Volume 301, number 2, 132–136 FEBS 10929 April 1992

Expression cloning of a sheep adreno-ferredoxin using the polymerase chain reaction

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Received 6 February 1992; revised version received 2 March 1992

In addition to the selective amplification of cDNA from total RNA by the PCR method, the distinctive properties of ferredoxin-expressing colonies can be used for cloning a ferredoxin cDNA. This strategy for cloning and expressing cDNA in *E. coli* was applied to a sheep adreno-ferredoxin. The expressed sheep ferredoxin showed a spectral pattern typical of [2Fe-2S] proteins. The amino acid sequence deduced from the DNA sequence showed that the mature form of sheep ferredoxin consists of 128 amino acid residues. This rapid and simple method for cloning and expressing cDNA can be applied to other ferredoxins.

Ferredoxin; Iron–sulfur protein; Nonheme iron protein; Mitochondrial cytochrome P-450-linked monooxygenase; PCR; Expression cloning

1. INTRODUCTION

The molecular cloning and expression of a cDNA for a protein of interest are becoming very important steps for many biological and biochemical studies on protein and nucleic acids, particularly in the fields of structure–function relationships and protein–protein interactions. However, these steps are laborious and time consuming. Recently, a PCR method has revolutionized the cloning strategy, in particular, the cloning of genes or cDNA without having to make or screen libraries has become possible [2,3]. Nevertheless, the identification of the gene or cDNA for a particular protein requires at least a partial DNA sequence of the clone or the assaying of a function of the expressed protein. Therefore, identification of the colony which has the cDNA for a protein of interest by a simple method makes the cloning more rapid and efficiently. This paper describes a rapid and efficient cloning and expression strategy for mammalian ferredoxins using the PCR method. This strategy was used to clone the cDNA for an adreno-ferredoxin* from sheep.

*The terms adreno- and hepato-ferredoxins (adrenal and hepatic ferredoxins) will be used synonymously in place of the popular terms, adrenodoxin and hepatoredoxin, in accordance with the recommendations of the IUPAC-IUB Commission in Biochemical Nomenclature [1].

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2. MATERIALS AND METHODS

2.1. *Extraction of RNA from sheep adrenocortex*

Total RNA was extracted from sheep adrenocortex (2-year-old, female) using guanidium thiocyanate, followed by centrifugation in cesium chloride solutions [4].

2.3. *Polymerase chain reaction*

Two primers were used for PCR. Primer FeBspHF, 5'TGATCATGAGCAGCTCAGAAGATAAA-3', is a 26-mer corresponding to the region between nucleotides 185 and 202, based on the nucleotide sequence of bovine ferredoxin [5,6], and has 8 extra bases in the 5' site which contains the *Bsp*HI recognition sequence. The *Bsp*HI cutting site is compatible with the *Nco*I cutting site of an expression vector, pKK233-2 (Toyobo Co., Japan) [7]. This makes the reading frame to be correct and start with the mature form of ferredoxin. The region between nucleotides 185 and 202 corresponds to the 6 amino acid residues between 59 and 64, which are the first 6 N-terminal residues of the mature ferredoxin. The amino acid sequence data showed that the first 6 amino acid residues of mature ferredoxin in sheep were identical to the bovine ones [8]. Primer Fe*Hin*dR, 5'-CCAAGCTTCCATGTTCTCATTTAATT-3', is a 26-mer complementary to the region between nucleotides 608 and 626, and has 8 extra bases in the 5' site which contains the *Hin*dIII recognition sequence. This region corresponds to the second poly(A) adenylation site of the ferredoxin [5,6].

Extracted total RNA (3.7 μg) was denatured for 10 min at 70°C, annealed with 0.2 μM primer Fe*Hin*dR, and then converted to cDNA by incubation for 1 h at 37°C with 1 U of M-MLV reverse transcriptase (BLR) in the presence of 82 U of human placental ribonuclease inhibitor (Takara Shuzo Co., Ltd., Japan). After the reverse transcriptase had been inactivated by heating for 5 min at 98°C, 0.2 μM primer Fe*Bsp*HF was added to the reaction mixture and then PCR was conducted with 1 U of Taq DNA polymerase (BRL) [2,3]. The conditions for annealing, polymerization, and denaturation were 58°C for 2 min, 72°C for 3 min, and 94°C for 1 min, respectively. The number of amplified cycles was 40.

