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Cloning, Expression, and Molecular Characterization of the Gene Encoding an Extremely Thermostable [4Fe-4S] Ferredoxin from the Hyperthermophilic Archaeon *Pyrococcus furiosus*

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The gene for ferredoxin from the hyperthermophilic archaeon *Pyrococcus furiosus* was cloned, sequenced, and expressed in *Escherichia coli*. The coding region confirmed the determined amino acid sequence. Putative archaeon-type transcriptional regulatory elements were identified. The *fdxA* gene appears to be an independent transcriptional unit. Recombinant ferredoxin was indistinguishable from the protein purified from *P. furiosus* in its thermal stability and in the potentiometric and spectroscopic properties of its [4Fe-4S] cluster.

In the last decade, a group of microorganisms that have the remarkable property of growing at temperatures near and even above 100°C have been isolated (1–3, 22, 23). The most studied of these so-called hyperthermophiles is *Pyrococcus furiosus* (14). In this organism, the redox protein ferredoxin (6) functions to accept electrons from a variety of unusual oxidoreductases and donate them to hydrogenase for H_2 production (2, 9). Ferredoxin is a small and extremely thermostable monomeric protein which contains a single [4Fe-4S] with unusual spectroscopic and cluster reactivity properties (10–12). These features together with its small size make it an ideal model protein with which to investigate both the mechanisms of hyperthermostability and the influence of protein structure on the properties of iron-sulfur clusters. A gene for a 4Fe-ferredoxin has not previously been cloned and expressed. Moreover, successful cloning and expression in *Escherichia coli* has been reported only for genes encoding *P. furiosus* α-amylase and *Pyrococcus woesei* glyceraldehyde-3-phosphate dehydrogenase (16, 26). We report here the molecular cloning and sequence analysis of the first gene for a 4Fe-type ferredoxin.

Cloning of the *P. furiosus* ferredoxin gene. Cloning of the predicted 198-bp ferredoxin gene from *P. furiosus* was accomplished with a PCR product by using 23-mer primer sets homologous to regions with minimal codon degeneracy as deduced from the known ferredoxin amino acid sequence (10). These primers contain at the 3′ end (underlined) all possible nucleotide permutations, whereas the 5′ end regions contain thymines (Ts) at each degenerate position. In order to generate a homologous nucleotide match to the ferredoxin target gene, four amino-terminal primers (N-1 through N-4 match the predicted nucleotide sequence from Trp-2 through Asp-9) were each combined with one of two carboxy-terminal primers (C-1 and C-2 match Met-53 through Ala-60). The primers used were as follows: N-1, 5′-TGGAATGTTTTTGTTGATCAAGA-3′; N-2, 5′-TGGAATGTTTTTGTTGATCAGGA-3′; N-3, 5′-TGGAATGTTTTTGTTGACCAAGA-3′; N-4, 5′-TGGAATGTTTTTGTTGACCAGGA-3′; C-1, 5′-GCTTTTACTGGTCATGCTTCCAT-3′; C-2, 5′-GCTTTTACTGGTCATGCCTCCAT-3′. The nucleotide sequence of a PCR product generated with N-3 and C-2 primers confirmed the predicted nucleotide sequence as deduced from the previously determined ferredoxin amino acid sequence (10). The ferredoxin-specific PCR fragment was used to probe *Eco*RI-digested *P. furiosus* genomic DNA. A 3.4-kb *Eco*RI *P. furiosus* genomic DNA fragment was subsequently isolated and inserted into the *Eco*RI site of pBluescript SK(−) (4). One clone (pAH007) carrying *fdxA* in the correct orientation from the pBluescript *lac* promoter was chosen for further analysis.

The complete nucleotide sequence of *fdxA* (Fig. 1) carried on pAH007 as translated is identical to the amino acid sequence of native ferredoxin (10). However, the *fdxA* coding region starts with an ATG initiation codon (methionine), a residue which is absent in ferredoxin protein isolated from *P. furiosus* (10). The G+C content of the ferredoxin coding region is 48.5%, higher than the genomic G+C value of 38% (14). Codon usage of the *fdxA* gene reveals the strong bias against CG dinucleotide-containing codons previously reported for archaea (13, 16, 26).

The flanking region upstream of *fdxA* (Fig. 1) reveals putative archaeon-type promoter elements (15). Possible *fdxA* regulatory elements include an A+T-rich promoter proximal element (15) and a box B structure (consensus, T/ATGC/A [19]) (Fig. 1). A putative box A promoter element (TTTATA) (Fig. 1) is found upstream of the *fdxA* coding region and shows strong homology to the recently published archaeal box A consensus sequence, 5′-(T/C)TTA(T/A)A-3′ (15, 18). A transcription start site(s) in archaeal systems usually involves a pyrimidine/purine dinucleotide motif, i.e., archaeal transcripts initiate almost exclusively at a purine following a pyrimidine (15). Two such potential start sites, located 27 to 30 bases downstream of the proposed box A element, are found at −8 and −11 of *fdxA* (Fig. 1). The nucleotide distance between the box A element and the proposed transcription start site(s) is within the proposed consensus distance of 25 to 28 bases on the basis of 84 mapped archaeal promoters (15). Interestingly, the *fdxA* proximal promoter region (−1 to −20) is strikingly

