FEBS Letters 351 (1994) 401–404

FEBS 14504

Cloning, sequencing and overexpression of the *Desulfovibrio gigas* ferredoxin gene in *E. coli*

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Received 14 July 1994

Abstract We have cloned the gene encoding *Desulfovibrio gigas* ferredoxin using a photodigoxigenin-labelled probe synthesized with the polymerase chain reaction. The DNA sequence of the gene predicts a polypeptide of 58 residues after removal of the initial formyl methionine (polypeptide M_r = 6,276). The ferredoxin gene was expressed in aerobically grown *E. coli* behind the *lac* promoter of pUC18 resulting in a high level of ferredoxin expression which comprises about 10% of the total cell protein. EPR analysis of recombinant ferredoxin revealed the presence of a [3Fe-4S] cluster which is characteristic of native *D. gigas* ferredoxin II.

Key words: Ferredoxin; Ferredoxin gene; Iron-sulfur cluster; *Desulfovibrio gigas*

1. Introduction

Ferredoxins are small iron-sulfur containing proteins involved in a variety of electron transfer reactions in different metabolic pathways. *Desulfovibrio gigas* ferredoxins were the first isolated from sulfate reducing bacteria [1] and have been extensively studied by different experimental approaches. Two distinct forms of ferredoxin, ferredoxin I and ferredoxin II have been identified in *D. gigas* [1–3]. Both contain the same polypeptide chain, but have different native molecular masses as a result of different states of oligomerization [4]. Ferredoxin I is a dimer and contains one (4Fe-4S] cluster per monomer while ferredoxin II is a tetramer and contains one [3Fe-4S] cluster per monomer [5]. The [3Fe-4S] core present in ferredoxin II can be converted in vitro to a [4Fe-4S] cluster [6] and the interconversion of the two oligomeric forms appears to be a unique feature of *D. gigas* ferredoxins.

The amino acid sequence of *D. gigas* native ferredoxin suggested that it was composed of 57 amino acids [7]. X-ray crystallography, however, disclosed an additional valine residue at position 56 and hence, a total of 58 residues [8]. Cysteine residues at positions 8, 11, 14 and 50 are involved in liganding the iron-sulfur cluster while cysteines at positions 18 and 42 form the only disulfide bond in the protein. Cysteine 11 has a crucial role in the process of cluster interconversion, tilted away towards the solvent in [3Fe-4S] ferredoxin II [8] and becoming a cluster ligand in [4Fe-4S] ferredoxin I [9].

In this paper we describe the cloning and sequencing of the gene encoding *D. gigas* ferredoxin. The nucleotide-derived amino acid sequence demonstrates that the amino acid at position 56 is not valine, but rather isoleucine. In addition, we have overexpressed the protein in *E. coli* and studied some spectroscopic properties of the recombinant ferredoxin.

2. Materials and methods

2.1. Cloning of the D. gigas ferredoxin gene

D. gigas was grown in basic sulfate medium [10], harvested by centrifugation at 4°C, and extracted for DNA by standard procedures [11]. Two primers with the least degeneracy were designed according to the published protein sequence of *D. gigas* ferredoxin [7]. The sequences of the probes were: 5′-AAQGAQGAQTGQATGGC-3′, DGpr1; and 5′-CCQTCQTCPTTCATQTCPAA-3′, DGpr2 (Q = C/T, P = G/A). Southern analysis showed that both probes hybridized to a 2 kb *Sma*I restriction fragment of *D. gigas* genomic DNA. Accordingly, a *Sma*I fragment pool was used to generate a subgenomic library in pUC18.

A large probe for screening the subgenomic library was synthesized by PCR with *D. gigas* genomic DNA as template. Purified PCR fragment was sequenced and the nucleotide-derived amino acid sequence was identical to the published amino acid sequence of *D. gigas* ferredoxin. The PCR reaction was repeated in the presence of DIG-11-dUTP to produce a functional 178 bp probe. The photodigoxigenin-labelled probe was used to screen the *D. gigas* subgenomic library using a Genius Labeling and Detection Kit (Boehringer Mannheim Corp., Indianapolis, IN). Clones containing the ferredoxin gene were identified and both strands of one such clone designated pUCGF-1 were sequenced by dideoxy sequencing [12] using synthesized primers.

