Biochemical and Biophysical Research Communications **260,** 209–215 (1999)
Article ID bbrc.1999.0748, available online at http://www.idealibrary.com on IDEAL®

Biochemical and Spectroscopic Characterization of Overexpressed Fuscoredoxin from *Escherichia coli*

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Received April 12, 1999

Fuscoredoxin is a unique iron containing protein of yet unknown function originally discovered in the sulfate reducers of the genus *Desulfovibrio.* It contains two iron-sulfur clusters: a cubane [4Fe-4S] and a mixed oxo- and sulfido-bridged 4Fe cluster of unprecedented structure. The recent determination of the genomic sequence of *Escherichia coli* (*E. coli*) has revealed a homologue of fuscoredoxin in this facultative microbe. The presence of this gene in *E. coli* raises interesting questions regarding the function of fuscoredoxin and whether this gene represents a structural homologue of the better-characterized *Desulfovibrio* proteins. In order to explore the latter, an overexpression system for the *E. coli* fuscoredoxin gene was devised. The gene was cloned from genomic DNA by use of the polymerase chain reaction into the expression vector pT7-7 and overexpressed in *E. coli* BL21(DE3) cells. After two chromatographic steps a good yield of recombinant protein was obtained (approximately 4 mg of pure protein per liter of culture). The purified protein exhibits an optical spectrum characteristic of the homologue from *D. desulfuricans,* indicating that cofactor assembly was accomplished. Iron analysis indicated that the protein contains circa 8 iron atoms/molecule which were shown by EPR and Mössbauer spectroscopies to be present as two multinuclear clusters, albeit with slightly altered spectroscopic features. A comparison of the primary sequences of fuscoredoxins is presented and differences on cluster coordination modes are discussed on the light of the spectroscopic data.

By definition, iron-sulfur (Fe-S) proteins contain iron-sulfur clusters in which the iron atoms are solely or predominantly coordinated by sulfur atoms. Found in all living organisms, these proteins can play diverse functional roles, including electron transfer, chemical catalysis, iron regulation, biological sensoring devices for Fe, O_2 and O_2^-, protein radical generation and protein structure stabilization (1–4). Fe-S clusters can have different nuclearities. The simplest one is the FeS_4 of the rubredoxin type in which a single iron atom is coordinated by four cysteinyl residues from the polypeptide chain. Examples of clusters with different nuclearities are the [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters. Although rare in biological systems, Fe-S clusters with higher nuclearities exist, such as the [8Fe-7S] or the [7Fe, Mo-6S] clusters in nitrogenase (5). Additional complex cofactors have been identified in sulfite reductase (6), CO dehydrogenase (7, 8), and hydrogenase (9), where Fe-S centers are covalently linked to other metals/groups.

Fuscoredoxin is a very unusual iron-sulfur protein isolated from the sulfate reducing bacteria *Desulfovibrio* (*D.*) *vulgaris* (Hildenborough) (10), and *D. desulfuricans* (ATCC 27774) (11). Initially, the protein was described to contain 6Fe cluster(s) (11–13). However, X-ray crystallography has recently shown that fuscoredoxin contains two tetranuclear clusters, a cubane-type [4Fe-4S] (cluster 1) and a cluster of novel structure (cluster 2) (14–16). The cubane [4Fe-4S] is coordinated by four cysteine residues in a Cys-X_2-Cys-X_8-Cys-X_5-Cys binding motif closely located in the N-terminus region of the protein (14). The Mössbauer parameters of this cluster are typical of a ferredoxin-type $[4Fe\text{-}4S]^{1+/2+}$ (11, 15, 16). The dithionite-reduced $[4Fe\text{-}4S]^{1+}$ cluster exhibits unusual magnetic properties which have been interpreted as a quantum-mechanically admixed $S = 3/2$, $S = 1/2$ state (14). Cluster 2 is unique among the Fe-S clusters described so far. The X-ray crystallographic data (14) indicate that cluster 2 has a mixed N, O, and S coordination environment, confirming previous assignment by Mössbauer spectroscopy (16). Cluster 2 has a [4Fe-2O, 3S] core, the structure of which can be described as a

