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Isolation of pea thioredoxin *f* precursor protein and characterization of its biochemical properties

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Abstract

Like many other soluble chloroplastic enzymes, thioredoxin *f* is nuclear-encoded and expressed as a precursor protein. After synthesis in the cytosol, it is imported into the chloroplast with subsequent cleavage of the transit sequence in the stroma. We report the expression and the partial purification of the recombinant precursor thioredoxin *f* protein. The prethioredoxin *f* was found to be located essentially in the insoluble *Echerichia coli* fraction, but could be renatured after urea treatment followed by dialysis. The renatured protein was active in the dithiothreitol- and thioredoxin-dependent activation of NADP malate dehydrogenase and also of fructose bisphosphatase and in the ferredoxin–thioredoxin-dependent fructose bisphosphatase activation. These data are discussed in relation with the known properties of mature thioredoxin *f*.

Abbreviations: DTT – dithiothreitol; FBPase – fructose-1,6-bisphosphatase; FTR – ferredoxin–thioredoxin reductase; NADP-MDH – NADP dependent malate dehydrogenase; Trx – thioredoxin

Introduction

In the chloroplasts of higher plants, most of the stromal enzymes are encoded by nuclear genes with Rubisco being a notable exception since only the small subunits are of nuclear origin while the large subunits are encoded by chloroplastic genes (Whitney et al. 1999). Most of the proteins synthesized in the cytosol are expressed as precursors which contain a N-terminal extension necessary for routing into organelles and notably plastids (Blobel 2000). This sequence varies in length between ca. 30 and 70 amino acids (Emanuelsson et al. 2000). In higher plants, it is highly biased with a high content of hydroxylated and basic residues and a low content or absence of acidic amino acids (Gavel and von Heijne 1990). This sequence known as the transit peptide allows recognition by the import machinery situated in the chloroplast envelope (Hinnah et al. 2002). It is believed that the precursor protein is imported via this machinery in an unfolded or partially unfolded conformation by presenting its N-terminal presequence first (Hirsch et al. 1994). Following import, the mature protein is refolded via chloroplastic chaperones and the transit peptide is cleaved by a stromal peptidase (Adam and Clarke 2002). If needed, post-processing steps occur to integrate prosthetic groups and reach an active functional conformation.

Thioredoxin *f* is a key regulator of chloroplastic carbon metabolism which is very specific for fructose-1,6-bisphosphatase (FBPase) (Aguilar et al. 1992). As described for other plant thioredoxins (Duek and Wolosiuk 2001), its function is to reduce disulfide bridges via its reactive dithiol which is present in the active site WCGPC sequence. The primary nucleophile of this thioredoxin has been identified in the

spinach enzyme as the first of the two cysteines of the active site (Brandes et al. 1993). Other than FBPase, recently identified molecular targets of thioredoxin *f* include acetyl-CoA carboxylase (Kozaki et al. 2000), phosphoribulokinase (Geck and Hartmann 2000) and Rubisco activase (Zhang and Portis 1999). Proteomics studies have produced a comprehensive list of enzymes that have the potential to interact with thioredoxin *f* (Balmer et al. 2003). The 3D structure of spinach thioredoxin *f* has been solved (Capitani et al. 2000) and evidence has been obtained that it can form heterodimers linked via a covalent disulfide bond with FBPase (Balmer and Schürmann 2001). This heterodisulfide involves Cys 155 of FBPase and the primary nucleophile of thioredoxin *f* (Cys 47 in the longer construction of the spinach enzyme). It is believed that the specificity of the FBPase/thioredoxin *f* interaction relies on both electrostatic and hydrophobic interactions (Wangensteen et al. 2001).