2.2. Overexpression and purification of recombinant ferredoxin

A 430 bp *Nae*I/*Msc*I fragment from pUCGF-1 (Fig. 1) which included 205 bp upstream of the initiator methionine codon was blunt end ligated into the *Sma*I site of pUC18. A positive clone in which the ferredoxin gene was in the same orientation as the *lac* promoter was identified. This clone, pUGN-1, was used for analysis of ferredoxin expression.

For pilot experiments, 50 ml aliquots of 2 × YT media containing 100 μg/ml ampicillin, 0.1% glucose and 0.5 mM $FeSO_4$, were inoculated with 0.5 ml of an overnight culture. Cultures were aerobically grown to an OD_{600} of 0.5 and then induced with 0.3 mM IPTG (isopropyl β-D-thiogalactoside). After 16 h, the harvested cells were disrupted by sonication and the debris removed at 12,000 × *g* for 30 min. The supernatant was collected and fractionated on a 15% non-denaturing polyacrylamide gel. Recombinant ferredoxin was visible as a dark brown band migrating close to the buffer front of the gel.

Large scale purifications were done with cells isolated from 1 l cultures. Cells were harvested, suspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) (1 g cell paste/5 ml) and frozen at −20°C overnight. The pellet (approx. 8 g) was thawed on ice and the cells were disrupted by ultrasonication. The sonicate was centrifuged at 12,000 × *g* for 30 min and the supernatant was further clarified at 165,000 × *g* for 1 h. The resulting supernatant was dialyzed overnight against 10 mM Tris-HCl, pH 7.5, loaded on a 5 ml Econo PacQ column (Bio-rad, Hercules, CA) and eluted with a step gradient of 100 ml each of 100, 200 and 400 mM Tris-HCl, pH 7.4. The fractions containing ferredoxin, which eluted at 400 mM Tris-HCl, were dialyzed against 10 mM Tris-HCl, pH 7.5, and then concentrated to 3 ml with an Amicon YM-3 membrane (Amicon, Berverly, MA). The sample was fraction-

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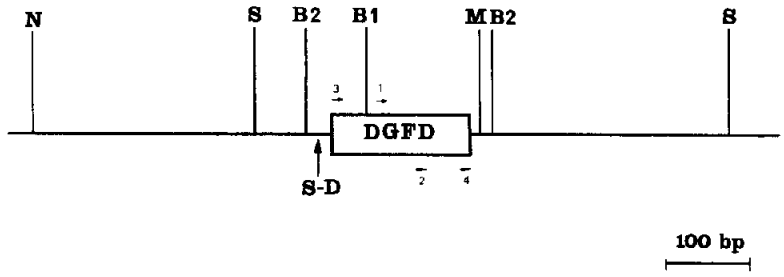

Fig. 1. Restriction map of the DNA fragment containing the *D. gigas* ferredoxin gene. The small arrows labeled 1, 2, 3 and 4 represent the four oligonucleotides used for cloning and sequencing. The large arrow indicates the ribosome binding site. Restriction sites: N, *Nae*I; S, *Sph*I; B1, *Bst*BI; B2, *Bst*EII; M, *Msc*I.

ated on a preparative 15% native polyacrylamide gel (Model 491 Prep Cell, Bio-Rad). Ferredoxin migrated as a brown band and was eluted from the bottom of the gel with 10 mM Tris-HCl, pH 7.5.

2.3. Analytical methods

Protein samples were analyzed by polyacrylamide gel electrophoresis using the buffer system of Laemmli [13] with or without SDS. Bromophenol blue was omitted from native gel electrophoresis loading buffer since it interfered with the visual identification of ferredoxin. The presence of disulfide bonds in the protein was assayed by treatment with iodoacetamide and β-mercaptoethanol as described [14]. N-terminal amino acid sequences were determined in the University of Georgia Molecular Genetics Facility with an Applied Biosystems 477A sequenator.