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[2Fe-2S] linked to a μ-oxo diiron unit by an oxo group and an unidentified ligand "X". Resonance Raman spectroscopy suggests that ligand X is a solvent-exchangeable oxygen group (17). The geometry of the two Fe atoms coordinated by sulfur ligands is described as tetrahedral while the geometry of the other two as trigonal bipyramidal. Interestingly, terminal ligation of one of the iron atoms is through a sulfur atom that appears to be part of a persulfido group (Cys-S-S$^-$) (14).

Both clusters were shown to be redox active (11, 15, 16). Cluster 1 is capable of transferring one electron at a relatively low midpoint redox potential similar to ferredoxin-type [4Fe-4S]$^{1+/2+}$ clusters. Cluster 2 on the other hand is able to accept multiple electrons and can be stabilized in at least four oxidation states. In its most oxidized form, cluster 2 is diamagnetic with all iron atoms in the ferric state. Introduction of one electron into the fully oxidized cluster 2 yields an $S = 9/2$ state with characteristic EPR and Mössbauer properties. Addition of another electron converts the $S = 9/2$ state into an integer spin state. Further reduction results in yet another half-integer spin state.

With the development of molecular biology techniques the fuscoredoxin-encoding gene has been identified in a wide range of microorganisms beside the *Desulfovibrio* species: *Thiobacillus ferrooxidans* (18), *Morganella morganii* (19), *Methanococcus jannaschii* (20), *Methanobacterium thermoautotrophicum* (DeltaH) (21), and *Escherichia* (*E.*) *coli.* (Blattner, F. R., Plunkett, G. III, Mayhew, G. F., Perna, N. T., and Glasner, F. D., unpublished data, Swiss-Prot #P75825). In this manuscript we report the expression in *E. coli* of the full-length gene encoding *E. coli* fuscoredoxin and the preliminary spectroscopic studies on the oxidized and dithionite-reduced forms of the recombinant protein.

EXPERIMENTAL PROCEDURES

Materials

The oligonucleotides used to amplify the fuscoredoxin-encoding gene were synthesized by the Molecular Biology Core Facility at the Mayo Clinic and Foundation. The expression vector pT7-7 was a gift of Dr. Stan Tabor (Harvard Medical School). Competent *E. coli* BL21(DE3) cells were purchased from Novagen Inc. (Madison, WI). Automated DNA sequencing was carried out by the Mayo Clinic Molecular Biology Facility. Source 15Q and Superdex 75 prep grade resins were purchased from Pharmacia Biotech. All other chemicals were of reagent grade or highest available purity. Optical spectra were recorded using a Shimadzu UV-160A or a Shimadzu UV-265FS spectrophotometer. EPR measurements were performed on an X-band Bruker EMX spectrometer equipped with a dual-mode cavity (Model ER 4116DM) and an Oxford Instruments continuous flow cryostat.

Methods

Construction of plasmid pTisa7-7. The fuscoredoxin gene was isolated from *E. coli* JM101 genomic DNA by the Polymerase Chain Reaction (PCR) using two oligonucleotide primers homologous to the 5′ and 3′ ends of non-coding strand of the fuscoredoxin gene respectively (5′-CCATGGATCCTCTAGAAGGAGATATACATATGATCATGTTTTGTGTGC-3′ and 5′-TTAAGCTTCTGCAGTCGACTTACGCGCTCAACAGTTG-3′, respectively). Both primers contain restriction endonuclease sites for subsequent subcloning. Also, primers were design in order to change the first amino acid from valine to methionine. Following PCR amplification, the resulting double stranded DNA fragment was purified by agarose gel electrophoresis and the resulting 1.7 kb fragment was digested with NdeI and PstI and ligated directly into pT7-7 digested similarly. Ligated plasmid was transformed into competent *E. coli* DH5-α cells and recombinant clones screened by restriction digest analysis of plasmid DNA. The complete fuscoredoxin encoding sequence of positive clones were verified by DNA sequencing of the entire gene. A positive clone was selected for subsequent protein expression described below.

