrophic and heterotrophic metabolism are indicated.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—**C. tepidum strain TLS was grown phototrophically in 20-liter Carboy bottles according to a modification of a method described previously (3). The cells were harvested anaerobically using continuous centrifugation at 35,000 × g at 4 °C under a flow of nitrogen gas. The harvested cells were washed twice with anaerobic 50 mM phosphate buffer, pH 7.0, containing 5 mM dithiothreitol. The cells were then resuspended in the same buffer (1 g of wet weight cells per 4 ml of buffer), after which the cell suspension was disrupted by 2 passes through a French pressure cell at 10,000 pounds/square inch under a flow of argon gas. Cell debris and broken cells were removed by centrifugation (20,000 × g, 10 min); the supernatant fraction was then centrifuged at 150,000 × g for 1.5 h. The supernatant fraction from this step was used for the purification.

**Protein Purification—**All purification procedures were carried out at room temperature under strictly anaerobic conditions using a Coy anaerobic chamber under an atmosphere of 85% nitrogen and 15% hydrogen. All buffers were repeatedly degassed and flushed with argon and were stored in the anaerobic chamber. Protein purification was performed in the Coy anaerobic chamber using the Biological Autoprotogram System from Bio-Rad. The supernatant after ultracentrifugation was loaded onto a DEAE-Sepharose fast flow column (2.5 × 20 cm), pre-equilibrated with 20 mM Tris-HCl, pH 7.8, 10 mM β-mercaptoethanol. This column was washed with 0.2 M NaCl at a flow rate of 7 ml per min, followed by elution with 600 ml of a linear gradient of 0.2–0.5 M NaCl prepared in the same buffer. Two dark brown colored proteins were eluted in distinct peaks and diluted 2-fold with salt-free buffer and separately applied to a Q-Sepharose high performance column (1.6 × 12 cm) pre-equilibrated with the same buffer. After loading, the column was washed with buffer and protein eluted using buffer containing 0.3 M NaCl followed by a gradient of buffer containing 0.3–0.5 M NaCl at a flow rate of 3 ml per min. At this stage, protein-containing fractions were concentrated using Centricon-5 cartridges (Millipore) and loaded onto a Superose-12 column (1.6 × 50 cm) pre-equilibrated with 20 mM Tris-HCl, pH 7.8, containing 0.2 M NaCl at a flow rate of 1.0 ml per min. Protein purity was established by SDS-PAGE analysis using gels containing 2% acrylamide. Subsequent analyses indicated that the two dark-brown pigmented proteins were distinct ferredoxins, Fd I and Fd II. C. tepidum PS/PFOR was also purified under anaerobic conditions from the same crude cell extract, as described elsewhere (2).

**Enzyme Assay—**The function of Fd as an electron acceptor for PFOR was determined spectrophotometrically either by direct measurement of Fd reduction or by coupling the reoxidation of reduced Fd to the reduction of methioninazalone (1-[2-hydroxyethyl]-2-methyl-5-nitroimidazole, MNZ). The standard assay mixture contained 10 mM pyruvate, 0.5 mM coenzyme A, PFOR (15 nM), 1 mM MgCl2, and 0.1 mM ThDP in 100 mM HEPES buffer in anaerobic cuvettes under argon, pH 7.6, at 45 °C (3). The steady-state kinetic parameters of PFOR for Fd I and Fd II were determined by measuring the MNZ-mediated reduction of Fd II catalyzed by PFOR. In the coupled assay, MNZ (100 μM) was added to the above reaction mixture, and the activity was measured by following MNZ reduction at 320 nm. Oxidized MNZ is reported to have a molar absorption coefficient of 9300 M−1 cm−1 (38), and it is assumed to be a one-electron process (39). The direct reduction of Fd I and Fd II by PFOR was measured at 390 nm. The molar absorption was used for calculating PFOR activity using Fd I and Fd II determined to be 10,110 and 8,030 M−1 cm−1 which were between fully oxidized and reduced forms, respectively, and 31,6000 and 30,900 M−1 cm−1 when oxidized. The specific activity was expressed as units/mg PFOR, where 1 unit is equivalent to the reduction of 1 μmol of Fd per min at 395 nm.

K.-S. Yoon and F. R. Tabita, manuscript in preparation. Preliminary results were presented at the Gordon Research Conference on the Molecular Basis of Microbial One-Carbon Metabolism, June 28–July 3, 1998, Henniker, NH.
PFOR had a specific activity of 6.3 units/mg when using methyl violo-
gen as an electron acceptor at 48 °C. Cytoplasmic hydrogenase was partially purified from the supernatant fraction obtained after ultra-
centrifugation followed by DEAE-Sepharose fast flow and Q-Sepharose high performance chromatography. Hydrogenase activity was deter-
mined spectrophotometrically using benzyl viologen as an electron ac-
ceptor in an anaerobic cell under argon at 48 °C (40).

