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Clonage et expression de la ferrédoxine : thiorédoxine réductase de chloroplastes d'épinard

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par

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UNIVERSITÉ DE NEUCHÂTEL

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Gaymard,E., Franchini,L., Manieri,W., Stutz,E. and Schürmann,P. A dicistronic construct for the expression of functional spinach chloroplast ferredoxin:thioredoxin reductase in *E.coli. Plant Sci.* 158:107-113, 2000.

Un exemplaire de la thèse se trouve à la bibliothèque du laboratoire de Biochimie.

CLONING AND EXPRESSION OF cDNAs CODING FOR THE SPINACH FERREDOXIN:THIOREDOXIN REDUCTASE

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1. Introduction

Ferredoxin:thioredoxin reductase (FTR) is the key enzyme of the ferredoxin/thioredoxin system, the light-dependent enzyme regulatory system in oxygenic photosynthesis (1). This nucleus encoded chloroplast enzyme reduces thioredoxins with electrons supplied by illuminated thylakoids. It is an iron-sulfur protein, composed of two dissimilar subunits of about equal size, one of which contains a [4Fe-4S] cluster and a redox-active disulfide bridge functional in the reduction of thioredoxins. This subunit is therefore called the catalytic subunit. It has been proposed to be rather well conserved since it is readily detected by heterologous antibodies (2,3). The other subunit is of variable size when comparing the FTR from different plant species and has no known catalytic function. We have sequenced both subunits of the spinach FTR on the level of the protein (4,5) and also isolated and characterized a cDNA coding for the catalytic subunit of the enzyme from corn (6). Here we report the isolation and characterization of full-length cDNAs encoding both subunits of FTR from spinach and the expression of a functional recombinant FTR in *E.coli*. The isolation cDNAs coding for spinach FTR, however different from ours, has been reported (7).

2. Materials and Methods

A 159 bp fragment isolated by PCR from a spinach cDNA library (6) and coding for part of the catalytic subunit of FTR was used as a probe to screen the spinach cDNA library (Gift of Prof.W.Gruissem, Berkeley) using standard procedures. Positive plaques were purified and one cDNA clone of 738 bp was isolated and cloned in pBluescript SK+ (Stratagene) and then characterized by sequencing using the chain termination technique with Sequenase (U.S. Biochemical Corp.). In a similar manner, we isolated a 286bp fragment coding for part of the variable subunit of FTR. The cDNA library was screened and a clone of 804bp was isolated and characterized. Using PCR we have made a di-cistronic construction carrying, in phase and on the same DNA strand, the coding parts of the genes for both subunits. The construction (pET(FTR)) cloned in PET-3a-d expression vector (8) was used to transform *E.coli* BL21(DE3). Transformed cells were grown in LB medium to a OD_{600nm} of 0.8 and expression induced with 2mM isopropyl thio-β-D-galactoside (IPTG) for 2.5 hours.
To test FTR expression bacterial extracts were prepared for SDS-PAGE (9): cultured cells were resuspended in water (0.1 $OD_{600nm}/4\mu l$) and lysed by freeze-thawing (-70°C, 15 min.). One volume of sample buffer was added and the lysate vigourosly vortexed for 5 min. Proteins were separated electrophoretically on a 15% gel and probed after membrane transfer with antibodies raised against total FTR and against each subunit. Secondary antibodies

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conjugated to horse-radish peroxydase were used together with H_2O_2 and 4-chloro-1-naphtol as chromogenic substrate to visualize the FTR .
Protein samples for testing FTR activity were prepared as follows: 200 ml of transformed BL21(DE3) cells grown as described above were harvested by centrifugation. After resuspension in 3 ml of 100 mM NaKPi pH 7.0 containing 0.1 mM phenylmethylsulfonyl fluoride, 14 mM β-mercaptoethanol they were lysed using lysozyme or sonication. Lysates were centrifuged and 10 µl supernatant tested for FTR activity as described elsewhere (10).

3. Results and Discussion

Figure 1 shows the nucleotide sequence and the translation of the entire cDNA coding for a protein of 174 residues. A comparison with the known protein sequence (4) indicates that residues 63 to 174 represent the mature variable subunit, whereas the first 62 residues apparently constitute the transit peptide which displays some typical features of chloroplast transit peptides (11). It starts with Met-Thr-Thr-, a sequence reported in ferredoxin:NADP reductase from spinach (12). The N-terminal part contains no Pro nor Gly, the central region is rich in Ser (30%) with a total Ser content of 20%. The C-terminal 10 amino acids exhibit a high probability for β-sheet formation. The cleavage site sequence, Ile-Cys-Cys↓Glu, follows the proposed consensus.