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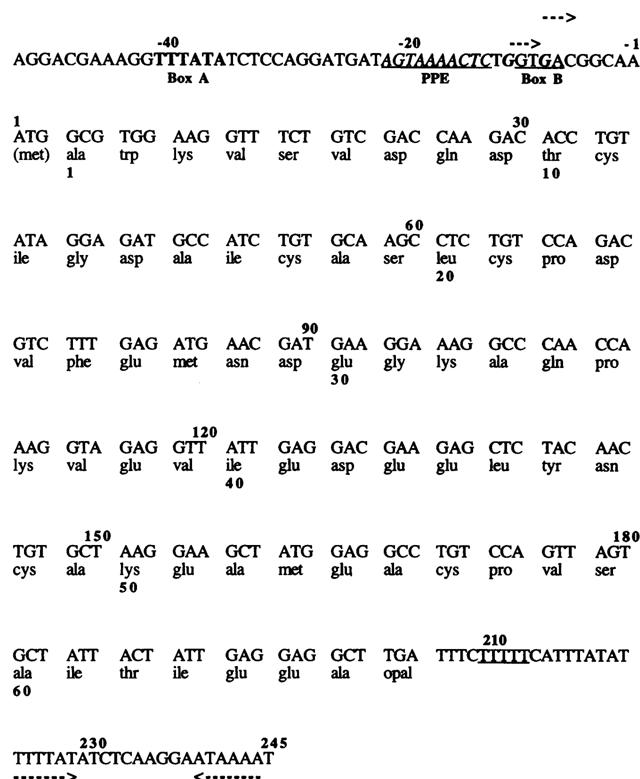

FIG. 1. Nucleotide sequence and derived amino acid sequence of the *P. furiosus* ferredoxin gene (*fdxA*) and its flanking regulatory regions. The *fdxA* DNA sequence numbering starts with the initiator methionine; however, since this residue is not part of the mature protein, amino acid numbering starts with alanine (Ala-1). Putative *fdxA* promoter elements are denoted as follows: box A is in boldfaced type, the proximal promoter element (PPE) is underlined and in italics, and box B is underlined. Possible transcriptional start sites are indicated in boldfaced italics and marked with horizontal arrows in the same direction. Downstream of the *fdxA* coding region, a possible transcriptional termination signal is underlined; a region capable of forming stem-loop structures is indicated with horizontal arrows facing each other.

homologous to the corresponding promoter region of the *P. woesei* glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene (26), possibly indicating common ribosome-binding sites and/or box B structures (Fig. 2). The initiation ATG codon of *fdxA* is preceded by a possible archaeon-type ribosome-binding site (GGTGA) (Fig. 2), a sequence which shows the highest degree of homology to the conserved 3′ end of archaeal 16S rRNA (8). If the proposed *fdxA* ribosome-binding site indeed functions as the initial binding site for the *P. furiosus* ribosome, transcriptional initiation at −8 seems unlikely. However, since the *fdxA* proximal promoter region from nucleotide −8 to −4 displays 60% homology to the *gapdh* ribosome-binding site (Fig. 2), it cannot be excluded that the latter sequence represents the *fdxA* ribosome-binding site. Finally, the recent finding that the 5′ noncoding region of the *P. furiosus* α-amylase gene lacks homology altogether to the archaeal consensus ribosome-binding site (16) suggests the possibility that the 3′ terminus of the *P. furiosus* 16S rRNA is different from the archaeal 16S rRNA termini used to compile the archaeal consensus ribosome-binding site (8). The highly conserved archaeal promoter structures upstream of the *fdxA* coding region suggest an efficiently functioning *fdxA* promoter in vivo, consistent with the relatively high yields of native ferredoxin from *P. furiosus* (6).

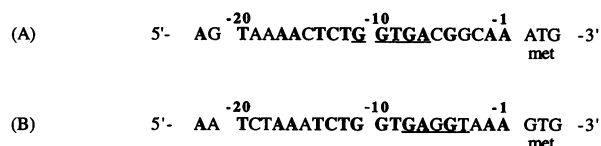

FIG. 2. Nucleotide sequence alignment of the proximal promoter regions from *P. furiosus fdxA* (A) and *P. woesei gapdh* (B) genes. Regions of homology are indicated in boldface, and proposed ribosome-binding sites of the *fdxA* and *gapdh* genes are underlined. See text for details.

A pyrimidine-rich region that includes a stretch of five Ts is found immediately downstream of the *fdxA* TGA stop codon (Fig. 1). This putative *fdxA* transcriptional termination region resembles the terminator structures of the SSV1 virus (20) and the *P. woesei gadph* (26) and *glnA* (24) genes. A region capable of forming stem-loop structures is found further downstream of the T block. However, it is not clear if these secondary structures have a significant biological function regarding transcription termination because of their probable lack of stability at the high growth temperatures used for *P. furiosus*. Upstream and downstream of the *fdxA* gene, two potential truncated open reading frames which do not overlap the putative regulatory regions of the *fdxA* gene are found (not shown).

