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Characteristic features of the heterologous functional synthesis in *Escherichia coli* of a 2[4Fe–4S] ferredoxin

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Different strategies have been used to express synthetic genes all encoding *Clostridium pasteurianum* 2[4Fe–4S] ferredoxin (Fd) in *Escherichia coli*. The polypeptide can be produced as the C-terminal addition to a hybrid Cro::Protein A fusion protein lacking the metallic centers. The incorporation of the [4Fe–4S] clusters into the cleaved apoFd cannot be carried out in the same conditions as those affording holoFd from purified *C. pasteurianum* apoFd. In contrast, fully functional Fds can be produced from non-fused synthetic genes under the dependence of strong promoters. The yields of recombinant Fd, although sufficient to purify significant quantities of protein, are limited by the very short half-life of the 2[4Fe–4S] Fd in *E. coli*, irrespective of the expression system used. These features are characteristic of 2[4Fe–4S] Fds when compared with the far more stable recombinant rubredoxin, and probably other small iron–sulfur proteins which have already been produced in high yields. The reasons for the high turnover of 2[4Fe–4S] Fds are discussed.

Keywords: synthetic genes, iron–sulfur, protein turnover, *Clostridium pasteurianum*

Introduction

It is now fully accepted that the possibility of expressing and manipulating heterologous genes in *Escherichia coli* has boosted our knowledge of a variety of proteins over the last decade. A number of efficient strategies have been devised to maximize expression (reviewed in Das 1990). Still, overexpression is not always a straightforward procedure since various drawbacks, such as formation of inclusion bodies or deleterious effects on the growth of the bacterial host, may affect the amount or the quality of the product. Yet additional problems often arise with the products of short genes for which stabilization at the RNA or protein levels is not easy to reach (e.g. Guo *et al.* 1984). Also, in the case of proteins requiring cofactors or metallic centers, the stability of the product often depends on the successful incorporation of these components.

Most of these and other potential difficulties are combined in the expression of 2[4Fe–4S] ferredoxins (Fds). These molecules of ~55 amino acids, including eight cysteines, are among the shortest metalloproteins known. They are intrinsically unstable and require the presence of their two [4Fe–4S] clusters to correctly fold (Hong & Rabinowitz 1970). The heterologous sythesis of 2[4Fe–4S] Fds in *E. coli* or other systems has been so far limited to those produced from the natural genes of *Clostridium pasteurianum* Fd (Baur *et al.* 1990) and of *Rhodobacter capsulatus* FdI (Grabau *et al.* 1991). For *C. pasteurianum* Fd, the production of a protein identical to the natural holoFd has also been reported from a synthetic gene (Davasse & Moulis 1992).

As an attempt to further characterize the expression of 2[4Fe–4S] Fd genes in *E. coli*, alternate strategies have been applied here to synthetic genes encoding *C. pasteurianum* Fd. The combined results obtained with these various approaches have allowed us to define some of the limiting steps in the production of this protein and should help in designing experiments aimed at overexpressing other small genes encoding related metalloproteins in *E. coli*.

Materials and methods

The versions of the synthetic gene have been assembled from a series of oligonucleotides as previously described (Davasse & Moulis 1992). Their correct insertion in the plasmids used has been checked by sequencing using the dideoxynucleotide termination method (Sanger *et al.* 1977).

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Production and purification of the Cro::Protein A::Fd fusion protein

E. coli N-4830-1 (Pharmacia, Biotech SA, St Quentin-Yvelines, France) bearing plasmid pRITFD (see Results and discussion) were grown at 30 °C up to the mid-log phase. Induction was started by shifting the temperature to 37 °C, cells were labeled with [^{35}S]cysteine 1 h after the beginning of induction and harvested 3 h later. Under these conditions, growth stopped 30 min after the onset of induction. The cells were disrupted by sonication and the light yellow supernatant was reacted with Sepharose–IgG (Pharmacia) for 20 h at 4 °C in 50 mM Tris–HCl, 0.15 M NaCl, pH 7.7 (buffer A). The resin poured in a column was washed, first with 10 volumes of buffer A, then with five volumes of 5 mM ammonium acetate, pH 5. The strongly interacting fusion protein was eluted with buffer A containing 7 M urea and dialyzed against 10 mM Tris–HCl, pH 7.5. In order to obtain a pure fusion protein, an anion exchange purification step was required. It was carried out either on a DEAE–cellulose resin (DE-52; Whatman Scientific Ltd, Maidstone, UK) or on a PL-SAX (Polymer Labs, Church Stretton, Salop, UK) high performance liquid chromatography column, developed with a NaCl gradient in 50 mM Tris–HCl, pH 7.5.