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                .         .         .         .         .         .
  1 ggaattcggcacgaggaacaaagaaagtaaaattatctgagacacagaaatccagtacct  60
 61 cttcacttcttctattccggataggaaaatcctcatctccatctcattattctcatctaa 120
121 aacaaatctaatctaaactcctccgatgacaacaggtgtggcagtaatgtcatcagcaac 180
                            M  T  T  G  V  A  V  M  S  S  A  T
181 agcagcatcaaccgcaaccgcaacggcggcggcgacggcgagaataccactcttcctaag 240
     A  A  S  T  A  T  A  T  A  A  A  T  A  R  I  P  L  F  L  S
241 ccgcaacaactcatcggccaccgtctgcagcaccctgaggtgcagaacaataacacgaac 300
     R  N  N  S  S  A  T  V  C  S  T  L  R  C  R  T  I  T  R  T
301 aagaaccagagccagactagcaatatgctgtgaagtagctttgaaatccgattcttcaac 360
     R  T  R  A  R  L  A  I  C  C  E  V  A  L  K  S  D  S  S  T
361 cgggttcgattcatcatcatcatcacctccagaagaagacgaggaattgaagaagaatct 420
     G  F  D  S  S  S  S  S  P  P  E  E  D  E  E  L  K  K  N  L
421 ggagaaagttggatgcaaggttaaggtgaaatcccctcttaaagtctaccatgttcctaa 480
     E  K  V  G  C  K  V  K  V  K  S  P  L  K  V  Y  H  V  P  K
481 attacctgaagttgaattaaccccagatatggttggggttattaagcagtatgttggatt 540
     L  P  E  V  E  L  T  P  D  M  V  G  V  I  K  Q  Y  V  G  F
541 ctggaaagggaaatacatttcccctaattatcctttcaaagttgagtaccgcatcgatgt 600
     W  K  G  K  Y  I  S  P  N  Y  P  F  K  V  E  Y  R  I  D  V
601 ccctgaccgtggttctgtcaaattagttgttcatcttaaagaagaagaattcgaaatcat 660
     P  D  R  G  S  V  K  L  V  V  H  L  K  E  E  E  F  E  I  I
661 agcagagtagaagtctaattattttctatttttattggtattgatttatgtatattgtct 720
     A  E  *
721 ttcttcttccttgttgtctatacatctgattactatggcagattggcaggatgtatcaaa 780
781 aaaaaaaaaaaaaaaaaactcgag 804
```

Figure 1. Nucleotide sequence and deduced protein sequence of the cDNA clone coding for the precursor peptide of spinach FTR subunit A. The putative transit peptide is given in italics.

Figure 2 shows the nucleotide sequence and the translation of the entire cDNA which codes for a protein of 144 residues. Residues 31 to 144 correspond to the mature catalytic subunit (5) and the first 31 residues apparently constitute the transit peptide, which again displays some typical features of a chloroplast transit peptides. It starts with Met-Arg-Ala, a sequence sofar never found in spinach transit peptides, but reported for *Chlamydomonas reinhardii* pre-apoplastocyanin (13). The 10 N-terminal residues contain one charge, but no Pro nor Gly. The central part is rich in Ser (40%), the total Ser content being 20%. The C-terminal 10 amino acids exhibit a high probability for beta sheet formation, but contrary to the consensus there are 4 charged amino acids (Arg) present. The cleavage site Ile-Arg-Ala-Gln↓Ala again follows closely the proposed consensus (Val/Ile)-X-(Ala/Cys)↓Ala (14). The translated sequences of both subunits match exactly the protein sequences.

```
                .         .         .         .         .         .