Overexpression and purification of fuscoredoxin. The pTisa7-7 expression plasmid described above was used to transform *E. coli* strain BL21(DE3). The expression protocol has been described elsewhere (22). Briefly, bacteria harboring the pTisa7-7 expression vector were grown in Luria Broth medium containing 100 μg/ml ampicillin, at 37°C with shaking. When the absorbance at 595 nm reached approximately 0.7, the expression of fuscoredoxin was induced by addition of IPTG to a final concentration of 1.0 mM. The cell culture was incubated for an additional 6 h, after which the cells were harvested by centrifugation at 3,000*g* for 15 min. The cell yield was typically in the 4–5 g/liter range (wet weight).

All purification steps were carried out at 4°C and by High Performance Liquid Chromatography. The cells were resuspended in a minimum volume of 0.01 M Tris–HCl, pH 7.6 and lysed by three passages through a French Press cell at 8,000 p.s.i. The cells debris were removed by centrifugation at 15,000*g* for 20 min and the resulting supernatant was further ultracentrifuged for 45 min at 180,000*g* to obtain the crude extract.

The crude extract was loaded onto a Source 15Q column (2.2 × 30 cm) previously equilibrated with 0.01 M Tris–HCl, pH 7.6. A linear gradient (0.01–0.5 M) was applied with a flow of 2 ml/min during 3 h. At about 0.2 M Tris–HCl a fraction was eluted exhibiting an optical spectrum typical of fuscoredoxin. After concentration on a Diaflo apparatus equipped with a YM30 membrane (Amicon), the protein fraction was loaded onto a Superdex 75 gel filtration column (2.6 × 70 cm) equilibrated with 0.05 M Tris–HCl, pH 7.0, 0.3 M NaCl and eluted with the same buffer. After this step the protein was considered pure as established by SDS–PAGE.

SDS–PAGE, protein determination, and iron content. SDS–PAGE was performed as described by Laemmli (23). 12.5% acrylamide gels were used and the protein bands were visualized with Coomassie Brilliant Blue R-250 staining. The Low Molecular Weight calibration kit from Pharmacia Biotech was used as protein markers. Protein concentrations were determined by the Lowry method using a mixture of human serum albumin and globulin (Sigma) as a standard (24). The iron content was determined chemically using 2,4,6-tripyridyl-*s*-triazine (Sigma) assay as described by Fischer and Price (25).

Spectroscopic quantifications. Spin quantitations of the $S = 1/2$ type EPR signals were determined by double integration of the first-derivative spectra relative to the spectrum of Cu(EDTA) recorded under non-saturating conditions.

RESULTS AND DISCUSSION

The complete coding sequence of the *E. coli* fuscoredoxin was cloned into the expression vector pT7-7 that carries a strong promoter and terminator sequences derived from phage T7. For this purpose the fuscoredoxin-encoding gene was amplified by the polymerase chain reaction using two synthetic oligonucleotides as primers and *E. coli* genomic DNA as template. The

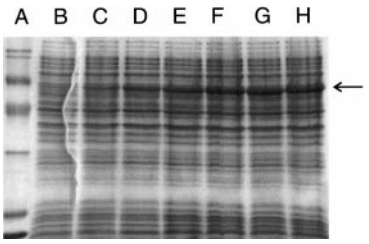

FIG. 1. SDS–PAGE (12.5%) analysis of fuscoredoxin expression in IPTG-induced *E. coli* cells before (lane B) and after (lanes C to H, 1 to 6 h, respectively) IPTG induction. The arrow indicates the 59-kDa protein band. Fuscoredoxin was expressed and subjected to SDS–PAGE as described under Methods. For comparison, molecular weight markers are shown in lane A.