2.3. *Cloning of the PCR products*

PCR reaction mixtures were digested with restriction endonu-

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cleases, *Bsp*HI and *Hin*dIII, and directly used for the ligation reaction with expression vector pKK233-2 digested with *Nco*I and *Hin*dIII. The ligated DNA was used to transform a host *E. coli*, XLI-Blue, by the method of Mandel and Higa [9].

2.4. *Selection of ferredoxin-expressing colonies*

Previously, we cloned bovine hepato-ferredoxin cDNA from a liver cDNA library and found it to be identical to the bovine adreno-ferredoxin [10]. Bovine ferredoxin was expressed in *E. coli* by inserting a cDNA into expression vector pKK233-2, in which the expression of cDNA controlled by the P_{trc} promoter (S. Miura et al., unpublished results). During the cloning and expressing the ferredoxin cDNA, we found two distinctive features of the expressed colonies. Firstly, the growth rate of bacteria in the medium containing 5 mM IPTG (isopropyl-β-D(−)-thiogalactopyranoside) was very poor, but not in the medium containing 125 μg/ml of ampicillin, probably because of the deleterious effect of the overexpressed ferredoxin on the host bacteria. Secondly, the ferredoxin-expressing cells collected by brief centrifugation, even without induction by IPTG, were red-brown in color, which reflected the color of ferredoxins. These two distinctive features were used for identifying the bacteria expressing the sheep ferredoxin.

2.5. *Induction of the expression of sheep ferredoxin in bacteria*

A single bacterial colony containing plasmid FeS-5 was inoculated into a 20 ml liquid culture containing 125 μg/ml of ampicillin, followed by incubation overnight at 37°C. Ten ml of the bacterial culture was added to a 1 liter liquid culture containing 125 μg/ml of ampicillin and then allowed to grow at 37°C. After 2 h, IPTG was added to the culture to the final concentration of 1 mM to induce the expression of ferredoxin. The culture was incubated with vigorous shaking for a further 14 h at 37°C.

2.6. *Partial purification of the sheep ferredoxin from the bacterial lysate*

To observe the spectral pattern in the visible region, the expressed ferredoxin was partially purified from the bacterial lysate by the method of Ichikawa [11]. Bacterial cells were collected by centrifugation at 10,000 × *g* (7,000 rpm) for 20 min at 4°C. The bacterial cell pellet was suspended in 25 ml of 75 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, homogenized twice for 5 min, sonicated 180 times for 1 s on ice, and then centrifuged at 93,000 × *g* (30,000 rpm) for 90 min at 4°C. The supernatant (25 ml) was adsorbed with DEAE-cellulose (8 ml, 16 × 40 mm) preequilibrated with 75 mM potassium phosphate buffer (pH 7.4). The column was washed with 32 ml of the same buffer and then washed with 20 ml of 75 mM potassium phosphate buffer (pH 7.4) containing 0.08 M KCl. The ferredoxin was eluted with 75 mM potassium phosphate buffer (pH 7.4) containing 0.25 M KCl. The eluted solution was used as the partially purified ferredoxin solution.

2.7. *Measurement of heme content*

The heme content in a sample was determined as pyridine hemochromes. Pyridine hemochrome spectra were measured in aqueous alkaline pyridine solution containing 250 mM NaOH and 25% (v/v) pyridine after reduction with sodium dithionite. The extinction coefficient of 34.4 $mM^{-1} \cdot cm^{-1}$ at 557 nm was used [12].

2.8. *Subcloning and DNA sequencing of the sheep ferredoxin cDNA*

DNA fragments of the sheep ferredoxin cDNA digested with *Eco*RI and *Xba*I were subcloned into plasmid Bluescript. The DNA sequence was determined by the dideoxy-chain termination method [13,14].

3. RESULTS

The cloning strategy for a ferredoxin cDNA from total RNA is outlined in Fig. 1. Total RNA extracted from sheep adrenocortex was converted to cDNA using primer Fe*Hin*dR, and adreno-ferredoxin cDNA was selectively amplified by means of PCR using primers Fe*Bsp*HF and Fe*Hin*dR (Fig. 2). The products of PCR were digested with *Bsp*HI and *Hin*dIII, and then inserted into the *Nco*I and *Hin*dIII sites of the expression vector, pKK233-2. The transformed bacteria were firstly plated on to the medium containing 125 μg/ml of ampicillin, and then the grown cells, which contained the plasmid, were secondly plated on the medium containing 5 mM IPTG and ampicillin. Cells which formed large colonies on the second plate were found not to express ferredoxin, because of the deleterious effect of the expressed ferredoxin on the growth rate. Therefore, the colonies that grew on the first plate but not on the second plate, approximately one in thirty colonies, were selected and were grown in liquid culture overnight. The color of the centrifuged cell pellet was inspected visually. The red-brown colored cells were identified as *E. coli* expressing ferredoxin and used for further analyses.