Other Spectral Determinations—Electron paramagnetic resonance (EPR) spectra were obtained with a Bruker Instruments ER 300 spect-
trometer equipped with ER 035M NMR gaußmeter and a Hewlett-
Packard 5352B microwave frequency counter. Double integration of the EPR spectra was performed with the ESP 300 software package from Bruker Instruments. Air-oxidized Fd was placed into quartz EPR tubes and frozen by slowly immersing the tubes in liquid nitrogen. Samples of reduced Fd were prepared under anaerobic conditions, using either an enzymatic protocol in which the activity of PFOR was used to reduce the Fd, or by chemical means by the addition of sodium dithionite. The reduced liquid samples were collected directly into EPR tubes and sealed with a rubber stopper; the samples were then frozen at specific times after mixing in a dry ice/acetone bath. EPR spectra were measured at 5–20 K using the following settings: 10 milliwatts microwave power, 10 G modulation amplitude, 100 kHz modulation frequency.

Circular dichroism (CD) spectra of oxidized Fds were measured at room temperature with an AVIV 62DS Spectropolarimeter. CD data were collected at a scan rate of 1 ms/s; spectra reported represent the average of three scans. UV-visible spectra were measured with a Cary 100 Bio UV-visible spectrophotometer (Varian Instruments).

Preparation of ApoFd—Purified holoFd I and Fd II (1 mg) were placed in argon-flushed stoppered tubes in an ice bath. To these prepara-
tions, an aliquot of 12 N HCl was added to a final concentration of 0.5 N; incubation was continued at 0 °C with continuous stirring under anaerobic argon for 45 min. The slurry was then centrifuged at 15,000 × g for 10 min, and the white pellet was quickly rinsed with demineralized distilled water and dissolved in degassed 100 mM Tris-HCl, pH 8.5. Treatment with HCl was repeated as before, and the resultant precip-
itate was dissolved in 200 μl of degassed 100 mM Tris-HCl, pH 8.5, 0.1 m NaCl and incubated for at least 30 min under anaerobic conditions under argon.

General Characterization of C. tepidum Fd I and Fd II—Midpoint reduction potentials of Fd I and Fd II (in 25 mM potassium phosphate, pH 7.5) were measured by cyclic voltammetry with a glassy carbon electrode. The scan rate was 20 mV/s over the potential range −200 to −900 mV (versus the Ag/AgCl electrode) at room temperature. The electrochemical cell and the procedure have been described (41). All values were referenced to the standard hydrogen electrode. The N-terminal amino acid sequences of Fd I and Fd II were determined at the University of California, Davis, Protein Sequencing Facility after blotting samples to a polyvinylidene difluoride membrane (42). The total amino acid compositions of Fd I and Fd II were determined at the Ohio State University Protein Sequencing Facility (43). Protein concentra-
tions were routinely estimated by established procedures (44). The iron content of the protein was determined by the o-phenanthroline proce-
dure (45). Labile sulfur was also determined using a colorimetric method (46). The molecular weights of Fd I and Fd II were determined by gel filtration using a Superose-12 HR (10/30) column and the Biological Autoprotocol System of Bio-Rad calibrated with β-amylase (M₆ = 200,000), bovine serum albumin (Mₑ = 67,000), carboxylic anhydride (Mₑ = 29,000), cytochrome c (Mₑ = 12,400), and aprotinin (Mₑ = 6,500).

RESULTS

Protein Purification—While investigating the structure-
function interactions of the key RTCA cycle enzymes PS/ PFOR and KGS/KGOR from C. tepidum, several small iron-
sulfur proteins including two rubredoxins, two ferredoxins, Fd I and Fd II, plus other proteins were isolated. These iron-sulfur proteins were all shown to be important for CO₂ fixation (via