entire fuscoredoxin gene following subcloning into pT7-7 was sequenced to assure the absence of errors introduced during PCR. Cells harboring the pTisa7-7 expression plasmid were induced by addition of IPTG to the LB culture. Expression was verified by SDS–PAGE analysis of protein extracts from recombinant *E. coli* cells (see Fig. 1). As shown in Fig. 1, fuscoredoxin was overproduced as a major protein among the soluble fraction. The recombinant fuscoredoxin was purified to homogeneity by two chromatographic steps. A yield of ≈4 mg of pure protein per liter of culture was obtained.

The molecular mass of the protein, determined by SDS–PAGE to be 59 ± 1 kDa, agrees with the values reported for the other fuscoredoxins described so far (11, 12). Moreover, this value compare well to the molecular mass as predicted from the gene sequence (60,276 Da).

The optical absorption spectrum of the recombinant fuscoredoxin (Fig. 2) is very similar to the one observed for the proteins isolated from *Desulfovibrio* species.

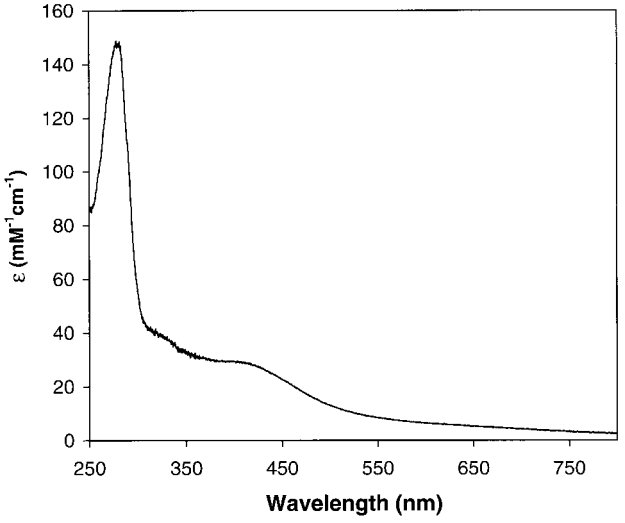

FIG. 2. Optical absorption spectrum of *E. coli* recombinant fuscoredoxin in 0.1 M Tris–HCl, pH 7.6.