  1 gaattcggcacgagatcaattttacaacaaaaattaatataccatgaaagctcttcaagc  60
                                                  M   K   A   L   Q   A
 61 ttcaatagcttacagcttccccatttcttctcctgcagcttctcccagacgtttctcccg 120
      S   I   A   Y   S   F   P   I   S   S   P   A   A   S   P   R   R   F   S   R
121 tgtaattcgtgctcaagcggatccttctgacaaatctatggaagtaatgaggaaattttc 180
      V   I   R   A   Q   A   D   P   S   D   K   S   M   E   V   M   R   K   F   S
181 tgagcagttctgtcgtaagtcagatacatacttttgtgttgataaaagtgtcactgccgt 240
      E   Q   F   C   R   K   S   D   T   Y   F   C   V   D   K   S   V   T   A   V
241 tgttatcaagggattagcagatcacagggacacattaggtgcacccctttgcccatgtcg 300
      V   I   K   G   L   A   D   H   R   D   T   L   G   A   P   L   C   P   C   R
301 gcattatgatgacaaagaagctgaagcaaaacagggtttttggaattgcccatgtgtgcc 360
      H   Y   D   D   K   E   A   E   A   K   Q   G   F   W   N   C   P   C   V   P
361 aatgagggagaggaaggagtgccactgtatgctgtttctgacccctgacaatgattttgc 420
      M   R   E   R   K   E   C   H   C   M   L   F   L   T   P   D   N   D   F   A
421 tggcaaggagcagactatcacattggatgaaattcgagaagttacatcaaatatgtaaaa 480
      G   K   E   Q   T   I   T   L   D   E   I   R   E   V   T   S   N   M   *
481 ttgtgatctgaagcctcttatgcgtagtaacagtaggcctgtacattacgtactatcacg 540
541 aggatgccaataactgttgtactgttatatgttgtatatactcttattctggagagcttt 600
601 gtcatctctaactcggctttatcaaggtatatagccatactcgtatctgagtggtgtatt 660
661 tggttttatgatactccgtatcatataatactgagtgttaaagaaagaaaaggtcaagaa 720
721 tgaagtactacaaaaaaa 738
```

Figure 2. Nucleotide sequence and deduced protein sequence of the cDNA clone coding for the precursor peptide of spinach FTR catalytic subunit. The putative transit peptide is given in italics.

Figure 3 shows by Western blot analysis of bacterial extracts the expression of recombinant FTR. These results clearly demonstrate that both subunits are expressed and recognized by the specific antibodies. In order to verify that functional recombinant FTR has been produced, bacterial extracts were tested for their capacity to reductively activate FBPase in the ferredoxin/thioredoxin system. Whereas in a control extract, from bacteria transformed with the expression vector carrying no FTR construct, there was background activity (4 nmol substrate hydrolized/min), the extracts from bacteria transformed with the FTR construct displayed very good activity (414 nmol substrate hydrolized/min). After partial purification the recombinant protein reveals the typical spectral properties known from the native FTR.

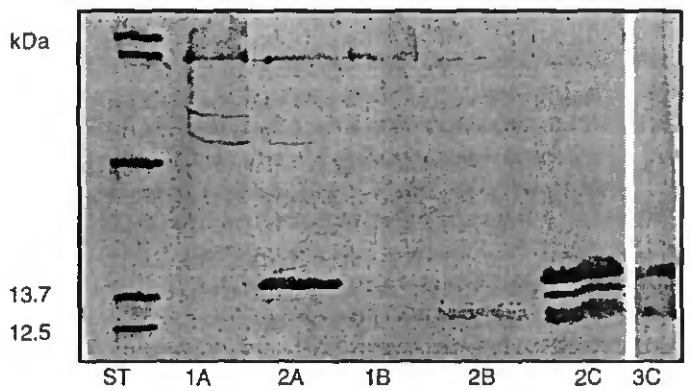

Figure 3. Western blot analysis of baterial extracts after expression of recombinant FTR. ST standard proteins; 1: extract from bacteria transformed with pET; 2: extract from bacteria transformed with pET(FTR); 3: native spinach FTR. The following antibodies were used: A - anti variable subunit, B - anti catalytic subunit, C - anti holoprotein.

