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Expression of human ferredoxin and assembly of the [2Fe-2S] center in *Escherichia coli*

(mitochondrial iron–sulfur protein/electron transfer/adrenodoxin)

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ABSTRACT **A cDNA fragment encoding human ferredoxin, a mitochondrial [2Fe-2S] protein, was introduced into *Escherichia coli* by using an expression vector based on the approach of Nagai and Thøgersen [Nagai, K. & Thøgersen, M. C. (1984) *Nature (London)* 309, 810–812]. Expression was under control of the λ P_L promoter and resulted in production of ferredoxin as a cleavable fusion protein with an amino-terminal fragment derived from bacteriophage λcII protein. The fusion protein was isolated from the soluble fraction of induced cells and was specifically cleaved to yield mature recombinant ferredoxin. The recombinant protein was shown to be identical in size to ferredoxin isolated from human placenta (13,546 Da) by $NaDodSO_4$/PAGE and partial amino acid sequencing. *E. coli* cells expressing human ferredoxin were brown in color, and absorbance and electron paramagnetic resonance spectra of the purified recombinant protein established that the [2Fe-2S] center was assembled and incorporated into ferredoxin *in vivo*. Recombinant ferredoxin was active in steroid hydroxylations when reconstituted with cytochromes $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ and exhibited rates comparable to those observed for ferredoxin isolated from human placenta. This expression system should be useful in production of native and structurally altered forms of human ferredoxin for studies of ferredoxin structure and function.**

The vertebrate ferredoxins are small (13–14 kDa) iron–sulfur proteins that occur in the mitochondria of steroid metabolizing tissues (1–4). They function to transfer reducing equivalents from NADPH-oxidoreductases to cytochrome P-450 enzymes involved in the biogenesis of steroid hormones, the production of bile acids, and the formation of vitamin D metabolites. Ferredoxins play a central role at regulated steps in these processes, and it is of particular interest to identify structural features that determine the properties of the iron–sulfur center and the specific interactions with steroid hydroxylase components.

The ferredoxin from bovine adrenal cortex, designated adrenodoxin, has been most extensively characterized, primarily because of its relative abundance in this tissue (5–8). Bovine adrenodoxin is synthesized in the cytoplasm with a large amino-terminal presequence, which is proteolytically removed upon mitochondrial uptake (9–12). A single [2Fe-2S] center is incorporated into the apoprotein, presumably within mitochondria, to produce the active holoprotein; it is not known whether incorporation of the prosthetic group occurs before or after removal of the presequence. Recently, we isolated ferredoxin from human placental mitochondria and showed that it is structurally and functionally similar to bovine adrenodoxin (13). Sequence analysis of human ferredoxin and of specific cDNAs from a human placental library revealed that the protein is synthesized as a 19,371-Da precursor that is processed to mature protein of 13,546 Da (13, 14). Complementary DNA from a human adrenal library (15) is identical to that found in the placenta, suggesting that a single gene product may function in all steroidogenic tissues within an organism. Ferredoxin structure must therefore be compatible with a variety of steroid hydroxylase components. Unfortunately, it is difficult to obtain human ferredoxin in amounts required for most structural analyses because of the low level present in placenta (13) and the lack of availability of sufficient amounts of other steroidogenic tissues.

In this report, we describe a system for high-level expression of human ferredoxin cDNA in *E. coli*. Using the approach developed by Nagai and Thøgersen (16), human ferredoxin is produced as a stable fusion protein with the bacteriophage λcII protein; the proteins are linked via a peptide, which includes a region that is recognized and cleaved by factor Xa, a sequence-specific protease. The ferredoxin fusion protein produced is soluble, and a [2Fe-2S] center is incorporated *in vivo*. Specific cleavage of the fusion protein yields fully active ferredoxin that is indistinguishable from the placental protein. This system allows for future investigations into the assembly and incorporation of the ferredoxin iron–sulfur center and provides a means by which mutant forms of the protein can be obtained for studies of ferredoxin structure and function.

MATERIALS AND METHODS

Materials. Restriction endonucleases and other enzymes used in plasmid construction were obtained from Boehringer Mannheim unless noted otherwise; reaction conditions were as specified by the supplier. Reagents for bacterial growth media were from Difco, and other chemicals were from Sigma.