PFOR and Q-Sepharose fast flow chromatography) and Q-Sepharose high performance chromatography. After Superose-12 chromatography, all performed in a Coy chamber under strictly anaer-
obic conditions at room temperature. The first purification step, DEAE-Sepharose fast flow chromatography, separated several types of colored proteins that were pink or dark-brown, typical of iron-sulfur proteins. To isolate the [4Fe-4S] cluster-
type Fd from C. tepidum, two major dark-brown proteins of low molecular weight, as determined by gel filtration, were further purified. The major portion of the two Fds eluted at a concentra-
tion of 0.35–0.40 m NaCl after DEAE-Sepharose chromatog-
raphy. After Q-Sepharose high performance column chroma-
tography, Fd I and Fd II eluted at 0.36–0.39 and 0.39–0.42 m NaCl, respectively, at a salt conductivity between 22.9 and 28.2 mS/cm (Fig. 1A). The proteins were concentrated to 2 ml and then loaded onto a Superose-12 column. The protein purity after Superose-12 chromatography was determined using 20% SDS-PAGE gels. Fd I and Fd II migrated with the dye front, even at this high concentration of acrylamide. However, apoFd I and apoFd II, prepared under strictly anaerobic conditions, exhibited molecular masses of ~8–8.5 kDa, respectively (Fig.

Fig. 1. A, chromatographic separation of C. tepidum Fd I and Fd II by Q-Sepharose high performance chromatography. Protein purification was performed in the Coy anaerobic chamber using the Biological Autoprotocol System from Bio-Rad. Column (1.0 × 10 cm) flow rate, 2 ml/min using a linear gradient from 0.3 to 0.5 m NaCl in 10 mM Tris-HCl, pH 7.8, contained 1 mM dithiothreitol. B, anaerobic SDS-
PAGE (20%) of purified Fd I and Fd II. Lane 1, standard marker proteins (66 to 6.5 kDa); lane 2, holoFd I; lane 3, apoFd I; lane 4, holoFd II; lane 5, apoFd II. ApoFds were prepared with 0.5 m HCl under anaerobic conditions at 4 °C.

Fig. 2. Thermal denaturation of C. tepidum Fd I (●, ○) and Fd II (◆, □) under anaerobic (●, ◆), or aerobic conditions (○, □). Fd I and Fd II were incubated at 75 °C in 20 mM Tris-HCl, pH 7.8, containing 0.2 m NaCl.

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The protein bands of holo- and apoFd II were less intense than those of Fd I using Coomassie R-250 staining, which is often the case for acidic low molecular weight proteins or peptides (19). ApoFds were prepared under strictly anaerobic conditions in an argon atmosphere in an ice bath. In the presence of air, apoFds were shown to be degraded during the iron removal procedure. The molecular weights of Fd I and Fd II, determined by gel filtration in the presence of 1.0 M NaCl, were estimated to be 9,000 and 1,000, respectively. In the presence of 0.1 M NaCl, the molecular weights increased to 20,000 and 19,000, respectively, indicating that Fd I and Fd II both probably exist as dimers in vivo at low ionic strength.

General Properties of *C. tepidum* Fd I and Fd II—*C. tepidum* grows optimally at 48 °C; however, Fd I and Fd II exhibit very high thermostability, up to 75 °C under anaerobic conditions (Fig. 2). By contrast, the Fe-S clusters of both Fds decomposed readily during air exposure even at room temperature or in an ice bath. The Fe-S chromophore of Fd II was more sensitive to air than the Fe-S cluster of Fd I. Indeed, after air exposure for a couple of hours at 4 °C, Fd II could be separated into two fractions, each of which was eluted at higher salt concentrations than normal after Q-Sepharose chromatography. Air-exposed Fd II possessed very low capacity to be used as an electron donor or acceptor for PS/PFOR using the assays described under “Experimental Procedures.” The iron content per monomer of purified Fd I and Fd II, as determined by colorimetric assay, was 7.8 ± 0.5 and 7.6 ± 0.5 g atoms iron/mol of protein for Fd I and Fd II, respectively. The amounts of acid-labile sulfide of Fd I and Fd II were consistently lower than the iron content, with values of 7.2 ± 0.5 and 7.1 ± 0.5 g atoms/mol for Fd I and Fd II, respectively. Based on the iron content and deduced amino acid sequences, both Fds possessed typical 2[4Fe-4S] clusters. The calculated isoelectric points (pI) of Fd I and Fd II, determined from either the directly measured amino acid composition or calculated from the deduced amino acid sequence, were 4.02 and 3.24, respectively.