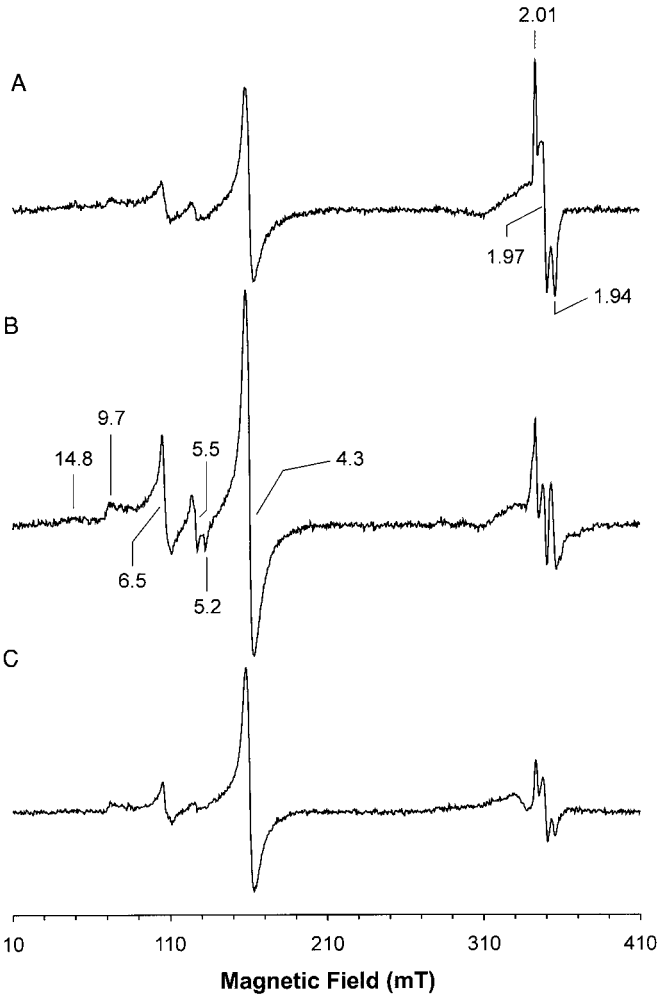

FIG. 3. EPR spectra of the as-isolated *E. coli* recombinant fuscoredoxin. The spectra are recorded at 4.2 K (A), 12 K (B), and 35 K (C). Other experimental conditions are: microwave frequency, 9.652 GHz; microwave power, 0.2 mW; modulation amplitude, 1 mT; receiver gain, 2×10^5.

The spectrum displays a broad feature around 400 nm, a shoulder at 305 nm and an absorption maximum at 280 nm, indicating that the recombinant protein is expressed containing Fe clusters. The molar extinction coefficient at 400 nm was determined to be 30 ± 1 $mM^{-1}\,cm^{-1}$ which is within the range determined previously for the *D. desulfuricans* fuscoredoxin (25–34 $mM^{-1}\,cm^{-1}$) (11).

The protein contains 8 ± 1Fe atoms/molecule as determined by the TPTZ assay and based on a protein concentration determined by the Lowry method, in agreement with the presence of two 4Fe clusters.

Figure 3 shows the EPR spectra of the *E. coli* recombinant fuscoredoxin in the as-isolated form recorded at different temperatures. The spectra are characterized by two major groups of resonance peaks, at high- and low-field regions, respectively. At high field, the spectra show an EPR signal typical of $S = 1/2$ species with

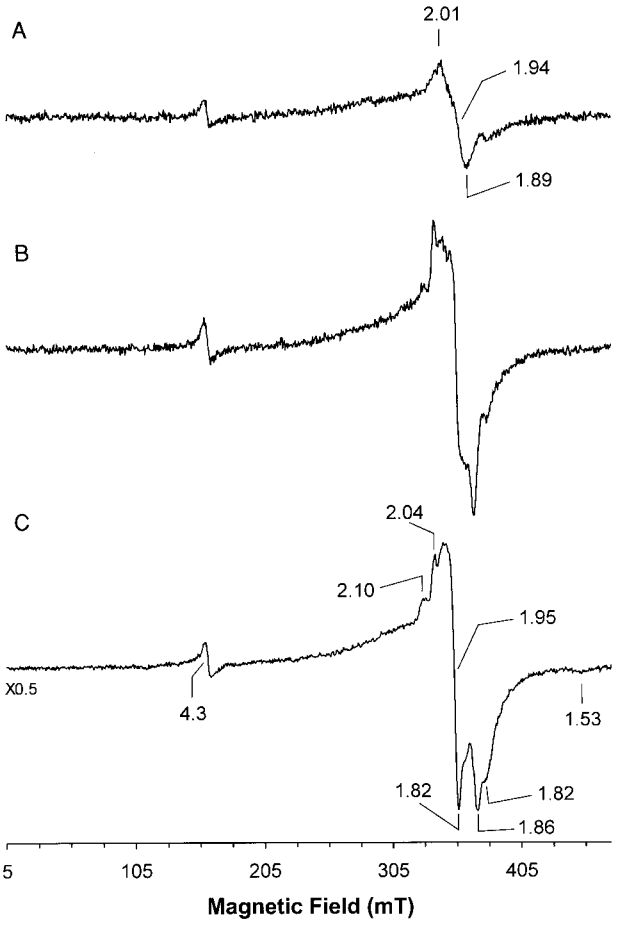

FIG. 4. EPR spectra of the dithionite-reduced *E. coli* recombinant fuscoredoxin. The spectra are recorded at 7 K (A), 15 K (B), and 25 K (C). Other expererimental conditions are: microwave frequency, 9.653 GHz; microwave power, 2 mW; modulation amplitude, 1 mT; receiver gain, 2×10^5.