Construction of pHFdx1. A 397-base-pair (bp) fragment containing a portion of human ferredoxin coding sequence was obtained by *Apa* I and *Xmn* I digestion of plasmid from a previously isolated cDNA clone (14). The fragment was treated with 10 units of mung bean nuclease (Stratagene) per μg of cDNA at 37°C for 30 min to produce blunt-ended fragments of various lengths. The plasmid pfX8 was provided by Charles Glabe (University of California, Irvine) and contains the factor Xa linker from M13mp11FX (16) inserted into the *Bam*HI site of pUC8. Plasmid pfX8 was linearized with *Stu* I and *Pst* I and treated with mung bean nuclease as described above, except 1 unit per μg of DNA was used. Blunt-ended pfX8 was dephosphorylated with alkaline phosphatase by using 12 units per μg of DNA for 30 min at 37°C. The dephosphorylated pfX8 was mixed and ligated to a 3-fold excess of blunt-ended ferredoxin cDNA fragments. The ligation mixture was used to transform competent *E. coli* HB101, and recombinants were screened on lifts with ^{32}P-

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Abbreviation: EPR, electron paramagnetic resonance.

labeled nick-translated ferredoxin cDNA (14). Plasmids were isolated (17) from selected positive colonies and mapped with various restriction endonucleases to select those having proper orientation of the ferredoxin sequence. *Bam*HI and *Hin*dIII digestion of these plasmids produced fragments containing the factor Xa site–human ferredoxin fusion sequence. These fragments were isolated, ligated into *Bam*HI/*Hin*dIII-cleaved M13mp19, and sequenced by the dideoxy method of Sanger *et al.* (18). A fragment was identified that had the codon for the first serine of mature ferredoxin immediately adjacent to the 3′ end of the factor Xa recognition sequence. This fragment was ligated into pMb3 (19) that had been cleaved with *Bam*HI and *Hin*dIII, and the mixture was used to transform *E. coli* HB101 cells. Plasmid isolated from transformed cells was designated pHFdx1.

Expression and Purification of Protein. Competent *E. coli* MZ-1 cells, a defective lysogenic strain (16), were transformed (17) with pHFdx1. Transformed cells were grown overnight at 30°C in 1 ml of Luria–Bertani medium (17) supplemented with ampicillin (50 μg/ml) (LB-amp). This culture was used to inoculate 1 liter of LB-amp in a 2-liter flask, and cells were grown at 30°C to an OD_{600} of ≈0.7. The culture was then heated in a 65°C water bath with rapid swirling to increase the temperature to 42°C and to induce synthesis of the ferredoxin fusion protein. The culture was incubated at 42°C for 2 hr and at 37°C for an additional 18 hr. All subsequent steps were performed at 4°C. Cells were harvested by centrifugation at 4000 × *g* for 5 min. The cellular pellet was resuspended in 40 ml of 50 mM Tris·HCl (pH 7.6) containing 0.1 mM EDTA, and the cells were lysed by two passages through a French press (10,000 psi; 1 psi = 6.9 kPa). Cellular debris was removed from the lysate by centrifugation at 35,000 × *g* for 15 min, yielding a straw-brown supernatant fluid.

The ferredoxin fusion protein was purified from the soluble extract as described for human placental ferredoxin (13). Briefly, the supernatant was loaded onto a DEAE-cellulose column and washed, and the brown ferredoxin-containing band was removed to a separate column and eluted with sodium chloride. The preparation was subjected to further chromatography on DEAE-agarose and Sephadex G-75. The purified fusion protein was treated with human factor Xa protease (Boehringer Mannheim) at a ratio of 1:50 (wt/wt) for 4 hr at 25°C in 50 mM Tris·HCl (pH 7.8) containing 0.1 mM EDTA. The processed mature ferredoxin was purified by ion-exchange chromatography on DEAE-agarose (2.5 × 20 cm) followed by gel filtration on Sephadex G-75 (1.0 × 40 cm).

Analytical Methods. Samples were analyzed by $NaDodSO_4$/PAGE using 15% gels and the buffer system of Laemmli (20). Protein concentrations were determined by the method of Lowry *et al.* (21). Gels were electroblotted onto polyvinyl difluoride membranes (Millipore) and stained with Coomassie blue R-250.