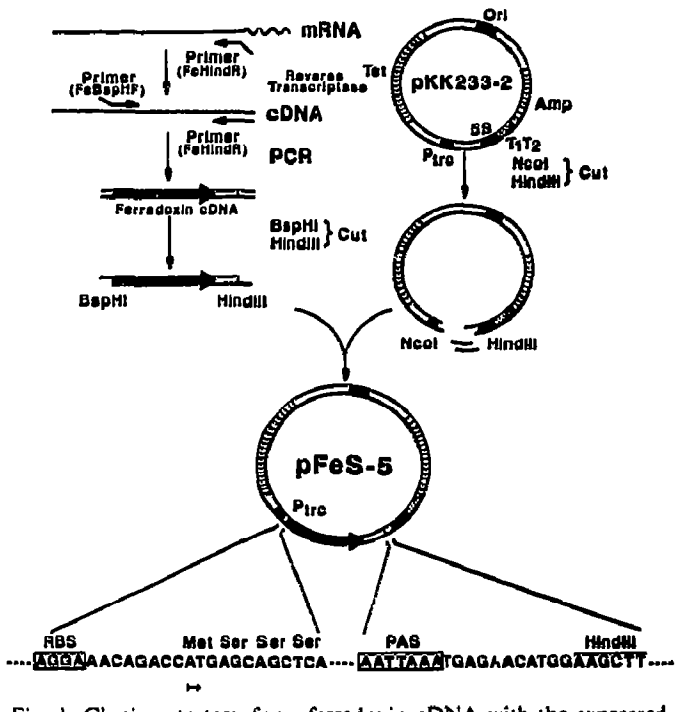

Fig. 1. Cloning strategy for a ferredoxin cDNA with the expressed form. RBS, ribosome binding site; PAS, poly(A) adenylation signal.

Expression of ferredoxin was induced by adding 1 mM IPTG and incubation for a further 14 h with vigorous shaking, as described previously [7,15]. One mM IPTG did not affect the growth of bacteria containing ferredoxin cDNA. Poor aeration resulted in a low yield. The expressed ferredoxin in *E. coli* was partially purified as described in section 2. The optical absorption spectra of the partially purified adreno-ferredoxin in the oxidized and dithionite-reduced forms are shown in Fig. 3. No detectable heme content, less than 0.001 nM, was observed in the sample of partially purified ferredoxin.

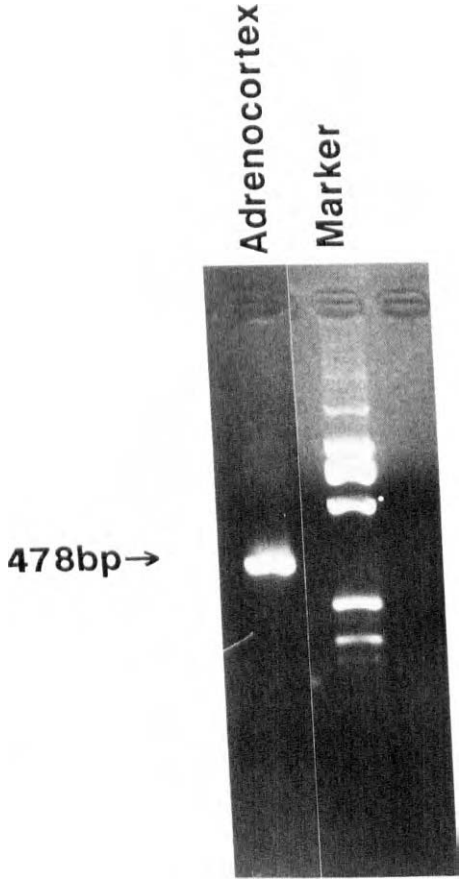

Fig. 2. PCR products from sheep adrenocortex cDNA separated by 2.0% agarose gel electrophoresis. The arrow shows the size of DNA fragment. The marker is plasmid Bluescript DNA digested with *Sau*3A.