As for other nuclear-encoded stromal chloroplastic enzymes, pea thioredoxin *f* is synthesized as a precursor of 182 amino acids which is subsequently cleaved to a mature protein of ca. 115 amino acids (Lepiniec et al. 1992). The exact N-terminus amino acid sequence of mature thioredoxin *f* is uncertain as the N-terminus of the spinach protein is blocked. Therefore, the recombinant enzyme has been produced as a short form (ca. 115 amino acids) or as an elongated form containing an additional 12 amino acids (Balmer and Schürmann 2001). Both forms are catalytically active but the elongated form appears to be more insoluble due to a series of hydrophobic amino acids in the N-terminus extension. Likewise, two constructs have been generated for pea thioredoxin *f*, one with the full-length sequence (unpublished) and the second one with a truncated sequence in which the transit peptide has been removed (Hodges et al. 1994). Only the short construction gave rise to a recombinant protein product which could be purified to homogeneity. We show here that the precursor of the pea thioredoxin *f* can also be produced as a recombinant product and partially purified. The catalytic properties of the precursor are also described.

Materials and methods

Chemicals and matrices

All chemicals were of the highest purity and either from Sigma, Euromedex or Merck. Chromatographic matrices were from LKB-Pharmacia or from IBF (ACA 44).

Escherichia coli *strains and plasmids*

E. coli strains DH5α and BL21(DE3) were routinely used for plasmid and protein production respectively. The construction of recombinant plasmid pETpea*f*2 (mature thioredoxin *f*) is described in Hodges et al. (1994). pETpea*f*1 (precursor protein) was produced in a similar fashion.

Expression and purification of the recombinant products

The expression and purification of mature thioredoxin *f* (pea *f*2) was described in Hodges et al. (1994). Expression and purification of sorghum NADP-MDH and pea FBPase were as in Jacquot et al. (1991, 1997). In order to express the sequence coding for the preprotein (pea *f*1), it was necessary to cotransform the cells with pETpea*f*1 and pSBET, a helper plasmid described in Schenk et al. (1995). Cotransformants were selected on plates containing both ampicillin and kanamycin as in Rouhier et al. (2002). One colony was successively amplified to 3 l in the presence of the two antibiotics and the protein expression was induced in the exponential phase at 37 °C by addition of 100 μM IPTG. After 3 h at 37 °C, bacteria were pelleted by centrifugation (15 min 5000 × *g*). The pellets were resuspended in TE buffer (Tris-HCl 30 mM pH 8.0, 1-mM EDTA) supplemented with 14 mM β-mercaptoethanol. The bacteria were broken by sonication and the insoluble fraction which contained the prethioredoxin *f* separated by centrifugation (20 min, 30,000 × *g*). The pellet was resuspended in ca. 10 ml TE supplemented with 8 M urea and dialyzed against an excess of TE buffer at 5 °C. After dialysis, the remaining insoluble fraction was eliminated by centrifugation (20 min, 30,000 × *g*). At this point, the precursor of thioredoxin *f* represents between ca. 30% and 50% of the total protein, depending on the preparation. Further purification of the precursor was attempted by using ACA 44 gel filtration, carboxymethyl and DEAE Sephacel chromatography.

Activity assays and SDS-PAGE

DTT-dependent activation of NADP-MDH

The enzyme was activated in 100 μl medium containing 50 mM Tris-HCl pH 8.0, 5 mM DTT, 2 μM NADP-MDH and variable (0–5 μM) amounts of

thioredoxin. After 5 min of incubation at 25 °C, an aliquot of 20 µl was taken to determine the activity of NADP-MDH in a reaction mixture (1 ml) containing 100 mM Tris-HCl pH 8.0, 750 µM oxaloacetic acid and 150 µM NADPH. The amount of NADPH oxidized by the enzyme was monitored as a decrease in absorbancy at 340 nm ($\epsilon M = 6200\,M^{-1}\,cm^{-1}$).