N-terminal amino acid sequences were determined by William T. Morgan (Bio-Technologies Unit, Louisiana State University Medical School, New Orleans) using automated Edman degradation on an Applied Biosystems 477A sequenator. Samples to be sequenced were denatured by boiling in $NaDodSO_4$/PAGE sample buffer, vacuum-blotted onto polyvinyl difluoride membranes, stained with Coomassie dye, and excised for direct analysis. C-terminal sequencing was performed with carboxypeptidase P as described (13).

Electron paramagnetic resonance (EPR) spectra were recorded by Gerard Jensen and Phillip Stevens (University of Southern California, Los Angeles) using a Bruker ESR-200D spectrometer equipped with an Oxford Instruments ESR-9 flow cryostat. An anaerobic 100 μM sample in 50 mM Tris·HCl, pH 7.4/0.1 mM EDTA was treated with 3 mM sodium dithionite. Spectra were obtained from 10–60 K at 9.5956 GHz using 6.3 μW power and 5 G modulation amplitude at 100 kHZ. Absorption spectra were recorded at ambient temperature in a Cary 17D spectrophotometer interfaced to a Zenith Z-100 computer adapted for data acquisition by On-Line Instrument Systems (Jefferson, GA). Concentrations of ferredoxin were calculated using $\varepsilon_{414} = 11$ $(mM \cdot cm)^{-1}$.

Assays for electron transfer from adenodoxin reductase to cytochrome *c*, cytochrome $P\text{-}450_{scc}$, and cytochrome $P\text{-}450_{11\beta}$ were conducted as described (13). The concentration of ferredoxin in each assay was approximately one-half the value K_m to ensure that the ferredoxin component was rate determining.

RESULTS

Construction of pHFdx1. The strategy used to construct a vector for expression of human ferredoxin is summarized in Fig. 1. To obtain the mature form of the protein, it was first necessary to obtain a cDNA fragment that was free of 5′ untranslated and presequence-encoding regions. This was achieved by using *Apa* I to cut plasmid from a previously isolated cDNA clone (14) within the last two codons of the ferredoxin presequence. *Xmn* I was used to blunt cut the cDNA 19 bp downstream from the termination codon, generating a 397-bp cDNA fragment. Partial digestion with mung bean nuclease was then used to remove the remaining presequence-encoding nucleotides from the 5′ end of this fragment. Sequence analysis revealed that approximately one-third of the treated fragments contained 5′ blunt ends which began with the codon for serine, the first amino acid of mature ferredoxin (13). Ligation of this fragment into *Stu* I/*Pst* I-cut pfX8 yielded a construct containing ferredoxin cDNA immediately downstream from a dodecanucleotide sequence encoding a factor Xa recognition site (X in Fig. 1). A DNA fragment containing the factor Xa site–ferredoxin fusion sequence was removed from this construct with *Bam*HI and *Hin*dIII and inserted in place of a similar fusion sequence in pMb3, a plasmid previously used to express high levels of myoglobin in *E. coli* (19). The resulting expression plasmid, designated pHFdx1, contained the mature ferredoxin coding sequence fused, via the factor Xa protease recognition sequence, to a region encoding the first 31 amino acids of the bacteriophage λcII protein. The amino-terminal cII protein segment is presumed to add stability to the fusion product in *E. coli*, and the factor Xa recognition site provides a means to specifically cleave the fusion protein to release mature recombinant ferredoxin (16, 22). Expression of the fusion product, henceforth referred to as cII–fX–ferredoxin, is under control of the bacteriophage λ P_L promoter (16).