For analysis on denaturing polyacrylamide gels, cells were suspended in 50 μl of 0.3 M sucrose–25 mM Tris–HCl–25 mM EDTA, pH 8 containing 1.7 mg ml^{-1} of lysozyme and incubated for 15 min at 4 °C. The suspensions were sonicated for 5 s and centrifuged for 5 min. Then 5 μl of the supernatants were added to 5 μl of 0.25 M Tris–HCl, pH 6.8, 4% SDS, 0.5% 2-mercaptoethanol, 20% glycerol (loading buffer) and denaturated for 5 min at 95 °C. After electrophoresis, the dried gel was analyzed by autoradiography.

Proteolytic release of Fd from the Cro::Protein A::Fd fusion protein

The peptide corresponding to *C. pasteurianum* Fd was cleaved by treatment with 2.5% (w/w) arginine-specific protease at 37 °C. The mixtures were separated on denaturing polyacrylamide gels and analyzed by autoradiography as above.

Expression of Fd from fd1 *or* fd2

Cells were grown at 37 °C in Luria–Bertani medium (Davasse & Moulis 1992) until they reached the 590 nm optical density of 1.85. They were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and harvested after labeling with 0.4 MBq of [^{35}S]cysteine. About 80 μg of the cell extract was solubilized in the gel loading buffer and analyzed by autoradiography as above. The integrated densitometric scans, measured with a dual wavelength flying spot scanner (CS9000; Shimadzu Corporation, Kyoto, Japan), of the band corresponding to recombinant Fd were used to quantitate the amount of protein produced.

Expression of Fd from fd3 *and determination of the half-lives of proteins*

E. coli K38 (Tabor 1990) containing plasmid pGP1-2 (Tabor & Richardson 1985) and *fd3* cloned into *Nde*I–*Hin*dIII-cleaved plasmid pT7-7 (Tabor 1990) were grown at 30 °C in Luria–Bertani medium containing 50 μg ml^{-1} of kanamycin and 100 μg ml^{-1} of ampicillin until they reached the 590 nm optical density of 1.35. T7 RNA polymerase synthesis was triggered by shifting the temperature to 41 °C for 30 min. At 20 min after the beginning of induction, 200 μg ml^{-1} rifampicine was added to the cultures. At 20 min after returning the cells to 30 °C, they were pulsed for between 5 and 40 min with 0.4 MBq of [^{35}S]cysteine and chased with 0.5% of L-cysteine for the indicated period (Figure 4). Samples were treated and analyzed as described above.

Results and discussion

Strategies used to express synthetic genes encoding C. pasteurianum *Fd in* E. coli

Three main options may be considered to express a foreign gene in *E. coli*: the protein can be produced alone or as a fusion or it can be excreted. The last option has not been tried in the present case since *C. pasteurianum* Fd contains eight cysteines which have to stay reduced in order to ligate the iron–sulfur clusters and are more likely to do so in the reduced cytoplasmic environment. Indeed, previous attempts at targeting a similar protein into the periplasmic space of *E. coli* failed to afford a product containing the metallic centers or to which they could be added *in vitro* (Bourdineaud *et al.* 1990).

A series of synthetic genes has thus been prepared (Figure 1) and studied for expression in the remaining options. Two versions differ slightly in the 5′ part of the coding strand; *fd1* has the initiation codon right after the *Eco*RI recognition site and the sequence around the start codon of *fd2* has been designed such as to follow the supposedly preferred base composition for efficient translation initiation in *E. coli* (deBoer & Hui 1990). *fd1* and *fd2* have been cloned into *Eco*RI–*Hin*dIII-digested plasmid pKK223-3 (Brosius & Holy 1984) to put them under the control of the hybrid *trp–lac* (*tac*) promoter (Amann *et al.* 1983).