Absorption spectra were recorded at room temperature with a Shimadzu UV-265 spectrophotometer. Protein concentrations were determined as described [15] using pure native *D. gigas* ferredoxin II as a standard to avoid the problem of low aromatic content. Ferredoxin II concentrations were calculated assuming $\varepsilon_{400} = 16\ mM^{-1} \cdot cm^{-1}$ [3,5]. Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker 200-tt spectrometer with an Oxford Instruments ESR-9 continuous flow helium cryostat.

3. Results

3.1. Cloning and sequences of the D. gigas *ferredoxin gene*

The DNA and nucleotide derived amino acid sequences of the cloned ferredoxin gene are shown in Fig. 2. Since the N-terminal sequence of the isolated protein indicated that the first methionine is removed, the amino acid sequence has been numbered with proline at position 1. The mature polypeptide is predicted to contain 58 amino acids with a molecular mass of 6,276 Da. The nucleotide derived amino acid sequence is identical to the X-ray revised amino acid sequence [7,8] except at position 56 where the nucleotide sequence predicts an isoleucine instead of valine. The isoleucine at position 56 has been confirmed in a second genomic clone, pUCGF-2 (data not shown). A putative Shine-Dalgarno sequence [16] occurs 6 bases upstream of the initiator methionine codon.

3.2. Overexpression of D. gigas *ferredoxin*

The ferredoxin gene in pUGN-1 was expressed in *E. coli* under regulation of the IPTG-inducible *lac* promoter. Plasmids containing the ferredoxin gene in the opposite orientation with respect to the *lac* promoter did not express protein (data not shown). Recombinant ferredoxin could be visualized as a brown band migrating close to the buffer front in 15% native polyacrylamide gels. The band also stained positively with a procedure specific for the presence of nonheme iron. Isolation of the recombinant ferredoxin yielded about 20 mg of purified protein per liter of culture (approximately a 200-fold overexpression compared to wild type cells). The recombinant ferredoxin was estimated to be about 10% of the total cell protein.

Polyacrylamide gel electrophoresis of the purified ferredoxin under denaturing conditions is shown in Fig. 3. The purified protein migrated in the same position as native *D. gigas* ferredoxin with an apparent M_r of about 6 kDa consistent with the size predicted by the gene sequence.

Amino-terminal sequencing of recombinant ferredoxin showed the presence of a single polypeptide and the first eight amino acids matched the nucleotide-derived amino acid sequence, as well as the amino acid sequence reported for native protein.

3.3. Spectral properties

The absorption spectrum of recombinant ferredoxin (Fig. 4) shows the general features of an iron-sulfur center with broad charge transfer bands around 410 and 300 nm. The 410 nm/280 nm absorption ratio is 0.6, a value close to the one reported for *D. gigas* ferredoxin II, 0.68 [4,5], indicating a high yield of cluster incorporation. The visible spectrum per se is not a diagnostic criterion for the presence of a [3Fe-4S] or a [4Fe-4S] cluster. Further identification of the cluster type was obtained by EPR spectroscopy. In the native (oxidized) state the recom-

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-20                  1
CCTAGCTTAAGGAGAGGACA ATG CCT ATC GAA GTC AAT GAC GAT
        S-D          (Met)Pro Ile Glu Val Asn Asp Asp
                          1

     30                                          60
TGC ATG GCC TGT GAA GCC TGT GTG GAA ATC TGC CCC GAC
Cys Met Ala Cys Glu Ala Cys Val Glu Ile Cys Pro Asp
        10                                       20

                                90
GTC TTC GAA ATG AAC GAA GAA GGC GAC AAG GCT GTG GTT
Val Phe Glu Met Asn Glu Glu Gly Asp Lys Ala Val Val
                                    30

                    120
ATC AAT CCC GAT TCC GAT CTG GAT TGC GTG GAA GAA GCC
Ile Asn Pro Asp Ser Asp Leu Asp Cys Val Glu Glu Ala
                         40

        150                                         180
ATC GAC AGC TGC CCT GCC GAA GCG ATC ATT CGG TCC TGA
Ile Asp Ser Cys Pro Ala Glu Ala Ile Ile Arg Ser  *
            50                                   58


AGTCGCTGTTCCCCAACGT
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Fig. 2. DNA sequence and deduced amino acid sequence of *D. gigas* ferredoxin. DNA sequence numbering begins with the predicted initiator methionine; since methionine is not present in the mature protein, amino acid sequence numbering begins with Pro-1. The putative ribosome binding site is underlined.