DTT-dependent activation of FBPase

The enzyme was activated in a 50 µl medium containing 100 mM Tris-HCl pH 8.0, 5 mM DTT, 2 µM FBPase and variable amounts of thioredoxin. After 5 min of incubation at 25 °C, an aliquot of 5 µl was taken to determine the activity of FBPase in a reaction mixture (1 ml) containing 100 mM Tris-HCl pH 8.0, 100 µM EDTA, 1.5 mM $MgSO_4$, 1 mM FBP-Na, 0.7 units glucose 6-phosphate dehydrogenase, 1.8 units phosphoglucoisomerase, 300 µM NADP and 1.4 mM β-mercaptoethanol. The activity was determined as the increase in absorbancy at 340 nm.

FTR-dependent activation of FBPase

The FBPase enzyme was activated in a 100 µl medium containing 100 mM Tris-HCl pH 8.0, 2 µM FBPase, 1 mM methyl viologen, 14 mM β-mercaptoethanol, 1 µM spinach FTR, 5 mM Na-dithionite and variable amounts of thioredoxin. After 15 min of incubation in anaerobic medium, in presence of argon, 5 µl were injected into the reaction mixture (1 ml) containing 100 mM Tris-HCl pH 7.9, 100 µM EGTA-Na, 1.5 mM $MgSO_4$, 1 mM FBP, 0.7 units glucose-6-phosphate dehydrogenase, 1.8 units phosphoglucoisomerase, 0.3 mM NADP and 1.4 mM β-mercaptoethanol. The activity was determined as the increase in absorbancy at 340 nm.

Polyacrylamide gel electrophoresis

Proteins were separated by SDS-PAGE following the method described in Hodges et al. (1994).

Results

Sequence comparison of plant thioredoxin f *precursors*

Several sequences are now available for the nuclear encoded precursor of chloroplastic thioredoxin *f*. Figure 1A shows an amino acid sequence comparison which includes the proteins from pea, spinach, rapeseed and two isoforms from *Arabidopsis thaliana*. There is extensive sequence homology between all sequences starting ca. 30 amino acids upstream of the conserved active site WCGPC. Preceding this high homology region there is an insertion present only in the spinach sequence and another region of lower homology ca. 10 amino acids upstream from this insertion region. As the active site of most thioredoxins is situated at position 30–35, this indicates that the cleavage site of the precursor is likely to be close to the spinach insertion. Consistent with this observation, the sequence homologies in the first 60 amino acids are very low, in agreement with the known properties of transit sequences. Nevertheless, using standard prediction programs such as PSORT (http://psort.nibb.ac.jp/form.html), it is extremely difficult to predict both the targeting of these sequences and the cleavage sites of the putative precursor proteins. Two of these sequences are predicted to be chloroplastic, two cytosolic and one mitochondrial, and the cleavage site of the proposed chloroplastic sequences is clearly not in agreement with the known biochemical properties of the protein. It is thus of interest to produce the precursor both to test its catalytic capacity and its subcellular localization following import.

Production and partial purification of the pea precursor

The nucleotide sequence of the precursor sequence shows the presence in the transit sequence of five arginine codons rarely used by *E. coli* (Figure 1B). Surprisingly, they are all localized in the transit sequence. One of these is coded by an AGA triplet, the others are encoded by AGG triplets (of particular importance is the presence of two consecutive rare AGG codons). The presence of these triplets and especially of consecutive AGG codons could affect the production of the precursor protein in *E. coli* BL21 (DE3) at the translational level (Brinkmann et al. 1989). To overcome this potential problem, the *E. coli* strain BL21 (DE3) was cotransformed by pETpea*f*1 and pSBET which contains the *arg U* gene encoding the rare $tRNA_{arg4}$ (Schenk et al. 1995). As detailed below, this system proved to be functional in the overproduction of the precursor protein.