In the third version of the gene (*fd3*, Figure 1), a *Nde*I recognition sequence has been engineered 5′ of the first Fd codon in order to substitute the coding sequence of the phage T7 protein 10 gene with the synthetic gene for Fd in plasmid pT7-7 (Tabor 1990). This kind of construction ensures an efficient transcription from the promoter (ϕ10) of the gene coding for bacteriophage T7 protein 10, selectively recognized by T7 RNA polymerase, and generally results in very high and almost exclusive expression of the gene of interest (Tabor & Richardson 1985).

As an alternative to direct expression, a modified version of the gene has been assembled (*fd4*, Figure 1) and cloned into *Eco*RI–*Pst*I-digested plasmid pRIT-2T (Nilsson *et al.* 1985) to give plasmid pRITFD. The synthetic

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                                M   A             E   *   *
fd1:          aggaaacagAATTC ATG GCG...........GAA TAA TGA
              tcctttgtcttaaG TAC CGC...........CTT ATT ACT TCGA
              rbs      EcoRI                              HindIII

                                M   A             E   *   *
fd2:        aggaaacagAATTC TT ATG GCT...........GAA TAA TGA
            tcctttgtcttaaG AA TAC CGA...........CTT ATT ACT TCGA
            rbs      EcoRI                              HindIII

                                M   A             E   *   *
fd3:          aaggagatatacaTATG GCT...........GAA TAA TGA
              ttcctctatatgtatAC CGA...........CTT ATT ACT TCGA
               rbs         NdeI                           HindIII

                                p  g  n  S   R   A         E   *   *
fd4:          tccggggAATTC CGT GCT...........GAA TAA TGA CTGCA
              aggcccctaaG GCA CGA...........CTT ATT ACT G
                        EcoRI                                PstI
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Figure 1. Sequences of the synthetic versions of the gene encoding *C. pasteurianum* Fd. Only the flanking sequences are given as the complete sequence of *fd2* has been reported elsewhere (Davasse & Moulis 1992) and has received Genbank Accession no. M83832. The protein sequences are given above the coding strand and the restriction sites below the complementary strand. *fd1* and *fd2* have been cloned into pKK223-3, *fd3* into pT7-7 and *fd4* into pRIT-2T to give plasmids pKCFd1, pKCFd2, pTFD and pRITFD, respectively (see text). The upstream sequences of the cloning vectors are indicated in lower case letters with the ribsome binding sites underlined for pKCFd1, pKCFd2 and pTFD. For pRITFD, the N-terminal aminoacid sequence of the fused Protein A fragment is also given in lower case letters.

gene was thus fused in frame with the 3′ end of a fragment of the *Staphylococcus aureus* gene encoding the immunoglobulin G (IgG)-binding domain of Protein A. A CGT codon for arginine was included in front of the first codon for Fd (Figure 1) in order to provide a convenient cleavage site. Indeed, efficient arginine-specific proteases are readily available and no such amino acids is present in the target sequence. The construction is likely to be efficiently transcribed as it contains the 5′ end of the *cro* gene encoding the repressor Cro protein of bacteriophage λ with the P_R promoter region (Zabeau & Stanley 1982).

Production of C. pasteurianum *Fd as a fusion protein*

The conditions leading to a significant production of the Cro::Protein A:: Fd protein have been studied by autoradiography of crude cell lysates after [^{35}S]cysteine labeling (Figure 2). A protein of the expected size (~ 36 kDa) was induced upon inactivation of the gene encoding the bacteriophage λ temperature-sensitive repressor *cI*ts857. Relatively low levels of the protein were produced in uninduced cells (lane C of Figure 2) and this amount increased sharply as the temperature of the culture was raised to 41 °C (lanes B, E and F). However, long induction periods failed to produce large amounts of protein (lanes I and J). Also, the addition of $FeSO_4$ did not increase the concentration of the fusion protein (lanes F and J). The autoradiographic pattern was the same whether the total lysate or only the soluble extract were analyzed (not shown). It may thus be confidently concluded that the product synthesized from pRITFD is a soluble protein.