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A dicistronic construct for the expression of functional spinach chloroplast ferredoxin:thioredoxin reductase in *Escherichia coli*

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Abstract

Ferredoxin:thioredoxin reductase (FTR) is a heterodimeric Fe–S containing disulfide reductase involved in the light-dependent activation of photosynthetic enzymes. We have designed a dicistronic construct for the heterologous expression of this nucleus encoded chloroplast protein in *Escherichia coli*. The coding sequences for the two mature subunits have been inserted in tandem into the expression vector pET-3d. This dicistronic construct is correctly translated yielding soluble, perfectly functional FTR. The recombinant enzyme is composed of both subunits, contains the correctly inserted Fe–S cluster as evidenced by its spectral properties and is indistinguishable from the enzyme isolated from leaves in its capacity to activate chloroplast fructose-1,6-bisphosphatase, one of the well known light activated enzymes of the Calvin cycle. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Spinach; Ferredoxin:thioredoxin reductase; Recombinant; Dicistron; Heterodimer; Fe–S protein; Heterologous expression

1. Introduction

Ferredoxin:thioredoxin reductase (FTR) is the key enzyme of the ferredoxin/thioredoxin system, the light-dependent enzyme regulatory system in oxygenic photosynthesis [1,2]. This nucleus-encoded enzyme reduces thioredoxins with electrons supplied through ferredoxin by illuminated thylakoids. It is a 25.5 kDa protein, composed of two dissimilar subunits, SUA and SUB, of about equal size, one of which contains a [4Fe–4S] cluster and a redox-active disulfide bridge functional in the reduction of thioredoxins. This subunit, SUB, is therefore also called the catalytic subunit. It is well conserved as evidenced by the fact that it cross reacts with heterologous anti FTR antibodies [3,4] and by a comparison of the FTR sequences from all organisms found in the data banks. The other subunit, SUA or variable subunit, is of variable size when compared with the FTR from other species and has no catalytic function. The primary structure of both subunits of spinach FTR has been reported [5,6]. Based on these sequencing data we were able to synthesize several primers successfully used to search for cDNA clones coding for the catalytic subunit of corn [7] and soybean [8] and for both subunits of spinach [9]. Falkenstein et al. have also reported clones coding for the spinach FTR subunits, which, however, are slightly different [10]. In view of a detailed analysis of this unique Fe–S protein it seemed warranted to use the clones coding for both FTR subunits from spinach to synthesize recombinant FTR.

In the following we describe: (a) the detailed protocol for the construction of a dicistronic expression vector; (b) the synthesis and purification of the recombinant spinach FTR; and (c) we show

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that the recombinant and the native FTR have identical physico-chemical and catalytic properties.

2. Material and methods

2.1. Biologicals, cDNA libraries, plasmids

Spinach (*Spinacia oleracea*) leaves were purchased from the local market. The cDNA library of spinach leaves was a gift from Professor Gruissem (Berkeley). Oligonucleotides were custom synthesized by Microsynth AG (Switzerland). pET-3d and its corresponding *Escherichia coli* strains BL21(DE3), BL21(DE3)/pLysS were purchased from Novagen and pTrc99A and its corresponding strain JM105 from Amersham Pharmacia. pET-3d and pBluescript (Stratagene) were replicated in XL1-Blue strain. Plasmid DNA was purified as described in [11].

2.2. Construction of the dicistron

In a first step the DNA regions coding for SUA and SUB (without the transit peptide) were separately tailored and amplified using the sense primers SUAmin, SUBmeg, respectively

SUAmin: CCATGGAAGTAGCTTTGAAATCCG

SUBmeg: GGTACCATAAGGAAACAGACATA TGGCAGATCCTTCTGACAAATCT

and the antisense primers SUAmeg and SUBmin.

SUAmeg: GGTACCATTAGACTTCTACTCTGC TATGATTTCGAATTC

SUBmin: GGATCCTTACATATTTGATGTAAC TTCTCG

We obtained thereby: (a) a SUA fragment with a NcoI (5′) and a KpnI (3′) restriction site; (b) a SUB fragment with a KpnI (5′) and a BamHI (3′) site including a ribosome binding site. The two fragments were directly ligated into the pET-3d expression vector previously digested with NcoI and BamHI. The recombinant plasmid carrying the dicistronic insert is called pET-3d/FTR as shown in Fig. 1. The ribosome binding site of SUA stems from the vector, while that from SUB was introduced by PCR. The ribosome binding sites are separated from the start codon ATG by eight nucleotides for SUA and nine for SUB [12].