Following the expression procedure described in 'Materials and methods', we could observe by SDS-PAGE a large overproduction of a polypeptide migrating with an apparent molecular mass of 19 kDa. This very prominent band was absent in untransformed

A

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pea             MALNLCTSPKWIG----TTVFDSASSSKP-SLASSFSTTSFSSSILCSKRVGLQRLS---
Brassica        MPLSLRLAPSP------TALSPTTGGFSPAKKQCRIPSYSGVATTT--RRIGLCSLDY--
Spinach         MALHLSLSHQS-----WTSPAHPITSSDPTRSSVPGTGLSRRVDFLGSCKINGVFVVKRK
Arab1           MPLSLRLSPSP------TALSPTTGGFGPSRKQCRIP-YSGVPTT----KIGFCSLDS--
Arab2           MPLSLRLAPSPTSFRYSPITSTGAGGFSPVKQHCRIP-NSGVATKIGFCSGGGGVLDS--
                * * *  .                     *          *           .   .

pea             -LRRSISVSVRSSLETAGP----TVTVGKVTEVNKDTFWPIVNAAGDKTVVLDMFTKWCG
Brassica        -VKRGDSSVVRCSLQTVN------VSVGQVTEVDKDTFWPIVKAAGEKIVVLDMYTQWCG
Spinach         DRRRMRGGEVRASMEQALGTQEMEAIVGKVTEVNKDTFWPIVKAAGDKPVVLDMFTQWCG
Arab1           -RKRGDSSVVRCSLETVN------VSVGQVTEVDKDTFWPIVKAAGEKLVVLDMYTQWCG
Arab2           -GRRIGSCVVRCSLETVN------VTVGQVTEVDKDTFWPIVKAAGDKIVVLDMYTQWCG
                  .*     **.*..           **.**** ******** ***.* *****.*.***

pea             PCKVIAPLYEELSQKYLDVVFLKLDCNQDNKSLAKELGIKVVPTFKILKDNKIVKEVTGA
Brassica        PCKVIAPKYKALSEKYEDVVFLKLDCNPENRPLAKELGIRVVPTFKILKDNQVVKEVTGA
Spinach         PCKAMAPKYEKLAEEYLDVIFLKLDCNQENKTLAKELGIRVVPTFKILKENSVVGEVTGA
Arab1           PCKVIAPKYKALSEKYDDVVFLKLDCNPDNRPLAKELGIRVVPTFKILKDNKVVKEVTGA
Arab2           PCKVIAPKYKELSEKYQDMVFLKLDCNQDNKPLAKELGIRVVPTFKILKDNKVVKEVTGA
                *** .** *  *.. * *..******* .*. *******.*********.* .* *****

pea             KFDDLVAAIDTVRSS----
Brassica        KYDDLVAAIETARSASSSG
Spinach         KYDKLLEAIQAARSS----
Arab1           KYDDLVAAIETARSAASG-
Arab2           KYEDLLAAIEAARSG----

                *.. *. ** . **
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B

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ATG GCT CTA AAT CTC TGC ACC TCC CCT AAA TGG ATC GGC ACC ACC GTC TTC GAT AGT GCT
 M   A   L   N   L   C   T   S   P   K   W   I   G   T   T   V   F   D   S   A
 1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18  19  20

TCT TCA TCG AAG CCA TCA CTT GCT TCT TCT TTT TCT ACT ACT TCA TTT TCT TCT TCC ATT
 S   S   S   K   P   S   L   A   S   S   F   S   T   T   S   F   S   S   S   I
 21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36  37  38  39  40

TTA TGT TCG AAG AGG GTT GGT CTA CAG AGG TTA AGC TTG AGG AGG AGT ATC AGT GTC AGT
 L   C   S   K   R   V   G   L   Q   R   L   S   L   R   R   S   I   S   V   S
 41  42  43  44  45  46  47  48  49  50  51  52  53  54  55  56  57  58  59  60

 GTA AGA TCC AGC TTG GAA ACT // GCG GGA CCC ACG GTG ACG
  V   R   S   S   L   E   T  //  A   G   P   T   V   T
  61  62  63  64  65  66  67      68  69  70  71  72  73
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Figure 1. Sequences of Pea thioredoxin *f* and related homologues in plants. (A) Amino acid sequence comparison. The active site sequences are underlined. (B) Nucleotide and derived amino acid sequence of the transit peptide of Pea thioredoxin *f*. The most probable cleavage sites are indicated by a double slash. The rare Arg codons are underlined. Identification and accession codes: Pea: *Pisum sativum* P29450; Brassica: *Brassica napus* THIF_BRANA; spinach: Spinacia oleracea P09856; arab 1: *Arabidopsis thaliana* AF144385.1; arab 2: *Arabidopsis thaliana* NM_121645.2.