Expression and Purification of Recombinant Ferredoxin. Plasmid pHFdx1 was used to transform the defective λ lysogen, *E. coli* MZ-1 (16). This strain has a temperature-sensitive mutation in the λ *c*I gene (*cI857*) so that transcription from the λ P_L promoter is repressed in cells grown at 30°C but not in cells grown at 42°C. Pellets of transformed cells grown at 42°C were brown in color, suggesting increased synthesis of an iron–sulfur protein. The results from analysis of total cellular protein (soluble and insoluble) by $NaDodSO_4$/PAGE are shown in Fig. 2 (lanes 1 and 2). The induced cells produced increased amounts of a protein that migrates with an apparent molecular mass of ≈20 kDa, a value slightly larger than expected for the cII–fX–ferredoxin fusion protein (17.6 kDa). Immunoblots using anti-adrenodoxin antibodies, previously shown to cross-react with human ferredoxin (13), identified the 20-kDa band with ferredoxin fusion protein (data not shown). Following disruption of induced cells and fractionation of their contents, most of the fusion product remained soluble; the remainder was associated with insoluble material and was discarded. The soluble cII–fX–ferre-

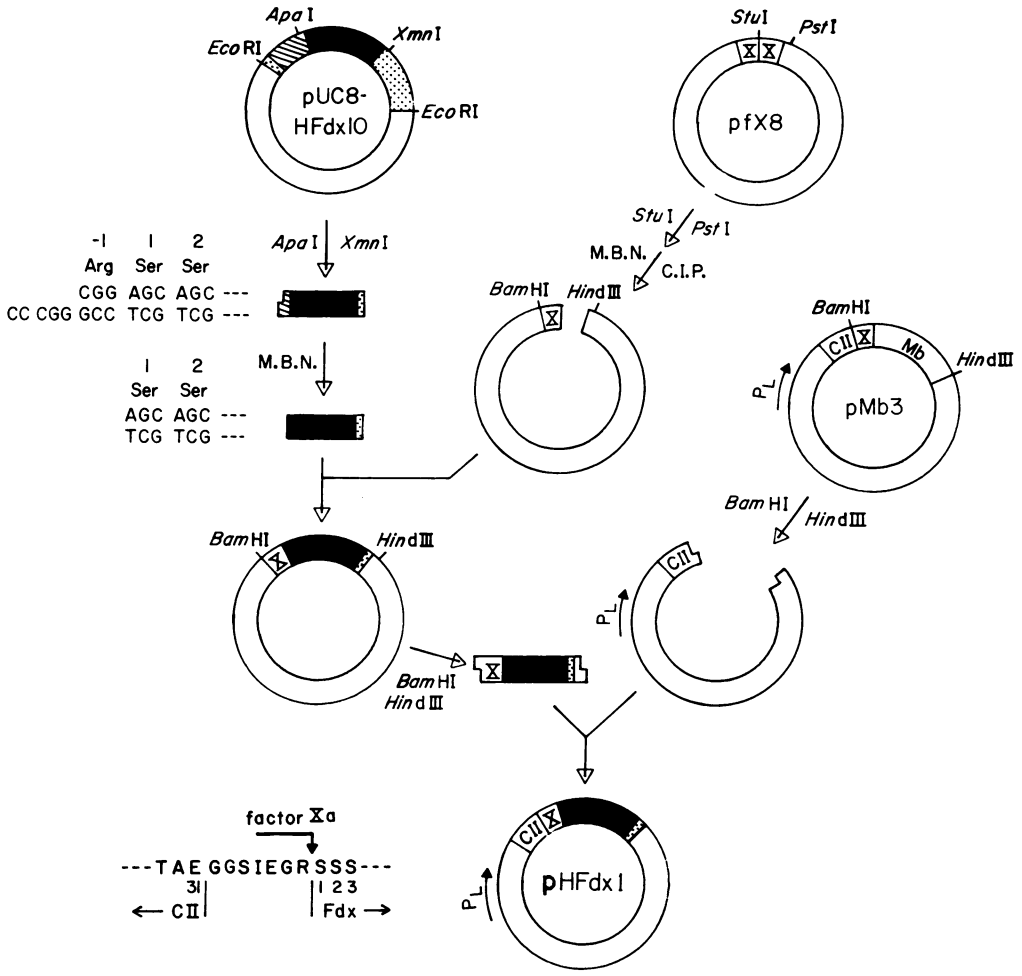

FIG. 1. Construction of human ferredoxin expression vector pHFdx1. The solid and hatched regions indicate mature and presequence coding regions of ferredoxin cDNA, respectively; the stippled sections represent untranslated flanking sequences. X, sequence encoding a factor Xa protease recognition site; CII, sequence encoding the first 31 amino acid residues of the λ cII protein.