2.3. Preparation of antibodies

Native FTR was purified as described [13]. To raise polyclonal rabbit-antibodies against SUA and SUB, FTR was separated by SDS-PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane (BA83, 0.2 mm, Schleicher and Schüll) and visualized with Ponceau red. Bands corresponding to each subunit were cut out, dissolved in DMSO and mixed with Freund's complete adjuvant. Rabbits were immunized by six consecutive, subcutaneous injections of about 200 μg of protein at two week intervals. The IgG fraction of the serum, taken two weeks after the last injection, was precipitated with ammonium sulfate at 45% saturation and passed through a DEAE–cellulose column in 5 mM phosphate buffer pH 8.0. The purified IgG fraction was dialyzed against 0.5% ammonium bicarbonate and concentrated with solid polyethylene glycol (PEG) 20 000. Antibodies were stored at −20°C after addition of glycerol to 50%. Antibodies against the holoenzyme were obtained by subcutaneous injection of pure FTR diluted with Freund's complete adjuvant.

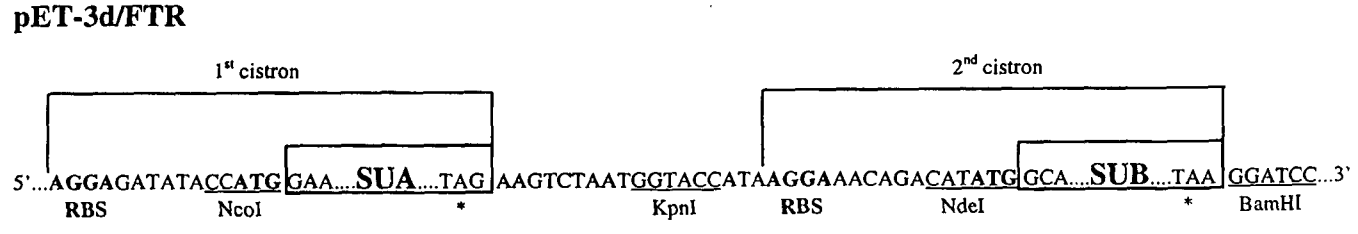

Fig. 1. Construction of the dicistronic FTR expression vector. The DNA fragments resulting from the amplification of cDNA SUA with the primers SUAmin and SUAmeg and from the amplification of cDNA SUB with the primers SUBmin and SUBmeg were directly ligated in pET-3d previously digested with NcoI and BamHI. The final construction is called pET-3d/FTR. **RBS** ribosome binding site (Shine–Dalgarno sequence); * stop of translation.

2.4. Analysis of FTR expression

200 ml of LB medium were inoculated with 1:10 of an overnight culture of BL21 (DE3) freshly transformed with pET-3d/FTR. The culture was grown at 37°C with shaking and when an A_{600nm} of 0.8–1.0 was reached, the expression induced with 0.5 mM isopropyl thio-β-D-galactoside (IPTG) and the culture continued for about 2.5 h.

To follow the expression by SDS-PAGE and immunoblotting 1 ml samples of culture were withdrawn at regular intervals. The cells were sedimented, washed with 500 μl of 0.1 M NaCl, 10 mM Tris–HCl, 1 mM EDTA pH 8.0 and resuspended in 4 μl of H_2O/0.1 of A_{600nm}. Lysis was achieved by freezing for 15 min at −70°C and thawing [14]. One volume of double strength loading buffer [15] was added, the lysate vortexed 5 min, then heated for 3 min at 100°C and centrifuged for 2 min at 16 000 × *g* before separation by SDS-PAGE. The protein bands were transferred to nitrocellulose and tested with antibodies raised against the holoenzyme and against each subunit. The horse radish peroxydase system was used to visualize the FTR-bands.

To reveal the presence of recombinant FTR by its activity the cells were resuspended in 100 mM phosphate buffer pH 7.0, 0.1 mM PMSF, 14 mM 2-mercaptoethanol (2-MET) and broken by sonication. The lysate was centrifuged 10 min at 39 000 × *g* and 4°C and the supernatant tested for activity [16]. The cell extract from a non-induced culture served as negative control.

2.5. Cell growth and harvest

In a 10 l fermentor (Bioengineering AG, Switzerland), 9.5 l of LB were inoculated with 500 ml of an overnight preculture of BL21 (DE3)pLysS freshly transformed with pET-3d/FTR. 1 ml of Antifoam A (Sigma) and 50 μg ml^{-1} of ampicillin and 34 μg ml^{-1} of chloramphenicol were added. The culture, kept at 37°C, was sparged with air at 16 l min^{-1} and stirred at 500 rpm. When an A_{600nm} of 0.8–1.0 was reached, the FTR expression was induced with 0.1 mM IPTG. After an additional 2.5 h the cells were harvested by centrifugation and resuspended in a minimum volume of 50 mM triethanolamine-Cl pH 7.3, 0.1 mM PMSF, 14 mM 2-MET and frozen at −20°C.