The Cro::Protein A::Fd protein has been purified in two chromatographic steps (see Materials and methods). The fused recombinant Fd (reFd) is colorless and lacks the [4Fe–4S] clusters. The absence of the metallic centers is

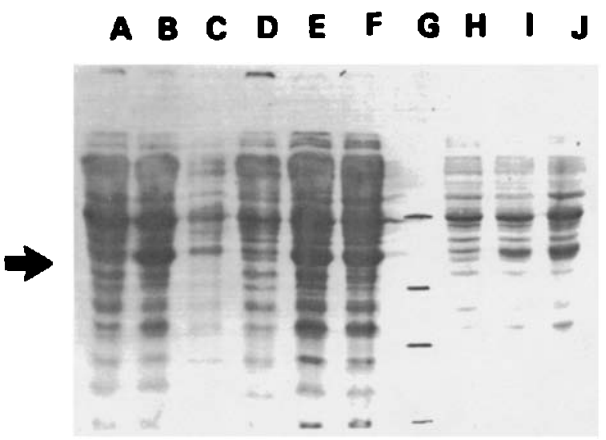

Figure 2. Expression of *fd4* in *E. coli* N4830-1. Cells bearing either pRIT-2T (without *fd4*) or pRITFD (with *fd4* cloned) were grown, induced and analyzed as described under Materials and methods. Lanes: A, pRIT-2T, 1 h after induction; B, pRITFD, 1 h after induction; C, pRITFD, uninduced; D, pRIT-2T, 4 h after induction; E, pRITFD, 4 h after induction; F, pRITFD, 2 h after induction in the presence of 50 μM $FeSO_4$; G, bars representing the positions of the 46 000, 30 000, 21 500 and 14 300 M_r markers; H, pRIT-2T, 20 h after induction; I, pRITFD, 20 h after induction; J, pRITFD, 20 h after induction in the presence of 50 μM $FeSO_4$. The arrow indicates the position of the Cro::Protein A::Fd protein.

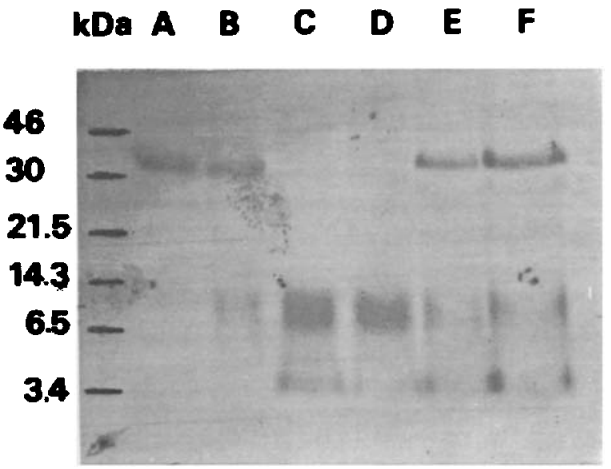

Figure 3. Cleavage of the Cro::Protein A::Fd protein. Lanes: A, pure protein; B, digested for 20 h with arginine-specific endoprotease from submaxillary glands of mice (Boehringer-Mannheim, Meylan, France); C, digested for 3 h with *Clostridium histolyticum* clostripain (Sigma L'Isle d'Abeau Chesnes, France); D, digested for 20 h with clostripain; E, digested for 3 h with clostripain in the presence of 6 M urea; F, digested for 20 h with clostripain in the presence of 6 M urea.

not the result of denaturation during purification since the faint color present in the crude extract can be separated from the Cro::Protein A::Fd fusion protein on an anion-exchange column before fractionation by affinity (not shown). It is more likely that the fusion protein does not bind the [4Fe–4S] clusters. This observation is to be compared with reports demonstrating the expression of holoFd of the [2Fe–2S] type as fusions in *E. coli* (Coghlan & Vickery 1989, Brandt *et al.* 1991). In these cases, the fusions involved the N-terminal addition of 31 amino acids from the cII protein of bacteriophage λ to 114 amino acids proteins (i.e. ~25% of the total length), instead of 265 amino acids added here to a 2[4Fe–4S] Fd of 55 amino acids. This very long N-terminal addition to *C. pasteurianum* Fd most probably triggers the folding of the fusion protein and accounts for the inefficiency of the *in vivo* incorporation of the [4Fe–4S] clusters.