doxin fusion protein typically represented ≈12% of the total soluble protein in induced cell extracts. Overnight growth at 37°C following induction at 42°C was found to increase yields of soluble cII–fX–ferredoxin, whereas supplementation of medium with 0.1% $FeSO_4$ did not affect the amount of fusion protein obtained. The acidic nature of the cII–fX–ferredoxin fusion protein is similar to that of other ferredoxins (pI ≈ 4), and previously established conditions for anion exchange (13) were utilized to isolate the fusion protein from the soluble fraction of induced cell lysates. Fig. 2 (lane 3) shows a partially purified preparation of cII–fX–ferredoxin fusion protein.

Properties of the Recombinant Protein. Because there are no factor Xa recognition sites within the mature ferredoxin sequence (14), this protease was used to cleave the partially purified fusion protein at the linkage site. The processed recombinant protein was then purified by using anion exchange and gel filtration (see *Materials and Methods*). Approximately 5 mg of purified recombinant ferredoxin was obtained per liter of induced cells. The processed protein migrates on $NaDodSO_4$/polyacrylamide gels as a single major band with an apparent molecular mass of ≈13.5 kDa (Fig. 2, lane 4), similar to full-length human ferredoxin isolated from placenta (lane 5).

Amino acid sequence analyses of the 13.5-kDa recombinant protein were performed to confirm that correct processing had occurred and was limited to the predicted site. The amino-terminal sequence was determined by automated Edman degradation. A sequence (yield in pmol) of Ser(38.8)-Ser(37.9)-Ser(36.7)-Glu(36.8)-Asp(32.6) was found, identical to the first five residues of ferredoxin obtained from human placenta (13). Carboxyl-terminal sequencing of the recombinant ferredoxin was performed with carboxypeptidase P. Successive release of serine, threonine, and then lysine was observed, corresponding to residues 122–124 at the carboxyl terminus of human placental ferredoxin (13, 14). These results are consistent with the recombinant protein being identical to full-length mature human ferredoxin.

The brown color of recombinant ferredoxin preparations suggested that the [2Fe-2S] cluster had been assembled and incorporated into the protein *in vivo*. This was confirmed by EPR and optical absorption spectroscopy. The EPR spectrum of reduced recombinant ferredoxin is shown in Fig. 3 *Upper*. The observed axial spectrum with $g_{||}$ greater than $g_{\perp}$ resembles that described for reduced adrenodoxin (2, 5) and the spectrum recorded for human placental mitochondria treated with dithionite (23). No other signals were observed from g 1–13. The absorption spectrum of oxidized recombinant ferredoxin is shown in Fig. 3 *Lower*. The visible spectrum is identical to spectra obtained for human placental (13) and bovine adrenal (4–6) ferredoxins, with characteristic maxima at 455, 414, and 322 nm. The recombinant ferredoxin exhibits low absorbance in the near ultraviolet due to the lack of tryptophan; fine structure in this region due to two tyrosine residues is apparent. The ratio $A_{414}/A_{276} = 0.82$ represents an

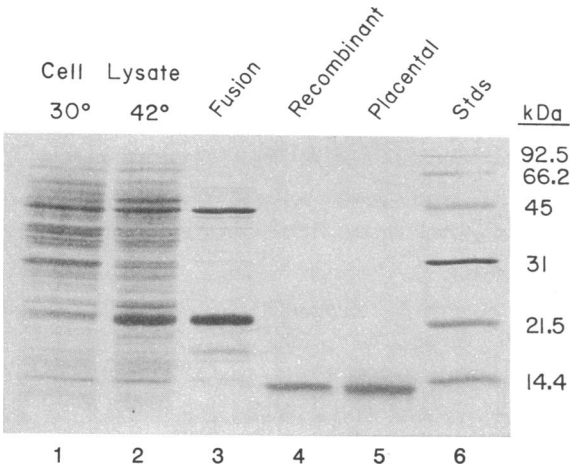

FIG. 2. Electrophoretic analysis of recombinant ferredoxin. Samples were separated by NaDodSO$_4$/PAGE in 15% gels, transferred to polyvinyl difluoride membrane, and stained with Coomassie blue. Lanes: 1 and 2, total cellular protein from transformed cells grown at 30°C or 42°C (50 μg each); 3, partially purified cII–fX–ferredoxin fusion protein (3 μg); 4, processed recombinant ferredoxin (3 μg); 5, ferredoxin isolated from human placenta (3 μg); 6, molecular mass markers (3 μg).

increase in purity over ferredoxin preparations previously obtained from human placenta (A_{414}/A_{276} = 0.60) (13).