2.6. Purification of the recombinant protein

All preparation steps were performed at 4°C. The FTR concentration was calculated using its absorption coefficient of 17 400 M^{-1} cm^{-1} at 408 nm [3].

1. Crude extract. Cells were thawed and sonicated 8 × 30 s. 2.5 U per ml of Benzonase (Merck, Darmstadt) and $MgCl_2$ to 10 mM were added and the extract incubated at 25°C until no longer viscous. Then the lysate was centrifuged at 140 000 × *g* and 4°C for 60 min.
2. Hydrophobic interaction chromatography. To the supernatant $(NH_4)_2SO_4$ was added to 50% saturation and the precipitate removed by centrifugation. After an additional filtration through a glass fiber filter (Whatman multigrade GMF 150, 1 μm) to remove lipoproteins the clarified solution was loaded on a 5.0 × 5.0 cm Phenyl-SepharoseFF (Amersham Pharmacia) column equilibrated with 100 mM phosphate buffer pH 7.3, 1.0 M $(NH_4)_2SO_4$ and 14 mM 2-MET. The column was washed with 10 column volumes of the same buffer followed by elution of the protein with 50 mM phosphate buffer pH 7.3 and 14 mM 2-MET. Active fractions were combined and the proteins concentrated by precipitation with ammonium sulfate at 90% saturation.
3. Gel filtration. The concentrated protein solution was clarified by centrifugation and chromatographed on a 5.0 × 90 cm Sephacryl S-100HR (Amersham Pharmacia) column equilibrated in 20 mM triethanolamine-Cl pH 7.3, 200 mM NaCl and 14 mM 2-MET.
4. Anion exchange chromatography. Active fractions from the previous step were combined and diluted with an equal volume of 20 mM triethanolamine-Cl pH 7.3 and 14 mM 2-MET and loaded on a 5.0 × 11 cm Q-SepharoseFF (Amersham Pharmacia) column equilibrated with 20 mM triethanolamine-Cl pH 7.3, 100 mM NaCl and 14 mM 2-MET. The proteins were eluted with a 2000 ml gradient from 100 to 500 mM NaCl in 20 mM triethanolamine-Cl pH 7.3 and 14 mM 2-MET. The active fractions were concentrated and diafiltered with 20 mM phosphate buffer pH 7.3 and 14 mM 2-MET.
5. Affinity chromatography. The final polishing was achieved by affinity chromatography on a

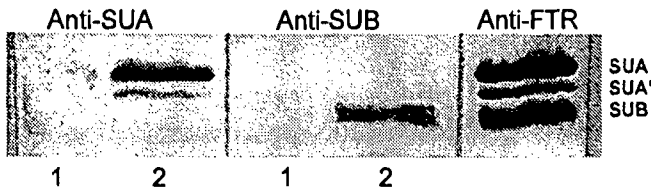

Fig. 2. Western blot analysis of extracts of *E. coli* cells transformed with pET-3d vector (1) or with vector carrying the FTR construct (2). The filters were probed with antibodies against the subunits (anti-SUA, anti-SUB) and against the holoenzyme (anti-FTR). SUA' is a truncated subunit (see text).

Table 1
FTR activity of bacterial extracts[a]

	Activity (nmol NADPH min^{-1})
Reaction mixture	
+bacterial extract	334
+control extract	3.6
+native FTR	457
−bacterial extract	3.7
Reaction mixture+bacterial extract	
−methylviologen	3.6
−thioredoxin *f*	0.3
−fructose-1,6-bisphosphatase	3.8

[a] FTR activity was tested with reduced methylviologen as electron donor and fructose-1,6-bisphosphatase as target enzyme [16] using 50 μl of bacterial extract. Fructose 1,6-bisphosphatase activity was measured after 15 min activation at 25°C. An extract from cells containing the pET-3d vector served as control.