E. coli cells, thus confirming that it was indeed the prethioredoxin *f*. However, the recombinant prethioredoxin *f* was present in the insoluble fraction (data not shown). When bacteria were grown in LB medium at low temperature (27 °C) without induction or in M9 medium, no significant amounts of

the soluble precursor protein were generated (data not shown).

A denaturation/renaturation treatment based on urea treatment followed by dialysis was thus required to solubilize the precursor thioredoxin *f*. After clarification by centrifugation, the extract was loaded onto a Sephadex G-50 column. Unfortunately, this gel filtration did not allow purifying the protein. DEAE or carboxymethyl chromatographies also turned out to be unsuited for the precursor purification as prethioredoxin *f* seemed to denature on the matrix. This result provides evidence that the precursor is rather unstable under the experimental conditions used. After renaturation and dialysis, the purity of the thioredoxin *f* precursor was about 50% for the best preparations and closer to 30% in Figure 2. The apparent molecular mass of the renatured precursor protein is in good agreement with the expected theoretical value, indicating that no significant proteolysis had occurred. Very large amounts of partially purified prethioredoxin *f* were obtained using this method (about 50 mg/l).

Catalytic properties

Two types of enzyme assays were used to compare the catalytic properties of the recombinant pea thioredoxin *f* precursor to those of the mature protein. Chloroplastic NADP-MDH and FBPase were activated either in the presence of DTT or in a reconstituted light activation system.

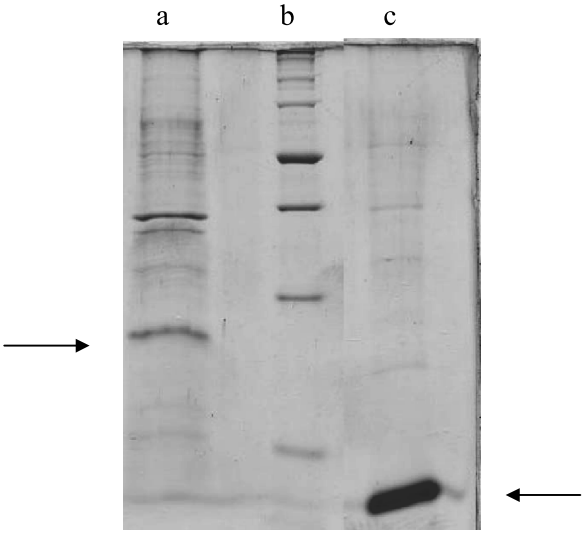

Figure 2. SDS gel electrophoresis of recombinant mature and prethioredoxin *f*. Lane a: Prethioredoxin *f*; Lane b: molecular weight markers; Lane 3: mature thioredoxin *f*. Markers are from bottom to top: 15, 25, 37, 50, 75 and 100 kDa. The positions of the recombinant proteins are indicated by arrows. The theoretical molecular mass of the initiator methionine excised prethioredoxin *f* is 19,625 Da.