The cleavage of the reFd polypeptide from the fusion protein has been studied with different proteases under various conditions (Figure 3). Clostripain from *Clostridium histolyticum* proved to digest the purified protein (lanes C and D) in a more efficient way than arginine-specific endoproteinase from submaxillary glands of mice (lane B) and no chaotropic agent appeared to be required to expose the cleavage site at the N-terminus of reFd (lanes E and F). Using a [^{35}S]cysteine-labeled and carboxy-methylated preparation, a peptide corresponding to the major radioactive band was sequenced and shown to have the first 17 amino acids expected from *C. pasteurianum* Fd. Hence, reFd fused to Protein A was efficiently produced in *E. coli* and could be recovered after cleavage at the engineered N-terminal arginine residue.

Attempts at inserting the inorganic centers *in vitro* have been carried out on the clostripain-cleaved fusion protein. Despite the use of various experimental conditions derived from a well established method (Hong & Rabinowitz 1970), genuine holoFd has not so far been purified from the reaction mixture. This failure is somewhat surprising as inclusion of the [4Fe–4S] clusters is a relatively straightforward procedure with this protein (Hong & Rabinowitz 1970, Moulis & Meyer 1982). The presence of other peptides generated by cleavage of the fusion protein might disturb the mechanism of Fe–S cluster assembly and the correct folding of the holoFd. The conditions under which *C. pasteurianum* apoFd expressed as a fusion protein may incorporate [4Fe–4S] clusters *in vitro* are still under study. Nevertheless, depending on the intended use of the Fd, the possibility of readily obtaining the peptide fused to Protein A may be of interest.

Direct production of C. pasteurianum *Fd in* E. coli

It has already been shown that gene *fd2* inserted in plasmid pKK223-3 produces in *E. coli* a reFd identical to that isolated from *C. pasteurianum* (Davasse & Moulis 1992). The expression of *fd2* in *E. coli* JM109 depends on the following parameters: (i) the concentration of reFd increases with cell density at the time of induction; (ii) reFd concentration increases during the first 2–3 h after induction but decreases beyond 4 h. $FeSO_4$ concentrations in the 0–50 μM range do not change this pattern; and (iii) reFd concentration increases with inducer concentration, maximal reFd production being almost reached at 1 mM.

Two versions of the synthetic gene (*fd1* and *fd2*, Figure 1) have been compared. Although *fd2* appears to direct a

slightly more efficient production of *C. pasteurianum* Fd, the differences observed are not very large. This suggests that the context around the start codon is of limited consequence to translation, as expected for a gene in which the building of mRNA secondary structure has been avoided by design (Davasse & Moulis 1992).

Expression of *fd3* from the T7 RNA polymerase-specific ϕ10 promoter shares most of the features described above for expression of *fd1* and *fd2* controlled by the *tac* promoter. In particular, holoFd can be isolated from K38/pGP1-2/pTFD in much the same way as described above for JM109/pKCFd2. Attempts at assembling [4Fe–4S] clusters into the potentially accumulated apoFd in crude extracts failed to provide a larger amount of holoFd, in contrast with results reported for *Anabaena* [2Fe–2S] Fd (Jacobson *et al.* 1992). Then, the final yields of reFd purified from cells bearing plasmids with the synthetic gene controlled by either the *tac* or ϕ10 promoters are roughly equivalent, generally between 0.3 and 0.6 mg l^{-1} of culture.

Protein turnover as a determining factor in the direct expression of C. pasteurianum *Fd*

The similar yields of reFd obtained with expression systems based on different promoters, *tac* from *E. coli* and ϕ10 from phage T7, seem to indicate that transcription of the synthetic genes, which does probably not occur at the same rate, is not limiting in these experiments. To further investigate the features determining the expression of the synthetic genes for reFd, the half-life of the product obtained from *fd3* has been evaluated. As shown in the upper part of Figure 4, the turnover of reFd after a 5 min pulse is very fast, with a half-life for the protein of the order of 5 min. This value compares with the lowest values reported for various proteins (Hargrove & Schmidt 1989) and explains the relatively low yield of reFd obtained.