g-values at 2.01, 1.97, and 1.94 that accounts for less than 0.1 spins/molecule. At low field, a group of resonances in the region between $g = 9.7$ and $g = 4.3$ is visible. The signal at $g = 4.3$ is typical of adventitiously bound ferric ions and may represent a minor impurity. The previous EPR and Mössbauer studies on the protein isolated from *D. desulfuricans* showed that the as-isolated fuscoredoxin exhibits an $S = 9/2$ EPR signal attributed to the one-electron reduced cluster 2 (11, 16). In addition to the resonances detected on the recombinant *E. coli* fuscoredoxin, the *D. desulfuricans* protein exhibits a resonance peak at $g = 15.3$, which has been assigned to the highest Kramer doublet ($S_z = \pm 1/2$) of the $S = 9/2$ spin multiplet. The resonances between $g = 9.7$ and $g = 4.3$ could be originated from the other excited doublets (for $D = -0.7\ \text{cm}^{-1}$ and $E/D = 0.062$). Similar observation have been made in the *D. vulgaris* protein (13).

The same assignment can be made for the signals observed between $g = 9.7$ and $g > 4.3$ for the *E. coli* protein. In Fig. 3, the spectra recorded at 4.2 K (A), 12 K (B), and 35 K (C) are shown. As the temperature increases, these EPR signals show a clear increase, followed by a decrease in their intensities, a property that is expected for the excited middle Kramer's doublets of the $S = 9/2$ spin multiplet. It appears that a larger energy splitting of the spin sublevels exist in the *E. coli* fuscoredoxin cluster 2 when compared to the *Desulfovibrio* proteins. The failure to observe the low-field signal of the highest doublet in the *E. coli* protein may be due to (i) the higher D value or (ii) g strain, or both.

Figure 4 shows the EPR spectra of the dithionite-reduced *E. coli* fuscoredoxin. The spectra are dominated by a complex signal centered around $g = 2.0$, whose components exhibit different temperature-dependent relaxation behavior. At least three sets of *g*-values are observed. A rhombic signal with *g*-values of 1.82, 1.53, and ≈1.3, detected up to 15 K, is attributed to the reduced cluster 1 as previously assigned by Arendensen *et al.* (14). The remaining resonances probably are originated from the magnetic interaction of clusters 1 and 2. Additional complexity may arise from the fact that cluster 2 exist in different conformations. At temperatures above 25 K only resonances at $g = 2.01$ and $g = 1.94$ can be observed. However, an important observation can be made. With the exception of a small resonance at $g = 4.3$ (probably due to an unreduced ferric impurity) no other resonances were observed in this region of the spectrum. So, in the case of *E. coli* fuscoredoxin, cluster 1 does not possess a quantum-mechanically admixed $S = 3/2$, $S = 1/2$ state. A structural difference resulting from an altered amino acid sequence of the N-terminus region, where the cysteinyl ligands of cluster 1 are located, might explain the difference in the magnetic behavior (see below).

Similar to that of the *D. desulfuricans* protein, the Mössbauer (data not shown) of the as-isolated *E. coli* fuscoredoxin also show two spectral components corresponding to two multinuclear iron clusters in two different electronic states. One component displays a large magnetic splitting similar to that observed for the one-electron-reduced cluster 2 of the *D. desulfuricans* fuscoredoxin (16). The other component displays diamagnetic property and can be attributed to the oxidized form of cluster 1.