The findings that recombinant ferredoxin was full length and that the prosthetic group was incorporated suggested that the protein may have assumed its native active conformation. To investigate this, the electron transfer ability of recombinant ferredoxin was assayed in three separate systems (Table 1). For coupling of NADPH-dependent oxidoreductase to cytochrome *c* reduction, both recombinant and human placental ferredoxins exhibited identical rates. For cytochrome P-450-catalyzed reactions, both cholesterol side-chain cleavage and deoxycorticosterone 11β-hydroxylation were supported by the recombinant protein at rates similar to those observed with preparations of ferredoxin obtained from human placenta. These findings establish that recombinant ferredoxin contains a functional [2Fe-2S] center and that regions of the protein that interact with ferredoxin reductase and cytochromes P-450$_{scc}$ and P-450$_{11\beta}$ maintain a properly folded structure. In separate experiments, the uncleaved cII–fX–ferredoxin fusion protein was assayed and also was found to be active in supporting P-450-catalyzed steroid hydroxylations (data not shown). This indicates that nonferredoxin sequences of the chimera do not interfere with ferredoxin–ferredoxin reductase or ferredoxin–cytochrome P-450 interactions.

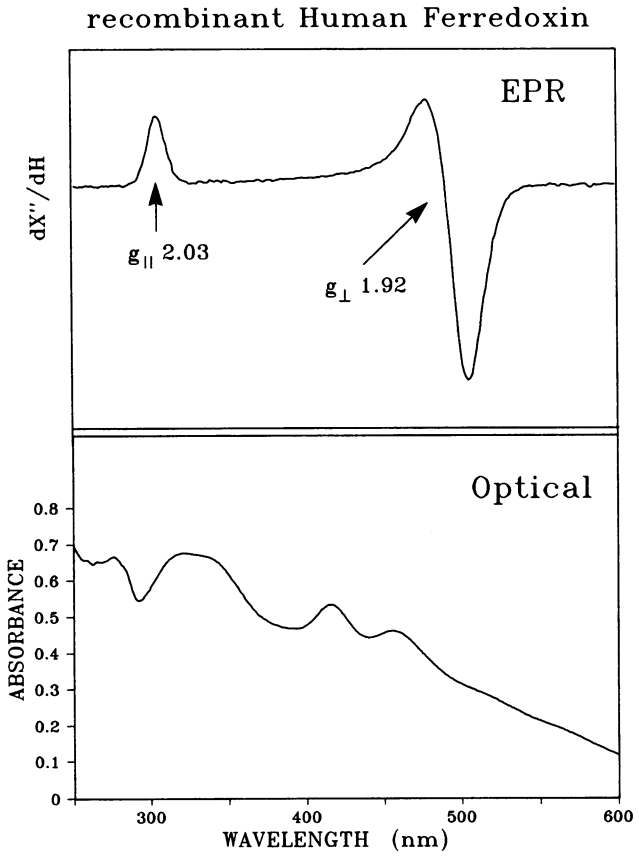

FIG. 3. Spectroscopic properties of recombinant ferredoxin. (*Upper*) EPR spectrum of dithionite reduced protein at 9.595 GHz, 10.2 K. (*Lower*) Absorption spectrum of resting (oxidized) protein at room temperature.

Table 1. Comparison of activities of human placental and recombinant ferredoxins

Assay (units)	Ferredoxin	
	Placental	Recombinant
Cytochrome *c* reduction (nmol of cyt *c* reduced per min)	0.87 ± 0.10	0.87 ± 0.10
Cholesterol side-chain cleavage (mol of pregnenolone per mol of P-450 per min)	4.80 ± 0.40	4.24 ± 0.40
Deoxycorticosterone 11β-hydroxylation (mol of corticosterone per mol of P-450 per min)	17.3 ± 0.1	18.8 ± 0.6

The concentration of ferredoxin used in each assay was approximately equal to one-half the K_m value for that system.