The combination of the transcriptional *fdxA* regulatory structures strongly suggests that the ferredoxin gene is not integrated into an operon with other *P. furiosus* coding elements; instead, it represents an independent transcription unit. Additional sequencing and transcriptional analyses are under way to confirm this.

Construction of a heterologous expression system. Preliminary attempts to express ferredoxin in *E. coli* with the *lac* promoter on pAH007 were inconclusive, which was attributed to the lack of an *E. coli* ribosome site upstream of the *fdxA* coding region. The structural gene of ferredoxin was, therefore, subcloned with a PCR fragment generated with a primer pair that is exactly homologous to the 5′ and 3′ ends of the ferredoxin gene. The N-terminal primer (35-mer, 5′-C GGG ATC CTA AGG AAA CAG ACC ATG GCG TGG AAG G-3′) is complementary to the ferredoxin region between nucleotides 1 and 13 and has 22 extra bases at the 5′ site containing an *Nco*I site (underlined) encompassing the *fdxA* initiator codon. The C-terminal primer (27-mer, 5′-CCC AAG CTT CCC GGG TCA AGC CTC CTC-3′) matches the ferredoxin sequence between nucleotides 193 and 204 and has 15 additional bases at the 5′ site containing the *Sma*I (underlined) recognition site. The *Nco*I-*Sma*I-digested PCR product was directly used in the ligation reaction with expression vector p*Trc*99A (5) digested with *Nco*I-*Sma*I. The ligation mix was used to transform JM105 cells (25). In this expression system (pAH1993), the *fdxA* gene was inserted into the *Nco*I site of the vector, which is juxtaposed next to the *lacZ* ribosome-binding site and the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *trc* promoter carried on p*Trc*99A; thus, ferredoxin expression in *E. coli* combines a strong, IPTG-inducible promoter and efficient *E. coli* translational initiation structures.

Expression and purification of recombinant ferredoxin. JM105 cells carrying pAH1993 were grown overnight under aerobic conditions in 2 liters of Luria-Bertani broth (21) supplemented with 100 μg of ampicillin per ml. This culture

was used to inoculate 80 liters of Luria-Bertani broth (supplemented with 100 μg of ampicillin per ml and 25 μM $FeCl_3$) in a 100-liter fermentor. Cells were grown aerobically at 30°C to an A_{600} of 0.45 prior to induction of the *trc* promoter with 1.5 mM IPTG. After 2.5 h of induction, cells were harvested, immediately frozen in liquid nitrogen, and stored at −80°C. Recombinant ferredoxin was purified anaerobically to avoid possible oxidative damage of the iron-sulfur centers (6, 11). *E. coli* cells were thawed on ice and resuspended in ice-cold lysis buffer (per gram of *E. coli* cells: 3 ml of 50 mM Tris-HCl [pH 8] containing 1 mM EDTA, 100 mM NaCl, 50 mM phenylmethylsulfonyl fluoride, and 0.25 mg of lysozyme per ml). The suspension was degassed, and sodium dithionite was added to a final concentration of 2 mM. Cells were lysed during incubation at 23°C for 1 h with stirring and then sonicated. Degassed solutions of RNase (5 μg/g of cells) and DNase (10 μg/g of cells) were added, and incubation was continued for 30 min. After centrifugation (45 min at 48,000 × *g* and 4°C), the supernatant was either immediately used for column chromatography or stored at −80°C.

Following disruption and fractionation of induced cells, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17) of soluble and insoluble total cellular protein showed that recombinant ferredoxin remained soluble in the *E. coli* cell extract (data not shown). Recombinant ferredoxin was purified in a two-step procedure based on that previously described to isolate native protein (6). Protein concentrations of pure ferredoxin samples were estimated from A_{390} by using a molar absorbance of 17,000 $M\ cm^{-1}$ (11). Purified recombinant protein represented approximately 0.15% of the total soluble protein in induced cell extracts.

Properties of recombinant ferredoxin. SDS-PAGE analysis showed that the electrophoretic properties of the native and recombinant proteins were identical (data not shown), consistent with them having the same molecular weight of 7,500 (10). The iron content was 3.8 ± 0.4 g atoms Fe/mol for native protein and 4.4 ± 0.4 g atoms Fe/mol for recombinant ferredoxin. The amino acid composition and N-terminal amino acid sequence of the recombinant were identical to those of native protein (10), including the absence of an N-terminal methionine. Both recombinant and native ferredoxins exhibited thermostability, being unaffected by 12 h at 95°C (6). The UV-visible absorption spectra of the air-oxidized and sodium dithionite-reduced states (data not shown) were virtually identical to those of native protein (6). Recombinant ferredoxin contained a single [4Fe-4S] cluster that had the same reduction potential and electron paramagnetic resonance properties as native protein (6, 11), and it functioned in an in vitro assay at 80°C as an electron acceptor for *P. furiosus* pyruvate ferredoxin oxidoreductase (7).

Nucleotide sequence accession number. The nucleotide sequence reported has been assigned GenBank accession number X79502.

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