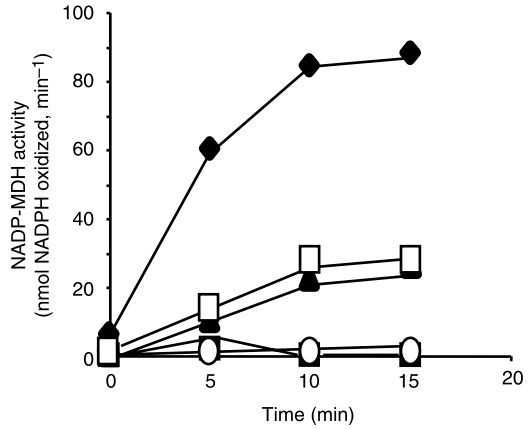

Figure 3. Activation of NADP-MDH by mature and prethioredoxin *f* in the presence of DTT. Closed diamonds: DTT and mature thioredoxin *f*; open squares: DTT and prethioredoxin *f*; closed triangles: DTT, prethioredoxin *f* and preimmuneserum; open circles: DTT alone; closed squares: DTT, prethioredoxin *f* and anti-thioredoxin *f* serum. Prethioredoxin *f* concentrations were estimated from the overall protein concentration of the sample, taking into account its degree of purity.

First, the two forms of thioredoxins were tested for their ability to activate the chloroplastic NADP-MDH in the presence of DTT. Figure 3 shows the time course activation of NADP-MDH in the presence of mature thioredoxin *f* or its precursor. As expected the mature thioredoxin *f* gave a high activation rate. Maximal activity was reached rapidly after a 10-min incubation. The prethioredoxin *f* also activated NADP-MDH but it appeared to be less efficient. The maximum activity represents only ca. 30% of the maximum activity obtained with the mature form under the same experimental conditions. The possibility that the measured activation rates could be due to endogenous *E. coli* thioredoxin (which is a good activator of this enzyme) was evaluated in two different ways. First, an untransformed *E. coli* sample was generated and partially purified under the same experimental conditions and tested as an activator of NADP-MDH. Using this sample, the rates of NADP-MDH activation were negligible in agreement with the soluble nature of the *E. coli* thioredoxins. In a second experiment, we showed that the addition of antibodies generated against mature pea thioredoxin *f* abolished NADP-MDH activation, while a preimmune serum had no effect. As DTT by itself cannot activate the NADP-MDH under these pH and concentration conditions,

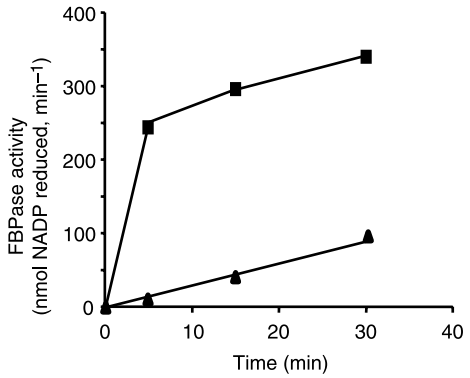

Figure 4. Time course of FBPase activation by mature and prethioredoxin *f* in the presence of DTT. Closed squares: DTT and mature thioredoxin *f*; closed triangles: DTT and prethioredoxin *f*. The rates with DTT alone are negligible.

these data prove that prethioredoxin *f* is indeed a catalyst for NADP-MDH reduction and activation.

The two forms of pea thioredoxin *f* were tested for their ability to activate chloroplastic FBPase in the presence of DTT (Figure 4). As seen before, mature thioredoxin *f* was able to activate FBPase with a high efficiency. After a 5-min incubation, full activity was reached. At approximately twice the mature thioredoxin *f* concentration, the precursor protein was a lot more sluggish in its activation properties. The rate of activation was lower and the reaction did not saturate but remained linear over 20 min. In addition, the DTT-dependent activation of FBPase in the presence of different concentrations of recombinant precursor protein was found to be linear up to a concentration of ca. 14 μM (Figure 5). Since no other enzyme in the *E. coli* extracts can activate FBPase, these experiments confirm that the precursor thioredoxin *f* is

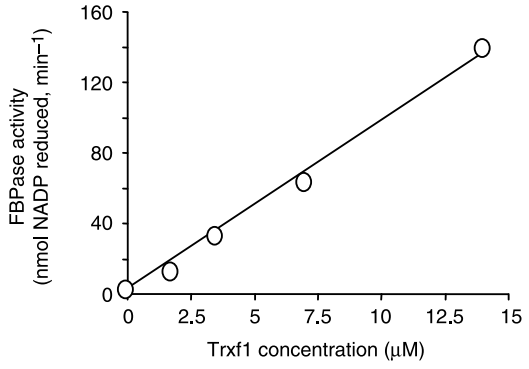

Figure 5. Activation of FBPase as a function of the prethioredoxin *f* concentration in the presence of DTT. Activities were determined after 20 min incubation with DTT and prethioredoxin *f*.