DISCUSSION

Using the approach of Nagai and Thøgersen (16), we have produced human ferredoxin as a cleavable fusion protein in *E. coli*. The majority of the ferredoxin fusion is produced as soluble protein, in contrast to most eukaryotic proteins previously expressed in *E. coli* by this approach (24). The highly acidic nature of the ferredoxin fusion protein and the fact that the ferredoxin portion correctly folds into a native conformation may contribute to its observed solubility. Partially purified fusion protein is specifically cleaved by factor Xa protease to yield the mature form of human ferredoxin. The yield of recombinant human ferredoxin from 1 liter of induced cells (≈5 mg) is greater than twice the amount of ferredoxin previously obtained from 40 human placentas (13). This high level of expression occurs even though ≈13% of the codons in human ferredoxin cDNA (14) are those that utilize tRNA species that are of low abundance in *E. coli* (25).

E. coli cells expressing the human ferredoxin fusion protein exhibit a distinct brown color, reflecting assembly of iron–sulfur clusters into the fusion protein *in vivo*, and spectroscopic characterization of purified recombinant ferredoxin confirms the identity of the [2Fe-2S]-type cluster with that found in ferredoxins of vertebrate mitochondria. *E. coli* has been found to contain both [2Fe-2S] and [4Fe-4S] proteins (26–28), but assembly of a [2Fe-2S] center into the foreign ferredoxin apoprotein was initially unexpected. Previous studies using the amidophosphoribosyltransferase gene from *Bacillus subtilis* have shown that *E. coli* have the capacity to assemble a foreign bacterial [4Fe-4S]-type protein (29). The mechanism of iron–sulfur protein biosynthesis has not been elucidated, but it has been proposed to require rhodanese to

provide a source of sulfide (30, 31). It is not known whether enzymes directly participate in the recognition of acceptor apoprotein or in the assembly of iron–sulfur clusters. The synthetic process may be spontaneous, requiring only apoprotein together with sulfur and iron in the proper redox state. A nonenzymatic mechanism for iron–sulfur protein synthesis is supported by reports of *in vitro* reconstitution of a number of iron–sulfur proteins, including adrenal ferredoxin (32, 33). Correct assembly in *E. coli* of iron–sulfur proteins as diverse as human ferredoxin and *B. subtilis* amidophosphoribosyltransferase suggests that the biosynthetic mechanism is not specific for the acceptor protein and that appropriate iron–sulfur centers may self-assemble *in vivo*.

Recombinant ferredoxin produced by this method exhibits full activity, implying that the [2Fe-2S] center and the folded protein assume a structure identical to the native placental protein. The activity of the recombinant protein also suggests that no posttranslational modifications of the polypeptide are required for human ferredoxin function, unless these modifications also occur in *E. coli*. The finding that the uncleaved cII–fX–ferredoxin fusion protein is active indicates that nonferredoxin sequences at the amino terminus of the chimera do not prevent proper folding of the apoprotein with the [2Fe-2S] center and also suggests that the amino-terminal region of mature ferredoxin is likely to be distant from sites of interaction with steroid hydroxylase components. In vertebrate cells, formation of mature human ferredoxin involves removal of the 60-residue amino-terminal presequence that directs mitochondrial import of the apoprotein; the effects of this presequence, which is highly basic, on protein folding and prosthetic group incorporation are not known.

This bacterial expression system should be useful for obtaining homogeneous preparations of human ferredoxin in amounts sufficient for extensive biochemical characterization. The atomic structure of a vertebrate ferredoxin has not been solved, and while crystallographic structures for an algal (34) and a bacterial (35) [2Fe-2S] ferredoxin have been determined, they show little sequence identity with vertebrate ferredoxins and may represent phylogenetically unrelated [2Fe-2S] ferredoxins (36). It is anticipated that recombinant preparations of human ferredoxin will be suitable for crystallographic and NMR analyses. The expression system also provides a means for obtaining mutant forms of ferredoxin produced by the techniques of site-specific mutagenesis.

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