Therefore the contribution of heme to the optical absorption spectra was negligible. Two peaks at 414 and 455 nm in the oxidized form, not seen in the reduced form, suggested the correct [2Fe-2S] structure of the expressed ferredoxin. These peaks were also observed for the purified native ferredoxin from sheep adrenocortex [8] and are characteristic of [2Fe-2S] proteins [16]. About 1000 nmol (14 mg) of adreno-ferredoxin from 1 liter of culture, as estimated from the absorption coefficient at 414 nm, 9.8 $mM^{-1} \cdot cm^{-1}$ [17], was obtained. Comparable yields, 5–6 mg/liter, were obtained in other studies [15,18].

The DNA sequence of sheep adreno-ferredoxin cDNA was determined. The sequencing strategy and nucleotide sequence of a cDNA clone (FeS-5) of sheep adreno-ferredoxin are shown in Figs. 4 and 5, respectively. The amino acid sequence deduced from that of cDNA was consistent with the amino acid sequence determined for the purified native ferredoxin except for at the 123rd and 128th positions [8]. On protein sequencing we could not detect the 128th amino acid residue in the C-terminal, however, the DNA sequence of the cDNA clone suggested the presence of a glutamate

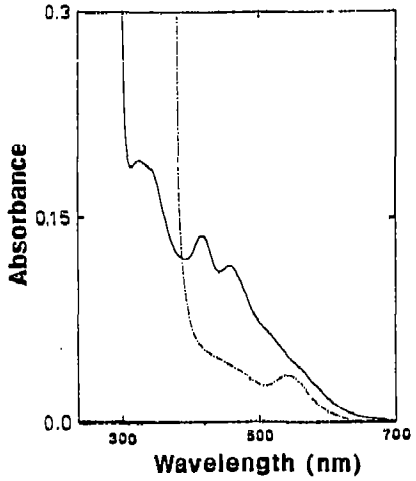

Fig. 3. Optical absorption spectra of the partially purified sheep adreno-ferredoxin in the oxidized and dithionite-reduced forms. The spectra were recorded in 75 mM potassium phosphate buffer, pH 7.4, at 25°C with a Shimadzu spectrophotometer, Model UV-240. Solid line, oxidized form; broken line, dithionite-reduced form; dotted line, base line.

at that position. When the DNA sequence of bovine adreno-ferredoxin cDNA was used for comparison, ten nucleotides were found to be different, of which 5 reflected the amino acid changes as shown in Fig. 5.

4. DISCUSSION

Human ferredoxin has been expressed in *E. coli* as a cleavable fusion protein [14]. The fusion protein was specifically cleaved by a factor, Xa, to yield mature recombinant ferredoxin. Combined with this expression system, a means of directional cloning of ferredoxin cDNA by amplifying it from a cDNA library using PCR was developed [18]. Chicken ferredoxin cDNA was cloned and expressed by this procedure. The cloning method described in this study showed that the ferredoxin cDNA was selectively amplified from total RNA and the characteristic features of the expressed cells were used to identify the cells expressing ferredoxin. Therefore, a ferredoxin cDNA can be cloned and expressed efficiently in *E. coli* without having to make or screen a cDNA library, very laborious and time con-

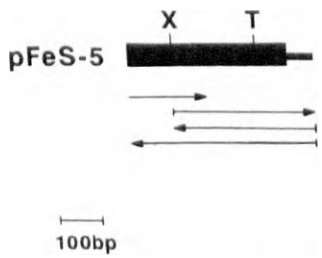

Fig. 4. Restiction map and sequencing strategy for a sheep adreno-ferredoxin cDNA clone (pFeS-5). X and T represent *Xba*I and *Taq*I sites, respectively.