Under identical conditions (Figure 4), *C. pasteurianum* rubredoxin, a protein of 54 amino acids taken as reference (Mathieu *et al.* 1992; Pétillot *et al.* 1993), is labeled after a 5 min pulse to a similar extent as reFd. This indicates that expression of both genes occurs at similar rates. However, the stability of rubredoxin is much greater ($t_{1/2} > 90$ min) than that of reFd. The detection of the long-lived rubredoxin after a chase of more than 1 h precludes the possibility of a general rapid turnover of proteins due to the shortage of some necessary component required for efficient expression in the conditions of Figure 4: as a result, at least 5 mg of rubredoxin are routinely purified per liter of culture (Pétillot *et al.* 1993), as compared to about 0.5 mg for reFd. It can then be concluded that the cellular concentration of reFd is not limited by the rates of mRNA or protein synthesis but rather by that of protein degradation.

Among the frequent causes of poor stability for recombinant products in *E. coli*, proteolytic degradation is very often involved but the use of a *lon*$^-$ mutant of *E. coli* lacking protease La failed to improve the yield of reFd produced from *fd2* (Davasse & Moulis 1992). In another

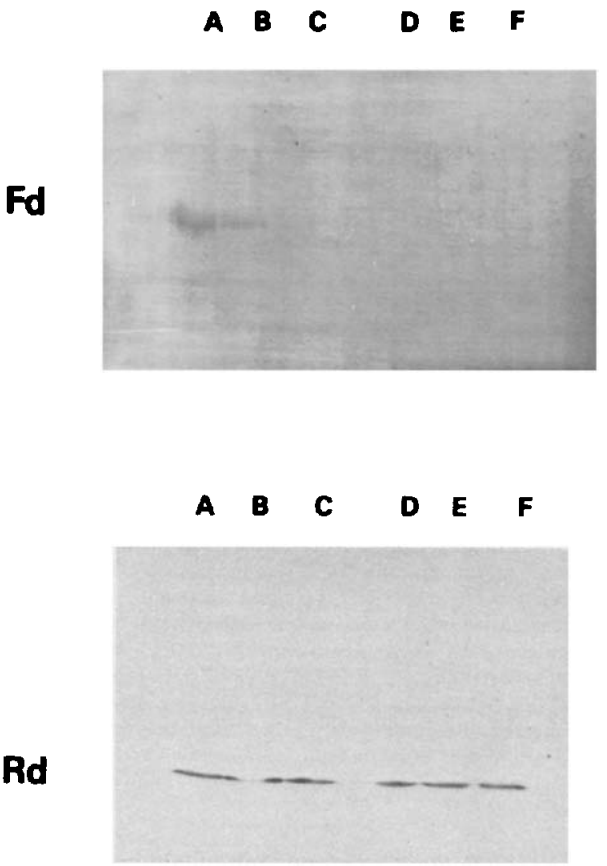

Figure 4. Compared half-lives of *C. pasteurianum* Fd and rubredoxin produced in *E. coli*. For Fd, expression from pTFD in K38/pGP1-2 was studied. For rubredoxin, the *Eco*RI–*Hin*dIII fragment of pCPRD2 (Mathieu *et al.* 1992) was cloned into pT7-5 (Tabor 1990) and used to transform K38/pGP1-2. Cells were treated as described under Materials and methods and the chase period was: lane A, 0; lane B, 5 min; lane C, 15 min; lane D, 30 min; lane E, 60 min; lane F, 90 min. Upper gel, Fd; lower gel, rubredoxin.

type of experiment, 6 μg of either purified apo- or holoFd have been incubated at 30 °C with cell-free extracts of *E. coli* K38/pGP1-2/pT7-7 corresponding to 10 ml of culture, i.e. the estimated relative quantities of proteins present when expressing *fd3* (see above). In both cases, it has been possible to detect apo- or holoFd in the incubation mixtures after 4 h by staining polyacrylamide gels with silver nitrate (not shown). These observations indicate that, on the one hand, no clear difference in stability can be demonstrated in cell-free extracts of *E. coli* between apo- and holoFd and, on the other hand, the fast turnover evidenced for reFd requires intact cells to take place.