Cyclic voltammograms of *C. tepidum* Fd I and Fd II (Fig. 3) indicated that Fd I and Fd II exhibited a single transition at approximately —514 and —584 mV, respectively, at pH 7.5 and 25 °C under strictly anaerobic conditions. Although Fd I and Fd
II have two different types of [4Fe-4S] clusters, the two clusters from each protein show a single wave intensity. The reduction potentials of C. tepidum Fd I and Fd II were thus more electronegative than any previously studied Fds, in which two [4Fe-4S] clusters showed a single transition. Interestingly, the two [4Fe-4S] clusters present in Fd I and Fd II were shown to be distinct. Reduction of Fd I and Fd II by 5 mM sodium dithionite was shown to cause a dramatic decrease in intensity of the spectra. In general, the overall optical properties of the clusters of these two proteins contribute to the CD spectra of 2[4Fe-4S] Fds, with the pair of CD bands around 370 and 420 nm believed to arise from excitation coupling of the strongest transitions. The two [4Fe-4S] clusters present in these proteins contribute to the CD spectra of 2[4Fe-4S] Fds, with the pair of CD bands around 370 (negative) and 420 nm (positive) are significant. The two [4Fe-4S] clusters present in these proteins contribute to the CD spectra of 2[4Fe-4S] Fds, with the pair of CD bands around 370 and 420 nm believed to arise from excitation coupling of the strongest transitions. These are observed by absorption spectra around 390 nm, which is assigned as S-Cys-Fe charge transfer bands (47).

Absorption, Circular Dichroism, and EPR Spectral Properties—The optical absorption spectra of oxidized and reduced C. tepidum Fd I and Fd II exhibited two identical peaks at 280 and 395 nm, with a shoulder at 312 nm (Fig. 4, A and B). The shoulder at 312 nm decreased gradually during air exposure. There was an approximate 31–33 and 25–27% decrease in absorbance at 395 nm of Fd I and Fd II, respectively, upon reduction of the FeS clusters after either the addition of sodium dithionite or through enzymatic reduction by means by PFOR catalysis. On the basis of a molecular mass of 7.6 kDa for Fd I and 7.4 kDa for Fd II, the molar absorption coefficients of Fd I and Fd II at 395 nm (ε_{395}) were calculated to be 31.6 and 30.9 m M^{-1} cm^{-1}, respectively, and the A_{395}/A_{280} ratios of Fd I and Fd II were in the range of 0.76–0.78 and 0.74–0.76, respectively.

CD determinations of oxidized C. tepidum Fd I and Fd II revealed unique spectral properties. These were characterized by intense positive bands of oxidized and reduced Fd I and Fd II, located at 330, 370, 420, and 570 nm for Fd I and 330, 370, 420, 570 nm for Fd II (Fig. 4, C and D), plus a broad negative band for both Fd I and Fd II at about 700 nm (data not shown). The overall optical properties of the clusters of these two proteins were similar; however, from 350 to 420 nm the CD spectra of Fd I and Fd II were shown to be distinct. Reduction of Fd I and Fd II by 5 mM sodium dithionite was shown to cause a dramatic decrease in intensity of the spectra. In general, the CD properties of oxidized Fd show a strong positive band around 425 nm and a relatively intense negative band around 370 nm; however, neither Fd I nor Fd II from C. tepidum exhibited the negative band at 370 nm. Fd I did not have a prominent CD band around 420 nm. The differences associated with the prominent bands around 370 (negative) and 420 nm (positive) are significant. The two [4Fe-4S] clusters present in these proteins contribute to the CD spectra of 2[4Fe-4S] Fds, with the pair of CD bands around 370 and 420 nm believed to arise from excitation coupling of the strongest transitions. These are observed by absorption spectra around 390 nm, which is assigned as S-Cys-Fe charge transfer bands (47).
signal centered on $g \sim 2$ with a sharp central feature and broad shoulders to both high and low field (not shown). Both Fd I and Fd II could be reduced by pyruvate via the coenzyme A-dependent PFOR activity or by addition of excess dithionite to yield EPR-active states with the signals shown in Fig. 5, A and B, respectively. At 20 K, the $g$ values obtained for Fd I and Fd II were essentially identical, with $g_{1,2,3} = 2.073, 1.938$, and 1.858. These $g$ values compare favorably with $g_{1,2,3} = 2.06, 1.93$, and 1.86 for aconitase (48) and 2.03, 1.94, and 1.86 for the Fd from P. furiosus (49) and are typical of an $S = 1/2$ system. Double integration of the signals for both Fd I and Fd II yielded $\sim 20$ spins per molecule, consistent with the occurrence of two $[4Fe-4S]$ clusters in each protein. Indeed, inspection of the spectra observed for both proteins, particularly in the $g_1$ and $g_2$ regions, suggests that the spectra for the two discrete centers in each protein, whereas obviously very similar, are not identical. Due to the close similarity, however, it was not possible to deconvolute the observed spectra into their components in any meaningful way. Below 10 K the spectra of both proteins grew more complex, with noticeable shoulders developing on the low field $g_1$ feature and a diminution of the $g_3$ feature (Fig. 5). At 5 K, the low field portion of the spectrum, below 250 milliteslas, was silent (data not shown), suggesting that the change in line shape with temperature was not due to spin interaction between the unpaired spins of the two reduced clusters, as half-field features would have been expected. Rather, it is more likely that the observed temperature dependence arises from thermal redistribution among $S = 1/2$ and higher spin states; such behavior is not uncommon with $[4Fe-4S]$ clusters (28).