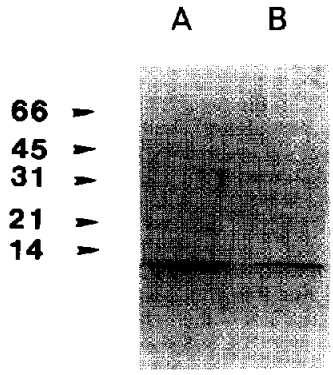

Fig. 3. Denaturing gel electrophoresis *D. gigas* ferredoxin expressed in *E. coli*. Purified samples were fractionated in a 20% SDS-polyacrylamide gel at pH 8.4. (A) Purified recombinant *D. gigas* ferredoxin; (B) purified native *D. gigas* ferredoxin II.

binant protein is EPR active. An almost isotropic EPR signal was observed at low temperature, centered around $g = 2.02$ (Fig. 5A). In the dithionite reduced state no major signals were observed in the $g = 2$ spectral region, excluding the presence of a small amount of remaining reduced [3Fe-4S] centers (in Fig. 5B, a minor contribution of a '$g = 1.94$' signal is observed that may have its origin in the interconversion of [3Fe-4S] centers under reducing conditions [6]). Double integration of the native EPR spectra (against *D. gigas* ferredoxin II) indicates 0.87 spins per 6 kDa.

4. Discussion

The *D. gigas* ferredoxin gene has been overexpressed about 200-fold in *E. coli*. As much as 20 mg of recombinant protein has been purified from 1 l of aerobically grown culture and EPR analysis showed that more than 90% of the protein incorporated iron-sulfur cluster. This high level of ferredoxin was present in the soluble fraction and not in inclusion bodies, yet not toxic to *E. coli* during 16 h of synthesis and accumulation.

The ferredoxin of *D. gigas* is unique in that it has been isolated from cells in two forms; one which contains a single [3Fe-4S] cluster per monomer, ferredoxin II, and a second which has one [4Fe-4S] cluster per monomer, ferredoxin I. Both genetic and amino acid sequence information indicate that there is a single ferredoxin gene in *D. gigas* and that ferredoxin I and ferredoxin II represent the same protein differing only in the type of iron-sulfur cluster present and their oligomerization state. It is not known for sure whether the two forms of ferredoxin exist in situ and represent a response to different cellular functions or physiological conditions, or if one is an artifact of isolation procedures. Recombinant *D. gigas* ferredoxin isolated under aerobic conditions from aerobically grown *E. coli* cells is exclusively [3Fe-4S] ferredoxin II. It could be contended that biosynthesis of ferredoxin under these conditions resulted in the expression of ferredoxin II and that expression in the reducing environment of anaerobically grown cells might result in the biosynthesis of ferredoxin I. We have, however, expressed the *D. africanus* ferredoxin I gene in *E. coli* under aerobic conditions (unpublished data) and interestingly over 90% of the apoprotein incorporates clusters which are exclusively of the [4Fe-4S] type indicating that assembly of a [4Fe-4S] cluster into a recombinant ferredoxin under aerobic conditions is possible in *E. coli*. Similarly, the recombinant ferredoxins from *Rhodobacter capsulatus* and *Clostridium pasteurianum* show the presence of [4Fe-4S] clusters [17,18]. To verify whether growth conditions affected the type of cluster incorporated into *D. gigas* ferredoxin, we purified ferredoxin from anaerobically grown *E. coli* cells. Unfortunately, though the level of recombinant apoprotein produced in anaerobically grown cells was equivalent to that of aerobically grown cells, iron-sulfur clusters were incorporated very inefficiently. This may be in part due to the inability to form disulfide bonds under highly reduced conditions. We have not yet been able to accumulate enough anaerobically expressed ferredoxin with sufficient cluster incorporation to perform EPR experiments.