Table 1. Effect of mature and prethioredoxin *f* on the FTR-linked activation of FBPase[a]

Thioredoxin	Concentration (μM)	FBPase activity (nmol NADP reduced min^{-1})
Trxf1	9	32
	13	79
Trxf2	1	400
No addition	0	4

[a] Trxf1: pea prethioredoxin *f*; Trxf2: mature pea thioredoxin *f*.

indeed catalytically active although less efficient than the mature protein. As observed with DTT, in the FTR dependent system, prethioredoxin *f* was also active but much less efficient than mature thioredoxin *f* (Table 1). These data indicate that the precursor protein could not only interact with FBPase but also with the ferredoxin–thioredoxin reductase.

Discussion

In order to produce successfully the pea thioredoxin *f* precursor, it was necessary to cotransform the *E. coli* strain BL21 (DE3) with pETpea*f*1 and pSBET, a helper plasmid which corrects for the deficit in tRNA corresponding to the rare AGG and AGA codons. The existence of multiple rare codons in transit peptides is not a universal feature and it appears that the high rate of rare codons observed in this pea presequence is rather the result of a random selection of the codons. Because of its insolubility and presumably instability, we could not purify the recombinant protein to homogeneity, but it could be solubilized in urea and renatured, resulting in a precursor-enriched preparation in which recombinant prethioredoxin *f* represented 30%–50% of the total proteins depending on the preparation.

In general, precursor proteins are believed to be maintained in an unfolded state in the cytosol via the action of chaperones prior to import. Whether the precursor proteins retain catalytic activity has not been thoroughly investigated. A few reports have shown that a precursor protein can be catalytically active, and examples include preadrenodoxin (Goder et al. 1988) and pre-alpha-lactalbumin (Raymond et al. 1982). In other cases such as limoene synthase, the presence of the transit sequence impaired catalysis (Williams et al. 1998). Our study clearly demonstrates that

prethioredoxin *f* is catalytically active although it is significantly less efficient than the mature protein. The precursor of thioredoxin *f* possesses at least three catalytic activities: the capacity to activate the regulatory enzymes NADP-MDH and FBPase, and the capacity to be reduced via ferredoxin–thioredoxin reductase.

The protein preparation that we have generated was refolded after urea treatment and dialysis. The catalytic properties of the enzyme indicate that the folding is sufficient to generate the secondary structures needed for substrate recognition. On the other hand, it is quite clear that the extra N-terminal sequence is a factor of instability for the protein as it was found to deteriorate upon purification and storage. Destabilization of the polypeptides by N-terminal presequences is not always true. The rat mitochondrial citrate carrier is actually more stable with its presequence (Zara et al. 2003). In this case however, the presequence did not seem to be implicated in targeting.

The data generated in this study lead to the following question: is the precursor protein active in the unfolded state when associated with the Hsp proteins? Whatever the answer, when the protein is translocated into the chloroplast stroma, after separating from the chloroplastic Hsp system and before cleavage through the stromal peptidase, there is a possibility that the precursor protein exists in a refolded state similar to that obtained in this study. The half-life of such species is not known and may depend on the nature of the presequence. If long enough, then these results are physiologically significant, suggesting that the precursor protein would be immediately active, before the ultimate processing.

The present study brings a new tool that may be of interest in two areas of research. Firstly, we can now use large amounts of the thioredoxin *f* precursor protein to test unequivocally whether it can be imported also into mitochondria as suggested by some prediction programs. Secondly, it opens the way for structural studies, as basically nothing is known about the 3D structure of such preproteins.

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