**Amino Acid Sequences and Modified Conformational Structure**—After the whole 2.1-Mb genome of C. tepidum was sequenced at The Institute for Genomics Research (www.tigr.org) and also illustrated at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) web site (50), N-terminal sequences of purified Fd I and Fd II were used to identify specific genes encoding each isolated protein. From the deduced amino acid sequence of C. tepidum Fd I and Fd II, these proteins were shown to be related to the chromatium-type of 2$[4Fe-4S]$ Fds, particularly with respect to sequence similarity at the second cluster motif. Chromatium-type Fds have 5–7 amino acid insertions between the second and third cysteine in the second cluster. C. tepidum Fd I and Fd II have a very high conservation of cysteine residues (Fig. 6). Although the second cluster motif of Fd I and Fd II resembled chromatium-type Fds, both C. tepidum Fds were more homologous overall to clostridial-type Fds than chromatium-type Fds (17, 18). Based on the deduced amino acid sequences, C. tepidum Fd I and Fd II were nearly identical to the 2$[4Fe-4S]$ Fd I and Fd II proteins from C. limicola.

Tertiary structures (Fig. 7) of C. tepidum Fd I and C. tepidum Fd II were modeled using the Protein Data Bank coordinates of C. vinosum Fd (CaFd; 1BLU), C. pasteurianum Fd (CpFd), P. asacharolyticus Fd (PaFd), and C. acidurici Fd (CaFd; 1FCA). The iron-sulfur cluster coordinates were imported from the CvFd structure and inserted based on an all atom alignment.

**FIG. 7.** The homology modeled structures were built using the Swiss-Model server version 3.5. C. tepidum Fd I and C. tepidum Fd II were modeled using the Protein Data Bank coordinates of C. vinosum Fd (CvFd; 1BLU), C. pasteurianum Fd (CpFd), P. asacharolyticus Fd (PaFd), and C. acidurici Fd (CaFd; 1FCA). The iron-sulfur cluster coordinates were imported from the CvFd structure and inserted based on an all atom alignment.

Anaerobically purified C. tepidum Fd I and Fd II was EPR-silent, as expected for the $S = 0$ oxidized state of $[4Fe-4S]$ clusters, although upon air oxidation both proteins exhibited a

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**Amino Acid Sequences and Modified Conformational Structure**—After the whole 2.1-Mb genome of C. tepidum was sequenced at The Institute for Genomics Research (www.tigr.org) and also illustrated at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) web site (50), N-terminal sequences of purified Fd I and Fd II were used to identify specific genes encoding each isolated protein. From the deduced amino acid sequence of C. tepidum Fd I and Fd II, these proteins were shown to be related to the chromatium-type of 2$[4Fe-4S]$ Fds, particularly with respect to sequence similarity at the second cluster motif. Chromatium-type Fds have 5–7 amino acid insertions between the second and third cysteine in the second cluster. C. tepidum Fd I and Fd II have a very high conservation of cysteine residues (Fig. 6). Although the second cluster motif of Fd I and Fd II resembled chromatium-type Fds, both C. tepidum Fds were more homologous overall to clostridial-type Fds than chromatium-type Fds (17, 18). Based on the deduced amino acid sequences, C. tepidum Fd I and Fd II were nearly identical to the 2$[4Fe-4S]$ Fd I and Fd II proteins from C. limicola.