Other post-translational events may then be considered to account for the high turnover of *C. pasteurianum* 2[4Fe–4S] Fd in the cytoplasm of *E. coli*. The major modification occurring after or during translation is the building of the metallic centers. One may argue that the peptide observed in Figure 4 is mainly apoFd which is not

processed to include the [4Fe–4S] clusters; however, no evidence for the presence of apoFd in the extracts to which clusters could be added has been found (see above). This suggests that the *fd1*, *fd2* and *fd3* genes analyzed here have all led to the synthesis of only fully functional Fd (Davasse & Moulis 1992). Also, no protein migrating like Fd on polyacrylamide gels has been detected in the particulate material of recombinant *E. coli* induced for the synthesis of this protein; this indicates that reFd is not sequestered in inclusion bodies or aggregated. In addition, increasing the pulse-labeling time up to 40 min in experiments like the one shown in the upper part of Figure 4 increases the period during which the protein can be detected, as labeling reached a plateau after ~ 20 min under such conditions. However, half-lives shorter than 15–20 min have always been determined. It then appears unlikely that the slow *in vivo* conversion of apo- to holoFd contributes significantly to the overall limited yield of recombinant protein (the former being supposedly less stable than the latter), since its turnover remains fast with increased labeling times and no accumulation has been observed for long expression periods (see above). Unfortunately, valuable data on the respective *in vivo* stabilities of apo- and holoFd are not available and cannot be easily obtained (see above).

Finally, as no endogenous 2[4Fe–4S] Fd has been described in *E. coli* (Ta & Vickery 1992), one might speculate that some essential factor for stability is missing. Nevertheless, even in the natural host, the yield of pure Fd is low, of the order of 0.5 $mg\,l^{-1}$ of *C. pasteurianum* culture (Moulis & Meyer 1982), and may also correlate with a high *in vivo* turnover. Indeed, an estimate of the degradation rate under conditions of iron limitation (Schönheit *et al.* 1979) points to a complete disappearance of this protein in 1 h at most. Given the presently available data, the relatively low yields of recombinant 2[4Fe–4S] Fd generally observed are probably linked to the intrinsic properties of this type of protein, among which the ready conversion to apoFd and resulting unfolding and degradation may be prominent.

General consequences for the expression of Fd genes

A general trend displayed by small iron–sulfur proteins like rubredoxins (Mathieu *et al.* 1992; see also above), [2Fe–2S] Fd (e.g. Coghlan & Vickery, 1989, Jacobson *et al.* 1992, Fujinaga & Meyer 1993) or 2[4Fe–4S] Fd (Baur *et al.* 1990, Grabau *et al.* 1991, Davasse & Moulis 1992) is that they do not require the addition of any special element to correctly fold as holoproteins in *E. coli* (Meyer *et al.* 1992). Whatever the origin, either natural or synthetic, of the gene used for the synthesis of these metalloproteins, no obvious need for a specific expression strategy is required. The straightest way to obtain such proteins is then certainly through the direct expression of the simplest possible gene encoding them, as demonstrated here.

However, the few reports describing the heterologous synthesis of 2[4Fe–4S] Fd in *E. coli* (Baur *et al.* 1990, Grabau *et al.* 1991, Davasse & Moulis 1992) set this family apart due to the relatively modest yields obtained. Significantly, very few studies of purified site-directed mutants has appeared (Gaillard *et al.* 1993). The lack of accumulating reFd is shown here to be due to the very short half-life of these proteins, irrespective of the expression system used in *E. coli*. It remains to be established whether the same phenomenon occurs in other potential hosts for the heterologous synthesis of 2[4Fe–4S] Fd, but the limited available data do not conclusively show that these proteins are more stable in their natural hosts. Indeed, these ubiquitous redox agents may fulfill additional roles to electron transfer, like the supply of iron under conditions of deprivation (Schönheit *et al.* 1979), and these regulatory functions might depend on the rapid *in vivo* turnover of 2[4Fe–4S] Fd.

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