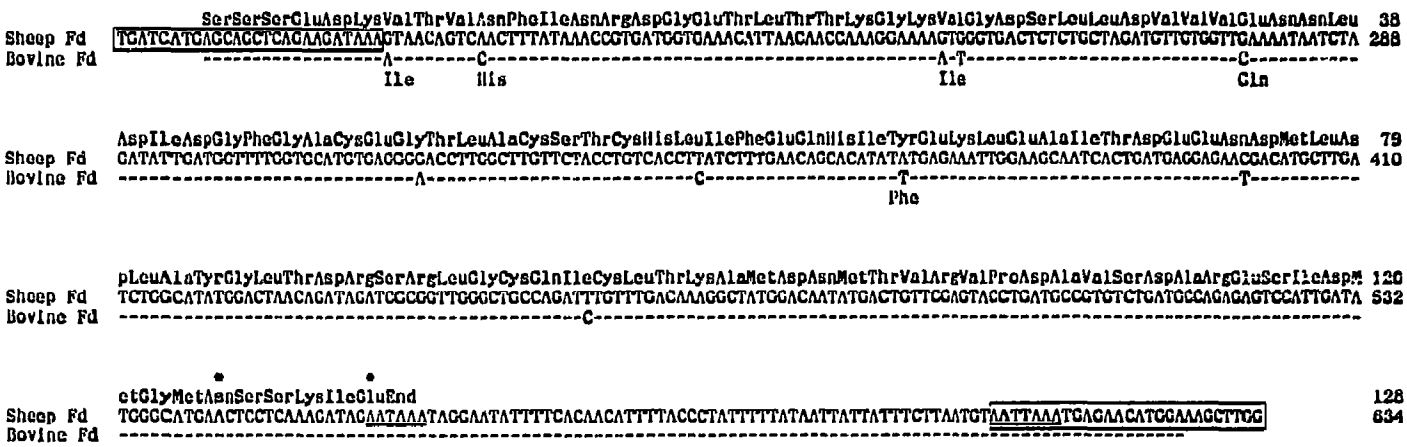

Fig. 5. Nucleotide sequences and deduced amino acid sequences of sheep and bovine ferredoxin cDNA clones. The nucleotide sequences of sheep and bovine ferredoxins are derived from the pFeS-5 and pBVL205 [10] clones, respectively. The amino acid residues of bovine ferredoxin different from those of the sheep one are shown below the nucleotide sequence of bovine ferredoxin. –, same nucleotides as in above row; * amino acid residue different from the protein sequence data; boxed regions, nucleotide sequences derived from synthesized primers; underlined region, poly(A) adenylation signal.

suming steps, and can be identified without knowing the DNA sequence.

The oligonucleotide primers used in this study were based on the DNA sequence of bovine ferredoxin cDNA, but can be applied to any other ferredoxin unless the primers anneal to homologous regions. In this respect, the sheep is a relatively closely related species, meaning less trouble regarding the annealing of the primers. The more distantly related ferredoxins are, the lower might be the stringency required for converting mRNA to cDNA and PCR. The first 6 N-terminal amino acid residues of mature ferredoxin in the region for annealing the 5′-primer are identical in cattle and sheep. Therefore, no amino acid change has occurred between the expressed and native sheep ferredoxins. However, when this method, with the same primers, is applied to the cloning and expression of other ferredoxins, of which the N-terminal amino acid sequences of the mature forms are unknown, the expressed ferredoxins would have the first 6 N-terminal amino acid residues identical to the bovine ferredoxin. If the expressed protein should be started with the mature and native form, the DNA sequence of the region for annealing the 5′- primer should be determined first, and then a second PCR with a primer synthesized based on the DNA sequence determined should be conducted. The 5′-primer for the first PCR should be synthesized according to the nucleotide sequence in the mitochondrial presequence region. In this case, usually less than ten extra amino acid residues would be attached to the N-terminal. However, neither the activity nor the structure of [2Fe-2S] would be disrupted. Actually, fusion ferredoxins with parts of lacZ or lcII proteins in the N-terminal site have been reported to be still active [15,18]. Therefore, the method for cloning described here can be also applied to fusion ferredoxins.

The amino acid sequence of the sheep adreno-ferredoxin deduced from the DNA sequence was consistent with the data obtained by sequencing the protein, except for 2 sites. The 123rd position was judged to be occupied by aspartate on protein sequencing but asparagine on DNA sequencing. This might be due to allelic variation. The other difference was the C-terminal residue. An isoleucine at the 127th position was the last residue determined on protein sequencing, however, glutamate at the 128th position was predicted to be the last residue on DNA sequencing. Similarly, the inconsistency has also been reported in bovine adreno- and hepato-ferredoxins. Purified ferredoxins from adrenocortex and liver were shorter and ended before the 128th position, which was predicted to be the last residue from the DNA sequence [5,6,10]. These are probably because of the processing of the C-terminal peptide in vivo or during purification of the protein [10,19,20].

Acknowledgements: We are grateful to the staff of the Iwate Meat Center for supplying the fresh sheep adrenal glands.

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