D. gigas ferredoxin II differs from *D. africanus* I ferredoxin in that it contains two additional cysteine residues which have been shown by biochemical analysis [7], combined NMR and Mössbauer studies [19] and X-ray crystallography [8] to form a disulfide bond. The recombinant *D. gigas* ferredoxin also showed the presence of a disulfide bond as judged by change in electrophoretic mobility after treatment with iodoacetamide [14]. Since the ferredoxin gene does not encode an amino-terminal signal peptide, it would appear that the disulfide bond is formed in the cytoplasm. This is somewhat unexpected as it is generally well documented that the majority of disulfide bonds are formed in the periplasm of most bacteria [14].

The crystal structure of *D. gigas* ferredoxin I has not been determined but the existence of a disulfide bridge is uncertain [6]. It is possible that the [4Fe-4S] cluster present in ferredoxin I in *D. gigas* is assembled in response to a specific factor/condition or protein association (in response to anaerobic conditions?) which may be absent in *E. coli*. We are currently attempting to answer some of these questions using in vitro reconstitution and site-directed mutagenesis.

Acknowledgements: This work is supported by National Institute of Health Research Grant GM41482-03 and Junta National de Investigação Científica e Tecnológica. We thank Dr. Jeff Robbins for his suggestions for cloning the *D. gigas* ferredoxin gene and Dr. J. LeGall

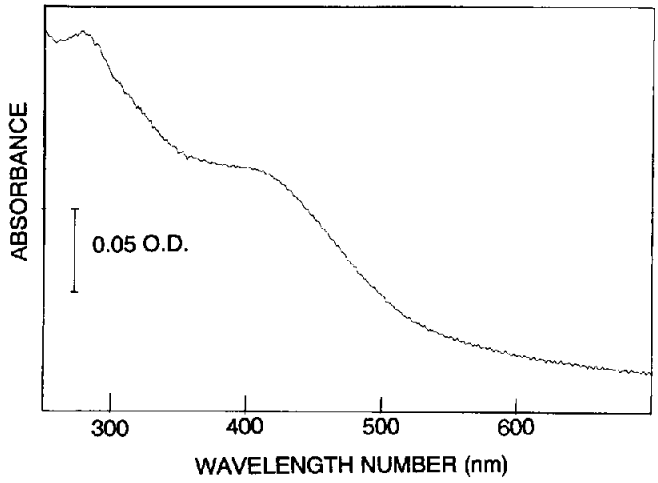

Fig. 4. Absorption spectrum of recombinant *D. gigas* ferredoxin. The UV/visible spectrum was recorded with a Shimadzu model 260 spectrophotometer. The protein (9.3 μM) was in 50 mM Tris-HCl buffer, pH 7.6.

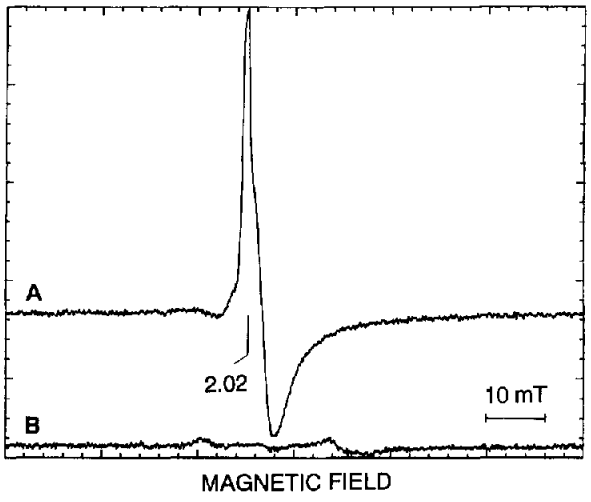

Fig. 5. X-band EPR spectra of recombinant *D. gigas* ferredoxin. (A) As isolated, and (B) dithionite reduced ferredoxin. Experimental conditions: protein concentration 47 μM (in 50 mM Tris-HCl, pH 7.6), temperature 8K, modulation amplitude 5 Gpp, gain 125,000, microwave frequency 9.43 GHz and microwave power 0.2 mW.

and Mr. Ming Liu for a sample of native *D. gigas* FdII and many stimulating discussions.

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