Tertiary structures (Fig. 7) of C. tepidum Fd I and C. tepidum Fd II were modeled using the Protein Data Bank coordinates of C. vinosum Fd, C. pasteurianum Fd, C. acidurici Fd, and Peptostreptococcus asacharolyticus Fd (32, 51–53). The homology modeled structures were built using the Swiss-Model server version 3.5 (54–56). The iron-sulfur cluster coordinates were imported from the C. vinosum Fd structure and inserted based on an all atom alignments. Only one structure of the chromatium-type of Fd has been solved to date, that from C. vinosum. The high percentage of sequence identity found in regions of C. vinosum Fd as well as clostridial Fd proteins from Clostridium pasteurianum, Clostridium acidurici, and P. asacharolyticus with C. tepidum Fd I and C. tepidum Fd II allowed us to construct practical models with which to visualize the proteins. As observed with the C. vinosum Fd structure, the C. tepidum Fd I and Fd II models exhibit core structures similar to those of Fds of the clostridial class. Also, as predicted by
sequence similarity to *C. vinosum* Fd, both *C. tepidum* Fd I and *C. tepidum* Fd II display a similar extended loop structure around the second FeS cluster (Fig. 7). This loop is composed of residues 42–49 in both *C. tepidum* Fd I and *C. tepidum* Fd II. Unique to *C. vinosum* Fd, hydrophobic residues in the loop (His-43 and Tyr-44) interact with residues of the extended C-terminal α-helix (Ile-71 and Tyr-75) (51). Interestingly, the same hydrophobic residues appear in the equivalent positions of *C. tepidum* Fd I, although in reversed order (Tyr-44 and His-45), despite the lack of an extended C terminus with which to interact.

**Functional Roles of Fd I and Fd II for PFOR Catalysis**—The dependence of *C. tepidum* PFOR on Fd I and Fd II was determined from the time-dependent change of the absorption spectra of the two ferredoxins (Fig. 8). As expected, given the observed catalytic reduction of Fd I and Fd II by PFOR as monitored by EPR (Fig. 5), *C. tepidum* PFOR catalysis resulted in the reduction of Fd I and Fd II. In the reaction mixture, pyruvate, coenzyme A, and PFOR were absolutely required for the reduction of both Fd I and Fd II. Subsequently, steady-state Michaelis-Menten kinetic parameters were determined by monitoring the direct reduction of Fd I and Fd II during PFOR catalysis. Activity was measured at 395 nm using the molar absorption coefficient differences (described under “Experimental Procedures”) of oxidized and reduced Fd I and Fd II. By using the PFOR assay, the $K_m$ values were ~17 and 37 μM for Fd I and Fd II, respectively. Despite differences in the $K_m$ values, $V_{max}$ values of Fd I and Fd II obtained using the MNZ-coupled system were higher than those obtained by the direct Fd reduction assay and much less variable (Table I). With MNZ, the $K_m$ values, $V_{max}$ values for Fd I and Fd II were 3.9 × 10$^6$ and 7.1 × 10$^6$ M$^{-1}$-min$^{-1}$, respectively. Thus, Fd I exhibited a 2.2-fold higher catalytic efficiency than Fd II under the present assay conditions. By using the MNZ-coupled assay system, reduced Fd is spontaneously reoxidized. The reduction of MNZ is essentially irreversible (38), and when MNZ is added in excess, Fd will remain largely oxidized in the steady state. The $V_{max}$ values of Fd I and Fd II obtained using the MNZ-coupled system were higher than those obtained by the direct Fd reduction assay and much less variable (Table I). With MNZ, the $K_m$ values of Fd I and Fd II by PFOR were ~11 and 26 μM, respectively. Again Fd II exhibited a higher $K_m$ value than Fd I, but the $V_{max}$ values for these two electron acceptors were shown to be the same, at ~0.39–0.41 units/mg ($k_{cat}$ of 1.01–1.03 × 10$^2$ min$^{-1}$). In agreement with the direct assay, the catalytic efficiency for Fd I was about 2.3-fold greater than that of Fd II. The $k_{cat}/K_m$ values determined for Fd I and Fd II were 9.2 × 10$^5$ and 3.9 × 10$^6$ M$^{-1}$-min$^{-1}$, respectively, for the MNZ assay. In this assay, MNZ may also be reduced directly by PFOR; kinetic data showing this effect in the absence of Fd are illustrated (Table I). This result suggests that PFOR alone also interacts with MNZ,
although the turnover rate is slow. The in vitro results also suggested that Fd I is a somewhat more effective electron acceptor and electron donor than Fd II for both the forward and reverse reactions catalyzed by PS/PFOR.

Finally, a cytoplasmic hydrogenase from *C. tepidum* was recently purified. Neither *C. tepidum* Fd I nor Fd II could serve as an electron acceptor for this cytoplasmic hydrogenase; rather this enzyme required NAD$^+$ as an electron acceptor.