1.5 × 6.5 cm ferredoxin-Sepharose column equilibrated with 20 mM phosphate buffer pH 7.3 [13]. After adsorption of the FTR the column was washed with several column volumes of equilibration buffer and eluted with the same buffer containing 300 mM NaCl. The FTR fractions were concentrated and diafiltered with 20 mM triethanolamine-Cl pH 7.3 by ultrafiltration on a YM-10 membrane and stored in liquid nitrogen.

3. Results and discussions

3.1. Construction and expression of the dicistronic vector

In order to express the heterodimeric FTR we opted for a dicistronic construct with the SUA and SUB genes arranged in tandem as shown in Fig. 1. The construction contains all the elements required for proper translation, i.e. upstream ribosome binding sites, start and stop codons for both genes.

The expression of the pET-3d/FTR vector was tested by Western blot analysis of the corresponding *E. coli* cell extracts. This revealed that both subunits of FTR are produced in soluble form in this expression system (Fig. 2). We have also inserted the same dicistronic construct into the pTrc99A expression vector but observed no expression in this system. It is noteworthy that the recombinant SUA shows two bands, SUA, SUA' as we have observed earlier with the native enzyme (see below).

To verify whether the recombinant FTR is functional, bacterial extracts were tested for their capacity to reductively activate fructose-1,6-bisphosphatase in the ferredoxin/thioredoxin system (Table 1). A control extract from bacteria transformed with the pET-3d vector and with the pET-3d/FTR construct displayed an activity of 4 and 334 nmol substrate hydrolyzed min^{-1}, respectively. The activity was strictly dependent on a reductant, either reduced methylviologen or ferredoxin, on thioredoxin *f* and on the target enzyme fructose-1,6-bisphosphatase. These results demonstrate that *E. coli* cells express a functional recombinant FTR, with correctly assembled subunits and containing the essential [4Fe–4S] cluster. The catalytic subunit (SUB) alone is not functional as was realized when testing extracts from *E. coli* cells that did not express SUA.

3.2. Purification of the recombinant FTR

The purification of the recombinant FTR was based on the methods developed for the isolation of the enzyme from spinach leaves [13]. The purpose of the first chromatographic step, on the Phenyl-Sepharose column, was essentially the removal of nucleic acid contaminants which are not retained by the hydrophobic interaction resin. The FTR, however, adsorbed to the column and was

released by a change of buffer. On the elution profile of the size exclusion separation, using a Sephacryl S-100HR column, the FTR already appeared as a prominent peak and the FTR containing fractions were recognizable by their color. For the initial purifications of the recombinant protein we have used a ceramic hydroxyapatite column as described for the chloroplast enzyme [13]. Subsequently this step was successfully replaced by the anion-exchange chromatography on Q-SepharoseFF. This column yielded already rather pure protein without the extreme dilution observed with the hydroxyapatite separation. As a final polishing step, removing a few contaminating proteins, the affinity chromatography on ferredoxin-Sepharose proved to be the most efficient. The purity of the FTR was assessed by calculating the A_{408}/A_{278} ratio [3,17]. We routinely obtained yields of about 1.5 mg pure FTR per liter of culture.

3.3. Characterization of the recombinant FTR

The purified recombinant protein shows the typical spectrum of FTR with a broad peak centered at 408 nm, identical to the spectrum of FTR isolated from spinach leaves (Fig. 3). The A_{408}/A_{278} spectral ratio of 0.37–0.38, which is a measure for the purity of the enzyme, is comparable to published values [3,17] indicating that the purified, recombinant protein is of equal quality. Recombinant FTR has already been used for spectroscopic experiments and was found to be indistinguishable from the native protein [18]. We have analyzed the FTR by FPLC on a calibrated Superdex-75 column. The enzyme chromatographed as a single protein band and eluted in a symmetrical peak corresponding to an apparent molecular weight of 31 000 ± 1000. We have observed comparable values for the enzyme isolated from chloroplasts. These numbers are markedly bigger than the molecular weight calculated from the sequence (25 500 Da) and must be due to the unusual shape of the molecule [19]. Analysis of the pure protein by SDS-PAGE shows the same pattern as in Fig. 2 which is typical for the spinach enzyme. A third band (SUA') is also present in the enzyme isolated from leaves and its amount increases upon storage and repeated freezing and thawing of the enzyme. We have shown earlier that the N-terminal extension of SUA, with its peculiar serine and proline rich sequence, is unstable and breaks apart giving rise to smaller peptides, one migrating as SUA' [20]. With the FTR isolated from maize, whose SUA has a much shorter N-terminus, we have not observed this degradation providing another indication that the particular N-terminal sequence of the spinach FTR is responsible for the instability of the subunit. The pure recombinant FTR is highly-active in the activation of target enzymes as shown for the fructose-1,6-bisphosphatase in Fig. 4. It could be reduced by methylviologen or ferredoxin, reduced chemically with dithionite, as in Fig. 4, or photochemically using thylakoids and light.