FIG. 5. Amino acid sequence comparison of known Fuscoredoxin and Fuscoredoxin-like genes from different organisms: (1) *D. desulfuricans* ATCC 27774, (2) *Morganella morganii*, (3) *Methanococcus jannaschii*, (4) *E. coli*, (5) *Thiobacillus ferrooxidans*, (6) *D. vulgaris*, and (7) *Methanobacterium thermoautotrophicum*. Cluster ligands are marked with ⇩ (cluster 1) and ✪ (cluster 2).

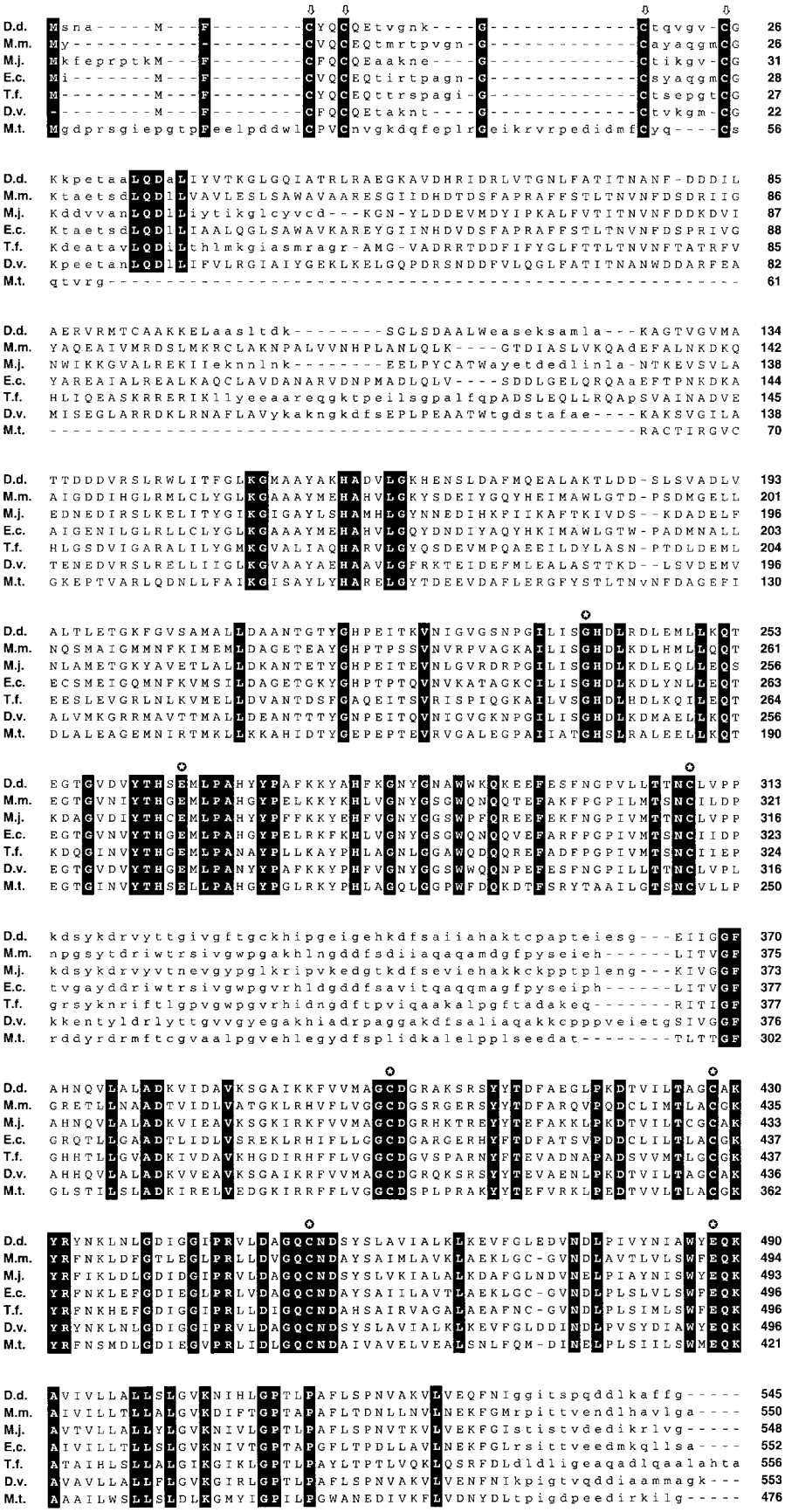
D.d.
M.m.
M.j.
E.c.
T.f.
D.v.
M.t.