**DISCUSSION**

The reduction potentials of iron-sulfur proteins may be classified according to their primary structures, which in turn reflect similarities or differences in visible, CD, and EPR spectra. Although the same basic [4Fe-4S]$_2$ cluster with four cysteine ligands is found in the redox active site of iron-sulfur proteins, the reduction potential for the cluster varies widely. Iron-sulfur proteins containing [4Fe-4S]$_{3\times2+}$ clusters have positive reduction potentials, ranging from 90 to 450 mV (17), whereas proteins with [4Fe-4S]$_{2\times2+}$ clusters have negative potentials from −280 to −715 mV (17, 23, 25, 31–33). *C. tepidum* Fd I and Fd II have typical two [4Fe-4S]$_{2\times2+}$ clusters that are coordinated in the first cluster via a C–C–C link, which are involved in the formation of salt bridges between redox partners for electron transfer, may not be involved in maintaining the unusually low reduction potential of Fd I. *C. tepidum* Fd II eluted at a higher salt concentration than *C. tepidum* Fd I during anion exchange chromatography; the calculated pI values of Fd I and Fd II are 4.02 and 3.24, respectively, which also confirm that Fd II is a more acidic protein than Fd I. The low pI of Fd II may explain why it stains poorly on SDS-PAGE gels. The greater frequency of acidic amino acid residues in Fd II relative to Fd I may account for the lower reduction potential of the former. Alternatively, there may be some other fundamental reason for this difference in potential. It appears clear, however, that differences in the reduction potentials of Fd I and Fd II cannot be explained on the basis of different amino acid residues near the [4Fe-4S] cluster. Comparisons of high resolution structures of *A. vinelandii* and *C. vinosum* Fds are unusually low, −633 and −653 mV, respectively (17, 23, 32), whereas *C. pasteurianum* and *C. acidurici* Fds have more positive redox values ranging from −403 to −434 mV, respectively (17, 31, 33). These values may be affected by parameters such as pH; nevertheless, the fact that the $E_0'$ values for the [4Fe-4S]$_{2\times2+}$ clusters in these different Fds may vary by over −200 mV suggests fundamental differences among these proteins related to unique structural arrangements. The eight amino acid groups that H-bond to the sulfur atoms of the cluster have been identified and shown to be highly conserved. This pattern is also typified by *C. tepidum* Fd I and Fd II, where the same sequence motifs that are involved in binding the [4Fe-4S]$_{2\times2+}$ cluster are found. Despite these similarities, it is apparent these conserved residues alone cannot be responsible for the considerable differences observed in the reduction potentials of the *C. tepidum* proteins (Fig. 3). Indeed, site-directed mutagenesis studies indicate that other residues contribute to the net reduction potential because alterations of the protein surface to a more negative charge near the [4Fe-4S] clusters have a great influence (57, 63). From deduced amino acid sequence comparisons (Fig. 6), *C. tepidum* Fd II did not contain any obvious negatively charged residues near the [4Fe-4S]$_{2\times2+}$ cluster that were different from *C. tepidum* Fd I. This suggests that aspartate and glutamate residues near the [4Fe-4S]$_{2\times2+}$ cluster, which are involved in the formation of salt bridges between redox partners for electron transfer, may not be involved in maintaining the unusually low reduction potential of Fd I. *C. tepidum* Fd II eluted at a higher salt concentration than *C. tepidum* Fd I during anion exchange chromatography; the calculated pI values of Fd I and Fd II are 4.02 and 3.24, respectively, which also confirm that Fd II is a more acidic protein than Fd I. The low pI of Fd II may explain why it stains poorly on SDS-PAGE gels. The greater frequency of acidic amino acid residues in Fd II relative to Fd I may account for the lower reduction potential of the former. Alternatively, there may be some other fundamental reason for this difference in potential. It appears clear, however, that differences in the reduction potentials of Fd I and Fd II cannot be explained on the basis of different amino acid residues near the [4Fe-4S] cluster. Comparisons of high resolution structures of *A. vinelandii* Fd and *P. asaccarolyticus* Fd implicate the importance of another factor, namely the relative solvent accessibility and protein folding near the [4Fe-4S]$_{2\times2+}$ clusters (63). With regard to *C. tepidum* Fd I and Fd II, there is no simple explanation for the different reduction potentials, although solvent accessibility and protein folding near the [4Fe-4S]$_{2\times2+}$ clusters are obvious candidates. In *C. tepidum* Fd I and Fd II, the resulting loop in the modeled *C. tepidum* Fd II structure shows a distinct deviation from that of *C. vinosum* Fd. Attempts to correct for this discrepancy using molecular mechanics were unsuccessful, suggesting a slightly different structure for *C. tepidum* Fd II in this loop. This may be responsible for the electrochemical dif-
ferences seen between C. tepidum Fd I and C. tepidum Fd II. Site-directed mutagenesis studies with C. vinosum Fd indicated that this loop contributes to the reduced potential of the second cluster compared with C. pasteurianum Fd (32). Thus, the fact that C. tepidum Fd II shows less sequence identity with C. vinosum Fd than C. tepidum Fd I in this loop may be significant.