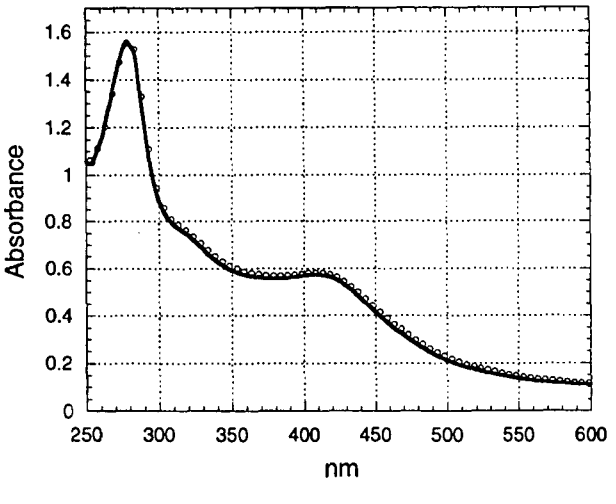

Fig. 3. Superposition of the absorption spectra of recombinant (open circles) and native (solid line) FTR. Spectra of the proteins in 20 mM phosphate buffer pH 7.3 were recorded with a Perkin–Elmer Lambda 16 spectrophotometer. The concentration of the native protein was 33 μM. The spectrum of the recombinant FTR was normalized at 278 nm.

In conclusion, we have designed a dicistronic construct containing the coding information for the two subunits of FTR in series separated by a ribosome binding site. Our results provide clear evidence that the *E. coli* proteosynthetic machinery translates this dicistronic message correctly into fully functional heterodimeric enzymes. The recombinant protein is composed of equal numbers of subunits as can be deduced from the estimated molecular mass and the catalytic subunit contains the essential Fe–S cluster correctly inserted as evidenced by the spectral properties. Our results also demonstrate that the presence of the variable subunit, SUA, is essential for a functional enzyme since an extract, which contained only SUB, was inactive. The availability of the recombinant spinach FTR facilitates further studies of

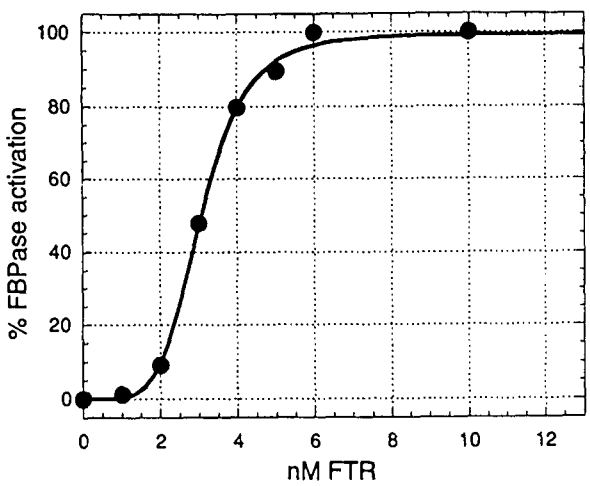

Fig. 4. FTR dependent activation of fructose-1,6-bisphosphatase. In a total volume of 100 μl 0.5 U of enzyme were incubated in presence of 100 mM Tris–Cl pH 7.9, 1 μM thioredoxin *f*, 5 μM ferredoxin, 14 mM 2-MET and varying concentrations of FTR, under argon. The activation was started by the addition of 10 mM sodium dithionite. After 5 min an aliquot of this activation mixture was transferred into a spectrophotometer cuvette containing the reaction mixture to measure the fructose-1,6-bisphosphatase activity at 25°C as described [16]. The degree of activation is expressed as percent maximal fructose-1,6-bisphosphatase activity.

structure and mechanism of this unique Fe–S protein.

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