Figure 5 shows the comparison of all known fuscoredoxin amino acid sequences. In the proposed alignment a total of 83 amino acids are conserved, including all the ligands for cluster 1 and 2. Such a degree of homology is surprising since seven different sequences, corresponding to six different genera, are compared. From this alignment, two patterns are evident. First, although the first two cysteines of the sequence motif that coordinates the [4Fe-4S] cluster have exactly two intervening residues, the number of residues which separate the other two coordinating cysteine residues falls into three groups: (*i*) pattern A, Cys-X_2-Cys-X_8-Cys-X_5-Cys-Gly, present in *D. desulfuricans*, *D. vulgaris*, and *Methanococcus jannaschii* fuscoredoxin sequences; (*ii*) pattern B, Cys-X_2-Cys-X_{11}-Cys-X_6-Cys-Gly, present in *E. coli*, *Morganella morganii*, and *Thiobacillus ferrooxidans* fuscoredoxin sequences; and (*iii*) pattern C, Cys-X_2-Cys-X_{25}-Cys-X_2-Cys-Gly, present only in *Methanobacterium thermoautotrophicum* fuscoredoxin sequence. Correlating the available EPR data with the current sequence comparison, we may tentatively associate pattern A to those clusters 1 that exhibit quantum mechanically admixed $S = 3/2$ and $S = 1/2$ state and pattern B to those clusters that exhibit only a $S = 1/2$ state in their reduced form. Second, besides the ligands for cluster 2, several amino acid residues capable of coordinating iron atoms are conserved in the sequence. For example, in *D. vulgaris* fuscoredoxin, besides E268, C459, and H244 which coordinate Fe7, other residues that reside in the highly conserved regions includes H164, D245, D248, Q255, H266, D461, E494, and E407. Although these residues do not form ligands to cluster 2 in the oxidation state used to obtain the X-ray structure, it is conceivable that they may displace a ligand or raise the coordination number of the iron sites in other oxidation states. Several examples of this type of structural flexibility are known and may be important for protein function. For example, it has been shown that the carboxylate ligands of the dinuclear iron clusters in soluble methane monooxygenase (26, 27) and in R2 subunit of *E. coli* ribonucleotide reductase (28, 29) can undergo carboxylate shifts between oxidized and reduced states, resulting in the acquisition of new ligands to the Fe ions. Also, Peters *et al.* have shown that the coordination environment of the 8Fe P cluster of nitrogenase is also redox sensitive (30).

In conclusion we report, in this communication, the overexpression, the isolation and the preliminary spectroscopic characterization of the first fuscoredoxin protein not purified from the *Desulfovibrio genus.* The study of both differences and similarities with the other two known *Desulfovibrio* fuscoredoxins may help on the understanding of the intricate magnetic and redox properties of this emerging class of proteins. Furthermore, the study of the *E. coli* system will be an important tool for revealing the biological significance of fuscoredoxin.

ACKNOWLEDGMENTS

This work was supported in part by a PRAXIS XXI grant (to I.M. and J.J.G.M.), funds provided by the Mayo Foundation (to F.R.), and a National Institutes of Health grant GM 47295 (to B.H.H.). J.J.G.M. also thanks the Mayo Foundation for support during his sabbatical leave in April 1997, when the overexpression of *E. coli* Fuscoredoxin was initiated.

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