The biological properties of these two C. tepidum Fds, determined from the in vitro experiments, indicated that both Fds probably exist in the form of a dimer in vivo at low ionic strength. This structural arrangement is required to support catalysis of C. tepidum PS/PFOR, a protein that also possesses a homodimeric structure. Thus, a complex of the two dimers may also be reduced using NADH or NAD(P)H in the presence of air; alternatively, the iron-sulfur centers of the EPR signals showed that C. tepidum Fd I and Fd II accept 2 electrons upon reduction. These results suggest that the C. tepidum Fd I and Fd II polyproteins contain two bound [4Fe-4S] clusters, which undoubtedly function as the biological electron donor or acceptor for C. tepidum PS/PFOR (3).

To obtain an accurate comparison of kinetic constants between the two Fds, we determined PFOR activity both by direct reduction of Fd and through the use of an MNZ-linked assay system. Fd I exhibited a 2-fold lower $K_m$ value than Fd II, but the $V_{\text{max}}$ values were essentially identical to one another, such that the $k_{\text{cat}}/K_m$ value of Fd I was ~2.3-fold higher than that measured for Fd II. In the MNZ-linked assay system, the $K_m$ value was lower; however, the $V_{\text{max}}$ value was higher than in the direct reduction assay. It is reasonable to assume that Fd reduced by PFOR is spontaneously oxidized by MNZ and that oxidized Fd remains constant during the catalytic reaction period. The reduction activity of both Fds did not show any inhibition at higher concentrations of Fd; this is different from C. pasteurianum PFOR where activity is inhibited even at Fd concentrations over 5 $\mu$M (64). Interestingly, C. tepidum PFOR alone was able to reduce MNZ during the pyruvate oxidation reaction, even though the $k_{\text{cat}}$ was very low (5.1 min$^{-1}$).

Thus far, the only attributed physiological role for C. tepidum Fd I and Fd II is as an electron acceptor or donor for PS/PFOR and KGS/KGOR (see Reactions 1 and 2). Unlike the situation with Fds from most bacteria, which may be reduced enzymatically with hydrogen in the presence of hydrogenase (16, 17, 65), neither C. tepidum Fd served as an electron acceptor for a recently isolated NAD-linked hydrogenase.

Fd may also be reduced using NADH or NADP+H in the presence of ferredoxin oxidoreductase (36). Related to this, it was recently reported that four different C. tepidum Fd fractions (A–D) could support the photoreduction of NADP+ in the presence of spinach Fd-NADP+ reductase and reaction center particles from C. tepidum (19). Detailed properties of these four different fractions were not presented; however, Fd B and Fd D appear similar to our Fd I and Fd II preparations and seem to be more specific for supporting the photoreduction of NADP+.

In our studies, we found Fd II to be more sensitive to air than purified Fd I. Indeed, after air exposure, Fd II could be separated into two different fractions that eluted from ion exchange columns at high salt concentrations. The latter observation is probably related to the appearance of the different peaks observed by Seo et al. (19) as these authors prepared their Fd fractions under an aerobic atmosphere, with some degradation of the absorbance at 395 nm noted. It is uncertain why Fd I was more air-stable than Fd II, but it may be related to decomposition of the sulfide bridge within the iron-sulfur clusters in the presence of air; alternatively, the iron-sulfur centers of Fd II may be more accessible to oxygen molecules than those of Fd I.

Finally, a phylogenetic tree of bacterial Fds has been developed (Fig. 9) in which the two [4Fe-4S] Fds can be divided into two types (50). In this context, the evolutionary implications of C. tepidum Fd I and Fd II may be considered. The classical model of Fd evolution suggests that the polypeptide chain of ancestral Fd was half as long as modern Fd and two 4Fe-4S domains are believed to have formed symmetrical homodimers. After gene duplication and fusion events, two 4Fe-4S clusters might then have formed a single polypeptide. Accordingly, Fds containing [2Fe-4S] proteins appear to have arisen from the symmetric or asymmetric division of the N- and C-terminal regions of the protein. The phylogenetic tree (Fig. 9) and other sequence and structure-function properties reported here suggest the very likely scenario that C. tepidum Fd I and Fd II may be an evolutionary intermediate of clostridial- or chromatium-type [2Fe-4S] cluster Fds. Presumably, these proteins were formed after gene duplication and fusion events.

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Spectroscopic and Functional Properties of Novel 2[4Fe-4S] Cluster-containing Ferredoxins from the Green Sulfur Bacterium *Chlorobium tepidum*

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